Supplementary Information

for

Efficient cell-specific uptake of binding proteins into the cytoplasm through engineered modular transport systems

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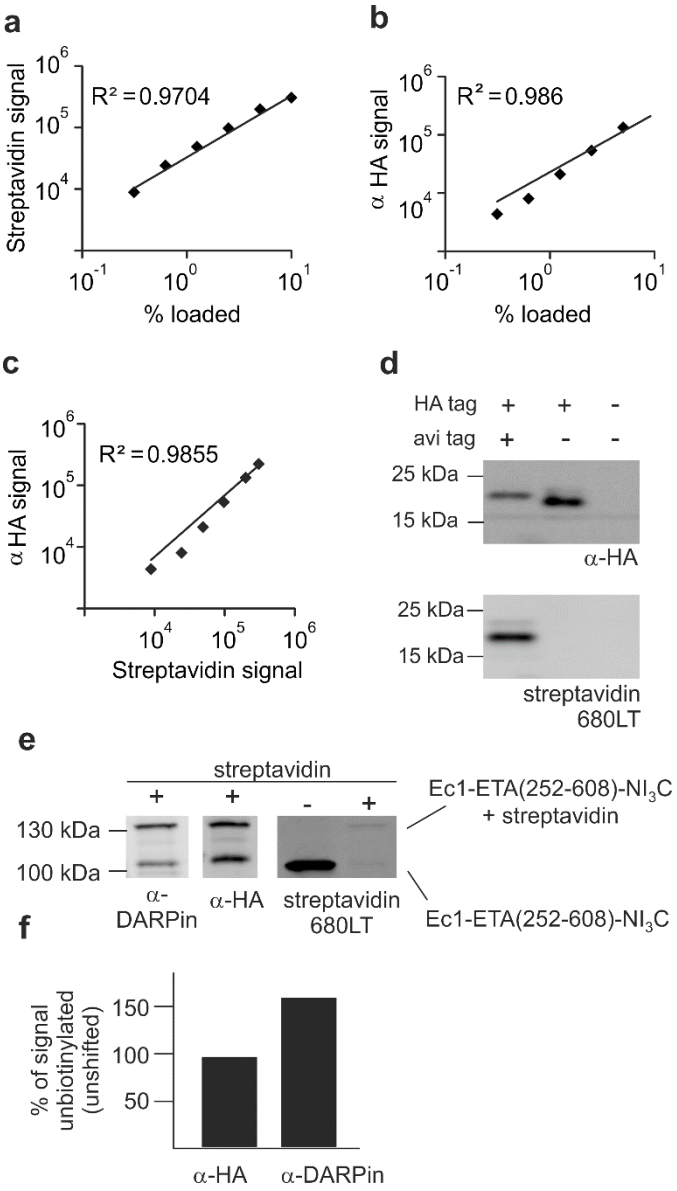
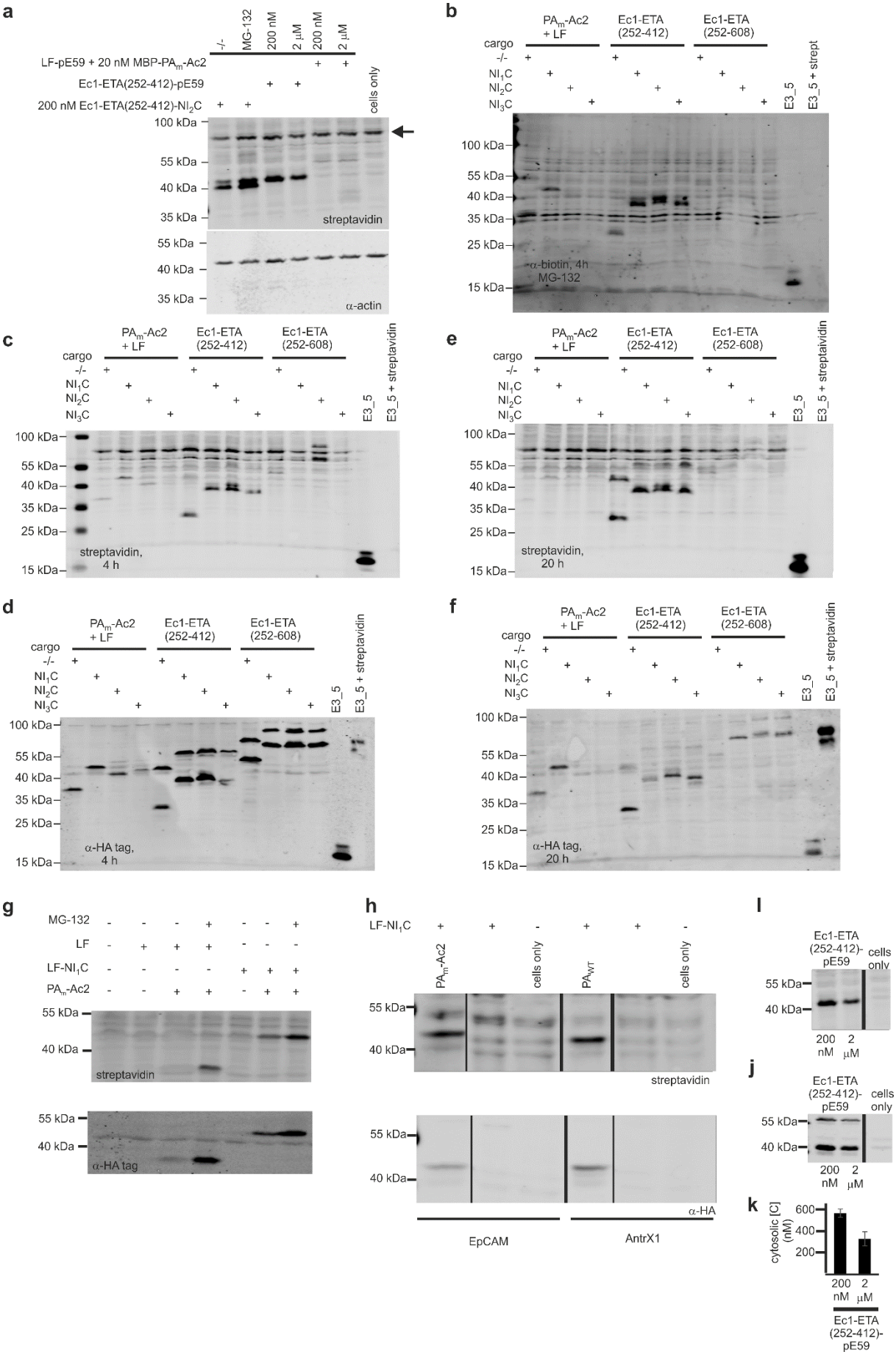
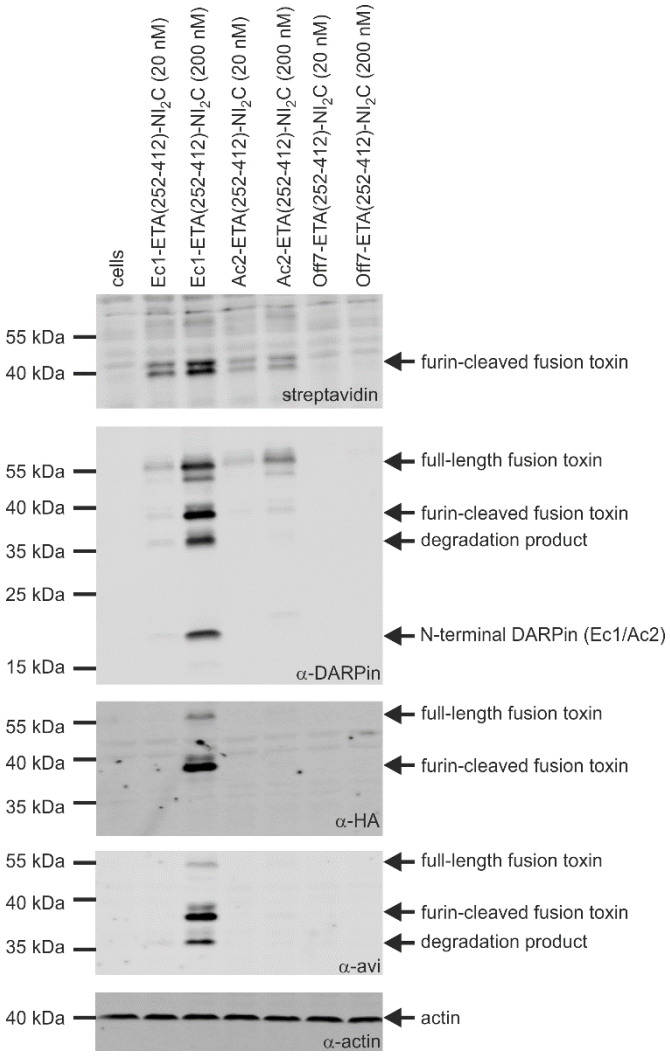


