

[0009] Although advances have been made in the engineering of complex antibody formats such as bispecific antibodies, the manufacturability of these in a single mammalian expression system often exhibits low performance. A low titer and insufficient product quality are two of the factors that contribute to making stable production of bsAb difficult. As a consequence, there is an urgent need to identify the limiting steps in the production system and to provide methods that allow for improved production of correctly assembled multimeric polypeptides, *e.g.*, in a eukaryotic or mammalian host cell system.

[0010] All references cited herein, including patent applications, patent publications, non-patent literature, and UniProtKB/Swiss-Prot Accession numbers are herein incorporated by reference in their entirety, as if each individual reference were specifically and individually indicated to be incorporated by reference.

SUMMARY

[0011] To meet these and other demands, provided herein are methods for producing multimeric polypeptides in a eukaryotic (*e.g.*, mammalian) host cell. These multimeric polypeptides comprise two or more subunits, with each subunit comprising two or more polypeptide chains (*e.g.*, as with a multispecific or bispecific antibody). Advantageously, these methods provide for more precise manipulation of the translational level of each polypeptide chain in the multimeric polypeptide, allowing for improved production of correctly assembled multimeric polypeptides.

[0012] Certain examples of the present disclosure relate to methods for producing a multimeric polypeptide in a eukaryotic host cell. In some examples, the multimeric polypeptide comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain. In some examples, the methods comprise: providing a eukaryotic host cell; culturing the eukaryotic host cell under conditions suitable for expression of the first, second, third, and fourth polypeptide chains, wherein upon expression the first, second, third, and fourth polypeptide chains form the multimeric polypeptide; and recovering the multimeric polypeptide produced by the eukaryotic host cell. In some examples, the eukaryotic host cell comprises a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain, a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain, a third polynucleotide comprising a third translation initiation sequence

operably linked to a third open-reading frame that encodes the third polypeptide chain, and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain. In some examples, the first subunit is expressed at a lower level than the second subunit when each subunit is expressed individually in the eukaryotic host cell, and one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence and the fourth translation initiation sequence. In some examples, the first subunit is expressed at a lower level than the second subunit when all subunits are expressed together in the same eukaryotic host cell, and one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence and the fourth translation initiation sequence. In some examples, the multimeric polypeptide is a bispecific antibody. In some examples, the multimeric polypeptide is a bispecific antibody, the first and third polypeptide chains are antibody heavy chains, the second and fourth polypeptide chains are antibody light chains, the first subunit is a first half-antibody that binds a first antigen, and the second subunit is a second half-antibody that binds a second antigen.

[0012a] In one aspect, the present disclosure refers to a method for producing a multimeric polypeptide in a eukaryotic host cell, wherein the multimeric polypeptide comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain, the method comprising: (a) providing the eukaryotic host cell, wherein the eukaryotic host cell comprises a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain, a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain, a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain, and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain, wherein the first subunit is expressed at a lower level than the second subunit when each subunit is expressed individually in the eukaryotic host cell, and wherein one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence and the fourth translation initiation sequence; (b) culturing the eukaryotic host cell under conditions suitable for expression of the first, second, third, and fourth polypeptide chains, wherein upon expression the first, second, third, and fourth polypeptide chains form the multimeric polypeptide; and (c) recovering the multimeric polypeptide produced by the eukaryotic host cell.

[0013] In another aspect, the present disclosure relates to a method for producing a bispecific antibody in a eukaryotic host cell, wherein the bispecific antibody comprises a first half antibody comprising a first antibody heavy chain and a first antibody light chain and a second half antibody comprising a second antibody heavy chain and a second antibody light chain, the method comprising: (a) providing the eukaryotic host cell, wherein the eukaryotic host cell comprises a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first antibody heavy chain, a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the first antibody light chain, a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the second antibody heavy chain, and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the second antibody light chain, wherein the first half antibody is expressed at a lower level than the second half antibody when each half antibody is expressed individually in the eukaryotic host cell, and wherein one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence

and the fourth translation initiation sequence; (b) culturing the eukaryotic host cell under conditions suitable for expression of the first and second antibody heavy and light chains, wherein the first and second antibody heavy and light chains form the bispecific antibody, and wherein the first half antibody binds a first antigen and the second half antibody binds a second antigen; and (c) recovering the bispecific antibody produced by the eukaryotic host cell.

