Supporting Information

Designed Ankyrin Repeat Proteins (DARPins) as Novel Isoform-Specific Intracellular Inhibitors of c-Jun N-Terminal Kinases

Petra Parizek[¶], Lutz Kummer[¶], Peter Rube[‡], Anke Prinz[‡], Friedrich W. Herberg[‡], and Andreas Plückthun[*,¶]

[¶]Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

[‡]Department of Biochemistry, University of Kassel, Heinrich-Plett-Strasse 40, 34132 Kassel, Germany

Supporting Information Figures

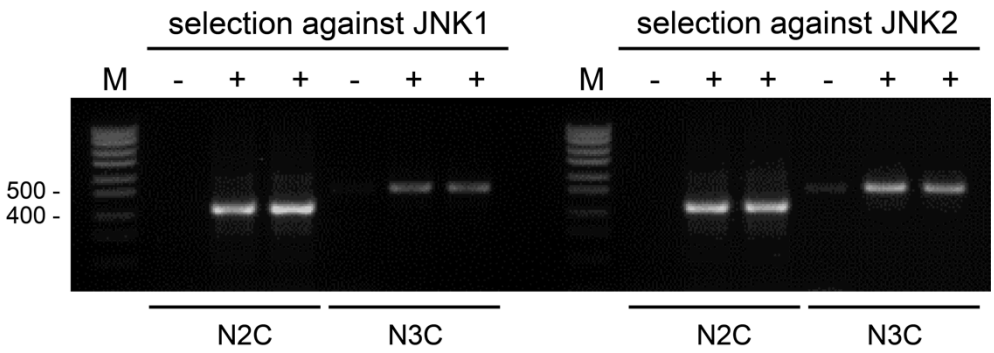

Figure S1. Enrichment and specificity after 3 ribosome display selection rounds.
The outcome of ribosome display selection was monitored as the product yield at the level of RT-PCR by agarose gel electrophoresis. The RT-PCR yields after the 3rd selection round are shown for the N2C and N3C DARPin library selected against pD_JNK1α1 and pD_JNK2α1 (+) or against neutravidin + BSA (-) as control.

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                 N-Cap                                                  1. repeat
                 1       5       10      15      20      25      30 1       5       10      15      20      25      30
                 . . | . . . | . . . . | . . . . | . . . . | . . . . | . . . . | | . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . .
Designed  N3C    G S D L G K K L L E A A R A G Q D D E V R I L M A N G A D V N A x D x x G x T P L H L A A x x G H L E I V E V L L K z G A D V N A

J1_2_32   N3C    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . S . Q L . I . . . . . V . I T . . . . . N . . . . . .
J1_4_7    N3C    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . I . A M . L . . . . . M R . . . . . Y . . . . . .
J1_8_27   N3C    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V . D Q . L . . . . . L D . . . . . Y . . . . . .
J2_3_29   N2C    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N . K N . V . . . . . W I . . . . . H . . . . . .
J1/2_2_25 N2C    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Y . D N . V . . . . . F L . . . . . Y . . . . . .
J1/2_2_3  N2C    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Y . D Y . V . . . . . T . F L . . . . . Y . . . . . .
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                 2. repeat                                              3. repeat
                 1       5       10      15      20      25      30 1       5       10      15      20      25      30
                 | . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . . | . . . | . . . . | . . . . | . . . . | . . . . | . . . . | . . .
Designed  N3C    x D x x G x T P L H L A A x x G H L E I V E V L L K z G A D V N A x D x x G x T P L H L A A x x G H L E I V E V L L K z G A D V N A

