



(43) International Publication Date
1 October 2015 (01.10.2015)

- (51) International Patent Classification:
C07K 14/525 (2006.01)
- (21) International Application Number:
PCT/US2015/021545
- (22) International Filing Date:
19 March 2015 (19.03.2015)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/969,582 24 March 2014 (24.03.2014) US
- (71) Applicant: **MEDIMMUNE, LLC** [US/US]; One
MedImmune Way, Gaithersburg, MD 20878 (US).
- (72) Inventor: **GILBRETH, Ryan**; C/o MedImmune, Llc, C/o
MedImmune Way, Gaithersburg, MD 20878 (US).
- (74) Agents: **MUKHERJEE, MITA** et al.; c/o MedImmune,
LLC, One MedImmune Way, Gaithersburg, MD 20878
(US).
- (81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,

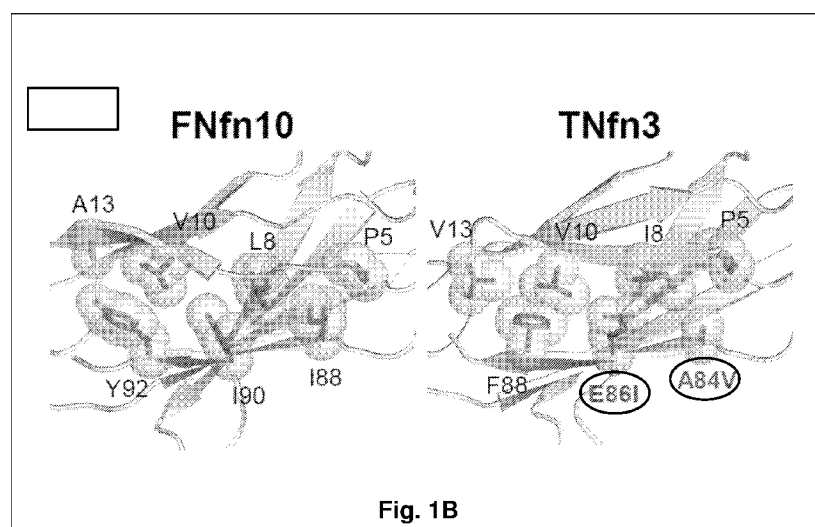
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- *without international search report and to be republished
upon receipt of that report (Rule 48.2(g))*

(54) Title: STABILIZED TNFN3 SCAFFOLD PROTEINS



(57) Abstract: Stabilized, target antigen-binding scaffold proteins derived from the third fibronectin domain of human tenascin C are provided.

STABILIZED TNFN3 SCAFFOLD PROTEINS

BACKGROUND

[0001] Biomolecules capable of specific binding to a desired target epitope are of great importance as therapeutics, research, and medical diagnostic tools. A well known example of this class of molecules is the antibody. Antibodies can be selected that bind specifically and with affinity to almost any structural epitope. However, classical antibodies are structurally complex heterotetrameric molecules with are difficult to express in simple eukaryotic systems. As a result, most antibodies are produced using complex and expensive mammalian cell expression systems.

[0002] Proteins having relatively defined three-dimensional structures, commonly referred to as protein scaffolds, may be used as reagents for the design of engineered products. These scaffolds typically contain one or more regions which are amenable to specific or random sequence variation, and such sequence randomization is often carried out to produce libraries of proteins from which desired products may be selected.

[0003] One particular area in which such scaffolds are useful is the field of antibody mimetic design. Antibody mimetics, *i.e.*, small, non-antibody protein therapeutics, capitalize on the advantages of antibodies and antibody fragments, such as high affinity binding of targets and low immunogenicity and toxicity, while avoiding some of the shortfalls, such as the tendency for antibody fragments to aggregate and be less stable than full-length IgGs.

[0004] One example of an scaffold-based antibody mimetic is based on the structure of a fibronectin module of type III (FnIII), a domain found widely across phyla and protein classes, such as in mammalian blood and structural proteins. The FnIII domain occurs often in various proteins, including fibronectins, tenascin, intracellular cytoskeletal proteins, cytokine receptors and prokaryotic enzymes (Bork and Doolittle, Proc. Natl. Acad. Sci. USA 89:8990-8894, 1992; Bork *et al.*, Nature Biotechnol. 15:553-557, 1997; Meinke *et al.*, J. Bacteriol. 175:1910-1918, 1993; Watanabe *et al.*, J. Biol. Chem. 265:15659-15665, 1990). PCT Publication No: WO 2009/058379 describes scaffolds based on the FnIII domain, in particular, the third FnIII domain of human tenascin C. Additional scaffolds based on the FnIII domain are described in PCT Publication No: WO 2011/130324, and in PCT

Publication No: WO 2011/130328. FnIII domains comprise seven beta strands, designated N-terminus to C-terminus A, B, C, D, E, F, and G strands, each strand separated by a loop region wherein the loop regions are designated N-terminus to C-terminus, AB, BC, CD, DE, EF, and FG loops. Although the FnIII domain is not an immunoglobulin, the overall fold of the third FnIII domain of human tenascin C domain is closely related to that of the smallest functional antibody fragment, the variable region of the heavy chain, which comprises the entire antigen recognition unit in camel and llama IgG. This makes it possible to display the three fibronectin loops on each opposite side of a FnIII domain, *e.g.*, the third FnIII domain of human tenascin C in relative orientations similar to those of CDRs in native antibodies.

[0005] The folding and stability of TNfn3 have been well-studied and some efforts to increase its stability have also been reported. Strickler *et al* used a computational algorithm to identify mutations that would stabilize TNfn3 by optimizing charge interactions on the protein surface (Strickler *et al.*, *Biochemistry*, 45:2761-2766 (2006)). From this approach the authors identified a quadruple mutant (Q7K, L19K, D49K, T89K) that exhibited an improved T_m of 66 °C. In other computational work, the entire TNfn3 sequence was redesigned to optimize it for the native backbone structure. These efforts also successfully yielded stabilized variants with T_m of 80-90 °C and $\Delta G_{\text{Unfolding}} = 8.7 - 11.9$ kcal/mol (Dantas *et al.* *J.Mol.Biol.* 332:449-460 (2003); Hu *et al.* *Structure* 16:1799-1805 (2008)). In additional related work, Jacobs *et al* used a consensus design strategy to generate highly stable FN3 domains (Jacobs *et al.* *Protein Eng.Des.Sel.* 25:107-117 (2012)). In this approach, a consensus sequence was derived from alignment of the 15 FN3 domains found in human tenascin-C. The tenascin consensus domain had significantly improved stability when compared to the natural FN3 domains from which it was derived ($T_m = 78$ °C, $\Delta G_{\text{Unfolding}} = 10.6$ kcal/mol) and this stability was further enhanced by additional point mutations ($T_m = 92.7$ °C, $\Delta G_{\text{Unfolding}} = 15.5$ kcal/mol).

[0006] Although effective, all of the approaches described above involved introducing several or many mutations at once. Thus, in all of these cases, the relative contributions of individual mutations remain unknown. As a result, it is unclear which mutations are required to maintain the enhanced stability and which mutations may be unnecessary. Because large portions of protein surface are often mutated when introducing novel binding

sites into small scaffolds such as TNFn3, understanding which positions must remain fixed to maintain enhanced stability can be advantageous. Furthermore, unnecessary mutations away from the wild-type human sequence could increase immunogenicity risks in cases where the variant is used as a scaffold in the development of therapeutic molecules.

[0007] Thus, there remains a need to engineer highly stable TNfn3 variants while also minimizing the number of mutations introduced, and having increased specificity, affinity, avidity, and stability for a variety of therapeutic and diagnostic applications, as well as screening methods for identifying such molecules.

[0008] Citation or discussion of a reference herein shall not be construed as an admission that such is prior art.

BRIEF SUMMARY

[0009] This disclosure provides a TNFn3 scaffold variant with high stability but with a minimum number of mutations. In certain aspects, a TNFn3 scaffold variant that includes the amino acid sequence: [A]-[ab]-[B]-[bc]-[C]-[cd]-[D]-[de]-[E]-[ef]-[F]-[fg]-[G], is provided, wherein [A], [B], [C], [D], [E], [F], and [G] are beta strands. In one embodiment, [A] is IEV (SEQ ID NO:6) or RLDAPX₇IEV, wherein X₇ is Q or K (SEQ ID NO:3); [B] is AX₁₉ITW, wherein X₁₉ is L or K (SEQ ID NO:7); [C] is X₃₂ELTYGI, wherein X₃₂ is I or F (SEQ ID NO:10); [D] is TTX₄₉L, wherein X₄₉ is D or K or N (SEQ ID NO:13); [E] is YSI (SEQ ID NO:16); [F] is YEVSLIS (SEQ ID NO:17); and [G] is KX₈₆TFX₈₉TX₉₁X₉₂, wherein X₈₆ is E or I or Q; X₈₉ is T or K; X₉₁ is any amino acid [G] or is deleted, and X₉₂ is any amino acid [L] or is deleted (SEQ ID NO:18). According to this embodiment, [ab], [bc], [cd], [de], [ef], and [fg] are variable loops each comprising at least 2 to no more than about 26 amino acids.

[0010] In certain embodiments, selected positions within the beta strands of the TNFn3 scaffold variant are randomized. In one such embodiment, [A] is IEV (SEQ ID NO:6) or RLDAPX₇IEV, wherein X₇ is Q or K (SEQ ID NO:3); [B] is AX₁₉ITW, wherein X₁₉ is L or K (SEQ ID NO:7); [C] is X₃₂X₃₃LTYGX₃₈, wherein X₃₂ is I or F, and X₃₃ and X₃₈ are independently any amino acid (SEQ ID NO:63); [D] is TX₄₇IX₄₉L, wherein X₄₇ and X₄₉ are independently any amino acid (SEQ ID NO:64); [E] is YSI (SEQ ID NO:16); [F] is

YEVSLIS (SEQ ID NO:17); and [G] is $KX_{86}TFX_{89}TX_{91}X_{92}$, wherein X_{86} is E or I or Q; X_{89} is T or K; X_{91} is any amino acid [G] or is deleted, and X_{92} is any amino acid [L] or is deleted (SEQ ID NO:18). According to this embodiment, [ab], [bc], [cd], [de], [ef], and [fg] are variable loops each comprising at least 2 to no more than about 26 amino acids. In another such embodiment, [A] is $RLDAPSX_7IX_9V$, wherein X_7 and X_9 are independently any amino acid (SEQ ID NO:65); [B] is $AX_{19}IX_{21}W$, wherein X_{19} and X_{49} are independently any amino acid (SEQ ID NO:66); [C] is $X_{32}ELTYGI$, wherein X_{32} is I or F (SEQ ID NO:10); [D] is $TTIX_{49}L$, wherein X_{49} is D or K or N (SEQ ID NO:13); [E] is $YX_{58}I$, wherein X_{58} is any amino acid (SEQ ID NO:68); [F] is YEVSLIS (SEQ ID NO:17); and [G] is $KX_{86}TFX_{89}TX_{91}X_{92}$, wherein X_{86} is E or I or Q; X_{89} is T or K; X_{91} is any amino acid [G] or is deleted, and X_{92} is any amino acid [L] or is deleted (SEQ ID NO:18). According to this embodiment, [ab], [bc], [cd], [de], [ef], and [fg] are variable loops each comprising at least 2 to no more than about 26 amino acids.

[0011] In certain aspects the TNFn3 scaffold variant amino acid sequence is not:

$RLDAPSKIEV[ab]AKITW[bc]IELTYGI[cd]TTIKL[de]YSI[ef]YEVSLIS[fg]KETFKTGA$
 ([SEQ ID NO:5]-[ab]-[SEQ ID NO:9]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:15]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:59]);

$RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KETFTTGL$
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

$IEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KETFTTGL$ ([SEQ ID
 NO:6]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-[SEQ ID
 NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

$RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KITFTTGL$
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:60]);

$RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTINL[de]YSI[ef]YEVSLIS[fg]KETFTTGL$
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:31]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KQTFTTGL
 ([SEQ ID NO:6]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:61]); or
 RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTINL[de]YSI[ef]YEVSLIS[fg]KETFTTGL
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:31]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:61]).

[0012] In certain aspects, a TNFn3 scaffold variant is provided in which beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is IELTYGI (SEQ ID NO:11), beta strand [D] is TTIDL (SEQ ID NO:14), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

[0013] In certain aspects, a TNFn3 scaffold variant is provided in which beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is IELTYGI (SEQ ID NO:11), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

[0014] In certain aspects, a TNFn3 scaffold variant is provided in which beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

[0015] In certain aspects loop [ab] comprises KDVTDDT (SEQ ID NO:23), or SEQ ID NO:23 with at least one, two, three, four, five, six, or seven amino acid substitutions, insertions, or deletions; loop [bc] comprises FKPLAEIDG (SEQ ID NO:24), or SEQ ID NO:24 with at least one, two, three, four, five, six, seven, eight, or nine amino acid substitutions, insertions, or deletions; loop [cd] comprises KDVPGR (SEQ ID NO:25), or SEQ ID NO:25 with at least one, two, three, four, five, six, or seven amino acid substitutions, insertions, or deletions; loop [de] comprises TEDENQ (SEQ ID NO:26), or SEQ ID NO:26 with at least one, two, three, four, five, or six, amino acid substitutions, insertions, or deletions; loop [ef] comprises GNLKPDTE (SEQ ID NO:27), or SEQ ID NO:27 with at least one, two, three, four, five, six, seven, or eight amino acid substitutions, insertions, or deletions; loop [fg] comprises $RX_{76}X_{77}X_{78}MSSNPX_{84}$ (SEQ ID NO:28), wherein X_{76} is any amino acid or is deleted [R]; X_{77} is any amino acid or is deleted [G]; X_{78}

is any amino acid or is deleted [D]; and X_{84} is A or V, or SEQ ID NO:28 with at least one, two, three, four, five, or six additional amino acid substitutions, insertions, or deletions; or any combination of loop sequences as described above.

[0016] In certain aspects, a TNFn3 scaffold variant is provided, which binds to a target antigen, e.g., with an affinity (K_D) of at least about 100 μ M. The target antigen can be, for example, a cell-surface antigen, a soluble antigen, an immobilized antigen, an immunosilent antigen, an intracellular antigen, an intranuclear antigen, a self antigen, a non-self antigen, a cancer antigen, a bacterial antigen, a viral antigen, or any combination thereof.

[0017] In certain aspects, a TNFn3 scaffold variant is provided, which exhibits enhanced stability, as measured by thermal melting temperature (T_m). In certain aspects the T_m of a TNFn3 scaffold variant provided herein is at least about 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C, as measured by differential scanning calorimetry (DSC) in 10 mM potassium phosphate, 50 mM sodium chloride, pH 7.4. In certain aspects the TNFn3 scaffold variant exhibits a T_m that is increased relative to the T_m observed for a TNFn3 scaffold protein consisting of SEQ ID NO:2 by least about 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or 30°C, as measured by differential scanning calorimetry (DSC).

[0018] In certain aspects, a TNFn3 scaffold variant as provided herein can be conjugated to a heterologous agent, e.g., a heterologous scaffold, a protein, a peptide, a protein domain, a linker, a drug, a toxin, a cytotoxic agent, an imaging agent, a radionuclide, a radioactive compound, an organic polymer, an inorganic polymer, polyethylene glycol (PEG), biotin, human serum albumin (HSA), a HSA FcRn binding portion, an antibody, a domain of an antibody, an antibody fragment, a single chain antibody, an albumin binding domain, an enzyme, a ligand, a receptor, a binding peptide, an epitope tag, a recombinant polypeptide polymer, a cytokine, or a combination of two or more of the heterologous agents. In certain aspects, a TNFn3 scaffold variant as provided herein can further comprise a heterologous epitope binding domain. In certain aspects, the heterologous epitope binding domain can be specific for a different antigen target, or different epitope of the same antigen target, than the TNFn3 scaffold variant.

- [0019] This disclosure further provides a multimeric scaffold comprising at least two TNFn3 scaffold variants as provided herein, or at least one TNFn3 scaffold variant of any one of claims 1 to 62 and a heterologous scaffold. In certain aspects a multimeric scaffold as provided herein can further comprise a heterologous epitope binding domain as described above. In certain aspects a multimeric scaffold is provided in which at least two TNFn3 scaffold variants are linked by another scaffold, an IgG molecule or fragment thereof, an Fc region, a dimerization domain, a chemical crosslinker, a disulfide bond, or an amino acid linker.
- [0020] This disclosure further provides an isolated polynucleotide comprising a nucleic acid molecule encoding a TNFn3 scaffold variant as provided herein, or a multimeric scaffold as provided herein. Also provided is a vector and a host cell comprising the isolated polynucleotide. The disclosure further provides a method of producing a TNFn3 scaffold variant or a multimeric scaffold comprising a TNFn3 scaffold variant comprising: culturing a host cell as provided herein under conditions in which a TNFn3 scaffold variant or a multimeric scaffold comprising the TNFn3 scaffold variant encoded by the polynucleotide is expressed, and recovering the TNFn3 scaffold variant or the multimeric scaffold comprising the TNFn3 scaffold variant.
- [0021] The disclosure further provides a composition, e.g., a pharmaceutical composition, comprising a TNFn3 scaffold variant as provided herein or a multimeric scaffold as provided herein, and a carrier or excipient.
- [0022] In another aspect, the disclosure provides a method of preventing, treating, managing, or ameliorating a disease or disorder in a subject comprising administering to a subject in need thereof an effective amount of a composition as provided herein. In certain aspects the disease is an autoimmune disease, an inflammatory disease, a proliferative disease, an infectious disease, a respiratory disease, a gastrointestinal disease, diabetes, lupus, or obesity.
- [0023] The disclosure further provides diagnostic methods and antigen capture methods.
- [0024] In certain aspects, the disclosure provides a method of constructing a binding molecule display library comprising: (a) providing a polynucleotide encoding the TNFn3 scaffold variant of any one of claims 1 to 62 or a polynucleotide encoding a multimeric

scaffold of any one of claims 63 to 68; (b) introducing random deletions or randomizing codon substitutions or insertions into the polynucleotide at selected positions in a region of the polynucleotide encoding at least one variable loop [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant; and (c) propagating copies of the polynucleotide to form the display library.

[0025] A polypeptide display library produced by this method is also provided. The TNFn3 scaffold variants of the library can include at least two variable loops [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant that are randomized by deletion, substitution, or addition of at least one amino acid. In certain aspects, two variable loops selected from [ab], [cd], and [ef], are randomized. In certain aspects, two variable loops selected from [bc], [de], and [fg] are randomized. In certain aspects at least three variable loops [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant are randomized by deletion, substitution, or addition of at least one amino acid. In certain aspects, variable loops [ab], [cd], and [ef] are randomized. In certain aspects, variable loops [bc], [de], and [fg] are randomized.

[0026] In certain aspects, the disclosure provides a method of constructing a binding molecule display library comprising: (a) providing a polynucleotide encoding the TNFn3 scaffold variant of any one of SEQ ID NOs: 69-71; (b) introducing randomizing codon substitutions or insertions into the polynucleotide at each position indicated with an "X"; and (c) propagating copies of the polynucleotide to form the display library.

[0027] A library as provided herein can be displayed on the surface of a ribosome, bacteriophage, virus, bacteria, or yeast and can, in certain aspects, have a sequence diversity of at least 10^6 . The disclosure further provides a collection of isolated polynucleotides encoding the library provided herein, and a plurality of expression vectors comprising such a collection polynucleotides.

[0028] The disclosure further provides a method of obtaining a binding molecule specific for a target antigen of interest, comprising: (a) contacting a target antigen with a library as provided herein under conditions that allow a scaffold-target antigen complex to form, and (b) recovering the scaffold that binds to the target antigen. In certain aspects the method can include further randomizing at least one variable loop or at least two or more variable loops

of the scaffold recovered in step (b) to generate a further randomized library and repeating steps (a) and (b) using the further randomized library.

[0029] In certain aspects, the variable loop that is randomized in the second operation was not randomized in the first operation of steps (a) and (b). The repetition of steps (a) and (b) can further comprises contacting a target antigen distinct from the target antigen of the first operation of step (a) and (b). The method can further comprise randomizing at least one beta strand of TNFn3 scaffold variant obtained in either the first or the second operation of step (b) to generate a further randomized library and repeating steps (a) and (b) using the further randomized library.

BRIEF DESCRIPTION OF THE FIGURES

[0030] **Figure 1 (A):** structure-based sequence alignment of TNfn3 and FNfn10. Regions corresponding to the seven β -strands of the FN3 fold are indicated with arrows and labeled A-G. Numbering is according to the TNfn3 sequence. Positions where FNfn10-inspired stabilizing mutations were introduced into TNfn3 are boxed. Positions of surface charge optimizing mutations are shaded gray. **Figure 1 (B):** comparison of A/G strand packing in FNfn10 and TNfn3. FNfn10 and TNfn3 are shown as gray cartoons with A/G strand residues shown as spheres/sticks. **Figure 1 (C):** comparison of W22 environments in FNfn10 and TNfn3. FNfn10 and TNfn3 are shown as gray cartoons with W22 and its local environment (residues having sidechain atoms within 5 Å of the W22 side chain) shown as sticks. W22 and the “capping” residues located at position 32 are indicated. In both panels B and C, positions where stabilizing mutations were introduced in TNfn3 are circled.

[0031] **Figure 2. (A)** Overlaid DSC curves for wild-type TNfn3, and CM4. **(B)** Guanidine thiocyanate denaturation curves for wild-type TNfn3 and CM4. **(C)** SDS-PAGE of wild-type TNfn3 and CM4 samples subjected to thermolysin treatment for varying periods of time.

[0032] **Figure 3.** Depicts the amino acid sequence of stabilized TNFn3. The positions which may be engineering for enhanced stability are designated as X₇, X₁₉, X₃₂, X₄₉, X₈₄, X₈₆, X₈₉, X₉₁, and X₉₂ wherein the number indicates the position within the scaffold as depicted. The positions of the loop regions are called out and the AB, CD and EF loops with

in the sequence are indicated with a single underline and the BC, DE and FG loops are double underlined. The integrin-binding “RDG” motif within the FG loop (at amino acid positions 76-78) is shown in lower case letters. It is contemplated that one or more residues within the RDG motif may be substituted or deleted with or without additional amino acid substitutions, insertions or deletions in the FG loop.

[0033] **Figure 4.** Depicts the amino acid sequence of the CM4 stabilized TNFn3 showing several different options for introducing randomization to generate libraries and screen for binding to desired targets. Panel A depicts particular sites within the loop which may be randomized (SEQ ID NO: 69). Panel B depicts sites within the concave portion of the scaffold (including sites within certain the C and D beta strands) which may be randomized (SEQ ID NO: 70). Panel C depicts sites with the flat portion of the scaffold (including sites within the A, B and E beta strands) which may be randomized. For the Flat randomization is specifically contemplated that one or more residues within the RDG motif loop (at amino acid positions 76-78) may be substituted or deleted (SEQ ID NO: 71). Each X may independently be any amino acid, however in certain embodiments is not C, E, F, H, I, K, M or Q; “m” is 1-4; “n” is 0-5; “z” is 0 or 1.

DETAILED DESCRIPTION

Definitions

[0034] This embodiments and claims provided in this disclosure are not limited to specific compositions or process steps, as such can vary. As used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the context clearly dictates otherwise. The terms “a” (or “an”), as well as the terms “one or more,” and “at least one” can be used interchangeably herein.

[0035] Furthermore, “and/or” where used herein encompasses the specific disclosure of each of the two specified features or components with or without the other. Thus, the term “and/or” as used in a phrase such as “A and/or B” herein is intended to include “A and B,” “A or B,” “A” (alone), and “B” (alone). Likewise, the term “and/or” as used in a phrase such as “A, B, and/or C” is intended to encompass each of the following embodiments: A, B, and

C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0036] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is directed. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0037] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or embodiments, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0038] Wherever embodiments or aspects are described with the language “comprising,” otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided.

[0039] Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, are referred to by their commonly accepted single-letter codes.

[0040] The term “epitope” as used herein refers to a determinant capable of specifically bound by a scaffold as provided herein. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

[0041] The term “target antigen” refers to a molecule that comprises one or more epitopes as described above. A target antigen can be a polypeptide, a nucleic acid, a polysaccharide, a lipid, or other structure.

