

Vinculin targeting by *Shigella* IpaA promotes stable cell adhesion independent of mechanotransduction

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32 ***Shigella*, the causative agent of bacillary dysentery, invades epithelial cells by injecting type**
33 **III effectors that locally reorganize the actin cytoskeleton¹. The type III effector IpaA targets the**
34 **focal adhesion protein vinculin to induce bacterial adhesion associated with the recruitment of**
35 **mature focal adhesion markers, despite the inability of bacteria to exert significant counter-forces²**
36 **³. Here, we show that three vinculin-binding sites (VBSs) exposed at the IpaA C-terminus act in a**
37 **cooperative manner to trigger a yet unreported mode of vinculin activation through specific**
38 **interactions with sites in the vinculin head sub-domain D2. Structural modeling based on the mass**
39 **spectrometry identification of interacting residues by cross-linking indicated that upon IpaA**
40 **binding to vinculin D2, vinculin head sub-domains undergo major conformational changes leading**
41 **to higher order heterocomplexes and vinculin homo-trimers. IpaA-mediated vinculin activation**
42 **induces the formation of large and stable focal adhesions resisting the action of actin relaxing**
43 **drugs. This property enables IpaA to rapidly elicit strong cell adhesion to fibronectin-coated**
44 **substrates. While *Shigella* IpaA promotes strong adhesion in the absence of mechanotransduction,**

its mode of vinculin activation likely reflects a key step during maturation of cell adhesions driven by acto-myosin contractility.

Integrin-mediated cell adhesion critically depends on the cytoskeletal linkers talin and vinculin⁴⁻⁶. During mechanotransduction, talin acts as a mechanosensor by exposing its VBSs as a function of the stretching force generated by acto-myosin and dependent on substrate stiffness⁷. Talin exposed VBSs recruit and activate vinculin, reinforcing anchorage to the actin cytoskeleton in response to mechanical load^{8, 9}. Vinculin contains three repetitions (D1-D3) of a conserved domain consisting of two bundles of four helices, and a fourth D4 domain containing only one helical bundle connected to a proline-rich unstructured region and the carboxyterminal F-actin binding domain¹⁰. Under its inactive folded form, intramolecular interactions between the vinculin head and tail domains prevent ligand binding.

The *Shigella* type III invasion effector IpaA contains 3 VBSs located in its carboxyterminal moiety¹¹⁻¹³. IpaA VBS1, as for all VBSs described to date to promote vinculin activation, interacts with the first helical bundle of the D1 domain, promoting major conformational changes that disrupt the D1-tail intramolecular interactions and free the vinculin F-actin binding region¹¹ (Fig. 1a). IpaA VBS2, in contrast, interacts with the second helical bundle of D1¹² (Fig. 1a) and its association with IpaA VBS1 results in a very high affinity and stable IpaA VBS1-2:D1 complex, with an estimated K_D in the femtoM range¹². While functional evidence indicates that IpaA VBS3 cooperates with IpaA VBS1-2 to stimulate bacterial invasion, the isolated peptide acts as IpaA VBS1 in promoting vinculin activation through interaction with the vinculin D1 first helical bundle and also forms a folded bundle with the talin H1-H4 helices^{13, 14}.

Here, we studied the effects of the IpaA subdomains containing VBS1-2 (A524) or VBS1-3 (A483) on vinculin activation (Fig. 1b). We performed SEC-MALS (Size Exclusion Chromatography-Multi-Angle Light Scattering) to analyze complexes formed upon incubation of A483 with HV₁₋₈₃₄ containing the D1-D4 domains, corresponding to full-length human vinculin (HV) devoid of the

carboxyterminal F-actin binding domain (Fig. 1b). In addition to the 1:1 D1D4: A483 complex, larger complexes were observed corresponding to a 2:1 heterocomplex and a 3:0 D1-D4 homo-trimer (Fig. 1c). Similar 2:1 and 3:0 complexes were observed when A483 was incubated with HV₁₋₄₈₄ containing only the vinculin D1 and D2 domains (D1D2) (Fig. 1d), indicating that vinculin homo-trimerization occurs exclusively through these vinculin head sub-domains. By contrast, when A524 was incubated with D1D2, 1:1 and 2:1 D1D2:A524 complexes were detected, but not the D1D2 homo-trimer suggesting a role for IpaA VBS3 in vinculin trimerization (Fig. 1d). Consistently, higher order D1D2 homo-complexes devoid of A483 and D1D2:A524 hetero-complexes were visualized by native PAGE (Fig. 1e and Suppl. Figs. 3c, d). These results suggest that binding of IpaA VBSs to vinculin triggers conformational changes in vinculin leading to the formation of an IpaA VBS3-dependent vinculin homo-trimer.

To further investigate initial interactions responsible for vinculin trimer formation, we performed binding assays with HV derivatives immobilized onto a solid phase to restrict conformational changes. These assays indicated that A483 and A524 bound to HV with a similar affinity, as estimated by their EC₅₀ (95% confidence interval) of 6.1 (4.2-9.0) and 3.7 (1.7-8.1) nM, respectively (Suppl. Fig. 1a). Strikingly, a large difference was observed in the binding plateau, indicating that HV presented more binding sites for A483 than for A524 (Suppl. Fig. 1a). Also, D1D2 presented more binding sites for HV₁₋₂₅₈ containing the D1 domain only, suggesting the presence of additional sites on the D2 domain (Suppl. Fig. 1b). Consistently, BN-PAGE showed the formation of 1:1, as well as a 1:2 D1D2:A483 complex, observed with increasing A483 molar ratio (Suppl. Fig. 1c). In contrast, single 1:1 complexes were observed for D1:A483, D1:A524 or D1D2-A524 (Suppl. Figs. 1c-h), indicating that IpaA VBS3 was required to reveal additional sites on the D2 domain. Of note, D1D2 homo-trimers observed in the SEC-MALS and native gel analysis (Figs. 1d, e and Ext Data Figs. 3c, d) were not detected in BN-PAGE, suggesting that Coomassie brilliant blue interfered with the formation of higher order D1D2 complexes. Together, these results suggested that the formation of vinculin trimers triggered by A483 required the IpaA VBS3 dependent exposure of binding sites on D2. These findings were

unexpected, since vinculin activating ligands have been described to bind to a single site on the D1 domain of vinculin.

To map interactions of A524 and A483 with D1D2, complexes were cross-linked, subjected to proteolysis and analyzed using Liquid Chromatography coupled to Mass Spectrometry (LC-MS) (Methods). Intermolecular links were identified from the characterization of cross-linked peptides (Suppl. Tables 1-3; Methods) and along with identified intramolecular links, used to produce structural models (Methods). The A524:D1 complex showed links consistent with a "canonical" conformer expected from established structures^{11, 12, 15} (Suppl. Fig. 2). Similar links were identified for the A524:D1D2 complex, with a majority of links observed with the D1 domain (Fig. 2a). For both complexes, the structure shows interactions between IpaA VBS1 and VBS2 with the D1 first and second bundles, respectively, leading to helical bundle reorganization of D1 associated with vinculin activation (Figs. 2c, d; Suppl. Fig. 2 and Suppl. Tables 1, 2). For the A483:D1D2 complex, structural modeling shows 2 major conformers accounting for the majority of links. In a first "closed" conformer, IpaA VBS1 and VBS2 interact with the D1 bundles in a similar manner as for A524, where the relative positioning of D1 and D2 is globally conserved compared to apo D1D2 in the A524-D1D2 complex (compare Figs. 2d and 2e). In this "closed" conformer, IpaA VBS3 interacts with an interface formed by the H5 (residues 128-149) and H8 (residues 222-250) helices in the second bundle of D1 and the H13 (residues 373-397) helix in the second bundle of the D2 domain (Figs. 2e and f). The second "open" conformer, however, shows a major re-orientation of D1 and D2 subdomains with their major axis forming an angle value of ca 82° compared to the 25° observed in the native vinculin structure or the first conformer (Fig. 2g), with IpaA VBS3 docking sidewise through extensive interaction with the H5 (residues 128-149) and H8 (residues 222-250) helices of the vinculin D1 domain. Because this latter conformer leads to major changes in bundle exposure in D1 and D2, we posit that it is involved in the formation of higher order D1D2 complexes and trimer induced by A483. To test this, we engineered a structural clamp by substituting residue Q68 in the first D1 bundle and A396 in the second D2 bundle for cysteine residues, expected to prevent the formation of the open

conformer upon disulfide bridge formation (Suppl. Figs. 3a, b). Consistently, cysteine clamped D1D2-Q68C A396C did not prevent the exposure of additional sites on D2 or 1:1 complex formation induced by A524 or A483, but prevented the formation of higher order complexes (Fig. 1 e and Suppl. Figs. 3c-e). We coined "supra-activation" the mode of vinculin activation induced by A483 involving major conformational changes in the vinculin head, to distinguish it from the canonical activation associated with the dissociation of vinculin head-tail domains.

We then characterized the effects of A524 and A483 expression by performing immunofluorescence staining of vinculin-containing adhesion structures. As shown in Figs 3a-c, C2.7 cells transfected with GFP-A524 formed more numerous and larger peripheral adhesions as well as actin-rich ruffles compared to control cells (Figs. 3a-d). Strikingly, GFP-A483 transfected cells formed even larger and more numerous adhesions than GFP-A524 transfected cells, but significantly less actin ruffles than GFP-A524 transfectants (Figs. 3a-d). These observations were confirmed by live TIRF microscopy showing the extreme stability of adhesions in GFP-A483 transfectants, with a median duration of at least 84 min, while GFP-A524 and control cells showed adhesions with a comparable median duration of less than 25 min (Fig. 3e; Suppl. movie 1). GFP-A524 and GFP-A483 transfectants showed slightly slower median instant rates of adhesion assembly than control cells (Suppl. Fig. 4; Suppl. movie 1) but significant 1.6-fold and 2-fold decrease in median instant rates of disassembly relative to control cells, respectively (Suppl. Fig. 4). The stability of GFP-A483-induced FAs was independent of acto-myosin contraction. Indeed, GFP-A483-induced FAs resisted the action of the Rho-kinase inhibitor Y27632 relaxing actin-myosin, with a five- and four-times slower median rate of disassembly of adhesions relative to control cells and GFP-A524 transfectants, respectively (Figs. 3f-i; Suppl. movie 2). Furthermore, large adhesions formed in GFP-A483 transfectants following addition of the inhibitor (Figs. 3f, h), a process that was not observed for the other samples, including cells transfected with GFP-A524 or GFP fused to the vinculin D1 domain (vD1) reported to delay talin refolding following stretching¹⁶⁻¹⁸ (Figs. 3g-i; Suppl. movie 2). In line with the stabilization of mature FAs through vinculin supra-activation, GFP-A483 but not GFP-vD1 also delayed the Y27632-induced

removal of the late adhesion marker VASP (Suppl. Fig. 5; Suppl. movie 3). Consistent with A483 bypassing mechanotransduction, GFP-A483 expression induced 5- and 1.6-fold higher yield of short term (≤ 10 min) cell adhesion to fibronectin substrates compared to control GFP and GFP-A524 transfectants, respectively (Suppl. Fig. 6a). By contrast, little difference in adhesion yield was detected at 15 min suggesting that canonical activation of vinculin also resulted in its supra-activation during mechanotransduction (Suppl. Fig. 6a). In line with this, MEF vinculin-null cells transfected with the clamped vinculin version formed fewer and smaller FAs than cells transfected with wild-type vinculin but more than mock-transfected cells (Suppl. Figs. 6b-d).

