

1 **A flexible loop in the paxillin LIM3 domain mediates direct binding to integrin $\beta 3$**

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52 **Abstract**

53 Integrins are fundamental for cell adhesion and the formation of focal adhesions (FA).
54 Accordingly, these receptors guide embryonic development, tissue maintenance and
55 haemostasis, but are also involved in cancer invasion and metastasis. A detailed
56 understanding of the molecular interactions that drive integrin activation, focal
57 adhesion assembly, and downstream signalling cascades is critical. Here, we reveal
58 a direct association of paxillin, a marker protein of focal adhesion sites, with the
59 cytoplasmic tails of the integrin $\beta 1$ and $\beta 3$ subunits. The binding interface resides in
60 paxillin's LIM3 domain, where based on the NMR structure and functional analyses a
61 flexible, seven amino acid loop engages the unstructured part of the integrin
62 cytoplasmic tail. Genetic manipulation of the involved residues in either paxillin or
63 integrin $\beta 3$ compromises cell adhesion and motility. This direct interaction between
64 paxillin and the integrin cytoplasmic domain identifies an alternative, kindlin-
65 independent mode of integrin outside-in signalling particularly important for integrin $\beta 3$
66 function.

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69 **Keywords:** Paxillin, LIM domain, integrin, kindlin2, talin, NMR spectroscopy, solution
70 structure, chemical shift perturbation mapping, cell adhesion

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73 Introduction

74 Integrins are specialized cell surface receptors of animal cells that sense the
75 extracellular matrix and coordinate cell adhesion with the organization of the
76 cytoskeleton (Sun et al., 2016). Integrins are transmembrane glycoproteins consisting
77 of an α and β subunit, with 18 distinct integrin α and 8 integrin β subunits encoded in
78 the human genome (Hynes, 2002). The high affinity, active conformation of these
79 heterodimers can be stabilized either by extracellular ligand binding (outside-in
80 signalling) or by distinct intracellular signalling processes, which allow the association
81 of the cytosolic scaffolding proteins talin and kindlin with the β subunit cytoplasmic tail
82 (inside-out signalling) (Sun et al., 2019). Active integrins together with their binding
83 partners talin and kindlin serve as the nucleus for the initiation of large, multimeric
84 protein complexes termed focal adhesions (FAs) (Geiger and Yamada, 2011; Moser
85 et al., 2009). Combined biochemical, genetic and microscopic analyses have revealed
86 the stratified layout of focal adhesions and identified the characteristic compendium of
87 signalling and adaptor proteins, the so-called integrin adhesome (Chastney et al.,
88 2020; Horton et al., 2015; Kanchanawong et al., 2010; Zaidel-Bar et al., 2007). While
89 the essential roles of talin and kindlin in initiating integrin-based adhesion sites in
90 various cell types have become clear (Bachmann et al., 2019), the function of other
91 core adhesome proteins during the initial steps of focal adhesion formation is still
92 debated.

93 For example, LIM (Lin-11, Isl1, MEC-3) domain containing adapter proteins are a
94 highly enriched subgroup of integrin adhesome proteins thought to be involved in
95 mechanosensing (Anderson et al., 2021; Horton et al., 2015; Schiller et al., 2011).
96 Individual LIM domains encompass ~60 amino acids forming a double zinc finger
97 motif, which mediates binding to other proteins or nucleic acids (Kadomas and

98 Beckerle, 2004; Matthews et al., 2009). A prominent member of this group of adapter
99 proteins is paxillin, which contains four LIM domains and is ubiquitously expressed in
100 mammalian tissues (Deakin et al., 2012; Deakin and Turner, 2008). Paxillin is
101 commonly employed as a marker for FAs and nascent focal complexes under various
102 conditions, even where the normal morphology, function and architecture of FAs is
103 disturbed. Paxillin is one of the first proteins recruited to FAs (Digman et al., 2008),
104 efficiently localizes there even in the absence of myosin-generated forces (Pasapera
105 et al., 2010), and can recruit vinculin to FAs in talin knockout cells (Atherton et al.,
106 2020). Paxillin is found in nanometer distance from the plasma membrane with the
107 carboxy-terminus detected in the same confined membrane-proximal layer as the
108 cytoplasmic domain of integrin αv (Kanchanawong et al., 2010). A main determinant
109 of paxillin's efficient recruitment to integrins seems to be its association with kindlin2,
110 which has been mapped to the amino-terminal LD domains and the carboxy-terminal
111 LIM4 domain of paxillin (Bottcher et al., 2017; Gao et al., 2017; Zhu et al., 2019).
112 Interestingly, initial work identified the paxillin LIM2 and LIM3 domains as being critical
113 for focal adhesion targeting (Brown et al., 1996). Moreover, paxillin can localize to FAs
114 in the absence of kindlins (Klaproth et al., 2019; Theodosiou et al., 2016) suggesting
115 additional, kindlin-independent mode(s) of integrin engagement, presumably involving
116 paxillin LIM2 and LIM3.

117 Here, we demonstrate that the paxillin LIM2 and LIM3 domains directly interact with
118 carboxy-terminal residues of the integrin β subunit. Biochemical analysis of
119 recombinant proteins, the NMR-based 3D-structure of the paxillin LIM domains, and
120 functional analysis of mutated paxillin and integrin $\beta 3$ in vitro and in the cellular context
121 reveal that this interaction is based on a clamp-like extension in paxillin's LIM3 domain,
122 which contributes to cellular responses towards integrin ligands.

123 **Results**

124 **Paxillin LIM2/3 domains can directly bind the cytoplasmic tails of integrin $\beta 1$ and**
125 **$\beta 3$**

126 In line with previous reports (Pasapera et al., 2010; Schiller et al., 2011), we recently
127 observed that paxillin can be recruited in the absence of force to clusters of the integrin
128 β CT, similar to the behaviour of known integrin binding partners such as talin and
129 kindlin2 (Baade et al., 2019). We wondered whether recruitment to clustered integrin
130 β tails is a general feature of LIM domain containing adhesome proteins. To this end,
131 we used CEACAM-integrin β CT fusion proteins (CEA-ITGB1 or CEA-ITGB3), which
132 can be engaged from the outside of the cell by multivalent CEACAM binding bacteria
133 (*Neisseria gonorrhoeae*). This process initiates microscale accumulation of free
134 integrin β tails mimicking nascent adhesion formation and was therefore named Opa
135 protein triggered integrin clustering (OPTIC) (Suppl. Fig. S1A) (Baade et al., 2019).
136 Interestingly, when co-expressed with CEA3-ITGB1 or CEA3-ITGB3, only paxillin and
137 the closely related proteins Hic-5 and leupaxin showed a significant enrichment
138 (Suppl. Fig. S1B-C). All other LIM domain proteins did not accumulate at clustered
139 integrin β tails (Suppl. Fig. 1C-D). Paxillin recruitment to integrin clusters was
140 dependent on the LIM domains, since the paxillin C-terminus encompassing LIM1-
141 LIM4, but not the isolated N-terminal LD1-LD5 domains, strongly accumulated at
142 integrin β cytoplasmic tails, and paxillin LIM1-LIM4, but not the LD1-5 domains,
143 displaced full length paxillin from focal adhesion sites (Fig. 1A and B). These results
144 suggest that paxillin, leupaxin and Hic-5 differ from other LIM domain containing
145 adhesome proteins by their ability to locate at clustered integrin β tails and confirm the
146 important role of the paxillin LIM domains for the specific subcellular localization.
147 Moreover, pull-down assays with purified, recombinant proteins demonstrated that,

148 similar to the talin F3 domain and Kindlin2, the paxillin LIM2/3 domains mediate a
149 direct interaction of this adapter protein with the cytoplasmic tails of integrin $\beta 1$ and $\beta 3$
150 (Fig. 1C).

151

152 **The solution structure of paxillin LIM2/3 reveals a flexible loop region in the LIM3
153 domain**

154 To investigate the direct interaction between paxillin and the integrin β subunit in more
155 detail, we used NMR spectroscopy to gain structural insight and to delineate the
156 binding interface. Since LIM3 and to a lesser extent LIM2 have been shown to be
157 mainly responsible for FA targeting of paxillin (Brown et al., 1996), we expressed the
158 LIM2/3 tandem domain (aa380-499) of human paxillin and determined its solution
159 structure based on heteronuclear multidimensional NMR experiments (Sattler et al.,
160 1999). Both the LIM2 and the LIM3 domain of paxillin exhibit the characteristic double
161 zinc finger motif as described for other LIM domain containing proteins and paxillin
162 family members (Kadomas and Beckerle, 2004; Kontaxis et al., 1998; Matthews et al.,
163 2009; Perez-Alvarado et al., 1994). Each domain comprises two orthogonally packed
164 β -hairpins, followed by an α -helix (Fig. 1D and E). Interestingly, the LIM2 and LIM3
165 domains are connected by a short linker of 4 amino acids (F438-K441) that provides
166 some degree of flexibility between both domains. This would fit into the previously
167 proposed scenario of the LIM domains as a sort of molecular ruler and/or tension
168 sensor (Schiller et al., 2011).

169 According to the measurements of the heteronuclear NOE between HN and N of the
170 amides, the linker between LIM2 and LIM3 shows only slightly higher flexibility on the
171 ps-to-ns timescale than the domains themselves (Fig. S2A). But there are less long-

172 range NOE contacts in this region, suggesting that the structure is less densely packed
173 here, resulting in differential relative orientations of the domains (Fig. 1D and E).

174 Interestingly, by analyzing the heteronuclear NOEs and the structural definition of the
175 final ensemble of conformers, we identified a 7 amino acid stretch (F475-F481) in the
176 second zinc-finger of the LIM3 domain that constitutes a flexible, surface exposed loop
177 in the free protein (Fig. 1E). This loop is flanked by F475 and F481 and is situated
178 adjacent to a hydrophobic patch or groove. In this region, the amino acid sequence
179 and in particular residues F475, F480 and F481 are highly conserved within paxillin
180 family members and across species (Suppl. Fig. S2B). We speculated that this flexible
181 loop might act in concert with its opposing residues (E451, N452 and Y453) of the first
182 zinc-finger and the hydrophobic patch to support a clamp-like mechanism for integrin
183 β CT binding.