[0042] The terms “fibronectin type III (FnIII) domain,” “FnIII domain” or “Fn3 domain” refer to polypeptides homologous to the human fibronectin type III domain having at least 7 beta strands which are distributed between two beta sheets, which themselves pack against each other to form the core of the protein, and further containing solvent exposed loops which connect the beta strands to each other. There are at least three such loops at each edge of the beta sheet sandwich, where the edge is the boundary of the protein perpendicular to the direction of the beta strands. In certain embodiments, an FnIII domain comprises 7 beta strands designated A, B, C, D, E, F, and G linked to six loop regions designated AB, BC, CD, DE, EF, and FG, wherein a loop region connects each beta strand. The loop regions are also referred to herein as structures [ab], [bc], [cd], [de], [ef], and [fg], each comprising at least 2 to no more than about 26 amino acids. The terms “fibronectin type III (FnIII) domain,” “FnIII domain” or “Fn3 domain” also comprise protein domains recognized to contain the Interpro IPR008957 fibronectin type III domain signature as determined using the InterProScan program, or recognized to contain the Pfam PF00041 fibronectin type III domain signature as determined using Pfam_scan, HMMER, or any other program known in the art capable of comparing a protein sequence to a Hidden Markov model describing an FnIII domain. In addition, the terms include functional fragments and engineered FnIII domains, *e.g.*, core-engineered FnIII domains (see, *e.g.*, Ng *et al.*, Nanotechnology 19: 384023, 2008).

[0043] This disclosure refers in particular to the third FnIII domain of human tenascin C, referred to herein as a “TNFn3 domain.”

[0044] The terms “Fibronectin type III (FnIII) scaffold,” “FnIII scaffold,” or “Fn3 scaffold” refer to a polypeptide comprising an Fn3 domain, or functional fragment thereof, wherein at least one loop is a non-naturally occurring variant of a Fn3 domain/scaffold of interest, and wherein the Fn3 scaffold, or functional fragment thereof is capable of specifically binding to an epitope on a target antigen. This disclosure refers in particular to scaffolds based on the third FnIII domain of human tenascin C. Such scaffolds are referred to herein as “TNFn3

scaffold variants.” A “non-naturally occurring variant” can vary by deletion, substitution or addition by at least one amino acid from the cognate sequences in a starting protein sequence (*e.g.*, an FnIII domain/scaffold such as a TNFn3 domain), which can be a native FnIII domain sequence or a previously identified FnIII scaffold sequence. In certain embodiments, the A beta strand is truncated, for example one or more N-terminal residues of the A beta strand can be absent. In certain embodiments, the G beta strand is truncated, for example one or more C-terminal residues of the G beta strand may be absent. In various embodiments provided herein, a TNFn3 scaffold variant comprises non-naturally occurring variants of one or more beta strands. In certain embodiments, the beta strands of the TNFn3 scaffold variants provided herein comprise one or more beta strand amino acid sequences presented as SEQ ID Nos. 3 to 22.

[0045] The term “fusion protein” as used in reference to a TNFn3 scaffold variant provided herein or a multimeric scaffold comprising a TNFn3 scaffold variant provided herein refers to protein that includes (i) one or more TNFn3 scaffold variants provided herein joined to (ii) a second, different protein (*i.e.*, a “heterologous” protein).

[0046] The term “heterologous moiety” is used herein to indicate the addition of a composition to a TNFn3 scaffold variant provided herein or a multimeric scaffold comprising a TNFn3 scaffold variant provided herein, wherein the composition is not normally part of an FnIII domain. Exemplary heterologous moieties include proteins, peptides, protein domains, linkers, drugs, toxins, imaging agents, radioactive compounds, organic and inorganic polymers, and any other compositions which might provide an activity that is not inherent in the TNFn3 domain itself, including, but are not limited to, polyethylene glycol (PEG), a cytotoxic agent, a radionuclide, an imaging agent, biotin, a dimerization domain (*e.g.* leucine zipper domain), human serum albumin (HSA) or an FcRn binding portion thereof, a domain or fragment of an antibody (*e.g.*, antibody variable domain, a CH1 domain, a Ckappa domain, a Clambda domain, a CH2, or a CH3 domain), a single chain antibody, an albumin binding domain, an IgG molecule, an enzyme, a ligand, a receptor, a binding peptide, a non-FnIII scaffold, an epitope tag, a recombinant polypeptide polymer, a cytokine, any related moieties, and any combination of such moieties.

- [0047] The term “linker” as used herein refers to any molecular assembly that joins or connects a TNFn3 scaffold variant provided herein to a heterologous moiety, or joins or connects two or more TNFn3 scaffold variants. The linker can be a molecule whose function is to act as a “spacer” between modules in a scaffold, or it can also be a molecule with additional function (*i.e.*, a “functional moiety”). A molecule included in the definition of “heterologous moiety” can also function as a linker.
- [0048] The terms “linked” and “fused” are used interchangeably. These terms refer to the joining together of two or more scaffolds, heterologous moieties, or linkers by whatever means including chemical conjugation or recombinant means.
- [0049] The terms “multimer,” “multimeric scaffold” and “multivalent scaffold” refer to a molecule that comprises at least two FnIII scaffolds in association. The scaffolds forming a multimeric scaffold can be linked through a linker that permits each scaffold to function independently. “Multimeric” and “multivalent” can be used interchangeably herein. A multivalent scaffold can be monospecific or bispecific.
- [0050] The terms “domain” or “protein domain” refer to a region of a protein that can fold into a stable three-dimensional structure, often independently of the rest of the protein, and which can be endowed with a particular function. This structure maintains a specific function associated with the domain's function within the original protein, *e.g.*, enzymatic activity, creation of a recognition motif for another molecule, or to provide necessary structural components for a protein to exist in a particular environment of proteins. Both within a protein family and within related protein superfamilies, protein domains can be evolutionarily conserved regions. When describing the components of a TNFn3 scaffold variant provided herein or a multimeric scaffold comprising a TNFn3 scaffold variant provided herein, the terms “domain,” “monomeric scaffold,” and “module” can be used to refer to a single Fn3 scaffold, *e.g.*, a TNFn3 scaffold variant provided herein, or a subregion thereof, *e.g.*, a beta strand or a loop region.. By “native FnIII domain” is meant any non-recombinant FnIII domain that is encoded by a living organism.
- [0051] The term “sequence homology” in relation to protein sequences refers to the similarity between two or more protein sequences, *i.e.*, the percentage of amino acid residues that are either identical or conservative amino acid substitutions.

- [0052] The terms “Percent (%) sequence similarity” and “Percent (%) homology” as used herein are considered equivalent and are defined as the percentage of amino acid residues in a candidate sequence that are identical with or conservative substitutions of the amino acid residues in a selected sequence, after aligning the amino acid sequences and introducing gaps in the candidate and/or selected sequences, if necessary, to achieve the maximum percent sequence similarity.
- [0053] “Percent (%) identity” is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a selected sequence, after aligning the sequences and introducing gaps in the candidate and/or selected sequence, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative amino acid substitutions as part of the sequence identity.
- [0054] The term “conservative substitution” as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic amino acid residue such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine, norleucine, or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine and vice versa, of glutamic acid for aspartic acid, and vice versa, glutamine for asparagine, and vice versa, and the like. Neutral hydrophilic amino acids which can be substituted for one another include asparagine, glutamine, serine and threonine. The term “conservative substitution” also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that the biologic activity of the peptide is maintained. Biological similarity between amino acid residues refers to similarities between properties such as, but not limited to, hydrophobicity, mutation frequency, charge, side chain length, size chain volume, pKa, polarity, aromaticity, solubility, surface area, peptide bond geometry, secondary structure propensity, average solvent accessibility, etc.
- [0055] Alignment for purposes of determining percent homology (*i.e.*, sequence similarity) or percent identity can be achieved in various ways that are within the skill in the art, for instance, using publicly or proprietary algorithms. For instance, sequence similarity can be determined using pairwise alignment methods, *e.g.*, BLAST, BLAST-2, ALIGN, or ALIGN-

2 or multiple sequence alignment methods such as Megalign (DNASTAR), ClustalW or T-Coffee software. Those skilled in the art can determine appropriate scoring functions, *e.g.*, gap penalties or scoring matrices for measuring alignment, including any algorithms needed to achieve optimal alignment quality over the full-length of the sequences being compared. Furthermore, those skilled in the art would appreciate that methods to identify proteins with a certain fold, *e.g.*, the FnIII fold, and to align the amino acid sequences of such proteins, include sequence-sequence methods, sequence-profile methods, and profile-profile methods. In addition, sequence alignment can be achieved using structural alignment methods (*e.g.*, methods using secondary or tertiary structure information to align two or more sequences), or hybrid methods combining sequence, structural, and phylogenetic information to identify and optimally align candidate protein sequences.

[0056] A “protein sequence” or “amino acid sequence” means a linear representation of the amino acid constituents in a polypeptide in an amino-terminal to carboxyl-terminal direction in which residues that neighbor each other in the representation are contiguous in the primary structure of the polypeptide.

[0057] The term “nucleic acid” refers to any two or more covalently bonded nucleotides or nucleotide analogs or derivatives. As used herein, this term includes, without limitation, DNA, RNA, and PNA. “Nucleic acid” and “polynucleotide” are used interchangeably herein.

[0058] The term “polynucleotide” is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA) or plasmid DNA (pDNA). The term “isolated” nucleic acid or polynucleotide is intended refers to a nucleic acid molecule, DNA or RNA, that has been removed from its native environment. For example, a recombinant polynucleotide encoding , *e.g.*, a TNFn3 scaffold variant contained in a vector is considered isolated. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides provided herein. Isolated polynucleotides or nucleic acids provided herein further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid can be

or can include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

[0059] The term “DNA” refers to a sequence of two or more covalently bonded, naturally occurring or modified deoxyribonucleotides.

[0060] By “RNA” is meant a sequence of two or more covalently bonded, naturally occurring or modified ribonucleotides. One example of a modified RNA included within this term is phosphorothioate RNA.

[0061] The term “pharmaceutically acceptable” refers to a compound or protein that can be administered to an animal (for example, a mammal) without significant adverse medical consequences.

[0062] The term “pharmaceutically acceptable carrier” refers to a carrier which does not have a significant detrimental impact on the treated host and which retains the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other pharmaceutically acceptable carriers and their formulations are known to one skilled in the art and are described, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, Pa., incorporated herein by reference.

[0063] By a “polypeptide” is meant any sequence of two or more amino acids linearly linked by amide bonds (peptide bonds) regardless of length, post-translation modification, or function. “Polypeptide,” “peptide,” and “protein” are used interchangeably herein. Thus, peptides, dipeptides, tripeptides, or oligopeptides are included within the definition of “polypeptide,” and the term “polypeptide” can be used instead of, or interchangeably with any of these terms. The term “polypeptide” is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide can be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. A polypeptide can be generated in any manner, including by chemical synthesis.

[0064] Also included as polypeptides in this disclosure are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. Variants can occur naturally or be non-naturally occurring. Non-naturally occurring variants can be produced using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions, or additions. Also included as “derivatives” are those peptides that contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids.

[0065] The term “derived from [*e.g.*, a protein or a polynucleotide]” means that a protein or polynucleotide is related to a reference protein or polynucleotide. The relation can be, for example, one of sequence or structural similarity. A protein or polynucleotide can be derived from a reference protein or polynucleotide via one or more of, *e.g.*, mutation (*e.g.*, deletion or substitution), chemical manipulation (*e.g.*, chemical conjugation of a scaffold to PEG or to another protein), genetic fusion (*e.g.*, genetic fusion of two or more scaffolds to a linker, a heterologous moiety, or combinations thereof), *de novo* synthesis based on sequence or structural similarity, or recombinant production in a heterologous organism.

[0066] By “randomized,” *e.g.*, “randomized polypeptide” is meant a polypeptide derived from a reference polypeptide and comprising one or more amino acid alterations, including deletions, substitutions or additions, relative to a reference sequence. By “randomizing” is meant the process of introducing, into a sequence, such an amino acid sequence or nucleic acid sequence, an alteration. Randomization can be accomplished through intentional, blind, or spontaneous sequence variation, generally of a nucleic acid coding sequence, and can occur by any technique, for example, PCR, error-prone PCR, or chemical DNA synthesis. In certain aspects, a library of polypeptides comprising randomized amino acid sequences can be generated by introducing randomizing codons into a nucleotide sequence encoding the amino acid sequence. Randomizing codons take advantage of degeneracy in the genetic code and need not be completely random. Randomizing codons include, but are not limited to NNS and NNK, where N is any nucleotide, S is G or C and K is G or T. The terms “randomizing”, “randomized”, “mutating”, “mutated” and the like are used interchangeably herein.

- [0067] The terms “scaffold” or “scaffolds” as used herein, refers to multimeric scaffolds as well as monomeric FnIII scaffolds, *e.g.*, TNFn3 scaffold variants. The term “specificity” as used herein, *e.g.*, in the terms “specifically binds” or “specific binding,” refers to the relative affinity by which a TNFn3 scaffold variant as provided herein binds to one or more target antigens via one or more antigen binding domains, and that binding entails some complementarity between one or more antigen binding domains and one or more antigens. According to this definition, a scaffold as provided herein is said to “specifically bind” to an epitope when it binds to that epitope more readily than it would bind to a random, unrelated epitope.
- [0068] The term “affinity” as used herein refers to a measure of the strength of the binding of a certain a TNFn3 scaffold variant as provided herein to an individual epitope or target antigen.
- [0069] The term “avidity” as used herein refers to the overall stability of the complex between a population of scaffolds or a multimeric scaffold and a certain epitope or target antigen, *i.e.*, the functionally combined strength of the binding of a plurality of scaffolds or a multimeric scaffold with the antigen. Avidity is related to both the affinity of individual antigen-binding domains with specific epitopes, and also the valency of the scaffold or multimeric scaffold.
- [0070] The term “valency” as used herein refers to the number of potential antigen-binding modules, *e.g.*, the number of FnIII modules, *e.g.*, TNFn3 scaffold variants in a scaffold as provided herein. When a scaffold, *e.g.*, a multimeric scaffold, comprises more than one antigen-binding module, each binding module can specifically bind, *e.g.*, the same epitope or a different epitope, in the same target antigen or different target antigens.
- [0071] The term “disulfide bond” as used herein includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group.
- [0072] The terms “TNFn3 scaffold” and “TNFn3 scaffold variant” as used herein, refer to a FnIII scaffold or stabilized variant (see, *e.g.*, the variants provided in Figure 3) thereof derived from the third FnIII domain of human tenascin C.

- [0073] The term “wild type TNFn3 scaffold” as used herein refers to an FnIII scaffold comprising SEQ ID NO: 1 (short form), or SEQ ID NO:2 (long form) derived from the 3rd FnIII of human tenascin C.
- [0074] The term “immunoglobulin” and “antibody” comprises various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon. It is the nature of this chain that determines the “class” of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. Modified versions of each of these classes are readily discernable to the skilled artisan. As used herein, the term “antibody” includes but not limited to an intact antibody, a modified antibody, an antibody VL or VL domain, a CH1 domain, a Ckappa domain, a Clambda domain, an Fc domain (see supra), a CH2, or a CH3 domain.
- [0075] As used herein, the term “modified antibody” includes synthetic forms of antibodies which are altered such that they are not naturally occurring, *e.g.*, antibodies that comprise at least two heavy chain portions but not two complete heavy chains (as, *e.g.*, domain deleted antibodies or minibodies); multispecific forms of antibodies (*e.g.*, bispecific, trispecific, etc.) altered to bind to two or more antigens or to different epitopes of a single antigen). In addition, the term “modified antibody” includes multivalent forms of antibodies (*e.g.*, trivalent, tetravalent, etc., antibodies that to three or more copies of the same antigen). (See, *e.g.*, Antibody Engineering, Kontermann & Dubel, eds., 2010 Springer Protocols, Springer).
- [0076] The term “expression” as used herein refers to a process by which a gene produces a biochemical, for example, a a TNFn3 scaffold variant provided herein or a multimeric scaffold comprising a TNFn3 scaffold variant provided herein. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into one or more mRNAs, and the translation of such mRNAs into one or more polypeptides. If the final desired product is a biochemical, expression includes the creation of that biochemical and any precursors.
- [0077] An “expression product” can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide. Expression products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation, or

polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

[0078] The term “vector” or “expression vector” is used herein to mean vectors used as a vehicle for introducing into and expressing a desired expression product in a host cell. As known to those skilled in the art, such vectors can easily be selected from the group consisting of plasmids, phages, viruses and retroviruses. In general, vectors can comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired nucleic acid and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

[0079] The term “host cell” refers to a cell that harbors a vector constructed using recombinant DNA techniques and encoding at least one expression product. In descriptions of processes for the isolation of an expression product from recombinant hosts, the terms “cell” and “cell culture” are used interchangeably to denote the source of the expression product unless it is clearly specified otherwise, *i.e.*, recovery of the expression product from the “cells” means either recovery from spun down whole cells, or recovery from the cell culture containing both the medium and the suspended cells.

[0080] The terms “treat” or “treatment” as used herein refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder in a subject, such as the progression of an inflammatory disease or condition. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable.

[0081] The term “treatment” also means prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

[0082] By “subject” or “individual” or “animal” or “patient” or “mammal,” is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, sports

animals, and zoo animals including, *e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, bears, and so on.

TNFn3 Scaffold Variants

[0083] This disclosure provides stabilized scaffold subunits derived from the third FnIII domain of human tenascin C (SEQ ID NO: 1 or SEQ ID NO:2). The TNFn3 scaffold variants provided herein are characterized by the same three dimensional structure, as SEQ ID NO:1 or SEQ ID NO:2, namely a beta-sandwich structure with three beta strands (A, B, and E) on one side and four beta strands (C, D, F, and G) on the other side, connected by six loop regions. These loop regions are designated according to the beta-strands connected to the N- and C- terminus of each loop. Accordingly, the AB loop (having an amino acid sequence [ab]) is located between beta strands A and B, the BC loop (having an amino acid sequence [bc]) is located between strands B and C, the CD loop (having an amino acid sequence [cd]) is located between beta strands C and D, the DE loop (having an amino acid sequence [de]) is located between beta strands D and E, the EF loop (having an amino acid sequence [ef]) is located between beta strands E and F, and the FG loop (having an amino acid sequence [fg]) is located between beta strands F and G. The solvent exposed loops regions are tolerant of randomization, which facilitates the generation of diverse pools of protein scaffolds capable of binding specific targets with high affinity. Amino acid sequences of exemplary, non-limiting loop variants for a TNFn3 scaffold variant as provided herein, methods to generate randomized loop variants, and methods to screen for TNFn3 scaffold variants that bind to a target antigen of interest can be found, *e.g.*, in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328, which are incorporated herein by reference in their entireties.

[0084] In certain embodiments, the disclosure provides a TNFn3 scaffold variant comprising the amino acid sequence: [A]-[ab]-[B]-[bc]-[C]-[cd]-[D]-[de]-[E]-[ef]-[F]-[fg]-[G]. [A], [B], [C], [D], [E], [F], and [G] represent the beta strands derived from TNFn3, and can include the following, non-limiting variations. In certain aspects, strand [A] is IEV (SEQ ID NO:6) or RLDAPSX₇IEV (SEQ ID NO:3), where X₇ can be Q or K; strand [B] is AX₁₉ITW (SEQ ID NO:7), where X₁₉ can be L or K; strand [C] is X₃₂ELTYGI (SEQ ID NO:10), where X₃₂

can be I or F; strand [D] is TTX₄₉L (SEQ ID NO:13), where X₄₉ can be D or K or N; strand [E] is YSI (SEQ ID NO:16); strand [F] is YEVSLLS (SEQ ID NO:17); and strand [G] is KX₈₆TFX₈₉TX₉₁X₉₂, (SEQ ID NO:18) wherein X₈₆ can be E or I or Q; X₈₉ can be T or K; X₉₁ can be any amino acid, for example, G, or can be deleted, and X₉₂ can be any amino acid, for example, L, or can be deleted. According to these embodiments, [ab], [bc], [cd], [de], [ef], and [fg] represent the variable loops AB, BC, CD, DE, EF, and FG, each comprising at least 2 to no more than about 26 amino acids. The amino acid sequence of stabilized TNFn3 scaffold variants is provided in Figure 3.

[0085] In certain embodiments, selected positions within the beta strands of the TNFn3 scaffold variant are randomized. In one such embodiment, [A] is IEV (SEQ ID NO:6) or RLDAPSX₇IEV, wherein X₇ is Q or K (SEQ ID NO:3); [B] is AX₁₉ITW, wherein X₁₉ is L or K (SEQ ID NO:7); [C] is X₃₂X₃₃LTYGX₃₈, wherein X₃₂ is I or F, and X₃₃ and X₃₈ are independently any amino acid (SEQ ID NO:63); [D] is TX₄₇IX₄₉L, wherein X₄₇ and X₄₉ are independently any amino acid (SEQ ID NO:64); [E] is YSI (SEQ ID NO:16); [F] is YEVSLLS (SEQ ID NO:17); and [G] is KX₈₆TFX₈₉TX₉₁X₉₂, wherein X₈₆ is E or I or Q; X₈₉ is T or K; X₉₁ is any amino acid [G] or is deleted, and X₉₂ is any amino acid [L] or is deleted (SEQ ID NO:18). According to this embodiment, [ab], [bc], [cd], [de], [ef], and [fg] are variable loops each comprising at least 2 to no more than about 26 amino acids. In another such embodiment, [A] is RLDAPSX₇IX₉V, wherein X₇ and X₉ are independently any amino acid (SEQ ID NO:65); [B] is AX₁₉IX₂₁W, wherein X₁₉ and X₄₉ are independently any amino acid (SEQ ID NO:66); [C] is X₃₂ELTYGI, wherein X₃₂ is I or F (SEQ ID NO:10); [D] is TTX₄₉L, wherein X₄₉ is D or K or N (SEQ ID NO:13); [E] is YX₅₈I, wherein X₅₈ is any amino acid (SEQ ID NO:68); [F] is YEVSLLS (SEQ ID NO:17); and [G] is KX₈₆TFX₈₉TX₉₁X₉₂, wherein X₈₆ is E or I or Q; X₈₉ is T or K; X₉₁ is any amino acid [G] or is deleted, and X₉₂ is any amino acid [L] or is deleted (SEQ ID NO:18). According to this embodiment, [ab], [bc], [cd], [de], [ef], and [fg] are variable loops each comprising at least 2 to no more than about 26 amino acids. The amino acid sequence of a representative stabilized TNFn3 scaffold variants in which selected positions within the beta strands are randomized is provided in Figures 4B and 4C.

[0086] In certain aspects of these embodiments the amino acid sequence of the TNFn3 scaffold variant is not:

RLDAPSKIEV[ab]AKITW[bc]IELTYGI[cd]TTIKL[de]YSI[ef]YEVSLIS[fg]KETFKTGA
 ([SEQ ID NO:5]-[ab]-[SEQ ID NO:9]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:15]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:59]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KETFTTGL
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

IEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KETFTTGL ([SEQ ID
 NO:6]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-[SEQ ID
 NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KITFTTGL
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:60]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTINL[de]YSI[ef]YEVSLIS[fg]KETFTTGL
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:31]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KQTFTTGL
 ([SEQ ID NO:6]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:61]); or

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTINL[de]YSI[ef]YEVSLIS[fg]KETFTTGL
 ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:31]-[de]-
 [SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:61]).

[0087] The loop regions sequences are represented herein as structures [ab], [bc], [cd], [de], [ef], and [fg], each comprising at least 2 to no more than about 26 amino acids. In certain non-limiting aspects, [ab] comprises KDVTDDT (SEQ ID NO:23), or SEQ ID NO:23 with at least one, two, three, four, five, six, or seven amino acid substitutions, insertions, or deletions; [bc] comprises FKPLAEIDG (SEQ ID NO:24), or SEQ ID NO:24 with at least one, two, three, four, five, six, seven, eight, or nine amino acid substitutions, insertions, or deletions; [cd] comprises KDVPDGR (SEQ ID NO:25), or SEQ ID NO:25 with at least one, two, three, four, five, six, or seven amino acid substitutions, insertions, or deletions; [de] comprises TEDENQ (SEQ ID NO:26), or SEQ ID NO:26 with at least one, two, three, four, five, or six, amino acid substitutions, insertions, or deletions; [ef] comprises GNLKPDTE (SEQ ID NO:27), or SEQ ID NO:27 with at least one, two, three, four, five, six, seven, or eight amino acid substitutions, insertions, or deletions; and/or [fg] comprises $RX_{76}X_{77}X_{78}MSSNPX_{84}$ (SEQ ID NO:28), wherein X_{76} is any amino acid, for example, R, or is deleted; X_{77} is any amino acid, for example, G, or is deleted; X_{78} is any amino acid, for example, D, or is deleted; and X_{84} is A or V, or SEQ ID NO:28 with at least one, two, three, four, five, or six additional amino acid substitutions, insertions, or deletion. In certain aspects, $X_{76}X_{77}X_{78}$ is not RGD. It is contemplated that in certain embodiments where the [fg] loop is randomized X_{84} may be any amino acid or may be held constant as an A or V.