[0014] In some examples, the first antibody heavy chain comprises a first antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the second antibody heavy chain comprises a second antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the CH3 domain of the first antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a smaller side chain volume, thereby generating a hole on the surface of the CH3 domain of the first antibody Fc region that interacts with the CH3 domain of the second antibody Fc region; and wherein the CH3 domain of the second antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a larger side chain volume, thereby generating a knob on the surface of the CH3 domain of the second antibody Fc region that interacts with the CH3 domain of the first antibody Fc region. In some examples, the first antibody heavy chain comprises a first antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the second antibody heavy chain comprises a second antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the CH3 domain of the second antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a smaller side chain volume, thereby generating a hole on the surface of the CH3 domain of the second antibody Fc region that interacts with the CH3 domain of the first antibody Fc region; and wherein the CH3 domain of the first antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a larger side chain volume, thereby generating a knob on the surface of the CH3 domain of the first antibody Fc region that interacts with the CH3 domain of the second antibody Fc region. In some examples, the knob mutation comprises at least one of: T366Y, T366W, T394W, and F405W, numbering based on human IgG1 according to EU index. In some examples, the hole mutation comprises at least one of: F405A, Y407T, Y407A, T366S, L368A, Y407V, and T394S, numbering based on human IgG1 according to EU index. In some examples, the knob mutation comprises T366W, and wherein the hole mutation

comprises at least one, at least two, or all three of T366S, L368A, and Y407V, numbering based on human IgG1 according to EU index. In some examples, the first antibody light chain comprises a first mutation, the first antibody heavy chain comprises a second mutation, and the first and second mutations promote selective association of the first antibody light chain with the first antibody heavy chain. In some examples, the first mutation comprises an amino acid substitution at V133, and/or the second mutation comprises an amino acid substitution at S183, numbering based on EU index. In some examples, the S183 substitution is selected from the group consisting of S183A, S183T, S183V, S183Y, S183F, S183H, S183N, S183D, S183E, S183R, and S183K; and/or the V133 substitution is selected from the group consisting of V133E, V133S, V133L, V133W, V133K, V133R, and V133D. In some examples, the second antibody light chain comprises a third mutation, the second antibody heavy chain comprises a fourth mutation, and the third and fourth mutations promote selective association of the second antibody light chain with the second antibody heavy chain. In some examples, the third mutation comprises an amino acid substitution at V133, and/or the fourth mutation comprises an amino acid substitution at S183, numbering based on EU index. In some examples, the S183 substitution is selected from the group consisting of S183A, S183T, S183V, S183Y, S183F, S183H, S183N, S183D, S183E, S183R, and S183K; and/or the V133 substitution is selected from the group consisting of V133E, V133S, V133L, V133W, V133K, V133R, and V133D. In some examples, the amino acid substitution at S183 results in a positively charged residue (*e.g.*, S183K), and the amino acid substitution at V133 results in a negatively charged residue (*e.g.*, V133E). In some examples, the amino acid substitution at S183 results in a negatively charged residue (*e.g.*, S183E), and the amino acid substitution at V133 results in a positively charged residue (*e.g.*, V133K).

[0015] In some examples, the method results in higher production of the multimeric polypeptide in the eukaryotic host cell, *e.g.*, as compared with production in which one or more, two or more, three or more, or four of the polynucleotides encoding polypeptide chains of a multimeric polypeptide comprise an open-reading frame operably linked to a native or unmodified translation initiation sequence, or production in which each of the polynucleotides encoding polypeptide chains of a multimeric polypeptide comprise the same translation initiation sequence. In some examples, the method results in fewer incorrectly paired side products in the eukaryotic host cell, *e.g.*, as compared with production in which one or more, two or more, three or more, or four of the polynucleotides encoding polypeptide chains of a multimeric polypeptide comprise an open-reading frame

