A modular toolkit to inhibit proline-rich motif–mediated protein–protein interactions

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Small-molecule competitors of protein–protein interactions are urgently needed for functional analysis of large-scale genomics and proteomics data. Particularly abundant, yet so far undruggable, targets include domains specialized in recognizing proline-rich segments, including Src-homology 3 (SH3), WW, GYF, and Drosophila enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) homology 1 (EVH1) domains. Here, we present a modular strategy to obtain an extendable toolkit of chemical fragments (ProMs) designed to replace pairs of conserved prolines in recognition motifs. As proof-of-principle, we developed a small, selective, peptidomimetic inhibitor of Ena/VASP EVH1 domain interactions. Highly invasive MDA MB 231 breast-cancer cells treated with this ligand showed displacement of VASP from focal adhesions, as well as from the front of lamellipodia, and strongly reduced cell invasion. General applicability of our strategy is illustrated by the design of an ErbB4-derived ligand containing two ProM-1 fragments, targeting the yes-associated protein 1 (YAP1)-WW domain with a fivefold higher affinity.

Ena | VASP | protein–protein interaction | actin cytoskeleton | cell migration

Proline-rich segments (PRSs) belong to the most abundant sequence motifs of the proteome, interacting frequently with PRS-recognizing domains (PRDs), such as EVH1, SH3, GYF, and WW. Although exhibiting different tertiary structures, PRDs expose clusters of aromatic residues, forming a shallower, corrugated binding groove with a hydrogen bond-donating residue (W, Y) in the central position. In the bound state, PRSs often show a conformation closely related to the ideal left-handed polyproline II (PPII) helix characterized by backbone angles of $\Phi = -78^\circ$ and $\Psi = +146^\circ$ (2). As a consequence of the axial symmetry of PPII helices, two different types of consensus motifs occur: one containing $\psi\phi$ specifically recognized by the EVH1 and SH3 domains, the other comprising $\chi\phi\chi\phi$, typical for motifs binding at WW and GYF domains. The conserved prolines represent the core of the consensus motifs and interact intimately with the exposed aromatic side chains. They cannot be replaced by any other natural amino acid without complete loss of affinity (2, 3). On the other hand, the core motif alone binds only very weakly to its PRD. Further interactions of flanking residues located outside the core motif contribute substantially to both affinity and specificity. Incorporation of nonnatural amino acids in place of such specificity-determining residues is therefore often beneficial for binding (4–9). However, peptide ligands display a number of disadvantages when used as competitors, among them metabolic instability and often low cell permeability. Cell-permeable small molecules that grant the ability to modulate the function of PRDs are still not available.

Here, we present a modular concept for the systematic development of such low-molecular weight compounds. It is based on molecular building blocks that can replace the conserved prolines within the core motif without any loss of affinity. Combinations of such building blocks allow complete replacement of the proline-rich core motifs. They may be supplemented with organic scaffolds addressing the flanking epitopes to obtain peptidomimetic inhibitors of PRDs, highly desirable for functional analysis of PRS-mediated protein–protein interactions.

As proof of concept, we developed a peptidomimetic inhibitor targeting the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family Ena/VASP homology 1 (EVH1) domains. This protein family is involved in modulation of the actin cytoskeleton, a complex and highly regulated process, which is the driving force of directed cell migration (10, 11) and plays important roles in disease-relevant processes like tumor metastasis (12, 13). The Ena/VASP family proteins [i.e., VASP, enabled homolog (EnaH), and Ena-VASP-like (EVL) (14–16)] are notably localized at focal adhesions and lamellipodia. Single Ena/VASP protein deletions are mostly compensated for the other members of the family (17); however, triple knock-out mice are embryonic lethal (18, 19). The proteins comprise EVH1 and Ena/VASP homology 2 (EVH2) domains, separated by a proline-rich region. Although EVH2 binds to the barbed ends of actin filaments, EVH1 interacts with proteins, like zyxin or lamellipodin (Lpd also called RAPH1), that contain the class 1 EVH1 consensus motif [FYWL]P$\psi\phi\phi$ (ψ is an aliphatic amino acid) (2, 20–22). Using our peptidomimetic inhibitor, we show that inhibition of the Ena/VASP

Significance

Protein–protein interactions mediated by proline-rich motifs are involved in regulation of many important signaling cascades. Protein domains specialized in recognition of these motifs expose a flat and relatively rigid binding site that preferentially interacts with sequences adopting a left-handed polyproline helix II. Here, we present a toolkit of new chemical entities that enables rational construction of selective small-molecule inhibitors for these protein domains. As proof of principle, we developed a selective, cell-permeable inhibitor of Drosophila enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) homology 1 (EVH1) domains of the Ena/VASP protein family. Invasive breast-cancer cells treated with our EVH1 inhibitor showed strongly reduced cell invasion.
family EVH1 domains strongly influences both cellular localization of VASP as well as cell migration.

Results
Design and Synthesis of ProM Building Blocks. We designed a novel class of conformationally restricted small-molecule fragments that should result in nonhydrolyzable yet cell-permeable, peptide-mimetic small-molecule interaction inhibitors of PRDs. These molecules, coined ProM-1, ProM-2, ProM-3, and ProM-4, maintain a carbonyl functionality within a rigidified, nonanomeric scaffold and were aimed to substitute the strictly conserved prolines within PRS consensus motifs. In this small but extendable toolkit of fragments, the scaffolds ProM-1 and ProM-2 replace a diproline motif in PPII conformation whereas ProM-3 and ProM-4 substitute for an xP motif (Fig. L4). Their frameworks show subtle conformational differences to satisfy individual steric requirements for a particular binding site. Similar to the foldamer concept (23), appropriate combinations of ProMs allow optimal complementarity of hydrophobic interactions between the domain surface and the ligand to be achieved. In a previously published pilot study, we showed that ProM-1 was able to replace a diproline motif in a peptide recognized by Fyn-SH3 whereas the respective complete PxxP recognition motif could not be replaced using a combination of this building block (24).

The new fragments were stereoselectively synthesized in Fmoc-protected form (Fig. 1B; see SI Appendix for characterization of ProMs). The modular strategy relies on the coupling of building blocks B1 to B4 with enantiopure cis- or trans-tet-butyl 5-vinyl-prolinate (ctPVP) (25), cyclization of the resulting dipeptides (DP1 to DP4) through Ru-catalyzed ring closing metathesis (26), and final replacement of the Boc-protecting group by Fmoc (Fig. 1B).

The preparation of the required building blocks was achieved on a multigram scale by exploiting, as a key step, either the Cu-catalyzed 1,4-addition of vinyl-MgBr to a cyclic dehydro-amino acid derivative (A1 and A4, respectively) (27), diastereoselective vinylation (formylation/ methylation) of the protected proline A2 (28), or the enantioselective Claisen rearrangement of the glycine derivative A3 (29). The Fmoc-protected ProMs can subsequently be used for ligand synthesis, exploiting established protocols for peptide coupling.

Development of an Ena/VASP EVH1 Inhibitor. Starting from peptide 1—derived from a segment of the surface protein ActA of Listeria monocytogenes that captures Ena/VASP proteins of the host cell via their EVH1 domains—we replaced stepwise its conserved sequence motif FPPP (Fig. 2A). In the course of this work, dissociation constants were determined by both isothermal titration calorimetry (ITC) and fluorescence titration (FT). Computational inspection of the binding site suggested introduction of a hydrophobic substituent at the ortho position of the phenylalanine ring within the core motif. The largest gain in affinity of all tested unnatural amino acids was observed for 2-chloro-L-phenylalanine (2-CI-F) (Fig. 2A and SI Appendix, Fig. S11 and Table S2). Replacement of the second pair of prolines within the FPPP motif in 1 by ProM-1 or ProM-3 indicated preference for ProM-1, with its five-membered ring next to R1 (Kd = 0.8 μM) (Fig. L4 and SI Appendix, Tables S3 and S4). Additional replacement of the first pair of prolines by ProM-2, which was found to satisfy the specific steric requirements at this interaction site, yielded compound 2, with the highest affinity to Ena/VASP EVH1 domains (Kd ranging from 0.2 to 0.6 μM) (Table 1). Reduction of 2 to the core motif led to the ligand Ac-[2-CI-F]-[ProM-2]-[ProM-1]-OH (4a). Analogous to the testing of ProM-3

Fig. 1. Design and synthesis of the PPII helix-mimetic fragments. (A) A simple vinylidene bridge between positions 3 and 5 (ProM-1) or between positions 2 and 4 (ProM-2) of two adjacent prolines restricts the relative orientations of the pyrrolidine rings. The overlay of the X-ray structures of ProM-1 (green), ProM-2 (magenta), and a diproline motif in an ideal left-handed PPII helix conformation (white) shows that both tricyclic scaffolds present the pyrrolidine rings in a similar position relative to the dipeptide whereas the orientation of the vinylidene bridge as found in ProM-1. The extension of ProM-1, with its five-membered ring next to R1 (ProM-2, blue) expands the portfolio of PPII-mimic modules while maintaining the orientation of the vinylidene bridge as found in ProM-1. The site, yielded compound 2, with the highest affinity to Ena/VASP EVH1 domains (Kd ranging from 0.2 to 0.6 μM) (Table 1). Reduction of 2 to the core motif led to the ligand Ac-[2-CI-F]-[ProM-2]-[ProM-1]-OH (4a). Analogous to the testing of ProM-3
described above, and again guided by structural considerations (acknowledging the fact that any aliphatic hydrophobic amino acid may be accommodated at the third proline position of the motif), we also tested ProM-4 (Fig. 1d), which contains a six-membered ring next to R1, as a replacement of the last two prolines. However, the compound containing ProM-4 was slightly less effective (Fig. 2A and SI Appendix, Table S5). Thus, the final 678-Da compound 4a represents the best low-molecular weight inhibitor targeting all three Ena/VASP EVH1 domains addressed in this study. A substantial increase in affinity of at least 180-fold (EVL- and Enah-EVH1) and 280-fold (VASP-EVH1) compared with the parent compound Ac-FPPP-TEDEL-NH₂ (Fig. 2A) indicates the importance of retaining a certain ligand length covering the full FPPPP motif (Fig. 2 and SI Appendix, Table S5).

**Esterification of 4a Yields Cell-Permeable Compound 4b.** Sufficient cell permeabilities of the compounds are necessary to study their cellular activities. We found that the N-terminal 7-nitro-2,1,3-benoxazo-diazol-4-yl-labeled (NBD-) compounds 2 and 4a were not able to penetrate the cell membrane. Assuming that the free carboxylic acid functions of NBD-2 (total charge −4) and NBD-4a (total charge −1) are responsible for poor membrane permeability, we synthesized the corresponding NBD-labeled ethyl ester NBD-4b, whose cellular uptake was indicated by a significant increase in cytosolic fluorescence (Fig. 2C and SI Appendix, Fig. S29). HPLC analysis of NBD-4b-treated colorectal cancer HCT 116 cells exhibited a time-dependent increase of compound NBD-4a in the cells, indicating ester cleavage (SI Appendix, Fig. S30). Furthermore, when incubated with 150 μM unlabeled ester 4b for 24 h, no significant loss of either HCT 116 or MDA MB 231 cell viability was observed (SI Appendix, Fig. S31).

**Ligand 4a Selectively Inhibits Ena/VASP EVH1 Domains.** Investigations concerning the selectivity of our ligands against different PRDs made us aware of the fact that the consensus motif of Ena/VASP EVH1 domains exhibits no overlap with those of other PRDs, apart from the occurrence of prolines (30). Therefore, we expected low cross-reactivity. Experimentally, we probed a possible cross-inhibition of other PRDs by NMR and ITC. In particular, the EVH1 class 2 domain of Homer1, which is most closely related to the Ena/VASP EVH1 class 1 domains, and the YAP1-WW, Fyn-SH3, and CD2BP2-GYF domains, as members of different PRD classes and containing the most similar PRSs, were analyzed (Table 2). Binding studies via ITC and 1H-15N-HSQC yielded large ΔG° values of around 400 μM and 330 μM for the interactions of 4a with Homer EVH1 and YAP1-WW, respectively. Fyn-SH3

![Figure 2](image.png)

**Fig. 2.** Development of a low-molecular weight EVH1 inhibitor. (A) Schematic representation of compound optimization. Ligand efficiency (LE = ∆G°/number of heavy atoms) and affinity were improved by replacing the prolines by the PPI-mimetic modules and introducing 2-chloro- and phenylalanine. Compound 4a exhibits the highest LE. Esterification yields the cell-permeable EVH1 inhibitor 4b. All K_d values shown are measured using ITC. (B) X-ray structure (1.7 Å) of the EnaH-EVH1-domain in complex with 4a. (Upper) Overlay of 4a with the FPPPP motif of 1evh. The FPPPP motif-containing peptide (white) and 4a (orange) exhibit the same binding mode (ProM-1, green; ProM-2, magenta). (Lower) Detailed view of the L-phenylalanine [1evh (31), white] binding pocket in comparison with bound 2-chloro-L-phenylalanine (orange). (C) Cell permeability of NBD-labeled 4a and 4b. NBD fluorescence is shown in green, and trypan blue stain (red) shows cell edges. Only NBD-4b yields significantly high NBD fluorescence in the cells. (Scale bars: 50 μm.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_d (μM)</th>
<th>LE</th>
<th>MIC (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VASP-EVH1</td>
<td>4a</td>
<td>2a</td>
<td>3a</td>
</tr>
<tr>
<td>K_d</td>
<td>22(1)</td>
<td>6.05(0.04)</td>
<td>1,880(400)</td>
</tr>
<tr>
<td>K_d</td>
<td>10(3)</td>
<td>0.15(0.06)</td>
<td>460(70)</td>
</tr>
<tr>
<td>K_d</td>
<td>20(1)</td>
<td>0.34(0.03)</td>
<td>450(60)</td>
</tr>
<tr>
<td>K_d</td>
<td>7(1)</td>
<td>0.19(0.05)</td>
<td>310(20)</td>
</tr>
<tr>
<td>K_d</td>
<td>10.4(0.4)</td>
<td>0.26(0.01)</td>
<td>700(300)</td>
</tr>
</tbody>
</table>

Values in parentheses represent SE.
and CD2BP2-GYF showed even weaker affinity (Table 2 and SI Appendix, Figs. S25–S28 and Table S7), clearly indicating a striking prevalence of 4a to inhibit the Ena/VASP EVH1 domains.