[0088] In certain aspects, some, but not all of the loop regions comprise variable sequences. For example, in some aspects loops AB, CD, and EF are maintained as the wild-type sequences, *i.e.*, [ab] is KDVTDDT (SEQ ID NO:23); [cd] is KDVPDGR (SEQ ID NO:25); and [ef] is GNLKPDTE (SEQ ID NO:27), while one or more of loops BC, DE, and FG are randomized by one or more amino acid insertions, deletions, or substitutions. According to these aspects, the TNFn3 scaffold variant can take the structure:

RLDAPSX₇IEV[ab]AX₁₉ITW[bc]X₃₂ELTYGI[cd]TTIX₄₉L[de]YSI[ef]YEVSLIS[fg]KX₈₆T
FX₈₉TX₉₁X₉₂ (long form); or

IEV[ab]AX₁₉ITW[bc]X₃₂ELTYGI[cd]TTIX₄₉L[de]YSI[ef]YEVSLIS[fg]KX₈₆TFX₈₉T
(short form); where the variable amino acids at positions 7, 19, 32, 49, 76, 77, 78, 84, 86, 91, and 92 are as described above.

[0089] In certain aspects, this disclosure provides a template TNFn3 scaffold variant in which the loop regions remain in the wild-type structure, for example, the disclosure provide a TNFn3 scaffold variant comprising the amino acid sequence RLDAPSX₇IEVKDVTDTTAX₁₉ITWFKPLAEIDGX₃₂ELTYGIKDVPGDRTTIX₄₉LTEDENQYSIGNLKPDTYEYVSLISRX₇₆X₇₇X₇₈MSSNPX₈₄KX₈₆TFX₈₉TX₉₁X₉₂ (SEQ ID NO:53), where the variable amino acids at positions 7, 19, 32, 49, 76, 77, 78, 84, 86, 91, and 92 are as described above. Binding molecules built from TNFn3 scaffold variant backbones can be constructed starting with this sequence.

[0090] In certain more specific aspects, one amino acid variations can be introduced into the beta strands A, B, C, D, and/or G of a TNFn3 scaffold variant. For example, each beta strand can be, interchangeably, the wild-type form or a variant form. For example, in certain aspects beta strand [A] can be RLDAPSQIEV (SEQ ID NO:4) (wild-type long form) or IEV (SEQ ID NO:6) (wild-type short form), RLDAPSKIEV (SEQ ID NO:5) (variant form). In certain aspects, beta strand [B] can be ALITW (SEQ ID NO:8) (wild type), or can be AKITW (SEQ ID NO:9) (variant form). In certain aspects beta strand [C] can be IELTYGI (SEQ ID NO:11) (wild type), or can be FELTYGI (SEQ ID NO:12) (variant form). In certain aspects beta strand [D] can be TTIDL (SEQ ID NO:14) (wild type), or can be TTIKL (SEQ ID NO:15) (variant form-a) or TTINL (SEQ ID NO:31) (variant form-b). In certain aspects beta strand [G] can be KETFTTX₉₁X₉₂ (SEQ ID NO:19) (wild type), or can be KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20) (variant form-86a) or KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32) (variant form-86b). In each of these forms of beta strand G, X₈₉ can be T or K, and X₉₁ and X₉₂ can each be, independently, any amino acid. In certain aspects, beta strand [G] can be KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21) (variant form-89), where X₈₆ can be E or I, and X₉₁ and X₉₂ can each be, independently, any amino acid. In certain aspects beta strand [G] can be KITFKTX₉₁X₉₂ (SEQ ID NO:22) (variant form 86a-89) or KQTFKTX₉₁X₉₂ (SEQ ID NO:33) (variant form 86b-89), where X₉₁ and X₉₂ can each be, independently, any amino acid.

[0091] In certain aspects two or more amino acid variations can be introduced into the beta strands of a TNFn3 scaffold variant. For example, in certain aspects beta strand [C] can be FELTYGI (SEQ ID NO:12), and beta strand [D] can be TTIKL (SEQ ID NO:15), or

alternatively SEQ ID NO:31. In other aspects, beta strand [C] can be FELTYGI (SEQ ID NO:12), and beta strand [D] can be TTINL (SEQ ID NO:31). In other aspects beta strand [C] can be FELTYGI (SEQ ID NO:12), and beta strand [G] can be KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20), or alternatively any one of SEQ ID Nos: 32, 21, 22, or 33. In certain aspects, beta strand [D] can be TTIKL (SEQ ID NO:15) or alternatively SEQ ID NO:31, and beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20), or alternatively any one of SEQ ID Nos: 32, 21, 22, or 33. In one aspect, a TNFn3 scaffold variant designated CM2 is provided, in which beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is IELTYGI (SEQ ID NO:11), beta strand [D] is TTIDL (SEQ ID NO:14), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

[0092] In certain aspects three or more amino acid variations can be introduced into the beta strands of a TNFn3 scaffold variant. For example in certain aspects, beta strand [C] can be FELTYGI (SEQ ID NO:12), beta strand [D] can be TTIKL (SEQ ID NO:15), or alternatively SEQ ID NO:31, and beta strand [G] can be KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20), or alternatively any one of SEQ ID Nos: 32, 21, 22, or 33. In one aspect, a TNFn3 scaffold variant designated CM3 is provided, in which beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is IELTYGI (SEQ ID NO:11), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

[0093] In certain aspects four or more amino acid substitutions can be introduced into the beta strands of a TNFn3 scaffold variant. For example in certain aspects, beta strand [C] can be FELTYGI (SEQ ID NO:12), beta strand [D] can be TTIKL (SEQ ID NO:15) or alternatively SEQ ID NO:31, and beta strand [G] is KITFKTGL (SEQ ID NO:34). In one aspect, a TNFn3 scaffold variant designated CM4 is provided, in which beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

[0094] A successful scaffold protein must have sufficient stability to tolerate the often extensive modification required to introduce a new binding site. Most non-antibody scaffolds are small, single domain proteins. As a result, a significant percentage of the protein surface

may be mutated in these systems, making a high level of starting stability all the more important.

[0095] The stability of a protein may be measured by the level of fluorescence exhibited by the protein under varying conditions. There is a positive correlation between the relative unfoldedness of a protein and a change in the internal fluorescence the protein exhibits under stress. Suitable protein stability assays to measure thermal characteristics include Differential Scanning Calorimetry (DSC) and Circular Dichroism (CD). When the protein demonstrates a sizable shift in parameters measured by DSC or CD, it correlates to an unfolded structure; the temperature at which this shift is made is termed the melting temperature or (T_m).

[0096] One generalized benchmark that has been suggested for starting stability in a small non-antibody scaffold is a $T_m > 60^\circ\text{C}$, as measured, *e.g.*, by differential scanning calorimetry (DSC) (Skerra, 2007). The most successful alternative scaffolds to date all meet this benchmark with some far surpassing it (Schlehuber and Skerra, 2002; Litvinovich and Ingham, 1995; Wetzel *et al.*, 2008; Koide *et al.*, 1998). One particular example of scaffold engineering is the introduction of at least one non-naturally occurring amino acid in an FnIII scaffold, *e.g.*, a TNFn3 scaffold variant, which results in improved stability, *e.g.*, a higher melting temperature (T_m) as measured by DSC. In certain aspects, a TNFn3 scaffold variant as provided herein has thermal melting temperature (T_m) of at least about 60°C , at least about 61°C , at least about 62°C , at least about 63°C , at least about 64°C , at least about 65°C , at least about 66°C , at least about 67°C , at least about 68°C , at least about 69°C , at least about 70°C , at least about 71°C , at least about 72°C , at least about 73°C , at least about 74°C , at least about 75°C , at least about 76°C , at least about 77°C , at least about 78°C , at least about 79°C , at least about 80°C , at least about 81°C , at least about 82°C , at least about 83°C , at least about 84°C , at least about 85°C , at least about 86°C , at least about 87°C , at least about 88°C , at least about 89°C , or at least about 90°C , as measured by DSC in 10 mM potassium phosphate, 50 mM sodium chloride, pH 7.4. In certain aspects a TNFn3 scaffold variant is provided which has a T_m of about 75°C (CM2), 81°C (CM3), or 83°C (CM4) as measured by DSC in 10 mM potassium phosphate, 50 mM sodium chloride, pH 7.4. In certain aspects a TNFn3 scaffold variant as provided herein exhibits a thermal melting temperature (T_m) that is increased relative to the T_m observed for a TNFn3 scaffold protein

consisting of SEQ ID NO:2 by at least about 1°C, at least about 2°C, at least about 3°C, at least about 4°C, at least about 5°C, at least about 6°C, at least about 7°C, at least about 8°C, at least about 9°C, at least about 10°C, at least about 11°C, at least about 12°C, at least about 13°C, at least about 14°C, at least about 15°C, at least about 16°C, at least about 17°C, at least about 18°C, at least about 19°C, at least about 20°C, at least about 21°C, at least about 22°C, at least about 23°C, at least about 24°C, at least about 25°C, at least about 26°C, at least about 27°C, at least about 28°C, at least about 29°C, or at least about 30°C, as measured by DSC.

[0097] In another embodiment, a TNFn3 scaffold variant as provided herein exhibits an increased melting temperature (T_m) of 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%, or at least 95% or more as compared to the T_m observed for a TNFn3 scaffold protein consisting of SEQ ID NO:2 under the same experimental conditions.

[0098] In certain aspects a TNFn3 scaffold variant as provided herein exhibits an increased $\Delta G_{\text{unfolding}}$ as compared to the wild-type TNFn3. For example, where the wild-type TNFn3 subunit exhibits a $\Delta G_{\text{unfolding}}$ of about 5.7 kcal/mol, as measured by denaturant titration using guanidine thiocyanate or guanidine HCl as monitored by intrinsic tryptophan fluorescence, a TNFn3 scaffold variant as provided herein can have a $\Delta G_{\text{unfolding}}$ of at least about 5.8 kcal/mol, at least about 6.0 kcal/mol, at least about 6.2 kcal/mol, at least about 6.4 kcal/mol, at least about 6.6 kcal/mol, at least about 6.8 kcal/mol, at least about 7.0 kcal/mol, at least about 7.2 kcal/mol, at least about 7.4 kcal/mol, at least about 7.6 kcal/mol, at least about 7.8 kcal/mol, at least about 8.0 kcal/mol, at least about 8.2 kcal/mol, at least about 8.4 kcal/mol, , at least about 8.6 kcal/mol, , at least about 8.8 kcal/mol, , at least about 9.0 kcal/mol, at least about 9.2 kcal/mol, at least about 9.4 kcal/mol, at least about 9.6 kcal/mol, or at least about 9.8 kcal/mol, as measured by denaturant titration using guanidine thiocyanate or guanidine HCl as monitored by intrinsic tryptophan fluorescence. In certain aspects the $\Delta G_{\text{unfolding}}$ is about 9.4 kcal/mol (CM4).

[0099] In certain aspects a TNFn3 scaffold variant as provided herein exhibits increased denaturation stability, *i.e.*, the TNFn3 scaffold variant exhibits 50% denaturation at a higher

concentration of guanidine thiocyanate [GdnSCN]_{50%} in 50 mM potassium phosphate, 50 mM NaCl, pH = 7.4 than a wild-type TNFn3 subunit. For example, where the wild-type TNFn3 subunit exhibits a [GdnSCN]_{50%} of about 0.79 M, a TNFn3 scaffold variant as provided herein can have a [GdnSCN]_{50%} of at least about 0.85 M, least about 0.90 M, least about 0.95 M, least about 1.00 M, least about 1.05 M, least about 1.10 M, least about 1.15 M, least about 1.20 M, least about 1.25 M, least about 1.30 M, least about 1.35 M, or least about 1.40 M, at 23 °C when measured by fluorescence emission at 310-400 nm using an excitation wavelength of 295 nm in 50 mM potassium phosphate, 50 mM NaCl, pH = 7.4.

[0100] In certain aspects, a TNFn3 scaffold variant as provided herein is more resistant to proteolysis than a wild type TNFn3 scaffold subunit. In certain aspects TNFn3 scaffold variants as provided herein remain resistant to 2 mg/ml thermolysin for at least 2 hours, at least 4 hours, at least 8 hours, at least 10 hours, at least 15 hours, at least 20 hours, and least 24 hours, or at least 48 hours, when incubated in 50 µM in 20 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH = 8.0, at 42 °C.

[0101] In certain aspects, a TNFn3 scaffold variant provided herein binds to a target antigen of interest. In certain embodiments, the TNFn3 scaffold binds to a target antigen of interest with an affinity (K_D) of at least 100 µM. Non-limiting examples of target antigens include cell-surface antigen, a soluble antigen, an immobilized antigen, an immunosilent antigen, an intracellular antigen, an intranuclear antigen, a self antigen, a non-self antigen, a cancer antigen, a bacterial antigen, a viral antigen, or any combination thereof. Exemplary target antigens for TNFn3 scaffold variants as provided herein can be found, *e.g.*, in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

[0102] In certain aspects, a TNFn3 scaffold variant as provided herein can bind to a target antigen of interest with a binding affinity characterized by a dissociation constant (K_D) of about 100 pM to about 0.1 pM as measured on a Kinetic Exclusion Assay (KinExA) 3000 platform.

[0103] In certain aspects, a TNFn3 scaffold variant as provided herein can bind to a target antigen of interest with a dissociation constant or K_D of less than 10^{-6} M, or of less than 10^{-7} M, or of less than 10^{-8} M, or of less than 10^{-9} M, or of less than 10^{-10} M, or of less than 10^{-11}

M, of less than 10^{-12} M, of less than 10^{-13} M, of less than 10^{-14} M, or of less than 10^{-15} M as measured, *e.g.*, by KINEXA® or BIACORE®.

[0104] In certain aspects, a TNFn3 scaffold variant as provided herein can bind to a target antigen of interest with a K_{off} of less than $1 \times 10^{-3} \text{ s}^{-1}$, or less than $2 \times 10^{-3} \text{ s}^{-1}$. In certain aspects, a TNFn3 scaffold variant as provided herein can bind to a target antigen of interest with a K_{off} of less than 10^{-3} s^{-1} , less than $5 \times 10^{-3} \text{ s}^{-1}$, less than 10^{-4} s^{-1} , less than $5 \times 10^{-4} \text{ s}^{-1}$, less than 10^{-5} s^{-1} , less than $5 \times 10^{-5} \text{ s}^{-1}$, less than 10^{-6} s^{-1} , less than $5 \times 10^{-6} \text{ s}^{-1}$, less than less than $5 \times 10^{-7} \text{ s}^{-1}$, less than 10^{-8} s^{-1} , less than $5 \times 10^{-8} \text{ s}^{-1}$, less than 10^{-9} s^{-1} , less than $5 \times 10^{-9} \text{ s}^{-1}$, or less than 10^{-10} s^{-1} as measured, *e.g.*, by KINEXA® or BIACORE®.

[0105] In certain aspects, a TNFn3 scaffold variant as provided herein can bind to a target antigen of interest with an association rate constant or k_{on} rate of at least $10^5 \text{ M}^{-1} \text{ s}^{-1}$, at least $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, at least $10^6 \text{ M}^{-1} \text{ s}^{-1}$, at least $5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, at least $10^7 \text{ M}^{-1} \text{ s}^{-1}$, at least $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, or at least $10^8 \text{ M}^{-1} \text{ s}^{-1}$, or at least $10^9 \text{ M}^{-1} \text{ s}^{-1}$ as measured, *e.g.*, by KINEXA® or BIACORE®.

[0106] The affinity or avidity of a TNFn3 scaffold variant as provided herein for a target antigen of interest can be determined experimentally using any suitable method well known in the art, *e.g.*, flow cytometry, enzyme-linked immunosorbent assay (ELISA), or radioimmunoassay (RIA), or kinetics (*e.g.*, KINEXA® or BIACORE™ analysis). Direct binding assays as well as competitive binding assay formats can be readily employed. (See, for example, Berzofsky *et al.*, "Antibody-Antigen Interactions," In Fundamental Immunology, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, Immunology, W. H. Freeman and Company: New York, N.Y. (1992); and methods described herein.) The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions (*e.g.*, salt concentration, pH, temperature). Thus, measurements of affinity and other antigen-binding parameters (*e.g.*, K_D or K_d , K_{on} , K_{off}) are made with standardized solutions of antibody and antigen, and a standardized buffer, as known in the art and such as the buffer described herein.

[0107] The loops connecting the various strands of the protein scaffold can be randomized for length and/or sequence diversity, in order to, *e.g.*, bind to a target antigen of interest. In one embodiment, a TNFn3 scaffold variant as provided herein comprises at least one loop

that is randomized for length and/or sequence diversity. In another embodiment, a TNFn3 scaffold variant as provided herein comprises at least one loop that is kept constant while at least one additional loop is randomized for length and/or sequence diversity. In another embodiment, a library of TNFn3 scaffold variants is provided where at least one of loops AB, CD, or EF is kept constant while at least one of loops BC, DE, or FG is randomized for length or sequence diversity. In another embodiment, a library of TNFn3 scaffold variants is provided where at least one of loops BC, DE, or FG is kept constant while at least one of loops AB, CD, or EF is randomized for length or sequence diversity.

[0108] Once randomized and selected for binding to a target antigen, the loops in a TNFn3 scaffold variant as provided herein can make contacts with targets equivalent to the contacts of the cognate CDR loops in antibodies. Accordingly, in some embodiments the AB, CD, and EF loops, alone or in combination, can be randomized and selected for binding to one or more target antigens. In some embodiments, this randomization and selection process may be performed in parallel with the randomization of one or more of the BC, DE, and FG loops, whereas in other embodiments this randomization and selection process can be performed in series.

Scaffold Multimers and Fusion Constructs

[0109] In one embodiment, a TNFn3 scaffold variant with improved stability is part of a multimeric scaffold comprising at least one TNFn3 scaffold variant, two or more tandem repeat TNFn3 scaffold variants, or a TNFn3 scaffold variant and heterologous scaffold subunits or other heterologous moieties. In some embodiments, the multimeric scaffold subunits or other heterologous moieties are fused by a peptide linker, thereby allowing expression as a single construct. In certain aspects, a multimeric scaffold as provided herein can comprise at least one TNFn3 scaffold variant as provided herein fused to at least one scaffold comprising the amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, or SEQ ID NO:39, or at least two or more scaffolds each independently comprising the amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, S SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, or SEQ ID NO:39.

- [0110] Accordingly, in certain aspects a TNFn3 scaffold variant as provided herein or a multimeric scaffold comprising a TNFn3 scaffold variant as provided herein can further comprise a heterologous epitope binding domain. In certain aspects, the heterologous epitope binding domain can be specific for a different antigen target, or different epitope of the same antigen target, than a TNFn3 scaffold variant monomer or one or two or more TNFn3 scaffold variants comprised in multimeric scaffold. Alternatively, the heterologous epitope binding domain can be specific for the same antigen target as a TNFn3 scaffold variant monomer or one or two or more TNFn3 scaffold variants comprised in multimeric scaffold.
- [0111] A multimeric scaffold as provided herein can comprise at least two TNFn3 scaffold variants linked to each other by, *e.g.*, another scaffold, an IgG molecule or fragment thereof, an Fc region, a dimerization domain, a chemical crosslinker, a disulfide bond, or an amino acid linker.
- [0112] The scaffold subunits that make up a multimeric scaffold as provided herein can correctly fold independently of each other, retain their binding specificity and affinity, and each of the scaffold subunits retains its functional properties. When FnIII scaffold subunits, *e.g.*, TNFn3 scaffold variants that make up a multimeric scaffold as provided herein are assembled in high valency multimeric scaffolds, *e.g.*, hexavalent or octavalent scaffolds, the scaffold subunits correctly fold independently of each other, retain their binding specificity and affinity, and each of the scaffold domains retains its functional properties.
- [0113] Multimeric scaffolds, including high valency scaffolds (*e.g.*, hexavalent or octavalent), fold correctly even when the topology of construct is not linear, *e.g.*, when the monomeric FnIII or multimeric FnIII scaffolds are assembled in complex branched structures (*e.g.*, Fc fusion constructs or antibody-like constructs).
- [0114] An advantage of the multimeric scaffolds is their ability to bind to multiple epitopes, *e.g.*, (i) binding to multiple epitopes in a single target, (ii) binding to a single epitope in multiple targets, (iii) binding to multiple epitopes located on different subunits of one target, or (iv) binding to multiple epitopes on multiple targets, thus increasing avidity.
- [0115] As a result of their ability to bind simultaneously to more than one target, multimeric scaffolds as provided herein can be used to modulate multiple pathways, cross-link receptors

on a cell surface, bind cell surface receptors on separate cells, and/or bind target molecules or cells to a substrate.

[0116] Any of the monomeric scaffold constructs described herein may be generated as dimers or multimers of scaffolds as a means to increase the valency and thus the avidity of antigen binding. Also, any of the scaffold constructs described herein may be generated as dimers or multimers of scaffolds as a means to increase the specificity of antigen binding (for example, scaffolds may be generated that bind distinct antigens). Such multimers (multimeric scaffolds or also known herein as multivalent scaffolds) may be generated through covalent binding between individual scaffold modules, for example, by the inclusion of an amino acid linker. In other methods, the multimeric scaffolds may be assembled through use of dimerization domains known in the art. In particular examples, covalently bonded scaffolds may be generated by constructing fusion genes that encode the monomeric scaffolds or, alternatively, by engineering codons for cysteine residues into monomer sequences and allowing disulfide bond formation to occur between the expression products.

[0117] Non-covalently bonded multimeric scaffolds may also be generated by a variety of techniques. These include the introduction, into monomer sequences, of codons corresponding to positively and/or negatively charged residues and allowing interactions between these residues in the expression products (and therefore between the monomers) to occur. This approach may be simplified by taking advantage of charged residues naturally present in a monomer subunit. Another means for generating non-covalently bonded scaffolds is to introduce, into the monomer scaffold gene (for example, at the amino- or carboxy-termini), the coding sequences for proteins or protein domains known to interact. Such proteins or protein domains include coil-coil motifs, leucine zipper motifs, and any of the numerous protein subunits (or fragments thereof) known to direct formation of dimers or higher order multimers.

[0118] In some embodiments, multimeric scaffolds provided herein comprise at least one scaffold fused to any domain (or fragment) of an antibody. In some embodiments, at least one scaffold is fused to an antibody variable domain, a CH1 domain, a Ckappa domain, a Clambda domain, a hinge domain, a CH2, and/or a CH3 domain. In other embodiments, at least one scaffold is fused to the hinge or CH2 domain of an antibody Fc. In such

embodiments, the resulting protein, when expressed will be bivalent for a particular target through the dimerization of the CH2 and CH3 regions of the antibody Fc fragment. In further embodiments, a TNFn3 scaffold variant as provided herein replaces the antibody variable region connected to the Fc fragment. In alternative embodiments, a TNFn3 scaffold variant as provided herein does not replace the antibody variable region connected to the CH1-Fc fragment, Ckappa or Clambda domains.

[0119] In further embodiments, multimeric scaffolds are constructed by fusing scaffolds to the CH1 and the Ckappa or Clambda regions of an antibody. In such embodiments, where the same scaffold is fused to the CH1 and Ckappa or Clambda regions the resulting proteins, when assembled, will be tetravalent for a single epitope. Where scaffolds recognizing different epitopes are fused to the CH1 and Ckappa or Clambda regions the resulting protein, when assembled, will be bivalent for two different epitopes. In some embodiments, a TNFn3 scaffold variant as provided herein replaces an antibody variable region fused to the CH1 and the Ckappa or Clambda regions of an antibody. In further embodiments, a TNFn3 scaffold variant as provided herein can be fused to the C-terminus of the light chain or heavy chain of an antibody. In other embodiments, a TNFn3 scaffold variant as provided herein can be fused to the N-terminus of the light chain or heavy chain of an antibody.

[0120] In some embodiments, multimeric scaffolds provided herein comprise scaffolds that are specific for the same epitope. In other embodiments, multimeric scaffolds provided herein comprise scaffolds that are specific for different epitopes otherwise known as an epitope binding domain. Multimeric scaffolds provided herein can be assembled and utilized as shown in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328. Such epitope binding domains can be selected from an antibody, an antibody fragment, a diabody, an scFv, a Fab, a Fv, or a binding peptide.

