DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display and engineered for multivalency

Nikolas Stefan [1, 3], Patricia Martin-Killias [1, 3], Sascha Wyss-Stoeckle[1], Annemarie Honegger[1], Uwe Zangemeister-Wittke [1,2] and Andreas Plückthun[1*]

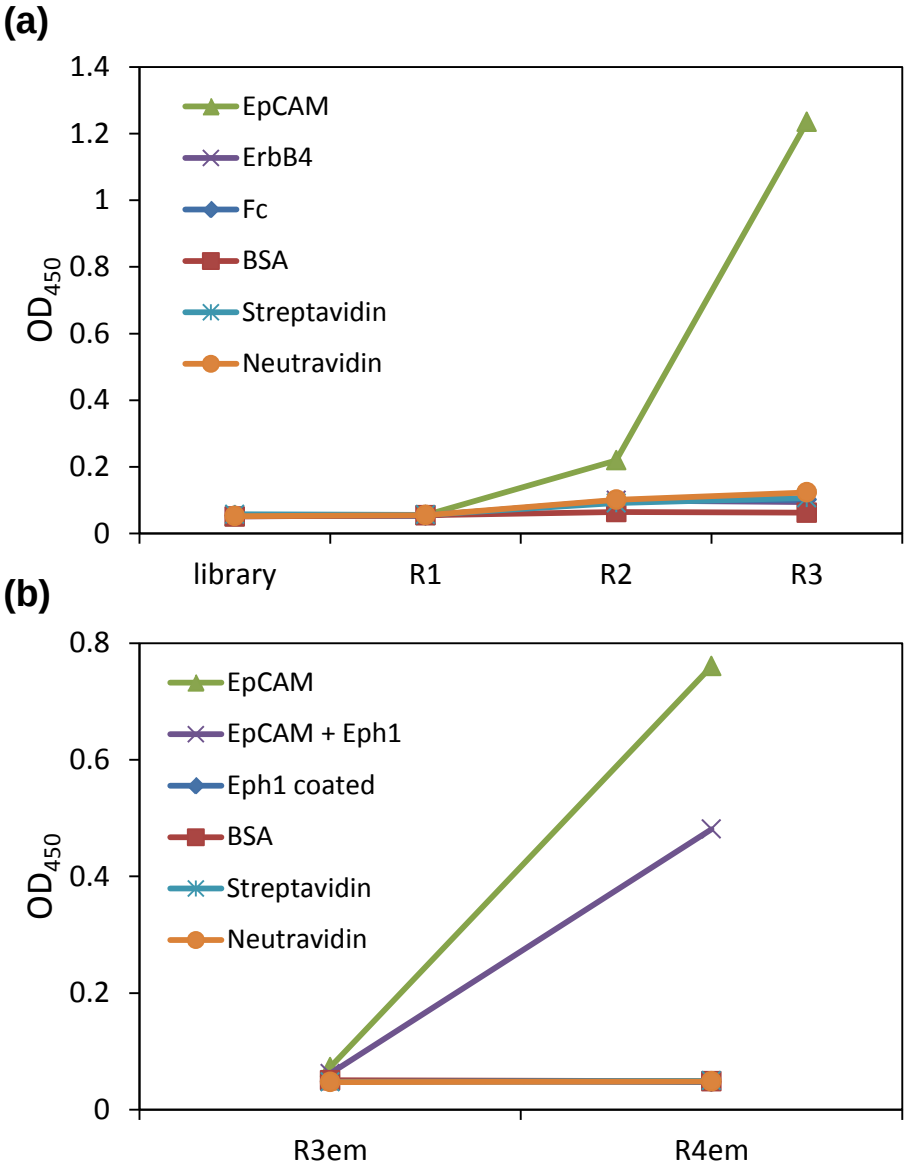
SUPPLEMENTARY FIGURES and LEGENDS

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

[2] Institute of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern

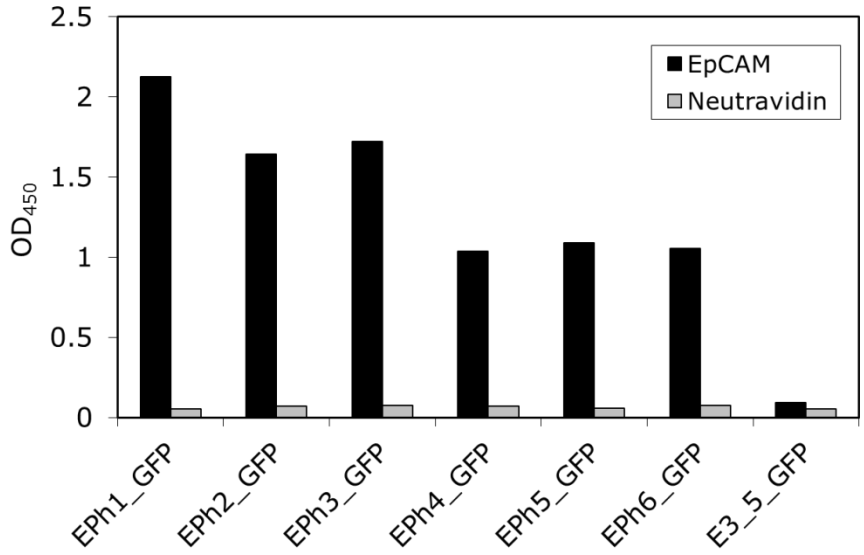
[3] These authors contributed equally to this work

* Corresponding author: Email: plueckthun@bioc.uzh.ch, Tel: +41-44-635 5570, FAX: +41-44-635 5712

