Non-Human Animals Expressing pH-Sensitive Immunoglobulin Sequences

FIELD OF THE INVENTION

[0001] A genetically modified non-human animal that expresses antibodies capable of binding to an antigen in a pH dependent manner. Genetically modified non-human animals that comprise immunoglobulin loci that are modified to contain at least one substitution or insertion of a codon encoding a protonatable amino acid. Genetically modified non-human animals that comprise immunoglobulin loci that are modified to contain at least one histidine substitution and/or at least one histidine insertion in an immunoglobulin heavy chain V, D, or J gene segment, or light chain V or J segment, or rearranged heavy chain VDJ region or rearranged light chain VJ region thereof. Genetically modified non-human animals that express immunoglobulins that exhibit pH sensitivity in antigen binding. Genetically modified animals that comprise B cell populations that are enriched with respect to immunoglobulin variable domains that comprise at least one histidine. Genetically modified non-human animals that comprise clusters of two or more histidines present as insertions and/or substitutions in an immunoglobulin heavy chain V, D, and/or J gene segment, and or a light chain V and/or J gene segment, and/or rearranged heavy chain VDJ sequences or rearranged light chain VJ sequences thereof.

[0002] Genetically modified immunoglobulin loci of non-human animals comprising an unrearranged human heavy chain variable region nucleotide sequence, wherein the unrearranged human heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon. Non-human animals, including rodents, *e.g.,* mice and rats, comprising a genetically modified immunoglobulin locus in their genome an unrearranged human heavy chain variable region nucleotide sequence, wherein the unrearranged human heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon. Genetically engineered non-human animals capable of expressing an antigen-binding protein that is characterized by pH-dependent antigen binding, improved recyclability, and/or enhanced serum half-life.

BACKGROUND

[0003] Immunoglobulin binding domains find therapeutic use in a wide variety of formats, including the traditional antibody format of a homodimeric immunoglobulin heavy chain associated with a cognate light chain. Many of these formats, including the traditional format, exhibit pharmacokinetic features *in vivo* that are suboptimal, due to a wide variety of

factors. In recent decades, disparate approaches have been tried to improve pharmacokinetics. These include, *e.g.,* increasing hydrodynamic radius to reduce renal clearance by conjugation to polymers (*e.g.,* PEG; reviewed in, *e.g.,* Duncan, R. (2006) Polymer conjugates as anticancer nanomedicines, Nat. Rev. Cancer 6:688-701); sialylation of N-glycans (reviewed in, *e.g.,* Stork, R. *et al.* N-glycosylation as novel strategy to improve pharmacokinetic properties of bispecific single-chain diabodies, J. Biol. Chem. 283(12):7804-7812); Fc modifications for promoting neutral pH Fc-FcRn binding while promoting release at endosomal pH and association with serum albumin (see, *e.g.,* Chuang *et al.* (2002) Pharmaceutical Strategies Utilizing Recombinant Serum Albumin, Pharm. Res. 19(5):569-577). In appropriate applications and for appropriate formats, each of these approaches may offer some benefits.

[0004] However, there remains a need in the art for improving therapeutic effects and modalities for biopharmaceuticals, including but not limited to manipulating immunoglobulin variable domain structures to engineer variable domains that exhibit pH-dependent binding. There is a need for variable domains for use in antigen-binding proteins of a variety of formats, wherein the variable domains (or antigen-binding fragments thereof) confer upon the antigen-binding protein pH sensitivity with respect to binding a target antigen or receptor. There is also a need in the art for systems and methods for generating pH-dependent immunoglobulin variable domains and antigen-binding fragments thereof. There is a need for biological systems that can generate a wide diversity of immunoglobulin variable domains, wherein the wide diversity is enriched with respect to titratable amino acids that may confer upon the variable domain pH sensitivity, *e.g.*, the ability to bind a target antigen or epitope at one pH (e.g., a neutral, or high pH), yet release the target antigen or epitope at a second pH (*e.g.*, a low, or endosomal, pH).

[0005] Immunoglobulin light chains in certain formats present unique challenges. Antibodies typically comprise a homodimeric heavy chain component, wherein each heavy chain monomer is associated with an identical light chain. Antibodies having a heterodimeric heavy chain component (*e.g.,* bispecific antibodies) are desirable as therapeutic antibodies. But making bispecific antibodies having a suitable light chain component that can satisfactorily associate with each of the heavy chains of a bispecific antibody has proved problematic.

[0006] In one approach, a light chain might be selected by surveying usage statistics for all light chain variable domains, identifying the most frequently employed light chain in human antibodies, and pairing that light chain *in vitro* with the two heavy chains of differing specificity.

[0007] In another approach, a light chain might be selected by observing light chain sequences in a phage display library (*e.g.,* a phage display library comprising human light chain variable region sequences, *e.g.,* a human scFv library) and selecting the most commonly used light chain variable region from the library. The light chain can then be tested on the two different heavy chains of interest.

[0008] In another approach, a light chain might be selected by assaying a phage display library of light chain variable sequences using the heavy chain variable sequences of both heavy chains of interest as probes. A light chain that associates with both heavy chain variable sequences might be selected as a light chain for the heavy chains.

[0009] In another approach, a candidate light chain might be aligned with the heavy chains' cognate light chains, and modifications are made in the light chain to more closely match sequence characteristics common to the cognate light chains of both heavy chains. If the chances of immunogenicity need to be minimized, the modifications preferably result in sequences that are present in known human light chain sequences, such that proteolytic processing is unlikely to generate a T cell epitope based on parameters and methods known in the art for assessing the likelihood of immunogenicity (i.e., *in silico* as well as wet assays).

[00010] All of such approaches rely on *in vitro* methods that subsume a number of *a priori* restraints, *e.g.,* sequence identity, ability to associate with specific pre-selected heavy chains, etc. There is a need in the art for compositions and methods that do not rely on manipulating *in vitro* conditions, but that instead employ more biologically sensible approaches to making human epitope-binding proteins that include a common light chain.

[00011] In addition, therapeutic antibodies, *e.g.,* bispecific therapeutic antibodies, have some limitations in that they often require high doses to achieve desired efficacy. This is partly due to the fact that antibody-antigen complexes are internalized into the endosome, and are targeted for lysosomal degradation in a process called target-mediated clearance. Thus, there is a need in the art for methods and compositions that lead to more efficient antibody recycling, *e.g.,* bispecific antibody recycling, and prevent degradation of the antibody by promoting dissociation of antibody-antigen complexes in the endosomal compartment without compromising the specificity and affinity of the antibody toward the antigen.

[00012] Drugs administered into the body, including therapeutic monoclonal antibodies, can be affected via various elimination mechanisms, including glomerular filtration (*e.g.*, into urine), secretion (*e.g.*, into the bile), and catabolism by cells. While small molecules are

cleared from the body via renal filtration, the majority of secreted antibodies (*e.g.*, IgG, which are too big to be filtered through glomeruli) are primarily removed from the body via cell-mediated catabolism, *e.g.*, fluid-phase endocytosis (phagocytosis) or receptor-mediated endocytosis. For example, soluble molecules with several repeated epitopes are bound by a plurality of circulating antibodies, and the resulting large antigen-antibody complexes are phagocytosed rapidly into cells for degradation. On the other hand, cell surface target receptors, which are bound by antibodies (*i.e.*, receptor-antibody complexes), undergo target-mediated endocytosis in a dose-dependent manner, which leads to formation of endosomes destined for lysosomal degradation inside cells. In some cases, the endocytosed receptor-antibody complexes bind neonatal Fc receptors (FcRn) inside the endosomes in a pH-dependent manner and are routed back to the cell surface for release into plasma or interstitial fluids upon exposure to a neutral extracellular pH (*e.g.*, pH 7.0-7.4).

[00013] There is a need in the art for systems, *e.g.*, non-human animals, cells, and genomic loci that generate antigen-binding proteins with titratable residues, *e.g.*, genetically modified loci that rearrange immunoglobulin gene segments to generate heavy chain variable domains that respond to changes in pH, *e.g.*, that donate or accept protons and, *e.g.*, whose binding characteristics differ according to protonation state.

[00014] There is also a need in the art for methods and compositions that can further increase recycling efficiency of endocytosed antigen-binding proteins by promoting dissociation of antigen-binding proteins from receptor-antigen-binding protein complexes or by increasing the affinity of antigen-binding proteins toward FcRn in an acidic endosomal compartment without compromising the specificity and affinity of the antigen-binding protein toward an antigen of interest.

SUMMARY

[00015] Compositions and methods are provided for making genetically modified animals that make immunoglobulin variable domains that comprise at least one histidine residue encoded by a germline modification of the non-human animal, wherein the germline modification comprises at least one of the insertion of a histidine codon into a heavy chain V, D, or J segment, insertion of a histidine codon into a light chain V or J segment, insertion of a histidine codon into a rearranged light chain VJ gene, substitution of a non-histidine codon with a histidine codon in a heavy chain V, D, or J segment, substitution of a non-histidine codon with a histidine codon in a light chain V or J segment, substitution of a non-histidine codon with a histidine codon in a rearranged light chain VJ sequence.

[00016] Compositions and methods are also provided for introducing clusters of histidine

codons in germline immmunoglobulin sequences of non-human animals.

[00017] Compositions and methods are also provided for introducing histidine insertions, or substitutions of non-histidine codons with histidine codons, in N-terminal-encoding regions of immunoglobulin genes, loop 4-encoding regions of immunoglobulin genes, CDR-encoding regions of immunoglobulin genes (*e.g.,* rearranged V(D)J sequences or V, (D), J gene segments).

[00018] Compositions and methods for making non-human animal progeny that comprise insertions of histidine codons and/or substitutions of non-histidine codons with histidine codons in both immunoglobulin heavy chain loci and in immunoglobulin light chain loci.

[00019] In one aspect, a genetically modified non-human animal comprising in its germline an immunoglobulin locus comprising a substitution or an insertion in an immunoglobulin variable locus of at least one non-histidine codon with a histidine codon. In one embodiment, the variable locus (*e.g.,* an unrearranged V(D)J segments locus) comprises at least a portion of a human variable (V(D)J segments) locus.

[00020] In one embodiment, the genetically modified non-human animal comprises in its germline a first variable locus (*e.g.,* an unrearranged immunoglobulin heavy chain (V(D)J segments locus) and a second variable locus (*e.g.*, an unrearranged immunoglobulin light chain (V,J segments locus; or a rearranged immunoglobulin light chain VJ sequence).

[00021] In one embodiment, the non-human animal comprises a first and a second variable locus, wherein at least the first or the second variable locus comprises an insertion of at least one histidine codon or a substitution of at least one non-histidine codon with a histidine codon.

[00022] In one embodiment, both the first and the second variable locus each comprise a substitution or insertion of at least one non-histidine codon with a histidine codon.

[00023] In one embodiment, the first variable locus comprises at least a functional portion of an unrearranged heavy chain variable locus (unrearranged V, D, J segments).

[00024] In one embodiment, the unrearranged heavy chain variable locus comprises at least a portion of a human locus (unrearranged V, D,J segments).

[00025] In one embodiment, the unrearranged heavy chain locus is a human locus comprising unrearranged V segments, a synthetic D segment that comprises a linker, and a

human J segment. In one embodiment, the synthetic D segment comprises at least one histidine codon.

[00026] In one embodiment, the second variable locus comprises at least a functional portion of an unrearranged light chain locus (unrearranged V,J segments).

[00027] In one embodiment, the second variable locus comprises a rearranged immunoglobulin light chain variable gene sequence (rearranged VJ sequence).

[00028] In one embodiment, the substitution of an non-histidine codon with a histidine codon and/or the insertion of a histidine codon is in a nucleic acid sequence that encodes a variable domain and the histidine is in a region selected from an N-terminal region of an immunoglobulin chain, a loop 4 region of an immunoglobulin chain, a CDR1 of a heavy chain, a CDR2 of a heavy chain, a CDR3 of a heavy chain, a CDR1 of a light chain, a CDR2 of a light chain, a CDR3 of a light chain, and a combination thereof.

[00029] In one embodiment, at least one of the first variable locus or the second variable locus is operably linked to an endogenous non-human constant region nucleic acid sequence at an endogenous non-human immunoglobulin locus.

[00030] In one embodiment the first variable locus (unrearranged human V, D, J segments) is operably linked to an endogenous non-human immunoglobulin heavy chain constant region nucleic acid sequence.

[00031] In one embodiment, the first variable locus (unrearrangd human V, D, J segments) is operably linked to the endogenous non-human immunoglobulin heavy chain constant region nucleic acid sequence at an endogenous non-human immunoglobulin locus.

[00032] In one embodiment, the second variable locus (unrearranged V, J segments) is operably linked to an endogenous non-human immunoglobulin light chain constant region sequence.

[00033] In one embodiment, the endogenous non-human immunoglobulin light chain constant region sequence is at an endogenous non-human immunoglobulin locus.

[00034] In one embodiment, the variable region sequence comprises a cluster of 2, 3, 4, or 5 histidines that are substituions of non-histidine codons with histidine codons and/or insertions of histidine codons.

[00035] In one embodiment, the unrearranged heavy chain locus comprises D gene

segments that are inverted with respect to the direction of orientation of the heavy chain locus. In one embodiment, the inverted D segments are in a hydrophilic reading frame.

[00036] In one aspect, a genetically modified non-human animal is provided, comprising at least a portion of a human unrearranged immunoglobulin heavy chain variable region nucleic acid sequence (unrearranged V, D, J segments) operably linked to a constant region gene sequence, wherein one or more of the V, D, and J gene segments comprise at least one substitution of a non-histidine codon for a histidine codon, or at least one histidine codon insertion; at least a portion of a human unrearranged immunoglobulin light chain variable region nucleic acid sequence (unrearranged V, J segments) operably linked to a constant region gene sequence, wherein one or more of the V and J gene segments comprise at least one substitution of a non-histidine codon for a histidine codon, or at least one histidine codon insertion; wherein the non-human animal expresses an immunoglobulin heavy chain variable domain and/or an immunoglobulin light chain variable domain that comprises a histidine derived from a histidine substitution or insertion in the germline of the mouse.

[00037] In one embodiment, the non-human animal is a mammal. In one embodiment, the mammal is a rodent. In one embodiment, the rodent is selected from the group consisting of a mouse, a rat, and a hamster.

[00038] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleic acid sequence is operably linked to a non-human constant region sequence.

[00039] In one embodiment, the non-human constant region nucleic acid sequence operably linked to the human unrearranged immunoglobulin heavy chain variable region nucleic acid sequence is at an endogenous non-human immunoglobulin locus in the germline of the non-human animal.

[00040] In one embodiment, the non-human constant region nucleic acid sequence operably linked to the human unrearranged immunoglobulin light chain variable region nucleic acid sequence is at an endogenous non-human immunoglobulin locus in the germline of the non-human animal.

[00041] In one aspect, a genetically modified non-human animal is provided, comprising at least a portion of a human unrearranged immunoglobulin heavy chain variable region nucleic acid sequence (unrearranged V, D, J segments) operably linked to a constant region gene sequence, wherein one or more of the unrearranged V, D, and J gene segments comprise at least one substitution of a non-histidine codon for a histidine codon, or at least

one histidine codon insertion; a human rearranged immunoglobulin light chain variable region nucleic acid sequence (rearranged VJ sequence) operably linked to a light chain constant region gene sequence, wherein the rearranged VJ sequence comprises at least one substitution of a non-histidine codon for a histidine codon, or at least one histidine codon insertion; wherein the non-human animal expresses an immunoglobulin heavy chain variable domain and/or an immunoglobulin light chain variable domain that comprises a histidine derived from a histidine substitution or insertion in the germline of the mouse.

[00042] In one embodiment, the genetically modified non-human animal is a mammal. In one embodiment, the mammal is a rodent. In one embodiment, the rodent is selected from the group consisting of a mouse, a rat, and a hamster.

[00043] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleic acid sequence is operably linked to a non-human constant region sequence. In one embodiment, the non-human constant region sequence operably linked to the human unrearranged immunoglobulin heavy chain variable region nucleic acid sequence is at an endogenous non-human immunoglobulin locus in the germline of the non-human animal. In one embodiment, the non-human constant region sequence operably linked to the human rearranged immunoglobulin light chain variable region nucleic acid sequence is at an endogenous non-human immunoglobulin locus in the germline of the non-human animal.

[00044] In one aspect, a genetically modified non-human animal is provided, wherein the animal comprises a B cell population that is characterized by an enhanced presence of histidine residues in immunoglobulin heavy and light chains of the B cell population as compared with a wild-type non-human animal. In one embodiment, the enhancement is about 2-4 fold. In one embodiment, the enhancement is about 2-10 fold.

[00045] In one aspect, a genetically modified non-human animal is provided that expresses immunoglobulin light and heavy chains that comprise histidines encoded by substitutions and/or insertions in germline immunoglobulin sequences of the non-human animal.

[00046] In one aspect, a method is provided for making a non-human animal that makes antibody variable domains with histidines encoded by germline histidine codons, comprising: modifying the non-human animal in its germline to comprise at least one substitution of histidine codon for a non-histidine codon, or insertion of a histidine codon, in an unrearranged immunoglobulin heavy chain variable (unrearranged V, D, J segments) locus;

and, modifying the non-human animal in its germline to comprise at least one substitution of a histidine codon for a non-histidine codon, or insertion of a histidine codon, in an unrearranged immunoglobulin light chain variable (unrearranged V, J segments) locus.

[00047] In one embodiment, the method comprises genetically modifying the germline of the mouse to comprise at least a portion of a human unrearranged immunoglobulin heavy chain variable (V, D, J segments) locus, and making the histidine substitution or insertion in the unrearranged immunoglobulin heavy chain variable (unrearranged V, D, J segments) human locus.

[00048] In one embodiment, the method comprises genetically modifying the germline of the mouse to comprise at least a portion of a human unrearranged immunoglobulin light chain (unrearranged V, J segments) locus, and making the histidine substitution or insertion in the unrearranged human immunoglobulin light chain locus.

[00049] In one embodiment of the method, the non-human animal is a rodent. In one embodiment, the rodent is selected from a mouse, a rat, and a hamster.

[00050] In one aspect, a method is provided for making a non-human animal that makes antibody variable domains with histidines encoded by germline histidine codons, comprising: modifying the non-human animal to comprise at least one substitution of histidine codon for a non-histidine codon, or insertion of a histidine codon, in an unrearranged immunoglobulin heavy chain variable (unrearranged V, D, J segments) locus; and, modifying the non-human animal to comprise at least one substitution of a histidine codon for a non-histidine codon, or insertion of a histidine codon, in a rearranged immunoglobulin light chain variable sequence (rearranged VJ sequence) in the germline.

[00051] In one embodiment of the method, the non-human animal is a rodent. In one embodiment, the rodent is selected from a mouse, a rat, and a hamster.

[00052] In various aspects and embodiments, the non-human animals are genetically modified by genetically modifying pluripotent or totipotent cells (*e.g.*, embryonic stem (ES) cells), and employing the genetically modified cells as donor cells with a host embryo in a surrogate mother to gestate an animal derived from the genetically modified donor cells. In various aspects and embodiments, the non-human animals are genetically modified by any other method known in the art.

[00053] Methods and compositions for making antibody variable domains that exhibit a pH-dependent antigen binding are provided. Modified antigen-binding proteins are provided,

as well as compositions and methods for making them, that bind target antigen with low affinity at a low (*e.g.,* endosomal) pH and that bind the same target antigen with high affinity at a higher (*e.g.,* extracellular), or neutral, pH.

[00054] In one aspect, a method for making an antibody that exhibits pH-dependent binding is provided, comprising modifying a sequence of a variable domain of the antibody to add a histidine residue, or to substitute an existing residue for a histidine residue, to form a histidine-modified variable domain. In one embodiment, the substitution is of a residue that is not critical for binding antigen (*e.g.,* at a neutral or extracellular pH).

[00055] In one embodiment, two, three, four, five, or six or more residues are substituted to histidines. In one embodiment, the two, three, four, five, or six or more residues substituted to histidines are in a cluster. In one embodiment, the cluster comprises two or more consecutive histidine substitutions. In one embodiment, the cluster comprises two or more histidine substitutions separated by one or more non-histidine residues. In one embodiment, the cluster is 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues in length, and all residues not critical for binding antigen (*e.g.,* at a neutral or extracellular pH) are modified to histidine.

[00056] In one embodiment the variable domain is a light chain variable domain (*e.g.,* κ or λ). In one embodiment, the variable domain is in a heavy chain variable domain. In one embodiment, the sequence of a light chain variable domain and a heavy chain variable domain are modified.

[00057] In one embodiment, the sequence of the variable domain is a CDR sequence. In one embodiment, the CDR sequence is a CDR sequence of a heavy chain. In one embodiment, the CDR sequence is a CDR sequence of a light chain. In one embodiment, the CDR sequence is a CDR sequence of a heavy chain and a CDR sequence of a light chain.

[00058] In one embodiment, the CDR sequence is a CDR3 sequence. In one embodiment, the CDR sequence is a CDR2 sequence. In one embodiment, the CDR sequence is a CDR3 sequence.

[00059] In one embodiment, the CDR sequence is a CDR1, a CDR2, and/or a CDR3 sequence of a light chain. In one embodiment, the CDR sequence is a CDR1, a CDR2, and/or a CDR3 sequence of a heavy chain.

[00060] In one embodiment, the sequence of the variable domain of the antibody is a loop 4 sequence. In one embodiment, the loop 4 sequence is a heavy chain loop 4

sequence. In one embodiment, the loop 4 sequence is a light chain loop 4 sequence.

[00061] In one embodiment, the sequence of the variable domain of the antibody is an N-terminal sequence. In one embodiment, the N-terminal sequence is a heavy chain N-terminal sequence. In one embodiment, the N-terminal sequence is a light chain N-terminal sequence.

[00062] In one embodiment, the sequence of the variable domain of the antibody is selected from a CDR sequence of a heavy chain, a CDR sequence of a light chain, a loop 4 sequence of a heavy chain, a loop 4 sequence of a light chain, an N-terminal sequence of a heavy chain, an N-terminal sequence of a light chain, and a combination thereof.

[00063] In one embodiment, the variable domain is from a heavy chain, and the sequence of the variable domain comprises a first CDR sequence and a sequence selected from an N-terminal sequence, a loop 4 sequence, a second CDR sequence, a third CDR sequence, and a combination thereof. In a specific embodiment, the first CDR sequence is a CDR3, and the sequence of the variable domain further comprises a sequence selected from an N-terminal sequence, a loop 4 sequence, a CDR2 sequence, a CDR1 sequence, and a combination thereof.

[00064] In one embodiment, the histidine-modified variable domain is from a heavy chain, and the histidine modification is in a loop 4 sequence and a sequence selected from a CDR1 or CDR2 or CDR3, an N-terminal sequence, and a combination thereof. In a specific embodiment, the histidine modification is in a loop 4 sequence and a CDR3 sequence. In a specific embodiment, the histidine modification is in a loop 4 sequence and a CDR3 sequence and an N-terminal sequence. In a specific embodiment, the histidine modification is in a loop 4 sequence and an N-terminal sequence.

[00065] In one aspect, a his-modified immunoglobulin variable domain as described herein is provided, wherein the his-modified immunoglobulin variable domain that does not bind an antigen of interest or that binds the antigen of interest at a first affinity at a pH of less than 6; and binds the same antigen of interest at a second affinity at a pH of about 7 or more. In one embodiment the first pH is less than 5.5, or less than 5. In one embodiment the first pH is 5.75. In one embodiment the second pH is about 7 or higher. In one embodiment, the second pH is an extracellular pH of a human. In one embodiment, the second pH is 7.2 to 7.4. In a specific embodiment, the second pH is 7.2.

[00066] In one embodiment, the his-modified variable domain comprises one, two, three, four, five, or six or more histidine substitutions in a sequence selected from a CDR, an N-

terminal, a loop 4, and a combination thereof. In a specific embodiment, the his-modified variable domain comprises a modification in a CDR3. In one embodiment, the his-modified variable domain comprises a modification selected from a modification of a CDR3 in a heavy chain, a modification of a CDR3 in a light chain, and a combination thereof. In one embodiment, the his-modified variable domain comprises at least one substitution in a CDR (*e.g.,* CDR3) and at least one substitution in a sequence selected from an N-terminal, a loop 4, and a combination thereof.

[00067] In one embodiment, the CDR is selected from the group consisting of a heavy chain CDR1, a heavy chain CDR2, a heavy chain CDR3, a light chain CDR1, a light chain CDR2, a light chain CDR3, and a combination thereof.

[00068] In one embodiment, the at least one CDR comprises a light chain CDR3. In one embodiment, the at least one CDR comprises a light chain CDR3 and a heavy chain CDR3.

[00069] In one embodiment, the his-modified immunoglobulin variable domain binds an antigen of interest at a neutral or basic pH (*e.g.,* pH 7-7.4) with a K_D of about 10^{-6} or less (*e.g.,* 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}), wherein the his-modified immunoglobulin variable domain comprises: a CDR1 wherein all non-antigen-binding amino acid residues are substituted with histidine, or wherein the CDR1 comprises a cluster of histidine substitutions. In one embodiment the variable domain does not bind the antigen of interest, or binds the antigen of interest 10^2-10^6-fold weaker at an acidic pH (*e.g.,* pH 5-6, in one embodiment, pH 6).

[00070] In one embodiment, the his-modified immunoglobulin variable domain binds an antigen of interest at a neutral or basic pH (*e.g.,* pH 7-7.4) with a K_D of about 10^{-6} or less (*e.g.,* 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}), wherein the his-modified immunoglobulin variable domain comprises a CDR2 wherein all non-antigen-binding amino acid residues are substituted with histidine, or wherein the CDR2 comprises a cluster of histidine substitutions. In one embodiment the variable domain does not bind the antigen of interest, or binds the antigen of interest 10^2-10^6-fold weaker at an acidic pH (*e.g.,* pH 5-6, in one embodiment, pH 6).

[00071] In one embodiment, the his-modified immunoglobulin variable domain binds an antigen of interest at a neutral or basic pH (*e.g.,* pH 7-7.4) with a K_D of about 10^{-6} or less (*e.g.,* 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}), wherein the his-modified immunoglobulin variable domain comprises a CDR3 wherein all non-antigen-binding amino acid residues are substituted with histidine, or wherein the CDR3 comprises a cluster of histidine substitutions.

In one embodiment the variable domain does not bind the antigen of interest, or binds the antigen of interest 10^2-10^6-fold weaker at an acidic pH (*e.g.,* pH 5-6, in one embodiment, pH 6).

[00072] In one aspect, a method is provided for making a human antigen-binding polypeptide comprising a his-modified domain, the method comprising modifying an immunoglobulin variable domain nucleotide sequence as described herein to encode one or more histidines to form a nucleic acid sequence encoding a his-modified domain, and fusing the nucleic acid sequence encoding the his-modified domain (directly or with a linker) to a human immunoglobulin sequence.

[00073] In one embodiment, the human immunoglobulin sequence is an immunoglobulin constant domain sequence. In a specific embodiment, the human immunoglobulin constant domain sequence encodes an amino acid sequence selected from the group consisting of a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof.

[00074] In one aspect, a cell that expresses a his-modified variable domain is provided, wherein the his-modified variable domain is modified as described herein. In one embodiment, the cell is a mammalian cell. In one embodiment, the cell is selected from a HeLa cell, a DU145 cell, a Lncap cell, a MCF-7 cell, a MDA-MB-438 cell, a PC3 cell, a T47D cell, a THP-1 cell, a U87 cell, a SHSY5Y (human neuroblastoma) cell, a Saos-2 cell, a Vero cell, a CHO cell, a GH3 cell, a PC12 cell, a human retinal cell (*e.g.,* a PERC.6™ cell) and a MC3T3 cell. In a specific embodiment, the cell is a CHO cell.

[00075] In one aspect, a his-modified immunoglobulin variable domain as described herein is provided, wherein the his-modified immunoglobulin variable domain does not bind an antigen of interest or binds the antigen of interest at a first affinity at a pH of 5-6 (*e.g.,* 5.75) and binds the same antigen of interest at a second affinity at a pH of 7-7.4 (*e.g.,* 7.2), wherein at least one CDR comprises two or more histidine substitutions, and at least one non-CDR sequence comprises one or more histidine substitutions wherein the at least one non-CDR sequence is selected from an N-terminal sequence, a loop 4 sequence, and a combination thereof.

[00076] In one embodiment, the first affinity is characterized by no binding, or a K_D of 10^{-6} or higher (*e.g.,* 10^{-3}), and the second affinity is characterized as being at least 2-fold, at least 5-fold, at least 10-fold, at least 10^2-fold, at least 10^3-fold, at least 10^4-fold, at least 10^5-fold, or at least 10^6-fold stronger than the first affinity.

[00077] In one embodiment, the non-CDR sequence is on the same polypeptide as the

at least one CDR sequence. In one embodiment, the non-CDR sequence is on a different polypeptide as the at least one CDR sequence.

[00078] In one embodiment, the at least one CDR is a CDR 3 of a heavy and/or light chain, and the CDR3 comprises a substitution of at least half of the non-antigen-binding amino acid residues to histidine. In a specific embodiment, all of the non-antigen-binding amino acid residues of the CDR3 are substituted to histidine.

[00079] In one embodiment, the at least one CDR is a CDR3 of a heavy and/or light chain, and the CDR3 comprises a substitution of three or more non-antigen-binding amino acid residues to histidine. In one embodiment, four or more of the non-antigen-binding amino acid residues are substituted to histidine.

[00080] In one embodiment, the at least one CDR is a CDR3 of a heavy and/or light chain, and the CDR3 comprises a substitution of two or more contiguous non-antigen-binding amino acid residues to histidine. In one embodiment, the CDR3 comprises a substitution of three or more contiguous non-antigen-binding amino acids residues to histidine.

[00081] In one embodiment, the at least one CDR is a CDR3 of a light and/or a heavy chain, and further comprises a CDR selected from a light chain CDR1, a light chain CDR2, and a combination thereof.

[00082] In one embodiment, the at least one CDR is a CDR3 of a light and/or a heavy chain, and further comprises a CDR selected from a heavy chain CDR1, a heavy chain CDR2, and a combination thereof.

[00083] In one embodiment, the CDR is selected from the group consisting of a heavy chain CDR1, a heavy chain CDR2, a heavy chain CDR3, a light chain CDR1, a light chain CDR2, a light chain CDR3, and a combination thereof.

[00084] In one embodiment, the at least one CDR comprises a light chain CDR3. In one embodiment, the at least one CDR comprises a light chain CDR3 and a heavy chain CDR3.

[00085] In one embodiment, the at least one CDR is a CDR3 of light and/or a heavy chain, and the at least one non-CDR sequence is a loop 4 sequence, wherein the loop 4 sequence comprises one or more histidine substitutions.

[00086] In one embodiment, the at least one CDR is a CDR3 of light and/or a heavy chain, and the at least one non-CDR sequence is an N-terminal sequence, wherein the N-

terminal sequence comprises one or more histidine substitutions.

[00087] In one embodiment, the at least one CDR is a CDR3 of a light chain, the at least one non-CDR sequence comprises an N-terminal sequence with one or more histidine substitutions and a loop 4 sequence with one or more histidine substitutions.

[00088] In one embodiment, the at least one CDR is a CDR3 of a heavy chain, the at least one non-CDR sequence comprises an N-terminal sequence with one or more histidine substitutions and a loop 4 sequence with one or more histidine substitutions.

[00089] In one embodiment, the his-modified immunoglobulin variable domain binds an antigen of interest at pH 7-7.4 (*e.g.,* pH 7.2) with a K_D of about 10-7 or less (*e.g.,* 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}), wherein the his-modified immunoglobulin variable domain comprises a CDR1 wherein all non-antigen-binding amino acid residues are substituted with histidine.

[00090] In one embodiment, the his-modified immunoglobulin variable domain binds an antigen of interest at pH 7-7.4 (e.g., pH 7.2) with a K_D of about 10^{-7} or less (*e.g.,* 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}), wherein the his-modified immunoglobulin variable domain comprises a CDR2 wherein all non-antigen-binding amino acid residues are substituted with histidine.

[00091] In one embodiment, the his-modified immunoglobulin variable domain binds an antigen of interest at pH 7-7.4 (*e.g.,* pH 7.2) with a K_D of about 10^{-7} or less (*e.g.,* 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}), wherein the his-modified immunoglobulin variable domain comprises a CDR3 wherein all non-antigen-binding amino acid residues are substituted with histidine.

[00092] In one aspect, use of a method as described herein in the manufacture of a medicament for treating a human disease or disorder is provided. In one embodiment, the medicament is an antibody. In a specific embodiment, the antibody is a human antibody.

[00093] In one aspect, use of a his-modified variable domain as described herein in the manufacture of a medicament for treating a human disease or disorder is provided. In one embodiment, the medicament is an antibody. In a specific embodiment, the antibody is a human antibody.

[00094] In one aspect, use of a method or his-modified variable domain as described herein in the manufacture of a medicament for treating a human disease or disorder is provided, wherein the medicament comprises an antigen-binding protein selected from an antibody, a multi-specific antibody (*e.g.,* a bi-specific antibody), an scFv, a bi-specific scFv, a diabody, a triabody, a tetrabody, a V-NAR, a VHH, a VL, a F(ab), a F(ab)2, a DVD (*i.e.,* dual variable domain antigen-binding protein), an SVD (*i.e.,* single variable domain antigen-

binding protein), or a bispecific T-cell engager (*i.e.,* a BiTE).

[00095] In one aspect, a method as described herein is employed to generate a heavy and a κ or a λ light chain variable region sequence for making a human antigen-binding protein, further comprising fusing heavy and/or light chain his-modified variable region sequences (directly or through a linker) to human heavy and light chain constant region sequences to form fused sequences, expressing the fused sequences in a cell, and recovering an expressed antigen-binding protein comprising the fused sequences. In various embodiments, the human heavy chain constant regions are selected from IgM, IgD, IgA, IgE and IgG. In various specific embodiments, the IgG is selected from an IgG1, an IgG2, an IgG3 and an IgG4. In various embodiments, the human heavy chain constant region is selected from a sequence comprising a C_H1, a hinge, a C_H2, a C_H3, a C_H4, and a combination thereof. In a specific embodiment the combination is a C_H1, a hinge, a C_H2, and a C_H3. In a specific embodiment the combination is a C_H1, a C_H2, and a C_H3. In a specific embodiment the combination is a hinge, a C_H2, and a C_H3. In a specific embodiment the combination is a hinge, a C_H2, and a C_H3.

[00096] In one aspect, a biological system is provided for generating an antibody or an antibody variable domain that binds a target antigen at a neutral pH but exhibits reduced binding of the same antigen at an acidic pH (*e.g.,* pH 5.0-6.0). The biological system comprises a non-human animal, *e.g.,* a rodent (*e.g,* a mouse or rat) that has a rearranged light chain sequence (*e.g.,* a rearranged V-J) that comprises one or more histidine modifications. In various aspects, the one or more histidine modifications are in the light chain CDR3 codon. In various aspects, the non-human animal comprises a human or humanized heavy chain immunoglobulin locus. In various aspects, the non-human animal comprises a replacement of endogenous non-human heavy chain variable gene segments with one or more human heavy chain V_H, D_H, and J_H segments, wherein the human segments are operably linked to a non-human immunoglobulin constant region. In various aspects, non-human animals with universal light chains comprising light chain variable domains with substitutions of non-histidine residues for histidine residues are provided. In various aspects these histidine-modified universal light chain non-human animals (*e.g.,* rodents, *e.g.,* mice) are referred to as histidine-universal light chain mice, histidine-ULC mice, or HULC mice.

[00097] Thus, in one aspect, provided herein is a genetically modified non-human animal that comprises in its germline an immunoglobulin light chain locus that comprises a single rearranged human immunoglobulin light chain variable region gene sequence comprising human V_L and J_L segment sequences, wherein the single rearranged human

immunoglobulin light chain variable region sequence comprises a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, the single rearranged human immunoglobulin variable region sequence is operably linked to an immunoglobulin light chain constant region gene sequence. In one embodiment, the immunoglobulin light chain constant region gene sequence is a non-human immunoglobulin light chain constant region gene sequence. In one embodiment, the non-human immunoglobulin light chain constant region gene sequence is an endogenous immunoglobulin light chain constant region gene sequence. In one embodiment, the non-human animal lacks a functional unrearranged immunoglobulin light chain variable region. In one embodiment, the immunoglobulin light chain locus is at an endogenous non-human immunoglobulin light chain locus.

[00098] In one embodiment, the animal further comprises in its germline an immunoglobulin heavy chain locus that comprises an unrearranged immunoglobulin heavy chain variable region gene sequence comprising human V_H, D_H, and J_H segments operably linked to an immunoglobulin heavy chain constant region gene sequence. In one embodiment, the immunoglobulin heavy chain constant region gene sequence is a non-human heavy chain constant region gene sequence. In one embodiment, the non-human heavy chain constant region gene sequence is an endogenous immunoglobulin heavy chain constant region gene sequence. In one embodiment, the immunoglobulin heavy chain locus is at an endogenous immunoglobulin heavy chain locus.

[00099] In one embodiment, the substitution of at least one non-histidine codon with a histidine codon is in the nucleotide sequence encoding a complementary determining region (CDR). In one embodiment, the substitution of at least one non-histidine codon with a histidine codon is in the nucleotide sequence encoding a CDR3. In one embodiment, the substitution is of one, two, three, four, or more CDR3 codons. In one aspect, the single rearranged human immunoglobulin light chain variable region sequence comprised at the immunoglobulin light chain locus is derived from a human Vκ1-39 or Vκ3-20 gene segment. In one embodiment, the single rearranged human immunoglobulin light chain variable region is derived from a rearranged Vκ1-39/Jκ5 or Vκ3-20/Jκ1 gene sequence. In one embodiment, the single rearranged human immunoglobulin light chain variable region is derived from a rearranged Vκ1-39/Jκ5 gene sequence, and the Vκ1-39/Jκ5 gene sequence comprises a replacement of at least one non-histidine codon with a histidine codon designed to express a histidine at a position selected from 105, 106, 108, 111, and a combination thereof. In another embodiment, the single rearranged human immunoglobulin light chain variable region is derived from a rearranged Vκ3-20/Jκ1 gene sequence, and the Vκ3-

20/Jκ1 gene sequence comprises a replacement of at least one non-histidine codon with a histidine codon designed to express a histidine at a position selected from 105, 106, 107, 109, and a combination thereof.

[000100] In one aspect, the non-human animal described herein comprises a population of B cells in response to an antigen of interest that is enriched for antibodies that exhibit a decrease in dissociative half-life ($t_{1/2}$) at an acidic pH as compared to neutral pH of at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold. In one embodiment, the decrease in $t_{1/2}$ at an acidic pH as compared to a neutral pH is about 30 fold or more.

[000101] In one embodiment, the animal expresses an antibody comprising a human immunoglobulin light chain variable domain with a substitution of at least one non-histidine residue with a histidine residue at an amino acid position encoded by the at least one codon substituted in the immunoglobulin light chain variable region gene sequence. In one embodiment, the animal expresses an antibody that retains a substitution of at least one non-histidine residue with a histidine residue in an expressed human immunoglobulin light chain variable domain, despite somatic hypermutations.

[000102] In one embodiment, the non-human animal is a mammal. In one embodiment, the mammal is a rodent, *e.g.,* a rat or a mouse. In one embodiment, the non-human animal is a mouse. Thus, in one aspect, also provided herein is a genetically modified mouse comprising in its germline an immunoglobulin light chain locus that comprises a single rearranged human immunoglobulin light chain variable region gene sequence comprising human V_L and J_L segment sequences, wherein the single rearranged human immunoglobulin light chain variable region sequence comprises a substitution of at least one non-histidine codon with a histidine. In one embodiment, the mouse lacks a functional unrearranged immunoglobulin light chain variable region.

[000103] In one embodiment, the single rearranged immunoglobulin light chain variable region gene sequence in the germline of the mouse is operably linked to an immunoglobulin light chain constant region gene sequence. In one embodiment, the immunoglobulin light chain constant region gene sequence is selected from a rat or a mouse immunoglobulin light chain constant region gene sequence. In one embodiment, the immunoglobulin light chain constant region gene sequence is a mouse sequence. In one embodiment, the immunoglobulin light chain locus is at an endogenous mouse immunoglobulin light chain locus.

[000104] In a further embodiment, the mouse also comprises in its germline an immunoglobulin heavy chain locus that comprises an unrearranged immunoglobulin heavy chain variable region sequence comprising human V_H, D_H, and J_H segments operably linked to an immunoglobulin heavy chain constant region gene sequence. In one aspect, the immunoglobulin heavy chain constant region gene sequence is a rat or a mouse heavy chain constant region gene sequence. In one embodiment, the immunoglobulin heavy chain constant region gene sequence is a mouse sequence. In one embodiment, the immunoglobulin heavy chain locus is at an endogenous mouse immunoglobulin heavy chain locus.

[000105] In one aspect, the mouse comprises a substitution of at least one non-histidine codon with a histidine codon wherein the substitution is in the nucleotide sequence encoding a CDR. In one embodiment, the substitution is in a CDR3 codon, *e.g.,* in one, two, three, four, or more CDR3 codons. In one embodiment, the immunoglobulin light chain locus of the mouse comprises the single rearranged human immunoglobulin light chain variable region sequence derived from a human Vκ1-39 or Vκ3-20 gene segment, *e.g.*, the single rearranged immunoglobulin light chain variable region sequence is derived from a rearranged Vκ1-39/Jκ5 or Vκ3-20/Jκ1 gene sequence. In one embodiment, the single rearranged immunoglobulin light chain variable region sequence is derived from a rearranged Vκ1-39/Jκ5 gene sequence and the Vκ1-39/Jκ5 sequence comprises a replacement of at least one non-histidine codon with a histidine codon designed to express a histidine at a position selected from 105, 106, 108, 111, and a combination thereof. In one embodiment, such replacement is designed to replace histidines at positions 105, 106, 108, and 111. In another embodiment, such replacement is designed to replace histidines at positions 106, 108, and 111.

[000106] In another embodiment, the single rearranged immunoglobulin light chain variable region sequence is derived from a rearranged Vκ3-20/Jκ1 gene sequence and the Vκ3-20/Jκ1 sequence comprises a replacement of at least one non-histidine codon with a histidine codon designed to express a histidine at a position selected from 105, 106, 107, 109, and a combination thereof. In one embodiment, such replacement is designed to replace histidines at positions 105, 106, 107, and 109. In another embodiment, such replacement is designed to replace histidines at positions 105, 106, and 109.

[000107] In one embodiment, the mouse described herein comprises a population of B cells in response to an antigen of interest that is enriched for antibodies that exhibit a decrease in dissociative half-life ($t_{1/2}$) at an acidic pH as compared to neutral pH of at least

about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold. In one embodiment, the decrease in $t_{1/2}$ at an acidic pH as compared to a neutral pH is about 30 fold or more.

