Supplementary Information for

"Epitope guided engineering of monobody binders for in vivo inhibition of Erk-2 signaling"

Jasdeep K. Mann[1], Jordan F. Wood[2], Anne Fleur Stephan[1], Emmanuel S. Tzanakakis[1,3,4], Denise M. Ferkey[2], Sheldon Park[1]

[1]Department of Chemical and Biological Engineering, [2]Department of Biological Sciences, [3]Department of Biomedical Engineering, and [4]Western New York Stem Cell Culture and Analysis Center, University at Buffalo, State University of New York, NY 14260

Reagents

The reagents used in the study include the following: Anti-cMyc antibody, 9E10 (AbD Serotec). Mouse monoclonal anti-FLAG antibody, M2 (Agilient). Rabbit monoclonal anti-FLAG antibody, anti-mouse IgG (whole molecule)–FITC antibody, anti-mouse IgG (whole molecule)–R-phycoerythrin antibody, anti-rabbit IgG (whole molecule)–FITC antibody, alkaline phosphatase (AP)-conjugated anti-mouse antibody (all from Sigma). Anti Elk1-pS383 antibody, anti ERK-pT183,pY185 antibody, anti-GFP antibody, anti-GAPDH antibody (all from Cell Signaling Technology). Streptavidin-R-phycoerythrin, SA-PE (Biolegend). Penicillin-streptomycin (Pen-Strep), nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Invitrogen). YPD, Dulbecco's Modified Eagle's Medium (DMEM) without glutamine and non-essential amino acids, fetal bovine serum (FBS) (Gibco).

Primers

Erk-2(NHN) was constructed using two mutagenic primers
5'-AACCTCCTGCTGAACACCACTAAAGATCTCAAGATCTGTGACTTT-3'
5'-ACAGCACCTCAGCAATGATAACATCTGCCATTTTCTTTATCAGATCCTGA-3',

Yeast media

The yeast growth medium (SD/-Trp) contains synthetic drop-out media supplement without tryptophan (1.92 g/L, Sigma), yeast nitrogen base (6.7 g/L, MP Biomedicals), dextrose (20 g/L, Fisher Scientific), citrate buffer (pH 4.5), and is supplemented with 50 µg/mL kanamycin and 100 U/mL pen-strep to retard the growth of bacteria. The yeast induction medium (SG/-Trp) contains drop-out media supplement without tryptophan (1.92 g/L), yeast nitrogen base (6.7 g/L), galactose (20 g/L, Sigma-Aldrich), and sodium phosphate (pH 6.0).

Affinity measurement

The affinity of the selected Fn3 clones was measured using a published protocol (1). The Fn3 displaying cells were incubated with different concentrations of Erk-2. The cells were concurrently labeled with anti-cMyc antibody to identify displaying cells. The Erk-2 binding was analyzed by fitting the mean fluorescence intensity (MFI) of the displaying population to a binding curve of the form, $MFI = MFI_{\min} + MFI_{range}\left(\frac{C}{C+K_d}\right)$, where C is the Erk-2

concentration, MFI_{min} is the intensity of the nondisplaying population, and MFI_{range} is the difference in MFI between the lowest and highest Erk-2 concentrations, by minimizing the sum of squared errors. The Erk-2 concentrations used in the study span 4 orders of magnitude straddling the final K_d value. The cell number and volume were adjusted such that Erk-2 is always in excess of Fn3 molecules on the cell surface.

Protein purification

Rat Erk-2 containing an N-terminal 6xHis tag and a C-terminal FLAG tag was purified from BL21(DE3) pLysS by inducing at OD_{600} = 0.8, 37 °C for 4 hr with 0.4 mM IPTG. The cells were lysed by sonication using Branson digital sonifier in a buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 1% triton X-100, 0.1% 2-mercaptoethanol, 2 mM PMSF, and 10% w/v glycerol. The lysate was incubated with pre-equilibrated Talon resin (Clontech) at 4 °C for 1 hr. The resin was washed in 50 mM sodium phosphate (pH 7.2), 300 mM NaCl, and 20 mM imidazole (Sigma), and the bound protein was eluted in 3 x 500 μl of 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, and 300 mM imidazole. The eluates were analyzed on a 14% SDS-PAGE gel and concentrated using 10 kDa Amicon centrifugal filters (Millipore). The mutant Erk-2(NHN) was constructed by PCR using two mutagenic primers and was verified by DNA sequencing. Biotinylation of Erk-2 was performed using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher Scientific).

The Fn3 genes were cloned into 6xHis tagged-pRSET-A vector digested with *NheI* and *XhoI* and transformed into BL21 (DE3) pLysS cells. Monobody synthesis was induced at OD_{600} = 0.8 with 0.4 mM IPTG for 4 hr at 37 °C. The cells were lysed using B-PER (Thermo scientific), and the monobody was purified using Talon resin and finally eluted in 300 mM imidazole in PBS. The protein was further purified by gel filtration chromatography on Superdex 75 10/300 GL (GE Healthcare) and buffer exchanged to PBS.

