Supplementary Information for:
Designed nanomolar small-molecule inhibitors of Ena/VASP EVH1 interaction impair invasion and extravasation of breast cancer cells

Matthias Baronea, Matthias Müllerb, Slim Chihab, Jiang Ren, Dominik Albatc, Arne Soikeb, Stephan Dohmenb, Marco Kleinb, Judith Brunsb, Maarten van Dinther, Robert Optizb, Peter Lindemannb, Monika Beerbaumb, Kathrin Motznya, Yvette Rosked, Peter Schmiederb, Rudolf Volkmer, Marc Nazaréa, Udo Heinemanna, Hartmut Oschkinatb, Peter ten Dijkec1, Hans-Günther Schmalzb,d, and Ronald Kühnea1

a Leibniz-Forschungsinstitut für Molekulare Pharmakologie, 13125 Berlin, Germany; b Department of Chemistry, University of Cologne, Greinstrasse 4, 50939 Köln, Germany; c Oncode Institute, Department of Cell and Chemical Biology, Leiden University Medical Center, 2333 Leiden, The Netherlands; d Max-Delbrück-Centrum für Molekulare Medizin, 13125 Berlin, Germany

1 To whom correspondence should be addressed. E-mail: p.ten_dijke@lumc.nl, schmalz@uni-koeln.de, kuehne@fmp-berlin.de.

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Methods.

Scaffold design and synthesis. Structure-based design was performed with ultra-high resolution structures using Sybyl or MOLOC. The stereoselective synthesis strategy via Ru-catalyzed ring closing metathesis and their assembly to synthetic inhibitors was performed according to the methods described before (1–8). In short, the ProM-based inhibitors were synthesized by liquid phase peptide coupling from the corresponding scaffolds according to established methodology and fully characterized (1H NMR, 13C NMR, HRMS, IR and optical rotation) as methyl esters (SI Appendix Figs. S27–S30). Ethyl esters were obtained by late stage transesterification, via saponification and re-esterification under Steglich conditions (EtOH, EDC-HCl, DMAP). For inhibitors that entered cellular assays, purity of the dissolved inhibitor batches was proven by LCMS, HPLC and MALDI analytics (SI Appendix Figs. S31–S34) while for the peptides, chimeras and inhibitors used in in vitro assays, characterization included HPLC traces and MALDI TOF (SI Appendix Figs. S35–S44).

Protein purification. BL21 DE3 transformed bacteria grown in 2YT medium over night were sonicated, centrifuged and supernatant loaded on glutathione sepharose high performance matrix (GE). GST-fusion protein was eluted with GSH and cleaved with thrombin over night. EVH1 domains were bound on SP sepharose ion exchange column (GE) and eluted with a NaCl gradient. Domains were re-buffered into crystallization buffer (20 mM NaCl, 5 mM TCEP, 10 mM HEPES pH 7.0) or affinity measurement buffer (40 mM sodium phosphate pH 7.3, 100 mM NaCl and 2 mM TCEP) by passing through a HiLoad 16/60 Superdex 75 size exclusion column (GE). Using vivaspin concentrators (Sartorius Stedim Biotech), Ena/VASP EVH1 domains were brought up to concentrations of 40 mg/ml and shock-frozen in liquid nitrogen for storage.

Affinity measurement: Fluorescence titration. FT experiments were carried out with a Jasco spectrofluorometer (FP-6500) at 25°C using a 10×4 mm precision cell made from quartz suprasi with conical bore for a stirring magnet (Hellma, 109.004F-QS), filled to 800 µl. Changes in the tryptophane fluorescence were detected at 340 nm, using slits opened to 1-3 nm (excitation) and 3-5 nm (emission) and a photomultiplier voltage to load the detector.
with around 30% arbitrary starting signal. Ligand was titrated with 20 injections to roughly five times nominal excess. Protein concentration was set to around 5 times $K_d$, and ligand stock solution prepared to inject $20 \times 5 \mu l$. Depending on the protein concentration, excitation wavelength was set between 298-305 nm to keep starting protein absorbance below 0.04 cm$^{-1}$. Data points were averaged during one second and spacing of one minute ensured homogeneous mixing of the cell content. In total three to four replicas were made, combined with two background measurements titrating ligand into buffer. Parameters were optimized iteratively until standard errors reached less than 20% for the $K_d$ and less than 0.5 kJ/mol for $\Delta G$.

A one-to-one model for ligand-bound protein was assumed:

$$[PL] = \frac{1}{2} \left( P_{tot} + L_{tot} + K_d - \sqrt{(P_{tot} + L_{tot} + K_d)^2 - 4 P_{tot} L_{tot}} \right)$$

The nominal ligand concentration was multiplied by a factor $0 < M \leq 1$ to determine the amount of active ligand and the fitting procedure was carried out by an script written in R language as described earlier (6). An exhaustive list of affinities for different inhibitor compositions can be found in SI Appendix Tabs. S7 and S8.

**Affinity measurement: Isothermal calorimetry.**  ITC experiments were performed at 25°C using 40 mM sodium phosphate pH 7.3, 100 mM NaCl and 1 mM DTT. Protein concentration was set to around 5 times $K_d$, and ligand stock solution prepared to inject $11 \times 2 \mu l$. Reference power was set to 18 μcal/sec with a stirring speed of 1000 s$^{-1}$ and 2 s filter period. In total three to four replicas were made, combined with two background measurements titrating ligand into buffer.

A one-to-one model was assumed and the nominal ligand concentration was multiplied by a factor $0 < M \leq 1$ to determine the amount of active ligand. The fitting procedure was carried out by an script written in R language as described earlier (6).

**SPOT substitution assay.**  SPOT array was prepared by an automated spot synthesis protocol on whatman-50-Cellulose (9). Membrane and bound peptides were washed with ethanol and washing buffer (3% (v/v) tween 20, 1xTBS pH 8.6) and subsequently blocked (5% (w/v) milk powder, 3% (v/v) tween 20, 1xTBS pH 8.6) for 3 min at room temperature. Each membrane was covered with GST-fused EVH1 domains (10μl/ml GST-VASP EVH1, GST-ENAH EVH1, GST-EVL EVH1, and GST) in sample buffer (3% (w/v) milk powder, 3% (v/v) tween 20, 1xTBS pH 8.6) and incubated at 7°C over night. After extensive washing, the anti GST antibody (Z-5 polyclonal rabbit IgG, Santa Cruz Biotech 200μg/ml) was added as 1:1000 (v/v) dilution in sample buffer and incubated over night at 4°C. Membranes were then washed and covered with anti-rabbit antibody (IRDye800, Santa Cruz Biotech), diluted 1:20000 (v/v) in sample buffer. After binding took place over 45 min at room temperature, excess antibody was washed away and membranes transferred into 1xTBS. Binding was detected with fluorescence signal on Odyssey infrared imaging system (Li-Cor, v2.0.40) at a resolution of 84μm.

**Crystallization and structure determination.**  Inhibitors were added to protein solution and mixture diluted to 8-26 mg/ml. Molar excess and final concentration depended on inhibitor affinity and activity. Crystals were grown by sitting vapor-diffusion method (Art Instruments Gryphon). For initial experiments, Quiagen Sciences Suites and equal volumes of reservoir and protein-ligand solutions were used. Single, well diffracting crystals suitable for high-resolution structures were achieved by switching to micro-seeding (volume ratio reservoir:protein:seed 3:2:1) and/or adjusting temperature. Crystallization plates were stored in plate hotels (Formulatrix RockImager), in an incubator or on a precision thermal plate for temperature drift experiments. Fully grown crystals were flash-frozen in mother liquor containing 20% glycerol as cryo protectant and measured at the BESSY-II radiation light source (MX-beamlines 14.1 and 14.2 in Adlershof, Berlin). Diffraction data was indexed, processed and scaled with XDS and/or adjusting temperature. Crystallization plates were stored in plate hotels (Formulatrix RockImager), in an incubator or on a precision thermal plate for temperature drift experiments. Fully grown crystals were flash-frozen in mother liquor containing 20% glycerol as cryo protectant and measured at the BESSY-II radiation light source (MX-beamlines 14.1 and 14.2 in Adlershof, Berlin). Diffraction data was indexed, processed and scaled with XDS (10–12). Molecular replacement started with unliganded EVH1 structures as search model using Phaser (13). Ligands were visible in the experimental map right after molecular replacement but were included in later rounds of model building. Non-natural ligands were built, minimized and docked with MOLOC (14) while the restraint files were generated on the PRODRG2 server (15). Peptidic chimeras were assembled in JLigand (16). All structures were refined with the PHENIX program suite (17) and rebuilt with COOT (18) and MOLOC. Of most complex structures existed multiple crystal data sets that were recorded during the process of crystal quality optimization. Isomorphous data sets were merged by XSCEALE and paired refinement was carried out with the unmerged and merged data sets.
at lowest resolution. For deposition, the most accurate model was chosen according to the free $R$ value calculated at the same resolution limit (11). Final complex structures were deposited in protein data bank (rcsb.org). Diffraction and refinement statistics of all 21 complex structures are listed in the SI Appendix Tabs. S1-S6.

**Graphical depiction.** Molecular structures were rendered by Chimera (19) (v1.10.1 and v1.13.1). Chains within asymmetric units were superposed in MOLOC using the atomic positions W23NE, W23N, F77CG, Y16CG, and Q79CD to calculate a rigid body match. Inhibitors were therefore positioned over the epitope-shaping amino acids as riding atoms. For better visibility the surveying superpositions contain only one protein complex each, while in detailed views shown in the SI Appendix contain all chains of the asymmetric unit.

**$^1$H-$^15$N HSQC-measurements.** Fluorescence titrations were performed prior to the HSQC NMR experiments to assess the activity of the ligand. To stably fit affinities from peak perturbations, 10-26 subsequent spectra were recorded with constant protein concentration (100-250 $\mu$M $^{15}$N-labeled protein) and increasing molar ligand excess. For this, an unbound and a nearly fully ligand-saturated protein sample were prepared and spectra recorded first. Protein saturation was then increased step by step by adding ligand-bound sample to the previous experiment while the rest of the samples were stored on ice. A Hamilton syringe with a customized long needle was used to remove the entire sample (typically 200 µl initial volume) from 3mm NMR tubes for homogeneous mixing in the low-bind Eppendorf tube of the unbound protein sample and transferring the new mixture back into the NMR tube. A script written in R language was used to optimize ligand consumption and protein saturation curves by adjusting maximal molar excess (final protein saturation), protein concentration and the ligand excess of the intermediate titration points.

HSQC spectra were recorded at 300 K with 16 scans at a resolution of 1024 ($^1$H) times 256 ($^15$N) complex points on an AV600 Bruker spectrometer (600 MHz proton frequency) using a 5 mm TCI cryoprobe equipped with self-shielded z-gradient and processed with TopSpin. Sparky-readable spectra were generated using bruk2ucsf to convert 2rr raw files to ucsf format and perturbations tracked in Sparky (v3.114) (20). Peak perturbation trajectories were calculated as euclidean distances of chemical shifts $\Delta\delta$ and fitted against the one-to-one binding model mentioned above:

$$f(L_{tot}) = \Delta\delta \propto |PL|$$

$$\sqrt{(\Delta\delta^\text{H})^2 + (0.1\Delta\delta^\text{N})^2} \propto |PL|$$

$$\sqrt{(\Delta\delta^\text{H})^2 + (0.1\Delta\delta^\text{N})^2} = A \left( P_{tot} + L_{tot} + K_d - \sqrt{(P_{tot} + L_{tot} + K_d)^2 - 4P_{tot}L_{tot}} \right)$$

The peak tables written by Sparky were processed, the trajectories calculated and fitted by a script written in R language. The script allowed sorting the HSQC data for certain exclusion criteria, such as minimal perturbation or goodness of the fits before producing the graphs seen in SI Appendix Figs. S13-S21.

**Cell culture.** MDA–MB–231 (ATCC HTB-26) were cultured in RPMI with 10% FBS at 37°C and 5% CO2 atmosphere. Cells were regularly tested for absence of mycoplasma and authenticity test was performed by SNP analysis (Eurofins Genomics). MDA–MB–231 cells stably expressing mCherry were obtained by transducing pLV-mCherry lentivirally. pLV-mCherry was a gift from Pantelis Tsoulfas (Addgene plasmid # 36084 ; http://n2t.net/addgene:36084 ; RRID:Addgene_36084).