Supplementary Figure S1: Supporting BirA assay data (a-c) Quantification of the signal intensities from western blot bands derived from lysates from HEK293T/17 cells transiently transfected with biotin ligase and HA-E3_5-avi from the same plasmid. The trendline was calculated before log transformation with an intercept set at zero. **(d)** The avi-tagged DARPin HA-E3_5 and HA-E3_5 without avi tag were transiently transfected in Flp-In 293 cells stably expressing BirA and EpCAM and lysates were probed with an α-HA antibody and streptavidin IRDye 680LT. **(e)** A partially biotinylated Ec1-ETA(252-608)-NI_3C construct was pre-incubated for 2 h at 4ºC with a 10-fold molar excess of streptavidin and analyzed via western blot using α-DARPin serum, an α-HA antibody, or via streptavidin 680LT (also without streptavidin pre-incubation). To highlight the performance of the detection systems, aliquots from the same protein sample were used. **(f)** Quantification of the α-DARPin and α-HA blots shown in **(e)**, measuring the signal of the shifted band at ca. 130 kDa (biotinylated Ec1-ETA(252-608)-NI_3C + streptavidin) after normalization to the signal of unbiotinylated Ec1-ETA(252-608)-

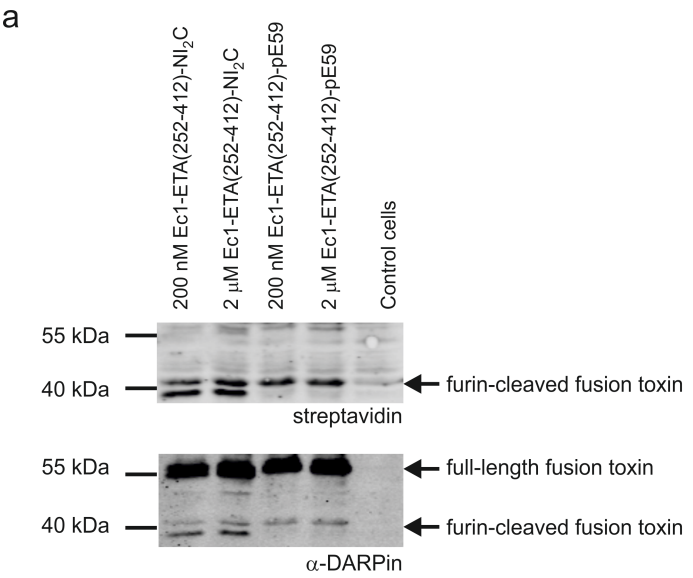
NI_3C from the same blot. The lower α-HA signal after normalization (i.e. compared to the α-DARPin signal) indicates a less efficient detection of the streptavidin-bound Ec1-ETA(252-608)-NI_3C via its HA tag compared to its unbound counterpart, suggesting interference of antibody and streptavidin binding to directly adjacent tags.

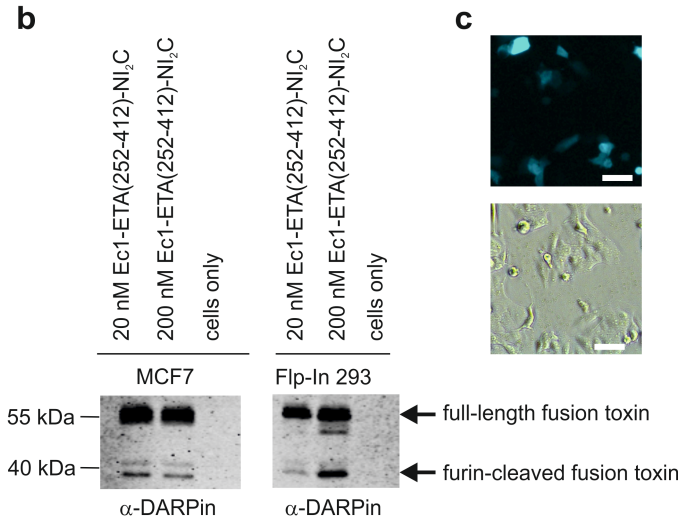

a
-/-
MG-132
200 nM
2 μM
200 nM
2 μM
LF-pE59 + 20 nM MBP-PAm-Ac2
Ec1-ETA(252-412)-pE59
200 nM Ec1-ETA(252-412)-NI2C
cells only
100 kDa
55 kDa
40 kDa
35 kDa
streptavidin
55 kDa
40 kDa
35 kDa
α-actin
b
PAm-Ac2 + LF
Ec1-ETA (252-412)
Ec1-ETA (252-608)
cargo
-/-
NI1C
NI2C
NI3C
E3_5
E3_5 + strept
100 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
α-biotin, 4h MG-132
c
PAm-Ac2 + LF
Ec1-ETA (252-412)
Ec1-ETA (252-608)
cargo
-/-
NI1C
NI2C
NI3C
E3_5
E3_5 + streptavidin
100 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
streptavidin, 4 h
e
PAm-Ac2 + LF
Ec1-ETA (252-412)
Ec1-ETA (252-608)
cargo
-/-
NI1C
NI2C
NI3C
E3_5
E3_5 + streptavidin
100 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
streptavidin, 20 h
d
PAm-Ac2 + LF
Ec1-ETA (252-412)
Ec1-ETA (252-608)
cargo
-/-
NI1C
NI2C
NI3C
E3_5
E3_5 + streptavidin
100 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
α-HA tag, 4 h
f
PAm-Ac2 + LF
Ec1-ETA (252-412)
Ec1-ETA (252-608)
cargo
-/-
NI1C
NI2C
NI3C
E3_5
E3_5 + streptavidin
100 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
α-HA tag, 20 h
g
MG-132
LF
LF-NI1C
PAm-Ac2
55 kDa
40 kDa
streptavidin
55 kDa
40 kDa
α-HA tag
h
LF-NI1C
PAm-Ac2
cells only
PAWT
cells only
55 kDa
40 kDa
streptavidin
55 kDa
40 kDa
α-HA
EpCAM
AntrX1
l
Ec1-ETA (252-412)-pE59
cells only
55 kDa
40 kDa
200 nM
2 μM
j
Ec1-ETA (252-412)-pE59
cells only
55 kDa
40 kDa
200 nM
2 μM
k
cytosolic [C] (nM)
600
400
200
200 nM
2 μM
Ec1-ETA (252-412)-pE59