GST-p38α was transformed in BL21 (DE3) pLysS and induced with 0.4 mM IPTG at OD_{600} = 0.6 for 4 hr at 37 °C. The cells were lysed in B-PER and 2 mM PMSF. The soluble fraction was then bound to reduced glutathione resin for 1 hr at 4 °C. The resin was washed four times with PBS and GST-p38α was eluted in 50 mM Tris, pH 8.0, containing 10 mM reduced glutathione (Sigma).

JNK1α1 was amplified from pCDNA3 and cloned into pRSET-A and transformed into BL21 (DE3) pLysS. Protein synthesis was induced at OD_{600} = 0.8 with 0.4 mM IPTG for 4 hr at 37 °C. The cells were lysed with B-PER and JNK1 was purified using Talon metal affinity resin using PBS containing 300 mM imidazole.

Cell culture

Human embryonic kidney (HEK) 293 cells were grown on tissue-culture treated flasks in DMEM with 10% FBS (Gibco). The cells were plated at a density of 6 x 10^5 cells per 4 cm^2. At 50 – 70% confluence, the cells were transfected with 1.6 μg of DNA and 4 μl of Lipofectamine 2000 (Invitrogen) in 200 μL of Optimem Reduced Serum for 4 hr. 800 μl of DMEM/10% FBS was added to the cells overnight and replaced with 1 ml of fresh media the following day.

Western blot

The HEK293 lysis buffer contained 50 mM Tris (pH 7.5), 150 mM NaCl, 1% v/v triton X-100, 10% v/v glycerol, 0.1% w/v SDS, 2 mM PMSF, 1x Halt protease inhibitor cocktail, and 1x Halt phosphatase inhibitor cocktail (Thermo Scientific). The Western blots were developed in the TBS Tween buffer containing tris buffered saline (pH 7.6) and 0.1% Tween-20. Prior to the binding of the AP-conjugated secondary antibody, the nitrocellulose membrane was blocked with 5% nonfat dry milk in TBS-Tween at room temperature for 1 hr. The following antibody dilutions were used in the study: anti Elk1-pS383 (1:1,000), anti ERK-pT183,pY185 (1:3,000), anti-GFP (1:5,000), anti-cMyc (1:2,000), anti-FLAG (1:2,000), anti-GAPDH (1:5,000). The bands were visualized using BCIP/NBT as substrates and quantified using ImageJ. All Western blots were repeated three times with independently prepared samples and representative blots are shown.

β galactosidase assay

To perform the β galactosidase assay, the yeast cells were resuspended in 500 µl of Z buffer containing 60 mM Na_2HPO_4, 40 mMNaH_2PO_4, pH. 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM mercaptoethanol, and 1% SDS. The enzyme activity was measured by adding 2-nitrophenyl β-D-galactopyranoside and incubating at 30 °C for 10 min. The reaction was stopped with 500 µl 1 M Na_2CO_3 and A_{420} was measured.

Plasmids used in *C. elegans* studies

lin-31 promoter (pFG126): ~4.3 kb of upstream genomic sequence was cloned from purified worm genomic DNA using primers JFW77 and JFW78. These primers incorporated 5' Sbf1 and 3' BamH1 restriction sites, respectively. The promoter was inserted into these sites of pPD49.26 (Fire Lab *C. elegans* Vector Kit, Addgene).

lin-31p::GFP (pFG127): GFP was cloned from pPD97.55 using primers JFW70 and DMF187 (Fire Lab *C. elegans* Vector Kit, Addgene). These primers incorporated 5' Nhe1 and 3' Sac1 sites, respectively. GFP was inserted into these sites in pFG126, downstream of the *lin-31* promoter.

lin-31p::Fn3 – (pFG128 – F0.1, pFG129 – F6.2, pFG130 – F7.9): F6.2 and F7.9 were cloned using primers JFW86 and JFW87. F0.1 was cloned using primers JFW90 and JFW91. These primers incorporated 5' Kpn1 and 3' Sac1 restriction sites respectively. Each Fn3 clone was isolated by KpnI/SacI digest of pFG and placed between the corresponding sites in pPD49.26, downstream of the *lin-31* promoter.

All constructs were sequenced following cloning.

