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Cell adhesion and spreading on fluid membranes through microtubules-dependent mechanotransduction

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Abstract

During cell adhesion, integrins form clusters that transmit mechanical forces to the substrate (mechanotransduction) and regulate biochemical signaling depending on substrate stiffness. In recent years, mechanotransduction studies significantly advanced our understanding of cell adhesion. Most studies were performed on rigid substrates such as glass, while more physiologically relevant fluid membranes have been less explored. In contrast to rigid substrates, integrins' ligands on fluid supported lipid bilayers (SLBs) are mobile and adhesive complexes cannot serve as anchoring points promoting cell spreading. Here, we demonstrate that cells spread on SLBs coated with Invasin, a high-affinity integrin ligand. We show that in contrast to SLBs functionalized with RGD peptides, integrin clusters grow and mature on Invasin-SLBs to a similar extent as on glass. While actomyosin contraction dominates adhesion maturation on stiff substrates, we find that integrin mechanotransduction and cell spreading on fluid SLBs rely on dynein pulling forces along microtubules, perpendicular to membranes, and microtubules pushing on adhesive complexes, respectively. Our findings, supported by a theoretical model, demonstrate a new mechanical role for microtubules in integrin clustering on fluid substrates. These forces may also occur on non-deformable surfaces, but have been overlooked.

41 Integrin-mediated adhesion is key to fundamental cellular processes such as cell
42 migration¹, differentiation², and the development of tissues and organs³. Integrin
43 clusters serve as communication hubs transmitting mechanical forces between cells
44 and substrates. During mechanotransduction, forces generated by a cell and
45 transmitted to a substrate regulate biochemical signaling as a function of substrate
46 stiffness⁴.

47

48 In recent years, studies on mechanotransduction have brought a wealth of information
49 and conceptual shifts in our understanding of cell adhesion⁵. A plethora of “adhesome
50 proteins” connecting integrins with cytoskeleton was identified and their role in
51 mechanotransduction and cell adhesion was elaborated⁶. The majority of these
52 studies, however, were performed on substrates with immobilized integrin ligands,
53 such as glass or deformable 2D-gels⁷. The actin cytoskeleton plays a crucial
54 mechanical role in adhesion reinforcement on stiff substrates⁸. Actin polymerization
55 is required to form small nascent adhesions (NAs)⁹, and actomyosin contraction
56 promotes their growth into large and dense focal adhesions (FAs) connected to stress
57 fibers¹⁰. However, much less is known about adhesion on soft substrates like 3D
58 matrices¹¹ or to the plasma membrane of other cells¹², relevant to the interaction
59 between immune and target cells^{13,14}, where integrin ligands are not immobilized but
60 embedded on fluid membranes.

61

62 Supported lipid bilayers (SLBs) are convenient model membranes for mimicking the
63 fluid characteristic of plasma membranes to study cell-cell adhesion¹⁵. Previous
64 studies with SLBs functionalized with canonical RGD peptides have shown that cells
65 do not spread, nor develop large and dense integrin adhesions like FAs and stress
66 fibers^{16,17}. The current understanding is that mobile integrin-ligand complexes on
67 bilayers cannot serve as anchoring points promoting cell spreading as on stiff
68 substrates¹⁰, and NAs cannot reinforce through mechanotransduction to promote
69 strong adhesion on fluid substrates¹⁸. However, previous studies have not addressed
70 the effect of the integrin receptor-ligand affinity that may significantly differ from
71 RGD peptides and could regulate cell adhesion^{19,20}.

72

73 To directly test the role of integrin receptor-ligand affinity, we used SLBs
74 functionalized with a high-affinity ligand, the *Yersinia* bacterial protein Invasin²¹, that
75 binds to a subset of β_1 -integrins, including the fibronectin receptor $\alpha_5\beta_1$. On these
76 SLBs, we seeded mouse embryonic fibroblasts (MEF) expressing a recombinant
77 integrin β_1 -subunit labeled with a Halotag at its ectodomain in place of the
78 endogenous β_1 -integrin²². This construct conjugated with membrane impermeable
79 Halotag-dyes allows studying β_1 -integrins primarily at the cell surface, excluding
80 signals from integrins inside the cell observed with genetic labeling. We used
81 confocal microscopy to detect β_1 -integrin clustering during the time course of
82 adhesion. We quantified cluster areas and integrin densities using fluorescent SLB
83 calibration standards¹⁵ and compared them for Invasin- and RGD-SLBs. Accordingly,
84 we provide evidence for mechanotransduction occurring on Invasin-SLBs, leading to
85 forming FA-like β_1 -integrin adhesions. Additionally, we found that cells spread on
86 fluid Invasin- but not on RGD-SLBs. Finally, we show that microtubules but not actin
87 play a significant role in mechanotransduction on these SLBs.

88

89 Results

90

91 Cell trembling and spreading on SLBs depend on ligands.

92 SLBs were functionalized with RGD peptides or Invasin, and their fluidity was
93 confirmed by FRAP (Supplementary Fig. S1). Following the addition of MEF cells
94 using brightfield microscopy, we observed cells with fluctuating edges that we called
95 "trembling" as well as "adherent" cells with immobile edges (Fig. 1A; Supplementary
96 videos V1-V2). The fraction of the latter increased over time, reaching 80% on RGD-
97 and Invasin-SLBs after 45 minutes (Fig. 1B). The fraction of "adherent" cells
98 increased faster on RGD- than on Invasin-SLBs (Fig. 1B), possibly due to higher
99 effective RGD densities on SLBs (20,000 RGD/ μm^2 vs. 600 Invasin/ μm^2) and/or to
100 the RGD binding to a larger range of integrin types that could facilitate integrin
101 activation²³. To further confirm this, we studied cell adhesion dynamics in the
102 presence of manganese (Mn^{2+}) that activates integrins²⁴. Strikingly, integrin activation
103 by Mn^{2+} had no effect on adhesion dynamics on RGD-SLBs. In contrast, it
104 significantly accelerated adhesion on Invasin-SLBs, which had a similar kinetics as
105 on RGD-SLBs (Fig. 1B). While similar cell edge fluctuations were previously
106 reported on glass²⁵, we found, however, that the adherent proportion on glass
107 remained constant and similar for both ligands with and without Mn^{2+}
108 (Supplementary Figs. S2A-C). These observations suggest that cell adhesion on SLBs
109 but not glass depends on the cell ligand's ability to activate integrins.

110

111 Next, we analyzed the morphology of cells spreading on SLBs as a proxy for cell
112 adhesion strength²⁶ by measuring their "projected areas" on the SLB surface, and their
113 "circularities", which characterize cell shape irregularity (Fig. 1C). Consistent with an
114 early adhesion stage, all "trembling" cells on both ligands had a relatively small and
115 round projected area, independently of Mn^{2+} treatment (Supplementary Figs. S2D-E).
116 Moreover, in agreement with previous studies on fluid substrates¹⁶, we found that
117 fibroblasts did not spread on RGD-SLBs, keeping small projected areas ($<200 \mu\text{m}^2$)
118 and remaining round ($0.8 < \text{circularity} < 1$), independently of Mn^{2+} treatment (Figs.
119 1D-E). In contrast, cells spread significantly more on Invasin-SLBs, with median
120 projected areas 1.5- and 2-fold higher than on RGD-SLBs for Mn^{2+} -untreated and
121 treated cells, respectively (Fig. 1E). Surprisingly, 35% of Mn^{2+} -untreated and 75% of
122 Mn^{2+} -treated cells on Invasin-SLBs had irregular shapes with multiple protrusions
123 (circularity < 0.8) and projected areas twice and 3-fold higher than the trembling
124 reference, respectively (Fig. 1C).

125

126 Altogether these results indicate that while integrin activation might be a limiting
127 factor on Invasin-SLBs, these can promote cell spreading upon integrin activation by
128 Mn^{2+} , in contrast to RGD-SLBs.

129

130 β_1 -integrin clusters are denser and larger on Invasin- than on RGD-SLBs.

131 We used confocal microscopy to study clustering of β_1 -integrins, labeled with
132 Alexa488-Halotag ligands, at the cell-SLB interface during the first hour of adhesion.
133 For both RGD- and Invasin-SLBs, we observed the formation of β_1 -integrin clusters
134 with isotropic shapes, morphologically different from FAs or actin-dependent
135 podosome-like structures, mainly composed of β_3 -integrins (Pr. Cheng-Han Yu,
136 private communication), and contrary to β_1 -integrin clusters, formed at low
137 actomyosin contractions¹⁷. We applied fluorescence calibration to determine integrin

138 density maps (Supplementary Fig. S3), and image segmentation programs to detect
139 integrin clusters and measure their areas (σ) and densities of integrins (ρ) (Methods).
140 Specifically, we segmented integrin density maps with a threshold of 300
141 integrins/ μm^2 , corresponding to the minimal spacing of 58 nm between integrin-
142 ligand pairs observed during mechanotransduction on glass²⁷.