184

185 **The C-terminal residues of integrin β 3 cytoplasmic tail are crucial for paxillin
186 binding**

187 To identify the binding region of paxillin LIM2/3 on the integrin β CT, we titrated
188 unlabeled paxillin LIM2/3 to ^{15}N labeled cytoplasmic tails of human integrin β 1 (aa758-
189 798) or β 3 (aa748-788), respectively. In both cases, significant chemical shift
190 perturbations (CSPs) of specific integrin residues could be observed (Fig. 2A and B,
191 and Suppl. Fig. S3A and B). A dissociation constant (K_D) of 52 ± 30 μM could be
192 determined for integrin β 1 (Suppl. Fig. S3A), while integrin β 3 showed a higher K_D
193 (528 ± 130 μM) (Fig. 2A). These findings are in line with our previous microscopic
194 observations, where recruitment of paxillin to CEA-ITGB1 was more pronounced
195 (Suppl. Fig. 1C). Surprisingly, when mapping the CSPs onto the primary sequence of

196 integrin β CTs, the interacting regions were distinct. While the highest CSPs in integrin
197 β 1 CT were distributed over the membrane proximal NPxY motif and a neighbouring
198 conserved TT motif (Suppl. Fig. S3B), the largest chemical shifts in integrin β 3 were
199 confined to the eight C-terminal amino acids, spanning the membrane distal NxxY
200 motif (Fig. 2B). Indeed, deleting eight amino acids from the C-terminus of integrin β 3
201 (Δ 8aa) completely abrogated paxillin LIM2/3 binding in pulldown experiments, while
202 deletion of the last three amino acids (Δ 3aa) significantly reduced paxillin binding (Fig.
203 2C). As expected, applying either of these mutant integrin β 3 CTs in titration
204 experiments with paxillin LIM2/3 yielded no significant CSPs, confirming the loss of
205 interaction (Fig. 2D and E and Suppl. Fig. S3C-D). The biochemical data were further
206 corroborated by OPTIC assays, where both the Δ 3aa and the Δ 8aa mutant, but not the
207 S778A mutation in the kindlin binding site, diminished paxillin recruitment to clustered
208 integrin β 3 tails (Suppl. Fig. S3E-F).

209

210 **Paxillin directly associates with the C-terminus of integrin β 3 to contribute to**
211 **cell spreading**

212 To study the physiological relevance of these mutations, we generated integrin β 3
213 knockout fibroblasts via CRISPR/Cas9 and stably re-introduced either full-length
214 integrin β 3 wt or one of the truncated integrin β 3 variants, Δ 8aa or Δ 3aa (Suppl. Fig.
215 S4A and B). While integrin β 3-deficient cells exhibited a strongly impaired initial
216 spreading on vitronectin- and fibronectin-coated substrates, the re-expression of
217 integrin β 3 wt reverted this phenotype (Fig. 3A and B). In contrast, integrin β 3 Δ 3aa,
218 and even more so integrin β 3 Δ 8aa re-expressing fibroblasts were still impaired in their
219 spreading ability (Fig. 3A and B). A similar spreading defect on integrin ligands has

220 also been reported for kindlin-deficient cells and deletions of the integrin carboxy-
221 terminus might also corrupt the kindlin binding site, indirectly affecting the recruitment
222 of paxillin. To substantiate our biochemical findings of a direct paxillin interaction with
223 the integrin β subunit, we employed kindlin1 and 2-deficient double knock-out cells
224 (Bottcher et al., 2017; Theodosiou et al., 2016). As these cells display strongly
225 diminished expression of integrin β 3, we introduced integrin β 3 wt, integrin β 3 Δ 8aa,
226 or integrin β 3 Δ 3aa into the kindlin1/2 KO cells (Suppl. Fig. S4C and D). As reported
227 before (Bottcher et al., 2017), the kindlin1/2 deficient fibroblasts hardly attached and
228 did not spread on the vitronectin-coated substrate (Fig. 3C). However, paxillin-positive
229 focal attachment sites appeared upon expression of integrin β 3 wt in the kindin1/2
230 deficient cells, whereas no such matrix contact sites were detectable in cells
231 expressing the truncated integrin β 3 mutants (Fig. 3C). The adhesions appeared upon
232 plating of the integrin β 3 wt cells onto vitronectin, but not upon plating onto poly-L-
233 lysine (Fig. 3C and Suppl. Fig. S4E), and these integrin β 3-mediated contacts also
234 stained positive for talin (Fig. 3D and Suppl. Fig. S4E). Altogether, our results
235 demonstrate that paxillin can localize to FAs in the absence of kindlins and that this
236 process requires the paxillin binding site in the integrin β 3 cytoplasmic tail.

237

238 **The LIM3 flexible loop mediates direct association with the integrin β 3
239 cytoplasmic tail**

240 To precisely identify the integrin binding site within the paxillin LIM2/3 domains, we
241 titrated the unlabeled CT of human integrin β 3 to ^{15}N -labelled paxillin LIM2/3. Similar
242 to the inverse titrations, the K_D value of this interaction was determined to 532 ± 239
243 μM (Suppl. Fig. S5A). Importantly, the most prominent CSPs in paxillin were recorded

244 within the second zinc-finger of the LIM3 domain, specifically in the flexible loop region
245 between F475 and F481 (Fig. 4A, B and C). To verify our NMR based epitope mapping
246 we individually mutated residues of the loop region. Interestingly, exchanging
247 phenylalanine F475, F480, or F481 for alanine caused a complete or partial unfolding
248 of the LIM3 domain (Suppl. Fig. S6A-F). Though these residues show strong CSPs
249 and participate in integrin binding, these phenylalanines are also essential for
250 maintaining the structure of the LIM domain. To gain further insight into the role of the
251 loop region, these phenylalanines were left intact, but instead the residues between
252 F475 and F480 were mutated to alanine (LIM2/3-4A). Paxillin LIM2/3-4A exhibited a
253 stable LIM domain fold, however, no saturable CSPs could be detected when titrating
254 up to millimolar concentrations of integrin $\beta 3$ to ^{15}N -labelled LIM2/3-4A (Suppl. Fig.
255 S7A and B). Pulldown assays with the paxillin LIM2/3-4A mutant confirmed diminished
256 binding to the integrin $\beta 3$ CT (Fig. 4D). Next, we introduced the LIM3 loop mutations
257 into full length paxillin (GFP-PXN-4A) to corrupt the direct engagement of integrin $\beta 3$
258 in intact cells. In addition, we generated a truncated paxillin lacking the LIM4 domain
259 (GFP-PXN Δ LIM4) to interfere with the kindlin-mediated indirect binding of paxillin to
260 the integrin beta subunit. GFP-tagged paxillin wt, GFP-PXN-4A, or GFP-PXN Δ LIM4,
261 were re-introduced into paxillin knockout fibroblasts and their cell spreading was
262 monitored (Fig. 4E and F). As expected, spreading of paxillin KO cells on vitronectin
263 was strongly impaired. Re-expression of GFP-paxillin wt rescued this phenotype,
264 whereas the GFP-PXN-4A mutant was not able to revert the spreading defect and
265 mimicked paxillin KO cells in the first 2 h after seeding on the substrate (Fig. 4E and
266 F). In contrast, paxillin lacking the LIM4 domain reconstituted cell spreading in a similar
267 manner as wildtype paxillin (Fig. 4E and F). Moreover, paxillin wildtype and PXN
268 Δ LIM4 reverted the round, circular morphology of the paxillin-knock-out fibroblasts to

269 the spindle shaped, pointed cell phenotype of the wildtype fibroblasts, whereas cells
270 re-expressing GFP-PXN-4A retained the elevated circularity of the paxillin knock-out
271 cells (Fig. 4E). Taken together, these results underscore the functional importance of
272 the direct interaction between paxillin and integrin $\beta 3$. The clamp-like mechanism
273 afforded by the flexible loop in paxillin's LIM3 domain could stabilize the association
274 with the integrin $\beta 3$ cytoplasmic domain and modulates integrin $\beta 3$ -initiated cellular
275 responses.

276

277 **Discussion**

278 Although paxillin was discovered more than 30 years ago and constitutes a core focal
279 adhesion protein, its mode of focal adhesion targeting has remained controversial.
280 Recent biochemical approaches have pointed to an indirect association of paxillin with
281 the integrin $\beta 1$ and $\beta 3$ subunits via the integrin binding partners kindlin1 or kindlin2
282 (Gao et al., 2017; Theodosiou et al., 2016). As the association of kindlin with paxillin
283 appears to rest on the paxillin LD repeats and the LIM4 domain (Bottcher et al., 2017;
284 Zhu et al., 2019), these findings do not explain the central role of the LIM3 domain for
285 focal adhesion localization, which has been delineated by microscopic observations in
286 intact cells (Brown et al., 1996). Here, we present evidence for a direct interaction
287 between the paxillin LIM3 domain and the cytoplasmic tails of integrin $\beta 1$ and integrin
288 $\beta 3$, respectively. Together with the indirect link provided by kindlin, the intimate
289 association of paxillin with the integrin β subunit now unveils the full spectrum of
290 paxillin's focal adhesion recruitment modalities and unmasks the fundamental building
291 principles of cellular attachment sites.

292 In solution, paxillin's LIM2 and LIM3 domains adopt an overall fold consistent with
293 available structural data for LIM domains of other proteins (Kadrymas and Beckerle,
294 2004; Kontaxis et al., 1998; Matthews et al., 2009; Perez-Alvarado et al., 1994).
295 However, our NMR structure reveals an intriguing detail, which is conserved in paxillin
296 orthologues from other species. Indeed, the LIM3 domain of paxillin harbours a
297 flexible, surface exposed loop, which, based on sequence homology, is also present
298 in the paxillin family members Hic-5 and leupaxin. This loop demarcates the integrin
299 binding site in the LIM3 domain and appears to function as a clasp to stabilize the
300 association of paxillin with the integrin cytoplasmic tail. This additional direct
301 interaction between the integrin β cytoplasmic tail and paxillin now consolidates an

302 emerging principle of focal adhesion organization: each core component of focal
303 adhesions, including talin, Kindlin, paxillin, FAK, vinculin, and α -actinin is able to
304 sustain multiple, independent interactions with other FA components. In analogy to a
305 steel frame construction, this kind of assembly not only allows a stepwise expansion
306 of the protein complex, but also provides a further mechanical reinforcement with
307 every incoming component. In the specific example of paxillin, this protein can
308 associate via its LIM4 domain with integrin-bound kindlin2 (Zhu et al., 2019), but then
309 paxillin will also be in place to exploit the clamping mechanism build in its LIM3 domain
310 to bind the integrin β subunit and to re-enforce this tripartite complex as a pre-requisite
311 for efficient initial cell spreading. Furthermore, as paxillin can interact via its LIM1/2
312 domains with the talin head (Ripamonti et al., 2021) and via its LD1 domain with the
313 talin rod region (Zacharchenko et al., 2016), there is the possibility that paxillin
314 strengthens the early integrin-associated protein complex beyond kindlin. Indeed, a
315 stabilization of the integrin-talin-kindlin nexus by paxillin has been observed (Gao et
316 al., 2017). Multiple reciprocal interactions between talin, kindlin, paxillin and the
317 integrin β tail would also increase the avidity and might demonstrate once more how
318 multivalent low-affinity interactions such as those observed for paxillin LIM3 and
319 integrin β 3 stabilize macromolecular networks. Indeed, the relatively low affinity
320 between LIM3 and integrin β 3 might be a prerequisite for efficient assembly and
321 disassembly of FAs.