Figure S1. A schematic view of mammalian MAPK pathways

Mitogen activated protein kinases (MAPKs) form evolutionarily conserved pathways in eukaryotes, and regulate changes in protein function and gene expression in response to stimuli at the cell surface. There are multiple MAPK pathways in humans, which collectively control cell proliferation, death, and other adaptive behavior of the cell. Correct activation of different MAPK pathways is crucial to generate appropriate biological responses, whereas overactive signaling results in a number of human diseases, including cancer and neurodegenerative diseases. MAPKs use modular binding interactions outside the catalytic site to achieve specificity of interaction. In particular, all MAPKs contain a common docking domain that is used to selectively bind a linear peptide on their substrates and regulators.

In humans, at least four MAPKs—Erk-1/2, p38, JNK, Erk-5—are involved in the regulation of embryogenesis and cellular response to growth factors, inflammatory cytokines, and stress signals (*2*). Three mammalian MAPK pathways are shown to illustrate the organization of the signaling enzymes. While MAPKs are functionally diverse, all MAPK pathways are organized in a three-tiered signal relay, in which a MAPK is phosphorylated by a dual specificity MAPK kinase (MEK) for activation, which is in turn phosphorylated by a MEK kinase (MEKK) (*3*). The hierarchical activation of the kinases is believed to increase the sensitivity of the network, lower the activation threshold, and amplify the strength of response (*4*). Despite sequence and structural similarities among the enzymes in each tier, there is high specificity of interaction. The specificity of signaling is important to generate appropriate biological responses and is mediated through specific protein-protein interaction.

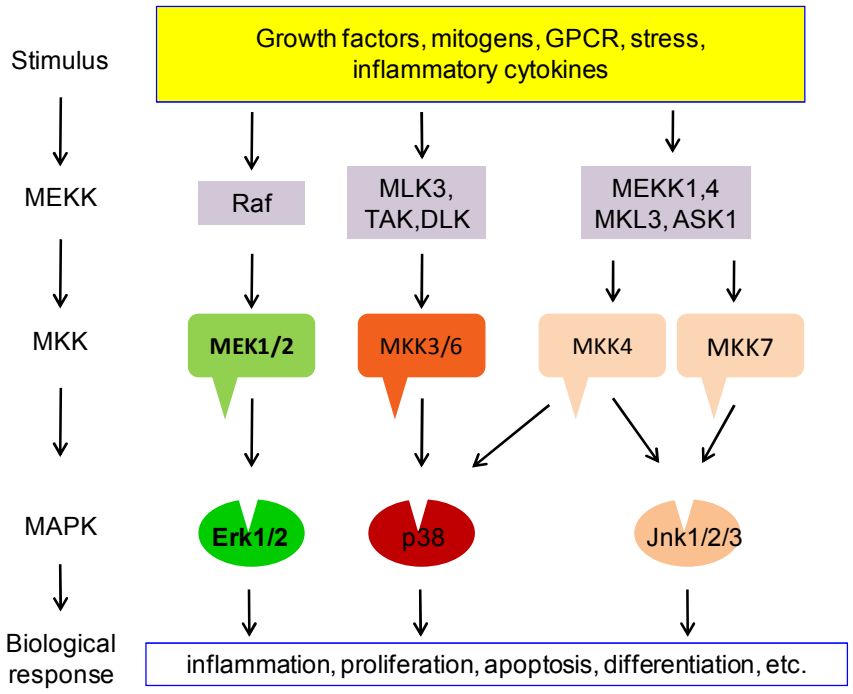


Figure S2. Principles of yeast display

Proteins are targeted for expression on the cell surface as fusions with Aga2. The displayed protein, e.g. Fn3, is bracketed by linear epitopes, HA and cMyc, that can be used to monitor the expression level. The binding activity of Fn3 is measured by incubating the cells with fluorescently labeled ligand.

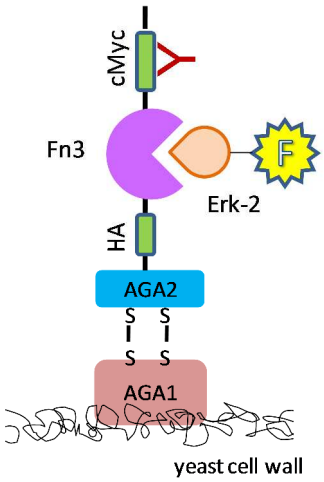


Figure S3. Sorting of the library

a. SDS-PAGE of Erk-2 purified from bacteria. Erk-2 was subsequently biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher Scientific). Although wild type Erk-2 has 22 lysines that can potentially be biotinylated, none of the lysines are located within the D-peptide docking site. Biotinylated-Erk-2 was used during the early rounds of sorting, when the average binding affinity is low, because the molecule can be efficiently and quantitatively labeled with SA-PE.