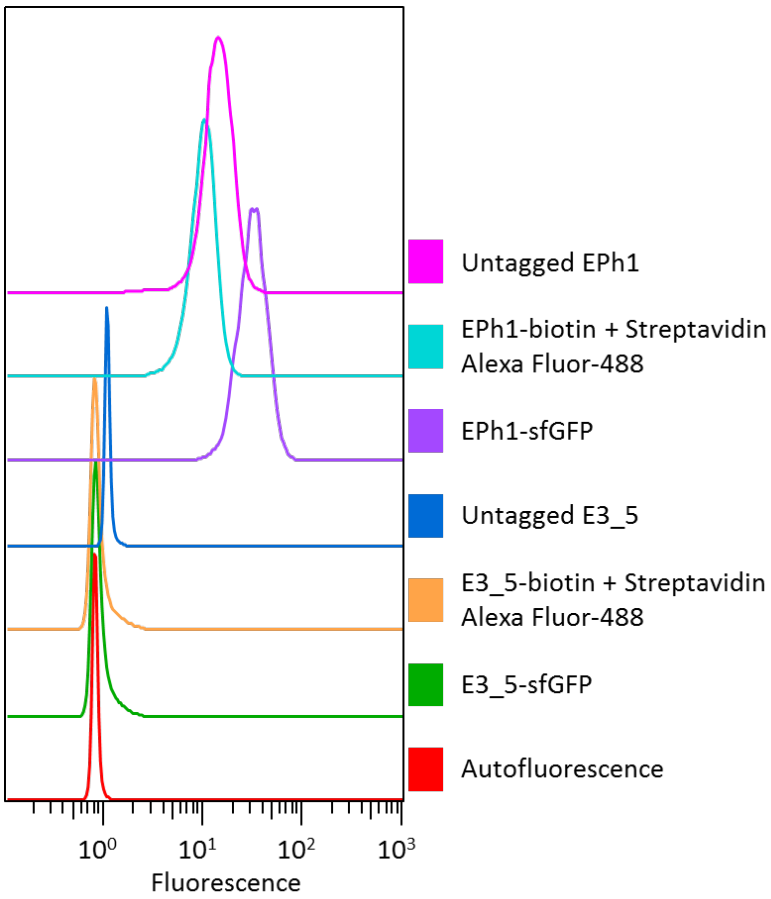


Supplementary Figure 1. (a) Enrichment of EpCAM binders by phage ELISA. BSA, streptavidin, neutravidin, biotinylated EpCAM or biotinylated ErbB4 (bound to streptavidin), or human IgG1 Fc were immobilized on a MaxiSorp 96-well plate (Nunc) and equal amounts of the initial phage library or of the output phages after each round (R1, R2 and R3) were added to the plate. The bound phages were detected using an anti-M13 antibody horseradish peroxidase (HRP) conjugate. (b) Enrichment of EpCAM binders after epitope masking by phage ELISA. Specific enrichment of phages from round 3em and 4em (epitope masking) was analyzed as in (a). In addition, enrichment of phages against EpCAM was tested in the presence of an excess of the blocking agent EPh1 (EpCAM + EPh1). Biotinylated EPh1 was coated to the neutravidin plates to test for enrichment against the blocking agent.

(a)

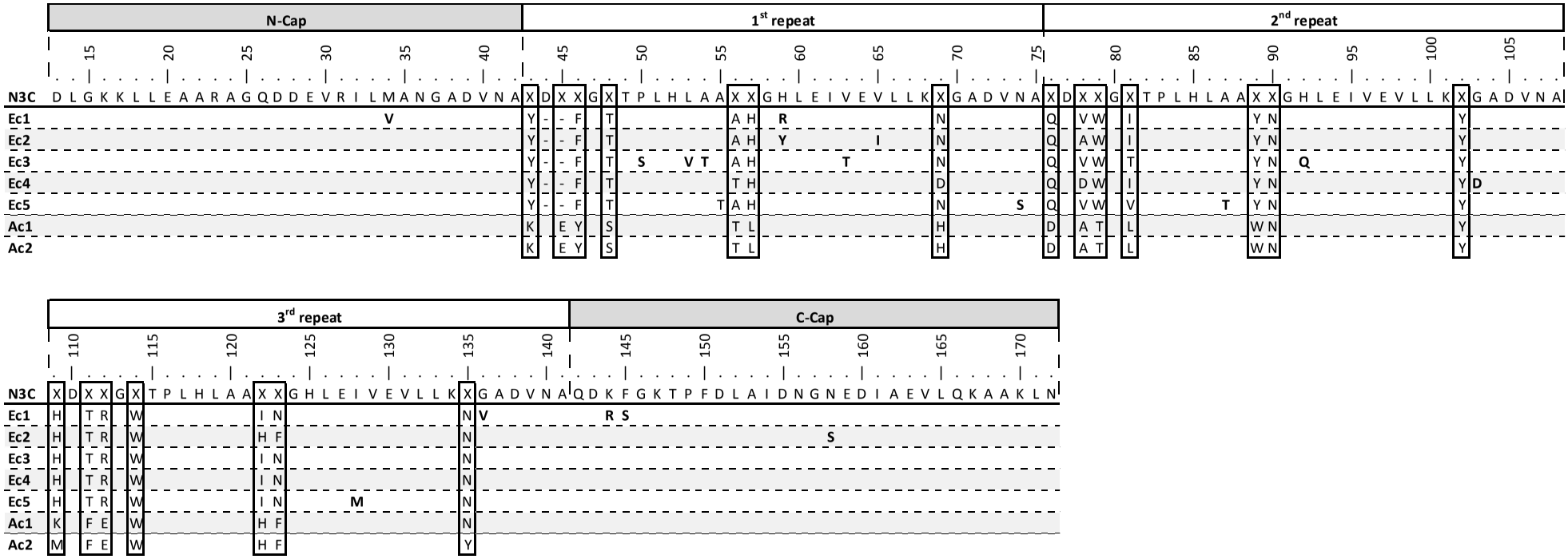


(b)