To confirm and extend these findings, cell adhesion strength was assessed measuring their detachment upon application of a controlled shear stress in a microfluidic chamber. When 1205Lu melanoma cells were allowed to adhere to fibronectin-coated surfaces for more than 25 min, little difference in resistance to shear stress could be detected among samples (Suppl. Fig. 7a). In contrast, when shear stress was applied less than 20 min following cell incubation, GFP-A483-transfected cells showed significantly higher resistance to shear stress up to $22.2 \text{ dynes.cm}^{-2}$ than GFP-A524- or GFP-transfected cells, with 1.7 ± 0.2 - and 0.9 ± 0.14 -fold enrichment $\pm$ SD of adherent cells for GFP-A483 and GFP-A524-transfected cells versus control GFP-transfected cells, respectively (Fig. 4b; Suppl. movie 4). In addition, similar to melanoma cells depleted for by siRNA treatment, cells transfected with the clamped vinculin version showed a decreased ability to adhere in comparison to WT vinculin-transfected cells (Suppl. Figs. 7a, b). These results are in full agreement with effects observed on adhesion structures and suggest that A483 interaction with vinculin leads to the bypass of mechanotransducing steps to promote strong adhesion.

Vinculin is paradoxically described as a prognostic marker favoring the migration of cancer cells or as a tumor suppressor stimulating cell anchorage¹⁹⁻²¹. These contradictory findings reflect its complex and poorly understood regulation, as well as different roles in 2D or 3D systems^{22, 23}. Also, an increase of the total pool of vinculin may not correlate with increased vinculin activation. We took advantage of the unique property of A483 to study the effects of vinculin supra-activation on the

motility and invasion of melanoma cells. In time-lapse microscopy experiments in 2D-chambers, GFP-A524 inhibited melanocyte motility compared to control cells, with a rate of Root Median Square Displacement (rMSD) of 3.16 and 15.6 $\mu\text{m}.\text{min}$, respectively (Figs. 4c, d). An even stronger inhibition was observed for GFP-A483-transfected cells (rMSD = 2.3 $\mu\text{m}.\text{min}$) (Figs. 4c, d). Transmigration of melanocytes in 3D-matrigels was similarly inhibited by A524 and A483 (Fig. 4e).

Bacteria invading through a triggering mode rely on a discrete number of T3SS-dependent contacts for which cytoskeletal tethering is likely critical for invasion². As opposed to physiological substrates, bacteria cannot sustain the range of counter-forces associated with integrin-mediated adhesion to the substrate. The *Shigella* type III effector IpaA provides an elegant solution to this problem by promoting strong adhesion without requirement for mechanotransduction. Through the joint action of its VBSs, IpaA induces major conformational changes of the vinculin head sub-domains. Understanding how these major vinculin conformational changes regulate the composition and properties of cell adhesions will bring important insights into cell adhesion processes and will be the focus of future investigations.

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AUTHOR CONTRIBUTIONS

CV-G and DIAS conceived and performed most of the experimental works, data analysis and wrote the manuscript. BCC and BM analyzed TIRF experiments. CB-N performed the SEC-MALS analysis. CB and NQD performed and analyzed experiments with melanocytes with the help of DJ and AM. BCC, AA and JF provided technical help for cell adhesion and microfluidics experiments. CM and JCR designed and performed the LC-MS analysis. DBL analyzed the cross-linked mass spectrometry data. HK and LM generated structural models. GTVN designed the project and wrote the manuscript.

FIGURES AND LEGENDS

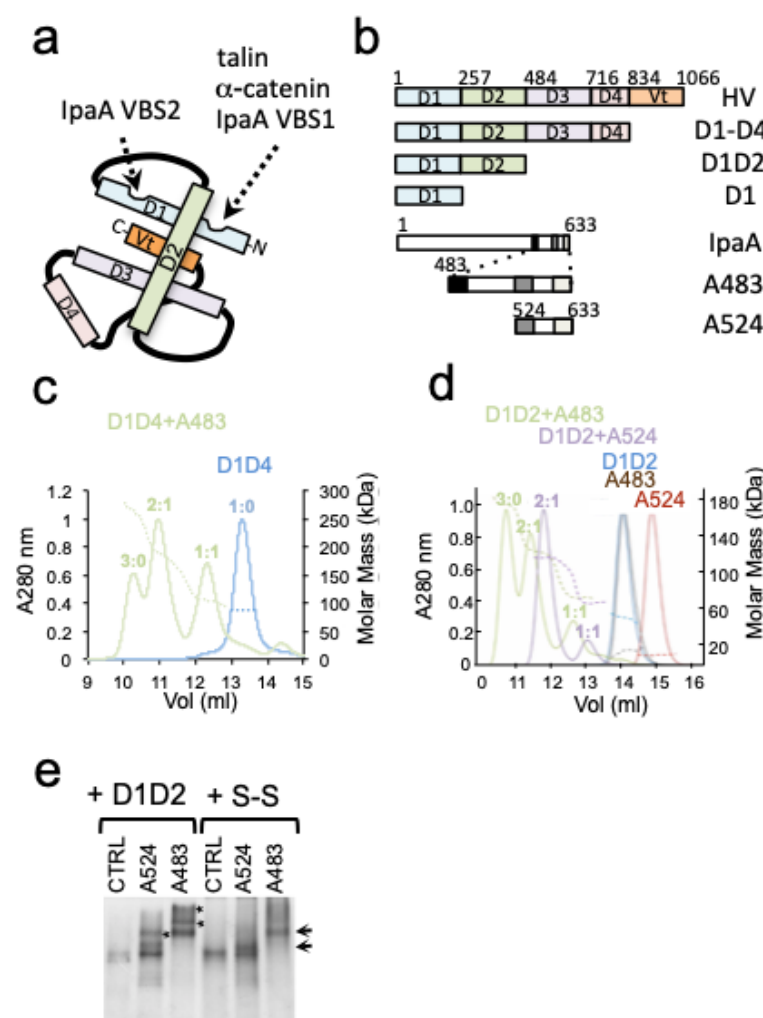
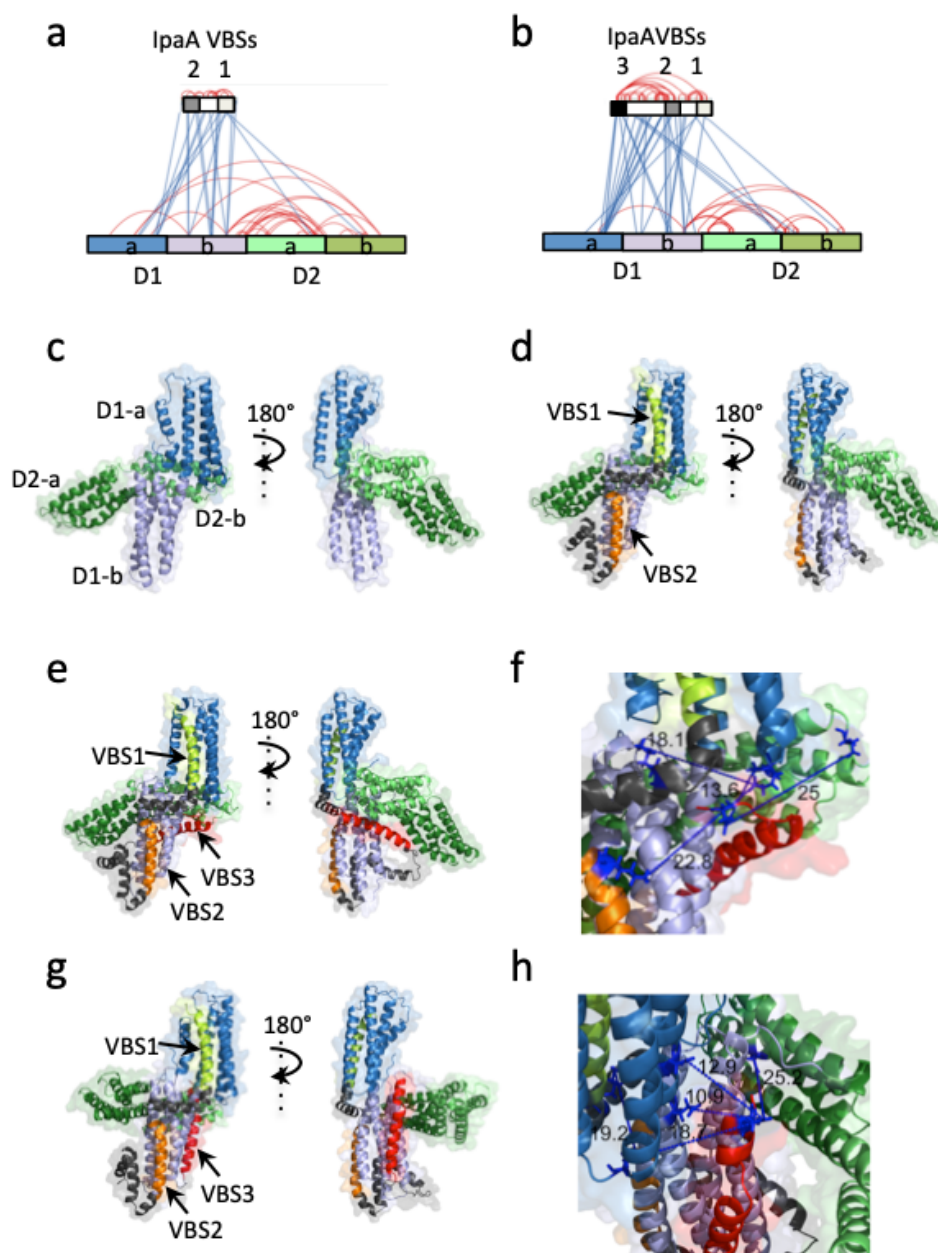


Figure 1 – IpaA reveals binding sites in vinculin head subdomains

a, Scheme of folded vinculin (HV). The binding sites and corresponding ligands are indicated. **b**, Scheme of HV and IpaA constructs. HV domains and IpaA VBSs are depicted as boxes. The numbers indicate the start residue of each domain. **c**, **d**, SEC elution profiles of complexes formed between A483 (green) or A524 (purple) and vinculin D1D4 (**c**) or D1D2 (**d**). The indicated complex stoichiometry was inferred from the molecular weight estimated by MALS. **e**, native gel analysis of vinculin D1D2 and IpaA derivatives. D1D2 or cysteine-clamped D1D2 (S-S) were incubated with the indicated IpaA derivatives and analyzed by native PAGE followed by Coomassie staining. Arrows: 1:1 complexes. *: higher order complexes. Note the absence of higher order complexes for cysteine-clamped D1D2.

