

method imposes a different selective filter on the antibody specificities that can be obtained. Alternatively, fully human antibodies can be isolated from libraries in eukaryotic systems, *e.g.*, yeast display, retroviral display, or expression in DNA viruses such as poxviruses. See, *e.g.*, U.S. Patent No. 7,858,559, and U.S. Patent Appl. Publication No. 2013-028892, which are incorporated herein by reference in their entireties.

[0003] Many important targets for therapeutic antibodies are integral membrane proteins (IMPs), *e.g.*, multi-pass membrane proteins (GPCRs, Ion Channels, etc.) that are difficult to express and purify in a conformationally-intact state. The absence of properly folded target proteins in an isolated state makes the identification and selection of antibodies to these targets challenging. While certain IMPs can be expressed on the surface of cells, *e.g.*, mammalian cells, whole cells are problematic for use in antibody discovery because they are complex antigen mixtures, target expression can be low, and because certain display packages used to construct antibody libraries (*e.g.*, vaccinia virus antibody libraries) can bind to whole cells non-specifically. There remains a need for new methods to express and display target IMPs of interest in their native conformation at a sufficient concentration and with minimal competition from other cell proteins to allow for identification and selection of therapeutic antibodies and antibody-like molecules from display libraries and from animal-based systems.

SUMMARY

[0004] This disclosure provides compositions and methods for expressing and displaying isolated integral membrane proteins (IMPs) or fragments thereof in a native conformation for use in the screening, selecting, and identifying of antibodies or antibody-like molecules that bind to a target IMP of interest.

[0004a] In one aspect, the disclosure provides an isolated polynucleotide comprising:
(a) a first nucleic acid fragment that encodes an integral membrane protein (IMP) or fragment thereof, wherein the IMP or fragment thereof comprises at least one extra-membrane region, at least one transmembrane domain and at least one intra-membrane region, and wherein a portion of the first nucleic acid fragment encoding at least one

intra-membrane region is situated at the 5' or 3' end of the first nucleic acid fragment; and
(b) a second nucleic acid fragment that encodes a fowlpox virus (FPV) FPV108
protein, a rabbit pox virus (RBXV) protein RBXV041 or functional fragment thereof,
wherein the second nucleic acid fragment is fused in frame to a portion of the first
nucleic acid fragment that encodes an intra-membrane region of the IMP; wherein a
poxvirus infected cell comprising the polynucleotide can express an IMP-FPV108 or
IMP-RBXV041 fusion protein as part of the outer envelope membrane of an
extracellular enveloped virion (EEV).

[0004b] In one aspect, the disclosure provides an FPX108 fusion protein encoded
by the polynucleotide as disclosed herein. In another aspect, the disclosure provides
an RBXV041 fusion protein encoded by the polynucleotide as disclosed herein. In
another aspect, the disclosure provides a poxvirus genome comprising the
polynucleotide as disclosed herein. In another aspect, the disclosure provides a
recombinant poxvirus EEV comprising the poxvirus genome as disclosed herein.

[0004c] In one aspect, the disclosure provides an *in vitro* method of producing the
recombinant poxvirus EEV as disclosed herein, comprising: (a) infecting a host cell
permissive for vaccinia virus, fowlpox virus, or rabbit pox virus infectivity with a
vaccinia virus, fowlpox virus or rabbit pox virus, respectively, wherein the vaccinia
virus comprises the poxvirus genome as disclosed herein, the fowlpox virus comprises
the poxvirus genome as disclosed herein, and the rabbit pox virus comprises the
poxvirus genome as disclosed herein; and (b) recovering EEV released from the host
cell.

[0004d] In one aspect, the disclosure provides an *in vitro* method to display an
integral membrane protein (IMP) or fragment thereof in a native conformation
comprising: (a) infecting host cells permissive for poxvirus infectivity with a
recombinant poxvirus that expresses the IMP or fragment thereof as a fusion protein
with the EEV-specific protein FPV108 or RBX041 or a membrane-associated
fragment thereof, wherein EEV produced by the infected host cell comprise the IMP
fusion protein as part of the EEV outer envelope membrane; (b) recovering EEV
released from the host cell wherein the IMP or fragment thereof displays on the surface
of the EEV in a native conformation.