J1_2_32   N3C    H . F V . T . . . . . . . F L . . . . . . . . . . . . Y . . . . . V . R D . L . . . . . I H . . . . . H . . . . . .
J1_4_7    N3C    E . Y Y . T . . . R . . . Y I . . . . . . . . . . . . Y . . . . . Y . I S . T . . . . . V L . . . . . Y . . . . . .
J1_8_27   N3C    H V D A . T . . . . . . . Y L . . . . . . . . . . . . N . . . . . A . NW . S . . . . . T F . . . . . N . . . . . .
J2_3_29   N2C    L . A W . S . . . . . . . M T . . . . . . . . . . . . Y . . . . . .
J1/2_2_25 N2C    A . S W . T . . . . . . . T W . . . . . . . . . . . . H . . . . . .
J1/2_2_3  N2C    A . T W . T . . . . . . V T F . . . . . . . . . . . . Y . . . . . .
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                 C-Cap
                 1       5       10      15      20      25
                 | . . . | . . . . | . . . . | . . . . | . . . . | . . .
Designed  N3C    Q D K F G K T A F D I S I D N G N E D L A E I L Q K L N

J1_2_32   N3C    . . . . . . . . . . . . . . . . . . . . . . . . . . . .
J1_4_7    N3C    . . . . . . . . . . . . . . . . . . . . . . . . . . . .
J1_8_27   N3C    . . . . . . . . . . . . . . . . . . . . . . . . . . . .
J2_3_29   N2C    . . . . . . . . . . . . . . . . . . . . . . . . . . . .
J1/2_2_25 N2C    . . . . . . . . . . . . . . . . . . . . . . . . . . . .
J1/2_2_3  N2C    . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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Figure S2. Sequences of DARPins selected against JNK1 and JNK2.
The amino acid sequences of the selected DARPins are shown in the alignment. The designed sequences for the N-Cap, the internal consensus repeat modules and the C-Cap are given above the selected sequences (x represents randomized potential interaction residues, where any amino acid was allowed except C, G, or P; z represents a randomized framework residue, where the three amino acids N, H or Y were allowed (*1*)). The names of the DARPins and their length are given on the left side of the respective sequence. In the alignment only differences to the designed sequence are highlighted and the tags are not included. DARPin J1/2_2_3 has already been published (original name JNK2_2_3; (*2*)) but is included in this alignment for direct comparison.