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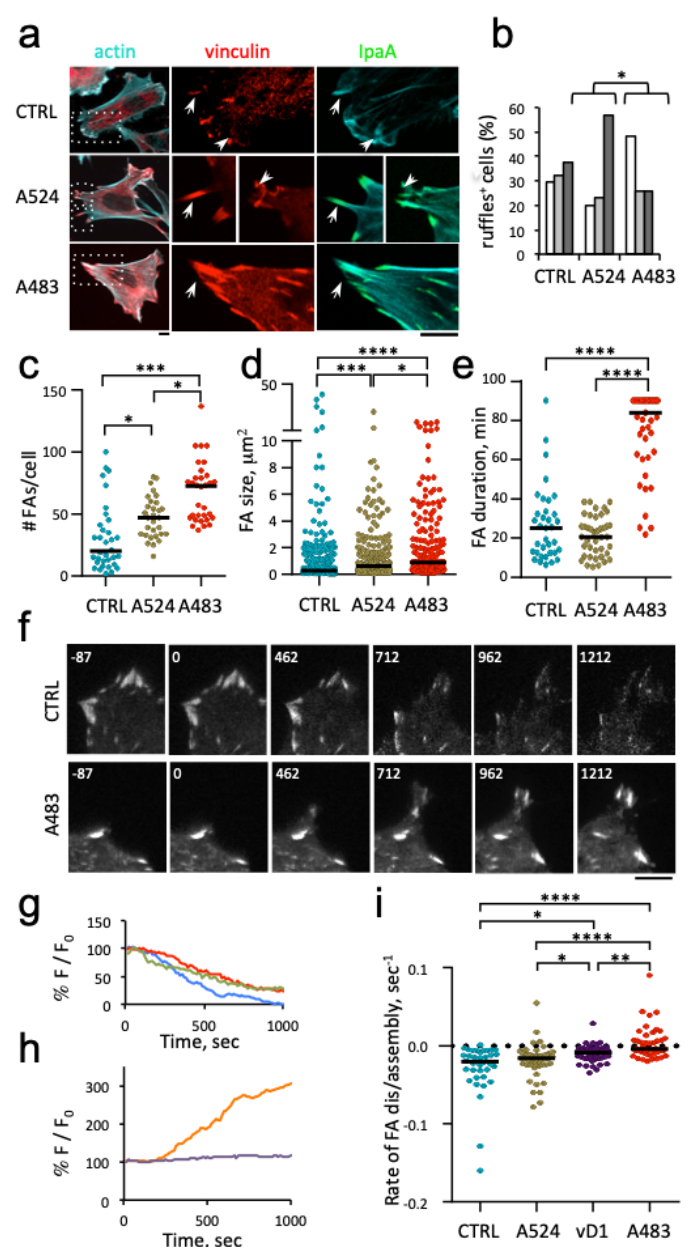


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272 **Figure 2 – Characterization of IpaA contact sites on vinculin.**

273 **a, b**, EDC cross-link map from mass spectrometry analysis (LC-MS/MS) of vinculin D1D2-A524 (**a**) and
 274 D1D2-A483 (**b**) following extraction of 1:1 complexes from BN-PAGE. Blue lines: inter-molecular links.
 275 Red lines: intra-molecular links. Note the links between IpaA VBS3 and the D2 second bundle. Cross-
 276 linked residues are detailed in Suppl. Table 1. **c-e**) Structural models of D1D2 (**c**), D1D2-A524 (**d**), D1D2-
 277 A483 “closed” conformer (**e**), D1D2-A483 “open” conformer (**g**). **f, h**, higher magnification of the IpaA

278 VBS3-D1D2 interaction in (e) and (g) showing the distance between residues in Å. IpaA VBS1-3 were
279 docked on the surface of Vinculin D1D2 using MS cross-link constraints. TX-MS protocol in combination
280 with MS constraints was used to unify and adjust the final model, which justifies over 100 cross-links.



281
282 **Figure 3 – A483 stabilizes vinculin in cell adhesions in the absence of mechanotransduction**
283 **a-c**, Immunofluorescence analysis of vinculin adhesions. CTRL: C2.7 cells. A524: GFP-A524
284 transfectants. A483: GFP-A483 transfectants. **a**, representative fluorescence micrographs. Arrows:
285 adhesions; arrowheads: ruffles. Green: GFP; red: vinculin; cyan: actin. **b**, percent of cells with ruffles $\pm$

SEM. Cells with: no ruffles (empty bars); small ruffles (light grey bars); large ruffles (dark grey bars). *: Pearson's Chi-squared test (N=3, n > 30, p = 0.036). **c**, **d**, vinculin containing FAs were detected using a semi-automated program. **c**, median number of FAs per cell; **d**, FA size. (N=3, n > 30). Dunn's multiple comparisons test. *: p < 0.05;***: p < 0.005. **e**, TIRF microscopy of C2.7 cells transfected with mCherry-vinculin alone (CTRL) or co-transfected with GFP-A524 (A524), GFP-vD1 (vD1), or GFP-A483 (A483). The duration of vinculin-containing adhesions was determined from time-lapse acquisitions. **f-i**, TIRF microscopy analysis of cells treated with 100 μ M Y-27632. **f**, Representative time series acquisitions. Numbers indicate the elapsed time in seconds, with the inhibitor added at t = 0. Scale bar = 5 μ m. **g**, **h**, F/F₀: normalized average fluorescence intensity of adhesions as a function of time. Representative traces corresponding to single adhesions for: **h**, control cells: blue, A524: red, vD1: green; **g**, A483: purple and orange. **i**, initial rates of adhesion disassembly inferred from linear fits. N = 5. Number of adhesions analyzed: CTRL: 84; vD1: 75; A524: 140; A483: 97. Dunn's multiple comparisons test. *: p < 0.05; **: p < 0.01;***: p < 0.005; ****: p < 0.001.

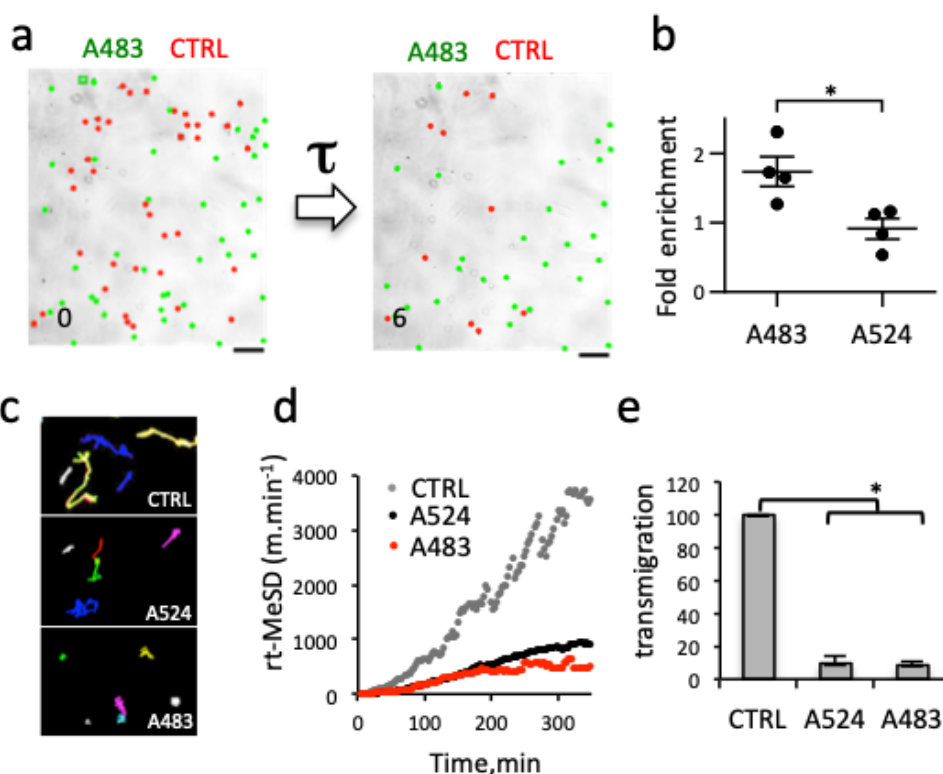


Figure 4. A483-mediated vinculin supra-activation stimulates strong cell adhesion and inhibits tumor cell invasion.

a, b, 1205Lu melanoma cells were transfected with the indicated constructs, labeled with calcein (methods) and mixed with the same ratio of control cells. Cells were perfused in a microfluidic chamber and allowed to adhere for 20 min prior to shear stress application. **a**, representative fields. The number indicates the elapsed time after shear stress application. **b**, Scatterplot of fold enrichment of: A483 (N = 4, n = 610) or A524 (N = 4, n = 433) transfected cells vs control cells (1594 cells, N = 4). Unpaired t test. *: p = 0.0229. **c, d**, cells were transfected with the indicated constructs, and analyzed by time-lapse videomicroscopy. **c**, representative single cell migration 20-hour tracks for indicated samples. **d**, Root of Median Square of displacement over time for control- (61 cells, N = 3), GFP-A524- (61 cells, N = 3) and GFP-A483 transfectants (64 cells, N = 3). ***p = 0.0007. The slopes were analyzed using a covariance test and found to be statistically different (ANCOVA, p < 2x10⁻¹⁶). **e**, 5 × 10⁴ cells were seeded in matrigel

314 chambers. The percent of transmigrated cells is indicated. (N = 3). Kruskal-Wallis test with Dunn's
315 multiple comparisons test. *: $p < 0.05$.
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METHODS

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Generation of constructs

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Human vinculin constructs were generated by polymerase chain reaction using the forward primer 5' GCGCATATGCCAGTGTTCATACG-3' and reverse primer 5'-CGTCGACTCACCAGGCATCTTCATCGGC-3' for D1 (residues 1-258) or 5'-CGTCGACTCAGTGACAGCTGCTTTG-3' for D2 (residues 1-492) using a plasmid containing full-length octahistidine-tagged human vinculin (residues 1–1,066), as template¹⁰, and cloned into the NdeI-Sall sites of pet15b (Novagen). The IpaA constructs GFP-A524 and GFP-A483 were generated by polymerase chain reaction (PCR) and cloning into pcDNA3.1 NT-GFP Topo TA (Invitrogen) using the 5'-TCAAAGGACATTACAAAATCC-3' and 5'-GCGATATCATGGCCAGCAAAGG-3' forward primers, respectively, and the 5'-GCGCGGCCGCTTAATCCTTATTGATATTC-3' reverse primer. The GST-A483A construct was generated by PCR using 5'-GGCGAATCCCGGAGACACATATTTAACACG-3' forward and 5'-GCCGTCGACTTAATCCTTATTGATATTCT-3' reverse primers and cloning into the *EcoRI-Sall* of pGEX-4T-2 (GE Lifesciences). pGST-A524 was previously described²⁴. The pGFP-vD1 plasmid was generated by polymerase chain reaction using the forward primer 5'-ACCCGGGATCCCGCC-3' and reverse primer 5'-ACCCGGGACCAGGCA-3', and cloned into pEGFP. The pmCherry-human vinculin (HV) and pmCherry-VASP plasmids were from Addgene. Stealth siRNA anti-human vinculin was from Invitrogen (reference number 1299001).