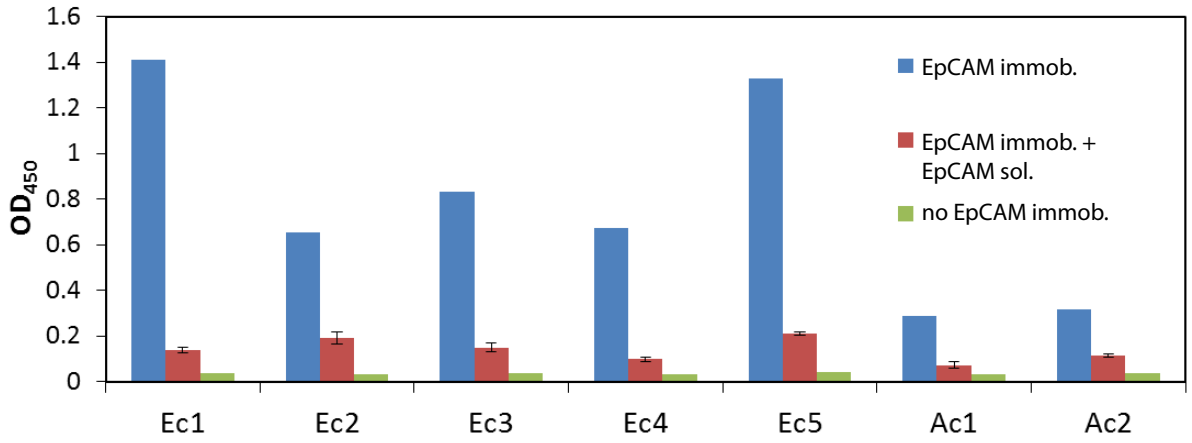


Supplementary Figure 2. (a) ELISA of EpCAM-specific binders fused to sfGFP. DARPins fused to superfolder GFP (sfGFP) were analyzed for EpCAM binding by ELISA. Biotinylated EpCAM (20 nM) was immobilized on neutravidin coated plates. Binding of DARPin-sfGFP fusion was detected using a

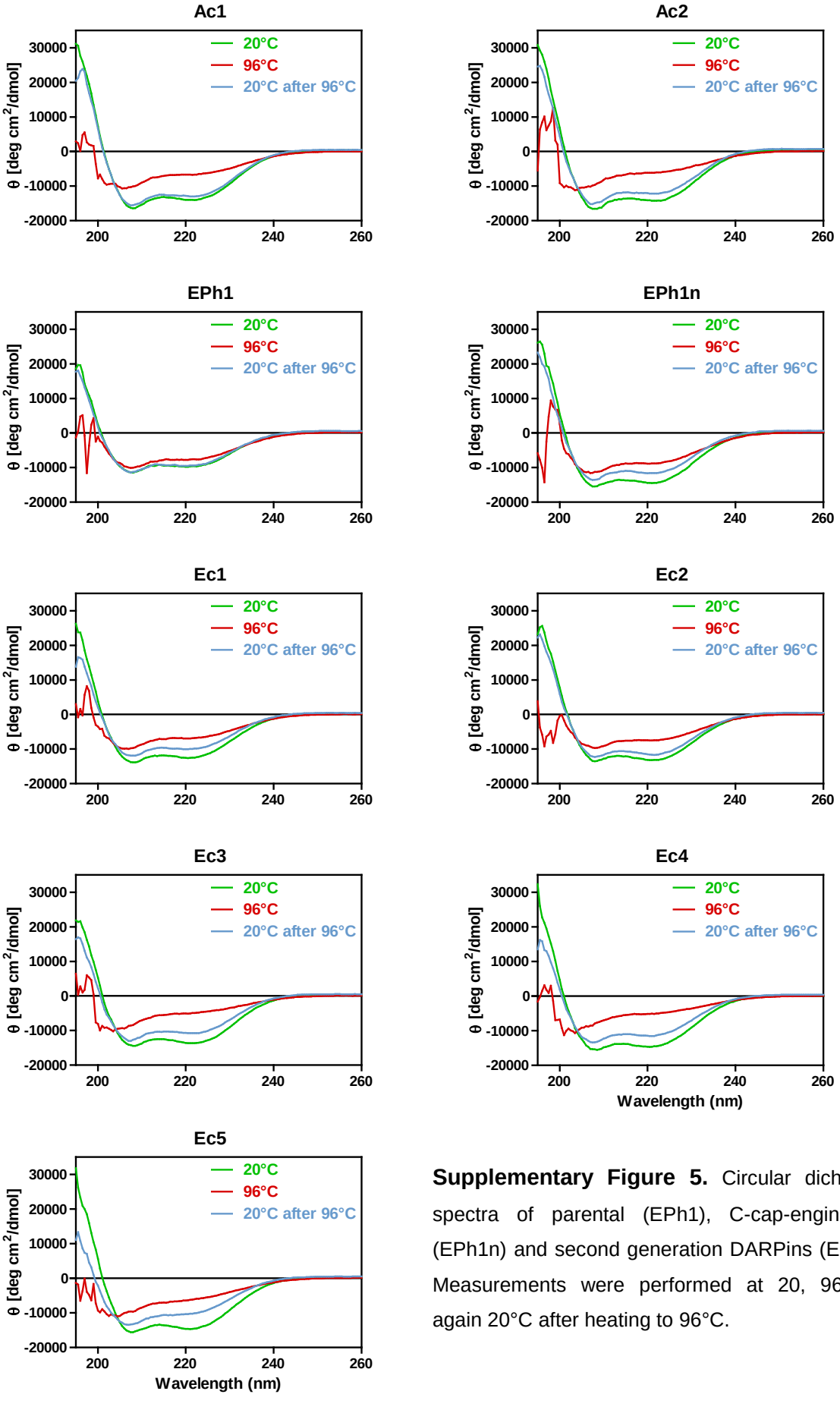
mouse anti-RGS(His)$_4$ as primary and alkaline phosphatase-coupled goat anti-mouse IgG as secondary antibody. E3_5-sfGFP, an unselected member of the library, is shown as a negative control. (b) Flow cytometry analysis of MCF-7 cells with EPh1-sfGFP, EPh1-biotin, and untagged Eph1. EPh1-sfGFP; EPh1-biotin (*in vivo* biotinylated at C-terminal Avi-tag, detected with streptavidin Alexa Fluor-488); and untagged EPh1 or, as control, an unselected member of the library E3_5 (E3_5-sfGFP, E3_5-biotin and untagged E3_5) were incubated with MCF-7 cells for 45 minutes at 4°C. Untagged EPh1 and E3_5 were detected with anti-RGS(His)$_4$ primary antibody and anti-mouse FITC labeled secondary antibody. Cells were analyzed by flow cytometry.