143

144 We observed that 45 minutes after cell seeding, clusters on Invasin-SLBs occupied
145 larger areas and were denser than on RGD-SLBs independent of Mn^{2+} (Figs. 2B-D).
146 Median densities of integrins per cell (ρ) reached about 100 integrins/ μm^2 for
147 trembling cells for both ligands (Fig. 2B; Supplementary Figs. S4A-B). However, for
148 adherent cells, median ρ increased significantly higher than for trembling cells: up to
149 160 integrins/ μm^2 and 450 integrins/ μm^2 on RGD- and Invasin-SLBs, respectively.
150 Noteworthy, ρ was significantly higher for Invasin- than RGD-SLBs, reaching values
151 comparable to glass²⁸, that were never observed for RGD-SLBs (Fig. 2B). Moreover,
152 individual integrin clusters were more than twice denser on Invasin- than on RGD-
153 SLBs, with mean values equal to 300 integrins/ μm^2 and 125 integrins/ μm^2 on Invasin
154 and RGD, respectively (Fig. 2C). Additionally, we compared areas of dense integrin
155 clusters σ_{300} , corresponding to $\rho > 300$ integrins/ μm^2 (Fig. 2D). Independent of Mn^{2+}
156 treatment, σ_{300} per cell was significantly higher on Invasin-SLBs, with 23% of
157 clusters larger than the theoretical diffraction limit, DL (183 nm; Methods), as
158 opposed to only 9% for RGD-SLBs (Fig. 2E).

159

160 Finally, we found that high integrin densities in integrin clusters correlated with high
161 spreading area and low cell circularity (Figs. 2F-G; Supplementary Fig. S4C). The
162 growth of adhesion clusters and increased cell spreading suggests that
163 mechanotransduction takes place on Invasin-SLBs.

164

165 **FA proteins are recruited to β_1 -integrin clusters.**

166 Integrin clusters grow and mature during cell adhesion on rigid substrates, recruiting
167 FA proteins in response to mechanotransduction driven by actomyosin
168 contractility^{6,29}. To determine whether β_1 -integrin adhesive clusters could also mature
169 on SLBs, we quantified the recruitment of FA proteins (Figs. 3A-B). “Early adhesion
170 proteins” like talin, kindlin-2, paxillin, vinculin and “late adhesion proteins” like
171 VASP and zyxin were recruited to β_1 -integrin clusters both on Invasin- and RGD-
172 SLBs. Except for talin, however, we observed a higher enrichment of all FA proteins
173 on Invasin- than on RGD-SLBs, consistent with higher mechanical forces applied on
174 Invasin-bound cells.

175

176 While integrin-mediated mechanotransduction on glass is generally associated with
177 the actin cytoskeleton, microtubules can stabilize and regulate protein turnover in
178 FAs^{30,31}. We first measured the association of F-actin to β_1 -integrin clusters in MEF
179 cells using lifeact-mScarlet (Fig. 3C). We found actin enrichment at β_1 -integrin
180 clusters for both ligands but did not observe actin stress fibers usually associated with
181 mechanotransduction (Figs. 3C; 3E). Furthermore, we found that F-actin was more
182 enriched at integrin adhesion clusters on RGD- than Invasin-SLBs (Fig. 3C). These
183 results contrast with the difference in the enrichment of FA proteins at these structures
184 and suggest that actin does not drive FA maturation on Invasin-SLBs. We then
185 investigated the role of the microtubule cytoskeleton using EMTB-iRFP. We found
186 that microtubules were organized in similar networks at the adhesion interface on
187 SLBs and glass (Supplementary Fig. S5A). However, microtubules were enriched

188 with a 17% higher median at β_1 -integrin clusters (Fig. 3D), with a 17% higher median
189 enrichment on Invasin- as compared to RGD-SLBs, suggesting their involvement in
190 maturation. Moreover, we observed that adaptor proteins that connect talin to
191 microtubules (Kank1³² and ELKS³³) were recruited to β_1 -integrin clusters
192 (Supplementary Figs. S5C-D).
193

194 **Dynein pulling along vertical microtubules leads to mechanosensitive growth of** 195 **integrin clusters.**

196
197 During mechanotransduction, integrin clusters are subjected to cellular mechanical
198 forces that significantly increase in magnitude if applied against rigid substrates¹⁰. On
199 fluid SLBs, lateral components of the forces are negligible compared to glass and are
200 only associated with membrane viscosity³⁴. However, SLBs can resist higher
201 magnitude normal forces leading to the maturation of integrin clusters. These normal
202 forces might lead to coupled local deformations of SLBs and cell plasma membranes.
203 We observed such deformations while imaging β_1 -integrin clusters above the SLB
204 focal plane (Fig. 4A; zoom 1; $0 < z < 1.5 \mu\text{m}$). Akin to clusters at SLB surfaces (Fig.
205 4A; zoom 2; $z = 0$), clusters in the cell volume had a larger total area in cells on
206 Invasin- than on RGD-SLBs (Fig. 4B). Some of them were associated with membrane
207 tubes that were pulled out of SLBs (Fig. 4A; zoom 2). The proportion of cells with
208 tubes and the number of tubes per cell were significantly higher on Invasin than on
209 RGD (Fig. 4C). These results are consistent with the notion that integrin clusters on
210 Invasin-SLBs are exposed to higher mechanical forces than those on RGD-SLBs,
211 leading to their growth in size and density, higher recruitment of FA proteins and
212 more tubes.
213

214 To characterize the origin of vertical forces pulling on integrin clusters, we tested the
215 effects of inhibitors of cytoskeleton polymerization and associated motors on the
216 proportion of cells with tubes and the number of tubes per cell on Invasin-SLBs since
217 they have a higher number of tubes compared to the ones on RGD-SLBs (Fig. 4D;
218 Supplementary Fig. S6A). We found that preventing actomyosin contractility by Rho
219 kinase inhibition (Y-27632) or blebbistatin did not influence the frequency of tube
220 formation, in agreement with previous studies on RGD-SLBs³⁵. Y-27632, however,
221 led to a significant decrease in β_1 -integrin cluster size and density (Figs. 4E-F).
222 Inhibition of formins and the Arp2/3 complex that nucleate actin polymerization using
223 SMIFH2 and CK666, respectively, also did not affect tube formation frequency (Fig.
224 4D; Supplementary Fig. S6A). Together, these results suggest that the actin
225 cytoskeleton is not involved or is not the only driver of vertical forces applied on
226 integrin clusters on SLBs.
227

228 Microtubules and associated molecular motors were reported to be involved in the
229 mechanosensitivity of FAs on rigid substrates^{36,37}. We thus investigated whether they
230 could be involved in tubular deformations of SLBs. We observed dense and large
231 integrin clusters at the SLB surface and on tubes associated with microtubules (Figs.
232 3D; 4A). When microtubules were depolymerized with nocodazole (NZ) or when
233 dynein activity was blocked with ciliobrevin D (CBD), the frequency and number of
234 tubes decreased drastically (Fig. 4D; Supplementary Fig. S6A). Similar findings were
235 observed in HeLa cells on Invasin-SLBs after dynein inhibition with CBD or
236 silencing of the dynactin subunit p150glued (Fig. 4G; Supplementary Figs. S6B-C).
237 These results suggest that vertical forces applied to β_1 -integrin clusters depend on

238 microtubules and are driven by dynein activity. Finally, NZ and CBD treatment
239 significantly decreased the total area of β_1 -integrin clusters and density (Figs. 4E-F).
240 Noteworthy, this NZ effect is opposite to that observed on rigid substrates, where it
241 was described to lead to an increase in size and density of FAs due to enhanced
242 actomyosin contractility when microtubules are depolymerized^{38,39}.

243

244 Globally, these findings suggest that mechanotransduction on SLBs results from the
245 pulling activity of the dynein motors on locally vertical microtubules, leading to the
246 growth and maturation of integrin clusters. Higher microtubule enrichment and higher
247 frequency of tube formation on Invasin- than on RGD-SLBs suggests affinity-
248 dependent mechanotransduction at integrin clusters on SLBs, in contrast with glass.
249 That is consistent with longer lifetime ligand-integrin bonds supporting more efficient
250 mechanotransduction. To further characterize the role of integrin-ligand affinity on
251 integrin clustering on fluid substrates, we propose a theoretical model (Supplementary
252 Material) that supports that ligand-receptor pairs with higher affinity form clusters of
253 larger density and can sustain larger vertical forces (Supplementary Fig. S7).

254

255 The model takes into account the concentrations of ligands, receptors, and bonds (i.e.,
256 bound ligand-receptor complexes), as well as the average distance between the cell
257 membrane and SLBs. SLB fluidity allows for ligand (and possibly bond) mobility,
258 which plays a dual role in the theory. First, ligand diffusion acts as a chemical contact
259 with a reservoir and promotes cluster formation. Second, the large tangential forces
260 exerted by the cytoskeleton tend to displace bonds rather than break them. Hence, the
261 vertical cytoskeletal forces are the primary driver of mechanotransduction in the
262 theory. We consider adhesion clusters as dense phase-separated regions of bonds.
263 Phase separation is driven by the interplay between diffusion and attractive bond-
264 bond interactions via two mechanisms. The first attractive mechanism is mediated by
265 the membranes⁴⁰⁻⁴² and the second by adaptor proteins in the adhesion site⁴³⁻⁴⁵.
266 Cytoskeletal forces drive conformational changes in adaptor proteins and increase the
267 effective attraction between bonds (e.g., via the exposure of vinculin binding sites on
268 talin^{46,47}). The formation of adhesion sites depends on affinity and vertical
269 cytoskeletal force in the following way: forces increase the bond-bond attraction, and
270 affinity increases the total number of bonds (Supplementary Fig. S7). The system is
271 predicted to phase separate for sufficiently numerous bonds and strong attraction,
272 implying adhesion clusters. The phase diagram obtained from the model agrees with
273 our experimental results of cell spreading on SLBs, for which the model explains the
274 differences in clustering between the two ligand types (Supplementary Fig. S7).

275

276 **Integrin clusters are pushed to the cell periphery in a dynein- and microtubule-**
277 **dependent manner.**

278 Cell spreading correlates with integrin clustering due to mechanotransduction on glass
279 and high viscosity RGD-SLBs³⁴. Similarly, we found that for Mn²⁺-treated cells
280 adhering on Invasin-SLBs, there was a correlation between cell spreading and the
281 presence of membrane tubes, consistent with mechanotransduction at integrin clusters
282 on SLBs (Fig. 5A).