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Protein purification

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BL21 (DE3) chemically competent *E. coli* (Life Technologies) was transformed with the expression constructs. D1 and D1D2 were purified essentially as described^{11, 25}. For the IpaA derivatives, bacteria grown until OD_{600nm} = 1.0 were induced with 0.5 mM IPTG and incubated for another 3 hrs. Bacteria were pelleted and washed in binding buffer 25 mM Tris PH 7.4, 100 mM NaCl and 1 mM beta-mercaptoethanol, containing CompleteTM protease inhibitor. Bacterial pellets were resuspended in

1/50th of the original culture volume and lyzed using a cell disruptor (One shot model, Constant System Inc.). Proteins were purified by affinity chromatography using a GStrap HP affinity column (GE Healthcare) and size exclusion chromatography (HiLoad S200, GE Healthcare). Samples were stored aliquoted at -80°C at concentrations ranging from 1 to 10 mg/ml.

Protein complex formation analysis

Proteins were incubated at a concentration of 30 µM in binding buffer for 60 min at 4°C. Samples were analyzed by SEC-MALS (Wyatt Technology Europe) using a 24 ml Superdex 200 Increase 10/300 GL filtration column and a MiniDAWN TREOS equipped with a quasi-elastic light scattering module and a refractometer Optilab T-rEX (Wyatt Technology). Data were analyzed using the ASTRA 6.1.7.17 software (Wyatt Technology Europe). Protein complex formation was visualized by PAGE under non-denaturing conditions using a 7.5% polyacrylamide gel, followed by Coomassie blue staining.

Solid-phase binding assay

96-well Maxisorp (Nunc) ELISA plates were coated with 30 nM of full-length vinculin, vinculin constructs or IpaA proteins at the indicated concentrations in binding buffer (25 mM Tris PH 7.4, 100 mM NaCl and 1 mM β-mercaptoethanol). Samples were blocked with PBS-BSA 2%, washed and incubated with IpaA or vinculin proteins in binding buffer containing 0.2% BSA at room temperature for one hour. After incubation, the plates were washed and incubated with an anti-IpaA (dilution 1/2000^e) polyclonal primary antibody³ or anti-vinculin (dilution 1/2000^e) Vin11Vin.5 monoclonal antibody (Sigma-Aldrich) in binding buffer containing 0.2% BSA for one hour at room temperature. Plates were washed and incubated with an HRP-coupled secondary anti-rabbit or anti-mouse IgG antibody (1/32000^e) (Jackson ImmunoResearch) for one hour. The reaction was revealed by adding 100 µl of tetramethylbenzidine (Sigma-Aldrich) for 15 min, stopped by adding 50 µl of 0.66N H₂SO₄ and the absorbance was read at 450 nm (Dynatech MR400).

BN-PAGE (Blue Native – Polyacrylamide Gel Electrophoresis) protein native gel analysis and complex cross-linking

25 μ M of vinculin constructs were incubated with different molar ratios of IpaA proteins in a 1X BN-PAGE buffer (250 mM ϵ -aminocaproic acid and 25mM Bis-Tris PH 7,0) at 4°C for one hour. The protein mixtures were separated in a one-dimension native BN-PAGE electrophoresis as described²⁶. For vinculin-IpaA protein ratio assay, vinculin-IpaA bands containing the complexes separated by BN-PAGE were cut, sliced and boiled in 2 x Laemmli SDS buffer followed by SDS-PAGE. The second dimension SDS-PAGE gels were stained (colloidal Coomassie staining) and the density of the bands was determined using Image J. The normalized vinculin:IpaA ratio of the complexes was compared using a non-parametric Kruskal-Wallis rank sum test (R statistical software).

For crosslinking vinculin-IpaA complex, bands containing the complexes were cut, sliced and electroeluted in native conditions (15 mM Bis-Tris pH 7.0 and 50 mM Tricine) inside a closed dialysis membrane (SpectraPor). The soluble complexes were recovered and their buffer exchanged twice into an amine-free cross-link buffer in 25 mM HEPES pH 7.0 containing 100 mM NaCl using 10MWCO ZEBA desalting columns (Thermo Scientific). The fractions containing the complexes were incubated for 1 hr at 4°C with 10 mM N-hydroxysulfosuccinimide and 5 mM EDC (Sigma-Aldrich) following the manufacturer's recommendations. The cross-linking reaction was stopped by adding 50 mM Tris pH 7.4 and incubating for 20 minutes. Samples were denaturated in 2x SDS Laemmli buffer for 5 min at 95°C and complexes were eluted from gel slices following SDS-PAGE.

Liquid Chromatography Mass spectrometry (LC-MS)

Complexes obtained after the cross-linking step were loaded onto a 4-20% polyacrylamide gradient gels and Coomassie stained. The bands containing the complexes were cut and submitted to tryptic digestion²⁷. The experiments were performed in duplicates for the 3 complexes D1:A524, D1D2:A524 and D1D2:A483. Peptides obtained after tryptic digestion were analyzed on a Q Exactive Plus instrument (Thermo Fisher Scientific, Bremen) coupled with an EASY nLC 1 000 chromatography system (Thermo

Fisher Scientific, Bremen). Sample was loaded on an in-house packed 50 cm nano-HPLC column (75 μm inner diameter) with C18 resin (1.9 μm particles, 100 Å pore size, Reprosil-Pur Basic C18-HD resin, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and equilibrated in 98 % solvent A (H_2O , 0.1 % FA) and 2 % solvent B (ACN, 0.1 % FA). A 120 minute-gradient of solvent B at 250 nL.min⁻¹ flow rate was applied to separate peptides. The instrument method for the Q Exactive Plus was set up in DDA mode (Data Dependent Acquisition). After a survey scan in the Orbitrap (resolution 70 000), the 10 most intense precursor ions were selected for HCD fragmentation with a normalized collision energy set up to 28. Charge state screening was enabled, and precursors with unknown charge state or a charge state of 1 and >7 were excluded. Dynamic exclusion was enabled for 35 or 45 seconds respectively.

Data analysis

The identification of cross-linked peptides from LC-MS data was performed using SIM-XL v. 1.3²⁸, with the following search parameters: EDC as cross-linker, a tolerance of 20 ppm for precursor and fragment ions, trypsin fully specific digestion with up to three missed cleavages. Carbamidomethylation of cysteines was considered as a fixed modification. All initial identification of cross-linked peptides required a primary score of SIM-XL greater than 2.5 for inter-links and 2.0 for intra-links or loop-links. As single incorrect cross-link identification might lead to a different model, a manual post-validation of the search engine results at the MS2 level was thus performed. A 2D-map showing the protein-protein interaction was generated as an output (Figs. 2a,b). Only peptides present in the 2 replicates are gathered in Supplementary Tables 1-3 and were used for the modeling.

Modeling

We used the constraints obtained from the cross-linking MS data (Suppl. Tables 1-3) to guide the protein structure modeling using the TX-MS protocol as described by Hauri, Khakzad et al.²⁹. In short, TX-MS uses the Rosetta comparative modeling protocol (RosettaCM)³⁰, and the flexible backbone docking protocol (RosettaDock)³¹ to generate models and evaluate how well each model explains the MS constraints using a novel scoring function. Here, a total of 100,000 models was generated, of which the

highest-scoring model is displayed in (Fig2e), supported by a total of 100 inter and intra-molecular cross-links.

Cell lines

C2.7 myoblasts³² and MEF vinculin null cells⁸ were routinely grown in DMEM 1 g / L glucose containing 10 % FCS in a 37°C incubator containing 10 % CO₂. 1205Lu melanoma cells³³ were grown in RPMI + Glutamax medium (RPMI1640) supplemented with 10 % fetal calf serum (FCS) and non-essential aminoacids in a 37°C incubator with 5 % CO₂.

Immunofluorescence staining

C2.7 mice myoblasts cells were seeded at 2.5×10^4 cells in 25 mm-diameter coverslips. Cells were transfected with 3 µg of pGFP-A524 or pGFP-A483 plasmids with 6 µl JetPEI transfection reagent (Polyplus) for 16 hours following the manufacturer's recommendations. Cells were fixed in PBS containing 3.7% paraformaldehyde for 20 min at 21°C and permeabilized with 0.1% Triton X-100 for 4 min at 21°C. Cells were processed for immunofluorescence staining using the Vin11.5 anti-vinculin monoclonal antibody (ref. V4505, Sigma-Aldrich) and anti-mouse IgG antibody coupled to Alexa 546 (Jackson Research) and Phalloidin-Alexa 633 (Invitrogen), as described previously³. Samples were analyzed using an Eclipse Ti inverted microscope (Nikon) equipped with a 60 x objective, a CSU-X1 spinning disk confocal head (Yokogawa), and a Coolsnap HQ2 camera (Roper Scientific Instruments), controlled by the Metamorph 7.7 software.

TIRF (Total Internal Reflection Microscopy) analysis

C2.7 cells were transfected with pmCherry-HV or pmCherry-VASP and the indicated plasmids as described above. Samples were mounted onto a TIRF microscopy chamber on a stage of an Eclipse Ti inverted microscope (Nikon) equipped with an Apo TIRF 100 x N.A. 1.49 oil objective heated at 37°C. TIRF analysis was performed using the Roper ILAS module and an Evolve EM-CCD camera (Roper Scientific

Instruments). When mentioned, Y-27632 was used at 100 μ M. Image acquisition was performed every 12.5 seconds for 30 to 90 minutes.

Live cell tracking

1205Lu melanoma cells were transfected with IpaA constructs or GFP alone (control) and transferred in microscopy chamber on a 37°C 5%-CO₂ stage in RPMI1640 medium containing 25 mM HEPES. For cell tracking, samples were analyzed using an inverted Leica DMiBe microscope and a 20 X phase contrast objective. Image acquisitions were performed every 3 min for 200 hrs. The mean velocity of migration was measured for all tracks followed for at least 5 hours. The root square of MdSD over time was plotted over time and fitted by linear regression. The slopes of the linear fit were compared using an ANCOVA test (linear model). The median cell surface was quantified as the mean of the surface for three time points (25%, 50% and 75%) of the whole cell track and dispersion measured by the Median absolute dispersion (MAD).

Invasion assays

Tissue culture Transwell inserts (8 μ m pore size; Falcon, Franklin Lakes, NJ) were coated for 3 hours with 10 μ g of Matrigel following the manufacturer's instructions (Biocoat, BD Biosciences, San Jose, CA). Inserts were placed into 24-well dishes containing 500 μ l of RPMI medium supplemented with 1% fetal calf serum. 5×10^4 melanoma cells were added to the upper chamber in 250 μ l of serum-free RPMI medium. After 24 hours, transmigrated cells were scored by bright field microscopy. Experiments were performed at least three times, each with duplicate samples.

Image processing and statistical analysis

For the quantification of the number of adhesion structures in C2.7 cells, a semi-automated protocol was developed using Icy software³⁴. Spinning-disk fluorescent microscopy planes were used to detect vinculin structures using HK means thresholding and overlaid binary masks obtained from the threshold projections of F-actin labeled images (Max-entropy method). FAs were detected as spots

positive for both vinculin mCherry and actin structures using Wavelet Spot Detector. The number of adhesions was analyzed using Dunn's multiple comparisons test. The statistical analysis of cell motility was performed in the R software. Medians were compared using a Wilcoxon rank sum test and dispersion by Median absolute dispersion (MAD) parameter.