322 Intriguingly, such reciprocal interactions between focal adhesion core components
323 could also be the basis for the astonishing flexibility in the temporal sequence, in which
324 these proteins can assemble at integrin cytoplasmic domains. For example, talin
325 binding to the integrin β tail appears as a pre-requisite for the recruitment of its binding
326 partner FAK (Wang et al., 2011; Zhang et al., 2008), while the opposite sequence of

327 assembly has also been reported (Lawson et al., 2012; Lawson and Schlaepfer, 2012).
328 This behaviour is mirrored by kindlin and paxillin, as kindlin is able to recruit paxillin,
329 while the direct binding of paxillin to the β subunit can turn this sequence of events on
330 its head and could potentially allow paxillin-dependent recruitment of kindlin.
331 Interestingly, one of the factors, which determines the order of assembly appears to
332 be the nature of the involved integrin heterodimer. In particular, differences between
333 integrin $\alpha 5\beta 1$ and integrin $\alpha v\beta 3$ do not only exist with regard to the ligand spectrum,
334 but also how they convey the ligand binding event into the cell (Bachmann et al., 2019).
335 In this regard, $\alpha 5\beta 1$ integrins are known to determine adhesion strength and form
336 catch bonds with their ligands, when increasing forces are applied (Kong et al., 2009;
337 Roca-Cusachs et al., 2009). The elevated binding affinity of paxillin for integrin $\beta 1$ and
338 its ability to associate with talin and kindlin might reflect this need to withstand high
339 forces. Though integrin $\alpha v\beta 3$ is not able to sustain the high binding strength of $\alpha 5\beta 1$
340 integrin, integrin $\alpha v\beta 3$ exhibits faster binding rates and stimulates integrin $\alpha 5\beta 1$ -
341 mediated binding (Bharadwaj et al., 2017; Schiller et al., 2013). Interestingly, the fast
342 binding rate of integrin $\alpha v\beta 3$ correlates with its increased ability to recruit paxillin and
343 its propensity to initiate larger paxillin-positive adhesion sites (Bharadwaj et al., 2017;
344 Missirlis et al., 2016). Furthermore, on patterned substrates, cell spreading and paxillin
345 recruitment preferentially occur via the vitronectin-binding integrin $\alpha v\beta 3$ (Pinon et al.,
346 2014). Together with our findings of a prominent recruitment of paxillin to integrin $\beta 3$
347 in kindlin-deficient cells and of reduced spreading of paxillin LIM3-4A expressing cells
348 on vitronectin, all these observations suggest a particularly prominent role for the direct
349 association of the paxillin LIM3 domain with integrin $\beta 3$. It is also interesting to note
350 that the paxillin LIM3 domain appears to latch onto a specific section of the integrin $\beta 3$
351 subunit at the far carboxy-terminus, which shows significant chemical shift

352 perturbations upon binding. The C-terminal amino acids of integrin β 3 differ from all
353 other β subunits making this a unique recognition site and helping to explain this
354 peculiar interaction mode of paxillin and integrin β 3.

355 Though further studies are needed to delineate the stoichiometry of talin, kindlin and
356 paxillin at clustered integrin β subunits, our structural elucidation of the paxillin LIM2
357 and LIM3 domains and their association with the integrin β carboxy-terminus now
358 provides the foundation to probe and manipulate the functional contribution of paxillin
359 to matrix adhesion and cell spreading.

360

361

362 **Material & Methods**

363 **Antibodies and dyes**

364 The following primary and secondary antibodies were used at indicated
365 concentrations: anti-human α -actinin1 (mouse monoclonal, BM75.2, Sigma Aldrich,
366 A5044; WB 1:1000), anti-human talin (mouse monoclonal, 8d4, Sigma Aldrich, T3287;
367 WB 1:750), anti-human FAK (rabbit polyclonal, A-17, Santa Cruz, sc-557; WB 1:250),
368 anti-human kindlin2 (mouse monoclonal, 3A3, Merck, MAB2617; WB 1:1000, IF
369 1:200), anti-mouse kindlin2 (rabbit polyclonal, 11453-1-AP, Proteintech; WB 1:2000),
370 anti-human cSRC (rabbit polyclonal, SRC2, Santa Cruz, sc-18; WB 1:1000), anti-
371 human ILK (rabbit monoclonal, EP1593Y, Epitomics; WB 1:1000), anti-human
372 p130Cas (rabbit polyclonal, N17, Santa Cruz; WB 1:1000), anti-human vinculin
373 (mouse monoclonal, VIN-1, Sigma Aldrich, V9131; WB 1:1000), anti-human Hic-5
374 (mouse monoclonal, 34, BD Biosciences, 611164; WB 1:500), anti-human paxillin
375 (mouse monoclonal, 5H11, Thermo Fisher Scientific, AHO0492; WB 1:1000, IF 1:200),
376 anti- GAPDH (mouse monoclonal, GA1R, Thermo Fisher Scientific, MA5-15738-
377 HRP), anti-human Rac (rabbit polyclonal, invitrogen, PA5-17519; WB 1:1000), anti-
378 human CEACAM1, 3, 4, 5, 6 (mouse monoclonal , D14HD11, Aldevron; WB 1:6000,
379 IF 1:200); mouse monoclonal anti 6xHis (mouse monoclonal, HIS.H8, Thermo Fisher
380 Scientific, MA1-21315; WB 1:2000), anti GFP (mouse monoclonal , JL8, Clontech;
381 WB: 1:6000), anti-human tubulin (mouse monoclonal, E7, purified from hybridoma cell
382 supernatants, Developmental Studies Hybridoma Bank, University of Iowa, USA; WB
383 1:1000), anti-mouse integrin β 1 (Armenian hamster monoclonal, Hmb1-1, Thermo
384 Fisher Scientific, 11-0291-82; FC 1:300), anti-mouse integrin β 3 (armenian hamster
385 monoclonal, 2C9.G3, Thermo Fisher Scientific, 13-0611-81; FC 1:200), anti-mouse
386 integrin α 5 (rat monoclonal, MFR5, BD Biosciences, 553319; FC 1:300), anti-mouse

387 integrin α V (rat monoclonal, RMV-7, BD Biosciences, 550024; FC 1:300). Secondary
388 antibodies used: horseradish peroxidase (HRP)-conjugated goat anti-mouse; WB:
389 1:10.000, horseradish peroxidase (HRP)-conjugated goat anti-rabbit; WB: 1:5000;
390 Cy5-conjugated goat anti-mouse; IF 1:200, Dylight 488 conjugated goat anti-mouse,
391 IF 1:200, Rhodamine Red-X conjugated goat anti-arm. Hamster; FC 1:300,
392 Rhodamine Red conjugated goat anti-rat; FC 1:300 (all from Jackson
393 ImmunoResearch Inc., Baltimore, USA). CellMask Orange Plasma membrane stain,
394 Thermo Fisher Scientific, C10045; IF 1:1000.

395

396 Cell culture and transient transfection

397 Human embryonic kidney 293T cells (293T; American Type Culture Collection CRL-
398 3216) were grown in DMEM supplemented with 10% calf serum. Flp-In™ 3T3 cells
399 (Thermo Fisher Scientific) were cultured in DMEM supplemented with 10% fetal calf
400 serum (FCS) and 1% non-essential amino acids. GFP-FAK expressing mouse
401 embryonic fibroblasts (GFP-FAK MEFs) derived from FAK/p53 -/- knockout MEFs
402 (Schlaepfer et al., 2007) were cultured in DMEM supplemented with 10% FCS and 1%
403 non-essential amino acids on gelatine-coated (0.1% in PBS) cell culture dishes. All
404 cells were maintained at 37°C, 5% CO₂, and subcultured every 2–3 days.

405 For transient transfection of 293T cells, cells were seeded at 25% confluence the day
406 before and transfected using the standard calcium phosphate method with a total
407 amount of 5 μ g plasmid DNA/dish. For transient transfection of Flp-In™ 3T3 cells, 1 x
408 10⁵ cells were seeded into 6 well plates the day before and transfected with using
409 jetPRIME® transfection reagent (Polyplus transfection, Illkirch, France), following

410 manufacturer's protocol. GFP-FAK MEFs were transiently transfected with
411 Lipofectamin 2000, according to manufacturer's recommendations.

412

413 Whole cell lysates (WCLs) and WB

414 WCLs were generated by lysing equal cell numbers in radioimmunoprecipitation assay
415 buffer (1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂,
416 1 mM EGTA, 0.1% wt/vol SDS, and 1% vol/vol deoxycholic acid) supplemented with
417 freshly added protease and phosphatase inhibitors (10 mM sodium pyrophosphate,
418 100 mM NaF, 1 mM sodium orthovanadate, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 10
419 µg/ml Pefabloc, 5 µg/ml pepstatin, and 10 µM benzamidine) and phosphatase
420 saturating substrate (para-nitrophenolphosphate [pNPP]; Sigma-Aldrich; 10 mM).