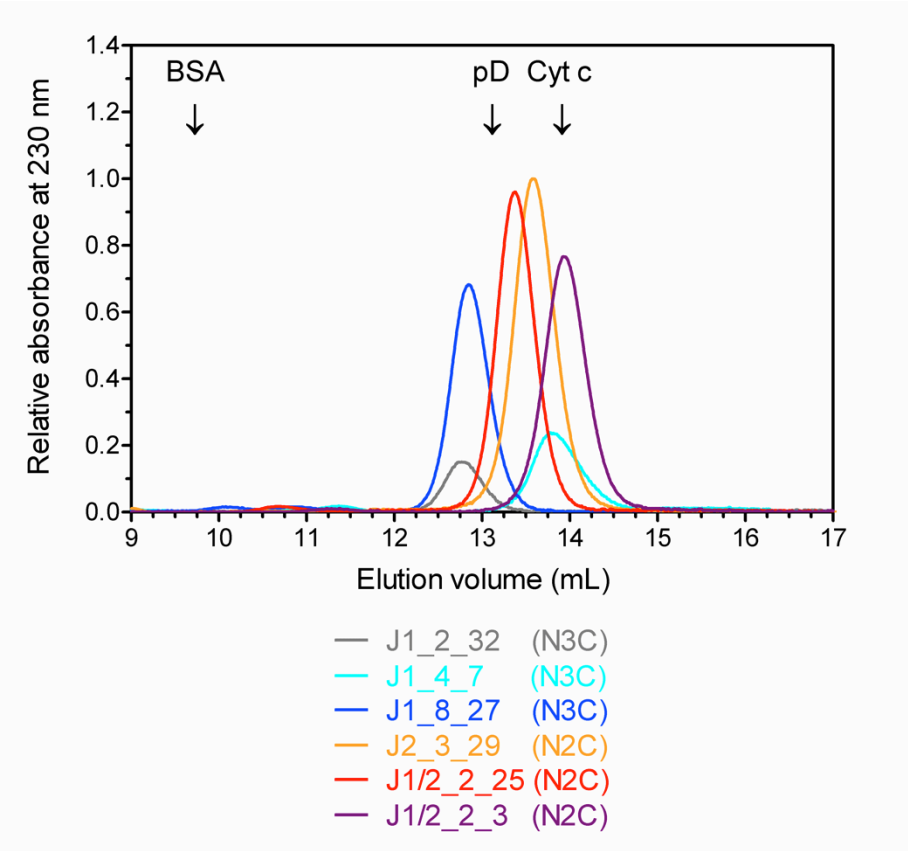

Figure S3. Size exclusion chromatography (SEC) of selected DARPins.
Elution profiles of selected DARPins were analyzed. Each DARPin (15 μM) was loaded on a Superdex 75 column and the absorbance at 230 nm was monitored. For each DARPin, the number of repeats is given in parentheses. Since the number of aromatic amino acids is different in these DARPins (Supplementary Figure S2), this leads to different signal intensities at 230 nm. The molecular mass standards, bovine serum albumin (BSA, 66 kDa), phage λ protein D (pD, 17.9 kDa) and cytochrome c (Cyt c, 12.4 kDa) are indicated by arrows.

