

isolation of cell lines producing that polypeptide. There is, however, a need in the art for new cell line development strategies that not only conserve resources but also generate cell lines exhibiting improved expression levels, product quality attributes, and production culture performance relative to conventional methodologies.

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SUMMARY

The presently disclosed subject matter relates to multi-vector recombinase mediated cassette exchange (multi-vector RMCE) approaches to achieve targeted integration of sequences of interest for the generation host cells expressing recombinant proteins, e.g.,
10 monoclonal antibodies, as well as compositions derived from the same, e.g., bispecific antibodies, and other complex format proteins, e.g., membrane protein complexes, and other difficult to express molecules.

In certain examples, the present disclosure is directed to TI host cells comprising an exogenous nucleotide sequence integrated at an integration site that is within a sequence: a)
15 at least about 90% homologous to all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of NW_006881296.1, all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054 of NW_006882936.1, or all or part of nucleotides 82214-
20 97705 of NW_003615411.1; or b) at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all
25 or part of nucleotides 97706-105117 of NW_003615411.1; and wherein the exogenous nucleotide sequence comprises four or more incompatible recombination recognition sequences (RRSs). In certain examples, the RRSs are selected from the group consisting of a LoxP sequence, a LoxP L3 sequence, a LoxP 2L sequence, a LoxFas sequence, a Lox5 l l sequence, a Lox2272 sequence, a Lox2372 sequence, a Lox5 l7l sequence, a Loxm2 sequence,
30 a Lox7l sequence, a Lox66 sequence, a FRT sequence, a Bxb1 attP sequence, a Bxb1 attB sequence, a ϕ C31 attP sequence, and a ϕ C31 attB sequence. In certain examples, the RRSs are recognized by recombinases selected from the group consisting of Cre recombinase, FLP recombinase, Bxb1 integrase, and a ϕ C31 integrase. In certain examples, the exogenous

nucleotide sequence comprises a selection marker located between the 5'-most RRS and the next RRS in the 3' direction. In certain examples, the selection marker is selected from the group consisting of aminoglycoside phosphotransferase (APH), hygromycin phosphotransferase (HYG), neomycin, G418 APH), dihydrofolate reductase (DHFR), thymidine kinase (TK), glutamine synthetase (GS), asparagine synthetase, tryptophan synthetase (indole), histidinol dehydrogenase (histidinol D), blasticidin, bleomycin, phleomycin, chloramphenicol, Zeocin, and mycophenolic acid. In certain examples, the cells comprise a second selection marker, wherein the first and the second selection markers are different. In certain examples, the second selection marker is selected from the group consisting of aminoglycoside phosphotransferase (APH), hygromycin phosphotransferase (HYG), neomycin, G418 APH), dihydrofolate reductase (DHFR), thymidine kinase (TK), glutamine synthetase (GS), asparagine synthetase, tryptophan synthetase (indole), histidinol dehydrogenase (histidinol D), blasticidin, bleomycin, phleomycin, chloramphenicol, Zeocin, and mycophenolic acid. In certain examples, the cells comprise a third selection marker and an internal ribosome entry site (IRES), wherein the IRES is operably linked to a third selection marker. In certain examples, the third selection marker is different from the first or the second selection marker. In certain examples, the third selection marker is selected from the group consisting of a green fluorescent protein (GFP) marker, an enhanced GFP (eGFP) marker, a synthetic GFP marker, a yellow fluorescent protein (YFP) marker, an enhanced YFP (eYFP) marker, a cyan fluorescent protein (CFP) marker, a mPlum marker, a mCherry marker, a tdTomato marker, a mStrawberry marker, a J-red marker, a DsRed-monomer marker, a mOrange marker, a mKO marker, a mCitrine marker, a Venus marker, a YPet marker, an Emerald6 marker, a CyPet marker, a mCFPm marker, a Cerulean marker, and a T-Sapphire marker. In certain examples, the TI host cell is a mammalian host cell. In certain examples, the TI host cell is a hamster host cell, a human host cell, a rat host cell, or a mouse host cell. In certain examples, the TI host cell is a Chinese hamster ovary (CHO) host cell, a CHO K1 host cell, a CHO K1 SV host cell, a DG44 host cell, a DUKXB-1 host cell, a CHOK1 S host cell, or a CHO K1M host cell.