Microfluidics cell adhesion assay

Analysis of cell detachment under shear stress was based on previous works³⁵. 1205Lu melanocytes were transfected with the indicated constructs, then labeled with 2 μM calcein-AM (Life Technologies) in serum-free DMEM for 20 minutes. Cells were detached by incubation with 2 μM Cytochalasin D (Sigma-Aldrich) for 40 minutes to disassemble FAs, followed by incubation in PBS containing 10 mM EDTA for 20 minutes. Cells were washed in EM buffer (120 mM NaCl, 7 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgCl_2 , 5 mM glucose and 25 mM HEPES at pH 7.3) by centrifugation and resuspended in the same buffer at a density of 1.5×10^6 cells/ml. Calcein-labeled transfected cells and control unlabeled cells were mixed at a 1:1 ratio and perfused onto a 25 mm-diameter glass coverslips (Marienfeld) previously coated with 20 $\mu\text{g/ml}$ fibronectin and blocked with PBS containing 2% BSA (Sigma-Aldrich) in a microfluidic chamber on a microscope stage at 37°C. We used a commercial microfluidic setup (Flow chamber system 1C, Provitro) and a Miniplus3 peristaltic pump (Gilson) to adjust the flow rate in the chamber. Microscopy analysis was performed using a LEICA DMRIBe inverted microscope equipped with a Cascade 512B camera and LED source lights (Roper Instruments), driven by the Metamorph 7.7 software (Universal imaging). Cells were allowed to settle for the indicated time prior to application of a 4 ml/min, flow corresponding to a wall shear stress of 22.2 dyn/cm^2 (2.22 Pa). Acquisition was performed using a 20 X objective using phase contrast and fluorescence illumination (excitation $480 \pm 20 \text{ nm}$, emission $527 \pm 30 \text{ nm}$). Fluorescent images were acquired before and after flushing to differentiate between target and control cells. Phase contrast images were acquired every 200 ms. Fold enrichment was defined as the ratio between of attached labeled and unlabeled cells.

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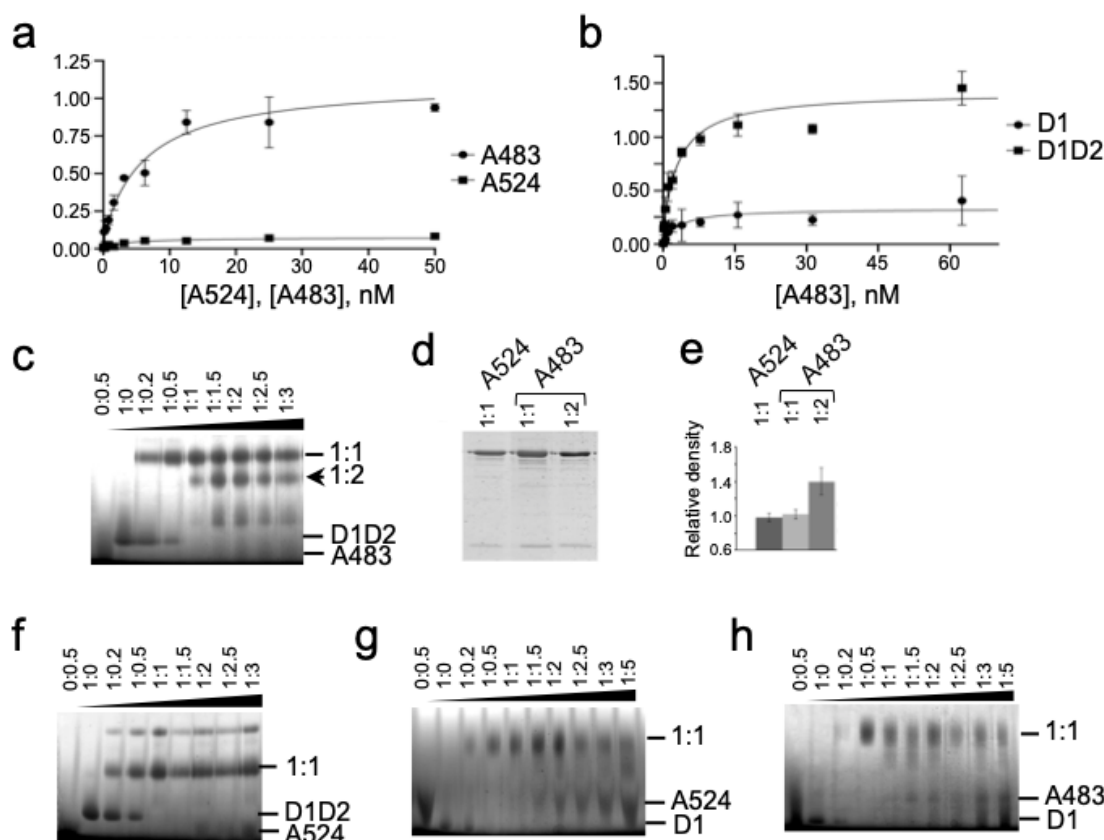
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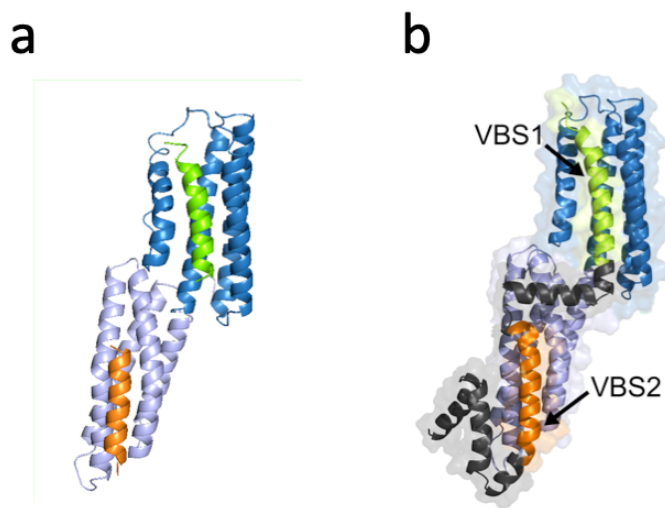
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SUPPLEMENTARY INFORMATION

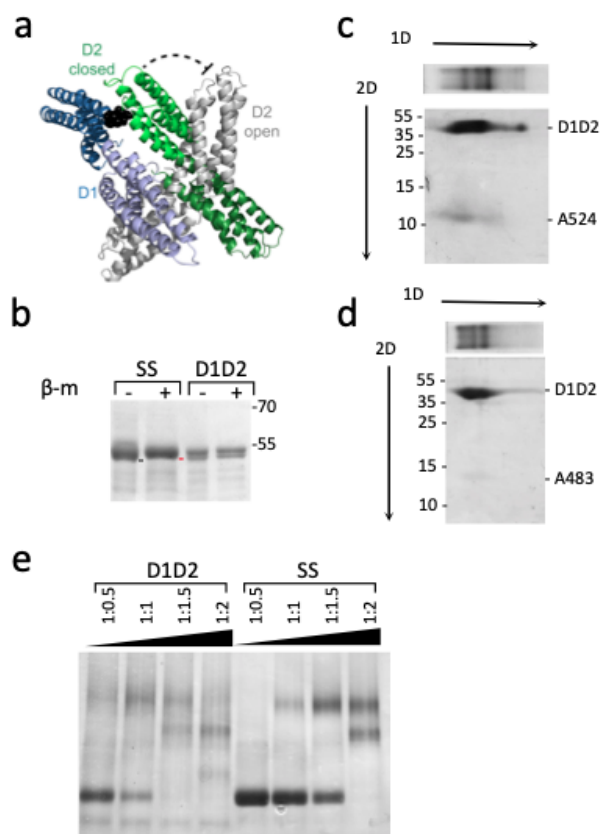


Supplementary Fig. 1. IpaA VBS3 reveals multiple binding sites on vinculin.

a, b, Solid phase binding assays. **a**, coating: HV; ligands: A483 (solid circles); A524 (solid squares). **b**, coating: D1 (solid circles) or HVD1D2 (solid squares); ligand: A483. **c, f-h**, BN-PAGE in 6-18% polyacrylamide gradient gels and Coomassie staining analysis of D1D2:A483 (**c**), D1D2:A524 (**f**), D1:A524 (**g**) or D1:A483 (**h**) complexes. The molar ratio is indicated above each lane. Arrowheads indicate protein alone, or complex migration at the indicated molar ratio. **d**, bands were recovered from BN-PAGE and analyzed in a second dimension SDS-PAGE in a 15% polyacrylamide gel and Coomassie staining. Bands were analyzed by densitometry. **e**, ratio of density values for the A524-D1D2 complex (empty bar) and A483-D1D2 complexes corresponding to the upper (light grey bar) or lower (dark grey bar) shifts.

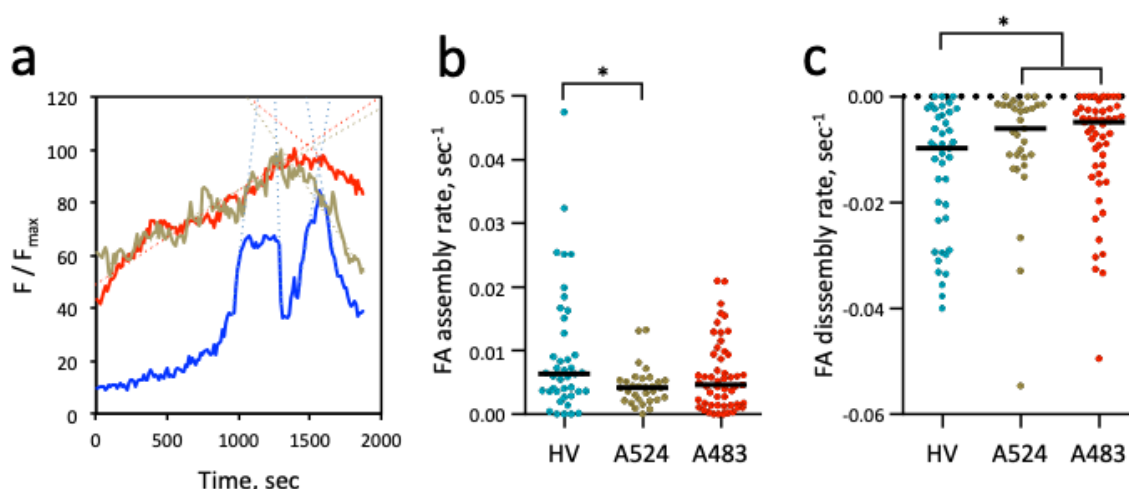


Supplementary Figure 2. Structural models of vD1:A524. **a**, Structure predicted from the resolved vD1: lpaA VBS1: and vD1:lpaA VBS2 crystal structures^{11, 12}. **b**, Structural model of vD1:A524. The model was established using RosettaCM protocol and accounts for 19 inter and intra-molecular cross-links out of 24 identified (Suppl. Table 1).