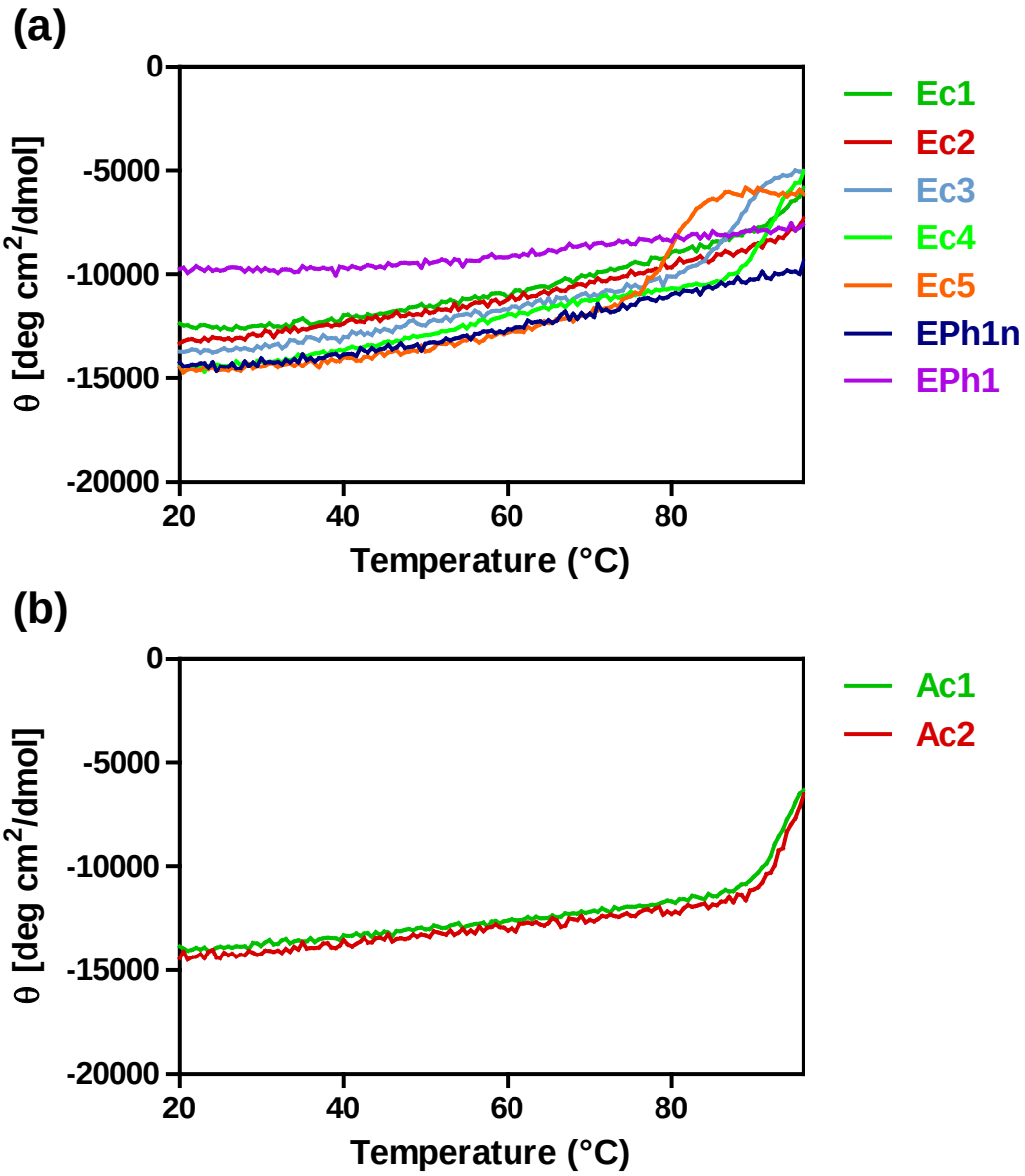
Supplementary Figure 3. Sequences of selected DARPins. The sequences of different DARPins are shown. Randomized positions in the N3C family sequence (first line) are indicated by X. In the lower lines, only residues that differ from the consensus sequence of an N3C DARPin are printed. Residues that had been randomized in the original design are boxed. Mutations that showed up at framework positions (outside the boxes) are printed in bold. Two major sequence families were found after optimization by ribosome display, termed Ec and Ac. The characteristic deletion of the Ec family missing two amino acids between the N-terminal capping repeat and the first internal repeat is indicated by dashes at positions 44 and 45.



