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Supporting information for article:

Structure of a hydrophobic leucinostatin derivative determined by host lattice display

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Supplementary Method 1. Synthesis of ZHAWOC8403.

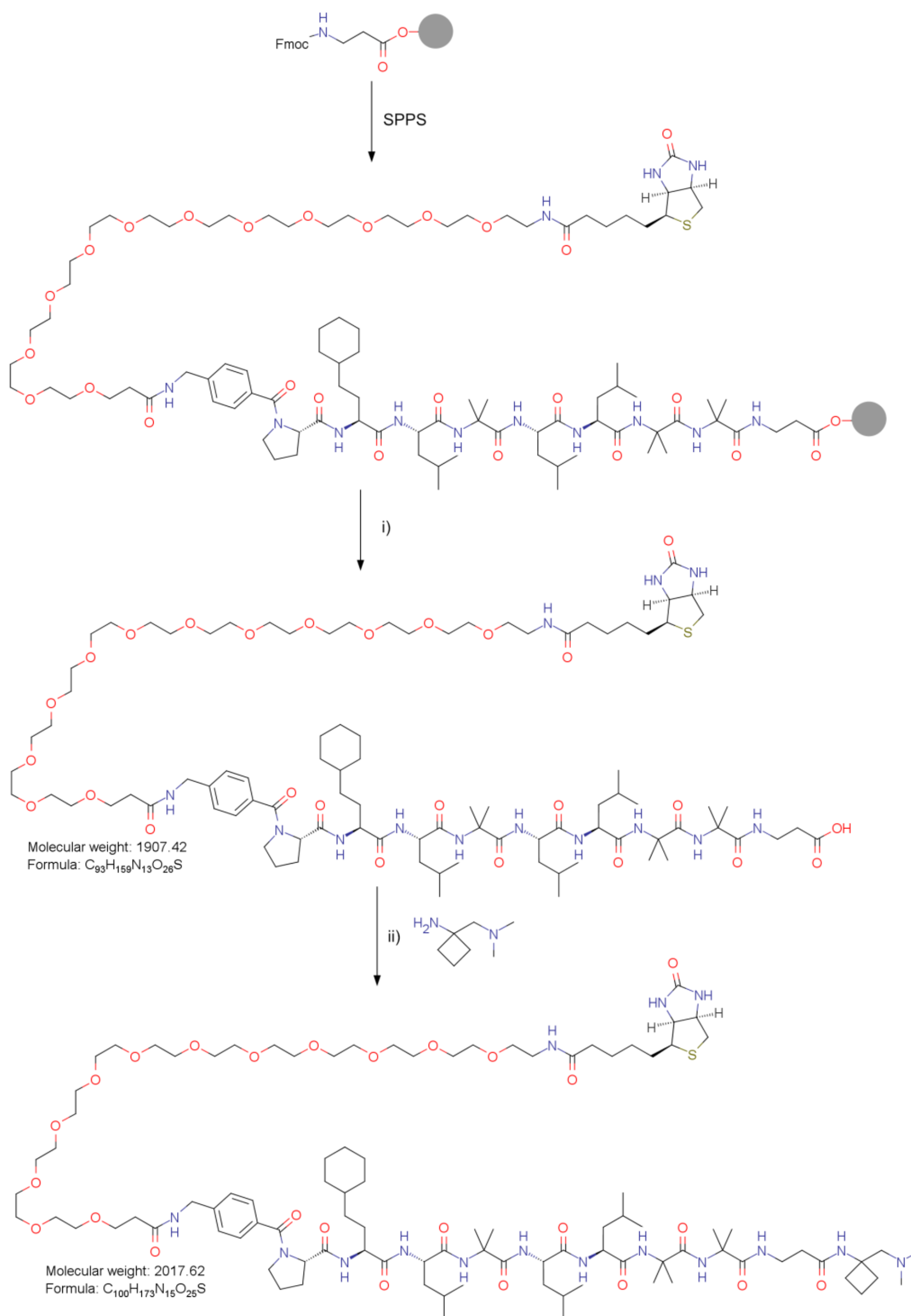
Amino acids and their derivatives were either purchased from Sigma Aldrich, Iris Biotech GmbH, Bachem or Senn Chemicals. Amino acids were used as the following derivatives: Fmoc-Aib-OH, Fmoc-Leu-OH, Fmoc-Pro-OH and Fmoc-(Cyhex)-OH. 4-Fluorobenzoic acid, COMU (((1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium-hexafluorophosphat)), triisopropylsilane, O-[2- (biotinyl-amino)-ethyl]-O′- (2-carboxy-ethyl)-undecaethylenglykol, and DIC (N,N'-diisopropylcarbodiimid) were purchased from Sigma Aldrich. The Fmoc-β-Ala-Wang resin (loading: 0.70-0.72 mmol g^{-1}; mesh size 100-200) and OxymaPure (hydroxyiminocyanoacetic acid ethyl ester) were purchased from Iris Biotech GmbH. 1-((Dimethylamino)methyl) cyclobutan-1-amine was purchased from Enamine. 4-Aminomethylbenzoic acid was purchased from Fluorochem and Fmoc-protected following the published method (Kim *et al.*, 2015). Solid phase peptide synthesis was performed on the Liberty Blue microwave (CEM) as outlined in Supplementary Figure 1. Purifications of the peptides were performed on Teledyne ISCO C18 reversed phase column using an Interchrom uptisphere strategy C18-HQ (5 µm, 250 x 21.2 mm, flow 19 mL/min) column.