Supplementary Figure 3. Cysteine-clamped vinculin D1D2 does not form high order complexes. **a**, Structural model of cysteine-clamped vinculin. Blue: D1 domain. Green: D2 domain in the closed conformer. Grey: D2 domain in the open conformer. Black: C68-C396 cysteine clamp preventing the switch from closed to open conformers. **b-e**, Coomassie blue staining. **b**, Disulfide bridge formation in D1D2. D1D2: wild-type sequence. SS: D1D2 Q68C A396C. SDS-PAGE analysis using a 10 % polyacrylamide gel. + β -metOH: samples were boiled in Laemmli sample loading buffer containing 5 mM beta-mercaptoethanol prior to SDS-PAGE. The molecular weight markers in kDa are indicated. The black and red bars point the respective migration of unreduced and reduced D1D2 Q68C A396C, respectively. **c**, **d**, gel strips of the native-PAGE corresponding to the A524 + D1D2 (**c**) or A483 + D1D2 (**d**) as shown in **Fig. 1e** were analyzed in a second dimension by SDS-PAGE using a 10 % polyacrylamide gel as depicted. **e**, BN-PAGE analysis in a 6-18% polyacrylamide gradient gel of D1D2, SS, A483 and A524.

D1D2:A483 (D1D2) and SS:A483 (SS) complexes. The molar ratio is indicated above each lane.
Arrowheads indicate protein alone, or complex migration



Supplementary Figure 4. TIRF analysis of FA dynamics. C2. 7 cells were transfected with HV-mCherry (HV), HV-mCherry and GFP-A524 (A524) or HV and GFP-A483 (A483). The dynamics of HV-mCherry-labeled FAs were analyzed by TIRF microscopy. **a**, traces correspond to the variations of average fluorescence intensity of a representative single FA (F) normalized to its maximal average fluorescence intensity over the analyzed period in seconds (F_{max}). Blue: HV; green: HV+A524; red: HV+A483. **b**, **c**, instant assembly (**b**) or disassembly (**c**) rates were inferred from the slopes of linear fits as depicted in **a**), with a Pearson correlation value > 0.85 . HV: $n = 41$, $N = 3$; HV+A524: $n = 31$, $N = 2$; HV+A483: $n = 55$, $N = 3$. Mann-Whitney U test. *: $p < 0.05$

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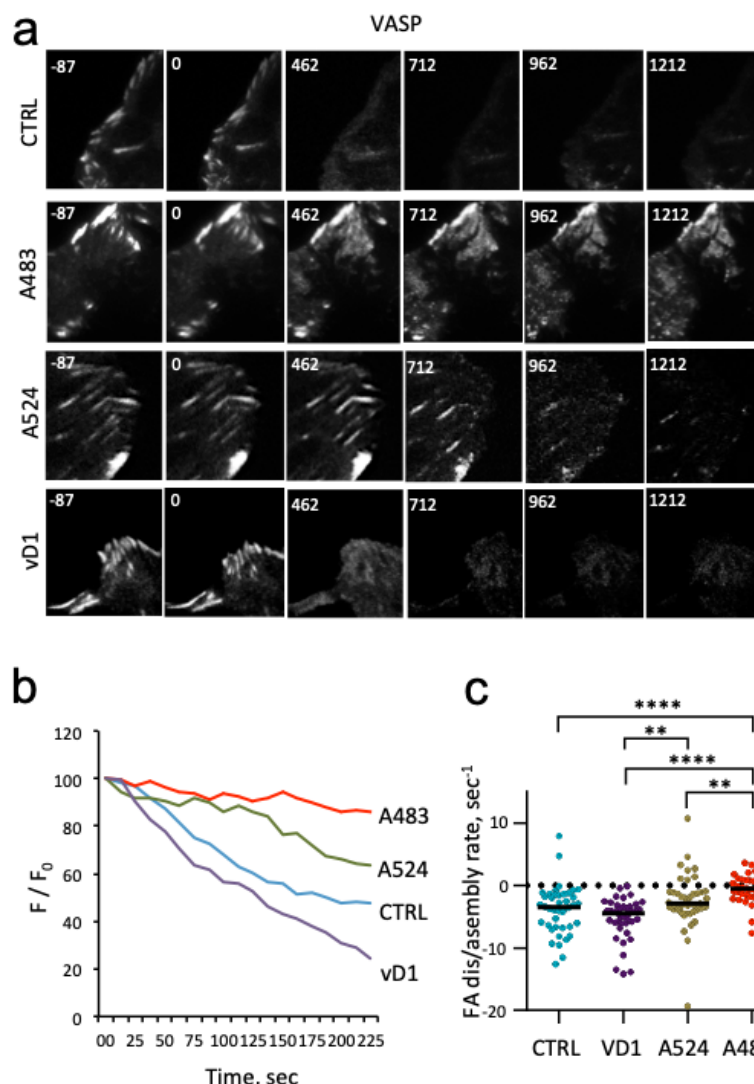
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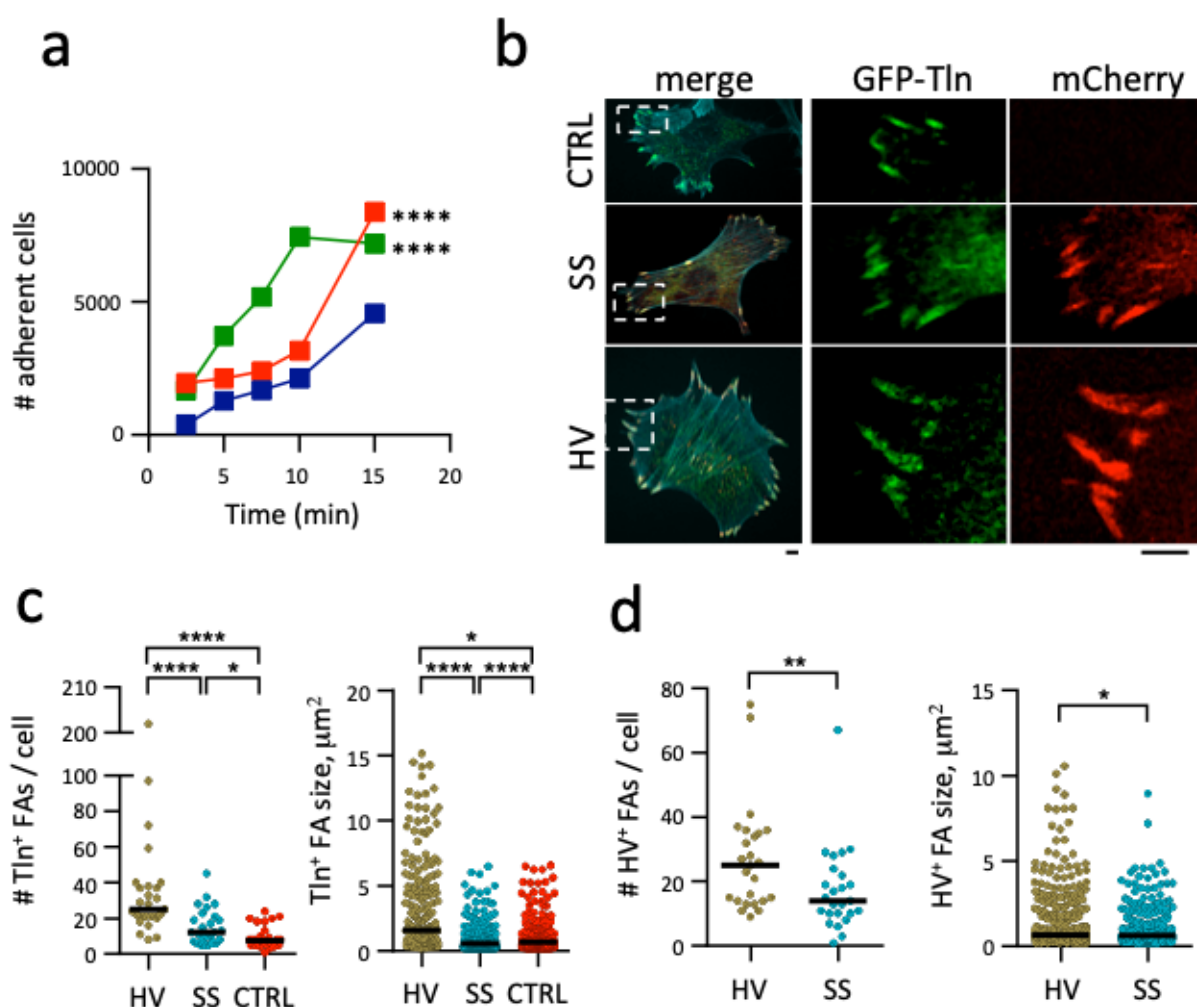
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Supplementary Figure 5. A483 stabilizes VASP-containing cell adhesions in the absence of mechanotransduction

TIRF microscopy of C2.7 cells transfected with mCherry-VASP alone (CTRL) or co-transfected with GFP-A524 (A524), GFP-vD1 (vD1), or GFP-A483 (A483). Adhesion kinetic parameters were determined from time-lapse acquisitions of cells co-transfected with mCherry-VASP and the indicated construct following treatment with 100 μM Y-27632. **a**) Representative time series acquisitions. Numbers indicated the elapsed time in seconds, with the inhibitor added at $t = 0$. Scale bar = 5 μm . **b**, $\Delta F/F_0$: normalized average fluorescence intensity of adhesions as a function of time. Representative traces corresponding to single adhesions for the indicate samples. **c**, initial rates of adhesion disassembly inferred from linear

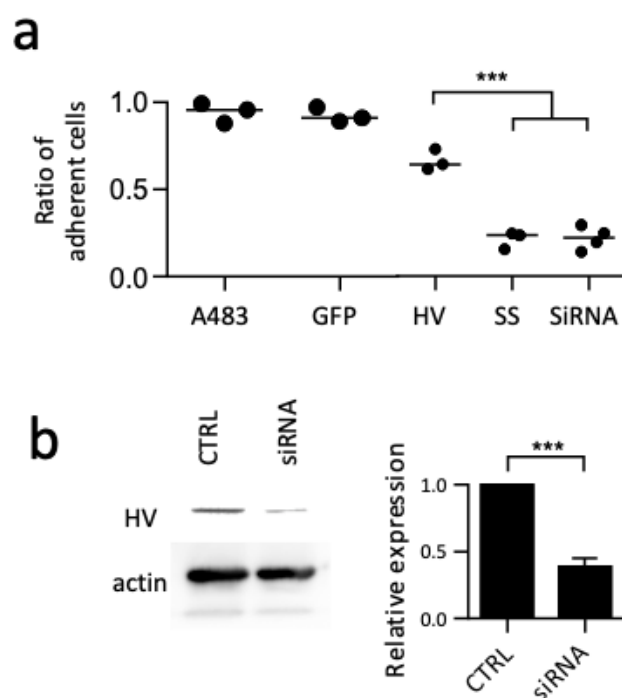
fits. N = 4. Number of adhesions analyzed: CTRL: 42; A483: 43; A524: 40; vD1: 40. Dunn's multiple comparisons test. *: $p < 0.05$; ****: $p < 0.001$.



