Supplementary Data

for

Modification of Adenovirus Receptor Specificity by Designed Multivalent Targeting Adapters

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Supplementary Methods:

Selection and affinity maturation of knob-binding DARPins.

Selection was performed using the DARPin libraries containing either two or three randomized repeats. [1, 2] The selection using ribosome display was performed essentially as previously described [3-6] with some minor modifications. [7] During the selection process the previously described RNA purification step after *in vitro* transcription using a LiCl/EtOH precipitation was replaced by purification of the RNA using Illustra G50 columns (GE Healthcare Biosciences, Pittsburg, PA, USA). For the selection on solid phase either streptavidin or neutravidin (100 µl at a concentration of 66 nM in TBS (Tris-buffered saline, 50 mM Tris, 150 mM NaCl, pH 7.6) was coated on a 96-well MaxiSorp polystyrene plate (Nalge Nunc International, Rochester, NY, USA) overnight at 4°C. The wells were washed twice with TBS/0.05% Tween-20 (TBST) and blocked with 0.5% BSA in TBST for 1 h at room temperature (RT). The biotinylated target protein, MRGS$(H)_6$-knobΔTAYT protein in rounds 1-3a in strategy 1, MA$(H)_6$-knobΔTAYT protein round 3b-6b in strategy 1 and all rounds in selection strategy 2, was immobilized to the wells (round 1 and 2 in strategy 1 and round 1 in strategy 2) in TBST at concentrations of 500 to 1000 nM for 1 h at 4°C. The wells were washed 3 times with TBST and once with WBT buffer [50 mM Tris acetate (pH 7.5, 4°C), 150 mM NaCl, 50 mM magnesium acetate, 0.05% Tween-20]. One hundred µl of the stopped translation mix containing the ternary DARPin/ribosome complexes was incubated for 1 h at 4°C.

Alternatively, for the selection in solution, the target protein was incubated with the DARPin/ribosome complexes in solution followed by capturing the DARPin/knob complexes using 40 to 80 µl MyOne T1 streptavidin coated magnetic beads (Invitrogen Ltd, Paisley, UK) previously blocked with BSA. For the selection in solution usually 250 µl of the stopped *in vitro* translation reaction was used in a total volume of 500 µl diluted with WBT buffer containing 0.5% BSA and 2.5 mg/ml heparin.

In general, the DARPin/ribosome complexes where exposed to the selection condition without the target for 30 to 60 min prior to the selection for binding to the mutated knob, except for round 1, where no pre-panning was carried out. The capture of the target-bound complexes was usually 30 – 60 min at 4°C. Washing of the beads in solution was performed in a volume of 500 µl WBT using 2 short washes of 1 minutes followed by 6 longer washes ranging from 2 – 15 min, except for round 1 where washing was performed twice with a short wash followed by 2 washes of 10 min (strategy 1) or 6 times for 5 minutes (strategy 2). RNA was eluted using buffer EB [50 mM Tris-acetate (pH 7.5 at 4°C), 150 mM NaCl, 25 mM EDTA] and purified using the High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany), including an on-column DNaseI digest following the manufacturer's instructions.

Error-prone PCR was performed using the dNTP analogues dPTP (6-(deoxy-β-D-erythro-pentofuranosyl)-3,4-dihydro-8H-pyrimido - [4,5-c][1,2]oxazine-7-one-5′-triphosphate) and 8-oxo-dGTP (8-oxo-2′-deoxyguanosine-5′-triphosphate) both ranging from 1 µM to 20 µM each, or no analogs. [8 9] For the selection, products from the different error-prone PCR reactions were pooled in equimolar amounts. Therefore,

the starting material for the next selection round contained a mixture of different mutational loads.