**Pulldown.** Pulldown experiments were performed by N-terminally GST-tagged ENAH EVH1 (100 pmol/condition) immobilized on glutathione Sepharose 4B beads (GE) using MDA–MB–231 cell lysate (200 µl/condition) with a total protein concentration of 2 mg/ml, measured by BCA Protein Assay Kit (Novagen) UV/VIS spectroscopy (NanoDrop 1000; Thermo Scientific). Displacement of RAPH1, RIAM and Zyxin was achieved by adding different concentrations of inhibitors 1 or 6 to the lysate incubating overnight on beads at 4°C. As a control, GST alone was immobilized on the beads and treated with lysate. The Western blot with target-specific antibody against RAPH1 (H-5, scbt), RIAM (EPR2806, Abcam), Zyxin (Z4751, Sigma) and GST (Z-5, scbt) and fluorescence of secondary antibody (IRDye 800: Licor) was measured on an infrared scanner (ODYSSEY; Licor). Fluorescence signals were then processed with a script written in R language. IC50 values were fitted using

$$f(x) = \min + (\max - \min) \left(1 + \left(x/IC50^{\exp}\right)^{-1}\right)$$

with $\min$ and $\max$ being the upper and lower baselines, $x$ the ligand concentration and $\exp$ the slope steepness around IC50. Statistical difference between the IC50 values obtained for inhibitor 1 and 7 were determined by the t-test ($P=1.4e-6$).

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**IncuCyte chemotaxis assay.** Migration of MDA–MB–231 cells toward serum was measured using the IncuCyte chemotaxis cell migration assay. We seeded 2000 cells per well in 40 µl DMEM high glucose (GIBCO, 41966) with 0.25% FBS (Biowest, S1860-500) in the upper chamber of an IncuCyte Clearview 96-well Cell Migration Plate (Essen Bioscience, 4582). The cells were left to settle for 15 minutes at ambient temperature. Next the insert was transferred to the plate containing the chemoattractant (DMEM high glucose with 1% FBS) and the compounds. The FBS gradient over the two chambers was optimized and is shown in SI Appendix Fig. S23. The experiment was set up as triplet for the inhibitor concentrations and quadruplets for the positive (PBS) and negative controls (20 µM PI3K inhibitor LY294002). Compounds were also added to the upper chamber so that the concentrations were equal in both chambers. Migration across the membrane was monitored by the IncuCyte S3 live cell imaging system (Essen Bioscience). Images were taken every three hours and the data was analyzed using the IncuCyte image analysis software (Essen Bioscience).

Raw data were processed with a script written in R language. Triplet and quadruplets of each condition were averaged without sorting for outliers nor zeroing the lower well signal at the beginning of the experiment. IC50 values were calculated only after the phase object signal started increasing linearly and stopped before confluency in the top well reached a plateau. At these time points, the IC50 was fitted using the same equation as mentioned above. Since the chemotactic response of MDA–MB–231 was lost at high inhibitor concentrations (6), \( \text{min} \) was forced to 0 to gain a degree of freedom (DOF) and only fits with \( P<0.05 \) for the fitted IC50 coefficient were taken into account. Statistical differences between the IC50 values obtained for inhibitor 1, 4a, 6, and 7 were determined by the t-test. \( P \) values are as follows: 1 vs. 4a \( P=6.8e-9 \), 1 vs. 6 \( P=8.4e-12 \), 4a vs. 6 \( P<2.2e-16 \), 6 vs. 7 \( P=0.55 \). For the IC50 curves shown in the main text, the dashed lines were calculated by using the upper and lower confidence intervals of the fitted coefficients at the 99.87th percentile (3 \( \sigma \)) and grouped DOF of 5 (8 inhibitor concentrations minus 3 fitting variables). Two graphs each using the upper or lower confidence interval were then plotted as dashed lines over the raw data and the fitted variables. For better visibility, each inhibitor signal was subsequently normalized to the fitted \( \text{max} \) value, even though these values varied only between 250 and 320 phase object count.

**Evaluation of cancer cell extravasation in embryonic zebrafish xenograft model.** Zebrafish experiments were performed at 48 hours post fertilization as described before (21). Approximately 400 mCherry-labeled MDA–MB–231 cells were injected into duct of curvier (DoC) of transgenic zebrafish embryos (fli:EGFP), which vasculatures are marked in green. After verification by microscope only correctly injected zebrafish were divided randomly and administrated with corresponding inhibitor treatment. Thereafter zebrafish were maintained at 34°C, a compromise for both the fish and the human cell lines. Five days after of DoC injection, the number of MDA–MB–231 cells that extravasated individually from circulation into the collagen fibres of the tail fin were analyzed under a confocal microscopy (SP5 STED, Leica Microsystems). Inhibitors were refreshed at the midterm of treatment. All experiments were repeated at least two times independently, and representative experiments are shown. Statistical differences between the control, inhibitor 1, 4a, 6, and 7 were determined by the t-test. \( P \) values are as follows: control vs. 1 \( P=0.26 \), control vs. 4a \( P=3.6e-9 \), control vs. 6 \( P=0.0035 \), control vs. 7 \( P=1.7e-8 \).

**Statistics.** Statistical analysis of the results was carried out with the two R packages ggplot2 and ggsignif. Differences were considered significant at confidence levels greater than 95% (two-tailed). Three levels of statistical significance are distinguished and plotted in the figures of the main text: * \( P<0.05 \); ** \( P<0.01 \); *** \( P<0.001 \).
Supplementary Tables and Figures.

Fig. S1. Antimetastatic drugs upstream of the actin interactome
Ena/VASP proteins are elongation factors of F-actin at the very end of tumorigenic pathways (blue and yellow). Antimetastatic drugs (red) inhibit signaling towards actin-associated proteins (green) that connect the actin cytoskeleton to the extracellular matrix via integrin-mediated adhesions and invadopodia. The actin interactome is governed by weak protein-protein interactions that are transient by necessity and is therefore viewed as undruggable.

Fig. S2. Overview and relations between ProM scaffold chemical structures
Related ProM scaffolds are sorted in columns. The two initial scaffolds ProM-1 and ProM-2 differ in the central ring size. Modifications of the N-terminal ring of ProM-1 yielded ProM-3 and ProM-4 (see section on page 23 of the SI Appendix). Modifications of the C-terminal ring of ProM-1 can be divided into two categories. Scaffolds ProM-12, ProM-15, ProM-17 in the upper row were in silico designed to establish a polar interaction on the protein surface via their C-terminus. ProM-9 and ProM-13 on the lower row represent directed modifications to increase contact area over an apolar patch of the protein surface.
| ENAH EVH1 | Ac–[2-Cl-Phe] | Ac–[2-Cl-Phe] | Ac–[2-Cl-Phe] | Ac–[2-Cl-Phe] |
|-----------|---------------|---------------|---------------|---------------|
| PPPP--OH  | PP[ProM-1]–OH | PP[ProM-1]–NH | [ProM-1][ProM-1]–OH |

**Diffraction statistics**

| Spacegroup | P1 | P1 | P3,21 | P2,2,2 |
|------------|----|----|-------|-------|
| Cell dimension [Å, °] | a=34.91, b=43.37, c=43.65, α=61.15, β=84.25, γ=84.33 | a=34.76, b=43.39, c=43.59, α=61.40, β=84.03, γ=84.10 | a=47.27, b=47.27, c=202.72, α=61.15, β=84.25, γ=84.33 | a=73.71, b=78.02, c=80.85 |
| Resolution [Å] | 38–1.49 | 38–1.16 | 41–1.80 | 45–1.46 |
| Unique reflections | 33987 (5476) | 71797 (1195) | 25337 (4010) | 81310 (12951) |
| Completeness [%] | 93.0 (92.6) | 93.1 (90.0) | 99.4 (99.7) | 99.7 (99.6) |
| Redundancy | 2.77 | 2.0 (1.9) | 5.4 (5.6) | 5.4 (5.5) |
| ⟨I/σ⟩mrgd | 12.58 (1.36) | 11.24 (1.86) | 14.61 (1.49) | 11.79 (1.66) |
| CC1/2 | 99.9 (59.5) | 99.9 (82.6) | 99.9 (58.1) | 99.8 (62.1) |
| Rmax b [%] | 5.5 (96.5) | 4.8 (51.7) | 8.6 (113) | 9.6 (101) |
| ISa | 26.18 | 36.57 | 45.41 | 21.62 |

**Refinement statistics**

| Solvent content [%] | 45.5 | 45.9 | 52.3 | 46.2 |
|---------------------|------|------|------|------|
| Vm [Å³ / Da] | 2.26 | 2.25 | 2.58 | 2.28 |
| Molecules per AU | 2 | 2 | 2 | 4 |
| Rfact c [%] | 17.9 | 13.9 | 20.21 | 17.8 |
| Rfree d [%] | 20.1 | 14.1 | 21.66 | 19.9 |
| rmsdback [Å] | 0.06 | 0.01 | 0.007 | 0.008 |
| rmsdangle [°] | 1.2 | 1.7 | 1.4 | 1.28 |
| PDB code | 5N91 | 5N9C | 5N9P | 5NAJ |

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Tab. S1. Diffraction and refinement statistics

Deposited crystal structures with ProM-1-based inhibitor compositions successfully replacing the core motif of ActA-derived peptide FPPPP.
ENAH EVH1

| Ac-[2-Cl-Phe]       | Ac-[2-Cl-Phe]       | Ac-[2-Cl-Phe]       | Ac-[2-Cl-Phe]       |
|---------------------|---------------------|---------------------|---------------------|
| [ProM-2]            | [ProM-2]            | [ProM-2]            | [ProM-2]            |
| [ProM-1]-NH₂        | [ProM-1]-OH         | [ProM-3]-OH         | [ProM-4]-OH         |

Inhibitor number 1b

**Diffraction statistics**

| Spacegroup       | C222,     | C222     | P2      | P1      |
|------------------|-----------|----------|---------|---------|
| Cell dimension [Å, °] | a=24.70  | a=89.83  | a=34.61 | a=34.72 |
|                  | b=60.69   | b=131.42 | b=38.25 | b=43.36 |
|                  | c=89.06   | c=35.56  | c=44.00 | c=44.18 |
|                  | β=90.59   |          |         |         |

| Resolution [Å]   | 46–1.36 (1.47–1.36) | 32–1.7 (1.74–1.70) | 44–1.15 (1.22–1.15) | 50–1.40 (1.48–1.40) |
| Unique reflections | 20643 (4246)       | 23659 (1704)       | 38436 (6060)       | 41864 (6668)       |
| Completeness [%] | 100 (100)          | 99.8 (99.9)        | 93.5 (92.0)        | 94.6 (93.3)        |
| Redundancy       | 14 (15)            | 4 (4.1)            | 2.2 (2.1)          | 2.3 (2.3)          |
| ⟨I/σ⟩ <sub>mrgd</sub> | 11.48 (0.61)   | 12.86 (2.35)       | 13.15 (1.44)       | 10.39 (1.12)       |
| CC<sub>1/2</sub> | 99.9 (27.3)       | 99.8 (76.8)        | 99.9 (59.1)        | 99.9 (60.2)        |
| R<sub>free</sub> | 13.1 (535)        | 8.2 (64.6)         | 4.5 (78.2)         | 7.3 (99.5)         |
| ISa              | 14.62            | 19.92              | 34.82              | 36.01              |

**Refinement statistics**

| Solvent content [%] | 33.3 | 40.9 | 47.2 | 46.3 |
| V<sub>M</sub> [Å³ / Da] | 1.84 | 2.08 | 2.33 | 2.29 |
| Molecules per AU | 1    | 2    | 1    | 2    |
| R<sub>free</sub> | 20.1 | 19.0 | 15.7 | 22.0 |
| R<sub>free</sub> | 23.6 | 23.6 | 18.6 | 26.0 |
| rmsd<sub>bond</sub> [Å] | 0.01 | 0.01 | 0.01 | 0.009 |
| rmsd<sub>angle</sub> [°] | 1.6 | 1.6 | 1.60 | 1.16 |
| PDB code | 7AKI | 4MY6 | 5NBF | 5NCF |