[0004e] In one aspect, the disclosure provides a fusion protein comprising: (a) the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11, wherein the fusion protein, when expressed by a recombinant poxvirus, appears on the surface of a poxvirus extracellular enveloped virion (EEV) in a native conformation.

[0004f] In one aspect, the disclosure provides a recombinant poxvirus EEV comprising the fusion protein as disclosed herein. In other aspect, the disclosure provides a recombinant poxvirus EEV comprising a heterologous IMP or fragment thereof fused to a fowlpox virus or rabbit pox virus EEV-specific protein or membrane-associated fragment thereof, wherein the fusion protein is situated in the EEV outer envelope membrane, wherein the IMP or fragment thereof displays on the surface of the EEV in its native conformation.

[0004g] In another aspect, the disclosure provides a method to select antibodies that bind to a multi-pass membrane protein (IMP) comprising: (a) attaching the recombinant EEV as described herein to a solid support; (b) providing an antibody display library, wherein the library comprises display packages displaying a plurality of antigen binding domains; (c) contacting the display library with the EEV such that display packages displaying antigen binding domains that specifically binds to the IMP expressed on the EEV can bind thereto; (d) removing unbound display packages; and (e) recovering display packages that display an antigen binding domain specific for the IMP expressed on the EEV.

[0004h] In another aspect, the disclosure provides a method to select antibodies that bind to a multi-pass membrane protein (IMP) comprising: (a) providing a first and second recombinant poxvirus EEV of claim 23, wherein the first and second recombinant poxvirus EEV are each generated in a different poxvirus, wherein an animal is to be immunized with the first recombinant poxvirus; (b) contacting a display library that comprises display packages displaying a plurality of antigen binding domains with the second recombinant poxvirus such that the display packages displaying antigen binding domains that specifically bind to the IMP expressed on the EEV can bind thereto, wherein said display library is generated from B cells which are to be isolated from the immunized animal; (c) removing

unbound display packages; and (d) recovering display packages that display an antigen binding domain specific for the IMP expressed on the second recombinant EEV.

[0004i] In another aspect, the disclosure provides a method to select antibodies or antigen-binding fragments thereof that bind to a multi-pass membrane protein (IMP), which comprises: (a) providing a recombinant poxvirus EEV as disclosed herein, wherein an animal is to be immunized with the recombinant poxvirus EEV; wherein optionally the animal is to be immunized with a second dose of the recombinant poxvirus EEV; (b) isolating serum from the immunized animal; and (c) isolating antibodies or antigen-binding fragments thereof that comprise an antigen binding domain specific for the IMP expressed on the recombinant poxvirus EEV.

[0005] In certain ~~embodiments~~examples, the disclosure provides an isolated polynucleotide that includes: a first nucleic acid fragment that encodes an integral membrane protein (IMP) or fragment thereof, where the IMP or fragment thereof includes at least one extra-membrane region, at least one transmembrane domain and at least one intra-membrane region, and where a portion of the first nucleic acid

fragment encoding at least one intra-membrane region is situated at the 5' or 3' end of the first nucleic acid fragment; and a second nucleic acid fragment that encodes a fowlpox virus FPV108 protein or functional fragment thereof or a rabbit pox virus RBXV041 protein or functional fragment thereof, where the second nucleic acid fragment is fused in frame to a portion of the first nucleic acid fragment that encodes an intra-membrane region of the IMP. According to these embodimentsexamples, a poxvirus infected cell containing the polynucleotide can express an IMP-FPV108 or IMP-RBXV041 fusion protein as part of the outer envelope membrane of an extracellular enveloped virion (EEV). In certain aspects-examples the IMP is a multi-pass membrane protein comprising at least two, at least three, at least four, at least five, at least six or at least seven transmembrane domains. In certain aspects-examples the IMP is a multi-pass membrane protein listed in Table 1.