421 Chromosomal DNA was mechanically sheared by passing through a metal needle.
422 DNA and cell debris were pelleted by addition of sepharose beads and centrifugation
423 (13,000 rpm, 30 min, 4°C). Supernatant was supplemented with 4× SDS sample buffer
424 (4% wt/vol SDS, 20% wt/vol glycerol, 125 mM Tris-HCl, 20% vol/vol β-
425 mercaptoethanol, and 1% wt/vol Bromophenol blue, pH 6.8) and boiled for 5 min at
426 95°C. Proteins were resolved on 10–18% SDS-PAGE. After separation, the proteins
427 were transferred to a polyvinylidene fluoride membrane (Merck Millipore), followed by
428 blocking in 2% BSA containing 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20,
429 pH 7.5 (TBS-T) buffer. The membrane was incubated with primary antibody in blocking
430 buffer overnight at 4°C, washed three times with TBS-T, and incubated with HRP-
431 conjugated secondary antibody in TBS-T for 1 h at RT. The chemiluminescent signal
432 of each blot was detected with ECL substrate (Thermo Fisher Scientific) on the
433 Chemidoc Touch Imaging System (Bio-Rad) in signal accumulation mode. Acquired

434 images were processed in Adobe Photoshop CS4 by adjusting illumination levels of
435 the whole image.

436

437 Recombinant DNA

438 The construction of His₆SUMO-tagged talin F3, eGFP-tagged full length talin,
439 His₆SUMO-kindlin2 as well as the generation of the Twin-Strep-tag vector for bacterial
440 expression has been described in detail (Grimm et al., 2020). The generation of CEA3-
441 ITGBct fusion constructs has been described previously (Baade et al., 2019). cDNA
442 of human paxillin isoform a (NM_002859.4) was kindly provided by Alexander
443 Bershadsky (Mechanobiology Institute, National University of Singapore, Singapore)
444 and was used as template for PCR amplification. His₆SUMO-PXN LIM2/3 was
445 generated by amplifying paxillin using primers: PXN LIM2/3 forward: 5'-
446 CCAGTGGGTCTCAGGTGGTCCCCGCGCTGCTAC-3'; PXN LIM2/3 reverse: 5'-
447 CTGATCCTCGAGTTACCCATTCTTGAAATATTCAAGGCGAGCCGCGCCGCTC-3'.
448 The product was then ligated into pET24a His-Sumo bacterial expression vector using
449 Eco31I and Xhol restriction sites.

450 Paxillin full length was amplified using primers PXN-fl forward: 5'-
451 ACTCCTCCCCGCCATGGACGACCTCGACGCCCTGCTG-3' and PXN-fl reverse:
452 5'-
453 CCCCCACTAACCCGCTAGCAGAAGAGCTTGAGGAAGCAGTTCTGACAGTAAGG-
454 3'. Paxillin LD1-5 was amplified using primers PXN-fl forward and PXN LD1-5 reverse:
455 5'-CCCCACTAACCCGCAGCTTGTTCAGGTCAG-3'. Paxillin LIM1-4 was amplified
456 using primers PXN-LIM1-4 forward: 5'-

457 ACTCCTCCCCGCCATGAAGCTGGGGTCGCCACAGTCGCCAAAG-3' and PXN-
458 fl reverse.

459 cDNAs encoding LIM domain proteins: Hic-5 cDNA (isoform 1, NM_001042454.3) was
460 kindly provided by Nicole Brimer (University of Virginia, Charlottesville, USA). Hic-5
461 was amplified using the primers Hic-5 forward: 5'-
462 ACTCCCCGCCATGGAGGACCTGGATGCC-3' and Hic-5 reverse: 5'-
463 CCCCCACTAACCGTCAGCCGAAGAGAGCTTCAGG-3'. Leupaxin was amplified from
464 pOTB7-LPXN (obtained from Harvard Medical School PlasmID Database;
465 HsCD00331641) using primers LPXN forward: 5'-
466 ACTCCTCCCCGCCATGGAAGAGAGTTAGATGCC-3' and LPXN reverse: 5'-
467 CCCCCACTAACCGGGCATTACAGTGGGAAGAGC-3'. PINCH-1 was amplified from
468 pDNR-LIB hLIMS1 (obtained from Harvard Medical School PlasmID Database,
469 HsCD00326503) using the primers PINCH forward: 5'-
470 ACTCCTCCCCGCCATGGCCAACGCCCTGGCCAGC-3' and PINCH reverse: 5'-
471 CCCCCACTAACCGTTCCCTAAGGTCTCAGC-3'. cDNA for LASP-1 was
472 provided by Elke Butt (Universitätsklinikum Würzburg, Würzburg, Germany) and
473 amplified using primers LASP forward: 5'-
474 ACTCCTCCCCGCCATGAACCCCAACTGCGCC-3' and LASP reverse: 5'-
475 CCCCCACTAACCGTCAGATGCCCTCACGTAGTTGG-3'.

476 The respective PCR products were cloned into the pDNR-Dual-LIC vector according
477 to the ligation independent cloning (LIC) strategy. The sequence verified constructs
478 were then subcloned into the expression vector pEGFP-C1 harbouring a loxP
479 recombination site via Cre-Lox recombination.

480 mEmerald-Migfilin was a gift from Michael Davidson (Addgene #54182) and was used
481 unmodified. pEGFP-Zyxin was described elsewhere (Agerer et al., 2005).

482

483 Site directed mutagenesis

484 The amino-acid residue of interest was changed using the overlap-extension PCR
485 mutagenesis procedure. Desalted oligonucleotide primers were purchased from
486 Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and can be found in following
487 table. Human Paxillin LIM2/3 was used as a template.

488	Mutation	Forward primer	Reverse primer
489	Paxillin LIM2/3 F475A	gcaGTGAACGGCAGCTTCTTC	TGGCGTGAAGCATTCCC
490	Paxillin LIM2/3 V476A	gcaAACGGCAGCTTCTTCGAGC	GAATGGCGTGAAGCATTCC
491	Paxillin LIM2/3 N477A	gcaGGCAGCTTCTTCGAGCAC	CACGAATGGCGTGAAGCATTCC
492	Paxillin LIM2/3 G478A	gcaAGCTTCTTCGAGCACGACG	GTTCACGAATGGCGTGAAGC
493	Paxillin LIM2/3 S479A	gcaTTCTTCGAGCACGACGG	GCCGTTACGAATGGCGTG
494	Paxillin LIM2/3 F480A	gcaTTCGAGCACGACGGGAG	GCTGCCGTTACGAATGG
495	Paxillin LIM2/3 F481A	gcaGAGCACGACGGGAGCCCTAC	GAAGCTGCCGTTACGAATGGC
496	Paxillin LIM2/3 4A	tgcagctTTCTTCGAGCACGACGG	gctgCGAATGGCGTGAAGCATT

497

498 Recombinant protein expression

499 Recombinant proteins with His₆-SUMO tag encoded on a pET24a vector were
500 expressed in *E. coli* Tuner (DE3) cells. Cells were cultured in lysogeny broth medium
501 containing 50 µg/mL kanamycin and 1% glucose (wt/vol) (for paxillin constructs

502 additionally 0.1 mM ZnCl₂) at 37 °C until an OD₆₀₀ value of 0.6-0.8 was reached.
503 Subsequently, overexpression was induced by addition of isopropyl β-D-
504 thiogalactoside (IPTG) to a final concentration of 0.5 mM (for integrin β1 1 mM
505 respectively). After 6-8 h incubation (for integrin-β1 overnight, respectively) at 30 °C
506 cells were harvested by centrifugation at 10 000 x g for 15 min at 4 °C and stored at -
507 80 °C. For isotopic labelling bacteria were cultured in M9-minimal medium containing
508 ¹⁵N ammonium chloride and/or ¹³C glucose as sole nitrogen and carbon sources.
509 Integrin cytoplasmic domains with a TwinStrepTagII tag encoded on a pET24a vector
510 were expressed in *E. coli* BL21(DE3) pRosetta cells. Cells were cultured in lysogeny
511 broth medium containing 50 µg/mL kanamycin. Expression conditions were identical
512 to His₆-SUMO integrin β cytoplasmic tails. His₆-SUMO, His₆-SUMO tagged talin F3
513 and kindlin2 were expressed in *E. coli* BL21(DE3). Bacteria were grown at 37 °C to an
514 OD of 0.6-0.8 and induced with 1 mM IPTG overnight at 30°C (His₆-SUMO and talin
515 F3) or 20 °C (kindlin2).

516

517 Protein purification

518 All steps were performed at 4 °C. Pelleted cells were slowly thawed on ice,
519 resuspended in 1:5 (wt/vol) lysis buffer (50 mM Tris, 300 mM NaCl, pH 8.0, protease
520 inhibitors) and lysed via a high-pressure homogenizer (Emulsiflex C3, Avestin Inc.,
521 Ottawa, Canada). The mixture was separated by ultracentrifugation at 100 000 x g for
522 30 min at 4 °C and supernatant was loaded onto a HisTrap HP column (GE Healthcare,
523 Freiburg, Germany) preequilibrated with 50 mM Tris, 10 mM imidazole and 300 mM
524 NaCl, pH 8.0. The loaded column was washed and eluted fractions, monitored by UV
525 absorbency at 280 nm, were pooled and dialyzed in 50 mM Tris, 300 mM NaCl, pH

526 8.0. After overnight cleavage with Ulp1 the His₆-SUMO tag was removed by
527 subsequent HisTrap purification and protein solution was subjected to size-exclusion
528 by using HiLoad 16/60 Superdex 30 (for integrin constructs) or Superdex 75 column
529 (for paxillin constructs, GE Healthcare, Freiburg, Germany) preequilibrated with 50
530 mM Na₂HPO₄, 150 mM NaCl, pH 6.2 (for integrin constructs) or 7.5 (for paxillin
531 constructs). For paxillin constructs all buffers contained also 0.1 mM ZnSO₄ and 1 mM
532 DTT respectively. Purified protein was checked by SDS-PAGE.