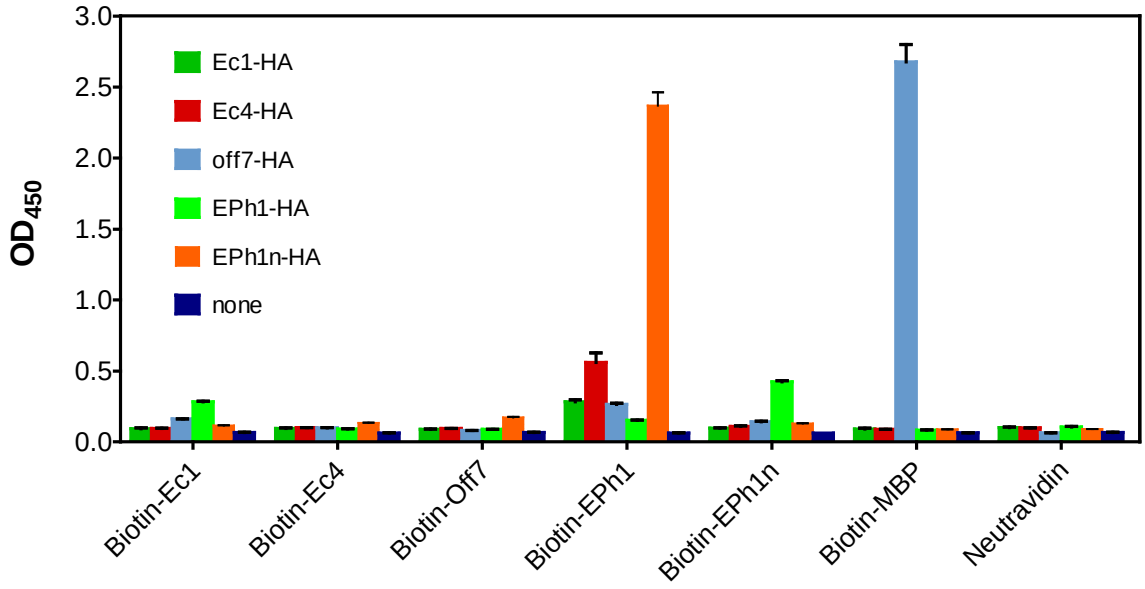
Supplementary Figure 4. Competition ELISA. Biotinylated EpCAM (10 nM) was immobilized on streptavidin-coated plates before EpCAM binders (10 nM) were allowed to bind for 10 min. For competition, binders were preincubated with a 10-fold excess of non-biotinylated EpCAM. Binding was detected with mouse anti-RGS(His)$_4$ as primary and alkaline phosphatase-coupled goat anti-mouse IgG as secondary antibody.



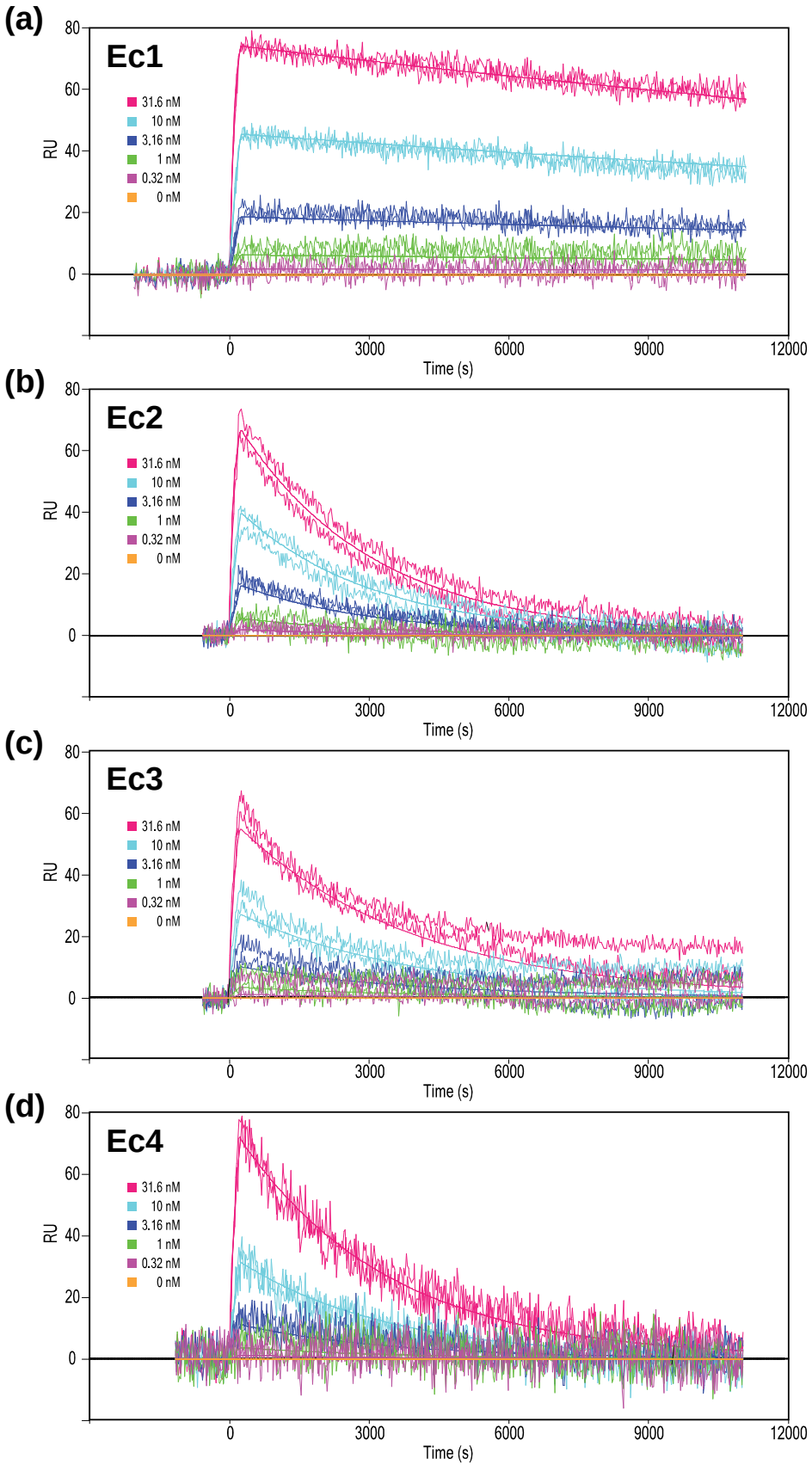
Supplementary Figure 5. Circular dichroism spectra of parental (EPh1), C-cap-engineered (EPh1n) and second generation DARPins (Ec1-5). Measurements were performed at 20, 96 and again 20°C after heating to 96°C.

