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(54) **METHODS AND COMPOSITION INVOLVING THERMOPHILIC FIBRONECTIN TYPE III (FN3) MONOBODIES**

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CPC ***C07K 14/78*** (2013.01); ***C40B 40/10*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

The current application describes various compositions and methods for the production of FN3-based binding proteins with improved stability properties. Aspects of the disclosure relate to polypeptides comprising a variant fibronectin type III (FN3) domain from *Sulfolobus tokodaii* or *Pyrococcus horikoshii* comprising one or more amino acid substitutions or insertions in a loop region of FN3, in a non-loop region of FN3, or in both.

8 Claims, 18 Drawing Sheets

Specification includes a Sequence Listing.

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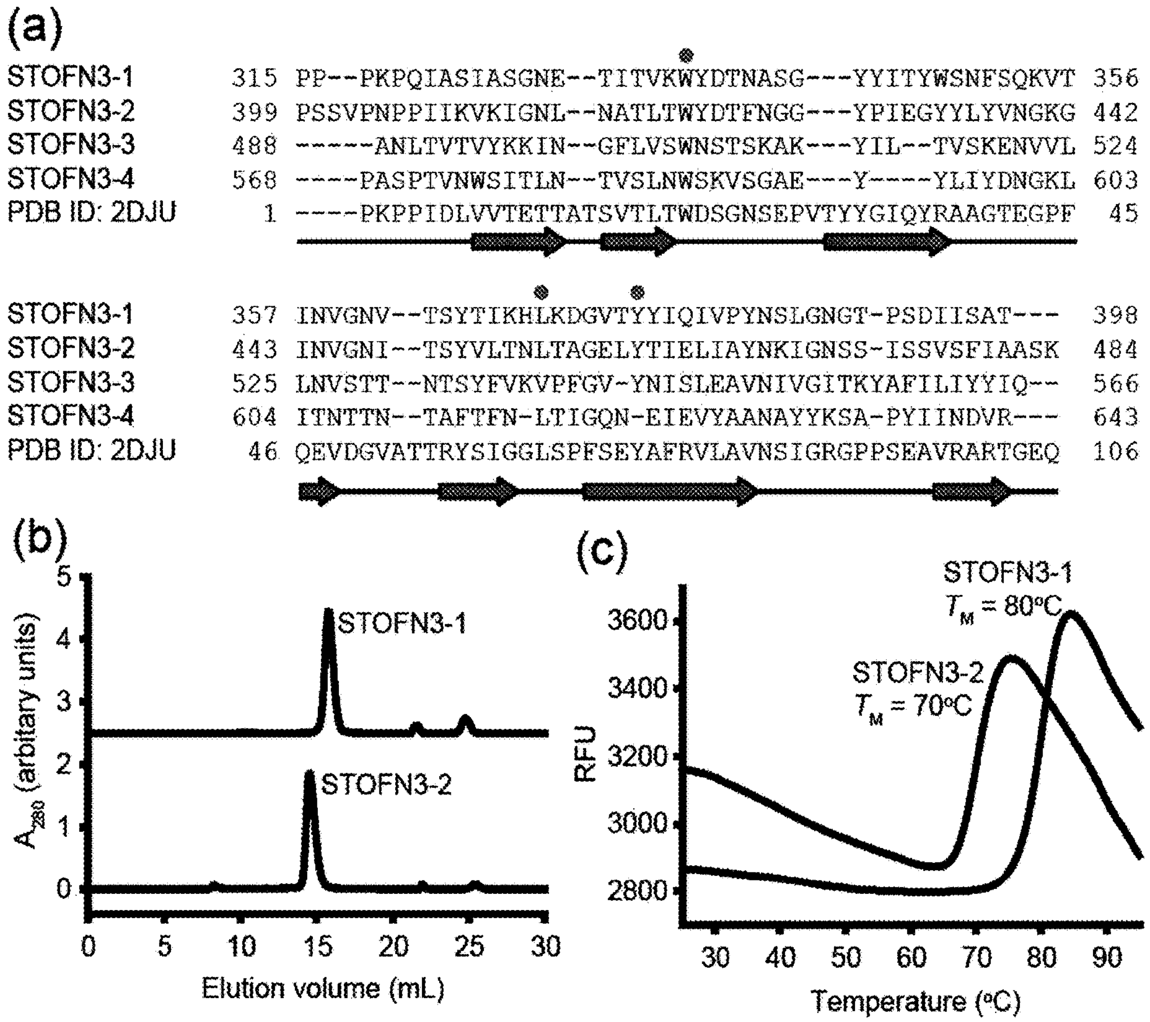

(a)
STOFN3-1 315 PP--PKPQIASIASGNE--TITVKWYDTNASG---YYITYWSNFSQKVT 356
STOFN3-2 399 PSSVPNPPIIKVKIGNL--NATLTWYDTFNGG---YPIEGYYLYVNGKG 442
STOFN3-3 488 ------ANLTVTVYKKIN--GFLVSWNSTSKAK---YIL--TVSKENVVL 524
STOFN3-4 568 ----PASPTVNWSITLN--TVSLNWSKVSGAE---Y----YLIYDNGKL 603
PDB ID: 2DJU 1 ----PKPPIDLVVTETTATSVTLTWDSGNSEPVTYYGIQYRAAGTEGPF 45
STOFN3-1 357 INVGNV--TSYTIKHLKDGVTYYIQIVPYNSLGNGT-PSDIISAT--- 398
STOFN3-2 443 INVGNI--TSYVLTNLTAGELYTIELIAYNKIGNSS-ISSVSFIAASK 484
STOFN3-3 525 LNVSTT--NTSYFVKVPFGV-YNISLEAVNIVGITKYAFILIYYIQ-- 566
STOFN3-4 604 ITNTTN--TAFTFN-LTIGQN-EIEVYAANAYYKSA-PYIINDVR--- 643
PDB ID: 2DJU 46 QEVDGVATTRYSIGGLSPFSEYAFRVLAVNSIGRGPPSEAVRARTGEQ 106
(b)
STOFN3-1
STOFN3-2
A280 (arbitary units)
Elution volume (mL)
(c)
STOFN3-1
TM = 80°C
STOFN3-2
TM = 70°C
RFU
Temperature (°C)

FIG. 1A-C

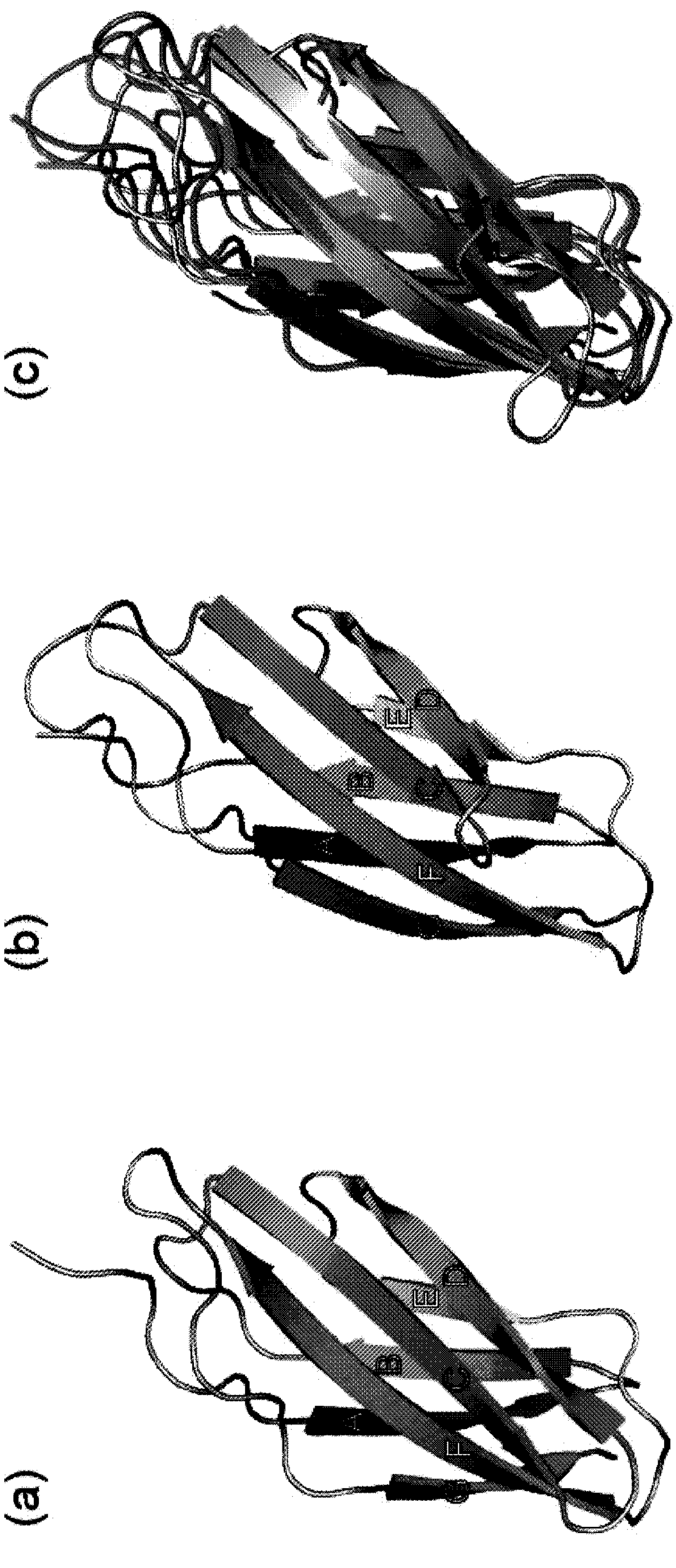
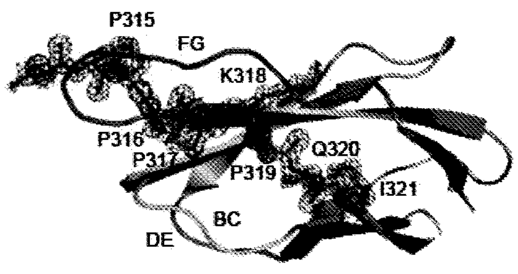


FIG. 2A-C

(a)



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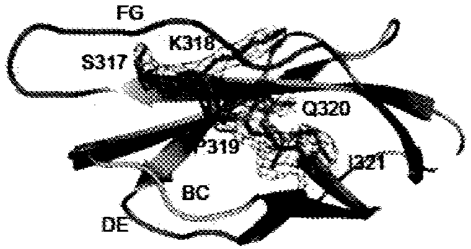
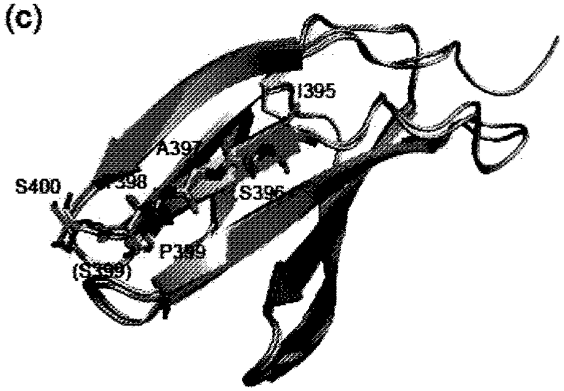


(c)



(d)

		T_M (°C)
FN3fn10 (1FNA)	-------RDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPI-SINYRTEI-	-
STOFN3-1 (STOTEMP1)	ssPPPKPQIASI-ASGNETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKDGVTYYIQIVPYNSL----GNGTPSDIISATss--	80.0
NPS	ssSSSKPQIASI-ASGNETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKDGVTYYIQIVPYNSL----GNGTPSDIISATss--	77.0
C+	ssPPPKPQIASI-ASGNETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKDGVTYYIQIVPYNSL----GNGTPSDIISATPSSs	87.3
C+/N328P	ssPPPKPQIASI-ASGPETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKDGVTYYIQIVPYNSL----GNGTPSDIISATPSSs	92.3
C+/D372P	ssPPPKPQIASI-ASGNETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKPGVTYYIQIVPYNSL----GNGTPSDIISATPSSs	>95.0
C+/N328P/D372P	ssPPPKPQIASI-ASGPETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKPGVTYYIQIVPYNSL----GNGTPSDIISATPSSs	>95.0
NPS/C+/N328P/D372P (STOTEMP2)	ssSSSKPQIASI-ASGPETITVKWYDT--NASGYYITYWS--NFSQKVTINVGN-VTSYTIKHLKPGVTYYIQIVPYNSL----GNGTPSDIISATPSSs	>95.0

FIG. 3A-D

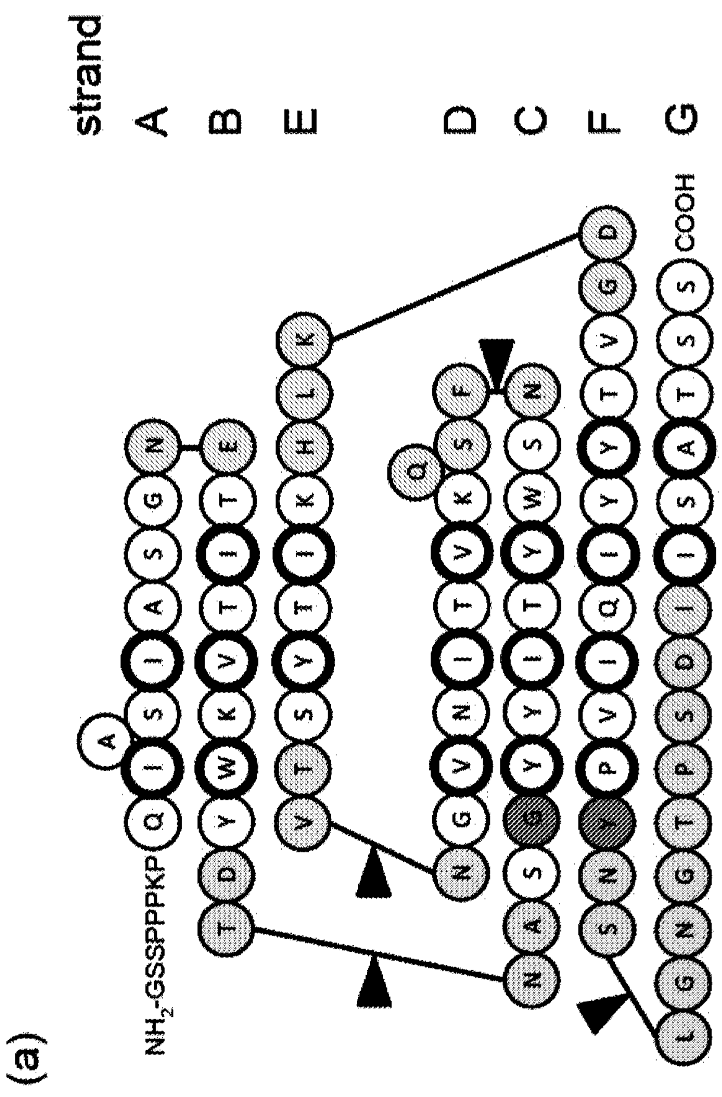


FIG. 4A

(b)

	AB	BC	CD	DE	EF	FG	T_M (°C)
STOFN3-1	ASGNETIT	WYDTNASG	WSNFSQK	VGNVTSY	IKHLKDGVT	PYNSLGNGTPSDII	80.0
STOFN3-1/ABSer	ASSSSSIT	WYDTNASG	WSNFSQK	VGNVTSY	IKHLKDGVT	PYNSLGNGTPSDII	79.0
STOFN3-1/BCSer	ASGNETIT	WYSSSSSG	WSNFSQK	VGNVTSY	IKHLKDGVT	PYNSLGNGTPSDII	74.7
STOFN3-1/CDSer	ASGNETIT	WYDTNASG	WSSSSQK	VGNVTSY	IKHLKDGVT	PYNSLGNGTPSDII	82.2
STOFN3-1/DESer1	ASGNETIT	WYDTNASG	WSNFSQK	VGSSSSY	IKHLKDGVT	PYNSLGNGTPSDII	81.7
STOFN3-1/DESer2	ASGNETIT	WYDTNASG	WSNFSQK	VSSSSSY	IKHLKDGVT	PYNSLGNGTPSDII	54.3
STOFN3-1/EFSer	ASGNETIT	WYDTNASG	WSNFSQK	VGNVTSY	IKSSSSSVT	PYNSLGNGTPSDII	64.0
STOFN3-1/FGSer1	ASGNETIT	WYDTNASG	WSNFSQK	VGNVTSY	IKHLKDGVT	PYSSSSSSSPSDII	66.7
STOFN3-1/FGSer2	ASGNETIT	WYDTNASG	WSNFSQK	VGNVTSY	IKHLKDGVT	PSSSSSSSSPSDII	60.0
STOFN3-1/FGSer3	ASGNETIT	WYDTNASG	WSNFSQK	VGNVTSY	IKHLKDGVT	PYSSSSSSSSSDII	63.2
STOFN3-1/FGSer4	ASGNETIT	WYDTNASG	WSNFSQK	VGNVTSY	IKHLKDGVT	PSSSSSSSSSSSII	62.0
STOTEMP3*	ASGPETIT	WYDTNASG	WSSSSQK	VGNVTSY	IKHLKPGVT	PYNSLGNGTPSDII	>95.0
STOTEMP4*	ASGPETIT	WYDTNASY	WSSSSQK	VGNVTSY	IKHLKPGVT	PVNSLGNGTPSDII	>95.0
STOTEMP4*/FGSer5	ASGPETIT	WYDTNASY	WSSSSQK	VGNVTSY	IKHLKPGVT	PVSSSSSSSPSDII	89.3
STOTEMP4*/FGSer6	ASGPETIT	WYDTNASY	WSSSSQK	VGNVTSY	IKHLKPGVT	PYSSSSSSSPSDII	85.8
STOTEMP4*/FGSer7	ASGPETIT	WYDTNASY	WSSSSQK	VGNVTSY	IKHLKPGVT	PSSSSSSSSPSDII	86.5

*C-terminal extented.

FIG. 4B

(c)

	BC	CD	DE	FG	T_M (°C)
STOFN3-1	WYDT--------NASG	WSN--------FSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	80.0
STOFN3-1/BC+2	WYDTSS------NASG	WSN--------FSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	80.3
STOFN3-1/BC+4	WYDTSSSS----NASG	WSN--------FSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	76.7
STOFN3-1/BC+8	WYDTSSSSSSSSNASG	WSN--------FSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	77.7
STOFN3-1/CD+2	WYDT--------NASG	WSNSS------FSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	71.7
STOFN3-1/CD+4	WYDT--------NASG	WSNSSSS----FSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	70.0
STOFN3-1/CD+8	WYDT--------NASG	WSNSSSSSSSSFSQ	VGN--------VTSY	PYNS--------LGNGTPSDII	67.7
STOFN3-1/DE+2	WYDT--------NASG	WSN--------FSQ	VGNSS------VTSY	PYNS--------LGNGTPSDII	55.8
STOFN3-1/DE+4	WYDT--------NASG	WSN--------FSQ	VGNSSSS----VTSY	PYNS--------LGNGTPSDII	55.0
STOFN3-1/DE+8	WYDT--------NASG	WSN--------FSQ	VGNSSSSSSSSVTSY	PYNS--------LGNGTPSDII	54.8
STOFN3-1/FG+2	WYDT--------NASG	WSN--------FSQ	VGN--------VTSY	PYNSSS------LGNGTPSDII	68.2
STOFN3-1/FG+4	WYDT--------NASG	WSN--------FSQ	VGN--------VTSY	PYNSSSSS----LGNGTPSDII	68.0
STOFN3-1/FG+8	WYDT--------NASG	WSN--------FSQ	VGN--------VTSY	PYNSSSSSSSSSLGNGTPSDII	67.2

FIG. 4C

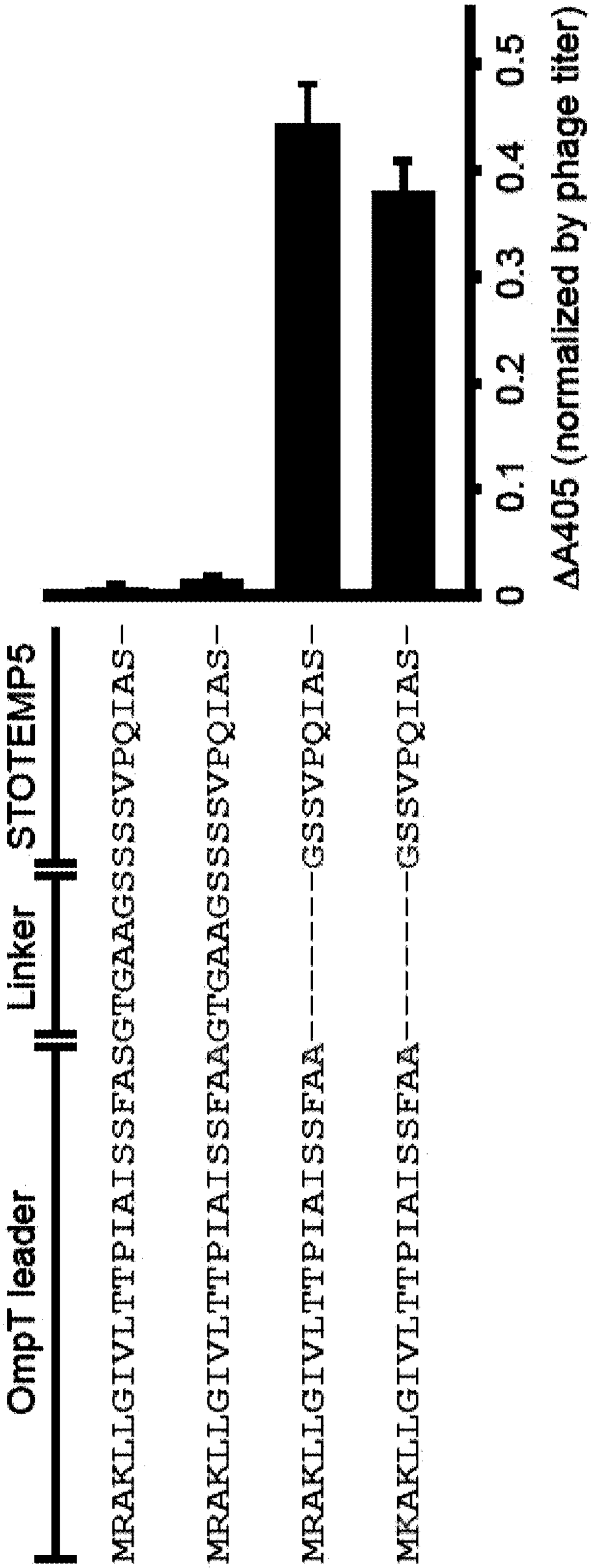
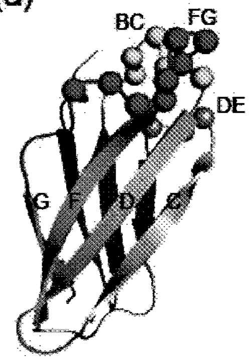

OmpT leader
Linker
STOTEMP5
MRAKLLGIVLTTPIAISSFASGTGAAGSSSSVPQIAS-
MRAKLLGIVLTTPIAISSFAAGTGAAGSSSSVPQIAS-
MRAKLLGIVLTTPIAISSFAA-------GSSVPQIAS-
MKAKLLGIVLTTPIAISSFAA-------GSSVPQIAS-
0
0.1
0.2
0.3
0.4
0.5
ΔA405 (normalized by phage titer)

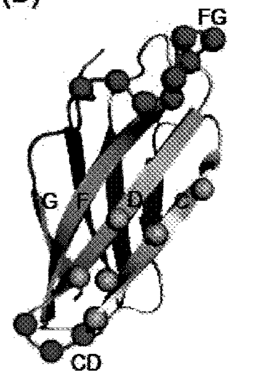
FIG. 5

(a)



Target	Clone	K_D (nM)	Amino acid sequece
			315 320 330 BC 350 DE 370 380 FG 400
	Library	-	sSSSVPQIASIASGPETIITVKWY(X_{5-9})----YYYITYWSSSSQKVTINVG(X_3)SYTIKHLKPGVTYYIQIVP(X_{6-13})-------PSDIISATPSSs
ySUMO	SUMOMbL03	63 ±18	sSSSVPQIASIASGPETIITVKWYDTNAS----YYYITYWSSSSQKVTINVGKPVSYTIKHLKPGVTYYIQIVPYYYEPSEDFWWGIPSDIISATPSSs
ySUMO	SUMOMbL14	44 ±15	sSSSVPQIASIASGPETIITVKWYDTNAS----YYYITYWSSSSQKVTINVGSPISYTIKHLKPGVTYYIQIVPYYTIGPDEYYYI-PSDIISATPSSs
MBP	MBPMbL08	12 ±3	sSSSVPQIASIASGPETIITVKWYWSSYYWSPFYYYITYWSSSSQKVTINVGNVTSYTIKHLKPGVTYYIQIVPYWYQYFPRYGY--PSDIISATPSSs
MBP	MBPMbL17	41±14	sSSSVPQIASIAVGPETIITVKWYRIPHAY---YYYITYWSSSSQKVTINVGNVTSYTIKHLKPGVTYYIQIVPYYPSPWYLSYGY-PSDIISATPSSs
EGFP	EGFPMbL24	58 ±9	sSSSVPQIASIASGPETIITVKWYHEYSNY---YYYITYWSSSSQKVTINVGNVTSYTIKHLKPGVTYYIQIVPDAGGYDKG-----PSDIISATPSSs
EGFP	EGFPMbL26	23 ±7	sSSSVPQIASIASGPETIITVKWYDAIYTVT--YYYITYWSSSSQKVTINVGNVTSYTIKHLKPGVTYYIQIVPESGWYSKW-----PSDIISATPSSs
AblSH2	AblSH2MbL03	105 ±11	sSSSVPQIASIASGPETIITVKWYPWYAYGD--YYYITYWSSSSQKVTINVGRRYSYTIKHLKPGVTYYIQIVPVNSLGNGT-----PSDIISATPSSs
AblSH2	AblSH2MbL04	78 ±18	sSSSVPQIASIASGPETIITVKWYYYSIDV---YYYITYWSSSSQKVTINVGPQTSYTIKHLKPGVTYYIQIVPYSFYSDSDYGYY-PSDIISATPSSs
NSH2	NSH2MbL06	81 ±24	sSSSVPQIASIASGPETIITVKWYPTGY-----YYYITYWSSSSQKVTINVGYRNSYTIKHLKPGVTYYIQIVPRYYLSSKGATP--PSDIISATPSSs
NSH2	NSH2MbL10	158 ±54	sSSSVPQIASIASGPETIITVKWYPTGY-----YYYITYWSSSSQKVTINVGYRNSYTIKHLKPGVTYYIQIVPASYVVPYDT----PSDIISATPSSs
CSH2	CSH2MbL01	67 ±17	sSSSVPQIASIASGPETIITVKWYDTNAS----YYYITYWSSSSQKVTINVGRNHSYTIKHLKPGVTYYIQIVPGGWADYAWLWY--PSDIISATPSSs
CSH2	CSH2MbL03	103 ±53	sSSSVPQIASIASGPETIITVKWYDTNAS----YYYITYWSSSSQKVTINVGRMYSYTIKHLKPGVTYYIQIVPGGWSSPTYTWY--PSDIISATPSSs

(b)



Target	Clone	K_D (nM)	Amino acid sequece
			315 320 330 βC CD βD 360 370 380 FG 400
	Library	-	sSSSVPQIASIASGPETIITVKWYDTNASYYYIBYUS(X_{3-5})ZZVZIZVGNVTSYTIKHLKPGVTYYIQIVP(X_{6-12})------PSDIISATPSSs
ySUMO	SUMOMbS08	65 ±16	sSSSVPQIASIASGPETIITVKWYDTNASYYYIYYWSLQYYREAVKIEVGNVTSYTIKHLKPGVTYYIQIVPFEEFGFGWYSGDPSDIISATPSSs
ySUMO	SUMOMbS20	95 ±26	sSSSVPQIASIAVGPETIITVKWYDTNASYYYIYYWSKHP--KKVKIEVGNVTSYTIKHLKPGVTYYIQIVPFISYYDLYYSGDPSDIISATPSSs
ySUMO	SUMOMbS34	20 ±8	sSSSVPQIASIASGPETIITVKWYDTNASYYYIYYWSGPYYYKKVTIIVGNVTSYTIKHLKPGVTYYIQIVPYHVHDWGYYYY-PSDIISATPSSs
MBP	MBPMbS01	33 ±13	sSSSVPQIASIASGPETIITVKWYDTNASYYYISYWSADYDSEAVAIAVGNVTSYTIKHLKPGVTYYIQIVPYKANSYYPGYGYPSDIISATPSSs
MBP	MBPMbS09	9 ±1	sSSSVPQIASIASGPETIITVKWYDTNASYYYISYWSYST--ATVAIAVGNVTSYTIKHLKPGVTYYIQIVPYYGYRPYPSYGYPSDIISATPSSs
MBP	MBPMbS12	484 ±66	sSSSVPQIASIASGPETIITVKWYDTNASYYYISYWSGQS--ETVAIEVGNVTSYTIKHLKPGVTYYIQIVPYYPGYYPVHYV-PSDIISATPSSs
EGFP	EGFPMbS04	7 ±2	sSSSVPQIASIAVGPETIITVKWYDTNASYYYIDYWSHGE--ETVEIAVGNVTSYTIKHLKPGVTYYIQIVPYYGDGHLGL---PSDIISATPSSs
EGFP	EGFPMbS05	8 ±1	sSSSVPQIASIASGPETIITVKWYDTNASYYYIDYWSYSK--ETVTIEVGNVTSYTIKHLKPGVTYYIQIVPYYGPSLGQ----PSDIISATPSSs
EGFP	EGFPMbS12	4 ±2	sSSSVPQIASIASGPETIITVKWYDTNASYYYIDYWSYGT--ETVTINVGNVTSYTIKHLKPGVTYYIQIVPYYYGVLHQ----PSDIISATPSSs
AblSH2	AblSH2MbS01	71 ±10	sSSSVPQIASIASGPETIITVKWYDTNASYYYIYYWSTWPVSEEVEINVGNVTSYTIKHLKPGVTYYIQIVPVNSLGNGT----PSDIISATPSSs
AblSH2	AblSH2MbS02	13 ±6	sSSSVPQIASIASGPETIITVKWYDTNASFYYIYYWSTYPVTEEVEIKVGNVTSYTIKHLKPGVTYYIQIVPQYWYLGAGY---PSDIISATPSSs
AblSH2	AblSH2MbS07	827 ±140	sSSSVPQIASIASGPETIITVKWYDTNASYYYIYYWSSYPVTEEVEIKVGNVTSYTIKHLKPGVTYYIQIVPGYYSYPYYLS--PSDIISATPSSs

FIG. 6A-B

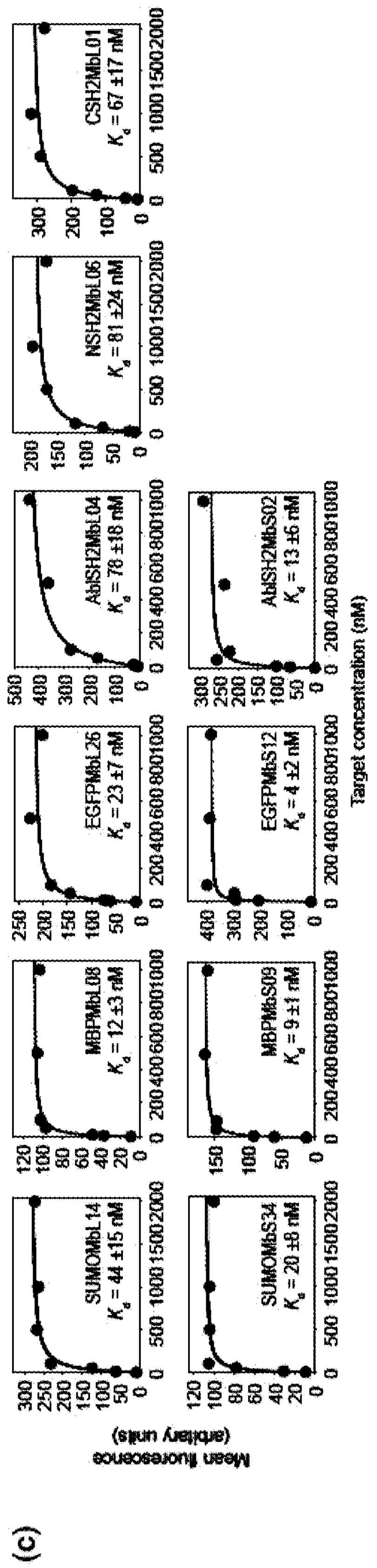

(c)
Mean fluorescence (arbitrary units)
Target concentration (nM)
SUMOMbL14
MBPMbL08
EGFPMbL26
AbISH2MbL04
NSH2MbL06
CSH2MbL01
SUMOMbS34
MBPMbS09
EGFPMbS12
AbISH2MbS02