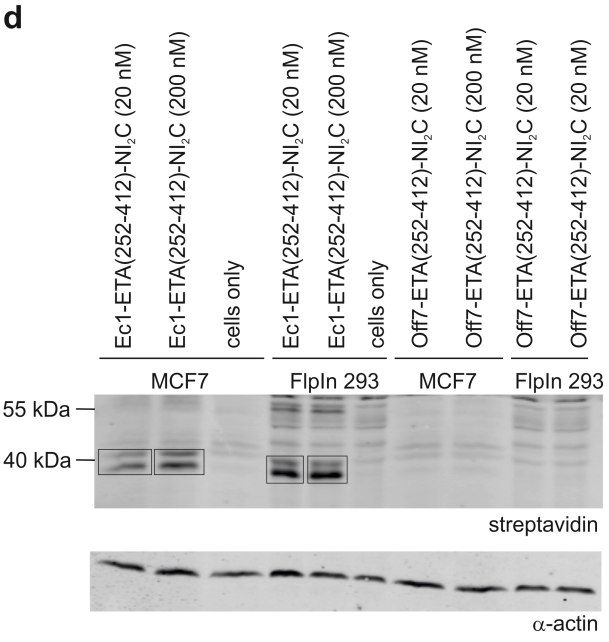
Supplementary Figure S2: Cytosolic delivery of DARPins via modular transport systems. (a) A comparison of signal intensities derived from the 70 kDa band (identified as unspecific Hsp-70, crossreacting with streptavidin IRDye 680LT) and an α-actin blot. The arrow in the upper panel denotes the Hsp-70 band. **(b)** An α-biotin Alexa Fluor 680 western blot of a 4-h-uptake experiment in Flp-In 293 cells stably expressing BirA and EpCAM. Avi-tagged toxins were added at 200 n*M* and MBP-PA_m-Ac2 at 20 n*M* in the presence of the proteasome inhibitor MG-132. **(c)** A streptavidin IRDye 680LT western blot of an uptake experiment as in **(b)**, but without proteasome inhibitor added. (**d)** A representative α-HA western blot with identical samples as in **(c). (e,f)** Representative streptavidin IRDye 680LT **(e)** and α-HA **(f)** western blots as in **(c)** and **(d)** for a 20-h uptake experiment. **(g)** 4-h-uptake experiment as described in **(b)** in the presence or absence of proteasome inhibition and/or MBP-PA_m-Ac2 as indicated. **(h)** Comparison of uptake after 4 h of LF-NI_1C via MBP-PA_m-Ac2 and MBP-PA_{wt} in HEK293T/17 cells transiently overexpressing either BirA and EpCAM or BirA and Anthrax toxin receptor 1. Concentrations were as described in **(b)**. A 4-h-uptake experiment of Ec1-ETA(252-412)-pE59 at distinct concentrations. Shown are the streptavidin IRDye 680LT western blot **(i),** the α-HA western blot **(j)** and the quantification based on two independent experiments **(k)**. Errors bars depict the range between duplicates.



Supplementary Figure S3: Cellular internalization and cytosolic delivery of DARPins via ETA(252-412) fused to Ec1, Ac2 or Off7. Flp-In 293 cells stably overexpressing BirA and EpCAM were incubated for 20 h with the indicated constructs at either 20 n*M* or 200 n*M*. Western blots using streptavidin IRDye 680LT or indicated antibodies are shown. Detection of actin was used as a loading control.


a
200 nM Ec1-ETA(252-412)-NI2C
2 µM Ec1-ETA(252-412)-NI2C
200 nM Ec1-ETA(252-412)-pE59
2 µM Ec1-ETA(252-412)-pE59
Control cells
55 kDa
40 kDa
furin-cleaved fusion toxin
streptavidin
55 kDa
40 kDa
full-length fusion toxin
furin-cleaved fusion toxin
α-DARPin


b
20 nM Ec1-ETA(252-412)-NI2C
200 nM Ec1-ETA(252-412)-NI2C
cells only
MCF7
20 nM Ec1-ETA(252-412)-NI2C
200 nM Ec1-ETA(252-412)-NI2C
cells only
Flp-In 293
55 kDa
40 kDa
full-length fusion toxin
furin-cleaved fusion toxin
α-DARPin
α-DARPin
c


d
Ec1-ETA(252-412)-NI2C (20 nM)
Ec1-ETA(252-412)-NI2C (200 nM)
cells only
Ec1-ETA(252-412)-NI2C (20 nM)
Ec1-ETA(252-412)-NI2C (200 nM)
cells only
Off7-ETA(252-412)-NI2C (20 nM)
Off7-ETA(252-412)-NI2C (200 nM)
Off7-ETA(252-412)-NI2C (20 nM)
Off7-ETA(252-412)-NI2C (20 nM)
MCF7
FlpIn 293
MCF7
FlpIn 293
55 kDa
40 kDa
streptavidin
α-actin