operably linked to a native or unmodified translation initiation sequence, or production in which each of the polynucleotides encoding polypeptide chains of a multimeric polypeptide comprise the same translation initiation sequence. For example, transient or stable transfectants can be cultured, and production of multimeric polypeptide can be assayed. Fed-batch or perfusion cultures are grown, and titers of product in the cell medium or on the cell surface can be assayed. For surface expression, cells can be stained with antibody to detect product and analyzed, *e.g.*, by flow cytometry. Product quality and/or purity can be assayed, *e.g.*, with electrophoresis and/or mass spectrometry. In some examples, one or both polypeptide chains of the first subunit is/are translated at a slower rate than one or both polypeptide chains of the second subunit when each subunit is expressed individually in the eukaryotic host cell. In some examples, one or both polypeptide chains of the first subunit fold(s) more slowly and/or less efficiently than one or both polypeptide chains of the second subunit when each subunit is expressed individually in the eukaryotic host cell. In some examples, the first subunit assembles at a slower rate than the second subunit when each subunit is expressed individually in the eukaryotic host cell. In some examples, the first and/or second translation initiation sequence is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, 5% to 30%, 5% to 50%, 5% to 75%, 10% to 30%, 10% to 50%, 10% to 75%, 25% to 50%, 25% to 75%, 25% to 100%, 50% to 75%, 50% to 100%, or 75% to 100% weaker than the third and/or fourth translation initiation sequence. In some examples, the first and/or second translation initiation sequence is at least 1.3-fold, at least 1.5-fold, at least 2-fold, at least 2.5-fold, at least 3-fold, at least 3.5-fold, at least 4-fold, at least 4.5-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, 1.3-fold to 3-fold, 1.5-fold to 3-fold, 2-fold to 10-fold, 2-fold to 5-fold, 3-fold to 5-fold, 3-fold to 10-fold, 5-fold to 10-fold, or 7-fold to 10-fold weaker than the third and/or fourth translation initiation sequence. In some examples, the first subunit or half antibody is expressed at a level that is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, 5% to 30%, 5% to 50%, 5% to 75%, 10% to 30%, 10% to 50%, 10% to 75%, 25% to 50%, 25% to 75%, 25% to 100%, 50% to 75%, 50% to 100%, or 75% to 100% lower than expression level of the second subunit or half antibody, *e.g.*, when each subunit or half antibody is

expressed individually in the eukaryotic host cell. In some examples, the first subunit or half antibody is expressed at a level that is at least 1.3-fold, at least 1.5-fold, at least 2-fold, at least 2.5-fold, at least 3-fold, at least 3.5-fold, at least 4-fold, at least 4.5-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, 1.3-fold to 3-fold, 1.5-fold to 3-fold, 2-fold to 10-fold, 2-fold to 5-fold, 3-fold to 5-fold, 3-fold to 10-fold, 5-fold to 10-fold, or 7-fold to 10-fold lower than expression level of the second subunit or half antibody, *e.g.*, when each subunit or half antibody is expressed individually in the eukaryotic host cell. In some examples, the first subunit or half antibody is expressed at a level that is 0.2-fold to 0.8-fold, less than 0.8-fold, less than 0.5-fold, or less than 0.3-fold that of expression level of the second subunit or half antibody, *e.g.*, when each subunit or half antibody is expressed individually in the eukaryotic host cell.

[0016] Other examples of the present disclosure relate to a plurality or composition of multimeric polypeptides or bispecific antibodies, wherein each multimeric polypeptide or bispecific antibody of the plurality or composition is produced according to the method of any one of the examples described herein. In one aspect, the present disclosure refers to a plurality of multimeric polypeptides, wherein each multimeric polypeptide of the plurality is produced according to any one of the methods described herein.