FIG. 6C

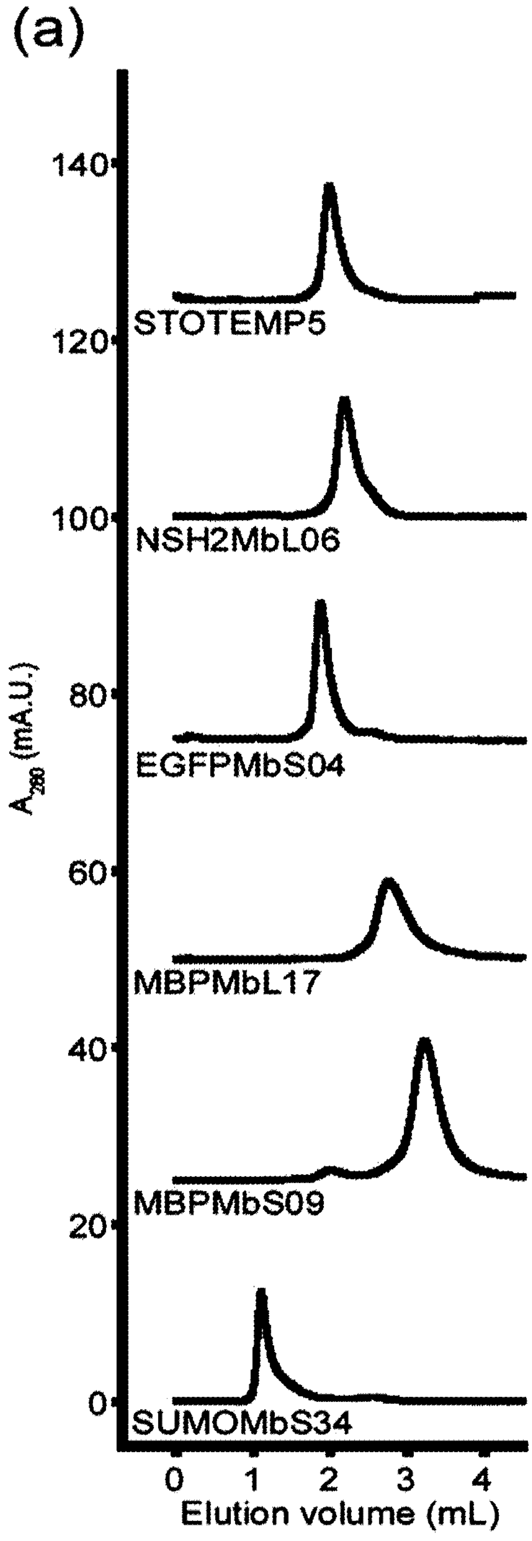

(a)
140
120
100
80
60
40
20
0
A_{280} (mA.U.)
STOTEMP5
NSH2MbL06
EGFPMbS04
MBPMbL17
MBPMbS09
SUMOMbS34
0
1
2
3
4
Elution volume (mL)

FIG. 7A

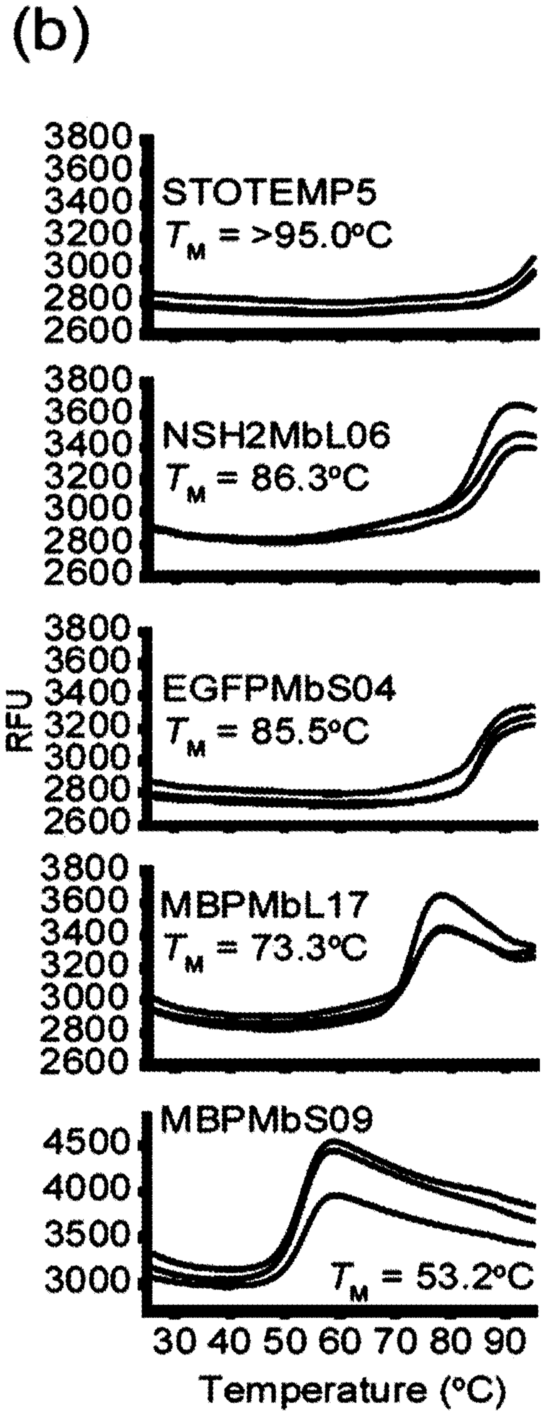

(b)
STOTEMP5
T_M = >95.0°C
NSH2MbL06
T_M = 86.3°C
EGFPMbS04
T_M = 85.5°C
MBPMbL17
T_M = 73.3°C
MBPMbS09
T_M = 53.2°C
RFU
3800
3600
3400
3200
3000
2800
2600
4500
4000
3500
3000
30 40 50 60 70 80 90
Temperature (°C)

FIG. 7B

(c)

	Oligomerization state	Themal stability (T_M, °C)
STOTEMP5	Monomeric	>95.0
SUMOMbL03	Monomeric	63.5
SUMOMbL14	Monomeric	63.5
MBPMbL08	Large aggregate	-
MBPMbL17	Late elution*	73.3
EGFPMbL24	Monomeric	54.2
EGFPMbL26	Late elution*	53.5
AblSH2MbL03	Monomeric	74.0
AblSH2MbL04	Monomeric	69.5
NSH2MbL06	Monomeric	86.3
NSH2MbL10	Monomeric	80.5
CSH2MbL01	Late elution*	64.8
CSH2MbL03	Late elution*	66.5
	Mean	68.1 ±10.1
SUMOMbS08	Large aggregate	-
SUMOMbS20	Monomeric	53.5
SUMOMbS34	Large aggregate	-
MBPMbS01	Late elution*	53.7
MBPMbS09	Late elution*	53.2
MBPMbS12	Monomeric	53.5
EGFPMbS04	Monomeric	85.5
EGFPMbS05	Monomeric	70.2
EGFPMbS12	Monomeric	66.0
AblSH2MbS01	Monomeric	71.0
AblSH2MbS02	Monomeric	53.5
AblSH2MbS07	Monomeric	50.5
	Mean	61.1 ±11.6

*Mono-dispersed single peak

FIG. 7C

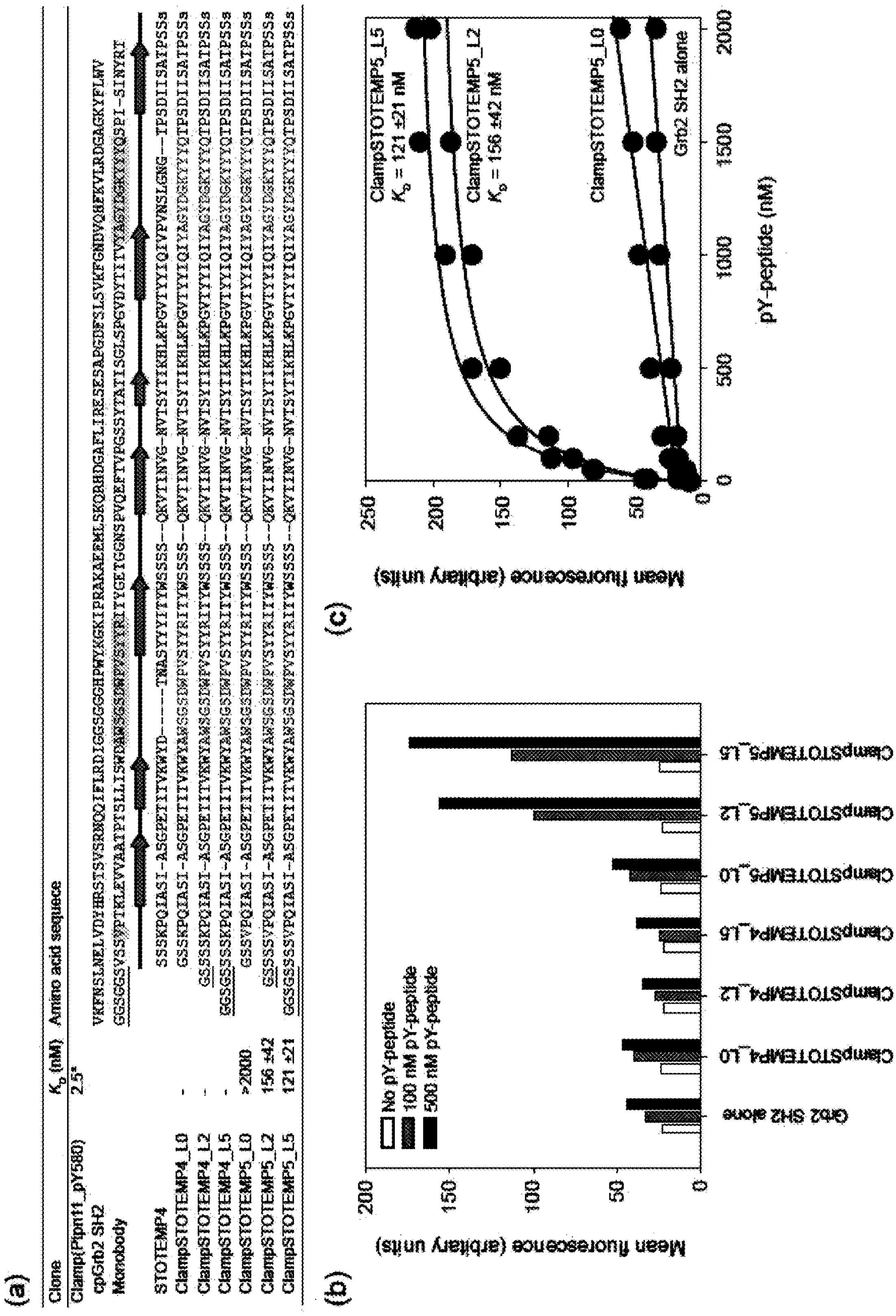

(a)
Clone
K_D (nM)
Amino acid sequece
Clamp(Ptpn11_pY580)
2.5*
cpGrb2 SH2
Monobody
STOTEMP4
ClampSTOTEMP4_L0
ClampSTOTEMP4_L2
ClampSTOTEMP4_L5
ClampSTOTEMP5_L0
>2000
ClampSTOTEMP5_L2
156 ±42
ClampSTOTEMP5_L5
121 ±21
(b)
No pY-peptide
100 nM pY-peptide
500 nM pY-peptide
Mean fluorescence (arbitary units)
Grb2 SH2 alone
ClampSTOTEMP4_L0
ClampSTOTEMP4_L2
ClampSTOTEMP4_L5
ClampSTOTEMP5_L0
ClampSTOTEMP5_L2
ClampSTOTEMP5_L5
(c)
ClampSTOTEMP5_L5
K_D = 121 ±21 nM
ClampSTOTEMP5_L2
K_D = 156 ±42 nM
ClampSTOTEMP5_L0
Grb2 SH2 alone
pY-peptide (nM)
Mean fluorescence (arbitary units)

FIG. 8A-C

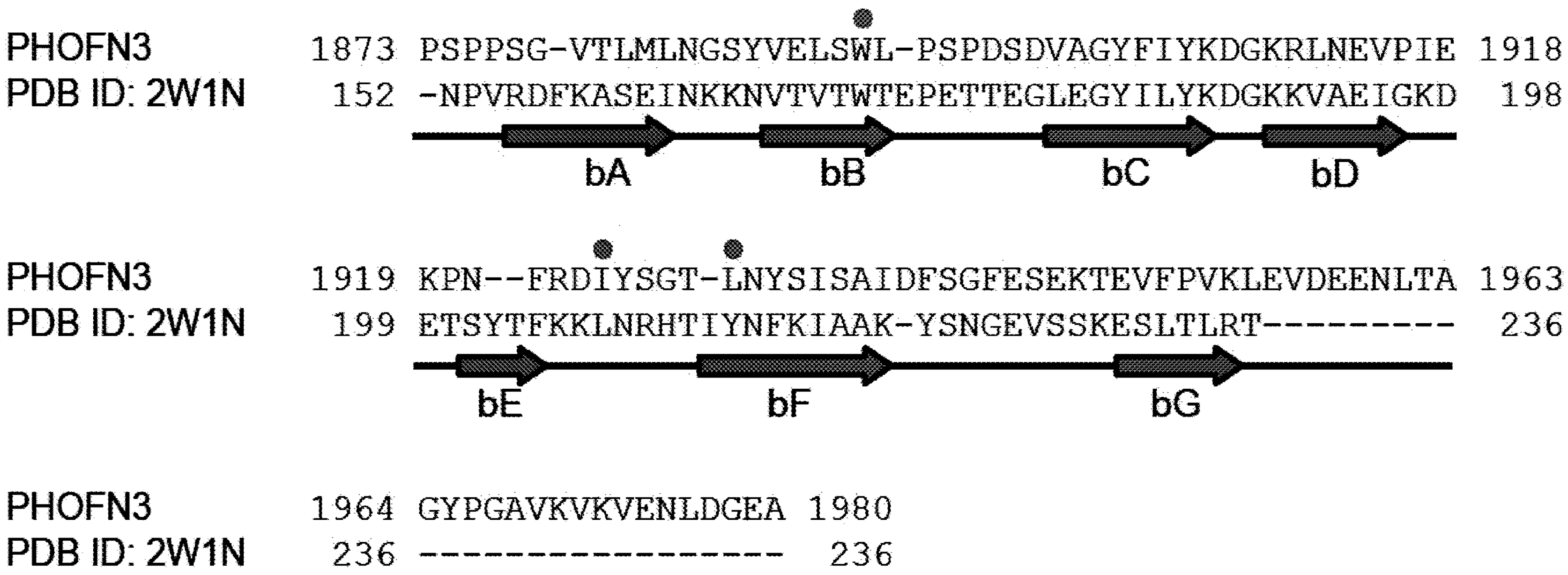

PHOFN3 1873 PSPPSG-VTLMLNGSYVELSWL-PSPDSDVAGYFIYKDGKRLNEVPIE 1918
PDB ID: 2W1N 152 -NPVRDFKASEINKKNVTVTWTEPETTEGLEGYILYKDGKKVAEIGKD 198
bA
bB
bC
bD
PHOFN3 1919 KPN--FRDIYSGT-LNYSISAIDFSGFESEKTEVFPVKLEVDEENLTA 1963
PDB ID: 2W1N 199 ETSYTFKKLNRHTIYNFKIAAK-YSNGEVSSKESLTLRT--------- 236
bE
bF
bG
PHOFN3 1964 GYPGAVKVKVENLDGEA 1980
PDB ID: 2W1N 236 ----------------- 236

FIG. 9

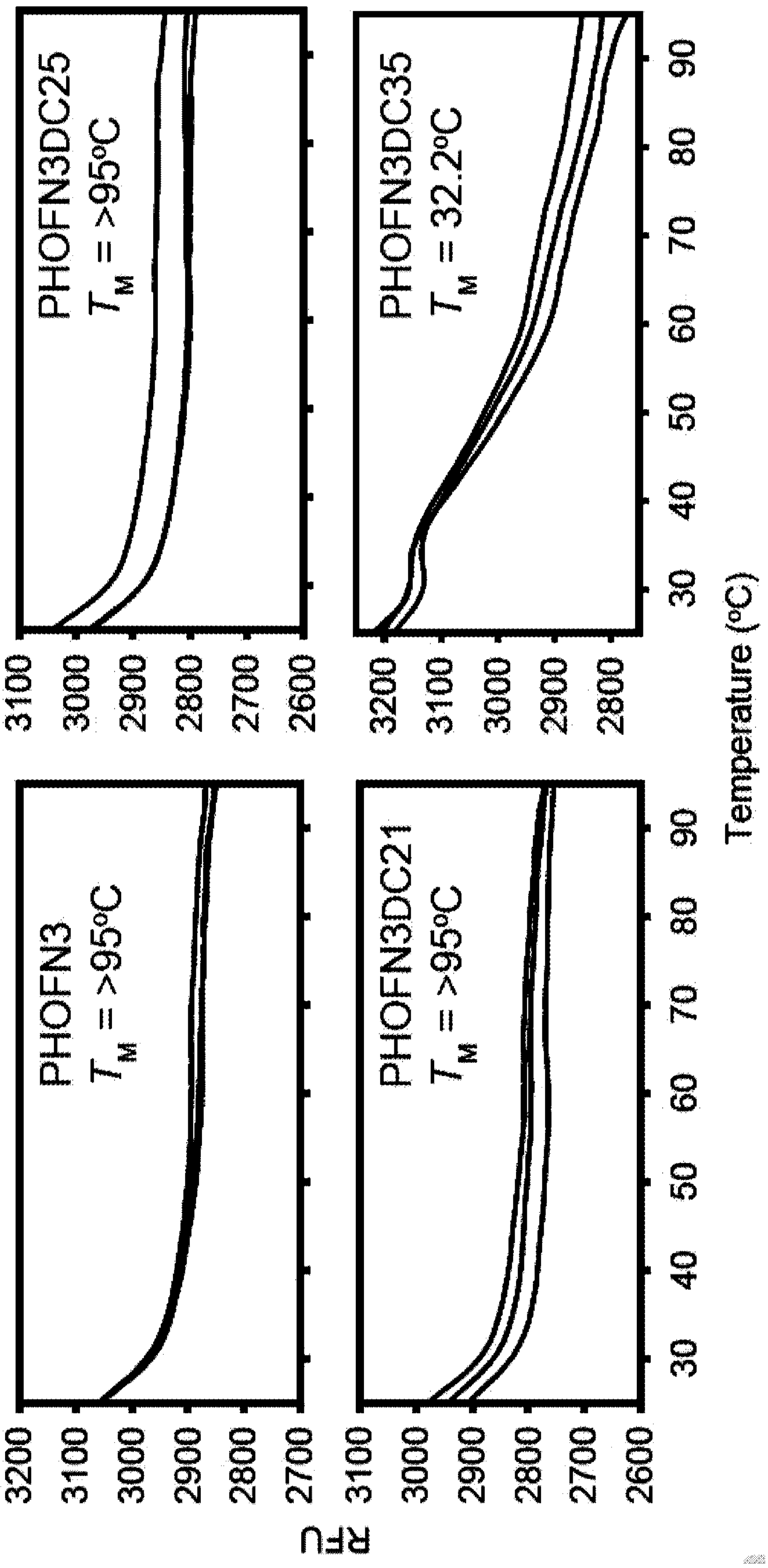

PHOFN3
T_M = >95°C
PHOFN3DC25
T_M = >95°C
PHOFN3DC21
T_M = >95°C
PHOFN3DC35
T_M = 32.2°C
RFU
Temperature (°C)

FIG. 10

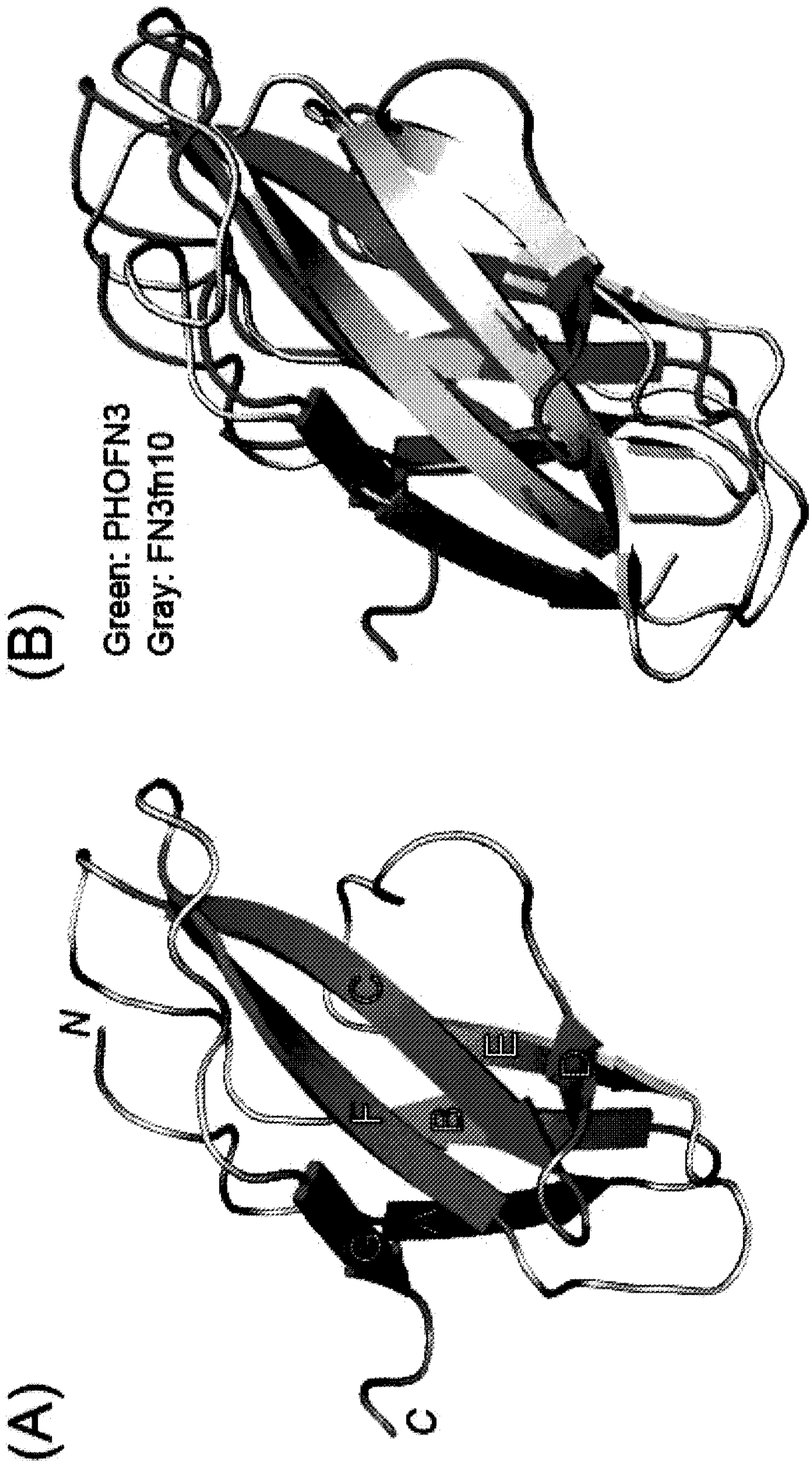


FIG. 11A-B

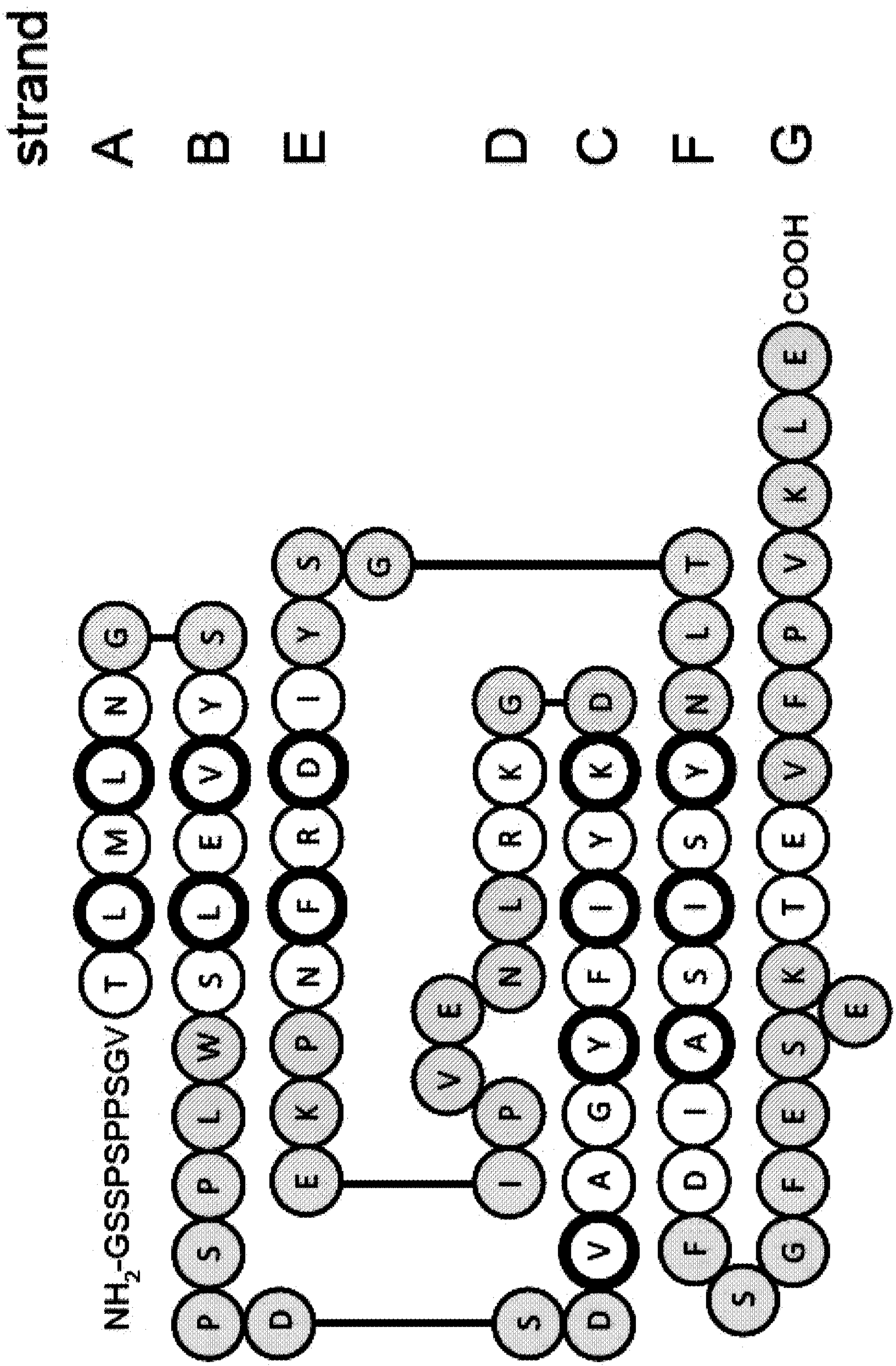


FIG. 12

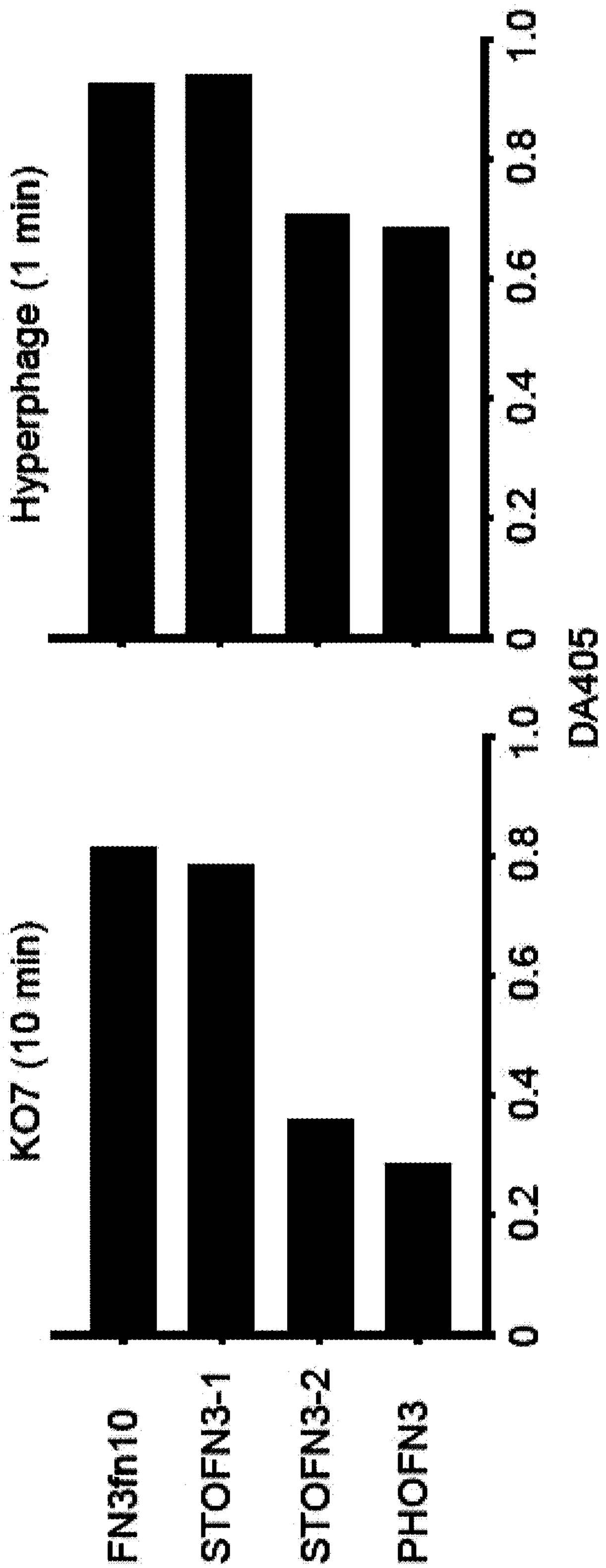

Hyperphage (1 min)
KO7 (10 min)
FN3fn10
STOFN3-1
STOFN3-2
PHOFN3
0
0.2
0.4
0.6
0.8
1.0
DA405

FIG. 13

METHODS AND COMPOSITION INVOLVING THERMOPHILIC FIBRONECTIN TYPE III (FN3) MONOBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national phase application under 35 U.S.C. § 371 of International Application No. PCT/US2019/018866 filed Feb. 21, 2019, which claims the benefit of priority of U.S. Provisional Patent Application No. 62/634,616 filed Feb. 23, 2018, all of which are hereby incorporated by reference in their entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under GM090324 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

The application contains a Sequence Listing prepared in compliance with ST.25 format and is hereby incorporated by reference in its entirety. Said Sequence Listing, created on Oct. 10, 2022 is named ARCD.P0616US_ST25 and is 114,037 bytes in size.

BACKGROUND OF THE INVENTION

Field of the Invention

Embodiments are directed generally to biology, medicine, and protein engineering.