<sup>a</sup> Numbers in parentheses correspond to outer shell
<sup>b</sup> R<sub>free</sub>: redundancy-independent R<sub>f</sub> factor
<sup>c</sup> R<sub>free</sub> = Σ|F<sub>0</sub> − F<sub>c</sub>| / Σ|F<sub>0</sub>| (22)
<sup>d</sup> the observed and calculated structure factors
<sup>e</sup> R<sub>free</sub> set contains 5% of total reflections (23)

Tab. S2. Diffraction and refinement statistics

Deposited crystal structures of ProM-2-based inhibitors composed of unsuccessful modifications on the N-terminal building blocks ProM-3 and ProM-4. These scaffolds are discussed in the SI Appendix on pages 23 and 24.
| ENAH EVH1 | Ac–WPPPTEDEL–NH₂ | Ac–[2-Cl-Phe]PPPPTEDEL–NH₂ | Ac–[2-Cl-Phe]PPPPTEDDL–NH₂ | Ac–[2-Cl-Phe]PPPPTEDEA–NH₂ |
|-----------|------------------|----------------------------|-----------------------------|-----------------------------|

**Diffraction statistics**

| Spacegroup | C2 | C2 | P1 | P1 |
|------------|----|----|----|----|
| Cell dimension [Å, °] | a=187.92 | a=147.78 | a=34.65 | a=34.74 |
| | b=34.42 | b=44.02 | b=44.28 | b=43.19 |
| | c=110.96 | c=34.67 | c=43.16 | 44.01 |
| | β=91.53 | β=102.13 | α=60.85 | α=61.04 |
| | | | β=84.04 | β=84.20 |
| | | | γ=84.01 | γ=84.21 |
| Resolution [Å] | 48–2.70 | 42–1.58 | 39–1.40 | 38–1.48 |
| Unique reflections | 20211 (2039) | 29131 (4618) | 42224 (6456) | 35565 (6688) |
| Completeness [%] | 98.8 (100) | 96.8 (95.7) | 96.0 (95.2) | 95.7 (93.5) |
| Redundancy | 5.3 (5.3) | 2.3 (2.3) | 3.8 (3.8) | 3.1 (2.8) |
| ⟨I/σ⟩<sub>mgd</sub> | 8.60 (1.53) | 10.23 (1.47) | 9.48 (1.00) | 3.86 (0.58) |
| CC<sub>1/2</sub> | 98.9 (58.7) | 99.8 (60.5) | 99.8 (47.9) | 98.4 (37.6) |
| R<sub>meas</sub> b [%] | 23.0 (153) | 8.5 (97.5) | 9.3 (155) | 22.1 (194) |
| ISa | 18.40 | 29.34 | 21.81 | 22.88 |

**Refinement statistics**

| Solvent content [%] | 65.2 | 44.2 | 45.6 | 45.5 |
| V<sub>M</sub> [Å<sup>3</sup>/Da] | 3.53 | 2.20 | 2.26 | 2.26 |
| Molecules per AU | 4 | 2 | 2 | 2 |
| R<sub>calc</sub> [%] | 20.1 | 24.6 | 20.1 | 24.6 |
| R<sub>free</sub> [%] | 27.0 | 19.9 | 23.1 | 26.9 |
| rmsd<sub>bond</sub> [Å] | 0.009 | 0.01 | 0.009 | 0.024 |
| rmsd<sub>angle</sub> [°] | 1.10 | 1.17 | 1.63 | 1.67 |
| PDB code | 5NC7 | 5NC2 | 6XVT | 6XXR |

* Numbers in parentheses correspond to outer shell

b R<sub>meas</sub>: redundancy-independent R factor

c R<sub>calc</sub> = \sum |F<sub>o</sub> – F<sub>c</sub>| / \sum |F<sub>o</sub>| (22)

d R<sub>free</sub> set contains 5% of total reflections (23)

Tab. S3. Diffraction and refinement statistics

Deposited crystal structures with C-terminally elongated ActA-derived peptide Ac–<sup>1</sup>FPPPPTEDDEL–NH₂ as well as the single point mutations from <sup>9</sup>Glu to <sup>9</sup>Asp and <sup>10</sup>Leu to <sup>10</sup>Ala (Relative affinity losses of these two peptides are found in SI Appendix Fig. S4c).
| Inhibitor number | 2ΔN |
|------------------|-----|

**Diffraction statistics**

| Spacegroup | C2 | C2 |
|------------|----|----|
| Cell dimension [Å, °] | a=149.74, b=44.24, c=34.83, β=101.47 | a=148.39, b=44.00, c=34.83, β=102.17 |
| Resolution [Å] | 42–1.45 (1.54–1.45) | 42–1.00 (1.06–1.00) |
| Unique reflections | 39592 (6309) | 118323 (18886) |
| Completeness [%] | 99.4 (99.2) | 99.9 (99.7) |
| Redundancy | 3.3 (3.1) | 14 (13) |
| ⟨I/σ⟩ | 7.69 (1.32) | 5.26 (0.0) |
| CC₁/₂ | 98.9 (58.7) | 98.8 (14.4) |
| Rₘₑₐ[s] [%] | 11.3 (104) | 23.9 (-99.9) |
| ISa | 13.37 | 23.36 |

**Refinement statistics**

| Solvent content [%] | 44.7 | 44.3 |
| Vᵣ [Å³ / Da] | 2.22 | 2.21 |
| Molecules per AU | 2 | 2 |
| Rₘₑₐ[s] [%] | 18.2 | 19.8 |
| Rₘₑ[s] [%] | 20.4 | 22.4 |
| rmsd bonds [Å] | 0.004 | 0.016 |
| rmsd angles [°] | 0.977 | 1.58 |
| PDB code | 5ND0 | 6RD2 |

- Numbers in parentheses correspond to outer shell
- Rₘₑₐ[s]: redundancy-independent R factor
- Rₘₑ[s] = ∑ |F₀ - Fₛ| / ∑ |F₀| (22)
  the observed and calculated structure factors
- Rₘₑ[s] set contains 5% of total reflections (23)

**Tab. S4. Diffraction and refinement statistics**
Deposited crystal structures with C-terminally elongated ActA-derived, ProM-containing chimeras.
| ENAH EVH1 | Ac–[2-Cl-Phe] | Ac–[2-Cl-Phe] | Ac–[2-Cl-Phe] | Ac–[2-Cl-Phe] |
|-----------|---------------|---------------|---------------|---------------|
|           | [ProM-2] | [ProM-12]-OH | [ProM-2] | [ProM-12]-OMe |
|           | [ProM-2] | [ProM-15]-OH | [ProM-2] | [ProM-15]-OMe |

**Inhibitor number**

| 3b | 3a | 4b | 4a |

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**Diffraction statistics**

| Spacegroup | C222 | C2 | C222 | C222 |
|------------|------|----|------|------|
| Cell dimension [Å, °] | a=44.14, b=141.37, c=34.71 | a=136.15, b=34.57, c=44.24 | a=43.38, b=141.46, c=34.80 | a=44.00, b=141.53, c=34.73 |
| Resolution [Å] | 42–1.65 (1.75–1.65) | 44–1.42 (1.50–1.42) | 41–1.10 (1.17–1.10) | 42–1.35 (1.43–1.35) |
| Unique reflections | 13548 (2144) | 38981 (5889) | 42538 (6649) | 24264 (3860) |
| Completeness [%] | 99.9 (100) | 99.9 (99.9) | 96.5 (94.4) | 99.4 (99.5) |
| Redundancy | 8.5 (8.4) | 6.4 (5.8) | 6.5 (6.6) | 6.5 (6.4) |
| ⟨I/σ⟩ mrgd | 9.14 (0.97) | 11.46 (1.25) | 9.43 (0.55) | 13.69 (1.21) |
| CC1/2 | 99.7 (43.3) | 99.9 (59.3) | 99.9 (27.8) | 99.9 (56.3) |
| Rmeas [%] | 20 (210) | 12 (170) | 9.3 (310) | 8.0 (143) |
| ISa | 19.13 | 17.26 | 18.54 | 27.72 |

**Refinement statistics**

| Solvent content [%] | 42.3 | 39.5 | 41.5 | 42.2 |
| Vm [Å³/Da] | 2.13 | 2.03 | 2.10 | 2.13 |
| Molecules per AU | 1 | 2 | 1 | 1 |
| Rmax [%] | 18.3 | 17.0 | 16.5 | 17.1 |
| Rfree [%] | 20.8 | 19.8 | 20.1 | 20.2 |
| rmsd [Å] | 0.009 | 0.02 | 0.019 | 0.020 |
| rmsd [°] | 1.27 | 1.86 | 1.69 | 1.88 |
| PDB code | 5NCP | 5NDU | 6RCF | 6RCJ |

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**Tab. S5. Diffraction and refinement statistics**

Deposited crystal structures with scaffolds yielded through structure-based drug design. These scaffolds allow replacement of the core motif of ActA-derived peptide Ac–FPPPPTEDEL–NH₂ and mimic the polar interaction of TEDEL.

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*a* Numbers in parentheses correspond to outer shell

*b* Rmeas: redundancy-independent R factor

*c* Rint = \( \sum |F_0 - F_c| / \sum |F_0| \) (22)

the observed and calculated structure factors

*d* Rfree set contains 5% of total reflections (23)
| ENAH EVH1 | Ac–[2-Cl-Phe][ProM-2] | Ac–[2-Cl-Phe][ProM-17]–OMe | Ac–[2-Cl-Phe][ProM-2] | Ac–[2-Cl-Phe][ProM-13]–OEt |
|-----------|---------------------|----------------------|---------------------|----------------------|
| Inhibitor number | 5a | 6c | 6b | 7 |

**Diffraction statistics**

| | C222, | C222 | P2,2,2, | P2 |
|---|---|---|---|---|
| Spacegroup | C222, | C222 | P2,2,2, | P2 |
| Cell dimension [Å, †] | a=35.15 | a=90.15 | a=35.02 | a=34.81 |
| | b=61.36 | b=131.52 | b=61.27 | b=44.36 |
| | c=88.94 | c=35.68 | c=89.26 | c=72.47 |
| | β=90.56 | | | |
| Resolution [Å] | 30–0.78 | 45–1.65 | 36–1.02 | 44–1.29 |
| | (0.85–0.78) | (1.75–1.65) | (1.08–1.02) | (1.39–1.29) |
| Unique reflections | 106735 (23284) | 25469 (3536) | 172743 (26288) | 53114 (9979) |
| Completeness [%] | 97.7 (94.7) | 97.3 (85.3) | 91.4 (86) | 99.4 (69.4) |
| Redundancy | 19.0 (15.8) | 4.1 (2.8) | 3.0 (2.9) | 4.7 (2.7) |
| | | | | |
| R<sub>free</sub> [%] | 23.1 (1.1) | 12.7 (1.7) | 12.4 (2.49) | 6.76 (1.94) |
| CC<sub>1/2</sub> | 100 (53.9) | 99.8 (60.7) | 99.9 (80.1) | 99.4 (76.4) |
| R<sub>max</sub> [%] | 5.1 (281) | 8.6 (74.6) | 6.2 (52.6) | 15.6 (65.6) |
| ISa | 26.65 | 21.54 | 25.51 | 19.34 |

**Refinement statistics**

| | 34.8 | 41.0 | 34.7 | 43.8 |
| Solvent content [%] | 1.89 | 2.08 | 1.88 | 2.19 |
| V<sub>M</sub> [Å<sup>3</sup> / Da] | 1 | 2 | 2 | 2 |
| Molecules per AU | 12.0 | 16.5 | 12.4 | 15.1 |
| R<sub>free</sub> [%] | 14.0 | 19.4 | 15.0 | 19.0 |
| R<sub>free</sub> [%] | 0.022 | 0.01 | 0.01 | 0.008 |
| rmsd<sub>model</sub> [Å] | 2.38 | 1.26 | 1.72 | 1.21 |
| rmsd<sub>angle</sub> [°] | | | | |
| PDB code | 7A5M | 5NBX | 5NC5 | 5NEG |

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Tab. S6. Diffraction and refinement statistics