533

534 Pulldown assays with integrin β cytoplasmic domains
535 2.5 μ g of TwinStrep-tagged integrins or 10 μ g of biotin-integrin peptides β 3wt aa742-
536 788 (Biotin-HDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT-OH),
537 β 3 Δ 3aa aa742-785 (Biotin-HDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITY-
538 OH) β 3 Δ 8aa aa742-780 (Biotin-HDRKEFAKFEEERARAKWDTANNPLYKEATSTFT-
539 OH); all from Novopep Limited) were loaded onto Strep-Tactin Sepharose beads (50%
540 suspension; IBA Lifesciences) or streptavidin agarose beads (50% suspension; 16-
541 126; Merck) in pulldown buffer (50 mM Tris, pH 8, 150 mM NaCl, 10% glycerol, 0.05%
542 Tween, 10 μ M ZnCl₂) for 30 min at RT under continuous rotation. After centrifugation
543 (2,700 g, 2 min, 4°C), samples were washed three times with pulldown buffer. Then
544 integrin-loaded beads were suspended in bait protein solution (2 μ M of protein diluted
545 in pulldown buffer) and incubated 2 h at 4°C under constant rotation. Samples were
546 centrifuged (2,700 g, 2 min, 4°C) and washed three times with pulldown buffer. Strep-
547 Tactin samples were eluted under native conditions by adding 30 μ l of buffer BXT (50
548 mM Tris, pH 8, 150 mM NaCl, 50 mM biotin). After 10 min incubation at RT under
549 constant rotation, samples were centrifuged. Supernatants were mixed with 4 \times SDS

550 and boiled for 5 min at 95°C before they were subjected to WB. Streptavidin agarose
551 beads were directly mixed with 2× SDS and boiled for 10 min at 95°C to elute proteins
552 from biotin-integrin peptides before they were subjected to WB.

553

554 Resonance assignment

555 All NMR-experiments for the resonance assignment and structure determination were
556 recorded on a Bruker Avance III 600 MHz spectrometer equipped with an H/C/N TCI
557 cryoprobe. Three-dimensional spectra were recorded using non-uniform sampling (25-
558 50% sparse sampling) and reconstructed by recursive multidimensional
559 decomposition (Topspin® v3.1-3.2). NMR-sample conditions: 500 μ M ^{13}C - ^{15}N -Paxillin-
560 LIM2/3, 150 mM NaCl, 50 mM Na₂HPO₄, 4 mM NaN₃, 1 mM DTT, 5% (or 100% D₂O),
561 pH 7.5. Recorded 3D-spectra in 5% D₂O: HNCO, HN(CA)CO, CBCANH,
562 CBCA(CO)NH, H(CCCO)NH, (H)C(CCO)NH, NOESY- ^{15}N -HSQC, NOESY- $^{13}\text{C}_{\text{ali.}}$ -
563 HSQC; in 100% D₂O: H(C)CH-TOCSY, (H)CCH-TOCSY, H(C)CH-COSY, NOESY-
564 $^{13}\text{C}_{\text{ali.}}$ -HSQC, NOESY- $^{13}\text{C}_{\text{aro.}}$ -HSQC (NOESY mixing time: 120 ms in all spectra).
565 Backbone resonance assignment was done semi-automatically using CARA v1.8.4.2
566 and Autolink II v0.8.7 (Masse and Keller, 2005). The sidechain resonances were
567 assigned manually. NOESY cross-peaks were picked and quantified using ATNOS
568 (Herrmann et al., 2002)(implemented in UNIO'10 v2.0.2 (Serrano et al., 2012)).
569 TALOS-N was used to calculate ϕ - und ψ - angles based on the backbone chemical
570 shifts (Shen and Bax, 2013). The resonance assignment of paxillin LIM2/3 has been
571 deposited to the BMRB (Entry 51154).

572

573 Structure calculation

574 Initial Structure calculation was done using Cyana v3.0 (Guntert, 2004), with the
575 protein sequence, the resonance assignment (CARA), NOESY-peaklists (ATNOS)
576 and backbone angular restraints (TALOS-N) as input. In later stages of the calculation
577 additional distance and angular constraints for a tetrahedral Zinc-coordination of the
578 respective amino acids were implemented. The coordination mode of the four
579 histidines (H403, H406, H462 and H492) was determined by the difference of chemical
580 shifts of $C^{\delta 2}$ and $C^{\varepsilon 1}$ and in all cases $\delta (C^{\varepsilon 1}) - \delta (C^{\delta 2})$ was larger than 17 ppm indicating
581 a coordination via $N^{\delta 1}$ for all histidines. The structures were visualized and analyzed
582 with PyMOL v1.3 (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger,
583 LLC). The coordinates of the final ensemble have been deposited to the PDB
584 (Accession code: 7QB0)

585 Table 1: Structural statistics

586 NMR constraints	
587 <i>Total unambiguous distance restraints</i>	1226 (100.0%)
588 Intraresidue (i, i)	303 (24.7%)
589 Sequential (i, i+1)	354 (28.9%)
590 Medium-range ($2 \leq i-j \leq 4$)	151 (12.3%)
591 Long-range ($ i-j > 4$)	418 (34.1%)
592 <i>Total dihedral angle restraints</i>	343
593 ϕ	100
594 ψ	100
595 χ_1	99
596 χ_2	31
597 χ_3	13
598 Ensemble statistics (20 structures)	
599 <i>Violation analysis</i>	

600	Maximum distance violation (Å)	0.61
601	Maximum dihedral angle violation region (deg.)	12.19
602		
603	<i>Target function</i>	
604	Mean CYANA target function	5.75±0.5
605		
606	<i>rmsd from mean structure</i>	
607	Backbone heavy atoms (Å)	0.77±0.17
608	All heavy-atoms (Å)	1.20±0.16
609		
610	<i>Ramachandran plot</i>	
611	Most-favorable regions (%)	77.4
612	Additionally allowed regions (%)	21.3
613	Generously allowed regions (%)	1.0
614	Disallowed regions (%)	0.4

615

616 Chemical shift perturbation mapping

617 ^1H - ^{15}N HSQC spectra were recorded on a Bruker Avance III 600 MHz spectrometer
618 equipped with a 5 mm BBI probe and a Bruker Avance NEO 500 MHz equipped with
619 a H/C/N TCI CryoProbe Prodigy (Bruker Biospin GmbH, Rheinstetten, Germany).
620 Chemical shifts were referenced to internal sodium 3-(Trimethylsilyl)propane-1-
621 sulfonat-d6 (DSS) at 0.0 ppm. The spectra were processed and analyzed with
622 Topspin® v2.1-4.0 (Bruker Biospin GmbH, Rheinstetten, Germany) and CARA (v.
623 1.9.1.5.). For NMR-experiments the proteins were concentrated by repeated
624 ultrafiltration (Amicon Ultra-4 Ultracel-3 kDa centrifugal filter device, Merck Millipore,
625 Burlington, USA).

626 Experiments were performed at 298 K in buffer containing 50 mM Na₂HPO₄, 150 mM
627 NaCl, 0.1 mM ZnSO₄, 1 mM DTT, 5% D₂O, pH = 6.2 (if integrin was ¹⁵N-labeled) or
628 7.5 (if paxillin was ¹⁵N-labeled). Experimental procedure: To a sample of ¹⁵N-labeled
629 protein a stock solution of unlabeled interaction partner up to a final concentration of
630 the respective constructs was added for collecting ¹H-¹⁵N HSQC spectra. The
631 resonance assignment of integrin β 1 was transferred from BMRB entry 16159
632 (Anthis et al., 2009) and for integrin β 3 from the BMRB entry 15552 (Oxley et al.,
633 2008). Chemical shift change ($\Delta\delta$) was calculated with the equation

634
$$\Delta\delta = \sqrt{0,5 * [\Delta\delta_H^2 + 0,14 * \Delta\delta_N^2]}$$

635 where $\Delta\delta$ [ppm] = $\delta_{\text{bound}} - \delta_{\text{free}}$. The titration curves were fitted in OriginPro® (v.
636 b9.5.5.409) using equation:

637
$$\Delta\delta([L], [P]) = \Delta\delta_{\text{max}} \frac{([P] + [L] + K_D) - \sqrt{([P] + [L] + K_D)^2 - 4[P][L]}}{2[P]}$$

638 A simultaneous fit for multiple signals was used, allowing individual $\Delta\delta_{\text{max}}$ values for
639 each residue, but a global value for the dissociation constant K_D .

640

641 sgRNA design and cloning

642 For the generation of recombinant sgRNA-expression vectors, we equipped the
643 pBluescript vector (pBS SK+, Agilent technologies, Santa Clara, CA, USA) with the
644 murine U6 promotor controlled sgRNA expression cassette from pSpCas9(BB)-2A-
645 GFP (PX458, a gift from Feng Zhang, Addgene plasmid # 48138) (Ran et al., 2013).
646 Therefore, we amplified the U6 controlled sgRNA expression cassette by polymerase
647 chain reaction (PCR) with the following primer pair: U6_sgRNA_forward: 5'-

648 ATAGGTACCGTGAGGGCCTATTCCC-3' U6_sgRNA_reverse: 5'-

649 ATACTCGAGGTCTGCAGAATTGGCGC-3'. The resulting construct was cloned into

650 pBS SK+ via Xhol and KpnI restriction sites. The sequence verified construct (pBS-

651 U6) was then digested with BbsI and ligated with the annealed primer pair:

652 MCS_oligo_forward:

653 5'-CACCGGGTCTCGATGGGCCAATCGAATACACGTGGTTGATTAAATGGG

654 CCCGAAGACCT-3'

655 MCS_oligo_reverse:

656 5'-AACACAGGTCTCGGGCCCATTAAATCAACCACGTGTATCGAATTGG

657 GCCCATCGAAGACCC-3'

658 to create pBS-U6 with a multiple cloning site (pBS-U6-MCS) within the BbsI restriction

659 sites. To generate the respective pBS-U6-Cer-sgRNA plasmid, the following sgRNA

660 Oligos Cer-KO forward: 5'-CACCGCCGTCCAGCTCGACCAGGA-3' and Cer-KO

661 reverse: 5'-AAACTCCTGGTCGAGCTGGACGGC-3' were annealed and ligated into

662 the pBS-U6-MCS vector via the BbsI restriction sites. To eliminate remaining pBS-U6-

663 MCS after the ligation step, samples were digested with BstBI. All constructs were

664 sequence verified by LGC Genomics.