Background

The fibronectin type III domain (FN3) has been established as an effective non-antibody, "alternative" scaffold for the generation of novel binding proteins. A member of the immunoglobulin superfamily, FN3 has three surface exposed loops at one end of the molecule which are analogous to antibody CDRs. Engineering strategies using this scaffold are based on combinatorial libraries created by diversifying both the length and amino acid sequence of these surface loops. From such libraries, FN3 variants capable of binding to a target of interest can be isolated using various selection methods. The utility of the FN3 scaffold has been demonstrated in producing high-affinity binding proteins to a number of different protein targets (reviewed in Bloom and Calabro, 2009). These binding proteins generated from this scaffold are referred to as monobodies. The FN3 scaffold offers many advantages compared to conventional antibodies or fragments thereof because it lacks disulfide bonds, can be readily and highly expressed in bacterial systems, and is relatively small.

The FN3 scaffold has produced high-affinity binding proteins to a number of distinct targets, achieving dissociation constants in the low- to mid-nanomolar range and as low as 1.1 picomolar (Bloom and Calabro, 2009; Hackel et al., 2008). Binding proteins based on the FN3 domain have also been used in a number of applications including, conformation specific purification of a target protein (Huang et al., 2006), probing protein conformational changes in cells (Koide et al., 2002), antagonism of a growth factor receptor for therapeutic use (Getmanova et al., 2006), inhibition of virus replication (Liao et al., 2009), and specifically inhibiting a protein-protein interaction in cells (Wojcik et al., 2010).

The ability to generate novel binding proteins capable of interacting with other proteins with high-affinity and/or specificity is important in biotechnology, medicine and molecular biology. Such designer binding proteins can be used in numerous applications. They can be used to label a protein of interest for detection and visualization, to purify a target protein from a complex mixture or to functionally perturb a target by blocking a functional site. Because of their broad utility, there is a need to develop strategies for producing novel binding proteins quickly and effectively and with improved stability prperties. To date, FN3-based binding proteins have been constructed by selections from combinatorial libraries in which loop regions are diversified (Bloom and Calabro, 2009).

SUMMARY OF THE INVENTION

The present application describes various compositions and methods for the production and use of FN3-based binding proteins with improved stability properties. Aspects of the disclosure relate to polypeptides comprising a variant fibronectin type III (FN3) domain from *Sulfolobus tokodaii* that includes one or more amino acid substitutions or insertions in a loop region of FN3, in a non-loop region of FN3, or in both.

In some embodiments, a polypeptide comprises a non-variant FN3 domain. In particular embodiments, the non-variant FN3 domain comprises at least 70% identity to SEQ ID NO:1. In some embodiments, the non-variant FN3 domain comprises SEQ ID NO: 1. In further embodiments, the non-variant FN3 domain comprises a polypeptide with at least 70% identity to SEQ ID NO:2 (STOFN3-2), 3 (STOFN3-3), or 4 (STOFN3-4). In some embodiments, the non-variant FN3 domain comprises SEQ ID NO:2, 3, or 4. In some embodiments, the amino acid substitution comprises one or more substitutions of the FN3 domain corresponding to amino acid positions 1, 2, and 3 of SEQ ID NO:1. In some embodiments, the amino acid substitutions at one or more positions corresponding to amino acid positions 1, 2, and/or 3 of SEQ ID NO:1 are a substitution with a hydrophilic amino acid or combinations thereof. In some embodiments, the amino acid insertion comprises one or more amino acid insertions of the FN3 domain after the amino acid corresponding to amino acid position 84 of SEQ ID NO:1. In some embodiments, the amino acid insertion comprises 1-3 amino acids. In some embodiments, the inserted amino acids are proline and serine or combinations thereof. In some embodiments, the substitution comprises one or more substitutions of the FN3 domain corresponding to amino acid positions 4, 14, 28, and 58 of SEQ ID NO:1. In some embodiments, the substitution corresponding to amino acid position 4 of SEQ ID NO:1 is with a valine. In some embodiments, the substitution corresponding to amino acid position 14 of SEQ ID NO:1 is with a proline. In some embodiments, the substitution corresponding to amino acid position 28 of SEQ ID NO:1 is with a tyrosine. In some embodiments, the substitution corresponding to amino acid position 58 of SEQ ID NO:1 is with a proline. In some embodiments, the one or more amino acid substitutions or insertions in the non-loop segment is in one or more of beta strand C or beta strand D. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 28, 29, 30, 31, 32, 33, 34, or 35 of SEQ ID NO:1. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 38, 39, 40, 41, 42, 43, 44, 45, or 46 of SEQ ID NO:1. In some embodiments, the one or more amino acid substitutions or insertions in the loop region of FN3 are in the BC loop, DE loop, FG loop and/or CD loop. In some embodiments, the DE loop does not comprise any inserted amino acids. In some embodiments, the EF loop comprises less than 3 substitutions or insertions. In some embodiments, the FN3 domain does not have substitutions at amino acid positions corresponding to positions 69 and 77 of SEQ ID NO:1. In some embodiments, the polypeptide further comprises a non-FN3 polypeptide that enhances the FN3 polypeptide binding affinity for a target molecule. In some embodiments, the polypeptide comprises or further comprises an insertion or deletion of at least 1 amino acids in at least one loop region of FN3. In some embodiments, at least one loop region of FN3 comprises an insertion of at least 2 amino acids. In some embodiments, at least one region of FN3 comprises an insertion of 2 to 25 amino acids in at least one loop region. In some embodiments, at least two loop regions comprise an insertion. In some embodiments, at least one loop region of FN3 comprises a deletion of at least 1 amino acid. In some embodiments, at least one loop region of FN3 comprises a deletion of 2 to 10 amino acids. In some embodiments, at least two loop regions comprise a deletion of at least 1 amino acid. In some embodiments, the polypeptide comprises at least 1 amino acid insertion and 1 amino acid deletion in at least one loop region. In some embodiments, the polypeptide comprises an insertion and deletion of at least 1 amino acid in the same loop region. In some embodiments, the polypeptide further comprises one or more serine residues immediately before amino acid 1 or immediately after amino acid 84 of SEQ ID NO:1.

In certain aspects, the insertion, deletion, or substitution, when described with respect to an amino acid at a position in SEQ ID NO:1 may be the corresponding amino acid at a corresponding position in SEQ ID NO:2, 3, and/or 4. The corresponding positions are those that are aligned with the amino acid in SEQ ID NO:1 in FIG. **1**.

In some embodiments, the AB, BC, CD, DE, EF, and/or FG loop or A, B, C, D, E, F, or G beta-strand may have at least, at most, or exactly 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acid substitutions, insertions and/or deletions (or combinations thereof). In certain aspects, beta strand variations can be used in conjunction with variations in the AB loop, the BC loop, the CD loop, the DE loop, and/or the FG loop of FN3 to generate a polypeptide library or a nucleic acid library encoding the same. FN3 polypeptides can be modified by inserting or deleting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 or more amino acids, or any range derivable therein, in a FN3 loop. In certain aspects, variations in loops AB, CD, and EF may be specifically excluded from invention, either individually or in various combinations. In some embodiments it is contemplated that an FN3 variant does not include a substitution, insertion and/or deletion in a bottom loop (loop AB, CD, EF). In further embodiments modifications in the bottom loop(s) is limited to 1, 2, 3, 4, or 5 or fewer substitutions, insertions, and/or deletions.

In certain embodiments, polypeptides comprise a variant fibronectin type III (FN3) domain comprising one or more amino acid substitutions in both a loop region of FN3 and in a non-loop region of FN3. In certain aspects, the one or more amino acid substitution in the non-loop segment is one or more substitution in beta strand C, beta strand D, beta strand F, and/or beta strand G.

In certain embodiments, the polypeptide can comprise 1, 2, 3, 4 or more insertions and/or deletions of amino acids corresponding to amino acids of SEQ ID NO:1. Insertions can include, but are not limited to stretches of poly-serine, poly-alanine, poly-valine, poly-threonine, or polymers of any other of the 20 amino acids, that is subsequently mutagenized or diversified for generating a combinatorial polypeptide library. Diversification of these inserted residues can include alteration to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 of the other natural amino acids. In certain aspects 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more contiguous amino acids are inserted into one or more of the AB, BC, CD, DE, EF, FG loops of a FN3 domain polypeptide. In a further aspect, the polypeptide can comprise an insertion, a deletion, or both an insertion and a deletion. The insertion and/or deletion can be at the beginning and/or end of the polypeptide. The insertion and deletion need not be located at the same position and may be located at sites distal or proximal to each other. The insertion and/or deletion can be in a loop or non-loop portion of the FN3 domain polypeptide. In certain aspects, at least one loop region of FN3 comprises an insertion of at least 2 amino acids. In a further aspect, at least one region of FN3 comprises an insertion of 2 to 25 amino acids in at least one loop region. In certain aspects at least 2, 3, or more loop regions comprise an insertion. In certain aspects, the polypeptide has at least one loop region of FN3 comprises a deletion of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids, including all values and ranges there between. In certain aspects, at least 2, 3, or 4 loop or non-loop segments, portions, or regions comprise a deletion of at least 1 amino acid. In certain aspects, the polypeptide comprises at least one insertion and one deletion in at least one loop or non-loop region. In a further aspect, the polypeptide comprises an insertion and a deletion in the same loop or non-loop region. The term region indicates the amino acids of a particular structural segment of the polypeptide as defined by secondary structure and/or crystal structure corresponding to the amino acids of SEQ ID NO:1 or its variants.

Further aspects relate to a polypeptide comprising a variant fibronectin type III (FN3) domain from *Pyrococcus horikoshii* comprising one or more amino acid substitutions or insertions in a loop region of FN3, in a non-loop region of FN3, or in both. In some embodiments, the non-variant FN3 domain comprises at least 70% identity to SEQ ID NO:6. In some embodiments, the non-variant FN3 domain comprises SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions in the non-loop segment is in one or more of beta strand C or beta strand D. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 13 and/or 14 of SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 20, 21, 22, 23, 24, 25, 26, and/or 27 of SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 36 and/or 37 of SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 40, 41, 42, 43, 44, 45, 46, 47, and/or 48 of SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 54, 55, 56, 57, 58, and/or 59 of SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions correspond to or after positions 67, 68, 69, 70. 71, 72, 73, and/or 74 of SEQ ID NO:6. In some embodiments, the one or more amino acid substitutions or insertions in the loop region of FN3 are in the AB loop, BC loop, CD loop, DE loop, EF loop, and/or FG loop. In some embodiments, the polypeptide further comprises a non-FN3 polypeptide that enhances the FN3 polypeptide binding affinity for a target molecule. In some embodiments, the polypeptide further comprises an insertion or deletion of at least 1 amino acids in at least one loop region of FN3. In some embodiments, at least one loop region of FN3 comprises an insertion of at least 2 amino acids. In some embodiments, at least one region of FN3 comprises an insertion of 2 to 25 amino acids in at least one loop region. In some embodiments, at least two loop regions comprise an insertion. In some embodiments, at least one loop region of FN3 comprises a deletion of at least 1 amino acid. In some embodiments, at least one loop region of FN3 comprises a deletion of 2 to 10 amino acids. In some embodiments, at least two loop regions comprise a deletion of at least 1 amino acid. In some embodiments, the polypeptide comprises at least 1 amino acid insertion and 1 amino acid deletion in at least one loop region. In some embodiments, the polypeptide comprises an insertion and deletion of at least 1 amino acid in the same loop region.

In certain aspects, the polypeptide is at least or at most 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical (or any derivable range therein) to a polypeptide sequence of the disclosure, including any of SEQ ID NOS:1-4 or 6.

In some embodiments, the polypeptide is isolated, recombinant, a non-natural polypeptide, engineered, and/or synthetic.

In some embodiments, the polypeptides of the disclosure bind specifically to a site on a target motif. In some embodiments, the polypeptides of the disclosure specifically bind a target motif. In some embodiments, the polypeptide further comprises a polypeptide comprising a biorecognition module including a molecular recognition domain; wherein the polypeptide comprising the biorecognition molecule and the polypeptide comprising the variant FN3 domain are operatively linked together either directly or indirectly via the linker, and are spatially oriented to bind the same, overlapping, or distinct sites on the target motif. In some embodiments, the polypeptide comprising the biorecognition molecule and the polypeptide comprising the variant FN3 domain bind overlapping sites on the target motif at the same time and are capable of forming a complex with the target motif. In some embodiments, the Kd for the polypeptide being equal to or lower than one μM. In some embodiments, the target motif is a peptide, a phosphorylated peptide or a methylated peptide. In some embodiments, the peptide is present within a protein. In some embodiments, the molecular recognition domain comprises an interaction domain or mutants of interaction domains. In some embodiments, the interaction domain is selected from the group consisting of PDZ, WW, SH2, PTB, SH3, Bromo, Chromo, PHD, Polo-box and FHA domains. In further embodiments, the polypeptide comprising the variant FN3 domain further comprises a first signaling moiety and the polypeptide comprising the biorecognition molecule further comprises a second signaling moiety and wherein the first and second signaling moieties are capable of interacting to produce a detectable signal. In some embodiments, the signaling moiety is a dye, a quencher, a reporter protein, or a quantum dot. In some embodiments, the first and second signaling moieties comprise a fluorescent resonance energy transfer (FRET) donor group and a FRET acceptor group, respectively, and binding of the first and second molecular recognition domains to the target motif results in a change in the FRET efficiency between the FRET donor and FRET acceptor groups.

The polypeptides of the invention can be comprised in a polypeptide library or encoded in a polynucleotide library that can be screened for particular polypeptide characteristics, e.g., biding affinity. One or more members of the library can then isolated from other members of the library and analyzed. In certain aspects the library comprises or encodes a plurality of those polypeptides described herein. In certain aspects, the polypeptide library is pre-selected to bind a target and those preselected members are then further diversified in selected amino acid position to generate a targeted library that is subsequently screened for a particular characteristic or property.

Certain aspects relate to a polypeptide library comprising a plurality of modified FN3 domain polypeptides comprising one or more amino acid substitutions, insertions, or deletions a loop region of FN3, in a non-loop region of FN3, or in both; wherein the unmodified FN3 domain comprises a polypeptide comprising the amino acid sequence of SEQ ID NOS:1-4 or 6. In some embodiments, the FN3 domain polypeptides comprises one or more amino acid substitutions corresponding to amino acid positions corresponding to positions 1, 2, 3, 4, 14, 28, and/or 58 of SEQ ID NO:1.

Certain aspects are directed to polynucleotides encoding one or more polypeptide described herein. In certain embodiments the polynucleotide is an expression cassette or an expression construct. The expression construct can be capable of expressing the encoded polypeptide in a host cell, such as a prokaryotic or eukaryotic cell line or strain. In certain aspects the expression construct is functional in one or more polypeptide expression systems known in the art. In a further aspect, the expression construct is functional in bacteria, yeast, insect cells or the like.

The polypeptide can further comprise a second FN3 domain that may or may not have been selected for affinity to a particular target. The second FN3 domain may or may not contain additional amino acid variations or diversification. In other aspects, the polypeptide can further comprise a non-FN3 polypeptide that enhances the FN3 polypeptide binding affinity for a target molecule. The non-FN3 polypeptide can include, but is not limited to domains involved in phospho-tyrosine binding (e.g., SH2, PTB), phosphoserine binding (e.g., UIM, GAT, CUE, BTB/POZ, VHS, UBA, RING, HECT, WW, 14-3-3, Polo-box), phosphothreonine binding (e.g., FHA, WW, Polo-box), proline-rich region binding (e.g., EVH1, SH3, GYF), acetylated lysine binding (e.g., Bromo), methylated lysine binding (e.g., Chromo, PHD), apoptosis (e.g., BIR, TRAF, DED, Death, CARD, BH), cytoskeleton modulation (e.g., ADF, GEL, DH, CH, FH2), or other cellular functions (e.g., EH, CC, VHL, TUDOR, PUF Repeat, PAS, MH1, LRR1 IQ, HEAT, GRIP, TUBBY, SNARE, TPR, TIR, START, SOCS Box, SAM, RGS, PDZ, PB1, LEVI, F-BOX, ENTH, EF-Hand, SHADOW, ARM, ANK).

In certain aspects, variants in any one or more of positions that correspond with amino acid position 13, 14, 15, 16, 22, 23, 24, 25, 26, 27, 36, 37, 47, 48, 49, 54, 55, 56, 57, 58, 59, 60, 71, 72, 73, 74, 75, 76, 77, 78, 79 and/or 80 of SEQ ID NO:1, including all ranges there between, can be specifically included in the claimed embodiments.

In other embodiments, variants in any one or more of positions that correspond with amino acid positions 13, 14, 20, 21, 22, 23, 24, 25, 26, 27, 36, 37, 40, 41, 42, 43, 44, 45, 46, 47, 48, 54, 55, 56, 57, 58, 59, 67, 68, 69, 70, 71, 72, 73 and 74 of SEQ ID NO:6, including all ranges there between, can be specifically included in the claimed embodiments.

In some embodiments, the polypeptide library comprises domain polypeptides are at least 50%, 60%, 70%, 80%, or 90% identical to SEQ ID NO:1, 2, 3, 4, or 6.

In some embodiments, the length of the polypeptide is at least, at most, or exactly about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 505, 510, 515, 520, 525, 530, 535, 540, 545, 550, 555, 560, 565, 570, 575, 580, 585, 590, 595, 600, 625, 650, 675, 700, 750, 800, or 900 amino acids (or any derivable range therein). In some embodiments, the polypeptide is truncated or not full length.

In some embodiments, the polypeptide library further comprises a non-FN3 polypeptide that enhances the FN3 polypeptide binding affinity for a target molecule. In some embodiments, the polypeptide library futher comprises an insertion or deletion of at least 1 amino acids in at least one loop region of the FN3 domain polypeptides. In some embodiments, at least one loop region of FN3 comprises an insertion of at least 2 amino acids. In some embodiments, at least one region of FN3 comprises an insertion of 2 to 25 amino acids in at least one loop region. In some embodiments, at least two loop regions comprise an insertion. In some embodiments, at least one loop region of FN3 comprises a deletion of at least 1 amino acid. In some embodiments, at least one loop region of FN3 comprises a deletion of 2 to 10 amino acids. In some embodiments, at least two loop regions comprise a deletion of at least 1 amino acid. In some embodiments, the polypeptide comprises at least 1 amino acid insertion and 1 amino acid deletion in at least one loop region. In some embodiments, the FN3 domain polypeptides comprise an insertion and deletion of at least 1 amino acid in the same loop region. In some embodiments, the library is pre-selected to bind a target.

Certain embodiments are directed to a monobody library comprising a plurality of polypeptides having a variant fibronectin type III (FN3) domain, compared to wildtype (SEQ ID NO:1-4 or 6), comprising one or more alterations or variants in a beta strand or a loop region. In some embodiments, the alterations are in one or more of beta strand C, beta strand D, BC loop, CD loop, DE loop, or and/or FG loop. In some embodiments, the monobody comprises one or more of the variant amino acids corresponding to position 1, 2, 3, 4, 14, 28, and/or 58 of SEQ ID NO:1. In some embodiments, the variant FN3 domains further comprise an insertion, substitution, or deletion of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 amino acids in at least one loop region of FN3.

The monobody library can also comprise variant FN3 domains comprising an amino acid insertion in loop FG. The monobody library can also comprise a plurality of those polypeptides described above.

Certain embodiments include methods of making a polypeptide or polynucleotide library comprising a plurality of FN3 variants. In certain aspects the library can contain 10, 100, 1000, 104, 105, 106, 107, 108, 109, 1010, 1011, 1012, 1013, 1014, 1015 or more different polypeptide or polynucleotide variants, including all values and ranges there between, though it will be understood that there may be duplicate variants. The methods of making such a polypeptide or polynucleotide include the engineering of various amino acids substitutions, deletions, and/or insertion described herein.

Certain embodiments include methods of selecting one or more FN3 variants comprising conducting one or more binding assays using a FN3 library having a plurality of different FN3 variants. In certain aspects the library can comprise FN3 polypeptides having amino acid variations in the FN3 loops, FN3 beta-strands, or both FN3 loops and beta-strands. After conducting the binding assay(s) one or more FN3 variants are selected that have a particular property, such as binding specificity and/or binding affinity to a target. In certain aspects, the amino acid or nucleic acid sequence of one or more of the selected library members can be determined using conventional methods. The sequence of the selected FN3 polypeptide(s) can then be used to produce a second library that introduces further variation of the selected sequences. The second library can then be screened for FN3 polypeptides having a particular property. The process can be repeated 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more times. Additional iterations would enrich the library as well as potentially include other variants.

In certain aspects the method for selecting a protein binding domain specific for a target comprises (a) detecting target specific binding of one or more members of a polypeptide library comprising a plurality of FN3 domain polypeptides as described herein; and (b) selecting the protein binding domain that specifically binds the target. The method can further comprise first preparing the plurality of FN3 domain polypeptide variants described herein. A polypeptide identified as exhibiting a particular characteristic can be isolated. In certain aspects, the method can further comprise determining the nucleic acid and/or the amino acid of sequence of the selected protein binding domain. The selected protein binding domain can then be synthesized or expressed.

Methods can further comprise conducting a first screen of a library having amino acid variations in only FN3 loops or only FN3 beta strands and conducting a second screen using variations in only FN3 loops or only FN3 beta strands. In certain aspects the first screen uses only variations in the FN3 loops and the second screen only uses variations in the FN3 beta-strands. In a further aspect, the second screen can use variations in both FN3 loops and beta-strands. In certain aspects, the FN3 amino acid residues varied in the first screen are or are not varied in the second screen.

Further aspects include methods of identifying a polypeptide that specifically binds a target comprising detecting specific binding of one or more polypeptides of a polypeptide library, the library comprising a plurality of fibronectin type III (FN3) polypeptides as described herein.

Further aspects include methods of detecting a target molecule comprising contacting a sample containing the target with a fibronectin type III (FN3) binding domain that specifically binds the target.

Yet further aspects relate to a method of making a polypeptide or polypeptide library according to the disclosure, the method comprising expressing a polynucleotide of the disclosure or a polynucleotide encoding such polypeptide in a host cell.

Certain aspects include methods of producing a fibronectin type III (FN3) variant comprising: (a) expressing a polypeptide comprising an amino acid sequence; and (b) isolating and/or purifying the expressed variant FN3 domain from a host cell expressing the variant FN3.

Certain embodiments are directed to kits. In certain aspects, a kit can comprise a plurality of polypeptides as described herein. In a further aspect, a kit can comprise a plurality of polynucleotides encoding FN3 domain variants as described herein.

Further aspects of the disclosure relate to methods for testing a polypeptide according to the embodiments described herein, the method comprising contacting the polypeptide with a target molecule and testing for binding activity between the polypeptide and the target molecule.

The term "fibronectin type III domain" or "FN3 domain" refers to a domain (region) from a wild-type fibronectin from any organism.

The term "fibronectin type III domain variant" or "FN3 variant domain" refers to a polypeptide region in which one or more amino acid substitutions, deletions, and/or insertions are present as compared to the amino acid sequence of a wildtype FN3 domain. In certain embodiments, the FN3 variant or FN3 variant domain has an alteration with respect to specifically the fibronectin type III domain of SEQ ID NO:1-4 or 6. The term "substitutional variant" includes the replacement of one or more amino acids in a peptide sequence with a conservative or non-conservative amino acid(s). In some embodiments, the FN3 domain variant has increased binding properties compared to the wildtype FN3 domain relative to a particular target.

The term "FN3-domain polypeptide" refers to a polypeptide that includes at least one FN3 domain. A "variant FN3 domain polypeptide" refers to a polypeptide that includes at least one variant FN3 domain. It is contemplated that such polypeptides are capable of specifically binding a polypeptide or protein.

A "non-FN3 binding sequence" refers to an amino acid sequence of more than 15 contiguous amino acid residues that is not present in an FN3 domain or an FN3 domain variant and that specifically binds to a protein or polypeptide. In some embodiments, a non-FN3 binding sequence is specifically a non-tenth module fibronectin type III domain binding sequence.

The β sheet is a form of regular secondary structure in proteins. Beta sheets consist of beta strands connected laterally by at least two or three backbone hydrogen bonds, forming a generally twisted, pleated sheet. A beta strand (also β strand) is a stretch of polypeptide chain typically 3 to 10 amino acids long with backbone in an almost fully extended conformation.

A loop is a less ordered, flexible stretch of amino acids (as compared to alpha helices and beta sheets) that typically connect other structural elements of a protein. In the context of FN3, the loops are designated by the beta-strands they connect, for example the loop connecting beta-strand A and beta-strand B is the AB loop.

Beta strand A refers to the amino acids preceding the AB loop

Beta strand B refers to the amino acids connecting the AB and BC loops

Beta strand C refers to the amino acids connecting the BC and CD loops.

Beta strand D refers to the amino acids connecting the CD and DE loops.

Beta strand E refers to the amino acids connecting the DE and EF loops.

Beta strand F refers to the amino acids connecting the EF and FG loops.

Beta strand G refers to the amino acids after the FG loop.

The term "binding protein" refers to a polypeptide that specifically binds another compound, such as a polypeptide through non-covalent chemical interactions.

As used herein, "monobody" is intended to mean a polypeptide having a sequence and structure related to the tenth module of the fibronectin type III domain (FN3) that includes a beta-strand domain lacking in disulfide bonds and containing a plurality of beta-strands, two or more loop regions each connecting one beta-strand to another beta-strand, and optionally an N-terminal tail, a C-terminal tail, or both, wherein at least one of the two or more loop regions, the N-terminal tail, and or the C-terminal tail is characterized by activity in binding a target protein or molecule. More specifically, in some embodiments such monobodies can include three or more loop regions or, even more specifically, four or more loop regions. The size of such polypeptide monobodies is preferably less than about 30 kDa, more preferably less than about 20 kDa.

The term "library" refers to a collection (e.g., to a plurality) of polypeptides having different amino acid sequences and different protein binding properties. In some embodiments there is a variant FN3 domain library comprising polypeptides having different variations of the FN3 domain. Unless otherwise noted, the library is an actual physical library of polypeptides or nucleic acids encoding the polypeptides. In further embodiments, there is a database that comprises information about a library that has been generated or a theoretical library that can be generated. This information may be a compound database comprising descriptions or structures of a plurality of potential variant FN3 domains. "FN3-based molecule" refers to a molecule having an amino acid sequence of an FN3 domain or FN3 variant domain.

The term "specifically binds" or "specific binding" refers to the measurable and reproducible ability of an FN3-based molecule to bind another molecule (such as a target), that is determinative of the presence of the target molecule in the presence of a heterogeneous population of molecules including biological molecules. For example, an FN3-based molecule that specifically or preferentially binds to a target is a polypeptide that binds this target with greater affinity, avidity, more readily, and/or with greater duration than it binds to most or all other molecules. "Specific binding" does not necessarily require (although it can include) exclusive binding.

An polypeptide that specifically binds to a target with an affinity of at least 1×10-6 M at room temperature under physiological salt and pH conditions, as measured by surface plasmon resonance.

The term "non-natural amino acid residue" refers to an amino acid residue that is not present in the naturally occurring FN3 domain in a mammal, such as a human. The term "non-natural polypeptide" refers to a polypeptide that is not wild-type and/or not found in nature.

The terms "tag", "epitope tag" or "affinity tag" are used interchangeably herein, and usually refers to a molecule or domain of a molecule that is specifically recognized by an antibody or other binding partner. The term also refers to the binding partner complex as well. Thus, for example, biotin or a biotin/avidin complex are both regarded as an affinity tag. In addition to epitopes recognized in epitope/antibody interactions, affinity tags also comprise "epitopes" recognized by other binding molecules (e.g., ligands bound by receptors), ligands bound by other ligands to form heterodimers or homodimers, His6 bound by Ni-NTA, biotin bound by avidin, streptavidin, or anti-biotin antibodies, and the like.

Epitope tags are well known to those of skill in the art. Moreover, antibodies specific to a wide variety of epitope tags are commercially available. These include but are not limited to antibodies against the DYKDDDDK epitope, c-myc antibodies (available from Sigma, St. Louis), the HNK-1 carbohydrate epitope, the HA epitope, the HSV epitope, the His4, His5, and His6 epitopes that are recognized by the His epitope specific antibodies (see, e.g., Qiagen), and the like. In addition, vectors for epitope tagging proteins are commercially available. A polypeptide can be tagged with the FLAG® epitope (N-terminal, C-terminal or internal tagging), the c-myc epitope (C-terminal) or both the FLAG (N-terminal) and c-myc (C-terminal) epitopes.

The term "conjugate" in the context of an FN3-based molecule refers to a chemical linkage between the FN3-based molecule and a non-FN3-based molecule. It is specifically contemplated that this excludes a regular peptide bond found between amino acid residues under physiologic conditions in some embodiments.

Other embodiments of the invention are discussed throughout this application. Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well and vice versa. The embodiments in the Example section are understood to be embodiments of the invention that are applicable to all aspects of the invention.

"Biorecognition module" and "recognition module" (used interchangeably), as used herein, refer to a biomolecule which makes up one module of the modular molecular affinity clamp embodying the principles of the invention. A biorecognition module contains a molecular recognition domain that has affinity for a target motif of interest.

"Molecular recognition domain" and "recognition domain", (used interchangeably), as used herein, refer to a binding domain within a biorecognition module that demonstrates an ability to bind to a target motif, i.e., has binding affinity for a target motif.

The terms "target" and "target molecule," as used herein, refer to a peptide, antigen or epitope that specifically binds to an FN3-based binding molecule or monobody described herein, or any biomolecule of interest for which a molecular affinity clamp is sought. Targets include, but are not limited to, epitopes present on proteins, peptides, carbohydrates, and/or lipids. Exemplary targets include, but are not limited to, secreted peptide growth factors, pharmaceutical agents, cell signaling molecules, blood proteins, portions of cell surface receptor molecules, portions of nuclear receptors, steroid molecules, viral proteins, carbohydrates, enzymes, active sites of enzymes, binding sites of enzymes, portions of enzymes, small molecule drugs, cells, bacterial cells, proteins, epitopes of proteins, surfaces of proteins involved in protein-protein interactions, cell surface epitopes, diagnostic proteins, diagnostic markers, plant proteins, peptides involved in protein-protein interactions, and foods. The target may be associated with a biological state, such as a disease or disorder in a plant or animal as well as the presence of a pathogen. When a target is "associated with" a certain biological state, the presence or absence of the target or the presence of a certain amount of target can identity the biological state.

A "target motif", as used herein, refers to any portion or sequence of a target of interest for which a molecular affinity clamp is sought, e.g., refers to a pattern of amino acid residues which is recognized by particular recognition domains. In accordance with the invention, the target motif can bind more than one recognition domain. In other words, a target motif is one to which an affinity clamp embodying the principles of the invention can bind with high affinity and specificity. Of particular importance are target motifs that are short peptides of about 2-100 amino acid residues, especially those of 3-10 amino acid residues. A target motif may be, be at least, or be at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 residues in length (or any range derivable therein), which may or may not be contiguous. A target motif may include 1, 2, 3, 4, 5 or more noncontiguous residues and/or regions of residues that may be, be at least, or be at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 residues in length (or any range derivable therein).

As used herein, the term "binds" in connection with the interaction between a target motif and a recognition domain indicates that the recognition domain associates with (e.g., interacts with or complexes with) the target motif to a statistically significant degree as compared to association with proteins generally (i.e., non-specific binding). Thus, the term "molecular recognition domain" is also understood to refer to a domain that has a statistically significant association or binding with a target motif.

In the context of a recognition domain binding to a target motif, the term "greater affinity" indicates that an affinity clamp binds more tightly than a reference domain, or than the same domain in a reference condition, i.e., with a lower dissociation constant. In particular embodiments, the greater affinity is at least 2-fold.

Also in the context of recognition domain binding to a target motif, the term "altered specificity" indicates that relative binding affinity of an affinity clamp is different from that exhibited by a biorecognition module alone. In other words, "altered binding specificity" may refer to an increased binding constant of the affinity clamp for one target motif without the same level of increase for another target, an unchanged binding constant for one target with a decreased binding constant for another, target or a combination thereof.