The peptides were synthesized by SPPS, purified by RP chromatography on the Teledyne ISCO and subsequently subjected to the final amide coupling with 1-((dimethylamino)methyl) cyclobutan-1-amine. The final products were purified by preparative HPLC. Peptides were synthesized on a 0.1-mmol scale on Fmoc-β-Ala Wang resin (0.7 mmol g^{-1}, 157 mg) using a Liberty Blue microwave peptide synthesizer (CEM Corp., Matthews, NC) employing Fmoc solid-phase techniques with repeated steps of coupling, deprotection, and washing. Coupling was performed as follows: Fmoc-L-amino acids or the capping group (5.0 eq, 0.2 M in DMF), DIC (5.0 eq, 0.5 M in DMF), and Oxyma (5.0 eq, 1.0 M in DMF) for 4 minutes with microwave irradiation at 90 °C. For the second Aib coupling, a double coupling with 15 minutes microwave irradiation each at 90 °C was performed. Fmoc deprotection was performed as follows: 10% piperazine in NMP/ethanol (9:1) for 1 minute with microwave irradiation at 90 °C. Following synthesis, the peptide was cleaved from the resin by treatment with a cleavage mixture (3 mL) consisting of TFA/triisopropylsilane/H_2O (95:2.5:2.5) for 90 minutes at ambient temperature. The suspended resin was removed by filtration and concentrated *in vacuo.* The crude peptide was purified by RP chromatography on Teledyne ISCO on a C18 column eluting with a gradient of 10 to 100% acetonitrile in water, unless otherwise stated. An aliquot of the carboxylic acid (1.0 eq) was stirred with COMU (2.0 eq), Et_3N (1.5 eq), and 1-((dimethylamino)methyl) cyclobutan-1-amine (1.5 eq) in DMF (0.4 mL) was stirred for 30

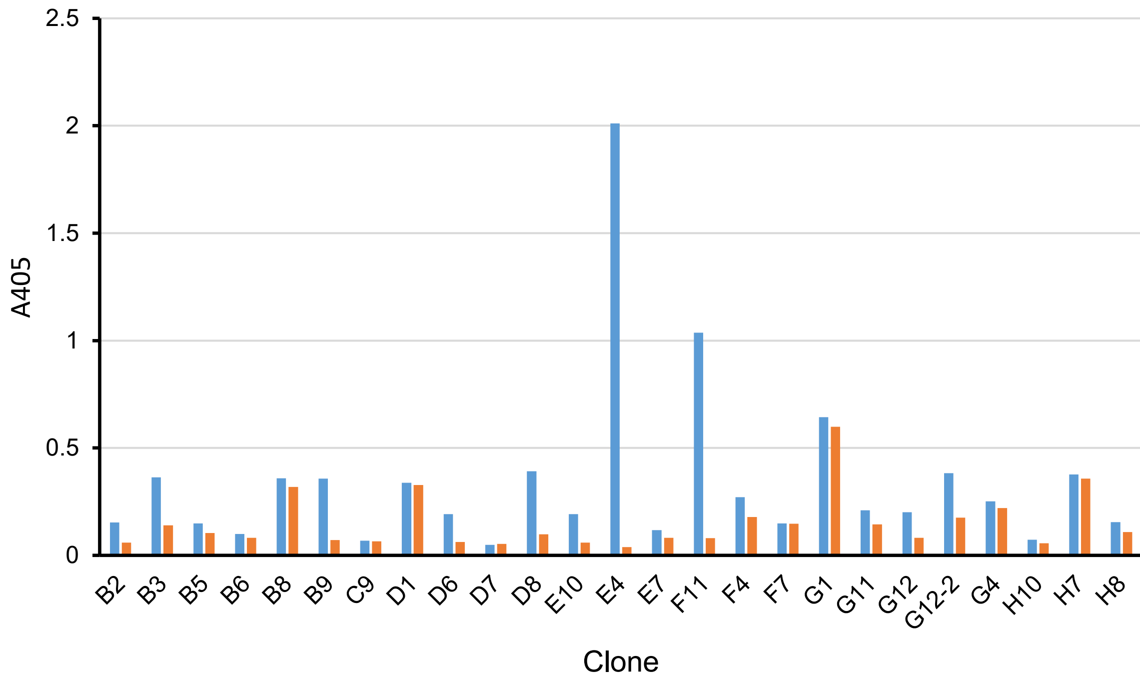
minutes at ambient temperature. The reaction mixture was purified on a semi-prep HPLC on a C18 column eluting with a gradient of 10% to 100% water in acetonitrile with 0.1% acetic acid. The product was isolated by lyophilization.