As template, the DNA encoding for the DARPins was inserted into the ribosome display plasmid pRDV (GenBank accession No. AY327136; please note the new entry with sequence corrections). The amplification was performed in a 50 µl reaction in the presence of 1 µM primers (T7B and tolAkurz), one unit of Platinum Taq DNA polymerase (Invitrogen Ltd, Paisley, UK), 1.5 mM $MgCl_2$ and 250 µM dNTPs each. A total of 25 cycles of PCR were performed, and the mutational load was determined by sequencing to be 0.75 (at 1 µM) and 1.6 (at 3 µM) mutations per N3C DARPin. Similar results had been previously observed, and it had additionally been found that the mutational load can be as high as 5.3 with 20 µM using Vent Polymerase Exo- (New England Biolabs, Ipswich, MA). [9]

Off-rate selection was performed as previously described [9, 10] by addition of an access of non-biotinylated mutant knob protein after the equilibration of the complex formation of DARPin and biotinylated mutant knob which was performed for 1-16 h at target concentrations ranging from 0.7-100 nM. Competition was performed for 2 to 20 h at 4°C at a molar excess of competitor ranging from 10- to 1000-fold before the DARPin-bound biotinylated mutant knobs were captured on streptavidin-coated magnetic beads.

Initial Screening of knob-binding DARPins.

DNA encoding for the DARPin pools after selection were subcloned via *Bam*HI and *Hind*III into a pQE30 derivative essentially as described previously. [11, 12] Single selected DARPins were expressed in 96-deep well plates and cells lysed using B-PERII (Pierce, Rockford, IL) as described previously. [12] Binders to the ΔTAYT knob protein were identified in an ELISA using a 1:2 to 1:10 dilution, in the later phase of affinity maturation a dilution of 1:1000 was used in order to distinguish binders of different affinities.

Different detection methods depending on the format of the biotinylated mutant knob protein that was available were applied. First, for the analysis of DARPins containing a C-terminal $(myc)_5$ tag, a MaxiSorp plate (Nalge Nunc International, Rochester, NY, USA) was coated with protein A (P6031, Sigma-Aldrich Corp., St. Louis, MO, USA; 1:100 from 1 mg/ml stock) in PBS overnight at 4°C, then washed with PBS/0.01% Tween-20 (PBS-T). After blocking with PBS-T with 0.2% BSA (PBS-TB) for 1 h at RT, plates were washed three times with PBS-T. The anti-myc mAb 9B11 (2276; Cell Signaling Technologies, Inc., Danvers, MA) was immobilized at a dilution of 1:300 in PBS-TB for 1 h at RT. Plates were washed three times with PBS-T and the DARPin lysates were incubated for 1 h at RT followed by washing. The biotinylated $MRGS(H)_6$ mutant knob was added in concentrations of 10 to 100 nM and detection was performed with a streptavidin-AP conjugate (A3562; Sigma-Aldrich Corp., St. Louis, MO, USA) at a dilution of 1:2000 and after addition of p-nitrophenyl phosphate as substrate (1:300 dilution of a 1 M stock).

Second, for the analysis of DARPins with a N-terminal $MRGS(H)_6$ fusion, the ELISA format was essentially as described. [12]

Construction of pBD001 and pBD002 vectors for the expression of biotinylated DARPins.

The expression plasmid pBD001 was constructed by a PCR-based strategy to replace the sequence comprising of the AviTag_MBP_MCS_$(His)_6$ tag of the vector pAT224. [1] The cloning of the DARPins via *BamH*I and *Hind*III in this vector version required a PCR amplification, allowing the in-frame cloning of the DARPin coding sequence with the N-terminal AviTag and the C-terminal $(His)_6$ tag. To avoid this PCR step, pBD002 was constructed (Supplementary Figure S1) which contains restriction sites to allow direct cloning of DARPins from pRDV (GenBank accession no. AY327136) or pDST72 [12] via *BamH*I and *Hind*III.

BioLayer Interferometry (BLI)

Affinity determination using the Octet QK system (FortéBIO Inc, Menlo Park, CA, USA) was performed at 30°C in PBS containing 0.1% Tween 20 (PBS-T). The streptavidin coated sensor tips were saturated in a 200 nM solution of biotinylated $MA(H)_6$ knobΔTAYT protein for 1 min. After washing of the tips in PBS-T DARPins were allowed to associate for 15 min in concentrations of 200, 100, 50 and 10 nM. Dissociation was followed for 20 min in PBS-T. Kinetic data analysis was performed using the Octet software.