The term "linked" refers to any method of functionally connecting peptides, particularly the two modules of the modular affinity clamps embodying the principles of the invention. "Linked" may also refer to non-covalent physical association. The biomolecular modules making up the biorecognition modules of the affinity clamps may be linked directly covalently, e.g., via a peptide linkage, or non-covalently, or indirectly via a linker.

A "linker" or "linker moiety," (used interchangeably) may refer to a peptide sequence of about 30 or more amino acid residues that is configured to associate two biorecognition modules in an orientation that facilitates binding of each module to a target motif. The linker, generally, is bifunctional in that it includes a functionality for linking the first biorecognition module and a functionality for linking the second biorecognition module.

By "binding site" is meant an area or region within a recognition domain where a biomolecule can bind non-covalently, i.e., interact with higher affinity than background interactions between molecules. Binding sites embody particular shapes and often can contain multiple binding pockets present within the binding site. The particular shapes are often conserved within a class of molecules, such as a protein family. Binding sites within a class also can contain conserved structures such as, for example, chemical moieties, the presence of a binding pocket, and/or an electrostatic charge at the binding site or some portion of the binding site, all of which can influence the shape of the binding site. It is noted that a molecular affinity clamp is distinguishable from other protein-based compositions that are multivalent—i.e., bind multiple but separate target motifs that are the same. Moreover, in some embodiments a molecular affinity clamp has one component that specifically binds to a target motif or an amino acid sequence that is distinct, overlapping, or the same as another target motif or amino acid sequence specifically bound by another component in the affinity clamp. The different binding components of a molecular affinity clamp do not compete against one another for binding to a target motif.

By "binding pocket" is meant a specific volume of space within a binding site that is available for occupation by a biomolecule. A binding pocket can often be a particular shape, indentation, groove, or cavity in the binding site. Binding pockets can contain particular chemical groups or structures that are important in the non-covalent binding of another molecule such as, for example, groups that contribute to ionic, hydrogen bonding, or van der Waals interactions between the molecules.

By "orientation" or "oriented," in reference to a biorecognition module bound to a target motif, is meant the spatial relationship of the biorecognition module, and at least some of its constituent atoms, to the atoms of the target motif.

By "assaying" is meant the creation of experimental conditions and the gathering of data regarding a particular result of the experimental conditions. For example, enzymes can be assayed based on their ability to act upon a detectable substrate. A particular target motif, in a test sample, can be assayed based on its ability to bind to a monobody or molecular affinity clamp.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably and mean polymers of amino acid monomers linked by peptide linkages between carboxyl (COOH) groups and amine (NH2) groups. A peptide may consist entirely of naturally occurring amino acid monomers, non-naturally occurring amino acids, or mixtures thereof. Unless denoted otherwise, whenever an amino acid sequence is represented, it will be understood that the amino acids are in N-terminal to C-terminal order from left to right. The term "polypeptide" may refer to small peptides, larger polypeptides, proteins containing single polypeptide chains, proteins containing multiple polypeptide chains, and multi-subunit proteins.

The term "amino acid", as used herein, refers to any amino acid, natural or non-natural, that may be incorporated, either enzymatically or synthetically, into a polypeptide or protein. Amino acids may also be altered. The term thus encompasses amino acids that have been modified naturally or by interaction. Examples may include, but are not limited to, phosphorylation, glycosylation, methylation, biotinylation, and any covalent and non-covalent additions to a protein that do not result in a change in amino acid sequence.

The term "label" as used herein refers to any tag, marker, or identifiable moiety. The skilled artisan will appreciate that many labels may be used in the methods of the invention. For example, labels include, but are not limited to, affinity tags, fluorophores, radioisotopes, chromogens, dyes, magnetic probes, magnetic particles, paramagnetic particles, electrophoretic molecules and particles, dielectrophoretic particles, phosphorescence groups, chemiluminescent, mobility modifiers, and particles that confer a dielectrophoretic change.

As used herein, the term "modulating" or "modulate" refers to an effect of altering a biological activity, especially a biological activity associated with a particular biomolecule. For example, an agonist or antagonist of a particular biomolecule modulates the activity of that biomolecule, e.g., an enzyme.

As used herein, the term "library" refers to any collection of two or more different polypeptides or proteins. In certain embodiments, a library may be a collection of polypeptides that have been modified to favor the inclusion of certain amino acid residues, or polypeptides of certain lengths.

As used herein, the term "variant" is meant to refer to a polypeptide differing from another polypeptide by one or more amino acid substitutions resulting from engineered mutations in the gene coding the polypeptide.

As used herein in connection with numerical values, the terms "approximately" and "about" are meant to encompass variations of ±20% to ±10% or less of the indicated value.

The terms "inhibiting," "reducing," or "preventing," or any variation of these terms, when used in the claims and/or the specification includes any measurable decrease or complete inhibition to achieve a desired result.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

It is contemplated that any embodiment discussed herein can be implemented with respect to any other embodiment discussed herein, and vice versa. Furthermore, compositions and kits can be used to achieve recited methods.

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." It is also contemplated that anything listed using the term "or" may also be specifically excluded.

As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. **1**A-C. Sequence alignment and biophysical properties of predicted STOFN3 domains. (a) Alignment of four predicted STOFN3 domains and the homologue FN3 domain of human receptor-type tyrosine-protein phosphatase F (PDB ID: 2DJU). Gaps are denoted as dashes. The human FN3 domain shows the sequence identities of 27, 29, 19 and 18% to STOFN3-1, STOFN3-2, STOFN3-3 and STOFN3-4, respectively. The ranges of the secondary structure of the human FN3 domain are shown below the sequences. Dots indicate the highly conserved residues of FN3 domains reported by Main et al. and Dickinson et al. (Main et al., 1992; Dickinson et al., 1994). Shown are SEQ ID NOS:8-17. (b) Size-exclusion chromatograms of STOFN 3-1 and STOFN3-2. The chromatographs are shown with vertical offsets for clarity. Both STOFN3 domains exhibited a mono-dispersed peak with the calculated molecular weight of ~12 kDa based on the calibration standards (not yet done, will do it). (c) Thermal stability of purified STOFN3-1 and STOFN3-2 monitored by DSF. One representative trace of three technical replicates is shown for each STOFN3 domains. The TM value is the mean of three replicates. The melting curves were measured by heating the samples at a rate of 0.5° C. per 30 seconds.

FIG. **2**A-C. The crystal structures of STOFN3-1 (a) and STOFN3-2 (b). For panel (a) and (b), the β-strands are labeled A-G. (c) Superposition of STOFN3-1, STOFN3-2 and FN3fn10 (PDBID: 1FNA). STOFN3-1, STOFN3-2 and FN3fn10 are colored green, cyan and gray, respectively.

FIG. **3**A-D. The structures of STOFN3-1 (STOTEMP1) and STOTEMP4 around the N-terminal (a-b) and C-terminal (c) regions. The 2Fo-Fc maps around the N-terminal regions of STOTEMP1 (a) and STOTEMP4 (b) are shown as mesh at the 1.5σ level. The N-terminal residues are indicated by green stick models, in which the oxygen and nitrogen atoms are colored red and blue, respectively. The BC-, DE- and FG-loops are colored yellow, pink and red. The low electron density of the segment N-terminal to P316S of STOTEMP4 indicates that this segment is conformationally disordered and dislodged from the folded portion. (c) Superposition of STOTEMP1 and STOTEMP4. STOTEMP1 is colored gray and the C-terminal residues are indicated as gray stick models. The C-terminal residues and A- and F-strands of STOTEMP4 are colored green, and the C-terminal residues are indicated as green stick models. The label of position 399 for STOTEMP1 is shown in parentheses. (d) Structure based alignment of STOFN3-1 with FN3fn10 (PDB ID: 1FNA) and amino acid sequences and melting temperatures of STOFN3-1 mutants. The ranges of the secondary structure of FN3fn10 are shown above the sequences. Mutated residues are colored in red. The mean TM values from three replicates are indicated. Shown are SEQ ID NOS:18-25.

FIG. **4**A-C. Schematic drawing of the amino acid sequence of STOFN3-1 in its secondary structure context (a). Loop residues as assigned by the program DSSP are shown in yellow. G342 and Y383 subjected to mutation in this work are also shown in cyan. An arrow marks the site at which poly-Serine residues were inserted. Residues of the β-strands whose side chain forms the hydrophobic core are enclosed in circles with thicker ring. (b)-(c) Amino acid sequences and melting temperatures of STOFN3-1 variants. Mutated residues are colored in red. The mean TM values from three replicates are indicated. FIG. **4**B shows SEQ ID NOS:26-41. FIG. **4**C shows SEQ ID NOS:42-54.

FIG. **5**. Optimization of the signal sequence and linker length for the efficient display of STOTEMP5. The amino acid sequences corresponding to OmpT leader, linker and STOTEMP5 are shown. Mutated residues are colored in red. Deleted residues are denoted as dashes. The display of STOTEMP5 on phage particle was measured by phage ELISA using an anti-V5 tag antibody and a HRP conjugated anti-M13 phage antibody. The absorbance changes at 405 nm after the reaction with 1-step Ultra TMB ELISA for 10 min at 25° C. are shown. For this measurement, aliquots of 50 μL of phage particles with normalized titers of 2×106 cfu/mL pre-blocked in 0.5% BSA/TBS were added per well. Shown are SEQ ID NOS:55-58.

FIG. **6**A-C. therMonobody library designs and generated clones. Amino acid sequences of therMonobodies generated from the loop-only library (a) and the side-and-loop library (b). The locations of diversified residues in the libraries are shown as spheres on the STOTEMP4 structure. "X" denotes a mixture of 30% Tyr, 13% Ser, 10% Gly, 5% Asp, 5% Leu, 5% Pro, 5% Trp and 2.5% each of all pther amino acids except for Cys and Met; "B", a mixture of Ala, Ser, Thr, Asn, Asp and Tyr; "U", a mixture of Ser, Thr, Asn and Tyr; "Z", a mixture of Ala, Glu, Lys and Thr. (c) Binding measurements by yeast surface display of representative therMonobodies for each combination of library and target. The mean fluorescence intensities of yeast cells displaying therMonobodies are plotted as a function of the target concentration. The error indicated are the standard deviations from curve fitting of the 1:1 binding model. FIG. **6**A shows SEQ ID NOS:59-71. FIG. **6**B shows SEQ ID NOS:72-84.

FIG. **7**A-C. Oligomerization state and thermal stability of generated therMonobodies. (a) Size-exclusion chromatograms of therMonobodies. The chromatographs are shown with vertical offsets for clarity. The labels show the identities of analyzed samples. MBPMbL17 and MBPMbS09 exhibited a mono-dispersed peak but appeared to interact with the chromatography media, resulting in late elution. SUMOMbS34 was eluted at the void volume fraction, indicating the large aggregate. (b) Thermal stability of therMonobodies monitored by DSF. The traces of three technical replicates are shown for each therMonobody. The TM value is the mean of three replicates. The melting curves were measured by heating the samples at a rate of 0.5° C. per 30 seconds. (c) Summary of oligomerization state and thermal stability measurements of 24 therMonobodies.

FIG. **8**A-C. Affinity clamp using therMonobody. (a) Amino acid sequences of Clamp(Ptpn11_pY580) and STOTEMP4 variants. The monobody segment of Clamp (Ptpn11_pY580) and STOTEMP4 variants are aligned based on structure based alignment of the monobody segment and STOTEMP4. The ranges of the secondary structure of the monobody segment of Clamp(Ptpn11_pY580) are shown below its sequence. Residues grafted to the structurally equivalent positions of STOTEMP4 are shaded in yellow. Mutated residues in STOTEMP4 are colored in red. Linkers are indicated by underlining. Shown in FIG. **8***a* are SEQ ID NOS:85-93. (b)-(c) pY-peptide binding properties of designed pY-clamps measured by yeast surface display. In panel (b), binding of the designed pY-clamps to the target pY-peptide (Ptpn11 pY580) at the concentration of 0, 100 and 500 nM is shown. In panel (c), binding titration curves and the dissociation constants (KD) of the designed pY-clamps are shown. The mean fluorescence intensities of yeast cells displaying the designed pY-clamp are plotted as a function of the target peptide concentration. The errors shown are the standard deviations from curve fitting of the 1:1 binding model.

FIG. **9**. Sequence alignment of predicted PHOFN3 and the bacterial FN3 domain in *Clostridium Perfringens* Glycoside Hydrolase Gh84c (PDB ID: 2W1N). Gaps are denoted as dashes. The bacterial FN3 domain shows the sequence identities of 25% to PHOFN3, which is the highest among PDB entries. The locations of beta-strands in the bacterial FN3 domain are shown as the green arrows. Red dots indicate the highly conserved residues of FN3 domains reported by Main et al. and Dickinson et al. (Main et al., 1992; Dickinson et al., 1994). Shown in FIG. **9** are SEQ ID NOS:94-98.

FIG. **10**. Thermal stability of purified PHOFN3 and its truncated variants monitored by DSF. Triplicate measurements are shown for each sample. The melting curves were measured by heating the samples at a rate of 0.5° C. per 30 seconds. Only PHOFN3□C35 exhibited an inflection point indicative of thermal denaturation. The TM value for PHOFN3□C35 is the mean of the inflection points from triplicates.

FIG. **11**A-B. A) The crystal structures of SeMet-labeled PHOFN3ΔC25. The seven β-strands are colored and labeled A-G. N- and C-terminus are labeled. B) Superposition of PHOFN3ΔC25 and FN3fn10 (PDBID:1FNA). PHOFN3ΔC25 and FN3fn10 are colored green and gray, respectively.

FIG. **12**. Schematic drawing of the amino acid sequence of PHOFN3ΔC25 in its secondary structure context. Loop residues as assigned by the program DSSP are shown in yellow. Residues of the β-strands whose side chain forms the hydrophobic core are enclosed in circles with thicker ring.

FIG. **13**. Phage ELISA analysis for surface display of PHOFN3 on phage particles. Binding of phage particles to an anti-V5 tag antibody. The V5 tag is located in the linker between PHOFN3 and the phage coat protein, P3, in the phage display vector. The absorbance changes at 405 nm after the HRP reaction with 1-step Ultra TMB ELISA for 10 min for phages produced with the M13KO7 helper phage or 1 min for hyperphage-produced phages are shown. For these measurements, 50 μL of 0.5% BSA/TBS containing 2.3×106 cfu/mL M13KO7-produced phages or 7.4×106 cfu/mL hyperphage-produced phages were added to each well and bound phages were detected with a HRP conjugated anti-M13 phage antibody.

DETAILED DESCRIPTION OF THE INVENTION

The fibronectin type III domain (FN3) has been particularly successful as a protein scaffold for generating synthetic binding proteins. Since the pioneering work by inventors on the tenth FN3 of human fibronectin (FNfn10), numerous binding proteins, termed monobodies, have been generated to diverse target molecules. To date, all FN3-based scaffolds have been derived from human proteins, primarily because of the prediction that molecules engineered from a human protein may have low immunogenicity, an important consideration in biotherapeutic development. However, immunogenicity concerns are less important in applications where synthetic binding proteins are not exposed directly to patients or consumers including chemical processing and research tools. Therefore, this current application is directed toward the development of a molecular scaffold for industrial applications using FN3's from non-human origins, in particular thermophiles.

Fibronectin Type III (FN3) Domain

A. *Sulfolobus tokodaii*

The inventors utilized the SMART database to explore FN3 domains from hyperthermophiles. The database predicted many FN3 domains in hyperthermophilic archaea and bacteria such as *Thermococcus kodakaraensis, Sulfolobus tokodaii, Pyrococcus horikoshii* and *Thermotoga lettingae*. The inventors first eliminated predicted domains that were shorter than the length of the shortest FN3 domains that had been structurally characterized (75 amino acids). Then, four predicted FN3 domains in the sequence of Kelch domain-containing protein ST0939 from the hyperthermophilic archaeon *Sulfolobus tokodaii* DSM 16993 were chosen as the candidate proteins, because of their detectably homology to a human FN3. In the predicted constructs, termed STOFN3-1, -2, -3 and -4.

STOFN3-1 comprises 84 amino acids at positions 315-398:

```
                                        (SEQ ID NO: 1)
PPPKPQIASIASGNETITVKWYDTNASGYYITYWSNFSQKVTINVGNVTS

YTIKHLKDGVTYYIQIVPYNSLGNGTPSDIISAT.
```

STOFN3-2 comprises 86 amino acids at positions 399-484:

```
                                        (SEQ ID NO: 2)
PSSVPNPPIIKVKIGNLNATLTWYDTFNGGYPIEGYYLYVNGKGINVGNI

TSYVLTNLTAGELYTIELIAYNKIGNSSISSVSFIA.
```

STOFN3-3 comprises 79 amino acids at positions 488-566:

```
                                        (SEQ ID NO: 3)
ANLTVTVYKKINGFLVSWNSTSKAKYILTVSKENVVLLNVSTTNTSYFVK

VPFGVYNISLEAVNIVGITKYAFILIYYI.
```

STOFN3-4 comprises 76 amino acids at positions 568-643:

```
                                        (SEQ ID NO: 4)
PASPTVNWSITLNTVSLNWSKVSGAEYYLIYDNGKLITNTTNTAFTFNLT

IGQNEIEVYAANAYYKSAPYIINDVR.
```

Each construct maintains at least two of the three highly conserved hydrophobic amino acids of FN3 domains (FIG. **1**A).

The full-length ST0939 comprises the sequence:

```
                                        (SEQ ID NO: 5)
MKRNTLLALVLVILIFPTLSTAYIEFTTSINQAIPDSLVYATSAYYDGKI

FLIGGENLYSTPVNSVYVYENGSWYLGPSLPFSLSSAGATVCNNTLYVVG

GANSTSIFGGILEFIGNGWKVITNSMPIPVYGAIVFSYDYKIYVIGGMNY

SGNSLVPPVNYIQVYNLKTNSWQIIGNAPLRLAYSAYYFNGSALFVVGGF

TQSATLTSSVFVYYPENNTWISLPSLPGVEAGGVLGYYNGYMYLVGGLYY

VSGAYQLGEILYYYNGTWRNTNIQEQIPTQFSTSVQIGNKLIILGGFGPG

NIPSNAMQTVSIYLPPPKPQIASIASGNETITVKWYDTNASGYYITYWSN
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FSQKVTINVGNVTSYTIKHLKDGVTYYIQIVPYNSLGNGTPSDIISATPS

SVPNPPIIKVKIGNLNATLTWYDTFNGGYPIEGYYLYVNGKGINVGNITS

YVLTNLTAGELYTIELIAYNKIGNSSISSVSFIAASKANLTVTVYKKING

FLVSWNSTSKAKYILTVSKENVVLLNVSTTNTSYFVKVPFGVYNISLEAV

NIVGITKYAFILIYYIQPASPTVNWSITLNTVSLNWSKVSGAEYYLIYDN

GKLITNTTNTAFTFNLTIGQNEIEVYAANAYYKSAPYIINDVRNYIVVVN

STAISISVPQIKVVSGENTDAPLQTNNIDLKSAIIVITVFVIALLMILVI

LRERSDNYW.
```

In certain aspects, the FN3 domain comprises beta strand A, beta strand B, beta strand C, beta strand D, beta strand E, beta strand F, and beta strand G. Connecting beta strands A, B, C, D, E, F, and G are loop regions AB, BC, CD, DE, EF, and FG. Beta strand A precedes the AB loop and beta strand G follows the FG loop. The loop regions correspond to the following amino acid positions in SEQ ID NO:1—AB (13-16), BC (22-27), CD (36-37), DE (47-49), EF (54-60), and FG (71-80). The loop regions in STOFN3-2, STOFN3-3, and STOFN3-4 are the corresponding amino acids in SEQ ID NO:2, 3, and 4, respectively. The corresponding amino acids can be determined from the alignment of FIG. **1**

FN3 polypeptides can be modified by inserting or deleting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 or more amino acids, or any range derivable therein, in a FN3 loop. Variants are discussed in U.S. Pat. No. 6,673,901, which is hereby incorporated by reference with respect to embodiments regarding FN3 monobodies.

B. *Pyrococcus horikoshii*

The inventors also characterized a FN3 domain in the sequence of putative uncharacterized protein PH0954 from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3, termed PHOFN3, as the candidate protein, because of its detectably homology to a bacterial FN3 domain in the *Clostridium Perfringens* Glycoside Hydrolase Gh84c whose FN3 fold has been experimentally confirmed. The SMART database predicted PHOFN3 with 108 (P1873-A1980) amino acid residues, but the C-terminal 26 residues (E1955-A1980) did not have detectable homology to the sequence of the bacterial homologue (FIG. **9**) and shown below:

```
                                            (SEQ ID NO: 6)
PSPPSGVTLMLNGSYVELSWLPSPDSDVAGYFIYKDGKRLNEVPIEKPNF

RDIYSGTLNYSISAIDFSGFESEKTEVFPVKLEVDEENLTAGYPGAVKVK

VENLDGEA.
```