The ProM-Toolkit Paves the Way to Develop Ligands also for Other PRD Families. To prove the potential of our current ProM toolkit and to test selectivity further, we developed new ligands exclusively addressing Homer1-EVH1, Fyn-SH3, YAP1-WW, and CD2BP2-GYF. Although for Fyn-SH3 the replacement of the diproline motif by ProM-1 resulted in a moderate loss of affinity, an almost fivefold higher affinity against YAP1-WW was observed with its ProM-modified ErbB4-derived ligand (Table 2, PP-ligand vs. ProM-ligand; and SI Appendix, Table S8). On the other hand, our ProM-toolkit was, so far, not suitable to substitute proline-rich motifs in peptides recognized by Homer1 EVH1 and CD2BP2-GYF (Table 2 and SI Appendix, Table S8). In this case, modeling studies based on available structures uncovered steric hindrances caused by the vinylidene bridges in the canonical binding mode.

X-Ray Structure of 4a in Complex with EnaH-EVH1 Verifies the Canonical Binding Mode. X-ray analysis of the EnaH-EVH1 domain in complex with compound 4a (resolution 1.7 Å) (Fig. 2 B and SI Appendix, Table S9 and Figs. S12–S16) confirmed the canonical binding mode (31), with the pyrrolidine rings of the two ProMs situated in the ligand-binding groove following the axis of the PP1II helix. As expected, the complex is stabilized by two hydrogen bonds between (i) the oxygen of the carbonyl group bridging ProM-2 and ProM-1 and the Ne-H of Trp23 and (ii) the carbonyl oxygen of the seven-membered ring of ProM-2 and an Ne-H of Gln79. Furthermore, a third hydrogen bond is observed between the carbonyl oxygen of the N-terminal acetyl group and the Nɛ-H of Arg81. Moreover, the vinylidene bridge of the ProM-1 fragment interacts with Phe77. The 2-Cl-F moiety resides in the hydrophobic pocket surrounded by residues Lys69, Asn71, Gln79, Arg81, and Val86. The chlorine atom fills the additional space in the pocket between Gln79, Arg81, and Val86, thereby substantially improving binding efficiency (Fig. 2B). NMR studies of VASP- and EVL-EVH1 domains and 4a confirmed similar binding modes (SI Appendix, Figs. S17–S20) for both interactions.

Ligands 2 and 4a Inhibit EVH1-Mediated Protein–Protein Interactions in Vitro. The potential of compounds 2 and 4a to interfere with binding and localization of two well-known Ena/VASP EVH1 interaction partners—zyxin and Lpd—was investigated using the highly invasive breast-cancer cell line MDA MB 231 and the slow-migrating colorectal cancer cell line HCT 116, respectively. Zyxin is abundant in focal adhesions of both cell lines whereas Lpd localizes mainly at the leading edge of MDA MB 231 but could not be detected in HCT 116 cells. Lpd targets VASP via its proline-rich motifs to the lamellipodial tips, causing faster migration (32). Western blot analysis of pull-down experiments with immobilized glutathione-(S)-transferase (GST)-tagged EnaH-EVH1 or VASP-EVH1 applied to the cell lysates revealed zyxin in both cell lines as an EVH1 interaction partner whereas interactions with Lpd were detected only in MDA MB 231 cells (Fig. 3 and SI Appendix, Fig. S32). Both compounds displaced zyxin and Lpd from the GST-fusion proteins in a concentration-dependent manner and corresponding to their respective \( K_d \) values. Despite the high level of homology among the Ena/VASP family EVH1 domains, the EnaH and VASP constructs, but not the GST-EVH1 construct, pulled zyxin and Lpd, although at different amounts. Restricted accessibility of ligand-binding sites in the immobilized domains, the recruitment of other proteins, or additional contributions by flanking epitopes of the FPPP motifs likely explain this behavior.

Inhibition of EVH1 Domains by 4b Reduces the Number of Stress Fibers and Inhibits Cell Invasion. To test cellular effects, we examined the response of both cell lines toward 4b. Untreated HCT 116 cells showed distinctive F-actin in the form of stress fibers. Treatment with 4b altered cell morphology: i.e., the number of cells exposing stress fibers was reduced by 50% (Fig. 4A, Left), and VASP is delocalized from focal adhesions, which are indicated by zyxin localization (Fig. 4A, Right).

Immunofluorescence staining of MDA MB 231 cells showed zyxin in focal adhesions and Lpd at the leading edge (Fig. 4B, columns 1 and 3, respectively). In both cases, these proteins colocalized with VASP (Fig. 4B, Bottom, columns 1 and 3). After treatment with 4b, we detected a strongly reduced presence of VASP at both focal adhesions and at the leading edge (Fig. 4B, columns 2 and 4) whereas localization of zyxin and Lpd remained unaltered (Fig. 4B, Top, columns 2 and 4). MDA MB 231 cells treated with 4b showed a remarkable two-thirds reduction of cell invasion, indicating the importance of EVH1 domain-dependent localization of VASP for this purpose (Fig. 4C). Due to the lack of Lpd in HCT 116, we only investigated interference of 4b with the formation of focal adhesion complexes in this cell line.

Table 2. \( K_d \) values of 4a and selected compounds to representatives of other PRDs

<table>
<thead>
<tr>
<th>Domain name</th>
<th>Consensus</th>
<th>4a</th>
<th>PP-ligand</th>
<th>ProM-ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>YAP1-WW</td>
<td>PP_Y</td>
<td>330(60)</td>
<td>Ac-LPPRYR-NH₂</td>
<td>21(2)</td>
</tr>
<tr>
<td>Homer1-EVH1</td>
<td>PP_F</td>
<td>400(100)</td>
<td>Ac-ALTPPSFRDS-NH₂</td>
<td>72(5)</td>
</tr>
<tr>
<td>CD2BP2-GFY</td>
<td>(QHR)_[L]P[LPP][GS][RH]</td>
<td>3,200(600)</td>
<td>Ac-EGFPPGWLGR-NH₂</td>
<td>6.3(0.5)</td>
</tr>
</tbody>
</table>

Consensus motifs of the domains are in italics. Prolines replaced by the ProM-fragments are in bold. N.D., not detectable.

Fig. 3. Displacement of zyxin and Lpd from EnaH- and VASP-EVH1. Pull-down experiments with lysates from MDA MB 231 cells and GST-tagged EnaH-EVH1 (Upper) and VASP-EVH1 (Lower) immobilized on glutathione Sepharose beads (Upper Frame: Lpd and zyxin in Western blot analysis; Lower Frame: loading control). With GST alone, no zyxin/Lpd was pulled. GST-EVH1 pulled zyxin/Lpd. Zyxin/Lpd displacement from GST-EVH1 domains was performed by adding different concentrations of compounds 2 or 4a to the lysate.
After treatment for 3 h with compound 4b, the number of cells exhibiting SF was reduced by 50% (Center) (SI Appendix, Table S10). (Scale bars: 10 μm.) (A, Right) Immunofluorescent stain of HCT 116 cells (F-Actin, green; VASP, red; zyxin, blue;ocolocalization, purple). Control (Upper row, 0.5% DMSO) and compound 4b-treated (Lower row) cells were incubated for 3 h. Control cells exhibit colocalization of VASP and zyxin in focal adhesions. Cells treated with 4b showed zyxin in focal adhesions, but the colocalization with VASP was reduced by 30% (three replicates, P < 0.001, Pearson’s r2 test) (SI Appendix, Table S11). (Scale bars: 10 μm.) (B, Left) Immunofluorescent stain of MDA MB 231. Control 0.5% DMSO (columns 1 and 3) and 100 μM compound 4b (columns 2 and 4) incubated for 3 h. (Top row) zyxin (ZYX,) and Lpd; (Middle row) VASP; (Lower row) merge (ZYX, blue; VASP, red; colocal., purple; Lpd, green; colocal., yellow). (B, Right) Compound 4b-treated cells showed a reduction of VASP location to focal adhesions (FAs) by ∼40% and to the leading edge by 30% (SI Appendix, Tables S12 and S13). FAs are marked by zyxin, the leading edge by Lpd. The localization of zyxin and Lpd remains unchanged. (Scale bars: 5 μm.) (C) Matrigel invasion assay. MDA MB 231 cells invaded through a matrigel-coated 8-μm-pore membrane on (i) an FBS gradient (DMSO and compound 4b) or (ii) in FBS without a gradient (DMSO) while incubating 100 μM 4b for 14 h. The invaded cells on the lower side of the membrane were counted. The control (DMSO, with gradient) was set to 100%. Compound 4b reduced the cell invasion by 64%.

**Discussion**

We synthesized a toolkit of four building blocks (ProM-1 to ProM-4) designed to replace the conserved PxxP and xPPx recognition motifs involved in PRS-mediated protein–protein interactions. As proof of concept, we developed a peptidomimetic, low-molecular weight inhibitor of the Ena/VASP family EVH1 domains that exhibits a fivefold higher affinity than the much larger ActA-derived peptide 1. In comparison with the isolated recognition sequence Ac-FFPPP-OH (3), the affinity is increased at least by a factor of 180 (Fig. 2 and Table 1). Remarkably, this dramatic effect is caused by introduction of only five additional heavy atoms. The progress achieved becomes apparent by comparing ligand efficiencies (LE = ΔG/number of heavy atoms) (33) of compounds 1, 2, and 4a. The gain of LE from 1 (−0.2 kJ·mol−1) over 2 (−0.3 kJ·mol−1) to 4a (−0.7 kJ·mol−1) represents a considerable success because the average LE of drug candidates targeting protein–protein interfaces is about −1.0 kJ·mol−1 (34).

The X-ray structure of the EnaH-EVH1 domain in complex with 4a indicates that ProMs mimic a PPII helix in the bound state, with the pyrrolidine rings of the ProMs perfectly matching the position of the conserved core motif prolines. The structural feature enhancing binding of 4a to Ena/VASP EVH1 domains is the vinylidene bridge in the tricyclic system that, together with the specific flanking residue, also boosts specificity. Selectivity of 4a is supported by improved PPII helix recognition in conjunction with optimized binding of flanking epitopes. Although the pure PPII helix recognition motif alone contributes little to affinity, it strongly affects the recognition, as indicated by the strong decrease in affinity detected for Ac-[2-Cl-F]-[ProM-2]-NH2 (Fig. 2A).

Western blot analysis of Ena/VASP EVH1 pull-down experiments showed that 4a is able to compete in a concentration-dependent manner with the focal adhesion protein zyxin, as well as with Lpd, a protein involved in directed cell migration and located at the front of lamellipodia. Although compound 4a is poorly cell-permeable, the ester derivative 4b can enter into cells, thereby allowing examination of its effect on cytoskeleton remodeling in cellular assays. Immunofluorescence staining of 4b-treated colorectal cancer cells HCT 116 exhibits reduction of stress fibers and delocalization of VASP from focal adhesions. Keeping in mind that our ligands inhibit all three Ena/VASP family EVH1 domains, this result is supported by Furman et al. (18), who showed that the number of stress fibers is strongly reduced in primary endothelial mouse cells in which all three Ena/VASP proteins are knocked out. Cells treated with 4b showed zyxin-rich spots indicating focal adhesions, but a strongly reduced presence of VASP in these locations. These findings correlate with the observation that cells with zyxin mutants lacking the FPPP motif do not show colocalization of VASP (21), indicating that the loss of an Ena/VASP EVH1-mediated protein–protein interaction is responsible for reduction of stress fibers. Treatment of highly invasive MDA MB 231 breast-cancer cells with 4b causes delocalization of VASP from the leading edge and from focal adhesions, thereby reducing strongly their ability for invasion. Hence, we suggest that EVH1-mediated protein–protein interactions play an important role in regulation of the dynamic remodeling of the actin cytoskeleton.
Compounds 4a and its produg 4b represent a successful proof of concept. To our knowledge, they are the first low-molecular weight inhibitors of Ena/VASP EVH1 domains. Compound 4b thereby represents a novel chemical probe that allows examination into the complex role of this protein family in the regulation of cytoskeletal remodeling events.

Our ProM toolkit enables construction of specific inhibitors for both ΨxΨ and ΨxxΨ recognizing domains as shown here for Ena/VASP EVH1 and YAP1-WW domains, respectively. Our results suggest the importance of flanking motifs outside the conserved prolines for specificity and affinity. Furthermore, the toolkit of ProM modules facilitates the fitting of the designed ligands in an optimal manner to the binding cavities. In the case of the YAP1-WW ligand, we identified a [ProM-1]2 module as the optimal replacement of the conserved xΨΨ motif whereas, for the Ena/VASP EVH1 domains, a [ProM-2][ProM-1] motif was better suited. The sensitivity of the different ProMs toward small differences between the proline-binding sites represents an opportunity for the design of specific ligands. A gain of affinity by enhanced flanking epitope binding together with selectivity against similar but nonetheless structurally distinct binding sites are the two critical factors that are likely to be exploited by our iterative approach of design, synthesis, and structure elucidation. The new inhibitors open new routes for pharmacological interference by directly modulating regulatory PRS-mediated protein–protein interactions in a specific manner.

Methods

Binding Studies. Dissociation constants were determined via FT and ITC. All experiments were done at 25 °C in 40 mM sodium phosphate, pH 7.3, 100 mM sodium chloride. For the Ena/VASP-EVH1 domains, the buffer additionally contained 1 mM DTT or 2 mM tris(2-carboxyethyl)phosphine (TCEP).

Crystallization. To freshly purified and concentrated protein, a 3:1 molar protein–interference by directly modulating regulatory PRS-mediated elucidation. The new inhibitors open new routes for pharmacological exploited by our iterative approach of design, synthesis, and structure elucidation. The new inhibitors open new routes for pharmacological interference by directly modulating regulatory PRS-mediated protein–protein interactions in a specific manner.

Migration Assay. The BD Matrigel invasion chamber 24-well plate (8.0 μm) cell-migration assay (BD Biosciences, Inc.) was performed as described in the manufacturer's guide. The experiments were done in triplicate with 50,000 cells (MDA MB 231) per well in (10%) (vol/vol) fetal serum bovine (FBS) gradient and (0) 10% (vol/vol) FBS without a gradient. After 14 h of incubation with 100 μM compound 4b—or 0.5% DMSO as a control—cells were fixed with p-formaldehyde and stained with crystal violet. The used microscope (Eclipse TS100; Nikon) had a 100.25 (air) objective (Nikon), and images were detected with a digital single camera (Nikon). Five random fields per well were taken, and cells were counted with ImageJ (imagej.nih.gov; NIH).