Deposited crystal structures with scaffolds yielded through structure-based drug design. These scaffolds allow replacement of the core motif of ActA-derived peptide Ac–FPFPPTEDEL–NH₂ and mimic the polar (ProM-17) or hydrophobic (ProM-9 and ProM-13) interaction of TEDEL.
**ENAH EVH1 + inhibitor composition**

| wt2 | Ac–SFE | F | PP | PP | TEDEL–NH₂ | $K_d, FT$ [µM] | $\Delta G$ [kJ/mol] |
|-----|---------|---|----|----|----------|----------------|-------------------|
|     | Ac–SFE  | [2-Cl-Phe] | PP | PP | TEDEL–NH₂ | 13 (1) | −27.9 (0.1) |
|     | Ac–SFE  | [2-Cl-Phe] | PP | PP | TEDDL–NH₂ | 0.79 (0.05) | −34.8 (0.2) |
|     | Ac– [2-Cl-Phe] | PP | PP | TEDEL–NH₂ | 1.43 (0.09) | −33.4 (0.1) |
|     | Ac– [2-Cl-Phe] | PP | PP | TEDDL–NH₂ | 1.25 (0.04) | −33.7 (0.07) |
|     | Ac– [2-Cl-Phe] | PP | PP | TEDEA–NH₂ | 3.8 (0.2) | −30.9 (0.1) |
|     | Ac– [2-Cl-Phe] | PP | PP | TEDDL–NH₂ | 3.5 (0.1) | −31.4 (0.1) |
|     | Ac– [2-Cl-Phe] | PP | [ProM-1] | TEDEL–NH₂ | 0.60 (0.04) | −35.5 (0.2) |
|     | Ac–SFE  | [2-Cl-Phe] | PP | PP | –OH | 4.6 (0.2) | −30.5 (0.1) |
|     | Ac–SFE  | F | [ProM-2] | PP | TEDEL–NH₂ | 12 (1) | −28.2 (0.3) |
|     | Ac–SFE  | F | [ProM-1] | PP | TEDEL–NH₂ | 8 (1) | −29.1 (0.3) |
|     | Ac– | F | PP | PP | –OH | 500 (70) | −19 (0.4) |
| wt1 | Ac– [2-Cl-Phe] | PP | PP | –OEt | 153 (8) | −21.8 (0.1) |
|     | Ac– [2-Cl-Phe] | PP | PP | –OH | 9.9 (0.8) | −28.6 (0.2) |
|     | 1c | Ac– [2-Cl-Phe] | PP | [ProM-1] | –OH | 2.4 (0.4) | −32.0 (0.4) |
|     | 1b | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-1] | –OH | 2.3 (0.2) | −32.2 (0.2) |
|     | 1 | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-1] | –OEt | 4.1 (0.3) | −30.8 (0.2) |
|     |     | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-4] | –OH | 3.0 (0.3) | −31.5 (0.2) |
|     |     | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-3] | –OH | 9.7 (0.8) | −28.6 (0.2) |
|     | Ac–SFE [2-Cl-Phe] | PP | [ProM-1] | TEDEL–NH₂ | 0.21 (0.05) | −38.1 (0.6) |
|     | 2 | Ac–SFE [2-Cl-Phe] | [ProM-2] | [ProM-1] | TEDEL–NH₂ | 0.15 (0.02) | −38.9 (0.2) |
|     | 6c | Ac– [2-Cl-Phe] | PP | [ProM-9] | –OH | 0.23 (0.05) | −37.9 (0.5) |
|     | 6b | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-9] | –OH | 0.12 (0.02) | −39.5 (0.3) |
|     | 6 | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-9] | –OEt | 0.38 (0.05) | −36.6 (0.3) |
|     | 7 | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-13] | –OEt | 0.12 (0.01) | −39.5 (0.3) |
|     | 3a | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-12] | –OMe | 15 (1) | −27.6 (0.2) |
|     | 3b | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-12] | –OH | 13.5 (0.5) | −27.8 (0.1) |
|     | 4a | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-15] | –OMe | 0.47 (0.03) | −36.1 (0.1) |
|     | 4b | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-15] | –OH | 0.32 (0.04) | −37.0 (0.3) |
|     | 5a | Ac– [2-Cl-Phe] | [ProM-2] | [ProM-17] | –OMe | 0.53 (0.09) | −35.8 (0.4) |

**Tab. S7. Affinities of inhibitor compositions binding to ENAH EVH1**

Binding studies based on fluorescence titration (FT) using ENAH EVH1 at 25°C. Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.
| VASP EVH1 + inhibitor composition | $K_{d, FT}$ [µM] | $\Delta G$ [kJ/mol] |
|----------------------------------|-----------------|------------------|
| wt2 Ac–SFE F PP PP TEDEL–NH$_2$   | 19 (2)          | –26.9 (0.3)      |
| Ac–SFE [2-Cl-Phe] PP PP TEDEL–NH$_2$ | 1.5 (0.3)     | –33.3 (0.5)      |
| Ac–SFE F [ProM-1] PP TEDEL–NH$_2$ | 40 (4)         | –25.1 (0.2)      |
| Ac–SFE F PP [ProM-1] TEDEL–NH$_2$ | 5.7 (0.3)      | –29.9 (0.1)      |
| Ac–SFE F [ProM-1] [ProM-1] TEDEL–NH$_2$ | 19 (6)       | –26.9 (0.8)      |
| Ac– F PP PP –OH                    | 800 (80)       | –17.7 (0.2)      |
| Ac– [2-Cl-Phe] PP PP –OH          | 13 (2)         | –27.8 (0.3)      |
| 1c Ac– [2-Cl-Phe] PP [ProM-1] –OH | 3.2 (0.3)      | –31.4 (0.3)      |
| 1b Ac– [2-Cl-Phe] [ProM-2] [ProM-1] –OH | 2.3 (0.1) | –32.2 (0.2)      |
| 1 Ac– [2-Cl-Phe] [ProM-2] [ProM-1] –OEt | 6.3 (0.6)     | –29.7 (0.3)      |
| Ac– [2-Cl-Phe] [ProM-2] [ProM-4] –OH | 1.6 (0.2)     | –33.1 (0.4)      |
| Ac–SFE [2-Cl-Phe] PP [ProM-1] TEDEL–NH$_2$ | 0.31 (0.05)  | –37.2 (0.4)      |
| 2 Ac–SFE [2-Cl-Phe] [ProM-2] [ProM-1] TEDEL–NH$_2$ | 0.25 (0.07) | –37.7 (0.7)      |
| Ac–SFE [2-Cl-Phe] PP [ProM-3] TEDEL–NH$_2$ | 1.5 (0.2)     | –33.2 (0.3)      |
| Ac–SFE [2-Cl-Phe] [ProM-1] [ProM-1] TEDEL–NH$_2$ | 0.3 (0.1)    | –37.1 (0.8)      |
| 6c Ac– [2-Cl-Phe] PP [ProM-9] –OH | 0.38 (0.09)    | –36.7 (0.5)      |
| 6b Ac– [2-Cl-Phe] [ProM-2] [ProM-9] –OH | 0.28 (0.06)  | –37.4 (0.5)      |
| 6 Ac– [2-Cl-Phe] [ProM-2] [ProM-9] –OEt | 0.78 (0.09)   | –34.9 (0.3)      |
| 7 Ac– [2-Cl-Phe] [ProM-2] [ProM-13] –OEt | 0.4 (0.1)     | –36.6 (0.6)      |

**Tab. S8. Affinities of inhibitor compositions binding to VASP EVH1**

Binding studies based on fluorescence titration (FT) using VASP EVH1 at 25°C. Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.
|       | 2    | 1    | 6    | 7    |
|-------|------|------|------|------|
| **VASP EVH1** |      |      |      |      |
| $K_d,\text{FT}$ [nM] | 250 (70) | 6200 (600) | 780 (90) | 400 (100) |
| $K_d,\text{ITC}$ [nM] | 560 (40) | 9400 (500) | 1400 (200) | 490 (60) |
| **ENAH EVH1** |      |      |      |      |
| $K_d,\text{FT}$ [nM] | 150 (60) | 4100 (300) | 380 (50) | 120 (10) |
| $K_d,\text{ITC}$ [nM] | 340 (30) | 7800 (400) | 800 (100) | 310 (40) |
| **EVL EVH1** |      |      |      |      |
| $K_d,\text{FT}$ [nM] | 190 (60) | 4100 (500) | 280 (50) | 130 (20) |
| $K_d,\text{ITC}$ [nM] | 260 (10) | 5800 (700) | 670 (70) | 280 (30) |

|       | 2    | 1    | 6    | 7    |
|-------|------|------|------|------|
| **VASP EVH1** |      |      |      |      |
| LE$_{\text{FT}}$ [J (mol HA)$^{-1}$] | –330 (6) | –590 (6) | –680 (6) | –700 (10) |
| LE$_{\text{ITC}}$ [J (mol HA)$^{-1}$] | –310 (2) | –570 (2) | –660 (6) | –690 (6) |
| **ENAH EVH1** |      |      |      |      |
| LE$_{\text{FT}}$ [J (mol HA)$^{-1}$] | –340 (9) | –620 (4) | –720 (6) | –760 (6) |
| LE$_{\text{ITC}}$ [J (mol HA)$^{-1}$] | –320 (2) | –580 (4) | –680 (4) | –710 (6) |
| **EVL EVH1** |      |      |      |      |
| LE$_{\text{FT}}$ [J (mol HA)$^{-1}$] | –330 (7) | –610 (6) | –730 (8) | –750 (8) |
| LE$_{\text{ITC}}$ [J (mol HA)$^{-1}$] | –327 (1) | –600 (8) | –690 (6) | –720 (6) |

**Tab. S9. Comparison of affinities and ligand efficiencies of final inhibitors 6 and 7 with parent inhibitor 1**

7 regained the affinity of the peptidic chimera 2 by introducing only two heavy atoms (HA). The improved ligand efficiency (LE: $\Delta G$ per HA) for both inhibitors 6 and 7 justified the increase of the molecular weight.
**SPOT substitution assay.** Single residue substitution experiments are reported only for VASP EVH1 (24). SPOT array substitution for all three Ena/VASP EVH1 paralogs revealed two conserved binding epitopes within the main binding groove that accepted other amino acids than the core motif sequence \(^1\)FPPPP. The first epitope, a deep pocket that EVH1 domains provide for \(^1\)Phe, was optimized earlier (6). Optimization of the second epitope, an apolar patch formed by Phe77 that contacts \(^4\)Pro (orange), was not successful. The structural insights of the scaffold modifications to probe this apolar patch are discussed here in the SI Appendix on pages 23-24.

**Fig. S3.** Substitution spot array of *L. monocytogenes*’ surface protein ActA-derived peptide SFEFPPPPTEDEL, color-coded according to the crystal structure of Ac-FPPPPT bound to Mena EVH1 (PDB code 1EVH). ENAH-, VASP-, and EVL EVH1 reveal a PxxP-binding mode in which the first and last proline can not be exchanged at all. Ena/VASP accepts for the preceding Phe of the core motif mainly aromatic residues but also Leu, while exchange to aliphatic residues are valid instead of the third proline. A more intimate contact of the terminal residues Glu-Leu of TEDEL is visible from more thinned out spots compared to the preceding TED, even though no clear recognition pattern is notable (best visible on EVL EVH1).
The conformation of TEDEL. Two of the crystallized ligands resembled the proline-rich core of the \textit{wt2} peptide, the other two ligand compositions contained the scaffold ProM-1 designated to mimic the additional interactions (SI Appendix Fig. S4a). Consistent with our finding, the secondary structure prediction server PSIPRED (25, 26) calculates helix propensity for amino acids succeeding Thr for all four proline-rich repeats (27, 28) of ActA. Within the flanking epitope, \textsuperscript{6}Thr is solvent-exposed but conserved in all four repeats of ActA, emphasizing its function as helix capping motif (29).

| Ligand composition                  | Resolution | PDB code |
|-------------------------------------|------------|----------|
| Ac–WPPPPTEDEL–NH\textsubscript{2}    | 2.8Å       | 5NC7     |
| Ac–[2-Cl-Phe]WPPPPTEDEL–NH\textsubscript{2} | 1.6Å       | 5NC2     |
| Ac–[2-Cl-Phe]PP[ProM-1]TEDEL–NH\textsubscript{2} | 1.45Å      | 5ND0     |
| \(2\Delta N\) Ac–[2-Cl-Phe][ProM-2][ProM-1]TEDEL–NH\textsubscript{2} | 1.00Å      | 6RD2     |

Fig. S4. Structure of the second binding epitope of \textit{L. monocytogenes'} surface protein ActA