Surface Plasmon Resonance (SPR)

SPR experiments were performed using a BIAcore 3000 system (GE Healthcare Biosciences, Pittsburg, PA, USA) and using a ProteOn XPR36 instrument (Bio-Rad Laboratories, Hercules, CA).

For the BIAcore 3000 system, biotinylated $MA(H)_6$ knob ΔTAYT protein was immobilized on a BIAcore streptavidin coated sensor chip. Measurements were performed at 25°C in HBST buffer (20 mM Hepes, 150 mM NaCl, 3 mM EDTA and 0.005 % Tween-20). Injection was performed for 1 min for F4 and 5 min for 2E6 at a flow rate of 30 µl/min and dissociation for 5 minutes for F4 and 35 min for 2E6.

For the ProteOn XPR36 instrument, a NeutrAvidin NLC sensor chip (Bio-Rad) was used for immobilization of the biotinylated knobΔTAYT protein to approximately 200 RU per vertical channel. All binding measurements were performed in PBS containing 0.005% Tween-20 at 20°C. For the analysis of monovalent DARPins 2E6 and 2E6-G5, bivalent DARPin $(2E6)_2$-G5 and trivalent DARPin $(2E6)_3$-G5 the corresponding DARPin was injected in the horizontal orientation of the fluidic system at a flow rate of 60 µl/min for 6 min, followed by a dissociation time of 60 min. Five different DARPin concentrations (0.4 to 50 nM) and running buffer as control were injected in channels 1 to 6.

Supplementary Figures:

Fig. S1: Vector for expressing biotinylated DARPins. DARPins can be directly cloned from pRDV (GenBank accession no. AY327136) or pDST72 [12] via *BamH*I and *Hind*III. The resulting DARPin will contain a N-terminal AviTag and a C-terminal $(His)_6$ tag.

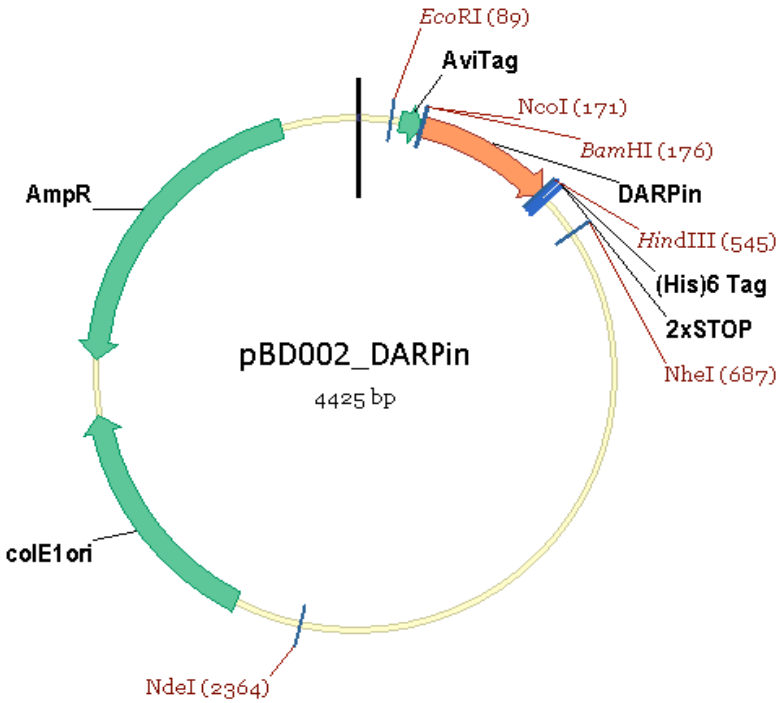


Fig. S2: Trimeric nature of the knobΔTAYT proteins. Size exclusion chromatography (SEC) coupled to multi angle light scattering (MALS) was used to determine the molecular weight (MW) of the knob preparations. Mutant knobs (ΔTAYT) were separated by SEC at a concentration of 5 µM in PBS. The peaks shown represent the UV signal recorded at 280 nm (left y-axis). The determined mass of the knob protein preparations are given on top of the according protein peaks (right y-axis). Blue: biotinylated MRGS$(H)_6$ knobΔTAYT; Green: biotinylated MA$(H)_6$ knobΔTAYT; Red: MA$(H)_6$ knobΔTAYT.