All other methods and materials are described in SI Appendix.
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1 Supplementary Methods

1.1 Characterization of ProM-1

TLC: 0.17 (CH₂Cl₂/MeOH 15:1).

Melting point: 140 °C.

\([\alpha]_D^{20} \approx -225.1^\circ \) (c = 0.450, CHCl₃).

\(^1\)H NMR (600 MHz, CDCl₃): rotamer 1 (60 %): \( \delta \) (ppm) = 1.74-1.64 (m, 1H, NCH₂CH), 1.97-1.81 (m, 2H, CHCHHCHHCHCO₂H), 2.16 (dt, \( J = 11.6, 5.8 \text{ Hz} \), 1H, NCH₂CHH), 2.36-2.23 (m, 1H, CHCHHCH₂CHCO₂H), 2.40 (d, \( J = 6.4 \text{ Hz} \), 1H, CHCH₂CHHCHCO₂H), 2.91 (s, 1H, NCH₂CH₂CH), 3.52 (td, \( J = 11.1, 5.4 \text{ Hz} \), 1H, NCHH), 3.79-3.72 (m, 1H, NCH), 4.28-4.24 (m, 1H, CHFmoc), 4.42 (d, \( J = 10.9 \text{ Hz} \), 1H, NCHCON), 4.53 (dd, \( J = 10.2, 6.3 \text{ Hz} \), 1H, CHHf), 4.68-4.59 (m, 2H, CHCH₂CH₂CHCO₂H, CHHf), 4.79 (d, \( J = 7.3 \text{ Hz} \), 1H, CHCH₂CH₂CHCO₂H), 5.55 (d, \( J = 11.1 \text{ Hz} \), 1H, NCH₂CH₂CHH), 5.79 (d, \( J = 11.2 \text{ Hz} \), 1H, CONCHCH), 7.33-7.26 (m, 2H, 2 × CHarom.Fmoc), 7.41-7.34 (m, 2H, 2 × CHarom.Fmoc), 7.63-7.52 (m, 2H, 2 × CHarom.Fmoc), 7.78-7.68 (m, 2H, 2 × CHarom.Fmoc). rotamer 2 (40 %): \( \delta \) (ppm) = 1.51-1.40 (m, 1H, NCH₂CHH), 1.97-1.81 (m, 2H, CHCHHCHHCHCO₂H), 2.05-1.99 (m, 1H, NCH₂CHH), 2.36-2.23 (m, 2H, CHCHHCHHCHCO₂H), 2.80 (s, 1H, NCH₂CH₂CH), 3.33 (td, \( J = 11.3, 5.3 \text{ Hz} \), 1H, NCHH), 3.79-3.72 (m, 1H, NCHH), 3.92 (d, \( J = 10.7 \text{ Hz} \), 1H, NCHCON), 4.14 (t, \( J = 5.1 \text{ Hz} \), 1H, CHFmoc), 4.21 (s, 1H, CHCH₂CH₂CHCO₂H), 4.33-4.27 (m, 1H, CHHFmoc), 4.35 (d, \( J = 7.0 \text{ Hz} \), 1H, CHCH₂CH₂CHCO₂H), 4.65-4.60 (m, 1H, CHHFmoc), 5.45 (d, \( J = 6.4 \text{ Hz} \), 1H, CHHf).
10.8 Hz, 1H, NCH₂CH₂CHCH), 5.70 (d, J 11.2, 1H, CONCHCH), 7.33-7.26 (m, 2H, 2 × CHₐrom. Fmoc), 7.41-7.34 (m, 2H, 2 × CHₐrom. Fmoc), 7.63-7.52 (m, 2H, 2 × CHₐrom. Fmoc), 7.78-7.68 (m, 2H, 2 × CHₐrom. Fmoc).

¹³C NMR (150 MHz, CDCl₃, mixture of rotamers): \( \delta \) (ppm) = 25.33/25.25 (t, CHCH₂CH₂-CCH₂-CCH₂-C), 31.29/30.60 (t, NCH₂CH₂), 33.37/33.26 (t, CHCH₂CH₂-CCH₂-C), 42.26/41.40 (d, NCH₂CH₂CH₂), 47.09/46.81 (t, NCH₂), 47.63/47.23 (d, CHFmoc), 58.26/58.01 (d, CHCH₂CH₂-CCH₂-C), 61.00/60.54 (d, CHFmoc), 62.06/61.64 (d, NCHCON), 67.67/66.01 (t, CH₂Fmoc), 119.95/119.66/119.56 (d, 2 × CHₐrom. Fmoc), 125.18/125.01/124.85/124.76 (d, 2 × CHₐrom. Fmoc), 127.07/127.03 (d, 2 × CHₐrom. Fmoc), 127.70/127.66/127.58/127.48 (d, 2 × CHₐrom. Fmoc), 128.80/128.65/128.54/128.28 (d, NCH₂CH₂CHCH, CHCH₂CH₂CHCH₂-C), 141.31/141.25 (s, Cₜₜₜ. Fmoc), 144.20/143.66 (s, Cₜₜₜ. Fmoc), 155.21/154.75 (s, NCO₂Fmoc), 172.22/172.15 (s, CHCO₂H), 172.83/172.63 (s, CHCON).

IR: \( \nu \) (cm⁻¹) = 3415 (m br), 3062 (w), 3035 (w), 3016 (w), 2967 (m), 2949 (m), 2884 (m), 2711 (w), 2626 (w), 2523 (w), 2248 (w), 1951 (w), 1914 (w), 1737 (s), 1697 (s), 1672 (s), 1556 (w), 1538 (w), 1477 (m), 1448 (s), 1420 (s), 1397 (w), 1350 (s), 1330 (m), 1304 (w), 1285 (w), 1273 (w), 1242 (m), 1222 (m), 1187 (s), 1169 (s), 1154 (w), 1121 (s), 1093 (w), 1073 (w), 1022 (w), 984 (w), 959 (w), 942 (w), 907 (s), 838 (w), 782 (w), 759 (s), 728 (s).

MS (EI, 70 eV): \( m/z \) (%) = 414 (1), 263 [M⁺]-C14H11O (1), 235 (1), 179 (30), 178 (100), 165 (5), 152 (5), 120 (5), 80 (5).

Supplementary Figure 1: $^1$H-NMR spectrum of ProM-1, measured at 600 MHz in CDCl$_3$; main rotamer.
Supplementary Figure 2: $^1$H-NMR spectrum of ProM-1, measured at 600 MHz in CDCl$_3$; minor rotamer.
Supplementary Figure 3: APT-NMR spectrum of ProM-1, measured at 150 MHz in CDCl₃.
1.2 Characterization of ProM-2

TLC: 0.20 (CH₂Cl₂/MeOH 15:1).

Melting point: 105.3 °C.

[^2]D: −117.8°, (c = 0.275, CHCl₃).

^1H NMR (600 MHz, CDCl₃, mixture of rotamers Rot1/Rot2 1.1/1): rotamer 1: δ (ppm) = 1.66-1.55 (m, 1H, H4’), 2.03-1.77 (m, 4H, H3’, H11’, H12), 2.16-2.08 (m, 1H, H4), 2.34-2.28 (m, 1H, H11), 2.42-2.35 (m, 1H, H3), 3.54-3.45 (m, 1H, H13’), 3.71-3.61 (m, 1H, H13), 4.33-4.16 (m, 3H, H5, H15’, H16), 4.41 (t, J = 8.7 Hz, 1H, H2), 4.54 (dd, J = 10.9, 6.0 Hz, 1H, H15), 5.69 (dd, J = 10.0, 2.4 Hz, 1H, H7), 5.87 (dd, J = 10.0, 1.1 Hz, 1H, H8), 7.32-7.26 (m, 2H, H19), 7.40-7.34 (m, 2H, H20), 7.63-7.50 (m, 2H, H18), 7.79-7.72 (m, 2H, H21); rotamer 2: δ (ppm) = 1.24-1.15 (m, 1H, H4’), 2.03-1.77 (m, 5H, H3’, H4, H11’, H12), 2.28-2.23 (m, 1H, H3), 2.34-2.29 (m, 1H, H11), 3.54-3.45 (m, 1H, H13’), 3.71-3.61 (m, 1H, H13), 4.03 (t, J = 5.6 Hz, 1H, H16), 4.17-4.11 (m, 1H, H5), 4.33-4.17 (m, 3H, H2, H15), 5.48 (dd, J = 9.8, 2.6 Hz, 1H, H7), 5.58 (d, J = 9.9 Hz, 1H, H8), 7.32-7.26 (m, 2H, H19), 7.40-7.34 (m, 2H, H20), 7.63-7.50 (m, 2H, H18), 7.79-7.72 (m, 2H, H21).

^13C NMR (150 MHz, CDCl₃, mixture of rotamers): δ (ppm) = 24.2/23.3 (t, C12), 29.5/29.5 (t, C3), 32.1/32.1 (t, C4), 40.4/39.5 (t, C11), 48.3 (d, C16), 49.9/49.4 (t, C13), 60.6/60.1 (d,
C5), 61.5/61.3 (d, C2), 65.8/65.7 (s, C9), 68.4/67.9 (t, C15), 121.0/120.9 (d, C21), 124.6/124.4 (d, C8), 126.1/126.1/126.0/125.8 (d, C18), 128.1/128.0/127.9 (d, C19), 128.8/128.7 (d, C20), 132.8/131.6 (d, C7), 142.6/142.5 (s, C22), 145.5/145.4, 145.1/145.0 (s, C17), 156.7/156.0 (s, C14), 170.2/170.1 (s, C10), 180.1/180.0 (s, C6).

IR:  \( \nu \) (cm\(^{-1}\)) = 3446 (w), 3040 (w), 2973 (w), 2876 (w), 2580 (w), 1736 (m), 1699 (s), 1659 (s), 1449 (s), 1412 (s), 1351 (s), 1300 (w), 1250 (w), 1213 (m), 1194 (m), 1151 (m), 1135 (m), 1033 (w), 936 (w), 879 (w), 758 (m), 740 (m).

HRMS ((-)ESI): calcd. for C\(_{27}\)H\(_{25}\)N\(_2\)O\(_5\): 457.17580; Found: 457.17578.

Supplementary Figure 4: \(^1\)H-NMR spectrum of ProM-2, measured at 600 MHz in CDCl\(_3\); main rotamer.
Supplementary Figure 5: $^1$H-NMR spectrum of ProM-2, measured at 600 MHz in CDCl$_3$; minor rotamer.
Supplementary Figure 6: APT-NMR spectrum of ProM-2, measured at 150 MHz in CDCl₃.
1.3 Characterization of ProM-3

**DC:**  $R_f = 0.20$ (DCM/MeOH 15:1).

$[\alpha]_{D}^{20} = -152.7^\circ$, (c = 0.345, CHCl$_3$).

**Melting point:**  105.3 °C.

$^1$H-NMR (500 MHz, CDCl$_3$):  $\delta$ (ppm) = 1.12 (d, $J = 7.1$ Hz, 3H, H10), 1.95-1.79 (m, 1H, H4'), 2.08-1.97 (m, 1H, H3'), 2.25-2.16 (m, 1H, H3), 2.35-2.26 (m, 1H, H4), 2.47 (s br, 1H, H9), 4.21 (t, $J = 7.3$ Hz, 1H, H15), 4.39-4.28 (m, 2H, H14), 4.74-4.57 (m, 3H, H2, H5, H11), 5.49 (d, $J = 11.7$ Hz, 1H, H8), 5.55 (d, $J = 11.7$ Hz, 1H, H7), 6.21 (d, $J = 8.9$ Hz, 1H, NH), 7.32-7.26 (m, 2H, H18), 7.41-7.35 (m, 2H, H19), 7.64-7.57 (m, 2H, H17), 7.74 (d, $J = 7.5$ Hz, 2H, H20), 10.53 (s, 1H, COOH).

$^{13}$C-NMR (125 MHz, CDCl$_3$):  $\delta$ (ppm) = 18.75 (q, C10), 26.89 (t, C3), 32.92 (t, C4), 35.78 (d, C9), 47.02 (d, C15), 55.60 (d, C11), 55.91 (d, C5), 60.32 (d, C2), 67.10 (t, C14), 119.83 (d, C20), 125.12 (d, C17), 126.96 (2xd, C17, C8), 127.57 (d, C19), 134.55 (d, C7), 141.14 (s, C21), 143.61/143.83 (s, C16), 156.61 (s, C13), 171.57 (s, C12), 174.42 (s, C6).

**IR (FT-ATR):**  $\nu$ (cm$^{-1}$) = 3284 (w), 3015 (w), 2971 (w), 2873 (w), 1715 (s), 1642 (s), 1530 (m), 1447 (s), 1319 (m), 1250 (s), 1220 (m), 1186 (m), 1104 (w), 1083 (m), 1033 (m), 979 (w), 943 (w), 871 (w), 830 (w), 757 (s), 740 (s), 666 (w).
HR-MS ((-)-ESI): calcd. for C_{26}H_{25}N_{2}O_{5}: 445.17581; Found: 445.17580.

Supplementary Figure 7: $^1$H-NMR spectrum of ProM-3, measured at 500 MHz in CDCl$_3$. 
Supplementary Figure 8: $^{13}$C-NMR spectrum of ProM-3, measured at 125 MHz in CDCl$_3$. 
1.4 Characterization of ProM-4

DC:  
$R_f = 0.13$ (MeOH/CH$_2$Cl$_2$ = 1:15).

$[\alpha]_D^{20} = -64.7^\circ$, (c = 0.295 CHCl$_3$).

Melting point:  157°C.