(a) Complex structures of four TEDEL-containing ligand compositions were crystallized. (b) The proline-rich portion of the 10-mer adopts PPII conformation (blue), whereas the \(7\)EDEL forms an \(\alpha\)-helical loop (yellow). Ramachandran inlay shows the according phi/psi backbone angles of the TEDEL residues. Superposition of 27 ENAH EVH1 chains of 12 asymmetric units reveals a stable and conserved protein epitope as well as TEDEL binding canonically to the domain, independent of the presence of ProM scaffolds (represented by ribbons calculated by the backbone atoms within). (c) Single point mutations from \textsuperscript{9}Glu to \textsuperscript{9}Asp, as well as \textsuperscript{10}Leu to \textsuperscript{10}Ala loose significant affinity for ENAH EVH1. The crystal structures of ENAH EVH1 with either point-mutated peptide reveal very weak electron density or even an unresolved \(\alpha\)-helical portion after Thr. Interestingly however, the conserved water bound to W23NH is clearly visible in both complex structures, indicating that the loop structures with either point mutation might be severely disturbed, allowing the water molecule to remain bound to W23NH in the crystal packing.
Ligands of Homer- and ENAH EVH1 use a diametric binding mode to access the hydrophobic patch. The mGluR-derived peptide TPPSPF is the only known ligand that interacts close to the hydrophobic patch that binds TEDEL. TPPSPF is recognized by Homer EVH1, a class II EVH1 domain displaying crucial point mutations in both epitopes that bind Phe and EL of PPPPTEDEL to ENAH EVH1. The C-terminal cleft hosting Phe is nonexistent on Homer1 EVH1 due to two mutations (R81A and Y16I) that flatten the surface of Homer EVH1 and allow TPPSPF to bind C-terminally shifted within the binding groove (SI Appendix Fig. S5a). The hydrophobic patch around of Phe77 and Met14 is also flattened due to an Asn90 to Gly89 mutation (SI Appendix Fig. S5b), which alters the epitope severely. TPPSPF adopts a unique conformation with Pro adopting cis-conformation, followed by a tight type VIa β-turn that brings Phe straight down over Homer EVH1 Gly89 (30). The tight turn within the ligand and the conserved Gly89 are crucial to provide enough space for Phe to access the patch (30). Like Gly89 in Homer EVH1, the solvent-exposed Asn90 is conserved among the Ena/VASP EVH1 domains and sterically blocks the volume needed for ligands accessing the hydrophobic patch with a binding mode seen by TPPSPF. Hence, the hydrophobic patch is reachable only from the other side and seems to require a second intermolecular contact via W23NH to stabilize the hydrophobic interaction. SI Appendix Fig. S5b highlights that Leu of TEDEL and Phe of TPPSPF bind to the same hydrophobic patch in close vicinity of the main binding groove.

Fig. S5. Class I ENAH EVH1 and class II Homer EVH1 use of the apolar patch to bind their ligands

(a) Solvent-accessible surface of Homer EVH1 bound to TPPSPF (green, PDB code 1DDV) in superposition with ENAH EVH1 bound to Ac–[2-Cl-Phe]PPPPTEDEL–NH₂ (white, PDB code 5NC2). TPPSPF binds Homer1 EVH1 C-terminally shifted and interacts with G89. (b) The same superposition and coloring but the solvent accessible surface of ENAH EVH1 added as mesh. The Gly to Asn mutation sterically blocks the hydrophobic patch for ligands with a binding modes like TPPSPF.
Fig. S6. $^1$H-$^{15}$N HSQC titration of ENAH EVH1 and Ac–WPPPPTEDEL–NH$_2$
Maximal euclidean distance of peak perturbations reached with a final 10-fold molar ligand excess. Perturbations further than 0.1 ppm mapped on the solvent accessible surface of ENAH EVH1 (PDB code 5NC7). Due to the flexibility of the loop, T30 and F32 could not be assigned.

Fig. S7. $^1$H-$^{15}$N HSQC titration of ENAH EVH1 and Ac–[2-Cl-Phe]PPPPTEDEL–NH$_2$
Maximal euclidean distance of peak perturbations reached with a final 4-fold molar excess. Perturbations further than 0.1 ppm mapped on the solvent accessible surface of ENAH EVH1 (PDB code 5NC2). Due to the flexibility of the loop, T30 and F32 could not be assigned.
Fig. S8. TEDEL-elongated ligands entirely shield the apolar patch from solvent
ProM-1 and $^{10}$Leu cover an apolar patch that extends from the rigidly shaped binding groove (Y16, W23, F77, M14). Drawn are the solvent accessible surfaces of the chimeric inhibitor 2ΔN (white) and ENAH EVH1 (blue-white-red for hydrophilic-hydrophobic, PDB code 6RD2). For better visibility amino acids 6TED are transparent.
Electron densities of the bound water contacted by ProM-12 and ProM-15-based inhibitors. The two scaffold modifications ProM-12 and ProM-15 aimed to establish contact to a conserved water molecule bound to Trp23 backbone (red sphere drawn with its electron density in SI Appendix Fig. S9). While inhibitors with ProM-12 (upper panel SI Appendix Fig. S9) showed relatively strong and clear difference signal for this water, ProM-15 seemed to compete the bound water off and occupancy dropped notably, which is visible in composite maps at 1σ (lower panel).

| Inhibitor composition | Occupancy | B-factor | Fig. S9 | PBD code |
|-----------------------|-----------|----------|---------|----------|
| 3b Ac–[2-Cl-Phe][ProM-2][ProM-12]-OH | 100% | 37 Å² | (a) | 5NCP |
| 4b Ac–[2-Cl-Phe][ProM-2][ProM-15]-OH | 95% | 31 Å² | (c) | 6RCF |
| 3a Ac–[2-Cl-Phe][ProM-2][ProM-12]-OMe | 73% | 24 Å² | (b) | 5NDU |
| 4a Ac–[2-Cl-Phe][ProM-2][ProM-15]-OMe | 62% | 27 Å² | (d) | 6RCJ |

Fig. S9. Electron densities of inhibitor compositions 3 and 4 designed to contact the bound water molecule. 2mF0-DFc composite (1σ, blue) and mF0-DFc difference (3σ, red/green) maps are calculated at (a) 3b 1.56 Å, (b) 3a 1.42 Å, (c) 4b 1.10 Å, (d) 4a 1.35 Å. Even though the resolution ranges are comparable, complex structures with inhibitor 4 reveal a significantly weaker signal for the water. The occupancy was lowered to roughly 60% to account for a partially bound water at this position. Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.
Fig. S10. Two ProM-9-based inhibitor compositions co-crystallized with ENAH EVH1
Compared to ProM-1, the methyl-substituted ProM-9 marginally influenced the positioning of the inhibitor, but allowed to cover more of the hydrophobic patch established by Phe77 and Met14 of ENAH EVH1. (a) Superposition of Ac-[2-Cl-Phe]PP[ProM-9]-OH (yellow, 6c) with the according reference composition Ac-[2-Cl-Phe]PP[ProM-1]-OH (white). (b) Superposition of Ac-[2-Cl-Phe][ProM-2][ProM-9]-OH (green, 6b) and Ac-[2-Cl-Phe][ProM-2][ProM-1]-OH (white, 1b). (c) Superposition of Ac-[2-Cl-Phe]PP[ProM-9]-OH (yellow, 6c) and Ac-[2-Cl-Phe][ProM-2][ProM-9]-OH (green, 6b) (left) and both in superposition with TEDEL-containing ligands drawn as white ribbons (different structures) confirmed that ProM-9 pointed towards the apolar patch contacted by the terminal Leu (right).
The hot-spot within the main binding groove of Ena/VASP EVH1. ProM-1 can not be shuffled within 1FPPP to replace any Pro-Pro tandem, and binding strength increases by −3 kJ/mol only if ProM-1 replaces 4PP. This boost is independent of the overall inhibitor composition; in the presence of N- and C-terminal flanking residues the boost is −3.3 (0.6), while the core motif pentamer gains −3.4 (0.4) kJ/mol of $\Delta G$ on ENAH EVH1 (SI Appendix Tab. 7). Similar boosts are measured on VASP EVH1 (SI Appendix Tab. 8) when ProM-1 is incorporated into the ActA 13-mer, −3.0 (0.3) kJ/mol, or the 2-Cl-Phe-equipped core recognition motif, −3.6 (0.4) kJ/mol.

As ITC data suggest, the gain of affinity is solely enthalpy driven (6). Hence, we assume that the measured affinity boost is based on the interaction of the scaffold’s vinylidene bridge with the underlying Phe77. Indeed, the crystal structures of ProM-1-containing complexes (PDB codes 4MY6, 5N9C, 5N9P or 5NAJ) confirmed that ProM-1 efficiently shielded Phe77 from the solvent (SI Appendix Fig. S11). Superposition of 4PP- and 4ProM-1-containing inhibitors (such as in the right panel of SI Appendix Fig. S11) highlight that the scaffold ProM-1 fit over the hydrophobic patch without affecting the backbone trace of the inhibitor.

![Fig. S11. Superposition of Ac—[2-Cl-Phe]PPPP–OH and Ac—[2-Cl-Phe]PP[ProM-1]–OH in complex with ENAH EVH1](image-url)

Left panel: Replacement of the second pair of prolines by ProM-1 (green, PDB code 5N9C) did not affect the backbone trace relative to 4PP (white, PDB code 5N91). Right panel: The vinylidene bridge snugly fit over Phe77 and shielded the hydrophobic patch from the solvent. Both complex structures contain two chains in the asymmetric unit, all are shown in the right panel. Chemical structure of ProM-1 is printed in SI Appendix Fig. S2.
Optimization of 4Pro by two scaffolds. The results of the spot array analysis showed that the third proline was exchangeable by aliphatic residues. ProM-1-based Xaa-Pro mimetica are interesting for other proline-rich segment recognizing domains apart from Ena/VASP EVH1, and could serve as templates for WW domains that recognize PLPPLP motifs. Like other Xaa-trans-Pro mimetica (31, 32), ProM-1 derivatives lack a freely rotating Xaa side chain as the vinylidene bridge fixates the Cβ in one conformation. Xaa-trans-Pro scaffolds however display an important alteration compared to Pro-Pro mimicking scaffolds, namely the unsubstituted, protonated backbone amide of the Xaa amino acid (ProM-3, top panel SI Appendix Fig. S12), which alters the chemical property of the scaffold compared to a Pro-Pro mimetica. In case of ProM-3, the protonated backbone amide lead to a unique crystal packing in which the scaffold itself was involved in a crystal contact. Several attempts to force ENAH EVH1 in complex with Ac–[2-Cl-Phe][ProM-2][ProM-3]–OH to grow in a different packing failed. Independent of the crystal space group used as seeds, the crystal packing could not be altered, suggesting that ProM-3 introduced dominant polar interactions on the solvent-exposed side of the bound inhibitor. Due to the increased interactions with the solvent, compositions with ProM-3 were therefore expected to loose affinity compared to ProM-1-containing inhibitors. Indeed, 4ProM-3-containing inhibitors bound to ENAH- and VASP EVH1 with the same affinity as 4PP, while parent compositions with 4ProM-1 bound 4-5 times stronger (SI Appendix Tabs. 7 and 8). The presence of possible intrachain hydrogen bonds by ProM-3 presumably weakened the stabilization by water (33) and increased the solvation of the inhibitor, which in turn tended to interact less strongly with other solutes (34). The hydration by more water molecules did not provide favorable conditions for PPII conformation (35) and the increased mobility of the scaffold finally raised the entropic penalty upon binding (34). The resulting weakened affinity confirms the hypothesis that backbone solvation is the major determinant of proline-rich segments to adopt pre-structured, extended helical conformations (35, 36).