[0017] Other examples of the present disclosure relate to a recombinant eukaryotic host cell for expression of a non-native multimeric polypeptide that comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain, the host cell comprising: a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain; a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain; a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain; and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain; wherein the first subunit is expressed at a lower level than the second subunit when each subunit is expressed individually in the recombinant eukaryotic host cell or when all subunits are expressed together in the same eukaryotic host cell; and wherein one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence and the fourth translation initiation sequence. In one aspect, the present disclosure refers to a recombinant eukaryotic host cell for expression of a non-native multimeric polypeptide that comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain, the recombinant cell comprising: (a) a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain; (b) a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain; (c) a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain; and (d) a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain; wherein the first subunit is expressed at a lower level than the second subunit when each subunit is expressed individually in the recombinant eukaryotic host cell; and wherein one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence and the fourth translation initiation sequence. In some examples, the multimeric polypeptide is a bispecific antibody, the first and third polypeptide chains

are antibody heavy chains, the second and fourth polypeptide chains are antibody light chains, the first subunit is a first half-antibody that binds a first antigen, and the second subunit is a second half-antibody that binds a second antigen.

[0018] In another aspect, the present disclosure relates to a recombinant eukaryotic host cell for expression of a bispecific antibody that comprises a first half antibody comprising a first antibody heavy chain and a first antibody light chain and a second half antibody comprising a second antibody heavy chain and a second antibody light chain, the recombinant cell comprising: (a) a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first antibody heavy chain; (b) a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the first antibody light chain; (c) a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the second antibody heavy chain; and (d) a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the second antibody light chain; wherein the first half antibody is expressed at a lower level than the second half antibody when each half antibody is expressed individually in the recombinant eukaryotic host cell; and wherein one or both of the first translation initiation sequence and the second translation initiation sequence is weaker than one or both of the third translation initiation sequence and the fourth translation initiation sequence. In some examples, the first and second antibody heavy and light chains form the bispecific antibody, and the first half antibody binds a first antigen and the second half antibody binds a second antigen.

[0019] In some examples, the first antibody heavy chain comprises a first antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the second antibody heavy chain comprises a second antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the CH3 domain of the first antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a smaller side chain volume, thereby generating a hole on the surface of the CH3 domain of the first antibody Fc region that interacts with the CH3 domain of the second antibody Fc region; and wherein the CH3 domain of the second antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a larger side chain volume, thereby generating a knob on the surface of the CH3 domain of the second antibody Fc region that interacts with the CH3 domain of the first antibody Fc region. In some examples, the first antibody heavy

chain comprises a first antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the second antibody heavy chain comprises a second antibody Fc region comprising a CH2 domain and a CH3 domain; wherein the CH3 domain of the second antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a smaller side chain volume, thereby generating a hole on the surface of the CH3 domain of the second antibody Fc region that interacts with the CH3 domain of the first antibody Fc region; and wherein the CH3 domain of the first antibody Fc region is altered so that within the CH3/CH3 interface one or more amino acid residues are replaced with amino acid residues having a larger side chain volume, thereby generating a knob on the surface of the CH3 domain of the first antibody Fc region that interacts with the CH3 domain of the second antibody Fc region. In some examples, the knob mutation comprises at least one of: T366Y, T366W, T394W, and F405W, numbering based on human IgG1 according to EU index. In some examples, the hole mutation comprises at least one of: F405A, Y407T, Y407A, T366S, L368A, Y407V, and T394S, numbering based on human IgG1 according to EU index. In some examples, the knob mutation comprises T366W, and wherein the hole mutation comprises at least one, at least two, or all three of T366S, L368A, and Y407V, numbering based on human IgG1 according to EU index. In some examples, the first antibody light chain comprises a first mutation, the first antibody heavy chain comprises a second mutation, and the first and second mutations promote selective association of the first antibody light chain with the first antibody heavy chain. In some examples, the first mutation comprises an amino acid substitution at V133, and/or the second mutation comprises an amino acid substitution at S183, numbering based on EU index. In some examples, the S183 substitution is selected from the group consisting of S183A, S183T, S183V, S183Y, S183F, S183H, S183N, S183D, S183E, S183R, and S183K; and/or the V133 substitution is selected from the group consisting of V133E, V133S, V133L, V133W, V133K, V133R, and V133D. In some examples, the second antibody light chain comprises a third mutation, the second antibody heavy chain comprises a fourth mutation, and the third and fourth mutations promote selective association of the second antibody light chain with the second antibody heavy chain. In some examples, the third mutation comprises an amino acid substitution at V133, and/or the fourth mutation comprises an amino acid substitution at S183, numbering based on EU index. In some examples, the S183 substitution is selected from the group consisting of S183A, S183T, S183V, S183Y, S183F, S183H, S183N, S183D, S183E, S183R, and S183K; and/or the V133 substitution is