$^1$H-NMR (mixture of rotamers, 1.5:1) (600 MHz, 258 K, CDCl$_3$):  $\delta$ (ppm) = 0.78 – 0.89 (m, 0.6 H, H-13$^{\text{rot1}}$), 1.36 – 1.41 (m, 0.4 H, H-13$^{\text{rot2}}$), 1.51 – 1.59; 1.69 – 2.16; 2.18 – 2.32 (3 x m, 7 H, H-3, H-4, H-12, H-13), 2.34 – 2.37 (m, 0.6 H, H-11$^{\text{rot1}}$), 2.61 – 2.67 (m, 0.4 H, H-11$^{\text{rot2}}$), 2.97 – 3.07 (m, 0.4 H, H-14$^{\text{rot2}}$), 2.37 – 3.45 (m, 0.6 H, H-14$^{\text{rot1}}$), 3.55 – 3.61 (m, 0.4 H, H-5$^{\text{rot2}}$), 3.81 – 3.85 (m, 0.4 H, H-10$^{\text{rot2}}$), 3.92 – 3.96 (m, 0.4 H, H-14$^{\text{rot2}}$), 4.02 – 4.06 (m, 0.6 H, H-14$^{\text{rot1}}$), 4.15 (m, 0.4 H, H-17$^{\text{rot2}}$), 4.28 – 4.33 (m, 1.2 H, H-16$^{\text{rot1}}$, H-17$^{\text{rot1}}$), 4.41 – 4.46 (m, 1 H, H-2$^{\text{rot2}}$, H-16$^{\text{rot1}}$), 4.56 – 4.58 (m, 0.4 H, H-16$^{\text{rot2}}$), 4.65 (d, $J = 11.6$ Hz, 0.6 H, H-10$^{\text{rot1}}$), 4.68 ? 4.74 (m, 0.6 H, H-5$^{\text{rot1}}$), 4.76 (d, $J = 8.6$ Hz, 0.6 H, H-2$^{\text{rot1}}$), 5.00 – 5.05 (m, 0.4 H, H-16$^{\text{rot2}}$), 5.36 (m, 0.8 H, H-7$^{\text{rot2}}$, H-8$^{\text{rot2}}$), 5.36 (m, 1.2 H, H-7$^{\text{rot1}}$, H-8$^{\text{rot1}}$), 7.32 – 7.35 (m, 2 H, H-20), 7.39 – 7.44 (m, 2 H, H-20), 7.56 – 7.63 (m, 2 H, H-21), 7.72 – 8.80 (m, 2 H, H-19), 7.32 – 7.35 (m, 2 H, H-22), 8.81 (bs, 1 H, -COOH).

$^{13}$C-NMR (mixture of rotamers, 1.5:1) (150 MHz, 258 K, CDCl$_3$):  $\delta$ (ppm) = 20.7, 21.5 (C13), 25.7; 25.8; 25.9; 26.2 (C3, C12), 32.8; 33.1 (C4), 32.9; 33.2 (C11), 37.1; 38.1 (C14),
46.8; 47.9 (C17), 56.2; 57.0 (C5), 58.4; 59.0 (C10), 59.9; 60.0 (C2), 62.2; 67.6 (C16), 119.6; 119.7; 119.9 (C22), 124.5; 124.8; 124.9; 125.1 (C19), 126.8; 126.9; 127.0; 127.1; 127.2; 127.5; 127.6 (C8, C20, C21), 132.2; 132.3 (C7), 141.0; 141.1; 141.2 (C23), 143.1; 143.4; 143.9; 144.4 (C18), 155.3; 156.2 (C15), 172.4; 172.8 (C9), 173.5; 173.6 (C6).

**IR:** \( \nu \text{ (cm}^{-1}\text{)} = 3013 \text{ (w), 2940 \text{ (w), 2866 \text{ (w), 2246 \text{ (w), 1696 \text{ (s), 1646 \text{ (s), 1446 \text{ (m), 1423 \text{ (m), 1350 \text{ (w), 1323 \text{ (w), 1280 \text{ (w), 1263 \text{ (w), 1216 \text{ (m), 1170 \text{ (m), 1126 \text{ (m), 1100 \text{ (w), 1080 \text{ (w), 1003 \text{ (w), 976 \text{ (w), 903 \text{ (s), 720 \text{ (s).}}}

**HRMS (ESI):** calcd. for [M-H]−: 471.19145; found 471.19216.

Supplementary Figure 9: \(^1\text{H-NMR spectrum of ProM-4, measured at 600 MHz in CDCl}_3\) at 258 K; mixture of rotamers 1.5 : 1.
Supplementary Figure 10: APT-NMR spectrum of ProM-4, measured at 150 MHz in CDCl$_3$ at 258 K; mixture of rotamers 1.5 : 1.
1.5 Plasmid construction, transformation, protein expression and purification

Plasmid coding for Glutathione-(S)-Transferase (GST)-fused human VASP-EVH1 (human vasodilator-stimulated phosphoprotein, aa1–115) was provided by Dr. Linda Ball (FMP, Berlin, Germany) [1]; expression construct for GST-Yap-WW (human Yes-associated protein, aa162–217) was a gift of Dr. Annette Diehl (FMP, Berlin, Germany). Construct for expression of N-terminally His-tagged GYF domain of the human CD2BP2 (human CD2 antigen cytoplasmic tail-binding protein 2, 280–341) was described previously [2]. The DNA fragments encoding human EnaH-EVH1 (human enabled homolog, aa1–111), EVL-EVH1 (human ena/VASP-like protein, aa1–111), Homer1-EVH1 (human Homer-1, aa1–117) and Fyn-SH3 (human tyrosine-protein kinase Fyn, aa83–152) were PCR-amplified (for primer sequences see Supplementary Table 1) and subcloned for expression into the vector pGEX-4T-1 (Clontech) using BamHI and NotI/XhoI/EcoRI. The cloned gene fragments were verified by sequencing. The double mutant C7S/C64S of the VASP-EVH1 domain was prepared via site-directed mutagenesis by overlap extension PCR using mutagenic primers (Supplementary Table 1), the vector specific primers pGex rev and pGex for, and the wild type VASP-EVH1 gene fragment as a template. Both mutations were introduced in succession and the final PCR fragment cloned into the BamHI and XhoI sites of pGEX 4T-1.

Transformation was performed via an electroporator (Bio-Rad). All proteins were expressed as GST-fusion proteins in *E. coli* BL21 (DE3) (Novagen) in 2YT medium (Carl Roth) supplemented with ampicillin (300 mg/l; Carl Roth) and induced at OD$_{600}$ of 0.5 to 1.0 using 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Carl Roth) at 18 °C for 18 h. The cells were lysed by sonication in the presence of benzonase (Merck) and purified from the soluble fraction via Profinia (Bio-Rad) by using a 5 ml glutathione-sepharose column (Bio-Rad), or by using affinity chromatography on a Glutathione Sepharose 4B (GE Healthcare) column in PBS buffer (pH 7.0 to 8.0) supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM 2-mercaptoethanol. If necessary GST moiety was cleaved overnight at 27 °C using thrombin protease (1 U/mL; Invitrogen), and subsequently gel-filtrated (Superdex75, GE Healthcare)
against 40 mM sodium phosphate, pH 7.3, 100 mM sodium chloride. 1 mM dithiothreitol (DTT) or 2 mM tris(2-carboxyethyl)phosphine (TCEP) was added if necessary. Samples were then concentrated using Vivaspin20 (5000 MWCO) concentrators (Satorius). His-tagged GYF domain purified using affinity chromatography on a prepacked HisTrap nickel agarose column (Amersham Biosciences) and subsequent gel filtration. Protein molecular masses, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), agreed with those predicted. Protein concentration was measured by UV/Vis spectroscopy, using the extinction coefficients at 280 nm, calculated from their sequence.

For crystallography experiments, an additional purification step using SP-sepharose was introduced after the thrombin digestion. EnaH-EVH1 was eluted from the column in 25 mM sodium phosphate buffer (pH 7.5), 20 mM sodium chloride, supplemented with 5 mM 2-mercaptethanol, with a gradient of 20 to 500 mM sodium chloride, and load on Superdex75 gel-filtration column using crystallization buffer (20 mM sodium chloride, 5 mM TCEP, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.0). Samples were concentrated up to 33 mg/ml, as described above. Purity was higher than 95 % as judged by SDS-PAGE.

The $^{15}$N- and $^{15}$N-$^{13}$C- labeled proteins were expressed in M9 medium supplemented with 1 g/L $^{15}$N–NH$_4$Cl and 2 to 5 g/L glucose or $^{13}$C–glucose, induced with 1 mM IPTG for 18 h at 18 °C and purified as described above.

1.6 Peptide synthesis

Peptides were synthesized automatically on an ABI 433A instrument (Applied Biosystems) following the standard solid-phase N$^\alpha$-9-fluorenylmethoxycarbonyl protocol in a batchwise mode as described in [3], or – if necessary – peptide synthesis was done manually. Peptides were purified by preparative reversed-phase high-performance liquid chromatography (HPLC) on an LC-10AD system (Shimadzu) operating at 220 nm. The purities of the final products were >95 % by analytical HPLC, and their expected molar masses were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Voyager-DE STR (Applied Biosys-
Stock solutions of peptides were prepared in Protein LoBind Tubes (Eppendorf). The amount of peptides were determined gravimetrically with a microbalance AT21 Comparator (Mettler Toledo).

The pH values of the peptide solutions were checked with a micro electrode InLab Micro (Mettler Toledo) and, if necessary, readjusted with monosodium or trisodium phosphate solutions (40 mM monosodium or trisodium phosphate, 100 mM sodium chloride, 1 mM DTT or 2 mM TCEP if necessary) to pH 7.3. Solutions were stored at –20 °C or below.

For cell assays, remaining trifluoroacetic acid was removed from compounds by lyophilizing them in 1 M HCl. Then they were solved in DMSO and stored at –20 °C.

1.7 Peptide SPOT array

Single substitutions of the ActA peptide were generated by semiautomated spot synthesis on Whatman 50 cellulose membranes as described in [4].

The washed membranes were blocked in PBS with 5 % m/v bovine serum albumin (BSA) and 0.05 % v/v Tween-20 and incubated with GST or GST-VASP-EVH1 (10 µg/ml) in PBS with 2 % m/v BSA and 0.05 % v/v Tween-20 for one hour. Bound GST was detected by antibodies (rabbit–anti–GST (1:1000) (Santa Cruz Biotechnology) and IRDye800 donkey anti–rabbit (1:20000) (Licor)) using an infrared scanner (ODYSSEY; Licor).

1.8 Binding studies

All experiments were done at 25 °C in 40 mM sodium phosphate, pH 7.3, 100 mM sodium chloride. For the Ena/VASP-EVH1 domains the buffer contained 1 mM DTT or 2 mM TCEP. Buffer was sterile-filtered and degassed before use. Each titration consists of at least three replicas.

Data analysis was done using R [5]. Functions used in particular were nls (using the algorithm plinear when ever possible) or nlsLM (package minpack.lm), and coeftest with the option vcov = sandwich (package sandwich) for computing robust variance-
covariance matrix estimators [6]. For the used model functions see Supplementary Equations.

1.8.1 Fluorescence Titrations

Like in all other proline-rich-motif recognition domains, all three Ena/VASP EVH1 domains contain a conserved tryptophane, which is directly involved in ligand binding [1]. Therefore we could use tryptophan based fluorescence titration methods to obtain the dissociation constant.

We used two different titration schemes: (i) fluorescence titration (FT): to a initial volume containing the protein, aliquots of ligand solution were added, (ii) continuous variation method (CVM): the concentrations of both the protein and ligand are varied simultaneously in opposite directions.

All experiments were carried out using an FP-6500 fluorescence spectrometer (Jasco) with an ADP-303T temperature-controller (Jasco) coupled to a water bath acting as a stable heat sink.

To minimize adsorption, stock solutions of protein and ligand were prepared in Protein LoBind Tubes (Eppendorf) and samples were prepared in low-retention 0.6 mL micro reaction vessels (G. Kisker), using low-retention tips (G. Kisker).

**Fluorescence Titration** For FT we used a 4 × 10 mm fluorescence cell made of Suprasil with a stirrer and cap (Hellma). We placed the cuvette with the smaller path length in the excitation beam, in order to reduce the primary filter effect [7].

To a initial volume of 800 µL, 5 µL aliquots of ligand solution were added. The total volume of added ligand solution was 100 µL. The stirrer was able to mix the entire volume homogeneously, as verified by adding a dye to the maximal used volume. After each aliquot was injected, the solution was incubated for one minute and then the fluorescence intensity was measured.

Concentration of the protein solution ranged from 5 to 100 µM, depending on the dissociation constant. The corresponding concentration of the ligand was calculated to be at least four times bigger than the protein concentration, when all ligand was added to the protein solution.

The excitation wavelength ranged from 298 to 305 nm (slit 1 nm), depending on the used
protein concentration in order to minimize the primary filter effect. Fluorescence intensity was
detected at 340 nm (slit 3 nm) for 0.5 to 1 sec. Photo bleaching effects were negligible.

In case of significant absorption at the excitation or emission wavelength, the measured intensi-
ty was corrected according to MacDonald et al. [8]:

\[
F_{\text{ideal}} = F_{\text{obs}} \times \frac{2.3 A_{\text{ex}} \Delta x 10^{A_{\text{ex}} x_1}}{1 - 10^{-A_{\text{ex}} \Delta x}} \times \frac{2.3 A_{\text{em}} \Delta y 10^{A_{\text{em}} y_1}}{1 - 10^{-A_{\text{em}} \Delta y}}
\]  

(1)

Here \(F_{\text{obs}}\) is the observed intensity, \(F_{\text{ideal}}\) the intensity after correction for the primary or sec-
ondary filter effect, \(A_{\text{ex}}\) is the absorption at the excitation wavelength, and \(A_{\text{em}}\) is the absorption
at the emission wavelength. The used parameter were \(x_1 = 0\) cm, \(\Delta x = 0.4\) cm, \(y_1 = 0.35\) cm and
\(\Delta y = 0.3\) cm.

In order to reduce adsorption of protein to plastic material, the same pipette tip was used
throughout each titration.

**Continuous variation method** For CVM a 3 × 3 mm ultra-micro fluorescence cell made of
Suprasil with a minimal volume of 50 µL (Hellma) was used.

Eleven independent samples with a volume of 60 µL were prepared for each titration. The
concentrations of both the protein and ligand were varied simultaneously but in opposite direc-
tions from 100 % to 0 %. The 100 % protein concentration was 10 µM, and the 100 % ligand
concentration ranged from 50 to 250 µM, depending on the dissociation constant.

An excitation wavelength of 298 nm (slit 3 nm to 5 nm) and a emission wavelength of 340 or
350 nm (slit 10 nm) was used. Fluorescence intensity was detected for 2 to 8 sec, after temperat-
ing the sample for 5 min.

**1.8.2 Isothermal titration calorimetry**

High-sensitivity isothermal titration calorimetry was performed on a VP-ITC or ITC200 (GE
Healthcare). For the VP-ITC 10 µL aliquots of ligand solution were injected into a 1.4 mL sam-
ple cell containing 50 to 100 µM protein; for the ITC200 1.4 to 2.49 µL aliquots were injected
into a 0.2 mL sample cell containing 50 to 250 µM protein. The background signal of injecting ligand into buffer was determined in separate measurement. Baseline correction of the thermogram, heat integration and data evaluation were done using R.