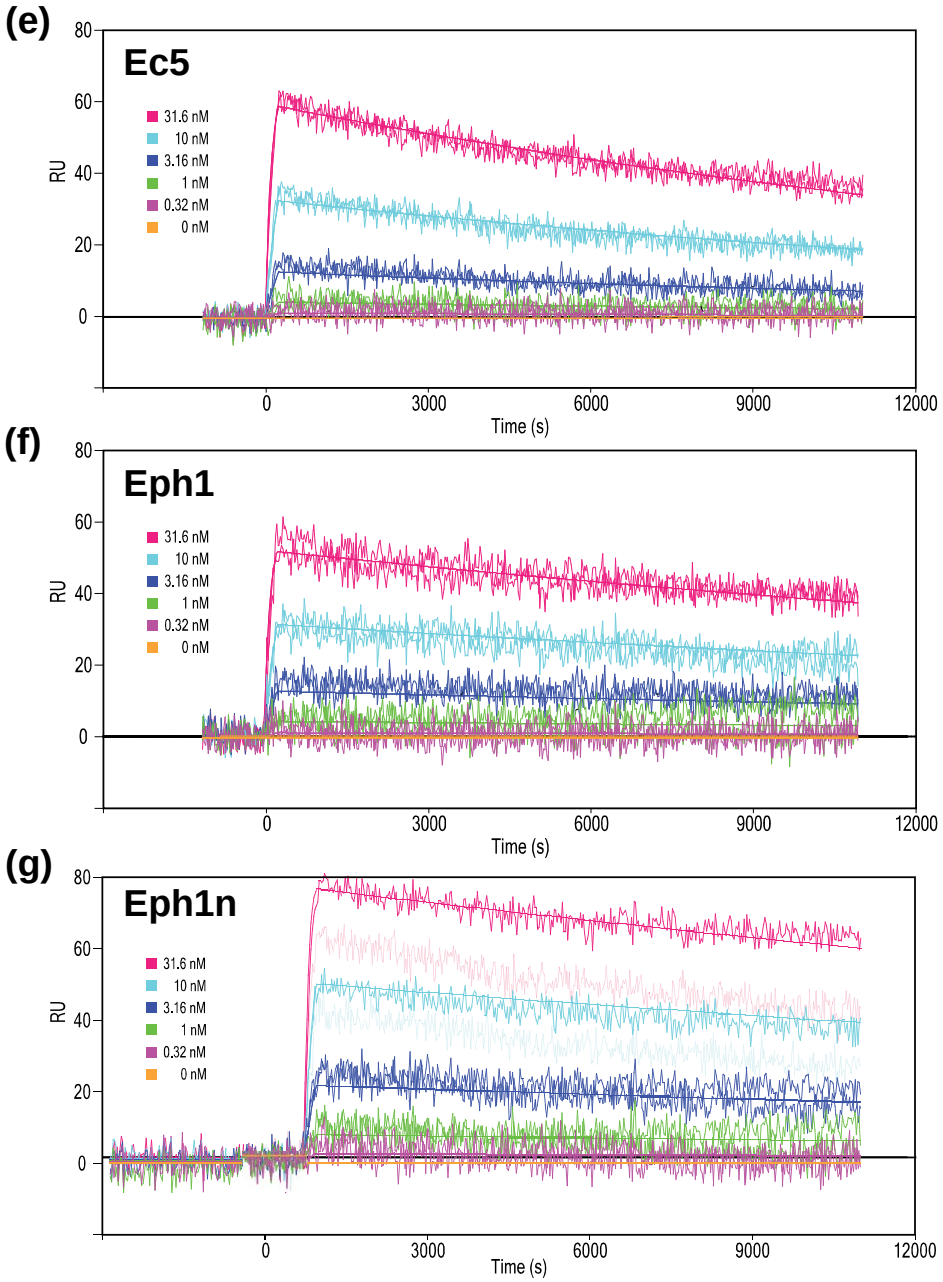


Supplementary Figure 6. Thermal denaturation of (a) parental (EPh1), C-cap-engineered (EPh1n) and second generation DARPins (Ec1-5) and (b) second generation DARPins of the Ac series. The denaturation was monitored by detecting the CD signal at 222 nm.