[0121] Choosing a suitable linker for a specific case where two or more scaffolds are to be connected can depend on a variety of parameters including, *e.g.* the nature of the monomer domains, and/or the stability of the peptide linker towards proteolysis and oxidation.

[0122] The linker polypeptide may predominantly include amino acid residues selected from the group consisting of Gly, Ser, Ala and Thr. For example, the peptide linker may contain at

least 75% (calculated on the basis of the total number of residues present in the peptide linker), such as at least 80%, *e.g.* at least 85% or at least 90% of amino acid residues selected from the group consisting of Gly, Ser, Ala and Thr. The peptide linker may also consist of Gly, Ser, Ala and/or Thr residues only. The linker polypeptide should have a length, which is adequate to link two or more monomer domains or two or more multimeric scaffolds in such a way that they assume the correct conformation relative to one another so that they retain the desired activity.

[0123] A suitable length for this purpose is a length of at least one and typically fewer than about 50 amino acid residues, such as 2-25 amino acid residues, 5-20 amino acid residues, 5-15 amino acid residues, 8-12 amino acid residues or 11 residues. Similarly, the polypeptide encoding a linker can range in size, *e.g.*, from about 2 to about 15 amino acids, from about 3 to about 15, from about 4 to about 12, about 10, about 8, or about 6 amino acids. In methods and compositions involving nucleic acids, such as DNA, RNA, or combinations of both, the polynucleotide containing the linker sequence can be, *e.g.*, between about 6 nucleotides and about 45 nucleotides, between about 9 nucleotides and about 45 nucleotides, between about 12 nucleotides and about 36 nucleotides, about 30 nucleotides, about 24 nucleotides, or about 18 nucleotides. Likewise, the amino acid residues selected for inclusion in the linker polypeptide should exhibit properties that do not interfere significantly with the activity or function of the polypeptide multimer. Thus, the peptide linker should on the whole not exhibit a charge which would be inconsistent with the activity or function of the polypeptide multimer, or interfere with internal folding, or form bonds or other interactions with amino acid residues in one or more of the monomer domains which would seriously impede the binding of the polypeptide multimer to specific targets.

[0124] The peptide linker may also be selected from a library where the amino acid residues in the peptide linker are randomized for a specific set of monomer domains in a particular polypeptide multimer. A flexible linker could be used to find suitable combinations of monomer domains, which is then optimized using this random library of variable linkers to obtain linkers with optimal length and geometry. The optimal linkers may contain the minimal number of amino acid residues of the right type that participate in the binding to the

target and restrict the movement of the monomer domains relative to each other in the polypeptide multimer when not bound to specific targets.

[0125] The use of naturally occurring as well as artificial peptide linkers to connect polypeptides into novel linked fusion polypeptides is well known in the literature (Hallewell *et al.* (1989), J. Biol. Chem. 264, 5260-5268; Alfthan *et al.* (1995), Protein Eng. 8, 725-731; Robinson & Sauer (1996), Biochemistry 35, 109-116; Khandekar *et al.* (1997), J. Biol. Chem. 272, 32190-32197; Fares *et al.* (1998), Endocrinology 139, 2459-2464; Smallshaw *et al.* (1999), Protein Eng. 12, 623-630; U.S. Pat. No. 5,856,456).

[0126] As mentioned above, it is generally preferred that the peptide linker possess at least some flexibility. Accordingly, in some embodiments, the peptide linker contains 1-25 glycine residues, 5-20 glycine residues, 5-15 glycine residues or 8-12 glycine residues. The peptide linker will typically contain at least 50% glycine residues, such as at least 75% glycine residues. In some embodiments, the peptide linker comprises glycine residues only.

[0127] In some cases it may be desirable or necessary to provide some rigidity into the peptide linker. This may be accomplished by including proline residues in the amino acid sequence of the peptide linker. Thus, in another embodiment, the peptide linker can comprise at least one proline residue in the amino acid sequence of the peptide linker. For example, the peptide linker has an amino acid sequence, wherein at least 25%, such as at least 50%, *e.g.* at least 75%, of the amino acid residues are proline residues. In one embodiment, the peptide linker comprises proline residues only.

[0128] In some embodiments, the peptide linker is modified in such a way that an amino acid residue comprising an attachment group for a non-polypeptide moiety is introduced. Examples of such amino acid residues may be a cysteine residue (to which the non-polypeptide moiety is then subsequently attached) or the amino acid sequence may include an *in vivo* N-glycosylation site (thereby attaching a sugar moiety (*in vivo*) to the peptide linker). An additional option is to genetically incorporate non-natural amino acids using evolved tRNAs and tRNA synthetases (see, *e.g.*, U.S. patent application Publication Ser. No. 2003/0082575) into the monomer domains or linkers. For example, insertion of keto-tyrosine allows for site-specific coupling to expressed monomer domains or multimers.

[0129] Sometimes, the amino acid sequences of all peptide linkers present in the polypeptide multimer will be identical. Alternatively, the amino acid sequences of all peptide linkers present in the polypeptide multimer may be different.

Scaffold Libraries

[0130] The scaffolds described herein may be used in any technique for evolving new or improved target antigen-binding proteins. In one particular example, the target antigen is immobilized on a solid support, such as a column resin or microtiter plate well, and is contacted with a library of candidate scaffold-based binding proteins. Such a library can consist of clones constructed from TNFn3 scaffold variants as provided herein through randomization of the sequence and/or the length of one or more loop regions. In certain embodiments, the library can be a phage, phagemid, virus, bacterial or yeast display or a ribosome display library. The selection and use of the various display library technologies is well-known to persons of ordinary skill in the art. Suitable technologies and methods for use with TNFn3 scaffold variants as provided herein can be found, *e.g.*, in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

[0131] The disclosure also provides libraries comprising TNFn3 scaffold variants as herein, or multimeric scaffolds comprising TNFn3 scaffold variants. In certain embodiments, a library as provided comprises TNFn3 scaffold variants comprising a beta strand domains, including variant beta strand domains engineered for increased stability, linked to a plurality of loop region sequences derived from a naturally occurring protein sequence, wherein one or more of the loop region sequences vary by deletion, substitution or addition by at least one amino acid from the corresponding loop sequences in the naturally occurring TNFn3 protein sequence.

[0132] This disclosure further provides a method of constructing a binding molecule display library comprising:

- (a) providing a polynucleotide encoding a TNFn3 scaffold variant as provided herein or a polynucleotide encoding the multimeric scaffold as provided herein;

- (b) introducing random deletions or randomizing codon substitutions or insertions into the polynucleotide at selected positions in a region of the polynucleotide encoding at least one variable loop [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant; and
- (c) propagating copies of the polynucleotide to form the display library.

[0133] In one embodiment, the disclosure provides a method of constructing a binding molecule display library comprising:

- (a) providing a polynucleotide encoding the TNFn3 scaffold variant of SEQ ID NOs: 54,
- (b) introducing random deletions or randomizing codon substitutions or insertions into the polynucleotide at selected positions in a region of the polynucleotide encoding at least one variable loop [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant; and
- (c) propagating copies of the polynucleotide to form the display library.

[0134] In certain aspects, the disclosure provides a method of constructing a binding molecule display library comprising:

- (a) providing a polynucleotide encoding the TNFn3 scaffold variant of any one of SEQ ID NOs: 69-71;
- (b) introducing randomizing codon substitutions or insertions into the polynucleotide at each position indicated with an "X"; and
- (c) propagating copies of the polynucleotide to form the display library.

[0135] In certain embodiments the randomizing codons can be NNS, NNK, NHT, or a combination thereof. In certain embodiments, each randomizing codon may encode any amino acid. In certain embodiments, the randomizing codons do not encode one or more of C, E, F, H, I, K, M or Q.

[0136] The library as provided herein can be incubated with target antigen of interest immobilized to a solid support, the support can be washed to remove non-specific binders, and the tightest binders can be eluted under very stringent conditions and subjected to PCR to recover the sequence information or to create a new library of binders which can be used to repeat the selection process, with or without further randomization of the sequence. A

number of rounds of selection can be performed until binders of sufficient affinity for the antigen are obtained.

[0137] Also provided are libraries comprising scaffolds comprising loop sequence diversity. One embodiment provides a library comprising scaffolds with at least one loop that contains at least one position that is randomized. One embodiment provides a library comprising scaffolds with at least one loop that comprises at least one position that is randomized while further comprising at least one position that is held constant. One embodiment provides a library comprising scaffolds with a loop that comprises at least one position that is subjected to a restricted randomization. One embodiment provides a library comprising scaffolds with at least one loop that comprises at least one position that is subjected to a restricted randomization and further comprises at least one position that is held constant. One embodiment provides a library comprising scaffolds with at least one loop that comprises at least one position that is subjected to a restricted randomization and further comprises at least one position that is randomized and at least one position that is held constant.

[0138] The loops connecting the various strands of the protein scaffold can be randomized for length and/or sequence diversity. One embodiment provides a library comprising scaffolds where at least one loop is randomized for length and/or sequence diversity. One embodiment provides a library comprising scaffolds where at least one loop is kept constant while at least one additional loop is randomized for length and/or sequence diversity. One embodiment provides a library comprising scaffolds where at least one, at least two, or all three of loops AB, CD, and EF are kept constant while at least one, at least two, or all three of loops BC, DE, and FG are randomized for length or sequence diversity. One embodiment provides a library comprising scaffolds where at least one, at least two, or at least all three of loops AB, CD, and EF are randomized while at least one, at least two, or all three of loops BC, DE, and FG are randomized for length or sequence diversity.

[0139] In certain aspects a library as provided herein can include at least three variable loops [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant, which can be randomized by deletion, substitution, or addition of at least one amino acid. In certain aspects, the loops AB, CD, and EF are randomized, while the remaining loops BC, DE, and FG are optionally left in

their wild-type form. In certain aspects the loops BC, DE, and FG are randomized, while the remaining loops AB, CD, and DE are optionally left in their wild-type form.

[0140] A scaffold library as provided herein can have a sequence diversity of at least 10^4 , at least 10^5 , at least 10^6 , at least 10^7 , at least 10^8 , at least 10^9 , at least 10^{10} , or at least 10^{11} .

[0141] In certain embodiments, a TNFn3 scaffold variant as provided herein can be subjected to affinity maturation. In this art-accepted process, a specific binding protein is subject to a scheme that selects for increased affinity for a specific target (see Wu *et al.* Proc Natl Acad Sci USA. May 1998 26;95(11):6037-42). The resultant scaffolds can exhibit binding characteristics as good or better than the scaffolds prior to affinity maturation.

[0142] In other embodiments, a TNFn3 scaffold variant as provided herein can be subjected to "loop grafting" analogous to CDR grafting for antibodies. In this art-accepted process, one or more CDRs from an antibody are "grafted" onto an acceptor antibody (or, in this example, a TNFn3 scaffold variant as provided herein (see Ewert *et al.* Methods:2004 Oct;34(2):184-99)). In another embodiment, at least one loop from another scaffold can be grafted onto a TNFn3 scaffold variant as provided herein.

[0143] The disclosure further provides a collection of isolated polynucleotides encoding a library as provided herein, a plurality of expression vectors comprising the collection polynucleotides, and/or a host cell culture comprising the vectors.

[0144] Also provided herein is a method of obtaining a binding molecule specific for a target antigen of interest, where the method includes one or more rounds of screening with one or more libraries. For example the disclosure provides a method of obtaining a binding molecule specific for a target antigen of interest, where the method includes"

(a) contacting a target antigen or interest with a TNFn3 scaffold variant library as provided hereein under conditions that allow a scaffold-target antigen complex to form, and

(b) recovering the scaffold that binds to the target antigen.

[0145] The method can further include randomizing at least one variable loop of the scaffold recovered in step (b) to generate a further randomized library and repeating steps (a) and (b) using the further randomized library. One or at least two, three, four five or six loops can be further randomized. In certain aspects, at least one variable loop randomized in the scaffold

recovered in step (b) was not randomized in the first operation of steps (a) and (b). In certain aspects, the repetition of steps (a) and (b) further comprises contacting a target antigen distinct from the target antigen of the first operation of step (a) and (b), *e.g.*, a target antigen presented in a different conformation or as part of a different compound. In certain aspects, at least one of variable loops AB, CD, or EF is randomized in the first operation of steps (a) and (b), and at least one of variable loops BC, DE, or FG is randomized in the second operation of steps (a) and (b). Alternatively, least one of variable loops BC, DE, or FG is randomized in the first operation of steps (a) and (b), and at least one of variable loops AB, CD, or DE is randomized in the second operation of steps (a) and (b).

[0146] The method described here can further comprise randomizing at least one beta strand of a TNFn3 scaffold variant obtained in either the first or the second operation of step (b) to generate a further randomized library and repeating steps (a) and (b) using the further randomized library.

[0147] This disclosure provides methods of identifying the amino acid sequence of a protein scaffold capable of binding to target antigen so as to form a scaffold:target antigen complex. In one embodiment, the method comprises the steps of: a) providing a polypeptide display library as described herein; b) contacting the polypeptide display library of (a) with an immobilized or separable target antigen; c) separating the scaffold:target antigen complexes from the free scaffolds; d) causing the replication of the separated scaffolds of (c) so as to result in a new polypeptide display library distinguished from that in (a) by having a lowered diversity and by being enriched in displayed scaffolds capable of binding the target antigen; e) optionally repeating steps (b), (c), and (d) with the new library of (d); and f) determining the nucleic acid sequence of the region encoding the displayed scaffold of a species from (d) and hence deducing the peptide sequence capable of binding to the target.

[0148] In another embodiment, TNFn3 scaffold variants as provided herein can be further randomized after identification from a library screen. One embodiment comprises further randomizing at least one, at least two, at least three, at least four, at least five or at least six loops of a scaffold identified from a library using a method described herein. In another embodiment, the further randomized scaffold is subjected to a subsequent method of identifying a scaffold capable of binding a target antigen, the method comprising (a)

contacting the further randomized scaffold with an immobilized or separable target antigen, (b) separating the further randomized scaffold:target antigen complexes from the free scaffolds, (c) causing the replication of the separated scaffolds of (b), optionally repeating steps (a)-(c), and (d) determining the nucleic acid sequence of the region encoding the further randomized scaffold and hence, deducing the peptide sequence capable of binding to the target. In a further embodiment, the further randomized scaffolds comprise at least one, at least two, at least three, at least four, at least five, or at least six further randomized loops that were previously randomized in the first library. In an alternate further embodiment, the further randomized scaffolds comprise at least one, at least two, at least three, at least four, at least five, or at least six randomized loops that were not previously randomized in the first library.

[0149] In another embodiment, one method of obtaining a TNFn3 scaffold variant as provided herein involves a first randomized loop selected from the BC, DE, and FG loops and a second loop not randomized in the library selected from the AB, CD, and EF loops. In yet another embodiment, another method of obtaining a scaffold involves a first randomized loop selected from the AB, CD, EF loops and a second loop not randomized selected from the BC, DE, and FG loops.

[0150] Also provided are methods of detecting a compound utilizing TNFn3 scaffold variants as provided herein. Based on the binding specificities of the scaffolds obtained by library screening, it is possible to use such scaffolds in assays to detect the specific target antigen in a sample, such as for diagnostic methods. In one embodiment, the method of detecting a compound comprises contacting the compound in a sample with a TNFn3 scaffold variant as provided herein under conditions that allow a compound:scaffold complex to form and detecting the scaffold, thereby detecting the compound in a sample. In certain embodiments, the scaffold can be labeled (*i.e.*, radiolabel, fluorescent, enzyme-linked or colorimetric label) to facilitate the detection of the compound.

[0151] Also provided are methods of capturing a compound utilizing TNFn3 scaffold variants as provided herein. Based on the binding specificities of the scaffolds obtained by library screening, it is possible to use such scaffolds in assays to capture the specific target in a sample, such as for purification methods. In one embodiment, the method of capturing a

compound in a sample comprises contacting the compound in a sample with a TNFn3 scaffold variant as provided herein under conditions that allow the formation of a compound:scaffold complex and removing the complex from the sample, thereby capturing the compound in the sample. In further embodiments, the scaffold is immobilized to facilitate the removing of the compound:scaffold complex.

[0152] In certain embodiments, TNFn3 scaffold variants isolated from libraries as provided herein comprise at least one, at least two, at least four, at least five, at least six, or more randomized loop regions. In some embodiments, isolated scaffold loop sequences can be swapped from a donor scaffold to any loop in a receiver scaffold (for example, an AB loop sequence from a donor scaffold can be transferred to any loop region in a receiver scaffold). In specific embodiments, an isolated loop sequence can be transferred to the cognate loop in the receiving scaffold (for example, an AB loop sequence from a donor scaffold can be transferred to a receiver scaffold in the AB loop position). In some embodiments, isolated loop sequences can be "mix and matched" randomly with various receiver scaffolds.

Fusions

[0153] The scaffolds described herein can be fused to other protein domains. For example, these scaffolds may be integrated with the human immune response by fusing the constant region of an IgG (Fc) with a scaffold, through the N or C-terminus. The Fc fusion molecule activates the complement component of the immune response and increases the therapeutic value of the protein scaffold. Similarly, a fusion between a scaffold and a complement protein, such as Clq, may be used to target cells, and a fusion between scaffold and a toxin may be used to specifically destroy cells that carry a particular antigen.

[0154] Additionally, various publications describe methods for obtaining physiologically active molecules whose half-lives are modified either by introducing an FcRn-binding polypeptide into the molecules (WO 97/43316; U.S. Pat. No. 5,869,046; U.S. Pat. No. 5,747,035; WO 96/32478; WO 91/14438) or by fusing the molecules with antibodies whose FcRn-binding affinities are preserved but affinities for other Fc receptors have been greatly reduced (WO 99/43713) or fusing with FcRn binding domains of antibodies (WO 00/09560; U.S. Pat. No. 4,703,039). Specific techniques and methods of increasing half-life of physiologically active molecules can also be found in U.S. Patent No. 7,083,784 granted Aug

1, 2006 entitled "Antibodies with Increased Half-lives" which is hereby incorporated by reference for all purposes. Specifically, it is contemplated that a TNFn3 scaffold variant as provided herein can be fused to an Fc region from an IgG, wherein the Fc region comprises amino acid residue mutations (as numbered by the EU index in Kabat): M252Y/S254T/T256E or H433K/N434F/Y436H.

[0155] In addition, a TNFn3 scaffold variant as provided herein can be fused with molecules that increases or extends *in vivo* or serum half life. In some embodiments, a TNFn3 scaffold variant as provided herein can associate with albumin, such as human serum albumin (HSA), polyethylene glycol (PEG), polysaccharides, immunoglobulin molecules (IgG), complement, hemoglobin, a binding peptide, lipoproteins and other factors to increase its half-life in the bloodstream and/or its tissue penetration. Any of these fusions may be generated by standard techniques, for example, by expression of the fusion protein from a recombinant fusion gene constructed using publicly available gene sequences.

[0156] Also, a TNFn3 scaffold variant as provided herein can bind or associate with molecules that increases or extends *in vivo* or serum half life. In some embodiments, a TNFn3 scaffold variant as provided herein can bind or associate with albumin, polyethylene glycol (PEG), polysaccharides, immunoglobulin molecules or immunoglobulin molecules including, immunoglobulins having Fc mutations that increase serum half life, complement, hemoglobin, lipoproteins and other factors to increase serum half life. TNFn3 scaffold variants that bind or associate with molecules that increase or extend *in vivo* half life can be generated, for example by screening the scaffold libraries described *supra* for a TNFn3 scaffold variant exhibiting the desired binding specificity.

[0157] The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moieties (*e.g.*, with thiol, triflate, tresylate, azirdine, oxirane, N-hydroxysuccinimide or a maleimide moiety). The term "PEG" is intended to indicate polyethylene glycol of a molecular weight between 500 and 150,000 Da, including analogues thereof, wherein for instance the terminal OH-group has been replaced by a methoxy group (referred to as mPEG).

[0158] In one embodiment, the scaffolds are derivatized with polyethylene glycol (PEG). PEG is a linear, water-soluble polymer of ethylene oxide repeating units with two terminal

hydroxyl groups. PEGs are classified by their molecular weights which typically range from about 500 daltons to about 40,000 daltons. In a presently preferred embodiment, the PEGs employed have molecular weights ranging from 5,000 daltons to about 20,000 daltons. PEGs coupled to a TNFn3 scaffold variant as provided herein can be either branched or unbranched. (See, for example, Monfardini, C. *et al.* 1995 Bioconjugate Chem 6:62-69). PEGs are commercially available from Nektar Inc., Sigma Chemical Co. and other companies. Such PEGs include, but are not limited to, monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S--NHS), monomethoxypolyethylene glycol-amine (MePEG-NH₂), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM).

[0159] Briefly, in one embodiment, the hydrophilic polymer which is employed, for example, PEG, is capped at one end by an unreactive group such as a methoxy or ethoxy group. Thereafter, the polymer is activated at the other end by reaction with a suitable activating agent, such as cyanuric halides (for example, cyanuric chloride, bromide or fluoride), diimadozle, an anhydride reagent (for example, a dihalosuccinic anhydride, such as dibromosuccinic anhydride), acyl azide, p-diazoiumbenzyl ether, 3-(p-diazoniumphenoxy)-2-hydroxypropylether) and the like. The activated polymer is then reacted with a polypeptide as described herein to produce a polypeptide derivatized with a polymer. Alternatively, a functional group in a TNFn3 scaffold variant as provided herein can be activated for reaction with the polymer, or the two groups can be joined in a concerted coupling reaction using known coupling methods.

[0160] In some embodiments, a TNFn3 scaffold variant as provided herein can be engineered to provide reactive groups for conjugation. In such scaffolds, the N-terminus and/or C-terminus may also serve to provide reactive groups for conjugation. In other embodiments, the N-terminus may be conjugated to one moiety (such as, but not limited to PEG) while the C-terminus is conjugated to another moiety (such as, but not limited to biotin), or vice versa.

[0161] The term "*in vivo* half-life" is used in its normal meaning, *i.e.*, the time at which 50% of the biological activity of the polypeptide is still present in the body/target organ, or the time at which the activity of the polypeptide is 50% of its initial value. As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, *i.e.*, the time at which 50% of the polypeptide molecules circulate in the plasma or bloodstream prior to being cleared. Determination of serum-half-life is often more simple than determining functional half-life and the magnitude of serum-half-life is usually a good indication of the magnitude of functional *in vivo* half-life. Alternative terms to serum half-life include plasma half-life, circulating half-life, circulatory half-life, serum clearance, plasma clearance, and clearance half-life. The functionality to be retained is normally selected from procoagulant, proteolytic, co-factor binding, receptor binding activity, or other type of biological activity associated with the particular protein.

[0162] The term "increased" with respect to the functional *in vivo* half-life or plasma half-life is used to indicate that the relevant half-life of the polypeptide is statistically significantly increased relative to that of a reference molecule (for example an unmodified polypeptide), as determined under comparable conditions. For instance the relevant half-life may be increased by at least about 25%, such as by at least about 50%, *e.g.*, by at least about 100%, at least about 150%, at least about 200%, at least about 250%, or at least about 500% compared to an unmodified reference molecule. In other embodiments, the half-life may be increased by about at least 1 fold, at least 2 fold, at least 3 fold, at least 4 fold, at least 5 fold, at least 10 fold, at least 20 fold, or at least 50 fold as compared to an unmodified reference molecule.

Scaffold Production

[0163] Recombinant expression of a TNFn3 scaffold variant as provided herein requires construction of an expression vector containing a polynucleotide that encodes the scaffold. Once a polynucleotide encoding a scaffold has been obtained, the vector for the production of scaffold may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing a scaffold encoding nucleotide sequence are described herein. Methods that are well known to

those skilled in the art can be used to construct expression vectors containing scaffold polypeptide coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The disclosure, thus, provides replicable vectors comprising a nucleotide sequence encoding a TNFn3 scaffold variant as provided herein, operably linked to a promoter.

[0164] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a TNFn3 scaffold variant as provided herein. Thus, the disclosure provides host cells containing a polynucleotide encoding a TNFn3 scaffold variant as provided herein, operably linked to a heterologous promoter. Suitable host cells include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli* and *B. subtilis*).

[0165] A variety of host-expression vector systems can be utilized to express a TNFn3 scaffold variant as provided herein. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which can, when transformed or transfected with the appropriate nucleotide coding sequences, express a TNFn3 scaffold variant as provided herein *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing scaffold coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing scaffold coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing scaffold coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing scaffold coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, NSO, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[0166] Expression vectors containing inserts of a gene encoding a TNFn3 scaffold variant as provided herein can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a gene encoding a peptide, polypeptide, protein or a fusion protein in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding the peptide, polypeptide, protein or the fusion protein, respectively. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a nucleotide sequence encoding an antibody or fusion protein in the vector. For example, if the nucleotide sequence encoding the scaffold is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the scaffold insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (*e.g.*, scaffold or multimer thereof) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the protein in *in vitro* assay systems, *e.g.*, binding, agonistic or antagonistic properties of the scaffold.