b. Sorting strategy. The Fn3 library displayed on the yeast surface has an initial diversity of 1.5 x 10^8. The library was sorted using a combination of MACS and FACS. We prepared 2 x 10^9 yeast cells displaying the library. The cells were washed and resuspended in 20 ml of PBSB (PBS pH 7.4, plus 0.5% bovine serum albumin). We added 1 μM of biotinylated Erk-2 (b-Erk-2) to the cells and incubated the cells at room temperature (~ 20 °C) for 1 hr. The unbound b-Erk-2 was removed by centrifugation and 100 μl of magnetic streptavidin beads (Miltenyi Biotec) were added to the cells to magnetically coat the Erk-2 binding cells. The mixture of cells and beads was then poured over a magnetic column (Miltenyi Biotec) to selectively retain b-Erk-2 binding cells, while allowing nonbinding cells to flow through. The unbound fraction was discarded and the cells that remained bound to the column were recovered by washing the column with 3 ml of yeast medium. Approximately 3 x 10^7 cells were recovered from the column. The collected cells were re-grown and induced to express Fn3. The

efficiency of MACS enrichment was tested by labeling the collected cells with 500 nM b-Erk-2 and SA-PE. A comparison with unsorted library shows that the Erk-2 binding population has increased from 0.21% to 2.4% (**Table S1**).

The cells were then labeled with 500 nM b-Erk-2 and SA-PE as well as 9E10 antibody to sort the cells by FACS (Round 1). The simultaneous labeling with anti-cMyc antibody allows the binding to be normalized with protein expression to allow quantitative sorting. The sorting was repeated with 250 nM, 50 nM, and 10 nM of b-Erk-2 to increase the stringency of selection and to identify the highest affinity clones (Round 2 – 4). Positive selection based on SA-PE labeling is indicated as green in the schematics. For Round 5 – 7, the cells were incubated with unmodified Erk-2 (i.e. unbiotinylated Erk-2) and labeled with anti-FLAG antibody M2. Positive selection for Erk-2 binding clones (Round 5 and 7) are colored blue, while negative selection for the clones that do not bind Erk-2(NHN) is colored red (Round 6).

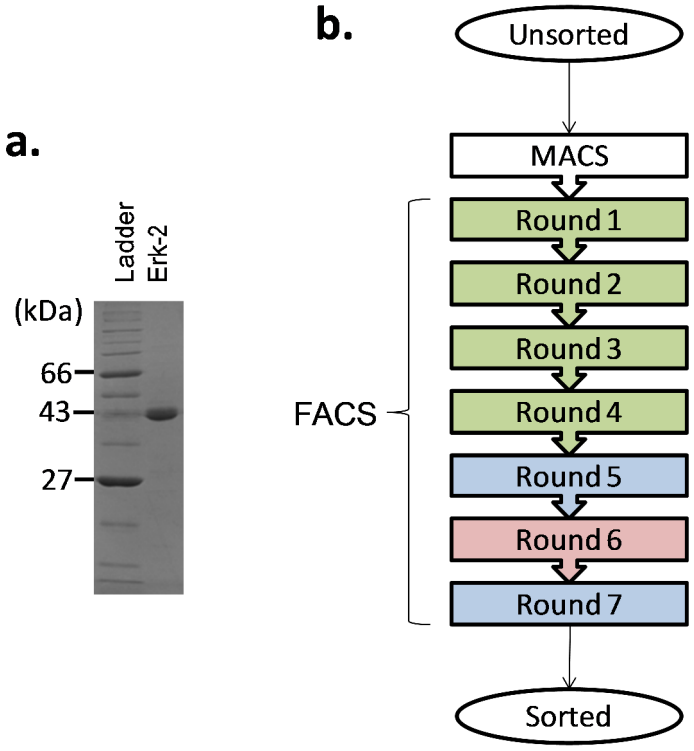


Table S1. The reagents used during each round of selection and the fraction of gated cells.

Round	Ligand	Labeling reagent	Gated fraction
MACS	b-Erk-2 1uM	SA-PE	0.21%
Round 1	b-Erk-2 500 nM	SA-PE	2.40%
Round 2	b-Erk-2 250 nM	SA-PE	7%
Round 3	b-Erk-2 50nM	SA-PE	13%
Round 4	b-Erk-2 10 nM	SA-PE	11.8%
Round 5	Erk-2 10 nM	anti-FLAG antibody	30%
Round 6	Erk-2 (NHN) 100 nM	anti-FLAG antibody	37.8%
Round 7	Erk-2 10 nM	anti-FLAG antibody	8.4%

Figure S4. Negative selection using rationally designed mutant

a. Three residues at the docking site that were rationally mutated in Erk-2(NHN). These mutations were designed based on the structure of Erk-2 bound to a D-peptide (2GPH). A monobody that binds at the CD domain of wt Erk-2 should not bind the mutant. By selecting the cells that do not bind Erk-2(NHN), therefore, we are enriching the Fn3 clones whose epitopes overlap with the CD domain and are likely to inhibit the binding of a D-peptide.