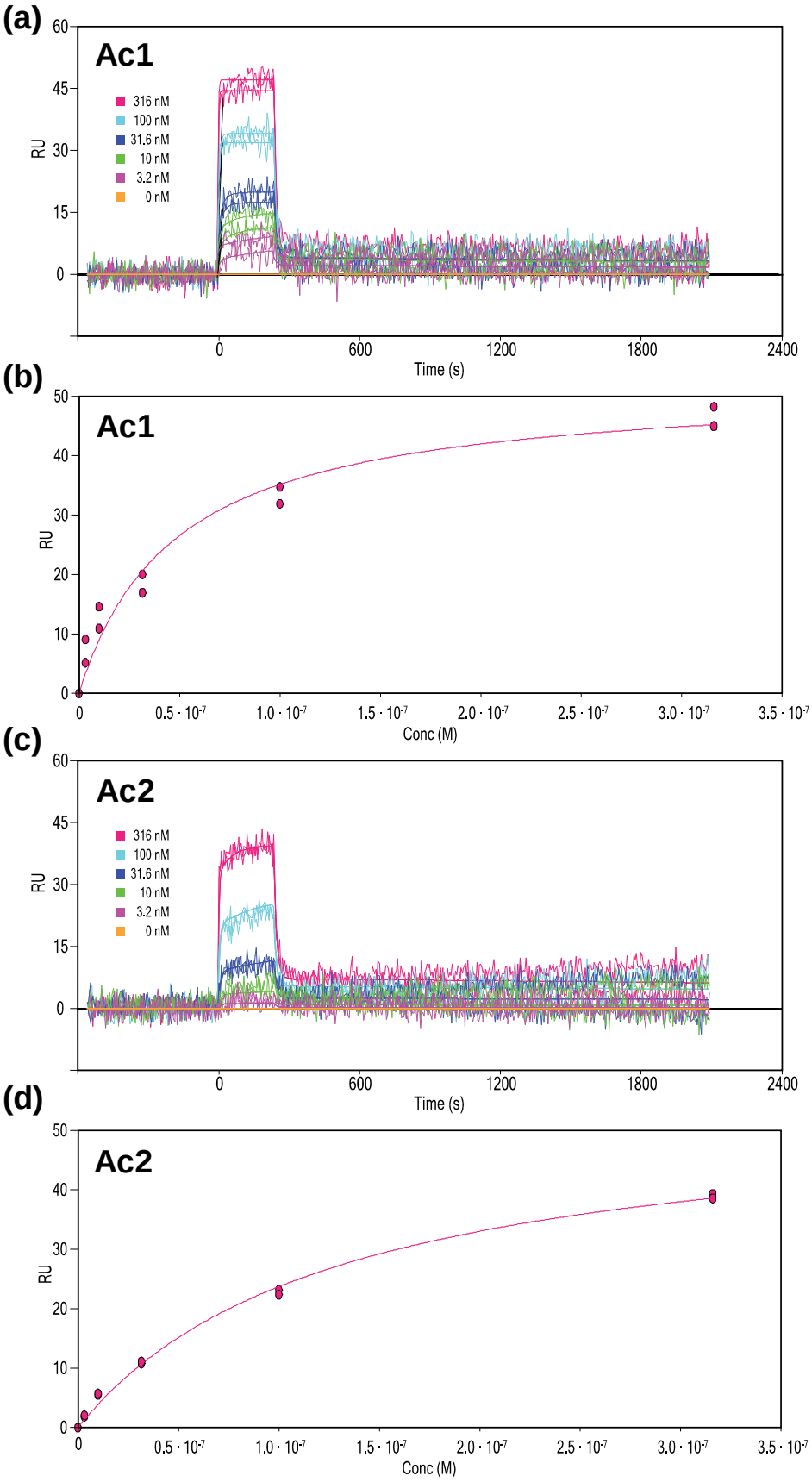


Supplementary Figure 7. ELISA self-binding assay. 100 nM DARPins *in vivo* biotinylated at an N-terminal Avi-tag and 100 nM DARPins with a C-terminal hemagglutinin (HA) tag were mixed and incubated overnight at 4°C. This DARPin mixture (100 µl) was then allowed to bind to neutravidin-coated wells for 1 h at 4°C. After washing, complexes were detected with an anti-HA antibody conjugated with horseradish peroxidase. The DARPin off7 with a C-terminal HA tag recognizing biotinylated maltose binding protein (MBP) was used as positive control.


(a)
Ec1
(b)
Ec2
(c)
Ec3
(d)
Ec4
31.6 nM
10 nM
3.16 nM
1 nM
0.32 nM
0 nM
RU
Time (s)
0
20
40
60
80
3000
6000
9000
12000