665 For targeting paxillin the sgRNA oligos targeting exon 2 PXN-KO sense: 5'-

666 CACCGACGGTGGTGGTGGACCGG-3' and PXN-KO reverse: 5'-

667 AAACCCGGTCCCACCACCAACCGTC-3' were annealed and ligated into

668 pSpCas9(BB)-2A-GFP (PX458-sgRNA mPXN)). For targeting murine integrin β 3, the

669 sgRNA oligos targeting exon 2 mITGB3-KO sense: 5'-

670 CACCGCGGACAGGATGCGAGCGCAG-3' and mITGB3-KO reverse: 5'-

671 AACCTGCGCTCGCATCCTGTCCGC-3' were annealed and ligated into pBS-U6-
672 MCS to generate pBS-U6-mITGB3-sgRNA.

673 All sgRNAs were designed with the help of the CRISPR design tool
674 (<http://crispr.mit.edu>) (Hsu et al., 2013) and E-CRISP (www.e-crisp.org/E-CRISP).

675

676 Generation of integrin β 3 and paxillin knockout cell lines

677 For the generation of integrin β 3 and paxillin knockout cell lines, Flp-In 3T3 cell line
678 (Invitrogen) was first stably transduced with a lentiviral vector encoding Histon2B
679 mCerulean. Therefore, human histone 2B cDNA (H2B, gift from Thomas U. Meyer,
680 University of Konstanz, Konstanz, Germany) was amplified by polymerase chain
681 reaction (PCR) with the following primer pair: hH2B_forward: 5'-
682 ATAGCTAGCACCATGCCAGAGCCAGCGAAGTC-3' hH2B_reverse: 5'-
683 ATAACCGGTTAGCGCTGGTGTACTTGG-3' and cloned into pmCerulean-C1 (gift
684 from David Piston, Vanderbilt University Medical Center, Nashville, USA) via NheI and
685 AgeI restriction sites. The resulting construct was again subjected to PCR amplification
686 with primers: H2B-Cer_forward: 5'-ATAGGATCCACCATGCCAGAGCCAGCGAAG-3'
687 and H2B-Cer_reverse: 5'-ATACTCGAGCTATTGTACAGTTCGTCCATGCCG-3'.
688 The PCR product was subcloned into pWZL Blasticidin (pWZLBlast, gift from Nicole
689 Brimer, University of Virginia, Charlottesville, USA) via BamHI and Xhol restriction
690 sites to generate pWZLBlast-H2B-Cer. For retroviral production, 80% confluent
691 Phoenix-Eco cells (Swift et al., 2001) were transfected with pWZLBlast-H2B-Cer and
692 cultured for 2 days. Afterwards, the supernatant was harvested, filtered through a 0.45
693 μ m pore-size filter unit (Minisart®, Sartorius Stedim Biotech GmbH, Göttingen,
694 Germany) and applied on previously seeded NIH3T3 Flp-In cells at a ratio of 1:1

695 (vol/vol, supernatant : NIH3T3 growth medium) together with 4 µg/ml Polybrene®
696 (Sigma-Aldrich). Transduced cells were cultured in regular growth medium
697 supplemented with 5 µg/ml blasticidin (Carl Roth GmbH + Co. KG, Karlsruhe,
698 Germany). Cerulean-positive cells were sorted by FACS and seeded as single cells
699 into 96-well plates to generate clonal Cerulean-positive NIH3T3 H2B-Cer Flp-In cell
700 lines.

701 Paxillin knockout cells were generated by transiently transfecting NIH3T3 H2B-Cer
702 Flp-In cells with a combination of PX458-sgRNA mPXN + pBS-U6-Cer-sgRNA at a
703 ratio of 1:5. Integrin β3 knockout cells were generated by transiently transfecting
704 NIH3T3 H2B-Cer Flp-In cells with a combination of PX458-sgRNA Cer + pBS-U6-
705 mITGB3-sgRNA at a ratio of 1:5. 10 days after transfection, single cerulean negative
706 cells were sorted into 96 well plates and clonal cell lines were expanded and knockout
707 of the target protein was verified by Western Blot.

708

709 Stable complementation of knockout cells
710 For complementation of integrin β3, cDNA of murine integrin β3 (gift from Michael
711 Davidson, addgene plasmid # 54130) was amplified by PCR using the following
712 primers mITGB3 forward: 5'-GATGACACTAGTGACCGCCATGCGAGCGCAGTG-3'
713 and mITGB3-fl reverse: 5'-
714 TCGGCAGCCCTCGAGCTAAGTCCCCCGGTAGGTGATATTG-3'; mITGB3 forward
715 and mITGB3Δ8aa reverse: 5'-
716 TCGGCAGCCCTCGAGCTAGAAGGTGGAGGTGGCCTCTTATAC-3'; mITGB3
717 forward and mITGB3Δ3aa reverse: 5'-
718 TCGGCAGCCCTCGAGCTAGTAGGTGATATTGGTGAAGGTGGAGGTG-3'.

719 The respective products were cloned into pEF5/FRT-DEST (gift from Rajat Rohatgi,
720 Addgene plasmid # 41008) using Spel and PspXI restriction sites.

721 For complementation of Flp-In paxillin KO cells, we equipped the expression vector
722 pEF5/FRT-DEST with a GFP-tag adjacent to a LoxP site for C-terminal protein tagging
723 via Cre-Lox recombination. Therefore, the respective sequence was amplified by PCR
724 from pEGFP C1 (Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France) with
725 the following primer pair: EGFP_forward: 5'-
726 GCCTAGACTAGTTAGCGCTACCGGTCGCCACCATG-3' EGFP_reverse: 5'-
727 GCAGCGCTCGAGGGCTGATTATGATCAGTTATCTAGATCC-3'. The resulting
728 construct was cloned into pEF5/FRT-DEST via Spel and PspXI restriction sites to
729 generate the expression vector pEF5/FRT EGFP C1 loxp.

730 The coding sequences (CDS) of paxillin was amplified by PCR with the following
731 primers:

732 PXN-fl forward: 5'-ACTCCTCCCCGCCATGGACGACCTCGACGCCCTGCTG-
733 3'

734 PXN-fl reverse: 5'-

735 CCCCCACTAACCCGCTAGCAGAAGAGCTTGAGGAAGCAGTTCTGACAGTAAG

736 G-3'. PXN Δ LIM4 forward: 5'-

737 ACTCCTCCCCGCCATGGACGACCTCGACGCCCTGCTG -3', PXN Δ LIM4

738 reverse: 5'-CCCCACTAACCCGCGAGCCGCGCCGCTCGTGGTAGTGC-3'; The

739 paxillin 4A mutant was generated by overlap extension PCR. In a first PCR two

740 fragments were generated using primers PXN-fl forward and PXN-4A reverse: 5'-

741 TGCTGCTGCAGCGAATGGCGTGAAGCATTCCGGCACACAAAG -3'. For the

742 second fragment primers PXN-4A forward: 5'-

743 GCTGCAGCAGCATTCTCGAGCACGACGGGCAGCCCTAC -3' and PXN-fl reverse
744 were used. In a second step the two fragments were annealed by overlap extension
745 PCR and amplified using primers PXN-fl forward and PXN-fl reverse.

746 The respective products were cloned into the pDNR-Dual-LIC vector according to the
747 ligation independent cloning (LIC) strategy. The sequence verified constructs were
748 then subcloned into the expression vector pEF5/FRT EGFP-C1 by Cre-Lox
749 recombination.

750 Respective knockout cell lines were complemented by transient transfection of 0.8 µg
751 cDNA coding for the gene of interest + 3.2 µg Flp recombinase expression vector
752 (pOG44) using jetPRIME® transfection reagent (Polyplus transfection, Illkirch,
753 France). After 3 days, positive cells were selected by addition of 250 µg/ml Hygromycin
754 B for 8 days.

755

756 Flow cytometry

757 Cells were trypsinized and suspended in growth medium. Samples were centrifuged
758 at 100g for 3 min and the resulting pellet was re-suspended in FACS buffer (PBS with
759 5% FCS, 2 mM EDTA). Cells were washed once in FACS buffer and 1x10⁶ cells per
760 sample were incubated with monoclonal anti-integrin antibodies as indicated for 1h at
761 4°C under constant rotation. Cells were washed three times with FACS buffer, followed
762 by incubation for 30 min with a Rhodamine-Red conjugated secondary antibody. Cells
763 were analysed by flow cytometry (BD LSRFortessa, FACSDiva™ software, BD
764 Biosciences, Heidelberg, Germany).

765

766 Cell spreading analysis

767 Sterile glass coverslips were coated over night at 4°C with 5µg/ml vitronectin or 5 µg/ml
768 fibronectin type III repeats 9-11 (FNIII9-11). Cells were starved overnight in starvation
769 medium (DMEM + 0.5% FCS). After 12h starvation cells were trypsinized, trypsin was
770 inactivated using soybean trypsin inhibitor (Applichem; 0.25 mg/ml in DMEM + 0.25%
771 BSA). Cells were pelleted by centrifugation (100g, 3 min, RT) and suspended in
772 DMEM + 0.25% BSA. Cells were kept in suspension for 30 min before seeding on
773 coated glass coverslips. After 30 and 120 min of adherence, cells were washed once
774 with PBS++ (0.5 mM MgCl₂, 0.9 mM CaCl₂), fixed with 4% PFA in PBS for 15 min at
775 RT, washed thrice in PBS, permeabilized with 0.4% Triton-X-100 in PBS for 5 min at
776 RT, washed thrice in PBS and blocked for 30 min in blocking buffer (10% heat
777 inactivated CS in PBS). Cells were stained with CellMask™ Orange (diluted to 5µg/ml
778 in blocking buffer) and DAPI (diluted to 0.2 µg/ml in blocking buffer) for 30 min at RT.
779 Images were analysed by a custom build ImageJ macro (Bioimaging Center,
780 University of Konstanz)

781

782 Fluorescent Microscopy and microscope settings

783 For confocal laser scanning microscopy all images were taken from fixed specimens
784 embedded in Dako fluorescent mounting medium (Dako Inc, Carpinteria, USA) on a
785 LEICA SP5 confocal microscope equipped with a 63.0x/1.40 NA oil HCX PL APO CS
786 UV objective and analyzed using LAS AF Lite software. All images were acquired in
787 xyz mode with 1024 x 1024 pixel format and 100 Hz scanning speed at 8 bit resolution.
788 Fluorochromes used are Pacific Blue (excitation 405 nm, emission bandwidth: 435 –
789 475 nm); CF405M (excitation 405 nm, emission bandwidth: 435 – 475) GFP (excitation

790 488 nm, emission bandwidth: 500 – 525 nm); CellMask Orange (excitation 561 nm,
791 emission bandwidth: 571 – 613 nm), RFP (excitation 561 nm. Emission bandwidth 571
792 – 613 nm) and Cy5 (excitation 633 nm, emission bandwidth: 640 -700 nm). Images
793 were processed using ImageJ by applying the same brightness/contrast adjustments
794 to all images within one experimental group.