Supplementary Figure S4: Cellular internalization and cytosolic delivery of ETA(252-412) fusions in MCF7 cells. (a) MCF7 cells were incubated for 20 h with 200 n*M* or 2 μ*M* of Ec1-ETA(252-412) fused to either DARPin NI_2C or pE59. Western blots using streptavidin IRDye 680LT for detection of biotin and α-DARPin serum for detection of DARPins are shown. To enable the biotinylation of the avi tag in the cytosol in MCF7 cells, which do not stably express BirA, cells were transiently transfected with a plasmid containing BirA. **(b)** Western blot comparing the total cellular uptake of Ec1-ETA(252-412)-NI_2C at 20 or 200 n*M* in MCF7 cells and Flp-In 293 cells after a 20-h incubation. The α-DARPin serum was used for detection. Equal amounts of protein were loaded on the basis of a BCA protein assay kit (Pierce). **(c)** Representative image showing the transfection efficiency of MCF7 cells transfected with a plasmid expressing eGFP as a fusion protein (E3_5-eGFP). The scale bar corresponds to 12 μm. **(d)** Western blot comparing the cytosolic delivery of Ec1-ETA(252-412)-NI_2C, and an Off7-fused control, at 20 or 200 n*M* in MCF7 cells and Flp-In 293 cells after a 20 h incubation. Streptavidin IRDye 680LT was used for detection of biotinylated proteins. The α-actin signal was included as a loading control. BirA was transiently transfected as in **(a)**. Equal amounts of protein were loaded on the basis of a BCA protein assay kit.

Supplementary Table ST1. List of used modular transport systems

Receptor-targeting DARPin	Translocation component	Cargo protein	Main results
Ec1	ETA(252-412)-KDEL	n/a	Figure 2
Ec1	ETA(252-412)-KDEL	NI_1C	Figure 2
Ec1	ETA(252-412)-KDEL	NI_2C	Figure 2
Ec1	ETA(252-412)-KDEL	NI_3C	Figure 2
Ec1	ETA(252-608)-KDEL	n/a	Figure 2
Ec1	ETA(252-608)-KDEL	NI_1C	Figure 2
Ec1	ETA(252-608)-KDEL	NI_2C	Figure 2
Ec1	ETA(252-608)-KDEL	NI_3C	Figure 2
Ac2	PA_m + LF1-254	n/a	Figure 2
Ac2	PA_m + LF1-254	NI_1C	Figure 2
Ac2	PA_m + LF1-254	NI_2C	Figure 2
Ac2	PA_m + LF1-254	NI_3C	Figure 2
Ac2	PA_m + LF1-254	Destabilized NI_2C (1-6)	Figure 3 (Table ST2)
Ac2	PA_m + LF1-254	Destabilized NI_3C (1-6)	Figure 3 (Table ST2)
Ac2	PA_m + LF1-254	NI_1C-allR	Figure 3
Ac2	PA_m + LF1-254	NI_2C-allR	Figure 3
Ac2	PA_m + LF1-254	Ubi-NI_1C	Figure 3
Ec1	ETA(252-412)-KDEL	pE59	Figure S2
Ac2	PA_m + LF1-254	pE59	Figure S2
n/a	PA_{wt}+ LF1-254	NI_1C	Figure S2
Ac2	ETA(252-412)-KDEL	NI_2C	Figure S3
Off7	ETA(252-412)-KDEL	NI_2C	Figure S3

Abbreviations: Ac2, Ec1, EpCAM-binding DARPins; ETA, *Pseudomonas* exotoxin A; KDEL, Lys-Asp-Glu-Leu tail; LF1-254, Anthrax lethal factor 1-254; $NI_{1/2/3}C$, consensus DARPins with 1, 2 or 3 internal repeats; $NI_{1/2}C$-allR, consensus DARPins with all lysines replaced with arginines; Off7, control DARPin binding to maltose-binding protein; $PA_{m,}$ protective antigen with mutations N682A and D683A, PA_{wt}, protective antigen with wild-type sequence; pE59, DARPin binding to phosphorylated ERK; ubi, arginine-only ubiquitin variant. n/a not applicable.