[0006] In certain aspects-examples the multi-pass IMP can have an odd number of transmembrane domains, the 5' end of the first nucleic acid fragment can encode an extra-membrane region, and the 3' end of the first nucleic acid fragment can encode an intra-membrane region fused to the 5' end of the second nucleic acid fragment. In certain aspects-examples the first nucleic acid fragment of this type can encode, *e.g.*, a G-protein coupled receptor (GPCR). In certain aspects the GPCR can be the human frizzled-4 protein (FZD4), or a fragment thereof, and the polynucleotide can encode a polypeptide that includes amino acids 20 to 892 of SEQ ID NO: 2. In certain aspects-examples the polypeptide can further include a signal peptide, *e.g.*, amino acids 1 to 19 of SEQ ID NO: 2. In certain aspects-examples the GPCR can be a CXC chemokine receptor, *e.g.*, CXCR4, or a fragment thereof, and the polynucleotide can encode a polypeptide that includes the amino acid sequence SEQ ID NO: 3.

[0007] In certain aspects-examples the multi-pass IMP can have an even number of transmembrane domains, and both the 5' and 3' ends of the first nucleic acid fragment can encode intra-membrane regions. In certain aspects-examples, the second nucleic acid fragment can be fused to 3' end of the first nucleic acid fragment. In certain aspects-examples the IMP can be, *e.g.*, human CD20 protein, or CD39 or a fragment thereof.

[0008] In certain ~~aspects~~examples, the first and second nucleic acid fragments of a polynucleotide provided herein can be directly fused. In certain ~~aspects~~examples the polynucleotide as provided herein can include a third nucleic acid fragment encoding a heterologous peptide, *e.g.*, a linker sequence, an amino acid tag or label, or a peptide or polypeptide sequence that facilitates purification, such as a histidine tag. In certain ~~aspects~~examples a polynucleotide as provided here can be operably associated with a poxvirus promoter, *e.g.*, a p7.5, a T7, or H5 promoter.

[0009] The disclosure further provides an FPV108 or RBXV041 fusion protein encoded by a polynucleotide as provided herein. The disclosure further provides a poxvirus genome, *e.g.*, a fowlpox virus genome or rabbit pox virus genome, that includes a polynucleotide as provided herein. The disclosure further provides a recombinant fowlpox virus EEV that includes a poxvirus genome as provided herein and a recombinant rabbit pox virus EEV that includes a poxvirus genome as provided herein.

[00010] The disclosure further provides a method of producing a recombinant pox virus EEV, such as a fowlpox virus EEV as provided herein where the method includes infecting a host cell permissive for fowlpox virus infectivity with a fowlpox virus comprising a poxvirus genome as provided herein, and recovering EEV released from the host cell. Similarly, the disclosure provides a method of producing a recombinant rabbit pox virus EEV as provided herein where the method includes infecting a host cell permissive for rabbit pox virus infectivity with a rabbit pox virus comprising a poxvirus genome as provided herein, and recovering EEV released from the host cell.

[00011] The disclosure further provides a method to display an integral membrane protein (IMP) or fragment thereof in a native conformation where the method includes infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus that expresses an IMP or fragment thereof as a fusion protein with poxvirus EEV-specific protein or membrane-associated fragment thereof, where EEV produced by the infected host cell comprise the IMP fusion protein as part of the EEV outer envelope membrane and recovering EEV released from the host cell. In certain aspects the IMP or fragment thereof displays on the surface of the EEV in a

native conformation. In certain aspects the EEV-specific protein can be the fowlpox virus FPV018 protein or the rabbit pox virus RBXV041 protein, any membrane-associated fragment thereof, or any combination thereof.

[00012] In certain aspects-examples the EEV-specific protein is F13L (SEQ ID NO: 1) or a functional fragment thereof. In certain aspects-examples the EEV-specific protein is FPV108 (SEQ ID NO: 2) or RBXV041 (SEQ ID NO:3). In certain aspects-examples the IMP is a multi-pass membrane protein that includes at least two, at least three, at least four, at least five, at least six or at least seven transmembrane domains. In certain aspects-examples the IMP can be a G-protein coupled receptor (GPCR), e.g., human FZD4 or CXCR4 as described above, that includes seven transmembrane domains, and the F13L, FPV108, or RBXV041 protein can be fused to the C-terminus of the IMP. In certain aspects-examples the IMP or fragment thereof can have an even number of transmembrane domains, e.g., human CD20 or CD39 as described above, where both the N-terminus and the C-terminus of the IMP or fragment thereof are intra-membrane, and the membrane-associated EEV-specific protein, e.g., FPV108 or RBXV041 can be fused to the N-terminus or the C-terminus of the IMP.