For the binding experiments of Ac-FPPP-OH to the Ena/VASP-EVH1 domains a competition assay was used [9]. To 100 µM protein with 1 mM Ac-FPPP-OH, 1.45 mM of compound 2 was titrated. The binding of ligand 4a to Fyn-SH3 and CD2BP2-GYF was measured also as a competition assay: to 50 µM Fyn-SH3 with 1 mM 4a, 0.6 mM Ac-VSLARRPLPPLP-NH2 [10] was titrated; to 100 µM CD2BP2-GYF with 1 mM 4a, 1.5 mM Ac-EFGPPGWLGR-NH2 [11] was titrated. Data evaluation was done according to Wang [12], see Supplementary Equations.

1.9 X-ray: Structure Determination

Diffraction data were collected at the BESSY beamline 14.1 at Wilhelm-Conrad-Roentgen Campus in Berlin on a MarMosaic 225 mm CCD detector. Indexing and scaling was performed with XDS [13]. Structure was solved by molecular replacement using the CCP4 [14] program MOLREP [15] and the structure 1evh of EnaH-EVH1 from Prehoda [16] (S2-L111) as search model. The structure was refined with the CCP4 program Refmac5 [17] and rebuilt with Moloc [18]. Although the experimental electron density of the ligands was visible from the beginning, the ligands were included in later refinement steps. The refined complex has a crystallographic R value 18.99 % and a free R factor of 23.58 %. Detailed statistics are displayed in Supplementary Table 9.

1.10 NMR

All samples produced for NMR spectroscopy were labelled according to the task (13C, 15N for the assignment, 15N for interaction studies). The buffer used for NMR spectroscopy contained 40 mM sodium phosphate, pH 7.3, 100 mM sodium chloride, for the Ena/VASP-EVH1 domains 1 mM DTT or 2 mM TCEP, and 10 % v/v deuterium oxide. All experiments were performed in ø 5 mm tubes (Norell), protein concentration was 0.5 to 1 mM for assignments, and 100 to
200 µM for titration experiments.

All NMR spectra were recorded at 297 K on AV600 Bruker spectrometers (600 MHz proton frequency) using either a RT-QXI probe, a cryo-TXI-probe or a cryo-TCI probe, all equipped with one-axis, self-shielded gradients.

VASP-EVH1 has been assigned previously [1] and the assignment was used for the interaction studies. For the two other proteins, EnaH-EVH1 and EVL-EVH1, an assignment of the backbone was necessary, which was performed using standard 3D and 4D triple resonance experiment [19, 20]. The following parameters were used for these experiments: EnaH-EVH1: 2D-HSQC: 4 scans, data size 512(1H) × 256(15N) complex points, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 85.0 ms; all 3D and 4D were recorded as BEST-version [21, 22]; 3D-HN(CA)CO: 16 scans, data size 512(1H) × 64(13C) complex points, constant time delay (13C) = 27 ms, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 15.9 ms, t_{C\text{max}} = 6.4 ms; constant time 3D-HNCA and 3D-HN(CO)CA: 8 scans, data size 512(1H) × 48(15N) × 64(13C) complex points, constant time delay (13C) = 27 ms, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 15.9 ms, t_{C\text{max}} = 12.8 ms; 3D-HN(CA)CO: 32 scans, data size 512(1H) × 48(15N) × 64(13C) complex points, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 15.9 ms, t_{C\text{max}} = 25.6 ms; 3D-HNCO: 8 scans, data size 512(1H) × 48(15N) × 64(13C) complex points, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 15.9 ms, t_{C\text{max}} = 25.6 ms. 4D-HNCOCA and 4D-HN(CA)COCA: 8 scans, 512(1H) × 24(15N) × 24(13C) × 23(13C) complex points and 25% non-uniform sampling, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 8.0 ms, t_{C_{\text{max}}\text{a}} = 4.8 ms, t_{CO\text{max}} = 9.1 ms. EVL-EVH1: 2D-HSQC: 4 scans, data size 512(1H) × 256(15N) complex points, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 85.0 ms; all 3D and 4D were recorded as BEST-version [21, 22]; 3D-HN(CA)CO: 16 scans, data size 512(1H) × 64(15N) × 64(13C) complex points, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 21.2 ms, t_{C_{\text{max}}\text{a}} = 6.4 ms; constant time 3D-HNCA: 16 scans, data size 512(1H) × 48(15N) × 64(13C) complex points, constant time delay (13C) = 27 ms, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 15.9 ms, t_{C_{\text{max}}\text{a}} = 12.8 ms; 4D-HNCO: 40 scans, 512(1H) × 24(15N) × 24(13C) × 23(13C) complex points and 25% non-uniform sampling, t_{H\text{max}} = 51.2 ms, t_{N\text{max}} = 8.0 ms, t_{C_{\text{max}}\text{a}} = 4.8 ms, t_{CO\text{max}} = 9.1 ms; 4D-HNCOCA: 16 scans, 512(1H) × 26(15N) × 24(13C) × 21(13C) complex points and 25% non-uniform sam-
pling, $t_{H_{\text{max}}} = 51.2$ ms, $t_{N_{\text{max}}} = 8.6$ ms, $t_{C_{\alpha_{\text{max}}}} = 4.8$ ms, $t_{CO_{\text{max}}} = 8.4$ ms.

$^1\text{H}-^{15}\text{N}$-HSQC experiments [23] for the interaction studies between the proteins and ligands were done with 8 to 16 scans, data size $512(1^H) \times 64(1^{15}N)$ complex points for all used proteins, except for Fyn-SH3 where $512(1^H) \times 128(1^{15}N)$ complex points were used. Ligand concentrations for the Ena/VASP-EVH1 titrations were calculated to yield 25%, 50%, 75% and $\geq 95\%$ protein–ligand complex, using the already determined $K_d$-values.

All spectra were processed using topspin 3.1 (Bruker Biospin). The processed data were first converted to UCSF-format [24] and subsequently transferred to CCPN [25] for assignment or Sparky [24] for interaction experiments.

### 1.11 Molecular graphics

Molecular graphics were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) [26].
2 Supplementary Equations

2.1 Regression model for Fluorescence Titration

We assume a one-to-one binding of ligand L to protein P:

\[ P + L \rightleftharpoons PL \]  \hspace{1cm} (2)

The total concentrations \( L_t \) and \( P_t \) of ligand and protein do not change during the reaction, therefore:

\[ P_t = P + PL \]
\[ L_t = L + PL \]  \hspace{1cm} (3)

The corresponding dissociation constant is defined as:

\[ K_d = \frac{[P][L]}{[PL]} \]  \hspace{1cm} (4)

The equilibrium concentration \([PL]\) of the protein–ligand complex can be calculated by insertion of (3) in (4), rearranging to obtain a quadratic equation, solving this quadratic equation, in which the physical meaningful solution is:

\[ [PL] = \frac{1}{2} \left( P_t + L_t + K_d - \sqrt{(P_t + L_t + K_d)^2 - 4P_t L_t} \right) \]  \hspace{1cm} (5)

While adding aliquots of ligand solution to the analyte, we increase the initial volume \( V_0 \) in the cuvette, and therefore dilute protein and ligand. The sum of all \( n \) added aliquots with the volume \( dV \) is the cumulative volume \( \Delta V = \sum_{i=0}^{n} dV_i \). The actual volume in the cuvette, after the \( n \)th aliquot is added, is therefore \( V_n = V_0 + \Delta V \).

We can assure, that the stirrer in the cuvette is able to mix the entire volume, homogeneously.
Hence the actual total concentration of protein and ligand after injection of the \( n \)th aliquot is:

\[
P_t = \frac{P^o}{V_0 + \Delta V} \]

\[
L_t = \frac{L^o}{V_0 + \Delta V} \tag{6}
\]

Here, \( P^o \) is the initial protein concentration in the cuvette, and \( L^o \) is the concentration of the added ligand solution.

The measured fluorescence intensity is the sum of four portions: the intensities for the free protein \( F_P \), free ligand \( F_L \), the protein–ligand complex \( F_{[PL]} \), and a fixed intensity for the background \( F_B \):

\[
F = F_P + F_L + F_{[PL]} + F_B \tag{7}
\]

We assume a simple linear relationship between the intensities \( F_P \), \( F_L \) and \( F_{[PL]} \) and their respective concentrations:

\[
F_P = \alpha P \\
F_L = \beta L \\
F_{[PL]} = \gamma [PL] \tag{8}
\]

Inserting (8) and (3) in (7) and rearranging yields:

\[
F - F_B = \alpha P_t + \beta L_t + \gamma [PL] (1 + \Delta v) \tag{9}
\]

Replacing \( P_t \) and \( L_t \) with (6), and abbreviate \( \Delta v = \Delta V/V_0 \), yields after rearranging:

\[
(F - F_B) (1 + \Delta v) = \alpha P^o_t + \beta L^o_t \Delta v + \delta [PL] (1 + \Delta v) \tag{10}
\]

The initial fluorescence intensity at \( \Delta v = 0 \) is \( F_0 = \alpha P^o_t \). Additionally we write \( m = \beta L^o_t \), which
represents the background intensity caused by the ligand. With that, we can write (10) as:

\[
\Delta F = (F - F_B) (1 + \Delta \nu) - F_0 - m \Delta \nu = \delta [PL] (1 + \Delta \nu)
\]

(11)

For the left side of (11) we use the abbreviation \(\Delta F\). With (6), (5), \(\bar{\delta} = \delta / 2\), we can write (11) then as:

\[
\Delta F = \bar{\delta} \left( P^o_t + L^o_t \Delta \nu + K_d (1 + \Delta \nu) - \sqrt{(P^o_t + L^o_t \Delta \nu + K_d (1 + \Delta \nu))^2 - 4P^o_t L^o_t \Delta \nu} \right)
\]

(12)

In regression model (12), \(\bar{\delta}\) is a conditionally linear parameter [27].

The ligand alone may contribute to the measured intensity. We determine this contribution by titrating the ligand into puffer solution, with the same ligand concentration, as for the titration experiment. With the same arguments as before, we can write for this experimental setup:

\[
(F - F_B) (1 + \Delta \nu) = \beta L^o_t \Delta \nu = m \Delta \nu
\]

(13)

This is a simple linear relationship, with \(m\) as the slope.

**Titration with unknown ligand concentration** Although we took great care in weighting our ligands, the correct concentration of the ligand is in question, as an unknown amount of trifluoroacetic acid, water and other components are part of the lyophilisate. Therefore we adapted the data analysis for isothermal titration calorimetry data for the FT.

In ITC data analysis, we can exclude the parameter for the concentration of the protein or the ligand by including this parameter to the list of fitted parameters [28]. In the most cases, this is done for the protein. In our case, we want to do this for the ligand concentration.

The condition needed for fitting the ligand concentration is described as the so called \(c\) value
The $c$ value has to be larger than one, depending on the quality of the data set. In our experience the $c$ value for the FT should be between one and 50, with an optimal value between 5 and 10. Then we can replace the parameter $L_t^o$ in equation (12) with:

$$L_t^o = M \times L_{t,nominal}^o$$ (15)

Here, $L_{t,nominal}^o$ is the nominal ligand concentration and $M$ is a correction factor; $M$ is part of the fitted parameters. For the influence of $M$ in the regression see Supplementary Figure 21.

### 2.2 Regression model for continuous variation method

For the CVM we keep the volume of each sample constant, and only the fraction of protein and ligand solution is changed. We describe that using the volume fraction $\phi_L$ of the ligand solution:

$$\phi_L = \frac{V_L}{V_L + V_P}$$ (16)

Here $V_L$ is the volume of the ligand solution, and $V_P$ is the volume of the protein solution. According to that we can substitute the total ligand and protein concentration with:

$$L_t = L_t^o \phi_L$$

$$P_t = P_t^o (1 - \phi_L)$$ (17)

Here, $P_t^o$ and $L_t^o$ are the concentration of the added protein or ligand solution.

Just as for the argumentation for the FT, we assume a one-to-one reaction, and the overall measured fluorescence intensity $F$ is a sum of four portion (see eq. (7)). Insertion of (8), (3) and
(17) in (7) yields for $F$:

$$F = \phi L (\beta L - \alpha P) + \alpha P + F_B + \delta [PL]$$  \hspace{1cm} (18)

The sum $F_{100\%P} = \alpha P + F_B$ is the intensity of the solution with the maximal (100 %) protein concentration. With $F_{100\%L} = \beta L + F_B$, – the intensity of the solution with the maximal (100 %) ligand concentration – the difference $\beta L - \alpha P = F_{100\%L} - F_{100\%P}$ is the slope of the background signal. Therefore we can write for $F$:

$$F = \phi L (F_{100\%L} - F_{100\%P}) + F_{100\%P} + \delta [PL]$$  \hspace{1cm} (19)

$F_{\text{lin}}$ is the background signal. We can calculate $F_{\text{lin}}$ simply by using the intensity at $\phi_L = 0$ (100 % protein, 0 % ligand) and at $\phi_L = 1$ (0 % protein, 100 % ligand) of the CVM titration data set. Subtracting then $F_{\text{lin}}$ from $F$ and insertion of (5) and (17), and using $\delta = \delta / 2$, yields the remaining intensity $\Delta F$:

$$\Delta F = F - F_{\text{lin}} = \bar{\delta} \left( L_{\phi_L} (1 - \phi_L) + K_d ight)$$

$$= \bar{\delta} \left( L_{\phi_L} (1 - \phi_L) + K_d \right) = \sqrt{(L_{\phi_L} (1 - \phi_L) + K_d)^2 - 4P_{\phi_L} L_{\phi_L} (1 - \phi_L)}$$  \hspace{1cm} (20)

In regression model (20), $\bar{\delta}$ is a conditionally linear parameter [27].

**Standardization** We standardized the intensity $F(\phi_L)$ of each CVM data set with:

$$F_{\text{std}} = 100 \left( \frac{F(\phi_L) - F(\phi_L = 1)}{F(\phi_L = 0) - F(\phi_L = 1)} \right)$$  \hspace{1cm} (21)

The intensities $F(\phi_L = 1)$ and $F(\phi_L = 0)$ are the measured intensities at $\phi_L = 1$ and 0, respectively. With the standardized intensity $F_{\text{std}}$ we can easily calculate the standardized reduced
intensity $\Delta F_{\text{std}}$:

$$\Delta F_{\text{std}} = F_{\text{std}} - 100 \left(1 - \phi_L\right) \quad (22)$$

We used then $\Delta F_{\text{std}}$ as $\Delta F$ in equation (20).