283

284 On glass, growing and maturing FAs move from the cell periphery towards the cell
285 center serving as platforms for cell protrusions nucleating F-actin at the cell
286 periphery⁴⁸. FAs also constitute holding points as cells protrude over them during cell
287 polarization⁴⁸, a function that they are unlikely to perform on fluid SLBs.

288 Unexpectedly, we observed that cells formed protrusions on Invasin-SLBs, leading to
289 their symmetrical spreading without polarization (Fig. 1C). In order to investigate this
290 peculiar spreading, we localized β_1 -integrin clusters and membrane tubes in cells with
291 respect to the proximity to cell protrusions (Fig. 5B). We found that tubes were more
292 frequent at the cell periphery than at the cell center (Fig. 5C). Furthermore, cells with
293 β_1 -integrin clusters in the cell center were significantly less spread and more circular
294 than cells with β_1 -integrin clusters at the cell periphery at the base of actin-rich
295 protrusions (Figs. 3E; 5D-E). These results, together with the finding that cell
296 spreading correlates with the density of β_1 -integrin clusters (Figs. 2F-H), suggest that
297 dense and mature β_1 -integrin clusters at the cell periphery play a role in cell
298 spreading.

300 Because microtubules and dyneins were crucial for integrin cluster growth and
301 densification (Figs. 4E-F), we hypothesized that when oriented parallel to the
302 substrate, they also played a role in the localization of clusters at the cell periphery, in
303 a similar manner as to what was described for cells spreading on glass^{49,50}. In line
304 with this, we often observed microtubule accumulation at peripheral integrin clusters,
305 at the base of actin protrusions (Fig. 3E; Invasin). Cell treatment with NZ or CBD
306 decreased integrin clustering (Figs. 4E-F) and completely abolished integrin cluster
307 localization at the cell periphery (Figs. 5F-G). Noteworthy, cells treated with NZ or
308 CBD did not spread (Figs. 5H-I). We interpret this result as a combination of
309 inhibition of microtubule and dynein pushing on adhesion clusters and an increase in
310 actomyosin contractility possibly linked to microtubule depolymerization³⁹.
311 Consistent with an antagonistic role of the actin cytoskeleton on cell spreading,
312 inhibition of Rho kinase with Y27632 led to even more spread cells⁵¹ (Figs. 5H-I).
313 Similarly to cells on glass⁵² and RGD-SLBs¹⁶, inhibition of formins by SMIFH2
314 impaired cell spreading on Invasin-SLBs and integrin cluster movement to the cell
315 periphery without significant effects on cluster growth (Figs. 4E-F; 5F-G). These
316 findings suggest that, due to dynein motor activity along microtubules, large and
317 mature β_1 -integrin clusters are pushed towards the cell periphery, contributing to cell
318 spreading on SLBs through actin-rich protrusions emanating from these adhesive
319 clusters.

320

321 **Discussion**

322 Our understanding of FAs is based primarily on studies on rigid substrates such as
323 glass, where mechanotransduction is mainly driven by actomyosin-dependent forces
324 transduced by actin fibers tangential to the cell basal membrane⁵³. Living tissues,
325 however, cover a wide range of stiffness⁵ and, instead of being immobilized, integrin
326 ligands can be present in cell membranes^{54,55} where they may diffuse laterally with
327 little constraints and may not exert the range of counter-forces required for
328 “canonical” mechanotransduction. Indeed, when cells adhere on SLBs, the absence of
329 actin stress fibers usually associated with FAs that planar forces on SLBs are
330 negligible compared to glass^{56,57}.

331

332 Using *Yersinia* Invasin as a high-affinity β_1 -integrin ligand, we show for the first time
333 that cells adhere, spread, and develop large and dense β_1 -integrin clusters on fluid
334 substrates (SLBs), similar to FAs on glass (Fig. 2B)²⁸. Contrary to glass, on SLBs,
335 actin-related forces tangential to the plasma membrane do not play a dominant role in
336 integrin adhesion due to substrate fluidity. In addition, instead of enhancing adhesion
337 and spreading³⁸, microtubule depolymerization strongly inhibits it. We show here that

338 adhesion maturation on bilayers relies on microtubule-dependent forces perpendicular
339 to the bilayer. We evidenced these forces by observing local SLB deformations/tubes
340 connecting the bilayer and the integrin cluster in the plasma membrane (Fig. 4A). The
341 existence of normal force components at integrin clusters was also reported on RGD-
342 SLBs using DNA-FRET force probes⁵⁸, but their origin was not identified.

343
344 On glass, microtubules orient vertically⁵⁹ and associate with mature FA at the cell
345 periphery via several adaptor proteins^{33,36}. Moreover, dyneins that stabilize
346 microtubule plus-ends at the plasma membrane⁶⁰ interact with integrin adhesion
347 clusters through paxillin and other FA proteins^{61,62}. Here, we present several pieces of
348 evidence supporting a role for microtubules in exerting forces on β_1 -integrin clusters,
349 normal to the SLB surface: i, microtubules are enriched at integrin clusters on SLBs
350 (Fig. 3D); ii, microtubule-talin adaptors KANK1 and ELKS are recruited at these
351 clusters (Supplementary Figs. S5D-E); iii, some microtubules have a vertical
352 orientation in association with integrin adhesions (Fig. 4A). In addition, we have
353 evidenced microtubule- and dynein-dependent (but not actin-dependent) local tubular
354 membrane deformations connected to the clusters (Fig. 4D) that could be related to
355 integrin endocytosis. Indeed, active β_1 -integrins participate in clathrin-dependent
356 endocytic pathway regulated by Arf6 and Rab5 GTPases that use microtubules as
357 tracks and dynein motors for integrin internalization^{63,64}. The tube formation that we
358 observe on SLBs could be viewed as part of that process. Indeed, similar membrane
359 tubulation was reported at invadopodia promoting integrin trafficking⁶⁵. Molecular
360 motors can collectively pull tubes from membranes by distributing the load on a
361 dynamically formed motor assembly, as shown *in vitro* for kinesins⁶⁶. Forces of the
362 order of 60 pN or less are necessary to pull tubes from SLBs on glass⁶⁷. Dimers of
363 dyneins exert forces of the order of 7 pN⁶⁸ and can then pull on integrin adhesion
364 complexes. When enough motors accumulate at the extremity of the microtubule, they
365 produce the force required to tubulate together the plasma membrane and the SLB
366 attached to it through the integrin-ligand links. This force induces conformational
367 changes in adaptor proteins leading to integrin clustering, which according to our
368 theoretical model, is more efficient for high-affinity Invasin than RGD. During
369 integrin internalization, these membrane tubes are cleaved by dynamin⁶⁹. However,
370 contrary to glass, a double membrane tube is formed during endocytosis on SLBs,
371 with the SLB membrane in the inner layer surrounded by the cell plasma membrane
372 as an outer layer (Figs. 4A; 6). Such double-layer tubes could resist more dynamin
373 scission, leading to the formation of stable tubes.

374
375 On rigid substrates, cells form protrusions to spread. In this process, mature FAs act
376 as anchoring points against which growing actin-rich protrusions push. The ability of
377 cells to spread on glass depends on the density of ligands on the surface, the size of
378 integrin adhesions, and their connection to the cytoskeleton⁷⁰. On SLBs, on the other
379 hand, mature integrin adhesions can move in the adhesion plane upon mechanical
380 stimuli. We show for the first time that cells spread on fluid SLBs coated with the
381 high-affinity ligand Invasin. Our results indicate that integrin adhesion clusters mature
382 on fluid substrates and are sufficiently constrained to serve as holding points to
383 support spreading, likely through their connection with microtubules.

384
385 Figure 6 summarizes our observations of cell adhesion on fluid substrates.
386 Microtubules were shown to be essential for cell spreading on relaxed collagen
387 networks and for forming dendritic extensions⁷¹. On Invasin-SLBs, integrin clusters

388 are dragged in the SLB plane towards the cell periphery in a microtubule and dynein-
389 dependent manner (Fig. 3E). Since dyneins are involved in the maturation of the
390 adhesions, essential for spreading (Fig. 5A), we cannot distinguish whether some
391 dyneins also push microtubules linked to adhesions parallel to the surface towards the
392 edge⁷² or whether microtubule dynamics is responsible for this. This movement is
393 likely antagonized by an inward force due to the actomyosin contraction of the cell
394 cortex^{73,74} (Figs. 3E; 5J), leading to stalling of integrin clusters at the cell edge where
395 they serve as anchoring centers from which actin-rich protrusions emanate^{71,75}. These
396 protrusions might result from a combination of pushing forces from actin and
397 microtubule polymerization and microtubule pushing through dynein activity. The
398 pushing forces are applied against the centrosome to which microtubules are
399 connected (Supplementary Fig. S5B). Remarkably, in contrast with cells on glass
400 where microtubules are essential for cell polarization^{76,77}, cells do not polarize on
401 SLBs and conserve their central symmetry despite the central role of microtubules
402 through a mechanism that remains to be characterized.