selected from the group consisting of V133E, V133S, V133L, V133W, V133K, V133R, and V133D. In some examples, the amino acid substitution at S183 results in a positively charged residue (*e.g.*, S183K), and the amino acid substitution at V133 results in a negatively charged residue (*e.g.*, V133E). In some examples, the amino acid substitution at S183 results in a negatively charged residue (*e.g.*, S183E), and the amino acid substitution at V133 results in a positively charged residue (*e.g.*, V133K). In some examples, the first antibody light chain comprises a V133K mutation, the first antibody heavy chain comprises an S183E mutation, the second antibody light chain comprises a V133E mutation, and the second antibody heavy chain comprises an S183K mutation, numbering based on EU index. In some examples, the first antibody heavy chain further comprises T366S, L368A, and Y407V mutations, and the second antibody heavy chain further comprises a T366W mutation, numbering based on human IgG1 according to EU index. In some examples, the second antibody light chain comprises a V133K mutation, the second antibody heavy chain comprises an S183E mutation, the first antibody light chain comprises a V133E mutation, and the first antibody heavy chain comprises an S183K mutation, numbering based on EU index. In some examples, the second antibody heavy chain further comprises T366S, L368A, and Y407V mutations, and the first antibody heavy chain further comprises a T366W mutation, numbering based on human IgG1 according to EU index.

[0020] Other examples of the present disclosure relate to methods for identifying a combination of translation initiation sequences for expressing a multimeric polypeptide in a eukaryotic host cell, wherein the multimeric polypeptide comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain. In some examples, the methods comprise: providing a library comprising a plurality of eukaryotic host cells, wherein each eukaryotic host cell in the plurality comprises: a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain, a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain, a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain, and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain, wherein a plurality of combinations of first, second, third, and

fourth translation initiation sequences are represented in the plurality of eukaryotic host cells; culturing the library of eukaryotic host cells under conditions suitable for expression of the multimeric polypeptide by the eukaryotic host cells of the plurality; measuring an amount of the multimeric polypeptide that is expressed by single eukaryotic host cells of the plurality or clones of single eukaryotic host cells of the plurality; and identifying the first, second, third, and fourth translation initiation sequences of one or more single eukaryotic host cells of the plurality or clones of single eukaryotic host cells of the plurality that express the multimeric polypeptide. In some examples, the first, second, third, and fourth translation initiation sequences of one or more single eukaryotic host cells of the plurality or clones of single eukaryotic host cells of the plurality that express the multimeric polypeptide are identified based on higher production of the multimeric polypeptide and/or a lower level of incorrectly paired side products, *e.g.*, as compared to a reference or different combination of four translation initiation sequences. In some examples, all of the first, second, third, and fourth translation initiation sequences in each host cell of the plurality comprise the sequence (from 5' to 3') NNNNNATGNGA, wherein N is C, G, A, or T/U (SEQ ID NO:1).

[0020a] In one aspect, the present disclosure refers to a method for identifying a combination of translation initiation sequences for expressing a multimeric polypeptide in a eukaryotic host cell, wherein the multimeric polypeptide comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain, the method comprising: (a) providing a library comprising a plurality of eukaryotic host cells, wherein each eukaryotic host cell in the plurality comprises: a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain, a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain, a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain, and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain, wherein a plurality of combinations of first, second, third, and fourth translation initiation sequences are represented in the plurality of eukaryotic host cells; (b) culturing the library of eukaryotic host cells under conditions suitable for expression of the multimeric polypeptide by the eukaryotic host cells of the plurality; (c) measuring an amount of the multimeric polypeptide that is expressed by single eukaryotic host cells of the plurality or clones of single eukaryotic host cells of the plurality; and (d) identifying the first, second, third, and fourth translation initiation sequences of one or more single eukaryotic host cells of the plurality or clones of single eukaryotic host cells of the plurality that express the multimeric polypeptide.