The hypothesis was supported by the crystal structure and affinity measurements of compositions containing ProM-4 (lower panel of SI Appendix Fig. S12). The cyclization of ProM-4 with a piperidine moiety conserved the amide N substitution within the inhibitor but caused a less rigid scaffold as compared to ProM-1. The piperidine moiety as N-terminal building block led to a different exit vector of ProM-4 and caused a slight rearrangement within the binding groove of ENAH EVH1 relative to the parent inhibitor. However, the ProM-4 composition bound not significantly worse compared to the ProM-1-containing reference inhibitor, neither to ENAH EVH1 nor VASP EVH1 (SI Appendix Tabs. S7 and S8). The notion that even bulkier substitutions for prolines are valid as long as the backbone amide remains substituted is in agreement with reported inhibitors for SH3 domains, where replacement of single prolines by amide N-substituted peptoids yields in high-affinity inhibitors (37–39). As we aimed to maximize the ligand efficiency, ProM-4-containing inhibitors were not investigated further.
Fig. S12. Superposition of crystal structures of ENAH EVH1 in complex with inhibitors Ac–[2-Cl-Phe][ProM-2][ProM-1]–OH (1b) and the related compositions R-[ProM-3]–OH and R-[ProM-4]–OH

Top panel: The scaffold ProM-3 was designed as Xaa-trans-Pro to mimic aliphatic interactions of 4Xaa as seen in the SPOT substitution Fig. S3. Formally removing the Cδ of the N-terminal ring of ProM-1 (1b, white) yielded in ProM-3 (green), which bound canonically within the main binding groove without any major rearrangement compared to inhibitor 1b.

Lower panel: The scaffold ProM-4 was designed to conserve the amide N-substitutions of Pro-trans-Pro while inducing some flexibility within the inhibitor by enlarging the N-terminal ring of ProM-1 (white). The piperidine moiety of ProM-4 (green) yields to a different exit vector that causes visible rearrangement of the whole scaffold on the C-terminal side. Solvent accessible surfaces of ENAH EVH1 are color coded by hydrophobicity (blue-white-red for hydrophilic-hydrophobic). Complex structure with Ac–[2-Cl-Phe][ProM-2][ProM-3]–OH contains one chain (PDB code 5NBF), inhibitor 1b (PDB code 4MY6) and Ac–[2-Cl-Phe][ProM-2][ProM-4]–OH (PDB code 5NCF) each contain two chains in the asymmetric unit, all are shown. Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.
Inhibitor selectivity. The procedure is described in the methods part on page 3. Here, 10 or 11 HMQC SOFAST spectra with 200 µM protein and different inhibitor excess were recorded. Each spectrum overlay was plotted with the \textit{nmrglue} module in python, drawn with the same contour level start value. The sparky peak lists were processed and perturbations fitted by a script written in R language. The same selection criteria were used for all 9 HMQC experiments (peak perturbation $>0.1$ ppm and $K_d$ with coefficient significance of $P<0.001$). Affinities meeting the criteria were plotted in a bar chart with continuous color gradient (colorRampPalette) and the same color palette used to create a command text string readable by Chimera to color the amino acids and sidechain atoms (marked as Trp NE, Asn ND or Gln NE in the following figures). Amino acid numbering was subsequently added in Photoshop (Adobe) and placed roughly at the position of the resonance.

For Yap1 WW1, the assignment was kindly gifted by Dr. Maria Macias. The sequence of the deposited NMR structure (PDB code 2LAY) was changed to align with the amino acid sequence of the peak table (renumbering D170 to D5). As the secondary structure of Yap1 WW1 seems to stabilize upon binding the ligand, some of the assigned peaks were not visible at low inhibitor excess. Roughly 5 of the assigned peaks had to be discarded for that reason.

For Fyn SH3, none of the deposited spectra in the Biological Magnetic Resonance Data Bank (BMRB) fit on the recorded spectrum of our construct. Clearly identifiable peaks had to be assigned manually using the spectrum shown by Mal et al. (40). For plotting the affinities, the solution NMR structure of Morton et al. (PDB code 1NYF) was chosen as the amino acid sequence numbering used by Mal matched with this structure. As our Fyn SH3 spectrum was almost identical to the one shown by Mal et al., 44 out of the 58 amino acids in the 1NYF structure could be tracked.

For Profilin, the assigned spectrum extracted from BRMB ID 4082 coincided almost perfectly with our spectrum. Of the 154 assigned peaks, 13 were not tracked due to ambiguity of their initial position. For plotting the affinities, however, the refined solution NMR structure of BRMB ID 4082 (PDB code 1PFL) was not used. Instead, the affinities were plotted onto a ternary complex of Profilin-Actin with the poly-proline sequence of VASP (PDB code 2PAV), which has the identical amino acid sequence as 1PFL.

\begin{tabular}{lccc}
\hline
 & \textbf{Inhibitor 1} & \textbf{Inhibitor 4a} & \textbf{Inhibitor 7} \\
 & $K_d$ [µM] & Decrease & $K_d$ [µM] & Decrease & $K_d$ [µM] & Decrease \\
\hline
ENAH EVH1 & 4.1 (ref.) & 0.47 (ref.) & 0.12 (ref.) \\
Fyn SH3 & 5400 $\times$1300 & 2400 $\times$5200 & 1900 $\times$16000 \\
Profilin & 780 $\times$190 & 630 $\times$1300 & 210 $\times$1800 \\
Yap1 WW1 & 1000 $\times$250 & 460 $\times$980 & 1100 $\times$9600 \\
\hline
\end{tabular}

Tab. S10. Mean affinities of fitted peak perturbations and relative affinity losses compared to ENAH EVH1

Mean affinities were calculated by transforming all fitted $K_d$ of the HSQC experiments first to the free Gibbs energy ($\Delta G$), which is assumed to be much closer normal distributed than the $K_d$. The arithmetic mean of $\Delta G$ values was then calculated back into the $K_d$ written in the table. Additionally, the table lists the fold decrease of binding strength of each inhibitor composition relative to ENAH EVH1 (reference, ref.), highlighting that the affinity-improved inhibitors bound worse to off-target proteins Fyn SH3, Profilin, and Yap1 WW1 compared to the initial inhibitor 1.
Fig. S13. $^1$H-$^15$N HSQC titration of Fyn SH3 and Ac–[2-Cl-Phe][ProM-2][ProM-1]–OEt
(a) Spectra overlay and detailed perturbation of T97N-HN and G131N-HN caused by titration of inhibitor 1. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Fyn SH3. The binding grooves of Fyn SH3 are formed by Y91, Y137, W119, Y91, Y93, and D100 (41).
Fig. S14. $^1$H-$^{15}$N HSQC titration of Fyn SH3 and Ac–[2-Cl-Phe][ProM-2][ProM-15]–OMe

(a) Spectra overlay and detailed perturbation of T97N-HN and G131N-HN caused by titration of inhibitor 4a. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Fyn SH3. The binding grooves of Fyn SH3 are formed by Y91, Y137, W119, Y91, Y93, and D100 (41).
Fig. S15. $^1$H-$^{15}$N HSQC titration of Fyn SH3 and Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt (6)
(a) Spectra overlay and detailed perturbation of T97N-HN and G131N-HN caused by titration of inhibitor 6. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Fyn SH3. The binding grooves of Fyn SH3 are formed by Y91, Y137, W119, Y91, Y93, and D100 (41).
Fig. S16. $^1$H-$^{15}$N HSQC titration of Profilin and Ac–[2-Chl-Phe][ProM-2][ProM-1]–OEt

(a) Spectra overlay and detailed perturbation of Y6N-HN and K107N-HN caused by titration of inhibitor 1. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Profilin. All perturbations are found close to the binding pocket recognizing the proline-rich motif of VASP.
Fig. S17. $^1$H-$^{15}$N HSQC titration of Profilin and Ac–-[2-Cl-Phe][ProM-2][ProM-15]–OMe
(a) Spectra overlay and detailed perturbation of I7N-HN and K107N-HN caused by titration of inhibitor 4a. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Profilin. Almost all perturbations are found close to the binding pocket recognizing the proline-rich motif of VASP.
Fig. S18. $^1$H-$^{15}$N HSQC titration of Profilin and Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt
(a) Spectra overlay and detailed perturbation of I7N-HN and K107N-HN caused by titration of inhibitor 6. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Profilin. All perturbations except a weak interaction by D75 are found close to the binding pocket recognizing the proline-rich motif of VASP.
Fig. S19. $^1$H-$^15$N HSQC titration of Yap1 WW1 and Ac–[2-Cl-Phe][ProM-2][ProM-1]–OEt
(a) Spectra overlay and detailed perturbation of T32N-HN and W42NE-HE caused by titration of inhibitor 1. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Yap1 WW1. The interaction site of Yap1 WW1 is formed by Q30, T32 Y23, W34, and H27 (42).
Fig. S20. $^1$H-$^{15}$N HSQC titration of Yap1 WW1 and Ac–[2-Cl-Phe][ProM-2][ProM-15]–OMe
(a) Spectra overlay and detailed perturbation of T32N-HN and K16N-HN caused by titration of inhibitor 4a. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Yap1 WW1. The interaction site of Yap1 WW1 is formed by Q30, T32 Y23, W34, and H27 (42).
**Fig. S21.** $^1$H-$^15$N HSQC titration of Yap1 WW1 and Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt

(a) Spectra overlay and detailed perturbation of T32N-HN and W34N-HN caused by titration of inhibitor 6. (b) Upper panel: Affinities of accepted fits of peak perturbations along the amino acid (AA) sequence. Plotted on the right panel are the two fitted peak perturbations shown in detail above. Lower panel: Color coding of the bar plot drawn on the solvent accessible surface of Yap1 WW1. The interaction site of Yap1 WW1 is formed by Q30, T32 Y23, W34, and H27 [(42)].
Fig. S22. Pulldown experiment of GST-tagged Fyn SH3 from Jurkat cell lysate
Natural binding partner ADAP binds to Fyn SH3 via proline-rich segments and is pulled from Jurkat cell lysate by bead-bound Fyn SH3 (43). ADAP is displaced from Fyn SH3 by the synthetic peptide Ac–RPLPPLP–NH$_2$ (44) but not by the inhibitors designed for Ena/VASP EVH1. Peptide and inhibitors were used at 50 µM active concentration, 1 mg/ml protein concentration of the lysate. Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.

Fig. S23. IncuCyte chemotaxis assay, set-up and FBS gradient optimization
(a) Schematic figure of the IncuCyte membrane-based migration assay. (b) The FBS gradient between upper and lower wells was optimized beforehand to maximize the relative chemotaxis, e.g the relative confluency of cells migrated. MDA–MB–231 migrated most efficiently over a gradient of 0.1 (upper) to 1% (lower). Steeper gradients and higher FBS concentrations in the upper well resulted in significantly less cells migrating to the lower well.
Fig. S24. Boyden chamber invasion assay
Left: MDA–MB–231 stably expressing mCherry were incubated inhibitors 1 or 7, which reduced invasion in a dose-dependent manner. (Representative fluorescence signals after 13 h and IC50 curve with 3σ dashed line). Right: IC50 were calculated during linear signal increase and plotted as group (P=2.4e-10). The calculated IC50 values confirm the IncuCyte chemotaxis assay mentioned in the main text with inhibitor 7 having a superior potency compared to 1 (mean IC50 12 µM vs 53 µM, P<0.0001).

Fig. S25. Cell viability assay
Cells were incubated for 24 h with inhibitors 1, 4a, 6, or 7 at 180 µM. Fluorescence of living-cell-mediated, reduced Alamar blue (Invitrogen) was measured indicating no toxicity up to an inhibitor concentration of 180 µM as used as maximal concentration in the IncuCyte chemotaxis assay. Three cell lines were tested: MDA–MB–231, B16F1 mouse melanoma wild-type and Ena/VASP triple knockout (3xKO), and mouse fibroblast cell line (MVD7) ENAH and VASP double (2xKO) and Ena/VASP triple knockout (3xKO). The mouse cell lines were kindly gifted by Prof. Dr. Jan Faix. Fluorescence signals normalized to the mean of the control without any inhibitor. Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.
Fig. S26. Duplicates of zebrafish assays
Zebrafish assays were performed twice for each inhibitor. Representative duplicates of a zebrafish extravasation assay under inhibitor treatment with 1 µM Ac–[2-Cl-Phe][ProM-2][ProM-1]–OEt (1) and Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt (7). Chemical structures of the mentioned ProM scaffolds are printed in SI Appendix Fig. S2.
**Inhibitor characterization.** Inhibitors were weighed out on fine scales (Sartorius ME36S-0CE or Mettler AT21 Comparator) and dissolved in sterile-filtered PBS or 40 mM sodium phosphate, 100 mM NaCl and titrated to pH 7.3. The batches designated for cellular experiments did not contain any reducing agents such as TCEP or DDT as mentioned in the affinity section of the methods part. FT-based affinity measurements were carried out to determine the amount of active substance, and activity-corrected concentrations were used in all cellular experiments.