The molar mass determined for the biotinylated MRGS$(H)_6$ knobΔTAYT was 79 kDa (theoretical Mw 73.33 kDa), for the biotinylated MA$(H)_6$ knobΔTAYT 76.27 kDa (theoretical Mw 71.75 kDa), and for the MA$(H)_6$ knobΔTAYT 66.65 kDa (theoretical Mw 65.61). Note that the biotinylated subunits contain an AviTag each.

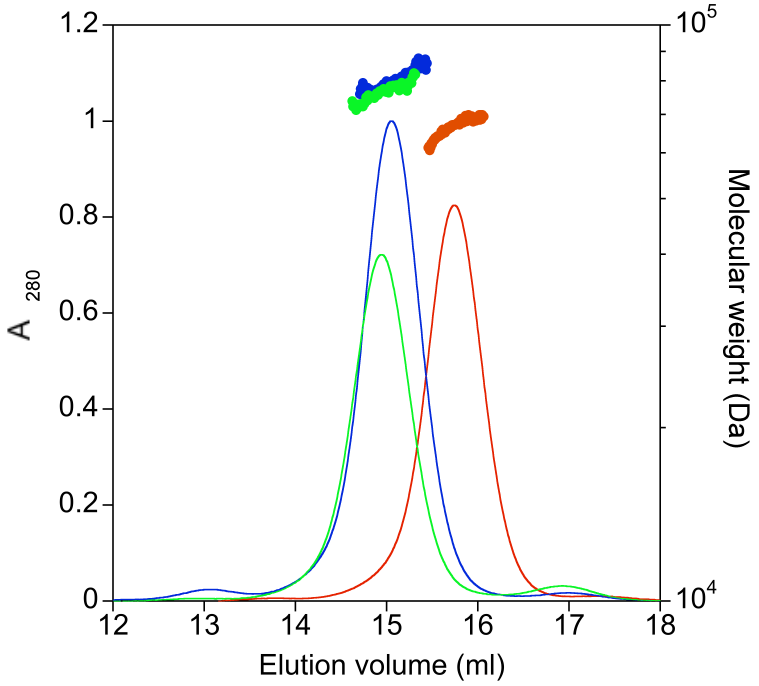


Fig. S3: Selection of knob-specific DARPins. The biotinylated variants of the mutant knob with the deletion of the TAYT amino acid sequence in the FG loop were used as target for ribosome display selections of DARPins specifically binding to the mutant knob. The selection was based on a mixture of non-stringent rounds and off-rate selection rounds. In addition, new diversity was introduced by the application of error-prone PCR using nucleotide analogues to incorporate random mutations. At different stages of the selection procedure single clones were analyzed for target binding from crude extract of *E. coli* expression cultures from single clones. In the early stages of the selection procedure only few clones resulted from the selection of strategy 1 round 3a, e.g. B1 (N4C containing four randomized internal repeats) and B2 (N3C containing three randomized internal repeats), which were estimated to have affinities in the µM range by using SPR measurements (data not shown).

To increase the affinity of the knob binders error-prone PCR (round 3b) in combination with an off-rate selection using a 500-fold excess of non-biotinylated knob was performed. Single DARPin clones, e.g. F4 and G12, both N3C DARPins, resulting after round 4b, had apparent affinities in the high nM range (for F4 see Supplementary Data Fig. S4a). Next, the clones B1, B2 and G12 were pooled and used as template for an additional error-prone PCR (round 5b). The selection pressure was increased by using an off-rate selection, where 1000-fold excess of competitor was used. In addition, selection strategy 2 was pursued in which error-prone PCR and off-rate selections were already incorporated at early stages of the selection. Both strategies resulted in knob binders in the low nM range, e.g. DARPin 2E6.