[0021] Other examples of the present disclosure relate to kits or articles of manufacture comprising polynucleotides for expression of a multimeric polypeptide that comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain. In some examples, the kit or article of manufacture comprises a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain; a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain; a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain; and a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain; wherein one or more of the first, second, third, and fourth translation initiation sequences is not operably linked with its respective open-reading frame as the respective open-reading frame exists in a naturally occurring host cell genome. In some examples, the multimeric polypeptide is a bispecific antibody, the first and third polypeptide chains are antibody heavy chains, the second and fourth polypeptide chains are

antibody light chains, the first subunit is a first half-antibody that binds a first antigen, and the second subunit is a second half-antibody that binds a second antigen.

[0021a] In one aspect, the present disclosure refers to a kit of polynucleotides for expression of a multimeric polypeptide that comprises a first subunit comprising a first polypeptide chain and a second polypeptide chain and a second subunit comprising a third polypeptide chain and a fourth polypeptide chain, the kit comprising: (a) a first polynucleotide comprising a first translation initiation sequence operably linked to a first open-reading frame that encodes the first polypeptide chain; (b) a second polynucleotide comprising a second translation initiation sequence operably linked to a second open-reading frame that encodes the second polypeptide chain; (c) a third polynucleotide comprising a third translation initiation sequence operably linked to a third open-reading frame that encodes the third polypeptide chain; and (d) a fourth polynucleotide comprising a fourth translation initiation sequence operably linked to a fourth open-reading frame that encodes the fourth polypeptide chain; wherein one or more of the first, second, third, and fourth translation initiation sequences is not operably linked with its respective open-reading frame as the respective open-reading frame exists in a naturally occurring host cell genome.

[0022] In some examples, one or both polypeptide chains of the first subunit are produced at a lower level than one or both polypeptide chains of the second subunit when each subunit is produced individually. In some examples, one or both polypeptide chains of the first subunit are produced at a lower level than one or both polypeptide chains of the second subunit when all subunits are produced by the same host cell. In some examples, the first subunit assembles with more incorrectly paired side products than the second subunit when each subunit is expressed individually in the eukaryotic host cell. In some examples, the first subunit assembles with more incorrectly paired side products than the second subunit when all subunits are expressed together in the same eukaryotic host cell.

[0023] In some examples described herein, all of the first, second, third, and fourth translation initiation sequences comprise the sequence (from 5' to 3') NNNNNATGNGA, wherein N is C, G, A, or T/U (SEQ ID NO:1). In some examples, one or both of the first translation initiation sequence and the second translation initiation sequence comprises a sequence selected from the group consisting of SEQ ID NOs:8-10. In some examples, one or both of the third translation initiation sequence and the fourth translation initiation sequence comprises the sequence ACCATGG (SEQ ID NO:3) or GAAGTATGA (SEQ ID NO:11). In some examples, the first translation initiation sequence comprises a sequence selected from the group consisting of SEQ ID NOs:8-10, the second translation initiation sequence comprises a sequence selected from the group consisting of SEQ ID NOs:8-10, the third translation initiation sequence comprises the sequence of SEQ ID NO:2, and the fourth translation initiation sequence comprises the sequence of SEQ ID NO:11. In some examples, the first translation initiation sequence comprises the sequence of SEQ ID NO:9, the second translation initiation sequence comprises the sequence of SEQ ID NO:9, the third translation initiation sequence comprises the sequence of SEQ ID NO:2, and the fourth translation initiation sequence comprises the sequence of SEQ ID NO:11. In some examples, each of the first, second, third, and fourth polynucleotides are operably linked to a promoter. In some examples, the first and the second polynucleotides are operably linked to the same promoter, and the third and the fourth polynucleotides are operably linked to the same promoter. In some examples, the first translation initiation sequence is weaker than the third translation initiation sequence. In some examples, the second translation initiation

sequence is weaker than the fourth translation initiation sequence. In some examples, the first translation initiation sequence is weaker than the fourth translation initiation sequence. In some examples, the second translation initiation sequence is weaker than the third translation initiation sequence. In some examples, the first translation initiation sequence is the same as the second translation initiation sequence. In some examples, the third translation initiation sequence is the same as the fourth translation initiation sequence. In some examples described herein, the multimeric polypeptide specifically binds one or more target antigens. In some examples, the multimeric polypeptide is a multispecific antigen binding protein.