Supplementary Method 2. Synthesis and regeneration of DARPin clamp R7 sfGFP affinity resin.

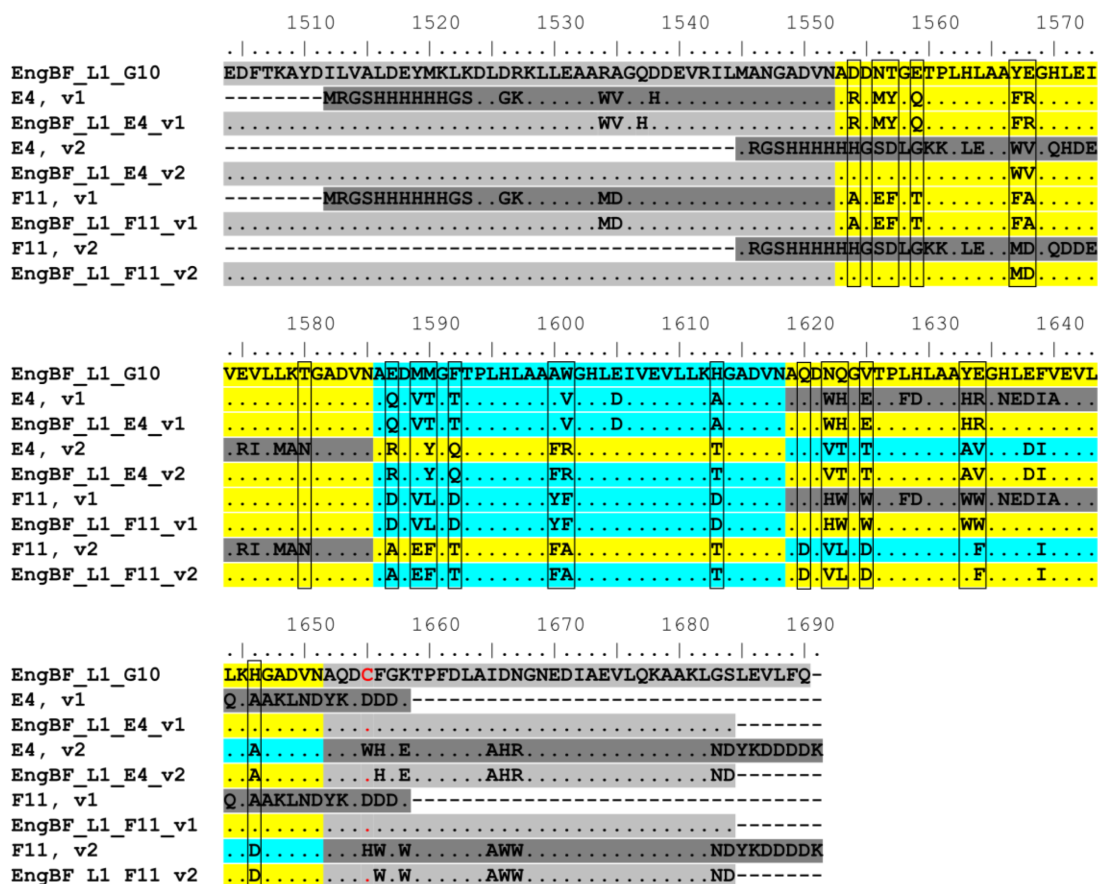
NHS-activated Sepharose beads (5 mL) were washed with 20 CV ice-cold 1 mM HCl in a PD10 plastic column to remove the storage solution. Subsequently, the column was equilibrated by adding 15 CV ice-cold PBS (pH 7.4) and the resin was resuspended in PBS (1:1 ratio). For each millilitre of resin, 2.3 mg anti-sfGFP DARPin clamp R7, which was purified as described in ref. (Hansen *et al.*, 2017), was added to the suspension before it was incubated overnight at 4 °C with constant agitation. The mixture was washed with 5 CV ice-cold 100 mM Tris solution (pH 8.0 measured at room temperature) to inactivate the remaining NHS groups. The resin was stored at 4 °C in PBS (pH 7.4) containing 20% (v/v) ethanol. Regeneration of the DARPin clamp R7 affinity resin was done by washing with 20 CV regeneration buffer (50 mM glycine/HCl, 6 M guanidinium hydrochloride, pH 1.5).



