

Supporting Information

All oligonucleotides were purchased from Integrated DNA Technologies unless otherwise stated. All oligos longer than 35 nucleotides were purified by urea PAGE. Creation of the 30 trillion member Fn library was described previously (1). Fnoligo1 and Fnoligo8 were used for amplification during selection.

Fnoligo1: 5'-TTCTAATACGACTCACTATAGGGACAATTACTATTTACAATTA-

CAATGCTCGAGGTCGTCG

Fnoligo8: 5'-GGAGCCGCTACCGGATCCGGTGCGGTAGTTGATGGAGATCGG

Fnoligo1 contains the T7 RNA polymerase promoter sequence, a portion of the TMV translation enhancer sequence, and anneals to the 5' end of the Fn library. Fnoligo8 anneals to the 3' end of the Fn library, contains a BamHI restriction site, and codes for a (Gly-Ser)₃ spacer. After *in vitro* transcription, mRNA pools were ligated to pF30P (Yale Keck Oligonucleotide synthesis facility) using Fn-pF30P-Splint.

pF30P: 5'-phospho-A₂₁-9₃-ACC-Pu

9 = phosphoramidite spacer 9, Pu = Puromycin (Glen Research)

Fn-pF30P-Splint: 5'-TTTTTTTTTTTTGGAGCCGCTACC

The pool 10 PCR product was cloned into pCR4-TOPO TA cloning vector (Invitrogen).

Eleven sequences were obtained:

Pool 10 sequences:

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WT10FnIII  MLEVVAATPTSLISWDAPAVTVRYRITYGETGGNSPVQEFVPGSKSTATISGLKPGVDYTTITVYAVTGRGDSPASSKPISINYRT
FnIkB10C19  .....PASSRWR.....QQLRQPKWRW.....
FnIkB10C08  .....PASSRWR.....L...QQLRQPKWRW.....
FnIkB10C04  .....PTSSRWR.....QQLRQPKWRW.....
FnIkB10C03  .....PASSRWR.....C...QQLHQPCKWRW.....
FnIkB10C05  .....PASSRWR.....G...K.....C...QQLHQPCKWRW.....
FnIkB10C16  .....PASSRWR.....C...QQLHQPCKWRW.....
FnIkB10C18  .....PASSRWR.....R.....C...QQLHQPCKWRW.....
FnIkB10C20  .....PASSRWR.....C...D...R.....C...QQLHQPCKWRW.....
FnIkB10C01  .....Q...PASSRWRH.....C...QQLHQPCKWRW.....
FnIkB10C09  .....V.....PASSRWR.....C...QQLHQPCKWRW.....
FnIkB10C17  .....V...A.....PASSRWR.....T.....S...QQLHQPCKWRW.....

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Fnoligo1 and Fnoligo8 were used to amplify 10C17 and 10C19 for *in vitro* transcription followed by *in vitro* translation to determine binding efficiencies.

All restriction enzymes and T4 DNA ligase were purchased from NEB. 10C17 and 10C19 were amplified with primers “Fnlibrary5Nhe” and “Fnlibrary3His” for cloning into pET11a.

Fnlibrary5NdeI: 5'-CTATTTACAATTCATATGCTCGAGGTCGTCG

Fnlibrary3His: 5'-GGTTGGTGATCAGTGGTGATGGTGGTGATGGGATCCGGTGCGGTAGTTG

Fnlibrary5NdeI contains an NdeI digestion sequence and anneals to the 5' end of the Fn library.

Fnlibrary3His anneals to the 3' end of the Fn library, contains a BamHI restriction site, codes for a (His)₆-tag, and contains a BclI restriction site. The PCR products were digested with NdeI and BclI, purified (QIAGEN, Qiagen), and were ligated into the NdeI and BamHI restriction sites of pET11a, creating vectors pAO5-10C17 and pAO5-10C19. Insertion of genes into the NdeI and BamHI restriction sites of the new vectors will result in addition of a C-terminal (His)₆-tag immediately followed by a stop codon. pAO5-10C17 and pAO5-10C19 were expressed in *E. Coli* BL21(DE3) but did not produce detectable soluble protein. 10C17-(His)₆ was purified under denaturing conditions but attempts to refold the protein were unsuccessful.