Supplementary Figure 6. Effects of A483 and vinculin supra-activation on cell adhesion kinetics

a, 1205Lu melanoma cells were transfected with GFP alone (blue), GFP-A524 (red) or GFP-A483 (green), lifted up by trypsinization and plated for the indicated time on Fn-coated coverslips. Samples were washed, fixed and adherent cells were scored microscopically. The total number of adherent cells scored is indicated. GFP: 3223 cells, N = 4; GFP-A524: n=7418, N = 4; GFP-A483: n = 5668, N = 4. Chi square corrected with Bonferroni multiple comparison correction. ****: $p < 0.001$. **b-d**, MEF vinculin null cells were co-transfected with GFP-talin (CTRL) and full length vinculin-mCherry (HV) or HV Q68C A396C-mCherry (SS). Samples were fixed and processed for fluorescence staining of F-actin. **b**, Representative fluorescence micrographs. Red: Vinculin-mCherry; green:

GFP-talin; cyan: F-actin. Scale bar = 1 μ m. **c, d**, FAs were scored using a semi-automatic detection program (Methods) and their number per cell (left) and size (right) are shown. Bar: median size. FAs labeled with: **c**, GFP-talin; **d**, HV or SS. CTRL: n=28, N=3; HV: n = 25 , N = 3; SS: n = 25, N = 3. Mann-Whitney test with Bonferroni multiple comparison correction. * p<0.05; ** p<0.01; *** p<0.005; **** p<0.001.



Supplementary Figure 7. Role of vinculin supra-activation on cell adhesion.

a, 1205Lu melanoma cells were transfected with the indicated constructs, labeled with calcein (methods) and mixed with the same ratio of control cells. Cells were perfused in a microfluidic chamber and allowed to adhere for 30 min (large solid circles) or 15 min (small solid circles) prior to shear stress application. Scatterplot of the ratio of adherent cells: A483 (N = 3, n = 557); GFP (N = 3, n = 490); HV: vinculin mCherry (N = 3, n = 481);

610 SS: vinculin Q68C A396C-mCherry (N = 3, n = 259); siRNA: cells treated with anti-vinculin siRNA (N = 3, n = 395).
 611 Unpaired t test. ***: $p < 0.005$. **b**, 1205Lu melanoma cells were mock-transfected (CTRL) or treated with anti-
 612 vinculin siRNA (siRNA, Methods). Right: anti-vinculin Western blot analysis. Left: average HV band intensity
 613 normalized to that of control cells. Unpaired t test. ***: $p = 0.005$.
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SUPPLEMENTARY TABLES

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#	Exp. MH ⁺	Primary Score	Peptide Sequence	A524	Hv D1
32670	4457.1	3.28	IDDTSAELLTDDISDLKNNNDITAENNNIYK - MAKMIDER	D604	K173
32798	1924.16	2.61	INNKLK - ELLPVLISAMK	K540	E200
33042	4613.23	3.70	IDDTSAELLTDDISDLKNNNDITAENNNIYK - NLGPGMTKMAK	D594	K170
33042	4613.23	3.70	IDDTSAELLTDDISDLKNNNDITAENNNIYK - NLGPGMTKMAK	D595	K170
33042	4613.23	4.11	IDDTSAELLTDDISDLKNNNDITAENNNIYK - NLGPGMTKMAK	D604	K170
33322	5310.64	4.48	IDDTSAELLTDDISDLKNNNDITAENNNIYK - VGKETVQTTEQILKR	K600	D67
33334	2347.28	2.51	DVTTSLSKVLK - MSAEINEIIR	K625	E240
33432	2138.26	2.88	VLKNINKD - ELLPVLISAMK	K628	E200
33496	2315.32	3.09	AAKDVTTSLSK - ELLPVLISAMK	K617	E200
34822	2642.55	2.68	AKEVSSALSKVLK - ELLPVLISAMK	K579	E200
38218	3685.86	3.87	NINKD - TIESILEPVAQQISHLVIMHEEGEVDGK	K632	E31
38218	3685.86	3.66	NINKD - TIESILEPVAQQISHLVIMHEEGEVDGK	K632	E28
#	Exp. MH ⁺	Primary Score	Peptide Sequence	Hv D1	
34075	2342.29	3	ELLPVLISAMK - NLGPGMTKMAK	E200	K170

36135	3088.67	2.62	NFTVE K MSAEINEIR - ELLPVLISAMK	K236	E200
#	Exp. MH ⁺	Primary Score	Peptide Sequence	A524	
14281	3051.56	3.52	NYVT E TNADTIDKNHAIYEK - INN K LK	E554	K540
15477	3342.67	2.74	NYVT E TNADTIDKNHAIYEK - AKEVSSALSK	E554	K571
17603	3141.54	2.71	NYVTETNADTIDKNHAIYEK - EVSSALSK	K562	K572
31359	4483.2	2.64	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AKEVSSALSK	E590	K571
31996	2965.5	2.94	IDDTSAELLTDDIS D LK - AAKDVTTSLSK	D598	K617
31996	2965.5	2.51	IDDTSAELLT D DISDLK - AAKDVTTSLSK	D594	K617
32705	4586.24	4.1	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AAKDVTTSLSK	E608	K617
33253	3291.74	3.81	IDDTSAELLTDD I DISDLK - AKEVSSALS K VLSK	D595	K579
33997	5328.54	4.76	IDDTSAELLTDDISDLKNNNDITAENNNIYK - ID D TSAELLTDDISDLK	K600	D586
34007	4655.33	4.78	IDDTSAELLT D DISDLKNNNDITAENNNIYK - DVTTSLS K VLK	D594	K625

Suppl. Table 1. Cross-linked residues characterized in the D1:A524 complex (XL-amino acids of each protein are bolded in the sequences).

#	Exp. MH ⁺	Primary	Peptide Sequence	A524	Hv D1D2
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		Score			
20480	3470.68	3.86	NYVTETNADTIDKNHAIYEK - NLGPGMTKMAK	E554	K170
23021	3883.92	3.71	NYVTETNADTIDKNHAIYEK - ETVQTTEQILKR	K562	E60
24707	3117.73	5.00	AKEVSSALSKVLSK - VGKETVQTTEQILK	K579	E66
36724	4612.22	4.82	IDDTSAELLTDDISDLKNNNDITAENNNIYK - NLGPGMTKMAK	D604	K170
39712	5255.49	3.79	IDDTSAELLTDDISDLKNNNDITAENNNIYK - NLGPGMTKMAKMIDER	D598	K173
39893	5162.6	4.51	IDDTSAELLTDDISDLKNNNDITAENNNIYK - ALAKQVATALQNLQTK	E590	K464
40011	2641.56	4.45	AKEVSSALSKVLSK - ELLPVLISAMK	K579	E200
41002	3047.64	3.99	LKVT DANIR - GILSGTSDLLTFDEAEVR	K542	E128
41440	3944.01	3.86	IDDTSAELLTDDISDLK - AQQVSQGLDVLTA KVENAAR	D598	K366
42699	3685.87	4.03	NINKD -TIESILEPVAQQISHLVIMHEEGEVDGK	K632	E31
#	Exp. MH ⁺	Primary Score	Peptide Sequence	Hv D1D2	
25162	3323.71	4.54	LNQAKGWL RDPSASPGDAGEQAIR - ALASIDSK	K281	D274
30025	3169.64	4.03	GQGSSPVAMQKAQQVSQGLDVLTA K - VENAAR	K352	E368
30045	3278.66	3.69	VLQLTSWDEDAWASK - KLEAMTNSKQSIK	D275	K381
30201	3695.87	3.82	LNQAKGWL RDPSASPGDAGEQAIR - MSAEINEIIR	K281	E240
31264	3251.77	5.03	ALAKQVATALQNLQTKTNR - RQGKGDSPEAR	K464	E458
31264	3251.77	4.50	ALAKQVATALQNLQTKTNR - RQGKGDSPEAR	K464	D455

31620	2516.36	3.44	ALASIDSKLNQAK - MSAEINEIIR	K295	E240
35256	3787.88	4.12	GILSGTSDLLLTFD E AEVR - GSSHHHHHHSSGLVPR	E128	G12
38115	3310.75	4.29	GILSGTSDLLLTFD E AEVR - AVANSRPAKAAVH	E147	K507
39508	3685.90	3.56	GQGSSPVAMQKKAQQVSQGLDVLTA K - MSAEINEIIR	K352	E240
40134	3129.61	2.19	NQGIEEALKNRNFTVEKMSAEINEIIR	E224	K236
40285	3184.71	4.81	AQQVSQGLDVLTA K VENAAR - SLGEISALT S K	K366	E437
40296	2280.32	3.69	ELLPV L ISAMK - LNQA K GWLR	E200	K281
40351	3255.72	4.00	AQQVSQGLDVLTA K VENAAR - MSAEINEIIR	K366	E240
40571	4536.51	4.44	GDSPEARALAKQVATALQNLQTK - SLGEISALT S KLADLRRQ G K	D455	K444
40881	3704.92	4.32	KIDAAQNW L ADPN G GGPEGE E EQIR - ELLPV L ISAMK	K387	E200
40923	3100.63	3.94	AQQVSQGLDVLTA K VENAAR - KLEAMTNSK	E368	K373
41473	3723.01	4.34	GQGSSPVAMQKKAQQVSQGLDVLTA K - ELLPV L ISAMK	K352	E200
41482	4633.41	3.54	TIESILEPVAQQISHLVIMHEEGEVDGK - KLEAMTNSKQ S IAK	E28	K381
42201	3294.83	3.66	AQQVSQGLDVLTA K VENAAR - ELLPV L ISAMK	K366	E200
#	Exp. MH ⁺	Primary Score	Peptide Sequence	A524	
15424	3050.56	3.57	NYVTETNADTIDKNHAIYEK - INNKLK	E554	K540
16531	3340.67	3.55	NYVTETNAD T IDKNHAIYEK - AKEVSSALSK	E558	K571

16865	3341.67	4.11	NYVT E TNADTIDKNHAIYEK - AKEVSSALSK	E554	K571
34602	4584.25	4.05	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AAKDVTTSLSK	E590	K617
35202	4586.25	5.2	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AAKDVTTSLSK	D594	K617
35824	4583.26	3.43	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AAKDVTTSLSK	E608	K617
39340	4911.48	4.37	IDDTSAELLTD D ISDLKNNNDITAENNNIYK - AKEVSSALS K VLSK	D595	K579
39340	4911.48	4.06	IDDTSAELLTDDIS D LKNNNDITAENNNIYK - AKEVSSALS K VLSK	D598	K579
39340	4911.48	3.83	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AKEVSSALS K VLSK	E590	K579
39730	3291.74	3.64	IDDTSAELLTD D ISDLK - AKEVSSALS K VLSK	D595	K571
40244	4655.32	4.35	IDDTSAELLTD D ISDLKNNNDITAENNNIYK - DVTTSLS K VLK	D594	K625
40244	4655.32	4.35	IDDTSAELLTD D ISDLKNNNDITAENNNIYK - DVTTSLS K VLK	D595	K625

Suppl. Table 2. Cross-linked residues characterized in the D1D2:A524 complex (XL-amino acids of each protein are bolded in the sequences).