Supplementary Table ST2. Estimated ΔΔG values for mutations introduced in consensus NI_2C and NI_3C DARPins

DARPin	**Mutation (s)**	**ΔΔG for 2QYJ-NI_2C[a] (ΔG = -248.7 ± 0.70[b])**	**ΔΔG for 2QYJ (NI_3C) (ΔG = -327.9 ± 0.87)**
NI_2C dest.1	L39G/L72G	13.66 ± 0.88	n/a
NI_2C dest.2	L39A/L72A	8.65 ± 0.78	n/a
NI_2C dest.3	L54G/L87G	12.24 ± 1.63	n/a
NI_2C dest.4	L54A/L87A	6.64 ± 1.21	n/a
NI_2C dest.5	L55G/L88G	11.57 ± 0.78	n/a
NI_2C dest.6	L55A/L88A	7.49 ± 0.98	n/a
NI_3C dest.1	L55A/L88A/L121A	n/a	12.47 ± 1.01
NI_3C dest.2	L55G/L88G/L121G	n/a	17.88 ± 1.01
NI_3C dest.3	L39A/L55A/L72A/L88A/L105A/L121A	n/a	24.63 ± 0.80
NI_3C dest.4	L39G/L55A/L72G/L88A/L105G/L121A	n/a	31.77 ± 0.99
NI_3C dest.5	L39A/L55G/L72A/L88G/L105A/L121G	n/a	29.96 ± 1.09
NI_3C dest.6	L39G/L55G/L72G/L88G/L105G/L121G	n/a	36.92 ± 1.04

[a]2QYJ-NI_2C was constructed from 2QYJ as described in the Materials and Methods section

[b] Indicated standard deviations were derived from 50 individual repacking runs

Supplementary Materials and Methods

Cellular uptake experiments. Flp-In 293 EpCAM/BirA cells were seeded at a density of 400,000 cells/well in a 24-well plate one day before the experiment. In uptake experiments where cells were transiently transfected, cells were seeded two days in advance (200,000 cells/well for HEK293/T17 and 125,000 cells/well for MCF7 cells). Approximately 24 h after seeding or transfection, cells were incubated with the avi-tagged fusion toxin for various times in 400 µl DMEM supplemented with 10 % fetal calf serum and 100 µ*M* biotin (Sigma-Aldrich). After the incubation, medium was removed and cells were trypsinized with 100 µL trypsin-EDTA solution (Sigma-Aldrich). After cells had detached, 800 µL full medium was added and cells were centrifuged for 3 min at 300 *g*. Cells were washed once with 1 mL PBS by another centrifugation step and the pellet was mixed with 50 µL preheated (~96°C) lysis buffer (100 m*M* Tris-HCl, pH 6.8, 4 % (w/v) SDS, 10 % (v/v) glycerol, 175 m*M* β-mercaptoethanol and 0.02 % (w/v) bromophenol blue) containing 20 µ*M* of a stabilized avi tag peptide containing 2 additional amino acids at either end (Ac-GGLNDIFEAQKIEWHED–NH_2; EMC Microcollections) as a competitive substrate and directly placed in a heating block at 96°C for 8 minutes in order to fully inactivate BirA. Samples were then frozen until further analysis. To confirm biotinylation activity of the EpCAM/BirA overexpressing cells, lysates from cells transfected with HA-E3_5-avi were diluted fourfold in non-denaturing and non-reducing loading buffer (0.5 *M* Tris-HCl, pH 6.8, 20 % (v/v) glycerol, 0.02 % (w/v) bromophenol blue). Unlabeled streptavidin (Promega) was then added to the lysate to a final concentration of 50 µg/mL, and mixtures were incubated for 2 h at 4°C. A complete band-shift on the α-Human influenza hemagglutinin (HA) tag western blot (see below) of the transfected protein (opposite terminus to avoid steric hindrance), confirmed all of the cytosolically expressed protein was biotinylated.

Western blotting. 10 µl of cellular lysates were separated via SDS-PAGE using 10 or 12 % (w/v) acrylamide according to standard procedures. When comparing uptake in Flp-In 293 and MCF7 cells, loaded volumes were adjusted on the basis of a BCA protein concentration assay. Proteins were transferred by wet blotting for 1 h at 100 V to an Immobilon-FL PDVF membrane (Millipore). Membranes were blocked for 20 min with casein blocking buffer (Sigma-Aldrich) and then incubated for 1 h at ambient temperature with streptavidin IRDye 680LT (1:10,000; Li-Cor), mouse monoclonal IgG 1k anti-biotin Alexa 680 (1:10,000; Jackson Immunoresearch), a polyclonal anti-HA antibody (1:1,000; Sigma-Aldrich), a polyclonal anti-DARPin rabbit serum (1:10,000, home-made, B. Dreier et

al., unpublished**)**, a rabbit anti-avi-tag antibody (Genscript) or a mouse monoclonal anti-actin antibody (1:2,000; A3853, Sigma-Aldrich) in PBS supplemented with 0.05 % (v/v) Tween-20 (PBS-T) and 1 x casein blocking buffer. Streptavidin IRDye 680LT incubations were performed in the presence of 0.1 % (w/v) SDS to reduce non-specific binding. As a secondary antibody recognizing the anti-HA and anti-avi-tag antibodies, or the anti-DARPin serum, a goat anti-rabbit IgG (H+L) conjugated to Alexa 680 (1:10,000; Invitrogen) was allowed to bind for 45 min at ambient temperature. A donkey-anti-mouse (H+L) IRDye 800 antibody (Rockland Immunochemicals, Inc.) was used to detect the mouse monoclonal anti-actin antibody. Between and after antibody incubations, blots were washed 4 x 5 min with PBS-T. Fluorescent signals were acquired with an Odyssey IR scanner (Li-Cor Biosciences) and band intensities were quantified with Image Studio Lite software (Li-Cor Biosciences). For streptavidin IRDye 680LT blots, the band intensity was corrected for loading on the basis of the detection of the 70 kDa band which has been previously identified to be Hsp70 [1] and performed similarly to anti-actin as a loading control (Supplementary Fig. S2a). Comparisons of the amount of internalized protein between experiments were performed by normalizing over the sum of band intensities for each experiment, provided the same samples were analyzed under identical conditions. Alternatively, a fully biotinylated maltose-binding protein (MBP; kindly provided by T. Reinberg) was loaded on the gel and used as a reference for quantitation. Full biotinylation of MBP was confirmed by incubating the protein with an excess of streptavidin and separating bound from unbound proteins via SDS-PAGE. Coomassie staining confirmed the full biotinylation, as the MBP completely shifted to higher molecular weight.