10C17 and subsequent variants were amplified under error prone conditions (7 mM MgCl₂, 0.05 mM MnCl₂) using Fnlibrary5NdeI primer and Fnoligo8. The resulting PCR products were digested with NdeI and BamHI and cloned into the GFP-reporter vector generously provided by Dr. Geoffrey S. Waldo (2). To test binding, selected variants were amplified with Fnoligo1 and Fnoligo8 for subsequent *in vitro* transcription and translation. 10C17B25 (see Figure 2) was created by amplifying 10C17B11 with 10C17oligoA10T followed by FnoligoNdeI.

10C17oligoA10T: CATATGCTCGAGGTCGTCGATGCATCTCCAACCAGCCTCCAGATCAGC

10C17B25 was used as the template for the third round of solubility evolution. 10C17C25 was created by amplifying 10C17C21 with 10C17oligoD6E.

10C17oligoD6E: GGAGATATACATATGCTCGAGGTCGTCGAAGCATCACCAACCAGC

The gene inserts from selected clones were digested directly from the miniprep GFP reporter plasmids using NdeI and BamHI, purified by agarose gel electrophoresis (QIAquick, Qiagen) and ligated into the NheI and BamHI sites of pAO5 for expression of C-terminal His-tagged proteins lacking GFP.

In order to increase the yields of soluble 10C17C25, two fusion vectors were created with MBP as an N-terminal (pAO7) or C-terminal fusion (pAO9). Both orientations were tested in order to determine if the enhancement in expression mediated by MBP is dependent on fusion orientation. For pAO7, MBP was amplified from pDW363C (3) in two steps using oligos MBP5N1 and MBP3N1 followed by MBP5N2 and MBP3N2.

MBP5N1: 5' -CATCATCACACGGTACCAGCAAAATCGAAGAAGGTAAACTGGTAATCTGG

MBP3N1: 5' -CTCGAGACGGCCGTCGATGGAGCTCGTCGAGCCATTAGTCTGCGCGTCTTTCAGG

MBP5N2: 5' -GAGTAGGCTTCACATATGCATCACCATCATCACACGGTACCAGC

MBP3N2: 5' -CATTACGTCGACCTAGGATCCACTAGTAAGCTTCTCGAGACGGCCGTCGATGG

The final PCR product was digested with NdeI and Sall. The modified pET28 vector containing the GFP reporter (2) was digested with NdeI and XhoI and agarose gel purified (QIAquick, Qiagen). Ligation of the digested PCR product into the modified pET28 vector results in a vector containing a (His)₆-tag, MBP, a peptide spacing linker, a factor Xa cleavage site, followed by a multiple cloning site (Figure S1).