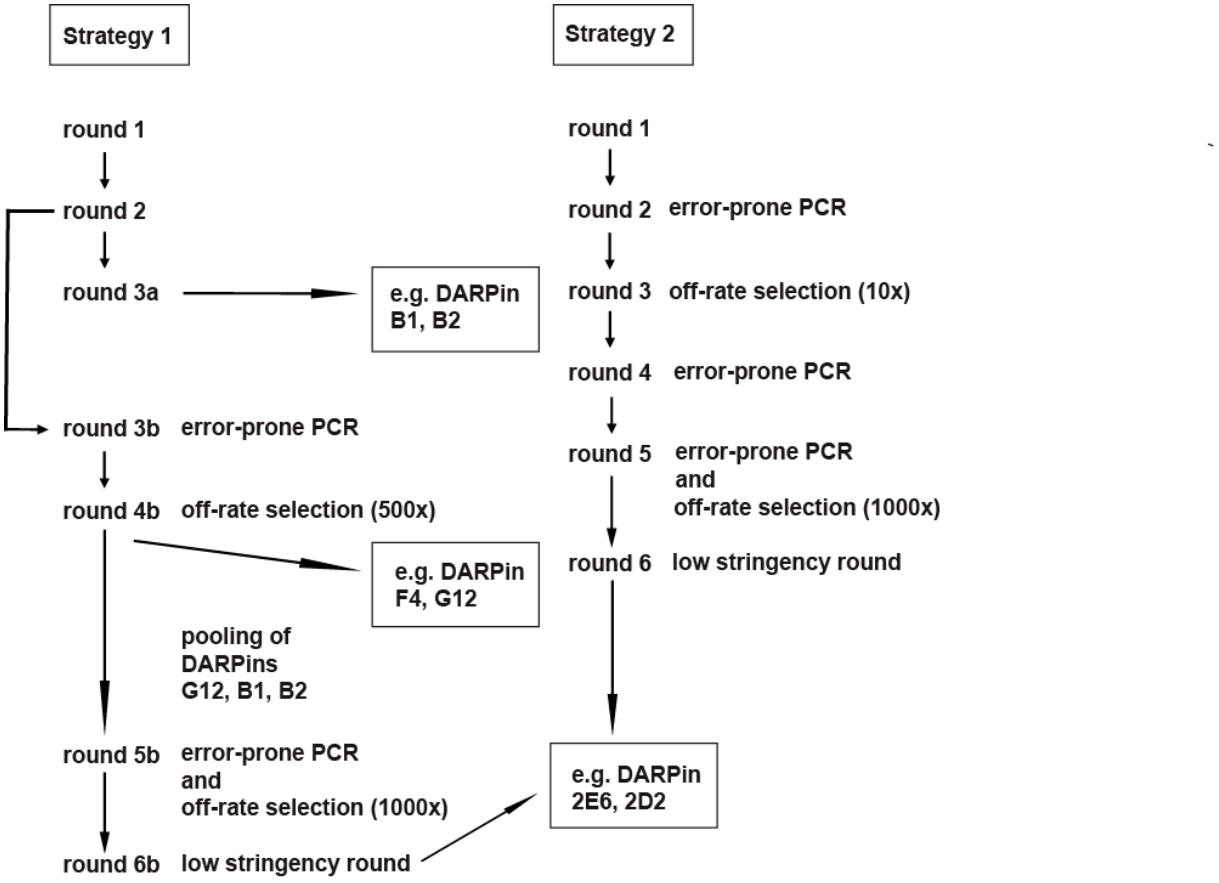


Fig. S4. Affinity of monovalent ΔTAYT knob-binding DARPins. SPR measurements on a BIAcore 3000 system were performed in duplicates, using a serial dilution of the monovalent DARPins. For DARPin F4 (**a**) concentrations of 2, 5, 10, 25, 50, 75, 100, 200 and 500 nM were used; for DARPin 2E6 (**b**) 1, 5, 10, 25, 50 and 100 nM. The curves were analyzed using Scrubber2 in combination with CLAMP XP version 3.5 to determine the affinity of the proteins. F4 could be analyzed with a 1:1 interaction model and the apparent affinity was calculated at 412 ± 0.07 nM with a k_a of (5.17 ± 0.07) x 10^5 M^{-1} s^{-1} and a k_d of 0.213 ± 0.02 s^{-1}. The binding kinetics for 2E6 could not be