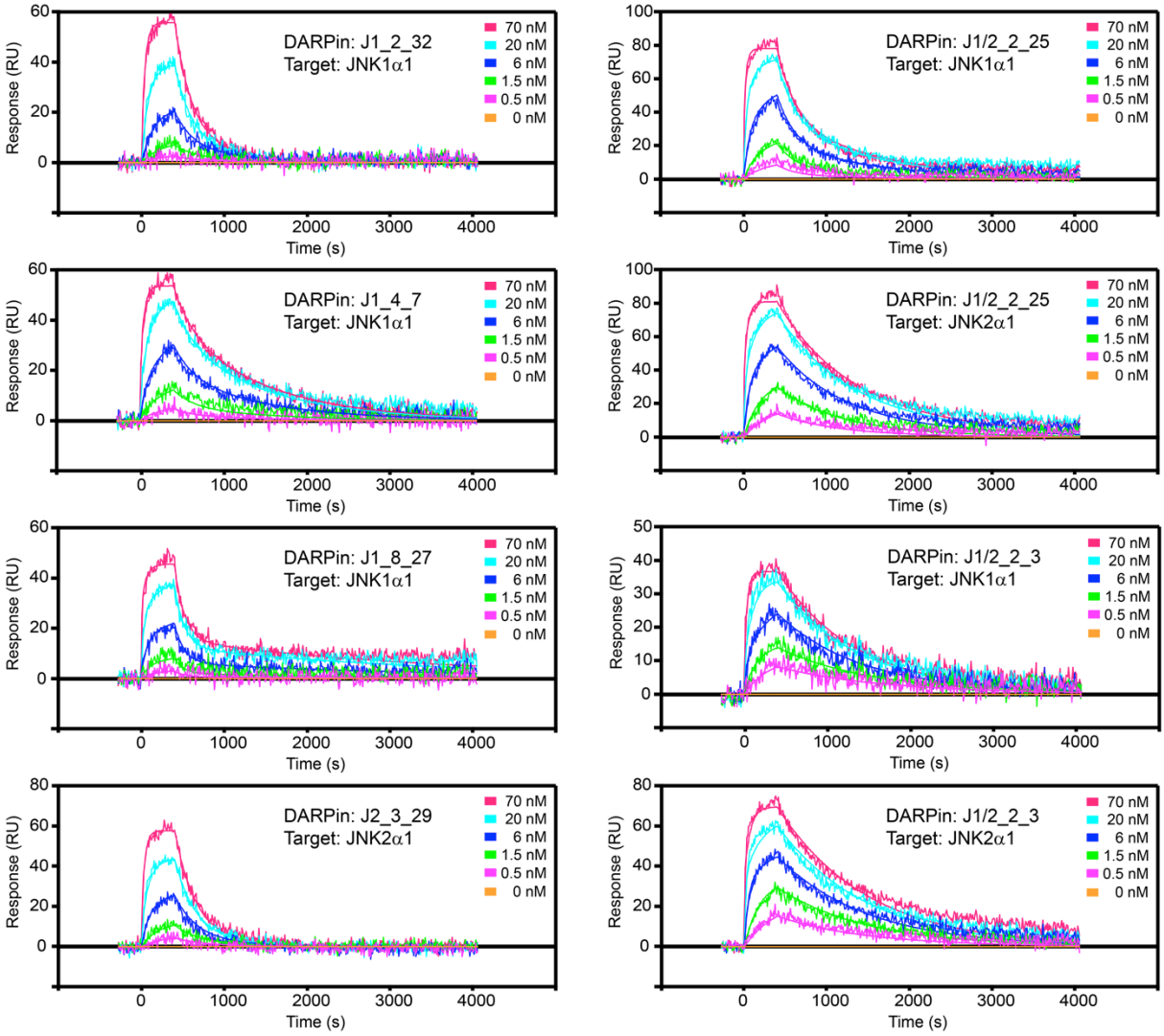

Figure S4. Affinity measurements of selected DARPins.
Binding kinetics of selected DARPins to immobilized targets pD_JNK1α1 and pD_JNK2α1 were measured on the ProteOn system using SRP technology. All measurements were performed at 15 °C due to the instability of the targets. All curves were analyzed with a heterogeneous ligand model using the integrated ProteOn Manager software (Bio-Rad). Both the signal of an uncoated channel reference interspot and the signal of a buffer injection were subtracted from the sensorgrams (double referencing). Due to the instability of JNK1, no duplicates within one experiment could be obtained. Therefore, one example of three independent experiments is shown, with the respective fits and the kinetic constants being summarized in Table 1.