The full-length PH0954 comprises the sequence:

```
                                            (SEQ ID NO: 7)
MINIKGLILTLILFISLIPPWALGEGSKDTKVFADYYLAGDSVVINATLYDAGSCNLTF

SVFSPIEAPNVSEISFTWMNLSEYIES ATEATYGEYLRDGNVIMREDDGYFIYELPFSL

NYFGREIKKIAVNTNGLIELLEEYEEPRIEDYYGIHEEGEFYESDVIFGLDEDLVTYDG

YLLLVNLQDKIVIEWLASTYEDYESEIVDNINFQVIINSNGTITWSYKSLEYSYHDYDL

FSGYYSKVSGDVKGFTKGEGKSFAIQVPLGTPKLYTYQVRESGSYLLTLPLSNYHVE

VFANCMDDPDLSNNLAEVGVWPGDYWVENASINNLIPGEFASINFKVRTTSKIPSAK

VKLLRNGVEEKIEYLSFYNGIAEGEISWLVQGGNYTLALLVEGKGDINSSNNIYLLGN

YNFPLPNFEVGNYSIDLPTCVDSTGEVRVNVTSTANWSIPVRLTLVYEEGNRSYTRYI

STKGEEESEVIFTPMIKAGTLEKVVIEIDPWNEVEESNESDNKVEVPYHIIIEKPDFTVK

SLNIPGNVSIGNLYEVNVTLDNLGGCYGRNVLVKLYENGTSKDWRRVRINNETNVT

LTWKPGNAGLVNLTVVVDPYSYVDEINEGNNRLSRLIFVNAPDFKISKVELLSFDGIA

GSKAKFNVTVKNEGEDYSGYFSIAVYGGLRSSIAYLRGIKSGEEKWTIISLPINGGNST

LIFVVDPHNVISETNEGNNVIFYNMGYIPKPNFVVKEISLPNNTVGYIPLNITIGNVGAP

YNATSYQVPVKIKTEYGWKVSYLRGIIRDNYTISIDSLAMLPPGSTINVTVNYNMKV

NETSYSDNSLIINYTTGYPDLELGIIPPSGELSAGKDVKITFLVKNVGNATLRIDRSSW

YSPYLGLYVTLEDENGKTHTLGRYELAPATLSPGANISQVVWITLNGGTNKIMGRIV

DEYENIYENNNDTLILTLEKPDFAILNYSIPDEILNGTAYLYKAYPIVLNISNLGGNFSD

GIRVDLFDNGIIKTSTSVYGLESGASRDVTLRYLPSSGKHNLSIVLDPYNRWIEENEEN

NNLTFSLSFGKPDLKVEGITWAPYNFTSGENVLFTIYVKNLGQPFLKSFTVRAEIWNG

TRKIYSTNAYPRNWSFGKGETKEFNWRWYNAKPGNLTVKIVVDYYNSIPEGNESNN

EFSAFLGNVGTPDFKLENLSVEDLAYGKFVRINATVKNLGDSIYRPITVLFNVSGERY

YRTVYGIKENESKSVTLPWYVDRVGEVRVKVEVDPGNRIVEGNESNNIIERTYYVES

PELMLSGYEWLEEEVRRGYLAYKVNVTNTGGDVYRGFYVQMFVDGEPKSSVWINK
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LLHGETAERTLRWRFSSGGRKEVRIVVDPQDYIPESNEDNNAIVENVTIVLPDIEVLSL

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LTIRPYPGNSTFKVVVDPTNAVVELNEDNNEISVRSYVKAPDIVVVSADLGNFTYPGE

MVNAKVRIRNSGDYKSGVYLLIRNKRRKLGSAYVDSITPGEEIEVNVPWLVDSGDY

NVSVIADPYNSVREWDEENNKLDIEVSVPSPDLTVENITHSGKEVAGEEIIIKVTVKNI

GESSKLPFYIVLYANSSFVGINRVTKIDKGESITLEFKWRASYGEYALRAIVDPYDEV

YEENESNNEGMVKVFIEDEEPPVLKLTYPENGTFTNKPYIGAYLRDEGSGVKFGEIEV

YREGTSVPGSTKFSGGWLIFQNSTPLLDGKYTVTVKAVDRAGNEITYSWNFTLDREP

PRIVCNLTDGTLYNGTVVPGVQVIDDNLDWYKVKVNGREFSGPIKLDGTYTLNVTA

KDKAGNLAEKIIRFTVNGVPSPPSGVTLMLNGSYVELSWLPSPDSDVAGYFIYKDGK

RLNEVPIEKPNFRDIYSGTLNYSISAIDFSGFESEKTEVFPVKLEVDEENLTAGYPGAV

KVKVENLDGEANGTLSIILIDEFGNEIEKLSRKVEVPRGRSSHEFVFMVPRGLTLIRGE

LKVGNSTARIIHRAKVREGENPEIRVGKLLAGFPGLVEVEIRNCGIVELNTSETLMKL

DNSSGELIEAPLTIPPGKKTVLRYKIVPPKKGSYNLTFRIADVEVRKIVNVSESVLNPIT

ISTENFIKGGKAKIYVSFRNIGSAPIFVKSIELNGMSKRLSIELPPNLSVEESFEYLISEEN

VEINATVNTDVGKFRKSLTLTAEQPEYNADVSVSSVYEVGKEILITGVAYNESGMLS

NVPVKVSIARGGFVREYIVTTNENGYFNLTFRPFKGESGHFIVSATHPKIELLERDAEF

DVVGIEVIPSLYLLTVPVEFNGTVRVRLINYWRASDVSVSVKAPPEYEVSIPKVLHLK

PGSNIINIGLSSKNAVNGSIMITFKARQLGLNITRSLTLKLKVLPPAPAIVTSPNFLDVG

VLTNETASAEVVVRNLGFTALRNVSIRSSIPWVKVVSNFTEVDPKDNESISLYIEPPRN

VTGTFKGEITISSSNYNPIKVPMRIRVTPNATGGVKVTVMDPNATRLENVKLTLYNG

YFHFEGYTNKNGTLEVENVPIGEYKLFASLEGYYGYSTSITIEVGVEKNVSIILTPSILE

VEWEVVPVTIQDVYIIKHEMWYSTHVPAPEIRMEGGDLEVYVDYEKLAEEGMLEFR

GQVIVRNTHQYISVYNVTFESGGSHYIDVEFGINRIDELKPGEAVIVPYVVRIYYSRSP

PINPCLHETKVFKLKAGVVCVEEAGKITLKAQRIHQIVVKPTCKGCWESVFPVAGKL

AFMAIAQKVGQALGNIDDTGVLSTLAGEALNNLESLFDAYNAYKANPTKENMENY

VKTFNSVKANLASLFMFDPVAYQEINSLQLTLIKTPKGDIAGFAVSRTATPVYALGM

GVGKIENGQLKVDYKKAVNIANGIVLNVMSKMGGALGGIASGVGLLQLLDKAAED

LPPYIAQLFLNCAICLMRNDCTLPEGEEIRPIQIIASGSLGGYPSMIPSGLAGGGDGGTA

VGRFTCGGLPTVKKSSTSMSCSTCSSEDVVKERVCRLFRESETHDEEEPSNTLHMCV

DLVLTIEQRLTFERQAFRASLKFTNTNRNYSLENVSVRVIFFDEEGNRVDDKFFVRLD

EKAGLSGSSLEPEKTAEMKWLIIPKVGAAEKFRARYYVMANITARVGSTKLVYETW

PAMIEVEPVPQLVLDYVLPSYVFGDDPYTPEKELPIPFIFGVRVKNVGYGTARKLRIA

SAQPKIERSNYPGVYIDFKIIGTLVNGKKVPNSLTIDFGDLNPGESSTAAWLMIAEVSG

KFLQYNATFKHSDELGGNETSLIKEVRTHFLIRAFNNTENDDGMLDFLVDDDGDGKP

EKIIDSRGFDYNVLLLNFTEVEEGSMRKIIPEMKTPFWVYFTVPFKGSVVRSDGKNPM

DQWMENGTLHVLDLGTPEFYILKSNQPPIPRIYVKEPVIANETVVLDGSLSYDPDGSII

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LKVEPQWGIVPFNLSITFNVTNVGDVSGEYSYIIKLGNSTIAEGSEIIESGRWKVINSTV

EIRKEGNYTVTANNLSKTVTAYRKVYGNLTENYIKEKDFGHYKSFYWNEFKRDFEG

WVEEALSTIELPKVNFKVLNYFPGNWSLLNYSEMLNITKGWGWINATYARRVRVEG

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LEEFKYLIVNVTQLVVLLGNATHELDESPPTLNVTPSSGIYSEIPKIQVRTCDETGITLV

WGAVGNYTKEFTEVESNGTCSTWEGIVPLNIGNNTVAIYAEDEFGNRGNVSLWIYLN

PEAPVIYIESPEEKVYNSREVMINYTVVNHDLVGVVAYLNGELISSNASYSGFIKLDY

GWHNFTIYAWDVSYNVSKSVIFRVNEPPSVDFSWEVDNLTVKFEANASDEDGISKYL

WDFGDNESSLLVNPTHTYRKGGRYNVTLTVWDSYNLSSSISKEVVVFGSSTLTMVK

EYSYTKDFGFYNTTSWKDFLKDFEVWVNLTLRNVTLPLEYFEEIIEVNVENWSLISVE

KNLKNDIGEMSAEYERNATIVGIMNYTRVTLKLTQEVILSGRARKVEDKIPPLVEILFP

RNMTYNETIREIKVRATDESGIANVTATINGESLSLEKVNETWIGRVELDDGKYELNV

FASDKWGNVGCSTVNFTINRSVKVRIINGTEIVTIPGDIKTRVYFEGDIIVEIVKESLRF

KIPSGGTLVIDERGRKDPWLLARINSTIENISKTSRIFEENGKKVHEIRYRISISRGYAIL

VVPLEGMKVSSIRIIKNGTVTRDEKHGNYYKLSKGYLFIFLSEDPIVEVTLSKIEKKDIF

RVLYYAGIIWERNYLRLKEEFIMKMSNETSQEAIRLHEEAEKYYLKGREYYPRIPSPS

AIYWYAVYMRKAYLTERKALELLSIS.
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In certain aspects, the FN3 domain comprises beta strand A, beta strand B, beta strand C, beta strand D, beta strand E, beta strand F, and beta strand G. Connecting beta strands A, B, C, D, E, F, and G are loop regions AB, BC, CD, DE, EF, and FG. Beta strand A precedes the AB loop and beta strand G follows the FG loop. The loop regions correspond to the following amino acid positions in SEQ ID NO:6—AB (13-14) BC (20-27), CD (36-37), DE (40-48), EF (54-59), and FG (67-74).

FN3 polypeptides can be modified by inserting or deleting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 or more amino acids, or any range derivable therein, in a FN3 loop. Variants are discussed in U.S. Pat. No. 6,673,901, which is hereby incorporated by reference with respect to embodiments regarding FN3 monobodies.

C. FN3 Library

A combinatorial library is a collection of diverse compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical “building blocks.” For example, a linear combinatorial chemical library such as a polypeptide (e.g., mutein or variant) library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length. Millions of compounds can be synthesized through such combinatorial mixing of chemical building blocks. For example, one commentator has observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (Gallop et al., 1994).

Embodiments of the disclosure are directed to a combinatorial library of FN3 domains. In certain aspects, polypeptides of the library include variations of amino acid sequence in one or more of the beta strands or body of the FN3 domains. In certain aspects, the library includes variations of amino acid sequences in one or more loops of the FN3 domains. In still further aspects, the library includes variation in both loops and beta strands of the FN3 domain.

FN3 variants can include alanine substitutions at one or more of amino acid positions. In certain aspects, any of the 19 other amino acids can be substituted for one or more amino acid of SEQ ID NO:1-4 or 6. Substitutions include, but are not limited to conservative substitutions that have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein.

In certain aspects, FN3 domains will have, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid substitutions that include, but are not limited to the following FN3 residue substitutions (corresponding to SEQ ID NO:1): K4V, N14P, D58P, G28Y, Y22A, Y22C, Y22D, Y22E, Y22F, Y22G, Y22H, Y22I, Y22K, Y22L, Y22M, Y22N, Y22P, Y22Q, Y22R, Y22S, Y22T, Y22V, Y22W, D23A, D23C, D23E, D23F, D23G, D23H, D23I, D23K, D23L, D23M, D23N, D23P, D23Q, D23R, D23S, D23T, D23V, D23W, D23Y, T24A, T24C, T24D, T24E, T24F, T24G, T24H, T24I, T24K, T24L, T24M, T24N, T24P, T24Q, T24R, T24S, T24V, T24W, T24Y, N25A, N25C, N25D, N25E, N25F, N25G, N25H, N25I, N25K, N25L, N25M, N25P, N25Q, N25R, N25S, N25T, N25V, N25W, N25Y, A26C, A26D, A26E, A26F, A26G, A26H, A26I, A26K, A26L, A26M, A26N, A26P, A26Q, A26R, A26S, A26T, A26V, A26W, A26Y, S27A, S27C, S27D, S27E, S27F, S27G, S27H, S27I, S27K, S27L, S27M, S27N, S27P, S27Q, S27R, S27T, S27V, S27W, S27Y, N47A, N47C, N47D, N47E, N47F, N47G, N47H, N47I, N47K, N47L, N47M, N47P, N47Q, N47R, N47S, N47T, N47V, N47W, N47Y, V48A, V48C, V48D, V48E, V48F, V48G, V48H, V48I, V48K, V48L, V48M, V48N, V48P, V48Q, V48R, V48S, V48T, V48W, V48Y, T49A, T49C, T49D, T49E, T49F, T49G, T49H, T49I, T49K, T49L, T49M, T49N, T49P, T49Q, T49R, T49S, T49V, T49W, T49Y, S71A, S71C, S71D, S71E, S71F, S71G, S71H, S71I, S71K, S71L, S71M, S71N, S71P, S71Q, S71R, S71T, S71V, S71W, S71Y, L72A, L72C, L72D, L72E, L72F, L72G, L72H, L72I, L72K, L72M, L72N, L72P, L72Q, L72R, L72S, L72T, L72V, L72W, L72Y, G73A, G73C, G73D, G73E, G73F, G73H, G73I, G73K, G73L, G73M, G73N, G73P, G73Q, G73R, G73S, G73T, G73V, G73W, G73Y, N74A, N74C, N74D, N74E, N74F, N74G, N74H, N74I, N74K, N74L, N74M, N74P, N74Q, N74R, N74S, N74T, N74V, N74W, N74Y, G75A, G75C, G75D, G75E, G75F, G75H, G75I, G75K, G75L, G75M, G75N, G75P, G75Q, G75R, G75S, G75T, G75V, G75W, G75Y, T76A, T76C, T76D, T76E, T76F, T76G, T76H, T76I, T76K, T76L, T76M, T76N, T76P,

T76Q, T76R, T76S, T76V, T76W, T76Y, P77A, P77C, P77D, P77E, P77F, P77G, P77H, P77I, P77K, P77L, P77M, P77N, P77Q, P77R, P77S, P77T, P77V, P77W, P77Y, S78A, S78C, S78D, S78E, S78F, S78G, S78H, S78I, S78K, S78L, S78M, S78N, S78P, S78Q, S78R, S78T, S78V, S78W, S78Y, D79A, D79C, D79E, D79F, D79G, D79H, D79I, D79K, D79L, D79M, D79N, D79P, D79Q, D79R, D79S, D79T, D79V, D79W, D79Y, I80A, I80C, I80D, I80E, I80F, I80G, I80H, I80K, I80L, I80M, I80N, I80P, I80Q, I80R, I80S, I80T, I80V, I80W, I80Y, N36A, N36C, N36D, N36E, N36F, N36G, N36H, N36I, N36K, N36L, N36M, N36P, N36Q, N36R, N36S, N36T, N36V, N36W, N36Y, F37A, F37C, F37D, F37E, F37G, F37H, F37I, F37K, F37L, F37M, F37N, F37P, F37Q, F37R, F37S, F37T, F37V, F37W, F37Y, G28A, G28C, G28D, G28E, G28F, G28H, G28I, G28K, G28L, G28M, G28N, G28P, G28Q, G28R, G28S, G28T, G28V, G28W, G28Y, Y29A, Y29C, Y29D, Y29E, Y29F, Y29G, Y29H, Y29I, Y29K, Y29L, Y29M, Y29N, Y29P, Y29Q, Y29R, Y29S, Y29T, Y29V, Y29W, Y30A, Y30C, Y30D, Y30E, Y30F, Y30G, Y30H, Y30I, Y30K, Y30L, Y30M, Y30N, Y30P, Y30Q, Y30R, Y30S, Y30T, Y30V, Y30W, I31A, I31C, I31D, I31E, I31F, I31G, I31H, I31K, I31L, I31M, I31N, I31P, I31Q, I31R, I31S, I31T, I31V, I31W, I31Y, T32A, T32C, T32D, T32E, T32F, T32G, T32H, T32I, T32K, T32L, T32M, T32N, T32P, T32Q, T32R, T32S, T32V, T32W, T32Y, Y33A, Y33C, Y33D, Y33E, Y33F, Y33G, Y33H, Y33I, Y33K, Y33L, Y33M, Y33N, Y33P, Y33Q, Y33R, Y33S, Y33T, Y33V, Y33W, W34A, W34C, W34D, W34E, W34F, W34G, W34H, W34I, W34K, W34L, W34M, W34N, W34P, W34Q, W34R, W34S, W34T, W34V, W34Y, S35A, S35C, S35D, S35E, S35F, S35G, S35H, S35I, S35K, S35L, S35M, S35N, S35P, S35Q, S35R, S35T, S35V, S35W, S35Y, S38A, S38C, S38D, S38E, S38F, S38G, S38H, S38I, S38K, S38L, S38M, S38N, S38P, S38Q, S38R, S38T, S38V, S38W, S38Y, Q39A, Q39C, Q39D, Q39E, Q39F, Q39G, Q39H, Q39I, Q39K, Q39L, Q39M, Q39N, Q39P, Q39R, Q39S, Q39T, Q39V, Q39W, Q39Y, K40A, K40C, K40D, K40E, K40F, K40G, K40H, K40I, K40L, K40M, K40N, K40P, K40Q, K40R, K40S, K40T, K40V, K40W, K40Y, V41A, V41C, V41D, V41E, V41F, V41G, V41H, V41I, V41K, V41L, V41M, V41N, V41P, V41Q, V41R, V41S, V41T, V41W, V41Y, T42A, T42C, T42D, T42E, T42F, T42G, T42H, T42I, T42K, T42L, T42M, T42N, T42P, T42Q, T42R, T42S, T42V, T42W, T42Y, I43A, I43C, I43D, I43E, I43F, I43G, I43H, I43K, I43L, I43M, I43N, I43P, I43Q, I43R, I43S, I43T, I43V, I43W, I43Y, N44A, N44C, N44D, N44E, N44F, N44G, N44H, N44I, N44K, N44L, N44M, N44P, N44Q, N44R, N44S, N44T, N44V, N44W, N44Y, V45A, V45C, V45D, V45E, V45F, V45G, V45H, V45I, V45K, V45L, V45M, V45N, V45P, V45Q, V45R, V45S, V45T, V45W, V45Y, G46A, G46C, G46D, G46E, G46F, G46H, G46I, G46K, G46L, G46M, G46N, G46P, G46Q, G46R, G46S, G46T, G46V, G46W, and/or G46Y, and combinations thereof. It is contemplated that one or more of these substitutions may be specifically excluded in embodiments described herein.

In still further embodiments other amino acid substitutions can be introduced before, during, or after introduction of those amino acid substitutions listed above. Further substitutions (corresponding to SEQ ID NO:1) include, but is not limited to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 of P1A, P1C, P1D, P1E, P1F, P1G, P1H, P1I, P1K, P1L, P1M, P1N, P1Q, P1R, P1S, P1T, P1V, P1W, P1Y, P2A, P2C, P2D, P2E, P2F, P2G, P2H, P2I, P2K, P2L, P2M, P2N, P2Q, P2R, P2S, P2T, P2V, P2W, P2Y, P3A, P3C, P3D, P3E, P3F, P3G, P3H, P3I, P3K, P3L, P3M, P3N, P3Q, P3R, P3S, P3T, P3V, P3W, P3Y, K4A, K4C, K4D, K4E, K4F, K4G, K4H, K4I, K4L, K4M, K4N, K4P, K4Q, K4R, K4S, K4T, K4V, K4W, K4Y, P5A, P5C, P5D, P5E, P5F, P5G, P5H, P5I, P5K, P5L, P5M, P5N, P5Q, P5R, P5S, P5T, P5V, P5W, P5Y, Q6A, Q6C, Q6D, Q6E, Q6F, Q6G, Q6H, Q6I, Q6K, Q6L, Q6M, Q6N, Q6P, Q6R, Q6S, Q6T, Q6V, Q6W, Q6Y, I7A, I7C, I7D, I7E, I7F, I7G, I7H, I7K, I7L, I7M, I7N, I7P, I7Q, I7R, I7S, I7T, I7V, I7W, I7Y, A8C, A8D, A8E, A8F, A8G, A8H, A8I, A8K, A8L, A8M, A8N, A8P, A8Q, A8R, A8S, A8T, A8V, A8W, ABY, S9A, S9C, S9D, S9E, S9F, S9G, S9H, S9I, S9K, S9L, S9M, S9N, S9P, S9Q, S9R, S9T, S9V, S9W, S9Y, I10A, I10C, I10D, I10E, I10F, I10G, I10H, I10K, I10L, I10M, I10N, I10P, I10Q, I10R, I10S, I10T, I10V, I10W, I10Y, A11C, A11D, A11E, A11F, A11G, A11H, A11I, A11K, A11L, A11M, A11N, A11P, A11Q, A11R, A11S, A11T, A11V, A11W, A11Y, S12A, S12C, S12D, S12E, S12F, S12G, S12H, S12I, S12K, S12L, S12M, S12N, S12P, S12Q, S12R, S12T, S12V, S12W, S12Y, G13A, G13C, G13D, G13E, G13F, G13H, G13I, G13K, G13L, G13M, G13N, G13P, G13Q, G13R, G13S, G13T, G13V, G13W, G13Y, N14A, N14C, N14D, N14E, N14F, N14G, N14H, N14I, N14K, N14L, N14M, N14P, N14Q, N14R, N14S, N14T, N14V, N14W, N14Y, E15A, E15C, E15D, E15F, E15G, E15H, E15I, E15K, E15L, E15M, E15N, E15P, E15Q, E15R, E15S, E15T, E15V, E15W, E15Y, T16A, T16C, T16D, T16E, T16F, T16G, T16H, T16I, T16K, T16L, T16M, T16N, T16P, T16Q, T16R, T16S, T16V, T16W, T16Y, I17A, I17C, I17D, I17E, I17F, I17G, I17H, I17K, I17L, I17M, I17N, I17P, I17Q, I17R, I17S, I17T, I17V, I17W, I17Y, T18A, T18C, T18D, T18E, T18F, T18G, T18H, T18I, T18K, T18L, T18M, T18N, T18P, T18Q, T18R, T18S, T18V, T18W, T18Y, V19A, V19C, V19D, V19E, V19F, V19G, V19H, V19I, V19K, V19L, V19M, V19N, V19P, V19Q, V19R, V19S, V19T, V19W, V19Y, K20A, K20C, K20D, K20E, K20F, K20G, K20H, K20I, K20L, K20M, K20N, K20P, K20Q, K20R, K20S, K20T, K20V, K20W, K20Y, W21A, W21C, W21D, W21E, W21F, W21G, W21H, W21I, W21K, W21L, W21M, W21N, W21P, W21Q, W21R, W21S, W21T, W21V, W21Y, S50A, S50C, S50D, S50E, S50F, S50G, S50H, S50I, S50K, S50L, S50M, S50N, S50P, S50Q, S50R, S50T, S50V, S50W, S50Y, Y51A, Y51C, Y51D, Y51E, Y51F, Y51G, Y51H, Y51I, Y51K, Y51L, Y51M, Y51N, Y51P, Y51Q, Y51R, Y51S, Y51T, Y51V, Y51W, T52A, T52C, T52D, T52E, T52F, T52G, T52H, T52I, T52K, T52L, T52M, T52N, T52P, T52Q, T52R, T52S, T52V, T52W, T52Y, I53A, I53C, I53D, I53E, I53F, I53G, I53H, I53K, I53L, I53M, I53N, I53P, I53Q, I53R, I53S, I53T, I53V, I53W, I53Y, K54A, K54C, K54D, K54E, K54F, K54G, K54H, K54I, K54L, K54M, K54N, K54P, K54Q, K54R, K54S, K54T, K54V, K54W, K54Y, H55A, H55C, H55D, H55E, H55F, H55G, H55I, H55K, H55L, H55M, H55N, H55P, H55Q, H55R, H55S, H55T, H55V, H55W, H55Y, L56A, L56C, L56D, L56E, L56F, L56G, L56H, L56I, L56K, L56M, L56N, L56P, L56Q, L56R, L56S, L56T, L56V, L56W, L56Y, K57A, K57C, K57D, K57E, K57F, K57G, K57H, K57I, K57L, K57M, K57N, K57P, K57Q, K57R, K57S, K57T, K57V, K57W, K57Y, D58A, D58C, D58E, D58F, D58G, D58H, D58I, D58K, D58L, D58M, D58N, D58P, D58Q, D58R, D58S, D58T, D58V, D58W, D58Y, G59A, G59C, G59D, G59E, G59F, G59H, G59I, G59K, G59L, G59M, G59N, G59P, G59Q, G59R, G59S, G59T, G59V, G59W, G59Y, V60A, V60C, V60D, V60E, V60F, V60G, V60H, V60I, V60K, V60L, V60M, V60N, V60P, V60Q, V60R, V60S, V60T, V60W, V60Y, T61A, T61C, T61D, T61E, T61F, T61G, T61H, T61I, T61K, T61L, T61M, T61N, T61P, T61Q, T61R, T61S, T61V, T61W,

T61Y, Y62A, Y62C, Y62D, Y62E, Y62F, Y62G, Y62H, Y62I, Y62K, Y62L, Y62M, Y62N, Y62P, Y62Q, Y62R, Y62S, Y62T, Y62V, Y62W, Y63A, Y63C, Y63D, Y63E, Y63F, Y63G, Y63H, Y63I, Y63K, Y63L, Y63M, Y63N, Y63P, Y63Q, Y63R, Y63S, Y63T, Y63V, Y63W, I64A, I64C, I64D, I64E, I64F, I64G, I64H, I64K, I64L, I64M, I64N, I64P, I64Q, I64R, I64S, I64T, I64V, I64W, I64Y, Q65A, Q65C, Q65D, Q65E, Q65F, Q65G, Q65H, Q65I, Q65K, Q65L, Q65M, Q65N, Q65P, Q65R, Q65S, Q65T, Q65V, Q65W, Q65Y, I66A, I66C, I66D, I66E, I66F, I66G, I66H, I66K, I66L, I66M, I66N, I66P, I66Q, I66R, I66S, I66T, I66V, I66W, I66Y, V67A, V67C, V67D, V67E, V67F, V67G, V67H, V67I, V67K, V67L, V67M, V67N, V67P, V67Q, V67R, V67S, V67T, V67W, V67Y, P68A, P68C, P68D, P68E, P68F, P68G, P68H, P68I, P68K, P68L, P68M, P68N, P68Q, P68R, P68S, P68T, P68V, P68W, P68Y, Y69A, Y69C, Y69D, Y69E, Y69F, Y69G, Y69H, Y69I, Y69K, Y69L, Y69M, Y69N, Y69P, Y69Q, Y69R, Y69S, Y69T, Y69V, Y69W, N70A, N70C, N70D, N70E, N70F, N70G, N70H, N70I, N70K, N70L, N70M, N70P, N70Q, N70R, N70S, N70T, N70V, N70W, N70Y, I81A, I81C, I81D, I81E, I81F, I81G, I81H, I81K, I81L, I81M, I81N, I81P, I81Q, I81R, I81S, I81T, I81V, I81W, I81Y, S82A, S82C, S82D, S82E, S82F, S82G, S82H, S82I, S82K, S82L, S82M, S82N, S82P, S82Q, S82R, S82T, S82V, S82W, S82Y, A83C, A83D, A83E, A83F, A83G, A83H, A83I, A83K, A83L, A83M, A83N, A83P, A83Q, A83R, A83S, A83T, A83V, A83W, A83Y, T84A, T84C, T84D, T84E, T84F, T84G, T84H, T84I, T84K, T84L, T84M, T84N, T84P, T84Q, T84R, T84S, T84T, T84V, T84W, or T84Y or combinations thereof. It is contemplated that one or more of these substitutions may be specifically excluded in embodiments described herein.

In certain aspects, FN3 domains will have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid substitutions that include, but are not limited to the following FN3 residue substitutions (corresponding to SEQ ID NO:6): G13A, G13C, G13D, G13E, G13F, G13H, G13I, G13K, G13L, G13M, G13N, G13P, G13Q, G13R, G13S, G13T, G13V, G13W, G13Y, S14A, S14C, S14D, S14E, S14F, S14G, S14H, S14I, S14K, S14L, S14M, S14N, S14P, S14Q, S14R, S14T, S14V, S14W, S14Y, Y15A, W20A, W20C, W20D, W20E, W20F, W20G, W20H, W20I, W20K, W20L, W20M, W20N, W20P, W20Q, W20R, W20S, W20T, W20V, W20Y, L21A, L21C, L21D, L21E, L21F, L21G, L21H, L21I, L21K, L21M, L21N, L21P, L21Q, L21R, L21S, L21T, L21V, L21W, L21Y, P22A, P22C, P22D, P22E, P22F, P22G, P22H, P22I, P22K, P22L, P22M, P22N, P22Q, P22R, P22S, P22T, P22V, P22W, P22Y, S23A, S23C, S23D, S23E, S23F, S23G, S23H, S23I, S23K, S23L, S23M, S23N, S23P, S23Q, S23R, S23T, S23V, S23W, S23Y, P24A, P24C, P24D, P24E, P24F, P24G, P24H, P24I, P24K, P24L, P24M, P24N, P24Q, P24R, P24S, P24T, P24V, P24W, P24Y, D25A, D25C, D25E, D25F, D25G, D25H, D25I, D25K, D25L, D25M, D25N, D25P, D25Q, D25R, D25S, D25T, D25V, D25W, D25Y, S26A, S26C, S26D, S26E, S26F, S26G, S26H, S26I, S26K, S26L, S26M, S26N, S26P, S26Q, S26R, S26T, S26V, S26W, S26Y, D27A, D27C, D27E, D27F, D27G, D27H, D27I, D27K, D27L, D27M, D27N, D27P, D27Q, D27R, D27S, D27T, D27V, D27W, D27Y, D36A, D36C, D36E, D36F, D36G, D36H, D36I, D36K, D36L, D36M, D36N, D36P, D36Q, D36R, D36S, D36T, D36V, D36W, D36Y, G37A, G37C, G37D, G37E, G37F, G37H, G37I, G37K, G37L, G37M, G37N, G37P, G37Q, G37R, G37S, G37T, G37V, G37W, G37Y, L40A, L40C, L40D, L40E, L40F, L40G, L40H, L40I, L40K, L40M, L40N, L40P, L40Q, L40R, L40S, L40T, L40V, L40W, L40Y, N41A, N41C, N41D, N41E, N41F, N41G, N41H, N41I, N41K, N41L, N41M, N41P, N41Q, N41R, N41S, N41T, N41V, N41W, N41Y, E42A, E42C, E42D, E42F, E42G, E42H, E42I, E42K, E42L, E42M, E42N, E42P, E42Q, E42R, E42S, E42T, E42V, E42W, E42Y, V43A, V43C, V43D, V43E, V43F, V43G, V43H, V43I, V43K, V43L, V43M, V43N, V43P, V43Q, V43R, V43S, V43T, V43W, V43Y, P44A, P44C, P44D, P44E, P44F, P44G, P44H, P44I, P44K, P44L, P44M, P44N, P44Q, P44R, P44S, P44T, P44V, P44W, P44Y, I45A, I45C, I45D, I45E, I45F, I45G, I45H, I45K, I45L, I45M, I45N, I45P, I45Q, I45R, I45S, I45T, I45V, I45W, I45Y, E46A, E46C, E46D, E46F, E46G, E46H, E46I, E46K, E46L, E46M, E46N, E46P, E46Q, E46R, E46S, E46T, E46V, E46W, E46Y, K47A, K47C, K47D, K47E, K47F, K47G, K47H, K47I, K47L, K47M, K47N, K47P, K47Q, K47R, K47S, K47T, K47V, K47W, K47Y, P48A, P48C, P48D, P48E, P48F, P48G, P48H, P48I, P48K, P48L, P48M, P48N, P48Q, P48R, P48S, P48T, P48V, P48W, P48Y, Y54A, Y54C, Y54D, Y54E, Y54F, Y54G, Y54H, Y54I, Y54K, Y54L, Y54M, Y54N, Y54P, Y54Q, Y54R, Y54S, Y54T, Y54V, Y54W, S55A, S55C, S55D, S55E, S55F, S55G, S55H, S55I, S55K, S55L, S55M, S55N, S55P, S55Q, S55R, S55T, S55V, S55W, S55Y, G56A, G56C, G56D, G56E, G56F, G56H, G56I, G56K, G56L, G56M, G56N, G56P, G56Q, G56R, G56S, G56T, G56V, G56W, G56Y, T57A, T57C, T57D, T57E, T57F, T57G, T57H, T57I, T57K, T57L, T57M, T57N, T57P, T57Q, T57R, T57S, T57V, T57W, T57Y, L58A, L58C, L58D, L58E, L58F, L58G, L58H, L58I, L58K, L58M, L58N, L58P, L58Q, L58R, L58S, L58T, L58V, L58W, L58Y, N59A, N59C, N59D, N59E, N59F, N59G, N59H, N59I, N59K, N59L, N59M, N59P, N59Q, N59R, N59S, N59T, N59V, N59W, N59Y, F67A, F67C, F67D, F67E, F67G, F67H, F67I, F67K, F67L, F67M, F67N, F67P, F67Q, F67R, F67S, F67T, F67V, F67W, F67Y, S68A, S68C, S68D, S68E, S68F, S68G, S68H, S68I, S68K, S68L, S68M, S68N, S68P, S68Q, S68R, S68T, S68V, S68W, S68Y, G69A, G69C, G69D, G69E, G69F, G69H, G69I, G69K, G69L, G69M, G69N, G69P, G69Q, G69R, G69S, G69T, G69V, G69W, G69Y, F70A, F70C, F70D, F70E, F70G, F70H, F70I, F70K, F70L, F70M, F70N, F70P, F70Q, F70R, F70S, F70T, F70V, F70W, F70Y, E71A, E71C, E71D, E71F, E71G, E71H, E71I, E71K, E71L, E71M, E71N, E71P, E71Q, E71R, E71S, E71T, E71V, E71W, E71Y, S72A, S72C, S72D, S72E, S72F, S72G, S72H, S72I, S72K, S72L, S72M, S72N, S72P, S72Q, S72R, S72T, S72V, S72W, S72Y, E73A, E73C, E73D, E73F, E73G, E73H, E73I, E73K, E73L, E73M, E73N, E73P, E73Q, E73R, E73S, E73T, E73V, E73W, E73Y, K74A, K74C, K74D, K74E, K74F, K74G, K74H, K74I, K74L, K74M, K74N, K74P, K74Q, K74R, K74S, K74T, K74V, K74W, and/or K74Y or combinations thereof. It is contemplated that one or more of these substitutions may be specifically excluded in embodiments described herein.

In still further embodiments other amino acid substitutions can be introduced before, during, or after introduction of those amino acid substitutions listed above. Further substitutions (corresponding to SEQ ID NO:6) include, but is not limited to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 of P1A, P1C, P1D, P1E, P1F, P1G, P1H, P1I, P1K, P1L, P1M, P1N, P1Q, P1R, P1S, P1T, P1V, P1W, P1Y, S3A, S3C, S3D, S3E, S3F, 53G, S3H, S3I, S3K, S3L, S3M, S3N, S3P, S3Q, 53R, S3T, S3V, S3W, S3Y, P4A, P4C, P4D, P4E, P4F, P4G, P4H, P4I, P4K, P4L, P4M, P4N, P4Q, P4R, P4S, P4T, P4V, P4W, P4Y, S5A, S5C, S5D, S5E, S5F, S5G,

S5H, S5I, S5K, S5L, S5M, S5N, 55P, S5Q, S5R, S5T, S5V, S5W, S5Y, G6A, G6C, G6D, G6E, G6F, G6H, G6I, G6K, G6L, G6M, G6N, G6P, G6Q, G6R, G6S, G6T, G6V, G6W, G6Y, V7A, V7C, V7D, V7E, V7F, V7G, V7H, V7I, V7K, V7L, V7M, V7N, V7P, V7Q, V7R, V7S, V7T, V7W, V7Y, T8A, T8C, T8D, T8E, T8F, T8G, T8H, T8I, T8K, T8L, T8M, T8N, T8P, T8Q, T8R, T8S, T8V, T8W, T8Y, L9A, L9C, L9D, L9E, L9F, L9G, L9H, L9I, L9K, L9M, L9N, L9P, L9Q, L9R, L9S, L9T, L9V, L9W, L9Y, M10A, M10C, M10D, M10E, M10F, M10G, M10H, M10I, M10K, M10L, M10N, M10P, M10Q, M10R, M10S, M10T, M10V, M10W, M10Y, L11A, LUC, L11D, L11E, L11F, L11G, L11H, L11I, L11K, L11M, L11N, L11P, L11Q, L11R, L11S, L11T, L11V, L11W, L11Y, N12A, N12C, N12D, N12E, N12F, N12G, N12H, N12I, N12K, N12L, N12M, N12P, N12Q, N12R, N12S, N12T, N12V, N12W, N12Y, Y15C, Y15D, Y15E, Y15F, Y15G, Y15H, Y15I, Y15K, Y15L, Y15M, Y15N, Y15P, Y15Q, Y15R, Y15S, Y15T, Y15V, Y15W, V16A, V16C, V16D, V16E, V16F, V16G, V16H, V16I, V16K, V16L, V16M, V16N, V16P, V16Q, V16R, V16S, V16T, V16W, V16Y, E17A, E17C, E17D, E17F, E17G, E17H, E17I, E17K, E17L, E17M, E17N, E17P, E17Q, E17R, E17S, E17T, E17V, E17W, E17Y, L18A, L18C, L18D, L18E, L18F, L18G, L18H, L18I, L18K, L18M, L18N, L18P, L18Q, L18R, L18S, L18T, L18V, L18W, L18Y, S19A, S19C, S19D, S19E, S19F, S19G, S19H, S19I, S19K, S19L, S19M, S19N, S19P, S19Q, S19R, S19T, S19V, S19W, S19Y, V28A, V28C, V28D, V28E, V28F, V28G, V28H, V28I, V28K, V28L, V28M, V28N, V28P, V28Q, V28R, V28S, V28T, V28W, V28Y, A29C, A29D, A29E, A29F, A29G, A29H, A29I, A29K, A29L, A29M, A29N, A29P, A29Q, A29R, A29S, A29T, A29V, A29W, A29Y, G30A, G30C, G30D, G30E, G30F, G30G, G30H, G30I, G30K, G30L, G30M, G30N, G30P, G30Q, G30R, G30S, G30T, G30V, G30W, G30Y, Y31A, Y31C, Y31D, Y31E, Y31F, Y31G, Y31H, Y31I, Y31K, Y31L, Y31M, Y31N, Y31P, Y31Q, Y31R, Y31S, Y31T, Y31V, Y31W, F32A, F32C, F32D, F32E, F32G, F32H, F32I, F32K, F32L, F32M, F32N, F32P, F32Q, F32R, F32S, F32T, F32V, F32W, F32Y, I33A, I33C, I33D, I33E, I33F, I33G, I33H, I33K, I33L, I33M, I33N, I33P, I33Q, I33R, I33S, I33T, I33V, I33W, I33Y, Y34A, Y34C, Y34D, Y34E, Y34F, Y34G, Y34H, Y34I, Y34K, Y34L, Y34M, Y34N, Y34P, Y34Q, Y34R, Y34S, Y34T, Y34V, Y34W, K35A, K35C, K35D, K35E, K35F, K35G, K35H, K35I, K35L, K35M, K35N, K35P, K35Q, K35R, K35S, K35T, K35V, K35W, K35Y, K38A, K38C, K38D, K38E, K38F, K38G, K38H, K38I, K38L, K38M, K38N, K38P, K38Q, K38R, K38S, K38T, K38V, K38W, K38Y, R39A, R39C, R39D, R39E, R39F, R39G, R39H, R39I, R39K, R39L, R39M, R39N, R39P, R39Q, R39S, R39T, R39V, R39W, R39Y, N49A, N49C, N49D, N49E, N49F, N49G, N49H, N49I, N49K, N49L, N49M, N49P, N49Q, N49R, N49S, N49T, N49V, N49W, N49Y, F50A, F50C, F50D, F50E, F50G, F50H, F50I, F50K, F50L, F50M, F50N, F50P, F50Q, F50R, F50S, F50T, F50V, F50W, F50Y, R51A, R51C, R51D, R51E, R51F, R51G, R51H, R51I, R51K, R51L, R51M, R51N, R51P, R51Q, R51S, R51T, R51V, R51W, R51Y, D52A, D52C, D52E, D52F, D52G, D52H, D52I, D52K, D52L, D52M, D52N, D52P, D52Q, D52R, D52S, D52T, D52V, D52W, D52Y, I53A, I53C, I53D, I53E, I53F, I53G, I53H, I53K, I53L, I53M, I53N, I53P, I53Q, I53R, I53S, I53T, I53V, I53W, I53Y, Y60A, Y60C, Y60D, Y60E, Y60F, Y60G, Y60H, Y60I, Y60K, Y60L, Y60M, Y60N, Y60P, Y60Q, Y60R, Y60S, Y60T, Y60V, Y60W, S61A, S61C, S61D, S61E, S61F, S61G, S61H, S61I, S61K, S61L, S61M, S61N, S61P, S61Q, S61R, S61T, S61V, S61W, S61Y, I62A, I62C, I62D, I62E, I62F, I62G, I62H, I62K, I62L, I62M, I62N, I62P, I62Q, I62R, I62S, I62T, I62V, I62W, I62Y, S63A, S63C, S63D, S63E, S63F, S63G, S63H, S63I, S63K, S63L, S63M, S63N, S63P, S63Q, S63R, S63T, S63V, S63W, S63Y, A64C, A64D, A64E, A64F, A64G, A64H, A64I, A64K, A64L, A64M, A64N, A64P, A64Q, A64R, A64S, A64T, A64V, A64W, A64Y, I65A, I65C, I65D, I65E, I65F, I65G, I65H, I65K, I65L, I65M, I65N, I65P, I65Q, I65R, I65S, I65T, I65V, I65W, I65Y, D66A, D66C, D66E, D66F, D66G, D66H, D66I, D66K, D66L, D66M, D66N, D66P, D66Q, D66R, D66S, D66T, D66V, D66W, D66Y, T75A, T75C, T75D, T75E, T75F, T75G, T75H, T75I, T75K, T75L, T75M, T75N, T75P, T75Q, T75R, T75S, T75V, T75W, T75Y, E76A, 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V99K, V99L, V99M, V99N, V99P, V99Q, V99R, V99S, V99T, V99W, V99Y, K100A, K100C, K100D, K100E, K100F, K100G, K100H, K100I, K100L, K100M, K100N, K100P, K100Q, K100R, K100S, K100T, K100V, K100W, V101Y, V101A, V101C, V101D, V101E, V101F, V101G, V101H, V101I, V101K, V101L, V101M, V101N, V101P, V101Q, V101R, V101S, V101T, V101W, V101Y, E102A, E102C, E102D, E102F, E102G, E102H, E102I, E102K, E102L, E102M, E102N, E102P, E102Q, E102R, E102S, E102T, E102V, E102W, E102Y, N103A, N103C, N103D, N103E, N103F, N103G, N103H, N103I, N103K, N103L, N103M, N103P, N103Q, N103R, N103S, N103T, N103V, N103W, N103Y, L104A, L104C, L104D, L104E, L104F, L104G, L104H, L104I, L104K, L104M, L104N, L104P, L104Q, L104R, L104S, L104T, L104V, L104W, L104Y, D105A, D105C, D105E, D105F, D105G, D105H, D105I, D105K, D105L, D105M, D105N, D105P, D105Q, D105R, D105S, D105T, D105V, D105W, D105Y, G106A, G106C, G106D, G106E, G106F, G106H, G106I, G106K, G106L, G106M, G106N, G106P, G106Q, G106R, G106S, G106T, G106V, G106W, G106Y, E107A, E107C, E107D, E107F, E107G, E107H, E107I, E107K, E107L, E107M, E107N, E107P, E107Q, E107R, E107S, E107T, E107V, E107W, E107Y, A108C, A108D, A108E, A108F, A108G, A108H, A108I, A108K, A108L, A108M, A108N, A108P, A108Q, A108R, A108S, A108T, A108V, A108W, and/or A108Y and combinations thereof. It is contemplated that one or more of these substitutions may be specifically excluded in embodiments described herein.

In certain aspects, the library comprises a variation in an amino acid corresponding to amino acid 1 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 2, 3, 4, 14, 28, and 58.

In a further aspect, the library comprises a variation in an amino acid corresponding to amino acid 2 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 1, 3, 4, 14, 28, and/or 58.

In a still a further aspect, the library comprises a variation in an amino acid corresponding to amino acid 3 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 1, 2, 4, 14, 28, and/or 58.

In a further aspect, the library comprises a variation in an amino acid corresponding to amino acid 4 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 1, 2, 3, 14, 28, and/or 58.

In a certain aspect, the library comprises a variation in an amino acid corresponding to amino acid 14 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 1, 2, 3, 4, 28, and/or 58.

In a further aspect, the library comprises a variation in an amino acid corresponding to amino acid 28 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 1, 2, 3, 4, 14, and/or 58.

In a further aspect, the library comprises a variation in an amino acid corresponding to amino acid 58 of SEQ ID NO:1 in combination with one or more residue corresponding to amino acid 1, 2, 3, 4, 14, and/or 28.

D. Library Screening

Library screening can be conducted in order to select FN3 variants that bind to specific ligands or targets. Combinatorial screening can easily produce and screen a large number of variants, which is not feasible with specific mutagenesis ("rational design") approaches. Amino acid variant at various amino acid positions in FN3 can be generated using a degenerate nucleotide sequence. FN3 variants with desired binding capabilities can be selected in vitro, recovered and amplified. The amino acid sequence of a selected clone can be identified readily by sequencing the nucleic acid encoding the selected FN3.

In some embodiments, a particular FN3-based molecule has an affinity for a target that is at least 2-fold greater than the affinity of the polypeptide prior to substitutions discussed herein. In some embodiments, the affinity is, is at least, or is at most about 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 15-, 20-, 25-, 30-, 35-, 40-, 45-, 50-, 60-, 70-, 80-, 90-, 100-fold increased (or any range derivable therein) compared to another FN3-based molecule.

Phage Display Library and Selection. A FN3 polypeptide library can be created using a "shaved" template containing polyserine sequence at locations to be diversified (Koide et al., 2007 and Wojcik et al., 2010). A synthetic DNA fragment that encodes signal sequence of DsbA (Steiner et al., 2006) can be fused to the gene for the template, and the fusion gene can be cloned into a phage display vector (Koide et al., 1998). A phage-display combinatorial library can be constructed by introducing codons for amino acid variation into the FN3 polypeptide. Library construction procedures have previously been described (Koide and Koide, 2007).

Phagemid particles can be prepared by growing XL1-Blue cells transfected with the phagemid library in the presence of IPTG and helper phage (Lo Conte et al., 1999; Fellouse et al., 2005). Phagemid library selection can be performed as follows. In the first round, 0.5 □M of a target protein modified with EZ-Link Sulfo-NHS-SS-Biotin (Sulfosuccinimidyl 2(biotinamido)-ethyl-1,3-dithiopropionate; Pierce) can be mixed with a sufficient amount of streptavidin-conjugated magnetic beads (Streptavidin MagneSphere Pramagnetic Particles; Promega, Z5481/2) in TBS (50 mM Tris HCl buffer pH 7.5 150 mM NaCl) containing 0.5% Tween20 (TBST). To this target solution, 1012-13 phagemids suspended in 1 ml TBST plus 0.5% BSA can be added, and the solution can then be mixed and incubated for 15 min at room temperature. After washing the beads twice with TBST, the beads suspension containing bound phagemids can be added to fresh *E. coli* culture. Phagemids were amplified as described before (Fellouse et al., 2005). In a second round, phagemids can be incubated with 0.1 □M target in TBST plus 0.5% BSA, and then captured by streptavidin-conjugated magnetic beads. Phagemids bound to the target protein can be eluted from the beads by cleaving the linker within the biotinylation reagent with 100 mM DTT in TBST. The phagemids can then be washed and recovered as described above. After amplification, the third round of selection may be performed using 0.02 □M target. Phage display is an established technique for generating binding members and has been described in detail in many publications such as Kontermann & Dubel (2001) and WO92/01047, each of which is incorporated herein by reference in its entirety.