Cloning of recombinant fusion toxins. *Anthrax Lethal factor (LF):* The gene encoding for the TEV protease site (GSENLYFQG) followed by LF 1-254, GGSGGGSG, a SpeI-AgeI cloning cassette for cargo, a GGSG linker and the avi tag was obtained from Eurofins and cloned C-terminally of His_6-MBP (for solubility enhancement) via BamHI and PstI sites in pQEMBP [2] (Genbank accession number: AY327141) and recloned in pQIQ, a pQE30(Qiagen)-derived vector with an added *lac* repressor gene under the control of a stronger $lacI^q$ promoter [3]. Consensus DARPins $NI_{1/2/3}C$ were PCR amplified from pSW_$NI_{1/2/3}C$ [4] and inserted via SpeI and AgeI sites. A C-terminal HA tag was subsequently introduced via a PCR amplification step. Destabilized NI_2C variants were purchased as strings (Geneart) and NI_3C variants as gBlocks (ITD, Inc.) and were cloned via SpeI and AgeI.

Pseudomonas aeruginosa Exotoxin A (ETA): Assembly PCR was used to generate Ec1-ETA(252-412)-His_6-KDEL and Ec1-ETA(252-608)-His_6-KDEL containing unique SalI and PstI sites directly before the His_6 site. The previously described Ec1-ETA(252-608)-His_6-KDEL was used as a template [5], where Ec1 is an EpCAM-binding DARPin. Site-directed mutagenesis was performed to generate Ec1-ETA(252-608;E553D)-His_6-KDEL. Model cargo DARPins $NI_{1/2/3}C$ with the avi and HA tag were PCR amplified from aforementioned LF constructs and inserted via the SalI and PstI restriction sites. Constructs without cargo were generated via insertion of adaptor DNA duplexes resulting in the linker sequence GGSG between ETA and the avi tag.

Protective antigen (PA). The gene for $PA_{N682A/D683A}$ (PA_m), followed by a SPGHKTQP linker and a BamH1-HindIII cloning cassette, was purchased from Genscript and cloned behind His-MBP-TEV in pQIQ using assembly PCR. The EpCAM-binding DARPin Ac2 was subsequently inserted via BamHI and HindIII and was directly followed by a myc and His_6-tag, generating MBP-PA_m-Ac2. MBP-PA_{wt} was obtained via site-directed mutagenesis, back-mutating the mutations N682A and D683A and removing the C-terminal Ac2-myc-His_6 via PCR amplification of the obtained construct and ligating the fragment using BglII and HindIII sites.

Expression of recombinant fusion toxin proteins. Origami B(DE3) cells were transformed with ETA constructs and single clones were picked and grown overnight with shaking at 37°C in 20 mL LB supplemented with 100 µg/mL ampicillin, 15 µg/mL kanamycin and 1 % (w/v) glucose. On the next day, 200 mL terrific broth (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 17 m*M* KH_2PO_4, and 72 m*M* K_2HPO_4) containing 0.8 % (w/v) glucose and the same antibiotic concentrations was inoculated with the overnight culture to an OD_{600} of 0.1. Cells were grown with shaking at 37°C until an OD_{600} of 0.6-0.8 was reached. The temperature was lowered to 25°C and expression was induced with 100 µ*M* IPTG. After 4 hours of expression, cells were centrifuged for 10 min at 5,000 *g* and pellets were snap-frozen in liquid N_2 and stored at -80°C until purification. Expression parameters for MBP-LF and MBP-PA_m-Ac2 constructs transformed in *E. coli* BL21(DE3) cells were as described for the ETA-based constructs, apart from the omission of kanamycin in BL21(DE3) expressions.