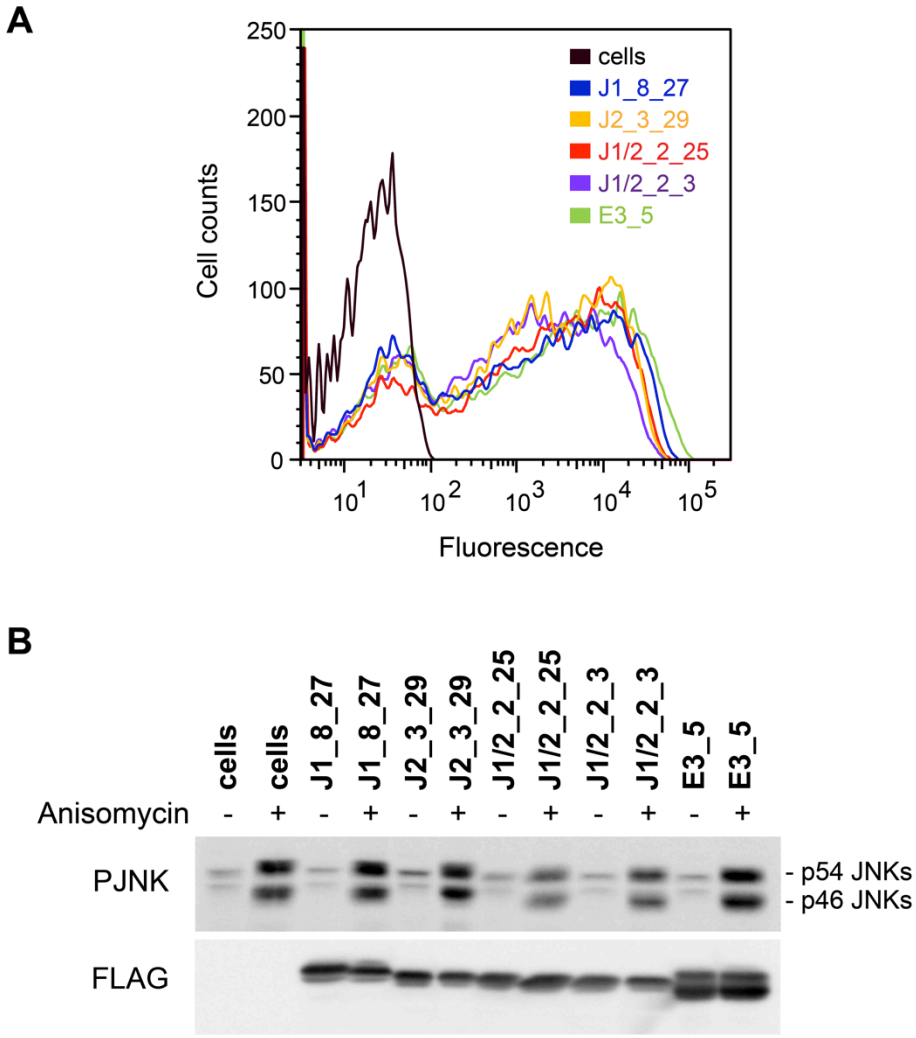

Figure S5. Expression of selected DARPins in cells.
(A) HEK 293T cells were transiently transfected with FLAG-DARPin-EGFP constructs and grown for 24 h. Then the expression levels were analyzed by flow cytometry detecting EGFP fluorescence. **(B)** Transfected cells as in (A) were either activated with anisomycin for 1 h or left untreated and the cell lysates were analyzed by Western blotting with antibodies against phosphorylated (P) JNK and the N-terminal FLAG tag of the DARPins.

Supporting Information Experimental Methods

Material

All chemicals were purchased from Fluka or Sigma-Aldrich. Primers were from Microsynth or from MWG Operon. Restriction enzymes, buffers and polymerases were from New England Biolabs or Fermentas.

Cloning and Production of Recombinant MAPK Proteins

To increase the expression yield and the solubility of JNKs, the genes were produced as bacteriophage lambda protein D (pD) fusions based on the vector pAT222 (GenBank accession AY327137) as described previously (*3, 4*). The following JNK isoforms were used as targets and controls: human JNK1α1 (pAT222_huJNK1α1, previously published as pAT222_JNK1), human JNK2α2 (pAT222_huJNK2α2, previously published as pAT222_JNK2) and human JNK2α1 (pAT222_huJNK2α1 as described below). To introduce the short splice variant JNK2α1 into the vector pAT222 the gene was amplified by PCR from the template pAT222_JNK2 using the primers *JNK2α1F* (5'-GGATCCGGTACCTCC-3') and *JNK2α1R* (5'-GCATAAGCTTGCTGCATCTGTGCTGAAGGCTGATCTTTTACAAC-3') and subcloned into the vector pAT222 via *Kpn*I/*Hind*III. All constructs contain an N-terminal avi-tag for *in vivo* biotinylation and a C-terminal $(His)_6$ tag for purification (avi_pD_JNKisoform_His_6).

For production of non-biotinylated pD_JNK proteins, *E. coli* XL1-Blue (Stratagene) were transformed with the corresponding plasmid and expression was induced for 4 h at 30 °C in 2YT medium. Biotinylated pD_JNKs were produced in the same way but using the plasmid pBirAcm (Avidity) for *in vivo* biotinylation according to the protocols of Avidity.

The pD_JNK proteins were purified at 4 °C using an IMAC column in the presence of 3 mM β-mercaptoethanol according to the manufacturer's instructions (Qiagen), followed by a preparative Superdex 200 (GE Healthcare Biosciences) size exclusion chromatography (SEC) step in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. For pD_JNK1α1 the fractions of the peak corresponding to the monomeric species were collected. pD_JNK2α1 and pD_JNK2α2 showed an additional peak corresponding to a dimeric state of the protein which could not be separated from the monomeric species due to reequilibration. Therefore, a mixture of monomers and dimers was obtained for the JNK2 splice variants after SEC. All proteins were confirmed by SDS-PAGE and mass spectrometry.