403
404 In HeLa cells on SLBs, we also observed dynein/microtubule-dependent formation of
405 tubes (Fig. 4G), suggesting that mechanotransduction associated with forces normal
406 to the adhesion plane occurs in various cell types. Functionalized SLBs allow to study
407 the early stages of cell adhesion on a fluid interface, which is relevant for cell-cell
408 interactions (i.e., brain cells, immune cells, etc.)⁴, but not accessible on substrates like
409 glass or gels. Moreover, functionalized SLBs reveal forces probably at play on stiff
410 substrates that cannot be observed with immobilized ligands. Using this platform, we
411 found a new role for dynein motors and microtubules in integrin adhesion cluster
412 growth and maturation on SLBs. In addition, our experiments and physical modeling
413 show that cell adhesion on a fluid interface can be strongly modulated by the
414 receptor-ligand affinity, in contrast with solid surfaces. That might be used as a
415 selective adhesion strategy by T- or B-cells, in which microtubules and dynein motors
416 are known to be involved in clustering TCR and BCR receptors, respectively^{78,79}.
417

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445 **Author contributions**

446 O.M., P.B. and G.T.V.N. conceived the study. P.B. and G.T.V.N. acquired funding and
447 equally contributed to this work. O.M. designed and performed experiments, analyzed data,
448 and wrote the original manuscript. A.S.M. and O.M. developed image analysis programs for
449 integrin cluster. F.D.F, J.M. and O.M. performed cloning, expression, purification and
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455

456 **Competing interests**

457 The authors declare no competing interests.

458

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665
666

667 **Figure Legends**

668

669 **Figure 1. MEF cells adhere faster RGD- than on Invasin-coated SLBs, but**
670 **adhered cells spread more on Invasin- than on RGD-coated SLBs.**

671 A) Brightfield images of an adherent (left) and a trembling (right) cell on Invasin-SLB with
672 corresponding kymographs describing cell edge movements over 2 minutes. Scale bars: 10
673 μm ; 1 μm .

674 B) Time evolution of fractions of adherent cells on SLBs functionalized with RGD (blue) and
675 Invasin (red) in presence (Mn^+ , full line) or absence (Mn^- , dashed line) of Mn^{2+} . Each
676 data point represents between 42 and 113 cells studied in at least 3 independent biological
677 experiments. A fraction of adherent cells 45 minutes after seeding on SLBs without
678 ligands (Ctrl-, green) in absence of Mn^{2+} . Line scatter plot, mean, SEM.

679 C) Brightfield images of cells with two different morphologies (I – round; II – not round) on
680 SLBs functionalized with RGD (blue) and Invasin (red). Scale bars: 10 μm .

681 D-E) Trembling (“Tr”, plots on grey background area) and adherent (“Ad”, 45 min – 1h after
682 seeding) cells in presence (Mn^+) or absence (Mn^-) of Mn^{2+} adhering on RGD- (blue) or
683 Invasin-coated (red) SLBs. Data from 44 cells, $N_{\text{exp}}=3$ (independent experiments) for
684 trembling cells on RGD; 57 cells, $N_{\text{exp}}=3$ for trembling cells on Invasin (both Mn^-); 49
685 cells, $N_{\text{exp}}=3$ for cells adhering on RGD (Mn^-); 53 cells, $N_{\text{exp}}=3$ for cells adhering on
686 Invasin (Mn^-); 58 cells, $N_{\text{exp}}=3$ for cells adhering on RGD (Mn^+) and 49 cells, $N_{\text{exp}}=5$
687 for cells adhering on Invasin (Mn^+).

688 D) Circularity of trembling and adherent cells, based on cell edge detection from brightfield
689 images, 45 minutes after seeding. 2 classes of cell morphologies are indicated: I
690 (circularity > 0.8), II (circularity < 0.8). Box plot.

691 E) Projected area of the cells described in D. Bar plot, median, 95% CI.

692

693 **Figure 2. β_1 -integrin clusters in MEF cells adhering on SLBs and relationship**
694 **between their density and cell spreading.**

695 A) Main panels: β_1 -integrin density maps, using the “physics” LUT of ImageJ below (cf.
696 Methods for the calibration derived from fluorescence images), showing β_1 -integrin cluster
697 organization in cells adhering on SLBs coated with RGD (upper left) and Invasin (upper
698 right). Images are taken at the focal plane of the SLBs. Lower left corners: corresponding
699 brightfield images.

700 Zoomed panels (white squares on the main panels): regions with β_1 -integrin clusters (black
701 arrows).

702 Bottom: β_1 -integrin density profiles along the white rectangles in the zoomed panels.

703 Scale bars: 5 μm (main panels); 1 μm (zoomed panels).

704 B-E) RGD-SLB (blue), Invasin-SLB (red). Data from 57 cells, $N_{\text{exp}}=3$ (independent
705 experiments) for trembling cells on Invasin; 44 cells, $N_{\text{exp}}=3$ for trembling cells on RGD
706 (both Mn^-); data from adhering cells 45 min – 1h after seeding : 83 cells, $N_{\text{exp}}=4$ for cells
707 adhering on Invasin (Mn^-); 48 cells, $N_{\text{exp}}=3$ for cells adhering on RGD (Mn^-); 78 cells,
708 $N_{\text{exp}}=5$ for cells adhering on Invasin (Mn^+) and 102 cells, $N_{\text{exp}}=4$ for cells adhering on
709 RGD (Mn^+).

710 B) Distributions of mean β_1 -integrin density per cell in trembling (“Tr”, plots on grey
711 background area) and adherent (“Ad”) cells in presence (Mn^+) or absence (Mn^-) of Mn^{2+}
712 adhering on RGD- or Invasin-coated SLBs. Box plot.

713 C) Histograms of integrin densities in individual clusters in Mn^{2+} treated cells adhering on
714 RGD- or Invasin-SLBs. Data from 11,441 clusters on Invasin and 16,115 clusters on
715 RGD.

716 D) Distributions of total area of β_1 -integrin clusters per cell in presence (Mn^+) or absence
717 (Mn^-) of Mn^{2+} in cells adhering on RGD- or Invasin-SLBs. Box plot.

718 E) Distributions of areas of individual β_1 -integrin clusters of density higher than 300 β_1 -
719 integrins/ μm^2 in Mn^{2+} -treated cells adhering on RGD (blue)- or Invasin (red)-coated

720 SLBs. 75-percentile of σ_{300} per cell distribution: 8-9 μm^2 for Invasin-SLBs and 0.1-0.15
721 μm^2 for RGD-SLBs. Data from 347 clusters for cells adhering on Invasin and 8,954
722 clusters for cells adhering on RGD. Box plot.
723 F-G) Mn^{2+} -treated MEF cells adhering on Invasin-SLB during 0 – 1h after seeding to
724 chambers with clusters of highest $\rho > 500$ (magenta); $300 < \rho < 500$ (green); and $\rho < 300$
725 β_1 -integrins/ μm^2 (black). Data from 157 cells studied in at least 3 independent biological
726 experiments ($N_{\text{exp}}=3$) for $\rho > 500$; 61 cells studied in at least $N_{\text{exp}}=3$ for $300 < \rho < 500$
727 and 87 cells studied in at least $N_{\text{exp}}=3$ for $\rho < 300$.
728 F) Cell circularity. Box plot.
729 G) Projected cell area (μm^2). Box plot.

730

731 **Figure 3. Focal adhesion proteins and cytoskeleton (actin and microtubules) are**
732 **enriched at β_1 -integrin clusters on SLBs.**

733

All cells are Mn^{2+} -treated and imaged 45 min – 1h after seeding in the chamber.

734

A) Main panels: fluorescence multi-channel images showing β_1 -integrin (cyan) and paxillin
735 (magenta) cluster organization in cells adhering on SLBs coated with RGD (blue frame,
736 left) and Invasin (red frame, right). Images are taken as max intensity z projections (in
737 Fiji) of the volume with 1 μm height (z coordinate) centered at the focal plane of the SLB.
738 Upper left corners: corresponding brightfield images.

739

Medium panels: zooms corresponding to the white squares on the main panels. Regions
740 with β_1 -integrin and paxillin clusters.

741

Bottom: β_1 -integrin and paxillin intensity profiles along the lines (white rectangles in the
742 zoomed panels).

743

Scale bars: 5 μm (main panels); 1 μm (zoomed panels).

744

B) Enrichment of FA proteins in β_1 -integrin clusters in cells adhering on SLBs functionalized
745 with RGD (blue) and Invasin (red) as a mean fluorescence intensity of the FA protein in
746 the region of β_1 -integrin clusters divided by the mean fluorescence intensity of the protein
747 in the cell. It was compared with “shuffled” control enrichments (black), enrichment
748 calculated at random pixels instead of β_1 -integrin clusters regions. Box plot.

749

Data for every FA protein from between 108 and 181 cells for the shuffled control
750 (shuffled), between 42 and 87 cells (RGD) and between 56 and 128 cells (Invasin). All the
751 cells are studied in at least 3 independent biological experiments (N_{exp}).

752

C) Enrichment of F-actin in β_1 -integrin clusters in cells adhering on SLBs functionalized with
753 RGD and Invasin. Box plot.

754

Data from 143 cells (shuffled), 58 cells (RGD) and 85 cells (Invasin). $N_{\text{exp}}=3$ at least.

755

D) Enrichment of microtubule in β_1 -integrin clusters in cells adhering on SLBs functionalized
756 with RGD (blue) and Invasin (red). Box plot.

757

Data from 107 cells (shuffled), 53 cells (RGD) and 54 cells (Invasin). $N_{\text{exp}}=3$ at least.

758

E) Fluorescence multi-channel images showing β_1 -integrin (yellow), actin (magenta) and
759 microtubules (cyan) in cells adhering on SLBs coated with RGD (blue frame, left) and
760 Invasin (red frame, right). Images are taken in the focal plane of the SLBs. β_1 -integrin
761 density, actin and microtubules intensity profiles along the lines (black rectangles in the
762 zoomed panels) are plotted for both cells. Scale bars: 5 μm , 1 μm .