Purification of recombinant fusion toxin proteins. *ETA-based constructs:* For purification of truncated ETA-DARPin fusion proteins, the bacteria were resuspended at 4°C in HBS-W (50 m*M* HEPES, pH 8.0, 150 m*M* NaCl, 1 m*M* $MgCl_2$, 20 m*M* imidazole)

containing 0.5 µg/ml lysozyme (Sigma-Aldrich), 10 U/mL benzonase (Sigma-Aldrich) and Complete EDTA-free protease inhibitor cocktail (Roche). All further steps were also performed at 4°C. Cells were lysed with a home-made French press, centrifuged for 40 min at 27,000 *g* and the supernatant was filtered (pore size 0.22 µm). His-tagged proteins were then purified via immobilized metal affinity chromatography (IMAC) as follows: Supernatants were incubated for 1 h with HBS-W-equilibrated Ni-NTA superflow resin before being washed with 20 column volumes (CV) of low-salt buffer (50 m*M* HEPES, pH 8.0, 20 m*M* NaCl, 20 m*M* imidazole), 20 CV of high-salt buffer (50 m*M* HEPES, pH 8.0, 1 *M* NaCl, 20 m*M* imidazole) and 10 CV of HBS-W. His-tagged proteins were eluted with PBS (pH 7.4) containing 300 m*M* imidazole. Eluted proteins were then either dialyzed overnight against PBS or run over a PD10 column (GE Healthcare) in order to remove imidazole. Proteins were then incubated with streptavidin beads (Genscript) or monomeric avidin beads (Pierce) for 30 min at 4°C while shaking in order to remove the biotinylated fraction, and the supernatant was concentrated via Amicon Ultra-4 (Millipore; MWCO 10,000 Da) tubes, snap-frozen in liquid N_2 in aliquots and stored at -80°C. Purity was confirmed by Coomassie staining and monomeric behavior was assessed by analytical size-exclusion chromatography using a Superdex Increase 5/150 GL (GE Healthcare) on an ÄKTA Micro system (GE Healthcare). All constructs containing ETA fragments were pure as assessed by Coomassie-stained SDS-PAGE and showed over 85 % monomeric fraction via analytical size-exclusion chromatography.

MBP-PA$_m$-Ac2: Purification of MBP-PA$_m$-Ac2 was similar until elution from the Ni-NTA resin. From this point, elution fractions were concentrated and separated via size-exclusion chromatography using a Superdex 200 10/300 GL (GE Healthcare). Fractions were collected, concentrated as described before, snap-frozen in liquid N_2 in aliquots and stored at -80°C. Purity was assessed as described for ETA-based constructs.

MBP-LF-based constructs: Purification of MBP-LF was similar as described above until the concentration step, at which point proteins were incubated o/n at 4°C in PBS with a 1:10 (w/w) ratio of his-tagged TEV protease to fusion toxin in order to cleave off His$_6$-MBP. His-tagged TEV protease and MBP were removed via reverse IMAC. The unbound fraction during reverse IMAC was loaded on a Superdex 200 10/300 GL (GE Healthcare) and the monomeric fractions were collected and biotinylated proteins removed as described above. The supernatant was concentrated as described, snap-frozen in liquid N_2 in aliquots and stored at -80°C. Purity was assessed as described above. The identity of several cleaved LF

constructs (LF and LF fused to $NI_{1/2/3}C$) was further corroborated by mass spectrometry, which yielded expected masses for all tested constructs.

Expression and purification of TEV protease. TEV protease was expressed in soluble form in the cytosol of BL21(DE3) from the vector pRK793 (kind gift from G. Hausammann). *E. coli* was grown in terrific broth supplemented with 50 µg/mL kanamycin at 37°C, expression was induced with 0.5 m*M* IPTG at OD_{600} ~ 0.8 and expression was allowed to continue for 5 h at 25°C. Pellets were centrifuged for 10 min at 5,000 *g* and snap-frozen. For purification, pellets were thawed and resuspended in lysis buffer (50 m*M* HEPES, pH 8.0, 150 m*M* NaCl, 0.2 % (w/v) NP-40 (Fluka), 4 m*M* β-mercaptoethanol and 1 m*M* phenylmethylsulfonyl fluoride (Roche) and lysed with a Constant Systems TS1.1 cell disrupter. The lysate was centrifuged for 30 min at 27,000 *g* and the supernatant was filtered (pore size 0.22 µm). The supernatant was passed twice over Ni-NTA superflow resin pre-equilibrated with lysis buffer in a bench-top setup at 4°C. The resin was washed with 20 CV high-salt wash buffer (20 m*M* HEPES, pH 8.0, 1 *M* NaCl, 4 m*M* β-mercaptoethanol, 10 m*M* imidazole), 20 CV medium-salt wash buffer (20 m*M* HEPES, pH 8.0, 150 m*M* NaCl, 4 m*M* β-mercaptoethanol, 50 m*M* imidazole) and eluted with 4 CV elution buffer (20 m*M* HEPES, pH 8.0, 150 m*M* NaCl, 4 m*M* β-mercaptoethanol, 350 m*M* imidazole and 10 % (v/v) glycerol). Elution fractions were dialyzed o/n against Tris-HCl (pH 7.6) with 150 m*M* NaCl and 4 m*M* β-mercaptoethanol. Glycerol was added to 10 % (v/v) and aliquots were snap-frozen and stored in liquid N_2. Purity was confirmed by Coomassie-stained SDS-PAGE.

Supplementary references

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