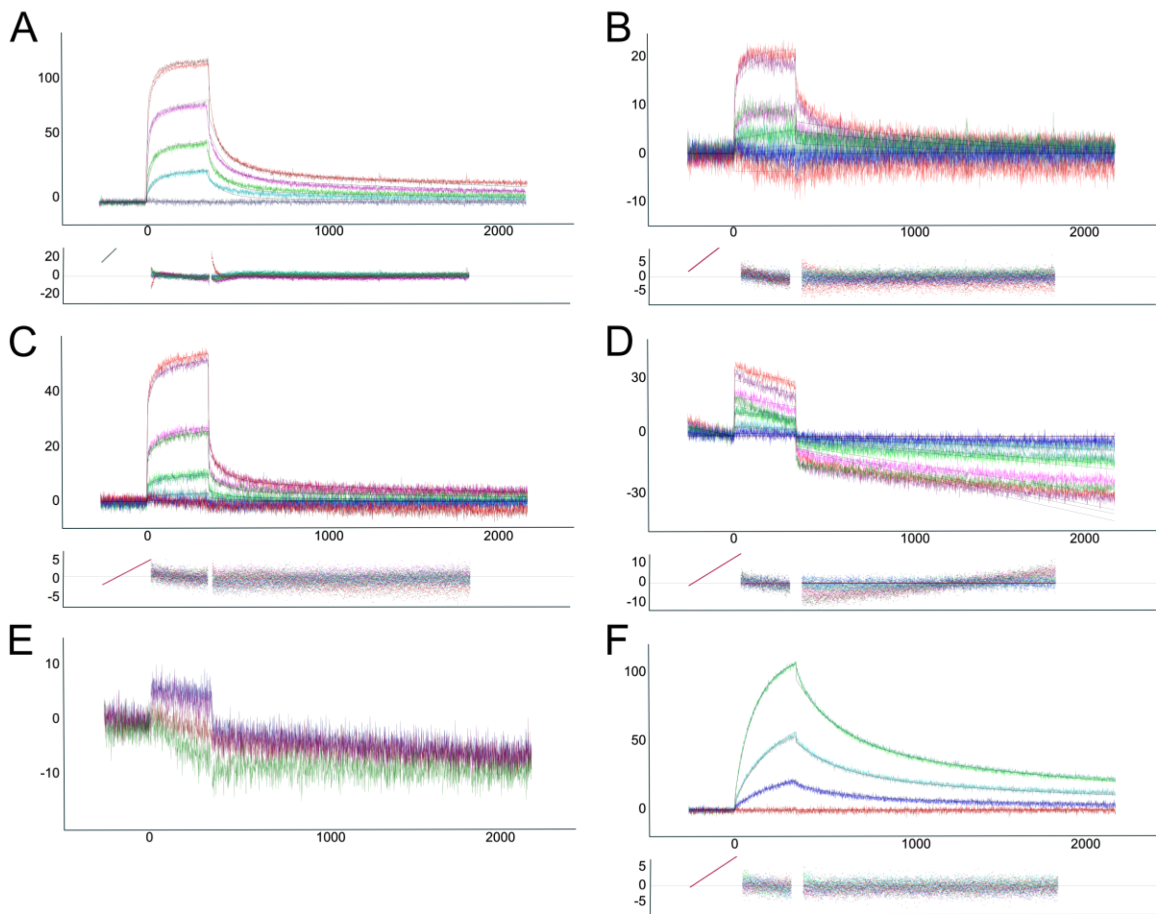
Supplementary Figure 1. Rection scheme for the synthesis of ZHAWOC8403. i) TFA, H_2O, triisopropylsilane, RT, 90 min, 48%. ii) 1-[(dimethylamino)methyl]cyclobutan-1-amine, COMU, Et_3N, DMF, RT, 30 min, 87%. The compound was synthesized according to the general procedure to yield a colourless solid (23 mg, yield 87%) and analysed by mass spectrometry on a MSQ Plus device (Thermo Scientific, Reinach, Switzerland). *m/z* (ES+) [Found: $(M+2H)^{2+}$ 1009.28, $C_{100}H_{175}N_{15}O_{25}S$, requires 2018.260].