763

764 **Figure 4. Tube formation at β_1 -integrin clusters is driven by microtubules and**
765 **dynein activity.**

766

All cells are Mn^{2+} -treated and imaged 45 min – 1h after seeding in the chamber on Invasin-
767 coated SLBs.

768

A) Top panel: fluorescence multi-channel image of a MEF cell showing the SLB labeled with
769 a TR-DHPE lipid, β_1 -integrin with Halotag-Alexa488, actin with lifeact-mScarlet and
770 microtubules (MT) with EMTB-iRFP. A brightfield image of the cell is in the upper left
771 corner of the merged image.

772

Zoomed panels: regions corresponding to white squares 1 and 2 on the main panels. Top: x-y.

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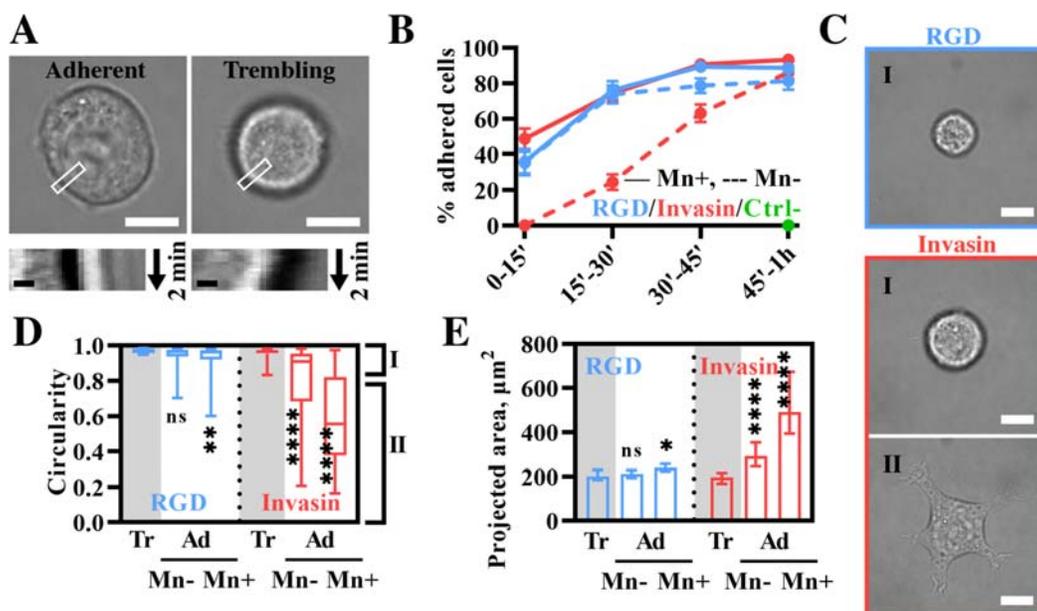
Bottom: x-z section corresponding the lines in x-y.

- 774 Scale bars: 10 μm (main panel); 2 μm (zoomed panel xy); 0.5 μm (zoomed panel xz).
775 B-C) Data from 78 cells, $N_{\text{exp}}=4$ on Invasin; 102 cells, $N_{\text{exp}}=4$ on RGD.
776 B) Distributions of total area of β_1 -integrin clusters per cell at the SLB level and in the
777 volume 1.5 μm above the SLB for MEF cells adhering on RGD- (blue) and Invasin-coated
778 (red) SLBs. Box plots.
779 C) Histograms of the number of detected tubes per cell in MEF cells adhering on RGD- (blue)
780 and Invasin-coated (red) SLBs.
781 D) Proportion of MEF cells with tubes. Cells adhere on Invasin-coated SLBs in presence of
782 drugs. Data from 78 cells, $N_{\text{exp}}=4$ for DMSO; 104 cells, $N_{\text{exp}}=4$ for CK666; 71 cells,
783 $N_{\text{exp}}=3$ for SMIFH2; 58 cells, $N_{\text{exp}}=3$ for CK666+SMIFH2; 72 cells, $N_{\text{exp}}=3$ for
784 Y27632; 86 cells, $N_{\text{exp}}=3$ for Nocodazole (NZ); 69 cells, $N_{\text{exp}}=4$ for Ciliobrevin D
785 (CBD). Bar plot: mean, SEM.
786 E-F) MEF cells treated with Nocodazole (NZ), Ciliobrevin D (CBD) and non-treated (DMSO).
787 Data from 86 cells, $N_{\text{exp}}=3$ for Nocodazole (NZ); 69 cells, $N_{\text{exp}}=4$ for Ciliobrevin D
788 (CBD); 78 cells, $N_{\text{exp}}=4$ for DMSO.
789 E) Distributions of the mean integrin density per cell. Box plot.
790 F) Distributions of the total area of β_1 -integrin clusters per cell. Box plot.
791 G) Proportion of HeLa cells with tubes. HeLa cells were treated with siRNA against
792 p150glued, siRNA scramble (as a negative control) and with CBD. Bar plot: mean, SEM.
793 Data from 58 cells, $N_{\text{exp}}=3$ for DMSO; 56 cells, $N_{\text{exp}}=3$ for siRNA scramble; 61 cells,
794 $N_{\text{exp}}=4$ for siRNA p150glued and 50 cells, $N_{\text{exp}}=4$ for CBD.
795

796 **Figure 5. Dyneins push integrin clusters along microtubules to the cell periphery**
797 **resulting in cell spreading on SLB.**

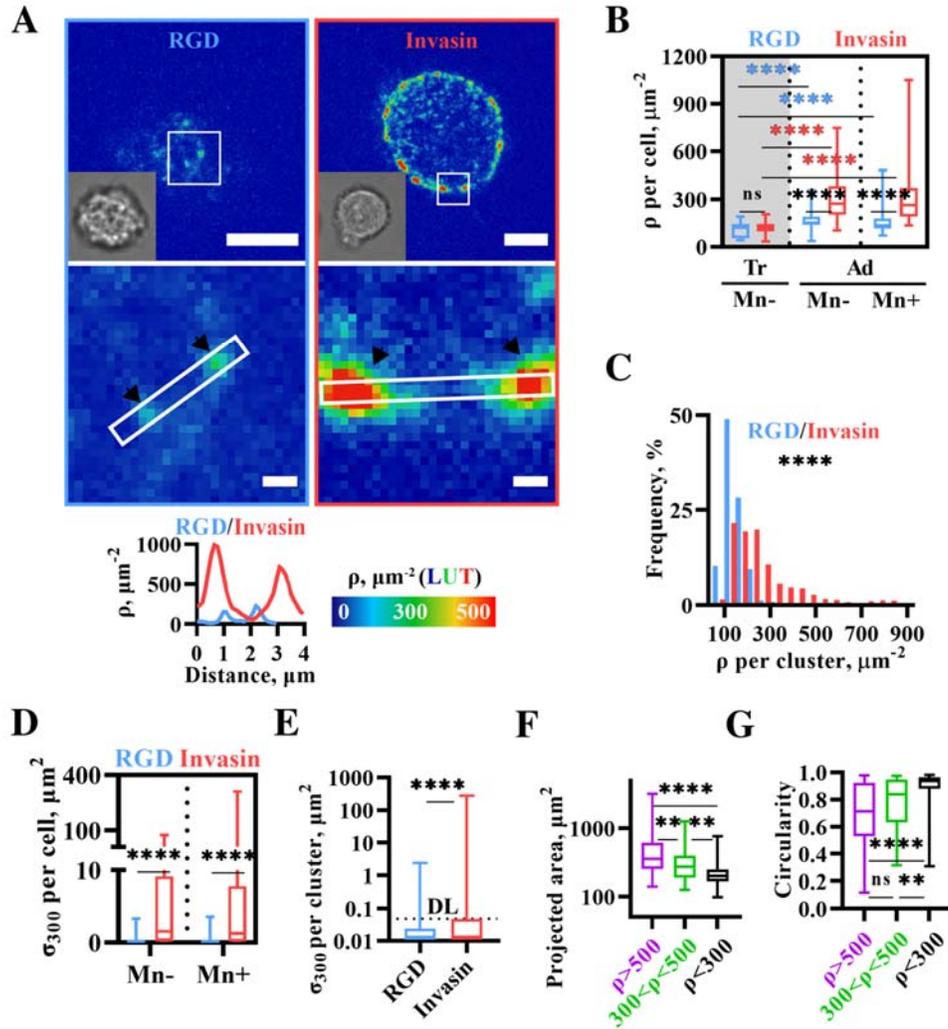
- 798 All cells are Mn^{2+} -treated MEF cells and imaged 45 min – 1h after seeding to the chamber
799 with Invasin-coated SLBs.
800 A&C-E) Data from 78 cells, 5 independent experiments.
801 A) Projected cell area (μm^2) of cells with and without detected tubes. Box plot.
802 B) Schematic representation of the zones in a spreading cell. Red: actin-rich protrusions
803 (lamellipodia-filopodia); green: cell periphery that corresponds to the band parallel to the
804 cell border (excluding actin-rich protrusions) with a width equal to 10% of the cell size
805 (length of the smallest side of the smallest rectangle containing the cell); grey: cell body,
806 which is the central cell area.
807 C) Projected cell area (μm^2) of cells that have higher β_1 -integrin cluster frequency at the cell
808 periphery or at the cell body. Box plot.
809 D) Cell circularity of cells that have higher β_1 -integrin cluster frequency at the cell periphery
810 or at the cell body. Box plot.
811 E) Tube spatial frequency distribution (number of tubes per μm^2) in the cell periphery zone vs
812 cell center zone. Box plot.
813 F) Comparative illustration of integrin cluster distribution for the cells treated with drugs
814 (CK666, SMIFH2, Y27632, NZ, CBD and DMSO for non-treated cells). β_1 -integrin
815 density maps (represented with “physics” LUT of Fiji, calibrated bar on the side). Images
816 are taken at the SLBs' plane. Corresponding brightfield images are in the upper right
817 corners. Scale bars: 10 μm .
818 G-I) Data from 78 cells, $N_{\text{exp}}=4$ with DMSO; 104 cells, $N_{\text{exp}}=4$ with CK666; 71 cells,
819 $N_{\text{exp}}=3$ with SMIFH2; 58 cells, $N_{\text{exp}}=3$ with CK666+SMIFH2; 72 cells, $N_{\text{exp}}=3$ with
820 Y27632; 86 cells, $N_{\text{exp}}=3$ with Nocodazole (NZ); 69 cells, $N_{\text{exp}}=4$ with Ciliobrevin D
821 (CBD).
822 G) Ratio of spatial frequencies P of integrin clusters in the periphery zone ($P_{\text{periphery}}$) to the
823 center zone (P_{center}). Cells with more clusters in the periphery have $P>1$ and are in the
824 green part of the graph where those with more clusters in the center have $P<1$ and are in
825 the grey part. Box plot.
826 H) Projected area (μm^2) of cells treated with drugs. Box plot.
827 I) Circularity of cells treated with drugs. Box plot.