[00013] In certain aspects-examples the membrane-associated EEV specific protein fragment can include or consist of the stalk, transmembrane, and intra-membrane domains of the vaccinia virus A56R protein, e.g., amino acids 108 to 314 of SEQ ID NO: 5.

[00014] A fusion protein as provided, when expressed by a recombinant poxvirus, e.g., a vaccinia virus, fowlpox virus, or rabbit pox virus can appear on the surface of the poxvirus extracellular enveloped virion (EEV) in a native conformation. A recombinant poxvirus EEV comprising the fusion protein is also provided. The disclosure further provides a recombinant poxvirus EEV that includes a heterologous IMP or fragment thereof fused to a poxvirus EEV-specific protein or membrane-associated fragment thereof, where the fusion protein is situated in the EEV outer envelope membrane, and where the IMP or fragment thereof displays on the surface of the EEV in its native conformation. In certain aspects-examples the recombinant poxvirus EEV is a fowlpox virus or rabbit pox virus EEV.

[00015] The disclosure also provides a method to select antibodies that bind to a multi-pass membrane protein (IMP) comprising: (a) providing a first and second

What is claimed is:

1. An isolated polynucleotide comprising: (a) a first nucleic acid fragment that encodes an integral membrane protein (IMP) or fragment thereof, wherein the IMP or fragment thereof comprises at least one extra-membrane region, at least one transmembrane domain and at least one intra-membrane region, and wherein a portion of the first nucleic acid fragment encoding at least one intra-membrane region is situated at the 5' or 3' end of the first nucleic acid fragment; and (b) a second nucleic acid fragment that encodes a fowlpox virus (FPV) FPV108 protein, a rabbit pox virus (RBXV) protein RBXV041 or functional fragment thereof, wherein the second nucleic acid fragment is fused in frame to a portion of the first nucleic acid fragment that encodes an intra-membrane region of the IMP; wherein a poxvirus infected cell comprising the polynucleotide can express an IMP-FPV108 or IMP-RBXV041 fusion protein as part of the outer envelope membrane of an extracellular enveloped virion (EEV).
2. The polynucleotide of claim 1, wherein the second nucleic acid encodes FPV108 protein comprising the amino acid sequence SEQ ID NO: 2 or a functional fragment thereof.
3. The polynucleotide of claim 1, wherein the second nucleic acid encodes RBXV041 protein comprising the amino acid sequence SEQ ID NO: 3 or a functional fragment thereof.
4. The polynucleotide of claim 1, wherein the IMP is a multi-pass membrane protein comprising at least two transmembrane domains.
5. The polynucleotide of claim 4, wherein the IMP has an odd number of transmembrane domains, wherein the 5' end of the first nucleic acid fragment encodes an extra-membrane region, wherein the 3' end of the first nucleic acid fragment encodes an intra-membrane region, and wherein the 5' end of the second polynucleotide is fused to the 3' end of the first nucleic acid fragment.
6. The polynucleotide of claim 5, wherein the IMP comprises a G-protein coupled receptor (GPCR).

7. The polynucleotide of claim 5, wherein the IMP is the human frizzled-4 protein (FZD4) or a CXC chemokine receptor (CXCR), or a fragment thereof.

~~8. The polynucleotide of claim 5, wherein the IMP is a CXC chemokine receptor CXCR, or a fragment thereof.~~

~~98. The polynucleotide of claim 87, wherein the IMP is the CXC chemokine receptor ~~is~~ CXCR4, or a fragment thereof.~~

~~109. The polynucleotide of claim 4, wherein the IMP has an even number of transmembrane domains, and wherein both the 5' and 3' ends of the first nucleic acid fragment encode intra-membrane regions, and wherein the second nucleic acid fragment is fused to 3' end of the first nucleic acid fragment.~~

~~110. The polynucleotide of claim 109, wherein the IMP is human CD20 or CD39 protein, or a fragment thereof.~~