[000108] In one embodiment, the mouse described herein expresses a population of antigen-specific antibodies in response to an antigen of interest wherein all antibodies comprise (a) immunoglobulin light chain variable domains derived from the same single rearranged human light chain variable region gene sequence which comprises a substitution of at least one non-histidine codon with a histidine codon, and (b) immunoglobulin heavy chains comprising heavy chain variable domains derived from a repertoire of human heavy chain V, D, and J segments.

[000109] Also provided herein is a non-human locus, *e.g.,* mouse locus, comprising a single rearranged human immunoglobulin light chain variable region gene sequence comprising human V_L and J_L segment sequences, wherein the single rearranged human immunoglobulin light chain variable region gene sequence comprises a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, the locus is comprised in the germline of a non-human animal. In one embodiment, the locus comprises the single rearranged human immunoglobulin light chain variable region gene sequence derived from a human Vκ1-39 or Vκ3-20 gene segment, *e.g.,* derived from a rearranged Vκ1-39/Jκ5 or Vκ3-20/Jκ1 gene sequence. In one embodiment, wherein the single rearranged human immunoglobulin light chain variable region gene sequence present in the locus is derived from the rearranged Vκ1-39/Jκ5 sequence, the substitution of at least one non-histidine codon with a histidine codon is designed to express a histidine at a position selected from 105, 106, 108, 111, and a combination thereof. In another embodiment, wherein the single rearranged human immunoglobulin light chain variable region gene sequence present in the locus is derived from the rearranged Vκ3-20/Jκ1 sequence, the substitution of at least one non-histidine codon with a histidine codon is designed to express a histidine at a position selected from 105, 106, 107, 109, and a combination thereof. In various embodiments, the non-human loci described herein may be generated using methods described below for making a genetically modified non-human animal.

[000110] In yet another aspect, provided herein is a method for making a non-human animal that comprises a genetically modified immunoglobulin light chain locus in its germline, wherein the method comprises modifying a genome of a non-human animal to delete or render non-functional endogenous immunoglobulin light chain V and J segments in

an immunoglobulin light chain locus, and placing in the genome a single rearranged human light chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, such method results in a genetically modified non-human animal that comprises a population of B cells enriched for antibodies exhibiting pH-dependent binding to the antigen of interest. In one embodiment, the single rearranged human immunoglobulin light chain variable region sequence placed in the genome is derived from a human Vκ1-39 or Vκ3-20, *e.g.,* a rearranged Vκ1-39/Jκ5 or Vκ3-20/Jκ1 gene sequence. Thus, in the embodiment wherein the single rearranged human immunoglobulin light chain variable region sequence is derived from a rearranged Vκ1-39/Jκ5, the substitution of at least one non-histidine codon with a histidine codon is designed to express a histidine at a position selected from 105, 106, 108, 111, and a combination thereof. In an embodiment wherein the single rearranged human immunoglobulin light chain variable region sequence is derived from a rearranged Vκ3-20/Jκ1, the substitution of at least one non-histidine codon with a histidine codon is designed to express a histidine at a position selected from 105, 106, 107, 109, and a combination thereof.

[000111] In another aspect, provided herein is a method of generating an antibody that exhibits pH-dependent binding to an antigen of interest comprising (a) generating a mouse described herein (*e.g.,* a mouse that comprises in its germline an immunoglobulin light chain locus that comprises a single rearranged human immunoglobulin light chain variable region sequence comprising human V_L and J_L segment sequences and a substitution of at least one non-histidine codon with a histidine codon in its rearranged light chain variable region sequence), (b) immunizing the mouse with an antigen of interest, and (c) selecting an antibody that binds to the antigen of interest with a desired affinity at a neutral pH while displaying reduced binding to the antigen at an acidic pH. In one embodiment, the method results in a generation of an antibody that exhibits $t_{1/2}$ at acidic pH and 37°C of about 2 minutes or less. In one embodiment, the method results in a generation of an antibody that displays a decrease in dissociative half-life ($t_{1/2}$) at an acidic pH as compared to neutral pH of at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold.

[000112] In other aspects, provided herein are additional methods of generating an antibody that exhibits pH-dependent binding to an antigen of interest. One such method comprises (a) selecting a first antibody that binds to an antigen of interest with a desired affinity, (b) modifying an immunoglobulin light chain nucleotide sequence of the first antibody to comprise a substitution of at least one non-histidine codon with a histidine codon, (c)

expressing an immunoglobulin heavy chain of the first antibody and the modified immunoglobulin light chain in a cell, and (d) selecting a second antibody expressed in the cell that retains a desired affinity for the antigen of interest at neutral pH and displays reduced binding to the antigen of interest at an acidic pH. In one embodiment, the immunoglobulin light chain nucleotide sequence of the first antibody comprises a single rearranged human immunoglobulin light chain variable region sequence. In one embodiment, the first antibody is generated in a non-human animal, *e.g.,* a mouse, comprising an immunoglobulin light chain sequence derived from a single rearranged human immunoglobulin light chain variable region sequence, and the modification of the immunoglobulin light chain is made in the single rearranged human immunoglobulin variable region sequence. In one embodiment, the first antibody is generated in a non-human animal, *e.g.,* a mouse, further comprising an immunoglobulin heavy chain sequence derived from a repertoire of human V_H, D_H, and J_H segments. In one embodiment, the single rearranged human immunoglobulin light chain variable region sequence is selected from Vκ1-39/Jκ5 and Vκ3-20/Jκ1 gene sequence. In an embodiment, wherein the single rearranged human immunoglobulin light chain variable region sequence is Vκ1-39/Jκ5, the modification in the immunoglobulin light chain nucleotide sequence of the first antibody is made in the CDR3 codon at a position selected from 105, 106, 108, 111, and a combination thereof. In an embodiment wherein the single rearranged human immunoglobulin light chain variable region sequence is Vκ3-20/Jκ1, the modification in the immunoglobulin light chain nucleotide sequence of the first antibody is made in the CDR3 codon at a position selected from 105, 106, 107, 109, and a combination thereof.

[000113] In one embodiment, the method of generating an antibody that exhibits pH-dependent binding to an antigen of interest described herein results in an antibody that displays a decrease in dissociative half-life ($t_{1/2}$) at an acidic pH as compared to neutral pH of at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold. In one embodiment, the method of generating the antibody results in an antibody that exhibits a $t_{1/2}$ at acidic pH and 37°C of about 2 minutes or less.

[000114] Genetically modified immunoglobulin heavy chain loci in the germline genome of non-human animals are provided, wherein the immunoglobulin heavy chain loci comprise a genetically modified unrearranged heavy chain variable region nucleotide sequence (*e.g.*, one or more genetically modified human V_H, D, and/or J_H gene segment), wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of at least one histidine codon or a substitution of at least one endogenous non-histidine codon

with a histidine codon. In various embodiments, the genetically modified unrearranged heavy chain variable region nucleotide sequence comprises at least one histidine codon in at least one reading frame that encodes an immunoglobulin heavy chain variable domain. In various embodiments, the unrearranged heavy chain variable region nucleotide sequence comprising the at least one histidine codon is operably linked to a human or non-human heavy chain constant region nucleotide sequence (*e.g.*, a heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgA, IgE, and IgG).

[000115] Non-human animals (mammals, *e.g.*, rodents such as mice, rats, or hamsters) are provided that are genetically engineered to contain immunoglobulin heavy chain genomic loci in their germline genome, wherein the genomic loci comprise an unrearranged heavy chain variable region nucleotide sequence (*e.g.*, one or more genetically modified human V_H, D, and/or J_H gene segments), wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of at least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon. In various embodiments, the genome of the non-human animals comprises a modification (i) that deletes or renders nonfunctional all, or substantially all, endogenous immunoglobulin V_H, D, and/or J_H gene segments (*e.g.*, via insertion of a nucleotide sequence, *e.g.*, an exogenous nucleotide sequence, in the immunoglobulin locus or via non-functional rearrangement or inversion of endogenous V_H, D, and/or J_H gene segments); and (ii) that introduces an unrearranged human heavy chain variable region nucleotide sequence (*e.g.*, genetically modified human V_H, D, or J_H gene segments), wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of at least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon. In various embodiments, the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous locus (*i.e.*, where the unrearranged heavy chain variable region nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin heavy chain locus in its genome) or within its endogenous locus (*e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome). In various embodiments, the immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence (*e.g.*, a heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgA, IgE, and IgG).

[000116] Genetically modified non-human animals are provided that are capable of

expressing a genetically modified immunoglobulin heavy variable domain comprising one or more histidines, wherein the one or more histidines are not encoded by a germline gene segment of a corresponding wild-type non-human animal.

[000117] Genetically modified non-human animals are provided that comprise a B cell population that is characterized by rearranged immunoglobulin heavy chain variable genes that encode an immunoglobulin heavy chain variable domain with one or more histidines that are not encoded by a germline gene segment of a corresponding wild-type non-human animal.

[000118] Methods and compositions are provided for making non-human animals that comprise a genetically modified immunoglobulin heavy chain variable locus comprising an unrearranged human heavy chain variable region nucleotide sequence containing one or more histidine codons in at least one reading frame that encodes a heavy chain variable domain.

[000119] Methods and compositions are provided for non-human animals that make antigen-binding proteins that exhibit a pH-dependent binding of antigen. Methods and compositions are provided for making non-human animals that have B cell populations, or antibody populations, that are enriched (as compared with corresponding wild-type animals) with antigen-binding proteins that are pH-dependent, *e.g.,* in particular, heavy chain variable domains, and/or antigen-binding fragments thereof.

[000120] In one aspect, a genetically modified immunoglobulin locus in a germline genome of a non-human animal is provided comprising an unrearranged human heavy chain variable region nucleotide sequence, wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000121] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster.

[000122] In one embodiment, the added or substituted histidine codon is present in an immunoglobulin heavy chain gene segment selected from a human V_H gene segment, a human D gene segment, a human J_H gene segment, and a combination thereof. In one embodiment, the immunoglobulin heavy chain gene segment is selected from a human germline V_H gene segment, a human germline D gene segment, a human germline J_H gene segment, and a combination thereof.

[000123] In one embodiment, the human V gene segment (V_H) is selected from the group consisting of V_H1-2, V_H1-3, V_H1-8, V_H1-18, V_H1-24, V_H1-45, V_H1-46, V_H1-58, V_H1-69, V_H2-5, V_H2-26, V_H2-70, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H3-20, V_H3-21, V_H3-23, V_H3-30, V_H3-30-3, V_H 3-30-5, V_H3-33, V_H3-35, V_H3-38, V_H3-43, V_H3-48, V_H3-49, V_H3-53, V_H3-64, V_H3-66, V_H3-72, V_H3-73, V_H3-74, V_H4-4, V_H4-28, V_H4-30-1, V_H4-30-2, V_H4-30-4, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H4-61, V_H5-51, V_H6-1, V_H7-4-1, V_H7-81, and a combination thereof.

[000124] In one embodiment, the human D gene segment is selected from the group consisting of D1-1, D1-7, D1-14, D1-20, D1-26, D2-2, D2-8, D2-15, D2-21, D3-3, D3-9, D3-10, D3-16, D3-22, D4-4, D4-11, D4-17, D4-23, D5-12, D5-5, D5-18, D5-24, D6-6, D6-13, D6-19, D6-25, D7-27, and a combination thereof.

[000125] In one embodiment, the human J gene segment is selected from the group consisting of J_H1, J_H2, J_H3, J_H4, J_H5, J_H6, and a combination thereof.

[000126] In one embodiment, the added or substituted histidine codon is present in the unrearranged heavy chain variable region nucleotide sequence that encodes an N-terminal region, a loop 4 region, a CDR1, a CDR2, a CDR3, or a combination thereof.

[000127] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprises 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, or 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more 35 or more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 41 or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more, 47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or more, 53 or more, 54 or more, 55 or more, 56 or more, 57 or more, 58 or more, 59 or more, 60 or more, or 61 or more of histidine codons.

[000128] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000129] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and

a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000130] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000131] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000132] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000133] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000134] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity

of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000135] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000136] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000137] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000138] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000139] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000140] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000141] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000142] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region

nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000143] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000144] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000145] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000146] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000147] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000148] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000149] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the

immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.,* at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000150] In one embodiment, the genetically modified immunoglobulin heavy chain locus comprises a modification that deletes or renders, all or substantially all, non-functional endogenous V_H, D, and J_H gene segments; and the genetically modified locus comprises an unrearranged heavy chain variable region nucleotide sequence comprising one or more human V_H, D, and/or J_H gene segments having one or more histidine codons, wherein the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000151] In one embodiment, the genetically modified immunoglobulin locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000152] In one embodiment, the genetically modified immunoglobulin locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000153] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant

region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000154] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (e.g., rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse λ locus.

[000155] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000156] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.,* at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000157] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of

less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000158] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain with one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000159] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification described herein.

[000160] **In one aspect**, a genetically modified immunoglobulin locus in a germline genome of a non-human animal is provided comprising an unrearranged human heavy chain

variable region nucleotide sequence, wherein the human unrearranged heavy chain variable region nucleotide sequence comprises a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000161] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster.

[000162] In one embodiment, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more, 35 or more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 41 or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more, 47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or more, 53 or more, 54 or more, 55 or more, 56 or more, 57 or more, 58 or more, 59 or more, 60 or more, or 61 or more of the endogenous non-histidine codons are replaced with histidine codons.

[000163] In one embodiment, the endogenous non-histone codon encodes the amino acid selected from Y, N, D, Q, S, W, and R.

[000164] In one embodiment, the endogenous non-histidine codon that is substituted by the histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes an immunoglobulin variable domain selected from an N-terminal region, a loop 4 region, a CDR1, a CDR2, a CDR3, a combination thereof.

[000165] In one embodiment, the substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a complementary determining region (CDR) selected from a CDR1, a CDR2, a CDR3, and a combination thereof.

[000166] In one embodiment, the substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a frame region (FR) selected from FR1, FR2, FR3, FR4, and a combination thereof.

[000167] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprises a genetically modified human V_H gene segment, wherein one or more endogenous non-histidine codon in at least one reading frame of the human V_H gene segment has been replaced with a histidine codon.

[000168] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a modification that replaces at least one endogenous non-histidine codon of a human V_H gene segment with a histidine codon, wherein the human V_H gene segment is selected from the group consisting of V_H1-2, V_H1-3, V_H1-8, V_H1-18, V_H1-24, V_H1-45, V_H1-46, V_H1-58, V_H1-69, V_H2-5, V_H2-26, V_H2-70, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H3-20, V_H3-21, V_H3-23, V_H3-30, V_H3-30-3, V_H 3-30-5, V_H3-33, V_H3-35, V_H3-38, V_H3-43, V_H3-48, V_H3-49, V_H3-53, V_H3-64, V_H3-66, V_H3-72, V_H3-73, V_H3-74, V_H4-4, V_H4-28, V_H4-30-1, V_H4-30-2, V_H4-30-4, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H4-61, V_H5-51, V_H6-1, V_H7-4-1, V_H7-81, and a combination thereof.

[000169] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a genetically modified human J_H gene segment, wherein one or more endogenous non-histidine codon in at least one reading frame of the human J_H gene segment has been replaced with a histidine codon.

[000170] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a modification that replaces at least one endogenous non-histidine codon of a human J_H segment with a histidine codon, wherein the human J_H gene segment is selected from the group consisting of J_H1, J_H2, J_H3, J_H4, J_H5, J_H6, and a combination thereof.

[000171] In one embodiment, the substituted histidine codon is present in a heavy chain variable region nucleotide sequence that encodes part of a CDR3. In one embodiment, the part of CDR3 comprises an amino acid sequence derived from a reading frame of a genetically modified human D gene segment comprising a modification that replaces at least one endogenous non-histidine codon in the reading frame with a histidine codon.

[000172] In one embodiment, the endogenous non-histidine codon that is substituted with a histidine codon encodes the amino acid selected from Y, N, D, Q, S, W, and R.

[000173] In one embodiment, the substituted histidine codon is present in at least one reading frame of the human D gene segment that is most frequently observed in VELOCIMMUNE® humanized immunoglobulin mice.

[000174] In one embodiment, the reading frame of the genetically modified human D gene segment that encodes part of CDR3 is selected from a hydrophobic frame, a stop frame, and a hydrophilic frame.

[000175] In one embodiment, the reading frame is a hydrophobic frame of a human D

gene segment.

[000176] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (GTTGT; SEQ ID NO: 88), D1-7 (GITGT; SEQ ID NO: 89), D1-20 (GITGT; SEQ ID NO: 89), and D1-26 (GIVGAT; SEQ ID NO: 90), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000177] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (DIVVVPAAI; SEQ ID NO: 92), D2-8 (DIVLMVYAI; SEQ ID NO: 94), D2-15 (DIVVVVAAT; SEQ ID NO: 95), and D2-21 (HIVVVTAI; SEQ ID NO: 97), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000178] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (ITIFGVVII; SEQ ID NO: 98), D3-9 (ITIF*LVII; SEQ ID NO: 99, SEQ ID NO:100), D3-10 (ITMVRGVII; SEQ ID NO:101), D3-16 (IMITFGGVIVI; SEQ ID NO:102), and D3-22 (ITMIVVVIT; SEQ ID NO:103), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon.

[000179] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (TTVT; SEQ ID NO: 105), D4-11 (TTVT; SEQ ID NO:105), D4-17 (TTVT; SEQ ID NO:105), D4-23 (TTVVT; SEQ ID NO: 106) and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000180] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (VDTAMV; SEQ ID NO: 107), D5-12 (VDIVATI; SEQ ID NO: 108), D5-18 (VDTAMV; SEQ ID NO:107), and D5-24 (VEMATI; SEQ ID NO:109), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000181] In one embodiment, the hydrophobic frame of the human D gene segment

comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (SIAAR; SEQ ID NO: 111), D6-13 (GIAAAG; SEQ ID NO: 113), and D6-19 (GIAVAG; SEQ ID NO:115), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000182] In one embodiment, the hydrophobic frame comprises a nucleotide sequence that encodes human D7-27 (LTG), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000183] In one embodiment, the reading frame is a stop reading frame of a human D gene segment.

[000184] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (VQLER; SEQ ID NO:8), D1-7(V*LEL), D1-20(V*LER), D1-26 (V*WELL; SEQ ID NO: 12), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000185] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (RIL**YQLLY; SEQ ID NO:14), D2-8 (RILY*WCMLY; SEQ ID NO:16 and SEQ ID NO: 17), D2-15 (RIL*WW*LLL), and D2-21 (SILWW*LLF; SEQ ID NO:19), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000186] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (VLRFLEWLLY; SEQ ID NO:21), D3-9 (VLRYFDWLL*; SEQ ID NO:23), D3-10 (VLLWFGELL*; SEQ ID NO:25), D3-16 (VL*LRLGELSLY; SEQ ID NO:27), and D3-22 (VLL***WLLL; SEQ ID NO:29), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000187] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the

group consisting of D4-4 (*LQ*L), D4-11 (*LQ*L), D4-17 (*LR*L), and D4-23 (*LRW*L), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000188] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (WIQLWL; SEQ ID NO:35); D5-12 (WI*WLRL; SEQ ID NO:37), D5-18 (WIQLWL; SEQ ID NO:35), and D5-24 (*RWLQL; SEQ ID NO:39), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000189] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (V*QLV), D6-13 (V*QQLV; SEQ ID NO:41), and D6-19 (V*QWLV; SEQ ID NO:43), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000190] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes D7-27 (*LG), and the human D gene segment further comprises a modification that replaces at least one endogenous codon of the human D gene segment in the nucleotide sequence with a histidine codon.

[000191] In one embodiment, the reading frame is a hydrophilic frame of a human D gene segment.

[000192] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (YNWND; SEQ ID NO: 45), D1-7 (YNWNY; SEQ ID NO: 47), D1-20 (YNWND; SEQ ID NO: 45), and D1-26 (YSGSYY; SEQ ID NO:49), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, and a combination thereof.

[000193] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (GYCSSTSCYT; SEQ ID NO:51), D2-8 (GYCTNGVCYT; SEQ

ID NO: 53), D2-15 (GYCSGGSCYS; SEQ ID NO:55), and D2-21 (AYCGGDCYS; SEQ ID NO:57), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, and a combination thereof.

[000194] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (YYDFWSGYYT; SEQ ID NO:59), D3-9 (YYDILTGYYN; SEQ ID NO:61), D3-10 (YYYGSGSYYN; SEQ ID NO:63), D3-16 (YYDYVWGSYRYT; SEQ ID NO:65), and D3-22 (YYYDSSGYYY; SEQ ID NO:67), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, and a combination thereof.

[000195] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (DYSNY; SEQ ID NO:69), D4-11 (DYSNY; SEQ ID NO:69), D4-17 (DYGDY; SEQ ID NO:71), and D4-23 (DYGGNS; SEQ ID NO:73), and the human D gene segment comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, and a combination thereof.

[000196] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (GYSYGY; SEQ ID NO:75), D5-12 (GYSGYDY; SEQ ID NO:77), D5-18 (GYSYGY; SEQ ID NO:75), and D5-24 (RDGYNY; SEQ ID NO:79), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, and a combination thereof.

[000197] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (EYSSSS; SEQ ID NO: 81), D6-13 (GYSSSWY; SEQ ID NO:83), and D6-19 (GYSSGWY; SEQ ID NO:85), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 76, and a combination thereof.

[000198] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes D7-27 (NWG), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence a histidine codon.

[000199] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, and a combination thereof.

[000200] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000201] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000202] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of

the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000203] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000204] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000205] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000206] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000207] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000208] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000209] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000210] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000211] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000212] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000213] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000214] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000215] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000216] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000217] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000218] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000219] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000220] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.,* at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000221] In one embodiment, the genetically modified locus comprises a modification that deletes or renders non-functional all or substantially all endogenous V_H, D, and J_H gene segments; and the genomic locus comprises the genetically modified, unrearranged human heavy chain variable region nucleotide sequence comprising a substitution of at least one

endogenous non-histidine codon with a histidine codon in at least one reading frame. In one embodiment, the genetically modified, unrearranged immunoglobulin heavy chain variable gene sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000222] In one embodiment, the genetically modified locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000223] In one embodiment, the genetically modified locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a mouse Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a mouse Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000224] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000225] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (*e.g.,* a rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the

CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment the mouse immunoglobulin light chain locus is a mouse λ locus.

[000226] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000227] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.,* at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification described herein.

[000228] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in

dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.,* pH of about 7.0 to about 7.4).

[000229] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000230] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000231] In one aspect, a genetically modified immunoglobulin locus of a non-human animal comprising a human V_H, D, and J_H gene segment is provided, wherein at least one of the human D gene segment has been inverted 5' to 3' with respect to a corresponding wild-type sequence, and wherein at least one reading frame of the inverted human D gene segment comprises a histidine codon.

[000232] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster

[000233] In one embodiment, the genetically modified immunoglobulin locus is present in a germline genome.

[000234] In one embodiment, the genetically modified immunoglobulin locus encodes an immunoglobulin heavy chain variable domain comprising one or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or

more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, or 34 or more of histidine residues.

[000235] In one embodiment, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, at least fifteen, at least sixteen, at least seventeen, at least eighteen, at least nineteen, at least twenty, at least twenty one, at least twenty two, at least twenty three, at least twenty four, or all or substantially all of functional human D gene segments have inverted orientation with respect to corresponding wild type sequences.

[000236] In one embodiment, all or substantially all of endogenous immunoglobulin V_H, D, J_H gene segments are deleted from the immunoglobulin heavy chain locus or rendered non-functional (*e.g.,* via insertion of a nucleotide sequence, *e.g.,* exogenous nucleotide sequence, in the immunoglobulin locus or via non-functional rearrangement or inversion of all, or substantially all, endogenous immunoglobulin V_H, D, J_H segments), and the genetically modified immunoglobulin locus comprises a human V_H, D, and J_H gene segments, wherein at least one of the human D gene segment is present in an inverted orientation with respect to a corresponding wild type sequence, and wherein at least one reading frame in the inverted human D gene segment comprises at least one histidine codon.

[000237] In one embodiment, the inverted human D gene segment is operably linked to a human V_H gene segment, and/or human J_H gene segment

[000238] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is selected from the group consisting of D1-1, D1-7, D1-20, D1-26, D2-2, D2-8, D2-15, D2-21, D3-3, D3-9, D3-10, D3-16, D3-22, D4-4, D4-11, D4-17, D4-23, D5-5, D5-12, D5-18, D5-24, D6-6, D6-13, D6-19, D7-27, and a combination thereof.

[000239] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D1 gene segment selected from the group consisting of D1-1, D1-7, D1-20, D1-26, and a combination thereof.

[000240] In one embodiment, the human D gene segment that is present in the inverted orientation relative a corresponding wild type sequence is a D2 gene segment selected from the group consisting of D2-2, D2-8, D2-15, D2-21, and a combination thereof.

[000241] In one embodiment, the human D gene segment that is present in the inverted

orientation relative to a corresponding wild type sequence is a D3 gene segment selected from the group consisting of D3-3, D3-9, D3-10, D3-16, D3-22, and a combination thereof.

[000242] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D4 gene segment selected from the group consisting of D4-4, D4-11, D4-17, D4-23, and a combination thereof.

[000243] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D5 gene segment selected from the group consisting of D5-5, D5-12, D5-18, D5-24, and a combination thereof.

[000244] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D6 gene segment selected from the group consisting of D6-6, D6-13, D6-19, and a combination thereof.

[000245] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is D7-27.

[000246] In one embodiment, the reading frame of the human D gene segment is selected from a stop reading frame, a hydrophilic reading frame, and a hydrophobic reading frame, and at least one reading frame of the inverted human D gene segment comprises a histidine codon.

[000247] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000248] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000249] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000250] In one embodiment, a heavy chain constant region nucleotide sequence is

present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000251] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000252] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000253] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000254] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000255] In one embodiment, the heavy chain constant region nucleotide sequence

encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000256] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000257] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000258] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000259] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000260] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000261] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000262] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the

second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000263] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000264] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000265] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000266] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000267] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000268] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000269] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about

98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.,* at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000270] In one embodiment, the genetically modified immunoglobulin heavy chain locus comprises a modification that deletes or renders, all or substantially all, non-functional endogenous V_H, D, and J_H gene segments; and the genetically modified locus comprises an unrearranged heavy chain variable region nucleotide sequence comprising at least one inverted human D gene segment as described herein wherein the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000271] In one embodiment, the genetically modified immunoglobulin locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000272] In one embodiment, the genetically modified immunoglobulin locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a mouse Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a mouse Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000273] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized,

unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000274] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (*e.g.,* a rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse immunoglobulin light chain locus is a mouse λ locus.

[000275] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000276] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.,* at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000277] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain

expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.,* pH of about 7.0 to about 7.4).

[000278] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000279] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000280] **In one aspect**, a non-human animal is provided comprising in its germline genome a genetically modified immunoglobulin locus comprising an unrearranged human heavy chain variable region nucleotide sequence, wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000281] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster.

[000282] In one embodiment, the added or substituted histidine codon is present in an immunoglobulin heavy chain gene segment selected from a human V_H gene segment, a human D gene segment, a human J_H gene segment, and a combination thereof. In one embodiment, the immunoglobulin heavy chain gene segment is selected from a human germline V_H gene segment, a human germline D gene segment, a human germline J_H gene segment, and a combination thereof.

[000283] In one embodiment, the human V_H gene segment is selected from the group consisting of V_H1-2, V_H1-3, V_H1-8, V_H1-18, V_H1-24, V_H1-45, V_H1-46, V_H1-58, V_H1-69, V_H2-5, V_H2-26, V_H2-70, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H3-20, V_H3-21, V_H3-23, V_H3-30, V_H3-30-3, V_H 3-30-5, V_H3-33, V_H3-35, V_H3-38, V_H3-43, V_H3-48, V_H3-49, V_H3-53, V_H3-64, V_H3-66, V_H3-72, V_H3-73, V_H3-74, V_H4-4, V_H4-28, V_H4-30-1, V_H4-30-2, V_H4-30-4, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H4-61, V_H5-51, V_H6-1, V_H7-4-1, V_H7-81, and a combination thereof.

[000284] In one embodiment, the human D gene segment is selected from the group consisting of D1-1, D1-7, D1-14, D1-20, D1-26, D2-2, D2-8, D2-15, D2-21, D3-3, D3-9, D3-10, D3-16, D3-22, D4-4, D4-11, D4-17, D4-23, D5-12, D5-5, D5-18, D5-24, D6-6, D6-13, D6-19, D6-25, D7-27, and a combination thereof.

[000285] In one embodiment, the human J_H gene segment is selected from the group consisting of J_H1, J_H2, J_H3, J_H4, J_H5, J_H6, and a combination thereof.

[000286] In one embodiment, the added or substituted histidine codon is present in the unrearranged heavy chain variable region nucleotide sequence encoding an N-terminal region, a loop 4 region, a CDR1, a CDR2, a CDR3, or a combination thereof.

[000287] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprises 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, or 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more 35 or more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 41 or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more, 47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or more, 53 or more, 54 or more, 55 or more, 56 or more, 57 or more, 58 or more, 59 or more, 60 or

more, or 61 or more of histidine codons.

[000288] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000289] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000290] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000291] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000292] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000293] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000294] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000295] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000296] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000297] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000298] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000299] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000300] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000301] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation

selected from the group consisting of H433K, N434Y, or both.

[000302] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000303] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000304] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000305] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000306] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000307] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000308] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000309] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.*, at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000310] In one embodiment, the genetically modified immunoglobulin heavy chain locus comprises a modification that deletes or renders, all or substantially all, non-functional endogenous V_H, D, and J_H gene segments; and the genetically modified locus comprises an unrearranged heavy chain variable region nucleotide sequence comprising one or more human V_H, D, and/or J_H gene segments having one or more histidine codons, wherein the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000311] In one embodiment, the genetically modified immunoglobulin locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000312] In one embodiment, the genetically modified immunoglobulin locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000313] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000314] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (e.g., a rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse λ locus.

[000315] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000316] In one embodiment, the non-human animal is heterozygous for the genetically modified immunoglobulin heavy chain locus, and the non-human animal is capable of expressing a human immunoglobulin heavy chain variable domain comprising at least one histidine residue derived predominantly from the genetically modified immunoglobulin heavy

chain locus as described herein.

[000317] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.,* at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000318] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.,* pH of about 7.0 to about 7.4).

[000319] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000320] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain

variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000321] In one aspect, a non-human animal comprising a genetically modified immunoglobulin locus is provided, wherein the genetically modified immunoglobulin locus comprises an unrearranged human heavy chain variable region nucleotide sequence, and wherein the human unrearranged heavy chain variable region nucleotide sequence comprises a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000322] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster.

[000323] In one embodiment, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more, 35 or more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 41 or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more, 47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or more, 53 or more, 54 or more, 55 or more, 56 or more, 57 or more, 58 or more, 59 or more, 60 or more, or 61 or more of the endogenous non-histidine codons are replaced with histidine codons.

[000324] In one embodiment, the endogenous non-histone codon encodes the amino acid selected from Y, N, D, Q, S, W, and R.

[000325] In one embodiment, the substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes an immunoglobulin variable domain selected from an N-terminal region, a loop 4 region, a

CDR1, a CDR2, a CDR3, a combination thereof.

[000326] In one embodiment, the substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a complementary determining region (CDR) selected from a CDR1, a CDR2, a CDR3, and a combination thereof.

[000327] In one embodiment, the substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a frame region (FR) selected from FR1, FR2, FR3, FR4, and a combination thereof.

[000328] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprises a genetically modified human V_H gene segment, wherein one or more endogenous non-histidine codon in at least one reading frame of the human V_H gene segment has been replaced with a histidine codon.

[000329] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a modification that replaces at least one endogenous non-histidine codon of a human V_H gene segment with a histidine codon, wherein the human V_H gene segment is selected from the group consisting of V_H1-2, V_H1-3, V_H1-8, V_H1-18, V_H1-24, V_H1-45, V_H1-46, V_H1-58, V_H1-69, V_H2-5, V_H2-26, V_H2-70, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H3-20, V_H3-21, V_H3-23, V_H3-30, V_H3-30-3, V_H 3-30-5, V_H3-33, V_H3-35, V_H3-38, V_H3-43, V_H3-48, V_H3-49, V_H3-53, V_H3-64, V_H3-66, V_H3-72, V_H3-73, V_H3-74, V_H4-4, V_H4-28, V_H4-30-1, V_H4-30-2, V_H4-30-4, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H4-61, V_H5-51, V_H6-1, V_H7-4-1, V_H7-81, and a combination thereof.

[000330] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a genetically modified human J_H gene segment, wherein one or more endogenous non-histidine codon in at least one reading frame of the human J_H gene segment has been replaced with a histidine codon.

[000331] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a modification that replaces at least one endogenous non-histidine codon of a human J_H segment with a histidine codon, wherein the human J_H gene segment is selected from the group consisting of J_H1, J_H2, J_H3, J_H4, J_H5, J_H6, and a combination thereof.

[000332] In one embodiment, the substituted histidine codon is present in a heavy chain variable region nucleotide sequence that encodes part of a CDR3. In one embodiment, the

part of CDR3 comprises an amino acid sequence derived from a reading frame of a genetically modified human D gene segment comprising a modification that replaces at least one endogenous non-histidine codon in the reading frame with a histidine codon.

[000333] In one embodiment, the endogenous non-histidine codon that is substituted with a histidine codon encodes the amino acid selected from Y, N, D, Q, S, W, and R.

[000334] In one embodiment, the substituted histidine codon is present in at least one reading frame of the human D gene segment that is most frequently observed in VELOCIMMUNE® humanized immunoglobulin mice.

[000335] In one embodiment, the reading frame of the genetically modified human D gene segment that encodes part of CDR3 is selected from a hydrophobic frame, a stop frame, and a hydrophilic frame.

[000336] In one embodiment, the reading frame is a hydrophobic frame of a human D gene segment.

[000337] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (GTTGT; SEQ ID NO: 88), D1-7 (GITGT; SEQ ID NO: 89), D1-20 (GITGT; SEQ ID NO: 89), and D1-26 (GIVGAT; SEQ ID NO:90), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000338] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (DIVVVPAAI; SEQ ID NO:92), D2-8 (DIVLMVYAI; SEQ ID NO: 94), D2-15 (DIVVVVAAT; SEQ ID NO:95), and D2-21 (HIVVVTAI; SEQ ID NO: 97), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000339] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (ITIFGVVII; SEQ ID NO:98), D3-9 (ITIF*LVII; SEQ ID NO:99, SEQ ID NO:100), D3-10 (ITMVRGVII; SEQ ID NO:101), D3-16 (IMITFGGVIVI; SEQ ID NO:102), and D3-22 (ITMIVVVIT; SEQ ID NO:103), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon.

[000340] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (TTVT; SEQ ID NO:105), D4-11 (TTVT; SEQ ID NO:105), D4-17 (TTVT; SEQ ID NO:105), D4-23 (TTVVT; SEQ ID NO: 106) and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000341] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (VDTAMV; SEQ ID NO: 107), D5-12 (VDIVATI; SEQ ID NO:108), D5-18 (VDTAMV; SEQ ID NO:107), and D5-24 (VEMATI; SEQ ID NO:109), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000342] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (SIAAR; SEQ ID NO:111), D6-13 (GIAAAG; SEQ ID NO:113), and D6-19 (GIAVAG; SEQ ID NO:115), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000343] In one embodiment, the hydrophobic frame comprises a nucleotide sequence that encodes human D7-27 (LTG), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000344] In one embodiment, the reading frame is a stop reading frame of a human D gene segment.

[000345] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (VQLER; SEQ ID NO:8), D1-7(V*LEL), D1-20(V*LER), D1-26 (V*WELL; SEQ ID NO:12), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000346] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (RIL**YQLLY; SEQ ID NO:14), D2-8 (RILY*WCMLY; SEQ ID

NO:16 and SEQ ID NO: 17), D2-15 (RIL*WW*LLL), and D2-21 (SILWW*LLF; SEQ ID NO:19), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000347] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (VLRFLEWLLY; SEQ ID NO:21), D3-9 (VLRYFDWLL*; SEQ ID NO:23), D3-10 (VLLWFGELL*; SEQ ID NO:25), D3-16 (VL*LRLGELSLY; SEQ ID NO:27), and D3-22 (VLL***WLLL; SEQ ID NO:29), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000348] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (*LQ*L), D4-11 (*LQ*L), D4-17 (*LR*L), and D4-23 (*LRW*L), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000349] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (WIQLWL; SEQ ID NO:35); D5-12 (WI*WLRL; SEQ ID NO:37), D5-18 (WIQLWL; SEQ ID NO:35), and D5-24 (*RWLQL; SEQ ID NO:39), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000350] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (V*QLV), D6-13 (V*QQLV; SEQ ID NO:41), and D6-19 (V*QWLV; SEQ ID NO:43), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000351] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes D7-27 (*LG), and the human D gene segment further comprises a modification that replaces at least one endogenous codon of the human D gene segment in the nucleotide sequence with a histidine codon.

[000352] In one embodiment, the reading frame is a hydrophilic frame of a human D gene

segment.

[000353] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (YNWND; SEQ ID NO: 45), D1-7 (YNWNY; SEQ ID NO: 47), D1-20 (YNWND; SEQ ID NO: 45), and D1-26 (YSGSYY; SEQ ID NO:49), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, and a combination thereof.

[000354] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (GYCSSTSCYT; SEQ ID NO:51), D2-8 (GYCTNGVCYT; SEQ ID NO: 53), D2-15 (GYCSGGSCYS; SEQ ID NO:55), and D2-21 (AYCGGDCYS; SEQ ID NO:57), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, and a combination thereof.

[000355] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (YYDFWSGYYT; SEQ ID NO:59), D3-9 (YYDILTGYYN; SEQ ID NO:61), D3-10 (YYYGSGSYYN; SEQ ID NO:63), D3-16 (YYDYVWGSYRYT; SEQ ID NO:65), and D3-22 (YYYDSSGYYY; SEQ ID NO:67), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, and a combination thereof.

[000356] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (DYSNY; SEQ ID NO:69), D4-11 (DYSNY; SEQ ID NO:69), D4-17 (DYGDY; SEQ ID NO:71), and D4-23 (DYGGNS; SEQ ID NO:73), and the human D

gene segment comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, and a combination thereof.

[000357] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (GYSYGY; SEQ ID NO:75), D5-12 (GYSGYDY; SEQ ID NO:77), D5-18 (GYSYGY; SEQ ID NO:75), and D5-24 (RDGYNY; SEQ ID NO:79), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, and a combination thereof.

[000358] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (EYSSSS; SEQ ID NO: 81), D6-13 (GYSSSWY; SEQ ID NO:83), and D6-19 (GYSSGWY; SEQ ID NO:85), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 76, and a combination thereof.

[000359] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes D7-27 (NWG), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence a histidine codon.

[000360] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, and a combination thereof.

[000361] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000362] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000363] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000364] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000365] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.*, L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000366] In one embodiment, the heavy chain constant region nucleotide sequence

encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000367] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000368] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000369] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000370] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000371] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000372] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000373] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000374] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000375] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000376] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000377] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000378] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000379] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000380] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000381] In one embodiment, the heavy chain constant region nucleotide sequence is a

human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000382] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.,* at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000383] In one embodiment, the genetically modified locus comprises a modification that deletes or renders non-functional all or substantially all endogenous V_H, D, and J_H gene segments; and the genomic locus comprises the genetically modified, unrearranged human heavy chain variable region nucleotide sequence comprising a substitution of at least one endogenous non-histidine codon with a histidine codon in at least one reading frame. In one embodiment, the genetically modified, unrearranged immunoglobulin heavy chain variable gene sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000384] In one embodiment, the genetically modified locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000385] In one embodiment, the genetically modified locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a mouse Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a mouse Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000386] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000387] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (e.g., rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment the mouse immunoglobulin light chain locus is a mouse λ locus.

[000388] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000389] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.,* at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without

the genetic modification.

[000390] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000391] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000392] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least

about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000393] In one embodiment, the non-human animal is heterozygous for the genetically modified immunoglobulin heavy chain locus, and the non-human animal is capable of expressing the human immunoglobulin heavy chain variable domain comprising at least one histidine residue derived predominantly from the genetically modified immunoglobulin heavy chain locus as described herein.

[000394] In one aspect, a non-human animal comprising a genetically modified immunoglobulin locus comprising a human V_H, D, and J_H gene segment is provided, wherein at least one of the human D gene segment has been inverted 5' to 3' with respect to a corresponding wild-type sequence, and wherein at least one reading frame of the inverted human D gene segment comprises a histidine codon.

[000395] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster

[000396] In one embodiment, the genetically modified immunoglobulin locus is present in a germline genome.

[000397] In one embodiment, wherein the reading frame of the inverted human D gene segment comprises one or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, or 34 or more of histidine codons.

[000398] In one embodiment, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, at least fifteen, at least sixteen, at least seventeen, at least eighteen, at least nineteen, at least twenty, at least twenty one, at least twenty two, at least twenty three, at least twenty four, or all or substantially all of functional human D gene segments have inverted orientation with respect to corresponding wild type sequences.

[000399] In one embodiment, all or substantially all of endogenous immunoglobulin V_H, D, J_H gene segments are deleted from the immunoglobulin heavy chain locus or rendered non-

functional (*e.g.,* via insertion of a nucleotide sequence, *e.g.,* exogenous nucleotide sequence, in the immunoglobulin locus or via non-functional rearrangement or inversion of all, or substantially all, endogenous immunoglobulin V_H, D, J_H segments), and the genetically modified immunoglobulin locus comprises a human V_H, D, and J_H gene segments, wherein at least one of the human D gene segment is present in an inverted orientation with respect to corresponding wild type sequences, and wherein at least one reading frame of the inverted human D gene segment comprises at least one histidine codon.

[000400] In one embodiment, the inverted human D gene segment is operably linked to a human V_H gene segment, and/or human J_H gene segment

[000401] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is selected from the group consisting of D1-1, D1-7, D1-20, D1-26, D2-2, D2-8, D2-15, D2-21, D3-3, D3-9, D3-10, D3-16, D3-22, D4-4, D4-11, D4-17, D4-23, D5-5, D5-12, D5-18, D5-24, D6-6, D6-13, D6-19, D7-27, and a combination thereof.

[000402] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D1 gene segment selected from the group consisting of D1-1, D1-7, D1-20, D1-26, and a combination thereof.

[000403] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequences is a D2 gene segment selected from the group consisting of D2-2, D2-8, D2-15, D2-21, and a combination thereof.

[000404] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D3 gene segment selected from the group consisting of D3-3, D3-9, D3-10, D3-16, D3-22, and a combination thereof.

[000405] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D4 gene segment selected from the group consisting of D4-4, D4-11, D4-17, D4-23, and a combination thereof.

[000406] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D5 gene segment selected from the group consisting of D5-5, D5-12, D5-18, D5-24, and a combination thereof.

[000407] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D6 gene segment selected from the group consisting of D6-6, D6-13, D6-19, and a combination thereof.

[000408] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is D7-27.

[000409] In one embodiment, the reading frame of the human D gene segment is selected from a stop reading frame, a hydrophilic reading frame, a hydrophobic reading frame, and a combination thereof, wherein at least one reading frame of the inverted human D gene segment comprises a histidine codon.