828

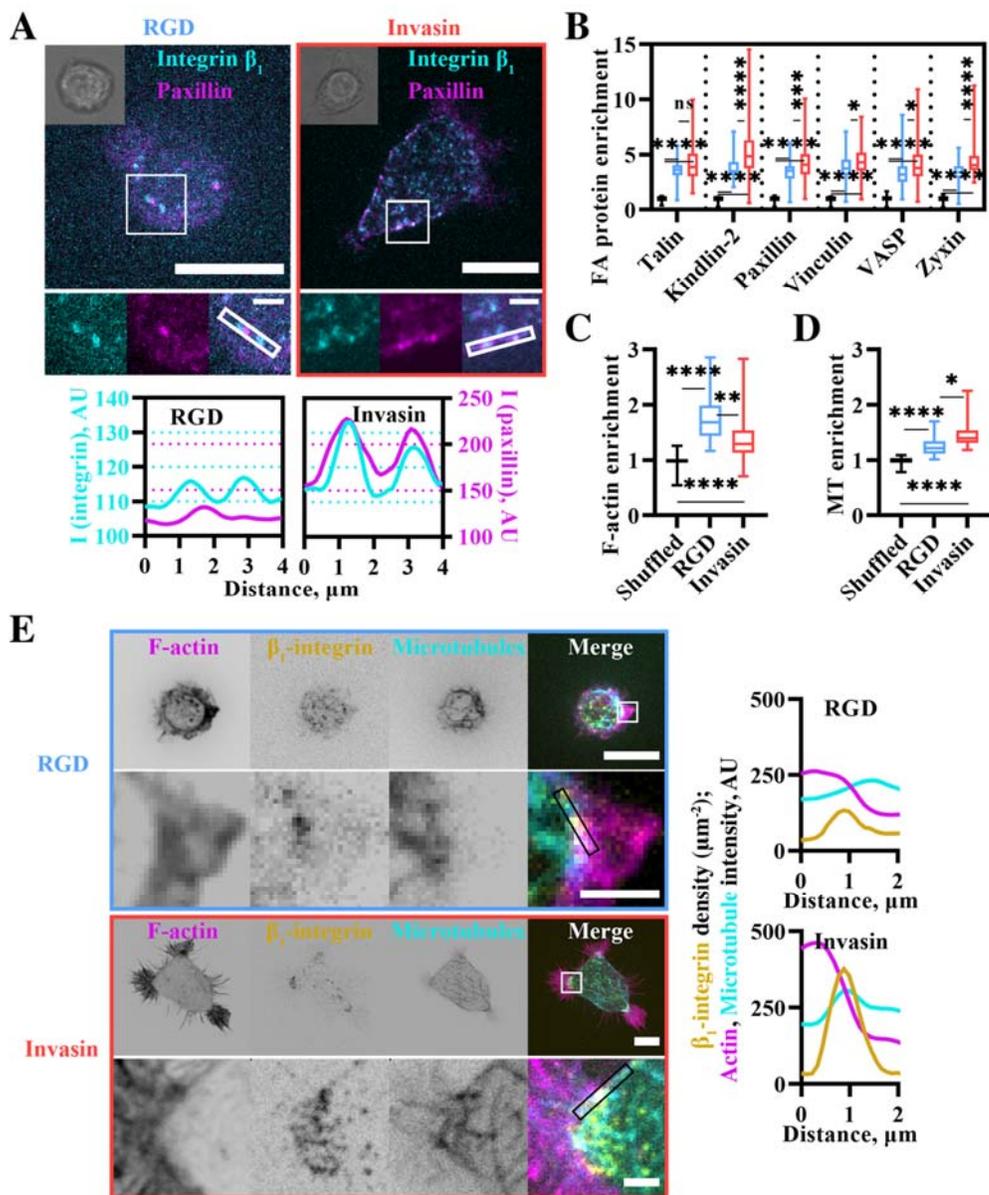


829

830 **Figure 1. MEF cells adhere faster RGD- than on Invasin-coated SLBs, but**
 831 **adhered cells spread more on Invasin- than on RGD-coated SLBs.**