**Adsorption of protein**  The concentration of the protein has to be varied over a large range (from 10 µM to 1 µM in this work). Therefore distortion of the linearity due to adsorption of protein to plastic material may play an important role. We describe this as:

$$P_t = P_t^o \left(1 - \phi_L\right) - P_{\text{ads}} \quad (23)$$

Here $P_{\text{ads}}$ is the amount of adsorbed protein. As $P_{\text{ads}}$ will itself depend on $\phi_L$ and an unknown association constant of the protein to the adsorber, we simplify by assuming that the unknown association constant will tend to infinity. Then $P_{\text{ads}}$ will be constant for $P_{\text{ads}} > P_t$, and for $P_{\text{ads}} \leq P_t$ all protein will be adsorbed. We can write this as:

$$P_t = \begin{cases} 
P_t^o \left(1 - \phi_L\right) - P_{\text{ads}} & \text{for } \phi_L < \left(1 - \frac{P_{\text{ads}}}{P_t^o}\right) \\
0 & \text{for } \phi_L \geq \left(1 - \frac{P_{\text{ads}}}{P_t^o}\right)
\end{cases} \quad (24)$$

For the influence of $P_{\text{ads}}$ on the regression see also Supplementary Figure 22.

### 2.3 Regression models for isothermal titration calorimetry

#### 2.3.1 One-to-one reaction

Just as for the fluorescence titrations, we assume a simple one-to-one binding of the ligand to the protein. Then, (2), (3), (4) and (5) also apply to the ITC data analysis.
According to the ITC-Manual the dilution of protein and ligand can be described as [30]:

\[
P_t = P^0_t \left( \frac{1 - \Delta V}{V_0} \right) \left( 1 + \frac{\Delta V}{2V_0} \right) \\
L_t = L^0_t \left( \frac{\Delta V}{V_0} \right) \left( 1 - \frac{\Delta V}{2V_0} \right)
\]  

(25)

Here \( P^0_t \) is the initial protein concentration in the ITC–cell, \( L^0_t \) is the ligand concentration in the syringe, \( V_0 \) is the volume of the ITC–cell, and \( \Delta V \) is the cumulative titration volume, as it has been used for the FT.

The change in the heat content \( \Delta Q_i \) of the \( i \)th injection can be described as in [30], with an additional term reflecting a constant heat of dilution \( Q_{dil} \) for every injection:

\[
\Delta Q_i = Q_i \left( 1 + \frac{dV_i}{2V_0} \right) - Q_{i-1} \left( 1 - \frac{dV_i}{2V_0} \right) + Q_{dil} dV_i
\]  

(26)

Here \( Q_i \) and \( Q_{i-1} \) are the total heat contents after the \( i \)th and \((i - 1)\)th injection, and \( dV_i \) is the volume of the \( i \)th injection. The total heat content can be described as \( Q_i = V_0 \Delta H_{[PL]} [PL]_i \), with \( \Delta H_{[PL]} \) the enthalpy of the reaction, and \([PL]_i \) the concentration of the protein–ligand complex after the \( i \)th injection. \([PL]_i \) can be described with equations (5) and (25). With that, and \( \bar{Q}_{dil} = Q_{dil} / \Delta H_{[PL]} \), we can describe the change in the heat content after the \( i \)th injection as:

\[
\Delta Q_i = \Delta H_{[PL]} \left( [PL]_i \left( V_0 + \frac{dV_i}{2} \right) - [PL]_{i-1} \left( V_0 - \frac{dV_i}{2} \right) \right) + \bar{Q}_{dil} dV_i
\]  

(27)

In this regression model, described with equation (27), \( \Delta H_{[PL]} \) is a conditionally linear parameter [27].

**Titration with unknown ligand concentration** For better regression results of ITC data, it is possible and mostly necessary to include an parameter for the adjustment of the protein or ligand concentration for the regression. Just as for the FT, we choose to set this parameter on the ligand concentration, simply because of the fact that the protein concentration is determined easily and
reliably via UV/Vis spectroscopy. Therefore we replaced $L_i^\circ$ with:

$$L_i^\circ = M \times L_{i,\text{nominal}}$$  \hspace{1cm} (28)

Here $L_{i,\text{nominal}}$ is the nominal ligand concentration in the syringe and $M$ is a correction factor; $M$ is part of the fitted parameters.

**Regression with a set of global and local parameters** Regression of replicated ITC-titration data sets could increase its precision for the important parameter $K_d$, if some of the fitted parameters were held local for each data set, namely the correction factor $M$ or the heat of dilution $Q_{\text{dil}}$. The reason for this could be, that some of the experiments could not to be done consecutively, but rather were done with different ligand batches *et cetera*. See Supplementary Figure 24 for an example.

### 2.3.2 Displacement

A weak protein–ligand interaction is difficult to observe directly via ITC. Therefore a competition assay is used where a strong binding ligand $A$ is titrated to the protein $P$ in complex with a weak binding ligand $B$. We have then two coupled reaction in the vessel:

$$P + A \rightleftharpoons PA$$  \hspace{1cm} (29)

$$P + B \rightleftharpoons PB$$

The total concentrations for the protein $P_t$ and both ligands $A_t$ and $B_t$ are constant, during the reaction:

$$P_t = P + PA + PB$$

$$A_t = A + PA$$  \hspace{1cm} (30)

$$B_t = B + PB$$
The dissociation constants of both reaction are defined as:

\[ K_{d, PA} = \frac{[P][A]}{[PA]} \]
\[ K_{d, PB} = \frac{[P][B]}{[PB]} \]  

(31)

According to Wang [12] the concentration of both protein–ligand complexes can be calculated with:

\[ [PA] = \frac{A_t \times d}{K_{d, PA} + d} \]
\[ [PB] = \frac{B_t \times d}{K_{d, PB} + d} \]  

(32)

The additional equations are:

\[ a = K_{d, PA} + K_{d, PB} + A_t + B_t - P_t \]
\[ b = K_{d, PB}(A_t - P_t) + K_{d, PA}(B_t - P_t) + K_{d, PA}K_{d, PB} \]
\[ c = -K_{d, PA}K_{d, PB}P_t \]
\[ \theta = \frac{1}{3} \arccos \left( \frac{-2a^3 + 9ab - 27c}{2\sqrt{(a^2 - 3b)^3}} \right) \]
\[ d = \frac{2}{3} \cos(\theta) \sqrt{a^2 - 3b - a} \]  

(33)

According to the ITC-Manual [30] we can describe the dilution of protein and both ligands as:

\[ P_t = P_t^0 \left( \frac{1 - \frac{\Delta V}{2V_0}}{1 + \frac{\Delta V}{2V_0}} \right) \]
\[ B_t = B_t^0 \left( \frac{1 - \frac{\Delta V}{2V_0}}{1 + \frac{\Delta V}{2V_0}} \right) \]
\[ A_t = A_t^0 \left( \frac{\Delta V}{V_0} \right) \left( 1 - \frac{\Delta V}{2V_0} \right) \]  

(34)

Here \( P_t^0 \) and \( B_t^0 \) are the initial concentration of the protein and the weak binding ligand in the
ITC-cell, and $A_i^\circ$ is the concentration of the strong binding ligand in the syringe.

Due to the fact that the total heat content $Q$ is additive, the change in the heat content of the reaction vessel after the $i$th injection can be described for the displacement as in equation (26) with $Q_i = Q_{PA,i} + Q_{PB,i}$:

$$
\Delta Q_i = \left( Q_{PA,i} + Q_{PB,i} \right) \left( 1 + \frac{dV_i}{2V_0} \right) - \left( Q_{PA,i-1} + Q_{PB,i-1} \right) \left( 1 - \frac{dV_i}{2V_0} \right) + Q_{dil} dV_i \tag{35}
$$

The total heat content of each separate reaction can be described as $Q_i = V_0 \Delta H_{[PL]} [PL]_i$, therefore the change in the total heat content after the $i$th injection is:

$$
\Delta Q_i = (\Delta H_{PA} \times [PA]_i + \Delta H_{PB} \times [PB]_i) \left( V_0 + \frac{dV_i}{2} \right) - (\Delta H_{PA} \times [PA]_{i-1} - \Delta H_{PB} \times [PB]_{i-1}) \left( V_0 - \frac{dV_i}{2} \right) + Q_{dil} dV_i \tag{36}
$$

**Titration with unknown ligand concentration**  
Like for the one-to-one model, we used a correction factor for the titrated ligand A. Therefore we replaced $A_i^\circ$ with:

$$
A_i^\circ = M \times A_i^\circ_{\text{nominal}} \tag{37}
$$

Here $A_i^\circ_{\text{nominal}}$ is the nominal ligand concentration in the syringe and $M$ is a correction factor; $M$ is part of the fitted parameters.

**2.4 Calculation of the free energy, the entropic energy and their uncertainties**

The free energy was calculated with:

$$
\Delta G^\circ = RT \ln \left( \frac{K_d}{1M} \right) \tag{38}
$$

38
According to the propagation of errors [31] the uncertainty $u_{\Delta G^o}$ of $\Delta G^o$ can then be calculated as:

$$u_{\Delta G^o} = \frac{RT}{K_d} u_{K_d}$$  \hspace{1cm} (39)

Here $u_{K_d}$ is the uncertainty of the dissociation constant, as computed via the variance–covariance matrix; $R$ is the gas constant and $T$ the absolute temperature.

The change in entropic energy $-T \Delta S$ can be calculated via the definition of the free energy, using the known dissociation constant $K_d$, the change in enthalpy $\Delta H$ and the absolute temperature $T$:

$$-T \Delta S = \Delta G^o - \Delta H = RT \ln \left( \frac{K_d}{1 \text{ M}} \right) - \Delta H$$  \hspace{1cm} (40)

The uncertainty $u_{-T \Delta S}$ of the change in entropic energy can be calculated via the propagation of error [31]:

$$u_{-T \Delta S} = \sqrt{ \left( \frac{RT}{K_d} u_{K_d} \right)^2 + u_{\Delta H}^2 - 2 \frac{RT}{K_d} u_{K_d, \Delta H} }$$  \hspace{1cm} (41)

Here $u_{\Delta H}$ is the variance of the change in enthalpy, $u_{K_d}$ is the uncertainty of the dissociation constant, and $u_{K_d, \Delta H}$ is the covariance of the dissociation constant and the change in enthalpy. All three were computed via the variance–covariance matrix.

### 2.5 Measure of goodness-of-fit

In case of available replica, the goodness-of-fit was measured using a $\hat{\chi}^2$-statistic:

$$\hat{\chi}^2 = \sum_{i=1}^{\bar{n}} \left( \frac{\bar{y}_i - f_i}{s_i} \right)^2 \approx \sum_{i=1}^{\bar{n}} \left( \frac{\bar{y}_i - f_i}{s_i} \right)^2$$  \hspace{1cm} (42)

Here, $\bar{y}_i$ is the mean of the replica of the $i$th data point, with its standard deviation $s_i$, $\bar{n}$ is the number of averaged data points, and $f_i$ is the theoretical value of the model function.

A known problem for the $\hat{\chi}^2$-statistic mentioned above is the number necessary to compute $s_i$: at least six replica are recommended, otherwise the calculated value for $s_i$ could be biased. In this study, the data sets consist of at least three to four replica. Therefore we used the more
robust lack-of-fit as an additional measure for the fit quality:

$$\hat{F} = \frac{1}{n-p} \sum_{i=1}^{n} n_i (\bar{y}_i - f_i)^2$$

$$= \frac{N-p}{N-n} \sum_{i=1}^{n} \sum_{j=1}^{n_i} (y_{ij} - \bar{y}_i)^2$$

(43)

Additionally to the above-mentioned parameters, $p$ is the number of parameters fitted, $n_i$ is the number of replica per averaged data point, $y_{ij}$ are the single values of each averaged data point, and $N$ is the overall number of all (unaveraged) data points.
3 Supplementary Figures

Supplementary Figure 11: Screening results for replacement of the important aromatic side chain of the consensus motif [FYWL]PφP via peptide SPOT array.

WT: SFEFPPPPTEDEL–[β-Ala]2–[membrane],
P1: SFE–Z–PPPPTEDEL–[β-Ala]2–[membrane],
P2: Z–PPPPTEDELIMR–[β-Ala]2–[membrane].

Shortening the WT peptide by the three amino acids SFE reduce the affinity to VASP-EVH1 [1]. Additionally: The free N-terminus of P2 reduces the affinity further (see Supplementary Table 6). Therefore, positive spots for sequence P2 showing only amino acids with a very strong improvement in affinity to VASP-EVH1. Here we found that L-2-chlorophenylalanine and L-1-naphtylalanine were the best candidates.
Supplementary Figure 12: Overall fold of EnaH-EVH1 bound to compound 4a.
Supplementary Figure 13: Overlay of complexes of EnaH-EVH1–4a with 1evh. Overlay of the complexes of EnaH-EVH1–4a (EnaH-EVH1: Chain B, orange; 4a: beige) with 1evh (EnaH-EVH1: blue; Ac-FPPPPT-NH$_2$: light-blue) of Prehoda et al. [16]. Atom-wise rigid-body match of atoms involved in H-bonding of ligand, shaping the binding groove, and atoms of the aromatic triad. Overall rmsd of 0.3513. Right image: semi-transparent surface showing the binding groove formed by strands 1, 5, 6, and 7.
Supplementary Figure 14: Comparison of ligand mediated crystal contacts in EnaH-EVH1 with compound 4a and Ac-FPPPPT-NH$_2$ of (1evh). Ca-$\alpha$-chain trace of complexes of EnaH-EVH1–4a (EnaH-EVH1: Chain B, orange; 4a: beige) and 1evh (EnaH-EVH1: blue; Peptide: light-blue) of Prehoda et al. [16]. An atom-wise rigid-body match of atoms forming the binding groove reveals the major difference in the crystal packing in a loop that connects strands 1 and 2. In the presented structure D18 of the neigboring loop (yellow) is involved in strong salt bridges that draw D18 and the whole loop closer to R81. Such a direct interaction causes R81 to adapt a side-on geometry towards the whole crystal contact, involving D18 and the backbone-carbonyl of D82, while maintaining one N$^\alpha$ to interact with the ligand’s acetyl-moiety.
Supplementary Figure 15: Orientation of the two ligands in the asymmetric unit. Bound compound 4a with 2Fo-Fc (1σ, blue) and mFo-DFc (3σ, green) electron density maps. The arrangement of EnaH-EVH1 in the asymmetric unit lead to a tightly packed “head-to-head” orientation and might explain all failed attempts to crystallize complexes with N-terminally elongated ligands.