analyzed by a 1:1 model. Since titration experiments analyzed by MALS also indicated that not all binding sites could be equally saturated, we used a heterogeneous ligand model. The apparent K_D for the first reaction was calculated at 1.35 ± 0.01 nM (k_{a1} of (1.28 ± 0.005) x 10^6 M^{-1} s^{-1} and a k_{d1} of (1.72 ± 0.005) x 10^{-3} s^{-1}), and for the second interaction at 18 ± 0.01 nM (k_{a2} of (2.82 ± 0.02) x 10^5 M^{-1} s^{-1} and a k_{d2} of (5.08 ± 0.02) x 10^{-3} s^{-1}). Black curves: duplicate measurements; red curve: respective fit. The statistical errors in the parameters given are those obtained from the best fit error.

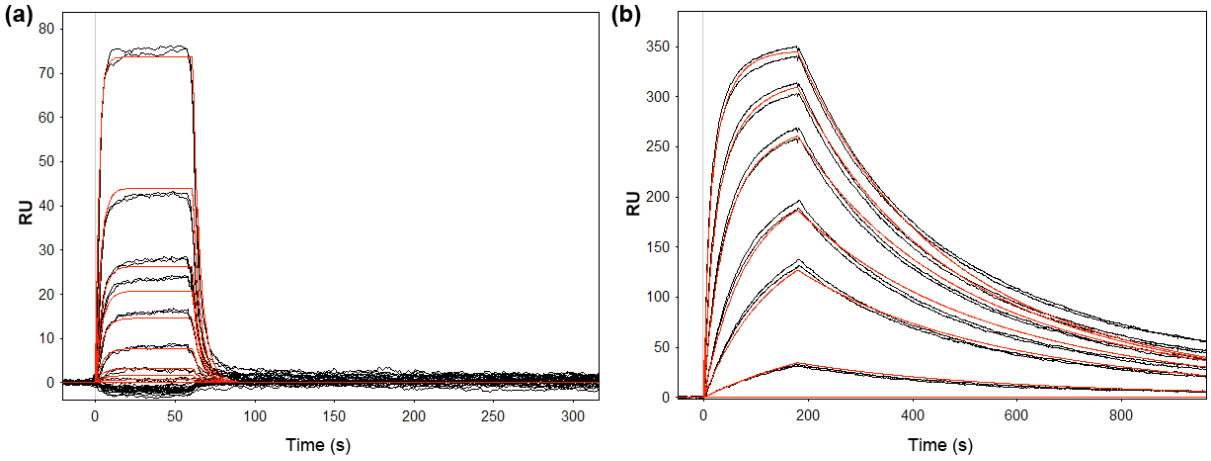
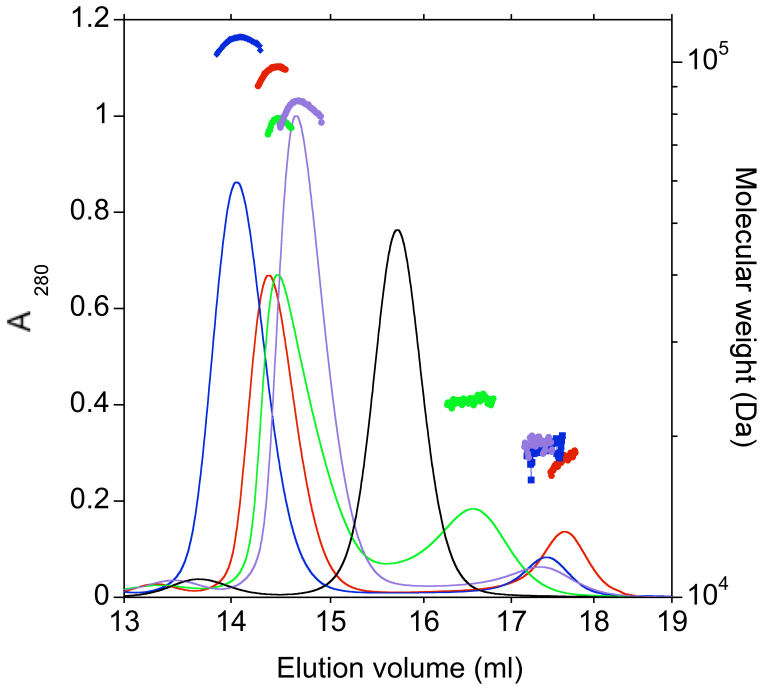