b. Wt and mutant Erk-2 (5 μM in 50 mM sodium phosphate, pH 7.4) were analyzed by circular dichroism spectroscopy at 25 °C using JASCO J-715 spectropolarimeter. Erk-2(NHN) has the same secondary structure as wt, suggesting that the mutations do not disrupt protein folding.

c. GST-pepHePTP binds and precipitates Erk-2 during GST pull-down but not Erk-2(NHN). The Erk-2 band was visualized by Western using anti-FLAG antibody. GST-pepHePTP also contains a FLAG tag at the C-terminus and appears as a dark band at the bottom of the gel. The weak intensity of the Erk-2 band on an SDS-PAGE gel is consistent with low affinity D-peptide-Erk-2 interaction (K_d ~ 5 μM) (*5*).

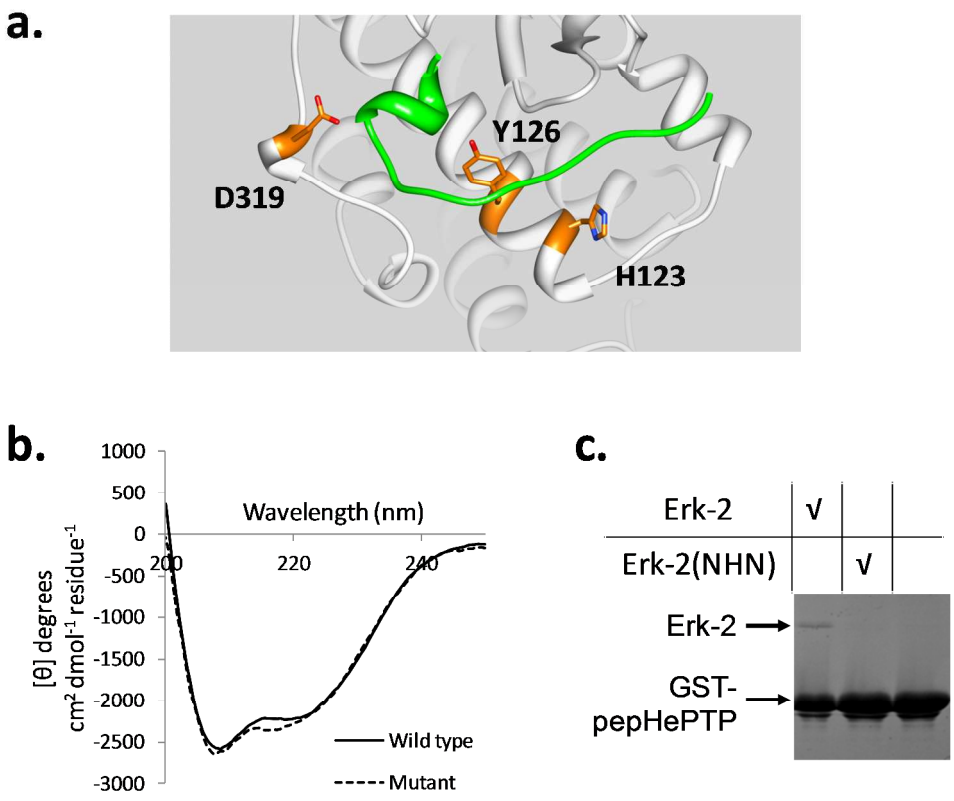


Figure S5. Specificity of interaction

Twenty clones were randomly selected from Round 6 and 7 and tested for binding to 10 nM Erk-2 and 10 nM Erk-2(NHN). Sixteen clones, including F6.2 and F7.1, selectively bound Erk-2 (blue) but not Erk-2(NHN) (red), suggesting that their epitopes overlap with the CD domain. However, three clones, including F7.4, bound both wt Erk-2 and Erk-2(NHN), and one clone did not bind either protein. The inclusion of the mutants with incorrect binding characteristics suggests that additional rounds of positive and negative sorting and/or the use of a tighter gate during each round of sorting may be needed to minimize false positives.