[000410] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000411] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000412] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000413] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000414] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.*, E or Q); 250 and 428 (*e.g.*, L or F); 252 (*e.g.*, L/Y/F/W or T), 254 (*e.g.*, S or T), and 256 (*e.g.*, S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.*, L/R/S/P/Q or K) and/or 434 (*e.g.*, H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.*, 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.*, M428L) and 434S (*e.g.*, N434S) modification; a 428L, 259I (*e.g.*, V259I), and a 308F (*e.g.*, V308F) modification; a 433K (*e.g.*,

H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000415] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000416] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000417] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000418] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000419] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000420] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000421] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000422] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000423] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000424] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000425] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000426] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000427] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000428] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M,

R409K, E419Q, and V422I by EU).

[000429] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000430] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000431] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.*, at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000432] In one embodiment, the genetically modified immunoglobulin heavy chain locus comprises a modification that deletes or renders, all or substantially all, non-functional endogenous V_H, D, and J_H gene segments; and the genetically modified locus comprises an unrearranged heavy chain variable region nucleotide sequence comprising at least one inverted human D gene segment as described herein wherein the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous location (*i.e.*, where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000433] In one embodiment, the genetically modified immunoglobulin locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000434] In one embodiment, the genetically modified immunoglobulin locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a mouse Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a mouse Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000435] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000436] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (e.g., a rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse immunoglobulin light chain locus is a mouse λ locus.

[000437] In one embodiment, the genetically modified immunoglobulin locus as described

herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000438] In one embodiment, the non-human animal is heterozygous for the genetically modified immunoglobulin heavy chain locus, and the non-human animal is capable of expressing the human immunoglobulin heavy chain variable domain comprising at least one histidine residue derived predominantly from the genetically modified immunoglobulin heavy chain locus as described herein.

[000439] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.*, at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000440] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000441] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000442] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000443] In one aspect, a non-human animal that is capable of expressing an antigen-binding protein with enhanced pH-dependent recyclability and/or enhanced serum half-life are provided, wherein the non-human animal comprises in its germline genome an unrearranged human immunoglobulin heavy chain variable region nucleotide sequence, wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon as described herein.

[000444] In one embodiment, the antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.*, at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000445] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified

immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000446] In one embodiment, the antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000447] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000448] In one aspect, a targeting construct is provided, comprising 5' and 3' targeting arms homologous to a genomic D region or genomic V and J region of a non-human animal,

wherein at least one V_H, D, or J_H gene segment comprises any of the modifications as described herein, *e.g.*, an addition of at least one histidine codon, a substitution of at least one endogenous non-histidine codon into a histidine codon, and/or inversion of at least one functional D gene segment with respect to a corresponding wild type sequence.

[000449] In one aspect, a hybridoma or quadroma is provided that is derived from a cell of any of the non-human animal as described herein. In one embodiment, the non-human animal is a rodent, *e.g.*, a mouse, a rat, or a hamster.

[000450] In one aspect, pluripotent, induced pluripotent, or totipotent stem cells derived form a non-human animal comprising the various genomic modifications of the described invention are provided. In a specific embodiment, the pluripotent, induced pluripotent, or totipotent stem cells are mouse or rat embryonic stem (ES) cells. In one embodiment, the pluripotent, induced pluripotent, or totipotent stem cells have an XX karyotype or an XY karyotype. In one embodiment, the pluripotent or induced pluripotent stem cells are hematopoietic stem cells.

[000451] In one aspect, cells that comprise a nucleus containing a genetic modification as described herein are also provided, *e.g.,* a modification introduced into a cell by pronuclear injection. In one embodiment, the pluripotent, induced pluripotent, or totipotent stem cells comprise a genetically modified immunoglobulin genomic locus, wherein the genomic locus comprises, from 5' to 3', (1) an FRT recombination site, (2) human V_H gene segments, (3) a mouse adam6 gene, (4) a loxP recombination site, (5) histidine-substituted human D gene segments, (6) human J_H gene segments, followed by (7) a mouse E_i (intronic enhancer), and (8) a mouse IgM constant region nucleotide sequence.

[000452] In one aspect, a lymphocyte isolated from a genetically modified non-human animal as described herein is provided. In one embodiment, the lymphocyte is a B cell, wherein the B cell comprises an immunoglobulin genomic locus comprising an unrearranged heavy chain variable region nucleotide sequence wherein the unrearranged heavy chain variable gene sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000453] In one aspect, a lymphocyte isolated from a genetically modified non-human animal as described herein is provided. In one embodiment, the lymphocyte is a B cell, wherein the B cell comprises an immunoglobulin locus that comprises a human V, D, and J gene segment, wherein at least one of the human D gene segment has been inverted 5' to 3' with respect to wild-type sequences, and wherein at least one reading frame of the

inverted human D gene segment encodes at least one histidine residue. In one embodiment, the B cell is capable of producing an antigen-binding protein comprising the genetically modified heavy chain variable domain as described herein. In one embodiment, the genetically modified heavy chain variable domain as described herein is operably linked to a heavy chain constant region amino acid sequence.

[000454] In one aspect, a B cell population is provided that are capable of expressing an antigen-binding protein wherein the antigen-binding protein comprises at least one histidine residue in a heavy chain variable domain, wherein the B cell population comprises any genetic modifications as described herein. In one embodiment, the at least one histidine residue is present in a heavy chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the at least one histidine residue is present in CDR3.

[000455] In one aspect, a B cell population is provided that are capable of expressing an antigen-binding protein with enhanced serum half-life and/or enhanced pH-dependent recyclability, wherein the B cell population comprises any genetic modifications as described herein.

[000456] In one aspect, a method for making a non-human animal comprising a genetically modified immunoglobulin heavy chain variable locus is provided, comprising: (a) modifying a genome of a non-human animal to delete or render non-functional endogenous immunoglobulin heavy chain V, D, and J gene segments (*e.g.*, via insertion of a nucleotide sequence, *e.g.*, an exogenous nucleotide sequence, in the immunoglobulin locus or via non-functional rearrangement or inversion of endogenous V_H, D, J_H segments); and (b) placing in the genome an unrearranged heavy chain variable region nucleotide sequence, wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon as described herein.

[000457] In one embodiment, the non-human animal is a mammal, including a rodent, e.g., a mouse, a rat, or a hamster.

[000458] In one embodiment, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more, 35 or more, 36 or more, 37 or

more, 38 or more, 39 or more, 40 or more, 41 or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more, 47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or more, 53 or more, 54 or more, 55 or more, 56 or more, 57 or more, 58 or more, 59 or more, 60 or more, or 61 or more of the endogenous non-histidine codons are replaced with histidine codons.

[000459] In one embodiment, the endogenous non-histone codon encodes the amino acid selected from Y, N, D, Q, S, W, and R.

[000460] In one embodiment, the added or substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes an immunoglobulin variable domain selected from an N-terminal region, a loop 4 region, a CDR1, a CDR2, a CDR3, a combination thereof.

[000461] In one embodiment, the added substituted histidine codon histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a complementary determining region (CDR) selected from a CDR1, a CDR2, a CDR3, and a combination thereof.

[000462] In one embodiment, the added or substituted histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a frame region (FR) selected from FR1, FR2, FR3, FR4, and a combination thereof.

[000463] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprises a genetically modified human V_H gene segment, wherein one or more endogenous non-histidine codon in at least one reading frame of the human V_H gene segment has been replaced with a histidine codon.

[000464] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a modification that replaces at least one endogenous non-histidine codon of a human V_H gene segment with a histidine codon, wherein the human V_H gene segment is selected from the group consisting of V_H1-2, V_H1-3, V_H1-8, V_H1-18, V_H1-24, V_H1-45, V_H1-46, V_H1-58, V_H1-69, V_H2-5, V_H2-26, V_H2-70, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H3-20, V_H3-21, V_H3-23, V_H3-30, V_H3-30-3, V_H 3-30-5, V_H3-33, V_H3-35, V_H3-38, V_H3-43, V_H3-48, V_H3-49, V_H3-53, V_H3-64, V_H3-66, V_H3-72, V_H3-73, V_H3-74, V_H4-4, V_H4-28, V_H4-30-1, V_H4-30-2, V_H4-30-4, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H4-61, V_H5-51, V_H6-1, V_H7-4-1, V_H7-81, and a combination thereof.

[000465] In one embodiment, the human unrearranged heavy chain variable region

nucleotide sequence comprises a genetically modified human J_H gene segment, wherein one or more endogenous non-histidine codon in at least one reading frame of the human J_H gene segment has been replaced with a histidine codon.

[000466] In one embodiment, the human unrearranged heavy chain variable region nucleotide sequence comprises a modification that replaces at least one endogenous non-histidine codon of a human J_H segment with a histidine codon, wherein the human J_H gene segment is selected from the group consisting of J_H1, J_H2, J_H3, J_H4, J_H5, J_H6, and a combination thereof.

[000467] In one embodiment, the added or substituted histidine codon is present in a heavy chain variable region nucleotide sequence that encodes part of a CDR3. In one embodiment, the part of CDR3 comprises an amino acid sequence derived from a reading frame of a genetically modified human D gene segment comprising a modification that replaces at least one endogenous non-histidine codon in the reading frame with a histidine codon.

[000468] In one embodiment, the endogenous non-histidine codon that is substituted with a histidine codon encodes the amino acid selected from Y, N, D, Q, S, W, and R.

[000469] In one embodiment, the added or substituted histidine codon is present in at least one reading frame of the human D gene segment that is most frequently observed in VELOCIMMUNE® humanized immunoglobulin mice.

[000470] In one embodiment, the reading frame of the genetically modified human D gene segment that encodes part of CDR3 is selected from a hydrophobic frame, a stop frame, and a hydrophilic frame.

[000471] In one embodiment, the reading frame is a hydrophobic frame of a human D gene segment.

[000472] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (GTTGT; SEQ ID NO: 88), D1-7 (GITGT; SEQ ID NO: 89), D1-20 (GITGT; SEQ ID NO: 89), and D1-26 (GIVGAT; SEQ ID NO:90), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000473] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the

group consisting of D2-2 (DIVVVPAAI; SEQ ID NO:92), D2-8 (DIVLMVYAI; SEQ ID NO: 94), D2-15 (DIVVVVAAT; SEQ ID NO:95), and D2-21 (HIVVVTAI; SEQ ID NO: 97), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000474] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (ITIFGVVII; SEQ ID NO:98), D3-9 (ITIF*LVII; SEQ ID NO:99, SEQ ID NO:100), D3-10 (ITMVRGVII; SEQ ID NO:101), D3-16 (IMITFGGVIVI; SEQ ID NO:102), and D3-22 (ITMIVVVIT; SEQ ID NO:103), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon.

[000475] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (TTVT; SEQ ID NO:105), D4-11 (TTVT; SEQ ID NO:105), D4-17 (TTVT; SEQ ID NO:105), D4-23 (TTVVT; SEQ ID NO: 106) and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000476] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (VDTAMV; SEQ ID NO: 107), D5-12 (VDIVATI; SEQ ID NO:108), D5-18 (VDTAMV; SEQ ID NO:107), and D5-24 (VEMATI; SEQ ID NO:109), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000477] In one embodiment, the hydrophobic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (SIAAR; SEQ ID NO:111), D6-13 (GIAAAG; SEQ ID NO:113), and D6-19 (GIAVAG; SEQ ID NO:115), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000478] In one embodiment, the hydrophobic frame comprises a nucleotide sequence that encodes human D7-27 (LTG), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000479] In one embodiment, the reading frame is a stop reading frame of a human D gene segment.

[000480] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (VQLER; SEQ ID NO:8), D1-7(V*LEL), D1-20(V*LER), D1-26 (V*WELL; SEQ ID NO:12), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000481] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (RIL**YQLLY; SEQ ID NO:14), D2-8 (RILY*WCMLY; SEQ ID NO:16 and SEQ ID NO: 17), D2-15 (RIL*WW*LLL), and D2-21 (SILWW*LLF; SEQ ID NO:19), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000482] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (VLRFLEWLLY; SEQ ID NO:21), D3-9 (VLRYFDWLL*; SEQ ID NO:23), D3-10 (VLLWFGELL*; SEQ ID NO:25), D3-16 (VL*LRLGELSLY; SEQ ID NO:27), and D3-22 (VLL***WLLL; SEQ ID NO:29), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000483] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (*LQ*L), D4-11 (*LQ*L), D4-17 (*LR*L), and D4-23 (*LRW*L), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000484] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (WIQLWL; SEQ ID NO:35); D5-12 (WI*WLRL; SEQ ID NO:37), D5-18 (WIQLWL; SEQ ID NO:35), and D5-24 (*RWLQL; SEQ ID NO:39), and the human D gene segment comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000485] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (V*QLV), D6-13 (V*QQLV; SEQ ID NO:41), and D6-19 (V*QWLV; SEQ ID NO:43), and the human D gene segment further comprises a modification that replaces at least one endogenous non-histidine codon in the nucleotide sequence with a histidine codon.

[000486] In one embodiment, the stop reading frame of the human D gene segment comprises a nucleotide sequence that encodes D7-27 (*LG), and the human D gene segment further comprises a modification that replaces at least one endogenous codon of the human D gene segment in the nucleotide sequence with a histidine codon.

[000487] In one embodiment, the reading frame is a hydrophilic frame of a human D gene segment.

[000488] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D1-1 (YNWND; SEQ ID NO: 45), D1-7 (YNWNY; SEQ ID NO: 47), D1-20 (YNWND; SEQ ID NO: 45), and D1-26 (YSGSYY; SEQ ID NO:49), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, and a combination thereof.

[000489] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D2-2 (GYCSSTSCYT; SEQ ID NO:51), D2-8 (GYCTNGVCYT; SEQ ID NO: 53), D2-15 (GYCSGGSCYS; SEQ ID NO:55), and D2-21 (AYCGGDCYS; SEQ ID NO:57), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, and a combination thereof.

[000490] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D3-3 (YYDFWSGYYT; SEQ ID NO:59), D3-9 (YYDILTGYYN; SEQ ID

NO:61), D3-10 (YYYGSGSYYN; SEQ ID NO:63), D3-16 (YYDYVWGSYRYT; SEQ ID NO:65), and D3-22 (YYYDSSGYYY; SEQ ID NO:67), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, and a combination thereof.

[000491] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D4-4 (DYSNY; SEQ ID NO:69), D4-11 (DYSNY; SEQ ID NO:69), D4-17 (DYGDY; SEQ ID NO:71), and D4-23 (DYGGNS; SEQ ID NO:73), and the human D gene segment comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, and a combination thereof.

[000492] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D5-5 (GYSYGY; SEQ ID NO:75), D5-12 (GYSGYDY; SEQ ID NO:77), D5-18 (GYSYGY; SEQ ID NO:75), and D5-24 (RDGYNY; SEQ ID NO:79), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, and a combination thereof.

[000493] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of D6-6 (EYSSSS; SEQ ID NO: 81), D6-13 (GYSSSWY; SEQ ID NO:83), and D6-19 (GYSSGWY; SEQ ID NO:85), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence with a histidine codon. In one embodiment, the hydrophilic frame comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 76, and a combination thereof.

[000494] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes D7-27 (NWG), and the human D gene segment further comprises a modification that replaces at least one endogenous codon in the nucleotide sequence a histidine codon.

[000495] In one embodiment, the hydrophilic frame of the human D gene segment comprises a nucleotide sequence that encodes the amino acid sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, and a combination thereof.

[000496] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000497] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000498] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000499] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000500] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or

T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0).

[000501] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000502] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000503] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000504] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000505] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000506] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A

mutation.

[000507] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000508] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000509] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000510] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000511] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second C_H3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000512] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000513] In one embodiment, the second CH_3 amino acid sequence is from a modified

human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000514] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000515] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000516] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000517] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.,* at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000518] In one embodiment, the genetically modified locus comprises a modification that deletes or renders non-functional all or substantially all endogenous V_H, D, and J_H gene segments; and the genomic locus comprises the genetically modified, unrearranged human heavy chain variable region nucleotide sequence comprising a substitution of at least one endogenous non-histidine codon with a histidine codon in at least one reading frame. In one embodiment, the genetically modified, unrearranged immunoglobulin heavy chain variable gene sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome or within its endogenous

locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000519] In one embodiment, the genetically modified locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000520] In one embodiment, the genetically modified locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a mouse Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a mouse Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000521] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000522] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (e.g., a rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ

light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment the mouse immunoglobulin light chain locus is a mouse λ locus.

[000523] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000524] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.*, at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000525] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000526] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000527] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000528] In one aspect, a method for making a non-human animal comprising a genetically modified immunoglobulin heavy chain variable locus is provided, comprising: (a) modifying a genome of a non-human animal to delete or render non-functional endogenous immunoglobulin heavy chain V, D, and J gene segments (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement or inversion of endogenous V_H, D, J_H segments); and (b) placing in the genome a human V_H, D, and J_H gene segment, wherein at least one of the human D gene segment has been inverted 5' to 3' with respect to a corresponding wild-type sequence, and wherein at least one reading frame of the inverted human D gene segment comprises a histidine codon.

[000529] In one embodiment, the non-human animal is a mammal, including a rodent, *e.g.,* a mouse, a rat, or a hamster

[000530] In one embodiment, the genetically modified immunoglobulin locus is present in a germline genome.

[000531] In one embodiment, the genetically modified immunoglobulin locus encodes an immunoglobulin heavy chain variable domain comprising one or more, 2 or more, 3 or more,

4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, or 34 or more of histidine residues.

[000532] In one embodiment, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, at least fifteen, at least sixteen, at least seventeen, at least eighteen, at least nineteen, at least twenty, at least twenty one, at least twenty two, at least twenty three, at least twenty four, or all or substantially all of functional human D gene segments have inverted orientation with respect to corresponding wild type sequences.

[000533] In one embodiment, all or substantially all of endogenous immunoglobulin V_H, D, J_H gene segments are deleted from the immunoglobulin heavy chain locus or rendered non-functional (*e.g.,* via insertion of a nucleotide sequence, *e.g.,* exogenous nucleotide sequence, in the immunoglobulin locus or via non-functional rearrangement or inversion of all, or substantially all, endogenous immunoglobulin V_H, D, J_H segments), and the genetically modified immunoglobulin locus comprises a human V_H, D, and J_H gene segments, wherein at least one of the human D gene segment is present in an inverted orientation with respect to a corresponding wild type sequence, and wherein at least one reading frame in the inverted human D gene segment comprises at least one histidine codon.

[000534] In one embodiment, the inverted human D gene segment is operably linked to a human V_H gene segment, and/or human J_H gene segment

[000535] In one embodiment, the human D gene segment that is present in the inverted orientation relative to wild type sequences is selected from the group consisting of D1-1, D1-7, D1-20, D1-26, D2-2, D2-8, D2-15, D2-21, D3-3, D3-9, D3-10, D3-16, D3-22, D4-4, D4-11, D4-17, D4-23, D5-5, D5-12, D5-18, D5-24, D6-6, D6-13, D6-19, D7-27, and a combination thereof.

[000536] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D1 gene segment selected from the group consisting of D1-1, D1-7, D1-20, D1-26, and a combination thereof.

[000537] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D2 gene segment selected from the group consisting of D2-2, D2-8, D2-15, D2-21, and a combination thereof.

[000538] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D3 gene segment selected from the group consisting of D3-3, D3-9, D3-10, D3-16, D3-22, and a combination thereof.

[000539] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D4 gene segment selected from the group consisting of D4-4, D4-11, D4-17, D4-23, and a combination thereof.

[000540] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D5 gene segment selected from the group consisting of D5-5, D5-12, D5-18, D5-24, and a combination thereof.

[000541] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is a D6 gene segment selected from the group consisting of D6-6, D6-13, D6-19, and a combination thereof.

[000542] In one embodiment, the human D gene segment that is present in the inverted orientation relative to a corresponding wild type sequence is D7-27.

[000543] In one embodiment, the reading frame of the human D gene segment is selected from a stop reading frame, a hydrophilic reading frame, a hydrophobic reading frame, and a combination thereof.

[000544] In one embodiment, the unrearranged heavy chain variable region nucleotide sequence comprising the inverted human D gene segment is operably linked to a human or non-human heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA.

[000545] In one embodiment, the human unrearranged immunoglobulin heavy chain variable region nucleotide sequence is operably linked to a human or non-human heavy chain constant region nucleotide sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof. In one embodiment, the heavy chain constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (C_H1-hinge-C_H2-C_H3).

[000546] In one embodiment, a heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000547] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000548] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.,* 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P), wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000549] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000550] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000551] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000552] In one embodiment, the heavy chain constant region nucleotide sequence

encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, N434S, and a combination thereof.

[000553] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof.

[000554] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising an N434A mutation.

[000555] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof.

[000556] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of T250Q, M248L, or both.

[000557] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human heavy chain constant region amino acid sequence comprising a mutation selected from the group consisting of H433K, N434Y, or both.

[000558] In one embodiment, the genetically modified immunoglobulin locus comprises: (1) a first allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a first heavy chain constant region nucleotide sequence encoding a first CH_3 amino acid sequence of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and (2) a second allele, wherein the unrearranged human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a second heavy chain constant region nucleotide sequence encoding a second C_H3 amino acid sequence of the human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, and wherein the second CH_3 amino acid sequence comprises a modification that reduces or eliminates binding for the second CH_3 amino acid sequence to Protein A (see, for example, US 2010/0331527A1).

[000559] In one embodiment, the second CH_3 amino acid sequence comprises an H95R modification (by IMGT exon numbering; H435R by EU numbering). In one embodiment the second CH_3 amino acid sequence further comprises an Y96F modification (by IMGT exon numbering; H436F by EU). In another embodiment, the second CH_3 amino acid sequence

comprises both an H95R modification (by IMGT exon numbering; H435R by EU numbering) and an Y96F modification (by IMGT exon numbering; H436F by EU).

[000560] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG1 and further comprises a mutation selected from the group consisting of D16E, L18M, N44S, K52N, V57M, and V82I (IMGT; D356E, L38M, N384S, K392N, V397M, and V422I by EU).

[000561] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG2 and further comprises a mutation selected from the group consisting of N44S, K52N, and V82I (IMGT: N384S, K392N, and V422I by EU).

[000562] In one embodiment, the second CH_3 amino acid sequence is from a modified human IgG4 and further comprises a mutation selected from the group consisting of Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (IMGT: Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU).

[000563] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000564] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000565] In one embodiment, all or substantially all endogenous V_H, D, and J_H gene segments are deleted from an immunoglobulin heavy chain locus or rendered non-functional (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement, or inversion, of the endogenous V_H, D, J_H segments). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous V_H, D, or J_H gene segments are deleted or rendered non-functional. In one embodiment, *e.g.*, at least 95%, 96%, 97%, 98%, or 99% of endogenous functional V, D, or J gene segments are deleted or rendered non-functional.

[000566] In one embodiment, the genetically modified immunoglobulin heavy chain locus

comprises a modification that deletes or renders, all or substantially all, non-functional endogenous V_H, D, and J_H gene segments; and the genetically modified locus comprises an unrearranged heavy chain variable region nucleotide sequence comprising at least one inverted human D gene segment as described herein wherein the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000567] In one embodiment, the genetically modified immunoglobulin locus comprises an endogenous Adam6a gene, Adam6b gene, or both, and the genetic modification does not affect the expression and/or function of the endogenous Adam6a gene, Adam6b gene, or both.

[000568] In one embodiment, the genetically modified immunoglobulin locus comprises an ectopically present Adam6a gene, Adam6b gene, or both. In one embodiment, the Adam6a gene is a non-human Adam6a gene. In one embodiment, the Adam6a gene is a mouse Adam6a gene. In one embodiment, the Adam6a gene is a human Adam6a gene. In one embodiment, the Adam6b gene is a non-human Adam6b gene. In one embodiment, the Adam6b gene is a mouse Adam6b gene. In one embodiment, the Adam6b gene is a human Adam6b gene.

[000569] In one embodiment, the genetically modified immunoglobulin locus further comprises a humanized, unrearranged λ and/or κ light chain variable gene sequence. In one embodiment, the humanized, unrearranged λ and/or κ light chain variable gene sequence is operably linked to an immunoglobulin light chain constant region nucleotide sequence selected from a λ light chain constant region nucleotide sequence and a κ light chain constant region nucleotide sequence. In one embodiment, the humanized, unrearranged λ light chain variable region nucleotide sequence is operably linked to a λ light chain constant region nucleotide sequence. In one embodiment, the λ light chain constant region nucleotide sequence is a mouse, rat, or human sequence. In one embodiment, the humanized, unrearranged κ light chain variable region nucleotide sequence is operably linked to a κ light chain constant region nucleotide sequence. In one embodiment, the κ light chain constant region nucleotide sequence is a mouse, rat, or human sequence.

[000570] In one embodiment, the genetically modified immunoglobulin locus comprises an unrearranged light chain variable gene sequence that contains at least one modification that

introduces at least one histidine codon in at least one reading frame encoding a light chain variable domain. In one embodiment, the genetically modified immunoglobulin locus comprises a rearranged (e.g., a rearranged λ or κ V/J sequence) sequence that comprises one, two, three, or four codons for histidine in a light chain CDR. In one embodiment, the CDR is a selected from a CDR1, CDR2, CDR3, and a combination thereof. In one embodiment, the unrearranged or rearranged light chain variable region nucleotide sequence is an unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence. In one embodiment, the unrearranged or rearranged human λ or κ light chain variable region nucleotide sequence is present at an endogenous mouse immunoglobulin light chain locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse κ locus. In one embodiment, the mouse immunoglobulin light chain locus is a mouse immunoglobulin light chain locus is a mouse λ locus.

[000571] In one embodiment, the genetically modified immunoglobulin locus as described herein is present in an immunoglobulin heavy chain locus of a mouse. In one embodiment, the genetically modified immunoglobulin locus is present in a humanized immunoglobulin heavy chain locus in a VELOCIMMUNE® mouse.

[000572] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein exhibits a weaker antigen binding at an acidic environment (*e.g.,* at a pH of about 5.5 to about 6.0) than a corresponding wild-type heavy chain variable domain without the genetic modification.

[000573] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-

binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000574] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000575] In one embodiment, the genetically modified immunoglobulin locus described herein comprises an enriched B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000576] In one aspect, a method for making a non-human animal that is capable of producing an immunoglobulin heavy chain variable domain with enhanced serum half-life and/or enhanced pH-dependent recyclability is provided, comprising (a) modifying a genome of a non-human animal to delete or render non-functional endogenous immunoglobulin heavy chain V, D, and J gene segments (*e.g.*, via insertion of a nucleotide sequence (*e.g.*, an exogenous nucleotide sequence) in the immunoglobulin locus or via non-functional rearrangement or inversion of endogenous V_H, D, J_H segments); and (b) placing in the genome an unrearranged human heavy chain variable region nucleotide sequence, wherein the unrearranged heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon

with a histidine codon, and wherein an antigen-binding protein comprising the immunoglobulin heavy chain variable domain produced by the non-human animal exhibits enhanced serum half-life and/or enhanced pH-dependent recyclability as compared to a wild-type immunoglobulin heavy chain domain.

[000577] In one embodiment, the non-human animal, upon contact with an antigen, can produce an enriched population of B cell repertoire that expresses an antigen-binding protein with enhanced serum half-life and/or enhanced pH-dependent recyclability, wherein the enriched B cell population comprises any genetic modifications as described herein.

[000578] In one embodiment, an antigen-binding protein produced by the genetically modified non-human animal is characterized by sufficient affinity to an antigen of interest at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4) and enhanced dissociation of the antibody from an antigen-antigen-binding protein complex at a pH less than the neutral pH (*e.g.*, at an endosomal pH, *e.g.* pH of about 5.5 to 6.0).

[000579] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.*, pH of about 7.0 to about 7.4).

[000580] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein is characterized by improved pH-dependent recyclability, enhanced serum half-life, or both as compared with a wild-type antigen-binding protein without the genetic modification.

[000581] In one embodiment, the genetically modified immunoglobulin locus described herein comprises a an enriched B cell population that, upon stimulation with an antigen of interest, is capable of producing antigen-binding proteins, *e.g.*, antibodies, comprising a heavy chain variable domain comprising one or more histidine residues. The antigen-binding proteins as described herein when administered into a subject, exhibits an increased serum half-life over a corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain. In some embodiments, the antigen-binding protein described herein exhibits an increased serum half-life that is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold higher than the corresponding wild-type antigen-binding protein, which possesses a similar or sufficiently similar amino acid sequence that encodes the heavy chain variable domain but does not comprise a histidine residue in the heavy chain variable domain.

[000582] In one embodiment, the antigen-binding protein comprises an immunoglobulin heavy chain variable domain that is capable of specifically binding an antigen of interest with an affinity (K_D) lower than 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12} at a neutral pH (pH of about 7.0 to about 7.4).

[000583] In one aspect, a method for obtaining an antigen-binding protein with enhanced recyclability and/or improved serum half-life is provided, comprising: (a) immunizing a non-human animal having a genetically modified immunoglobulin locus as described herein wherein the non-human animal comprises an unrearranged human heavy chain variable region nucleotide sequence comprising an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon; (b) allowing the non-human animal to mount an immune response; (c) harvesting a lymphocyte (*e.g.*, a B cell) from the immunized non-human animal; (d) fusing the lymphocyte with a myeloma cell to form a hybridoma cell, and (e) obtaining an antigen-binding protein produced by the hybridoma cell, wherein the antigen-binding protein exhibits enhanced recyclability and/or serum stability.

[000584] In one aspect, a genetically modified immunoglobulin heavy chain locus

obtainable by any of the methods as described herein is provided.

[000585] In one aspect, a genetically modified non-human animal obtainable by any of the methods as described herein is provided.

[000586] In various embodiments, the non-human animal is a mammal. In one embodiment, the mammal is a rodent, *e.g.*, a mouse, a rat, or a hamster.

[000587] In various embodiments, the genetically modified immunoglobulin loci as described herein are present in the germline genome of a non-human animal, *e.g.*, a mammal, e.g., a rodent, *e.g.*, a mouse, a rat, or a hamster.

BRIEF DESCRIPTION OF THE DRAWINGS

[000588] FIGs. 1A and 1B illustrate the amino acid sequences encoded by the three reading frames (*i.e.*, stop, hydrophilic, and hydrophobic reading frames) of human D gene segments (D) and the amino acid sequences encoded by the three reading frames of histidine-substituted human D gene segments (HD). Introduction of histidine codons (typed in bold) in the hydrophilic reading frame also changed many stop codons in the stop reading frame to Ser codons (typed in bold) but introduced few changes in the hydrophobic reading frame. The "*" symbol represents a stop codon, and the comma between the two SEQ ID NOs indicates that there are two amino acid sequences separated by the stop codon.

[000589] FIG. 2 illustrates schemes for targeting pLMa0174 containing a spectinomycin selection cassette into the 5' end of MAID 1116 (Step 1. BHR (Spec)). In Step 1, a chloramphenicol selection cassette, a neomycin selection cassette, a loxP site, two V_H gene segments (hV_H1-3 and hV_H1-2), the human Adam6 gene, all of which are located upstream of hV_H6-1, were deleted from the clone and replaced by a spectinomycin cassette to yield the VI433 clone. In Step 2 (BHR (Hyg + Spec)), pNTu0002 containing a hygromycin cassette flanked by FRT sites was targeted into a region comprising human immunoglobulin D gene segments. Via Step 2, all human D gene segments were deleted from VI433 and replaced with the hygromycin cassette to yield MAID6011 VI 434 (clone 1).

[000590] FIG. 3 illustrates schemes for assembling histidine-substituted human D gene segments via sequential ligation.

[000591] FIG. 4 illustrates the introduction of pre-assembled, histidine-substituted human D gene segments containing a neomycin cassette into a region between the most D-proximal V_H gene segment (V_H 6-1) and the most D-proximal J_H gene segment (J_H1) via enzyme-mediated digestion (PI-SceI and I-CeuI) and ligation. This process removes the

hygromycin cassette from MAID 6011 VI434 and introduces pre-assembled human histidine-substituted D gene segments into the clone. Bacterial cells comprising a successfully targeted clone are selected based on both neomycin and spectinomycin resistance. The resulting clone (MAID6012 VI469) comprises, from 5' to 3', (1) a spectinomycin selection cassette, (2) a 50 kb arm comprising a human V_H gene segment (V_H 6-1), (3) a neomycin cassette flanked by loxP sites, (4) human D gene segments containing histidine substitutions (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)), (5) about 25 kb of a genomic region containing human J_H gene segments, (6) a mouse E_i sequence (SEQ ID NO: 5; an intronic enhancer that promotes V_H to DJ_H rearrangement in developing B cells), and (7) a mouse IgM constant region nucleotide sequence (mIgM exon 1; SEQ ID NO: 7).

[000592] FIG. 5 illustrates schemes for deleting the human immunoglobulin heavy chain D gene region from the MAID 1460 heterozygous ES cells by targeting the 129 strain-derived chromosome of MAID 1460 het with the hygromycin selection cassette in MAID 6011 VI434.

[000593] FIG. 6 shows a list of primers and probes used to confirm a loss of allele (LOA), a gain of allele (GOA), or a parental allele (Parental) in the screening assays for identifying MAID 6011.

[000594] FIG. 7 illustrates schemes for constructing MAID 6012 het by targeting MAID 6011 heterozygous ES cells with MAID 6012 VI469. Electroporation of the MAID 6012 VI469 construct into the MAID 6011 heterozygous ES cells yielded MAID 6012 heterozygous ES cells in which the 129 strain-derived chromosome is modified to contain, from 5' to 3' direction, an FRT site, human V_H gene segments, a mouse genomic region comprising adam6 genes, a floxed neomycin selection cassette, human D gene segments comprising histidine substitutions (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)), human J_H gene segments, a mouse E_i sequence (SEQ ID NO: 5; an intronic enhancer that promotes V_H to DJ_H rearrangement in developing B cells), and a mouse IgM constant region nucleotide sequence (mIgM exon 1; SEQ ID NO: 7).

[000595] FIG. 8 shows a list of primers and probes used to confirm a loss of allele (LOA), a gain of allele (GOA), or a parental allele (Parental) in the screening assay for identifying MAID 6012.

[000596] FIG. 9 illustrates schemes for removing a neomycin cassette from MAID 6012

heterozygous ES cells. Electroporation of a Cre-expressing plasmid into the MAID 6012 ES cells lead to recombination and deletion of the floxed neomycin cassette, yielding MAID 6013 heterozygous ES cells.

[000597] FIGs. 10A-10E illustrate human D gene segment nucleotide sequences with translations for each of the six reading frames, *i.e.*, three reading frames for direct 5' to 3' orientation and three reading frames for inverted orientation (3' to 5' orientation). The "*" symbol represents a stop codon, and the comma between two SEQ ID NOs indicates that there are two amino acid sequences separated by the stop codon.

[000598] FIGs. 11-13 illustrate mRNA sequences and their encoded protein sequences expressed by 6013 F0 heterozygous mice, which comprise histidine-substituted human D gene segments (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)) in the immunoglobulin heavy chain locus in their 129 strain-derived chromosome. The boxed sequences in each figure indicate the presence of histidine codons in the CDR3 sequences derived from the genetically modified immunoglobulin heavy chain locus comprising the histidine-substituted human D gene segments. FWR represents frame region and CDR represents complementarity determining region. In the alignment, the dot "." indicates a sequence identical to the query sequence, and the dash "-" indicates a gap in the sequence.

[000599] FIG. 14 illustrates histidine incorporation frequency in immunoglobulin heavy chain CDR3 sequences. The X-axis represents the number of histidine codons appeared in each CDR3 sequence, and the Y-axis represents the correspondingproportion of reads. The "6013 F0 het" indicates CDR3 sequences expressed by the 6013 heterozygous mice comprising histidine-substituted D gene segments. The "VI3-Adam6" indicates CDR3 sequences obtained from control mice comprising human VH, D, and JH gene segments without the histidine modification as described herein. The "ASAP" indicates CDR3 sequences obtained from the Regeneron antibody database, which was used as another control.

[000600] FIG. 15 illustrates an amino acid alignment of human Vκ1-39-derived light chains from various antigen-specific antibodies (A-K antibodies). Histidine (H) residues located within each light chain sequence are in bold. Various light chain regions (Framework and CDR) are indicated above the alignment.

[000601] FIG. 16 illustrates the combinations and locations of histidine residues engineered in the CDR3 region of human Vκ1-39-derived light chains by mutagenesis.

Corresponding nucleic acid sequences are included. Histidine residues introduced through mutagenesis and corresponding nucleic acid residues are shown in bold. Amino acid positions (105, 106, *etc.*) are based on a unique numbering described in Lefranc *et al.* (2003) Dev. Comp. Immunol. 27:55-77, and can also be viewed on www.imgt.org.

[000602] FIG. 17 illustrates the level of antibody expression in ng/mL detected in the supernatants of CHO cells transfected with nucleic acids encoding five (1-5) different heavy chains and Vκ1-39-derived light chains having histidine residues engineered at indicated locations (see Y axis) in the CDR3.

[000603] FIG. 18 is a western blot showing expression of selected antigen-specific human antibodies containing histidine engineered light chains in CHO cell supernatants.

[000604] FIGs. 19A-19J shows the binding kinetics for selected heavy chains from antigen-specific antibodies paired with various histidine engineered light chains at a neutral (7.4) and acidic (5.5) pH.

[000605] FIGs. 20A-20E show the binding kinetics for selected heavy chains (1-5) from antigen-specific antibodies paired with various histidine engineered light chains at a neutral (7.4) and acidic (5.75) pH. Various kinetic parameters including k_a, k_d, K_D, and $t_{1/2}$ are shown. NB=no binding.

[000606] FIG. 21 shows kinetic parameters (K_D and $t_{1/2}$) for antibodies comprising parental universal light chain or histidine-modified universal light chain paired with indicated heavy chains (2, 3, and 6). Histidine substitutions lead to strong pH dependence in several antibodies. Histidine substitutions were made in CDR3 to convert the sequence $_{105}$QQSYSTP$_{111}$ (SEQ ID NO:3) to $_{105}$HHSYSTH$_{111}$ (SEQ ID NO:329). Note that NB = no binding detected (K_D > 10 micromolar).

[000607] FIG. 22 shows the sequence and properties (%GC content, N, % mismatch, Tm) of selected mutagenesis primers used to engineer histidine residues into CDR3 of a rearranged human Vκ1-39/Jκ5 light chain sequence. SEQ ID NOs for these primers used in the Sequence Listing are included in the Table below. F=forward primer, R=reverse primer.

[000608] FIGs. 23A – 23B show a general strategy for construction of targeting vectors for engineering of histidine residues into a rearranged human light chain variable region sequence derived from Vκ1-39/Jκ5 variable region for making a genetically modified mouse that expresses antibodies containing the modified human light chain. FIGs. 23C-23D show introduction of the targeting vector for ULC-H105/106/108/111 substitutions into ES cells and

generation of heterozygous mice from the same; while FIGs. 23E-23F show introduction of the targeting vector for ULC-H106/108/111 substitutions into ES cells and generation of heterozygous mice from the same. The diagrams are not presented to scale. Unless indicated otherwise, filled shapes and solid lines represent mouse sequence, empty shapes and double lines represent human sequence.

[000609] FIG. 24 shows antiserum titers against immunogen from mice heterozygous for histidine universal light chain (HULC) (with 4 His substitutions – HULC 1927 mice; with 3 His substitutions – HULC 1930 mice) and wild type animals in a second bleed.

[000610] FIG. 25 is a comparison of the number of total antigen positive clones and the number of antigen positive clones displaying pH sensitive antigen binding obtained from hybridoma fusions from HULC (1927 vs 1930) and WT mice. Figure includes data for two mice for each mouse type ("mouse 1" and "mouse 2").

[000611] FIGs. 26A-26C show sensorgrams from surface plasmon resonance binding experiments in which monoclonal antibodies (AA, BB, CC, DD, HH, GG, NN, and OO) from either heterozygous HULC or WT mice were allowed to associate with the immunogen at neutral pH (pH 7.4) followed by a shift to a buffer with pH of either 7.4 or 6.0 for the dissociation phase. The individual lines in each graph represent the binding responses at different concentrations of the respective antibodies. All experiments were carried out at 25°C. Dissociative half-life values (t½) are noted above the respective sensorgrams, and fold change in t½ is included to the right of each sensorgram. Antibodies AA, BB, CC, DD, HH, and GG were from HULC 1927 mice using His-substituted light chain, NN is from HULC 1927 mouse using WT light chain, and OO is from a WT mouse (See Table 5 for clarification).

[000612] FIG. 27 shows positions of histidine residues engineered in the CDR3 region of human Vκ3-20-derived light chains by mutagenesis. Histidine residues introduced through mutagenesis and corresponding nucleic acid residues are shown in bold. Amino acid positions (105, 106, etc.) are based on a unique numbering described in Lefranc *et al.* (2003) Dev. Comp. Immunol. 27:55-77, and can also be viewed on www.imgt.org.

[000613] FIG. 28 shows the sequence and properties (%GC content, N, % mismatch, Tm) of selected mutagenesis primers used to engineer histidine residues into CDR3 of a rearranged human Vκ3-20/Jκ1 light chain sequence. SEQ ID NOs for these primers used in the Sequence Listing are included in the Table below. F=forward primer, R=reverse primer.

[000614] FIGs. 29A – 29B show a general strategy for construction of targeting vectors for

the engineering of histidine residues into a rearranged human light chain variable region sequence derived from Vκ3-20/Jκ1 light chain variable region for making a genetically modified mouse that expresses antibodies containing the modified human light chain. FIG 29C shows introduction of the targeting vector for ULC-Q105H/Q106H/Y107H/S109H substitutions into ES cells and generation of heterozygous mice from the same; while FIG. 29D shows introduction of the targeting vector for ULC-Q105H/Q106H/S109H substitutions into ES cells and generation of heterozygous mice from the same. The diagrams are not presented to scale. Unless indicated otherwise, filled shapes and solid lines represent mouse sequence, empty shapes and double lines represent human sequence.

DETAILED DESCRIPTION

[000615] This invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention is defined by the claims.

[000616] Unless defined otherwise, all terms and phrases used herein include the meanings that the terms and phrases have attained in the art, unless the contrary is clearly indicated or clearly apparent from the context in which the term or phrase is used. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, particular methods and materials are now described.

Definitions

[000617] The term "antibody", as used herein, includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable domain and a heavy chain constant region (C_H). The heavy chain constant region comprises three domains, C_H1, C_H2 and C_H3. Each light chain comprises a light chain variable domain and a light chain constant region (C_L). The heavy chain and light chain variable domains can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each heavy and light chain variable domain comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (heavy chain CDRs may be abbreviated as HCDR1, HCDR2 and HCDR3; light chain CDRs may be abbreviated as LCDR1, LCDR2 and LCDR3. The term “high affinity” antibody refers to an antibody that has a K_D with respect to its target

epitope about of 10^{-9} M or lower (*e.g.,* about 1 x 10^{-9} M, 1 x 10^{-10} M, 1 x 10^{-11} M, or about 1 x 10^{-12} M). In one embodiment, K_D is measured by surface plasmon resonance, *e.g.,* BIACORE™; in another embodiment, K_D is measured by ELISA.