Yeast Surface Display. Yeast surface experiments can be performed according to Boder and Wittrup (2000) with minor modifications. The Express-tag in the yeast display vector, pYD1, (Invitrogen) may be removed, since it can cross-react with anti-FLAG antibodies (Sigma). The genes for monobodies in the phagemid library after three rounds of selection can be amplified using PCR and mixed with the modified pYD1 cut with EcoRI and XhoI, and yeast EBY100 cells can transformed with this mixture. The transformed yeast cells can grown in the SD-CAA media at 30□C for two days, and then monobody expression can be induced by growing the cells in the SG-CAA media at 30□C for 24 h.

Sorting of monobody-displaying yeast cells may be performed as follows. The yeast cells may be incubated with a biotinylated target (50 nM) and mouse anti-V5 antibody (Sigma), then after washing incubated with anti-mouse antibody-FITC conjugate (Sigma) and neutravidin-PE conjugate (Invitrogen). The stained cells can be sorted based on the FITC and PE intensities. Typically, cells exhibiting the top □1% PE intensity and top 10% FITC intensity are recovered.

After FACS sorting, individual clones can be analyzed. Approximate Kd values can be determined from a titration curve by FACS analysis (Boder and Wittrup, 2000). Amino acid sequences can be deduced from DNA sequencing.

Effects of *E. coli* lysate on monobody-target interaction can be tested by comparing binding in the presence and absence of *E. coli* lysate prepared from cell suspension with OD600 of 50.

Protein Expression and Purification. The nucleic acid encoding any targets can be cloned in the appropriate expression vector. In one example, genes for monobodies can be cloned in the expression vector, pHFT2, which is a derivative of pHFT1 (Huang et al., 2006) in which the His-6 tag had been replaced with a His-10 tag. Protein expression and purification can be performed as described previously (Huang et al., 2006).

An expression vector comprising cDNA encoding a FN3 polypeptide or a target molecule is introduced into *Escherichia coli*, yeast, an insect cell, an animal cell or the like for expression to obtain the polypeptide. Polypeptides used in the methods and compositions of the disclosure can be produced, for example, by expressing a DNA encoding it in a host cell using a method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) or the like. A recombinant vector is produced by inserting a cDNA downstream of a promoter in an appropriate expression vector. The vector is then introduced into a host cell suitable for the expression vector. The host cell can be any cell so long as it can express the gene of interest, and includes bacteria (e.g., *Escherichia coli*), an animal cell and the like. Expression vector can replicate autonomously in the host cell to be used or vectors which can be integrated into a chromosome comprising an appropriate promoter at such a position that the DNA encoding the polypeptide can be transcribed.

Affinity Claims

Embodiments of the disclosure, particularly those comprising a polypeptide comprising a biorecognition module including a molecular recognition domain relate to a polypeptide capable of forming an "affinity clamp" to a target motif.

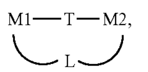
It is informative to compare characteristics of molecular affinity clamps with those of antibodies, the gold standard of affinity reagents. Antibodies are general and versatile affinity reagents. The immune system can produce an antibody to virtually any molecule. The diversity of the immunoglobulin repertoire is 1010-12, which is similar in size to the diversity of a typical phage display library (1010). This versatility of the antibodies, however, also means that the antibody repertoire is not focused and that only a small subset of the naive repertoire is available to bind to a particular class of antigen. For example, antibodies that bind to lysozyme and those that bind to a phospho-Ser peptide are distinct subsets of the same repertoire.

Economical and scalable production is another important area of consideration for affinity reagents. As noted above, polyclonal antibodies cannot be reproduced, once the original stock is depleted. Monoclonal antibodies can be reproduced, but the maintenance and large-scale culture of hybridoma cells are cumbersome and expensive. Antibodies can also be produced by recombinant technologies, but the natural diversity throughout the antibody molecules (i.e., framework diversity in addition to the extensive diversity within the antigen binding loops) makes formatting them for different applications fundamentally low throughput.

Moreover, because of the presence of critical disulfide bonds, recombinant production of antibodies is not straightforward. For this reason, a number of alternative "molecular scaffolds" for engineering affinity reagents have been developed that are small and devoid of disulfide bonds (8-10). Although these new-generation affinity reagents generally have good affinity and specificity, developing affinity reagents for short peptide motifs remains a major challenge in the field, because of the fundamental difficulties stated hereinabove.

In contrast, molecular affinity clamps in accordance with the disclosure are affinity reagents directed to a pre-defined motif. In one aspect, molecular affinity clamps are built with a particular biorecognition module comprising an interaction domain that is specific primarily to the class of target motifs that the interaction domain recognizes. Because of this pre-defined binding specificity, repertoire diversity can then be used to enhance the properties of affinity reagents rather than to blindly search for initial hits. This distinctive feature of the invention may lead to an increased success rate of producing high-affinity reagents for a motif of interest. In another embodiment, the molecular affinity clamps are build with one or more variant FN3 domains.

The two polypeptide molecules of the affinity clamp (e.g. the first polypeptide comprising the variant FN3 domain and the second polypeptide comprising the biorecognition module, which can include an interaction domain or a second variant FN3 domain) are spatially oriented to bind distinct, overlapping, or the same sites within target motif of a target. The configuration of the two biorecognition modules about the target motif is clamp-like or clamshell-like, i.e., the target motif is "clamped" between the two biorecognition modules. The two biorecognition modules of the affinity clamp are capable of together binding a single target motif on a single target. The first polypeptide molecule and second polypeptide molecules may bind at least overlapping portions the target motif on a target. This is unlike other binding molecules, such as an antibody, where the binding molecules recognize non-overlapping target motifs, different target motifs, or the same target motif located on more than one target. In another aspect, the affinity clamp is suitably described as a ternary complex composition of the type:



wherein M1 and M2 are independently the polypeptide comprising the variant FN3 domain and the polypeptide comprising the biorecognition module, L is a direct bond or linker moiety used for tethering the first and second biorecognition modules, and T is a target motif. M1 includes a variant FN3 domain bound to a first site of the target motif, and M2 includes a molecular recognition domain bound to a second site of the target motif (or vice versa) without disrupting the binding of the variant FN3 domain. The first and second sites can be the same, overlapping, or distinct sites within the target motif. L as a linker is selected from the group consisting of a peptide which is equal to or shorter than 30 residues, a group capable of disulfide bonding, and a chemical crosslinker.

B. Target Motif

A target motif suitable in accordance with the disclosure may be any motif which can be recognized by a biorecognition module, e.g., an interaction domain. Such target motifs include peptides and covalently modified peptides, including but not limited to peptides that are phosphorylated, methylated, acetylated, ubiquinated, SUMOylated, ISGylated, glycosylated, acylated, prenylated, ribosylated, gammacarboxylated, or sulfated.

C. Biorecognition Module

Among the commonly occurring domains identified in signaling proteins are the so-called "interaction domains." Interaction domains are typically small (usually less than ~100 amino acids) and autonomously folded. Many of them bind to short peptide motifs that often contain modified amino acids. It has been found that a primary binding domain, i.e., the molecular recognition domain, of the biorecognition module is suitably an interaction domain. With molecular affinity clamp technology, the interaction domains as the biorecognition modules can be engineered in such a way that the enhancer domain can be connected in a proper orientation. The bifunctional module architecture of the molecular affinity clamps in accordance with the invention, after optimization, significantly increases the surface areas of the peptide-binding interface by forming the clamshell architecture, leading to higher affinity and/or specificity. Use of interaction domains as the primary binding domain is based on the following common features of these domains: a target peptide motif binds to a shallow groove on the interaction domain surface, and the peptide is still highly exposed; there are turns and/or loops located close to the peptide-binding site; and the N- and C-termini are juxtaposed in space so that they could be connected and a new set of termini could be created elsewhere.

In short, molecular affinity clamp technology makes it possible to define the primary specificity of affinity reagents in advance (e.g., using the specificity of the interaction domain), and then, enhance that affinity and/or specificity. This modular architecture in accordance with the disclosure transforms affinity reagent development from an "unguided fishing expedition" to a focused, rational and robust process.

Interaction domains, suitable as the recognition domain, include, but are not limited to, domains involved in phoshotyrosine binding (e.g. SH2, PTB), phospho-serine binding (e.g. UIM, GAT, CUE, BTB/POZ, VHS, UBA, RING, HECT, WW, 14-3-3, Polo-box), phospho-threonine binding (e.g. FHA, WW, Polo-box), proline-rich region binding (e.g. EVH1, SH3, GYF), acetylated lysine binding (e.g. Bromo), methylated lysine binding (e.g. Chromo, PHD), apoptosis (e.g. BIR, TRAF, DED, Death, CARD, BH), cytoskeleton modulation (e.g. ADF, GEL, DH, CH, FH2), or other cellular functions (e.g. EH, CC, VHL, TUDOR, PUF Repeat, PAS, MH1, LRR, IQ, HEAT, GRIP, TUBBY, SNARE, TPR, TIR, START, SOCS Box, SAM, RGS, PDZ, PB1, LIM, F-BOX, ENTH, EF-Hand, SHADOW, ARM, ANK).

D. Linkers

The variant FN3 polypeptide and polypeptide comprising the biorecognition module may be linked together either directly, e.g., bound together with a peptide sequence via a tail from one of the modules, or indirectly via a linker. As to the latter, the linker generally is bifunctional in that it includes a functionality for linking the biorecognition module and a functionality for linking variant FN3 polypeptide. The linker may suitably be a specific moiety, such as an amino acid sequence of about 30 or fewer residues. It is also contemplated that the two polypeptide domains may be linked non-covalently through a high affinity binding interaction or physical association such as the interaction mediated by coiled-coil peptides.

E. Detection of Affinity Clamp Binding

In general, the different conformational states of modular affinity clamps used in accordance with the disclosure will correspond to different separation distances between the polypeptide modules, whereby changes in conformation may be conveniently monitored by means of a separation sensitive signal.

Various forms of separation sensitive signal systems may be used with the affinity clamps of the disclosure. In such embodiments, the biorecognition module includes a first signaling moiety and the variant FN3 polypeptide includes a second signaling moiety, and the first and second signaling moieties are capable of interacting to produce a detectable signal. The signaling moieties may include dyes, quenchers, reporter proteins and quantum dots. Particularly useful are embodiments in which the polypeptide domains include optical signaling pairs that can produce a detectable signal when the proximity of the modules with respect to each other changes with the binding of the polypeptide domains. Suitably, the first and second signaling modules are a fluorescence resonance energy (FRET) donor group and a receptor group, respectively. The change in proximity of the FRET groups produces an optical signal which differs between when the target motif is present and not present.

It will also be appreciated that various other means may be used for "reading" the presence of target motif binding to a modular affinity clamp, and/or the resultant change in conformational state of the affinity clamp structure. Many different labeling systems may be used, such as fluorophore labeling (including quantum dot), radio-labeling, and redox labeling.

F. Use of Affinity Clamps as Biosensors

Molecular affinity clamps in accordance with the disclosure may be suitably used as a biosensor wherein the polypeptide modules are each labeled with paired signaling moieties as described above.

A plurality of affinity clamps described herein may be immobilized, directly or indirectly to a support or substrate to form an array of clamps or an array of biosensors. Supports or substrates can take a variety of forms such as polymers, glasses, metal and those with coating therein. Arrays are ordered arrangements of elements, allowing them to be displayed and examined in parallel. Arrays of immobilized affinity clamps can be used to detect the target motif and demonstrate the binding reaction. Certain array formats are sometimes referred to as "biochips." Biochips may include a plurality of locations configured so that each location is spatially addressable. Typically, the clamp format is configured in a row and column format with regular spacing between locations, wherein each location has machine-readable (e.g., computer-readable) information to identify the location on the surface of the substrate.

The affinity clamp technology provides a method of detecting the presence and amount of a target motif in a sample by using the affinity clamp as a biosensor. Specifically, a sample is contacted under specific conditions with a biosensor. Fluorescence events are sensed with the binding of the polypeptide modules to the target motif in the sample and in the absence of the sample, and the fluorescence sensing in the absence of the target motif is correlated with a change in the FRET signal in the presence of the target motif. Thus, absence of the target motif generates a specific FRET signal in terms of the wavelength and amplitude of the emission, and the presence of the target motif generates a modulated FRET signal emission in terms of either the wavelength or amplitude or both. Samples may include blood, saliva or tissue.

Accordingly, an affinity clamp array as a biosensor array includes a plurality of affinity clamps or biosensors anchored to the surface of a substrate, each at an addressable site on the substrate.

G. Construction of a Modular Molecular Affinity Clamp

The general engineering of a molecular affinity clamp is given basically in four steps. Step 1 involves identifying the potential locations for attachment, via a linker, of the variant FN3 polypeptide to the biorecognition module by visual inspection of the interaction domain structure and/or from sequence variability among interaction domain family members, and testing the tolerance of identified locations for extensive modifications, for example, by inserting four Gly residues.

Step 2 includes two sub-steps, Step 2a and 2b. Step 2a is included if circular permutations are performed to construct new termini closer to the interaction domain binding site. In some embodiments, Step 2a is not needed. In Step 2a, if circular permutation is performed, a domain is constructed by joining the original termini and cutting the polypeptide at a location closer to the target-binding site of the interaction domain that tolerates mutations. Then, in Step 2b, the variant FN3 polypeptide is attached to the C-terminus of the circularly permutated domain or the natural C-terminus (in the case where no circular permutation is performed.) The N-terminus of FN3 is located close to its functional loops, and thus, connecting the FN3 N-terminus to the interaction domain ensures that the FN3 binding loops are facing the target motif-binding site.

In Step 3, amino acid diversity is introduced in FN3 loops to construct a large combinatorial library of mutated polypeptides, and in Step 4, library sorting is performed to optimize the enhancer domain for a specific target motif.

Further embodiments of the affinity clamp are described in WO/2009/062170, which is herein incorporated by reference.

Polypeptide Compositions

The polypeptides or polynucleotides of the disclosure may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 or more variant amino acids or nucleic acid substitutions or be at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% similar, identical, or homologous with at least, or at most 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 300, 400, 500, 550, 1000 or more contiguous amino acids or nucleic acids, or any range derivable therein, of SEQ ID NOs:1-7.

The polypeptides or polynucleotides of the disclosure may include 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 300, 400, 500, 550, 1000 or more contiguous amino acids, or any range derivable therein, of SEQ ID NO:1-7.

In some aspects there is a nucleic acid molecule or polypeptide starting at position 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 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605, 606, 607, 608, 609, 610, 611, 612, 613, 614, or 615 of any of SEQ ID NOS:1-7 and comprising 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 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604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, or 615 contiguous nucleotides or polyeptpdies of any of SEQ ID NOS:1-7.

The polypeptides and nucleic acids of the disclosure may include at least, at most, or exactly 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, or 615 substitutions.

The substitution may be at amino acid position or nucleic acid position 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, or 615 of one of SEQ ID NO:1-7.

Embodiments include polypeptides and polynucleotides with at least, at most, or exactly 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% (or any derivable range therein) identity, similarity, or homology to one of SEQ ID NO:1-7.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, with or without the loss of other functions or properties. Substitutions may be conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine. Alternatively, substitutions may be non-conservative such that a function or activity of the polypeptide is affected. Non-conservative changes typically involve substituting a residue with one that is chemically dissimilar, such as a polar or charged amino acid for a nonpolar or uncharged amino acid, and vice versa.

The current disclosure concerns methods and compositions related to the identification and use of variants of FN3 and libraries containing the same. As used herein, a "polypeptide" generally is defined herein to refer to a peptide sequence of about 10 to about 1,000 or more amino acid residues.

The polypeptides included in the methods set forth herein are variants in that they comprise a FN3 amino acid sequence that has been altered by substitution, insertion and/or deletion of one or more amino acid. The polypeptides set forth herein may demonstrate a selective and/or specific binding affinity for particular target molecules or portions thereof.

In certain embodiments, the polypeptide is a fusion polypeptide that includes a variant FN3 amino acid sequence linked at the N- or C-terminus to a second peptide or polypeptide. In other embodiments, the polypeptide comprises a linker interposed between the FN3 amino acid sequence and the second peptide or polypeptide sequence. Linkers are discussed in greater detail in the specification below.

Furthermore, the polypeptides set forth herein may comprise a sequence of any number of additional amino acid residues at either the N-terminus or C-terminus of the amino acid sequence that includes the variant FN3 amino acid sequence. For example, there may be an amino acid sequence of about 3 to about 1,000 or more amino acid residues at either the N-terminus, the C-terminus, or both the N-terminus and C-terminus of the amino acid sequence that includes the variant FN3 amino acid sequence.

The polypeptide may include the addition of an antibody epitope or other tag, to facilitate identification, targeting, and/or purification of the polypeptide. The use of 6×His and GST (glutathione S transferase) as tags is well known. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other amino acid sequences that may be included in the polypeptide include functional domains, such as active sites from enzymes such as a hydrolase, glycosylation domains, cellular targeting signals or transmembrane regions. The polypeptide may further include one or more additional tissue-targeting moieties.

Polypeptides may possess deletions and/or substitutions of amino acids relative to the native sequence. Sequences with amino acid substitutions are contemplated, as are sequences with a deletion, and sequences with a deletion and a substitution. In some embodiments, these polypeptides may further include insertions or added amino acids.

Substitutional or replacement variants typically contain the exchange of one amino acid for another at one or more sites within the protein and may be designed to modulate one or more properties of the polypeptide, particularly to increase its efficacy or specificity. Substitutions of this kind may or may not be conservative substitutions. Conservative substitution is when one amino acid is replaced with one of similar shape and charge. Being that the libraries of variant FN3 domains serves to provide a diversity of amino acid sequences and binding selectivity conservative substitutions are not required. However, if used, conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine. Changes other than those discussed above are generally considered not to be conservative substitutions. It is specifically contemplated that one or more of the conservative substitutions above may be included as embodiments. In other embodiments, such substitutions are specifically excluded. Furthermore, in additional embodiments, substitutions that are not conservative are employed in variants.

In addition to a deletion or substitution, the polypeptides may possess an insertion of one or more residues.

The variant FN3 amino acid sequence may be structurally equivalent to the native counterparts. For example, the variant FN3 amino acid sequence forms the appropriate structure and conformation for binding targets, proteins, or peptide segments.

The following is a discussion based upon changing of the amino acids of a polypeptide to create a library of molecules or a second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a polypeptide without appreciable loss of function, such as ability to interact with a target peptide sequence. Since it is the interactive capacity and nature of a polypeptide that defines that polypeptide's functional activity, certain amino acid substitutions can be made in a polypeptide sequence and nevertheless produce a polypeptide with like properties.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. However, in some aspects a non-conservative substitution is contemplated. In certain aspects a random substitution is also contemplated. Exemplary substitutions that take into consideration the various foregoing characteristics are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Polynucleotides

Aspects of the disclosure relate to polypeptides and polynucleotides encoding such polypeptides. The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, dsRNA, siRNA, miRNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the disclosure that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

The term "complementary" as used herein refers to Watson-Crick base pairing between nucleotides and specifically refers to nucleotides hydrogen bonded to one another with thymine or uracil residues linked to adenine residues by two hydrogen bonds and cytosine and guanine residues linked by three hydrogen bonds. In general, a nucleic acid includes a nucleotide sequence described as having a "percent complementarity" to a specified second nucleotide sequence. For example, a nucleotide sequence may have 80%, 90%, or 100% complementarity to a specified second nucleotide sequence, indicating that 8 of 10, 9 of 10 or 10 of 10 nucleotides of a sequence are complementary to the specified second nucleotide sequence. For instance, the nucleotide sequence 3'-TCGA-5' is 100% complementary to the nucleotide sequence 5'-AGCT-3'. Further, the nucleotide sequence 3'-TCGA- is 100% complementary to a region of the nucleotide sequence 5'-TTAGCTGG-3'. It will be recognized by one of skill in the art that two complementary nucleotide sequences include a sense strand and an antisense strand.

Polypeptides may be encoded by a nucleic acid molecule in the composition. In certain embodiments, the nucleic acid molecule can be in the form of a nucleic acid vector. The term "vector" is used to refer to a carrier nucleic acid molecule into which a heterologous nucleic acid sequence can be inserted for introduction into a cell where it can be replicated and expressed. A nucleic acid sequence can be "heterologous," which means that it is in a context foreign to the cell in which the vector is being introduced or to the nucleic acid in which is incorporated, which includes a sequence homologous to a sequence in the cell or nucleic acid but in a position within the host cell or nucleic acid where it is ordinarily not found. Vectors include DNAs, RNAs, plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (e.g., YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (for example Sambrook et al., 2001; Ausubel et al., 1996, both incorporated herein by reference). Vectors may be used in a host cell to produce an antibody.

The term “expression vector” refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed or stably integrate into a host cell’s genome and subsequently be transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described herein. It is contemplated that expression vectors that express a marker may be useful in the methods and compositions of the disclosure. In other embodiments, the marker is encoded on an mRNA and not in an expression vector.

A “promoter” is a control sequence. The promoter is typically a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases “operatively positioned,” “operatively linked,” “under control,” and “under transcriptional control” mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and expression of that sequence. A promoter may or may not be used in conjunction with an “enhancer,” which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

The particular promoter that is employed to control the expression of a peptide or protein encoding polynucleotide is not believed to be critical, so long as it is capable of expressing the polynucleotide in a targeted cell, preferably a bacterial cell. Where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a bacterial, human or viral promoter. In some embodiments, the host cell is an eukaryotic cell. In some embodiments, using eukaryotic cells is beneficial, as it provides for secondary modifications that may not be present in certain prokaryotic systems.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals.

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. (See Carbonelli et al., 1999, Levenson et al., 1998, and Cocea, 1997, incorporated herein by reference.)

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression. (See Chandler et al., 1997, incorporated herein by reference.)

The vectors or constructs will generally comprise at least one termination signal. A “termination signal” or “terminator” is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary in vivo to achieve desirable message levels. In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message.

In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript.

In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed “ori”), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

The polynucleotides and polypeptides of the disclosure may be transfected of transformed into host cells or expressed in host cells. As used herein, the terms “cell,” “cell line,” and “cell culture” may be used interchangeably. All of these terms also include both freshly isolated cells and ex vivo cultured, activated or expanded cells. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, “host cell” refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of replicating a vector or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors or viruses. A host cell may be “transfected” or “transformed,” which refers to a process by which exogenous nucleic acid, such as a recombinant protein-encoding sequence, is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. Common host cells include bacteria (such as *E. coli, B. subtilis, S. viofoceoruber*), yeast (such as *S. cerevisiae, P. pastoris*), fungi (such as *A. oryzae*) or eukaryotic cells.

Kits

Kits are also contemplated as being made or used in certain aspects of the present disclosure. For instance, a polypeptide or nucleic acid of the disclosure can be included in a kit or in a library provided in a kit. A kit can be included in a sealed container. Non-limiting examples of containers include a microtiter plate, a bottle, a metal tube, a laminate tube, a plastic tube, a dispenser, a pressurized container, a barrier container, a package, a compartment, or other types of containers such as injection or blow-molded plastic containers into which the dispersions or compositions or desired bottles, dispensers, or packages are retained. Other examples of containers include glass or plastic vials or bottles. The kit and/or container can include indicia on its surface. The indicia, for example, can be a word, a phrase, an abbreviation, a picture, or a symbol.