795

796 TIRF microscopy

797 Cells were starved overnight in starvation medium (DMEM + 0.5% FCS). After 12h
798 starvation cells were trypsinized, trypsin was inactivated using soybean trypsin
799 inhibitor (Applichem; 0.25 mg/ml in DMEM + 0.25% BSA). Cells were pelleted by
800 centrifugation (100g, 3 min, RT) and suspended in DMEM + 0.25% BSA. Cells were
801 kept in suspension for 30 min before seeding on Wilco dishes, coated with 5 µg/ml
802 vitronectin. Cells were imaged with a GE DeltaVision OMX Blazev4, equipped with a
803 60x/1.49 UIS2 APON TIRFM objective in Ring TIRF mode. Settings were adjusted to
804 reach clean TIRF illumination without epifluorescence. A separate sCMOS camera
805 was used for each channel and images were later aligned using OMX image alignment
806 calibration and softWoRx 7.0. Fluorophores used were GFP (excitation wavelength
807 488 nm, emission bandwidth: 528/48 nm) and Cy5 (excitation wavelength 647 nm,
808 emission bandwidth: 683/40 nm).

809

810 Opa-protein triggered integrin clustering (OPTIC)

811 OPTIC was performed as described previously (Baade et al., 2019). Briefly, 293T cells
812 were transfected with pcDNA3.1 CEACAM3-ITGB fusion constructs together with
813 cDNA coding for the protein of interest fused to eGFP. 48 h post-transfection, cells

814 were seeded on coverslips coated with 10 µg/ml poly-L-lysine in suspension medium
815 (DMEM + 0.25% BSA). After 2h, adherent cells were infected with Pacific Blue-stained
816 *Neisseria gonorrhoeae* (Opa52-expressing, non-piliated *N. gonorrhoeae* MS11-B2.1,
817 kindly provided by T. Meyer, Berlin, Germany) at MOI 20 for 1h in suspension medium.
818 After 1h cells were fixed for 15 min with 4% paraformaldehyde in PBS at room
819 temperature followed by 5 min permeabilization with 0.1% Triton X-100 in PBS. After
820 washing with PBS, cells were incubated for 10 min in blocking solution (10% heat
821 inactivated calf serum in PBS) and stained for CEACAM3. After washing, cells were
822 again incubated for 10 min in blocking solution followed by secondary antibody
823 staining. Coverslips were mounted on glass slides using Dako fluorescent mounting
824 medium (Dako Inc, Carpinteria, USA).

825

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837

838 **Author contributions**

839 H.M.M. and C.R.H. conceived the study. H.M.M., C.R.H., T.B., M.M., A.P., C.P. and
840 N.K. designed the experiments. T.B., C.P. and L.S. cloned constructs, established cell
841 lines and performed OPTIC assays. T.B. performed cell-spreading experiments, TIRF
842 microscopy and pulldown assays. M.M., A.P., N.K. and R.N. cloned and prepared
843 paxillin and integrin proteins and peptides and performed NMR experiments. All
844 authors analyzed and interpreted data. T.B., M.M., H.M.M. and C.R.H wrote the
845 manuscript.

846

847 **Conflict of Interest**

848 The authors declare that they have no conflict of interest.

849 **References**

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1021

1022 **Figure legends**

1023 **Figure 1: Paxillin LIM2/3 domains can directly bind the cytoplasmic tails of**
1024 **integrin $\beta 1$ and $\beta 3$**

1025 (A) Stable GFP-Paxillin expressing Flp-In 3T3 cells were transiently transfected with
1026 RFP-tagged LIM1-4 or LD1-5 domains. (B) Paxillin localization at FAs was evaluated
1027 by measuring the GFP fluorescence intensity in presence of either overexpressed LIM
1028 or LD domains. Shown are mean values of GFP-intensity from three independent
1029 experiments. The total number of analysed FAs is given in brackets under each
1030 sample. Error bars represent 5 and 95 percentiles. Significance was calculated using
1031 one-way ANOVA, followed by Bonferroni Multiple Comparison Test (** p<0.0001, ns
1032 = not significant). (C) In vitro pulldown using recombinant Twin-Strep-tag integrin β
1033 cytoplasmic tails and recombinant talin1 F3 domain, full length kindlin2 as well as
1034 paxillin LIM2/3 domain fused to His₆-Sumo, or His₆-Sumo only as negative control. (D)
1035 and (E) Solution structure of paxillin LIM2/3. The final ensemble of ten conformers with
1036 lowest target function is shown fitted to the LIM2 domain (residues P381 to F438) (D)
1037 and fitted to the LIM3-domain (residues P440 to R497) (E), shown in ribbon
1038 representations. In the fitted part, α -helices are colored cyan and β -strands magenta.
1039 Zinc ions are shown as grey spheres. The flexible loop of the LIM3 domain (residue
1040 T473 to E482) is shown in blue. The domain that was not used for fitting is shown in
1041 light grey.

1042

1043 **Figure 2: The C-terminal residues of integrin $\beta 3$ cytoplasmic tail are crucial for**
1044 **paxillin binding**

1045 (A) ^{15}N -HSQC titration of 300 μM ^{15}N integrin $\beta 3$ ct (ITGB3 ct) with paxillin LIM2/3.
1046 Paxillin was added in concentrations up to 900 μM . Boxes show a selection of signals
1047 affected by CSPs (residues N782, I783 & R786) in the presence of 0 μM (black), 150
1048 μM (green), 300 μM (blue) and 900 μM (red) paxillin LIM2/3. Insets show the
1049 concentration dependence of combined amide CSPs globally fitted to a one site
1050 binding model. (B) Combined amide CSPs of 300 μM ^{15}N integrin $\beta 3$ ct in the presence
1051 of 760 μM paxillin LIM2/3 vs residue number of integrin $\beta 3$ ct. Lines indicate average
1052 $\delta\Delta + 1\text{x s.d.}$ (yellow), $\delta\Delta + 2\text{x s.d.}$ (orange) and $\delta\Delta + 3\text{x s.d.}$ (red). (C) Representative
1053 Western Blot of in vitro pulldown using biotinylated integrin β peptides and
1054 recombinant His₆-SUMO or His₆-SUMO-paxillin LIM2/3 (PXN LIM2/3). A decreased
1055 binding of paxillin to truncated integrin $\beta 3$ peptides is visible. Lower panel:
1056 Densitometric quantification of the Western Blots in (A) (n=3). Statistical significance
1057 was calculated using One sample t-test to calculate if samples mean are significantly
1058 different from a hypothetical value of 1 (* $p<0.05$, ** $p< 0.01$). (D) ^{15}N -HSQC titration
1059 of 300 μM ^{15}N integrin $\beta 3$ $\Delta 3\text{aa}$ (ITGB3 $\Delta 3\text{aa}$) with paxillin LIM2/3 (PXN LIM2/3).
1060 Paxillin was added up to a concentration of 750 μM . (E) Combined amide CSPs of 300
1061 μM ^{15}N integrin $\beta 3$ ct $\Delta 3\text{aa}$ in the presence of 750 μM paxillin LIM2/3 vs residue
1062 number of integrin $\beta 3$ ct $\Delta 3\text{aa}$.

1063

1064 **Figure 3: C-terminally truncated integrin $\beta 3$ causes defective cell spreading.**

1065 (A) and (B) Serum starved Flp-In 3T3 integrin $\beta 3$ knockout fibroblasts or knockout cells
1066 re-expressing integrin $\beta 3$ wt or C-terminally truncated integrin $\beta 3$ mutants were seeded
1067 onto glass slides coated with 5 $\mu\text{g/ml}$ fibronectin (A) or 5 $\mu\text{g/ml}$ vitronectin (B) for 30
1068 min and cell area was measured. Shown are mean values and 95% confidence

1069 intervals of n=60 cells per sample from 3 independent experiments. Statistical
1070 significance was calculated using one-way ANOVA followed by Bonferroni Multiple
1071 Comparison Test. (C) Serum starved kindlin1/2 deficient fibroblasts (Kind^{KO}) cells
1072 stably expressing full length integrin β 3 or truncated mutants were seeded on glass
1073 coverslips coated with 50 μ g/ml vitronectin (left panel) or poly-Lysin (right panel) for 4
1074 h. Cells were fixed and stained for endogenous paxillin. (D) Serum starved Kind^{KO}
1075 cells stably expressing full length integrin β 3 or truncated mutants were seeded on
1076 glass coverslips coated with 50 μ g/ml vitronectin for 4 h. Cells were fixed and stained
1077 for endogenous talin.