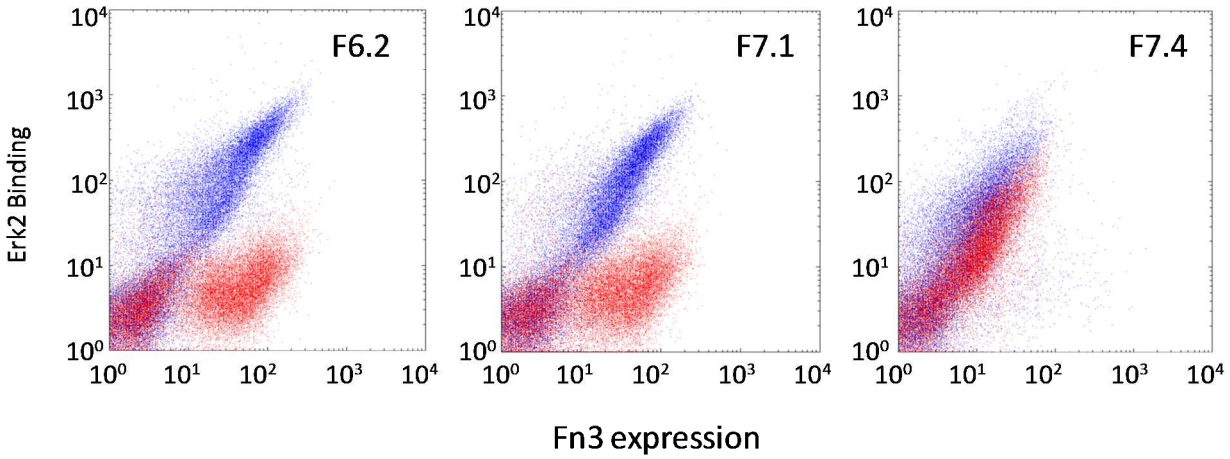


Figure S6. Biophysical characterization of isolated monobodies

a. The monobodies were purified from BL21 (DE3) pLysS using Talon resin and analyzed on a reducing SDS-PAGE gel. The dominant band, indicated by an arrow, likely corresponds to a truncated version of full length F6.2 (*), from which the flexible N- and C-termini have been proteolyzed. The gel corresponds to an eluate following 6xHis affinity chromatography and shows high molecular weight bands that correspond to either aggregations or nonspecific binding to the resin. All proteins were further purified by gel filtration chromatography before analysis.

b. Stability measurement of F6.2 by circular dichroism spectroscopy. The folded fraction was fitted to obtain the denaturation temperature T_m = 69 °C.

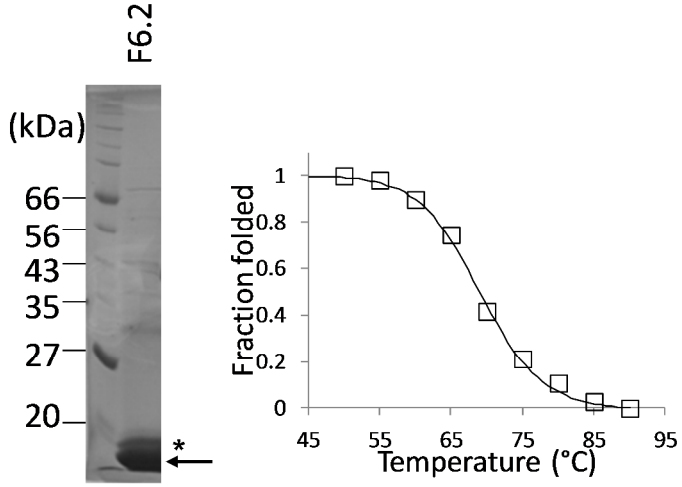


Figure S7. Cellular expression of EGFP-Elk1(307-428)

a. A dual expression vector used to heterologously express Fn3 and Elk1 in HEK293. The drawing is not to the scale. The vector pCMVFn3-IEGFP-Elk1(307-428) was constructed as follows: First, the Fn3 gene was extracted from the selected clones by digesting with NheI and XhoI and ligated into pIRES2-EGFP cut with the same enzymes. Next, the gene corresponding to human Elk1 (307-428) was PCR amplified from pCMV5FELK (A. Sharrocks) and inserted after EGFP using BsrGI and NotI. The inserted portions of the vector were sequenced to confirm that there are no errors in the sequence.

b. Expression of EGFP and EGFP-Elk1(307-428) in transfected HEK293 cells. The cells were transfected with pCMVFn3-IEGFP or with pCMVFn3-IEGFP-Elk1(307-428). The successfully transfected cells can be identified by the presence of EGFP under a fluorescence microscope. The integrity of the EGFP-Elk1 fusion was checked by Western blot with anti-GFP antibody. The fusion (MW = 40 kDa) can be clearly distinguished from EGFP (MW = 27 kDa).

