

[13] The present disclosure provides a new method for generating conditionally active polypeptides, taking advantage of directed mutagenesis for more efficiently production. The present disclosure also provides new conditionally active polypeptides produced by this method.

SUMMARY OF THE DISCLOSURE

[14] Disclosed is a conditionally active polypeptide obtainable by increasing a net charge of a parent polypeptide, wherein said conditionally active polypeptide exhibits a decrease in activity in a first assay at a first value of a condition compared to the same activity in a second assay at a second value of the same condition. Charged amino acid residues may be selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and, optionally, Histidine.

[15] In each of the foregoing embodiments, the net charge may be increased through one or more techniques selected from increasing a total number of the charged amino acid residues in the polypeptide, decreasing a total number of uncharged amino acid residues in the polypeptide or a combination thereof. In each of the foregoing embodiments, the condition may be selected from pH, temperature, osmotic pressure, osmolality, oxidative stress, electrolyte concentration, concentration of a protein and combinations of two or more of these.

[16] In each of the foregoing embodiments, the condition may be pH, and the conditionally active polypeptide may exhibit the decreased activity at a normal physiological pH and the increased activity at an aberrant pH that differs from the normal physiological pH.

[17] In each of the foregoing embodiments, the conditional activity may be assayed in the presence in the presence of at least one species having a molecular weight of less than 900 a.m.u. and a pKa up to 2 units, or up to 3 units away from the pH at which the increased activity is desired. In the foregoing embodiments, the pKa of the species may be between the first value of the pH and the second value of the pH.

[18] In each of the foregoing embodiments, the conditional activity may be assayed in the presence in the presence of at least one species selected from, histidine, histamine, hydrogenated adenosine diphosphate, hydrogen sulfide, hydrogenate adenosine triphosphate, citrate, acetate, lactate, bisulfide, ammonium, bicarbonate, dihydrogen phosphate and combinations thereof.

[19] In each of the foregoing embodiments, the first assay and the second assay may be conducted in assay solutions in the absence of serum.

[20] In each of the foregoing embodiments, the net charge may be increased by introducing one or more charged amino residues into the parent polypeptide through amino acid residue substitutions, amino acid insertions, or a combination thereof.

[21] In each of the foregoing embodiments, the one or more charged amino residues may be introduced into an active site of the parent polypeptide. In each of the foregoing embodiments, the one or more charged amino residues may be introduced into a region outside of an active site of the parent polypeptide. In each of the foregoing embodiments, the conditionally active polypeptide may be a conditionally active antibody and

the one or more charged amino acid residues may be introduced into at least one of complementarity determining regions of the conditionally active antibody.

[22] In each of the forgoing embodiments, the one or more charged amino acid residues may be less than five charged amino acid residues, or less than four charged amino acid residues, or less than three charged amino acid residues, or less than two charged amino acid residues. In each of the forgoing embodiments, the one or more charged amino acid residues may be selected from Aspartic acid and Glutamic acid.

[23] In each of the forgoing embodiments, the conditionally active polypeptide may be reversibly inactivated at the first value of the condition.

[24] In each of the forgoing embodiments, the first and second assays may be conducted in the presence of a species selected from histidine, histamine, hydrogenated adenosine diphosphate, hydrogen sulfide, hydrogenate adenosine triphosphate, citrate, acetate, lactate, bicarbonate, bisulfide, ammonium, dihydrogen phosphate and combinations thereof.

[25] In each of the forgoing embodiments, the first value of the condition may be a value of a normal physiological condition that is within a normal range of the physiological condition at a site of administration of the conditionally active polypeptide to a subject, or at a tissue or organ at a site of action of the conditionally active polypeptide of a subject, and the second value of the condition may be a value of an aberrant condition that deviates from the normal range of the physiological condition at the site of administration of the conditionally active polypeptide, or at the tissue or organ at the site of action of the conditionally active polypeptide.

[26] In each of the forgoing embodiments, the conditionally active polypeptide may be a protein or protein fragment. In each of the forgoing embodiments, the conditionally active polypeptide may be an antibody, a single chain antibody, and an antibody fragment and the activity is a binding activity to an antigen, an Fc region of an antibody, or an enzyme and the activity is an enzymatic activity, or a receptor, a regulatory protein, a soluble protein, a cytokine and a fragment of a receptor, a regulatory protein, a stress protein, a vault-related protein, a neuron protein, a digestive tract protein, a growth factor, a mitochondrial protein, a cytosolic protein, an animal protein, a structural protein, and a plant protein.

[27] Disclosed is a pharmaceutical composition comprising an effective amount of the conditionally active polypeptide of any one of the forgoing embodiments and a pharmaceutically acceptable carrier.