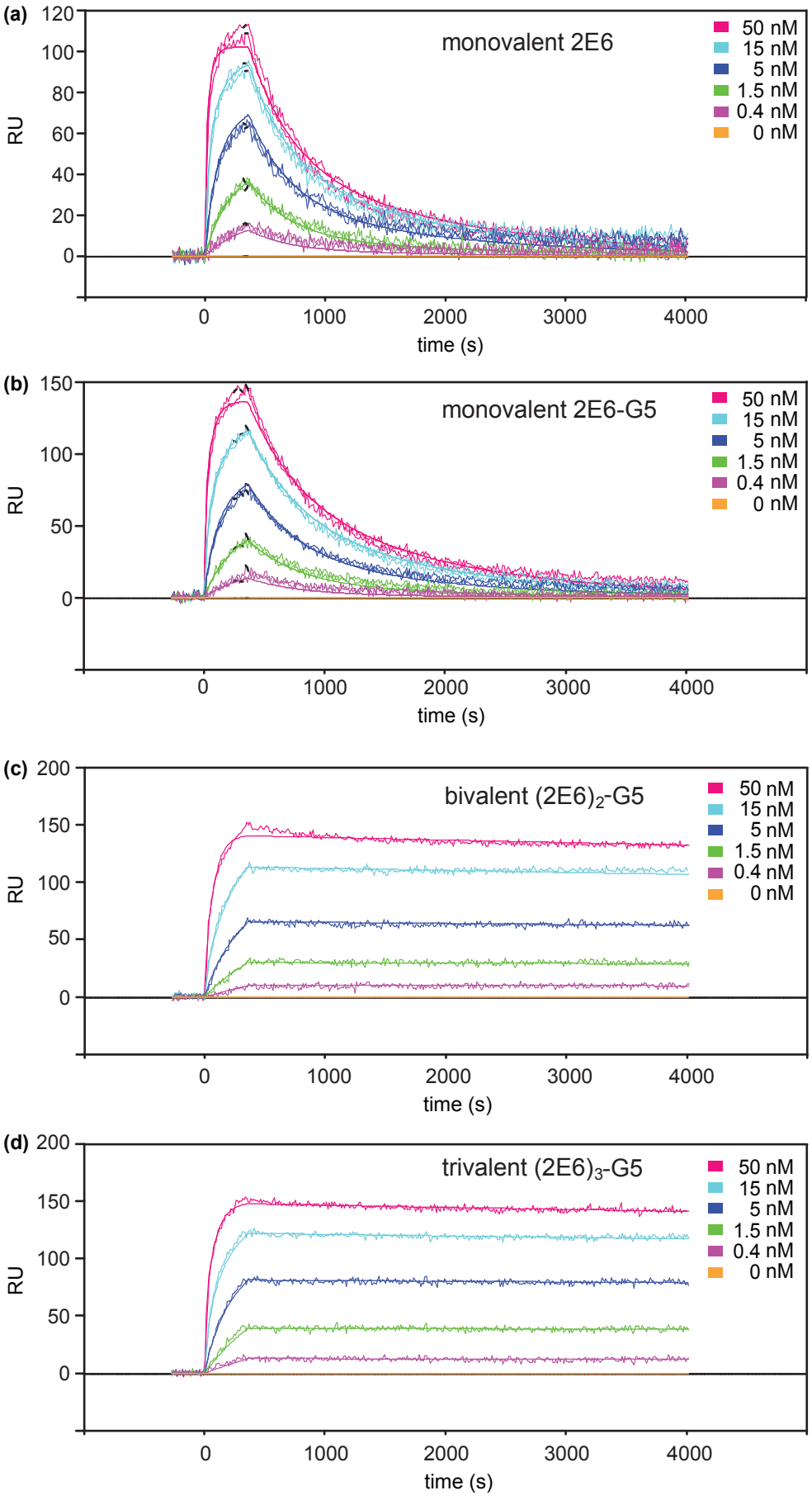
Fig. S5: Dependence of the observed stoichiometry of the DARPin/knob complex formation on affinity. Size exclusion chromatography coupled to multi-angle light scattering was used to determine the molecular weight (MW) of DARPin/knob complexes. Free trimeric knobΔTAYT was injected at a concentration of 10 µM corresponding to a subunit concentration of 30 µM, or preincubated for at least 1 h with 45 µM of DARPin before separation by size exclusion chromatography in PBS. The curves shown represent the UV signal recorded at 280 nm (left y-axis). The determined mass of the proteins and complexes are given above the respective protein peaks (right y-axis). Black: free trimeric knob; blue: 2E6 complex; red: 2D2 complex; green: B1 complex; magenta: G12 complex. While 2E6, 2D2, and G12 are N3C DARPins containing three randomized repeats, B1 is a N4C with four internal repeats. The observed ratio of DARPins per knob trimer in the complex is proportional to the estimated affinity of the DARPins to the ΔTAYT knob protein.