[000618] The phrase "bispecific antibody" includes an antibody capable of selectively binding two or more epitopes. Bispecific antibodies generally comprise two nonidentical heavy chains, with each heavy chain specifically binding a different epitope—either on two different molecules (*e.g.,* different epitopes on two different immunogens) or on the same molecule (*e.g.,* different epitopes on the same immunogen). If a bispecific antibody is capable of selectively binding two different epitopes (a first epitope and a second epitope), the affinity of the first heavy chain for the first epitope will generally be at least one to two or three or four or more orders of magnitude lower than the affinity of the first heavy chain for the second epitope, and vice versa. Epitopes specifically bound by the bispecific antibody can be on the same or a different target (*e.g.,* on the same or a different protein). Exemplary bispecific antibodies include those with a first heavy chain specific for a tumor antigen and a second heavy chain specific for a cytotoxic marker, *e.g.,* an Fc receptor (*e.g.,* FcγRI, FcγRII, FcγRIII, *etc.*) or a T cell marker (*e.g.,* CD3, CD28, *etc.*). Further, the second heavy chain variable domain can be substituted with a heavy chain variable domain having a different desired specificity. For example, a bispecific antibody with a first heavy chain specific for a tumor antigen and a second heavy chain specific for a toxin can be paired so as to deliver a toxin (*e.g.,* saporin, vinca alkaloid, *etc.*) to a tumor cell. Other exemplary bispecific antibodies include those with a first heavy chain specific for an activating receptor (*e.g.,* B cell receptor, FcγRI, FcγRIIA, FcγRIIIA, FcαRI, T cell receptor, *etc.*) and a second heavy chain specific for an inhibitory receptor (*e.g.,* FcγRIIB, CD5, CD22, CD72, CD300a, *etc.*). Such bispecific antibodies can be constructed for therapeutic conditions associated with cell activation (*e.g.* allergy and asthma). Bispecific antibodies can be made, for example, by combining heavy chains that recognize different epitopes of the same immunogen. For example, nucleic acid sequences encoding heavy chain variable sequences that recognize different epitopes of the same immunogen can be fused to nucleic acid sequences encoding the same or different heavy chain constant regions, and such sequences can be expressed in a cell that expresses an immunoglobulin light chain. A typical bispecific antibody has two heavy chains each having three heavy chain CDRs, followed by (N-terminal to C-terminal) a C_H1 domain, a hinge, a C_H2 domain, and a C_H3 domain, and an immunoglobulin light chain that either does not confer epitope-binding specificity but that can associate with each heavy chain, or that can associate with each heavy chain and that can bind one or more of the epitopes bound by the heavy chain epitope-binding regions, or that can associate with each heavy chain and enable binding of one or both of the heavy chains to one or both epitopes.

[000619] The term "cell" includes any cell that is suitable for expressing a recombinant nucleic acid sequence. Cells include those of prokaryotes and eukaryotes (single-cell or multiple-cell), bacterial cells (*e.g.,* strains of *E. coli, Bacillus spp., Streptomyces spp., etc.*), mycobacteria cells, fungal cells, yeast cells (*e.g., S. cerevisiae, S. pombe, P. pastoris, P. methanolica, etc.*), plant cells, insect cells (*e.g.,* SF-9, SF-21, baculovirus-infected insect cells, *Trichoplusia ni, etc.*), non-human animal cells, human cells, or cell fusions such as, for example, hybridomas or quadromas. In some embodiments, the cell is a human, monkey, ape, hamster, rat, or mouse cell. In some embodiments, the cell is eukaryotic and is selected from the following cells: CHO (*e.g.,* CHO K1, DXB-11 CHO, Veggie-CHO), COS (e.g., COS-7), retinal cell, Vero, CV1, kidney (*e.g.,* HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK), HeLa, HepG2, WI38, MRC 5, Colo205, HB 8065, HL-60, (e.g., BHK21), Jurkat, Daudi, A431 (epidermal), CV-1, U937, 3T3, L cell, C127 cell, SP2/0, NS-0, MMT 060562, Sertoli cell, BRL 3A cell, HT1080 cell, myeloma cell, tumor cell, and a cell line derived from an aforementioned cell. In some embodiments, the cell comprises one or more viral genes, *e.g.,* a retinal cell that expresses a viral gene (*e.g.,* a PER.C6™ cell).

[000620] The term "complementary determining region" or "CDR," as used herein, includes an amino acid sequence encoded by a nucleic acid sequence of an organism's immunoglobulin genes that normally (*i.e.,* in a wild type animal) appears between two framework regions in a variable region of a light or a heavy chain of an immunoglobulin molecule (*e.g.,* an antibody or a T cell receptor). A CDR can be encoded by, for example, a germline sequence or a rearranged sequence, and, for example, by a naïve or a mature B cell or a T cell. A CDR can be somatically mutated (*e.g.,* vary from a sequence encoded in an animal's germline), humanized, and/or modified with amino acid substitutions, additions, or deletions. In some circumstances (*e.g.,* for a CDR3), CDRs can be encoded by two or more sequences (*e.g.,* germline sequences) that are not contiguous (*e.g.,* in an unrearranged nucleic acid sequence) but are contiguous in a B cell nucleic acid sequence, *e.g.,* as a result of splicing or connecting the sequences (*e.g.,* V-D-J recombination to form a heavy chain CDR3).

[000621] The term "conservative," when used to describe a conservative amino acid substitution, includes substitution of an amino acid residue by another amino acid residue having a side chain R group with similar chemical properties (*e.g.,* charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of interest of a protein, for example, the ability of a variable region to specifically bind a target epitope with a desired affinity. Examples of groups of amino acids that have side chains with similar chemical properties include aliphatic side chains such as glycine,

alanine, valine, leucine, and isoleucine; aliphatic-hydroxyl side chains such as serine and threonine; amide-containing side chains such as asparagine and glutamine; aromatic side chains such as phenylalanine, tyrosine, and tryptophan; basic side chains such as lysine, arginine, and histidine; acidic side chains such as aspartic acid and glutamic acid; and, sulfur-containing side chains such as cysteine and methionine. Conservative amino acids substitution groups include, for example, valine/leucine/isoleucine, phenylalanine/tyrosine, lysine/arginine, alanine/valine, glutamate/aspartate, and asparagine/glutamine. In some embodiments, a conservative amino acid substitution can be substitution of any native residue in a protein with alanine, as used in, for example, alanine scanning mutagenesis. In some embodiments, a conservative substitution is made that has a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet et al. (1992) Exhaustive Matching of the Entire Protein Sequence Database, Science 256:1443-45. In some embodiments, the substitution is a moderately conservative substitution wherein the substitution has a nonnegative value in the PAM250 log-likelihood matrix.

[000622] In some embodiments, residue positions in an immunoglobulin light chain or heavy chain differ by one or more conservative amino acid substitutions. In some embodiments, residue positions in an immunoglobulin light chain or functional fragment thereof (*e.g.,* a fragment that allows expression and secretion from, *e.g.,* a B cell) are not identical to a light chain whose amino acid sequence is listed herein, but differs by one or more conservative amino acid substitutions.

[000623] The term "dissociative half-life" or "$t_{1/2}$" as used herein refers to the value calculated by the following formula: $t_{1/2}$ (min)=(ln2/k_d)/60, wherein k_d represents a dissociation rate constant.

[000624] The phrase "epitope-binding protein" includes a protein having at least one CDR and that is capable of selectively recognizing an epitope, *e.g.,* is capable of binding an epitope with a K_D that is at about one micromolar or lower (*e.g.,* a K_D that is about 1 x 10^{-6} M, 1 x 10^{-7} M, 1 x 10^{-9} M, 1 x 10^{-9} M, 1 x 10^{-10} M, 1 x 10^{-11} M, or about 1 x 10^{-12} M). Therapeutic epitope-binding proteins (*e.g.,* therapeutic antibodies) frequently require a K_D that is in the nanomolar or the picomolar range.

[000625] The term "functional" as used herein, *e.g.,* in reference to a functional polypeptide, includes a polypeptide that retains at least one biological activity normally associated with the native protein. In another instance, a functional immunoglobulin gene segment may include a variable gene segment that is capable of productive rearrangement to generate a rearranged immunoglobulin gene sequence.

[000626] The phrase "functional fragment" includes fragments of epitope-binding proteins that can be expressed, secreted, and specifically bind to an epitope with a K_D in the micromolar, nanomolar, or picomolar range. Specific recognition includes having a K_D that is at least in the micromolar range, the nanomolar range, or the picomolar range.

[000627] The term "germline" as used herein, in reference to an immunoglobulin nucleic acid sequence, includes reference to nucleic acid sequences that can be passed to progeny.

[000628] The phrase "heavy chain," or "immunoglobulin heavy chain" includes an immunoglobulin heavy chain sequence, including immunoglobulin heavy chain constant region sequence, from any organism. Heavy chain variable domains include three heavy chain CDRs and four FR regions, unless otherwise specified. Fragments of heavy chains include CDRs, CDRs and FRs, and combinations thereof. A typical heavy chain has, following the variable domain (from N-terminal to C-terminal), a C_H1 domain, a hinge, a C_H2 domain, and a C_H3 domain. A functional fragment of a heavy chain includes a fragment that is capable of specifically recognizing an epitope (*e.g.,* recognizing the epitope with a K_D in the micromolar, nanomolar, or picomolar range), that is capable of expressing and secreting from a cell, and that comprises at least one CDR. Heavy chain variable domains are encoded by variable region nucleotide sequence, which generally comprises V_H, D_H, and J_H segments derived from a repertoire of V_H, D_H, and J_H segments present in the germline. Sequences, locations and nomenclature for V, D, and J heavy chain segments for various organisms can be found in IMGT database, which is accessible via the internet on the world wide web (www) at the URL "imgt.org."

[000629] The term "identity" when used in connection with sequence, includes identity as determined by a number of different algorithms known in the art that can be used to measure nucleotide and/or amino acid sequence identity. In some embodiments described herein, identities are determined using a ClustalW v. 1.83 (slow) alignment employing an open gap penalty of 10.0, an extend gap penalty of 0.1, and using a Gonnet similarity matrix (MACVECTOR™ 10.0.2, MacVector Inc., 2008). The length of the sequences compared with respect to identity of sequences will depend upon the particular sequences, but in the case of a light chain constant domain, the length should contain sequence of sufficient length to fold into a light chain constant domain that is capable of self-association to form a canonical light chain constant domain, *e.g.,* capable of forming two beta sheets comprising beta strands and capable of interacting with at least one C_H1 domain of a human or a mouse. In the case of a C_H1 domain, the length of sequence should contain sequence of sufficient length to fold into a C_H1 domain that is capable of forming two beta sheets comprising beta strands and capable of interacting with at least one light chain constant

domain of a mouse or a human.

[000630] The phrase "immunoglobulin molecule" includes two immunoglobulin heavy chains and two immunoglobulin light chains. The heavy chains may be identical or different, and the light chains may be identical or different.

[000631] The phrase "light chain" includes an immunoglobulin light chain sequence from any organism, and unless otherwise specified includes human kappa (κ) and lambda (λ) light chains and a VpreB, as well as surrogate light chains. Light chain variable domains typically include three light chain CDRs and four framework (FR) regions, unless otherwise specified. Generally, a full-length light chain includes, from amino terminus to carboxyl terminus, a variable domain that includes FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, and a light chain constant region amino acid sequence. Light chain variable domains are encoded by the light chain variable region nucleotide sequence, which generally comprises light chain V_L and light chain J_L gene segments, derived from a repertoire of light chain V and J gene segments present in the germline. Sequences, locations and nomenclature for light chain V and J gene segments for various organisms can be found in IMGT database, which is accessible via the internet on the world wide web (www) at the URL "imgt.org." Light chains include those, *e.g.*, that do not selectively bind either a first or a second epitope selectively bound by the epitope-binding protein in which they appear. Light chains also include those that bind and recognize, or assist the heavy chain with binding and recognizing, one or more epitopes selectively bound by the epitope-binding protein in which they appear. Light chains also include those that bind and recognize, or assist the heavy chain with binding and recognizing, one or more epitopes selectively bound by the epitope-binding protein in which they appear. Common or universal light chains include those derived from a human Vκ1-39Jκ5 gene or a human Vκ3-20Jκ1 gene, and include somatically mutated (*e.g.*, affinity matured) versions of the same.

[000632] The phrase "micromolar range" is intended to mean 1-999 micromolar; the phrase "nanomolar range" is intended to mean 1-999 nanomolar; the phrase "picomolar range" is intended to mean 1-999 picomolar.

[000633] "Neutral pH" includes pH between about 7.0 and about 8.0, e.g., pH between about 7.0 and about 7.4, *e.g.*, between about 7.2 and about 7.4, *e.g.*, physiological pH in, *e.g.*, a mouse or a human. "Acidic pH" includes pH of 6.0 or lower, *e.g.*, pH between about 5.0 and about 6.0, pH between about 5.75 and about 6.0, *e.g.*, pH of endosomal or lysosomal compartments.

[000634] The term "operably linked" refers to a relationship wherein the components operably linked function in their intended manner. In one instance, a nucleic acid sequence encoding a protein may be operably linked to regulatory sequences (*e.g.,* promoter, enhancer, silencer sequence, *etc.*) so as to retain proper transcriptional regulation. In one instance, a nucleic acid sequence of an immunoglobulin variable region (or V(D)J segments) may be operably linked to a nucleic acid sequence of an immunoglobulin constant region so as to allow proper recombination between the sequences into an immunoglobulin heavy or light chain sequence.

[000635] The phrase "somatically mutated," as used herein, includes reference to a nucleic acid sequence from a B cell that has undergone class-switching, wherein the nucleic acid sequence of an immunoglobulin variable region, *e.g.,* a heavy chain variable region (*e.g.,* a heavy chain variable domain or including a heavy chain CDR or FR sequence) in the class-switched B cell is not identical to the nucleic acid sequence in the B cell prior to class-switching, such as, for example a difference in a CDR or a framework nucleic acid sequence between a B cell that has not undergone class-switching and a B cell that has undergone class-switching. The phrase "somatically mutated" includes reference to nucleic acid sequences from affinity-matured B cells that are not identical to corresponding immunoglobulin variable region nucleotide sequences in B cells that are not affinity-matured (*i.e.,* sequences in the genome of germline cells). The phrase "somatically matured" also includes reference to an immunoglobulin variable region nucleic acid sequence from a B cell after exposure of the B cell to an epitope of interest, wherein the nucleic acid sequence differs from the corresponding nucleic acid sequence prior to exposure of the B cell to the epitope of interest. The term "somatically mutated" also refers to sequences from antibodies that have been generated in an animal, *e.g.,* a mouse having human immunoglobulin variable region nucleic acid sequences, in response to an immunogen challenge, and that result from the selection processes inherently operative in such an animal.

[000636] The term "unrearranged," with reference to a nucleic acid sequence, includes nucleic acid sequences that exist in the germline of an animal cell.

[000637] The phrase "variable domain" includes an amino acid sequence of an immunoglobulin light or heavy chain (modified as desired) that comprises the following amino acid regions, in sequence from N-terminal to C-terminal (unless otherwise indicated): FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[000638] The phrase "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. In

one instance, a nucleic acid sequence encoding a protein may be operably linked to regulatory sequences (*e.g.,* promoter, enhancer, silencer sequence, *etc.*) so as to retain proper transcriptional regulation. In one instance, a nucleic acid sequence of an immunoglobulin variable region (or V(D)J segments) may be operably linked to a nucleic acid sequence of an immunoglobulin constant region so as to allow proper recombination between the sequences into an immunoglobulin heavy or light chain sequence.

[000639] The term "replacement" in reference to gene replacement refers to placing exogenous genetic material at an endogenous genetic locus, thereby replacing all or a portion of the endogenous gene with an orthologous or homologous nucleic acid sequence.

[000640] The term "functional" as used herein, *e.g.,* in reference to a functional polypeptide, includes a polypeptide that retains at least one biological activity normally associated with the native protein. In another instance, a functional immunoglobulin gene segment may include a variable gene segment that is capable of productive rearrangement to generate a rearranged immunoglobulin gene sequence.

Variable Domains with Histidine Substitutions

[000641] The design of human immunoglobulin-based therapeutics is a well-studied phenomenon, yet certain unsolved problems persist in making such therapeutics with optimal characteristics, *e.g.,* extending serum half-life of such therapeutics or otherwise improving their ability to bind more target per therapeutic molecule. Much work over the last couple of decades aimed at elucidating serum immunoglobulin turnover has focused on ways to increase serum half-life of therapeutically important antibodies, or immunoglobulin-based therapeutics, by modifying antibody structure. For the most part, this modification work has focused on the interaction of the constant domains of antibodies with the neonatal Fc receptor (FcRn). The neonatal Fc receptor on the extracellular surface binds circulating antibodies through their Fc regions to form an antibody-FcRn complex that is incorporated, or endocytosed, into the cell where the ligand and antibody part ways and the antibody-FcRn complex undergoes a cycling process that brings the antibody and the FcRn back to the cell's surface where the antibody is released and can re-bind a new target molecule. Cycling of the antibody-FcRn complex became an area of intense interest following the discovery of general mechanisms of receptor cycling.

[000642] Receptor cycling can proceed by a variety of mechanisms. Receptor-mediated endocytosis provides an endosomal pathway for a regulated recycling of cell surface receptors and (in some cases, *e.g.,* FcRns) their ligands. (Pinocytosed molecules are

otherwise typically shuttled through an endosomal pathway that ends in degradation.) The discovery of the mechanism of receptor-mediated endocytosis and the body of work concerning recycling of membrane receptors provided a framework for a detailed understanding of receptor-ligand turnover in general (for a review see, *e.g.,* Brown, M.S., Anderson, R.G.W., and Goldstein, J.L. (1983) Recycling Receptors: The Round-Trip Itinerary of Migrant Membrane Proteins, Cell 32:663-667; see also, Goldstein, J.L. and Brown, M.S. (2009) The LDL Receptor, Arterioscler. Thromb. Vasc. Biol. 29:431-438; Basu, S.K. (1984) Receptor-mediated endocytosis: An overview of a dynamic process, J. Biosci. 6(4):535-542). Other work on endosomal sorting helped properly frame the question of the fate of circulating immunoglobulins and the phenomenon of immunoglobulin receptor recycling and pharmacokinetics of antibody drugs. This work revealed a complex antibody-FcRn complex cycling process that appears to be primarily responsible for the relatively long half-life of IgG molecules in serum. Indeed, even rather early work in this area established that endosomes are the most plentiful in vivo source of FcRn (see, Roberts, D.M. *et al.* (1990) Isolation and Characterization of the Fc Receptor from the Fetal Yolk Sac of the Rat, J. Cell. Biol. 111:1867-1876). And it had long been observed that receptor-positive endosomal fractions are in large part not headed for lysosomal degradation (see, *e.g.,* Brown, M.S. *et al.* (1983) Recycling Receptors: The Round-Trip Itinerary of Migrant Membrane Proteins, Cell 32:663-667; see also, von Figura *et al.* (1984) Antibody to mannos 6-phosphate specific receptor induces receptor deficiency in human fibroblasts, EMBO J. 3(6):1281-1286), in the absence of aggregation (see, *e.g.,* Dunn, K.W. *et al.* (1989) Iterative Fractionation of Recycling Receptors from Lysosomally Destined Ligands in an Early Sorting Endosome, J. Cell. Biol. 109(6):3303-3314). It is this endosomal system that participates in a cycling process that ensures that antibodies that bind FcRn well under acidic conditions (*e.g.,* human IgG1 antibodies) persist for an extended period of time in serum.

[000643] According to some reports, the recycling mechanism of FcRn-containing endosomes is novel and unusual; it does not involve ubiquitin-dependent complete organelle merging but rather resembles incomplete merging mediated by tubular extensions more similar to a kiss-and-linger model (Gan, Z. *et al.* (2009) Analyses of the recycling receptor, FcRn, in live cells reveal novel pathways for lysosomal delivery, Traffic 10(5):600; see also, Tzaban, S. *et al.* (2009) The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity, J. Cell Biol. 185(4):673-684). Thus, the antibody-FcRn cycling model appears to be distinct from other endosomal pathways.

[000644] The antibody-FcRn endosomal cycling mechanism assures that antibodies that bind well to FcRn are able to sustain prolonged presence in serum through a more or less

continuous FcRn-protective process that entails sequestering bound antibody in an endosomal compartment where the binding of antibody to FcRn is maintained, preventing lysosomal degradation of antibody bound to FcRn. Typically, circulating antibody molecules bind FcRn on the cell surface. Antibody-FcRn complexes appear in endosomes as the result of a continuous endocytosis process. FcRn-bound molecules (*e.g.,* antibodies, or Fc fusion proteins) remain associated with FcRn in the acidic endosomal compartment through acid-stable Fc-FcRn interaction. Molecules not bound to the endosomal surface (through, *e.g.,* FcRn or another receptor) are shuttled to the lysosomal pathway and degraded, whereas receptor-bound molecules are recycled to the plasma membrane when the endosome fuses with the plasma membrane. Upon fusion with the plasma membrane, the acid-stable Fc-FcRn interaction is exposed to a near neutral extracellular pH where the Fc readily dissociates from the FcRn. It is the pH binding differential of the Fc, coupled with a differential thermal stability of FcRn that varies with protonation state, that is believed to be primarily responsible for the ability of certain Fc's to sustain serum concentrations through binding FcRn. A key to the endosomal cycling mechanism is ligand release by receptors in the acidic endosomal compartment (reviewed, *e.g.,* in Brown, M.S. *et al.* (1983)).

[000645] IgG1 Fc moieties bind FcRn with high affinity at pH 6.0 to about 6.5; binding at pH 7.0 to about pH 7.5 is about two orders of magnitude weaker, presumably due to titration of histidine residues near the region of the Fc that binds FcRn, residues 310-433, and an FcRn intramolecular thermal stability differential mediated by protonation state (Raghaven, M. *et al.* (1995) Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants, Biochemistry 34:14649-14657; Vaughn, D.E. and Bjorkman, P.J. (1998) Structural basis of pH-dependent antibody binding by the neonatal Fc receptor, Structure 6:63-73); it has been demonstrated that rat FcRn exhibits a better thermal stability profile at pH 6.0 than at pH 8.0 (Raghavan, M. *et al.* (1993) The class I MHC-related Fc receptor shows pH dependent stability differences correlating with immunoglobulin binding and release, Biochemistry 32:8654-8660).

[000646] Although nature gave rise to Fc structures that bind FcRn differentially, the science of Fc engineering arose to design Fc structures that would result in tighter binding to FcRn and—presumably—longer serum half-life. Many such structures were designed and tested, far too numerous to review here, with varying degrees of success. Mutating immunoglobulin constant region sequences to promote recycling of antibody by modifying FcRn binding characteristics has a long and varied history. To date, most if not all effort to identify mutations have focused on residues believed to be critical in binding or interacting with FcRn, i.e., residues whose modification affect affinity of the Fc for FcRn.

[000647] But binding of Fc to FcRn is itself a complex matter. Different types of therapeutic antibodies (humanized, chimeric, and mouse), and even within types (*e.g.,* comparing different humanized antibodies to one another, comparing IgG1 isotype antibodies to one another, *etc.*), exhibit dissociation constants with respect to FcRn that vary as much as about two-fold (see, *e.g.,* Suzuki, T. *et al.* (2010) Importance of Neonatal FcR in Regulating the Serum Half-Life of Therapeutic Proteins Containing the Fc Domain of Human IgG1: A Comparative Study of the Affinity of Monoclonal Antibodies and Fc-Fusion Proteins to Human Neonatal FcR, J. Immunol. 184:1968-1976). This observation permits an inference that the primary structure of the constant region may not account for all pharmacokinetic behavior. Others have postulated that overall isoelectric point (pI) of an antibody, keeping the constant region primary structure fixed, is an important determinant of serum half-life—presumably through an unspecified non-FcRn-dependent mechanism (Igawa, T. *et al.* (2010) Reduced elimination of IgG antibodies by engineering the variable region, Protein Engineering, Design & Selection 23(5):385-392). Under this view, the lower the pI of the antibody, the tighter the binding to FcRn (*Id.*). For at least one IgG4 isotype antibody, a change in pI from 9.2 to 7.2 correlated with a 2.4-fold increase in half-life and a 4.4-fold reduction in clearance (*Id.*), consistent with an inference that a nonspecific lowering of pI by modifying residues in both the heavy and light chain variable regions together can significantly impact pharmacokinetic behavior. In that report, residue modification did not follow any particular pattern and no residue was substituted to histidine, although at least one residue in a light chain CDR2 was changed from a histidine residue to a glutamate residue (*Id.,* at Fig. 5, p. 390). Further, an odd paradox may erupt when comparing *in vitro* FcRn binding and in vivo pharmacokinetics: for at least one clinically important IgG1 antibody with multiple substitutions in the Fc region that interacts with FcRn, *in vitro* FcRn binding did not correlate with *in vivo* pharmacokinetic behavior (see, Petkova, S.B. *et al.* (2006) Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease, Int'l Immunol. 18(12):1759-1769). Finally, release of Fc ligand from FcRn upon fusion with the plasma membrane appears to occur in two phases—a rapid phase and an extended phase—of unknown mechanism (see, Ober R.J. *et al.* (2004) Exocytosis of IgG as mediated by the receptor FcRn: an analysis at the single-molecule level, Proc. Natl Acad. Sci. USA 101:11076-11081).

[000648] Finally, extending half-life of antibodies in serum is one way to enhance efficiency of antibody therapy. Improved efficacy, or improved availability of the same antibody or variable domain to bind and eliminate two or three or more target molecules are not necessarily addressed by improving FcRn binding and turnover that affects target

antigen. Modifications that increase affinity of an Fc to FcRN are expected to increase turnover and thus improve pharmacokinetics of a therapeutic antibody. Antigen-antibody complexes bind FcRn tightly, resulting in the antigen-antibody complex cycling back into extracellular space rather than being degraded by a lysosomal pathway. In this scenario, however, the antigen, or target, may largely remain complexed to the antibody and recycled together with the antibody into the extracellular space. For therapeutic antibodies, this phenomenon can be very undesirable.

[000649] However, antibodies whose interaction with antigen are pH-dependent, *i.e.,* antibodies engineered to bind antigen with lower affinity at an endosomal pH, would not recycle antigen in an FcRn-dependent manner due to instability of the antigen-antibody complex in the endosomal compartment. This is because in the acidic environment of the endosome, the antigen will disengage from the antibody-FcRn complex, and the antibody-bound FcRn will recycle to the surface of the cell, whereas disengaged free antigen will shuttle to a lysosomal degradation pathway. In this way, pH-dependent antigen binding can provide enhanced efficacy and/or pharmacokinetics within the context of FcRn-mediated cycling (but not directly depending on the Fc-FcRn interaction) by freeing cycled antibody to bind antigen, bind FcRn, cycle through endosomes, and re-enter the extracellular space to bind more antigen and shuttle more antigen to a lysosomal degradation pathway.

[000650] Capitalizing on the observation that ligands will frequently dissociate from their receptors at an endosomal pH, it had been suggested to search for antibodies that effectively release antigen at an endosomal pH in order to make certain specific multifunctional molecules that target specific cells in order to import toxins into the cells and release the toxins within endosomes (see, *e.g.,* U.S. Pat. No. 5,599,908 and U.S. Pat. No. 5,603,931). But that does not address antigen-antibody cycling, in particular for human therapeutics.

[000651] To leverage antibody structure for shuttling endosomally disengaged antigen through a lysosomal pathway while maintaining FcRn-dependent cycling of antigen-FcRn complexes, certain approaches to pH-dependent antigen binding have been explored. Such approaches include a generalized histidine-scanning over the variable region to substitute residues with histidine and test to see whether the generalized approach of histidine replacement yields an antibody with desired pH-dependent antigen binding (see, *e.g.,* Igawa, T. *et al.* (2010) Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization, Nature Biotech. 28(11):1203-1208; see also, U.S. Patent Application Publication No. 2011/0111406 A1)). A likely disadvantage of this approach is that modifying residues important for antigen binding are likely to disrupt binding

at either an acidic or neutral pH, which can eliminate any leverage due to the pH differential between the endosomal compartment and the extracellular space.

[000652] In various aspects, compositions and methods are provided for making one or more histidine substitutions at a few judiciously-selected regions in an antibody variable region (heavy chain and/or light chain variable domain) provides a method for making antibody variable domains that bind a target antigen in a pH-dependent manner, *e.g.,* variable domains that bind an antigen of interest with a first affinity at a neutral or basic or extracellular pH, yet bind the same antigen of interest with a second affinity at an acidic pH, wherein the first affinity is high and wherein the second affinity is low.

[000653] In various aspects, the one or more histidine substitutions are in a CDR1, a CDR2, a CDR3, an N-terminal, and/or a loop 4 sequence.

[000654] In some aspects the one or more histidine substitutions are in a CDR1, a CDR2, and/or a CDR3.

[000655] In some aspects, the one or more histidine substitutions are in a CDR3 and a loop 4 sequence. In a further embodiment, the substitutions are also in an N-terminal sequence.

[000656] In some aspects, the one or more histidine substitutions are in a CDR3 and an N-terminal sequence. In a further embodiment, the substitutions are also in a loop 4 sequence.

[000657] In some aspects, the one or more histidine substitutions are in a CDR2 sequence and a loop 4 sequence. In a further embodiment, the substitutions are also in an N-terminal sequence.

[000658] In some aspects, the loop 4 sequence is for a λ light chain variable domain residues 83-88; for a κ light chain variable domain residues 83-88; and for a heavy chain variable region 82-88 (IMGT numbering).

[000659] In some aspects, the N-terminal sequence for a light chain variable domain or a heavy chain variable domain are residues 1-26 (IMGT numbering). In one embodiment, the N-terminal sequence that comprises one or more (*e.g.,* clustered) histidine substitutions is residues 1-5, in one embodiment residues 1-10, in one embodiment 1-15, in one embodiment 1-20, in one embodiment 1-25, in one embodiment 5-10, in one embodiment 10-15, in one embodiment 15-20, in one embodiment 20-25, in one embodiment 5-15, in one embodiment 10-20, in one embodiment 5-20. In one embodiment, the histidine substitutions

are two or more (*e.g.,* three, four, five, or six or more), and at least two or more of the histidine substitutions are made within a stretch of N-terminal sequence that is about 3 residues, 4 residues, five residues, or six residues or more. In one embodiment, a plurality of histidine substitutions are made in the N-terminal, and the histidine substitutions comprise clusters of at least two, at least three, or at least four histidine substitutions. In one embodiment, at least one cluster of histidine substitutions comprises histidine substitutions that are separated by one or more non-histidine substitutions.

[000660] In some aspects, the one or more histidine substitutions the CDR are two, three, four, five, or six substitutions within the CDR. In one embodiment, all residues in the CDR that are not critical for binding at a neutral pH are substituted with a histidine. In one embodiment, the two, three, four, five, or six substitutions are contiguous; in one embodiment, one or more of the two, three, four, five, or six substitutions are present in a cluster, wherein the cluster comprises at least one non-histidine residue; in one embodiment, the cluster comprises two non-histidine residues; in one embodiment, the cluster comprises three non-histidine residues; in one embodiment, the cluster comprises four non-histidine residues.

[000661] In some aspects, the one or more histidine substitutions in the N-terminal are one, two, three, four, five, or six substitutions. In one embodiment, all residues in the N-terminal that do not reduce antigen binding at a neutral pH (*e.g.,* by more than 1%, 2%, 3%, 4%, or 5%), are substituted with a histidine. In one embodiment, the two, three, four, five, or six substitutions are contiguous; in one embodiment, one or more of the two, three, four, five, or six substitutions are present in a cluster, wherein the cluster comprises at least one non-histidine residue; in one embodiment, the cluster comprises two non-histidine residues; in one embodiment, the cluster comprises three non-histidine residues; in one embodiment, the cluster comprises four non-histidine residues.

[000662] In some aspects, the method comprises modifying a variable domain to comprise a cluster of histidine substitutions (*e.g.,* as described herein, contiguous or interrupted with one or more non-histidine residues) in a region selected from a CDR1, a CDR2, a CDR3, an N-terminal, a loop 4, and a combination thereof. In some aspects, the cluster is a sequence bounded upstream by a first histidine residue, and downstream by a second histidine residue, and comprises one or more residues between the first and second histidine residues. In one embodiment, the one or more residues between the first and second histidine residues are 1, 2, 3, 4, 5, or 6 or more non-histidine residues. In one embodiment, the one or more residues between the first and second histidine residues are 1, 2, 3, 4, 5, or six histidine residues. In one embodiment, the cluster is 3 residues, in one

embodiment 4 residues, in one embodiment 5 residues, in one embodiment 6 residues, in one embodiment 7 residues, in one embodiment 8 residues or more.

[000663] In various aspects, the method comprises identifying sequences in an antibody variable domain (heavy and/or light chain) that are critical for binding antigen (*e.g.,* at a neutral pH, *e.g.,* pH 7-7,4, *e.g.,* pH 7.2, *e.g.,* an extracellular pH), and substituting one or more residues within the sequence to histidine, wherein the substitution to histidine does not eliminate binding of the variable domain to a target antigen at a neutral pH. In various aspects, a cluster of two or more, three or more, four or more, or five or more residues that are not critical for binding at a neutral pH are substituted with histidine residues. In various aspects, the cluster of histidine residues is within a CDR, a loop 4, an N-terminal, or a combination thereof.

[000664] In various aspects, a residue that is critical for binding is identified as a residue that when substituted with a substitute amino acid at about a neutral (or extracellular) pH, reduces binding of the variable domain by in one embodiment at least 5%, in one embodiment at least 10%, in one embodiment at least 20%, in one embodiment at least 30%, in one embodiment at least 40%, in one embodiment at least 50%, in one embodiment at least 60%, in one embodiment at least 70%, in one embodiment at least 80%, in one embodiment at least 90%, in one embodiment results in no detectable binding. In one embodiment, the substitute amino acid is a histidine. In one embodiment, the substitute amino acid is an alanine.

[000665] In one aspect, a method is provided for making an antibody variable domain that binds and antigen weaker at an acidic pH than it binds the same antigen at a neutral or basic pH, wherein the method comprises substituting one or more amino acid residues of the variable region with one or more histidine residues. In one embodiment, the binding at the acidic pH is negligible or zero.

[000666] In one embodiment, the one or more amino acid residues substituted are in a light chain. In a specific embodiment, the one or more residues are in an N-terminal region of a light chain. In a specific embodiment, the N-terminal residues are selected from 1-26, 1-20, 1-15, 1-10, 1-6, or 1-5 (IMGT numbering). In one embodiment, the one or more residues are in loop 3. In a specific embodiment, the loop 4 residues are 83-88 in Vκ or Vλ, and 82-88 in V_H (IMGT numbering).

[000667] In one embodiment, the one or more residues are in a heavy chain. In a specific embodiment, the one or more residues are in an N-terminal region of a heavy chain. In a

specific embodiment, the N-terminal residues are selected from 1-26, 1-20, 1-15, 1-10, 1-6, or 1-5 (IMGT numbering). In one embodiment, the one or more residues are in loop 4. In a specific embodiment, the loop 3 residues are selected from (for Vκ and/or Vλ) 83, 84, 85, 86, 87, 88, and a combination thereof (IMGT numbering); or (for V_H) 82, 83, 84, 85, 86, 87, 88, and a combination thereof (IMGT numbering); and, a combination thereof.

[000668] In one embodiment, the one or more residues are in a CDR selected from a CDR1, a CDR2, and a CDR3; and the one or more residues when substituted (*e.g.,* with alanine or with histidine) do not result in decreased binding of the target antigen at a neutral or a basic pH. In a specific embodiment, decreased binding of the target antigen at neutral or basic pH as the result of substitution (*e.g.,* by alanine or histidine substitution) is no more than 5%, no more than 10%, no more than 15%, or no more than 20%, no more than 25%, or no more than 30% as compared with non-substituted variable domain.

[000669] In some aspects, the his-modified variable domain complexed with the target antigen exhibits a half-life of at least about 20 minutes at an elevated pH (*e.g.,* an extracellular pH, or a pH from 7-7.4, *e.g.*, pH 7.2) and exhibits a half-life of less than 5 minutes, less than 4 minutes, less than 3 minutes, less than 2 minutes, or less than a minute at an endosomal pH, or a pH from *e.g.,* pH 5-6, *e.g.,* pH 5.75. In one embodiment, the his-modified variable domain complexed with the target antigen exhibits a half-life of at least about 20 minutes at the elevated pH, and exhibits a half-life at an endosomal pH of less than 60 seconds, less than 30 seconds, less than 10 seconds, less than 5 seconds, less than 4 seconds, less than 3 seconds, or less than 2 seconds. In one embodiment, the his-modified variable domain complexed with the target antigen exhibits a half-life of at least about 20 minutes at the elevated pH (*e.g.*, pH 7-7.4, *e.g.,* pH 7.2), and exhibits a half-life at an endosomal pH of less than about a second, less than 0.5 second, less than 0.1 second, or less than 0.05 second. In one embodiment, half-life at an endosomal pH is measured using a BIACORE™ assay in which his-modified variable domain complexed with target antigen is equilibrated on the surface of a BIACORE™ chip at neutral or elevated pH, and buffer at an endosomal pH (or, *e.g.,* a pH of 5-6, *e.g.,* pH 5.75) is flowed over the complex.

[000670] In various aspects, a method for making an antibody variable domain that binds a target antigen with a first affinity at an extracellular pH, and that does not bind the target antigen or binds the target antigen with a second affinity at an endosomal pH, wherein the first affinity is about 10, 10^2-, 10^3-, 10^4-, 10^5-, 10^6-, 10^7-, 10^8-, 10^9, 10^{10}-, 10^{11}-, or 10^{12}- fold (or higher-fold) than the second affinity. In some aspects, the first affinity is in the picomolar to nanomolar range (*e.g.,* K_D is 10^{-12} to 10^{-9}), and the second affinity is in the micromolar or higher range (*e.g.,* $K_D = 10^{-6}$ or greater, *e.g.,* 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, or higher). In some

aspects, the first affinity is in the range of about K_D 10-9 to about K_D 10^{-12}, and the second affinity is in the range of about K_D 10^{-3} to about 1 or larger. In one embodiment, the first affinity is in the range of about K_D 10^{-9} to about K_D 10^{-12}, and the second affinity is characterized by a K_D > 1; in a specific embodiment, the second affinity is characterized by a K_D >> 1 (e.g., 10, 10^2, 10^3 or higher). In a specific embodiment, the first affinity is characterized by a K_D from about 10^{-9} to about 10^{-12}, and the second affinity is characterized by an inability to detect binding over background in a BIACORE™ binding assay.

[000671] Various aspects are illustrated by a particular case in which a human light chain variable sequence is modified to contain a one or more, including a cluster, of histidines in a light chain CDR3, and the light chain is expressed in a CHO cell with a cognate human heavy chain. The identity of the antigen to which the histidine-modified antibody binds is unimportant, as is the particular sequence of the light chain variable domain. The principles illustrated in the Examples are applicable to CDR3, CDR2, CDR1, the N-terminal region, or loop 4. For example, residues in the cited regions can be substituted for histidine, alone or in clusters of 2, 3, 4, or 5, *e.g.,* and the resulting antibodies tested for pH-dependent antigen binding.

[000672] Methods for engineering antibodies that are capable of binding to an antigen in a pH dependent manner can be made by making modifications of an immunoglobulin light chain variable region at one or more positions along the sequence of the light chain as described (*e.g.,* in a CDR3, CDR2, CDR1, loop 4, N-terminal). Histidines are tolerated in CDR regions; light chains, typically show somatic hypermutation along the variable region sequence, and, in some cases, such mutations can result in a substitution of histidine residues in CDRs (FIG. 15).

[000673] In the Examples, histidine substitutions have been identified at one to four positions in the light chain CDR3 region, at residues not critical for binding target antigen at a neutral pH, from which fifteen mutant constructs were made. The particular light chain shown—with a variety of different but cognate heavy chains—is derived from a single Vκ and a single Jκ segment (Vκ1-39/Jκ5). Such mutants when expressed confer upon the antibody (in conjunction with a cognate heavy chain) the property of pH-dependent antigen binding. The mutant constructs were made using antigen-specific antibody variable domains and tested for expression and antigen binding at approximately a neutral pH and release at low pH ("catch-and-release"). In certain examples shown the locations of the four identified residues (where mutation to histidine is not critical for binding at neutral pH) are Q105H, Q106H, Y108H and P111H. For an antibody that binds a different target antigen, or for an antibody comprising a different rearranged V-J sequence, his-mutatable residues for making

a pH-dependent variable domain are found by identifying which residues are not critical for binding at neutral pH, then modifying one or more of those residues (*e.g.,* in clusters) and expressing an antibody comprising the mutations, and testing for binding (and/or release time, *e.g.,* $t_{1/2}$) at a neutral pH (*e.g.,* an extracellular pH) and at an acidic pH (*e.g.,* an endosomal pH). Although the data shown here are for a Vκ1-39/Jκ5 light chain, other light chains, including those derived from a Vκ3-20/Jκ1 rearrangement, are amenable to the approach described herein, as are heavy chains.

[000674] All of the histidine-engineered light chain constructs that were made in this experiment expressed well in conjunction with heavy chains. Further, binding of the antibodies to antigen in a pH-dependent manner was demonstrated from BIACORE™ assay data showing the binding of antigen at around a neutral pH and at an acidic pH for the 15 mutants with five different heavy chains that specifically recognize the same cell surface antigen (FIG.19A-J).

[000675] The methods described, and those particular methods used for purposes of illustration in certain of the examples and figures herein, are useful to generate variable regions of antibodies that can be used to make, *e.g.,* human therapeutic binding proteins that bind their targets by human immunoglobulin variable domains that comprise the histidines in a CDR3. The altered binding at a lower pH will in some circumstances allow faster turnover because the therapeutic will bind a target on a cell's surface, be internalized in an endosome, and more readily or more rapidly dissociate from the target in the endosome, so that the therapeutic can be recycled to bind yet another molecule of target (*e.g.,* on another cell or the same cell). In various embodiments, this will result in the ability to dose the therapeutic at a lower dose, or dose the therapeutic less frequently. This is particularly useful where it is not desirable to dose frequently, or to administer above a certain dosage, for safety or toxicity reasons. For example, the half-life of an antibody therapeutic in the serum of a subject will be increased as a result.

[000676] Thus, in various embodiments codons in a gene encoding a rearranged human light chain can be made at positions 105, 106, 108, 111, or a combination thereof. For example, position 105 in conjunction with one or more of 106, 108, and 111; position 106 in conjunction with one or more of 105, 108, and 111; position 108 in conjunction with one or more of 105, 106, and 111; position 111 in conjunction with one or more of 105, 106, and 108. Corresponding positions in other light chains (*i.e.,* derived from other V-J rearrangements) are included in various embodiments.