In certain examples, the present disclosure is directed to methods of preparing a TI host cell expressing SOIs comprising: a) providing a TI host cell comprising an exogenous nucleotide sequence integrated at an integration site that is within a sequence: (A) at least about 90% homologous to all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of

NW_006881296.1, all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054 of NW_006882936.1, or all or part of nucleotides 82214-97705 of NW_003615411.1; or (B) at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all or part of nucleotides 97706-105117 of NW_003615411.1; and wherein the exogenous nucleotide sequence comprises four or more incompatible RRSs; b) introducing into the cell provided in a) at least three vectors, each vector comprising: (A) two RRSs matching two sequentially oriented RRSs on the integrated exogenous nucleotide sequence; and (B) each pair of RRSs flanking at least one exogenous SOI and at least one second selection marker; c) introducing recombinases or nucleic acids encoding recombinases, wherein the recombinases recognize the RRSs; and d) selecting for TI cells expressing the selection markers to thereby isolate a TI host cell expressing the SOIs. In certain examples, the recombinases are selected from Cre recombinase, FLP recombinase, Bxb1 integrase, and a ϕ C31 integrase. In certain examples, the SOIs encode a single chain antibody, an antibody light chain, an antibody heavy chain, a single-chain Fv fragment (scFv), or an Fc fusion protein. In certain examples, the TI host cell is a mammalian host cell. In certain examples, the TI host cell is a hamster host cell, a human host cell, a rat host cell, or a mouse host cell. In certain examples, the TI host cell is a CHO host cell, a CHO K1 host cell, a CHO K1SV host cell, a DG44 host cell, a DUKXB-11 host cell, a CHOK1S host cell, or a CHO K1M host cell. In certain examples, the vector is selected from the group consisting of an adenovirus vector, an adeno-associated virus vector, a lentivirus vector, a retrovirus vector, an integrating phage vector, a non-viral vector, a transposon and/or transposase vector, an integrase substrate, and a plasmid.

In one aspect, the disclosure provides a method of preparing a TI host cell expressing sequences of interest (SOIs) comprising:

a) providing a TI host cell comprising an exogenous nucleotide sequence integrated at an integration site that is within a sequence: a. at least about 90% homologous to all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of NW_006881296.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054 of NW_006882936.1, or all or part of nucleotides 82214-97705 of NW_003615411.1; or b. at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all or part of nucleotides 97706-105117 of NW_003615411.1; and wherein the exogenous nucleotide sequence comprises four or more incompatible recombination recognition sequences (RRSs);

b) introducing into the cell provided in a) at least three vectors, each vector comprising: a. a pair of two RRSs matching two sequentially oriented RRSs on the integrated exogenous nucleotide sequence; and b. each pair of RRSs flanking at least one exogenous SOI and at least one selection marker;

c) introducing recombinases or nucleic acids encoding recombinases, wherein the recombinases recognize the RRSs; and

d) selecting for TI host cells expressing the selection markers to thereby isolate a TI host cell expressing the SOIs.

In certain examples, the present disclosure is directed to methods for expressing SOIs comprising: a) providing a TI host cell comprising an exogenous nucleotide sequence integrated at an integration site that is within a sequence: (A) at least about 90% homologous to all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of NW_006881296.1, all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054

of NW_006882936.1, or all or part of nucleotides 82214-97705 of NW_003615411.1; or (B) at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all or part of nucleotides 97706-105117 of NW_003615411.1; and wherein the exogenous nucleotide sequence comprises four or more incompatible RRSs; b) introducing into the cell provided in a) at least three vectors, each vector comprising: (A) two RRSs matching two sequentially oriented RRSs on the integrated exogenous nucleotide sequence; and (B) each pair of RRSs flanking at least one exogenous SOI and at least one second selection marker; c) introducing recombinases or nucleic acids encoding recombinases, wherein the recombinases recognize the RRSs; d) selecting for TI cells expressing the selection markers to thereby isolate a TI host cell expressing the SOIs; and e) culturing the cell in d) under conditions suitable for expressing the SOIs and recovering the expressed protein therefrom. In certain examples, the recombinases are selected from Cre recombinase, FLP recombinase, Bxb1 integrase, and a ϕ C31 integrase. In certain examples, the SOIs encode a single chain antibody, an antibody light chain, an antibody heavy chain, a single-chain Fv fragment (scFv), or an Fc fusion protein. In certain examples, the TI host cell is a mammalian host cell. In certain examples, the TI host cell is a hamster host cell, a human host cell, a rat host cell, or a mouse host cell. In certain examples, the TI host cell is a CHO host cell, a CHO K1 host cell, a CHO K1SV host cell, a DG44 host cell, a DUKXB-1 host cell, a CHOK1S host cell, or a CHO K1M host cell. In certain examples, the vector is selected from the group consisting of an adenovirus vector, an adeno-associated virus vector, a lentivirus vector, a retrovirus vector, an integrating phage vector, a non-viral vector, a transposon and/or transposase vector, an integrase substrate, and a plasmid.