~~121. The polynucleotide of any one of claims 1 to 110, which is operably associated with a poxvirus promoter.~~

~~132. The polynucleotide of any one of claims 1 to 121, wherein the first and second nucleic acid fragments are directly fused.~~

~~143. The polynucleotide of any one of claims 1 to 132, further comprising a third nucleic acid fragment encoding a heterologous peptide.~~

~~154. The polynucleotide of claim 143, wherein the heterologous peptide comprises a linker sequence, an amino acid tag or label, or a peptide or polypeptide sequence that facilitates purification.~~

~~16. The polynucleotide of claim 15, wherein the heterologous peptide comprises a histidine tag.~~

~~17~~15. The polynucleotide of any one of claims 1 to ~~16~~14, which is operably associated with a poxvirus promoter.

~~18~~16. The polynucleotide of claim ~~17~~15, wherein the poxvirus promoter is p7.5 or H5 or T7.

~~19~~17. An FPX108 fusion protein encoded by the polynucleotide of any one of claims 1 to ~~18~~16.

~~20~~18. An RBXV041 fusion protein encoded by the polynucleotide of any one of claims 1 to ~~18~~16.

~~21~~19. A poxvirus genome comprising the polynucleotide of any one of claims 1 to ~~18~~16.

~~22~~20. The poxvirus genome of claim ~~21~~19, which is a vaccinia virus genome.

~~23~~21. The poxvirus genome of claim ~~21~~19, which is a fowlpox virus genome.

~~24~~22. The poxvirus genome of claim ~~21~~19, which is a rabbit pox virus genome.

~~25~~23. A recombinant poxvirus EEV comprising the poxvirus genome of any one of claims ~~22~~20 to ~~22~~22.

~~26~~24. An *in vitro* method of producing the recombinant poxvirus EEV of claim ~~25~~23, comprising: (a) infecting a host cell permissive for vaccinia virus, fowlpox virus, or rabbit pox virus infectivity with a vaccinia virus, fowlpox virus or rabbit pox virus, respectively, wherein the vaccinia virus comprises the poxvirus genome of claim ~~22~~20, the fowlpox virus comprises the poxvirus genome of claim ~~23~~21, and the rabbit pox virus comprises the poxvirus genome of claim ~~24~~22; and (b) recovering EEV released from the host cell.

~~27~~25. An *in vitro* method to display an integral membrane protein (IMP) or fragment thereof in a

native conformation comprising: (a) infecting host cells permissive for poxvirus infectivity with a recombinant poxvirus that expresses the IMP or fragment thereof as a fusion protein with the EEV-specific protein FPV108 or RBX041 or a membrane-associated fragment thereof, wherein EEV produced by the infected host cell comprise the IMP fusion protein as part of the EEV outer envelope membrane; (b) recovering EEV released from the host cell wherein the IMP or fragment thereof displays on the surface of the EEV in a native conformation.

2826. The *in vitro* method of claim 2725, wherein the EEV-specific protein is FPV108 or a functional fragment thereof.

2927. The *in vitro* method of claim 2725, wherein the EEV-specific protein is RBXV041 or a functional fragment thereof.

3028. The *in vitro* method of any one of claims 2725 to 2927, wherein the IMP is a multi-pass membrane protein comprising at least two transmembrane domains.

3129. The *in vitro* method of claim 3028, wherein the IMP comprises a G-protein coupled receptor (GPCR) comprising seven transmembrane domains, and wherein FPV108 or RBX041 is fused to the C-terminus of the IMP.

3230. The *in vitro* method of claim 3129, wherein the IMP is the human frizzled-4 protein (FZD4), a CXC chemokine receptor or a fragment thereof.

~~33. The method of claim 30, wherein the IMP is a CXC chemokine receptor.~~

3431. The *in vitro* method of claim 3330, wherein the IMP is the CXC chemokine receptor is CXCR4, or a fragment thereof.

3532. The *in vitro* method of any one of claims 2725 to 2927, wherein the IMP or fragment thereof has an even number of transmembrane domains, and wherein both the N-terminus and the C-terminus of the IMP or fragment thereof are intra-membrane.