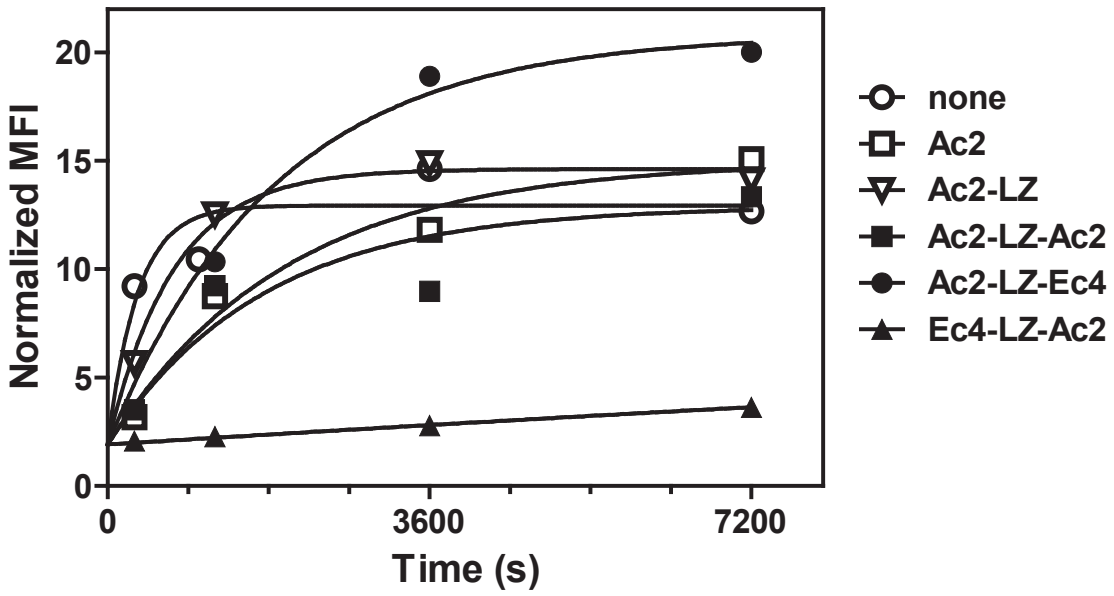


Supplementary Figure 8. Affinity determination by surface plasmon resonance. The EpCAM-binding affinity of the Ec-family DARPins, (a) - (e), and parental DARPins, (f) and (g), was measured by surface plasmon resonance using a ProteOn XPR36 (Bio-Rad Laboratories). One ligand channel of a neutravidin (NLC) sensor chip was coated with 300 resonance units (RU) of EpCAM. Kinetic data were obtained by parallel injection of different concentrations of DARPins ranging from 0.32 to 31.6 nM at a buffer flow rate of 60 µl/min in PBS pH 7.4 containing 3 mM EDTA and 0.005% Tween-20. Data evaluation was performed using the ProteOn Manager software (Bio-Rad). Results are summarized in Table 1 in the main text.

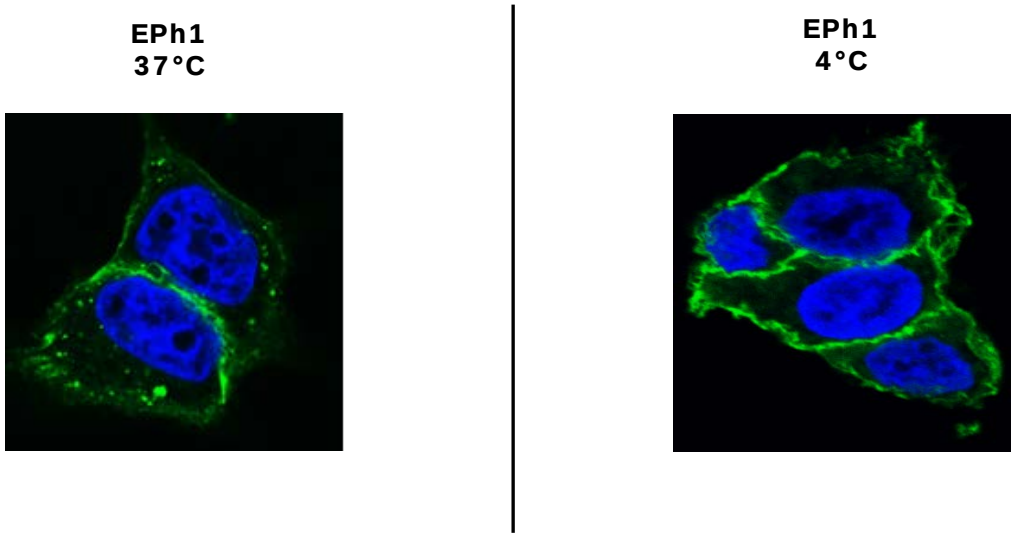


Supplementary Figure 9. Affinity determination by surface plasmon resonance. The EpCAM-binding affinity of the Ac-family DARPins was measured by surface plasmon resonance using a ProteOn XPR36 (Bio-Rad Laboratories). One ligand channel of a neutravidin (NLC) sensor chip was

coated with 300 resonance units (RU) of EpCAM. Kinetic data were obtained by parallel injection of different concentrations of DARPins ranging from 3.16 to 316 nM at a buffer flow rate of 60 µl/min in PBS pH 7.4 containing 3 mM EDTA and 0.005% Tween-20. Kinetic parameters, k_{on1}, k_{on2}, k_{off1}, and k_{off2}, were calculated from association and dissociation phases of Ac1 (a) and Ac2 (c) using the heterogeneous ligand model. A fast dissociation phase and a very slow dissociation phase (after 300 s) can clearly be discerned. This slow phase represents specific binding and probably different forms of the epitopes, also seen on cells, as discussed in the main text. Additionally, the equilibrium dissociation constant K_D representing the fast phase of Ac1 (b) and Ac2 (d) was calculated by steady-state analysis. Data evaluation was performed using the ProteOn Manager software (Bio-Rad). Results are summarized in Table 2 in the main text.



Supplementary Figure 10. Indirect measurement of dissociation rates of multivalent DARPins from MCF-7 cells using Ac2-sfGFP as competitor. $6 \cdot 10^5$ cells in 1 ml were saturated for 1 h at 4°C with 200 nM unlabeled DARPin, as indicated in the legend. Then, cells were centrifuged and resuspended with 2 µM Ac2-sfGFP. Association of the labeled monovalent binder was measured at different time points between 0 to 2 h.



Supplementary Figure 11. EPh1 internalization visualized by confocal microscopy. EpCAM-positive MCF-7 cells were incubated with EPh1 labeled with Alexa Fluor-488 at 37°C or 4°C. After 1 h of incubation, cells were washed, fixed with 4% paraformaldehyde, and imaged using a confocal scanning fluorescence microscope. Nuclei were counterstained with DAPI. Images were produced with a confocal plane selected about in the middle of the cells and processed using the Imaris 3D software.