Non-Human Animals that Express Immunoglobulin Heavy Chain Variable Domain

Comprising Histidine Residues

[000677] The described invention provides genetically modified non-human animals that can produce antigen-binding proteins with pH-dependent antigen binding characteristics. In various embodiments, the antigen-binding proteins produced by the genetically modified non-human animals as described herein exhibit increased pH-dependent recycling efficiency and/or enhanced serum half-life. In particular, the described invention employs genetic modifications in the immunoglobulin heavy chain locus to introduce histidine codons into a human heavy chain variable region nucleotide sequence and, optionally, to introduce a mutation(s) in a constant region nucleotide sequence that encodes C_H2 and/or C_H3 domains that increases the binding of the antibody constant region to an FcRn receptor, which facilitates recycling of the antigen-binding protein. Antigen-binding proteins comprising the modification may more loosely bind its target in an acidic intracellular compartment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0) than in an extracellular environment or at the surface of a cell (*i.e.*, at a physiological pH, *e.g.*, a pH ranging from about 7.0 to about 7.4) due to protonated histidine residues located in the antigen binding sites. Therefore, the antigen-biding proteins comprising the genetic modifications as described herein would be able to be recycled more rapidly or efficiently than wild-type antigen-binding proteins that do not comprise such genetic modifications following target-mediated endocytosis. Furthermore, since the modified histidine residues are protonated only in an acidic environment, but not at a neutral pH, it is expected that such modification would not affect binding affinity and/or specificity of the antigen-binding protein toward an antigen of interest at a physiological pH.

[000678] In various aspects, non-human animals are provided comprising immunoglobulin heavy chain loci that comprise an unrearranged human heavy chain variable region nucleotide sequence, wherein the unrearranged human heavy chain variable region nucleotide sequence comprises an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000679] In various aspects, methods of making and using the non-human animals are also provided. When immunized with an antigen of interest, the genetically modified non-human animals are capable of generating B cell populations that produce antigen-binding proteins comprising heavy chain variable domains with histidine residues, wherein the antigen-binding proteins exhibit enhanced pH-dependent recycling and/or increased serum half-life. In various embodiments, the non-human animals generate B cell populations that express human heavy chain variable domains along with cognate human light chain variable domains. In various embodiments, the genetically modified immunoglobulin heavy chain loci

are present in a germline genome of the non-human animal.

[000680] In various embodiments, the genetically modified immunoglobulin heavy chain locus comprises a modification that deletes or renders, all or substantially all, non-functional endogenous V_H, D, and J_H gene segments; and the genetically modified locus comprises an unrearranged heavy chain variable region nucleotide sequence comprising one or more human V_H, D, and/or J_H gene segments having one or more histidine codons, wherein the unrearranged heavy chain variable region nucleotide sequence is present at an endogenous location (*i.e.,* where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.,* at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome). In one embodiment, *e.g.*, about 80% or more, about 85% or more, about 90% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% or more of all endogenous heavy chain V, D, or J gene segments are deleted or rendered non-functional. In one embodiment, *e.g.,* at least 95%, 96%, 97%, 98%, or 99% of endogenous functional heavy chain V, D, or J gene segments are deleted or rendered non-functional.

[000681] In one embodiment, the non-human animal is a mammal. Although embodiments directed to introducing histidine codons into an unrearranged human heavy chain variable gene sequence in a mouse are extensively discussed herein, other non-human animals are also provided that comprise a genetically modified immunoglobulin locus containing an unrearranged human heavy chain variable region nucleotide sequence comprising an addition of least one histidine codon or a substitution of at least one endogenous non-histidine codon with a histidine codon. Such non-human animals include any of those which can be genetically modified to express the histidine-containing heavy chain variable domain as disclosed herein, including, *e.g.*, mouse, rat, rabbit, pig, bovine (*e.g.*, cow, bull, buffalo), deer, sheep, goat, chicken, cat, dog, ferret, primate (*e.g.*, marmoset, rhesus monkey), etc. For example, for those non-human animals for which suitable genetically modifiable ES cells are not readily available, other methods are employed to make a non-human animal comprising the genetic modification. Such methods include, *e.g.*, modifying a non-ES cell genome (*e.g.*, a fibroblast or an induced pluripotent cell) and employing somatic cell nuclear transfer (SCNT) to transfer the genetically modified genome to a suitable cell, *e.g.,* an enucleated oocyte, and gestating the modified cell (*e.g.*, the modified oocyte) in a non-human animal under suitable conditions to form an embryo. Methods for modifying a non-human animal genome (*e.g.*, a pig, cow, rodent, chicken, etc.

genome) include, *e.g.*, employing a zinc finger nuclease (ZFN) or a transcription activator-like effector nuclease (TALEN) to modify a genome to include a nucleotides sequence that encodes

[000682] In one embodiment, the non-human animal is a small mammal, *e.g.*, of the superfamily *Dipodoidea* or *Muroidea*. In one embodiment, the genetically modified animal is a rodent. In one embodiment, the rodent is selected from a mouse, a rat, and a hamster. In one embodiment, the rodent is selected from the superfamily *Muroidea*. In one embodiment, the genetically modified animal is from a family selected from *Calomyscidae* (*e.g.*, mouse-like hamsters), *Cricetidae* (*e.g.*, hamster, New World rats and mice, voles), *Muridae* (true mice and rats, gerbils, spiny mice, crested rats), *Nesomyidae* (climbing mice, rock mice, with-tailed rats, Malagasy rats and mice), *Platacanthomyidae* (*e.g.*, spiny dormice), and *Spalacidae* (*e.g.*, mole rates, bamboo rats, and zokors). In a specific embodiment, the genetically modified rodent is selected from a true mouse or rat (family *Muridae*), a gerbil, a spiny mouse, and a crested rat. In one embodiment, the genetically modified mouse is from a member of the family *Muridae*. In one embodiment, the animal is a rodent. In a specific embodiment, the rodent is selected from a mouse and a rat. In one embodiment, the non-human animal is a mouse.

[000683] In one embodiment, the non-human animal is a rodent that is a mouse of a C57BL strain selected from C57BL/A, C57BL/An, C57BL/GrFa, C57BL/KaLwN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6N, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, and C57BL/Ola. In another embodiment, the mouse is a 129 strain. In one embodiment, the 129 strain is selected from the group consisting of 129P1, 129P2, 129P3, 129X1, 129S1 (*e.g.*, 129S1/SV, 129S1/SvIm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, 129T2 (see, *e.g.*, Festing *et al.* (1999) Revised nomenclature for strain 129 mice, Mammalian Genome 10:836, see also, Auerbach *et al.* (2000) Establishment and Chimera Analysis of 129/SvEv- and C57BL/6-Derived Mouse Embryonic Stem Cell Lines). In one embodiment, the genetically modified mouse is a mix of an aforementioned 129 strain and an aforementioned C57BL strain (*e.g.*, a C57BL/6 strain). In another embodiment, the mouse is a mix of aforementioned 129 strains, or a mix of aforementioned C57BL/6 strains. In one embodiment, the 129 strain of the mix is a 129S6 (129/SvEvTac) strain. In another embodiment, the mouse is a mix of a 129/SvEv- and a C57BL/6-derived strain. In a specific embodiment, the mouse is a mix of a 129/SvEv- and a C57BL/6-derived strain as described in Auerbach *et al.* 2000 *BioTechniques* 29:1024-1032. In another embodiment, the mouse is a BALB strain, *e.g.*, BALB/c strain. In another embodiment, the mouse is a mix of a BALB strain (*e.g.*, BALB/c strain) and another

aforementioned strain.

[000684] In one embodiment, the non-human animal is a rat. In one embodiment, the rat is selected from a Wistar rat, an LEA strain, a Sprague Dawley strain, a Fischer strain, F344, F6, and Dark Agouti. In one embodiment, the rat strain is a mix of two or more of a strain selected from the group consisting of Wistar, LEA, Sprague Dawley, Fischer, F344, F6, and Dark Agouti.

[000685] In one embodiment, the non-human animal is a mouse. In one embodiment, the mouse is a VELOCIMMUNE® humanized mouse.

[000686] VELOCIMMUNE® humanized mice (see, *e.g.,* US 6,596,541, US 7,105,348, and US20120322108A1), which contain a precise replacement of mouse immunoglobulin variable regions with human immunoglobulin variable regions at the endogenous mouse loci, display a surprising and remarkable similarity to wild-type mice with respect to B cell development. VELOCIMMUNE® humanized mice display an essentially normal, wild-type response to immunization that differed only in one significant respect from wild-type mice - the variable regions generated in response to immunization are fully human.

[000687] VELOCIMMUNE® humanized mice contain a precise, large-scale replacement of germline variable region nucleotide sequences of mouse immunoglobulin heavy chain (IgH) and immunoglobulin light chain (e.g., κ light chain, Igκ) with corresponding human immunoglobulin variable region nucleotide sequences, at the endogenous loci (see, *e.g.,* US 6,596,541, US 7,105,348, US 20120322108A1). In total, about six megabases of mouse loci are replaced with about 1.5 megabases of human genomic sequence. This precise replacement results in a mouse with hybrid immunoglobulin loci that make heavy and light chains that have a human variable regions and a mouse constant region. The precise replacement of mouse V_H-D-J_H and Vκ-Jκ segments leave flanking mouse sequences intact and functional at the hybrid immunoglobulin loci. The humoral immune system of the mouse functions like that of a wild-type mouse. B cell development is unhindered in any significant respect and a rich diversity of human variable regions is generated in the mouse upon antigen challenge.

[000688] VELOCIMMUNE® humanized mice are possible because immunoglobulin gene segments for heavy and κ light chains rearrange similarly in humans and mice, which is not to say that their loci are the same or even nearly so—clearly they are not. However, the loci are similar enough that humanization of the heavy chain variable gene locus can be accomplished by replacing about three million base pairs of contiguous mouse sequence

that contains all the V_H, D, and J_H gene segments with about one million bases of contiguous human genomic sequence covering basically the equivalent sequence from a human immunoglobulin locus.

[000689] In some embodiments, further replacement of certain mouse constant region nucleotide sequences with human constant region nucleotide sequences (*e.g.*, replacement of mouse heavy chain C_H1 nucleotide sequence with human heavy chain C_H1 nucleotide sequence, and replacement of mouse light chain constant region nucleotide sequence with human light chain constant region nucleotide sequence) results in mice with hybrid immunoglobulin loci that make antibodies that have human variable regions and partly human constant regions, suitable for, *e.g.*, making fully human antibody fragments, *e.g.*, fully human Fab's. Mice with hybrid immunoglobulin loci exhibit normal variable gene segment rearrangement, normal somatic hypermutation frequencies, and normal class switching. These mice exhibit a humoral immune system that is indistinguishable from wild type mice, and display normal cell populations at all stages of B cell development and normal lymphoid organ structures—even where the mice lack a full repertoire of human variable region nucleotide segments. Immunizing these mice results in robust humoral responses that display a wide diversity of variable gene segment usage.

[000690] The precise replacement of the mouse germline variable region nucleotide sequence allows for making mice that have partly human immunoglobulin loci. Because the partly human immunoglobulin loci rearrange, hypermutate, and class switch normally, the partly human immunoglobulin loci generate antibodies in a mouse that comprise human variable regions. Nucleotide sequences that encode the variable regions can be identified and cloned, then fused (*e.g.*, in an *in vitro* system) with any sequences of choice, *e.g.*, any immunoglobulin isotype suitable for a particular use, resulting in an antibody or antigen-binding protein derived wholly from human sequences.

[000691] In various embodiments, at least one histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes an N-terminal region, a loop 4 region, a CDR1, a CDR2, a CDR3, or a combination thereof.

[000692] In various embodiments, at least one histidine codon is present in an unrearranged heavy chain variable region nucleotide sequence that encodes a framework region (FR) selected from the group consisting of FR1, FR2, FR3, and FR4.

[000693] In various aspects, the genetically modified immunoglobulin locus comprises a nucleotide sequence wherein at least one codon has been replaced with a histidine codon.

[000694] In various aspects, the genetically modified immunoglobulin locus comprises an unrearranged human heavy chain variable region nucleotide sequence comprising a substitution of at least one endogenous non-histidine codon with a histidine codon.

[000695] In one embodiment, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, 25 or more, 26 or more, 27 or more, 28 or more, 29 or more, 30 or more, 31 or more, 32 or more, 33 or more, 34 or more, 35 or more, 36 or more, 37 or more, 38 or more, 39 or more, 40 or more, 41 or more, 42 or more, 43 or more, 44 or more, 45 or more, 46 or more, 47 or more, 48 or more, 49 or more, 50 or more, 51 or more, 52 or more, 53 or more, 54 or more, 55 or more, 56 or more, 57 or more, 58 or more, 59 or more, 60 or more, or 61 or more of the endogenous non-histidine codons are replaced with histidine codons.

[000696] Previous studies on reading frame usage of human immunoglobulin D gene segments have shown that, of the three reading frames (*i.e.,* stop, hydrophobic, and hydrophilic), the stop frame is used very infrequently. Apparently, some stop frames are chewed back and result in expression. However, stop reading frames are used at such a low frequency that for the purposes of engineering histidine codons, it is more efficient not to use the stop reading frame. As between hydrophilic and hydrophobic reading frames, the hydrophilic reading frame appears to be preferred. Thus, in one embodiment, the hydrophilic reading frame of human D gene segments is engineered to contain one or more histidine codons (as compared with the stop frame or with the hydrophobic frame).

[000697] Methods of introducing a mutation *in vitro*, *e.g.*, site-directed mutagenesis, are well known in the art. In some embodiments of the described invention, histidine codons are enriched by designing histidine-substituted human D gene segments *in silico* (*e.g.,* mutation of Y, D, and N codons to H codons, *e.g.*, CAT, CAC), which are synthesized (*e.g.,* chemical synthesis) with (unique) restriction enzyme sites for ligating them back together. The synthesized D gene segments are made with the appropriate recombination signal sequences (RSS) upstream and downstream. In one embodiment, when ligated to one another, the synthesized histidine-substituted D gene segments include the intergenic sequences observed in a human between each D gene segment.

[000698] It is understood that the codons that encode the one or more histidines, upon rearrangement and/or somatic hypermutation, may change such that one or more of the histidines will be changed to another amino acid. However, this may not occur for each and

every codon encoding histidine, in each and every rearrangement in the non-human animal. If such changes occur, the changes may occur in some but not all B cells or in some but not all heavy chain variable sequences.

[000699] In various aspects, the genetically modified immunoglobulin locus comprises a human heavy chain V, D, and J gene segment, wherein at least one of the human D gene segment has been inverted 5' to 3' with respect to a corresponding wild-type sequence, and wherein at least one reading frame of the inverted human D gene segment comprises a histidine codon.

[000700] In various embodiments, the nucleotide sequence comprises one or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 21 or more, 22 or more, 23 or more, 24 or more, or 25 or more of histidine codons.

[000701] There are 25 functional human D gene segments in 6 families of 3-5 members each (one family- the D7 family- has a single member). Direct recombination of human D gene segments is much more frequent than inversion, although inverted reading frames exhibit more histidine codons. Certain D gene segments and reading frames are used more frequently than others. All three direct reading frames and all three inverted orientation reading frames for all the functional D gene segments are presented in FIGs. 10A-10E. As shown in FIGs. 10A-10E, there are many more histidine codons in inverted reading frames than in direct reading frames. More specifically, there are 34 histidines in inverted reading frames and only four in direct reading frames. In addition, of the four in direct reading frames, three histidines are encoded by pseudogenes or present in alternate alleles. Therefore, there is only a single direct reading frame of a germline human D gene segment that contains a histidine codon, with further histidine codons possibly encountered in alternate alleles (presumably in subsets of the human population).

[000702] Inverted D rearrangements are extremely rare. Tuaillon et al. (J. Immunol., 154(12): 5453-6465) showed that usage of inverted reading frames (as measured by limiting dilution PCT) is very rare, *i.e.*, that the ratio of direct to indirect rearrangements are, in most cases, 100 to 1000. To the extent that the ratio of direct to indirect rearrangement was low, it was only observed in those D segments that exhibit very low usage. It was also shown that D gene segment family 7, which is located adjacent to J1 (far down from other D family members) is mostly used in fetuses, but exhibits a low usage in adults (Schroeder et al., Immunology 30, 2006, 119-135). Therefore, in one embodiment, D family 7 sequences are

not inverted 5' to 3'.

[000703] In one embodiment, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, at least fifteen, at least sixteen, at least seventeen, at least eighteen, at least nineteen, at least twenty, at least twenty one, at least twenty two, at least twenty three, at least twenty four, or all or substantially all of the human functional D gene segments are inverted 5' to 3' with respect to corresponding wild type sequences.

[000704] In one embodiment, the human immunoglobulin heavy chain variable domain comprising at least one non-naturally occurring histidine residue exhibits pH-dependent antigen binding characteristics. For example, an antibody comprising the modified immunoglobulin heavy chain variable domain binds a target with sufficient affinity at around a neutral pH (*e.g.*, pH of about 7.0 to about 7.4), but either does not bind or binds weaker to the same target at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0). In one embodiment, the acidic pH is selected from about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, and about 6.0. In one embodiment, the neutral pH is selected from about 7.0, about 7.1, about 7.2, about 7.3, and about 7.4.

[000705] In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 2 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 25°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin heavy chain locus as described herein has a dissociative half-life ($t_{1/2}$) of less than 1 min at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) at 37°C. In one embodiment, an antigen-binding protein comprising a heavy chain variable domain expressed by the genetically modified immunoglobulin locus as described herein has at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold decrease in dissociative half-life ($t_{1/2}$) at an acidic pH (*e.g.*, pH of about 5.5 to about 6.0) as compared to

the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH (*e.g.,* pH of about 7.0 to about 7.4).

[000706] In one embodiment, antigen binding proteins comprising the genetically modified human immunoglobulin heavy chain variable domain is capable of specifically binding an antigen of interest with an affinity (K_D) lower than 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} or 10^{-10}, 10^{-11}, 10^{-12} at a neutral or physiological pH (pH of about 7.0 to about 7.4).

[000707] The altered binding property of the immunoglobulin heavy chain variable domain at an acidic pH (*e.g.,* pH of about 5.5 to about 6.0) would, in some circumstances, allow faster turnover of the antibody because the therapeutic antibody will bind a target on a cell's surface, be internalized into an endosome, and more readily or more rapidly dissociate from the target in the endosome, so that the therapeutic can be recycled to bind yet another molecule of target present in another cell. This would allow one to administer a therapeutic antibody at a lower dose, or administer the therapeutic antibody less frequently. This is particularly useful in a situation where it is not desirable to administer a therapeutic antibody frequently, or administer at a level above a certain dosage for safety or toxicity reasons.

[000708] In various embodiments, the human immunoglobulin heavy chain variable region nucleotide sequence as described herein is operably linked to a human or non-human heavy chain constant region nucleotide sequence (*e.g.*, a heavy chain constant region nucleotide sequence that encodes an immunoglobulin isotype selected from IgM, IgD, IgG, IgE, and IgA). In various embodiments, the human or non-human heavy chain constant region nucleotide sequence is selected from the group consisting of a C_H1, a hinge, a C_{H2}, a C_{H3}, and a combination thereof. In one embodiment, the constant region nucleotide sequence comprises a C_H1, a hinge, a C_H2, and a C_H3 (*e.g.*, C_H1-hinge-a C_H2-C_H3).

[000709] In various embodiments, the heavy chain constant region nucleotide sequence is present at an endogenous locus (*i.e.*, where the nucleotide sequence is located in a wild-type non-human animal) or present ectopically (*e.g.*, at a locus different from the endogenous immunoglobulin chain locus in its genome, or within its endogenous locus, *e.g.*, within an immunoglobulin variable locus, wherein the endogenous locus is placed or moved to a different location in the genome).

[000710] In one embodiment, the heavy chain constant region nucleotide sequence comprises a modification in a C_H2 or a C_H3, wherein the modification increases the affinity of the heavy chain constant region amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000711] The neonatal Fc receptor for IgG (FcRn) has been well characterized in the transfer of passive humoral immunity from a mother to her fetus across the placenta and proximal small intestine (Roopenian, D. and Akilesh, S., Nat. Rev. Immun., 2007, 7:715-725,). FcRn binds to the Fc portion of IgG at a site that is distinct from the binding sites of the classical FcγRs or the C1q component of complement, which initiates the classical pathway of complement activation. More specifically, it was shown that FcRn binds the C_H2-C_H3 hinge region of IgG antibodies - a versatile region of Fc that also binds *Staphylococcal* protein A, *Streptococcal* protein G, and the rheumatoid factor. In contrast to other Fc-binding proteins, however, FcRn binds the Fc region of IgG in a strictly pH-dependent manner; at physiological pH 7.4, FcRn does not bind IgG, whereas at the acidic pH of the endosome (*e.g.*, where the pH ranges from about 5.5 to about 6.0), FcRn exhibits a low micromolar to nanomolar affinity for the Fc region of IgG. This pH-dependent interaction has been shown to be mediated by the titration of histidine residues in the C_H2-C_H3 region of IgG and their subsequent interaction with acidic residue on the surface of FcRn (Roopenian, D. and Akilesh, S., Nat. Rev. Immun., 2007, 7:715-725).

[000712] Various mutations in the C_H2-C_H3 region of IgG that can increase the affinity of Fc region to FcRn at an acidic pH are known in the art. These include, but are not limited to, modification at position 250 (*e.g.,* E or Q); 250 and 428 (*e.g.,* L or F); 252 (*e.g.,* L/Y/F/W or T), 254 (*e.g.,* S or T), and 256 (*e.g.,* S/R/Q/E/D or T); or a modification at 428 and/or 433 (*e.g.,* L/R/S/P/Q or K) and/or 434 (*e.g.,* H/F or Y); or a modification at 250 and/or 428; or a modification at 307 or 308 (*e.g.,* 308F, V308F), and 434. In another example, the modification can comprise a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and a 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 52Y, 254T, and 256E) modification; a 250Q and 428L modification, or a combination thereof.

[000713] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 252 and 257, wherein the modification increases the affinity of the human C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000714] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H2 amino acid sequence comprising at least one modification between amino acid residues at positions 307 and 311, wherein the modification increases the affinity of the C_H2 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000715] In one embodiment, the heavy chain constant region nucleotide sequence encodes a human C_H3 amino acid sequence, wherein the C_H3 amino acid sequence comprises at least one modification between amino acid residues at positions 433 and 436, wherein the modification increases the affinity of the C_H3 amino acid sequence to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0).

[000716] In one embodiment, the human constant region amino acid sequence encoded by the heavy chain constant region nucleotide sequence described herein comprises a mutation selected from the group consisting of M428L, N434S, and a combination thereof. In one embodiment, the human constant region amino acid sequence comprises a mutation selected from the group consisting of M428L, V259I, V308F, and a combination thereof. In one embodiment, the human constant region amino acid sequence comprises an N434A mutation. In one embodiment, the human constant region amino acid sequence comprises a mutation selected from the group consisting of M252Y, S254T, T256E, and a combination thereof. In one embodiment, the human constant region amino acid sequence comprises a mutation selected from the group consisting of T250Q, M248L, or both. In one embodiment, the human constant region amino acid sequence comprises a mutation selected from the group consisting of H433K, N434Y, or both.

[000717] In one embodiment, the heavy chain constant region amino acid sequence is a non-human constant region amino acid sequence, and the heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

[000718] In one embodiment, the heavy chain constant region nucleotide sequence is a human heavy chain constant region amino acid sequence, and the human heavy chain constant region amino acid sequence comprises one or more of any of the types of modifications described above.

Engineered Histidine Residues In Immunoglobulin Light Chain Genes

[000719] In various embodiments, genetically modified non-human animals (*e.g.,* mammals, *e.g.,* mice, rats, rabbits, *etc.*) are provided that comprise in their genome, *e.g.,* in their germline, nucleotide sequence(s) encoding human antibody molecules that exhibit pH-dependent antigen binding, *e.g.,* a nucleotide sequence of immunoglobulin light chain comprising rearranged human immunoglobulin light chain variable region sequence encoding antibodies that exhibits pH-dependent antigen binding; embryos, cells, and tissues comprising the same; methods of making the same; as well as methods of using the same.

[000720] The inventors have discovered that non-human animals that express antibodies that are capable of binding to an antigen in a pH dependent manner can be made by making modifications of an immunoglobulin light chain variable region at one or more positions along the sequence of the light chain. Methods of making modifications in the germline of a non-human animal so that the animal would express histidines in CDRs of antibodies are described. In particular, methods for making modifications in an immunoglobulin light chain variable sequence in the germline of the mouse are described. Variable region sequence, *e.g.,* of light chains, typically show somatic hypermutation along the variable region sequence, and, in some cases, such mutations can result in a substitution of histidine residues (see, *e.g.,* FIG. 15). Such mutations can even occur in complementary determining regions (CDRs), which are the regions of variable domains responsible for antigen binding. In some cases, such mutations can result in antibodies that display pH-dependent antigen binding, *e.g.,* reduced antigen binding at an acidic pH as compared to antigen binding at a neutral pH. Such pH-dependent antigen binding is desired because it may enable the antibody to bind to the antigen outside the cell, and, when internalized into an endosome, release the antigen and recycle back to the surface to bind another antigen, avoiding target-mediated clearance. Approaches for introducing histidine residues to achieve this effect by using a *random* his-scanning mutagenesis to engineer pH-dependent binding properties in anti-IL-6R antibodies have been reported (US 2011/0111406 A1). However, random mutagenesis of antibody residues may result in decreased affinity of antibody to the antigen. A non-human animal genetically modified to express a histidine substitution in antibody sequence enables generation of high-affinity antibodies in response to an antigen of interest that, due to histidine modification(s), would also display pH-dependent antigen binding.

[000721] Thus, in various embodiments, provided herein is a genetically modified non-human animal (*e.g.,* rodent, *e.g.,* a mouse or a rat) that comprises in its genome, *e.g.,* its germline, a human immunoglobulin light chain variable region sequence comprising modifications that result in the animal expressing antibodies capable of binding to antigens in a pH-dependent manner. In one embodiment, the non-human animal comprises modifications in the human immunoglobulin light chain variable region sequence (*e.g.,* V_L and/or J_L segment sequence) that comprise substitutions in at least one non-histidine codon with a histidine codon (in some cases, also may be referred to as "histidine substitution," "histidine codon substitution," or the like). In one embodiment, the animal comprises at least one substitution of a non-histidine codon with a histidine codon in a nucleotide sequence of a complementary determining region (CDR; *e.g.,* CDR1, CDR2, and/or CDR3) of a human immunoglobulin light chain. In one embodiment, the substitution is in a CDR3 codon. In one embodiment, the light chain is a κ light chain. In one embodiment, the animal expresses an

immunoglobulin light chain, *e.g.,* a light chain CDR, *e.g.,* a light chain CDR3, comprising a substitution of at least one amino acid with a histidine. In another embodiment, the light chain is a λ light chain. In yet another embodiment, the mouse comprises a substitution of at least one non-histidine codon with a histidine codon in both κ and λ light chains.

[000722] A histidine residue is encoded by two different codons, CAT and CAC (deoxyribonucleic acid residues). Thus, a non-histidine codon may be substituted with a CAT or a CAC. The substitution is engineered in a codon that in its germline configuration (*i.e.,* non-somatically mutated state) does not encode a histidine residue.

[000723] In one embodiment a light chain is a universal light chain (also termed a common light chain). As described in U.S. Patent Application Nos. 13/022,759, 13/093,156, 13/412,936 and 13/488,628 (U.S. Application Publication Nos. 2011/0195454, 2012/0021409, 2012/0192300 and 2013/0045492), a non-human animal (e.g., a mouse) that selects a common light chain for a plurality of heavy chains has a practical utility. In various embodiments, antibodies expressed in a non-human animal comprising only a common light chain will have heavy chains that can associate and express with an identical or substantially identical light chain. This is particularly useful in making bispecific antibodies. For example, such an animal can be immunized with a first immunogen to generate a B cell that expresses an antibody that specifically binds a first epitope. The animal (or an animal genetically the same) can be immunized with a second immunogen to generate a B cell that expresses an antibody that specifically binds the second epitope. Variable heavy chain regions can be cloned from the B cells and expressed with the same heavy chain constant region and the same light chain (e.g., a common light chain) in a cell to make a bispecific antibody, wherein the heavy chain component of the bispecific antibody has been selected by an animal to associate and express with the same light chain component. In various embodiments described, the variable regions of the genetically engineered mice are human variable regions.

[000724] Thus, a mouse was engineered that is capable of generating immunoglobulin light chains that will suitably pair with a rather diverse family of heavy chains, including heavy chains whose human variable regions depart from germline sequences, *e.g.,* affinity matured or somatically mutated variable regions. In various embodiments, the mouse is devised to pair human light chain variable domains with human heavy chain variable domains that comprise somatic mutations, thus enabling a route to high affinity binding proteins suitable for use as human therapeutics.

[000725] The genetically engineered mouse, through the long and complex process of

antibody selection within an organism, makes biologically appropriate choices in pairing a diverse collection of human heavy chain variable domains with a limited number of human light chain options. In order to achieve this, the mouse is engineered to present a limited number of human light chain variable domain options in conjunction with a wide diversity of human heavy chain variable domain options. Upon challenge with an immunogen, the mouse maximizes the number of solutions in its repertoire to develop an antibody to the immunogen, limited largely or solely by the number or light chain options in its repertoire. In various embodiments, this includes allowing the mouse to achieve suitable and compatible somatic mutations of the light chain variable domain that will nonetheless be compatible with a relatively large variety of human heavy chain variable domains, including in particular somatically mutated human heavy chain variable domains.

[000726] The engineered common light chain mouse described in U.S. Application Publication Nos. 2011/0195454, 2012/0021409, 2012/0192300 and 2013/0045492 comprised nucleic acid sequence encoding a limited repertoire of light chain options, e.g., common or universal light chain "ULC" that comprised no more than two V_L segments or a single rearranged human immunoglobulin light chain variable region sequence. To achieve such limited repertoire, a mouse was engineered to render nonfunctional or substantially nonfunctional its ability to make, or rearrange, a native mouse light chain variable domain. In one aspect, this was achieved, *e.g.,* by deleting the mouse's light chain variable region gene segments. As previously described, the endogenous mouse locus can then be modified by exogenous suitable human light chain variable region gene segments of choice, operably linked to the endogenous mouse light chain constant domain, in a manner such that the exogenous human variable region gene segments can combine with the endogenous mouse light chain constant region gene and form a rearranged reverse chimeric light chain gene (human variable, mouse constant). In various embodiments, the light chain variable region is capable of being somatically mutated. In various embodiments, to maximize ability of the light chain variable region to acquire somatic mutations, the appropriate enhancer(s) is retained in the mouse. In one aspect, in modifying a mouse κ light chain locus to replace endogenous mouse κ light chain gene segments with human κ light chain gene segments, the mouse κ intronic enhancer and mouse κ 3' enhancer are functionally maintained, or undisrupted.

[000727] Thus, provided was a genetically engineered mouse that expresses a limited repertoire of reverse chimeric (human variable, mouse constant) light chains associated with a diversity of reverse chimeric (human variable, mouse constant) heavy chains. In various embodiments, the endogenous mouse κ light chain gene segments are deleted and

replaced with a single (or two) rearranged human light chain region, operably linked to the endogenous mouse Cκ gene. In embodiments for maximizing somatic hypermutation of the rearranged human light chain region, the mouse κ intronic enhancer and the mouse κ 3' enhancer are maintained. In various embodiments, the mouse also comprises a nonfunctional λ light chain locus, or a deletion thereof or a deletion that renders the locus unable to make a λ light chain.

[000728] The universal light chain mouse generated antibodies in response to various antigens that were capable of utilizing a diverse repertoire of heavy chain variable region sequences, comprising a diverse repertoire of V_H, D_H, and J_H segments. Antibodies generated in such genetically engineered ULC mouse are useful for designing bispecific therapeutic antibodies; however, as with any other antibody, each bispecific antibody may only bind to one target during its lifetime in the plasma; the antibody is internalized into an endosome and targeted for lysosomal degradation. Studies have shown that MHC-class-I-like Fcγ receptor FcRn is capable of rescuing immunoglobulins from lysosomal degradation by recycling it back to the cell surface from the sorting endosome. Simister and Mostov (1989) An Fc receptor structurally related to MHC class I antigens. Nature 337: 184-87. As explained above, to improve efficiency of antibody recycling, further modifications to antibody sequences, e.g., modifications that result in decreased antigen binding at acidic pH (e.g., pH of the endosome), while retaining antibody-antigen affinity and specificity at neutral pH (e.g., pH of body fluids such as blood) are beneficial. The non-human animals described herein, wherein histidine residues are substituted for non-histidine residues in the a universal light chain sequence are beneficial because they are capable of producing high-affinity antibodies based on universal light chain format that also display pH-dependent binding, e.g., display reduced binding to the antigen at acidic versus neutral pH.

[000729] Thus, in one embodiment, provided herein is a non-human animal (e.g., a rodent, e.g., a mouse or a rat) that comprises in its genome, e.g., in its germline, a limited repertoire of human light chain variable regions, or a single human light chain variable region, from a limited repertoire of human light chain variable gene segments, wherein the human light chain variable region(s) comprise at least one substitution of a non-histidine codon for a histidine codon. In some embodiments, provided non-human animals are genetically engineered to include a single unrearranged human light chain variable region gene segment (or two human light chain variable region gene segments) that rearranges to form a rearranged human light chain variable region gene (or two rearranged light chain variable region genes) that expresses a single light chain (or that express either or both of two light chains), wherein the light chain variable region gene(s) comprise a substitution of at

least one non-histidine codon with a histidine codon. The rearranged human light chain variable domains encoded by these histidine-substituted light chain variable region gene(s) are capable of pairing with a plurality of affinity-matured human heavy chains selected by the animals, wherein the heavy chain variable regions specifically bind different epitopes. In various embodiments, the at least one substitution of a non-histidine residue with a histidine residue results in a rearranged human light chain that, when expressed with a cognate heavy chain, binds to its antigen in a pH-dependent manner.

[000730] Genetically engineered animals are provided that express a limited repertoire of human light chain variable domains, or a single human light chain variable domain, from a limited repertoire of human light chain variable region gene sequences, wherein the variable region gene sequences comprise at least one substitution of a non-histidine codon with a histidine codon. In some embodiments, provided animals are genetically engineered to include a single V/J human light chain sequence (or two V/J sequences) that comprises a substitution of at least one non-histidine codon with a histidine codon and expresses a variable region of a single light chain (or that express either or both of two variable regions). In one aspect, a light chain comprising the variable sequence is capable of pairing with a plurality of affinity-matured human heavy chains clonally selected by the animal, wherein the heavy chain variable regions specifically bind different epitopes. In one embodiment, the antibody binds to its antigen(s) in a pH-dependent manner. In one embodiment, the single V/J human light chain sequence is selected from Vκ1-39Jκ5 and Vκ3-20Jκ1. In one embodiment, the two V/J sequences are Vκ1-39Jκ5 and Vκ3-20Jκ1. In one embodiment, the Vκ1-39Jκ5 and Vκ3-20Jκ1 sequences are rearranged V/J sequences.

[000731] In one aspect, a genetically modified non-human animal is provided that comprises a single human immunoglobulin light chain V_L gene segment that is capable of rearranging with a human J_L gene segment (selected from one or a plurality of J_L segments) and encoding a human variable domain of an immunoglobulin light chain, wherein the single human immunoglobulin light chain V_L gene segment and/or human J_L gene segment comprise a substitution of at least one non-histidine codon with a histidine codon. In another aspect, a genetically modified mouse is provided that comprises no more than two human V_L gene segments, each of which is capable of rearranging with a human J_L gene segment (selected from one or a plurality of J_L segments) and encoding a human variable domain of an immunoglobulin light chain, wherein each of the no more than two V_L gene segments and/or the J_L gene segment comprise a substitution of at least one non-histidine residue with a histidine residue.

[000732] Also provided herein is a genetically modified non-human animal that comprises

in its genome, e.g., in its germline, a single rearranged human immunoglobulin light chain variable region sequence comprising a human V_L and J_L sequences wherein the single rearranged human immunoglobulin light chain variable region comprises a substitution of at least one non-histidine codon with a histidine codon. In one aspect, the single rearranged human immunoglobulin light chain variable region sequence is derived from human germline V_L and J_L gene sequences, but for the histidine substitution(s). In one embodiment, the human immunoglobulin light chain is a human immunoglobulin κ chain. Thus, in one embodiment, the human V_L gene sequence is selected from Vκ1-39 and Vκ3-20. In one embodiment, the single rearranged human immunoglobulin light chain variable region sequence comprises rearranged Vκ1-39/J or Vκ3-20/J sequence. In one embodiment, the human J_L gene sequence is selected from Jκ1, Jκ2, Jκ3, Jκ4, and Jκ5. In one embodiment the human J_L sequence is selected from Jκ1 and Jκ5. In one embodiment, the single rearranged human immunoglobulin light chain variable region sequence is selected from Vκ1-39Jκ5 and Vκ3-20Jκ1 (e.g., but for the histidine substitution(s)). In an alternative embodiment, the human immunoglobulin light chain is a human λ chain.

[000733] In one embodiment, the substitution of at least one non-histidine codon for a histidine codon is in the nucleotide sequence encoding a complementary determining region (CDR) of the light chain variable domain. In one embodiment, the substitution of at least one non-histidine codon for a histidine codon is in the nucleotide sequence encoding CDR1, CDR2 or CDR3 of the light chain variable domain. In one specific embodiment, the substitution is in the nucleotide sequence encoding CDR3.

[000734] In one aspect, the substitution is of at least one non-histidine codon for a histidine codon in the CDR3 codon of the human light chain variable region gene sequence. In one embodiment, the substitution is of one, two, three, four, or more CDR3 codons. In the embodiment wherein the single rearranged human immunoglobulin light chain variable region is a Vκ1-39Jκ5 variable region, the replacement of at least one non-histidine codon with a histidine codon comprises a replacement at a position in the immunoglobulin light chain gene sequence encoding CDR3 designed to express histidine at position selected from 105, 106, 108, 111, and a combination thereof. In one embodiment, the replacement is designed to express histidine at positions 105 and 106. In one embodiment, the replacement is designed to express histidine at positions 105 and 111. In one embodiment, the replacement is designed to express histidine at positions 105 and 108. In one embodiment, the replacement is designed to express histidine at positions 105, 108 and 111. In one embodiment, the replacement is designed to express histidine at positions 105, 106, and 108. In one embodiment, the replacement is designed to express histidine at

positions 106 and 108. In one embodiment, the replacement is designed to express histidine at positions 106 and 111. In one embodiment, the replacement is designed to express histidine at positions 108 and 111. In one embodiment, the replacement is designed to express histidine at positions 106, 108, and 111. In yet another embodiment, the replacement is designed to express histidine at positions 106, 108 and 111. In one embodiment, the replacement is designed to express histidine at positions 105, 106, and 111. In one embodiment, the replacement is designed to express histidine at positions 105, 106, 108, and 111. The nucleic acid and amino acid sequences of the histidine-substituted regions are depicted in sequence alignment of FIG. 16 and set forth in SEQ ID NOs: 327-357.

[000735] In the embodiment wherein the single rearranged human immunoglobulin light chain variable region is a $V_\kappa 3\text{-}20J_\kappa 1$ variable region, the replacement of at least one non-histidine codon with a histidine codon comprises a replacement at a position in the immunoglobulin light chain gene sequence encoding CDR3 region that is designed to express histidine at position selected from 105, 106, 107, 109, and a combination thereof. In one embodiment, the replacement is designed to express histidine at positions 105 and 106. In one embodiment, the replacement is designed to express histidine at positions 105 and 107. In one embodiment, the replacement is designed to express histidine at positions 105 and 109. In one embodiment, the replacement is designed to express histidine at positions 106 and 107. In one embodiment, the replacement is designed to express histidine at positions 106 and 109. In one embodiment, the replacement is designed to express histidine at positions 107 and 109. In one embodiment, the replacement is designed to express histidine at positions 105, 106, and 107. In one embodiment, the replacement is designed to express histidine at positions 105, 107, and 109. In one embodiment, the replacement is designed to express histidine at positions 106, 108, and 111. In one embodiment, the replacement is designed to express histidine at positions 105, 106 and 109. In another embodiment, the replacement is designed to express histidine at positions 105, 106, 107, and 109. The nucleic acid and amino acid sequences of exemplary histidine-substituted regions are depicted in sequence alignment of FIG. 27 and set forth in SEQ ID NOs: 398-403.

[000736] Amino acid positions (105, 106, etc.) are based on a unique numbering described in Lefranc et al. (2003) Dev. Comp. Immunol. 27:55-77, and can also be viewed on www.imgt.org.

[000737] In one embodiment, the human V_L gene segment is operably linked to a human or non-human leader sequence. In one embodiment, the leader sequence is a non-human

leader sequence. In a specific embodiment, the non-human leader sequence is a mouse Vκ3-7 leader sequence. In a specific embodiment, the leader sequence is operably linked to an unrearranged human V_L gene segment. In a specific embodiment, the leader sequence is operably linked to a rearranged human V_L/J_L sequence. Thus, in one specific embodiment, the single rearranged Vκ1-39/Jκ5 or Vκ3-20/Jκ1 variable region gene sequence comprising at least one histidine substitution is operably linked to a mouse Vκ3-7 leader sequence.

[000738] In one embodiment, the V_L gene segment is operably linked to an immunoglobulin promoter sequence. In one embodiment, the promoter sequence is a human promoter sequence. In a specific embodiment, the human immunoglobulin promoter is a human Vκ3-15 promoter. In a specific embodiment, the promoter is operably linked to an unrearranged human V_L gene segment. In a specific embodiment, the promoter is operably linked to a rearranged human V_L/J_L sequence. Thus, in one specific embodiment, the single rearranged Vκ1-39/Jκ5 or Vκ3-20/Jκ1 variable region gene sequence comprising at least one histidine substitution is operably linked to the human Vκ3-15 promoter.

[000739] In one embodiment, the light chain locus comprises a leader sequence flanked 5' (with respect to transcriptional direction of a V_L gene segment) with a human immunoglobulin promoter and flanked 3' with a human V_L gene segment that rearranges with a human J_L segment and encodes a variable domain of a reverse chimeric light chain comprising an endogenous non-human light chain constant region (C_L). In a specific embodiment, the V_L and J_L gene segments are at the non-human Vκ locus, and the non-human C_L is a non-human Cκ (*e.g.,* mouse Cκ). In one specific embodiment, the variable region sequence is operably linked to the non-human constant region sequence, *e.g.,* the non-human Cκ gene sequence.