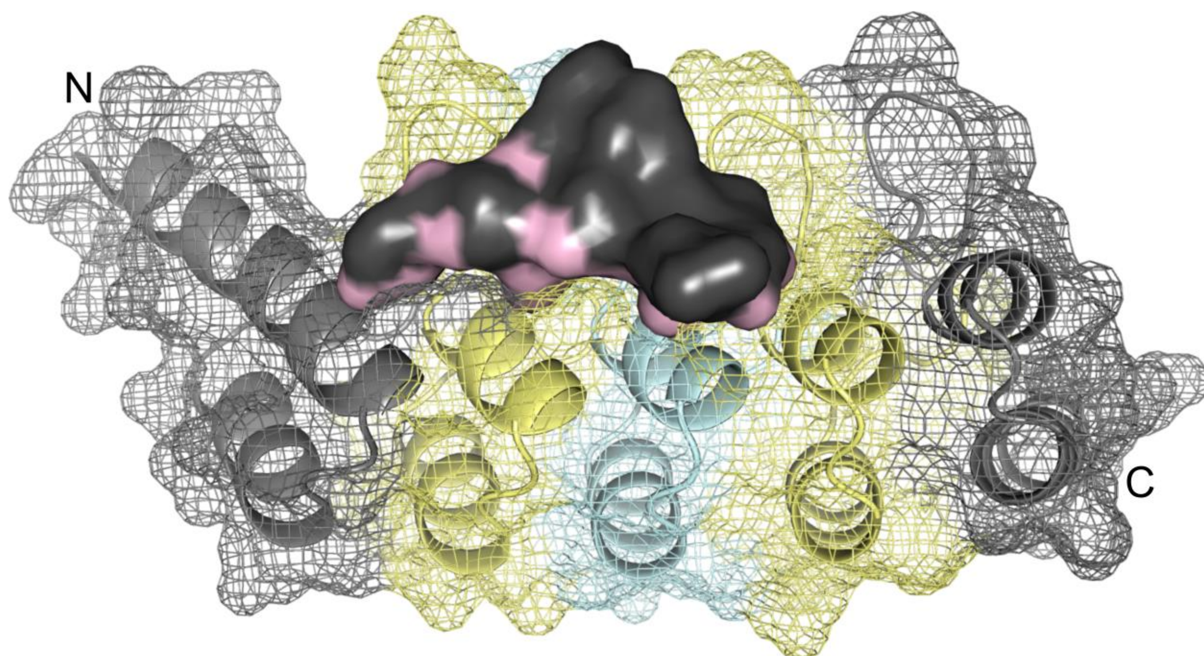
Supplementary Figure 2. ELISA of purified DARPin hits. Biotinylated ZHAWOC8403 was immobilized on a neutravidin-coated MTP384 plate, and DARPins were detected with an anti-FLAG-M2 antibody and a secondary antibody coupled to alkaline phosphatase. The absorption at a wavelength of 405 nm with (blue) and without (orange) immobilized ZHAWOC8403 is indicated.