[0167] Methods useful for the production of a TNFn3 scaffold variant as provided herein are disclosed, for example, in in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

Scaffold Purification

[0168] Once a TNFn3 scaffold variant as provided herein has been produced by recombinant expression, it may be purified by any method known in the art for purification of a protein, for example, by chromatography (*e.g.*, metal-chelate chromatography, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

[0169] The highly stable nature of a TNFn3 scaffold variant as provided herein allows for variations on purification schemes. For example, the thermal stability exhibited by a TNFn3

scaffold variant as provided herein allows for the heating of the crude lysate comprising the scaffolds to remove the bulk of the host cell proteins by denaturation..

[0170] Production of a TNFn3 scaffold variant as provided herein in the research laboratory can be scaled up to produce scaffolds in analytical scale reactors or production scale reactors, as described in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

Labeling or Conjugation of Scaffolds

[0171] A TNFn3 scaffold variant as provided herein can be used in non-conjugated form or conjugated to at least one of a variety of heterologous moieties to facilitate target detection or for imaging or therapy. The scaffolds of the can be labeled or conjugated either before or after purification, when purification is performed.

[0172] Many heterologous moieties lack suitable functional groups to which a TNFn3 scaffold variant as provided herein can be linked. Thus, in some embodiments, the effector molecule is attached to the scaffold through a linker, wherein the linker contains reactive groups for conjugation. In some embodiments, the heterologous moiety conjugated to a TNFn3 scaffold variant as provided herein can function as a linker. In other embodiments, the moiety is conjugated to the scaffold via a linker that can be cleavable or non-cleavable. In one embodiment, the cleavable linking molecule is a redox cleavable linking molecule, such that the linking molecule is cleavable in environments with a lower redox potential, such as the cytoplasm and other regions with higher concentrations of molecules with free sulfhydryl groups. Examples of linking molecules that may be cleaved due to a change in redox potential include those containing disulfides.

[0173] In some embodiments, a TNFn3 scaffold variant as provided herein can be engineered to provide reactive groups for conjugation. In such scaffolds, the N-terminus and/or C-terminus can also serve to provide reactive groups for conjugation. In other embodiments, the N-terminus can be conjugated to one moiety (such as, but not limited to PEG) while the C-terminus is conjugated to another moiety (such as, but not limited to biotin), or vice versa.

[0174] The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moieties (*e.g.*, with thiol, triflate, tresylate, aziridine, oxirane, N-hydroxysuccinimide or a maleimide moiety). The term "PEG" is intended to indicate polyethylene glycol of a molecular weight between 500 and 150,000 Da, including analogues thereof, wherein for instance the terminal OH-group has been replaced by a methoxy group (referred to as mPEG).

[0175] A TNFn3 scaffold variant as provided herein can be derivatized with polyethylene glycol (PEG). PEG is a linear, water-soluble polymer of ethylene oxide repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights which typically range from about 500 daltons to about 40,000 daltons. In a specific embodiment, the PEGs employed have molecular weights ranging from 5,000 daltons to about 20,000 daltons. PEGs coupled to a TNFn3 scaffold variant as provided herein can be either branched or unbranched. PEGs are commercially available from Nektar Inc., Sigma Chemical Co. and other companies.

[0176] Briefly, the hydrophilic polymer which is employed, for example, PEG, is capped at one end by an unreactive group such as a methoxy or ethoxy group. Thereafter, the polymer is activated at the other end by reaction with a suitable activating agent, such as cyanuric halides (for example, cyanuric chloride, bromide or fluoride), carbonyldiimidazole, an anhydride reagent (for example, a dihalo succinic anhydride, such as dibromosuccinic anhydride), acyl azide, p-diazoniumbenzyl ether, 3-(p-diazoniumphenoxy)-2-hydroxypropylether) and the like. The activated polymer is then reacted with a polypeptide as described herein to produce a polypeptide derivatized with a polymer. Alternatively, a functional group in a TNFn3 scaffold variant as provided herein can be activated for reaction with the polymer, or the two groups can be joined in a concerted coupling reaction using known coupling methods.

[0177] In other embodiments, a TNFn3 scaffold variant as provided herein can be conjugated to a diagnostic or detectable agent. Such scaffolds can be useful for monitoring or prognosing the development or progression of a disease as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can be accomplished by coupling the scaffold to detectable substances including, but not limited to

various enzymes, such as but not limited to horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as but not limited to streptavidin/biotin and avidin/biotin; fluorescent materials, such as but not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as, but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials; positron emitting metals using various positron emission tomographies, nonradioactive paramagnetic metal ions, and molecules that are radiolabeled or conjugated to specific radioisotopes.

[0178] The present disclosure further provides uses of scaffolds conjugated to a therapeutic moiety. A scaffold may be conjugated to a therapeutic moiety such as a cytotoxin, *e.g.*, a cytostatic or cytotoxic agent, a therapeutic agent or a radioactive metal ion, *e.g.*, alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Exemplary therapeutic moieties can be found, *e.g.*, in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

[0179] Further, a scaffold may be conjugated to a therapeutic moiety or drug moiety that modifies a given biological response. Therapeutic moieties or drug moieties are not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Exemplary therapeutic moieties for conjugation or fusion to a TNFn3 scaffold or multimeric scaffold as provided herein can be found in, *e.g.*, PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

[0180] The therapeutic moiety or drug conjugated to a TNFn3 scaffold variant as provided herein can be chosen to achieve the desired prophylactic or therapeutic effect(s) for a particular disorder in a subject. A clinician or other medical personnel should consider the following when deciding on which therapeutic moiety or drug to conjugate to a scaffold: the nature of the disease, the severity of the disease, and the condition of the subject.

Pharmaceutical Compositions

[0181] In another aspect, the present disclosure provides a composition, for example, a pharmaceutical composition, containing one or a combination of scaffolds or multimeric

scaffolds as provided herein, formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of, for example, but not limited to two or more different TNFn3 scaffold variants as provided herein. For example, a pharmaceutical composition can comprise a combination of scaffolds that bind to different epitopes on the target antigen or that have complementary activities. In a specific embodiment, a pharmaceutical composition comprises a multimeric scaffold.

[0182] Pharmaceutical compositions can also be administered in combination therapy, such as, combined with other agents. For example, the combination therapy can include a TNFn3 scaffold variant as provided herein combined with at least one other therapy wherein the therapy may be immunotherapy, chemotherapy, radiation treatment, or drug therapy.

[0183] The pharmaceutical compounds provided herein can include one or more pharmaceutically acceptable salts. Examples of such salts include acid addition salts and base addition salts.

[0184] A pharmaceutical composition provided herein can also include a pharmaceutically acceptable antioxidant. Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil- soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[0185] Examples of suitable aqueous and non-aqueous carriers that can be employed in the pharmaceutical compositions provided herein include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[0186] These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be

ensured both by sterilization procedures and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[0187] Suitable and exemplary compositions and formulations, and methods of preparing such compositions and formulations can be found, *e.g.*, in PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328.

Methods of Using Scaffolds

[0188] TNFn3 scaffold variants as provided herein have *in vitro* and *in vivo* diagnostic and therapeutic utilities. For example, these molecules can be administered to cells in culture, *e.g.* *in vitro* or *ex vivo*, or in a subject, *e.g.*, *in vivo*, to treat, prevent or diagnose a variety of disorders.

[0189] The disclosure also provides methods of using a TNFn3 scaffold variant as provided herein. The present disclosure also encompasses the use of a TNFn3 scaffold variant as provided herein for the prevention, diagnosis, management, treatment or amelioration of one or more symptoms associated with diseases, disorders of diseases or disorders, including but not limited to cancer, inflammatory and autoimmune diseases, infectious diseases either alone or in combination with other therapies. The disclosure also encompasses the use of a TNFn3 scaffold variant as provided herein conjugated or fused to a moiety (*e.g.*, therapeutic agent or drug) for prevention, management, treatment or amelioration of one or more symptoms associated with a disease, disorder, or infection, including but not limited to an autoimmune disease, an inflammatory disease, a proliferative disease such as cancer, an infectious disease, a respiratory disease, a gastrointestinal disease, diabetes, lupus, or obesity either alone or in combination with other therapies.

[0190] The disclosure also provides methods of targeting epitopes not easily accomplished with traditional antibodies. For example, in one embodiment, a TNFn3 scaffold variant as

provided herein can be used to first target an adjacent antigen and while binding, another binding domain may engage the cryptic antigen.

[0191] The disclosure also provides methods of using the scaffolds to bring together distinct cell types. In one embodiment, a TNFn3 scaffold variant as provided herein can bind a target cell with one binding domain and recruit another cell via another binding domain. In another embodiment, the first cell may be a cancer cell and the second cell is an immune effector cell such as an NK cell. In another embodiment, a TNFn3 scaffold variant as provided herein can be used to strengthen the interaction between two distinct cells, such as an antigen presenting cell and a T cell to possibly boost the immune response.

[0192] The disclosure also provides methods of diagnosing diseases. TNFn3 scaffold variant as provided herein that bind a specific target associated with a disease can be implemented in a method used to diagnose the disease. In one embodiment, a TNFn3 scaffold variant as provided herein can be used in a method to diagnose a disease in a subject, the method comprising obtaining a sample from the subject, contacting the target with the scaffold in the sample under conditions that allow the target:scaffold interaction to form, identifying the target: scaffold complex and thereby detecting the target in the sample.

[0193] In some embodiments, the target is an antigen associated with disease. In another embodiment, the target is a cytokine, inflammatory mediator, and intracellular antigen, a self-antigen, a non-self antigen, an intranuclear antigen, a cell-surface antigen, a bacterial antigen, a viral antigen or a fungal antigen. In other embodiments, the disease to be diagnosed is described herein.

[0194] The disclosure also provides methods of imaging specific targets. In one embodiment, a TNFn3 scaffold variant as provided herein conjugated to imaging agents such as green fluorescent proteins, other fluorescent tags (Cy3, Cy5, Rhodamine and others), biotin, or radionuclides may be used in methods to image the presence, location, or progression of a specific target. In some embodiments, the method of imaging a target comprising a TNFn3 scaffold variant as provided herein is performed *in vitro*. In other embodiments, the method of imaging a target comprising a TNFn3 scaffold variant as provided herein is performed *in vivo*. In other embodiments, the method of imaging a target comprising a TNFn3 scaffold

variant as provided herein is performed by MRI, PET scanning, X-ray, fluorescence detection or by other detection methods known in the art.

[0195] The disclosure also provides methods of monitoring disease progression, relapse, treatment, or amelioration using a TNFn3 scaffold variant as provided herein. In one embodiment, methods of monitoring disease progression, relapse, treatment, or amelioration is accomplished by the methods of imaging, diagnosing, or contacting a compound/target with a TNFn3 scaffold variant as provided herein.

Kits

[0196] Also within the scope of the disclosure are kits comprising a compositions as provided herein (*e.g.* scaffolds,) and instructions for use. The kit can further contain at least one additional reagent, or one or more additional scaffolds. Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

Equivalents

[0197] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

[0198] All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. This application claims the benefit of priority to U.S. Provisional Application No.: 61/323,708 filed April 13, 2010, the entire contents of which are incorporated herein by reference. Additionally, PCT Application No. PCT/US2008/012398, filed on October 10, 2008 and published as International Publication No. WO 2009/058379 is hereby incorporated by reference herein in its entirety for all purposes.

[0199] The practice of the appended claims will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art.

Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, ed. (1989) *Molecular Cloning A Laboratory Manual* (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook *et al.*, ed. (1992) *Molecular Cloning: A Laboratory Manual*, (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) *DNA Cloning*, Volumes I and II; Gait, ed. (1984) *Oligonucleotide Synthesis*; Mullis *et al.* U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) *Nucleic Acid Hybridization*; Hames and Higgins, eds. (1984) *Transcription And Translation*; Freshney (1987) *Culture Of Animal Cells* (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) *A Practical Guide To Molecular Cloning*; the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) *Gene Transfer Vectors For Mammalian Cells*, (Cold Spring Harbor Laboratory); Wu *et al.*, eds., *Methods In Enzymology*, Vols. 154 and 155; Mayer and Walker, eds. (1987) *Immunochemical Methods In Cell And Molecular Biology* (Academic Press, London); Weir and Blackwell, eds., (1986) *Handbook Of Experimental Immunology*, Volumes I-IV; *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); and in Ausubel *et al.* (1989) *Current Protocols in Molecular Biology* (John Wiley and Sons, Baltimore, Md.).

[0200] General principles of antibody engineering are set forth in Borrebaeck, ed. (1995) *Antibody Engineering* (2nd ed.; Oxford Univ. Press). General principles of protein engineering are set forth in Rickwood *et al.*, eds. (1995) *Protein Engineering, A Practical Approach* (IRL Press at Oxford Univ. Press, Oxford, Eng.). General principles of antibodies and antibody-hapten binding are set forth in: Nisonoff (1984) *Molecular Immunology* (2nd ed.; Sinauer Associates, Sunderland, Mass.); and Steward (1984) *Antibodies, Their Structure and Function* (Chapman and Hall, New York, N.Y.). Additionally, standard methods in immunology known in the art and not specifically described are generally followed as in *Current Protocols in Immunology*, John Wiley & Sons, New York; Stites *et al.*, eds. (1994) *Basic and Clinical Immunology* (8th ed; Appleton & Lange, Norwalk, Conn.) and Mishell and Shiigi (eds) (1980) *Selected Methods in Cellular Immunology* (W.H. Freeman and Co., NY).

[0201] Standard reference works setting forth general principles of immunology include *Current Protocols in Immunology*, John Wiley & Sons, New York; Klein (1982) J.,

Immunology: The Science of Self-Nonself Discrimination (John Wiley & Sons, NY); Kennett *et al.*, eds. (1980) Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses (Plenum Press, NY); Campbell (1984) "Monoclonal Antibody Technology" in Laboratory Techniques in Biochemistry and Molecular Biology, ed. Burden *et al.*, (Elsevier, Amsterdam); Goldsby *et al.*, eds. (2000) Kuby Immunology (4th ed.; H. Freeman & Co.); Roitt *et al.* (2001) Immunology (6th ed.; London: Mosby); Abbas *et al.* (2005) Cellular and Molecular Immunology (5th ed.; Elsevier Health Sciences Division); Kontermann and Dubel (2001) Antibody Engineering (Springer Verlag); Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press); Lewin (2003) Genes VIII (Prentice Hall 2003); Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Press); Dieffenbach and Dveksler (2003) PCR Primer (Cold Spring Harbor Press).

[0202] All of the references cited above, as well as all references cited herein, are incorporated herein by reference in their entireties.

[0203] The following examples are offered by way of illustration and not by way of limitation.

Examples

Example 1: Construction and characterization of stabilized TNFn3 scaffold variants

[0204] This example demonstrates the construction of highly stable TNfn3 scaffold variants with a minimal number (up to four) of mutations introduced. Structural differences between TNfn3 and the more stable structural homolog FNfn10 were identified. We then designed mutations at these positions intended to mimic the structure of the more stable FNfn10. We also identified the most stabilizing mutations from among the four previously identified by Strickler *et al.*, *Biochemistry*, 45:2761-2766 (2006). By combining just 3 or 4 of these mutations, we increased the T_m of TNfn3 to as high as 83 °C and $\Delta G_{\text{Unfolding}}$ to as high as 9.4 kcal/mol.

Materials and Methods

Protein Expression and Purification

[0205] Genes encoding wild-type TNfn3 and all described variants were cloned into a pET22b-based bacterial expression vector (Novagen) and sequences of all constructs were confirmed by DNA sequencing. The wild-type TNfn3 protein sequence from which all other constructs were derived was as follows:

ARLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTEDENQYSIGNLK
PDTEYEVSLISRRGDMSSNPAKETFTTGL[GGSGGTLEHHHHHH] (SEQ ID NO:40)

[0206] Residues enclosed in brackets constitute a tag sequence used to facilitate purification by Ni affinity chromatography. An extra N-terminal alanine (underlined, italics) was also present due to a restriction site used for cloning. The initiator methionine was efficiently removed by bacterial processing and its absence in expressed proteins was confirmed by mass spectrometry.

[0207] Proteins were expressed in *E. Coli* BL21(DE3) (Lucigen) by overnight growth in auto-inducing Magic Media (Invitrogen). Proteins were initially purified by His-tag using Ni-NTA Superflow resin (Qiagen) and further purified by ion exchange using a HiTrap Q HP column (GE Healthcare). Protein purity was confirmed by SDS-PAGE and protein masses were confirmed by mass spectrometry.

Size Exclusion Chromatography

[0208] 25 μ l of 1 mg/mL protein in 10 or 50 mM potassium phosphate, 50 mM NaCl pH = 7.4 was loaded onto a Superdex75 5/150 GL column (GE Healthcare) equilibrated with 20 mM potassium phosphate, 150 mM NaCl pH = 7.4. Proteins were eluted at a flowrate of 0.15 mL/min using the same buffer.

Differential Scanning Calorimetry

[0209] For DSC analysis, proteins were dialyzed into 10 mM potassium phosphate, 50 mM NaCl, pH = 7.4, and diluted to a final concentration of 100 μ M in the same buffer. These samples were then dialyzed into 10 mM HEPES, 50 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine (TCEP), pH = 7.4 and concentrations adjusted to 100 μ M. Samples were analyzed using a VP-DSC (MicroCal, LLC). Samples were heated from 30 °C to 110

°C using a scan rate of 90 °C/hr. Buffer blank and progress baselines were subtracted from all data. All samples exhibited > 80% reversibility, as assayed by cooling, reheating and comparing recovery of ΔH_{cal} values derived from integration of the transition peak. All samples showed $\Delta H_{\text{cal}}/\Delta H_{\text{VH}}$ (Van't Hoff Enthalpy) ~ 1 , so for final analysis, curves were fit to a simple 2-state model to derive T_m and ΔH_m using analysis software provided by the manufacturer.

Denaturant Titrations

[0210] Protein samples were dialyzed into 50 mM potassium phosphate, 50 mM NaCl, pH = 7.4 and diluted to a final concentration of 5 μM in varying concentrations (0-3M) of guanidine thiocyanate (GdnSCN), 50 mM potassium phosphate, 50 mM NaCl, pH = 7.4. After mixing, all samples were incubated at room temperature (23 °C) for at least 24 hours. Fluorescence emission spectra (310-400 nm) were then recorded with a Fluoromax-4 Spectrofluorometer (Horiba Jobin Yvon) at 23 °C using an excitation wavelength of 295 nm. Denaturation curves were constructed by plotting fluorescence intensity vs. [GdnSCN] at the wavelength that showed the largest change in intensity over the course of the titration (360-370 nm). Data were then fit to a two-state model as previously described to derive m -values, $\Delta G_{\text{unfolding}}$, and $[D]_{50\%}$ (Santoro and Bolen, 1988).

Protease Stability Assays

[0211] Proteins were diluted to a final concentration of 50 μM in 20 mM Tris, 150 mM NaCl, 10 mM CaCl_2 , pH = 8.0 and thermolysin (MP Biopharmaceuticals) was added at a final concentration of 0.2 mg/mL. Samples were then incubated at 42 °C for varying amounts of time. At each time point, reactions were quenched by addition of EDTA to a final concentration of 100 mM. Samples were then analyzed by reducing SDS-PAGE.

Results

Stabilizing TNfn3 by Point Mutation

[0212] Although TNfn3 and the more stable homolog FNfn10 share only ~24% sequence identity, they share an essentially identical overall structure (**Figure 1A, B, C**). Thus, we first looked to FNfn10 to provide clues as to how TNfn3 might be made more stable. Previous

studies have shown that swapping the hydrophobic core residues of FNfn10 with those of TNfn3 results in significant destabilization, suggesting that features of the FNfn10 core contribute to its increased stability (Billings *et al.*, *J.Mol.Biol.* 375:60-571 (2008)). While this same work implicated surface residues as important contributors to stability as well, we chose to focus our efforts toward residues in the hydrophobic core. Focusing stabilizing mutations to the hydrophobic core carries the added benefits of masking the mutations from host B-cells and leaving surface positions available for mutation when introducing novel binding sites.

[0213] By many measures, the hydrophobic cores of TNfn3 and FNfn10 are very similar. Most core residues bury a similar percentage of their surface in the two proteins and also exhibit a similar number of contacts. Furthermore, the TNfn3 and FNfn10 cores bury similar amounts of total surface (Billings *et al.*, *J.Mol.Biol.* 375:60-571 (2008)). However, the two homologs do show some structural differences. Perhaps most striking, are differences in A/G strand packing (**Figure 1B, C**). In FNfn10, the A and G strands pack together efficiently through tightly interdigitating hydrophobic residues. In contrast, in TNfn3, a charged glutamate is substituted at position 86 in the center of the G strand, despite the fact that this position points inward toward the hydrophobic core. This is the most non-conservative substitution in the TNfn3 core compared to FNfn10. E86 is unsurprisingly less buried than the equivalent I90 in FNfn10 since its carboxylic acid group is pointed outward toward solvent and the result is an apparent “peeling apart” of the β -sandwich in this area. Consistent with an unfavorable effect of glutamic acid at position 86, previous studies showed that an E86A mutation stabilizes TNfn3 by ~1.6 kcal/mol (Cota *et al.*, *J.Mol.Biol.* 302:13-725 (2000)). Additionally, Jacobs *et al.* have cited unpublished data indicating that the E86I mutation increases the T_m of TNfn3, though no further description or characterization of this mutant was reported (Jacobs *et al. Protein Eng.Des.Sel.* 25:107-117 (2012)). Packing in the A/G strand region is seemingly made worse by an alanine substitution at position 84 that appears unable to pack as densely as the bulkier isoleucine at the equivalent position 88 in FNfn10. Flawed packing at positions 84 and 86 of TNfn3 is also quantitatively supported by data from a previous analysis carried out by Cota *et al* which showed that A84 and E86 make

fewer interresidue contacts and bury a smaller percentage of their surface area than their counterparts in FNfn10 (Cota *et al.*, *J.Mol.Biol.* 302:13-725 (2000)).

[0214] In view of these data, we made and characterized the stability of two TNfn3 mutants, A84V and E86I, designed to improve A/G strand packing and mimic the seemingly more optimal structure of FNfn10 (**Figure 1A, B, C**). DSC experiments showed that A84V improved the stability of TNfn3 only slightly, increasing the T_m by ~1 °C. However, E86I substantially improved stability, increasing the T_m by ~11 °C (**Table 1**).

Table 1: Differential Scanning Calorimetry Parameters of TNfn3 Variants

Clone	T_m (°C)	ΔH_m (kcal/mol)	ΔS_m (cal/mol·K)
Wild-type TNfn3	58.7	73	220
Q7K	59.9	69	207
L19K	56.7	67	203
D49K	63.7	86	255
T89K	63.9	79	234
A84V	59.8	71	213
E86I	69.9	90	262
I32F	61.0	80	239
E86I, T89K (CM2)	74.9	95	272
E86I, T89K, D49K (CM3)	80.5	100	283
E86I, T89K, D49K, I32F (CM4)	82.6	106	298

[0215] The TNfn3 and FNfn10 core structures also differ near the heavily buried W22. W22 is the most conserved residue among FN3 family members, and even conservative substitution at this position is substantially or catastrophically destabilizing (Cota *et al.*, *J.Mol.Biol.* 302:13-725 (2000)). W22 makes extensive contacts with other core residues and is thought to be a part of the common “folding nucleus” among FN3 domains (Cota *et al.*, *J.Mol.Biol.* 305:1185-1194 (2001)). Because of its importance, we reasoned that differences in the local environment and packing of W22 might have significant effects on TNfn3 stability. Although W22 is buried to a similar extent in TNfn3 and FNfn10, it makes fewer interresidue contacts in TNfn3 (Cota *et al.*, *J.Mol.Biol.* 302:13-725 (2000)). Structural examination shows that this is mostly due to a “capping” interaction that exists between Y32

and W22 in FNfn10 that is absent in TNfn3 due to a Y to I substitution (**Figure 1C**). Across FN3 family members, Y/F is favored at position 32 and F in particular is favored among other FN3s in human tenascin. Thus, we reasoned that an I32F mutation might stabilize TNfn3 by improved packing with W22. DSC experiments revealed that the I32F mutant indeed modestly stabilized TNfn3 by 2.3 °C (**Table 1**).