***In Vitro* Selections of JNK Isoform-Specific DARPins with Ribosome Display**

DARPin libraries N2C and N3C (*1*) were transcribed and 3 rounds of ribosome display (RD) selections were carried out as previously described (*3, 5*). MaxiSorp plates (Nunc) were coated alternately with neutravidin or streptavidin (66 nM) to prevent selection of binders to these proteins and then blocked with bovine serum albumin (BSA). To obtain a high percentage of JNK1 isoform-specific DARPins, biotinylated pD_JNK1α1 (1 µM) was immobilized on plates and the other isoform pD_JNK2α1 (non-biotinylated, 1 µM) was used as competitor in solution during the panning step in round 2 and 3. For the selection of JNK2 isoform-specific DARPins, biotinylated pD_JNK2α1 was immobilized and non-biotinylated pD_JNK1α1 was used as competitor. To prevent formation of disulfide bonds in the targets, both panning and washing buffers contained additionally 1 mM DTT. After each RD round the RT-PCR products of correct size were excised from an agarose gel and used as templates for the next round.

Production of Selected Binders

From the RT-PCR pools that showed enrichment for the specific target JNK1 and JNK2, respectively, DNA fragments encoding the DARPin sequences were subcloned into a pQE30-derived vector (pQE30ss (*6*), containing an N-terminal MRGS(His)$_6$- tag and a C-terminal double stop codon) using the restriction enzymes *Bam*HI and *Hind*III.

Single DARPin clones were picked and expressed in *E. coli* XL1-Blue in 96-deep well plates and the crude extracts were analyzed for specific binding to JNK1 and JNK2 in an ELISA-based assay as described (*7*) with some modifications (see below). The DNA of the selected binders was sequenced and analyzed (Supplementary Figure S2).

For further analysis, the selected DARPins were expressed from pQE30ss plasmids in *E. coli* XL1-Blue on a 1 liter scale and purified by IMAC as described (*1*) and dialyzed against 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT to apply the same buffer conditions as for the kinases. As controls, the unselected DARPin E3_5 was produced from pQIss_E3_5 (pQE30ss containing additionally a *lacI* gene, kindly provided by A. Mohr) and DARPin J1/2_2_3 (original name JNK2_2_3) from pQE30_JNK2_2_3 (*3*).

ELISA

To determine the binding specificity of the selected DARPins, crude extract ELISA and quantitative ELISA were performed as described (*3, 7*) with some modifications. All steps were performed at 4 °C due to the instability of some kinases. MaxiSorp plates (Nunc) were coated with neutravidin (66 nM) and blocked with 0.5% (w/v) BSA. Biotinylated MAPKs (100 nM/well) were immobilized for 1 h in 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5% (w/v) BSA. For the screening of single clones for binding to JNK1 and JNK2, crude extracts (100 µL each) were added to the wells and incubated for 1 h. DARPin binding was detected by an anti-

RGS-His_6 antibody (Qiagen, 1:5000) followed by goat anti-mouse IgG fused to alkaline phosphatase (Sigma, 1:10000). After addition of the substrate (*p*-nitrophenylphosphate, Fluka) and incubation for approximately 1 h at 37 °C, the $A_{405-540}$ signal was measured in a plate reader. For the quantitative ELISA with different MAPKs, purified DARPins (200 nM) were added to the wells instead of crude extract and the DARPin binding was detected in the same manner.

Size Exclusion Chromatography (SEC) of DARPins and Multi-Angle (Static) Light Scattering (SEC-MALS) of JNK-DARPin Complexes

For all selected DARPins, size exclusion chromatography was performed at RT on an Agilent LC1100 HPLC system (Agilent Technologies). 50 µL of each purified DARPin (15 µM) were loaded on a 24 mL Superdex 75 column (GE Healthcare Biosciences) with a flow rate of 0.5 mL/min and 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT as running buffer.