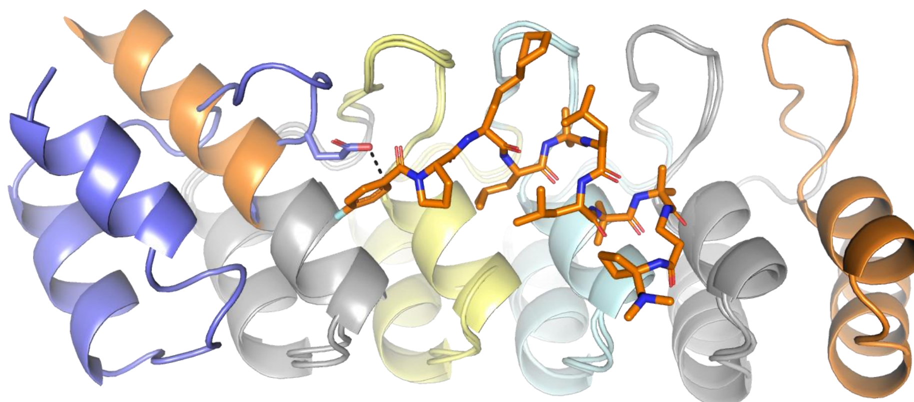
Supplementary Figure 3. Grafting of DARPins E4 and -F11 on EngBF_L1_G10 using two different alignment registers (v1 and v2). Capping repeats and the EngBF framework are shown in dark and light grey, respectively. Internal repeats 1 and 3 are highlighted in yellow, and internal repeat 2 in cyan. Cys1655, which is crucial for the crystal contact, is emphasized in red. Vertical boxes indicate residues at randomized positions. EngBF residues (up to residue number 1503), which are identical in all constructs, have been omitted for clarity.



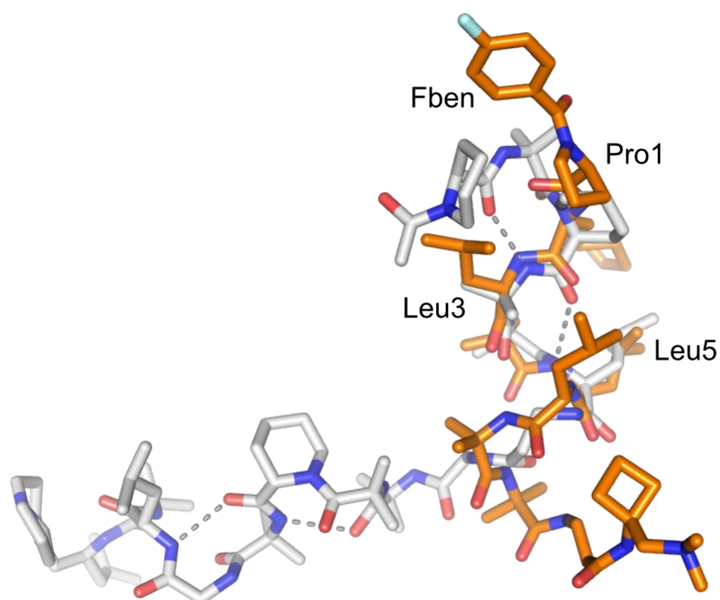
Supplementary Figure 4. Surface plasmon resonance analysis of selected DARPins and EngBF fusion proteins. Sensogram data (in colour) and residuals of fitted curves are shown. The X- and Y-axis indicate time (seconds) and response units, respectively. The experimental data are in colour and the fitted curves are given in black and the corresponding kinetic data are shown in Table 1. (A) DARPin E4, (B) DARPin F11, (C) EngBF_L1_E4_v1, (D) EngBF_L1_F11_v1, (E) EngBF_L1_E4_v2 without residuals, and (F) EngBF_L1_F11_v2.



Supplementary Figure 5. EngBF_L1_E4_v1:ZHAWOC6027 complex structure. The DARPin E4 domain is shown as a cartoon and its molecular surface as a mesh. Coloring according to Supplementary Figure 3. ZHAWOC6027 is shown as a grey surface. Surface areas which are contacting EngBF_L1_E4_v1 are highlighted in pink (distance smaller than 3.8 Å).



Supplementary Figure 6. Superposition of EngBF_L1_E4_v2 without ligand on EngBF_L1_E4_v1:ZHAWOC6027 based on the parental DARPin E4 sequence. ZHAWOC6027 is shown as sticks and the fusion proteins as cartoons. Coloring according to DARPin E4 with the N/C-caps in grey, 1st internal repeat in light yellow and 2nd internal repeat in cyan. EngBF_L1 framework residues from the v1- and v2 registers are shown in orange and blue, respectively. Glu1559 from the EngBF_L1_E4_v2 framework is shown as blue sticks and close contacts as black dotted lines.



Supplementary Figure 7. Superposition of ZHAWOC6027 with orange carbons on efrapeptidin (PDB ID: 1EFR) with grey carbons. Grey dotted lines indicate H-bonds following the (n to n+3) pattern in the bovine F_1-ATPase:efrapeptin complex (Abrahams *et al.*, 1996). Residue numbering according to the EngBF_L1_E4_v1:ZHAWOC6027 structure.

Supplementary References

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