~~36~~33. The *in vitro* method of claim ~~35~~32, wherein FPV108 or RBX041 is fused to the C-terminus of the IMP.

~~37~~34. The *in vitro* method of claim ~~36~~33, wherein the IMP is human CD20, or a fragment thereof.

~~38~~35. A fusion protein comprising: (a) the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11, wherein the fusion protein, when expressed by a recombinant poxvirus, appears on the surface of a poxvirus extracellular enveloped virion (EEV) in a native conformation.

~~39~~36. A recombinant poxvirus EEV comprising the fusion protein of claim ~~38~~35.

~~40~~37. A recombinant poxvirus EEV comprising a heterologous IMP or fragment thereof fused to a fowlpox virus or rabbit pox virus EEV-specific protein or membrane-associated fragment thereof, wherein the fusion protein is situated in the EEV outer envelope membrane, wherein the IMP or fragment thereof displays on the surface of the EEV in its native conformation.

~~41~~38. The recombinant poxvirus EEV of claim ~~40~~37, wherein the poxvirus EEV is a fowlpox virus EEV.

~~42~~39. A method to select antibodies that bind to a multi-pass membrane protein (IMP) comprising: (a) attaching the recombinant EEV of any one of claims ~~25, 39, 40 or 41~~23, 36, 37, or 38 to a solid support; (b) providing an antibody display library, wherein the library comprises display packages displaying a plurality of antigen binding domains; (c) contacting the display library with the EEV such that display packages displaying antigen binding domains that specifically binds to the IMP expressed on the EEV can bind thereto; (d) removing unbound display packages; and (e) recovering display packages that display an antigen binding domain specific for the IMP expressed on the EEV.

~~43. The method of claim 42 wherein the recombinant EEV are inactivated prior to attachment to the solid support.~~

~~44. The method of claim 43, wherein the EEV are inactivated by incubation with Psoralen (Trioxsalen, 4'-aminomethyl-, hydrochloride) in the presence of UV irradiation.~~

~~45. The method of any one of claims 42 to 44, wherein the EEV are attached to the solid surface via reaction with tosyl groups attached to the surface.~~

~~46. The method of claim 45, wherein the solid surface is tosyl-activated magnetic beads.~~

~~47. The method of any one of claims 42 to 44, wherein the EEV are biotinylated and attached to a streptavidin-coated solid surface.~~

~~48. The method of claim 47, wherein the solid surface is streptavidin-coated magnetic beads.~~

4940. A method to select antibodies that bind to a multi-pass membrane protein (IMP) comprising: (a) providing a first and second recombinant poxvirus EEV of claim 2523, wherein the first and second recombinant poxvirus EEV are each generated in a different poxvirus; ~~(b) immunizing wherein an animal is to be immunized~~ with the first recombinant poxvirus; (b) contacting a display library that comprises display packages displaying a plurality of antigen binding domains with the second recombinant poxvirus such that the display packages displaying antigen binding domains that specifically bind to the IMP expressed on the EEV can bind thereto, wherein said display library is generated from B cells which are to be isolated from the immunized ~~mammal~~ animal; (c) removing unbound display packages; and (d) recovering display packages that display an antigen binding domain specific for the IMP expressed on the second recombinant EEV.

5041. The method of claim 4940, wherein the first recombinant poxvirus EEV is a vaccinia virus EEV or a fowlpox virus EEV.

51. The method of claim 50, wherein the second recombinant poxvirus EEV is a fowlpox virus EEV.

5242. A method to select antibodies or antigen-binding fragments thereof that bind to a multi-pass membrane protein (IMP), which comprises: (a) providing a recombinant poxvirus EEV of claim 2523; ~~(b), wherein an animal is to be immunized~~ immunizing a mammal with the recombinant poxvirus EEV; ~~(c) wherein optionally immunizing the mammal~~ the animal is to be immunized with a second dose of the recombinant poxvirus EEV; ~~(d)~~ (b) isolating serum from the immunized animal; and ~~(e)~~ (c) isolating antibodies or antigen-binding fragments thereof that comprise an antigen binding domain specific for the IMP expressed on the recombinant poxvirus EEV.