To determine the molar mass of each JNK-DARPin complex, SEC-MALS measurements were performed essentially as described above using a 24 mL Superdex 200 column (GE Healthcare Biosciences) followed by a miniDAWN three-angle light-scattering detector (Wyatt Technology) and an Optilab rEX differential refractometer (Wyatt Technology). This method is independent of the elution profile, since for the calculation of the molar mass only the scattered light (Rayleigh ratio) measured by the MALS detector and the concentration of the protein obtained from the dRI-detector, using the differential refractive index increment (dn/dc) of the protein, are taken into account. pD_JNK1α1 or pD_JNK2α1 (15 µM) were injected directly (60 µL) or first premixed with the corresponding DARPin (37.5 µM) at a molar ratio of 1:2.5. The data were recorded and processed using the ASTRA V software (Wyatt Technology). For the calculation of the molar masses a differential refractive index increment (dn/dc) value of 0.186 mL/g was used.

Surface Plasmon Resonance (SPR) Measurements

SPR was measured using a ProteOn XPR36 instrument (Bio-Rad). All binding measurements were performed in Tris buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing 0.005% (v/v) Tween-20 at 15 °C.

For coating, one channel of a neutravidin NLC sensor chip (Bio-Rad) was immobilized with biotinylated pD_JNK1α1 and a second channel with biotinylated pD_JNK2α1 to approximately 800 RU in the vertical orientation of the fluidic system at a flow rate of 25 µL/min. Five different DARPin concentrations and running buffer as control were injected in the horizontal channels 1 to 6 at a flow rate of 60 µL/min. The association time was measured for 400 s, followed by a dissociation time of 60 min at the same flow rate.

All sensorgrams were processed and analyzed using the integrated ProteOn Manager software (Bio-Rad). Both the signal of an uncoated channel reference interspot and the signal of a buffer injection were subtracted from the sensorgrams

(double referencing). Binding curves were fitted using the heterogeneous ligand model (global fit) (Supplementary Figure S4).

Pull Down Experiments

To investigate binding of the selected DARPins to endogenous JNKs, pull-down experiments were performed as described previously (*4*) with some modifications. In each assay, 4 µg of purified DARPin were coupled to Ni-NTA agarose beads (20 µL of a 50% suspension (Qiagen)). The beads were blocked with 0.5% (w/v) BSA and incubated with 500 µL cold cell lysate (1 mg of total protein from HEK 293T cells lysed in 50 mM Tris pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1% (v/v) NP-40) for 2 h at 4 °C. Bound proteins were eluted and the endogenous JNKs and the recombinant DARPins were detected by Western blotting using primary antibodies against JNK1 (F-3) (Santa Cruz Biotechnology, 1:200), against JNK2 (long splice forms) (Cell Signaling, 1:2000) and against the RGS-His-tag (1:5000), followed by corresponding secondary antibodies against rabbit and mouse IgGs, respectively.

Cloning of BRET Constructs and Optimization

DARPins were amplified from plasmids pQE30ss_8_27, pQE30ss_3_29, pQE30ss_2_25, pQE30ss_JNK2_2_3 (anti-JNK-DARPins), and pQIss_E3_5 (control-DARPin) using the primer combination *darpin2f-Bgl*II (5´-GGAAGATCTATGGACCTGGGTAAGAAACTGCTG-3´) and *darpin2r-Hind*III (5´-TCCAAGCTTTTGCAGGATTTCAGCCAGGT-3´) and subcloned into the vector pCMV-GFP^2-N3 (PerkinElmer Life Sciences) with indicated restriction enzymes. To optimize the BRET constructs, we mutated the methionine present at the beginning of GFP to alanine, employing site-directed mutagenesis using the primers: 5´-CGGGATCCACCGGTCGCCACCGCGGTGAGCAAGGGC-3´ and 3´-GCCGTAGGTGGCCAGCGGTGGCGCCACTCGTTCCCG-5´. This significantly reduced the translation of GFP^2 without the fusion partner. We went on to shorten the linker sequence of the DARPin-containing vectors by cutting the linker region with *Kpn*I and *Age*I, treatment with T4 polymerase to generate blunt ends followed by re-ligation. The linker region was thus shortened by seven amino acids and reads now: KLAPVAT. This improved the BRET ratio (see next section) by ~ 10% without changing the specific interactions of the DARPins investigated (data not shown).