[0216] As a supplement to the mutations identified above, we also dissected the individual contributions of four previously reported surface charge optimizing mutations described by Strickler *et al* (Q7K, L19K, D49K and T89K) (**Figure 1A**) (Strickler *et al.*, Biochemistry, 45:2761-2766 (2006)). Although simultaneous introduction of these mutations increased the T_m of TNfn3 to 66 °C, previous work did not characterize the individual effects of these mutations, leaving open the possibility that one or more might be destabilizing. DSC analysis revealed that D49K and T89K were clearly stabilizing, each increasing the T_m by ~5 °C compared to wild-type (**Table 1**). Q7K was slightly stabilizing, increasing the T_m by ~1 °C and L19K was slightly destabilizing, decreasing the T_m by ~2 °C. These results suggest that the 10 °C increase in T_m previously reported for the quadruple mutant is due mostly to D49K and T89K, with a small additional positive contribution from Q7K and a small negative contribution from L19K.

[0217] We next examined whether TNfn3 could be stabilized to a greater extent by combining multiple stabilizing mutations. We began with the most stabilizing mutation, E86I, and added the next most stabilizing mutations in succession. We chose FNfn10 as an approximate benchmark for stability given its success as an alternative scaffold and similarity to TNfn3. We therefore aimed for a $T_m > \sim 80$ °C using the fewest mutations possible. DSC analysis showed that a triple mutant termed CM3 (E86I, T89K, D49K) and a quadruple mutant termed CM4 (E86I, T89K, D49K, I32F) met this criteria with T_m values of 81 and 83 °C respectively (**Table 1**).

Denaturation and protease inhibition studies

[0218] The point mutation strategy resulted in TNFn3 scaffold variants that were highly stabilized, exhibiting T_m values up to 82.6 for CM4 (**Table 1**, **Figure 2A**). In order to further examine the nature of the stability increases, and to assess whether the increases in T_m translated to improved folding free energies, we performed a denaturant titration for CM4

using guanidine thiocyanate (GdnSCN) or guanidine HCl (GdnHCl), and monitored unfolding by intrinsic tryptophan fluorescence (Figure 2B, Table 2). CM4 had an increased $[\text{GdnSCN}]_{50\%}$ value as compared to wild-type, and a $\Delta G_{\text{unfolding}}$ value of 9.45 kcal/mol (Table 2).

Table 2: Chemical Denaturation Parameters of TNfn3 Variant CM4

Clone	$\Delta G_{\text{Unfolding}}$ (kcal/mol) [†]	<i>m</i> -value (kcal/mol·M) [†]	$[\text{GdnSCN}]_{50\%}$ (M) [†]
WT TNfn3	5.70 ± 0.50	7.18 ± 0.65	0.79 ± 0.01
CM4	9.46 ± 1.14	7.46 ± 0.89	1.27 ± 0.01

[0219] To further probe the nature of increased stability in CM4, we assessed its resistance to proteolysis. CM4 and WT TNfn3 were incubated with thermolysin for varying periods of time and their degree of degradation was assessed by SDS-PAGE. Because thermolysin preferentially cleaves at hydrophobic residues most often found in the hydrophobic core (*i.e.* I,L,F,V) thermolysin resistance can be reasonably regarded as reflecting the “foldedness” of a protein sample (Park and Marqusee, 2005; Minde *et al.*, 2012; Heinrikson, 1977). We observed that all the WT and CM4 samples showed a fast cleavage event which resulted in a small mass shift at early time points (Figure 2C). Mass spectrometry analyses revealed that this cleavage was the same for all samples including wild-type and corresponded to the loss of a C-terminal tag sequence present in all of the constructs (data not shown). Thus, this initial cleavage event does not reflect the stability of the FN3 domain itself. At longer timescales, wild-type TNfn3 was almost completely degraded within 4 hours (Figure 2C). In contrast, CM4 remained almost completely intact even after 24 hours. Thus, these proteolysis experiments are consistent with chemical denaturation and DSC data and indicate that CM4 is substantially more stable than wild-type TNfn3.

[0220] In this Example, we produced highly stable TNfn3 variants using just 3 or 4 total mutations. This small level of perturbation stands in contrast to other efforts that, although successful, have yielded proteins that are extensively mutated compared to wild-type TNfn3

(Jacobs *et al. Protein Eng.Des.Sel.* 25:107-117 (2012); Hu *et al. Structure* 16:1799-1805 (2008); Dantas *et al. J.Mol.Biol.* 332:449-460 (2003)). Interestingly, the variants reported here exhibit similar levels of stability as those generated by these other approaches, suggesting that many of the mutations introduced in these strategies may have neutral or negative effects on stability and may, therefore, be unnecessary.

[0221] The I32F, A84V and E86I mutations were intended to improve packing of the A/G strands as well as optimize the environment of W22 in a way similar to that observed in the structure of FNfn10. Denaturant *m*-values are known to correlate with the amount of hydrophobic surface buried in the native state (Myers *et al.*, 1995). Although modest, the increased *m*-value of CM4 relative to wild-type TNfn3 is thus, consistent with improved/expanded core packing (**Table 2**). While all three of the FNfn10-inspired mutations were stabilizing to some degree, the E86I mutation was particularly effective, increasing *T_m* by ~11 °C. The strong stabilizing effect of E86I suggests that differences in A/G strand interactions might be a significant contributor to the difference in stability between TNfn3 and FNfn10. Consistent with this notion, previous studies by Cota *et al* identified stark differences between the two proteins in structural dynamics and response to mutation in this region (Cota *et al.*, *J.Mol.Biol.* 302:13-725 (2000)).

[0222] The Q7K, L19K, D49K and T89K mutations examined here were previously reported, however, their effects had only been investigated in combination and their individual effects on stability had not been characterized (Strickler *et al.*, *Biochemistry*, 45:2761-2766 (2006)). While Q7K, D49K and T89K were all confirmed to be stabilizing, L19K was found to be mildly destabilizing. L19 is a part of a β -bulge structure comprised by positions 11, 12 and 19 that is conserved among FN3 domains. A previous study exploring the sequence space of the β -bulge residues in FNfn10 showed that positions 11, 12 and 19 favor a β -X- β , β -X-X or X-X- β sequence motif where β is a β -branched amino acid and X is any amino acid (Dutta *et al. J.Mol.Biol.* 382:721-733 (2008)). Variants lacking one of these motifs were frequently observed to be destabilized. The L19K mutation in the context of TNfn3 eliminates an existing X-X- β motif. Thus, we speculate that while L19K may optimize surface charge interactions, it may destabilize the β -bulge structure leading to the observed decrease in *T_m*. Among the surface charge mutations investigated, D49K and T89K

were particularly effective. Examining the TNfn3 structure, we suspect that both of these mutations act by neutralizing clusters of like charge. D49 is located across from E33 in the neighboring β -strand, possibly resulting in electrostatic repulsion. A similar juxtaposition of negatively charged sidechains in FNfn10 was found to be destabilizing, but could be ameliorated if one of the charges was reversed (Koide *et al. Biochemistry* 40:10326-10333 (2001)). Similarly, T89 is located near the C-terminus and a cluster of negative charge previously implicated as potentially destabilizing (Meekhof *et al. J.Mol.Biol.* 282:181-194 (1998)). While not wishing to be bound by theory, introduction of a positive charge into this cluster might help to alleviate charge repulsions.

Example 2. Construction of a 2 loop library on the a stabilized TNFn3 scaffold

[0223] A new library can be constructed based upon any of the TNFn3 scaffold variants provided herein. This example provides a method for generation of a 2 loop a library based on the CM4 scaffold variant (SEQ ID NO:41) in phage. In this example diversity is introduced into the BC and FG loops. The BC loop diversity is introduced using PCR and the FG loop diversity is introduced using Kunkel mutagenesis (Table 3). A library of at least 1.0×10^{10} members can be constructed.

Table 3: Degenerate oligonucleotides for CM4 library construction

Oligo	Loop	Sequence	Seq ID
BC9	BC	ACCGCGCTGATTACCTGGTTTNNKSCGNNKGSTNNKNNKN NKGGCTTTGAACTGACCTATGGC	42
BC11	BC	ACCGCGCTGATTACCTGGTTTNNKCCGBSTNNKNNKNNKN NKNNKNNKGACGGCTTTGAACTGACCTATGGC	43
BC12	BC	ACCGCGCTGATTACCTGGTTTVMACCGNNKNNKNNKRRRC RGCNNKATTNNKGGTTTTGAACTGACCTATGGC	44
FG9	FG	TATGAAGTGAGCCTGATTAGCNNKAMSNNKNNKGGTNNK NNKNNKGYCAAAATAACCTTTAAAACCGGTCTC	45
FG10	FG	TATGAAGTGAGCCTGATTTGCNNKAMSNNKNNKNNKNNK RGCAACCCGGYGAAAATAACCTTTAAAACCGGTCTC	46
FG11	FG	TATGAAGTGAGCCTGATTTGCNNKAMSNNKNNKGGTNNK	47

		NNKAGCAACCCGGYGAAAATAACCTTTAAAACCGGTCTC	
DErev	DE	CCGGTTTCAGGTTACCAATGCTATAMNNMNNMNNMNNM NNMNNCAGTTTTATGGTGGTGCGATCGCC	55
FG9 rev	FG	GAGACCGGTTTTAAAGGTTATTTTCRCMNNMNNMNNACC MNNMNNNSKTMNNGGAAATCAGGCTCACTTCATATTCGGT	56
FG10 rev	FG	GAGACCGGTTTTAAAGGTTATTTTCRCCGGGTTGCTMNN MNNMNNMNNNSKTMNNGGAAATCAGGCTCACTTCATATT CGGT	57
FG11 rev	FG	GAGACCGGTTTTAAAGGTTATTTTCRCCGGGTTGCTMNNM NNACCMNNMNNNSKTMNNGGAAATCAGGCTCACTTCATAT TCGGT	58

Experimental Procedure

[0224] The BC loop diversity is made by using primers which randomize one or more position with the BC loop and/or introduces additional amino acids with in the loop. Exemplary, non-limiting primers BC9, 11, or 12 which can be used are provided in Table 3. These primers anneal on their 3' ends to the TNFn3 DNA and the degeneracy forms a library upon completion of the PCR. These PCR products are amplified with flanking primers to make a complete TNFn3-CM4 gene flanked by restriction sites (e.g., *NcoI* and *KpnI*) which is then digested with the appropriate restriction enzymes (e.g., *NcoI* and *KpnI*) and ligated into a phage display vector. The DNA is transformed into *E. coli* by electroporation. The final diversity of the resulting BC library is expected to be about 3.0×10^9 members.

[0225] After electroporation, the BC library is incubated for about 1 hour at 37°C with shaking. A helper phage (e.g., M13K07 helper phage) is added and after about one hour the cells are diluted to a larger volume and grown at 37°C with shaking overnight. The next day phage are removed and concentrated from the supernatant, for example by precipitation with PEG 8000. BC library phage are used to infect *E. coli* (e.g., CJ236 *E. coli*). After a one hour infection, cells are diluted into 2xYT with 100 µg/mL carbenicillum and grown overnight with shaking at 37°C. The next day phage are removed and concentrated from the

supernatant, for example, by precipitation with PEG 8000. The BC library may be screened directly or may be used as a template for randomization of additional loops.

[0226] Where additional loops are to be randomized single stranded DNA is recovered by using a Qiagen (Valencia, CA) QIAprep spin M13 kit. This DNA can serve as the template for Kunkel mutagenesis using primers which randomize one or more additional loop regions. (Kunkel TA *et al.*, *Methods Enzym.* 204, 125, 1991). For example, the exemplary, non-limiting primers FG9, FG10 and FG11 provided in Table 3 may be used to introduce diversity into the FG loop. The resulting libraries may be screening using methods well known in the art to identify specific clones which bind to a particular antigen.

Example 3. Construction of a 3 loop library on the a stabilized TNFn3 scaffold

[0227] A three loop library can readily be constructed based upon any of the TNFn3 scaffold variants provided herein. This example provides a method for generation of a BC, DE, FG three loop library based on the CM4 scaffold variant (SEQ ID NO:41) in phage. In this example diversity is introduced by randomizing the sequences of the BC, DE, and FG loops. Exemplary, non-limiting primers, DErev, FG9rev, FG10rev, and FG11rev, are shown in Table 3. Briefly, single stranded DNA from a two loop CM4 scaffold BC loop library (e.g., the library in Example 2 above) is used as a template for a PCR with the DE rev primer in Table 3. This PCR will generate a product that contains a portion of the CM4 scaffold with BC and DE randomization. A second PCR is performed using the BC, DE loop randomized PCR product as template for amplification with the FG9rev, FG10rev and FG11rev primers listed in Table 3. The resulting PCR products are amplified with flanking primers to make a complete CM4 gene flanked by restriction enzyme sites (e.g., *NcoI* and *KpnI*) which is then cut with the appropriate restriction enzymes (e.g., *NcoI* and *KpnI*) and ligated into a vector (e.g., phage display vector) for expression and screening. The DNA is transformed into *E. coli* by electroporation.

[0228] After electroporation, the library is incubated for about 1 hour at 37°C with shaking. Helper phage (e.g., M13K07 helper phage) is added and after about one hour the cells are diluted to a larger volume and grown at 37°C with shaking overnight. The next day the phage are purified from the culture supernatant, for example, by precipitation with a saline PEG 8000 solution. The final diversity of the resulting three loop library is expected to be at

least about 1.5×10^9 members. The resulting libraries may be screening using methods well known in the art to identify specific clones which bind to a particular antigen.

Example 4. Design of Randomized Library using AB, CD and EF loops

[0229] As with loops on the opposite side of the TNFn3 molecule, the AB, CD and EF loops vary in length and sequence composition for different Fn3 domains. The AB and CD loops are usually 5 to 9 amino acids long, although exceptions occur for some Fn3 domains which have AB and/or CD loops longer or shorter than this. The most common length within this data set was 6 residues for the CD loop (31% of sequences), and 7 residues for the AB loop (61% of sequences). Length variation occurs less frequently for the EF loop, and an 8 residue loop is most commonly observed (80% of sequences). Both the AB and CD loops show significant diversity in sequence and do not show overt preferences for specific amino acids in particular positions. An exception is the final position in the AB loop which is often Ser or Thr (58/103 sequences). The sequences of EF loops reveals strongly preferred amino acids at specific positions, though this is restricted to those that are 8 residues long. A Leu at position 3 within these loops is strongly conserved (76/82 sequences), and given the sidechain of this residue is buried in each of the structures, it is likely to be important for the structural integrity of the scaffold. A Pro residue is also commonly observed at position 5 (44/82 sequences), while Gly, Asn, Asp and Ser are often in position 2 (71/82 sequences) and Thr in position 7 (40/82).

[0230] A further practical consideration in the design of these Tn3 libraries is to identify an alternative to the “NNK” (N = A, G, T, C; K = G, T) mixed codon scheme typically used in degenerate oligonucleotides to code for any amino acid. Although the “NNK” mixture gives 32 different codons which code for all 20 amino acids, they are not encoded equally (Table 4). For instance, 3/32 codons in the “NNK” scheme code for Leu (CTG, CTT, TTG), but only 1/32 codes for Asp (GAT). In addition, the “NNK” mixture encodes one stop codon (TAG) and a Cys codon (TGT), neither of which is desirable when generating naïve libraries. In considering an alternative scheme, we took note of the fact that synthetic antibody libraries have been described which encode CDR sequences composed of a small subset of amino acids. Antibody libraries with CDR's composed of just 4 amino acids (Tyr, Ala, Asp, Ser), or even a binary pair (Tyr, Ser) have been shown to yield specific high affinity mAbs to

protein antigens (Fellouse *et al.*, Proc. Natl. Acad. Sci. USA. 2004, 101: 12467-72; J. Mol. Biol. 2005, 348: 1153-62). Similarly, a library of scaffold proteins with randomized loop sequences comprising just Tyr and Ser also yielded specific binders to a protein target (Koide *et al.*, Proc. Natl. Acad. Sci. USA. 2007, 104: 6632-7). Although libraries containing highly restricted sets of amino acids are able to produce specific binding proteins, it is likely that the diversity of binders that are obtained from such a library will be limited. We therefore designed an alternate "NHT" mixed codon scheme for introducing diversity into a TNFn3 library (H = A, T, C). "NHT" mixes (Table 5) code for a reasonable subset of the 20 amino acids, but avoid the disadvantages described with "NNK" mixed codons (Table 4). This scheme generates 12 codons that code for 12/20 amino acids, that is, each codon codes for a unique amino acid. Moreover, there are no stop or Cys codons.

Table 4: Amino acids encoded by "NNK" codon mixtures

A	AAG = Lys	ATG = Met	ACG = Thr	AGG = Arg	G
	AAT = Asn	ATT = Ile	ACT = Thr	AGT = Ser	T
G	GAG = Glu	GTG = Val	GCG = Ala	GGG = Gly	G
	GAT = Asp	GTT = Val	GCT = Ala	GGT = Gly	T
C	CAG = Gln	CTG = Leu	CCG = Pro	CGG = Arg	G
	CAT = His	CTT = Leu	CCT = Pro	CGT = Arg	T
T	TAG = STOP	TTG = Leu	TCG = Ser	TGG = Trp	G
	TAT = Tyr	TTT = Phe	TCT = Ser	TGT = Cys	T
	A	T	C	G	

Table 5: Amino acids encoded by "NHT" codon mixtures

A	AAT = Asn	ATT = Ile	ACT = Thr
G	GAT = Asp	GTT = Val	GCT = Ala
C	CAT = His	CTT = Leu	CCT = Pro
T	TAT = Tyr	TTT = Phe	TCT = Ser



[0231] A final design for TNFn3 libraries containing randomized AB, CD and EF loops is shown below. This design incorporates diversity observed in natural Fn3 sequences, two different lengths for the AB and CD loops, and uses “NHT” codon mixes.

AB loop (7 and 9 residues):

TNFn3 wild type amino acid sequence: **KDVTDTT** (SEQ ID NO:23)

Library amino acid sequence (7 aa): **Kxxxxxa**

DNA sequence: AAA-NHT-NHT-NHT-NHT-NHT-RST (SEQ ID NO:48)

Library amino acid sequence (9 aa): **Kxxxxxxxxa**

DNA sequence:AAA-NHT-NHT-NHT-NHT-NHT-NHT-NHT-RST (SEQ ID NO:49)

CD loop (7 and 9 residues):

Tn3 wild type amino acid sequence: **KDVPGDR** (SEQ ID NO:25)

Library amino acid sequence (7 aa): **xxxxxxx**

DNA sequence: NHT-NHT-NHT-NHT-NHT-NHT-NHT (SEQ ID NO:50)

Library amino acid sequence (9 aa): **xxxxxxxxxx**

DNA sequence: NHT-NHT-NHT-NHT-NHT-NHT-NHT-NHT-NHT (SEQ ID NO:51)

EF loop (8 residues):

Tn3 wild type amino acid sequence: **GNLKPDE** (SEQ ID NO:27)

Library amino acid sequence: **xbLxPxcx**

DNA sequence: NHT-RRB-CTG-NHT-CCG-NHT-RBT-NHT (SEQ ID NO:52)

Amino acid codes: x = N/D/H/Y/I/V/L/F/T/A/P/S; a = S/T/A/G; b = N/K/S/R/D/E/G;
c = I/T/S/V/A/G

Nucleotide codes: N = G/A/T/C; H = A/T/C; R = A/G; S = G/C; B = T/C/G

[0232] Incorporation of these degenerate sequences into the AB, CD, and EF loops of TNFn3 scaffold variants as provided herein can be accomplished according to standard methods. See *e.g.*, PCT Publication No: WO 2009/058379, in PCT Publication No: WO 2011/130324, and in PCT Publication No: WO 2011/130328 and Examples 2 and 3 disclosed

herein. Similar degeneracies can be introduced into one, two, three, four, five, or six loops of a TNFn3 scaffold variant provided herein.

TABLE 6.: Sequences and SEQ ID Nos of molecular components to assemble representative stabilized scaffolds of the invention:

Name/Brief Description	Sequence	SEQ ID NO
3rd FnIII of tenascin C WT SHORT	IEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDR TTIDLTEDENQYSIGNLKPDPTEYEVSLISRRGDMSSNP AKETFTT	1
3rd FnIII of tenascin C WT LONG	RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGI KDVPGDRTTIDLTEDENQYSIGNLKPDPTEYEVSLISRR GDMSSNPAKETFTT <u>X₉₁X₉₂</u> X ₉₁ is any amino acid or is deleted, and X ₉₂ is any amino acid or is deleted. Underlined residues add stability	2
TNFn3 beta strand A long	RLDAPSX ₇ IEV, wherein X ₇ is Q or K	3
TNFn3 beta strand A long WT	RLDAPS <u>Q</u> IEV	4
TNFn3 beta strand A long var	RLDAPSK <u>I</u> EV	5
TNFn3 beta strand A short	IEV	6
TNFn3 beta strand B	AX ₁₉ ITW, wherein X ₁₉ is L or K	7
TNFn3 beta strand B WT	A <u>L</u> ITW	8
TNFn3 beta strand B var	A <u>K</u> ITW	9
TNFn3 beta strand C	X ₃₂ ELTYGI, wherein X ₃₂ is I or F	10
TNFn3 beta strand C WT	<u>I</u> ELTYGI	11
TNFn3 beta strand C var	<u>F</u> ELTYGI	12
TNFn3 beta strand D	TTIX ₄₉ L, wherein X ₄₉ is D or K or N	13
TNFn3 beta strand D WT	TTI <u>D</u> L	14
TNFn3 beta strand D var 49a	TTI <u>K</u> L	15
TNFn3 beta strand E	YSI	16
TNFn3 beta strand F	YEVSLIS	17

Name/Brief Description	Sequence	SEQ ID NO
TNFn3 beta strand G	KX ₈₆ TFX ₈₉ TX ₉₁ X ₉₂ , wherein X ₈₆ is E or I or Q; X ₈₉ is T or K; X ₉₁ is any amino acid or is deleted, and X ₉₂ is any amino acid or is deleted	18
TNFn3 beta strand G WT	K <u>E</u> TF <u>T</u> TGL	19
TNFn3 beta strand G var 86a	K <u>I</u> TFX ₈₉ TX ₉₁ X ₉₂	20
TNFn3 beta strand G var 89	KX ₈₆ TF <u>K</u> TX ₉₁ X ₉₂	21
TNFn3 beta strand G var 86a-89	K <u>I</u> TF <u>K</u> TX ₉₁ X ₉	22
AB loop WT	KDVTDDT	23
BC loop WT	FKPLAEIDG	24
CD loop WT	KDVPGR	25
DE loop WT	TEDENQ	26
EF loop WT	GNLKPDE	27
FG loop	RX ₇₆ X ₇₇ X ₇₈ MSSNPX ₈₄ (SEQ ID NO:28), wherein X ₇₆ is any amino acid or is deleted [R]; X ₇₇ is any amino acid or is deleted [G]; X ₇₈ is any amino acid or is deleted [D]; and X ₈₄ is A or V	28
FG loop WT	RRDGMSSNPA	29
FG loop var	RX ₇₆ X ₇₇ X ₇₈ MSSNPV	30
TNFn3 beta strand D var49b	TTI <u>N</u> L	31
TNFn3 beta strand G var 86b	K <u>Q</u> TFX ₈₉ TX ₉₁ X ₉₂	32
TNFn3 beta strand G var 86b-89	K <u>Q</u> TF <u>K</u> TX ₉₁ X ₉₂	33
TNFn3 beta strand G var 86a-89-GL	K <u>I</u> TF <u>K</u> T <u>G</u> L	34
TNFn3 beta strand G var 86b-89-GL	K <u>Q</u> TF <u>K</u> T <u>G</u> L	35
TNFn3 quadruple mutation identified by Strickler <i>et al.</i>	RLDAPSKIEVKDVTDTTAKITWFKPLAEIDGIELTYGI KDVPGRRTTIKLTEDENQYSIGNLKPDEYEVSLSRR GDMSSNPAKETFKTGL	36
TNFn3 E86I mutation identified by Jacobs <i>et al.</i>	RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGI KDVPGRRTTIDLTEDENQYSIGNLKPDEYEVSLSRR GDMSSNPAKITFTTGL	37

Name/Brief Description	Sequence	SEQ ID NO
TNFn3 charge mutant D49N from PCT Publication No: WO 2009/058379	IEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDR TTINLTEDENQYSIGNLKPDEYEVSLSRRGDMSSNP AKETFTT	38
TNFn3 charge mutant E86Q from PCT Publication No: WO 2009/058379	IEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDR TTIDLTEDEENQYSIGNLKPDEYEVSLSRRGDMSSNP AKQTFTT	39
WT TNFn3 expression construct	ARLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYG IKDVPGDRTTIDLTEDEENQYSIGNLKPDEYEVSLSR RGDMSSNPAKETFTTGLGGSGGTLEHHHHHH <u>Underlined N- and C-term residues are not part of TNFn3 scaffold</u>	40
CM4 starting backbone	RLDAPSQIEVKDVTDTTALITWFKPLAEIDG F ELTYGI KDVPGDRTTI K LTEDEENQYSIGNLKPDEYEVSLSR R G DMSSNPAK I TF K TGL	41
TNFn3 scaffold variant with FG loop mutations	RLDAPSX ₇ IEVKDVTDTTAX ₁₉ ITWFKPLAEIDGX ₃₂ ELT YGIKDVPGDRTTIX ₄₉ LTEDEENQYSIGNLKPDEYEVSLSR ISRX ₇₆ X ₇₇ X ₇₈ MSSNPX ₈₄ KX ₈₆ TFX ₈₉ TX ₉₁ X ₉₂	53
TNFn3 scaffold variant with partial FG loop mutations	RLDAPSX ₇ IEVKDVTDTTAX ₁₉ ITWFKPLAEIDGX ₃₂ ELT YGIKDVPGDRTTIX ₄₉ LTEDEENQYSIGNLKPDEYEVSLSR ISRRGDMSSNPX ₈₄ KX ₈₆ TFX ₈₉ TX ₉₁ X ₉₂	54
Strickler G Strand	KETFKTGA	59
Jacobs G strand	KITFTTGL	60
G Strand E86Q	KQTFTTGL	61
TNFn3 charge mutant D49N /E86Q from PCT Publication No: WO 2009/058379	IEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDR TTINLTEDENQYSIGNLKPDEYEVSLSRRGDMSSNP AKQTFTT	62
TNFn3 beta strand C var with randomization	X ₃₂ X ₃₃ LTYGX ₃₈ , wherein X ₃₂ is I or F, and X ₃₃ and X ₃₈ are independently any amino acid	63
TNFn3 beta strand D with randomization	TX ₄₇ IX ₄₉ L, wherein X ₄₇ and X ₄₉ are independently any amino acid	64
TNFn3 beta strand A long with randomization	RLDAPSX ₇ IX ₉ V, wherein X ₇ and X ₉ are independently any amino acid	65
TNFn3 beta strand B with randomization	AX ₁₉ IX ₂₁ W, wherein X ₁₉ and X ₄₉ are independently any amino acid	66
TNFn3 beta strand E with randomization	YX ₅₈ I, wherein X ₅₈ is any amino acid	68

Name/Brief Description	Sequence	SEQ ID NO
CM4 Loop Randomization	RLDAPSQIEVKDVTDTTALITWXXPXX[X] _m IXXFELTY GIKDVPGDRRTTIKLXXXXXXXXYSIGNLKPDTEYEVSLIS XXXXXX[X] _n NPAKITFKTGL	69
CM4 Concave Randomization	RLDAPSQIEVKDVTDTTALITWFKPLAEIXXFXLTYGX XXXXXX[X] _z TXIXLTEDENQYSIGNLKPDTEYEVSLI SXXXXXX[X] _n NPAKITFKTLG	70
CM4 Flat Randomization	RLDAPSXIXVXXVXXXXXAXIXWXKPLAEIDGFELTYGI KDVPGBDRRTTIKLTEDXXXYXIXNLKPDTEYEVSLISRR DGMSSNPAKITFKTGL	71

[0233] The foregoing description will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present disclosure. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[0234] The breadth and scope of the claimed invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the claims and their equivalents.