The substance batches that went into cellular and *in vivo* experiments were analyzed in detail and listed first. Thereafter follow analytic data of inhibitors mentioned in the main text. Two general trends notable in nearly all of the batches are discussed here and not further mentioned:

1. MALDI TOF as well as LCMS generally show a small impurity with a molecular weight offset by +14 Da from the \([\text{M+H}]+\) fragment as well as \([\text{M+Na}]+\) and \([\text{M+K}]+\). As the synthesis strategy does not allow any side reaction that would add an additional CH2 group, the most likely impurity is a mixture between ethyl and propyl esters (for –OEt compositions) or methyl and ethyl esters (for –OMe compositions), respectively. We assume that the mixture of ester functions may have formed during masking the C-terminal scaffold by traces of ethanol and propanol in the according solvents. Furthermore, as the difference between \([\text{M+Na+14}]+\) and \([\text{M+K}]+\) is only 2 Da, we suggest that only a minor fraction of the recorded \([\text{M+K}]+\) ions contributed to the impurity. Given the similar affinities of –OMe and –OEt compositions (SI Appendix Tabs. 7 and 8), we do not expect that the impurity had any effect in the cellular assays.

2. Optical activity at 280 and 254 nm is low, and inhibitors are best visible at 220 nm, where only minor impurities are detectable. UV/Vis spectrograms generally reveal a distinct double-peak elution pattern of the inhibitors. Investigation of this behavior by solution NMR and LCMS revealed that each fraction splits again into a double-peak when stored at room temperature. Furthermore, upon heating the column, the double-peak pattern of the analytical HPLC merges into one peak with an area roughly the size of the double-peak. Hence, we suggest that the observed double-peak elution pattern originates from two rotamers of the product with slightly different retention times, supposedly formed by the slow *cis-trans* isomerization of the [2-Cl-Phe–ProM-2] peptide bond.
Ac-[1-2Cl-F]-[ProM-2]-[ProM-1]-OMe (1a)

M(C₃H₄ClN₅O₇) 692.210 g/mol.

Yield 87% (528 mg, 76 µmol).

Rf 0.29 (MeOH/CH₂Cl₂, 1:15).

HR-MS (ESI): m/z = [M+H]⁺ calculated: 692.2846; determined: 692.2848; [M+Na]⁺ calculated 714.2665; determined: 714.2673.

¹H NMR (600 MHz, CDCl₃, mixture of rotamers): δ [ppm] = 7.38 - 7.30 (m, 1H, H-33), 7.26 - 7.14 (m, 3H, H-30/31/32), 6.41 (d, J = 8.1 Hz, 0.3H, -NH₁), 6.16 (dd, J = 41.5 Hz, 9.0 Hz, 0.7H, -NH₂), 5.94 - 5.74 (m, 2H, H-17/18), 5.67 - 5.52 (m, 1H, H-5 o. 6), 5.22 - 5.14 (m, 1H, H-11), 4.99; 4.94 (2×s, J = 8.2 Hz, 8.0 Hz, 0.7H, H-23), 4.85 (t, J = 8.4 Hz, 0.3H, H-23), 4.80 - 4.70 (m, 2H, H-5 o. 6/19), 4.60 - 4.53 (m, 1H, H-16), 4.41 - 4.33 (m, 1H, H-7), 4.18 - 4.04 (m, 1H, H-13), 3.99 - 3.89 (m, 1H, H-1), 3.84 - 3.77 (m, 0.7H, H-1), 3.71; 3.70 (2×s, 3H, H-25), 3.59 - 3.48 (m, 1H, H-13), 3.38 (dd, J = 14.2 Hz, 3.8 Hz, 0.3H, H-10), 3.26 - 3.10 (m, 1.4H, H-25/26/27/28), 3.04 - 2.95 (m, 1H, H-15), 2.85 (dd, J = 14.2 Hz, 10.7 Hz, 0.6H, H-28), 2.50 - 2.44 (m, 0.6H, H-10), 2.41 - 2.36 (m, 0.4H, H-10), 2.35 - 2.24 (m, 2H, H-3/22), 2.22 - 1.70 (m, 14H, H-23/29/30/32/41/42/43/44).

¹³C NMR (150 MHz, CDCl₃, mixture of rotamers): δ [ppm] = 172.3 (C-8), 171.1; 170.9 (C-20), 170.1 (C-26), 169.6; 169.2 (C-24), 169.0; 168.9 (C-35), 166.9; 166.8 (C-12), 134.6; 134.5 (C-29), 134.4 (C-34), 132.0; 131.9 (C-17), 131.4; 130.9 (C-5), 129.0; 129.5 (C-6), 129.4; 129.4 (C-33), 128.5 (C-18), 128.4; 128.3 (C-32), 126.6; 126.5 (C-31), 123.1; 122.9 (C-30), 65.3; 65.2 (C-4), 62.1; 62.0 (C-25), 59.4; 59.3 (C-16), 59.0; 57.5 (C-11), 57.3; 56.8 (C-19), 56.2; 56.1 (C-7), 52.2 (C-25), 50.6; 50.44 (C-27), 48.6; 48.5 (C-1), 47.1; 47.0 (C-13), 40.4 (C-15), 38.1; 37.7 (C-3), 37.3; 36.0 (C-21), 32.9 (C-28), 31.6; 31.5 (C-9), 31.3; 31.0 (C-14), 28.1; 28.0 (C-22), 27.1; 26.9 (C-10), 23.9; 23.6 (C-2), 23.3; 23.0 (C-36).

IR (ATR) ν (cm⁻¹) = 3313 (bs), 3056 (w), 2956 (w), 1744 (s), 1637 (s), 1524 (w), 1437 (m), 1324 (w), 1265 (s), 1207 (m), 1053 (w), 896 (w), 731 (s), 702 (s).

[α]₂⁰₂⁰ (c = 0.515, CHCl₃) = -456.3 ° (436 nm), -259.2 ° (546 nm), -226.6 ° (579 nm), -218.1 ° (589 nm).

Fig. S27. Representative full characterization of inhibitor 1a
Fig. S28. 1D $^1$H NMR of inhibitors 1a and 3a
Fig. S29. 1D $^{13}$C NMR and small molecule crystal structure of inhibitor 1a.
Fig. S30. 1D $^1$H NMR of inhibitors 4a and 7a.
Ac–[2-Cl-Phe][ProM-2][ProM-1]–OEt. Inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-1]–OEt (1) has molecular weight of 706.24 Da. Shown here is the analysis of the inhibitor batch used in cellular experiments. MALDI TOF as well as LCMS show a small impurity with a molecular weight offset by 14 Da from [M+H]^+ as well as [M+Na]^+ and [M+K]^+ as mentioned before. UV/Vis spectrograms reveal a distinct double-peak elution of the inhibitor as mentioned before. The synthesis batch of 1 contained minor traces of Fmoc-[2-Cl-Phe][ProM-2][ProM-1]–OEt. These impurities are visible only when dissolved in acetonitrile-water mixtures (such as the LCMS and MALDI TOF of SI Appendix Fig. S31a and c). As Fmoc-compositions are not water soluble, the supernatant of the dissolved batches used for the cellular studies was free of Fmoc-impurities (see HPLC SI Appendix Fig. S31b and SI Appendix Tab 11).

| Ret. time [min] | Relative peak area [%] |
|-----------------|------------------------|
| 4.851           | 0.1                    |
| 5.493           | 0.1                    |
| 5.888           | 0.4                    |
| 5.966           | 0.4                    |
| 6.165           | 0.3                    |
| 6.45            | 1.8                    |
| 6.679           | 0.7                    |
| 6.88            | 1.6                    |
| 7.175           | 1.6                    |
| 7.69            | 91.9                   |
| 8.532           | 0.7                    |
| 8.706           | 0.1                    |
| 8.983           | 0.4                    |
| total           | 100                    |

Tab. S11. HPLC peak table of inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-1]–OEt at 220 nm
Peak areas of the HPLC run visible in SI Appendix Fig. S31b left panel, relative to the total area under the 220 nm spectrogram.
Fig. S31. Analysis of Ac–[2-Cl-Phe]–ProM–2–ProM–1–OEt (1)
(a) LCMS. TIC scan and UV spectrograms, both normalized to the maximal signal during acquisition time (minutes). (b) Analytical HPLC. UV spectrograms on absolute scale (left) and normalized to the according maximal OD (right) during acquisition time (minutes). (c) MALDI TOF with assigned fragments.
Ac–[2-Cl-Phe][ProM-2][ProM-15]–OMe. Inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-15]–OMe (4a) has molecular weight of 694.23 Da. Shown here is the analysis of the inhibitor batch used in cellular experiments. Generally, the impurity offset by 14 Da from [M+H]$^+$ as well as [M+Na]$^+$ and [M+K]$^+$ as mentioned before is more dominant for methyl ester-masked inhibitors compared to the ethyl ester compositions. Indeed, scanning for Ac–[2-Cl-Phe][ProM-2][ProM-15]–OEt ([M+H]$^+$ of 708.27 Da) reveals a notable signal in the total ion current (TIC) chromatogram profile (brown line SI Appendix Fig. S32a) but no further impurities. UV/Vis spectrograms reveal a distinct double-peak elution of the inhibitor as mentioned before. The supernatant used for cellular studies, as analyzed by MALDI TOF (SI Appendix Fig. S32c) is virtually free of Fmoc-bound intermediates.

| Ret. time [min] | Relative peak area [%] |
|-----------------|------------------------|
| 3.581           | 0.1                    |
| 5.252           | 0.1                    |
| 5.42            | 0.1                    |
| 5.963           | 0.1                    |
| 6.098           | 0.1                    |
| 6.474           | 0.2                    |
| 7.509           | 1.7                    |
| 7.8             | 96.6                   |
| 9.027           | 0.6                    |
| 9.775           | 0.3                    |
| 10.037          | 0.2                    |
| total           | 100                    |

Tab. S12. HPLC peak table of inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-15]–OMe at 220 nm
Peak areas of the HPLC run visible in SI Appendix Fig. S32b left panel, relative to the total area under the 220 nm spectrogram.
Fig. S32. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-15]–OMe (4a)
(a) LCMS. TIC scan and UV spectrograms, both normalized to the maximal signal during acquisition time (minutes). (b) Analytical HPLC. UV spectrograms on absolute scale (left) and normalized to the according maximal OD (right) during acquisition time (minutes). (c) MALDI TOF with assigned fragments.
Ac–[2-Cl-Phe][ProM-2][ProM-9]–OEt. Inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-9]–OEt (6) has molecular weight of 720.26 Da. Shown here is the analysis of the inhibitor batch used in cellular experiments. MALDI TOF as well as LCMS show a small impurity with a molecular weight offset by 14 Da from [M+H]+ as well as [M+Na]+ and [M+K]+ as mentioned before. UV/Vis spectrograms reveal a distinct double-peak elution of the inhibitor as mentioned before. Even though LCMS and HPLC spectrograms reveal more peaks leading and trailing the double-peak, no ions other than [M+H]+, [M+Na]+, and [M+K]+ were found (in accordance to the MALDI TOF).

| Ret. time [min] | Relative peak area [%] |
|-----------------|------------------------|
| 7.597           | 2.8                    |
| 7.856           | 4.3                    |
| 8.271           | 92.5                   |
| 10.348          | 0.1                    |
| 10.723          | 0.2                    |
| **total**       | **100**                |

Tab. S13. HPLC peak table of inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-9]–OEt at 220 nm
Peak areas of the HPLC run visible in SI Appendix Fig. S33b left panel, relative to the total area under the 220 nm spectrogram.
Fig. S33. Analysis of Ac−[2-Cl-Phe][ProM-2][ProM-9]−OEt (6)
(a) LCMS. TIC scan and UV spectrograms, both normalized to the maximal signal during acquisition time (minutes). (b) Analytical HPLC. UV spectrograms on absolute scale (left) and normalized to the according maximal OD (right) during acquisition time (minutes). (c) MALDI TOF with assigned fragments.
Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt. Inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt (7) has molecular weight of 734.29 Da. Shown here is the analysis of the inhibitor batch used in cellular experiments. MALDI TOF as well as LCMS show a small impurity with a molecular weight offset by 14 Da from [M+H]^+ as well as [M+Na]^+ and [M+K]^+ as mentioned before. UV/Vis spectrograms reveal a distinct double-peak elution of the inhibitor as mentioned before.