[000740] In one embodiment, the light chain locus comprises a leader sequence flanked 5' (with respect to transcriptional direction of a V_L gene segment) with a human immunoglobulin promoter and flanked 3' with a rearranged human variable region sequence (V_L/J_L sequence) and encodes a variable domain of a reverse chimeric light chain comprising an endogenous non-human light chain constant region (C_L). In a specific embodiment, the rearranged human V_L/J_L sequence is at the non-human kappa (κ) locus, and the non-human C_L is a non-human Cκ. In one specific embodiment, the rearranged human variable region sequence is operably linked to the non-human immunoglobulin light chain constant region sequence, *e.g.,* the non-human Cκ gene sequence. In one embodiment, the non-human immunoglobulin light chain constant region sequence is an

endogenous non-human sequence. In one embodiment, the non-human animal is a mouse and the Cκ gene sequence is a mouse Cκ gene sequence. In one embodiment, the rearranged human immunoglobulin light chain variable region sequence comprising a substitution of at least one non-histidine codon with a histidine codon is at the endogenous non-human (*e.g.,* mouse) immunoglobulin light chain locus (κ locus). Exemplary embodiments of the locus are presented in FIGs. 23C, 23E, 29C, and 29D.

[000741] In one embodiment, the genetically modified non-human animal is a mouse, and the variable region locus of the mouse is a κ light chain locus, and the κ light chain locus comprises a mouse κ intronic enhancer, a mouse κ 3' enhancer, or both an intronic enhancer and a 3' enhancer.

[000742] In one embodiment, the non-human animal (*e.g.,* a rodent, *e.g.,* a rat or a mouse) comprises a nonfunctional immunoglobulin lambda (λ) light chain locus. In a specific embodiment, the λ light chain locus comprises a deletion of one or more sequences of the locus, wherein the one or more deletions renders the λ light chain locus incapable of rearranging to form a light chain gene. In another embodiment, all or substantially all of the V_L gene segments of the λ light chain locus are deleted. In one embodiment, the non-human animal (*e.g.,* rodent, *e.g.* mouse or rat) comprises a rearranged human immunoglobulin light chain variable region sequence comprising a substitution of at least one non-histidine codon with a histidine codon, and lacks a functional unrearranged immunoglobulin light chain variable region, e.g., endogenous unrearranged light chain variable region. In one embodiment, the rearranged, histidine-substituted human immunoglobulin light chain variable region gene sequence replaces endogenous unrearranged immunoglobulin light chain variable region gene sequence.

[000743] In one embodiment, the animal makes a light chain that comprises a somatically mutated variable domain derived from a human variable region sequence that comprises a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, the light chain comprises a somatically mutated variable domain derived from a human variable region sequence that comprises a substitution of at least one non-histidine codon with a histidine codon, and a non-human Cκ region. In one embodiment, the non-human animal does not express a λ light chain.

[000744] One skilled in the art would appreciate that although substitution(s) of at least one non-histidine residue with a histidine residue is genetically engineered into the human immunoglobulin light chain variable region, due to somatic hypermutations, not all antibodies

that are generated in the genetically modified non-human animal will harbor that histidine residue(s) at engineered position(s). However, generation of a wide repertoire of antibodies in the non-human animal will allow to select for *in vivo* generated antigen-specific antibodies that display high affinity for an antigen of interest while retaining histidine modifications introduced into the germline and, thus, exhibiting pH-dependent antigen binding.

[000745] Thus, in one embodiment, the animal retains at least one substitution of a non-histidine amino acid with a histidine. In one embodiment, the animal retains all or substantially all histidine substitutions in its somatically mutated light chain variable domain that were introduced into its variable region gene.

[000746] In one embodiment, the genetically modified non-human animal described herein also comprises in its genome, *e.g.,* its germline, an unrearranged immunoglobulin heavy chain variable region comprising V_H, D_H, and J_H gene segment sequences. In one embodiment, the V_H, D_H, and J_H gene segment sequences are human V_H, D_H, and J_H gene segment sequences, and the unrearranged immunoglobulin heavy chain variable region is a human heavy chain variable region. In one embodiment, the human V_H, D_H, and J_H gene segment sequences are operably linked to non-human heavy chain constant region sequence. In one embodiment, the non-human heavy chain constant region sequence is an endogenous non-human heavy chain constant region sequence. In one embodiment, the human heavy chain gene segment sequences are at the endogenous non-human immunoglobulin heavy chain locus. In one embodiment, the human immunoglobulin heavy chain variable region sequence comprised in a non-human animal also comprises a substitution of at least one non-histidine codon for a histidine codon.

[000747] In one embodiment, the non-human animal described herein expresses an immunoglobulin light chain that comprises a non-human light chain constant region sequence. In one embodiment, the non-human animal expresses an immunoglobulin light chain that comprises a human light chain constant region sequence.

[000748] In one embodiment, the non-human animal described herein expresses an immunoglobulin heavy chain that comprises a non-human sequence selected from a C_H1 sequence, a hinge sequence, a C_H2 sequence, a C_H3 sequence, and a combination thereof.

[000749] In one embodiment, the non-human animal expresses an immunoglobulin heavy chain that comprises a human sequence selected from a C_H1 sequence, a hinge sequence, a C_H2 sequence, a C_H3 sequence, and a combination thereof.

[000750] In the embodiment where the animal comprises a single rearranged

immunoglobulin light chain variable region comprising a substitution of at least one non-histidine codon with a histidine codon, the rearranged immunoglobulin light chain sequence in the germline of the animal is at an endogenous non-human immunoglobulin light chain locus. In a specific embodiment, the rearranged immunoglobulin light chain sequence comprising a substitution of at least one non-histidine codon with a histidine codon in the germline of the animal replaces all or substantially all endogenous non-human light chain V and J segment sequences at the endogenous non-human immunoglobulin light chain locus.

[000751] In one embodiment, the non-human animal comprises a replacement of endogenous V_H gene segments with one or more human V_H gene segments, wherein the human V_H gene segments are operably linked to a non-human C_H region gene, such that the non-human animal rearranges the human V_H gene segments and expresses a reverse chimeric immunoglobulin heavy chain that comprises a human V_H domain and a non-human C_H. In one embodiment, 90-100% of unrearranged non-human V_H gene segments are replaced with at least one unrearranged human V_H gene segment. In a specific embodiment, all or substantially all (*e.g.,* 90-100%) of the endogenous non-human V_H gene segments are replaced with at least one unrearranged human V_H gene segment. In one embodiment, the replacement is with at least 19, at least 39, or at least 80 or 81 unrearranged human V_H gene segments. In one embodiment, the replacement is with at least 12 functional unrearranged human V_H gene segments, at least 25 functional unrearranged human V_H gene segments, or at least 43 functional unrearranged human V_H gene segments. In one embodiment, the non-human animal comprises a replacement of all non-human D_H and J_H segments with at least one unrearranged human D_H segment and at least one unrearranged human J_H segment. In one embodiment, the non-human animal comprises a replacement of all non-human D_H and J_H segments with all unrearranged human D_H segments and all unrearranged human J_H segments.

[000752] A non-human animal, *e.g.,* a mouse, comprising in its genome, *e.g.,* its germline, a limited repertoire of human immunoglobulin light chain variable regions, *e.g.,* a single rearranged human immunoglobulin light chain variable region (*e.g.,* Vκ1-39/Jκ5 or Vκ3-20/Jκ1), with a substitution of at least one non-histidine codon with a histidine codon and a diverse repertoire of unrearranged human V_H, D_H, and J_H segments is capable of generating antigen binding proteins encoded by heavy chain variable region sequences derived from various permutations of unrearranged human V_H, D_H, and J_H segments, wherein the V_H, D_H, and J_H segments present in the heavy chain variable sequences are derived from all or substantially all functional human V_H, D_H, and J_H segments present in the genome of the animal. Various available possibilities for heavy chain variable domain sequences

expressed in the cells, *e.g.*, B cells, of the genetically modified animals described herein (*i.e.,* derived from combinations of various functional human V, D, and J segments) are described in U.S. Application Publication Nos. 2011/0195454, 2012/0021409, 2012/0192300 and 2013/0045492. In various embodiments, the rearranged human immunoglobulin light chain variable region sequence comprising substitution(s) of at least one non-histidine codon with a histidine codon and the unrearranged human immunoglobulin heavy chain variable region sequence are comprised in the germline of the non-human animal.

[000753] In one embodiment, the non-human animal comprises one copy of one or both of the rearranged human immunoglobulin light chain variable region sequence comprising substitution(s) of at least one non-histidine codon with a histidine codon and the unrearranged human immunoglobulin heavy chain variable region sequence. In another embodiment, the non-human animal comprises two copies of one or both of the rearranged human immunoglobulin light chain variable region sequence comprising substitution(s) of at least one non-histidine codon with a histidine codon and the unrearranged human immunoglobulin heavy chain variable region sequence. Thus, the non-human animal may be homozygous or heterozygous for one or both the rearranged human immunoglobulin light chain variable region sequence comprising substitution(s) of at least one non-histidine codon with a histidine codon and the unrearranged human immunoglobulin heavy chain variable region sequence.

[000754] In addition to genetically modified non-human animals comprising in their genome an immunoglobulin light chain variable region gene sequence (e.g., a single rearranged immunoglobulin light chain variable region gene sequence) comprising substitution of at least one non-histidine codon with a histidine codon (*e.g.,* in CDR3 of the light chain), also provided herein are genetically modified non-human animals comprising an immunoglobulin light chain variable region gene sequence with one or more additions of histidine codon(s), such that the expressed variable domain comprises an additional amino acid(s) which, if not subject to somatic hypermutation, is a histidine.

[000755] The genetically modified non-human animal comprising a human immunoglobulin light chain variable region gene sequence with a substitution of at least one non-histidine codon with a histidine codon described herein may be selected from a group consisting of a mouse, rat, rabbit, pig, bovine (*e.g.,* cow, bull, buffalo), deer, sheep, goat, chicken, cat, dog, ferret, primate (*e.g.,* marmoset, rhesus monkey). For the non-human animals where suitable genetically modifiable ES cells are not readily available, methods distinct from those described herein are employed to make a non-human animal comprising the genetic modification. Such methods include, *e.g.,* modifying a non-ES cell genome (*e.g.,*

a fibroblast or an induced pluripotent cell) and employing nuclear transfer to transfer the modified genome to a suitable cell, *e.g.,* an oocyte, and gestating the modified cell (*e.g.,* the modified oocyte) in a non-human animal under suitable conditions to form an embryo.

[000756] In one aspect, the non-human animal is a mammal. In one aspect, the non-human animal is a small mammal, *e.g.,* of the superfamily *Dipodoidea* or *Muroidea*. In one embodiment, the genetically modified animal is a rodent. In one embodiment, the rodent is selected from a mouse, a rat, and a hamster. In one embodiment, the rodent is selected from the superfamily *Muroidea*. In one embodiment, the genetically modified animal is from a family selected from *Calomyscidae* (e.g., mouse-like hamsters), *Cricetidae* (*e.g.,* hamster, New World rats and mice, voles), *Muridae* (true mice and rats, gerbils, spiny mice, crested rats), *Nesomyidae* (climbing mice, rock mice, with-tailed rats, Malagasy rats and mice), *Platacanthomyidae* (*e.g.,* spiny dormice), and *Spalacidae* (*e.g.,* mole rates, bamboo rats, and zokors). In a specific embodiment, the genetically modified rodent is selected from a true mouse or rat (family *Muridae*), a gerbil, a spiny mouse, and a crested rat. In one embodiment, the genetically modified mouse is from a member of the family *Muridae*. In one embodiment, the animal is a rodent. In a specific embodiment, the rodent is selected from a mouse and a rat. In one embodiment, the non-human animal is a mouse.

[000757] In a specific embodiment, the non-human animal is a rodent that is a mouse of a C57BL strain selected from C57BL/A, C57BL/An, C57BL/GrFa, C57BL/KaLwN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, and C57BL/Ola. In another embodiment, the mouse is a 129 strain selected from the group consisting of a strain that is 129P1, 129P2, 129P3, 129X1, 129S1 (*e.g.*, 129S1/SV, 129S1/SvIm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, 129T2 (see, *e.g.*, Festing *et al.* (1999) Revised nomenclature for strain 129 mice, Mammalian Genome 10:836, *see also*, Auerbach et al (2000) Establishment and Chimera Analysis of 129/SvEv- and C57BL/6-Derived Mouse Embryonic Stem Cell Lines). In a specific embodiment, the genetically modified mouse is a mix of an aforementioned 129 strain and an aforementioned C57BL/6 strain. In another specific embodiment, the mouse is a mix of aforementioned 129 strains, or a mix of aforementioned BL/6 strains. In a specific embodiment, the 129 strain of the mix is a 129S6 (129/SvEvTac) strain. In another embodiment, the mouse is a BALB strain, *e.g.,* BALB/c strain. In yet another embodiment, the mouse is a mix of a BALB strain and another aforementioned strain.

[000758] In one embodiment, the non-human animal is a rat. In one embodiment, the rat is selected from a Wistar rat, an LEA strain, a Sprague Dawley strain, a Fischer strain, F344, F6, and Dark Agouti. In one embodiment, the rat strain is a mix of two or more strains

selected from the group consisting of Wistar, LEA, Sprague Dawley, Fischer, F344, F6, and Dark Agouti.

[000759] Thus, in one embodiment, the genetically modified non-human animal is a rodent. In one embodiment, the genetically modified non-human animal is a rat or a mouse. In one embodiment, the animal is a mouse. Thus, in one embodiment, provided herein is a genetically modified mouse comprising in its genome, *e.g.,* its germline, a single rearranged human immunoglobulin light chain variable region comprising human V_L and J_L gene sequences, wherein the single rearranged human immunoglobulin light chain variable region comprises a substitution of at least non-histidine codon with a histidine codon. In one embodiment, the mouse lacks a functional unrearranged immunoglobulin light chain variable region (*e.g.,* lacks functional unrearranged V and J gene segment sequences). In one embodiment, the rearranged human immunoglobulin light chain variable region with histidine codon substitution(s) is Vκ1-39/Jκ or Vκ3-20/Jκ variable region. In one embodiment the J segment sequence is selected from Jκ1, Jκ2, Jκ3, Jκ4, and Jκ5. In one embodiment the J segment sequence is Jκ1 or Jκ5. In one embodiment, the substitution of at least one non-histidine codon with a histidine codon is in the nucleotide sequence encoding a CDR3 region. In one embodiment, wherein the rearranged variable region sequence is Vκ1-39/Jκ5 sequence, the histidine substitution(s) is designed to express at a position selected from 105, 106, 108, 111, and a combination thereof. In another embodiment, wherein the rearranged variable region sequence is Vκ3-20/Jκ1 sequence, the histidine substitution(s) is designed to express at a position selected from 105, 106, 107, 109, and a combination thereof. In one embodiment, the rearranged immunoglobulin light chain variable region with substituted histidine codon(s) is operably linked to an endogenous mouse immunoglobulin constant region gene sequence (e.g., Cκ gene sequence). In one embodiment, the mouse further comprises in its genome, *e.g.,* its germline, an unrearranged immunoglobulin heavy chain variable region comprising human V_H, D_H, and J_H segments. In one embodiment, human V_H, D_H, and J_H segments are operably linked to an endogenous mouse immunoglobulin heavy chain constant region gene sequence. In various embodiments, the rearranged human immunoglobulin light chain variable region sequence comprising substitution(s) of at least one non-histidine codon with a histidine codon and the unrearranged human immunoglobulin heavy chain variable region sequence are comprised in the germline of the mouse.

[000760] Also provided herein are targeting vectors for generating genetically modified non-human animals, *e.g.,* mice, described herein. In one aspect, provided is a targeting vector comprising, from 5' to 3' in transcriptional direction with reference to the sequences of

the 5' and 3' mouse homology arms of the vector, a 5' mouse homology arm, a human or mouse immunoglobulin promoter, a human or mouse leader sequence, a human variable region selected from a rearranged human Vκ1-39Jκ5 or a rearranged human Vκ3-20Jκ1 and comprising a substitution of at least one non-histidine codon with a histidine codon, and a 3' mouse homology arm. In one embodiment, the 5' and 3' homology arms target the vector to a sequence 5' with respect to an enhancer sequence that is present 5' and proximal to the mouse Cκ gene. In another embodiment, the targeting vector comprises a 5' mouse homology arm followed by a selection cassette flanked by recombination sites, human or mouse immunoglobulin promoter, human or mouse leader sequence, a human variable region selected from a rearranged human Vκ1-39Jκ5 or a rearranged human Vκ3-20Jκ1 and comprising a substitution of at least one non-histidine codon with a histidine codon, followed by the 3' mouse homology arm that comprises mouse enhancers and constant region (Cκ) sequences.

[000761] A selection cassette is a nucleotide sequence inserted into a targeting construct to facilitate selection of cells (*e.g.,* ES cells) that have integrated the construct of interest. A number of suitable selection cassettes are known in the art. Commonly, a selection cassette enables positive selection in the presence of a particular antibiotic (*e.g.,* Neo, Hyg, Pur, CM, Spec, *etc.*). In addition, a selection cassette may be flanked by recombination sites, which allow deletion of the selection cassette upon treatment with recombinase enzymes. Commonly used recombination sites are *lox*P and *Frt*, recognized by Cre and Flp enzymes, respectively, but others are known in the art.

[000762] In one embodiment, the promoter is a human immunoglobulin variable region gene segment promoter. In a specific embodiment, the promoter is a human Vκ3-15 promoter. In one embodiment, the leader sequence is a mouse leader sequence. In a specific embodiment, the mouse leader sequence is a mouse Vκ3-7 leader sequence. Exemplary embodiments of the targeting vectors are presented in FIGs. 23B and 29B.

[000763] In one aspect, a targeting vector is provided as described above, but in place of the 5' mouse homology arm the human or mouse promoter is flanked 5' with a site-specific recombinase recognition site (SRRS), and in place of the 3' mouse homology arm the human V_L region is flanked 3' with an SRRS.

[000764] Also provided herein are methods of making genetically modified non-human animals (*e.g.,* rodents, *e.g.,* mice or rats) described herein. In one aspect, the method for making a genetically modified non-human animal described herein utilizes a targeting vector, made using VELOCIGENE® technology, introducing the construct into ES cells, and

introducing targeted ES cell clones into a mouse embryo using VELOCIMOUSE® technology, as described in the Examples. Histidine modifications may be introduced into the targeting vector using a variety of molecular biology techniques, *e.g.,* site directed mutagenesis or de novo DNA synthesis. Upon completion of gene targeting, ES cells of genetically modified non-human animals are screened to confirm successful incorporation of exogenous nucleotide sequence of interest or expression of exogenous polypeptide. Numerous techniques are known to those skilled in the art, and include (but are not limited to) Southern blotting, long PCR, quantitative PCT (*e.g.,* real-time PCR using TAQMAN®), fluorescence *in situ* hybridization, Northern blotting, flow cytometry, Western analysis, immunocytochemistry, immunohistochemistry, etc. In one example, non-human animals (*e.g.,* mice) bearing the genetic modification of interest can be identified by screening for loss of mouse allele and/or gain of human allele using a modification of allele assay described in Valenzuela *et al.* (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis, Nature Biotech. 21(6):652-659. Other assays that identify a specific nucleotide or amino acid sequence in the genetically modified animals are known to those skilled in the art.

[000765] Thus, in one embodiment, the method of generating genetically modified non-human animals comprises replacing an immunoglobulin light chain variable region gene sequence in the animal with a human immunoglobulin light chain variable region gene sequence (comprising human V_L and J_L gene segments) wherein the human immunoglobulin variable region gene sequence comprises a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, the substitution of at least one non-histidine codon with a histidine codon is in the nucleotide sequence encoding a CDR region, *e.g.*, a CDR3 region.

[000766] In one embodiment, the method of generating genetically modified non-human animals described herein comprises replacing an immunoglobulin light chain variable region gene sequence in the animal with a single rearranged human immunoglobulin light chain variable region gene sequence comprising human V_L and J_L gene segment sequences, wherein the single rearranged human immunoglobulin variable region gene sequence comprises a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, the substitution is in a CDR codon. In one embodiment, the substitution is of one, two, three, four, or more CDR3 codon(s). In one embodiment, the single rearranged human immunoglobulin light chain variable region gene sequence is based on the human germline rearranged light chain variable region sequence selected from V_κ1-39J_κ5 and V_κ3-20J_κ1. Thus, in one embodiment, where the single rearranged human immunoglobulin light

chain variable region gene sequence is derived from Vκ1-39Jκ5, replacement of at least one non-histidine codon with histidine codon is designed to express a histidine at positions selected from 105, 106, 108, 111, and a combination thereof. In one embodiment, where the single rearranged human immunoglobulin light chain variable region gene sequence is derived from Vκ3-20κ1, replacement of at least one non-histidine codon with a histidine codon is designed to express a histidine at position selected from 105, 106, 107, 109, and a combination thereof.

[000767] In another embodiment, the method of generating a non-human animal described herein (i.e., comprising a genetically modified immunoglobulin light chain locus described herein) comprises modifying a genome of a non-human animal to delete or render non-functional endogenous immunoglobulin light chain V and J segments in an immunoglobulin light chain locus, and placing in the genome a single rearranged human light chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon. In one embodiment, the method results in a genetically modified non-human animal that comprises a population of B cells enriched for antibodies exhibiting pH dependent binding to an antigen of interest.

[000768] In some embodiments, the methods of generating genetically modified non-human animals described herein comprise replacing an immunoglobulin light chain variable region gene sequence with human sequence in the animal that also comprises a replacement of endogenous non-human immunoglobulin heavy chain variable region gene sequence with a human immunoglobulin heavy chain variable region gene sequence comprising at least one of each or a repertoire of human V_H, D_H, and J_H sequences, as described above. In one embodiment, in order to generate a non-human animal comprising a replacement of endogenous immunoglobulin light chain variable region gene sequence human light chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon and a replacement of endogenous non-human immunoglobulin heavy chain variable region gene sequence with a human immunoglobulin heavy chain variable region gene sequence, the animal with replacement of light chain variable region gene sequence is bred to an animal with replacement of heavy chain variable region gene sequence.

[000769] Inventors presently provide genetically engineered non-human animals (*e.g.,* rodents, *e.g.,* rats or mice) that express antigen-binding proteins, *e.g.,* antibodies, that comprise a universal light chain, *e.g.,* a human universal light chain (*e.g.,* a light chain derived from a single rearranged human immunoglobulin light chain variable region) that comprises one or more histidine modifications, wherein the antigen-binding proteins exhibit a

pH-dependent antigen binding of a target antigen. The animals are genetically engineered to include a light chain CDR3 that comprises one or more histidine modifications. In various embodiments, the light chain CDR3 comprises two, three, or four or more histidine residues in a cluster.

[000770] In one embodiment, provided herein is a genetically engineered non-human animal that comprises a population of antigen-specific antibodies that express histidine residue(s) as a result of codon modifications in the light chain variable region gene sequence, and display pH-dependent binding of target antigen. In one embodiment, these animals comprise a population of B cells that are enriched for antibodies, *e.g.,* antigen-specific antibodies, that display pH-dependent binding properties (*e.g.,* decreased dissociative half-life ($t_{1/2}$), at acidic pH vs neutral pH) as compared to a population of antigen-specific antibodies generated in animals that do not comprise a substitution of at least one non-histidine codon with a histidine codon in immunoglobulin light chain variable region described herein. In one embodiment, the enrichment of antigen-specific antibodies displaying pH-dependent antigen binding properties generated in the genetically engineered animals described herein as compared to similar animals that do comprise histidine substitutions in light chain variable region is greater than about 2 fold, *e.g.,* greater than about 5 fold, *e.g.,* greater than about 10 fold. Thus, the genetically modified animals of the invention are enriched for antibodies with improved antibody recycling properties, which is desired in order to reduce target-mediated clearance as well as to reduce the dose and/or dosing frequency of a therapeutic antigen-binding protein developed based on such *in vivo* generated antibody format.

[000771] Thus, provided herein is an antigen-binding protein, generated in genetically modified non-human animals described herein, wherein the antigen-binding protein displays pH-dependent antigen binding. In one embodiment, the antigen-binding protein is an antibody, *e.g.,* antigen-specific antibody. In one embodiment, the antibody comprises a light chain which comprises a human light chain variable domain derived from a rearrangement of human immunoglobulin light chain variable gene segments where at least one non-histidine codon was substituted for a histidine codon in the germline gene sequence, and wherein the antibody retains at least one histidine substitution in its expressed human light chain variable domain. In one embodiment, the antibody comprises a light chain which comprises a human light chain variable domain derived from a single rearranged human light chain variable region gene sequence, wherein the single rearranged light chain variable region gene sequence comprises a substitution of at least one non-histidine codon with a histidine codon, and wherein the antibody retains at least one histidine substitution in its expressed light

chain variable domain. In one embodiment, the antibody comprises a light chain derived from a human Vκ1-39Jκ5 or Vκ3-20Jκ1 rearrangement, wherein the human Vκ1-39Jκ5 or Vκ3-20Jκ1 gene sequence comprises a substitution of at least one non-histidine codon with a histidine codon, and wherein the antibody retains at least one histidine substitution in its expressed light chain variable domain. In some embodiments, the antibody retains all or substantially all histidine substitutions in its expressed light chain variable domain. In one embodiment, the substitution is of three non-histidine codons with three histidine codons in the nucleotide sequence encoding CDR3 of the light chain variable region gene sequence, and the antibody retains all three histidine substitutions in its expressed light chain variable domain. In one embodiment, the substitution is of four non-histidine codons with four histidine codons in the nucleotide sequence encoding CDR3 of the light chain variable region gene sequence, and the antibody retains three or four histidine substitutions in its expressed light chain variable domain.

[000772] In one embodiment, the light chain of the antibody further comprises a non-human light chain constant region amino acid sequence, *e.g.,* endogenous light chain constant region amino acid sequence. In addition, the antibody, *e.g.,* antigen-specific antibody, generated in a genetically modified non-human animal described herein also comprises a heavy chain which comprises a human heavy chain variable domain derived from a rearrangement of human heavy chain V, D, and J segments. Human heavy chain V, D, and J segments may be selected from a repertoire of human heavy chain segments present at the endogenous non-human heavy chain locus, *e.g.,* at least one functional V, at least one functional D, and at least one functional J segment, *e.g.,* up to a complete repertoire of functional human V, D, and J segments. Exemplary possible rearrangements of human heavy chain variable segments may be gleaned from a listing of functional human V, D, and J segments in IMGT database, and from U.S. Application Publication Nos. 2011/0195454, 2012/0021409, 2012/0192309, and 2013/0045492. Furthermore, in one embodiment, the heavy chain of the antibody comprises a non-human heavy chain constant region amino acid sequence, *e.g.,* an endogenous non-human heavy chain constant region amino acid sequence. In one embodiment, the non-human heavy chain constant region comprises C_H1, hinge, C_H2, and C_H3 domains. In one embodiment, the antibody is an IgG, IgE, IgD, IgM, or IgA isotype.

[000773] Thus, in one embodiment, provided herein is a binding protein generated in the genetically modified non-human animals described herein, wherein the binding protein comprises a reverse chimeric light chain comprising (a) a light chain variable domain derived from a human Vκ1-39Jκ5 rearrangement comprising a substitution of at least one non-

histidine codon with a histidine codon, wherein the light chain retains at least one histidine substitution in its expressed light chain variable domain and (b) a non-human, *e.g.,* a mouse, light chain constant region amino acid sequence, wherein the light chain is associated with a reverse chimeric heavy chain comprising (a) a heavy chain variable domain derived from a rearrangement of human V, D, and J segments, wherein the V, D, and J segments are selected from a repertoire of human V, D, and J segments present in the animal, and (b) a non-human, *e.g.,* mouse, heavy chain constant region amino acid sequence. In one embodiment, the repertoire of human V, D, and J segments comprises at least one functional V, at least one functional D, and at least one functional J segment, *e.g.,* up to a complete repertoire of functional human V, D, and J segments. In one embodiment, the heavy and the light chain constant domains are endogenous heavy and light chain constant regions. In one embodiment, the heavy and light chain variable domains are somatically mutated domains. In one embodiment, the somatically mutated light chain domain retains at least one histidine substitution introduced into the germline sequence. In some embodiments, the somatically mutated light chain domain retains all or substantially all histidine substitutions introduced into the germline sequence. In one embodiment, the antigen-binding protein displays pH-dependent antigen binding properties.

[000774] In another embodiment, provided herein is a binding protein generated in the genetically modified non-human animals described herein, wherein the binding protein comprises a reverse chimeric light chain comprising (a) a light chain variable domain derived from a human Vκ3-20Jκ1 rearrangement comprising a substitution of at least one non-histidine codon with a histidine codon, wherein the light chain retains at least one histidine substitution in its expressed light chain variable domain and (b) a non-human, *e.g.,* a mouse, light chain constant region amino acid sequence, wherein the light chain is associated with a reverse chimeric heavy chain comprising (a) a heavy chain variable domain derived from a rearrangement of human V, D, and J segments, wherein the V, D, and J segments are selected from a repertoire of human V, D, and J segments present in the animal, and (b) a non-human, *e.g.,* mouse, heavy chain constant region amino acid sequence. In one embodiment, the repertoire of human V, D, and J segments comprises at least one functional V, at least one functional D, and at least one functional J segment, *e.g.,* up to a complete repertoire of functional human V, D, and J segments. In one embodiment, the heavy and the light chain constant regions are endogenous heavy and light chain constant regions. In one embodiment, the heavy and light chain variable domains are somatically mutated domains. In one embodiment, the somatically mutated light chain domain retains at least one histidine substitution introduced into the germline sequence. In some embodiments, the somatically mutated light chain domain retains all or substantially all

histidine substitutions introduced into the germline sequence. In one embodiment, the antigen-binding protein displays pH-dependent antigen binding properties.

[000775] In one embodiment, also provided herein is a B cell of the genetically modified animal described herein, that comprises in its germline a histidine-modified human light chain variable region sequence, *e.g.,* a histidine-modified single rearranged human light chain variable region sequence, described herein, and expresses an antigen-binding protein described herein. In one embodiment, the antigen-binding protein, *e.g.,* an antibody, expressed in the B cell retains at least one histidine residue introduced into the germline, and displays pH-dependent antigen-binding properties. In some embodiments, the antigen-binding protein, *e.g.,* an antibody, expressed in the B cell retains all or substantially all histidine residues introduced into the germline, and displays pH-dependent antigen-binding properties.

[000776] In various embodiments, the genetically modified non-human animal described herein comprises a human light chain variable region gene sequence, e.g., a single rearranged human light chain variable region gene sequence (*e.g.,* Vκ1-39Jκ5 or Vκ3-20Jκ1 sequence) that comprises a substitution of at least one non-histidine codon with a histidine codon (or an addition of a histidine codon into the germline sequence). These additions or substitutions result in a non-human animal that comprises a population of B cells enriched for antigen-binding proteins with pH dependent binding properties for their antigens. In one embodiment, antigen-binding proteins, e.*g.,* antibodies, generated in the non-human animals described herein in response to antigen stimulation display pH dependent antigen binding while exhibiting high affinity for the antigen at neutral pH, *e.g.,* pH between about 7.0 and about 8.0, *e.g.*, pH between about 7.0 and about 7.4, *e.g.,* between about 7.2 and about 7.4, *e.g.,* pH of the body fluids such as blood. In one embodiment, the affinity of the antigen-binding protein to its antigen, expressed as a dissociation constant (K_D) at a neutral pH is less than 10^{-6} M, *e.g.,* less than 10^{-8} M, *e.g.,* less than 10^{-9} M, *e.g.,* less than 10^{-10} M, *e.g.,* less than 10^{-11} M, *e.g.,* less than 10^{-12} M.

[000777] In one embodiment, an antigen-binding protein, *e.g.,* an antibody, generated in the genetically modified non-human animal described herein, exhibits reduced binding to its antigen in acidic pH (*e.g.,* pH of 6.0 or lower, *e.g.,* pH between about 5.0 and about 6.0, pH between about 5.75 and about 6.0, *e.g.*, pH of endosomal or lysosomal compartments) as compared to neutral pH. In one embodiment, the antigen-binding protein, *e.g.,* the antibody, generated in the genetically modified non-human animal described herein, exhibits no binding to the antigen in acidic pH, while retaining binding to the antigen at neutral pH. In one embodiment, an antigen-binding protein generated by the genetically modified non-

human animal described herein, has a decrease in dissociative half-life ($t_{1/2}$) at an acidic pH as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH of at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold. In one embodiment, an antigen-binding protein expressed by the genetically modified non-human animal described herein has a $t_{1/2}$ at an acidic pH and 37°C of about 2 min or less. In one embodiment, an antigen-binding protein expressed by the genetically modified non-human animal described herein has a $t_{1/2}$ at an acidic pH and 37°C of less than about 1 min. In one embodiment, an antigen-binding protein expressed by the genetically modified non-human animal described herein has a $t_{1/2}$ at an acidic pH and 25°C of about 2 min or less. In one embodiment, an antigen-binding protein expressed by the genetically modified non-human animal described herein has a $t_{1/2}$ at an acidic pH and 25°C of less than about 1 min.

[000778] Kinetic parameters, such as equilibrium dissociation constants (K_D) and dissociative half-lives ($t_{½}$) can be calculated from kinetic rate constant as: K_D (M) = k_d / k_a; and $t_{½}$ (min) = ln2/(60*k_d).

[000779] In one embodiment, the antigen-binding protein, *e.g.,* an antibody, generated in the genetically modified non-human animals described herein, exhibits increased binding to FcRn molecule. As described above, FcRn is a receptor present inside the endosomal compartment that is capable of binding immunoglobulins at an acidic pH and recycling them back to the surface. Screening antibody molecules in the genetically modified non-human animals described herein presents a unique opportunity to select for antibodies with three beneficial parameters: high affinity for an antigen, pH-dependent antigen binding (with weaker antigen binding at acidic pH) and increased binding to FcRn.

[000780] In one embodiment, a genetically modified non-human animal described herein comprises a population of B cells in response to an antigen that produces and is enriched for antigen-binding proteins, *e.g.,* antibodies, that, when reformatted into therapeutics, exhibit increased serum half life upon administration of a therapeutic dose to a subject over an equivalent B cell population produced in response to the same antigen in non-human animals that do not comprise histidine modification(s) in their human light chain variable region gene sequences. Thus, in one embodiment, an antigen-binding protein, *e.g.*, an antibody, produced in response to an antigen of interest in a genetically modified non-human animal described herein, when reformatted into a therapeutic, exhibits increased serum half life upon administration of a therapeutic dose to a subject over a serum half life of an antigen-binding protein (when reformatted into a therapeutic and administered at the same

therapeutic dose) that was produced in response to the same antigen in a non-human animal that does not comprise histidine modification(s) in its human light chain variable region gene sequence. In some embodiments, the increase in serum half life is about 2 fold, *e.g.,* about 5 fold, *e.g.,* about 10 fold, *e.g.,* about 15 fold, *e.g.,* about 20 fold, or greater.

[000781] In one aspect, a pluripotent, induced pluripotent, or totipotent cell derived from a non-human as described herein is provided. In a specific embodiment, the cell is an embryonic stem (ES) cell.

[000782] In one aspect, a tissue derived from a non-human animal as described herein is provided. In one embodiment, the tissue is derived from spleen, lymph node or bone marrow of a non-human animal as described herein.

[000783] In one aspect, a nucleus derived from a non-human animal as described herein is provided. In one embodiment, the nucleus is from a diploid cell that is not a B cell.

[000784] In one aspect, a non-human cell is provided that is isolated from a non-human animal (*e.g.,* a rodent, *e.g.,* a mouse or a rat) as described herein. In one embodiment, the cell is an ES cell. In one embodiment, the cell is a lymphocyte. In one embodiment, the lymphocyte is a B cell. In one embodiment, the B cell expresses a chimeric heavy chain comprising a variable domain derived from a human gene segment; and a light chain derived from a rearranged human V_K1-39/J sequence with a substitution of at least one non-histidine codon with histidine codon, rearranged human V_K3-20/J sequence with a substitution of at least one non-histidine codon with histidine codon, or a combination thereof and further comprising a substitution of at least one amino acid encoded in the germline for a histidine; wherein the heavy chain variable domain is fused to a non-human constant region and the light chain variable domain is fused to a non-human or a human constant region.

[000785] In one aspect, a hybridoma is provided, wherein the hybridoma is made with a B cell of a non-human animal as described herein. In a specific embodiment, the B cell is from a mouse as described herein that has been immunized with an immunogen comprising an epitope of interest, and the B cell expresses a binding protein that binds the epitope of interest, the binding protein has a somatically mutated human variable heavy chain domain and a mouse C_H, and has a human variable light chain domain derived from a rearranged human V_K1-39J_K5 with a substitution of at least one non-histidine codon with histidine codon or a rearranged human V_K3-20J_K1 with a substitution of at least one non-histidine codon with histidine codon and a mouse C_L, wherein the human light chain domain comprises a

substitution of at least one amino acid encoded in the germline with a histidine.

[000786] Also provided is a cell expressing an antigen-binding protein generated in the non-human animals described herein. In one embodiment, the cell is selected from CHO, COS, 293, HeLa, and a retinal cell expressing a viral nucleic acid sequence (*e.g.,* a PERC.6™ cell).

[000787] In one aspect, a non-human embryo is provided, wherein the embryo comprises a donor ES cell that is derived from a non-human animal as described herein.

[000788] The non-human animals described herein are useful to generate B cells that express antibodies having histidines in a CDR3. An animal that places histidines in a CDR3 is useful for making antibodies in general, and in particular useful for developing antibodies that bind a target with sufficient affinity at or around a neutral pH, but that either do not bind or that bind weaker to the same target at an acidic pH.

[000789] The non-human animal is useful to generate variable regions of antibodies that can be used to make, *e.g.,* human therapeutic binding proteins that bind their targets by human immunoglobulin variable domains that comprise the histidines in a CDR3. The altered binding at a lower pH will in some circumstances allow faster turnover because the therapeutic will bind a target on a cell's surface, be internalized in an endosome, and more readily or more rapidly dissociate from the target in the endosome, so that the therapeutic can be recycled to bind yet another molecule of target (*e.g.,* on another cell or the same cell). In some circumstances, this will result in the ability to dose the therapeutic at a lower dose, or dose the therapeutic less frequently. This is particularly useful where it is not desirable to dose frequently, or to administer above a certain dosage, for safety or toxicity reasons. As a result, the serum half life of the antibody therapeutic when administered to a subject will be increased.

[000790] The non-human animal, *e.g.,* rodent, *e.g.,* mouse or rat, is useful in a method for increasing the number of B cells in a animal that exhibit an antibody variable region having a CDR3 with one or more histidines in it. The non-human animal is useful for generating antibody sequences that will exhibit pH-dependent antigen binding. The non-human animal is useful for generating a greater number of antibody sequences, resulting from a single immunization, wherein the antibodies will exhibit a pH-dependent antigen binding.

Antigen-Binding Proteins and Methods of Generating the Same

[000791] In one aspect, also provided herein are methods for generating human antigen-binding proteins, *e.g.,* antibodies, which exhibit pH-dependent antigen binding, from the

genetically modified non-human animals described herein with standard methods used in the art.

[000792] Several techniques for the producing antibodies have been described. For example, in various embodiments chimeric antibodies are produced in mice as described herein. Antibodies can be isolated directly from B cells of an immunized mouse (*e.g.*, see U.S. 2007/0280945A1) and/or the B cells of the immunized mouse can be used to make hybridomas (Kohler and Milstein, 1975, *Nature* 256:495-497). DNA encoding the antibodies (human heavy and/or light chains) from non-human animals as described herein is readily isolated and sequenced using conventional techniques. Hybridoma and/or B cells derived from non-human animals as described herein serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the non-human sequences. Thus, once nucleic acid sequences of antibodies with desired characteristics, e.g., affinity, epitope, pH-dependent antigen binding, *etc.*, are determined, the non-human constant region gene sequences are replaced with a desired human constant region sequences to generate a fully human antibody containing a non-IgM isotype, for example, IgG1, IgG2, IgG3 or IgG4.

[000793] Thus, in one embodiment provided herein is a method of generating an antibody that exhibits pH-dependent antigen binding properties comprising generating a non-human animal (*e.g.,* a mouse) as described herein, immunizing a mouse with an antigen of interest, allowing a non-human animal to mount an immune response to the antigen, and selecting in the non-human animal an antigen-specific antibody that exhibits pH dependent antigen binding properties, *e.g.,* weaker binding to the antigen at an acidic than at neutral pH.

[000794] Also provided herein are methods of making multi-specific antigen binding proteins, *e.g.,* bispecific antigen-binding proteins. These are molecules capable of binding more than one epitope with high affinity. Advantages of the invention include the ability to select suitably high binding (*e.g.,* affinity matured) heavy chain immunoglobulin chains each of which will associate with a single light chain. In addition, advantages of the invention include the ability to generate a multi-specific, *e.g.,* a bispecific, antigen-binding protein that exhibits pH-dependent antigen binding.

[000795] Because of the dual nature of bispecific antibodies (*i.e.*, may be specific for different epitopes of one polypeptide or may contain antigen-binding domains specific for

more than one target polypeptide, see, *e.g.*, Tutt *et al.*, 1991, *J. Immunol.* 147:60-69; Kufer *et al.*, 2004, *Trends Biotechnol.* 22:238-244), they offer many useful advantages for therapeutic application. For example, the bispecific antibodies can be used for redirected cytotoxicity (*e.g.*, to kill tumor cells), as a vaccine adjuvant, for delivering thrombolytic agents to clots, for converting enzyme activated prodrugs at a target site (*e.g.*, a tumor), for treating infectious diseases, targeting immune complexes to cell surface receptors, or for delivering immunotoxins to tumor cells.

[000796] The bispecific antibodies described herein can also be used in several therapeutic and non-therapeutic and/or diagnostic assay methods, such as, enzyme immunoassays, two-site immunoassays, *in vitro* or *in vivo* immunodiagnosis of various diseases (*e.g.*, cancer), competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Other uses for the bispecific antibodies will be apparent to those skilled in the art.

[000797] Several techniques for making bispecific antibody fragments from recombinant cell culture have been reported. However, synthesis and expression of bispecific binding proteins has been problematic, in part due to issues associated with identifying a suitable light chain that can associate and express with two different heavy chains, and in part due to isolation issues. In various embodiments, compositions and methods described herein provide the advantage of full length bispecific antibodies that do not require special modification(s) to maintain traditional immunoglobulin structure by increasing stability/interaction of the components. In various embodiments, such modification(s) has proven cumbersome and served as an obstacle to development of bispecific antibody technology and their potential use in treating for human disease. Thus, in various embodiments, through providing a natural immunoglobulin structure (*i.e.,* full length) having the added property of multiple specificities, full length bispecific antibodies maintain their critical effector functions that previous bispecific fragments lacked, and further provide therapeutics that demonstrate the important pharmacokinetic parameter of a longer half-life.