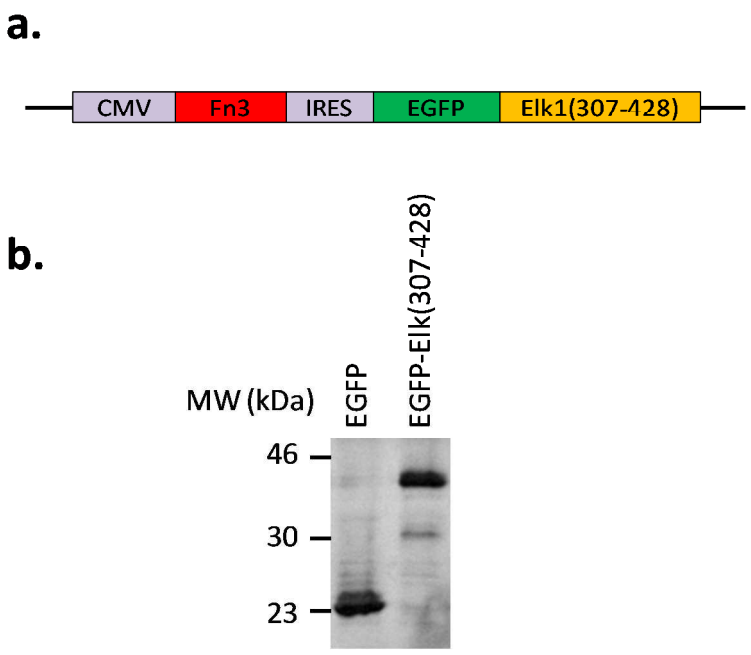


Figure S8. Inhibition of the p38 pathway in HEK293

HEK293 cells expressing different Fn3 clones were stimulated with 10 µg/ml anisomycin for 0 – 60 min. There is sustained p38 phosphorylation over the time period of 15 – 60 min, indicating that the p38 pathway is stimulated by the treatment. The p38-dependent phosphorylation of its substrate ATF-2 was independent of the Fn3 clone expressed in the cells, including F0.1, F6.2, F7.1 and F7.9. The expression level of Fn3 was comparable in all cases.

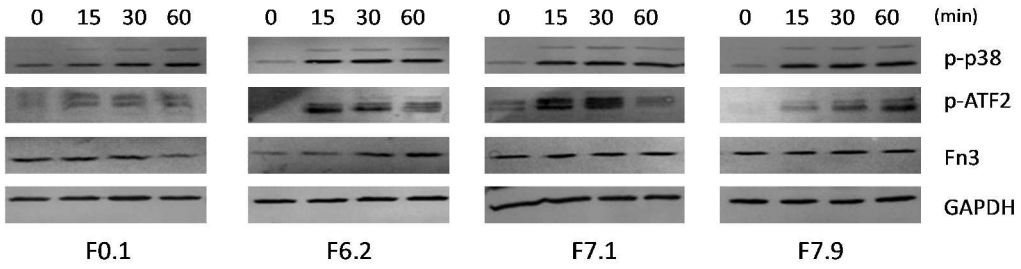


Figure S9. A comparison of mammalian Erk-2 and yeast Fus3, Kss1, and Hog1

a. The CD domain of Erk-2 corresponds to approximately 5% of its entire solvent accessible surface area (SA) (800 $Å^2$ of 17,104 $Å^2$). The change in solvent accessibility varies among the 28 residues within 5 Å of bound D-peptide (green) and may be used to identify the residues that are most important for docking.

b. Mammalian Erk-2 and yeast Fus3 sequences were aligned using ClustalW to obtain a sequence conservation score of 0 to 3 for each residue. The surface of Erk-2 was colored based on the degree of sequence conservation. The CD domain residues are highly conserved in Fus3, which explains how the engineered monobodies may be able to inhibit the Fus3 pathway in yeast.

c. Although the overall sequence identity is the same for the Erk-2-Kss1 pair as for Erk-2-Fus3, the distribution of conserved residues in Kss1 is different. For example, two areas within and near the CD domain (arrows) have greater sequence divergence in Kss1. T116 is conserved in Fus3 but corresponds to N in Kss1. Kss1 also contains an insertion of five residues between Q117 and H118 of Erk2. These differences may have contributed to the difference in the measured difference in the monobody activity. Depending on how each monobody binds, some sequence differences may be more important than others regarding how they affect monobody binding.

d. Hog1 is an ortholog of p38 and has lower sequence similarity with Erk-2 than Fus3 or Kss1. Importantly, the CD residues are poorly conserved in Hog1, including some residues with large buried SA (oval), e.g. Q117 (41 $Å^2$) and H123 (40 $Å^2$), which may have contributed to the observed specificity of engineered Fn3 among yeast MAPKs.