| Ret. time [min] | Relative peak area [%] |
|-----------------|------------------------|
| 2.133           | 0.1                    |
| 3.588           | 0.3                    |
| 5.252           | 0.3                    |
| 5.418           | 0.5                    |
| 5.968           | 0.2                    |
| 6.454           | 0.4                    |
| 6.646           | 0.2                    |
| 7.552           | 0.2                    |
| 7.757           | 0.8                    |
| 7.915           | 0.2                    |
| 8.064           | 0.5                    |
| 8.439           | 1.8                    |
| 8.8             | 94.6                   |
| total           | 100                    |

Tab. S14. HPLC peak table of inhibitor Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt at 220 nm
Peak areas of the HPLC run visible in SI Appendix Fig. S34b left panel, relative to the total area under the 220 nm spectrogram.
Fig. S34. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-13]–OEt (7) (a) LCMS. TIC scan and UV spectrograms, both normalized to the maximal signal during acquisition time (minutes). (b) Analytical HPLC. UV spectrograms on absolute scale (left) and normalized to the according maximal OD (right) during acquisition time (minutes). (c) MALDI TOF with assigned fragments.
Fig. S35. Analysis of Ac–FPPPP–OEt
HPLC and MALDI TOF of peptide wt1, molecular weight 623.75 Da. The peptide was synthesized as free acid, followed by Steglich esterification. Ac–FPPPP–OH is detected as [M+Na]+ (618.7 Da) and [M+K]+ (634.7 Da). The MALDI TOF ion with 730.75 Da could not be assigned. One ion, the hexapeptide Ac–FPPPPP–OH, [M+Na]+ (728.34 Da) would have a similar molecular weight but is not detected in the HPLC. Instead, the UV/Vis spur reveals a hydrophobic substance eluting at the end of the acetonitrile gradient. However, no Fmoc-containing composition fit to 730.75 Da.
Fig. S36. Analysis of Ac–SFEFPFPFPTEDEL–NH₂
HPLC and MALDI TOF of peptide wt2, molecular weight 1545.67 Da, visible as [M+Na]+.
Fig. S37. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-1]–OH HPLC and ESI-FTMS of inhibitor 1b, molecular weight 678.18 Da. MALDI TOF reveals [M+H]^+ as well as the deacetylated fragment (636.2 Da). The small impurity with 980.8 Da could originate from a fraction of Fmoc-bound precursor (858.4 Da).
Fig. S38. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-1]TEDEL–NH₂
HPLC and MALDI TOF of compound 2AN, molecular weight 1264.78 Da. Beside the expected ions, MALDI TOF reveals a significantly smaller ion (1040.8 Da). We suspect that this side product originated from unsuccessful coupling of Fmoc-ProM-2 to the growing chimera.

The deacetylated fragment Ac–[2-Cl-Phe][ProM-1]TEDEL–NH₂ weighs 1004 Da, its [M+K]⁺ 1043.5 Da. Due to the absence of two prolines within the core recognition motif, such a composition would not bind in in vitro assays.
Fig. S39. Analysis of Ac–SFE[2-Cl-Phe][ProM-2][ProM-1]–OEt

HPLC and MALDI TOF of compound 2ΔC, molecular weight 1069.61 Da. HPLC elution profile suggest several termination products. Some of the low molecular weight ions suggest that the ProM scaffolds might not have bound to the resin: The [M+K]⁺ of the deacetylated fragment Ac–SFE–OEt (448.43 Da), Fmoc–SFE–OEt (631.7 Da), or Ac–SFE[2-Cl-Phe]–OEt (633.1 Da) all closely match the found ions in MALDI. The ion with 1118.8 Da mass should be the deacetylated product fragment (1014.5 Da) as no other composition matches that ion mass. Hydrophobic impurities were spun down after diluting the substance in phosphate buffer. Despite a low activity, the affinity could be determined accurately (percentage standard error $K_d$ 5.7%).
Fig. S40. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-12]–OMe

HPLC and ESI-FTMS of inhibitor 3a, molecular weight 692.21 Da, visible as [M+Na]^+. The HPLC shows the typical double peak pattern mentioned before.
Fig. S41. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-17]–OMe ESI-FTMS and assigned 1H and 13C NMR chemical shifts (spectra printed on next page) of inhibitor 5a, molecular weight 722.28 Da, visible as [M+Na]⁺.
Fig. S42. Analysis of Ac–[L-2-Cl-F][ProM-2][ProM-17]–OMe

1D $^1$H and $^{13}$C NMR spectra of inhibitor 5a. Chemical shift tables printed on previous page lower panel.

**Figure 1:** $^1$H-NMR spectrum of Ac–[L-2-Cl-F][ProM-2][ProM-17]–OMe, 500 MHz.

**Figure 2:** $^{13}$C-NMR spectrum of Ac–[L-2-Cl-F][ProM-2][ProM-17]–OMe, 125 MHz.

Fig. S42. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-17]–OMe
1D $^1$H and $^{13}$C NMR spectra of inhibitor 5a. Chemical shift tables printed on previous page lower panel.
Fig. S43. Analysis of Ac–[2-Cl-Phe][ProM-2][ProM-9]–OH
HPLC and MALDI TOF of inhibitor 6b, molecular weight 692.21 Da, visible as [M+H]^+ and [M+Na]^+. The additional small weight ion might belong to the termination product [ProM-2][ProM-9]–OH ([M+H]^+ 468.55 Da)
Fig. S44. Analysis of Ac–[2-Cl-Phe]PP[ProM-9]–OH
HPLC and MALDI TOF of inhibitor 6c, molecular weight 668.19 Da, visible as [M+H]$^+$.

The additional small weight ion might belong to the termination product PP[ProM-9]–OH ([M+H]$^+$ 444.52 Da)
1. Zaminer J, et al. (2010) Addressing protein-protein interactions with small molecules: a pro-pro dipetide mimic with a ppi helix conformation as a module for the synthesis of grid-binding ligands. *Angew Chem Int Ed* 49:711–715.

2. Reuter C, Neudörfl J, Schmalz HG (2010) Potassium (1-methoxy-carbonyl-2-methyl-prop-2-en-2-yl-idene)aziridin. *Acta Crystallographica Sect E Struct Rep E66*.

3. Huy P, Neudörfl J, Schmalz HG (2011) A practical synthesis of Trans-3-substituted proline derivatives through 1,4-addition. *Org. Lett.* 13(2):216–219.

4. Hack V, et al. (2013) Efficient α-helix induction in a linear peptide chain by n-capping with a bridged tricyclic dipropyl analogue. *Angew Chem Int Ed* 52:9539–9543.

5. Reuter C, et al. (2015) Design and stereoselective synthesis of prom-2: A spirocyclic dipropyl mimetic with polyproline type ii (ppi) helix conformation. *Chem Eur J* 21(3):844–8470.

6. Opitz R, et al. (2015) A modular toolkit to inhibit proline-rich motif-mediated protein-protein interactions. *Proc Natl Acad Sci U S A* 112:5011–5016.

7. Chiha S, et al. (2018) Design and synthesis of building blocks for ppi-helix secondary-stucture mimetics: A stereoselective entry to 4-substituted 5-vinylprolines. *Eur J Org Chem* 4455–460.

8. Dohmen S, et al. (2020) Pd-Catalyzed Asymmetric N-Allylation of Amino Acid Esters with Exceptional Levels of Catalyst Control: Stereo-Divergent Synthesis of ProM-15 and Related Bicyclic Dipetide Mimetics. *Chem Eur J* 26(14):3049–3053.

9. Heine HN (2000) Ph.D. thesis (Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin).

10. Kabsch W (1993) Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. *J Appl Crystallogr.* 26:795–800.

11. Karplus PA, Diederichs K (2012) Linking crystallographic model and data quality. *Science* 336:1030–1033.

12. Karplus PA, Diederichs K (2015) Assessing and maximizing data quality in macromolecular crystallography. *Curr Opin Struct Biol* 22:60–68.

13. McCoy AJ, et al. (2007) Phaser crystallographic software. *J Appl Crystallogr.* 40:658–674.

14. Johannesson P (1991) Moloc: using prolog for conceptual modelling. [http://www.moloc.ch](http://www.moloc.ch).

15. Schlüttelkopf AW, van Aalten DMF (2004) Prodrugs: a tool for high-throughput crystallographic studies of protein-ligand complexes. *Acta Crystallographica D Biol Crystallography* 60:1355–1363.

16. Lebedev AA, et al. (2012) Jigand: a graphical tool for the cp4 template- restraint library. *Acta Crystallographica D Biol Crystallography* 68:431–440.

17. Adams PD, et al. (2010) Phenix: a comprehensive python-based system for high-throughput crystallography. *Acta Crystallographica D Biol Crystallography* 66:213–221.

18. Emsley P, Cowtan K (2004) Coot: building macromolecular models with graphics. *Acta Crystallographica D* 60:2126–2132.

19. Pettersen EF, et al. (2004) UCSF Chimera-a visualization system for exploratory research and analysis. *J Comput Chem* 25(13):1605–1612.

20. Goddard, T. D. and Kneller DG (year?) Sparky 3. University of California, San Francisco.

21. Smith GA, Theriot JA, Portnoy DA (1996) The tandem repeat domain in the listeria monocytogenes actA protein controls the rate of actin-based motility, the percentage of moving bacteria, and the localization of vasodilator-stimulated phosphoprotein and profilin. *J Cell Biol* 135(3):647–660.

22. Aurora, R and Rose (1996) Helix capping. *Protein Sci* 7:21–38.

23. Benecken J, et al. (2000) Structure of the homer evh1 domain-peptide complex reveals a new twist in polyproline recognition. *Neuron* 26(1):143–154.

24. Seneci P, et al. (2009) Rational design, synthesis and characterization of potent, non-peptidic smac mimics/xiap inhibitors as proapoptotic agents for cancer therapy. *Biorg. Med. Chem* 17(16):5834–5856.

25. Allard B, et al. (2015) Synthesis and evaluation of a [3R,6S,9S]-2-oxo-1-azabicyclo[4.3.0]nonane scaffold as a mimic of xaa-trans-pro in poly-l-proline type ii helix conformation. *Org Biomol Chem* 13(15):4562–4569.

26. Bochicchio B, Tamburro AM (2002) Polyproline ii helix in proteins: identification by chiroptical spectroscopies, stability, and functions. *Chirality* 14(10):782–792.

27. Williamson MP (1994) The structure and function of polyproline-rich regions in proteins. *Biochem. J* 297:249–260.

28. Adzhubei IA, Sternberg MJK, Makarov AA (2013) Polyproline ii helix in proteins: structure and function. *J. Mol. Biol.* 425(12):2100–2132.

29. Creamer T, Campbell M (2002) Determinants of the polyproline ii helix from modeling studies. *Adv Protein Chem* 62:263–282.

30. Nguyen, J, Turk C, Cohen F, Zuckermann R, Lim W (1998) Exploiting the basis of polyproline recognition by sh3 and ww domains: design of n-substituted inhibitors. *Soi 282:2088–2092.

31. Nguyen JT, et al. (2000) Improving sh3 domain ligand selectivity using a non-natural scaffold. *Chem Biol* 7:463–473.

32. Li H, Lawrence D (2005) Acquisition of fyn-selective sh3 domain ligands via a combinatorial library strategy. *Chem Biol* 12:905–912.

33. Mal TK, Matthews SJ, Kovacs H, Campbell ID, Boyd J (1998) Some nmr experiments and a structure determination employing a [15n,2h] enriched protein. *J Biomol NMR* 12:259–276.

34. Feng S, Kasahara C, Riddles RJ, Schreiber SL (1995) Specific interactions outside the proline-rich core of two classes of src homology 3 ligands. *Proc. Natl. Acad. Sci. U.S.A.* 92(26):12408–12415.

35. Iglesias-Bexiga M, et al. (2015) WW domains of the yes-kinase-associated-protein (YAP) transcriptional regulator behave as independent units with different binding preferences for PpY motif-containing ligands. *PLoS ONE* 10(1):e0113828.

36. da Silva AJ, et al. (1997) Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leucocyte protein 76 and modulates interleukin 2 production. *Proc. Natl. Acad. Sci. U.S.A.* 94(14):7493–7498.

37. Sparks AB, Quilliam LA, Thom JM, Der CJ, Kay BK (1994) Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries. *J. Biol. Chem.* 269(39):23853–23856.