Supplementary information

Thermodynamic Stability Is a Strong Predictor for Delivery of DARPin to the Cytosol via Anthrax Toxin

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Figure S1. Western blot and quantification showing delivery of different LFs-cargo constructs with PA-w-sANTXR-Ac2. (a, c, e, g, i) Cargo proteins delivered to the cytosol are biotinylated by cytoplasmic BirA and stained with Streptavidin IRDye 680LT (b, d, f, h, j) Quantification of Western blot bands from (a, c, e, g, i). The dashed lines represent background signals (i.e. cells only). NI1C and eGFP are used as delivery controls for maximum signal intensity of cytosolic uptake (NI1C) and no cytosolic localization (eGFP). Destabilized (dest) DARPins have been described previously [1] and the location of mutations is listed in Table 1 in the main text and Supplementary Figure S3.
Figure S2. (a-e) Total cellular uptake measured via HA-tag of the LFv-cargo. This corresponds to the samples of the BirA assay in Figure S1. Destabilized (dest) DARPins (b, c) have been described previously [1] and the location of mutations is listed in Table 1 in the main text and Supplementary Figure S3.
Figure S3. Destabilizing mutations in DARPin framework as published previously [1]. (a) Positions of framework mutation of each internal repeat are represented in green (L8) and orange (L24). The respective DARPin target binding area is represented by the grey area, spatially distant to the framework mutations. N and C termini are labelled in red or black, respectively; structural representation adapted from PDB ID 1svx. (b) Position and amino acid substitution of destabilized DARPin of each internal repeat; each position was mutated in all three internal repeats.
Figure S4. Western blot and quantification showing delivery of different LF\textsubscript{N}-cargo constructs of the consensus DARPin NI3C and variants of the NI3C dest1 with PA\textsubscript{wt}-sANTXR-Ac2. Variants with the L24A mutation are mutated only in the first (NI3C dest11), only the second (NI3C dest12), only the third (NI3C dest13), first and second (NI3C dest11,2) or second and third (NI3C dest12,3) internal repeat. Western Blots (a, b) and respective quantification (c) of the biotin ligase assay, showing efficient cytosolic translocation only for the fully destabilized variant NI3C dest1.
Figure S5. SPR measurement of off7 and off7 dest1 (L24A mutation in all three internal repeats) showing a similar KD for target binding.
Figure S6. pH titration of different DARPin(s). NI1C, NI2C, NI3C, J1/2_2_25 and J1_2_32 were incubated overnight in PBS at pH 7.4 (a), MES at pH 6.5 (b), MES at pH 6.0 (c), sodium acetate at pH 5.5 (d), sodium acetate at pH 5.0 (e), sodium acetate at pH 4.5 (f), sodium acetate at pH 4.0 (g), citric acid at pH 3.5 (h); all buffers were used at 50 mM and supplemented with 150 mM NaCl, except PBS. Subsequently, proteins were analyzed via circular dichroism (CD).
Figure S7. GdnHCl-induced equilibrium unfolding at pH 6 of Ni1C, Ni2C, Ni3C, Ni3C dest1, J1/2_2_25 and J1_2_32 in 50 mM MES (pH 6.0) supplemented with 150 mM NaCl at 20 °C analyzed by CD spectroscopy; (b) Denaturation midpoints (D_m) were taken from Table 1 (PBS, pH 7.4) and by fitting Equation 1 to the data from (a). Values marked with an asterisk (*) were estimated by a nonlinear fit (GraphPad Prism 8.0, X is concentration) to experimental data since fitting Equation 1 was not possible due to too few datapoints.
Figure S8. (a) GdnHCl-induced equilibrium unfolding of LFN-NI₁C, LFN-NI₂C and LFN-NI₃C in PBS (pH 7.4) at 20 °C analyzed by CD spectroscopy. (b) The denaturation midpoint (Dₘ,₁) for LFN-NI₁C was determined by fitting Equation 1 to the data obtained in (a). The two denaturation midpoints Dₘ,₁ and Dₘ,₂ of LFN-NI₂C and LFN-NI₃C, marked with an asterisk (*), were estimated by fitting a nonlinear biphasic fit (GraphPad Prism 8.0) to the data.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dₘ,₁</th>
<th>Dₘ,₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFN-NI₁C</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>LFN-NI₂C</td>
<td>0.9*</td>
<td>3.5*</td>
</tr>
<tr>
<td>LFN-NI₃C</td>
<td>0.9*</td>
<td>4.9*</td>
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Figure S9. Correlation of normalized cytosolic update intensity of LFN-DARPin cargo and the denaturation midpoint (a) or $\Delta G_0$ (b) of the DARPin. The coefficient of determination $R^2$ was calculated using GraphPad Prism 8.0.