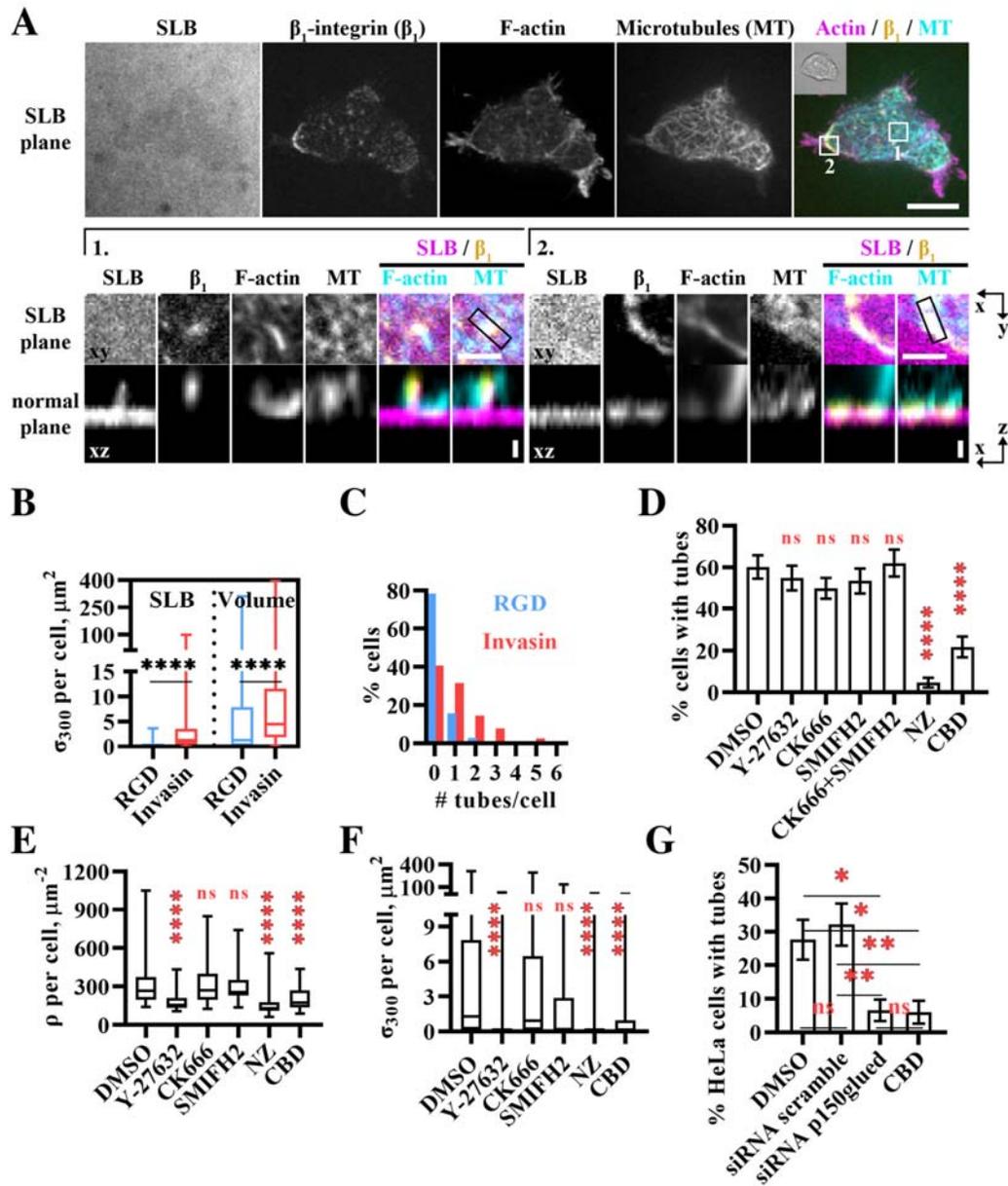


832
 833 **Figure 2. β_1 -integrin clusters in MEF cells adhering on SLBs and relationship**
 834 **between their density and cell spreading.**
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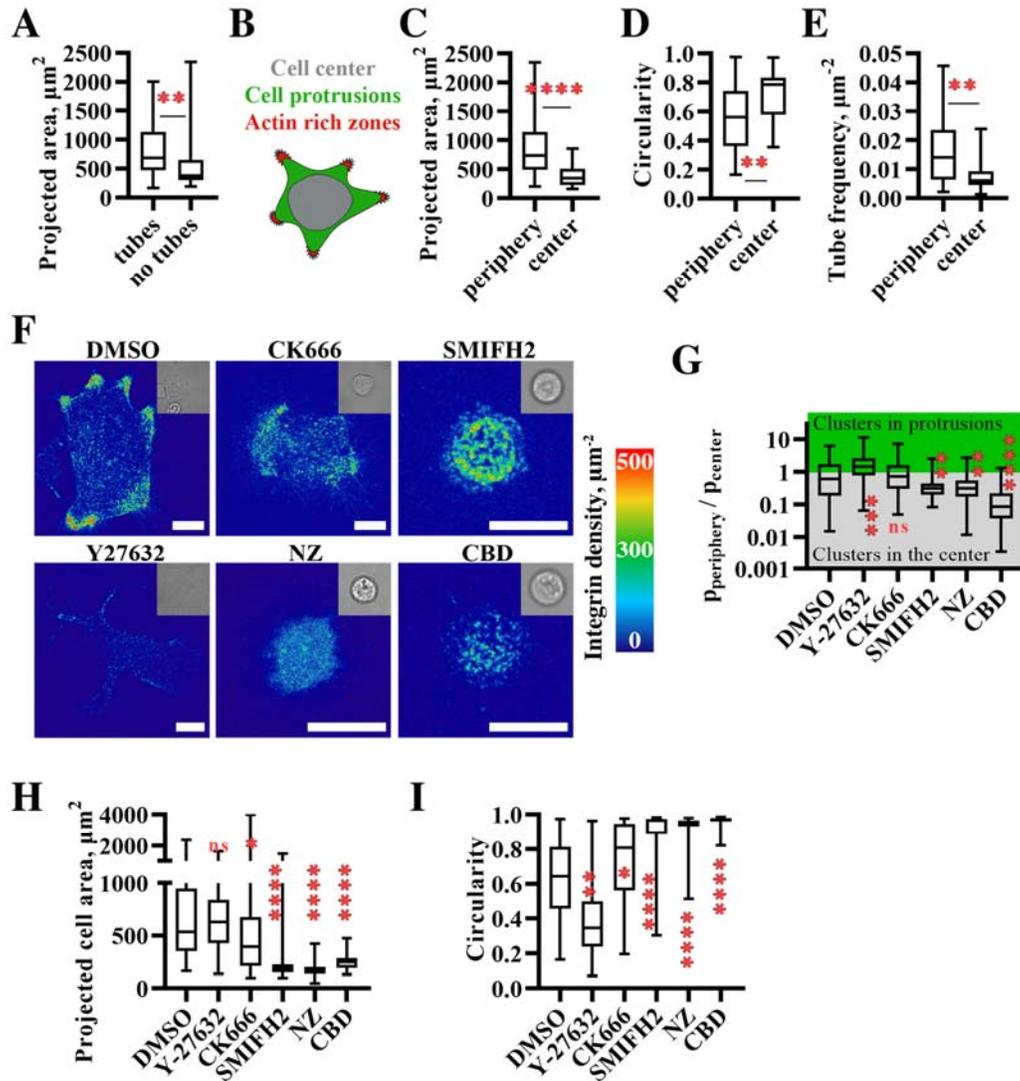
Figure 3. Focal adhesion proteins and cytoskeleton (actin and microtubules) are enriched at β_1 -integrin clusters on SLBs.



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Figure 4. Tube formation at β_1 -integrin clusters is driven by microtubules and dynein activity.

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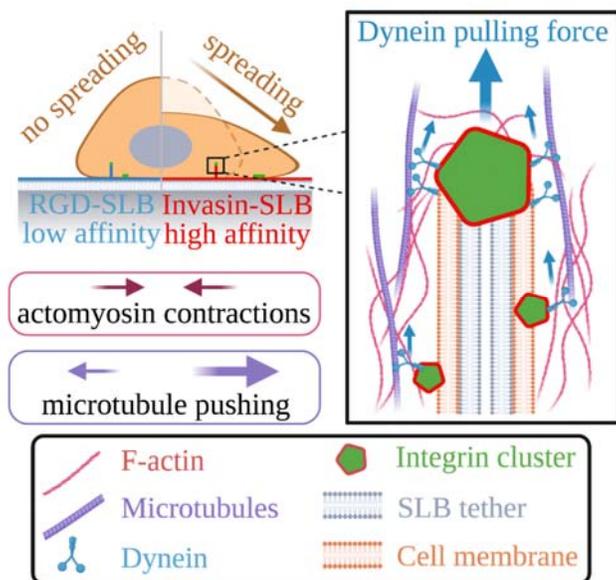
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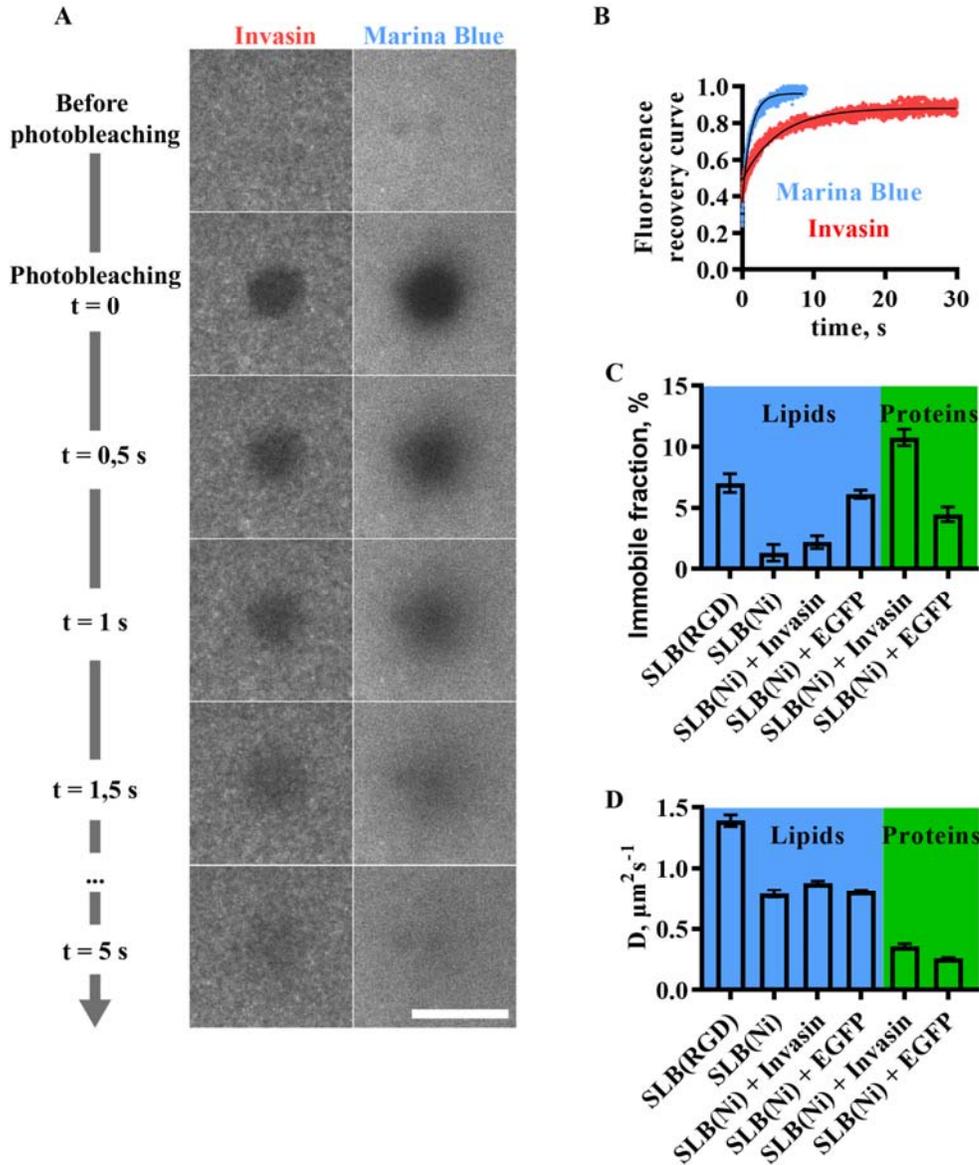
Figure 5. Dyneins push integrin clusters along microtubules to the cell periphery resulting in cell spreading on SLB.



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Figure 6. Summary: cell spreading and mechanotransduction during adhesion on SLBs depends on receptor-ligand affinity.

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Supplementary Figure S1. SLB fluidity verified with FRAP.

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For each sample we have measured 10 FRAP curves in 2 independent experiments from a photobleached circular zone of approximately 20 μm .

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A) Illustration of fluorescence recovery after photobleaching. Scale bar: 10 μm .

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B) Fluorescence recovery curves normalized by the fluorescence of the neighboring unbleached regions (blue – lipids labeled with Marina Blue, red – Invasin labeled with JF549).