In one aspect, the disclosure provides a method for expressing SOIs comprising:
a) providing a TI host cell comprising an exogenous nucleotide sequence integrated at an integration site that is within a sequence: a. at least about 90% homologous to all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of NW_006881296.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054 of NW_006882936.1, or all or part of nucleotides 82214-97705 of NW_003615411.1; or b. at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all or part of nucleotides 97706-105117 of NW_003615411.1; and wherein the exogenous nucleotide sequence comprises four or more incompatible RRSs;
b) introducing into the cell provided in a) at least three vectors, each vector comprising: a. a pair of two RRSs matching two sequentially oriented RRSs on the integrated exogenous nucleotide sequence; and b. each pair of RRSs flanking at least one exogenous SOI and at least one selection marker;
c) introducing recombinases or nucleic acids encoding recombinases, wherein the recombinases recognize the RRSs;
d) selecting for TI host cells expressing the selection markers to thereby isolate a TI host cell expressing the SOIs; and
e) culturing the cell in d) under conditions suitable for expressing the SOIs and recovering the expressed protein therefrom.

In certain examples, the present disclosure is directed to methods for producing a recombinant mammalian cell comprising a nucleic acid encoding an antibody, comprising:
a) providing a mammalian cell comprising at least a single exogenous nucleic acid incorporated at a predetermined locus of the genome of the mammalian cell comprising four or more incompatible RRSs; b) introducing into the recombinant mammalian cell of a), at least three vectors, each vector comprising a pair of incompatible RRSs matching two of the incompatible RRSs comprised in the exogenous nucleic acid incorporated at a predetermined locus of the

genome of the mammalian cell and each pair of incompatible RRSs flank one or more SOIs where the SOIs encode an antibody and/or one or more selection markers; c) introducing one or more recombinases, simultaneously or sequentially, with the introduction of the at least three vectors comprising the SOIs and/or selection markers; and d) selecting for cells expressing one or more of the SOIs and/or selection markers, thereby producing a recombinant mammalian cell comprising nucleic acid SOIs encoding the antibody. In certain examples, the exogenous nucleic acid is incorporated at a locus: (A) at least about 90% homologous to all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of NW_006881296.1, all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054 of NW_006882936.1, or all or part of nucleotides 82214-97705 of NW_003615411.1; or (B) at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all or part of nucleotides 97706-105117 of NW_003615411.1. In certain examples, the recombinases are selected from Cre recombinase, FLP recombinase, Bxb1 integrase, and a ϕ C31 integrase. In certain examples, the SOIs encode a single chain antibody, an antibody light chain, an antibody heavy chain, a single-chain Fv fragment (scFv), or an Fc fusion protein. In certain examples, the mammalian cell is a hamster host cell, a human host cell, a rat host cell, or a mouse host cell. In certain examples, the mammalian cell is a CHO host cell, a CHO Kl host cell, a CHO K1SV host cell, a DG44 host cell, a DUKXB-1 host cell, a CHOK1S host cell, or a CHO K1M host cell. In certain examples, the vectors are selected from the group consisting of adenovirus vectors, adeno-associated virus vectors, lentivirus vectors, retrovirus vectors, integrating phage vectors, non-viral vectors, transposon and/or transposase vectors, integrase substrates, and plasmids.

In one aspect, the disclosure provides a method for producing a recombinant mammalian cell comprising a nucleic acid encoding an antibody, comprising:

- a) providing a recombinant mammalian cell comprising at least a single exogenous nucleic acid incorporated at a predetermined locus of the genome of the mammalian cell comprising four or more incompatible RRSs;
- b) introducing into the recombinant mammalian cell of a) at least three vectors, each vector comprising a pair of incompatible RRSs matching two of the incompatible RRSs comprised in the exogenous nucleic acid incorporated at a predetermined locus of the genome of the mammalian cell and each pair of incompatible RRSs flank one or more SOIs, where the SOIs encode an antibody, and/or one or more selection markers;
- c) introducing one or more recombinases, simultaneously or sequentially, with the introduction of the at least three vectors comprising the SOIs and/or selection markers; and
- d) selecting for cells expressing one or more of the SOIs and/or selection markers, thereby producing a recombinant mammalian cell comprising nucleic acid SOIs encoding the antibody.