Blue: 2E6 (N3C, K_D = 1.34 nM)
Red: 2D2 (N3C, K_D ≈ 50 nM)
Magenta: G12 (N3C, K_D ≈ 400 nM)
Green: B1 (N4C, K_D ≈ µM)
Black: knob alone

DARPins per knob trimer: 2.5
DARPins per knob trimer: 1.7
DARPins per knob trimer: 0.96
DARPins per knob trimer: 0.45

Fig. S6: Dissociation rate constants of monovalent, bivalent and trivalent knob-binding DARPins. Binding kinetics of DARPins to the immobilized knobΔTAYT protein were measured on the ProteOn system using SPR technology. For the monovalent DARPins 2E6 **(a)** and 2E6-G5 **(b)** measurements are shown in duplicates with the corresponding fits. For the bivalent adapter $(2E6)_2$-G5 **(c)** and trivalent DARPin $(2E6)_3$-G5 **(d)** one example of two independent experiments is shown with the respective fit, since no duplicates could be measured in the same channel due to incomplete dissociation. 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). The k_d for the first reaction was calculated to be $(2.94 \pm 0.02) \times 10^{-3}$ s^{-1} for monovalent 2E6 and $(2.5 \pm 0.01) \times 10^{-3}$ s^{-1} for monovalent 2E6-G5, respectively. Bivalent DARPin $(2E6)_2$-G5 showed a dissociation rate constant of $\leq (5.4 \pm 0.07) \times 10^{-5}$ s^{-1} and trivalent DARPin $(2E6)_3$-G5 a dissociation rate constant of $\leq (2 \pm 0.02) \times 10^{-5}$ s^{-1}.


(a)
monovalent 2E6
50 nM
15 nM
5 nM
1.5 nM
0.4 nM
0 nM
RU
time (s)
(b)
monovalent 2E6-G5
(c)
bivalent (2E6)2-G5
(d)
trivalent (2E6)3-G5

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