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C-D) Different types of SLB surfaces:

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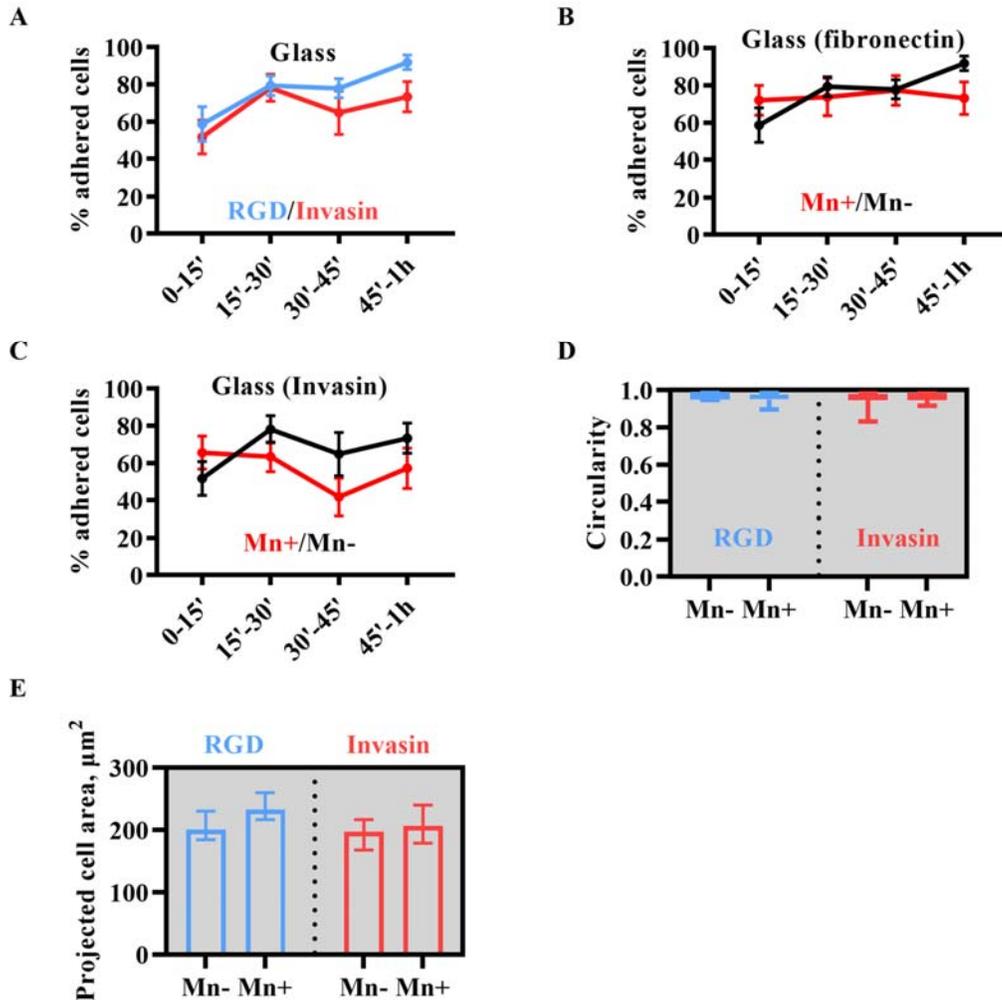
C) Immobile fractions of proteins and lipids.

866

D) Diffusion coefficients of proteins and lipids.

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870

Supplementary Figure S2. Percentage of “adherent” (“non-trembling”) MEF cells on glass over time and morphology of “trembling” MEF cells on SLBs.

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872

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A-C) Time evolution of fractions of adherent cells on.

874

For fibronectin (Mn-) each data point represents between 29 and 63 cells studied in at least 3

875

independent biological experiments ($N_{exp}=3$). For fibronectin (Mn+) : between 19 and 32

876

cells studied in at least $N_{exp}=3$. For Invasin (Mn-) : between 17 and 32 cells studied in at

877

least $N_{exp}=3$. For Invasin (Mn+) : between 21 and 30 cells studied in at least $N_{exp}=3$. Line

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scatter plots, mean, SEM.

879

A) glass functionalized with fibronectin (RGD, blue) and Invasin (red) in absence of

880

Mn^{2+} .

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B) glass functionalized with fibronectin (RGD) in presence (Mn+, red) and in absence

882

(Mn-, black) of Mn^{2+} .

883

C) glass functionalized with Invasin in presence (Mn+, red) and in absence (Mn-, black)

884

of Mn^{2+} .

885

D-E) Cell morphology of “trembling” MEF on SLBs during 1h after seeding to chambers.

886

Data from 44 cells studied in at least 3 independent biological experiments ($N_{exp}=3$) for

887

RGD-SLB (Mn-); 35 cells studied in at least $N_{exp}=3$ for RGD-SLB (Mn+); 57 cells studied

888

in at least 3 independent biological experiments ($N_{exp}=3$) for Invasin-SLB (Mn-); 46 cells

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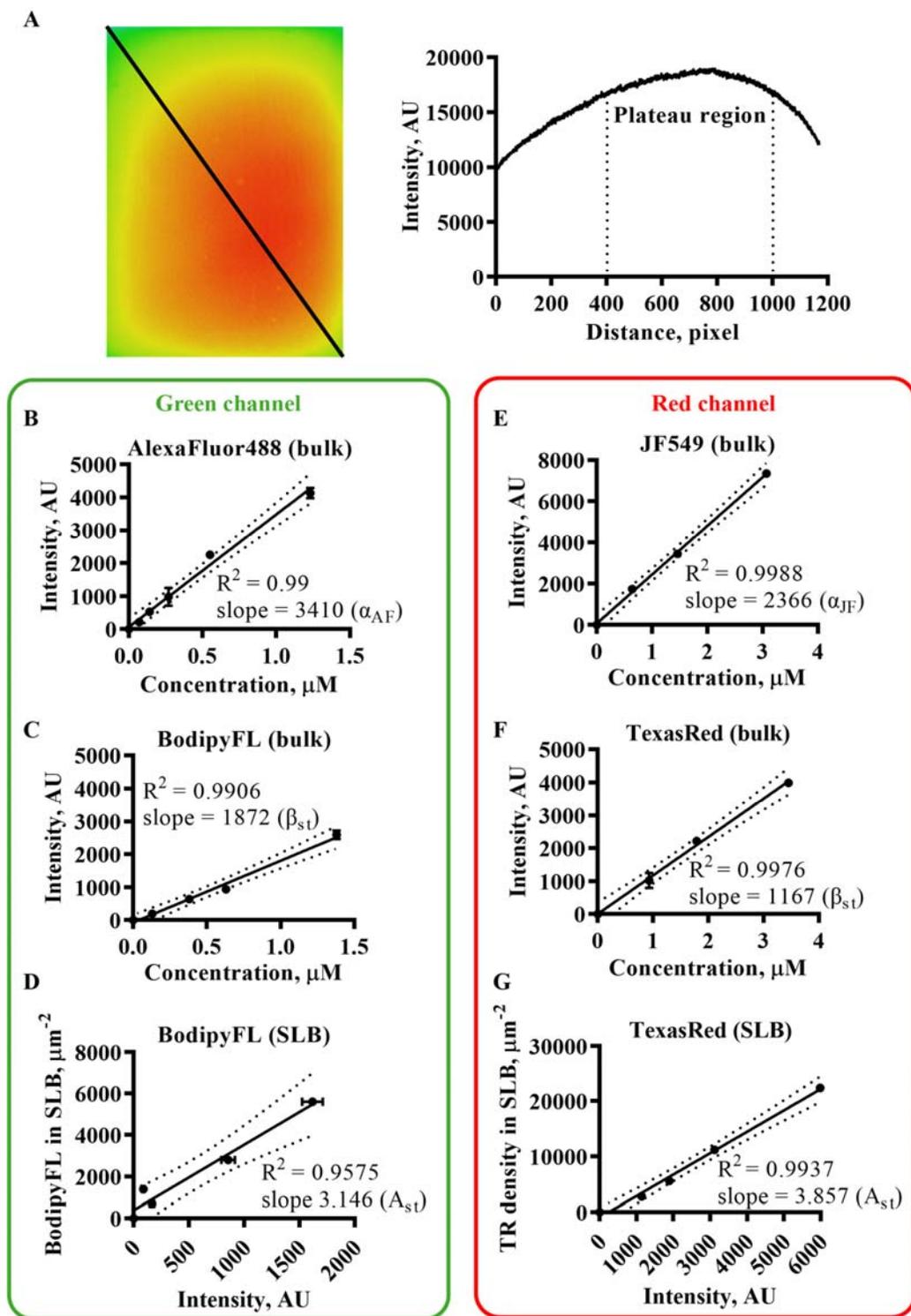
studied in at least $N_{exp}=3$ for Invasin-SLB (Mn+).

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D) Cell circularity. Box plots.

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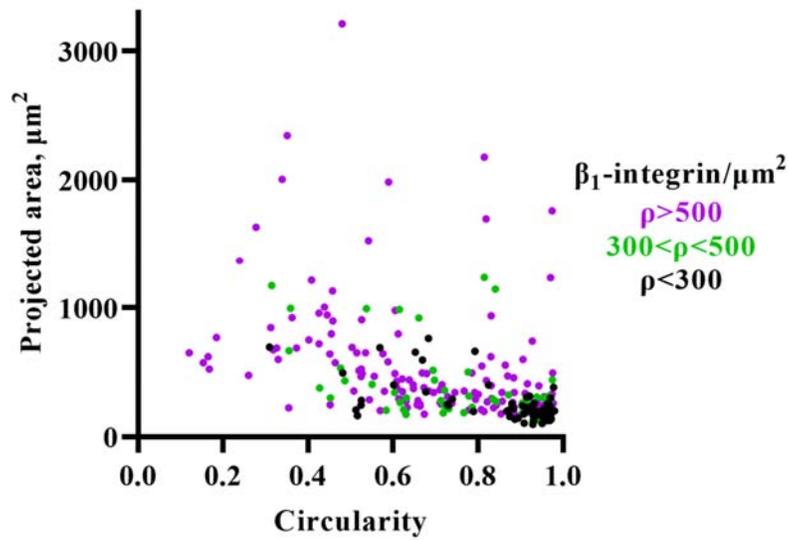
E) