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Supplemental Information

A Library-Based Screening Strategy for the Identification of DARPins as Ligands for Receptor-Targeted AAV and Lentiviral Vectors

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Supplementary Figures

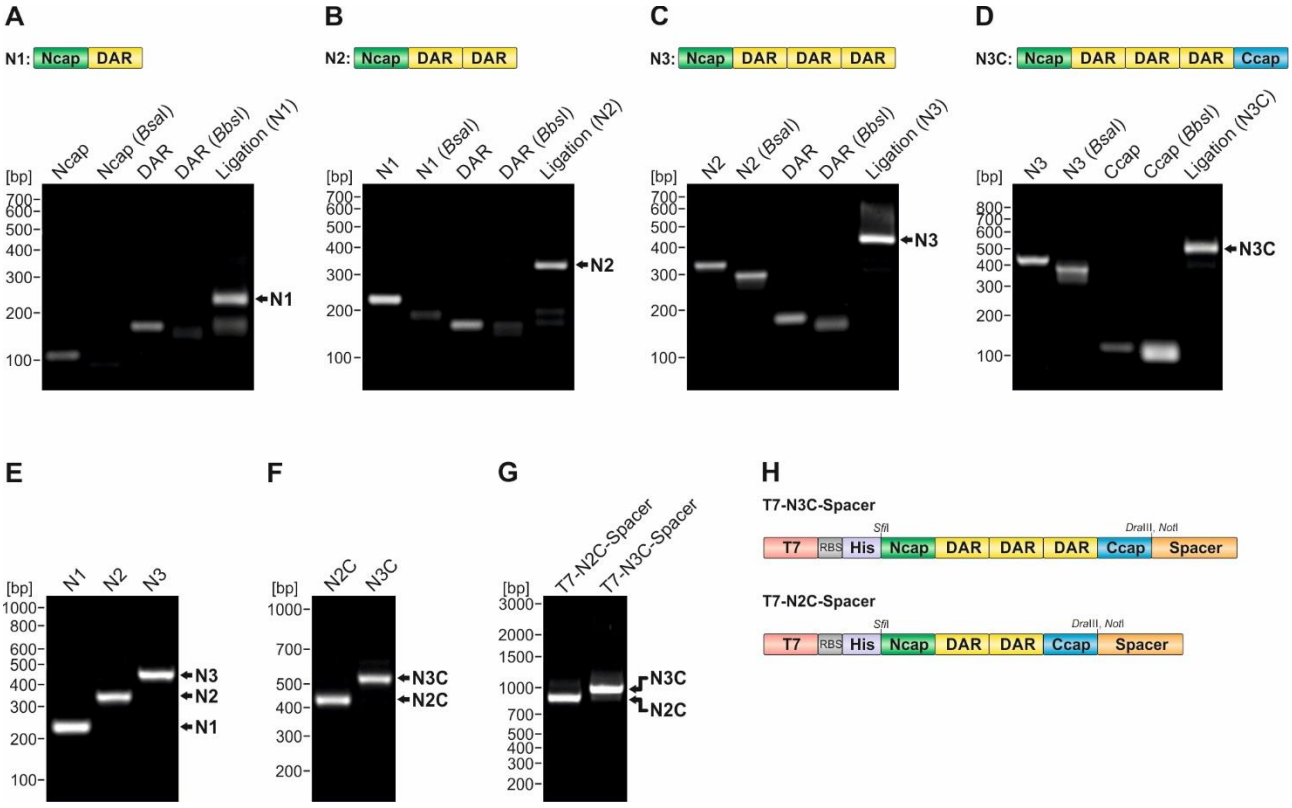

Figure S1: Stepwise generation of the VV-N2C and VV-N3C DARPin libraries.

DARPin libraries were assembled from DNA fragments encoding a constant N-terminal capping domain (Ncap), a constant C-terminal capping domain (Ccap) and diversified designed ankyrin repeat (DAR) elements generated with degenerated oligonucleotides. **(A-D)** 200 ng of the undigested and digested PCR fragments and the product of the ligation reaction, respectively, were separated on 2% agarose gels. A schematic drawing of the resulting DNA fragment of each assembly step is depicted above each gel. **(E-G)** Agarose gel analysis of purified DARPin DNA after PCR amplification of the corresponding ligation product. On a 2 % agarose gel 200 ng of DNA were separated and stained with ethidium bromide. **(H)** Schematic representation of the DARPin libraries. The N2C and N3C DARPin libraries are flanked by a T7 promoter (T7), a ribosome binding site (RBS) and a hexahistidin tag (His) as well as a spacer sequence encoding an unstructured region from the *E. coli* TolA protein without stop codon. Positions of the added restriction sites are indicated.

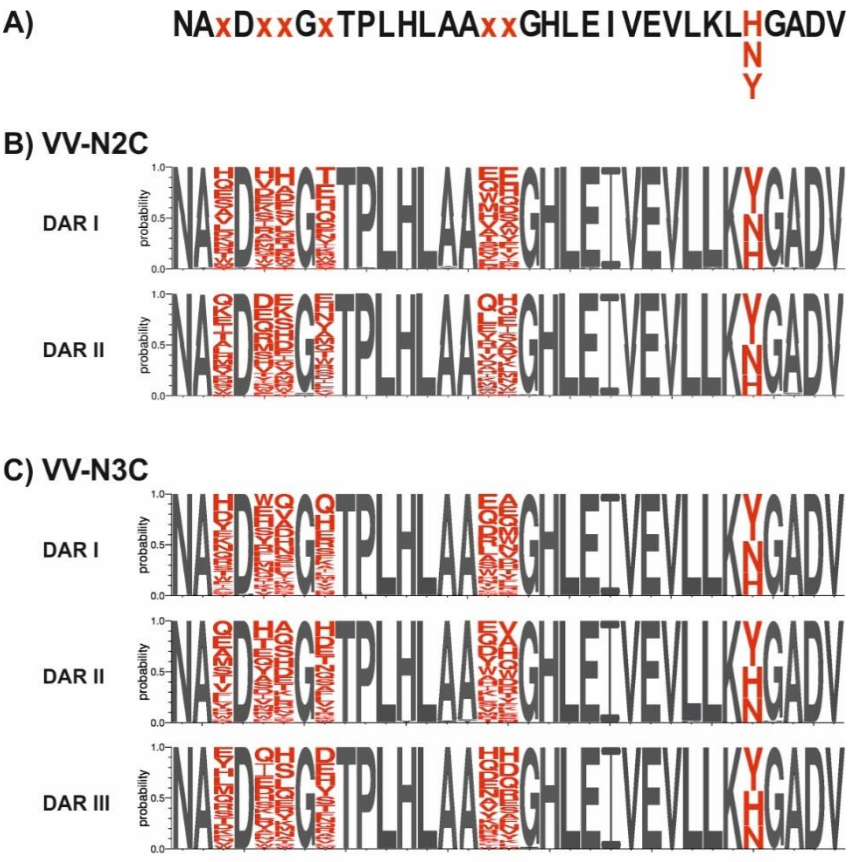

Figure S2: Sequence analysis of the generated DARPin libraries.

(A) Consensus sequence of one designed ankyrin repeat (DAR) element on protein level. Each DAR harbors six diversified positions (red x), where any amino acid is allowed except of glycine, proline or cysteine, and one diversified positions being a histidine, asparagine or tyrosine. **(B, C)** Sequence logo of the generated VV-N2C (B) and VV-N3C (C) DARPin library covering the DAR elements. To verify the frame work and diversified positions of the generated DARPin libraries, 45 ng of each library DNA was cloned into the plasmid pJet1.2 and transformed into *E. coli* TOP10. 100 individual clones per library were analyzed by sequencing. 76 (N2C) and 58 (N3C) sequences encoded a DARPin and were used to create the corresponding sequence logo using WebLogo 3 (http://weblogo.threeplusone.com/create.cgi).

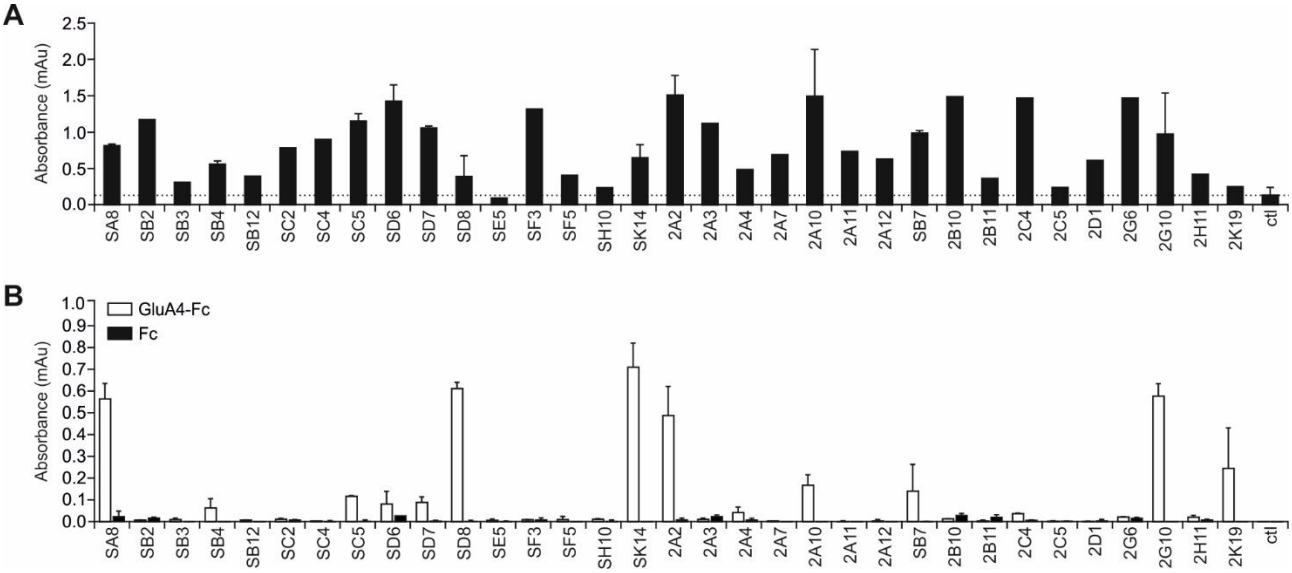

Figure S3: Screening and characterization of ribosome display selected GluA4-DARPins.

Crude *E. coli* extracts of randomly picked clones were analyzed for the expression of soluble DARPins (**A**) and tested for binding to recombinant GluA4-Fc (**B**) by ELISA. (**A**) 1 µl of crude *E. coli* extracts were coated on immunoplates. DARPins were detected using a biotinylated anti-His antibody in conjunction with HPR-conjugated streptavidin. (**B**) Recombinant GluA4-Fc or recombinant Fc protein was used to determine DARPins specific for GluA4 or the constant region of human IgG1 (huIgG1-Fc), which is present in the GluA4-Fc protein. DARPins were detected using an anti-His antibody in conjunction with a HRP-conjugated anti-mouse antibody.

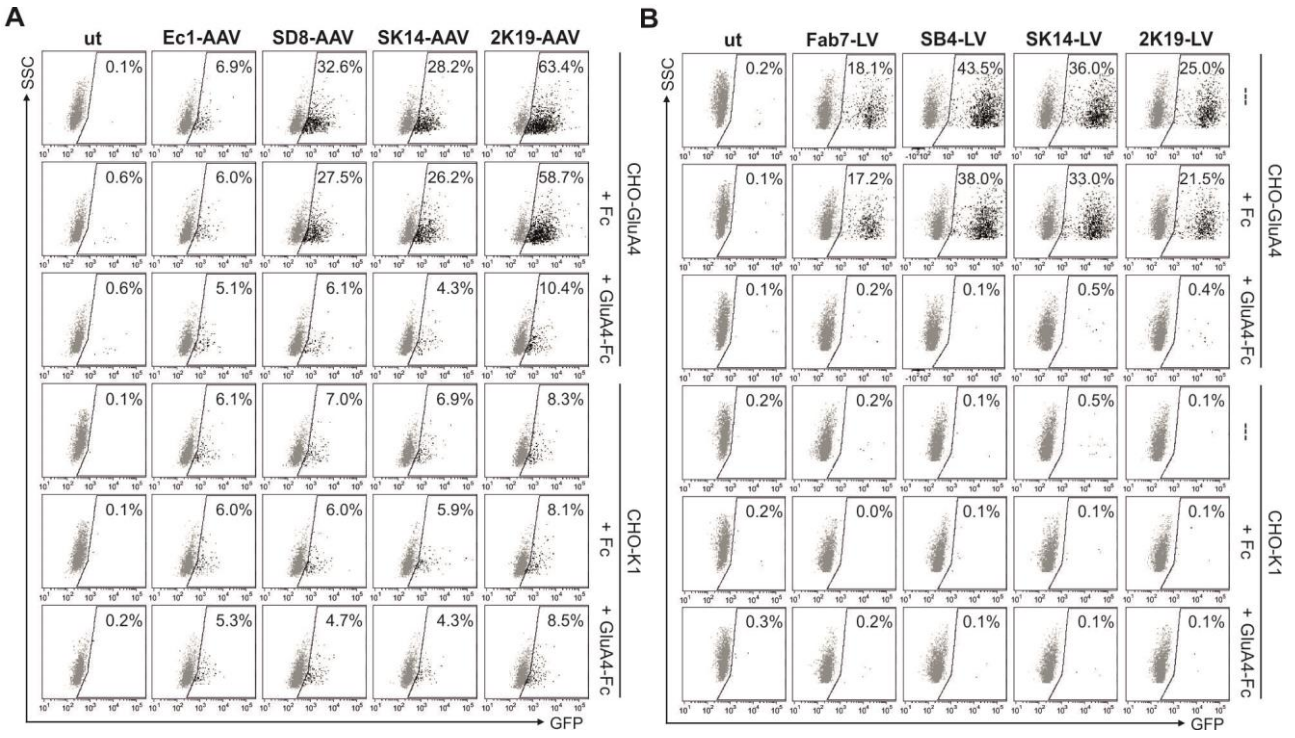

Figure S4: Competition assay evaluating DARPin mediated transduction.

For the best performing AAV (**A**) and LV (**B**) particles displaying the indicated DARPins a competition assay was performed by incubation of the vector particles with recombinant GluA4-Fc and Fc proteins, respectively or buffer as control prior to transduction of CHO-K1 or CHO-GluA4 cells. The cells were analyzed for GFP expression 72 h post transduction by flow cytometry. Untransduced cells and AAV particles displaying the EpCam-specific DARPin Ec1 were used as negative control. For LVs, particles displaying the scFv Fab7 were used as positive control. Unmodified AAV particles and VSV-G pseudotyped LV particles are used as control. Each transduction experiment was performed at least three times with individually produced vector particles, showing mean values and standard deviations (SD). Representative dot plots are shown. The percentage of GFP positive cells is indicated.

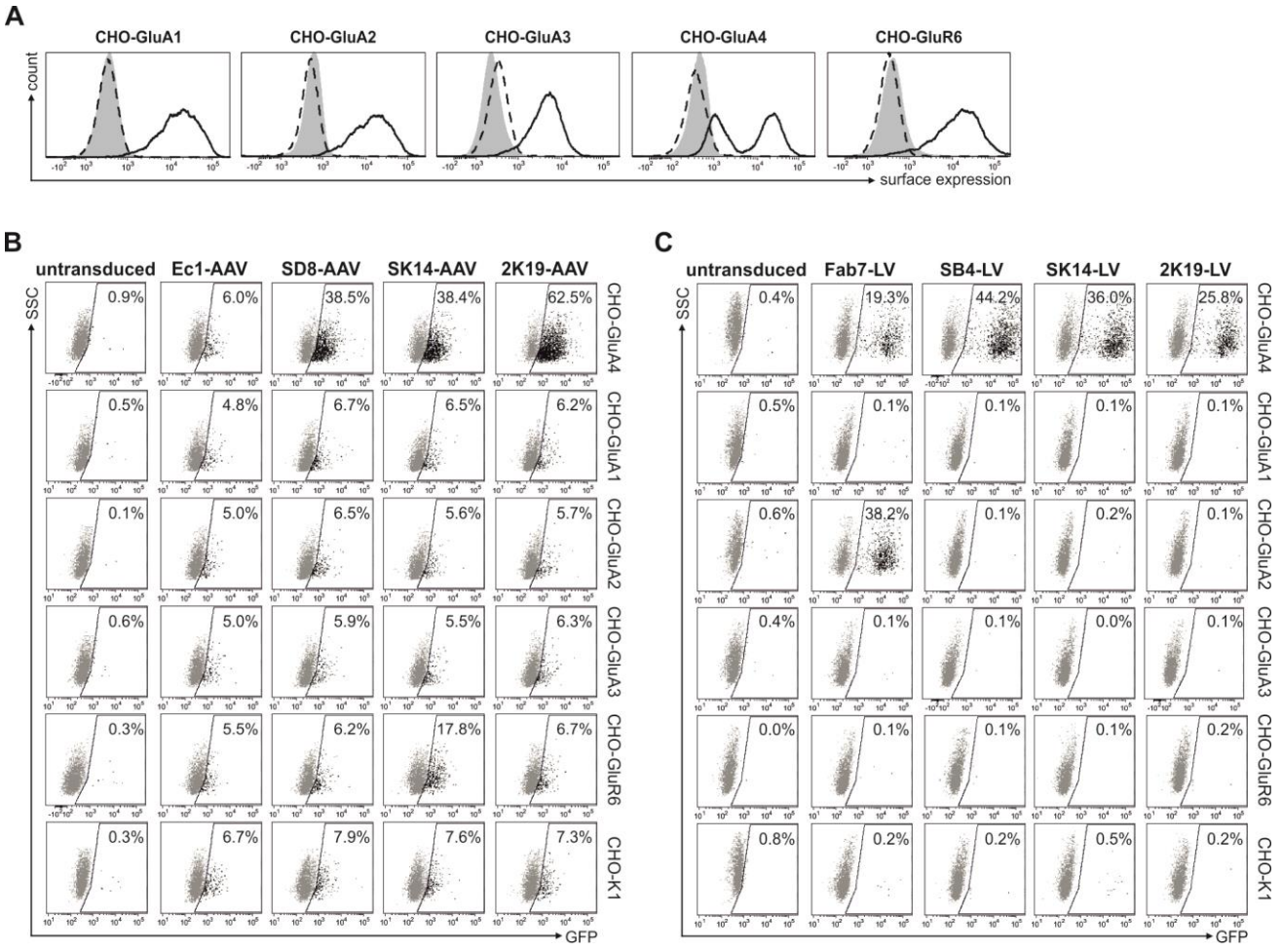

Figure S5: Transduction of a panel of CHO cells expressing various glutamate receptors.

(A) Representative flow cytometry histograms showing the cell surface expression of glutamate receptors as indicated after transduction of CHO-K1 cells with lentiviral vectors encoding the corresponding glutamate receptor. Cells were stained with a PE coupled myc tag specific antibody (solid black line) in the case of CHO-GluA4 cells and with a PE coupled Flag tag specific antibody (solid black line) in the case of CHO-GluA1, CHO-GluA2, CHO-GluA3 and CHO-GluR6 cells. As control cells were incubated with buffer only (filled curves) and compared to CHO-K1 cells stained with the respective fluorophore coupled antibody (dashed black lines). (**B-C**) CHO-K1 cells expressing GluA1-4 or GluR6 as well as the parental cell line were incubated with AAV (**B**) or LV (**C**) particles displaying the indicated DARPins. The cells were analyzed for GFP expression 72 h post transduction by flow cytometry. Untransduced cells and AAV particles displaying the EpCAM-specific DARPin Ec1 (**B**) or LV particles displaying the GluA2/4-specific scFv Fab7 (**C**) served as controls. Each transduction experiment was performed at least three times with individually produced vector particles, showing mean values and standard deviations. Representative dot plots are shown in the bottom panel. The percentage of GFP-positive cells is indicated.

A) S-N3C

```
     <------------------Ncap--------------------->|<------------AR1---------------->
SA8  MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASDWRGRTPLHAAAQKGHLEIVDVLLTHGADV
SB4  MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASDWRGRTPLHAAAQKGHLEIVDVLLAHGADV
SC5  MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASNWTGATPLHAAADWGHLEIVDVLLANGADV
SD6  MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASDWRGRTPLHAAAQKGHLEIVDVLLAHGADV
SD7  MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASDWRGRTPLHAAAQKGHLEIVDVLLAHGADV
SD8  MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASDWDGKTPLYLAARNGHLEIVDVLLAHGADV
SK14 MRGSHHHHHHAAQPAKDLGKKLLDAASAGQDDEVRILMANGADV|NASTWAGDTPLHLAARNGHLEIVDVLLAHGADV

     <------------AR2----------------->|<------------AR3----------------->|<-----------Ccap------------->
SA8  NASDHYGWTPLHTAAAYGHLEIVDVLLAHGADV|                                  |NANTYSGKTPFDLAIDNGNEDIAEVLQKAA
SB4  NASDSNGKTTLHVAAADGHLEIVDVLLANGADV|NASDHYGWTPLHTAAAYGHLEIVDVLLAHGADV|NANTYSGKTPFDLAIDNGNEDIAEVLQKAA
SC5  NASDTNGSTPLHAAASSGHLEIVDVLLANDADV|NASNHYGWTPLHTAASYGHLEIVDVLLAYGADV|NANTYSGKTPFDLAIDNGNEDIAEVLQKAA
SD6  NASDSNGKTTLHVAAADGHLEIVDVLLANGADV|NASNHYGWTPLHTAASYGHLEIVDVLLAYGADV|NANSYSGKTPFDLAIDNGNEDIAEVLQKAA
SD7  NASDTNGSTPLHAAASSGHLEIVDVLLANGADV|NASNHYGWTPLHTAASYGHLEIVDVLLAYGADV|NANTYSGKTPFDLAIDNGNEDIAEVLQKAA
SD8  NASDTNGSTPLHAAASSGHLEIVDVLLANGADV|NASNHYGWTPLHTAASYGHLEIVDVLLAYGADV|NANTYSGKTPFDLAIDNGNEDIAEVLQKAA
SK14 NASDTNGSTPLHAAASSGHLEIVDVLLANGADV|NASNHYGWTPLHTAAAWGHLEIVDVLLANGADV|NANSYSGKTPFDLAIDNGNEDIAEVLQKAA
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B) VV-N2C

```
     <------------------Ncap--------------------->|<------------AR1---------------->
2A2  MRGSHHHHHHAAQPADLGKKLLEAARAGQDDEVRILMANGADV|NAQDWEGRTPLHLAAHNSHLEIVEVLLKHGADV
2A10 MRGSHHHHHHAAQPADLGKKLLEAARAGQDDEVRILMANGADV|NALDHWGTTPLHLAAWSGHLEIVEVLLKNGADV
2B7  MRGSHHHHHHAAQPADLGKKLLEAARVGQDDEVRILMANGADV|NANDYHGSTPLHLAADYGHLEIVEVLLKYGADV
2G10 MRGSHHHHHHAAQPADLGKKLLEAARAGQDDEVRILMANGADV|NAQDWEGRTPLHLAAHNSHLEIVEVLLKHGADV
2K19 MRGSHHHHHHAAQPADLGKKLLEAARAGQNDEVRILMANGADV|NAIDMAGRTPLHLAAWSGHLEIVEVLLKYDADV

     <------------AR2----------------->|<-----------Ccap------------->
2A2  NAFDWYGNTPLHQAAAQGHLEIVEVLLKYGEDV|NAQDKFGKTPFDLAIDNGNEDIAEVLQKAA
2A10 NATDYQGRTPLHLAAVMGHLEIVEVLLKNGADV|NAQDKFGKTPFDLAIDNGMKILPKCCRKQ
2B7  NALDNMGMTPLHLAAQWGHLEIVEVLLKNGADV|NAQDKFGKTPFDLAIDNGNEDIAEVLQKAA
2G10 NAFDWYGNTPLHQAAAQGHLEIVEVLLKYGEDV|NAQDKFGKTPFDLAIDNGNEDIAEVPESSA
2K19                                  |NATDHFGLTPLHLAASDGHLDIAEVLQKAA
```

Figure S6: Sequence alignment of 12 DARPins selected for GluA4 binding via ribosome display.

Sequence alignment of DARPin candidates selected from the S-N3C library (A) and the VV-N3C library (B). The amino acid sequence was determined by sequencing of the corresponding pQE plasmid DNA. The diversified positions are highlighted in bold. Color code indicates the amino acid (AA) property (red, small and hydrophobic AA including aromatic AA; blue, negatively charged AA; lilac, positively charged AA; green, polar AA including tyrosine and histidine). Point mutations within the framework regions are shown in italic-black.

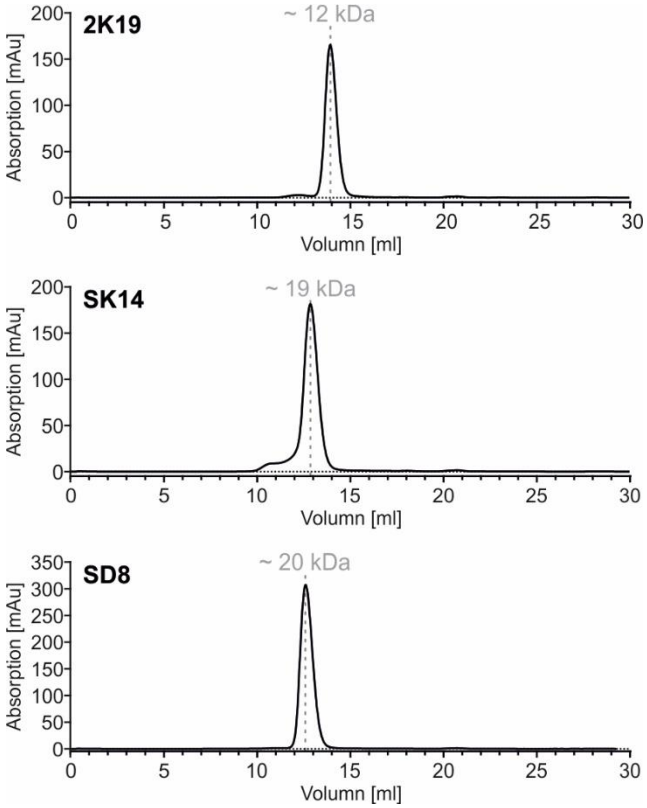

Figure S7: Size exclusion chromatography of purified DARPins.

Size exclusion chromatogram of the indicated DARPins expressed in *E. coli* and purified via His-tag affinity chromatography. The calculated molecular weight of the corresponding peak is indicated.

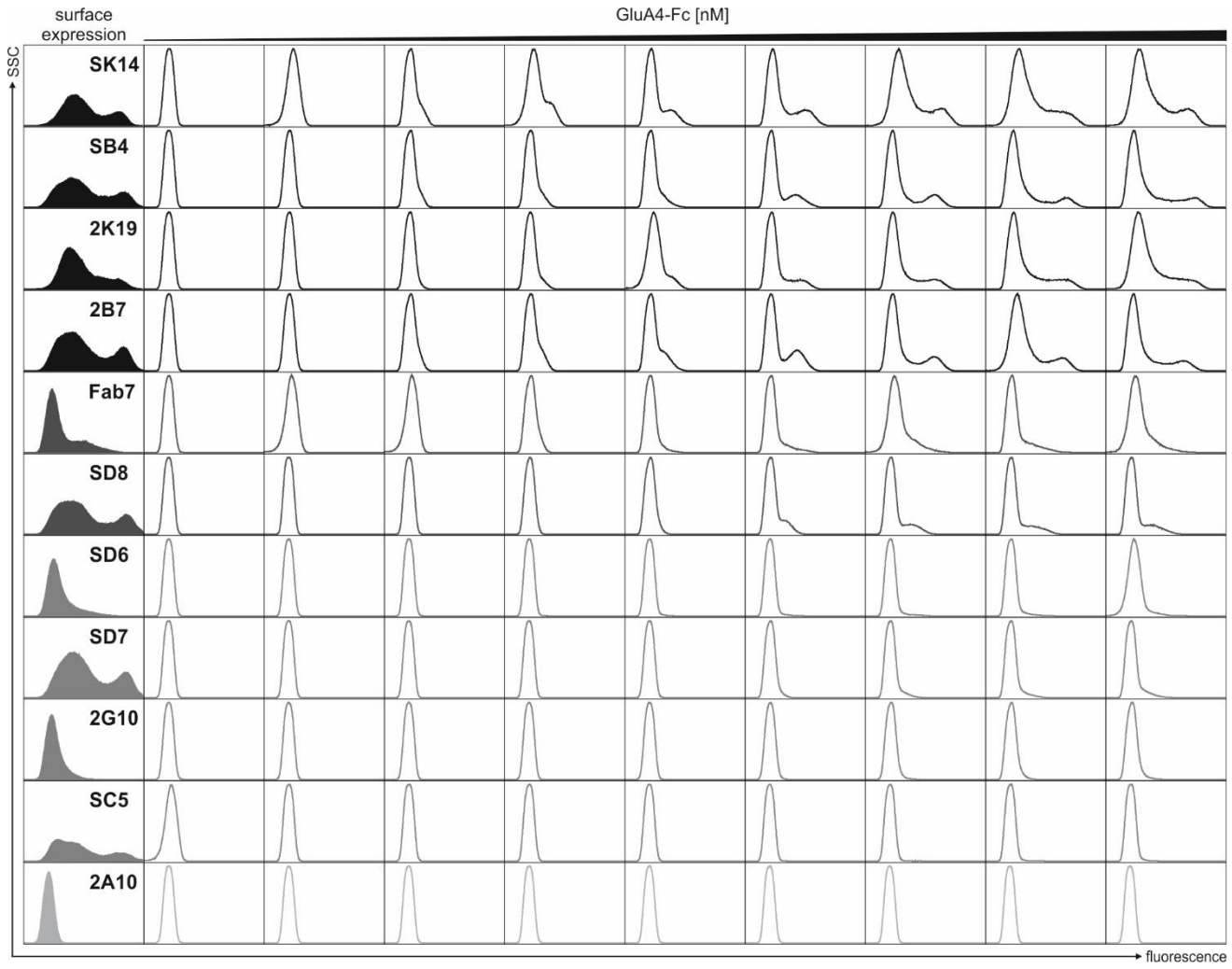

Figure S8: Binding of recombinant GluA4 to selected GluA4-DARPins.

Representative flow cytometry histograms obtained after transient transfection of HEK293T cells with expression plasmids encoding the fusion protein composed of H and the indicated DARPin. The Fab7-H fusion protein served as positive control. Transfected cells were either stained with a PE-coupled His-tag specific antibody to determine the surface expression or were incubated with increasing molar concentrations of recombinant GluA4-Fc protein before staining with a huIgG1-Fc specific antibody (rows 2-10).

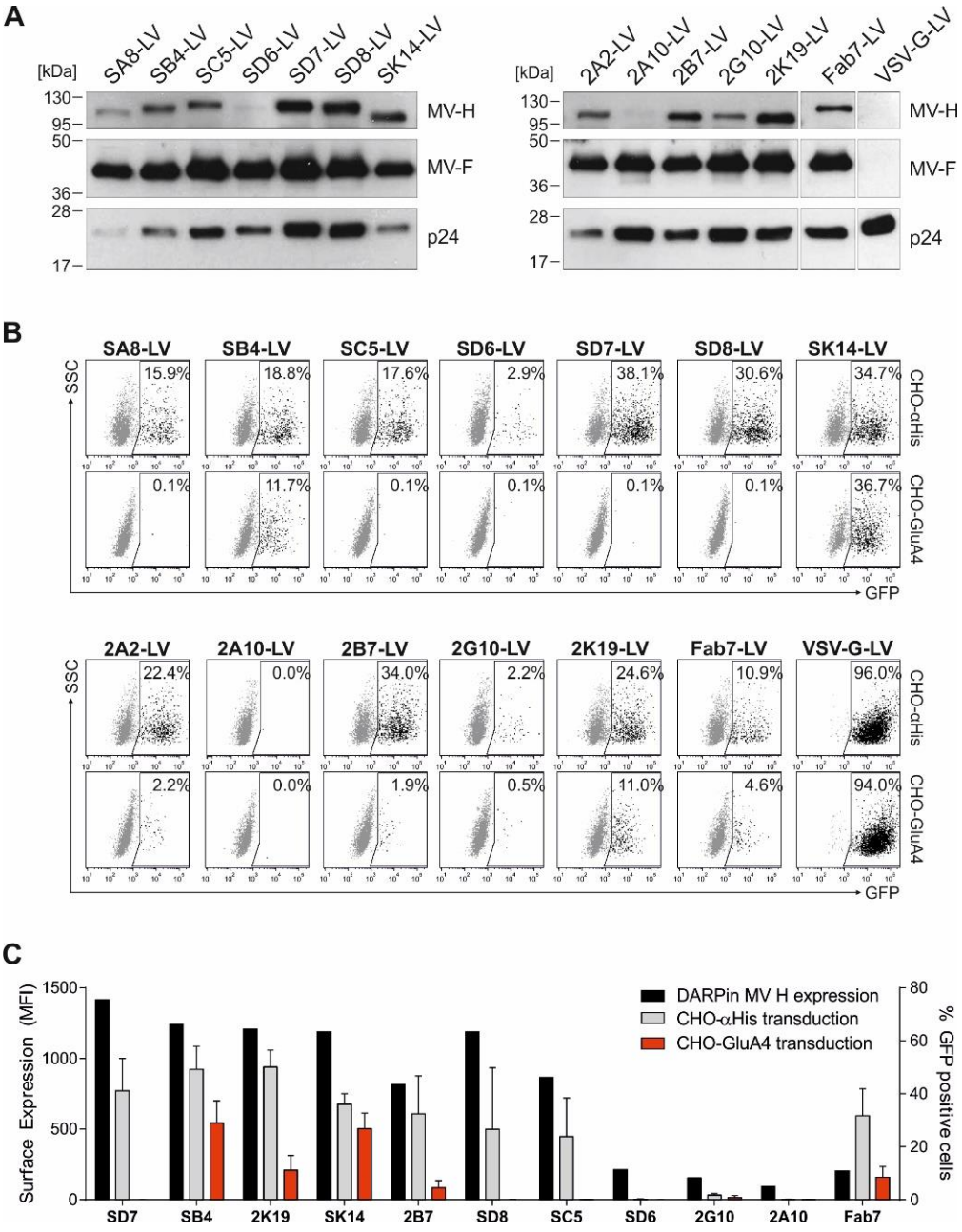

Figure S9: Comparision of H-DARPin and F incorporation into LV vectors and vector transduction efficacy.

(A) Western Blot analysis of the indicated LVs. Particle amounts corresponding to 5 µl concentrated vector stock were loaded onto a 10% SDS gel. Proteins were detected utilizing anti-MV-F, anti-MV-H, or anti-p24 antibodies. LV particles displaying the scFv Fab7 instead of a DARPin or pseudotyped VSV-G were used as controls. **(B)** Transduction of CHO-αHis or CHO-GluA4 cells with 1 µl of indicated concentrated vector stocks used for western blot analysis in (A). Cells were analyzed for GFP expression 72 h post transduction by flow cytometry. The percentage of GFP positive cells is indicated. **(C)** Bar diagram of DARPin-H cell surface expression and transduction of CHO-αHis or CHO-GluA4 cells of indicated DARPins and the scFv Fab7 from Figure 7. Left scale: median fluorescent intensities (MFI) of surface stained DARPin-H or Fab7-H proteins after transient transfection of HEK293T cells with the corresponding expression plasmids. Right scale: percentage of GFP-positive CHO-αHis or CHO-GluA4 cells transduced with LV particles displaying the indicated DARPins or Fab7. Each transduction experiment was performed at least three times with individually small scale produced vector particles, showing mean values and standard deviations.

Supplementary Tables

Table S1: Calculated diversity of the assembled DARPin libraries and intermediates.

Fragment[1]	Size	Amount of DNA[2]	Diversity[3]
Ncap-DAR (N1)	228 bp	910 ng	$3.89x10^{12}$
Ncap-DAR-DAR (N2)	327 bp	5835 ng	$1.74x10^{13}$
Ncap-DAR-DAR-DAR (N3)	426 bp	8364 ng	$1.90x10^{13}$
Ncap-DAR-DAR-Ccap (N2C)	393 bp	5736 ng	$1.42x10^{13}$
Ncap-DAR-DAR-DAR-Ccap (N3C)	492 bp	9240 ng	$1.83x10^{13}$
T7-N2C-Spacer	1300 bp	40.8 µg	$3.06x10^{13}$
T7-N3C-Spacer	1399 bp	53.8 µg	$3.75x10^{13}$

[1] For product nomenclature refer to Fig. S1

[2] Recovered ligation products after gel extraction

[3] Calculated theoretical diversity based on the fragment size and amount of recovered DNA.

Table S2: Primer sequences for plasmid construction.

Primer	No.	Sequence (5' to 3')
huFc for	1	CTAGCTAGCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAAC
huFc-G4S-Avi rev	2	CGAGGCTGATCAGCGAGCTTCTAGATATTATTCATGCCACTCAATCTTCTGAGCTTCGA AAATGTCGTTAAGGCCGGAGCCCCCTCCGCCTTTACCCGGGGACAGGGAG
BirA for	3	TATCAGGGCCCAGCCGGCCAGATCTATGAAGGATAACACCGTGCCACTG
BirA-ER rev	4	CCGCTCGAGCGGCCGCGTCGACTCACAGCTCGTCCTTTGAACCCCCAGATCCAGATGT AGA CCCTTTTTCTGCACTACGCAGGGATATTTC
BirA rev	5	GAGGCTGATCTCGAGCGGCCGCGTCGACTCATTTTTCTGCACTACGCAGGGATATTTC
HA-Oligo for	6	AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCACCATCACCATCACGG TTCTTATCCATATGATGTTCCAGATTATGCTGCGGCCCAGC
HA-Oligo rev	7	GGGCCGCAGCATAATCTGGAACATCATATGGATAAGAACCGTGATGGTGATGGTGAT GCGATCCTCTCATAGTTAATTTCTCCTCTTTAATG
3xSTOP Oligo for	8	CGGCCCAGGTCCAGCTGCAGGAATCCGGGCCCAGATCTGCCGGCCGCGGCCGCTTAA TTGATTGACACGCAGTGTGATTGATTGAGCCAGTGGCGTGGTTAAT
3xSTOP Oligo rev	9	TAACCACGCCACTGGCTCAATCAATCACACTGCGTGTCAATCAATTAAGCGGCCGCGG CCGGCAGATCTGGGCCCGGATTCCTGCAGCTGGACCTGGGCCGGCT
CD015-DARPin for	10	AAATTTGGCCCAGCCGGCCGACCTGGGTAAGAAACTGCTGGAAG
CD105-DARPin rev	11	TGTACAGAGCGGCCGCATTAAGCTTTTGCAGGATTT

Table S3: DNA sequence of codon optimized DNA fragments used for DARPin library assembly.

Fragment	Sequence (5' to 3')[1]
Ncap	GGCCCAGCCGGCC**GACCTGGGTAAAAAACTGCTGGAAGCAGCACGTGCAGGTCAGGATGATGAAGT TCGTATTCTGATGGCAAATGGTGCAGAC**GTGAGACCTTAGGAATTC
Ccap	GCTAGCTAGGAAGACCT**GACGTTAATGCCCAGGATAAATTTGGTAAAACACCGTTTGATCTGGCCA TTGATAATGGCAATGAAGATATTGCCGAAGTGCTGCAGAAAGCAGCA**GCGGCCGCTCACGCAGTGG AATTC
AR	GGATCCTAGGAAGACCT**GACGTTAACGCA*NNN*GAT*NNNNNN*GGT*NNN*ACACCGCTGCATCTGGCAG CA*NNNNNN*GGTCATCTGGAAATTGTTGAAGTGCTGCTGAAA*NNN*GGTGCAGACGTG**AGACCTTAGA AGCTT

[1]Coding regions are depicted in bold.

Table S4: Primer sequences for DARPin library generation.

Primer	**No.**	**Sequence (5’ to 3’)**
Ncap for	1	CGCGGATCCGACCTGGGTAAAAAAC
AR rev	2	AGATCTAGGCCTTCTAGACCCAAGC
Lib for	3	AAATTTGGCCCAGCCGGCCGACCTGGGTAAAAAAC
Lib rev	4	CCGGAATTCCACTGCGTGAGCGGCCGCTGCTGCTTTCTGCAGCACTTCGGCAATATC
T7 for	5	AGGGAGAAAGGCGGACAGGTATCCGGTAAGCG
T7 rev	6	AACTATCAGGGCCGGCTGGGCCGCGTGATG
Spacer for	7	ATAAGAATGCGGCCGCTCACGCAGTGGAATTCGGATCTGGTGGCCAGAAG
Spacer rev	8	AAAGGGAATAAGGGCGACACGG
T7B	9	ATACGAAATTAATACGACTCACTATAGGGAGACCACAACGG
TolAk	10	CCGCACACCAGTAAGGTGTGCGGTTTCAGTTGCCGCTTTCTTTCT

Supplementary Methods

DARPin detection in crude cell extract and binding assay by ELISA – Detection of DARPins within crude *E. coli* extracts and DARPin binding analysis was performed on the basis of previously described protocols[1,2]. In brief for detection of DARPins within crude *E. coli* extracts, 96-well Maxisorb plates (Nunc) were coated with 1 µl of crude DARPin containing *E. coli* extracts in a total volume of 100 µl TBS overnight at 4°C. After washing the wells three times with 300 µl TBS-T (TBS, 0.1% Tween-20), unspecific binding was blocked by addition of 300 µl TBS-TB (TBS, 0.1% Tween-20, 0.5% BSA) per well for 1 h at room temperature. Subsequently, wells were incubated with a biotin-conjugated mouse anti-PentaHis antibody (1:2000; Qiagen, 34440) and HRP-conjugated streptavidin (1:500; Jackson ImmunoResearch, 016-030-084) for 1 h at room temperature. The plates were washed three times with TBS-T after each antibody incubation step. The bound antibodies were detected using SureBlue TBM substrate (KPL) and 1 N H_2SO_4. The reaction product was quantified in a microtiter plate reader at 450 nm.

To determine target binding of DARPins within crude *E. coli extracts,* 96-well Maxisorb plates (Nunc) were coated with 20 µM neutravidin diluted in TBS for 1 h at room temperature. After washing the wells three times with 300 µl TBS-T, unspecific binding was blocked by addition of 300 µl TBS-TB per well for 1 h at room temperature. Next, wells were incubated with 20 nM recombinant target protein diluted in TBS-TB overnight at 4°C. After washing the wells three times with 300 µl TBS-T, each well was incubated with 2.5 µl of crude DARPin containing *E. coli* extracts in a total volume of 100 µl TBS for 1 h at 4°C. Wells were washed three times with 300 µl TBS-T before subsequent incubation with a mouse anti-RGSHis antibody (1:2000; Qiagen, 34650) and a HRP conjugated rabbit anti-mouse antibody (1:2000; DAKO, P0260) for 1 h at room temperature. The plates were washed three times with TBS-T after each antibody incubation step. The bound antibodies were detected using SureBlue TMB substrate (KPL) and 1 N H_2SO_4. The reaction product was quantified in a microtiter plate reader at 450 nm.

Surface Expression – Flow cytometry analysis was performed on the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). Surface expression of GluA1-4 and GluR6 on the respective CHO cell line was detected by staining with PE-coupled myc antibody (clone 9B11; 1:100; Cell Signaling Technology) in case of CHO-GluA4, or PE-coupled FLAG antibody (clone M2; 1:100; Abcam) for all other investigated cell lines. As control, cells were incubated without antibody.

LV particle production for SDS-PAGE – Lentiviral vector were generated by transient transfection using PEI as described previously with some minor modifications as indicated. In brief, 24 h prior to transfection, $9x10^6$ HEK-293T cells were seeded in one T75 flask. On the day of transfection, the cell culture medium was replaced by 10 ml DMEM+FCS. For the transfection mix, 15 µg of total DNA was mixed with 990 µl of DMEM without additives and added to 930 µl DMEM supplemented with 60 µl of 18 mM PEI solution per T75 flask. After incubation for 20 minutes at room temperature, the transfection mix was added to the HEK-293T cells. 24 h later, the medium was replaced by 10 ml fresh cell culture medium. At day two post transfection, the cell supernatant was filtered (0.45 µm filter) and concentrated and purified by centrifugation at 4,500x g over a 20% sucrose cushion for at least 24 hours at 4°C. The pellet was resuspended in 50 µl PBS. For LV-targeting vectors, 580 ng of DARPin-H or scFv-H plasmid, 1.73 µg of MV-F plasmid, 6.2 µg of the packaging plasmid pCMVΔR8.9 and 6.5 µg pSEW were used for transfection. LV particles pseudotyped with VSV-G were produced by co-transfection of 2.6 µg pMD.G2 along with 4.9 µg pCMVΔR8.9 and 7.5 µg pSEW.

Supplementary References

1. Dreier, B. and Plückthun, A. (2012). Rapid selection of high-affinity binders using ribosome display. Methods Mol. Biol. 805, 261–286.
2. Steiner, D., Forrer, P. and Plückthun, A. (2008). Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. J. Mol. Biol. 382, 1211–1227.