[000798] Methods and compositions described herein allow for a genetically modified mouse to select, through otherwise natural processes, a suitable light chain that can associate and express with more than one heavy chain, including heavy chains that are somatically mutated (*e.g.,* affinity matured), wherein the light chain further confers upon the antigen-binding protein its pH-dependent antigen binding property. Human heavy and light chain variable region sequences from suitable B cells of immunized mice as described herein that express affinity matured antibodies having reverse chimeric heavy chains (i.e., human variable and mouse constant) can be identified and cloned in frame in an expression

vector with a suitable human constant region gene sequence (*e.g.,* a human IgG1). Two such constructs can be prepared, wherein each construct encodes a human heavy chain variable domain that binds a different epitope. One of the human light chain variable regions (*e.g.,* human Vκ1-39Jκ5 or human Vκ3-20Jκ1), comprising a substitution of at least one non-histidine codon with a histidine codon, can be fused in frame to a suitable human light chain constant region gene (*e.g.,* a human κ constant gene). These three fully human heavy and light constructs can be placed in a suitable cell for expression. The cell will express two major species: a homodimeric heavy chain with the identical light chain, and a heterodimeric heavy chain with the identical light chain. To allow for a facile separation of these major species, one of the heavy chains is modified to omit a Protein A-binding determinant, resulting in a differential affinity of a homodimeric binding protein from a heterodimeric binding protein. Compositions and methods that address this issue are described in USSN 12/832,838, filed 25 June 2010, entitled "Readily Isolated Bispecific Antibodies with Native Immunoglobulin Format," published as US 2010/0331527A1. Once the specie comprising heterodimeric heavy chain with an identical light chain is selected, this bi-specific antigen binding protein can be screened to confirm the retention of its pH-dependent antigen binding property.

[000799] In one aspect, an epitope-binding protein as described herein is provided, wherein human light chain and heavy chain variable region sequences are derived from animals described herein that have been immunized with an antigen comprising an epitope of interest.

[000800] In one embodiment, an epitope-binding protein is provided that comprises a first and a second polypeptide, the first polypeptide comprising, from N-terminal to C-terminal, a first epitope-binding region that selectively binds a first epitope, followed by a constant region that comprises a first C_H3 region of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof; and, a second polypeptide comprising, from N-terminal to C-terminal, a second epitope-binding region that selectively binds a second epitope, followed by a constant region that comprises a second C_H3 region of a human IgG selected from IgG1, IgG2, IgG4, and a combination thereof, wherein the second C_H3 region comprises a modification that reduces or eliminates binding of the second C_H3 domain to protein A. Various such modifications are described in, *e.g.,* U.S. Application Publication Nos. 2010/0331527 and 2011/0195454.

[000801] One method for making an epitope-binding protein that binds more than one epitope and exhibits pH-dependent epitope binding property is to immunize a first mouse in accordance with the invention with an antigen that comprises a first epitope of interest,

wherein the mouse comprises (1) an endogenous immunoglobulin light chain variable region locus that does not contain an endogenous mouse light chain variable region gene sequence that is capable of rearranging and forming a light chain, wherein at the endogenous mouse immunoglobulin light chain variable region locus is a single rearranged human light chain variable region operably linked to the mouse endogenous light chain constant region gene, and the rearranged human light chain variable region is selected from a human Vκ1-39Jκ5 and a human Vκ3-20Jκ1 comprising a substitution of at least one non-histidine codon with a histidine condon, and (2) the endogenous mouse V_H gene segments have been replaced in whole or in part with human V_H gene segments, such that immunoglobulin heavy chains made by the mouse are solely or substantially heavy chains that comprise human variable domains and mouse constant domains. When immunized, such a mouse will make a reverse chimeric antibody, comprising only one of two human light chain variable domains (*e.g.,* one of human Vκ1-39Jκ5 or human Vκ3-20Jκ1, *e.g.,* comprising a substitution of at least one amino acid with a histidine). Commonly, at least some of the substituted histidine residues introduced into the germline sequence will be retained in the reverse chimeric antibody. Once a B cell is identified that encodes a heavy chain variable domain that binds the epitope of interest and expresses an antibody that exhibits pH-dependent antigen binding properties, the nucleotide sequence of the heavy chain variable region (and, optionally, the light chain variable region) can be retrieved (*e.g.,* by PCR) and cloned into an expression construct in frame with a suitable human immunoglobulin heavy chain constant region sequence. This process can be repeated to identify a second heavy chain variable domain that binds a second epitope, and a second heavy chain variable region gene sequence can be retrieved and cloned into an expression vector in frame to a second suitable human immunoglobulin heavy chain constant region sequence. The first and the second immunoglobulin constant domains encoded by the constant region gene sequence can be the same or different isotype, and one of the immunoglobulin constant domains (but not the other) can be modified as described herein or in US 2010/0331527A1, and epitope-binding protein can be expressed in a suitable cell and isolated based on its differential affinity for Protein A as compared to a homodimeric epitope-binding protein, *e.g.,* as described in US 2010/0331527A1.

[000802] Thus, in various embodiments, following isolation of the DNA and selection of the first and second nucleic acid sequences that encode the first and second human heavy chain variable domains having the desired specificities/affinities, and a third nucleic acid sequence that encodes a human light chain domain (a germline rearranged sequence or a light chain sequence isolated from a non-human animal as described herein) and comprises a substitution of at least one non-histidine codon with a histidine codon, the three nucleic

acids sequences encoding the molecules are expressed to form the bispecific antibody using recombinant techniques which are widely available in the art. Often, the expression system of choice will involve a mammalian cell expression vector and host so that the bispecific antibody is appropriately glycosylated (*e.g.*, in the case of bispecific antibodies comprising antibody domains which are glycosylated). However, the molecules can also be produced in the prokaryotic expression systems. Normally, the host cell will be transformed with DNA encoding both the first human heavy chain variable domain, the second human heavy chain variable domain, the human light chain domain on a single vector or independent vectors. However, it is possible to express the first human heavy chain variable domain, second human heavy chain variable domain, and human light chain domain (the bispecific antibody components) in independent expression systems and couple the expressed polypeptides in vitro. In various embodiments, the human light chain domain derived from a germline sequence but for the substitution of at least one non-histidine coding with a histidine codon, *e.g.,* in a CDR codon. In various embodiments, the human light chain domain comprises no more than one, no more than two, no more than three, no more than four, or no more than five somatic hypermutations within the light chain variable sequence of the light chain domain. In some embodiments, the somatic hypermutations do not alter the presence of at least one histidine residue introduced into the germline sequence of the light chain variable region.

[000803] In various embodiments, the nucleic acid(s) (*e.g.*, cDNA or genomic DNA) encoding the two heavy chains and single human light chain with a substitution of at least one non-histidine with a histidine is inserted into a replicable vector for further cloning (amplification of the DNA) and/or for expression. Many vectors are available, and generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Each component may be selected individually or based on a host cell choice or other criteria determined experimentally. Several examples of each component are known in the art.

[000804] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid sequences that encode each or all the components of the bispecific antibody. A large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to bispecific antibody-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

[000805] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant,

animal, human, or nucleated cells from other multicellular organisms) may also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the bispecific antibody components. Suitable expression vectors for various embodiments include those that provide for the transient expression in mammalian cells of DNA encoding the bispecific antibody. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of bispecific antibodies having desired binding specificities/affinities or the desired gel migration characteristics relative to the parental antibodies having homodimers of the first or second human heavy chain variable domains.

[000806] In various embodiments, once the DNA encoding the components of the bispecific antibody are assembled into the desired vector(s) as described above, they are introduced into a suitable host cell for expression and recovery. Transfecting host cells can be accomplished using standard techniques known in the art appropriate to the host cell selected (*e.g.*, electroporation, nuclear microinjection, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, *etc.*).

[000807] A host cell is chosen, in various embodiments, that best suits the expression vector containing the components and allows for the most efficient and favorable production of the bispecific antibody species. Exemplary host cells for expression include those of prokaryotes and eukaryotes (single-cell or multiple-cell), bacterial cells (*e.g.*, strains of E. coli, *Bacillus spp.*, *Streptomyces spp.*, *etc.*), mycobacteria cells, fungal cells, yeast cells (*e.g.*, *S. cerevisiae*, *S. pombe*, *P. pastoris*, *P. methanolica*, *etc.*), plant cells, insect cells (*e.g.*, SF-9, SF-21, baculovirus-infected insect cells, *Trichoplusia ni*, *etc.*), non-human animal cells, human cells, or cell fusions such as, for example, hybridomas or quadromas. In various embodiments, the cell is a human, monkey, ape, hamster, rat, or mouse cell. In various embodiments, the cell is eukaryotic cell selected from CHO (*e.g.*, CHO K1, DXB-11 CHO, Veggie-CHO), COS (*e.g.*, COS-7), retinal cell, Vero, CV1, kidney (*e.g.*, HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK), HeLa, HepG2, WI38, MRC 5, Colo205, HB 8065, HL-60, (*e.g.*, BHK21), Jurkat, Daudi, A431 (epidermal), CV-1, U937, 3T3, L cell, C127 cell,

SP2/0, NS-0, MMT 060562, Sertoli cell, BRL 3A cell, HT1080 cell, myeloma cell, tumor cell, and a cell line derived from an aforementioned cell. In various embodiments, the cell comprises one or more viral genes, *e.g.* a retinal cell that expresses a viral gene (*e.g.*, a PER.C6™ cell).

[000808] Mammalian host cells used to produce the bispecific antibody may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. Media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other supplements may also be included at appropriate concentrations as known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are, in various embodiments, those previously used with the host cell selected for expression, and will be apparent to those skilled in the art.

[000809] The bispecific antibody may be recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysate when directly produced without a secretory signal. If the bispecific antibody is membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.*, Triton-X 100).

[000810] Following isolation, a bispecific antibody comprising a two human heavy chains and a single human light chain derived from a rearranged human light chain variable region gene sequence selected from Vκ1-39Jκ5 and Vκ3-20Jκ1 sequences that comprise a substitution of at least one non-histidine codon with a histidine codon, is screened for its ability to exhibit pH dependent binding of one, preferably both of its antigens. The ability of bispecific antibodies to bind its antigens differently at neutral and acidic pH's (*e.g.,* their ability to demonstrate decreased $t_{1/2}$ at acidic pH compared to neutral pH) can be determined by a variety of techniques available in the art and described in the following examples, *e.g.,* BIACORE™ assay.

Additional Methods for Generating Antigen-Binding Proteins with pH-Dependent Antigen Binding

[000811] Various methods of generating antigen-binding proteins with pH-dependent antigen binding properties in genetically modified non-human animals described herein are

provided. Also provided are methods of generating antigen binding proteins with pH-dependent antigen binding properties *in vitro*. Such methods may involve generating various components of the antigen-binding proteins *in vivo* in genetically modified non-human animals, and then modifying them and reassembling them *in vitro* outside an organism as protein complexes expressed in mammalian cell culture.

[000812] In one embodiment, the method of generating antigen-binding proteins with pH-dependent antigen binding properties utilizes an antigen-binding protein sequence, *e.g.,* an antibody sequence, that is generated in a mouse comprising a limited repertoire of light chain variable region V and J segments, *e.g.,* human light chain variable region V and J segments, "universal light chain" or "common light chain" mouse ("ULC" mouse), such as the mouse described in U.S. Application Publication Nos. 2011/0195454, 2012/0021409, 2012/0192300 and 2013/0045492. In one embodiment, the method of generating antigen-binding proteins with pH-dependent antigen binding properties utilizes an antigen binding protein sequence that is generated in a mouse comprising a single rearranged human light chain variable region gene sequence. In one embodiment, the method utilizes an antigen binding protein generated in a mouse comprising a single rearranged human light chain variable region gene sequence selected from human Vκ1-39Jκ5 and human Vκ3-20Jκ1.

[000813] In one embodiment, the method for generating an antigen-binding protein, *e.g.,* an antibody, with pH dependent antigen binding properties comprises selecting a first antibody that binds to an antigen of interest (*e.g.,* binds to an antigen of interest with a desired affinity), modifying an immunoglobulin light chain nucleotide sequence of the first antibody to comprise a substitution of at least one non-histidine codon with a histidine codon, expressing an immunoglobulin heavy chain of the first antibody and the modified immunoglobulin light chain in a cells, and selecting a second antibody expressed in the cell that retains binding to the antigen of interest (*e.g.,* retains desired affinity for the antigen of interest) at neutral pH and displays reduced binding to the antigen of interest at an acidic pH.

[000814] In one embodiment, the method for generating an antigen-binding protein, *e.g.,* an antibody, with pH dependent antigen binding properties comprises selecting an immunoglobulin heavy chain from an antibody (*e.g.,* obtained from a non-human animal, *e.g.,* a mouse, *e.g.,* a ULC mouse) that comprises an immunoglobulin light chain having a single rearranged human immunoglobulin light chain variable region sequence wherein the antibody binds to an antigen of interest (*e.g.,* binds to an antigen of interest with a desired affinity); modifying the nucleic acid sequence of the immunoglobulin light chain such that the single rearranged human immunoglobulin light chain variable region sequence comprises a

substitution of at least one non-histidine codon with a histidine codon; expressing the selected immunoglobulin heavy chain and the immunoglobulin light chain comprising the substitution of at least one amino acid with a histidine in its variable domain; and selecting an antibody that retains binding to the antigen of interest at a neutral pH (*e.g.,* retains desired affinity to the antigen of interest) while displaying reduced binding to the antigen of interest at an acidic pH. In various embodiments, the immunoglobulin heavy chain is derived from a rearrangement of human heavy chain variable gene segments (human V, D, and J segments).

[000815] In one embodiment, the method for generating an antigen-binding protein, *e.g.,* an antibody, with pH-dependent antigen binding properties comprises (1) immunizing a non-human animal, *e.g.,* a mouse, comprising a single rearranged human light chain variable region gene sequence and a repertoire of unrearranged human heavy chain variable gene segments (V, D, and J segments) with an antigen of interest and allowing a mouse to mount an immune response to said antigen, (2) selecting in the non-human animal, *e.g.,* in the mouse, an antibody that binds to the antigen of interest with a desired affinity, (3) isolating from the non-human animal, *e.g.,* from the mouse, a nucleotide sequence of an immunoglobulin heavy chain of the antibody that binds to the antigen of interest with a desired affinity, (4) determining the nucleotide sequence of said heavy chain, (5) modifying a nucleotide sequence of an immunoglobulin light chain containing the single rearranged human immunoglobulin light chain variable region to comprise a substitution of at least one non-histidine codon with a histidine codon, (6) expressing the immunoglobulin heavy chain of the antibody that binds to the antigen of interest with desired affinity and the immunoglobulin light chain comprising the histidine modification in a cell, and (7) determining whether the antibody expressed in the cell retains binding to the antigen at a neutral pH while displaying reduced binding at an acidic pH. In one embodiment, the antibody expressed in the cell exhibits desired affinity to the antigen at neutral pH. In various embodiments, the immunoglobulin heavy chain is derived from a rearrangement of human heavy chain variable gene segments (human V, D, and J segments).

[000816] In one embodiment, the mouse comprising a single rearranged human light chain variable region gene sequence is a universal light chain or common light chain "ULC" mouse described in, e.g., U.S. Application Publication Nos. 2011/0195454, 2012/0021409, 2012/0192300 and 2013/0045492. In one embodiment, the single rearranged human light chain variable region gene sequence is selected from human Vκ1-39Jκ5 and human Vκ3-20Jκ1 sequence.

[000817] In one embodiment, the antigen of interest is selected from a soluble antigen, a

cell surface antigen (*e.g.*, a tumor antigen) and a cell surface receptor. In a specific embodiment, the cell surface receptor is an immunoglobulin receptor. In a specific embodiment, the immunoglobulin receptor is an Fc receptor.

[000818] In one embodiment, the desired affinity of an antibody for an antigen expressed as a dissociation constant (K_D) at a neutral pH is less than 10^{-6} M, *e.g.*, less than 10^{-8} M, *e.g.*, less than 10^{-9} M, *e.g.*, less than 10^{-10} M, *e.g.*, less than 10^{-11} M, *e.g.*, less than 10^{-12} M.

[000819] As explained above, the ULC mice, in one embodiment, comprise a single rearranged human immunoglobulin light chain variable gene sequence, and express antibodies in response to the antigen where the affinity of antibodies to the antigen is primarily mediated through the heavy chains of their antibodies. These mice comprise a repertoire of human heavy chain variable (V, D, and J) segments, that rearrange to encode a human heavy chain variable domain of an antibody that also comprises the light chain derived from the single rearranged human light chain variable sequence. In one embodiment, upon antigen exposure, these mice utilize the diverse repertoire of human heavy chain variable (V, D, and J) segments to generate an antibody with affinity to and specificity for the antigen. Thus, upon exposure to the antigen, the nucleotide sequence of an immunoglobulin heavy chain of the antibody generated in the ULC mice may be isolated and utilized to generate a desired binding protein also comprising an immunoglobulin light chain derived from the single rearranged human immunoglobulin light chain variable region sequence (*e.g.,* the single rearranged human immunoglobulin light chain variable region sequence with a substitution of at least one non-histidine codon with a histidine codon).

[000820] In one embodiment of the ULC mice, 90-100% of unrearranged non-human V_H gene segments are replaced with at least one unrearranged human V_H gene segment. In a specific embodiment, all or substantially all (*e.g.*, 90-100%) of the endogenous non-human V_H gene segments are replaced with at least one unrearranged human V_H gene segment. In one embodiment, the replacement is with at least 19, at least 39, or at least 80 or 81 unrearranged human V_H gene segments. In one embodiment, the replacement is with at least 12 functional unrearranged human V_H gene segments, at least 25 functional unrearranged human V_H gene segments, or at least 43 functional unrearranged human V_H gene segments. In one embodiment, the non-human animal comprises a replacement of all non-human D_H and J_H segments with at least one unrearranged human D_H segment and at least one unrearranged human J_H segment. In one embodiment, the non-human animal comprises a replacement of all non-human D_H and J_H segments with all unrearranged human D_H segments and all unrearranged human J_H segments. Thus, the ULC mouse utilizes a diverse repertoire of human variable region gene segments (V, D, and J segments)

to generate an antibody in response to the antigen of interest.

[000821] Once the heavy chain of the antibody that binds to the antigen of interest with the desired affinity is determined, the nucleotide sequence of the heavy chain is isolated and sequenced. The sequence is cloned into a vector for expression in suitable host cells, *e.g.,* eukaryotic cells, *e.g.,* CHO cells. In one embodiment, the sequence of a human heavy chain constant region is cloned downstream of the human heavy chain variable region sequence isolated from the mouse (*e.g.*, the ULC mouse).

[000822] In one embodiment, the generating an antigen-binding protein with pH-dependent antigen-binding properties comprises modifying a nucleotide sequence the immunoglobulin light chain, particularly the sequence of the single rearranged human immunoglobulin light chain variable region, to comprise a substitution of at least one non-histidine codon with a histidine codon. Various techniques for modifying a nucleotide sequence are known in the art, *e.g.,* site directed mutagenesis. In addition, a nucleotide sequence comprising the desired histidine substitution may be synthesized de novo.

[000823] In one embodiment, the substitution of at least one non-histidine codon with a histidine codon comprises a substitution resulting in expression of one, two, three, four, or more histidine residues. In one embodiment, the substitution(s) results in expression of three or four histidine residues. In one embodiment, the substitution(s) is in the immunoglobulin light chain variable region. In one embodiment, the substitution(s) is in the CDR codon, *e.g.,* CDR1, CDR3, and/or CDR3. In one embodiment, the substitution(s) is in the CDR3 codon.

[000824] In one embodiment, wherein the immunoglobulin light chain nucleic acid sequence comprises $V\kappa1$-39$J\kappa5$ gene sequence, and the substitution(s) is in the CDR3 codon, the substitution results in expression of histidine at position selected from 105, 106, 108, 111, and combinations thereof. In one embodiment, the substitutions result in expression of histidines at positions 105, 106, 108, and 111. In one embodiment, the substitutions result in expression of histidines at positions 105 and 106. In one embodiment, the substitutions result in expression of histidines at positions 105 and 108. In one embodiment, the substitutions result in expression of histidines at positions 105 and 111. In one embodiment, the substitutions result in expression of histidines at positions 106 and 108. In one embodiment, the substitutions result in expression of histidines at positions 106 and 111. In one embodiment, the substitutions result in expression of histidines at positions 108 and 111. In one embodiment, the substitutions result in expression of histidines at positions 105, 106, and 108. In one embodiment, the substitutions result in expression of

histidines at positions 105, 106, and 111. In one embodiment, the substitutions result in expression of histidines at positions 105, 108, and 111. In one embodiment, the substitutions result in expression of histidines at positions 106, 108, and 111. Amino acid and nucleic acid sequences of Vκ1-39Jκ5 CDR3 regions comprising various histidine substitutions are depicted in FIG. 16 and included in the sequence listing.

[000825] In one embodiment, wherein the immunoglobulin light chain nucleic acid sequence comprises Vκ3-20Jκ1 gene sequence, and the substitution(s) is in the CDR3 codon, the substitution results in expression of histidine at position selected from 105, 106, 107, 109, and combinations thereof. In one embodiment, the substitutions result in expression of histidines at positions 105, 106, 107, and 109. In one embodiment, the substitutions result in expression of histidines at positions 105 and 106. In one embodiment, the substitutions result in expression of histidines at positions 105 and 107. In one embodiment, the substitutions result in expression of histidines at positions 105 and 109. In one embodiment, the substitutions result in expression of histidines at positions 106 and 107. In one embodiment, the substitutions result in expression of histidines at positions 106 and 109. In one embodiment, the substitutions result in expression of histidines at positions 107 and 109. In one embodiment, the substitutions result in expression of histidines at positions 105, 106, and 107. In one embodiment, the substitutions result in expression of histidines at positions 105, 106, and 109. In one embodiment, the substitutions result in expression of histidines at positions 105, 107, and 109. In one embodiment, the substitutions result in expression of histidines at positions 106, 107, and 109. Selected amino acid and nucleic acid sequences of Vκ3-20Jκ1 CDR3 regions comprising various histidine substitutions are depicted in FIG. 27 and included in the sequence listing.

[000826] Once the sequence of immunoglobulin light chain, *e.g.,* human immunoglobulin light chain variable domain, is modified to include histidine residues at desired positions, the nucleotide sequence of the light chain is cloned into a vector for expression in suitable host cells, *e.g.,* eukaryotic cells, *e.g.,* CHO cells. In one embodiment, the sequence of a human light chain constant region is cloned downstream of the modified nucleotide sequence of human variable region.

[000827] In one embodiment, vectors comprising nucleotide sequence encoding modified human immunoglobulin light chain and selected human immunoglobulin heavy chain are co-expressed in a suitable host cell, *e.g.,* eukaryotic host cell, *e.g.,* CHO cell, to generate an antigen-binding protein. Various host cells that can be used for expression are known in the art and are mentioned throughout this specification.

[000828] An antigen-binding protein, *e.g.,* an antibody, generated in the host cell may be secreted into cell supernatant, which is screened for proper expression and affinity for the original antigen at neutral pH. The antigen-binding protein may also be recovered from cell lysate, or, if membrane bound, released from the membrane using a suitable detergent (*e.g.,* Triton-X). The antigen-binding protein with desired characteristics may be purified.

[000829] In one embodiment, the antigen-binding protein comprising histidine modification(s) retains the affinity to the antigen that is comparable to the affinity to the antigen of the same (original) antigen-binding protein that does not comprise histidine modification(s). In one embodiment, the affinity of the histidine-modified antigen-binding protein for the antigen of interest expressed as a dissociation constant (K_D) at a neutral pH is less than 10^{-6} M, *e.g.*, less than 10^{-8} M, *e.g.*, less than 10^{-9} M, *e.g.,* less than 10^{-10} M, *e.g.,* less than 10^{-11} M, *e.g.,* less than 10^{-12} M.

[000830] In one embodiment, the antigen-binding protein, *e.g.,* an antibody, comprising histidine modifications described herein exhibits pH dependent antigen binding properties. In one embodiment, the antigen-binding protein comprising histidine modifications possesses enhanced pH dependent properties over an equivalent antigen-binding protein without the histidine modifications (antigen-binding protein of the same amino acid sequence but for the histidine modifications). In one embodiment, the antigen-binding protein described herein retains binding to the antigen at neutral pH (*e.g.,* retains desired affinity for the antigen at neutral pH) while displaying reduced binding at an acidic pH. In one embodiment, the antigen-binding protein, *e.g.,* the antibody, described herein, exhibits no binding to the antigen in acidic pH, while retaining binding to the antigen at neutral pH. In one embodiment, an antigen-binding protein described herein, has a decrease in dissociative half-life ($t_{1/2}$) at an acidic pH as compared to the dissociative half-life ($t_{1/2}$) of the antigen-binding protein at a neutral pH of at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, or at least about 30-fold. In one embodiment, an antigen-binding protein described herein has a $t_{1/2}$ at an acidic pH and 37°C of about 2 min or less. In one embodiment, an antigen-binding protein described herein has a $t_{1/2}$ at an acidic pH and 37°C of less than about 1 min. In one embodiment, an antigen-binding protein described herein has a $t_{1/2}$ at an acidic pH and 25°C of about 2 min or less. In one embodiment, an antigen-binding protein described herein has a $t_{1/2}$ at an acidic pH and 25°C of less than about 1 min.

[000831] In one embodiment, the antigen-binding protein *e.g.,* the antibody, comprising histidine modifications described herein, exhibits increased serum half life upon

administration of a therapeutic dose to a subject as compared to a serum half life upon administration of an equivalent therapeutic dose of antigen-binding protein that does not comprise histidine modifications (*e.g.,* the original antigen-binding protein that does not comprise histidine modifications). In some embodiments, the increase in serum half life upon administration of a dose of the antigen-binding protein comprising histidine modifications described herein over a serum half life upon administration of the same dose of the antigen-binding protein not comprising histidine modifications is about 2 fold, *e.g.,* about 5 fold, *e.g.,* about 10 fold, *e.g.,* about 15 fold, *e.g.,* about 20 fold, or greater. In one embodiment, serum half-life is at least about 1 day, *e.g.,* at least about 2 days, *e.g.,* at least about 7 days, *e.g.,* at least about 14 days, *e.g.,* at least about 30 days, *e.g.*, at least about 60 days.

[000832] In addition to the *in vitro* methods for generating antigen-binding proteins with pH-dependent antigen binding properties described above, also provided herein are antigen-binding proteins, *e.g.*, antibodies, generated by said method. In addition, said method may be utilized to generate multi-specific, *e.g.*, bispecific, antigen-binding proteins, by selecting two different human immunoglobulin heavy chains that bind to a common (universal) light chain in a mouse, determining nucleotide sequences of the heavy chains, modifying universal light chain to comprise histidine substitutions as described above, and co-expressing two human heavy chains with a single histidine-modified universal light chain in a host cell. Various steps for generating an antigen-binding protein described above may be applicable to the method of generating a bispecific antigen-binding protein. Bispecific antigen binding protein, confirmed to possess desired affinity for the antigen(s) and pH-dependent antigen binding properties may be purified. Thus, bispecific antibodies comprising two human heavy chains and a single human light chain comprising a human light chain variable domain sequence encoded by a human variable region gene, e.g., Vκ1-39Jκ5 or Vκ3-20Jκ1 variable region gene comprising a substitution of at least one non-histidine codon with a histidine codon, is provided.

[000833] Also provided are constructs utilized in making an antigen-binding protein comprising human immunoglobulin heavy chain and human immunoglobulin light chain comprising histidine substitutions. Host cells expressing antigen-binding proteins, *e.g.*, antibodies, described herein are also provided.

EXAMPLES

[000834] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present

invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1. Construction of Humanized Immunoglobulin Heavy Chain Loci Comprising Histidine-Substituted D Gene Segments

[000835] Construction of immunoglobulin heavy chain loci comprising histidine-substituted human D gene segments was carried out by series of homologous recombination reactions in bacterial cells (BHR) using Bacterial Artificial Chromosome (BAC) DNA. Several targeting constructs for creation of a genetically engineered mouse that expresses a heavy chain variable domain comprising one or more histidine residues were generated using VELOCIGENE® genetic engineering technology (see, *e.g.*, US Pat. No. 6,586,251 and Valenzuela, D. M. *et al.* (2003), High-throughput engineering of the mouse genome coupled with high-resolution expression analysis, *Nature Biotechnology* 21(6):652-659).

[000836] Initially, human D gene segments were synthesized *in silico* as four pieces (4 repeats) in which the codons encoding tyrosine (Y), asparagine (N), serine (S), glycine (G), and aspartate (D) in the hydrophilic frame were substituted with histidine codons (hereinafter "histidine-substituted human D gene segments", *i.e.*, HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4) (FIG. 3). The four repeats also contained unique restriction enzyme sites at the ends for ligating them back together. The specific location of the histidine substitutions (labeled in bold type) in each human D gene segment is shown in FIGs. 1A and 1B in the column labeled "Hydrophilic." As shown in FIG. 1, while the modification introduced histidine codons in the hydrophilic reading frame, it also changed some stop codons to serine codons in the "Stop" reading frame. The modification, however, made few changes in the "Hydrophobic" reading frame. The detailed procedure for ligating the four synthesized D segment repeats is illustrated in FIG. 3 (sequential ligation). The resulting clone contained, from 5' to 3', a 5' mouse homology arm, a floxed neomycin cassette, human D gene segments comprising histidine substitutions (*i.e.*, HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)), a chloramphenicol selection cassette, and a 3' homology arm.

[000837] The following six genetic modifications were carried out in order to replace the endogenous human D gene segments in the VELOCIMMUNE® humanized mouse with the histidine-substituted human D gene segments described above.

[000838] First, pLMa0174, containing a spectinomycin selection cassette and an AsiSI restriction site, was targeted into the 5' end of the MAID 1116 clone (Step 1. BHR (Spec); FIG. 2). During Step 1, a chloramphenicol selection cassette, a neomycin selection cassette, a loxP site, two V_H gene segments (hV_H1-3 and hV_H1-2), and the human Adam6p gene, all of which are located 5' upstream of hV_H6-1, were deleted from the MAID 1116 clone and replaced by a spectinomycin cassette to yield the VI433 clone.

[000839] Second, in Step 2 (BHR (Hyg + Spec); FIG. 2), pNTu0002 containing a hygromycin cassette flanked by FRT sites was targeted into a region comprising human immunoglobulin D_H gene segments. During Step 2, all human heavy chain D gene segments were deleted from VI433 and replaced with the hygromycin cassette to yield MAID6011 VI 434 (clone 1). The modification also introduced the PI-SceI and the I-CeuI restriction sites at the 5' and 3' end of the hygromycin cassette.

[000840] Third, the genomic region comprising histidine-substituted human D gene segments (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)) were introduced into a region between the PI-SceI and the I-CeuI sites of MAID 6011 VI434 via restriction digestion and ligation (PI-SceI/I-CeuI Ligation modified 1116 (Kan + Spec); Fig. 4). This yielded MAID6012 VI469 containing, from 5' to 3', a spectinomycin cassette, about 50 kb of a genomic region comprising V_H6-1, a floxed neomycin cassette, about 40 kb of the histidine-substituted human D gene segments (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)), and about 25 kb of a genomic region containing human J_H gene segments, followed by a mouse E_i (mIgH intronic enhancer; SEQ ID NO: 5), a mouse switch region (SEQ ID NO: 6), and a mouse IgM constant region nucleotide sequence (mIgM exon 1; SEQ ID NO: 7). Bacterial cells containing the modification were selected based on Kanamycin and Spectinomycin selection.

[000841] Fourth, MAID 1460 heterozygous mouse ES cells were targeted with MAID 6011 VI434 via electroporation in order to remove all endogenous human D gene segments from the MAID 1460 clone as illustrated in Fig. 5. This yielded MAID 6011 heterozygous mouse ES cells comprising in its immunoglobulin heavy chain locus (at the 129 strain-derived chromosome), from 5' to 3', an FRT site, human V_H gene segments, a mouse genomic

region encompassing adam6a/b genes, a hygromycin cassette flanked by FRT sites, and human J_H segments, followed by a mouse E_i sequence and an IgM constant region nucleotide sequence. The genetic modification of MAID 6011 (a loss of alleles, a gain of alleles, and presence of parental alleles) was confirmed by using the probes and primers as shown in Fig 6.

[000842] Fifth, MAID 6011 heterozygous mouse ES cells were electroporated with MAID 6012 VI469 in order to introduce histidine-substituted human D gene segments (*i.e.*, HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)) into MAID 6011. The targeting step removed the floxed hygromycin selection cassette from MAID 6011 and replaced the sequence with the histidine-substituted human D gene segments. This lead to MAID 6012 hetrozygous ES cells comprising a wild-type C57BL/6 strain-derived chromosome and a genetically modified 129 strain-derived chromosome comprising human wild-type V_H and J_H gene segments and the histidine-substituted human D gene segments described herein. In addition, the ES cells contained a mouse genomic region encompassing adam6a/b genes and a floxed neomycin cassette between the V_H and D segments (Fig. 7). The genetic modification of MAID 6012 (a loss of alleles, a gain of alleles, and presence of parental alleles) was confirmed by using the probes and primers as shown in Fig 8.

[000843] Lastly, MAID 6012 ES cells were electroporated with a plasmid that expresses a Cre recombinase in order to remove the neomycin selection cassette from the MAID 6012 ES cells, resulting in MAID 6013 heterozygous ES cells (Fig. 9). The final MAID 6013 heterozygous ("MAID 6013 het") ES cell contains a wild-type C57BL/6 strain-derived chromosome and a genetically modified, 129 strain-derived chromosome comprising in its immunoglobulin heavy chain locus, from 5' to 3', (1) an FRT site; (2) human V_H gene segments; (3) a mouse genomic region encompassing adam6a/b genes; (4) a floxed neomycin selection cassette; (5) histidine-substituted human D gene segments (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4)); (6) human J_H gene segments; followed by (7) a mouse E_i sequence (mIgH intronic enhancer; SEQ ID NO: 5), (8) a switch region (SEQ ID NO: 6); and (9) a mouse IgM constant region nucleotide sequence (mIgM exon 1; SEQ ID NO: 7) as illustrated in Fig. 9.

[000844] The targeted ES cells (MAID 6013) described above were used as donor ES cells and introduced into an 8-cell stage mouse embryo by the VELOCIMOUSE® method (see, *e.g.*, US 7,576,259, US 7,659,442, US 7,294,754, US 2008-0078000 A1). Mice bearing the genetically modified immunoglobulin heavy chain locus comprising the histidine-

substituted human heavy chain D gene segments described herein were identified by genotyping using the primers and probes set forth in Fig. 8. The resulting genetically modified F0 mouse was crossed to a wild-type mouse to obtain F1 offspring. F1 pups were genotyped, and the F1 pups that are heterozygous for the genetically modified immunoglobulin locus comprising histidine-substituted human heavy chain D gene segments were selected for further characterization.

Example 2. Analysis of Rearranged Heavy Chain Variable Region Nucleotide Sequences

[000845] Next, it was examined whether the genetically modified mouse comprising histidine-substituted human D gene segments described herein, *e.g.*, 6013 F0 heterozygous mouse, which comprises in its germline a 129 strain-derived chromosome comprising human V_H, J_H gene segments, and histidine-substituted human D gene segments (HD 1.1-6.6 (9586bp; SEQ ID NO: 1), HD 1.7-6.13 (9268bp; SEQ ID NO: 2), HD 1.14-6.19 (9441bp; SEQ ID NO: 3), and HD 1.20-6.25, 1.26 (11592bp; SEQ ID NO: 4), can express rearranged heavy chain V(D)J sequences comprising one or more histidine codons derived from the genetically modified immunoglobulin heavy chain locus.

[000846] To this end, mRNA sequences isolated from splenic B cells of the 6013 F0 heterozygous mice were analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) for the presence of IgM CDR3 sequences derived from the histidine-substituted human D gene segments.

[000847] Briefly, spleens were harvested and homogenized in 1xPBS (Gibco) using glass slides. Cells were pelleted in a centrifuge (500xg for 5 minutes), and red blood cells were lysed in ACK Lysis buffer (Gibco) for 3 minutes. Cells were washed with 1xPBS and filtered using a 0.7µm cell strainer. B-cells were isolated from spleen cells using MACS magnetic positive selection for CD19 (Miltenyi Biotec). Total RNA was isolated from pelleted B-cells using the RNeasy Plus kit(Qiagen). PolyA+ mRNA was isolated from total RNA using the Oligotex® Direct mRNA mini kit (Qiagen).

[000848] Double-stranded cDNA was prepared from splenic B cell mRNA by 5' RACE using the SMARTer™ Pico cDNA Synthesis Kit (Clontech). The Clontech reverse transcriptase and dNTPs were substituted with Superscript II and dNTPs from Invitrogen. Heavy chain variable region (V_H) antibody repertoires were amplified from the cDNA using primers specific for IgM constant regions and the SMARTer™ 5' RACE primer (Table 1). PCR products were cleaned up using a QIAquick® PCR Purification Kit (Qiagen). A second

round of PCR was done using the same 5' RACE primer and a nested 3' primer specific for the IgM constant regions (Table 2). Second round PCR products were purified using a SizeSelect™ E-gel® system (Invitrogen). A third PCR was performed with primers that added 454 adapters and barcodes. Third round PCR products were purified using Agencourt® AMPure® XP Beads. Purified PCR products were quantified by SYBR®-qPCR using a KAPA Library Quantification Kit (KAPA Biosystems). Pooled libraries were subjected to emulsion PCR (emPCR) using the 454 GS Junior Titanium Series Lib-A emPCR Kit (Roche Diagnostics) and bidirectional sequencing using Roche 454 GS Junior instrument according to the manufacturer's protocols.

[000849] Table 1.

NAME	SEQUENCE
3' mIgM CH1 outer	TCTTATCAGACAGGGGGCTCTC (SEQ ID NO:318)

[000850] Table 2.

NAME	
3' mIgM CH1 inner	GGAAGACATTTGGGAAGGACTG (SEQ ID NO:319)

[000851] Bioinfomatic Analysis

[000852] The 454 sequences were sorted based on the sample barcode perfect match and trimmed for quality. Sequences were annotated based on alignment of rearranged Ig sequences to human germline V,D and J segments database using local installation of igblast (NCBI, v2.2.25+). A sequence was marked as ambiguous and removed from analysis when multiple best hits with identical score were detected. A set of perl scripts was developed to analyze results and store data in mysql database. The CDR3 region was defined between conserved C codon and FGXG motif (SEQ ID NO: 320) for light chains and WGXG motif (SEQ ID NO: 321) for heavy chains. CDR3 length was determined using only productive antibodies.

[000853] As shown in Figs. 11-13, 6013 F0 heterozygous mice expressed a diverse repertoire of rearranged heavy chain variable region mRNA sequences (rearranged V-D-J sequences) encoding one or more histidine codons in CDR3. The sequencing data suggested that the histidine codons appeared in CDR3 were derived from various histidine-

substituted human D gene segments present in the genetically modified immunoglobulin heavy chain locus of the 6013 mice described herein.

Example 3. Histidine Usage in Antigen-Specific Human Light Chains

[000854] Amino acid sequences of selected light chains from antigen-specific human antibodies were aligned. Histidine mutations in the CDRs of human Vκ1-39-derived light chains for a selected number of antigen-specific human antibodies were identified (FIG. 15). The human Vκ1-39-derived light chains were isolated from immunized mice engineered to contain a single rearranged human Vκ1-39 light chain (see US 2011/0195454A1), and bear somatic hypermutations as generated in the antibody repertoire of the mouse.

[000855] Histidine residues were engineered into a rearranged human Vκ1-39 light chain using molecular mutagenesis techniques known in the art. Locations of the engineered residues are shown in FIG. 16.

[000856] Human Vκ1-39-derived light chain variable regions containing engineered histidine residues were constructed and paired with various human heavy chain variable regions in an antibody format, specific for a human cell surface receptor, to analyze expression in CHO cells.

[000857] CHO cells having a particular heavy chain and a light chain with indicated his modifications (*e.g.,* 105, 106, 108, 111) were seeded into wells of a 48-well plate. The next day, DNA corresponding to heavy chain and light chain, in equal weight (400 ng), were mixed with transfection reagent (Lipofectin 2000), allowed to form a complex by incubation, and the complex added to the plated cells. Four days later, media was collected. The media contained the expressed antibody.

[000858] CHO cells having different heavy chains paired with the same light chain having one or more his substitutions in CDR3 express well. Level (ng/mL) of antibody expression in ng/mL detected in supernatants of CHO cells transfected with antibody genes having histidine residues engineered at selected locations in the CDR3 of the light chains was determined.

[000859] Expression in supernatants of CHO cells of paired antigen-specific heavy chains with histidine engineered light chains using selected heavy chains, measured by protein blots, is shown in FIG. 18. ULC refers to a rearranged human Vκ1-39-derived light chain.

[000860] An aliquot of media was subjected to analysis on a BIACORE™ instrument using the target antigen for the antibody (a cell surface receptor sequence). Antibody was

captured on the chip. Antibody capture level is shown in FIG. 19A-19J as RU. Captured antibody on the BIACORE™ chip was subjected to flow containing the sequence of the target antigen. Antibody capture of the target antigen was measured, as well as association rate and other parameters as shown. Antigen flow was stopped and dissociation rate was determined as antigen disengaged from the bound antibody.

[000861] Equilibrium dissociation constants (K_D) (apparent) for selected antibody supernatants were determined by SPR (Surface Plasmon Resonance) using a BIACORE™ T100 instrument (GE Healthcare). Kinetics were measured at pH 7.4 and at pH 5.75. Results are shown in FIG. 19A-19J.

[000862] As shown in FIG. 19A-19J, data for antibody binding to a cell surface receptor, where the light chains have been modified to encode histidine residues a specific positions in a CDR, for Vκ1-30/Jκ5 light chains paired with the indicated heavy chains, demonstrates that the histidine modifications directly influence binding of the antigen (*e.g.,* a cell surface receptor) with different affinities at pH 7.4 and pH 5.75. Histidine modifications that retain binding at pH 7.4, but that exhibit a low binding or no detectable binding at pH 5.75, are desirable.

Example 4. Identification of Histidine Residues in Antigen-specific Human Light Chains.

[000863] Generation of a common light chain mouse (*e.g.,* Vκ1-39 or Vκ3-20 common light chain mouse) and antigen-specific antibodies in those mice is described in U.S. Patent Application Nos. 13/022,759, 13/093,156, and 13/412,936 (Publication Nos. 2011/0195454, 2012/0021409, and 2012/0192300, respectively). Briefly, rearranged human germline light chain targeting vector was made using VELOCIGENE® technology (see, *e.g.,* US Pat. No. 6,586,251 and Valenzuela *et al.* (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis, Nature Biotech. 21(6): 652-659) to modify mouse genomic Bacterial Artificial Chromosome (BAC) clones, and genomic constructs were engineered to contain a single rearranged human germline light chain region and inserted into an endogenous κ light chain locus that was previously modified to delete the endogenous κ variable and joining gene segments. Targeted BAC DNA was then used to electroporate mouse ES cells to create modified ES cells for generating chimeric mice that express a rearranged human germline Vκ1-39Jκ5 or Vκ3-20Jκ1 region. Targeted ES cells were used as donor ES cells and introduced into an 8-cell stage mouse embryo by the VELOCIMOUSE® method (see, *e.g.,* US Pat. No. 7,294,754 and Poueymirou *et al.* (2007) F0 generation mice that are essentially fully derived from the donor gene-targeted ES cells

allowing immediate phenotypic analyses Nature Biotech. 25(1): 91-99). VELOCIMICE® independently bearing an engineered human germline Vκ1-39Jκ5 or Vκ3-20Jκ1 light chain region were identified by genotyping using a modification of allele assay (Valenzuela *et al., supra*) that detects the presence of the unique rearranged human germline light chain region.