WHAT IS CLAIMED IS:

1. A TNFn3 scaffold variant comprising the amino acid sequence: [A]-[ab]-[B]-[bc]-[C]-[cd]-[D]-[de]-[E]-[ef]-[F]-[fg]-[G], wherein:

[A], [B], [C], [D], [E], [F], and [G] are beta strands; wherein

[A] is IEV (SEQ ID NO:6) or RLDAPSX₇IEV, wherein X₇ is Q or K (SEQ ID NO:3) or RLDAPSX₇IX₉V, wherein X₇ and X₉ are independently any amino acid (SEQ ID NO: 65);

[B] is AX₁₉ITW, wherein X₁₉ is L or K (SEQ ID NO:7) or AX₁₉IX₂₁W, wherein X₁₉ and X₄₉ are independently any amino acid (SEQ ID NO: 66);

[C] is X₃₂ELTYGI, wherein X₃₂ is I or F (SEQ ID NO:10), or X₃₂X₃₃LTYGX₃₈, wherein X₃₂ is I or F, and X₃₃ and X₃₈ are independently any amino acid (SEQ ID NO: 63);

[D] is TTX₄₉L, wherein X₄₉ is D or K or N (SEQ ID NO:13) or TX₄₇IX₄₉L, wherein X₄₇ and X₄₉ are independently any amino acid (SEQ ID NO: 64);

[E] is YSI (SEQ ID NO:16) or YX₅₈I, wherein X₅₈ is any amino acid (SEQ ID NO: 68);

[F] is YEVSLIS (SEQ ID NO:17); and

[G] is KX₈₆TFX₈₉TX₉₁X₉₂, wherein X₈₆ is E or I or Q; X₈₉ is T or K; X₉₁ is any amino acid [G] or is deleted, and X₉₂ is any amino acid [L] or is deleted (SEQ ID NO:18);

wherein [ab], [bc], [cd], [de], [ef], and [fg] are variable loops each comprising at least 2 to no more than about 26 amino acids; and

wherein the amino acid sequence is not:

RLDAPSKIIEV[ab]AKITW[bc]IELTYGI[cd]TTIKL[de]YSI[ef]YEVSLIS[fg]KETFKTGA ([SEQ ID NO:5]-[ab]-[SEQ ID NO:9]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:15]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:59]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KETFTTGL ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

IEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KETFTTGL ([SEQ ID NO:6]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KITFTTGL ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:60]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTINL[de]YSI[ef]YEVSLIS[fg]KETFTTGL ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:31]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:19]);

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTIDL[de]YSI[ef]YEVSLIS[fg]KQTFTTGL ([SEQ ID NO:6]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:14]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:61]); or

RLDAPSQIEV[ab]ALITW[bc]IELTYGI[cd]TTINL[de]YSI[ef]YEVSLIS[fg]KETFTTGL ([SEQ ID NO:4]-[ab]-[SEQ ID NO:8]-[bc]-[SEQ ID NO:11]-[cd]-[SEQ ID NO:31]-[de]-[SEQ ID NO:16]-[ef]-[SEQ ID NO:17]-[fg]-[SEQ ID NO:61]).

2. The TNFn3 scaffold variant of claim 1, wherein

[ab] comprises KDVTDTT (SEQ ID NO:23), or SEQ ID NO:23 with at least one, two, three, four, five, six, or seven amino acid substitutions, insertions, or deletions;

[bc] comprises FKPLAEIDG (SEQ ID NO:24), or SEQ ID NO:24 with at least one, two, three, four, five, six, seven, eight, or nine amino acid substitutions, insertions, or deletions;

[cd] comprises KDVPGR (SEQ ID NO:25), or SEQ ID NO:25 with at least one, two, three, four, five, six, or seven amino acid substitutions, insertions, or deletions;

[de] comprises TEDENQ (SEQ ID NO:26), or SEQ ID NO:26 with at least one, two, three, four, five, or six, amino acid substitutions, insertions, or deletions;

[ef] comprises GNLKPDTE (SEQ ID NO:27), or SEQ ID NO:27 with at least one, two, three, four, five, six, seven, or eight amino acid substitutions, insertions, or deletions;

[fg] comprises $RX_{76}X_{77}X_{78}MSSNPX_{84}$ (SEQ ID NO:28), wherein X_{76} is any amino acid or is deleted [R]; X_{77} is any amino acid or is deleted [G]; X_{78} is any amino acid or is deleted [D]; and X_{84} is A or V, or SEQ ID NO:28 with at least one, two, three, four, five, or six additional amino acid substitutions, insertions, or deletions; or

any combination thereof.

3. The TNFn3 scaffold variant of claim 1 or claim 2, wherein

[bc] is FKPLAEIDG (SEQ ID NO:24);

[de] is TEDENQ (SEQ ID NO:26); and

[fg] is $RX_{76}X_{77}X_{78}MSSNPX_{84}$ (SEQ ID NO:28), wherein $X_{76} - X_{77} - X_{78}$ is not R-G-D, and wherein X_{84} is A or V.

4. The TNFn3 scaffold of claim 3, wherein X_{84} is A.

5. The TNFn3 scaffold of claim 3, wherein X_{84} is V.

6. The TNFn3 scaffold variant of claim 1 or claim 2, wherein

[ab] is KDVTDTT (SEQ ID NO:23);

[cd] is KDVPGR (SEQ ID NO:25); and

[ef] is GNLKPDTE (SEQ ID NO:27).

7. The TNFn3 scaffold variant of any one of claims 1 to 6, comprising the amino acid sequence:

RLDAPSX₇IEV[ab]AX₁₉ITW[bc]X₃₂ELTYGI[cd]TTIX₄₉L[de]YSI[ef]YEVSLIS[fg]KX₈₆TFX₈₉TX₉₁X₉₂; or

IEV[ab]AX₁₉ITW[bc]X₃₂ELTYGI[cd]TTIX₄₉L[de]YSI[ef]YEVSLIS[fg]KX₈₆TFX₈₉TX₉₁X₉₂

;

wherein X₉₁ is any amino acid, and X₉₂ is any amino acid.

8. The TNFn3 scaffold variant of claim 7, comprising the amino acid sequence
RLDAPSX₇IEVKDVTDTTAX₁₉ITWFKPLAEIDGX₃₂ELTYGIKDVPDRTTIX₄₉LTEDENQYSI
GNLKPDEYEVSLISRX₇₆X₇₇X₇₈MSSNPX₈₄KX₈₆TFX₈₉TX₉₁X₉₂ (SEQ ID NO:3).

9. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [A] is
RLDAPSQIEV (SEQ ID NO:4) or IEV (SEQ ID NO:6).

10. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [A] is
RLDAPSKIEV (SEQ ID NO:5).

11. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [B] is
ALITW (SEQ ID NO:8).

12. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [B] is
AKITW (SEQ ID NO:9).

13. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [C] is
IELTYGI (SEQ ID NO:11).

14. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [C] is FELTYGI (SEQ ID NO:12).

15. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [D] is TTIDL (SEQ ID NO:14).

16. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [D] is TTIKL (SEQ ID NO:15).

17. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [D] is TTINL (SEQ ID NO:31).

18. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [G] is KETFTTX₉₁X₉₂ (SEQ ID NO:19).

19. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20).

20. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [G] is KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32).

21. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [G] is KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21).

22. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [G] is KITFKTX₉₁X₉₂ (SEQ ID NO:22).

23. The TNFn3 scaffold variant of any one of claims 1 to 8, wherein beta strand [G] is KQTFKTX₉₁X₉₂ (SEQ ID NO:33).

24. The TNFn3 scaffold variant of claim 14 or claim 16, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), and beta strand [D] is TTIKL (SEQ ID NO:15).

25. The TNFn3 scaffold variant of claim 14 or claim 17, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), and beta strand [D] is TTINL (SEQ ID NO:31).

26. The TNFn3 scaffold variant of claim 14 or claim 19, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), and beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20).

27. The TNFn3 scaffold variant of claim 14 or claim 20, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), and beta strand [G] is KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32).

28. The TNFn3 scaffold variant of claim 14 or claim 21_, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), and beta strand [G] is KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21).

29. The TNFn3 scaffold variant of claim 16 or claim 19, wherein beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20).

30. The TNFn3 scaffold variant of claim 17 or claim 19, wherein beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20).

31. The TNFn3 scaffold variant of claim 16 or claim 20, wherein beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32).

32. The TNFn3 scaffold variant of claim 17 or claim 20, wherein beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32).

33. The TNFn3 scaffold variant of claim 16 or claim 21, wherein beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21).

34. The TNFn3 scaffold variant of claim 17 or claim 21, wherein beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21).

35. The TNFn3 scaffold variant of any one of claims 1-9, 11, 13, 15, 19, 21, or 22, wherein beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is IELTYGI (SEQ ID NO:11), beta strand [D] is TTIDL (SEQ ID NO:14), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

36. The TNFn3 scaffold variant of any one of claims 1-12, 14, 16, 19, 24, 26, or 29, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20).

37. The TNFn3 scaffold variant of any one of claims 1-12, 14, 17, 19, 25, 26, or 30, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KITFX₈₉TX₉₁X₉₂ (SEQ ID NO:20).
38. The TNFn3 scaffold variant of any one of claims 1-12, 14, 16, 20, 24, 27, or 31, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32).
39. The TNFn3 scaffold variant of any one of claims 1-12, 14, 17, 20, 25, 27, or 32, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KQTFX₈₉TX₉₁X₉₂ (SEQ ID NO:32).
40. The TNFn3 scaffold variant of any one of claims 1-12, 14, 16, 21, 24, 28, or 33, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21).
41. The TNFn3 scaffold variant of any one of claims 1-12, 14, 17, 21, 21, 25, 28, or 34, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KX₈₆TFKTX₉₁X₉₂ (SEQ ID NO:21).
42. The TNFn3 scaffold variant of any one of claims 1-14, 16, or 22, wherein beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTX₉₁X₉₂ (SEQ ID NO:22).
43. The TNFn3 scaffold variant of any one of claims 1-9, 11, 13, 16, 19, 21, 22, 29, or 33, wherein beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is IELTYGI (SEQ ID NO:11), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

44. The TNFn3 scaffold variant of any one of claims 1-14, 17, 22, or 25, wherein beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KITFKTX₉₁X₉₂ (SEQ ID NO:22).

45. The TNFn3 scaffold variant of any one of claims 1-14, 16, 20, 21, 23, 24, 27, 28, 31, or 33, wherein beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KQTFKTX₉₁X₉₂ (SEQ ID NO:33).

46. The TNFn3 scaffold variant of any one of claims 1-14, 17, 20, 21, 23, 25, 27, 28, 32, or 34, wherein beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KQTFKTX₉₁X₉₂ (SEQ ID NO:33).

47. The TNFn3 scaffold variant of any one of claims 1-12, 14, 16, 19, 21, 22, 24, 28, 29, or 33, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTX₉₁X₉₂ (SEQ ID NO:22).

48. The TNFn3 scaffold variant of any one of claims 1-9, 11, 14, 16, 19, 21, 22, 24, 28, 33, 40, 42, or 47, wherein beta strand [A] is RLDAPSQIEV (SEQ ID NO:4), beta strand [B] is ALITW (SEQ ID NO:8), beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KITFKTGL (SEQ ID NO:34).

49. The TNFn3 scaffold variant of claim 44, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KITFKTX₉₁X₉₂ (SEQ ID NO:22).

50. The TNFn3 scaffold variant of claim 45, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTIKL (SEQ ID NO:15), and beta strand [G] is KQTFKTX₉₁X₉₂ (SEQ ID NO:33).

51. The TNFn3 scaffold variant of claim 46, wherein beta strand [C] is FELTYGI (SEQ ID NO:12), beta strand [D] is TTINL (SEQ ID NO:31), and beta strand [G] is KQTFKTX₉₁X₉₂ (SEQ ID NO:33).

52. The TNFn3 scaffold variant of any one of claims 1 to 51, wherein X₉₁ - X₉₂ is G-L.

53. The TNFn3 scaffold variant of any one of claims 1 to 52, which binds to a target antigen.

54. The TNFn3 scaffold variant of claim 53, which bind to the target antigen with an affinity (K_D) of at least 100 μ M.

55. The TNFn3 scaffold variant of claim 53 or claim 54, wherein the target antigen is a cell-surface antigen, a soluble antigen, an immobilized antigen, an immunosilent antigen, an intracellular antigen, an intranuclear antigen, a self antigen, a non-self antigen, a cancer antigen, a bacterial antigen, a viral antigen, or any combination thereof.

56. The TNFn3 scaffold variant of any one of claims 1 to 55, which exhibits a thermal melting temperature (T_m) of at least about 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C, as measured by differential scanning calorimetry (DSC) in 10 mM potassium phosphate, 50 mM sodium chloride, pH 7.4.

57. The TNFn3 scaffold variant of any one of claims 1 to 55, which exhibits a thermal melting temperature (T_m) that is increased relative to the T_m observed for a TNFn3 scaffold protein

consisting of SEQ ID NO:2 by least about 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or 30°C, as measured by differential scanning calorimetry (DSC).

58. The TNFn3 scaffold variant of any one of claims 1 to 57, which is conjugated to a heterologous agent

59. The TNFn3 scaffold variant of claim 58, wherein the heterologous agent is a heterologous scaffold, a protein, a peptide, a protein domain, a linker, a drug, a toxin, a cytotoxic agent, an imaging agent, a radionuclide, a radioactive compound, an organic polymer, an inorganic polymer, polyethylene glycol (PEG), biotin, human serum albumin (HSA), a HSA FcRn binding portion, an antibody, a domain of an antibody, an antibody fragment, a single chain antibody, an albumin binding domain, an enzyme, a ligand, a receptor, a binding peptide, an epitope tag, a recombinant polypeptide polymer, a cytokine, or a combination of two or more of the heterologous agents.

60. The TNFn3 scaffold variant of any one of claims 1 to 59, further comprising a heterologous epitope binding domain.

61. The TNFn3 scaffold variant of claim 60, wherein the heterologous epitope binding domain is specific for a different antigen target, or different epitope of the same antigen target, than the TNFn3 scaffold variant.

62. The TNFn3 scaffold variant of claim 61, wherein the heterologous epitope binding domain is specific for the same antigen target as the TNFn3 scaffold variant.

63. A multimeric scaffold comprising at least two TNFn3 scaffold variants of any one of claims 1 to 62.

64. A multimeric scaffold comprising at least one TNFn3 scaffold variant of any one of claims 1 to 62 and a heterologous scaffold.

65. The multimeric scaffold of claim 63 or claim 64, further comprising a heterologous epitope binding domain.

66. The multimeric scaffold of claim 65, wherein the heterologous epitope binding domain is specific for a different antigen target, or different epitope of the same antigen target, than the at least two TNFn3 scaffold variants.

67. The multimeric scaffold of claim 66, wherein the heterologous epitope binding domain is specific for the same antigen target as at least one of the at least two TNFn3 scaffold variants.

68. The multimeric scaffold of any one of claims 63 to 67, wherein at least two TNFn3 scaffold variants are linked by another scaffold, an IgG molecule or fragment thereof, an Fc region, a dimerization domain, a chemical crosslinker, a disulfide bond, or an amino acid linker.

69. An isolated polynucleotide comprising a nucleic acid molecule encoding the TNFn3 scaffold variant of any one of claims 1 to 62, or the multimeric scaffold of any one of claims 63 to 68.

70. A vector comprising the polynucleotide of claim 69.

71. The vector of claim 70, which is an expression vector capable of facilitating transcription, translation, or transcription and translation of the polynucleotide of claim 69.

72. A host cell comprising the vector of claim 70 or claim 71.

73. A method of producing a TNFn3 scaffold variant or a multimeric scaffold comprising a TNFn3 scaffold variant comprising:

culturing the host cell of claim 72 under conditions in which the TNFn3 scaffold variant or the multimeric scaffold comprising the TNFn3 scaffold variant encoded by the polynucleotide is expressed, and

recovering the TNFn3 scaffold variant or the multimeric scaffold comprising the TNFn3 scaffold variant.

74. A composition comprising the TNFn3 scaffold variant of any one of claims 1 to 62 or the multimeric scaffold of any one of claims 63 to 68, and a carrier or excipient.

75. A method of preventing, treating, managing, or ameliorating a disease or disorder in a subject comprising administering to a subject in need thereof an effective amount of the composition of claim 74.

76. The method of claim 75, wherein the disease is an autoimmune disease, an inflammatory disease, a proliferative disease, an infectious disease, a respiratory disease, a gastrointestinal disease, diabetes, lupus, or obesity.

77. A method of detecting a target antigen in a sample comprising providing a TNFn3 scaffold variant of any one of claims 1 to 62 or a multimeric scaffold of any one of claims 63 to 68

specific for a target antigen of interest, labeling the scaffold variant or multimeric scaffold, contacting the labeled scaffold variant or multimeric scaffold with a sample, and detecting complex formation between the scaffold variant or multimeric scaffold and the target antigen.

78. A method of capturing a target antigen in a sample, comprising providing a TNFn3 scaffold variant of any one of claims 1 to 62 or a multimeric scaffold of any one of claims 63 to 68 specific for a target antigen of interest, contacting the scaffold variant or multimeric scaffold with a sample under conditions that allow complex formation between the scaffold variant or multimeric scaffold and the target antigen, and recovering the complex.

79. A method of constructing a binding molecule display library comprising:

- (a) providing a polynucleotide encoding the TNFn3 scaffold variant of any one of claims 1 to 62 or a polynucleotide encoding a multimeric scaffold of any one of claims 63 to 68;
- (b) introducing random deletions or randomizing codon substitutions or insertions into the polynucleotide at selected positions in a region of the polynucleotide encoding at least one variable loop [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant; and
- (c) propagating copies of the polynucleotide to form the display library.

80. The method of claim 79, wherein the randomizing codons are NNS, NNK, NHT, or a combination thereof.

81. A polypeptide display library produced by the method of claim 79 or claim 80.

82. The library of claim 81, wherein at least two variable loops [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant are randomized by deletion, substitution, or addition of at least one amino acid.

83. The library of claim 82, wherein two variable loops selected from [ab], [cd], and [ef], are randomized.

84. The library of claim 82, wherein two variable loops selected from [bc], [de], and [fg] are randomized.

85. The library of claim 81, wherein at least three variable loops [ab], [bc], [cd], [de], [ef], or [fg] of the TNFn3 scaffold variant are randomized by deletion, substitution, or addition of at least one amino acid.

86. The library of claim 85, wherein variable loops [ab], [cd], and [ef] are randomized.

87. The library of claim 85, wherein variable loops [bc], [de], and [fg] are randomized.

88. The library of any one of claims 81 to 87, wherein the TNFn3 scaffold variant or multimeric scaffold is displayed on the surface of a ribosome, bacteriophage, virus, bacteria, or yeast.

89. The library of claim 88, which has a sequence diversity of at least 10^6 .

90. A collection of isolated polynucleotides encoding the library of any one of claims 81 to 88.

91. A plurality of expression vectors comprising the collection polynucleotides of claim 90.
92. A method of obtaining a binding molecule specific for a target antigen, comprising:
- (a) contacting a target antigen with the library of any one of claims 81 to 89 under conditions that allow a scaffold-target antigen complex to form, and
 - (b) recovering the scaffold that binds to the target antigen.
93. The method of claim 92, further comprising randomizing at least one variable loop of the scaffold recovered in step (b) to generate a further randomized library and repeating steps (a) and (b) using the further randomized library.
94. The method of claim 93, wherein at least two loops are further randomized.
95. The method of claim 94, wherein at least three of the loops are further randomized.
96. The method of any one of claims 93 to 95, wherein the at least one variable loop randomized was not randomized in the first operation of steps (a) and (b).
97. The method of any one of claims 92 to 96, wherein the repetition of steps (a) and (b) further comprises contacting a target antigen distinct from the target antigen of the first operation of step (a) and (b).

98. The method of claim 97, wherein at least one of variable loops [ab], [cd], or [ef] are randomized in the first operation of steps (a) and (b), and at least one of variable loops [bc], [de], or [fg] are randomized in the second operation of steps (a) and (b).

99. The method of claim 97, wherein at least one of variable loops [bc], [de], or [fg] are randomized in the first operation of steps (a) and (b), and at least one of variable loops [ab], [cd], or [ef] are randomized in the second operation of steps (a) and (b).

100. The method of any one of claims 92 to 99, further comprising randomizing at least one beta strand of TNFn3 scaffold variant obtained in either the first or the second operation of step (b) to generate a further randomized library and repeating steps (a) and (b) using the further randomized library.