[28] In each of the forgoing embodiments, the pharmaceutical composition may further comprise at least one anti-cancer agent. In each of the forgoing embodiments, the at least one anti-cancer agent may be a chemotherapeutic agent, a radiation therapeutic agent, a hormonal therapeutic agent, a toxin or an immunotherapeutic agent. In each of the forgoing embodiments, the at least one anti-cancer agent may be a toxin selected from a taxane, a maytansinoid, an auristatin, a calicheamicin, a duocramycin, a mmae, a soporific, a duomycin, an anthracycline, a CC-1065 analog, docetaxel, a cathepsin, ricin, gelonin, Pseudomonas exotoxin, diphtheria toxin, RNase, and a toxic radioisotope. In each of the forgoing

embodiments the conditionally active polypeptide may be conjugated to the at least one anti-cancer agent, or at least four anti-cancer agent, or at least six anti-cancer agents.

[29] Disclosed is a use of the conditionally active polypeptide of each of the forgoing embodiments for treatment of solid tumors, inflamed joints, or brain diseases or disorders, or removal of senescent cells from a subject.

[30] Disclosed is a method of treatment of solid tumors, inflamed joints, or brain diseases or disorders comprising a step of administering a conditionally active polypeptide of each of the forgoing embodiments.

[31] Disclosed is a nucleic acid molecule that encodes the conditionally active polypeptide of each of forgoing embodiments. Disclosed is an expression vector comprising the nucleic acid molecule of the forgoing embodiments or a host cell comprising such an expression vector.

[32] Disclosed is a method for diagnosing the presence of cancer in a subject, comprising steps of (a) contacting cells or tissue of the subject with a detectably labeled conditionally active polypeptide of each of the forgoing embodiments, wherein the conditionally active polypeptide binds to a target on cancer cells; (b) determining whether the conditionally active polypeptide binds to the cell or tissue of the subject.

[33] In each of the forgoing embodiments, the contacting of the cells or tissue with the conditionally active polypeptide may occur *in vitro*. In each of the forgoing embodiments, the contacting of the cells or tissue with the conditionally active polypeptide may occur within said subject.

[34] Disclosed is a bispecific antibody comprising at least one binding domain that contains at least one of the conditionally active polypeptides of the forgoing embodiments. Disclosed is a chimeric protein containing two different binding or catalytic domains, wherein one or both of the domains comprise at least one of the conditionally active polypeptides of the forgoing embodiments. Disclosed is an oncolytic virus comprising at least one of the conditionally active polypeptides of the forgoing embodiments.

[35] Disclosed is a method of preparing a conditionally active polypeptide from a parent polypeptide. In the method, a DNA encoding the parent polypeptide is evolved to increase the net charge of the polypeptide using one or more techniques selected from increasing a total number of codons of charged amino acid residues in the DNA, decreasing a total number of codons of uncharged amino acid residues in the DNA or a combination thereof to create mutant DNAs. The mutant DNAs are expressed to obtain mutant polypeptides; and the conditionally active polypeptide is selected from the mutant polypeptides. The selected conditionally active polypeptide exhibits a decrease in activity in a first assay at a first value of a condition compared to the same activity in a second assay at a second value of the same condition. The charged amino acid residues are selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and Histidine.

[35a] In one aspect, there is provided a method of preparing a conditionally active polypeptide from a parent polypeptide, said method comprising steps of: i. evolving a DNA encoding the parent polypeptide to increase the net charge of the parent polypeptide using one or more techniques selected from increasing a total number of codons of charged amino acid residues in the DNA, decreasing a total number of codons of uncharged amino acid residues in the DNA and a combination thereof, to create mutant DNAs; ii. expressing the mutant DNAs to obtain mutant polypeptides; and iii. selecting the conditionally active polypeptide from the mutant polypeptides which exhibits a decrease in activity in a first assay at a first value of a condition compared to the same activity in a second assay at a second value of the same condition and the charged amino acid residues are selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and Histidine.

[36] In each of the foregoing embodiments, the evolving step may employ a site directed mutagenesis technique, optionally selected from oligonucleotide-mediated mutagenesis, PCR mutagenesis, and cassette mutagenesis. In each of the foregoing embodiments, the evolving step may employ codon substitution or codon insertion.

[37] In each of the foregoing embodiments, the charged amino acid residues may be selected from Aspartic acid and Glutamic acid.

[38] In each of the foregoing embodiments, the one or more codons may be introduced into a region of the DNA that encodes an active site of the parent polypeptide. In each of the foregoing embodiments, the parent polypeptide may be a parent antibody and the active site may be a complementarity determining region of the parent antibody.

[39] In each of the foregoing embodiments, the the one or more charged amino residues may be introduced into a region outside of an active site of the parent polypeptide.

[40] In each of the foregoing embodiments, the region outside of the active site may be a constant region of an antibody, or a framework region of the antibody.

[41] In each of the foregoing embodiments, the conditionally active polypeptide may be reversibly inactivated under the first value of the condition.

[42] In each of the foregoing embodiments, the condition may be selected from pH, temperature, osmotic pressure, osmolality, oxidative stress, electrolyte concentration, and concentration of a protein.