The containers can dispense or contain a pre-determined amount of a composition of the present disclosure. The composition can be dispensed as a liquid, a fluid, or a semi-solid. A kit can also include instructions for using the kit and/or compositions. Instructions can include an explanation of how to use and maintain the compositions.

EXAMPLES

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently representative of particular embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: Thermonobodies, Synthetic Binding Proteins Based on a Hyperthermophilic Fibronectin Type III Domain

A. Identification of FN3 Domain from Hyperthermophiles

The inventors utilized the SMART database to explore FN3 domains from hyperthermophiles. The database predicted many FN3 domains in hyperthermophilic archaea and bacteria such as *Thermococcus kodakaraensis, Sulfolobus tokodaii, Pyrococcus horikoshii* and *Thermotoga lettingae*. The inventors first eliminated predicted domains that were shorter than the length of the shortest FN3 domains that had been structurally characterized (75 amino acids). Then, four FN3 domains in the sequence of Kelch domain-containing protein ST0939 from the hyperthermophilic archaeon *Sulfolobus tokodaii* DSM 16993 were chosen as the candidate proteins, because of their detectably homology to a human FN3. In the predicted constructs, termed STOFN3-1, -2, -3 and -4, consist of 84 (positions 315-398), 86 (399-484), 79 (488-566) and 76 (568-643) amino acids, respectively, and each construct maintain at least two of the three highly conserved hydrophobic amino acids of FN3 domains (FIG. **1**A).

Expression vectors for these constructs were constructed using synthetic genes. It was found that only the N-terminal two domains (STOFN3-1 and -2) were robustly produced in *E. coli* as soluble proteins that were predominantly monomeric (FIG. **1**B). We detected little protein for the third domain, and the fourth domain was expressed mostly as insoluble proteins. These results demonstrate the challenge of predicting constructs that behave well as purified proteins, even for members of the well characterized FN3 domain. Melting temperatures obtained by differential scanning fluorimetry (DSF) were 80° C. for STOFN3-1 and 70° C. for STOFN3-2, respectively, confirming that they were indeed thermostable proteins (FIG. **1**C).

To confirm that STOFN3-1 and STOFN3-2 had the FN3 fold, we determined their crystal structures at 1.28 and 2.45 Å resolutions, respectively. Both segments indeed adopt the FN3 fold consisting of seven anti-parallel β-strands and six loops (FIG. **2**A, **2**B). Superposition with the FN3fn10 (PDBID: 1FNA), the well characterized FN3 scaffold, demonstrated that the structures of the segments are highly similar to FN3fn10 with an average RMSD value of 1.9 Å for STOFN3-1 and 1.8 Å for STOFN3-2, respectively, for aligned backbone Ca atoms (excluding loops) (FIG. **2**C). STOFN3-1 was then chosen for further experiments, because of its superior biophysical properties to STOFN3-2 in terms of expression level, thermal stability, solubility and the ease to obtain high-resolution structure.

In the crystal structure, three continuous Pro residues (P315, P316 and P317) in the N-terminal region of STOFN3-1 were highly ordered and positioned between the BC and FG loops, the loops commonly utilized for presenting diversified residues (FIG. **3**A). Because this N-terminal segment may interfere with creating a target binding site by presenting BC and/or FG loops, the Pro residues were substituted with Ser residues to reduce hydrophobicity and increase flexibility. The high-resolution crystal structure showed that the segment N-terminal to P317S of the mutant had low electron density indicative of conformational disorder (FIG. **3**B), strongly suggesting that this segment was dislodged from the folded portion within the construct. The apparent dissociation of this fragment only slightly decreased the stability FIG. **3**D).

The crystal structure of STOFN3-1 also revealed that, surprisingly, the last residues of the FN3 domain according to the SMART database prediction (T398) was located in the middle of the last β-strand (G strand). The β-strand continued by incorporating a Ser residue we added as an artificial extension to the predicted C-terminus (FIG. **3**C), suggesting that the predicted boundaries did not accurately match the structural boundary of the domain. We thus extended the C-terminus by adding four residues (P399, 5400, 5401 and an extra Serine residue) of ST0939, of which P399-S401 were predicted to be N-terminal residues of STOFN3-2 by the SMART database. The crystal structure of this extended construct confirmed that the added segment was properly incorporated into the β-sheet (FIG. **3**C), and the construct was more thermostable than the original one (FIG. **3**D).

The stability of STOFN3-1 was further improved by structure-guided design. Based on a structure-guided alignment with FN3 domains, it was found that STOFN3-1 lacked highly conserved Proline residues in FN3 domains. Replacing these residues with Pro (N328P in the AB loop and D372P in the EF loop) improved the thermostability (FIG. **3**D). The variant containing all the improvements, namely, the N-terminal substitution, C-terminal extension and Pro mutations now exhibited very high thermal stability with the melting temperature exceeding 95° C. (FIG. **3**D). This construct was used as the template for constructing combinatorial libraries (termed STOTEMP2).

B. Identification of Positions Permissive to Amino Acid Diversification

In parallel to the scaffold improvements described above, the inventors identified positions of STOFN3-1 that are tolerant to amino acid diversification so that library designs avoid mutating positions critical for maintaining thermal stability and high solubility. A series of point mutations and insertions were introduced to the STOFN3-1 construct and their thermal stabilities were measured (FIG. 4A-C).

On the bottom end of the molecule as depicted in FIG. 2A, the poly-Ser mutations of the AB and CD loops resulted in little destabilization. In contrast, whereas that of EF loop destabilized STOFN3-1 by 16° C. The sensitivity of the EF loop to mutations is consistent with previous reports for other FN3 domains, which can be rationalized by the presence of the structurally important “Tyrosine corner” motif in the EF loop of STOFN3-1. On the top end of the molecule, the mutation of the BC loop slightly destabilized STOFN3-1 and that of the DE loop showed no destabilizing effect. The serine mutation of G360 immediately N-terminal to the DE loop resulted in inclusion body formation in *E. coli* and also dramatically decreased the stability (Tm reduction by more than 15° C.). Four sets of poly-Serine mutations of the FG loop all showed karge destabilization effect (13-20° C.), and all but FGser1 were expressed predominantly as inclusion bodies. This result suggested that both Y383 and P391, residues not mutated in the FGser1 mutant, were critical for efficient folding of STOFN3-1.

In addition to substation mutations, insertions of 2, 4 and 8 Serine residues into the BC, CD, DE and FG loops, the loops that tolerated substitution mutations were tested (FIG. 4C). Of these loops, the BC loop highly tolerated the insertions, whereas insertions in the CD and FG loops were destabilizing but these mutants still maintained the Tm of around 70° C. A very large destabilizing effect was observed for the DE-loop elongation. Even an insertion of two residues decreased the stability by 25° C. None of these insertion mutants resulted in substantial inclusion body formation. Taken together, these systematic mutation experiments identified that DE, EF and FG loops are less permissive to mutations than the other loops, but the most destablized mutants still had Tm higher than 50° C.

In the crystal structure, the side chain of Y383, the structurally important Tyr in the F strand, interacts with G342 in the C strand, apparently providing the “Aromatic rescue” of the destabilizing Gly residue in a □-strand (Regan ref). The impact of Y383 mutation completely disappeared when we substituted G342 with Tyr, an equivalent residue in FN3fn10 (FIG. 3D and FIG. 4B). Because it was envisioned that introducing Tyr, an amino acid particularly suitable for binding, at position 342 and the ability to diversify position 383 would both positively contribute to creating molecular recognition surfaces, the G342Y mutation was incorporated in the scaffold.

C. Phage and Yeast Display of STOFN3-1

Efficient display of a scaffold on the phage particle is a prerequisite for efficient selection of binding proteins using phage display. Highly stable, rapidly folding proteins present challenges in phage display, because phage display requires that the displayed protein fused to a phage coat protein be translocated into the periplasm of *E. coli* and highly stable proteins are not efficiently translocated across the *E. coli* inner membrane using a conventional, posttranslational secretion signal such as OmpT. An elegant solution was to use a co-translational secretion signal sequence such as the DsbA signal. Different signal sequences were examined for robust display of STOFN3-1. For this examination, the variant of STOTEMP4 with K318V mutation (STOTEMP5) was used because this mutation was crucial to function as the enhancer domain of affinity clamp (see below). Unexpectedly, it was found that an OmpT-based system achieved high levels of display of STOFN3-1 on the phage after optimization of the signal sequence and a linker length between the signal and STOFN3-1 with use of the C-terminal domain of M13 pIII. Notably, a single point mutation of the signal peptidase cleavage site from -SSFA/S- found in the vector pET12a (where the slash denotes the cleavage site) to -SSFA/A- and no additional residues between this signal sequence and the first residue of STOFN3-1 were crucial for the efficient display (FIG. 5). Unlike in a previous report, the R2K mutation of the OmpT signal did not improve surface display. For yeast surface display of STOFN3-1, a standard Aga2-mediated system as previously described that robustly displayed the protein as confirmed using flow cytometric analysis was used (data not shown).

D. Design and Evaluation of Combinatorial Libraries

Following previous designs of combinatorial libraries of FNfn10, two distinct libraries were constructed. One library is “loop only” library where positions in the BC, DE, and FG loops are utilized (FIG. 6A). The other library is “side-and-loop library” where residues in the C strand (residues 346 and 348) and the D strand (residues 352-354, 356 and 358) as well as residues in the CD and FG loops are utilized (FIG. 6B). Both libraries used highly biased amino acid diversity that emphasized Tyr, Ser, Gly and excluded Cys and Met, similar to designs used for FNfn10 and Fab libraries. In both libraries, the length of the FG loop was varied. In the “loop only” library, the length of the BC loop was varied. In the “side-and-loop” library, the same design of diversification as previous studies was applied to residues in the C and D strands and the CD loop. For diversifying the position of W348 on the C-strand, two sets of oligos were used for each length of CD-loop (3, 4, and 5 residues). One contains a codon for Tryptophan and the other contains codons for Serine, Threonine, Asparagine and Tyrosine so that this position could be diversified to five amino acids. Both libraries were constructed using the STOTEMP5 as a template scaffold in the phage-display format described above with estimated numbers of independent sequences of 2.5×109 and 1.7×109 for the loop only and side-and-loop libraries, respectively.

The inventors evaluated the performance of the two libraries using a total of six target proteins, yeast small ubiquitin-like modifier (ySUMO), maltose-binding protein (MBP), enhanced green fluorescent protein (GFP), Abl SH2, SHP2 N- and C-SH2 domains. For each combination of target and library, the inventors first enriched binding clones from the phage-display library, performed gene shuffling among the enriched population and identified high-affinity clones using yeast surface display. The inventors successfully generated binding proteins, called therMonobodies (thermophilic Monobodies), to ySUMO, MBP, EGFP and AblSH2 from both libraries and SHP2 N- and C-SH2 from the loop-only library (FIG. 6A, 6B). Most therMonobodies had Kd values in the low nanomolar range as measured in the yeast-display format (FIG. 6C). Residues in the FG loop were mutated in almost all the therMonobodies derived from either library, suggesting the importance of residues in the FG loop in target binding. Only exceptions were tMb (ABLSH2_L03) and tMb(ABLSH2_S01) that had no mutations in the FG loop, suggesting the possibility of achieving high affinity without utilizing the FG loop. We identified loop lenths that were not encoded in our designs. tMb (SUMO_L03) had 13 residues in the FG oop, one residue longer than the longest design, and tMb(NSH2_L06) and tMb(NSH2_L10) had four residues in the BC loop, one residue shorter than the shorted design. These sequences probably arose from errors in DNA synthesis and/or PCR errors, and their high functionality indicates that we could potentially expand the range of loop lengths in our library designs. For position 348, only Trp was selected, even though the position was diversified to a combination of five amino acids, Trp, Ser, Thr, Asn and Tyr. Subsequent mutation analysis showed that the replacement of W348 with Tyr or Ser destabilized STOTEMP5 by >18° C. and >25° C., respectively, indicating the importance of W348. In the crystal structure, the indole ring of W348 interacts with Y377 on the adjacent F-strand and also form a cation-7c interaction with K354 on the adjacent D-strand. Replacement of W348 may well disrupt or weaken these interactions, and this destabilization effect may lead to the low occurrence frequencies.

These results clearly demonstrate that the combinatorial libraries constructed on the newly developed scaffold and the selection strategy can generate high affinity binding proteins.

E. Biophysical Characterization of therMonobodies

The inventors characterized the oligomerization state and thermal stability of purified therMonobodies (FIG. **7**). Of 24 therMonobodies tested, 15 were predominantly monomeric as assayed using size-exclusion chromatography (SEC). Six eluted from the SEC as a monodispersed peak at the volume corresponding to a molecular weight much smaller than expected, suggesting that they were predominantly monomeric but weakly interacted with the column. The remaining three proteins eluted at the void volume, indicative of large aggregates. Thus, the vast majority (21/24) of these therMonobodies were produced as soluble, monomeric species. The thermal stability of the monomeric therMonobodies was assessed using DSF (FIG. **7**B, C). The therMonobodies derived from the loop only library had a mean Tm value of 68±10° C. with the highest and lowest of 86.3 and 53.5° C., respectively. Those generated from the side-and-loop library had a mean Tm value of 59.2±11.3° C. with the highest and lowest of 85.5 and 50.5° C., respectively. Interestingly, the most stable clones, tMb(NSH2_L06) and tMb(NSH2_L10), had four residues in the BC loop, which is one residue shorter than the designed range of the BC loop length. This finding suggests a new library design that better maintain the high stability of the underlying STOFN3-1 scaffold.

The highest Tm value of 86° C. and the lowest Tm value of 50° C. are both 13° C. higher than those of previously generated monobodies built from the FNfn10 scaffold (ref. Vazquez-Lombardi, 2015, Drug Discov Today). Thus, these results suggest that the therMonobodies system is better suited for generating thermostable binding proteins.

F. therMonobody Functions as the Enhancer Domain of Affinity Clamps

The affinity clamping technology involves connecting a FN3 domain to a peptide-binding domain (such as an interaction domain or another FN3 domain) and subsequent optimization of the FN3 domain via directed evolution so as to create clamshell architecture that "clamps" a target motif in the newly generated interface between the two domains. The enlarged ligand interaction interface relative to that afforded by either domain achieves high specificity and high affinity. In this context, the FN3 portion is termed as the enhancer domain. To determine whether therMonobody can be used as the enhancer domain for affinity clamping technology, the FNfn10-based monobody was replaced in an affinity clamp directed to a phosphotyrosine (pY)-containing peptide with a therMonobody. The pY-clamp consists of an engineered Grb2 SH2 domain linked, via short linker, to a FNfn10 monobody. STOFN3-1 was structurally aligned with the FNfn10 monobody segment in the pY-clamp, clamp(Ptpn11_pY580). Residues of the monobody segment that were located within 5A from the Grb2 SH2 domain or 6A from the target pY-peptide were grafted to the structurally equivalent positions of STOTEMP4 (FIG. **8**A). Because V4 located in the N-terminal tail of the monobody segment participates in the interaction with the Grb2 SH2 domain in the pY-clamp, the inventors also prepared a construct in which structurally equivalent K318 of STOTEMP4 was mutated to Val.

The inventors first examined biophysical properties of the designed therMonobody segments of the designed pY-clamps in isolation, i.e. not linked to the engineered SH2 domain. The therMonobody segments with and without the K318V mutation were expressed as >50% of soluble proteins and showed monodispersed size-exclusion chromatography profiles with an elution time consistent with that of their parent templates. The Tm values obtained from DSF were 68.7 and 63.5° C. for the variants without and with K318V mutation, respectively, indicating that both variants retained high stability. Together with the results mentioned in the above section, these results demonstrate the high tolerance of therMonobody template to extensive mutations.

Next the inventors examined binding properties of the designed pY-clamps to the target pY-peptide (Ptpn11 pY580). A total of six constructs were tested by combining the presence and absence of the K318V mutation with linker lengths of 0, 2 and 5 amino acids between the Grb2 SH2 domain and the therMonobody segment. Two of them, with the K318V mutation and linker lengths of 2 or 5 residues showed significantly higher binding to the pY-peptide than the original Grb2 SH2 domain alone (FIG. **8**B), with KD values of 156±42 and 121±21 nM for the 2- and 5-residue linkers, respectively (FIG. **8**C). For comparison, clamp (Ptpn11_pY580) with the optimized enhancer domain based on FNfn10 had a 6 amino acids linker between the Grb2 SH2 domain and the monobody segment and a KD value of 2.5 nM. Although the affinity of the therMonobody versions of the pY-clamp was lower, these results clearly indicates that therMonobody can readily be used as a building block for generating affinity clamps.

Example 2: Hyperthermophilic Fn3 Domain from *Pyrococcus horikoshii* OT3

The inventors utilized the SMART database to explore FN3 domains from hyperthermophiles. The database predicted many FN3 domains in hyperthermophilic archaea and bacteria such as *Thermococcus kodakaraensis, Sulfolobus tokodaii, Pyrococcus horikoshii* and *Thermotoga lettingae*. Predicted domains that were shorter than the length of the shortest FN3 domains that had been structurally characterized (75 amino acids) were first eliminated. Then, a FN3 domain in the sequence of putative uncharacterized protein PH0954 from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3, termed PHOFN3 was chosen as the candidate protein, because of its detectably homology to a bacterial FN3 domain in the *Clostridium Perfringens* Glycoside Hydrolase Gh84c whose FN3 fold has been experimentally confirmed (PDBID: 2W1N). The SMART database predicted PHOFN3 with 108 (P1873-A1980) amino acid residues, but the C-terminal 26 residues (E1955-A1980) did not have detectable homology to the sequence of the bacterial homologue (FIG. **9**).

In order to confirm whether the C-terminal extended region is a part of the core structure of PHOFN3, the inventors constructed expression vectors for different segments of PHOFN3 with the same N-terminus but with different truncations from the C-terminus, PHOFN3 (P1873-

A1980), PHOFN3ΔC21 (P1873-E1959), PHOFN3ΔC25 (P1873-E1955) and PHOFN3ΔC35 (P1873-E1945), and analyzed their soluble expressions in *E. coli* and thermal stabilities. It was found that PHOFN3, PHOFN3ΔC21 and PHOFN3ΔC25 were robustly produced in *E. coli* as soluble proteins that were predominantly monomeric. Melting temperatures obtained by differential scanning fluorimetry (DSF) were >95° C. for PHOFN3, PHOFN3ΔC21 and PHOFN3ΔC25 at pH 7.4, confirming that they were highly thermostable proteins (FIG. **10**). These results suggested that the C-terminal extended region, which was not aligned with the bacterial homologue, was not a part of the core structure of PHOFN3. In contrast, PHOFN3ΔC35 was expressed mostly as insoluble proteins and the melting temperature was 32.2° C. at pH 7.4 (FIG. **10**), suggesting that the region of K1946-E1955 was important for folding a proper structure and for thermal stability as the region was aligned with the last β-strand (G strand) of the bacterial homologue.

To confirm that PHOFN3 had the FN3 fold, the inventors determined the crystal structure of PHOFN3ΔC25 at 1.7 Å resolution with Se-Met labeled at M1882 and I1905M. The PHOFN3ΔC25 segment indeed adopts the FN3 fold consisting of seven anti-parallel β-strands and six loops (FIG. **11**A). Superposition with the FN3fn10 (PDBID: 1FNA), the most well characterized FN3 domain, demonstrated that the structure of the segment is highly similar to FN3fn10 with an average RMSD value of 2.2 Å for aligned backbone Ca atoms excluding residues in loops (FIG. **11**B). Figure E shows the schematic drawing of the amino acid sequence of PHOFN3ΔC25 in its secondary structure context.

Phage display of PHOFN3. A vector for phage display of PHOFN3 (without C-terminal deletion, P1873-A1980) was constructed based on the previously reported DsbA-based vector containing the V5 tag sequence for display detection (Wojcik et al., NSMB). Phage particles were produced using helper phage, M13K07 or hyperphage, and the display of PHOFN3 on phage particles was examined by phage ELISA using an anti-V5 tag antibody, a HRP conjugated anti-M13 phage antibody and 1-step Ultra TMB ELISA (Thermo Scientific). The absorbance changes at 405 nm after the HRP reaction for 10 min for M13KO7-produced phages or 1 min for hyperphage-produced phages are shown in FIG. **12**. FIG. **13** shows the significant levels of display of PHOFN3 on phage particles. The phage ELISA signals for PHOFN3 with M13K07 and hyperphage were lower than those for FN3fn10 only by 2.9 and 1.3-folds, respectively, strongly suggesting that a combinatorial library similar to those for FNfn10 can be constructed using phage display from which novel binding proteins can be identified.

Although certain embodiments have been described above with a certain degree of particularity, or with reference to one or more individual embodiments, those skilled in the art could make numerous alterations to the disclosed embodiments without departing from the scope of this invention. Further, where appropriate, aspects of any of the examples described above may be combined with aspects of any of the other examples described to form further examples having comparable or different properties and addressing the same or different problems. Similarly, it will be understood that the benefits and advantages described above may relate to one embodiment or may relate to several embodiments. Any reference to a patent publication or other publication is a herein a specific incorporation by reference of the disclosure of that publication. The claims are not to be interpreted as including means-plus- or step-plus-function limitations, unless such a limitation is explicitly recited in a given claim using the phrase(s) "means for" or "step for," respectively.

SEQUENCE LISTING

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Tyr Trp Ser Asn Phe Ser Gln Lys Val Thr Ile Asn Val Gly Asn Val
        35                  40                  45

Thr Ser Tyr Thr Ile Lys His Leu Lys Asp Gly Val Thr Tyr Tyr Ile
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Asn Ile Thr Ser Tyr Val Leu Thr Asn Leu Thr Ala Gly Glu Leu Tyr
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20 25 30

Glu Asn Val Val Leu Leu Asn Val Ser Thr Thr Asn Thr Ser Tyr Phe
35 40 45

Val Lys Val Pro Phe Gly Val Tyr Asn Ile Ser Leu Glu Ala Val Asn
50 55 60

Ile Val Gly Ile Thr Lys Tyr Ala Phe Ile Leu Ile Tyr Tyr Ile
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Asn Gly Lys Leu Ile Thr Asn Thr Thr Asn Thr Ala Phe Thr Phe Asn
35 40 45

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Tyr Lys Ser Ala Pro Tyr Ile Ile Asn Asp Val Arg
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20 25 30

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65                  70                  75                  80

Pro Phe Ser Leu Ser Ser Ala Gly Ala Thr Val Cys Asn Asn Thr Leu
                85                  90                  95

Tyr Val Val Gly Gly Ala Asn Ser Thr Ser Ile Phe Gly Gly Ile Leu
            100                 105                 110

Glu Phe Ile Gly Asn Gly Trp Lys Val Ile Thr Asn Ser Met Pro Ile
        115                 120                 125

Pro Val Tyr Gly Ala Ile Val Phe Ser Tyr Asp Tyr Lys Ile Tyr Val
    130                 135                 140

Ile Gly Gly Met Asn Tyr Ser Gly Asn Ser Leu Val Pro Pro Val Asn
145                 150                 155                 160

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Ala Leu Phe Val Val Gly Gly Phe Thr Gln Ser Ala Thr Leu Thr Ser
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Ser Val Phe Val Tyr Tyr Pro Glu Asn Asn Thr Trp Ile Ser Leu Pro
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Ser Leu Pro Gly Val Glu Ala Gly Gly Val Leu Gly Tyr Tyr Asn Gly
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Gln Lys Val Thr Ile Asn Val Gly Asn Val Thr Ser Tyr Thr Ile Lys
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His Leu Lys Asp Gly Val Thr Tyr Tyr Ile Gln Ile Val Pro Tyr Asn
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Ser Val Pro Asn Pro Pro Ile Ile Lys Val Lys Ile Gly Asn Leu Asn
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Lys Ile Asn Gly Phe Leu Val Ser Trp Asn Ser Thr Ser Lys Ala Lys
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    530                 535                 540

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                565                 570                 575

Ile Thr Leu Asn Thr Val Ser Leu Asn Trp Ser Lys Val Ser Gly Ala
            580                 585                 590

Glu Tyr Tyr Leu Ile Tyr Asp Asn Gly Lys Leu Ile Thr Asn Thr Thr
        595                 600                 605

Asn Thr Ala Phe Thr Phe Asn Leu Thr Ile Gly Gln Asn Glu Ile Glu
    610                 615                 620

Val Tyr Ala Ala Asn Ala Tyr Tyr Lys Ser Ala Pro Tyr Ile Ile Asn
625                 630                 635                 640

Asp Val Arg Asn Tyr Ile Val Val Val Asn Ser Thr Ala Ile Ser Ile
                645                 650                 655

Ser Val Pro Gln Ile Lys Val Val Ser Gly Glu Asn Thr Asp Ala Pro
            660                 665                 670

Leu Gln Thr Asn Asn Ile Asp Leu Lys Ser Ala Ile Ile Val Ile Thr
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        35                  40                  45

Asn Phe Arg Asp Ile Tyr Ser Gly Thr Leu Asn Tyr Ser Ile Ser Ala
    50                  55                  60

Ile Asp Phe Ser Gly Phe Glu Ser Glu Lys Thr Glu Val Phe Pro Val
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Leu Tyr Asp Ala Gly Ser Cys Asn Leu Thr Phe Ser Val Phe Ser Pro
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Lys Ser Phe Ala Ile Gln Val Pro Leu Gly Thr Pro Lys Leu Tyr Thr
260 265 270

Tyr Gln Val Arg Glu Ser Gly Ser Tyr Leu Leu Thr Leu Pro Leu Ser
275 280 285

Asn Tyr His Val Glu Val Phe Ala Asn Cys Met Asp Asp Pro Asp Leu
290 295 300

Ser Asn Asn Leu Ala Glu Val Gly Val Trp Pro Gly Asp Tyr Trp Val
305 310 315 320

Glu Asn Ala Ser Ile Asn Asn Leu Ile Pro Gly Glu Phe Ala Ser Ile
325 330 335

Asn Phe Lys Val Arg Thr Thr Ser Lys Ile Pro Ser Ala Lys Val Lys
340 345 350

Leu Leu Arg Asn Gly Val Glu Glu Lys Ile Glu Tyr Leu Ser Phe Tyr
355 360 365

Asn Gly Ile Ala Glu Gly Glu Ile Ser Trp Leu Val Gln Gly Gly Asn
370 375 380

Tyr Thr Leu Ala Leu Leu Val Glu Gly Lys Gly Asp Ile Asn Ser Ser

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385                 390                 395                 400

Asn Asn Ile Tyr Leu Leu Gly Asn Tyr Asn Phe Pro Leu Pro Asn Phe
                405                 410                 415

Glu Val Gly Asn Tyr Ser Ile Asp Leu Pro Thr Cys Val Asp Ser Thr
            420                 425                 430

Gly Glu Val Arg Val Asn Val Thr Ser Thr Ala Asn Trp Ser Ile Pro
        435                 440                 445

Val Arg Leu Thr Leu Val Tyr Glu Glu Gly Asn Arg Ser Tyr Thr Arg
    450                 455                 460

Tyr Ile Ser Thr Lys Gly Glu Glu Glu Ser Glu Val Ile Phe Thr Pro
465                 470                 475                 480

Met Ile Lys Ala Gly Thr Leu Glu Lys Val Val Ile Glu Ile Asp Pro
                485                 490                 495

Trp Asn Glu Val Glu Glu Ser Asn Glu Ser Asp Asn Lys Val Glu Val
            500                 505                 510

Pro Tyr His Ile Ile Ile Glu Lys Pro Asp Phe Thr Val Lys Ser Leu
        515                 520                 525

Asn Ile Pro Gly Asn Val Ser Ile Gly Asn Leu Tyr Glu Val Asn Val
    530                 535                 540

Thr Leu Asp Asn Leu Gly Gly Cys Tyr Gly Arg Asn Val Leu Val Lys
545                 550                 555                 560

Leu Tyr Glu Asn Gly Thr Ser Lys Asp Trp Arg Arg Val Arg Ile Asn
                565                 570                 575

Asn Glu Thr Asn Val Thr Leu Thr Trp Lys Pro Gly Asn Ala Gly Leu
            580                 585                 590

Val Asn Leu Thr Val Val Val Asp Pro Tyr Ser Tyr Val Asp Glu Ile
        595                 600                 605

Asn Glu Gly Asn Asn Arg Leu Ser Arg Leu Ile Phe Val Asn Ala Pro
    610                 615                 620

Asp Phe Lys Ile Ser Lys Val Glu Leu Leu Ser Phe Asp Gly Ile Ala
625                 630                 635                 640

Gly Ser Lys Ala Lys Phe Asn Val Thr Val Lys Asn Glu Gly Glu Asp
                645                 650                 655

Tyr Ser Gly Tyr Phe Ser Ile Ala Val Tyr Gly Gly Leu Arg Ser Ser
            660                 665                 670

Ile Ala Tyr Leu Arg Gly Ile Lys Ser Gly Glu Glu Lys Trp Thr Ile
        675                 680                 685

Ile Ser Leu Pro Ile Asn Gly Gly Asn Ser Thr Leu Ile Phe Val Val