Supplementary Figure 16: Well defined binding groove and the aromatic triad. 2Fo-Fc (1σ, blue) and mFo-DFc (3σ, green) electron density maps of the underlaying epitope that is mainly shaped by well defined aromatic triad composed of Y16, W23, and F77 of the binding groove of EnaH-EVH1. View from the N-terminus (left) and C-terminus (right, without the densities of the water molecules) of the ligand.
Supplementary Figure 17: Results for the HSQC interaction experiments for ε-NH W23 of VASP-EVH1 and the ActA peptide and other ligands:

- free protein
- PPPP Ac-SFEE-PPPP-TEDEL-NH₂
- PP[ProM-1] Ac-SFEE-PP[ProM-1]-TEDEL-NH₂
- [ProM-1]PP Ac-SFEE-[ProM-1]PP-TEDEL-NH₂
- [ProM-1][ProM-1] Ac-SFEE-[ProM-1][ProM-1]-TEDEL-NH₂
- [ProM-2][ProM-1] Ac-SFEE-[ProM-2][ProM-1]-TEDEL-NH₂

Tryptophan W23 of VASP-EVH1 is directly involved in binding the proline-rich ligands; one of the two hydrogen bonds of the poly-proline core is between the carbonyl oxygen of the peptide to ε-NH of W23. This results in a strong shift, when the ligand is titrated to the protein. Compare also with Supplementary Table 3.
Supplementary Figure 18: Results for the HSQC interaction experiments for ε-NH W23 of VASP-EVH1 and the ActA peptide and other ligands:

- free protein
- FPPPP
- [2-Cl-F][ProM-2][ProM-1]
- Ac-SFE-[2-Cl-F][ProM-1][ProM-1]-TEDEL-NH₂
- Ac-SFE-[2-Cl-F][ProM-2][ProM-1]-TEDEL-NH₂

The observed different behavior for ProM-1 and ProM-2 in Supplementary Figure 17 is unaltered, when the important aromatic residue is changed to 2-Cl-F. But it seems, that for [2-Cl-F][ProM-2][ProM-1] the chemical shift is increased, when compared with the FPPPP-peptide. We found a slow exchange regime for the chemical shift of ε-NH W23 for all tested ligands with 2-Cl-F.
Supplementary Figure 19: Chemical shifts of $^1$H-$^{15}$N-HSQC interaction experiments of $\epsilon$-NH W23 of VASP-, EnaH- and $\epsilon$-NH W25 of EVL-EVH1 and 4a. Red: no ligand; Green: plus ActA peptide; Blue: plus 4a. The complex of compound 4a or the ActA peptide with all three Ena/VASP EVH1 domains shows the same up-field shift for $\epsilon$-NH of W23 (VASP-EVH1), W23 (EnaH-EVH1) and W25 (EVL-EVH1).
Supplementary Figure 20: Plotted chemical shifts of $^1$H-$^{15}$N-HSQC titration experiments of VASP-, EnaH- and EVL-EVH1 with compound 4a. For VASP- and EVL-EVH1 structures 1eqx [1] and 1q6 [32] were used.
Supplementary Figure 21: FT of EnaH-EVH1 with compound 4a. Effect of the parameter $M$ – the correction factor of the ligand concentration – on the results of the data fit (left side: data fit without $M$; right side: data fit with $M$).
Supplementary Figure 22: CVM of VASP-EVH1 with Ac-SFEPFP[ProM-1]TEDEL-NH₂. 10 µM VASP-EVH1 was titrated with 50 µM ligand. Effect of the parameter $P_{\text{ads}}$ – the correction factor for protein adsorption – on the results of the data fit (left: data fit without $P_{\text{ads}}$; right: data fit with $P_{\text{ads}}$).
Supplementary Figure 23: ITC analysis for the titration of VASP-EVH1 with 4a. ITC analysis of only one data set is shown, for reasons of clarity. In practice all replicas were analyzed simultaneously. Top: thermograms for titration of ligand to protein (left) and ligand to buffer (right) after baseline subtraction. As the buffer contained DTT, a strong signal for the background titration was measured. Middle: Plot of the fitted data (circle, red line), the background signal (triangle up, green line) and the titration data without subtraction of background (triangle down, blue line). Outliers were detected automatically and excluded from the fitted data (here: crossed circle). The ligand concentration was used as a adjustable parameter in the data fit, as the protein concentration was easier to determine. Here only half of the nominal ligand concentration was found to be active. We can exclude a second binding site of VASP-EVH1 for 4a by $^{15}$N-HSQC-NMR spectra and X-ray crystallography. Bottom: Standardized residuals of the fitted data plotted against the molar ratio.
Supplementary Figure 24: Data fit with global and local fitted parameters for the ITC of EVL-EVH1 with compound 2. For the regression the parameter $K_d$ and $\Delta H$ were held global for all data sets, while for each data set a separate $M$ and $Q_{dil}$ were fitted.
Supplementary Figure 25: $^1$H-$^{15}$N-HSQC spectra for the titration of Fyn-SH3 with 4a. 100 µM protein were titrated with 0 µM (blue), 200 µM (magenta), 500 µM (green), and 1000 µM (red) of ligand 4a. Shifts for the backbone NH of Thr97 and Arg98 – part of the RT-loop – are visible.
Supplementary Figure 26: $^1$H-$^{15}$N-HSQC spectra for the titration of YAP1-WW with 4a. 100 µM protein were titrated with 0 µM (blue), 200 µM (magenta), 500 µM (green), and 1000 µM (red) of ligand 4a. The Peaks for T17 NH, L25 NH and T33 NH appear with increasing ligand concentration.
Supplementary Figure 27: $^1$H-$^{15}$N-HSQC spectra for the titration of Homer1-EVH1 with 4a. 100 µM protein were titrated with 0 µM (blue), 250 µM (magenta), 500 µM (green), and 1000 µM (red) of ligand 4a.
Supplementary Figure 28: $^1$H-$^{15}$N-HSQC spectra for the titration of CD2BP2-GYF with 4a. 100 µM protein were titrated with 0 µM (blue), 200 µM (magenta), 500 µM (green), and 1000 µM (red) of ligand 4a.
Supplementary Figure 29: Cell permeability of compounds. NBD-fluorescence is shown in green an trypan blue stain (red) shows cell edges. Upper row: HCT 116 cells; lower row: MDA MB 231 cells. Untreated control cells are shown left, cells incubated with NBD-4a in the middle and cells incubated with NBD-4b on the right. Compound NBD-4b is the only compound that showed high NBD-fluorescence in the cells. The white bar represents 50 µm.
Supplementary Figure 30: 4b is intracellular de-esterified to 4a. Top: HPLC chromatographs of comp. NBD-4a (red) and NBD-4b (black). Both compounds differ in their retention times and are thus to distinguish. Bottom: HPLC analysis of NBD-4b-treated HCT 116 cells. HCT 116 shows an endogenous de-esterification of compound NBD-4b in a time-dependent manner.
Supplementary Figure 31: Viability assay. Compound 4b was incubated on HCT 116 or MDA MB 231 cells for 24 h. Fluorescence of living-cell-mediated, reduced alamarblue (Invitrogen) was measured indicating that 4b has no toxic effect on HCT 116 nor on MDA MB 231 cells within 24 h up to a concentration of 150 µM.
**Supplementary Figure 32: Displacement of Zyxin (from HCT 116) from EnaH- and VASP-EVH1.** Pull down experiments with lysate from HCT 116 and GST-tagged EnaH-EVH1 (upper panel) and VASP-EVH1 (lower panel) immobilized on glutathione rephrase beads. Compound 2 and 4a displace zyxin in a concentration-dependent manner.
## 4 Supplementary Tables

<table>
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<th>Primer</th>
<th>Sequence</th>
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**Supplementary Table 1: Used primers in this study.**
Ac–SFE–[7]-PPPTEDEL–NH₂

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Supplementary Table 2: Results of binding studies for VASP-EVH1 and ligands with replacement of the important aromatic residue of the consensus motif (W/F) PxΦPx. The best replacements found with the peptide spot array – L-2-chlorophenylalanine (2-Cl-Phe) and L-1-naphtylalanine (1-Nal); see also Supplementary Figure 11 – reduced the dissociation constant up to a factor of 13. Additionally to its lower increase in affinity, the 1-Nal containing ligand – but not the 2-Cl-Phe containing ligand – showed in ITC experiments a very slow kinetic, probably due to a slow cis–trans isomerization rate of the (1-Nal)–Pro pair [33]. We investigated other ortho substituted phenylalanine derivates (R = CF₃, CH₃, F), which yielded the subsequent order in affinity: Cl ≈ CF₃ < CH₃ < F < H. The attempt to increase the affinity further by using L-2,6-di-chlorophenylalanine failed tremendously: the K_d dropped to 1 mM. As a result, out of all substitutions tested, 2-Cl-Phe was the most affine and simplest derivate of all tested compounds. For the FT of the substituted phenylalanines the mutant C7S/C64S-VASP-EVH1 was used. This mutant has the same fold, and almost the same thermal stability, as the WT protein, as confirmed via CD spectroscopy. Furthermore, the mutant bound to the 2-Cl-Phe containing ligand with the same affinity, as the WT protein.
Supplementary Table 3: Results of the binding studies for VASP-EVH1 and ligands with replaced Pro–Pro pairs by ProM-1 and ProM-2. The scaffold ProM-1 was able to replace all four prolines without loss, but also without gain in affinity. However, replacing only the last two prolines with ProM-1 increased the affinity. This also altered the observed thermodynamic binding profile: the binding is more exothermic and less entropically favored, as ligands without ProM-1 replacing the last two prolines. This indicates the contribution of the vinylidene-bridge to the binding. Molecular modeling revealed that the vinylidene-bridge of ProM-1 and the aromatic ring of Tyr16 of VASP-EVH1 form a steric clash, when ProM-1 substituted the first two prolines. Because of that, we designed ProM-2 from ProM-1, which avoids these clashes. This is reflected by the observed dissociation constants.

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</table>
Supplementary Table 4: Results of the binding studies for VASP-EVH1 and ligands with L-
2-chlorophenylalaine (2-Cl-F) and replaced Pro–Pro pairs by ProM-1, ProM-2 and ProM-
3. Introduction of 2-Cl-F reduced the dissociation constant of the ProM-1 and ProM-2 contain-
ing ligand by a factor of more than ten (compare with Supplementary Table 3). Replacing the
last two prolines with ProM-3 did not decrease nor increase the affinity, compared with a Pro–
Pro pair. Therefore ProM-3 could not outcompete ProM-1. With the most of these ligands we
observed a difference in the results for $K_d$ for the FT and the ITC, which is also observable in
Supplementary Table 3. We interpret this results with the difference in the experimental setup
for both methods: for FT we observe any change in the chemical environment around W23 of
VASP-EVH1, whereas for the ITC any reaction with a sufficient change in enthalpy is observ-
able. With 2-Cl-F, ProM-1 and ProM-2 we increase the affinity around W23, while for the ITC
also other binding events contribute to the $K_d$. Therefore is the ITC-$K_d$ greater than the FT-$K_d$. 

<table>
<thead>
<tr>
<th></th>
<th>FPPP</th>
<th>[2-Cl-F]PPP</th>
<th>[2-Cl-F][ProM-1]</th>
<th>[2-Cl-F][ProM-3]</th>
<th>[2-Cl-F][ProM-1][ProM-1]</th>
<th>[2-Cl-F][ProM-2][ProM-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ [µM]</td>
<td>19 (2)</td>
<td>1.5 (0.3)</td>
<td>0.31 (0.05)</td>
<td>1.5 (0.2)</td>
<td>0.31 (0.1)</td>
<td>0.28 (0.08)</td>
</tr>
<tr>
<td>$\Delta G^{\circ}$ [kJ/mol]</td>
<td>–26.9 (0.3)</td>
<td>–33.3 (0.5)</td>
<td>–37.2 (0.4)</td>
<td>–33.2 (0.3)</td>
<td>–37.1 (0.8)</td>
<td>–37.4 (0.7)</td>
</tr>
<tr>
<td>ITC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ [µM]</td>
<td>22 (1)</td>
<td>1.6 (0.5)</td>
<td>0.77 (0.03)</td>
<td>3.2 (0.1)</td>
<td>1.3 (0.1)</td>
<td>0.56 (0.04)</td>
</tr>
<tr>
<td>$\Delta G^{\circ}$ [kJ/mol]</td>
<td>–26.6 (0.2)</td>
<td>–33.1 (0.1)</td>
<td>–34.9 (0.1)</td>
<td>–31.4 (0.1)</td>
<td>–33.6 (0.1)</td>
<td>–35.7 (0.2)</td>
</tr>
<tr>
<td>$\Delta H$ [kJ/mol]</td>
<td>–14 (2)</td>
<td>–19.9 (0.2)</td>
<td>–29 (4)</td>
<td>–29.3 (0.3)</td>
<td>–25 (1)</td>
<td>–27.5 (0.3)</td>
</tr>
<tr>
<td>$-T \Delta S$ [kJ/mol]</td>
<td>–12 (2)</td>
<td>–14.8 (0.3)</td>
<td>–5 (4)</td>
<td>–2.1 (0.4)</td>
<td>–8 (1)</td>
<td>–8.2 (0.5)</td>
</tr>
</tbody>
</table>
Supplementary Table 5: Results of the binding studies for VASP-EVH1 and ligands which are reduced to the core motif. Pro–Pro pairs were replaced with ProM-1, ProM-2 and ProM-4. Replacing the phenylalanine with 2-Cl-F and the prolines with ProM-1 and ProM-2 resulted in a decrease of the dissociation constant by a factor of more than 200. Although scaffold ProM-4 bound slightly better to VASP-EVH1 for the FT studies, the ITC data suggest, that ProM-4 could not outcompete ProM-1. For ligand Ac-[2-Cl-Phe][ProM-2]-NH₂, a competition binding assay was used. Due to uncertainty of the concentration of the small ligand, a lower limit (active ligand concentration is only 10% of the nominal concentration) and a upper limit (active ligand concentration is the same as nominal concentration) is reported.
<table>
<thead>
<tr>
<th></th>
<th>Ac-FPPPTEDEL-NH₂</th>
<th>FPPPTEDEL-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ [µM]</td>
<td>71 (7)</td>
<td>210 (60)</td>
</tr>
<tr>
<td>$\Delta G^\circ$ [kJ/mol]</td>
<td>$-23.7 (0.3)$</td>
<td>$-20.9 (0.7)$</td>
</tr>
<tr>
<td>Ac-[2-Cl-F]PPPPTEDDEL-NH₂</td>
<td>[2-Cl-F]PPPPTEDDEL-NH₂</td>
<td></td>
</tr>
<tr>
<td>FT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ [µM]</td>
<td>2 (1)</td>
<td>9 (1)</td>
</tr>
<tr>
<td>$\Delta G^\circ$ [kJ/mol]</td>
<td>$-33 (1)$</td>
<td>$-28.9 (0.2)$</td>
</tr>
<tr>
<td>ITC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ [µM]</td>
<td>3.6 (0.1)</td>
<td>8 (1)</td>
</tr>
<tr>
<td>$\Delta G^\circ$ [kJ/mol]</td>
<td>$-31.1 (0.1)$</td>
<td>$-29.1 (0.3)$</td>
</tr>
<tr>
<td>$\Delta H$ [kJ/mol]</td>
<td>$-20.8 (0.1)$</td>
<td>$-10.1 (0.3)$</td>
</tr>
<tr>
<td>$-T \Delta S$ [kJ/mol]</td>
<td>$-10.3 (0.2)$</td>
<td>$-19.0 (0.7)$</td>
</tr>
</tbody>
</table>

Supplementary Table 6: Acetylation of the N-terminus is important for binding of N-terminal shortened ligands by VASP-EVH1.
Supplementary Table 7: ITC results for binding of 4a to Fyn-SH3, YAP1-WW, Homer1-EVH1 and CD2BP2-GYF. For Fyn-SH3 and CD2BP2-GYF a competition assay was used (see supplementary table 8 for the used ligands); for YAP1-WW and Homer1-EVH1 a direct titration of ligand 4a was possible.