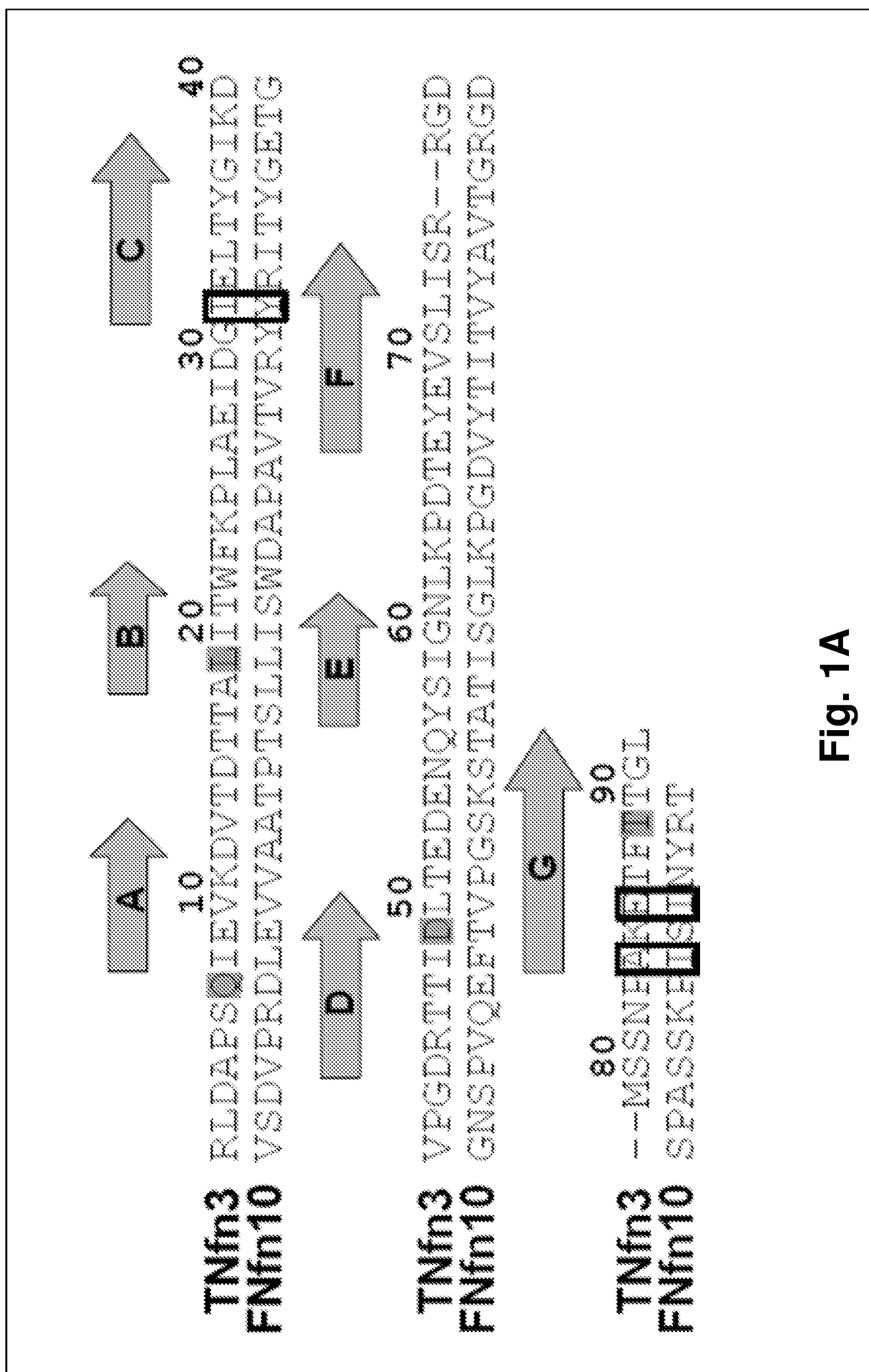


Fig. 1A

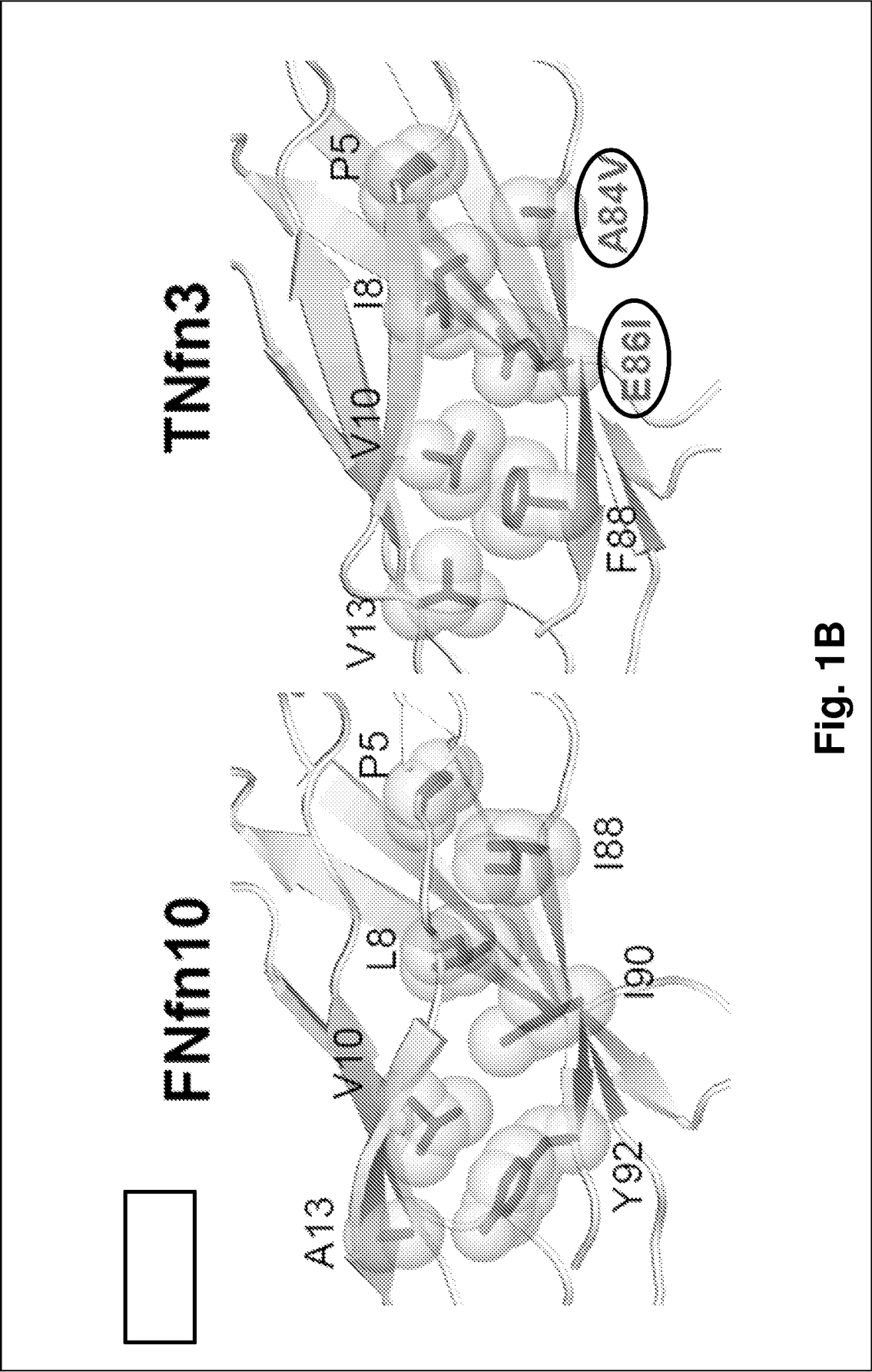


Fig. 1B

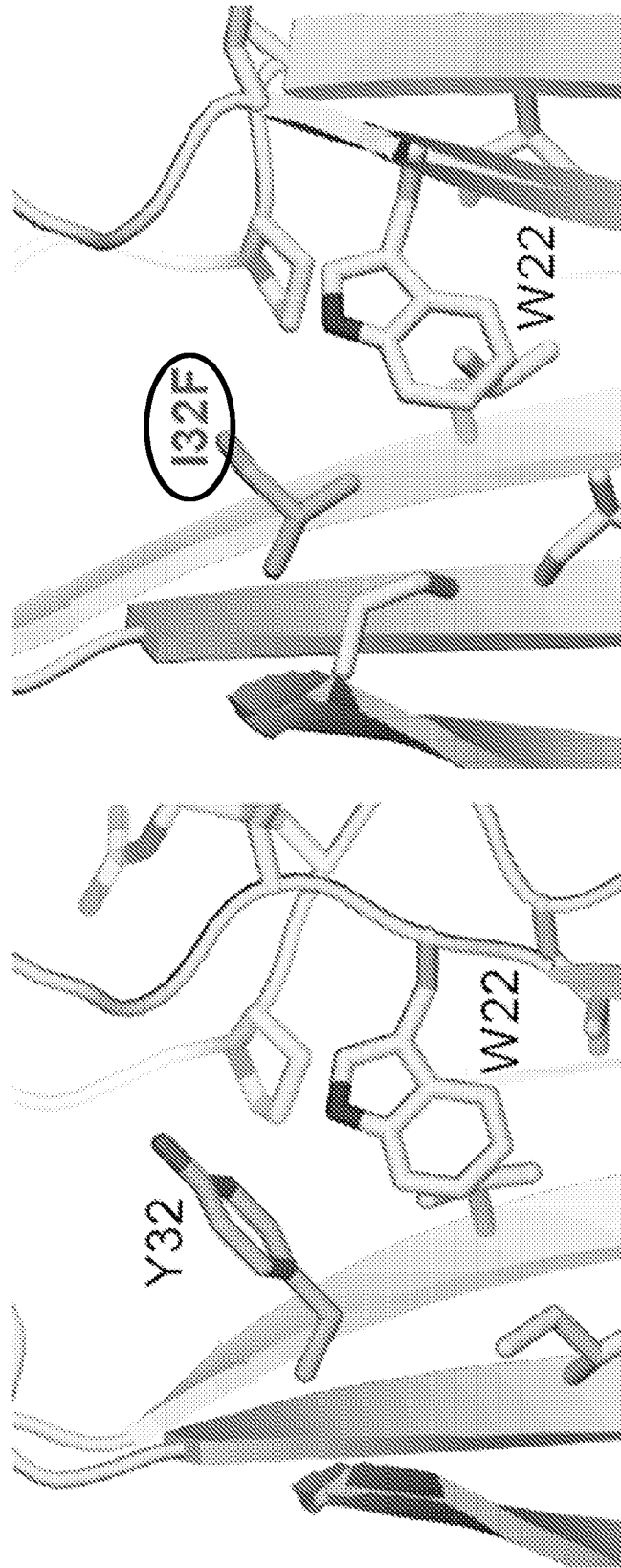


Fig. 1C

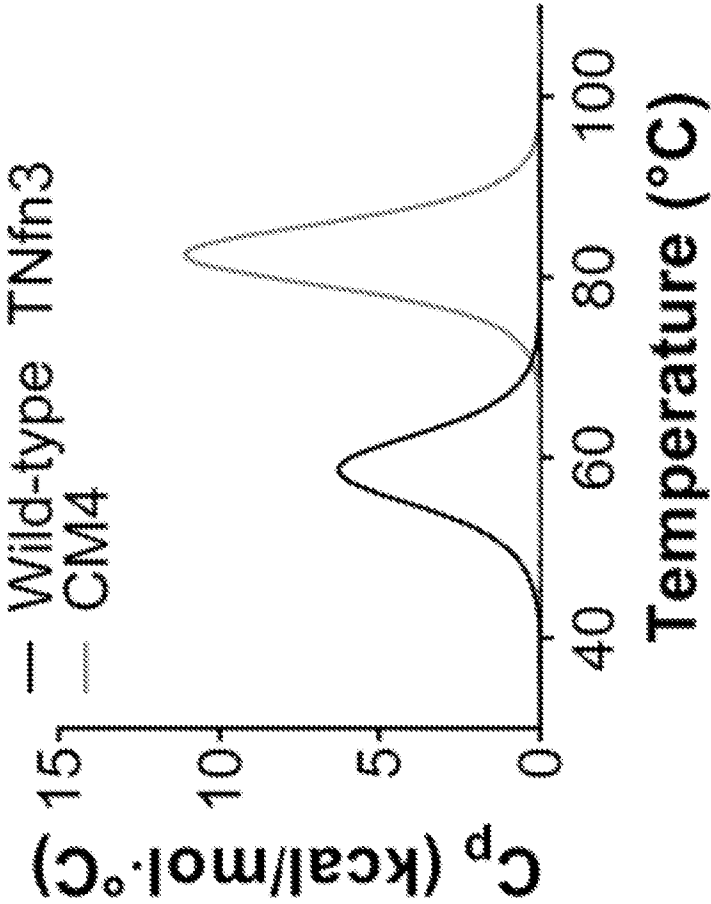


Fig. 2A

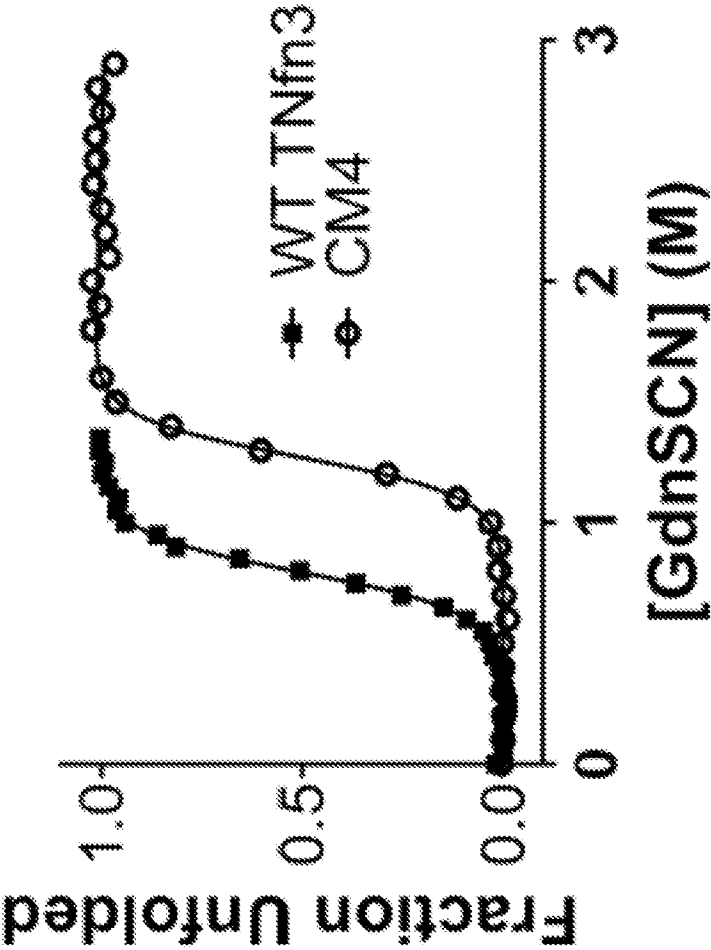


Fig. 2B

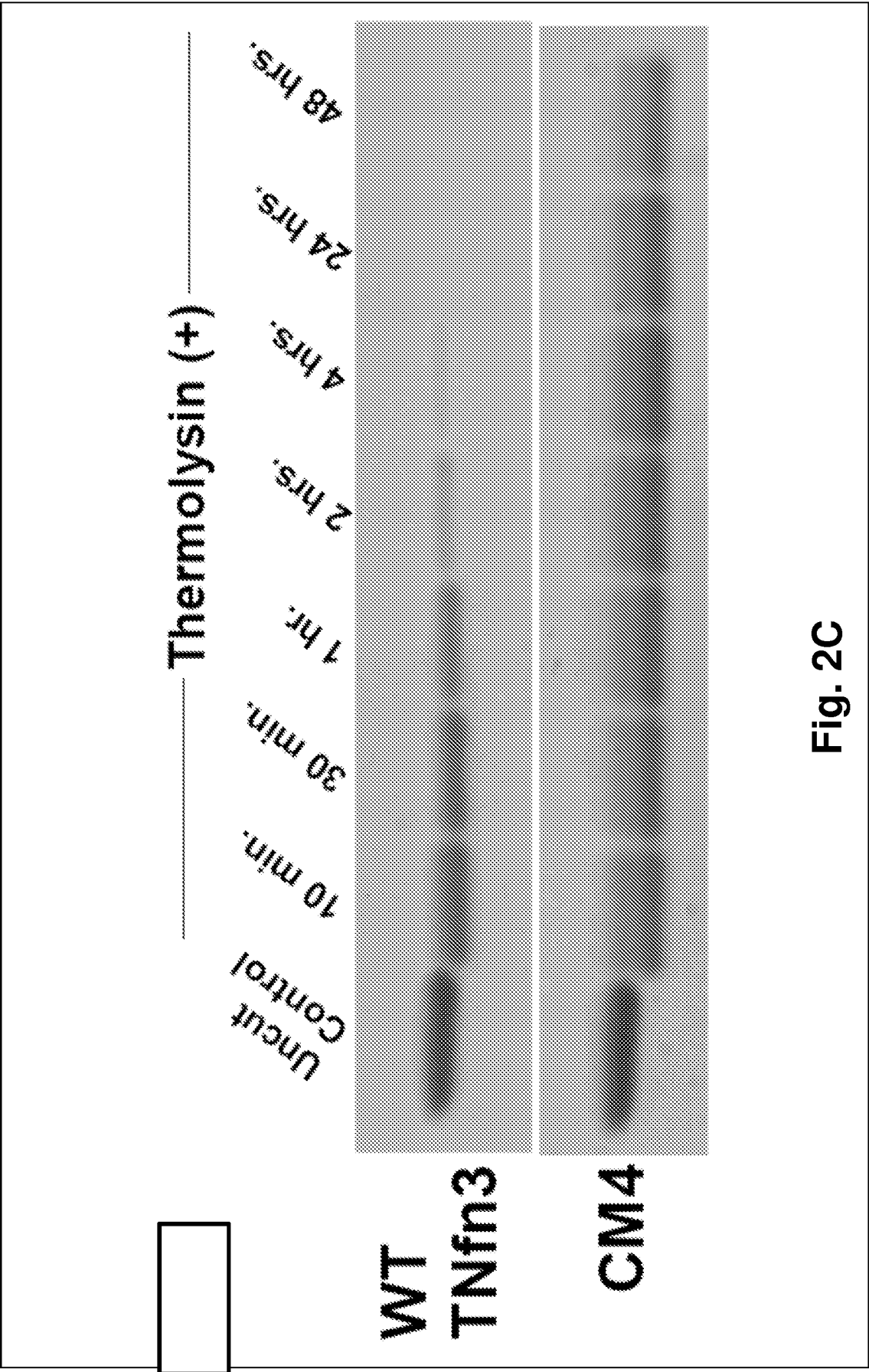
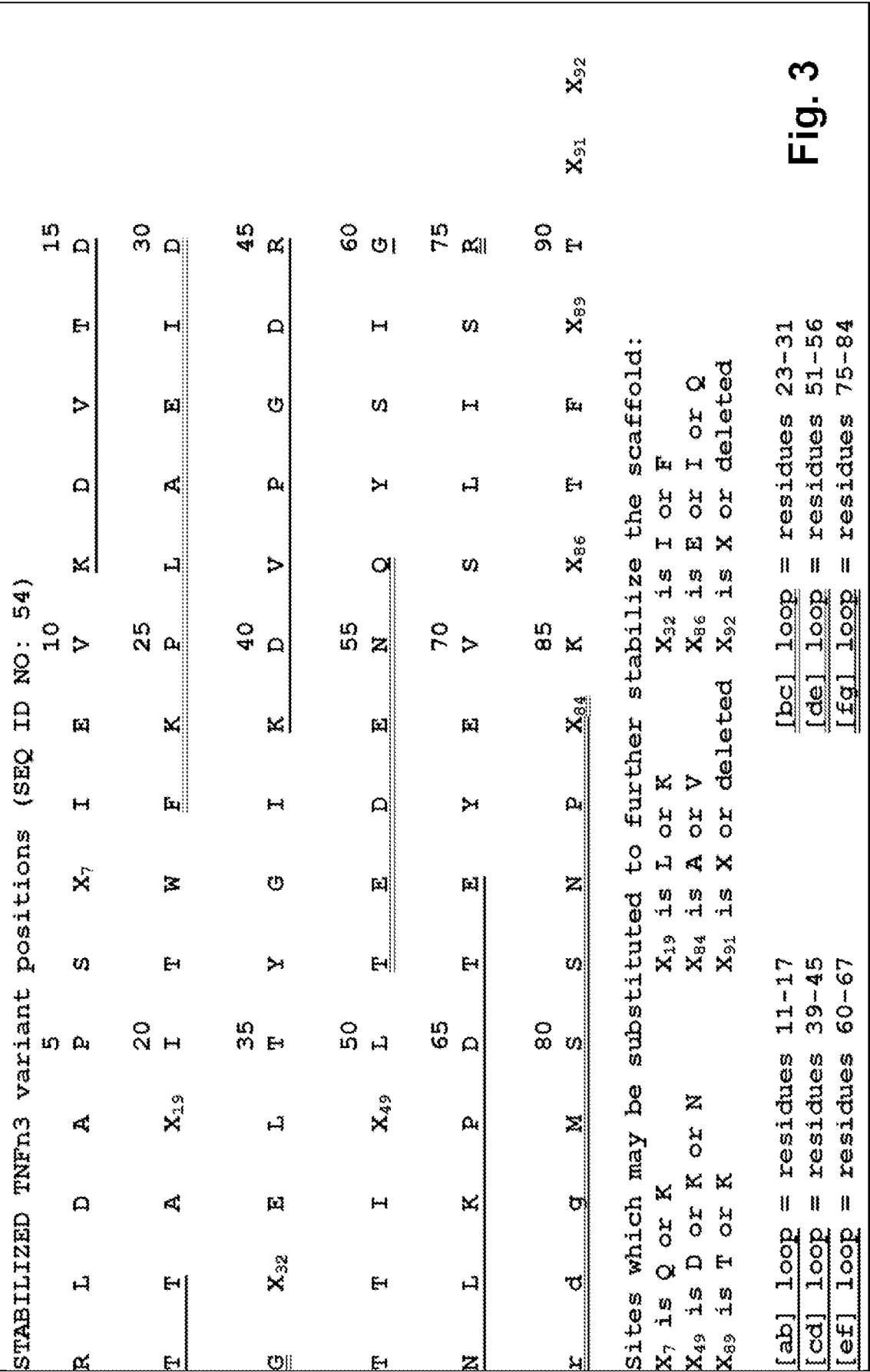


Fig. 2C



CM4 Loop Randomization (SEQ ID NO: 69)															
R	L	D	A	P	5	S	Q	I	E	V	10	D	V	T	15
T	T	A	L	I	20	T	W	X	X	P	25	X	[X] _m	I	30
X	F	E	L	T	35	Y	G	I	K	D	40	P	G	D	45
T	T	I	K	L	50	X	X	X	X	X	55	Y	S	I	60
N	L	K	P	D	65	T	E	Y	E	V	70	L	I	S	75
X	X	X	X	X	80	[X] _n	N	P	A	K	85	T	F	K	90
AB loop = residues 11-17 DE loop = residues 51-56															
BC loop = residues 23-31 EF loop = residues 60-67															
CD loop = residues 39-45 FG loop = residues 75-84															

Fig. 4A

CM4 Conceave Randomization (SEQ ID NO: 70)															
R	L	D	A	P	S	Q	I	E	V	K	D	V	T		15
															D
T	T	A	L	I	T	W	F	K	P	L	A	E	I	X	30
															X
X	F	X	L	T	Y	G	X	X	X	X	X	X	X	[X] _z	45
T	X	I	X	L	T	E	D	E	N	Q	Y	S	I	G	60
N	L	K	P	D	T	E	Y	E	V	S	L	I	S	X	75
X	X	X	X	X	[X] _n	N	P	A	K	I	T	F	K	T	90
															L
															G

AB loop = residues 11-17 BC loop = residues 23-31 CD loop = residues 39-45
DE loop = residues 51-56 EF loop = residues 60-67 FG loop = residues 75-84

Fig. 4B

CM4 Flat Randomization (SEQ ID NO: 71)															
R	L	D	A	P	S	X	I	X	V	X	X	V	X	X	15
X	X	A	X	I	X	W	X	K	P	L	A	E	I	D	30
G	F	E	L	T	Y	G	I	K	D	V	P	G	D	R	45
T	T	I	K	L	T	E	D	X	X	X	Y	X	I	X	60
N	L	K	P	D	T	E	Y	E	V	S	L	I	S	R	75
r	d	g	M	S	S	N	P	A	K	I	T	F	K	T	90
AB loop = residues 11-17 DE loop = residues 51-56															
BC loop = residues 23-31 EF loop = residues 60-67															
CD loop = residues 39-45 FG loop = residues 75-84															

Fig. 4C