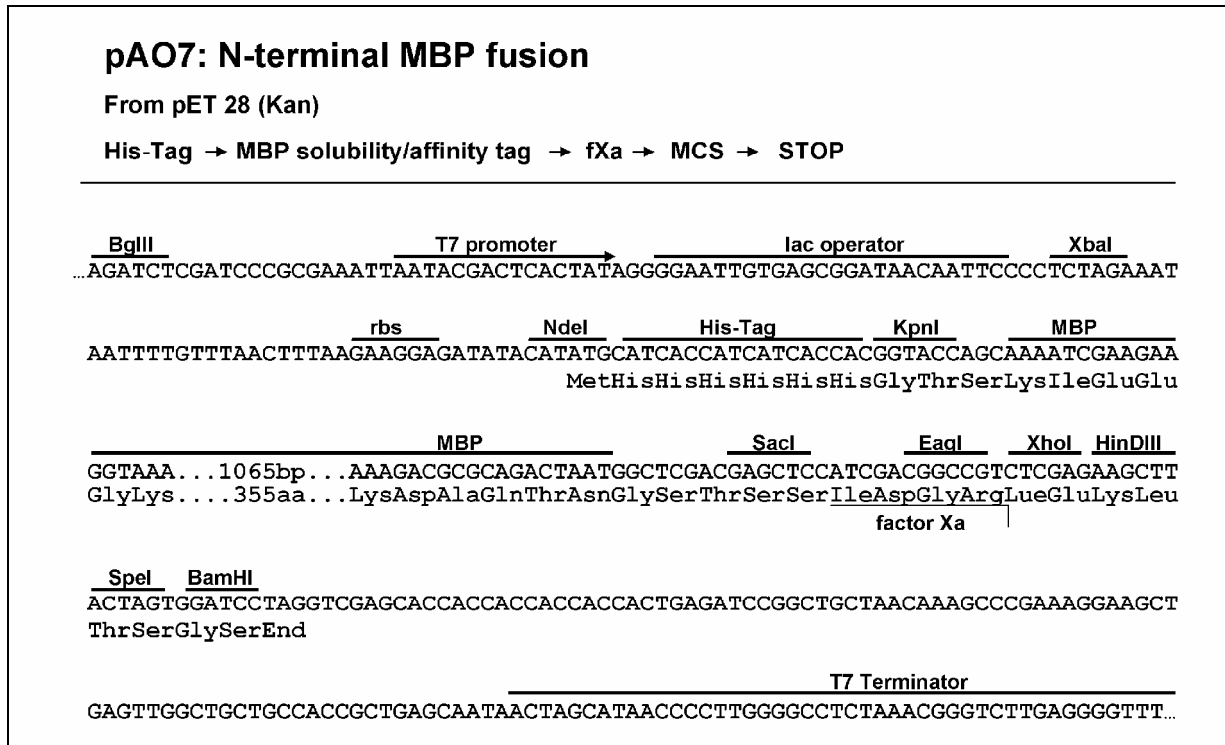


Figure S1. pAO7 vector.

For pAO9, MBP was amplified in two steps with MBP5C1 and MBP3C followed by MBP5C2 and MBP3C.

MBP5C1: ACGACGATAAGGGGAGCTCCAAAATCGAAGAAGGTAAACTGGTAATCTGG

MBP3C: GATTAGCTCGAGGCCATTAGTCTGCGCGTCTTTCAGG

MBP5C2: CTAAGCGGATCCGATTACAAGGATGACGACGATAAGGGGAGCTCC

The final PCR product and the pET28-GFP reporter were digested with BamHI and XhoI for ligation. The vector used for this ligation is identical to the one described by Waldo et al. except that the original multiple cloning site was replaced (See figure S3). The resulting pAO9 vector contains the multiple cloning site followed by a Flag-tag, enterokinase cut site, MBP, and (His)₆-tag figure S2.

pAO9: C-terminal MBP fusion

From pET28 vector (Kan)

MCS → FLAG-Tag/Enterokinase Cleavage site → MBP solubility/affinity tag → His-Tag → STOP

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BglII                T7 promoter → lac operator                XbaI
...AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAAT

                rbs                NdeI NheI SpeI                HinDIII BamHI Flag-Tag
AATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCACTAGTTGAGTAGTAAGCTTGGATCCGATTACAAG
                MetAlaSerThrSer-----LysLeuGlySerAspTyrLys

Flag-Tag SacI MBP                MBP                XhoI
GATGACGACGATAAAGGGGAGCTCCAAAATCGAAGAAGGTAAA...1065bp...AAAGACGCGCAGACTAATGGCCTCGAG
AspAspAspAspLysGlySerSerLysIleGluGluGlyLys... 355aa...LysAspAlaGlnThrAsnGlyLeuGlu
enterokinase |

His-Tag
CACCACCACCACCACCCTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGA
HisHisHisHisHisHisEnd

                T7 Terminator
GCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG...

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Figure S2. pAO9 vector.

pAO5-10C17C25 plasmid was digested with either XhoI and BamHI or NdeI and BamHI and the 10C17C25 fragments were agarose gel purified. These fragments were ligated into purified pAO7 or pAO9 digested with XhoI and BamHI or NdeI and BamHI respectively. We found that the orientation of the MBP fusion does not affect the amount of soluble protein expressed and chose pAO9-10C17C25 for large-scale expression.

For mammalian cell culture expression, 10C17C25-GFP was amplified in two steps (from the GFP reporter vector) with Fn5pIRES and GFP3FLAG1 followed by amplification with Fn5pIRES and GFP3FLAG2.

Fn5pIRES: 5' -CTTCTAGCGCCGCCACCATGCTCGAGGTCGTCG

GFP3FLAG1: 5' -CGTCCTTGAGTACCAGAGCCTTTGTAGAGCTCATCCATGCCATGTG

GFP3FLAG2: 5' -GTGACCTGATCACTTATCGTCATCGTCCTTGTAGTACCAGAGC

Fn5pIRES contains a Not I restriction site, encodes a Kozak sequence, and anneals to the 5' end of the Fn library. GFP3FLAG1 anneals to the 3' end of GFP and encodes a Flag sequence.

ECFP was digested with NdeI and BamHI. EYFP was digested with BamHI and SalI. pET21a was digested with NdeI and XhoI. The three fragments were ligated together to produce pKC1 (figure S3).