1078

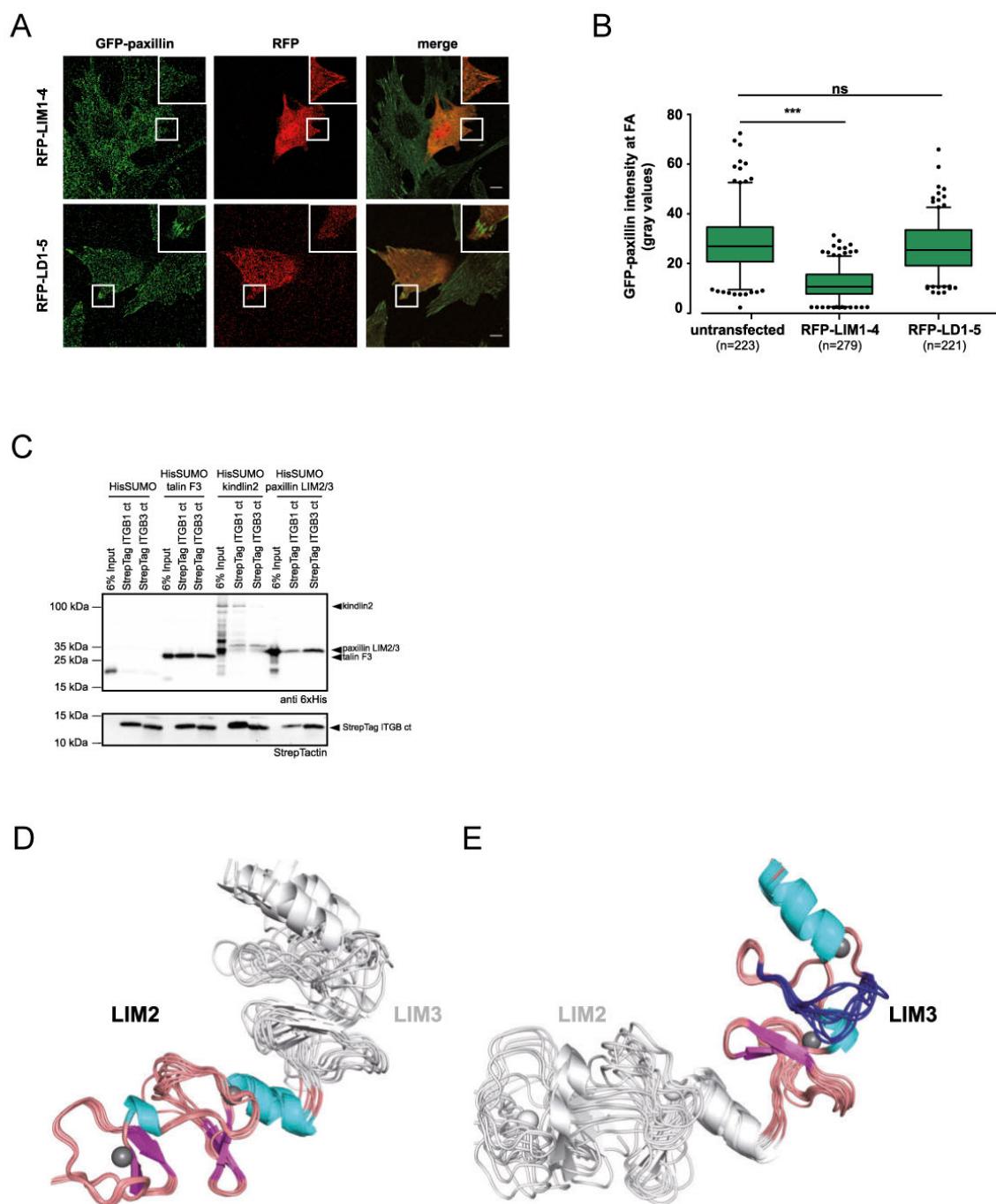
1079 **Figure 4: The LIM3 flexible loop mediates binding to the integrin β cytoplasmic
1080 tail**

1081 (A) Mapping of combined amide CSPs when binding to integrin β 3 onto the solution
1082 structure of paxillin LIM2/3 shown as surface representation from two perspectives.
1083 Residues showing CSPs larger than average $\delta\Delta + 3 \times$ s.d. are colored red, residues
1084 for which [average $\delta\Delta + 3 \times$ s.d. < $\delta\Delta$ < average $\delta\Delta + 2 \times$ s.d.] are colored orange, and
1085 residues for which [average $\delta\Delta + 2 \times$ s.d. < $\delta\Delta$ < average $\delta\Delta + 1 \times$ s.d.] are colored
1086 yellow. Residues with $\delta\Delta <$ average $+ 1 \times$ s.d. are colored grey. The boxed region is
1087 also shown in stick representation including the flexible loop of the LIM3 domain using
1088 the same color code. Residues experiencing significant CSPs are labelled by amino
1089 acid type and position. (B) Combined amide CSPs of 250 μ M ^{15}N paxillin LIM2/3 in the
1090 presence of 750 μ M integrin β 3 vs residue number of paxillin. Lines indicate average
1091 $\delta\Delta + 1 \times$ s.d. (yellow), $\delta\Delta + 2 \times$ s.d. (orange) and $\delta\Delta + 3 \times$ s.d. (red). (C) ^{15}N -HSQC
1092 titration of 250 μ M ^{15}N paxillin LIM2/3 wt (PXN LIM2/3 wt) with integrin β 3. Integrin was

1093 added in concentrations up to 2420 μ M. Boxes show a selection of signals affected by
1094 CSPs (residues F480 & F481) in the presence of 0 μ M (black), 200 μ M (green), 600
1095 μ M (blue) and 2420 μ M (red) integrin β 3 ct. (D) Recombinantly expressed His₆-SUMO
1096 PXN LIM2/3 wt or His₆-SUMO PXN LIM2/3 4A were pulled down using Twin-Strep-
1097 Tag integrin β 3 ct. PXN LIM2/3 4A shows reduced binding to integrin β 3 ct. (E)
1098 Outside-in signalling dependent cell spreading of paxillin knockout cells, stably re-
1099 expressing ctrl vector (PXN KO), GFP-paxillin wt (PXN wt) or paxillin mutants PXN
1100 Δ LIM4 and PXN-4A. Cells were starved overnight and seeded for 30 or 120 min,
1101 respectively on the integrin β 3 ligand vitronectin in the absence of serum. Cells were
1102 fixed and the cell membrane was stained with CellMask Orange. Scale bar represents
1103 20 μ m. (F) Quantification of cell area from images (E). Shown are mean values with
1104 95% confidence intervals from 3 independent experiments for 30min timepoint or from
1105 2 independent experiments for 120 min timepoint. Sample sizes are given in brackets.
1106 Statistical significance was calculated using one-way ANOVA followed by Bonferroni
1107 Multiple Comparison Test (ns: not significant; *** $p \leq 0.0001$; ** $p \leq 0.01$).
1108

1109 **Figures**

Baade et al. Figure 1

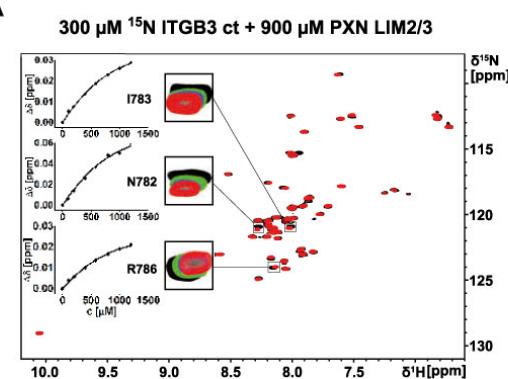


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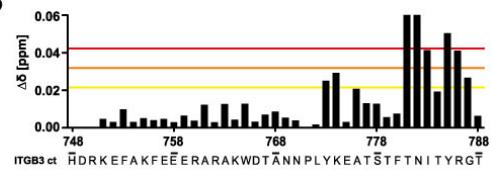
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Baade et al. Figure 2

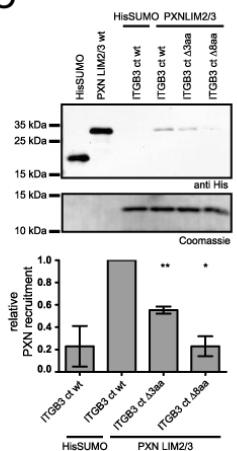
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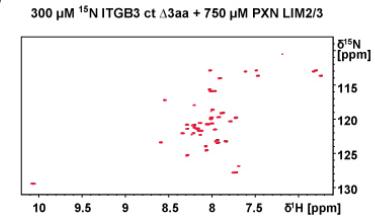
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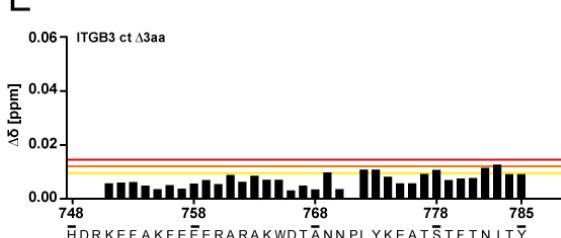
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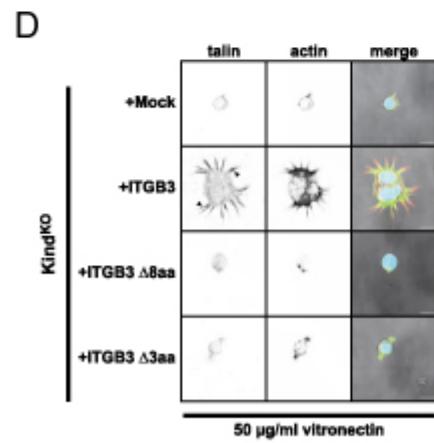
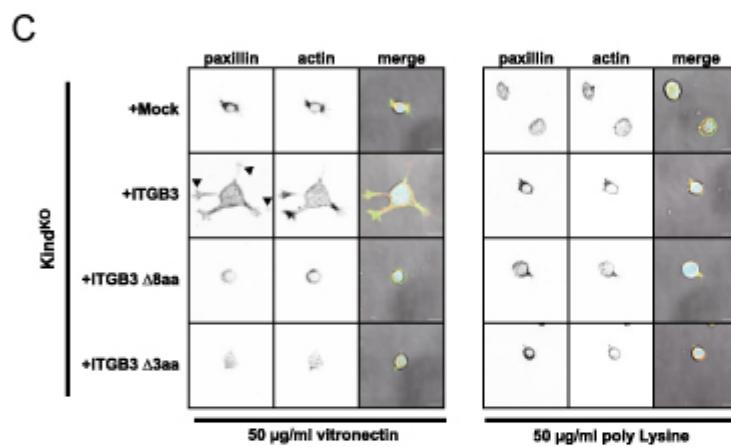
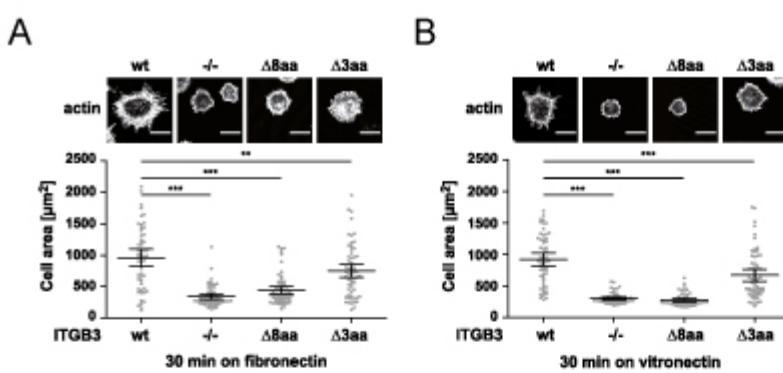
E



1112

1113

Baade et al. Figure 3



Baade et al. Figure 4