<table>
<thead>
<tr>
<th></th>
<th>Fyn-SH3</th>
<th>YAP1-WW</th>
<th>Homer1-EVH1</th>
<th>CD2BP2-GYF</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ [µM]</td>
<td>5900 (900)</td>
<td>340 (60)</td>
<td>400 (100)</td>
<td>3200 (600)</td>
</tr>
<tr>
<td>$\Delta G^o$ [kJ/mol]</td>
<td>−12.7 (0.4)</td>
<td>−19.8 (0.5)</td>
<td>−19.2 (0.6)</td>
<td>−14.3 (0.4)</td>
</tr>
<tr>
<td>$\Delta H$ [kJ/mol]</td>
<td>−24 (9)</td>
<td>−11 (2)</td>
<td>−8 (2)</td>
<td>−2 (2)</td>
</tr>
<tr>
<td>$−T \Delta S$ [kJ/mol]</td>
<td>+11 (9)</td>
<td>−9 (2)</td>
<td>−11 (2)</td>
<td>−13 (2)</td>
</tr>
</tbody>
</table>
### Supplementary Table 8: ITC results for used ligands for Fyn-SH3, CD2BP2-GYF, YAP1-WW and Homer1-EVH1.

The peptide ligands for Fyn-SH3 [10] and CD2BP2-GYF [11] were used for the competition assay (see also supplementary table 7).

<table>
<thead>
<tr>
<th></th>
<th>Fyn-SH3 + Ac-VSLARRPLPPLP-NH₂</th>
<th>CD2BP2-GYF + Ac-EFGPPGWLGR-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ [µM]</td>
<td>0.26 (0.02)</td>
<td>6.3 (0.5)</td>
</tr>
<tr>
<td>$\Delta G^\circ$ [kJ/mol]</td>
<td>–37.6 (0.2)</td>
<td>–29.7 (0.2)</td>
</tr>
<tr>
<td>$\Delta H$ [kJ/mol]</td>
<td>–66.1 (0.3)</td>
<td>–40.1 (0.6)</td>
</tr>
<tr>
<td>$-T \Delta S$ [kJ/mol]</td>
<td>+28.5 (0.5)</td>
<td>+10.4 (0.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>YAP1-WW + Ac-LPPPYRHR-NH₂</th>
<th>Homer1-EVH1 + Ac-ALTPSPFRDS-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ [µM]</td>
<td>21 (2)</td>
<td>72 (5)</td>
</tr>
<tr>
<td>$\Delta G^\circ$ [kJ/mol]</td>
<td>–26.8 (0.2)</td>
<td>–23.7 (0.2)</td>
</tr>
<tr>
<td>$\Delta H$ [kJ/mol]</td>
<td>–62.5 (0.9)</td>
<td>–26.1 (0.4)</td>
</tr>
<tr>
<td>$-T \Delta S$ [kJ/mol]</td>
<td>+36 (1)</td>
<td>+2.5 (0.6)</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ [µM]</td>
<td>21 (2)</td>
<td>800 (100)</td>
</tr>
<tr>
<td>$\Delta G^\circ$ [kJ/mol]</td>
<td>–30.57 (0.08)</td>
<td>–17.8 (0.3)</td>
</tr>
<tr>
<td>$\Delta H$ [kJ/mol]</td>
<td>–82.3 (0.6)</td>
<td>–14.4 (0.5)</td>
</tr>
<tr>
<td>$-T \Delta S$ [kJ/mol]</td>
<td>+51.7 (0.6)</td>
<td>–3.4 (0.8)</td>
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</tbody>
</table>
**Diffraction statistics**

<table>
<thead>
<tr>
<th>Crystal System</th>
<th>Orthorhombic</th>
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</thead>
<tbody>
<tr>
<td>Space group</td>
<td>C222</td>
</tr>
<tr>
<td>Cell dimensions [Å, °]</td>
<td>(a = 89.830) (b = 131.420) (c = 35.560), (\alpha = \beta = \gamma = 90)</td>
</tr>
<tr>
<td>Resolution [Å]</td>
<td>35-1.7 (1.74-1.7)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>23659 (1704)</td>
</tr>
<tr>
<td>Completeness [%]</td>
<td>99.8 (99.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4 (4.1)</td>
</tr>
<tr>
<td>(I/\sigma(I))</td>
<td>12.86 (2.35)</td>
</tr>
<tr>
<td>(R_{\text{obs}}) [%]</td>
<td>7.1 (58.9)</td>
</tr>
<tr>
<td>(R_{\text{meas}}) [%]</td>
<td>8.2 (64.6)</td>
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<tr>
<td>ISa</td>
<td>19.92</td>
</tr>
</tbody>
</table>

**Refinement statistics**

| \(V_M\) [Å\(^3\)/Da] | 2.06 |
| Molecules per asymmetric unit | 2 |
| \(R_{\text{fact}}\) [%] | 18.99 |
| \(R_{\text{free}}\) [%] | 23.58 |
| Average B-factor [Å\(^2\)] | 19.120 |
| rmsd\(_{\text{bond-length}}\) [Å] | 0.0128 |
| rmsd\(_{\text{bond-angle}}\) [°] | 1.601 |
| PDB code | 4my6 |

\(a\) Numbers in perenthesis correspond to outer shell  
\(b\) \(R_{\text{meas}}\): redundancy independent R-factor  
\(c\) \(R_{\text{fact}} = \sum |F_0 - F_c|/\sum |F_0|\), where \(F_0\) and \(F_c\) are the observed and calculated structure factors  
\(d\) \(R_{\text{free}}\) set contains 5% of total reflections

**Supplementary Table 9:** X-Ray statistics for the complex of EnaH-EVH1 with compound 4a.
Supplementary Table 10: Statistical analysis of stress fiber (SF) reduction in HCT 116 by 4b. Cells were treated with DMSO or with 4b solved in DMSO; the DMSO concentration was 0.5 % (v/v) for both treatments. The cells were divided into two different phenotypes: with or without stress fibers. Results for all three replicas are shown. Each replica was tested for no difference of both treatments, using Pearson’s $\chi^2$-Test. Results and $p$-values for each replica are shown. The sum of all single $\chi^2$-values is $\chi^2 = 121.5231$. With a degree of freedom of df = 3 a over-all $p$-value of $p = 3.626 \times 10^{-26}$ was calculated.

\[ \chi^2 = 6.5587, \ p = 0.01044 \]

<table>
<thead>
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<th>Phenotypes</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with SF</td>
<td>without SF</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>55</td>
<td>37</td>
<td>92</td>
</tr>
<tr>
<td>50 µM 4b</td>
<td>39</td>
<td>56</td>
<td>95</td>
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<tr>
<td>Total</td>
<td>94</td>
<td>93</td>
<td>187</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 37.969, \ p = 7.188 \times 10^{-10} \]

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<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>with SF</td>
<td>without SF</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>80</td>
<td>48</td>
<td>128</td>
</tr>
<tr>
<td>50 µM 4b</td>
<td>37</td>
<td>108</td>
<td>145</td>
</tr>
<tr>
<td>Total</td>
<td>117</td>
<td>156</td>
<td>273</td>
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</tbody>
</table>

\[ \chi^2 = 76.9954, \ p < 2.2 \times 10^{-16} \]

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<td>without SF</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>111</td>
<td>62</td>
<td>173</td>
</tr>
<tr>
<td>50 µM 4b</td>
<td>38</td>
<td>162</td>
<td>200</td>
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<tr>
<td>Total</td>
<td>149</td>
<td>224</td>
<td>373</td>
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</tbody>
</table>
\[ \chi^2 = 43.7053, \ p = 3.817 \times 10^{-11} \]

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<td>without VASP</td>
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<tr>
<td>DMSO</td>
<td>179</td>
<td>11</td>
<td></td>
<td>190</td>
</tr>
<tr>
<td>50 \mu M 4b</td>
<td>147</td>
<td>69</td>
<td></td>
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<tr>
<td>Total</td>
<td>326</td>
<td>80</td>
<td></td>
<td>406</td>
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\[ \chi^2 = 48.3542, \ p = 3.558 \times 10^{-12} \]

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<td>without VASP</td>
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<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>136</td>
<td>9</td>
<td></td>
<td>145</td>
</tr>
<tr>
<td>50 \mu M 4b</td>
<td>78</td>
<td>55</td>
<td></td>
<td>133</td>
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<tr>
<td>Total</td>
<td>214</td>
<td>64</td>
<td></td>
<td>278</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 52.7209, \ p = 3.845 \times 10^{-13} \]

<table>
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<td></td>
<td>with VASP</td>
<td>without VASP</td>
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<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>163</td>
<td>5</td>
<td></td>
<td>168</td>
</tr>
<tr>
<td>50 \mu M 4b</td>
<td>120</td>
<td>60</td>
<td></td>
<td>180</td>
</tr>
<tr>
<td>Total</td>
<td>283</td>
<td>65</td>
<td></td>
<td>348</td>
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</table>

**Supplementary Table 11: Statistical analysis of VASP-reduction in focal adhesion (FA) in HCT 116 by 4b.** Cells were treated with DMSO or with 4b solved in DMSO; the DMSO concentration was 0.5 % (v/v) for both treatments. The cells were divided into two different phenotypes: FA with or without VASP; FA were detected via zyxin. Results for all three replicas are shown. Each replica was tested for no difference of both treatments, using Pearson’s \( \chi^2 \)-Test. Results and \( p \)-values for each replica are shown. The sum of all single \( \chi^2 \)-values is \( \chi^2 = 144.7804 \). With a degree of freedom of df = 3 a over-all \( p \)-value of \( p = 3.520 \times 10^{-31} \) was calculated.
\[ \chi^2 = 39.5848, \ p = 3.141 \times 10^{-10} \]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FA Phenotypes with VASP</th>
<th>FA Phenotypes without VASP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>95</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td>100 µM 4b</td>
<td>65</td>
<td>37</td>
<td>102</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>38</td>
<td>198</td>
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</tbody>
</table>

\[ \chi^2 = 35.3781, \ p = 2.715 \times 10^{-9} \]

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<th>Treatment</th>
<th>FA Phenotypes with VASP</th>
<th>FA Phenotypes without VASP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>62</td>
<td>1</td>
<td>63</td>
</tr>
<tr>
<td>100 µM 4b</td>
<td>31</td>
<td>28</td>
<td>59</td>
</tr>
<tr>
<td>Total</td>
<td>93</td>
<td>29</td>
<td>122</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 20.685, \ p = 5.414 \times 10^{-6} \]

<table>
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<tr>
<th>Treatment</th>
<th>FA Phenotypes with VASP</th>
<th>FA Phenotypes without VASP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>82</td>
<td>3</td>
<td>85</td>
</tr>
<tr>
<td>100 µM 4b</td>
<td>24</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>15</td>
<td>121</td>
</tr>
</tbody>
</table>

Supplementary Table 12: Statistical analysis of VASP-reduction in focal adhesion (FA) in MDA MB 231 by 4b. Cells were treated with DMSO or with 4b solved in DMSO; the DMSO concentration was 0.5 % (v/v) for both treatments. The cells were divided into two different phenotypes: FA with or without VASP; FA were detected via zyxin. Results for all three replicas are shown. Each replica was tested for no difference of both treatments, using Pearson’s \( \chi^2 \)-Test. Results and \( p \)-values for each replica are shown. The sum of all single \( \chi^2 \)-values is \( \chi^2 = 95.6479 \). With a degree of freedom of df = 3 a over-all \( p \)-value of \( p = 1.34 \times 10^{-20} \) was calculated.
\[
\chi^2 = 4.8431, \ p = 0.08878
\]

<table>
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<th>Phenotypes</th>
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<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
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<td>without Lpd</td>
<td>with Lpd</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>without VASP</td>
<td>with VASP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>5</td>
<td>4</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>100 µM 4b</td>
<td>4</td>
<td>7</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>11</td>
<td>36</td>
<td>56</td>
</tr>
</tbody>
</table>

\[
\chi^2 = 11.583, \ p = 0.003053
\]

<table>
<thead>
<tr>
<th>Treatment</th>
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<td>23</td>
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\[
\chi^2 = 5.9022, \ p = 0.05228
\]

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**Supplementary Table 13: Statistical analysis of VASP-reduction in focal adhesion (FA) in MDA MB 231 by 4b.** Cells were treated with DMSO or with 4b solved in DMSO; the DMSO concentration was 0.5% (v/v) for both treatments. The cells were divided into three different phenotypes: leading edge with or without Lpd, and leading edge with Ldp and with or without VASP. Results for all three replicas are shown. Each replica was tested for no difference of both treatments, using Pearson’s \( \chi^2 \)-Test. Results and \( p \)-values for each replica are shown. The sum of all single \( \chi^2 \)-values is \( \chi^2 = 22.3283 \). With a degree of freedom of \( df = 3 \) a over-all \( p \)-value of \( p = 5.57 \times 10^{-5} \) was calculated.
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