The oligos used to create the IKK FRET sensors are:

IKK1F: 5' -GGTTGGAAGCTTGGAGCGTCTGCTCGACGATCGCCATGACAGCGGACTGG

IKK1R: 5' -GGTGGTCGGCCGATTCTCATCCTTCATAGAATCCAGTCCGCTGTCATGG

IKK2F: 5' -GGTGGTCTCGAGCGGAAGCAACGGTGAAGCGAGCGTCTGCTCGACGATCGCCATGACAGC

IKK2R: 5' -GGTGGTGGATCCAGATTCTCATCCTTCATAGAATCCAGTCCGCTGTCATGGCGATCGTCCG

C25XhoI5S: 5' -GGTGGTCTCGAGCATGCTGGAGGTCGTCGAAGCATC

C25XhoI5L: 5' -GGTGGTCTCGAGCGGAAGCAACGGTGAAGCAACGGGTCGTCTATGCTGGAGG-
TCGTCGAAGC

C25HindIII5: 5' -GGTTGGAAGCTTGGGCGGTATGCTCGAGGTCGTCGAAGC

FnHindIII5: 5' - GGTTGGAAGCTTGGGCGGTATGCTCGAGGTCGTCGCTGC

FnEagI3S: 5' - GGTGGTCGGCCGAGGTGCGGTAGTTGATGGAGATCG

FnEagI3L: 5' - GGTGGTCGGCCGACCCGTTGCTTCCACCGTTGCTTCCGCTAGAGGTGCGGTAGTT-
GATGGAGATCG

First, the IKK substrate sequences were assembled by extending either IKK1F and IKK1R or IKK2F and IKK2R with Klenow Fragment (3'→5' exo-) per the manufacturer's instructions (NEB). To create IKK FS C25 1S and 1L, the IKK1 fragment was digested with HindIII and EagI and ligated into the HindIII and EagI restriction sites of pKC1. To create IKK FS C25 2S and 2L, the IKK2 fragment was digested with XhoI and BamHI and ligated into the XhoI and BamHI sites of pKC1. 10C17C25 was amplified with either C25XhoI5S and Fnoligo8 or C25XhoI5L and Fnoligo8. Both PCR products were digested with XhoI and BamHI and ligated into pKC1 containing the IKK1 substrate sequence to create IKK FS C25 1S and 1L (See Figure 4b and Figure S4). 10C17C25 was next amplified with either C25HindIII and FnEagI3S or

C25HindIII and FnEagI3L. A non-selected control, Fn04, was also amplified with FnHindIII5 and FnEagI3S. All three PCR products were digested with HindIII and EagI and ligated into pKC1 containing the IKK2 substrate sequence to create IKK FS C25 2S, 2L and control FS Fn04. All spacer sequences are listed in figure S4.

C25 1S	CFP	<u>SSSL</u>	IKK SUBSTRATE	<u>SAGGSNGSS</u>	Fn10C17C25	GSS	YFP
C25 1L	CFP	<u>SSSL</u>	IKK SUBSTRATE	<u>SAGGSNGSSSGSNGSS</u>	Fn10C17C25	GSS	YFP
C25 2S	CFP	<u>SSSLGG</u>	Fn10C17C25	<u>SAGGSNGSSSGSNGGS</u>	IKK SUBSTRATE	SGSS	YFP
C25 2L	CFP	<u>SSSLGG</u>	Fn10C17C25	<u>SSGSNGGSNGSAGGSNGSSSGSNGGS</u>	IKK SUBSTRATE	SGSS	YFP
Fn04	CFP	<u>SSSLGG</u>	Control Fn04	<u>SAGGSNGSSSGSNGGS</u>	IKK SUBSTRATE	SGSS	YFP

Figure S4. IKK FRET sensors. The underlined spacer sequences are encoded by pKC1.

REFERENCES

- (1) Olson, C. A., and Roberts, R. W. (2007) Design, expression, and stability of a diverse protein library based on the human fibronectin type III domain. *Protein Sci* 16, 476-84.
- (2) Waldo, G. S., Standish, B. M., Berendzen, J., and Terwilliger, T. C. (1999) Rapid protein-folding assay using green fluorescent protein. *Nat. Biotechnol.* 17, 691-5.
- (3) Ja, W. W., Adhikari, A., Austin, R. J., Sprang, S. R., and Roberts, R. W. (2005) A peptide core motif for binding to heterotrimeric G protein alpha subunits. *J Biol Chem* 280, 32057-60.