[0024] Also provided herein is a polynucleotide comprising an open-reading frame operably linked with a translation initiation sequence selected from the group consisting of SEQ ID NOs:8-11. Further provided herein is a set of polynucleotides comprising: a first translation initiation sequence that comprises a sequence selected from the group consisting of SEQ ID NOs:8-10 operably linked with a first open-reading frame, a second translation initiation sequence that comprises a sequence selected from the group consisting of SEQ ID NOs:8-10 operably linked with a second open-reading frame, a third translation initiation sequence that comprises the sequence of SEQ ID NO:2 operably linked with a third open-reading frame, and a fourth translation initiation sequence that comprises the sequence of SEQ ID NO:11 operably linked with a fourth open-reading frame. In some examples, the first translation initiation sequence comprises the sequence of SEQ ID NO:9, the second translation initiation sequence comprises the sequence of SEQ ID NO:9, the third translation initiation sequence comprises the sequence of SEQ ID NO:2, and the fourth translation initiation sequence comprises the sequence of SEQ ID NO:11. Yet further provided herein are host cells comprising the polynucleotide or set of polynucleotides according to any one of the examples described herein.

[0025] In some examples described herein, the first, second, third, and fourth polynucleotides are integrated into one or more chromosomes of the eukaryotic host cell. In some examples, the first, second, third, and fourth polynucleotides are integrated into the same chromosomal locus of the eukaryotic host cell. In some examples, the first, second, third, and fourth polynucleotides are part of one of more extrachromosomal polynucleotides in the eukaryotic host cell. In some examples, the eukaryotic host cell is a mammalian host cell. In some examples, the mammalian host cell is a Chinese hamster ovary (CHO) cell.

[0026] It is to be understood that one, some, or all of the properties of the various examples described herein may be combined to form other examples of the present disclosure. These and other examples of the disclosure will become apparent to one of skill in the art. These and other examples of the disclosure are further described by the detailed description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0028] **FIG. 1** shows the names and nucleotide sequences of the Kozak (Kz) sequence variants designed and analyzed by transient transfection. The start codon is gray and underlined. Sequences shown correspond to SEQ ID NOs:3-7 (top to bottom).

[0029] **FIG. 2** provides the normalized titer of Fc-fusion proteins A and B with different Kozak sequence variants tested by transient transfection followed by homogeneous time resolved fluorescence (HTRF). Data are presented as mean values, and error bars represent the standard deviation (SD) of four independent transfections. Variations in the Kozak consensus sequence modulated the Fc-fusion protein titer.

[0030] **FIG. 3** illustrates the processes for the design of a Kozak library, transformation of *E.coli* cells with the library, sequencing to determine the nucleotide sequence of variants, screening of variants by transient transfection, categorizing within ranges and narrowing down variants, and finally the selection of the variants that cover the entire range of expression.

[0031] **FIG. 4** shows the wild-type (WT) Kozak sequence in the upper line. In the lower line, the designed Kozak library is shown. N is either A, T, G or C. The start codon is gray and underlined. Positions -3 and +4 from start codon are indicated. Sequences shown correspond to (top to bottom) SEQ ID NOs:23 and 1, respectively.

[0032] **FIG. 5** shows the normalized antibody titer of 111 Kozak sequence variants that were transiently transfected into CHO cells. Data are presented as mean values and error bars represent the standard deviation (SD) of two independent transfections. A nearly continuous range of translation from 0.05 to 1.54 units of normalized titer was observed (1.0 corresponds with titer produced using the Wt Kozak sequence).

[0033] **FIG. 6** provides the nucleotide distribution of 108 colonies of the Kozak library. The percentage of each nucleotide for each position is shown. The nucleotide distribution for