    690                 695                 700

Asp Pro His Asn Val Ile Ser Glu Thr Asn Glu Gly Asn Asn Val Ile
705                 710                 715                 720

Phe Tyr Asn Met Gly Tyr Ile Pro Lys Pro Asn Phe Val Val Lys Glu
                725                 730                 735

Ile Ser Leu Pro Asn Asn Thr Val Gly Tyr Ile Pro Leu Asn Ile Thr
            740                 745                 750

Ile Gly Asn Val Gly Ala Pro Tyr Asn Ala Thr Ser Tyr Gln Val Pro
        755                 760                 765

Val Lys Ile Lys Thr Glu Tyr Gly Trp Lys Val Ser Tyr Leu Arg Gly
    770                 775                 780

Ile Ile Arg Asp Asn Tyr Thr Ile Ser Ile Asp Ser Leu Ala Met Leu
785                 790                 795                 800

Pro Pro Gly Ser Thr Ile Asn Val Thr Val Asn Tyr Asn Met Lys Val
                805                 810                 815
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Asn Glu Thr Ser Tyr Ser Asp Asn Ser Leu Ile Ile Asn Tyr Thr Thr
            820                 825                 830

Gly Tyr Pro Asp Leu Glu Leu Gly Ile Ile Pro Pro Ser Gly Glu Leu
        835                 840                 845

Ser Ala Gly Lys Asp Val Lys Ile Thr Phe Leu Val Lys Asn Val Gly
    850                 855                 860

Asn Ala Thr Leu Arg Ile Asp Arg Ser Ser Trp Tyr Ser Pro Tyr Leu
865                 870                 875                 880

Gly Leu Tyr Val Thr Leu Glu Asp Glu Asn Gly Lys Thr His Thr Leu
                885                 890                 895

Gly Arg Tyr Glu Leu Ala Pro Ala Thr Leu Ser Pro Gly Ala Asn Ile
            900                 905                 910

Ser Gln Val Val Trp Ile Thr Leu Asn Gly Gly Thr Asn Lys Ile Met
        915                 920                 925

Gly Arg Ile Val Asp Glu Tyr Glu Asn Ile Tyr Glu Asn Asn Asn Asp
    930                 935                 940

Thr Leu Ile Leu Thr Leu Glu Lys Pro Asp Phe Ala Ile Leu Asn Tyr
945                 950                 955                 960

Ser Ile Pro Asp Glu Ile Leu Asn Gly Thr Ala Tyr Leu Tyr Lys Ala
                965                 970                 975

Tyr Pro Ile Val Leu Asn Ile Ser Asn Leu Gly Gly Asn Phe Ser Asp
            980                 985                 990

Gly Ile Arg Val Asp Leu Phe Asp  Asn Gly Ile Ile Lys  Thr Ser Thr
        995                 1000                1005

Ser Val  Tyr Gly Leu Glu Ser  Gly Ala Ser Arg Asp  Val Thr Leu
    1010                1015                1020

Arg Tyr  Leu Pro Ser Ser Gly  Lys His Asn Leu Ser  Ile Val Leu
    1025                1030                1035

Asp Pro  Tyr Asn Arg Trp Ile  Glu Glu Asn Glu Glu  Asn Asn Asn
    1040                1045                1050

Leu Thr  Phe Ser Leu Ser Phe  Gly Lys Pro Asp Leu  Lys Val Glu
    1055                1060                1065

Gly Ile  Thr Trp Ala Pro Tyr  Asn Phe Thr Ser Gly  Glu Asn Val
    1070                1075                1080

Leu Phe  Thr Ile Tyr Val Lys  Asn Leu Gly Gln Pro  Phe Leu Lys
    1085                1090                1095

Ser Phe  Thr Val Arg Ala Glu  Ile Trp Asn Gly Thr  Arg Lys Ile
    1100                1105                1110

Tyr Ser  Thr Asn Ala Tyr Pro  Arg Asn Trp Ser Phe  Gly Lys Gly
    1115                1120                1125

Glu Thr  Lys Glu Phe Asn Trp  Arg Trp Tyr Asn Ala  Lys Pro Gly
    1130                1135                1140

Asn Leu  Thr Val Lys Ile Val  Val Asp Tyr Tyr Asn  Ser Ile Pro
    1145                1150                1155

Glu Gly  Asn Glu Ser Asn Asn  Glu Phe Ser Ala Phe  Leu Gly Asn
    1160                1165                1170

Val Gly  Thr Pro Asp Phe Lys  Leu Glu Asn Leu Ser  Val Glu Asp
    1175                1180                1185

Leu Ala  Tyr Gly Lys Phe Val  Arg Ile Asn Ala Thr  Val Lys Asn
    1190                1195                1200

Leu Gly  Asp Ser Ile Tyr Arg  Pro Ile Thr Val Leu  Phe Asn Val
    1205                1210                1215
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Ser Gly Glu Arg Tyr Tyr Arg Thr Val Tyr Gly Ile Lys Glu Asn
    1220                1225                1230

Glu Ser Lys Ser Val Thr Leu Pro Trp Tyr Val Asp Arg Val Gly
    1235                1240                1245

Glu Val Arg Val Lys Val Glu Val Asp Pro Gly Asn Arg Ile Val
    1250                1255                1260

Glu Gly Asn Glu Ser Asn Asn Ile Ile Glu Arg Thr Tyr Tyr Val
    1265                1270                1275

Glu Ser Pro Glu Leu Met Leu Ser Gly Tyr Glu Trp Leu Glu Glu
    1280                1285                1290

Glu Val Arg Arg Gly Tyr Leu Ala Tyr Lys Val Asn Val Thr Asn
    1295                1300                1305

Thr Gly Gly Asp Val Tyr Arg Gly Phe Tyr Val Gln Met Phe Val
    1310                1315                1320

Asp Gly Glu Pro Lys Ser Ser Val Trp Ile Asn Lys Leu Leu His
    1325                1330                1335

Gly Glu Thr Ala Glu Arg Thr Leu Arg Trp Arg Phe Ser Ser Gly
    1340                1345                1350

Gly Arg Lys Glu Val Arg Ile Val Val Asp Pro Gln Asp Tyr Ile
    1355                1360                1365

Pro Glu Ser Asn Glu Asp Asn Asn Ala Ile Val Glu Asn Val Thr
    1370                1375                1380

Ile Val Leu Pro Asp Ile Glu Val Leu Ser Leu Asn Ile Pro Ser
    1385                1390                1395

Met His Ala Asn Ser Tyr Phe Lys Val Asn Ala Thr Ile Lys Asn
    1400                1405                1410

Ser Gly Gly Gln Asp Val Lys Arg Ile Phe Tyr Val Ser Leu Tyr
    1415                1420                1425

Gln Asp Gly Lys Leu Leu Gly Ser Ala Pro Val Tyr Ser Leu Ala
    1430                1435                1440

Ser Gly Glu Val Lys Glu Val Thr Leu Thr Ile Arg Pro Tyr Pro
    1445                1450                1455

Gly Asn Ser Thr Phe Lys Val Val Val Asp Pro Thr Asn Ala Val
    1460                1465                1470

Val Glu Leu Asn Glu Asp Asn Asn Glu Ile Ser Val Arg Ser Tyr
    1475                1480                1485

Val Lys Ala Pro Asp Ile Val Val Val Ser Ala Asp Leu Gly Asn
    1490                1495                1500

Phe Thr Tyr Pro Gly Glu Met Val Asn Ala Lys Val Arg Ile Arg
    1505                1510                1515

Asn Ser Gly Asp Tyr Lys Ser Gly Val Tyr Leu Leu Ile Arg Asn
    1520                1525                1530

Lys Arg Arg Lys Leu Gly Ser Ala Tyr Val Asp Ser Ile Thr Pro
    1535                1540                1545

Gly Glu Glu Ile Glu Val Asn Val Pro Trp Leu Val Asp Ser Gly
    1550                1555                1560

Asp Tyr Asn Val Ser Val Ile Ala Asp Pro Tyr Asn Ser Val Arg
    1565                1570                1575

Glu Trp Asp Glu Glu Asn Asn Lys Leu Asp Ile Glu Val Ser Val
    1580                1585                1590

Pro Ser Pro Asp Leu Thr Val Glu Asn Ile Thr His Ser Gly Lys
    1595                1600                1605

Glu Val Ala Gly Glu Glu Ile Ile Ile Lys Val Thr Val Lys Asn
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    1610                1615                1620

Ile Gly  Glu Ser Ser Lys Leu  Pro Phe Tyr Ile Val  Leu Tyr Ala
    1625                1630                1635

Asn Ser  Ser Phe Val Gly Ile  Asn Arg Val Thr Lys  Ile Asp Lys
    1640                1645                1650

Gly Glu  Ser Ile Thr Leu Glu  Phe Lys Trp Arg Ala  Ser Tyr Gly
    1655                1660                1665

Glu Tyr  Ala Leu Arg Ala Ile  Val Asp Pro Tyr Asp  Glu Val Tyr
    1670                1675                1680

Glu Glu  Asn Glu Ser Asn Asn  Glu Gly Met Val Lys  Val Phe Ile
    1685                1690                1695

Glu Asp  Glu Glu Pro Pro Val  Leu Lys Leu Thr Tyr  Pro Glu Asn
    1700                1705                1710

Gly Thr  Phe Thr Asn Lys Pro  Tyr Ile Gly Ala Tyr  Leu Arg Asp
    1715                1720                1725

Glu Gly  Ser Gly Val Lys Phe  Gly Glu Ile Glu Val  Tyr Arg Glu
    1730                1735                1740

Gly Thr  Ser Val Pro Gly Ser  Thr Lys Phe Ser Gly  Gly Trp Leu
    1745                1750                1755

Ile Phe  Gln Asn Ser Thr Pro  Leu Leu Asp Gly Lys  Tyr Thr Val
    1760                1765                1770

Thr Val  Lys Ala Val Asp Arg  Ala Gly Asn Glu Ile  Thr Tyr Ser
    1775                1780                1785

Trp Asn  Phe Thr Leu Asp Arg  Glu Pro Pro Arg Ile  Val Cys Asn
    1790                1795                1800

Leu Thr  Asp Gly Thr Leu Tyr  Asn Gly Thr Val Val  Pro Gly Val
    1805                1810                1815

Gln Val  Ile Asp Asp Asn Leu  Asp Trp Tyr Lys Val  Lys Val Asn
    1820                1825                1830

Gly Arg  Glu Phe Ser Gly Pro  Ile Lys Leu Asp Gly  Thr Tyr Thr
    1835                1840                1845

Leu Asn  Val Thr Ala Lys Asp  Lys Ala Gly Asn Leu  Ala Glu Lys
    1850                1855                1860

Ile Ile  Arg Phe Thr Val Asn  Gly Val Pro Ser Pro  Pro Ser Gly
    1865                1870                1875

Val Thr  Leu Met Leu Asn Gly  Ser Tyr Val Glu Leu  Ser Trp Leu
    1880                1885                1890

Pro Ser  Pro Asp Ser Asp Val  Ala Gly Tyr Phe Ile  Tyr Lys Asp
    1895                1900                1905

Gly Lys  Arg Leu Asn Glu Val  Pro Ile Glu Lys Pro  Asn Phe Arg
    1910                1915                1920

Asp Ile  Tyr Ser Gly Thr Leu  Asn Tyr Ser Ile Ser  Ala Ile Asp
    1925                1930                1935

Phe Ser  Gly Phe Glu Ser Glu  Lys Thr Glu Val Phe  Pro Val Lys
    1940                1945                1950

Leu Glu  Val Asp Glu Glu Asn  Leu Thr Ala Gly Tyr  Pro Gly Ala
    1955                1960                1965

Val Lys  Val Lys Val Glu Asn  Leu Asp Gly Glu Ala  Asn Gly Thr
    1970                1975                1980

Leu Ser  Ile Ile Leu Ile Asp  Glu Phe Gly Asn Glu  Ile Glu Lys
    1985                1990                1995

Leu Ser  Arg Lys Val Glu Val  Pro Arg Gly Arg Ser  Ser His Glu
    2000                2005                2010
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Phe Val  Phe Met Val Pro Arg  Gly Leu Thr Leu Ile  Arg Gly Glu
    2015                 2020                 2025

Leu Lys  Val Gly Asn Ser Thr  Ala Arg Ile Ile His  Arg Ala Lys
    2030                 2035                 2040

Val Arg  Glu Gly Glu Asn Pro  Glu Ile Arg Val Gly  Lys Leu Leu
    2045                 2050                 2055

Ala Gly  Phe Pro Gly Leu Val  Glu Val Glu Ile Arg  Asn Cys Gly
    2060                 2065                 2070

Ile Val  Glu Leu Asn Thr Ser  Glu Thr Leu Met Lys  Leu Asp Asn
    2075                 2080                 2085

Ser Ser  Gly Glu Leu Ile Glu  Ala Pro Leu Thr Ile  Pro Pro Gly
    2090                 2095                 2100

Lys Lys  Thr Val Leu Arg Tyr  Lys Ile Val Pro Pro  Lys Lys Gly
    2105                 2110                 2115

Ser Tyr  Asn Leu Thr Phe Arg  Ile Ala Asp Val Glu  Val Arg Lys
    2120                 2125                 2130

Ile Val  Asn Val Ser Glu Ser  Val Leu Asn Pro Ile  Thr Ile Ser
    2135                 2140                 2145

Thr Glu  Asn Phe Ile Lys Gly  Gly Lys Ala Lys Ile  Tyr Val Ser
    2150                 2155                 2160

Phe Arg  Asn Ile Gly Ser Ala  Pro Ile Phe Val Lys  Ser Ile Glu
    2165                 2170                 2175

Leu Asn  Gly Met Ser Lys Arg  Leu Ser Ile Glu Leu  Pro Pro Asn
    2180                 2185                 2190

Leu Ser  Val Glu Glu Ser Phe  Glu Tyr Leu Ile Ser  Glu Glu Asn
    2195                 2200                 2205

Val Glu  Ile Asn Ala Thr Val  Asn Thr Asp Val Gly  Lys Phe Arg
    2210                 2215                 2220

Lys Ser  Leu Thr Leu Thr Ala  Glu Gln Pro Glu Tyr  Asn Ala Asp
    2225                 2230                 2235

Val Ser  Val Ser Ser Val Tyr  Glu Val Gly Lys Glu  Ile Leu Ile
    2240                 2245                 2250

Thr Gly  Val Ala Tyr Asn Glu  Ser Gly Met Leu Ser  Asn Val Pro
    2255                 2260                 2265

Val Lys  Val Ser Ile Ala Arg  Gly Gly Phe Val Arg  Glu Tyr Ile
    2270                 2275                 2280

Val Thr  Thr Asn Glu Asn Gly  Tyr Phe Asn Leu Thr  Phe Arg Pro
    2285                 2290                 2295

Phe Lys  Gly Glu Ser Gly His  Phe Ile Val Ser Ala  Thr His Pro
    2300                 2305                 2310

Lys Ile  Glu Leu Leu Glu Arg  Asp Ala Glu Phe Asp  Val Val Gly
    2315                 2320                 2325

Ile Glu  Val Ile Pro Ser Leu  Tyr Leu Leu Thr Val  Pro Val Glu
    2330                 2335                 2340

Phe Asn  Gly Thr Val Arg Val  Arg Leu Ile Asn Tyr  Trp Arg Ala
    2345                 2350                 2355

Ser Asp  Val Ser Val Ser Val  Lys Ala Pro Pro Glu  Tyr Glu Val
    2360                 2365                 2370

Ser Ile  Pro Lys Val Leu His  Leu Lys Pro Gly Ser  Asn Ile Ile
    2375                 2380                 2385

Asn Ile  Gly Leu Ser Ser Lys  Asn Ala Val Asn Gly  Ser Ile Met
    2390                 2395                 2400
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Ile Thr  Phe Lys Ala Arg Gln  Leu Gly Leu Asn Ile  Thr Arg Ser
    2405                2410                2415

Leu Thr  Leu Lys Leu Lys Val  Leu Pro Pro Ala Pro  Ala Ile Val
    2420                2425                2430

Thr Ser  Pro Asn Phe Leu Asp  Val Gly Val Leu Thr  Asn Glu Thr
    2435                2440                2445

Ala Ser  Ala Glu Val Val Val  Arg Asn Leu Gly Phe  Thr Ala Leu
    2450                2455                2460

Arg Asn  Val Ser Ile Arg Ser  Ser Ile Pro Trp Val  Lys Val Val
    2465                2470                2475

Ser Asn  Phe Thr Glu Val Asp  Pro Lys Asp Asn Glu  Ser Ile Ser
    2480                2485                2490

Leu Tyr  Ile Glu Pro Pro Arg  Asn Val Thr Gly Thr  Phe Lys Gly
    2495                2500                2505

Glu Ile  Thr Ile Ser Ser Ser  Asn Tyr Asn Pro Ile  Lys Val Pro
    2510                2515                2520

Met Arg  Ile Arg Val Thr Pro  Asn Ala Thr Gly Gly  Val Lys Val
    2525                2530                2535

Thr Val  Met Asp Pro Asn Ala  Thr Arg Leu Glu Asn  Val Lys Leu
    2540                2545                2550

Thr Leu  Tyr Asn Gly Tyr Phe  His Phe Glu Gly Tyr  Thr Asn Lys
    2555                2560                2565

Asn Gly  Thr Leu Glu Val Glu  Asn Val Pro Ile Gly  Glu Tyr Lys
    2570                2575                2580

Leu Phe  Ala Ser Leu Glu Gly  Tyr Tyr Gly Tyr Ser  Thr Ser Ile
    2585                2590                2595

Thr Ile  Glu Val Gly Val Glu  Lys Asn Val Ser Ile  Ile Leu Thr
    2600                2605                2610

Pro Ser  Ile Leu Glu Val Glu  Trp Glu Val Val Pro  Val Thr Ile
    2615                2620                2625

Gln Asp  Val Tyr Ile Ile Lys  His Glu Met Trp Tyr  Ser Thr His
    2630                2635                2640

Val Pro  Ala Pro Glu Ile Arg  Met Glu Gly Gly Asp  Leu Glu Val
    2645                2650                2655

Tyr Val  Asp Tyr Glu Lys Leu  Ala Glu Glu Gly Met  Leu Glu Phe
    2660                2665                2670

Arg Gly  Gln Val Ile Val Arg  Asn Thr His Gln Tyr  Ile Ser Val
    2675                2680                2685

Tyr Asn  Val Thr Phe Glu Ser  Gly Gly Ser His Tyr  Ile Asp Val
    2690                2695                2700

Glu Phe  Gly Ile Asn Arg Ile  Asp Glu Leu Lys Pro  Gly Glu Ala
    2705                2710                2715

Val Ile  Val Pro Tyr Val Val  Arg Ile Tyr Tyr Ser  Arg Ser Pro
    2720                2725                2730

Pro Ile  Asn Pro Cys Leu His  Glu Thr Lys Val Phe  Lys Leu Lys
    2735                2740                2745

Ala Gly  Val Val Cys Val Glu  Glu Ala Gly Lys Ile  Thr Leu Lys
    2750                2755                2760

Ala Gln  Arg Ile His Gln Ile  Val Val Lys Pro Thr  Cys Lys Gly
    2765                2770                2775

Cys Trp  Glu Ser Val Phe Pro  Val Ala Gly Lys Leu  Ala Phe Met
    2780                2785                2790

Ala Ile  Ala Gln Lys Val Gly  Gln Ala Leu Gly Asn  Ile Asp Asp
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    2795                2800                2805

Thr Gly  Val Leu Ser Thr Leu  Ala Gly Glu Ala Leu  Asn Asn Leu
    2810                2815                2820

Glu Ser  Leu Phe Asp Ala Tyr  Asn Ala Tyr Lys Ala  Asn Pro Thr
    2825                2830                2835

Lys Glu  Asn Met Glu Asn Tyr  Val Lys Thr Phe Asn  Ser Val Lys
    2840                2845                2850

Ala Asn  Leu Ala Ser Leu Phe  Met Phe Asp Pro Val  Ala Tyr Gln
    2855                2860                2865

Glu Ile  Asn Ser Leu Gln Leu  Thr Leu Ile Lys Thr  Pro Lys Gly
    2870                2875                2880

Asp Ile  Ala Gly Phe Ala Val  Ser Arg Thr Ala Thr  Pro Val Tyr
    2885                2890                2895

Ala Leu  Gly Met Gly Val Gly  Lys Ile Glu Asn Gly  Gln Leu Lys
    2900                2905                2910

Val Asp  Tyr Lys Lys Ala Val  Asn Ile Ala Asn Gly  Ile Val Leu
    2915                2920                2925

Asn Val  Met Ser Lys Met Gly  Gly Ala Leu Gly Gly  Ile Ala Ser
    2930                2935                2940

Gly Val  Gly Leu Leu Gln Leu  Leu Asp Lys Ala Ala  Glu Asp Leu
    2945                2950                2955

Pro Pro  Tyr Ile Ala Gln Leu  Phe Leu Asn Cys Ala  Ile Cys Leu
    2960                2965                2970

Met Arg  Asn Asp Cys Thr Leu  Pro Glu Gly Glu Glu  Ile Arg Pro
    2975                2980                2985

Ile Gln  Ile Ile Ala Ser Gly  Ser Leu Gly Gly Tyr  Pro Ser Met
    2990                2995                3000

Ile Pro  Ser Gly Leu Ala Gly  Gly Gly Asp Gly Gly  Thr Ala Val
    3005                3010                3015

Gly Arg  Phe Thr Cys Gly Gly  Leu Pro Thr Val Lys  Lys Ser Ser
    3020                3025                3030

Thr Ser  Met Ser Cys Ser Thr  Cys Ser Ser Glu Asp  Val Val Lys
    3035                3040                3045

Glu Arg  Val Cys Arg Leu Phe  Arg Glu Ser Glu Thr  His Asp Glu
    3050                3055                3060

Glu Glu  Pro Ser Asn Thr Leu  His Met Cys Val Asp  Leu Val Leu
    3065                3070                3075

Thr Ile  Glu Gln Arg Leu Thr  Phe Glu Arg Gln Ala  Phe Arg Ala
    3080                3085                3090

Ser Leu  Lys Phe Thr Asn Thr  Asn Arg Asn Tyr Ser  Leu Glu Asn
    3095                3100                3105

Val Ser  Val Arg Val Ile Phe  Phe Asp Glu Glu Gly  Asn Arg Val
    3110                3115                3120

Asp Asp  Lys Phe Phe Val Arg  Leu Asp Glu Lys Ala  Gly Leu Ser
    3125                3130                3135

Gly Ser  Ser Leu Glu Pro Glu  Lys Thr Ala Glu Met  Lys Trp Leu
    3140                3145                3150

Ile Ile  Pro Lys Val Gly Ala  Ala Glu Lys Phe Arg  Ala Arg Tyr
    3155                3160                3165

Tyr Val  Met Ala Asn Ile Thr  Ala Arg Val Gly Ser  Thr Lys Leu
    3170                3175                3180

Val Tyr  Glu Thr Trp Pro Ala  Met Ile Glu Val Glu  Pro Val Pro
    3185                3190                3195
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Gln Leu Val Leu Asp Tyr Val Leu Pro Ser Tyr Val Phe Gly Asp
    3200                3205                3210

Asp Pro Tyr Thr Pro Glu Lys Glu Leu Pro Ile Pro Phe Ile Phe
    3215                3220                3225

Gly Val Arg Val Lys Asn Val Gly Tyr Gly Thr Ala Arg Lys Leu
    3230                3235                3240

Arg Ile Ala Ser Ala Gln Pro Lys Ile Glu Arg Ser Asn Tyr Pro
    3245                3250                3255

Gly Val Tyr Ile Asp Phe Lys Ile Ile Gly Thr Leu Val Asn Gly
    3260                3265                3270

Lys Lys Val Pro Asn Ser Leu Thr Ile Asp Phe Gly Asp Leu Asn
    3275                3280                3285

Pro Gly Glu Ser Ser Thr Ala Ala Trp Leu Met Ile Ala Glu Val
    3290                3295                3300

Ser Gly Lys Phe Leu Gln Tyr Asn Ala Thr Phe Lys His Ser Asp
    3305                3310                3315

Glu Leu Gly Gly Asn Glu Thr Ser Leu Ile Lys Glu Val Arg Thr
    3320                3325                3330

His Phe Leu Ile Arg Ala Phe Asn Asn Thr Glu Asn Asp Asp Gly
    3335                3340                3345

Met Leu Asp Phe Leu Val Asp Asp Asp Gly Asp Gly Lys Pro Glu
    3350                3355                3360

Lys Ile Ile Asp Ser Arg Gly Phe Asp Tyr Asn Val Leu Leu Leu
    3365                3370                3375

Asn Phe Thr Glu Val Glu Glu Gly Ser Met Arg Lys Ile Ile Pro
    3380                3385                3390

Glu Met Lys Thr Pro Phe Trp Val Tyr Phe Thr Val Pro Phe Lys
    3395                3400                3405

Gly Ser Val Val Arg Ser Asp Gly Lys Asn Pro Met Asp Gln Trp
    3410                3415                3420

Met Glu Asn Gly Thr Leu His Val Leu Asp Leu Gly Thr Pro Glu
    3425                3430                3435

Phe Tyr Ile Leu Lys Ser Asn Gln Pro Pro Ile Pro Arg Ile Tyr
    3440                3445                3450

Val Lys Glu Pro Val Ile Ala Asn Glu Thr Val Val Leu Asp Gly
    3455                3460                3465

Ser Leu Ser Tyr Asp Pro Asp Gly Ser Ile Ile Ala Tyr Thr Trp
    3470                3475                3480

Lys Ile Gly Asn Glu Ser Phe Val Gly Asp Lys Val Ser Tyr Val
    3485                3490                3495

Phe Arg Glu Pro Gly Thr Tyr Asn Val Thr Leu Thr Val Arg Asp
    3500                3505                3510

Asp Lys Gly Thr Glu Ser Ser Lys Thr Met Glu Ile Lys Val Tyr
    3515                3520                3525

Leu Gly Pro Lys Phe Asn Glu Ser Leu Lys Val Glu Pro Gln Trp
    3530                3535                3540

Gly Ile Val Pro Phe Asn Leu Ser Ile Thr Phe Asn Val Thr Asn
    3545                3550                3555

Val Gly Asp Val Ser Gly Glu Tyr Ser Tyr Ile Ile Lys Leu Gly
    3560                3565                3570

Asn Ser Thr Ile Ala Glu Gly Ser Glu Ile Ile Glu Ser Gly Arg
    3575                3580                3585
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Trp Lys  Val Ile Asn Ser Thr  Val Glu Ile Arg Lys  Glu Gly Asn
    3590                 3595                 3600

Tyr Thr  Val Thr Ala Asn Asn  Leu Ser Lys Thr Val  Thr Ala Tyr
    3605                 3610                 3615

Arg Lys  Val Tyr Gly Asn Leu  Thr Glu Asn Tyr Ile  Lys Glu Lys
    3620                 3625                 3630

Asp Phe  Gly His Tyr Lys Ser  Phe Tyr Trp Asn Glu  Phe Lys Arg
    3635                 3640                 3645

Asp Phe  Glu Gly Trp Val Glu  Glu Ala Leu Ser Thr  Ile Glu Leu
    3650                 3655                 3660

Pro Lys  Val Asn Phe Lys Val  Leu Asn Tyr Phe Pro  Gly Asn Trp
    3665                 3670                 3675

Ser Leu  Leu Asn Tyr Ser Glu  Met Leu Asn Ile Thr  Lys Gly Trp
    3680                 3685                 3690

Gly Trp  Ile Asn Ala Thr Tyr  Ala Arg Arg Val Arg  Val Glu Gly
    3695                 3700                 3705

Leu Glu  Glu Phe Lys Tyr Leu  Ile Val Asn Val Thr  Gln Leu Val
    3710                 3715                 3720

Val Leu  Leu Gly Asn Ala Thr  His Glu Leu Asp Glu  Ser Pro Pro
    3725                 3730                 3735

Thr Leu  Asn Val Thr Pro Ser  Ser Gly Ile Tyr Ser  Glu Ile Pro
    3740                 3745                 3750

Lys Ile  Gln Val Arg Thr Cys  Asp Glu Thr Gly Ile  Thr Leu Val
    3755                 3760                 3765

Trp Gly  Ala Val Gly Asn Tyr  Thr Lys Glu Phe Thr  Glu Val Glu
    3770                 3775                 3780

Ser Asn  Gly Thr Cys Ser Thr  Trp Glu Gly Ile Val  Pro Leu Asn
    3785                 3790                 3795

Ile Gly  Asn Asn Thr Val Ala  Ile Tyr Ala Glu Asp  Glu Phe Gly
    3800                 3805                 3810

Asn Arg  Gly Asn Val Ser Leu  Trp Ile Tyr Leu Asn  Pro Glu Ala
    3815                 3820                 3825

Pro Val  Ile Tyr Ile Glu Ser  Pro Glu Glu Lys Val  Tyr Asn Ser
    3830                 3835                 3840

Arg Glu  Val Met Ile Asn Tyr  Thr Val Val Asn His  Asp Leu Val
    3845                 3850                 3855

Gly Val  Val Ala Tyr Leu Asn  Gly Glu Leu Ile Ser  Ser Asn Ala
    3860                 3865                 3870

Ser Tyr  Ser Gly Phe Ile Lys  Leu Asp Tyr Gly Trp  His Asn Phe
    3875                 3880                 3885

Thr Ile  Tyr Ala Trp Asp Val  Ser Tyr Asn Val Ser  Lys Ser Val
    3890                 3895                 3900

Ile Phe  Arg Val Asn Glu Pro  Pro Ser Val Asp Phe  Ser Trp Glu
    3905                 3910                 3915

Val Asp  Asn Leu Thr Val Lys  Phe Glu Ala Asn Ala  Ser Asp Glu
    3920                 3925                 3930

Asp Gly  Ile Ser Lys Tyr Leu  Trp Asp Phe Gly Asp  Asn Glu Ser
    3935                 3940                 3945

Ser Leu  Leu Val Asn Pro Thr  His Thr Tyr Arg Lys  Gly Gly Arg
    3950                 3955                 3960

Tyr Asn  Val Thr Leu Thr Val  Trp Asp Ser Tyr Asn  Leu Ser Ser
    3965                 3970                 3975

Ser Ile  Ser Lys Glu Val Val  Val Phe Gly Ser Ser  Thr Leu Thr
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    3980                3985                3990

Met Val  Lys Glu Tyr Ser Tyr  Thr Lys Asp Phe Gly  Phe Tyr Asn
    3995                4000                4005

Thr Thr  Ser Trp Lys Asp Phe  Leu Lys Asp Phe Glu  Val Trp Val
    4010                4015                4020

Asn Leu  Thr Leu Arg Asn Val  Thr Leu Pro Leu Glu  Tyr Phe Glu
    4025                4030                4035

Glu Ile  Ile Glu Val Asn Val  Glu Asn Trp Ser Leu  Ile Ser Val
    4040                4045                4050

Glu Lys  Asn Leu Lys Asn Asp  Ile Gly Glu Met Ser  Ala Glu Tyr
    4055                4060                4065

Glu Arg  Asn Ala Thr Ile Val  Gly Ile Met Asn Tyr  Thr Arg Val
    4070                4075                4080

Thr Leu  Lys Leu Thr Gln Glu  Val Ile Leu Ser Gly  Arg Ala Arg
    4085                4090                4095

Lys Val  Glu Asp Lys Ile Pro  Pro Leu Val Glu Ile  Leu Phe Pro
    4100                4105                4110

Arg Asn  Met Thr Tyr Asn Glu  Thr Ile Arg Glu Ile  Lys Val Arg
    4115                4120                4125

Ala Thr  Asp Glu Ser Gly Ile  Ala Asn Val Thr Ala  Thr Ile Asn
    4130                4135                4140

Gly Glu  Ser Leu Ser Leu Glu  Lys Val Asn Glu Thr  Trp Ile Gly
    4145                4150                4155

Arg Val  Glu Leu Asp Asp Gly  Lys Tyr Glu Leu Asn  Val Phe Ala
    4160                4165                4170

Ser Asp  Lys Trp Gly Asn Val  Gly Cys Ser Thr Val  Asn Phe Thr
    4175                4180                4185

Ile Asn  Arg Ser Val Lys Val  Arg Ile Ile Asn Gly  Thr Glu Ile
    4190                4195                4200

Val Thr  Ile Pro Gly Asp Ile  Lys Thr Arg Val Tyr  Phe Glu Gly
    4205                4210                4215

Asp Ile  Ile Val Glu Ile Val  Lys Glu Ser Leu Arg  Phe Lys Ile
    4220                4225                4230

Pro Ser  Gly Gly Thr Leu Val  Ile Asp Glu Arg Gly  Arg Lys Asp
    4235                4240                4245

Pro Trp  Leu Leu Ala Arg Ile  Asn Ser Thr Ile Glu  Asn Ile Ser
    4250                4255                4260

Lys Thr  Ser Arg Ile Phe Glu  Glu Asn Gly Lys Lys  Val His Glu
    4265                4270                4275

Ile Arg  Tyr Arg Ile Ser Ile  Ser Arg Gly Tyr Ala  Ile Leu Val
    4280                4285                4290

Val Pro  Leu Glu Gly Met Lys  Val Ser Ser Ile Arg  Ile Ile Lys
    4295                4300                4305

Asn Gly  Thr Val Thr Arg Asp  Glu Lys His Gly Asn  Tyr Tyr Lys
    4310                4315                4320

Leu Ser  Lys Gly Tyr Leu Phe  Ile Phe Leu Ser Glu  Asp Pro Ile
    4325                4330                4335

Val Glu  Val Thr Leu Ser Lys  Ile Glu Lys Lys Asp  Ile Phe Arg
    4340                4345                4350

Val Leu  Tyr Tyr Ala Gly Ile  Ile Trp Glu Arg Asn  Tyr Leu Arg
    4355                4360                4365

Leu Lys  Glu Glu Phe Ile Met  Lys Met Ser Asn Glu  Thr Ser Gln
    4370                4375                4380
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Glu Ala  Ile Arg Leu His Glu  Glu Ala Glu Lys Tyr  Tyr Leu Lys
    4385                 4390                 4395

Gly Arg  Glu Tyr Tyr Pro Arg  Ile Pro Ser Pro Ser  Ala Ile Tyr
    4400                 4405                 4410

Trp Tyr  Ala Val Tyr Met Arg  Lys Ala Tyr Leu Thr  Glu Arg Lys
    4415                 4420                 4425

Ala Leu  Glu Leu Leu Ser Ile  Ser
    4430                 4435
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The invention claimed is:

1. A polypeptide comprising a region corresponding to the beta strand A region of SEQ ID NO:1, a region corresponding to the AB loop region of SEQ ID NO:1, a region corresponding to the EF loop region of SEQ ID NO:1, and a region corresponding to the beta strand G region of SEQ ID NO:1, wherein the region corresponding to the beta strand A region of SEQ ID NO:1 comprises amino acids 1-12 of SEQ ID NO:1 with P1S, P2S, and P3S substitutions and optionally a K4V substitution, the region corresponding to the AB loop region of SEQ ID NO:1 comprises amino acids 13-16 of SEQ ID NO:1 with a substitution of N14P; the region corresponding to the EF loop region of SEQ ID NO:1 comprises amino acids 54-60 of SEQ ID NO:1 with a substitution of D58P; and wherein the region corresponding to the beta strand G region of SEQ ID NO:1 comprises amino acids 81-84 of SEQ ID NO:1 with an insertion of the amino acids P5S after amino acid 84.

2. The polypeptide of claim **1**, wherein the polypeptide comprises a region corresponding to the beta strand C region of SEQ ID NO:1, which comprises amino acids 28-35 of SEQ ID NO:1, a region corresponding to the CD loop region of SEQ ID NO:1, which comprises amino acids 36-37 of SEQ ID NO:1, a region corresponding to the beta strand D region of SEQ ID NO:1, which comprises amino acids 38-46 of SEQ ID NO:1, and a region corresponding to the FG loop region of SEQ ID NO:1, which comprises amino acids 71-80 of SEQ ID NO:1, wherein the regions corresponding to the beta strand C region, CD loop region, beta strand D region, and/or FG loop region of SEQ ID NO:1 comprises one or more amino acid substitutions or insertions relative to the beta strand C region, CD loop region, beta strand D region, and FG loop regions of SEQ ID NO:1.

3. The polypeptide of claim **1**, wherein the polypeptide comprises a region corresponding to the BC loop region of SEQ ID NO:1, which comprises amino acids 22-27 of SEQ ID NO:1, a region corresponding to the DE loop region of SEQ ID NO:1, which comprises amino acids 47-49 of SEQ ID NO:1, and a region corresponding to the FG loop region of SEQ ID NO:1, which comprises amino acids 71-80 of SEQ ID NO:1; wherein the BC, DE, and/or FE loop regions comprises one or more amino acid substitutions or insertions relative to the BC, DE, and FE loop regions of SEQ ID NO:1.

4. The polypeptide of claim **1**, wherein the polypeptide comprises a substitution at the amino acid position corresponding to amino acid 28 of SEQ ID NO:1.

5. The polypeptide of claim **4**, wherein the substitution corresponding to amino acid position 28 of SEQ ID NO:1 is with a tyrosine.

6. The polypeptide of claim **1**, wherein the polypeptide is recombinant.

7. The polypeptide of claim **1**, wherein the polypeptide is synthetic.

8. A kit comprising the polypeptide of claim **1**.

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