[43] In each of the foregoing embodiments, the first and second assays may be performed in assay solutions comprising a protein found in blood. In each of the foregoing embodiments, the blood protein may be albumin.

[44] In each of the foregoing embodiments, the first and second assays may be performed in assay solutions in absence of serum.

[45] In each of the foregoing embodiments, the value of the second condition may be a value of a normal physiological condition that is within a normal range of the physiological condition at a site of administration of the conditionally active polypeptide to a subject, or at a tissue or organ at a site of action of the conditionally active polypeptide of a subject, and the value of the first condition may be a value of an aberrant condition that deviates from the normal range of the physiological condition at the site of administration of the conditionally active polypeptide, or at the tissue or organ at the site of action of the conditionally active polypeptide.

[46] In each of the foregoing embodiments, the condition may be pH and the first and second assays may be performed in assay media including at least one species having a molecular weight of less than 900 a.m.u. and a pKa up to 3 units away from the first value of pH.

[47] In each of the foregoing embodiments, the condition may be pH and the first and second assays may be performed in assay media including at least one species having a molecular weight of less than 900 a.m.u. and a pKa between the second value of pH and the first value of pH.

WHAT IS CLAIMED IS:

1. A method of preparing a conditionally active polypeptide from a parent polypeptide, said method comprising steps of:
 - i. evolving a DNA encoding the parent polypeptide to increase the net charge of the parent polypeptide using one or more techniques selected from increasing a total number of codons of charged amino acid residues in the DNA, decreasing a total number of codons of uncharged amino acid residues in the DNA and a combination thereof, to create mutant DNAs;
 - ii. expressing the mutant DNAs to obtain mutant polypeptides; and
 - iii. selecting the conditionally active polypeptide from the mutant polypeptides which exhibits a decrease in activity in a first assay at a first value of a condition compared to the same activity in a second assay at a second value of the same condition and the charged amino acid residues are selected from Aspartic acid, Glutamic acid, Arginine, Lysine, and Histidine.
2. The method of claim 1, wherein the evolving step employs site directed mutagenesis selected from oligonucleotide-mediated mutagenesis, PCR mutagenesis, and cassette mutagenesis.
3. The method of any one of claims 1-2, wherein the evolving step comprising introducing one or more codons of charged amino acid residues into the DNA.
4. The method of any one of claims 1-3, wherein the charged amino acid residues are selected from Aspartic acid and Glutamic acid.
5. The method of any one of claims 3-4, wherein the one or more codons are introduced into a region of the DNA that encodes an active site of the parent polypeptide.
6. The method of claim 5, wherein the parent polypeptide is a parent antibody and the active site is a complementarity determining region of the parent antibody.
7. The method of any one of claims 3-4, wherein the one or more charged amino residues are introduced into a region outside of an active site of the parent polypeptide.
8. The method of claim 7, wherein the region outside of the active site is a constant region of an antibody, or a framework region of the antibody.
9. The method of any one of claims 1-8, wherein the conditionally active polypeptide is reversibly inactivated under the first value of the condition.

10. The method of any one of claims 1-9, wherein the condition is selected from pH, temperature, osmotic pressure, osmolality, oxidative stress, electrolyte concentration, and concentration of a protein.
11. The method of any one of claims 1-10, wherein the first and second assays are performed in assay solutions comprising a protein found in blood.
12. The method of claim 11, wherein the blood protein is albumin.
13. The method of any one of claims 1-12, wherein the first and second assays are performed in assay solutions in the absence of serum.
14. The method of any one of claims 1-13, wherein the value of the first condition is a value of a normal physiological condition that is within a normal range of the physiological condition at a site of administration of the conditionally active polypeptide to a subject, or at a tissue or organ at a site of action of the conditionally active polypeptide of a subject, and the value of the second condition is a value of an aberrant condition that deviates from the normal range of the physiological condition at the site of administration of the conditionally active polypeptide, or at the tissue or organ at the site of action of the conditionally active polypeptide.
15. The method of any one of claims 1-14, wherein the parent polypeptide is selected from an antibody, a single chain antibody, and an antibody fragment and the activity is a binding activity to an antigen.
16. The method of any one of claims 1-14, wherein the parent polypeptide is an enzyme and the activity is an enzymatic activity.
17. The method of any one of claims 1-14, wherein the parent polypeptide is selected from a receptor, a regulatory protein, a soluble protein, a cytokine and a fragment of a receptor, a regulatory protein, a stress protein, a vault-related protein, a neuron protein, a digestive tract protein, a growth factor, a mitochondrial protein, a cytosolic protein, an animal protein, a structural protein, and a plant protein.
18. The method of any one of claims 1-17, wherein the selecting step further comprises selecting the conditionally active polypeptide based on a property selected from affinity, expression level, and humanization,
19. The method of any one of claims 1-18, where the expression step employs phage display or a eukaryotic cell production host.

20. The method of claim 1-3 and 5-19, wherein the charged amino acid residues selected from Aspartic acid, Glutamic acid, Arginine and Lysine.