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#	Exp. MH ⁺	Primary Score	Peptide Sequence	A483	Hv D1D2
13237	3014.59	3.29	DITKSTTEHR - VGKETVQTTEDEQILKR	K530	E66
14671	2518.38	2.49	SKDITK - VGKETVQTTEDEQILKR	K526	E66
14791	2556.44	2.35	INNKLK - VGKETVQTTEDEQILKR	K540	E66
17806	2621.29	3.10	GSPGIPGDTYLTR - QQELTHQEHR	G517	E181
20039	2571.40	3.08	LKVTDANIR - ETVQTTEDEQILKR	K542	E66
20526	3651.79	3.37	NYVTETNADTIDKNHAIYEK - NSKNQGIEEALK	E554	K219
20985	3469.67	3.01	NYVTETNADTIDKNHAIYEK - NLGPGMTKMAK	D558	K170
22936	3468.66	2.62	NYVTETNADTIDKNHAIYEK - NLGPGMTKMAK	E554	K170
23137	4011	3.29	NYVTETNADTIDKNHAIYEK - VGKETVQTTEDEQILK	D561	K59
25418	3725.82	3.27	NYVTETNADTIDKNHAIYEK - ETVQTTEDEQILK	K562	E66
25418	3725.82	3.72	NYVTETNADTIDKNHAIYEK - ETVQTTEDEQILK	K562	E60
27618	3161.66	3.21	GSPGIPGDTYLTR - VGKETVQTTEDEQILKR	G517	E60
27618	3161.66	3.61	GSPGIPGDTYLTR - VGKETVQTTEDEQILKR	G517	D67
28366	2918.61	3.18	EVSSALS K LSK - VGKETVQTTEDEQILK -	K579	E66
29996	1977.96	3.06	GSPGIPGDTYLTR - MIDER	G517	D176
36497	2772.45	3.78	GSPGIPGDTYLTR - AQQVSQGLDVLTA K	G517	D361
36615	4613.23	3.68	IDDTSAELLTD D DISDLKNNNDITAENNNIYK - NLGPGMTKMAK	D594	K170
36632	2214.27	3.22	AKEVSSALS K - ELLPVLISAMK -	K571	E200
37536	3824.86	2.64	GSPGIPGDTYLTR - KIDAAQNW L ADPN G GGPEGE E EQIR	G517	D389

40263	3413.78	3.79	GSPGIPG D TYLTR - AQQVSQGLDVLTA K VENAAR	D484	K366
40432	5992.94	5.1	IDDTSAELLTDDISDLKNNNDITAENNNIYK - GQGSSPVAMQKAQQVSQGLDVLTA K	E590	K362
40679	2708.42	3.25	S KDITK - GILSGTSDLLLT F DEAEVR	K526	E128
40994	3074.69	3.12	K VTNSLSNLISLIGTK - ETVQTTEDQILK	K498	E66
41187	4612.22	2.86	IDDTSAELLTDDISDLKNNNDITAENNNIYK - NLGPGMT K MAK	E590	K170
41816	2387.41	3.65	DVTTSLS K VLK - ELLPVLISAMK	K625	E200
43326	3137.65	3.41	AAKDVTTSLSK - GILSGTSDLLLT F DEAEVR	K625	E128
43537	3542.88	3.63	IDDTSAELLT D DISDLK - ALAKQVATALQNLQTK	D594	K464
43612	3604.91	3.34	VTNSLSNLISLIGT K SGTQER - ETVQTTEDQILK	K513	E66
45081	3350.71	3.48	G SPGIPGDTYLTR - GILSGTSD L LLTFDEAEVR	G517	D121
53474	5745.03	4.09	NIN K D - TIESILEPVAQQISHLVIMHEEG E VDGKAIPDLTAPV AAVQAAVSNLVR	K632	E31
#	Exp. MH ⁺	Primary Score	Peptide Sequence	Hv D1D2	
17582	1670.89	3.51	VGKETVQTTEDQILK	K59	E66
17582	1670.89	3.38	VGKETVQTTED D QILK	K59	D67
26000	3322.71	4.03	LNQAKGWLDRPSASPGDAGEQAIR - ALAS I DSK	K281	D374
30901	3169.64	3.79	GQGSSPVAMQ K AQQVSQGLDVLTA K - VENAAR	K352	E368
31031	3695.87	3.81	LNQAKGWLDRPSASPGDAGEQAIR - MSAEINEI R	K281	E240
35827	3665.8	3.75	K IDAAQNWLDAPNGGPEGEEQIR - MSAEINEI R	K387	E240

38215	3040.71	3.43	VGKETVQTTEDEQILKR - ELLPVLISAMK	K59	K200
39734	3614.9	4.15	GQGSSPVAMQKAQQVSQGLDVLTA - SLGEISALTSK	K352	E437
40218	3580.72	3.88	VLQLTSWDEDAWASKDTEAMK - MSAEINEIR	K261	E240
40418	2881.63	3.77	QVATALQNLQTKTNR - ELLPVLISAMK	K476	E200
40559	3184.71	4.71	AQQVSQGLDVLTAKEVENAAR - SLGEISALTSK	K366	E437
40560	2280.32	3.35	ELLPVLISAMK - LNQAKGWLR	E200	K281
40604	2553.46	3.78	ALASIDSKLNQAK - ELLPVLISAMK	K276	E200
41140	2892.69	3.97	ALAKQVATALQNLQTK - ELLPVLISAMK	K464	E200
41598	4173.16	5.49	GQGSSPVAMQKAQQVSQGLDVLTAKEVENAAR - KLEAMTNSK	K366	E375
41598	4173.16	5.8	GQGSSPVAMQKAQQVSQGLDVLTAKEVENAAR - KLEAMTNSK	E368	K373
41598	4173.16	4.98	GQGSSPVAMQKAQQVSQGLDVLTAKEVENAAR - KLEAMTNSK	D361	K373
41682	3726.02	3.87	GQGSSPVAMQKAQQVSQGLDVLTAKEVENAAR - ELLPVLISAMK	K352	E200
42387	3293.83	4.63	AQQVSQGLDVLTAKEVENAAR - ELLPVLISAMK	K366	E200
42387	3293.83	3.3	AQQVSQGLDVLTAKEVENAARK - LNQAKGWLR	E368	K281
#	Exp. MH ⁺	Primary Score	Peptide Sequence	A483	
17398	3339.66	3.31	NYVTETNADTIDKNHAIYEK - AKEVSSALSK	D561	K571
17630	3341.68	3.44	NYVTETNADTIDKNHAIYEK - AKEVSSALSK	D558	K571
17630	3341.68	4.19	NYVTETNADTIDKNHAIYEK - AKEVSSALSK	E554	K571

20071	3141.53	3.53	NYVTETNADTID KN HAIYEK - AKEVSSALSK	K562	E572
20150	3349.69	4.44	NYVTETNAD T IDKNHAIYEK - LKVTDANIR	D558	K542
20150	3349.69	4.03	NYVTETNADTID K NHAIYEK - LKVTDANIR	D561	K542
35163	4584.24	3.59	IDDTSAELLT D DISDLKNNNDITAENNNIYK - AAKDVTTSLSK	D594	K617
35163	4584.24	3.49	IDDTSAELLT D ISDLKNNNDITAENNNIYK - AAKDVTTSLSK	D595	K617
35382	4586.24	3.35	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AAKDVTTSLSK	E590	K617
35967	4584.24	4.42	IDDTSAELLTDDIS D LKNNNDITAENNNIYK - AAKDVTTSLSK	D598	K617
35967	4584.24	4.95	IDDTSAELLTDDISDLKNNNDITA E NNNIYK - AAKDVTTSLSK	E608	K617
35967	4584.24	4.52	IDDTSAELLTDDISDLKNNNDITAENNNIYK - AAK D VTTSLSK	K600	D618
35967	4584.24	4.22	IDDTSAELLTDDISDLKNNNDITAENNNIY K - AAK D VTTSLSK	K614	D618
35967	4584.24	4.94	IDDTSAELLTDDISDLKNNN D ITAENNNIYK - AAKDVTTSLSK	D604	K617
39881	2845.52	4.73	VTNSLSNLISLIGT K SGTQER - ELQ E K	K513	E520
39881	2845.52	4.62	VTNSLSNLISLIGT K SGTQER - ELQ E K	K513	E523
40044	4655.33	5.07	IDDTSAELLT D DISDLKNNNDITAENNNIYK - DVTTSLSKV LK	D594	K625
40044	4655.33	4.86	IDDTSAELLT D ISDLKNNNDITAENNNIYK - DVTTSLSKV LK	D595	K625

40044	4655.33	4.45	IDDTSAELLTDDIS DL KNNNDITAENNNIYK - DVTTSLSKV LK	D598	K625
40044	4655.33	4.44	IDDTSAELLTDDIS DL KNNNDITAENNNIYK - DVTTSLSKV LK	E590	K625
40065	3092.6	3.51	IDDTSAELLT DD ISDLK - EVSSALS SKVLSK	D594	K579
40065	3092.6	3.38	IDDTSAELLT DD ISDLK - EVSSALS SKVLSK	D594	K579
40069	3093.61	3.36	IDDTSAELLTDDIS DL K - EVSSALS SKVLSK	D598	K579
40095	2988.61	4.48	VTNSLSNLISLIG TK SGTQER - VT D ANIR	K513	D545
40448	3252.74	3.81	VTNSLSNLISLIG TK SGTQER - ETIF E ASKK	K513	E494
40603	5153.62	4.36	IDDTSAELLT DD ISDLKNNNDITAENNNIYK - KVTNSLSNLISLIG TK	D594	K498
40603	5153.62	4.46	IDDTSAELLT DD ISDLKNNNDITAENNNIYK - KVTNSLSNLISLIG TK	D595	K498
40603	5153.62	4.56	IDDTSAELLTDDIS DL KNNNDITAENNNIYK - KVTNSLSNLISLIG TK	D598	K498
40603	5153.62	3.99	IDDTSAELLTDDISDLKNNND I TAENNNIYK - KVTNSLSNLISLIG TK	D604	K498
40606	5153.62	3.53	IDDTSAELLTDDISDLKNNNDITAENNNIYK - KVTNSLSNLISLIG TK	E590	K498
41116	2593.44	3.85	KVTNSLSNLISLIG TK - ETIF E ASK	K498	E490
41142	3909.09	4	ETIF E ASKKVTNSLSNLISLIG TK - G SPGIPGDTYL R	E490	G477

Suppl. Table 3. Cross-linked residues characterized in the D1D2 -A483 1:1 complex (XL-amino acids of each protein are bolded in the sequences)

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Suppl. movie 1. TIRF analysis of HV-mCherry expressing C2.7 cells co-transfected with a GFP fusion to the indicated construct. The time is indicated in seconds.

Suppl. movie 2. TIRF analysis of HV-mCherry expressing C2.7 cells co-transfected with a GFP fusion to the indicated construct. The time is indicated in seconds. At time "0", addition of the Rho kinase inhibitor Y-27632 was added at 100 μ M final concentration.

Suppl. movie3. TIRF analysis of VASP-mCherry expressing C2.7 cells co-transfected with a GFP fusion to the indicated construct. The time is indicated in seconds. At time "0", addition of the Rho kinase inhibitor Y-27632 was added at 100 μ M final concentration.

Suppl. Movie 4. 1205Lu melanoma cells 1205Lu melanoma cells were transfected with the indicated constructs. Cells were perfused in a microfluidic chamber and allowed to adhere for 20 min prior to application of shear stress reaching 22.2 dynes.cm⁻². The elapsed time is indicated in seconds.