In certain examples, the present disclosure is directed to methods for producing a recombinant mammalian cell comprising nucleic acids encoding one or more antibodies, comprising: a) providing a mammalian cell comprising at least two exogenous nucleic acid incorporated at predetermined loci of the genome of the mammalian cell comprising, each exogenous nucleic acid comprising four or more incompatible RRSs, where the exogenous

nucleic acids can comprise the same or different RRSs; b) introducing into the recombinant mammalian cell of a), at least three vectors, each vector comprising a pair of incompatible RRSs matching two of the incompatible RRSs comprised in one or both of the exogenous nucleic acids incorporated at predetermined loci of the genome of the mammalian cell and each pair of incompatible RRSs flank one or more SOIs where the SOIs encode an antibody and/or one or more selection markers; c) introducing one or more recombinases, simultaneously or sequentially, with the introduction of the at least three vectors comprising the SOIs and/or selection markers; and d) selecting for cells expressing one or more of the SOIs and/or selection markers,

thereby producing a recombinant mammalian cell comprising nucleic acid SOIs encoding the antibody. In certain examples, the exogenous nucleic acids are incorporated at distinct loci selected from loci: (A) at least about 90% homologous to all or part of nucleotides 41190-45269 of NW_006874047.1, all or part of nucleotides 63590-207911 of NW_006884592.1, all or part of nucleotides 253831-491909 of NW_006881296.1, all or part of nucleotides 69303-79768 of NW_003616412.1, all or part of nucleotides 293481-315265 of NW_003615063.1, all or part of nucleotides 2650443-2662054 of NW_006882936.1, or all or part of nucleotides 82214-97705 of NW_003615411.1; or (B) at least about 90% homologous to all or part of nucleotides 45270-45490 of NW_006874047.1, all or part of nucleotides 207912-792374 of NW_006884592.1, all or part of nucleotides 491910-667813 of NW_006881296.1, all or part of nucleotides 79769-100059 of NW_003616412.1, all or part of nucleotides 315266-362442 of NW_003615063.1, all or part of nucleotides 2662055-2701768 of NW_006882936.1, or all or part of nucleotides 97706-105117 of NW_003615411.1. In certain examples, the recombinases are selected from Cre recombinase, FLP recombinase, Bxb1 integrase, and a ϕ C31 integrase. In certain examples, the SOIs encode a single chain antibody, an antibody light chain, an antibody heavy chain, a single-chain Fv fragment (scFv), or an Fc fusion protein. In certain examples, the mammalian cell is a hamster host cell, a human host cell, a rat host cell, or a mouse host cell. In certain examples, the mammalian cell is a CHO host cell, a CHO K1 host cell, a CHO K1SV host cell, a DG44 host cell, a DUKXB-1 host cell, a CHOK1S host cell, or a CHO K1M host cell. In certain examples, the vectors are selected from the group consisting of adenovirus vectors, adeno-associated virus vectors, lentivirus vectors, retrovirus vectors, integrating phage vectors, non-viral vectors, transposon and/or transposase vectors, integrase substrates, and plasmids.

In one aspect, the disclosure provides a method for producing a recombinant mammalian cell comprising nucleic acids encoding one or more antibodies, comprising:

a) providing a recombinant mammalian cell comprising at least two exogenous nucleic acid incorporated at predetermined loci of the genome of the mammalian cell comprising, each exogenous nucleic acid comprising four or more incompatible RRSs, where the exogenous nucleic acids can comprise the same or different RRSs;
b) introducing into the recombinant mammalian cell of a) at least three vectors, each vector comprising a pair of incompatible RRSs matching two of the incompatible RRSs comprised in one or both of the exogenous nucleic acids incorporated at predetermined loci of the genome of the mammalian cell and each pair of incompatible RRSs flank one or more SOIs, where the SOIs encode an antibody, and/or one or more selection markers;
c) introducing one or more recombinases, simultaneously or sequentially, with the introduction of the at least three vectors comprising the SOIs and/or selection markers; and
d) selecting for cells expressing one or more of the SOIs and/or selection markers, thereby producing a recombinant mammalian cell comprising nucleic acid SOIs encoding the antibody.