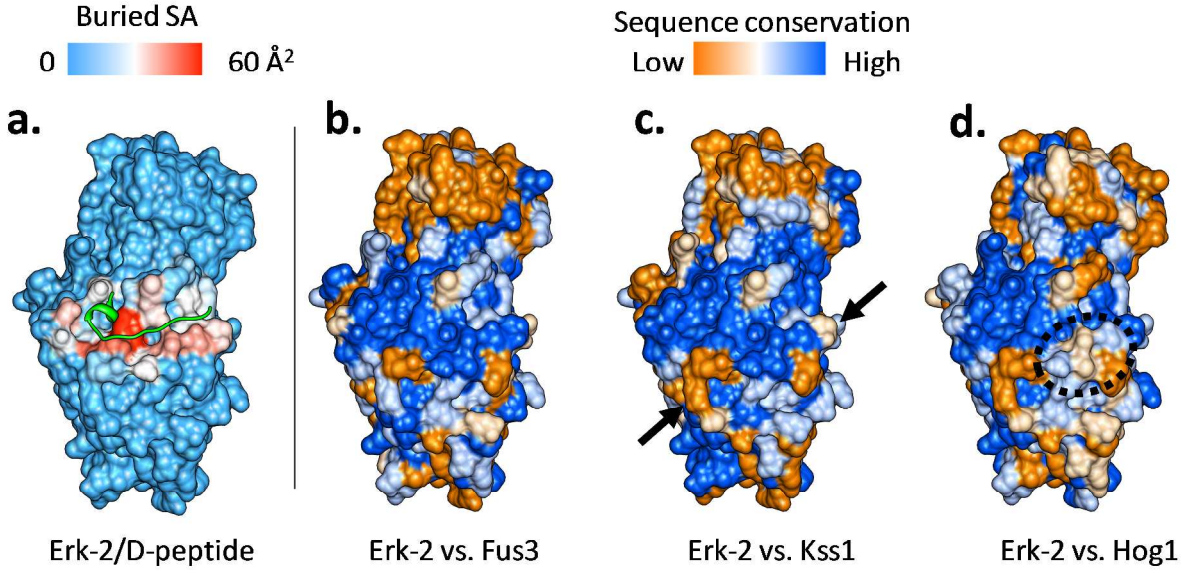
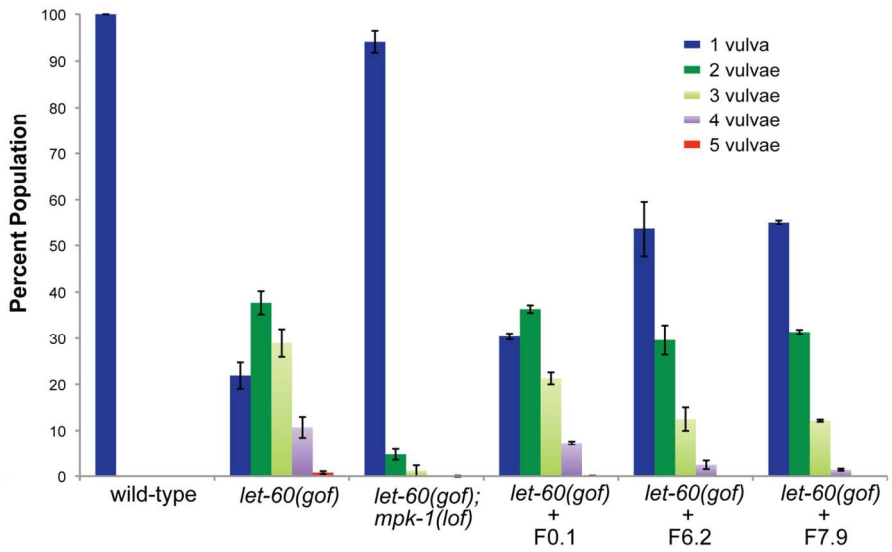


Figure S10. Functional studies of Fn3 in transgenic *C. elegans*

Wild type (N2) animals consistently display a single, properly positioned and functional vulva, while Ce-Ras *let-60(n1046)* gain of function worms are multivulval (Muv) and display a range of additional (from 1 to 4) ectopic pseudovulvae. The majority of *let-60(gof)* animals display at least one ectopic pseudovulva, while many have two or three in addition to the vulva. *let-60(gof);mpk-1(lof)* are predominantly non-Muv when compared to *let-60(gof)* worms, indicating the *let-60(gof)* Muv phenotype is mediated primarily through downstream Ce-MPK-1 activity. To test if Fn3 would inhibit MAPK activity in *C. elegans* vulval precursor cells (VPCs), Fn3 clones were expressed under the VPC-specific *lin-31* promoter. For vulval quantification, adult transgenic *let-60(gof)* worms were selected by *myo-2p::GFP* expression (Fn3 co-injection marker) and thus selected blindly with regards to the animals' respective Muv phenotypes. Monobody expression in *let-60(gof)* worms reproducibly reduced the prevalence of ectopic pseudovulvae when compared with those expressing F0.1 and *let-60(gof)*. Therefore, F6.2 and F7.9 function within *let-60(gof)* VPCs to inhibit Ce-MPK-1 activity and prevent ectopic vulval development. The mean percentages of assayed populations displaying a given number of vulvae are presented together with the standard error of the mean. The combined data of 2 independent transgenic lines were used to compile the statistics. Alleles used: *let-60(n1064)* gain of function and *mpk-1(n2521)* loss of function.



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