Since it has been reported that in most instances the *jnk1* gene produces predominantly a 46 kDa protein product whereas the *jnk2* gene produces predominantly a 54 kDa protein (*8-11*), the JNK1 short splice form (JNK1α1) and the JNK2 long splice form (JNK2α2) were chosen. Human JNK1α1 was amplified using the primer pair *jnk1f-Xho*I (5´-GGACTCGAGATGTCCCGTAGCAAGCGT-3´) and *jnk1rstop-Hind*III (5´-TCCAAGCTTCTACTATTGCTGCACCTGTGCTAA-3´) and pAT222_huJNK1α1 as the template. The PCR fragments were digested with *Xho*I and *Hind*III and subcloned into pCMV-Rluc-C2. Human JNK2α2 was amplified using the primer pair *jnk2f-Xho*I (5'-GCACTCGAGATGTCCGACTCTAAATGTGA-3') and *jnk2rstop-Hind*III (5'-TCCAAGCTTCTATCGACAGCCTTCAAGG-3´) and

pAT222_huJNK2α2 as the template. The PCR fragments were again digested with *Xho*I and *Hind*III and subcloned into pCMV-Rluc-C2.

Rat ERK2 was amplified from pLK1_ERK2 using the primers *erk2f-Xho*I (5´-GGACTCGAGCCATGGGCGCGG-3´) and *erk2rstop-Hind*III (5´-TCCAAGCTTCTAAGATCTGTATCCTGGCTG-3´), cut with *Xho*I and *Hind*III to allow ligation into the vector pCMV-Rluc-C3.

Cloning of Eukaryotic Expression Vectors for Inhibition Studies In Cells

For inhibition studies in mammalian cells, FLAG-DARPin-$(G_4S)_2$linker-EGFP constructs were generated. The EGFP gene was amplified by PCR from the pEGFP-N2 vector (Clontech) with primer *ForBgl*II*EGFPdMV* (5‘-CATGCAGATCTAGCAAGGGCGAGGAGCTGTTC-3‘) introducing a *Bgl*II restriction site and deleting the first 2 amino acids of EGFP to avoid an alternative start site and primer *RevBsa*I*EGFP* (5‘-GCATCGTAGCTTGAGACCCTTGTACAGCTCGTCCATGCCG-3‘) introducing a *Bsa*I restriction site. This product was subcloned into pQIBi_1_1 vector (kindly provided by C. Gehringer) via *Bgl*II/*Bsa*I resulting in pQIBi_EGFPdeltaMV. The expression cassette of pQiBi_EGFPdeltaMV was amplified with primer *ForAfl*II*Bi* (5‘-AGACTCTTAAGGCCACCATGGACTACAAGGACGACGACGACAAGGGATCCGGT GGTACCCCGG-3‘) introducing an *Afl*II restriction site followed by an optimal Kozak sequence and a FLAG-tag (coding for DYKDDDDK) at the N-terminus and primer *RevNot*I*BiEGFP* (5‘-AGCATGCGGCCGCCTATCATTAATTTAGCTTGAGACCCTTGTACAGC-3‘) adding a *Not*I restriction site at the C-terminus. Cloning into the vector pcDNA3.1(+) (Invitrogen) via *Afl*II/*Not*I resulted in pcDNA3.1(+)Bi_EGFPdeltaMV (Flag-$(G_4S)_2$-EGFP). DARPin sequences were optimized for GC content and mammalian codon usage by GENEART prior to cloning into pcDNA3.1(+)Bi_EGFPdeltaMV via *Bam*HI and *Hind*III. The resulting plasmids pcDNA3.1(+)Bi_DARPin#opt_EGFPdeltaMV contain a certain DARPin with a FLAG-tag at the N-terminus and a linker followed by EGFP at the C-terminus (FLAG-DARPin-$(G_4S)_2$-EGFP).

Supplemental References

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