[000864] Mice bearing an engineered human germline light chain locus (ULC mice) were bred with mice that contain a replacement of the endogenous mouse heavy chain variable gene locus with the human heavy chain variable gene locus (see US 6,596,541; the VELOCIMMUNE® mouse, Regeneron Pharmaceuticals, Inc.).

[000865] VELOCIMMUNE® mouse containing a single rearranged human germline light chain region is challenged with an antigen of interest and antibodies comprising a universal light chain (e.g., Vκ1-39Jκ5) are isolated and sequenced. Amino acid sequences of selected light chains (A-K) from antigen-specific human antibodies generated in a common Vκ1-39Jκ5 light chain mouse were aligned. Histidine mutations in the CDRs of human Vκ1-39-derived light chains for a selected number of antigen-specific human antibodies were identified (FIG. 15). The partial amino acid sequence of germline Vκ1-39Jκ5 variable domain is shown above the alignments and set forth in SEQ ID NO:325, the complete variable domain amino acid sequence is set forth in SEQ ID NO:404.

Example 5. Engineering and Characterization of Histidine-Substituted Human Universal Light Chain Antibodies

Example 5.1. Engineering of Histidine Residues into a Germline Human Rearranged Light Chain

[000866] Histidine residues were engineered into a rearranged human Vκ1-39Jκ5 light chain using site directed mutagenesis primers specifically designed to introduce engineered histidine residues at Q105, Q106, Y108, and P111 positions of the human Vκ1-39Jκ5 light chain. Site directed mutagenesis was performed using molecular techniques known in the art (e.g., QuikChange II XL Site Directed Mutagenesis Kit, Agilent Technologies). Locations of the engineered residues in the CDR3 are shown in FIG. 16, the nucleic acid sequences of histidine-substituted CDR3's depicted in FIG. 16 are set forth in SEQ ID NOs: 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, and 356 (corresponding amino acid sequences are set forth in SEQ ID NOs: 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, and 357). The nucleic acid and amino acid sequences of germline rearranged Vκ1-39Jκ5 CDR3 are set forth in SEQ ID NOs: 326 and 327, respectively.

Example 5.2. Construction and Expression of Histidine Engineered Light Chains

[000867] Human Vκ1-39-derived light chains containing germline engineered histidine residues made according to Example 2 were constructed and paired with various human heavy chains (labeled 1-5), specific for a human cell surface receptor, to analyze expression in CHO cells. The five human heavy chains specific for a human cell surface receptor that were paired with histidine-substituted Vκ1-39-derived light chains were obtained from mice that have a single rearranged human light chain (a human Vκ1-39/Jκ5 rearranged light chain; see US2011/0195454A1).

[000868] *Enzyme-linked Immunosorbent assay (ELISA):* Antibody secretion from CHO cells was detected using an Fc ELISA, for light chains with indicated histidine modifications with five different heavy chains. The light and heavy chain sequences (but for the modifications) were generated in mice that have a single rearranged human light chain (*e.g.,* a human Vκ1-39/Jκ5 rearranged light chain; see US2011/0195454A1). Capture antibody was goat anti-human IgG and detection antibody was goat anti-human (Fc gamma-specific)-HRP. The results are shown in FIG. 17. ULC + heavy: specific heavy chain and unmodified human Vκ1-39-derived light chain. As shown in FIG. 17, expression was detected in about all mutants.

[000869] *Protein Immunoblot.* Expression in supernatants of CHO cells of paired antigen-specific heavy chains with histidine engineered light chains was further analyzed by western blot. Samples were run on a 4-12% tris-glycine gel. Results using a selected heavy chain (heavy chain 3) are shown in FIG. 18. ULC refers to a rearranged human Vκ1-39-derived light chain (as described above).

Example 5.3. Determination of Binding Affinity of Histidine Engineered Light Chains

[000870] Equilibrium dissociation constants (K_D), dissociative half-lives ($t_{1/2}$), and other kinetic parameters for selected antibody supernatants were determined by SPR (Surface Plasmon Resonance) using a BIACORE™ T200 instrument (GE Healthcare). Kinetics were measured at pH 7.4 and at pH 5.75. Results are shown in FIGs. 20A – 20E.

[000871] Numerical values for the kinetic binding properties (e.g., k_a, k_d, K_D, $t_{½}$, etc.) of antibodies binding to immunogen at neutral pH (pH 7.4) and at acidic pH (pH 5.75) were obtained using a real-time surface plasmon resonance biosensor (Biacore T200.) A Biacore CM5 sensor chip was derivatized with a mouse anti-human Fc antibody to capture antibodies from the supernatant. A single concentration (50nM) of immunogen was then

injected over the antibody-captured surface at a flow rate of 30 µl/min. Antibody-antigen association was monitored for 2.5 minutes and then the dissociation of antigen from the captured antibody was monitored for 8 minutes. Kinetic association (ka) and dissociation (kd) rate constants were determined by processing and fitting the data to a 1:1 binding with a mass transport model using Biacore T200 Evaluation software version 1.0. Equilibrium dissociation constants (K_D) and dissociative half-lives ($t_{½}$) were calculated from the kinetic rate constants as: K_D (M) = k_d / k_a; and $t_{½}$ (min) = (ln2/(60*k_d).

[000872] As shown in FIG. 20, in a binding assay of antibody to a cell surface receptor, two out of five antibodies with histidine-modified common light chains (histidine modified CDR3's of Vκ1-39/Jκ5 light chains) that were paired with the antigen-specific human heavy chains, exhibited binding to the antigen (*e.g.,* to a cell surface receptor) with different affinities at pH 7.4 and pH 5.75. Antibodies with histidine modifications that retain binding at pH 7.4, but that exhibit a low binding or no detectable binding at pH 5.75, are desirable. Antibodies with histidine modification that exhibit reduced $t_{1/2}$ at pH 5.75 as compared to pH 7.4 are desirable.

[000873] Antigen binding data for three antibodies comprising histidine-modified common light chains and three antigen-specific heavy chains (labeled 2, 3, and 6) at different pHs is summarized further in FIG. 21. These antibodies exhibited significant drop in antigen binding at pH 5.75 in comparison to pH 7.4, as demonstrated, e.g., by reduction in $t_{1/2}$ or no binding detected at pH 5.75.

Example 6. Engineering and Characterization of Genetically Modified Mouse Comprising a Histidine-Substituted Vκ1-39Jκ5 Universal Light Chain

Example 6.1. Constructing of Targeting Vector for Engineering Histidine Residues in a Rearranged Human Light Chain Variable Region

[000874] A genetically modified mouse containing a rearranged human light chain gene having histidine residues engineered into a CDR region of the human light chain is made using targeting vectors made by standard molecular cloning techniques known in the art.

[000875] Briefly, various rearranged human germline light chain targeting vectors are made using VELOCIGENE® technology (see, *e.g.,* US Pat. No. 6,586,251 and Valenzuela *et al.* (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis, Nature Biotech. 21(6):652-659) to modify mouse genomic Bacterial Artificial Chromosome (BAC) DNA to contain a single rearranged human germline light chain region and inserted into an endogenous κ light chain locus that was previously modified to

delete the endogenous κ variable and joining gene segments. The rearranged human germline light chain region is modified at one or more nucleotide positions within the sequence of the light chain to encode histidine residues that are not normally present at the respective locations of the germline sequence. The targeting vectors are electroporated into mouse embryonic stem (ES) cells to create and confirmed using a quantitative PCR assay (*e.g.*, TAQMAN™).

[000876] Specifically, a strategy for constructing these targeting vectors is shown in FIGs. 23A – 23F. A plasmid used for generating a targeting vector for common (universal) light chain mouse ("ULC mouse," described in, e.g., US2011/0195454A1), containing pBS + FRT-Ub-Hyg-FRT + mouse Vκ3-7 leader +human Vκ1-39Jκ5 was modified by site directed mutagenesis (QuickChange II XL Kit) to replace Q105, Q106, Y108 and P111 or Q106, Y108 and P111 with histidine residues in the CDR3 region using site-directed mutagenesis primers shown in FIG. 22 (See FIG. 23A for this engineering step). Resultant vectors (H105/106/108/111 and H106/108/111) were modified further and ligated into a vector comprising mouse Igκ constant region, mouse enhancers, a mouse 3' homology arm and a SPEC cassette (FIG. 23B). Further modification involved ligation into a vector carrying 5' mouse arm and comprising Frt-Ub-NEO-Frt cassette (FIG. 23B). Resultant targeting vectors were electroporated into ES cells comprising deletion of the mouse Igκ variable locus (comprising κ variable and joining gene segments) (FIGs. 23C-23F).

[000877] Positive ES cell clones were confirmed by using a modification of allele assay (Valenzuela et al.) using probes specific for the engineered Vκ1-39Jκ5 light chain region inserted into the endogenous κ light chain locus. Primers and probes used in the assay are shown in Table 3 below and set forth in the Sequence Listing; the locations of the probes are depicted in FIGs. 23C-23F.

Table 3: Primers and Probes Used for ES Cell Screening

Probe Name	Assay	Probe Sequence	5' Primer	3' Primer
Neo	GOA	TGGGCACAAC AGACAATCGGC TG (SEQ ID NO:362)	GGTGGAGAG GCTATTCGGC (SEQ ID NO:363)	GAACACGGCGG CATCAG (SEQ ID NO:364)
ULC-m1	GOA	CCATTATGATG CTCCATGCCTC TCTGTTC (SEQ ID NO:365)	AGGTGAGGG TACAGATAAG TGTTATGAG (SEQ ID	TGACAAATGCCC TAATTATAGTGAT CA (SEQ ID NO:367)

Probe Name	Assay	Probe Sequence	5' Primer	3' Primer
			NO:366)	
1633h2 (Vκ1-39Jκ5-specific)	GOA	ATCAGCAGAAA CCAGGGAAAGC CCCT (SEQ ID NO:368)	GGGCAAGTC AGAGCATTAG CA (SEQ ID NO:369)	TGCAAACTGGAT GCAGCATAG (SEQ ID NO:370)
mIgKd2	Retentio n	GGCCACATTCC ATGGGTTC (SEQ ID NO:371)	GCAAACAAAA ACCACTGGCC (SEQ ID NO:372)	CTGTTCCTCTAAA ACTGGACTCCAC AGTAAATGGAAA (SEQ ID NO:373)
mIgKp15	Retentio n	GGGCACTGGAT ACGATGTATGG (SEQ ID NO:374)	CACAGCTTGT GCAGCCTCC (SEQ ID NO:375)	AGAAGAAGCCTG TACTACAGCATC CGTTTTACAGTCA (SEQ ID NO:376)

[000878] The NEO selection cassette introduced by the targeting constructs was deleted by transfecting ES cells with a plasmid that expresses FLP (FIGs. 23C and 23E). Optionally, the neomycin cassette may be removed by breeding to mice that express FLP recombinase (*e.g.,* US 6,774,279). Optionally, the neomycin cassette is retained in the mice.

[000879] Targeted ES cells described above were used as donor ES cells and introduced into an 8-cell stage mouse embryo by the VELOCIMOUSE® method (see, *e.g.,* US Pat. No. 7,294,754 and Poueymirou *et al.* (2007) F0 generation mice that are essentially fully derived from the donor gene-targeted ES cells allowing immediate phenotypic analyses *Nature Biotech.* 25(1):91-99. VELOCIMICE® independently bearing an engineered human light chain gene that contains histidine residues mutated into one or more positions along the sequence were made from the targeted ES cells described above.

[000880] Pups were genotyped and pups heterozygous for the engineered histidine-modified human light chain were selected for characterizing expression of the light chain and binding capabilities of the expressed antibodies. Primers and probes for genotyping of mice specifically comprising a universal light chain gene with either three (H106/108/111; "1930") or four (H105/105/108/111; "1927") histidine modifications are listed in Table 4 below and set forth in the Sequence Listing. Mice containing histidine modification in their universal light chains are referred herein as "HULC" mice (histidine universal light chain mice).

[000881] Table 4: Primers and Probes Used for Genotyping

Probe Name	Assay	Probe Sequence	5' Primer	3' Primer
1927jxn3	GOA 1927 (4 His) mouse-specific	ACCATAGTCACAG TACCCA (SEQ ID NO:377)	AGCAGTCTGCAAC CTGAAGATTT (SEQ ID NO:378)	CCCTTGGCCGAAG GTGAT (SEQ ID NO:379)
1930jxn3	GOA 1930 (3 His) mouse-specific	ATAGTCACAGTAC CCATCC (SEQ ID NO:380)	AGTCTGCAACCTG AAGATTTTGC (SEQ ID NO:381)	CCCTTGGCCGAAG GTGAT (SEQ ID NO:382)

Example 6.2. Analysis of Immune Response to Antigen in Mice with Histidine-Substituted Universal Light Chains

[000882] Cell surface receptor ("Antigen A") was used as the immunogen to immunize mice that were either heterozygous for expression of a pre-arranged human kappa light chain utilizing Vk1-39 and Jk5 that has 4 histidine substitutions in CDR3 (hereinafter "HULC 1927") or heterozygous for expression of a pre-arranged human kappa light chain utilizing Vk1-39 and Jk5 that has 3 histidine substitutions in CDR3 (hereinafter "HULC1930"), or homozygous WT mice. Pre-immune serum was collected from the mice prior to the initiation of immunization. The immunogen was administered at 2.35 µg of protein for the initial priming immunization mixed with 10 µg of CpG oligonucleotide as an adjuvant (Invivogen) in a volume of 25 µl via footpad (f.p.). Subsequently, mice were boosted via the same route with 2.35 µg of Antigen A along with 10 µg of CpG and 25 µg of Adju-Phos (Brenntag) as adjuvants on days 3, 6, 11, 13, 17, 20 for a total of 6 boosts. The mice were bled on days 15 and 22 after the 4^{th} and 6^{th} boost, respectively. Their antiserum was assayed for antibody titers to Antigen A.

[000883] Antibody serum titers against immunogen were determined by a standard ELISA. To perform the ELISA, 96-well microtiter plates (Thermo Scientific) were coated at 2 µg/ml with Antigen A in phosphate-buffered saline (PBS, Irvine Scientific) overnight at 4°C. The next day, plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T, Sigma-Aldrich) four times using a plate washer (Molecular Devices). Plates were then blocked with 250 µl of 0.5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS and incubated for 1 hour at room temperature. The plates were then washed four times with PBS-T. Sera from immunized mice and pre-immune sera were serially diluted three-fold in 0.5% BSA-PBS starting at 1:300 or 1:1000, added to the blocked plates in duplicate, and then incubated for 1 hour at room temperature. The last two wells were left blank to be used as a secondary antibody control (background control). The plates were again washed four

times with PBS-T in a plate washer. Goat anti-mouse IgG-Fc- Horse Radish Peroxidase (HRP) conjugated secondary antibody (Jackson Immunoresearch) was then added to the plates at 1:5000/1:10,000 dilution and incubated for 1 hour at room temperature. Plates were then washed eight times with PBS-T and developed using TMB/H_2O_2 as substrate. The substrate was incubated for 20 min and the reaction was stopped with 2 N sulfuric acid (H_2SO_4, VWR, cat# BDH3500-1) or 1 N phosphoric acid (JT Baker, Cat# 7664-38-2). Plates were read on a spectrophotometer (Victor, Perkin Elmer) at 450nm. Antibody titers were computed using Graphpad PRISM software.

[000884] The immune response induced in mice to the injected immunogen is represented as antibody titers, which is defined as the reciprocal of the highest serum dilution at which antigen binding absorbance is two-fold higher over background. Therefore, the higher the number, the greater the humoral immune response to the immunogen. Antibody titers induced to the immunogen were very high in both strains of HULC mice and in the WT mice, with no significant differences observed among the strains (FIG. 24).

Example 6.3. Generation of pH-Sensitive Monoclonal Antibodies

[000885] When a desired immune response to the immunogen was achieved in both strains of HULC mice and in the WT mice, splenocytes from each mouse strain were harvested and fused with mouse myeloma cells to generate hybridoma cells, which were allowed to grow in 96-well plates. After 10 days of growth, supernatants from each hybridoma cell-containing well were screened via immunogen-specific ELISA to identify positive antigen binding samples. For the ELISA, 96 well micro-titer plates were coated with 1ug/mL of an anti-myc polyclonal antibody (Novus Biologicals, #NB600-34) overnight at 4°C to immobilize the myc-tagged antigen, followed by blocking with a solution of 0.5% (w/v) BSA in PBS. The plates were washed, the antigen solutions were added to the plates at a concentration of 1 μg/mL and allowed to bind to the coated plate for 1 hour at room temperature. Subsequently, supernatants from hybridoma cells were added to the wells at 1:50 dilution and allowed to bind for 1 hour at room temperature. The plate bound antibodies were detected using an anti-mouse IgG polyclonal antibody conjugated with HRP (Jackson Immunoresearch, #115-035-164). TMB substrates were added to the plates (BD Biosciences, # 51-2606KC/51-2607KC) and colorimetric signals were developed according to manufacturer recommended protocol. The absorbance was recorded at 450nm on a Victor Wallac plate reader. Antigen positive samples defined as having an OD equal to or greater than 0.5 (with the baseline having OD of about 0.1) were subject to affinity screening using a real-time surface plasmon resonance biosensor (Biacore 4000).

[000886] Kinetic binding parameters (e.g., k_a, k_d, K_D, $t_{½}$, etc.) for antibody binding to the immunogen at neutral pH (pH 7.4) and at acidic pH (pH 6.0) were recorded. A Biacore CM4 sensor chip was derivatized with a polyclonal goat anti-mouse Fc antibody to capture antibodies from the supernatant. A single concentration (100nM) of immunogen was then injected over the antibody-captured surface at a flow rate of 30 µl/min. Antibody-antigen association was monitored for 1.5 minutes and then the dissociation of antigen from the captured antibody was monitored for 2.5 minutes. Kinetic association (k_a) and dissociation (k_d) rate constants were determined by processing and fitting the data to a 1:1 binding with a mass transport model using Biacore 4000 Evaluation software version 1.0. Equilibrium dissociation constants (K_D) and dissociative half-lives ($t_{½}$) were calculated from the kinetic rate constants as: K_D (M) = k_d / k_a; and $t_{½}$ (min) = ln2/(60*k_d). A set of samples that displayed decreased binding at pH 6.0 as compared to that at pH 7.4 (pH sensitive) as well as a set of control samples that displayed no significant rate changes between the pH 7.4 and pH 6.0 (pH insensitive controls) were selected to be produced clonally. FIG. 25 depicts comparison of the number of total antigen positives and the number of antigen positives displaying pH-sensitive antigen binding from HULC and WT mice.

[000887] Among the antigen positives, 18 and 7 clones isolated from two heterozygous HULC1927 mice and two HULC1930 respectively, and 1 clone from the WT mouse, were made monoclonal. Supernatants of the monoclonal hybridomas were subject to neutral and low pH antigen dissociation rate (off-rate) analysis and cell pellets were used for light chain variable domain DNA sequencing.

Example 6.4. Sequencing and Somatic Hypermutations in CDR3 Region of Vκ1-39Jκ5-based Histidine Universal Light Chain Mice

[000888] Cell pellets from monoclonal hybridomas from HULC and WT mice were used for light chain variable domain DNA sequencing. From the 26 clones made monoclonal (see Example 3.3 above) and subjected to sequencing, 15 were confirmed as using either a HULC or WT mouse light chain (MM and NN, see Table 4). 14 clones were derived from HULC heterozygous mice (1927 or 1930 mice) and 1 was derived from a WT mouse.

[000889] From the 14 antigen positive samples derived from HULC heterozygous mice, 12 of the monoclonal antibodies utilized their corresponding HULC light chain, while 2 utilized a WT mouse light chain. All but one of the HULC utilizing antibodies retained all of the introduced histidine mutations as shown in Table 3 (italicized antibody). Sequencing of clone AA produced 2 different HULC sequences, which is reflected by two entries in Table 5.

Table 5: Number of conserved histidine insertions and somatic hypermutations in light sequences from clones utilizing the HULC light chain

		Light Chain Sequences from mice utilizing HULC		
Clone Name	**Mouse Strain**	**# Conserved His Mutations in CDR3**	**# Somatic Hypermutations in Framework**	**# Somatic Hypermutations in CDRs**
AA (Sequence 1)	1927	4	3	0
AA (Sequence 2)	1927	4	1	1
BB	1927	4	3	3
CC	1927	4	0	0
DD	*1927*	*3*	*1*	*1*
EE	1927	4	2	2
FF	1927	4	0	1
GG	1927	4	1	1
HH	1927	4	2	0
II	1930	3	1	1
JJ	1930	3	4	5
KK	1930	3	1	2
LL	1930	3	1	0

Example 6.5. pH-Dependent Binding of Monoclonal Antibodies Generated in Vκ1-39Jκ5-based Histidine Universal Light Chain Mice

[000890] In order to further assess the pH-dependent binding characteristics of the monoclonal antibodies isolated from HULC and WT mice, binding experiments were carried out in which the antibody/antigen association phase was observed at neutral pH and the antibody/antigen dissociation phase was observed at either neutral or acidic pHs.

[000891] A Biacore CM4 sensor chip was derivatized with a polyclonal rabbit anti-mouse Fc antibody. Monoclonal antibody supernatants were captured onto the anti-mouse Fc sensor surface. Two concentrations, 50 nM (in duplicate) and 16.7 nM, of the immunogen were injected over the monoclonal antibody captured surface at a flow rate of 30 µl/min. Antibody-antigen association was monitored at pH 7.4 for 4 minutes and then the dissociation of antigen from the captured monoclonal antibody was monitored for 15 minutes at either pH 7.4 or 6.0. Dissociation (k_d) rate constants were determined by processing and fitting the data using Scrubber version 2.0 curve fitting software and are shown in Table 6.

Dissociative half-lives ($t_{1/2}$) were calculated from the dissociation rate constants as: $t_{1/2}$ (min) = (ln2/k_d)/60, and are shown in Table 6. Sensorgrams depicting the association/dissociation characteristics of several antibodies listed in Table 4 under the various pH conditions are shown graphically in FIG 26. The individual lines in each graph represent the binding responses at different concentrations of the respective antibodies. All experiments were carried out at 25°C. Dissociative half-life values (t½) are noted above the respective sensorgrams. Response is measured in RU.

Table 6. Dissociation (k_d) rate constants and dissociative half-lives ($t_{1/2}$) of monoclonal HULC or WT antibodies binding to their immunogen at neutral and low pH.

		pH 7.4 Association/pH 7.4 Dissociation				pH 7.4 Association/pH 6.0 Dissociation				pH 6.0/pH7.4 ratio	
Clone Name	Light chain used	neutral mAb cap-ture	50nM immuno-gen bound (RU)	k_d (1/s)	t½ (min)	low mab cap-ture	50nM immuno-gen bound (RU)	k_d (1/s)	t½ (min)	k_d	t½
AA	HULC (1927)	129	70	5.60E-05	206	122	73	2.18E-04	53	3.9	0.3
BB	HULC (1927)	350	165	6.00E-04	19	378	185	2.20E-03	5	3.7	0.3
CC	HULC (1927)	611	251	2.03E-04	57	545	226	6.68E-03	2	33.0	0.03
DD	HULC (1927)	182	75	3.55E-04	33	168	74	6.44E-04	18	1.8	0.6
HH	HULC (1927)	268	92	1.36E-04	85	251	91	5.39E-04	21	4.0	0.3
GG	HULC (1927)	353	110	2.78E-04	42	328	102	8.97E-04	13	3.2	0.3
FF	HULC (1927)	334	202	4.79E-05	241	364	220	6.90E-05	167	1.4	0.7
EE	HULC (1927)	339	124	5.08E-04	23	299	120	4.66E-04	25	0.9	1.1
II	HULC (1930)	387	174	1.22E-04	95	334	147	2.14E-04	54	1.8	0.6
JJ	HULC (1930)	363	14	9.83E-04	12	333	12	5.30E-04	22	0.5	1.9
KK	HULC (1930)	490	303	7.41E-05	156	484	295	1.29E-04	90	1.7	0.6
LL	HULC (1930)	636	41	3.09E-04	37	597	36	5.77E-04	20	1.9	0.5

		pH 7.4 Association/pH 7.4 Dissociation				pH 7.4 Association/pH 6.0 Dissociation				pH 6.0/pH7.4 ratio	
Clone Name	Light chain used	neutral mAb capture	50nM immunogen bound (RU)	k_d (1/s)	t½ (min)	low mab capture	50nM immunogen bound (RU)	k_d (1/s)	t½ (min)	k_d	t½
MM*	WT (from 1927 mouse)	245	6	NA	NA	203	6	NA	NA	NA	NA
NN	WT (from 1927 mouse)	394	231	5.26E-04	22	378	231	9.35E-04	12	1.8	0.6
OO	WT	413	89	2.94E-04	39	400	83	3.57E-04	32	1.2	0.8

*k_d and $t_{1/2}$ values could not be determined due to low antigen binding signal

Example 7. Engineering of Genetically Modified Mouse Comprising a Histidine-Substituted Vκ3-20Jκ1 Universal Light Chain

[000892] A mouse comprising a common Vκ3-20Jκ1 light chain was generated as described in, e.g., U.S. Patent Application Nos. 13/022,759, 13/093,156, 13/412,936, and 13/488,628 (Publication Nos. 2011/0195454, 2012/0021409, 2012/0192300, and 2013/0045492, respectively), and in Example 1 above. The amino acid sequence of the germline universal Vκ3-20Jκ1 light chain variable domain is set forth in SEQ ID NO:383.

[000893] Histidine substitutions were introduced into the Vκ3-20Jκ1 universal light chain targeting vector and mice generated from the same using a similar strategy to the one described above in Example 3 for Vκ1-39Jκ5 histidine modified universal light chain mice (HULC 1927 and 1930).

[000894] Briefly, the strategy for generating a histidine-modified Vκ3-20Jκ1 universal light chain targeting vector is summarized in FIGs. 29A-129D. A plasmid used for generating a targeting vector for common (universal) light chain mouse ("ULC mouse," described in, e.g., US2011/0195454A1), containing pBS + FRT-Ub-Hyg-FRT + mouse Vκ3-7 leader +human Vκ3-20Jκ1 was modified by site directed mutagenesis (QuickChange Lightning Kit) to replace Q105, Q106, Y107 and S109 or Q105, Q106 and S109 (see alignment in FIG. 27) with histidine residues in the CDR3 region using site-directed mutagenesis primers shown in FIG. 28 (See FIG. 29A for this engineering step). Resultant vectors (H105/106/107/109 and H105/106/109) were modified further and ligated into a vector comprising mouse Igκ

constant region, mouse enhancers, a mouse 3' homology arm and a SPEC cassette (FIG. 29B). Further modification involved ligation into a vector carrying 5' mouse arm and comprising Frt-UB-NEO-Frt cassette (FIG. 29B). Resultant targeting vectors were electroporated into ES cells comprising deletion of the mouse Igκ variable locus (comprising κ variable and joining gene segments) (FIGs. 29C-29D).

[000895] Positive ES cell clones were confirmed by using a modification of allele assay (Valenzuela et al.) using probes specific for the engineered Vκ3-20κJ1 light chain region inserted into the endogenous κ light chain locus. Primers and probes used in the assay are shown in Table 7 below and set forth in the Sequence Listing; the locations of the probes are depicted in FIGs. 29C-29D.

Table 7: Primers and Probes Used for ES Cell Screening

Probe Name	Assay	Probe Sequence	5' Primer	3' Primer
Neo	GOA	TGGGCACAAC AGACAATCGG CTG (SEQ ID NO:362)	GGTGGAGAG GCTATTCGGC (SEQ ID NO:363)	GAACACGGC GGCATCAG (SEQ ID NO:364)
ULC-m1	GOA	CCATTATGATG CTCCATGCCT CTCTGTTC (SEQ ID NO:365)	AGGTGAGGG TACAGATAAG TGTTATGAG (SEQ ID NO:366)	TGACAAATGC CCTAATTATA GTGATCA (SEQ ID NO:367)
1635h2 (Vκ3-20Jκ1 specific)	GOA	AAAGAGCCAC CCTCTCCTGC AGGG (SEQ ID NO:389)	TCCAGGCACC CTGTCTTTG (SEQ ID NO:390)	AAGTAGCTGC TGCTAACACT CTGACT (SEQ ID NO:391)
mIgKd2	Retention	GGCCACATTC CATGGGTTC (SEQ ID NO:371)	GCAAACAAAA ACCACTGGCC (SEQ ID NO:372)	CTGTTCCTCT AAAACTGGAC TCCACAGTAA ATGGAAA (SEQ ID NO:373)

Probe Name	Assay	Probe Sequence	5' Primer	3' Primer
mIgKp15	Retention	GGGCACTGGA TACGATGTATG G (SEQ ID NO:374)	CACAGCTTGT GCAGCCTCC (SEQ ID NO:375)	AGAAGAAGCC TGTACTACAG CATCCGTTTT ACAGTCA (SEQ ID NO:376)

[000896] The NEO selection cassette introduced by the targeting constructs is deleted by transfecting ES cells with a plasmid that expresses FLP (FIGs. 29C and 29D). Optionally, the neomycin cassette may be removed by breeding to mice that express FLP recombinase (*e.g.,* US 6,774,279). Optionally, the neomycin cassette is retained in the mice.

[000897] Targeted ES cells described above are used as donor ES cells and introduced into an 8-cell stage mouse embryo by the VELOCIMOUSE® method (see, *e.g.,* US Pat. No. 7,294,754 and Poueymirou *et al.* (2007) F0 generation mice that are essentially fully derived from the donor gene-targeted ES cells allowing immediate phenotypic analyses *Nature Biotech.* 25(1):91-99). VELOCIMICE® independently bearing an engineered human light chain gene that contains histidine residues mutated into one or more positions along the sequence are made from the targeted ES cells described above.

[000898] Pups are genotyped and pups heterozygous for the engineered histidine-modified human light chain are selected for characterizing expression of the light chain and binding capabilities of the expressed antibodies. Primers and probes for genotyping of mice specifically comprising a universal light chain gene with either three (H105/106/109; "6183") or four (H105/105/108/111; "6181") histidine modifications are listed in Table 6 below and set forth in the Sequence Listing. Mice containing histidine modification in their universal light chains are referred herein as "HULC" mice (histidine universal light chain mice).

[000899] Table 8: Primers and Probes Used for Genotyping

Probe Name	Assay	Probe Sequence	5' Primer	3' Primer
hVl494-1	GOA 6181 (4 His) mouse-specific	CTGTCATCACC ATGG (SEQ ID NO:392)	GCAGACTGGA GCCTGAAGAT TTT (SEQ ID NO:393)	CCGAACGTCCAA GGTGAGTG (SEQ ID NO:394)
hVl495-1	GOA 6183 (3 His)	TACTGTCATCA	GCAGACTGGA	CCGAACGTCCAA

	mouse-specific	CTATGG (SEQ ID NO:395)	GCCTGAAGAT TT (SEQ ID NO:396)	GGTGAGTG (SEQ ID NO:397)

[000900] Mice are immunized with antigen of interest and tested for ability to generate antibodies with pH-dependent binding.

Example 8. Breeding of Mice Comprising a Histidine-Substituted Single Rearranged Human Universal Light Chain Mouse (HULC)

[000901] This Example describes several other genetically modified mouse strains that can be bred to any one of the HULC mice described herein to create multiple genetically modified mouse strains harboring multiple genetically modified immunoglobulin loci.

[000902] *Endogenous Igλ Knockout (KO).* To optimize the usage of the engineered light chain locus, any one of the HULC animals described above (e.g., comprising Vκ1-39Jκ5 or Vκ3-20Jκ1 histidine-substituted universal light chain) may be bred to another mouse containing a deletion in the endogenous λ light chain locus. In this manner, the progeny obtained will express, as their only light chain, the rearranged histidine-substituted human germline light chain region as described in Examples 3 and 4 above. Breeding is performed by standard techniques recognized in the art and, alternatively, by a commercial breeder (*e.g.,* The Jackson Laboratory). Mouse strains bearing an engineered histidine-substituted light chain locus and a deletion of the endogenous λ light chain locus are screened for presence of the unique light chain region and absence of endogenous mouse λ light chains.

[000903] *Humanized Endogenous Heavy Chain Locus.* Mice bearing an engineered human germline light chain locus (HULC mice) are bred with mice that contain a replacement of the endogenous mouse heavy chain variable gene locus with the human heavy chain variable gene locus (see US 6,596,541; the VELOCIMMUNE® mouse, Regeneron Pharmaceuticals, Inc.). The VELOCIMMUNE® mouse comprises a genome comprising human heavy chain variable regions operably linked to endogenous mouse constant region loci such that the mouse produces antibodies comprising a human heavy chain variable domain and a mouse heavy chain constant region in response to antigenic stimulation.

[000904] Mice bearing a replacement of the endogenous mouse heavy chain variable region locus with the human heavy chain variable region locus and a histidine-substituted single rearranged human light chain variable region at the endogenous κ light chain locus

are obtained. Reverse chimeric antibodies containing somatically mutated heavy chains (human heavy chain variable domain and mouse C_H) with a histidine-substituted single human light chain (HULC, human light chain variable domain and mouse C_L) are obtained upon immunization with an antigen of interest. pH-dependent human antibodies generated in such mice are identified using antibody isolation and screening methods known in the art or described above. Variable light and heavy chain region nucleotide sequences of B cells expressing the antibodies, e.g., pH-sensitive antibodies, are identified, and fully human antibodies are made by fusion of the variable heavy and light chain region nucleotide sequences to human C_H and C_L nucleotide sequences, respectively, in a suitable expression system.

Example 9. Progeny of Genetically Modified Mice

[000905] Mice bearing an engineered human germline light chain locus comprising a limited repertoire of rearranged human light chain variable region sequences or a single rearranged human light chain variable region sequence (HULC mice) described herein are bred with mice that contain a histidine-modified human heavy chain variable gene locus described herein. Mice are obtained, and the presence of a light chain sequence containing histidine-modified human light chain variable region and a heavy chain sequence containing a histidine-modified human heavy chain variable region is confirmed by genotyping.

[000906] Reverse chimeric antibodies containing histidine-modified heavy chains (human histidine-modified heavy chain variable domain and mouse C_H) and histidine-modified single human light chain (HULC, human histidine-modified light chain variable domain and mouse C_L) are obtained upon immunization with an antigen of interest. pH-dependent human antibodies generated in such mice are identified using antibody isolation and screening methods known in the art or described above. Variable light and heavy chain region nucleotide sequences of B cells expressing the antibodies, e.g., pH-sensitive antibodies, are identified, and fully human antibodies are made by fusion of the variable heavy and light chain region nucleotide sequences to human C_H and C_L nucleotide sequences, respectively, in a suitable expression system.

Equivalents

[000907] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. While the described invention has been described with reference to the specific

embodiments thereof it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adopt a particular situation, material, composition of matter, process, process step or steps, to the objective spirit and scope of the described invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

1. A method of obtaining a cell that expresses an antigen-specific immunoglobulin variable domain with histidines encoded by germline histidine codons, comprising harvesting a lymphocyte from a non- human animal; wherein the non-human animal comprises in its germline genome either or both (i) an unrearranged immunoglobulin heavy chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon or an insertion of at least one histidine codon, and (ii) an unrearranged immunoglobulin light chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon or an insertion of at least one histidine codon; wherein the non-human animal further comprises *in vivo* a plurality of diverse antigen-binding proteins that display high affinity for an antigen of interest and comprise an immunoglobulin heavy chain variable domain that retains at least one histidine amino acid derived from the substituted or inserted histidine codon in the germline genome and/or an immunoglobulin light chain variable domain that retains at least one histidine amino acid derived from the substituted or inserted histidine codon in the germline genome; and wherein the lymphocyte expresses one of the plurality of diverse antigen-binding proteins.

2. The method of claim 1, wherein the unrearranged immunoglobulin heavy chain variable region gene sequence is an unrearranged human immunoglobulin heavy chain variable region nucleic acid sequence comprising (i) unrearranged human V_H, unrearranged human D_H, and unrearranged human J_H gene segments, and (ii) the substituted or inserted histidine codon, and/or wherein the unrearranged immunoglobulin light chain variable region gene sequence is an unrearranged human immunoglobulin light chain variable region nucleic acid sequence comprising (i) unrearranged human V_L and unrearranged human J_L gene segments, and (ii) the substituted or inserted histidine codon.

3. The method of claim 1, wherein the unrearranged immunoglobulin heavy chain variable region gene sequence is an unrearranged human immunoglobulin heavy chain variable region nucleic acid sequence comprising (i) unrearranged human V_H gene segments, a synthetic D segment that comprises a linker, an unrearranged human J_H gene segment, and (ii) the substituted or inserted histidine codon, and/or wherein the unrearranged immunoglobulin light chain variable region gene sequence is an unrearranged human immunoglobulin light chain variable region nucleic acid sequence comprising (i) unrearranged human V_L and unrearranged human J_L gene segments, and (ii) the substituted or inserted histidine codon.

4. The method of any one of claims 1 to 3, wherein an endogenous non-human immunoglobulin heavy chain variable region gene is replaced with the unrearranged immunoglobulin heavy chain variable region gene sequence, which is optionally operably linked to an endogenous non-human immunoglobulin heavy chain constant region gene sequence, and/or wherein an endogenous non-

human immunoglobulin light chain variable region gene is replaced with the unrearranged immunoglobulin light chain variable region gene sequence, which is optionally operably linked to an endogenous non-human immunoglobulin light chain constant region gene sequence.

5. The method of any one of claims 1 to 4, wherein the substituted or inserted histidine codon is in a CDR1 encoding sequence, a CDR2 encoding sequence, a CDR3 encoding sequence, an N terminal encoding sequence or a loop encoding sequence; optionally a CDR3 encoding sequence.

6. The method of any one of claims 1 to 5, wherein the non-human animal is a rodent; optionally a rat, a mouse, or a hamster; optionally a mouse.

7. The method of any one of claims 1 to 6, further comprising the step of producing a hybridoma from the harvested lymphocyte.

8. The method of any one of claims 1 to 7, further comprising as last steps obtaining from the harvested lymphocyte, or a hybridoma produced therefrom, a first nucleotide sequence that encodes the immunoglobulin heavy chain variable domain that retains at least one histidine amino acid derived from the substituted or inserted histidine codon in the germline genome and/or a second nucleotide sequence that encodes the immunoglobulin light chain variable domain that retains at least one histidine amino acid derived from the substituted or inserted histidine codon in the germline genome; and expressing in a cell a first nucleic acid operably linked to a human heavy chain constant region gene and/or a second nucleic acid operably linked to a human light chain constant region gene, wherein the first nucleic acid comprises a sequence identical to or substantially identical to the first nucleotide sequence, and wherein the second nucleic acid comprises a sequence identical to or substantially identical to the second nucleotide sequence.

9. A method of obtaining a cell that expresses an antigen-specific immunoglobulin variable domain with histidines encoded by germline histidine codons, comprising harvesting a lymphocyte from a non- human animal; wherein the non-human animal comprises in its germline genome both (i) an unrearranged immunoglobulin heavy chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon or an insertion of at least one histidine codon, and (ii) a rearranged immunoglobulin light chain variable region gene sequence comprising a substitution of at least one non-histidine codon with a histidine codon or an insertion of at least one histidine codon; wherein the non-human animal further comprises in vivo a plurality of diverse antigen-binding proteins that display high affinity for an antigen of interest and comprises an immunoglobulin light chain variable domain that is encoded by the rearranged immunoglobulin light chain variable region gene sequence and retains at least one histidine amino acid derived from the

substituted or inserted histidine codon in the germline genome; and wherein the lymphocyte expresses one of the plurality of diverse antigen-binding proteins.

10. The method of claim 9, wherein the unrearranged immunoglobulin heavy chain variable region gene sequence is an unrearranged human immunoglobulin heavy chain variable region nucleic acid sequence comprising (i) unrearranged human V_H, unrearranged human D_H, and unrearranged human J_H gene segments, and the substituted or inserted histidine codon, or (ii) unrearranged human V_H gene segments, a synthetic D segment that comprises a linker, an unrearranged human J_H gene segment, and the substituted or inserted histidine codon; and wherein the rearranged immunoglobulin light chain variable region gene sequence is a rearranged human immunoglobulin light chain variable region nucleic acid sequence comprising a human V_L gene segment rearranged with a human J_L gene segment and the substituted or inserted histidine codon.

11. The method of claim 9 or claim10, wherein an endogenous non-human immunoglobulin heavy chain variable region gene is replaced with the unrearranged immunoglobulin heavy chain variable region gene sequence, which is optionally operably linked to an endogenous non-human immunoglobulin heavy chain constant region gene sequence, and wherein an endogenous non-human immunoglobulin light chain variable region gene is replaced with the rearranged immunoglobulin light chain variable region gene sequence, which is optionally operably linked to an endogenous non-human immunoglobulin light chain constant region gene sequence.

12. The method of any one of claims 9 to11, wherein the rearranged immunoglobulin light chain variable region gene sequence is derived from a human Vk1-39 gene segment or a human Vk3-20 gene segment.

13. The method of any one of claims 9 to12, wherein the substituted or inserted histidine codon is in a CDR1 encoding sequence, a CDR2 encoding sequence, a CDR3 encoding sequence, an N terminal encoding sequence or a loop encoding sequence; optionally a CDR3 encoding sequence.

14. The method of any one of claims 9 to13, wherein the non-human animal is a rodent; optionally a rat, a mouse, or a hamster; optionally a mouse.

15. The method of any one of claims 9 to14, further comprising the step of producing a hybridoma from the harvested lymphocyte.

16. The method of any one claims 9 to15, further comprising as last steps obtaining from the

harvested lymphocyte, or a hybridoma produced therefrom, a first nucleotide sequence that encodes an immunoglobulin heavy chain variable domain that is cognate to the immunoglobulin light chain variable domain, and optionally comprises at least one histidine amino acid that is derived from the substituted or inserted histidine codon in the germline; and expressing in a cell a first nucleic acid operably linked to a human heavy chain constant region gene and a second nucleic acid operably linked to a human light chain constant region gene, wherein the first nucleic acid comprises a sequence identical to or substantially identical to the first nucleotide sequence, and wherein the second nucleic acid comprises a sequence identical to or substantially identical to the rearranged immunoglobulin light chain variable region gene sequence.

17. The method of claim16, wherein the cell further comprises a third nucleic acid operably linked to a human heavy chain constant region gene, wherein the third nucleic acid encodes a different immunoglobulin heavy chain variable domain that is cognate to the immunoglobulin light chain variable domain, and wherein the cell expresses the first, second and third nucleic acids as a bi-specific antigen- binding protein.

18. The method of claim17, wherein one of the human heavy chain constant region genes is modified to omit a Protein A-binding determinant.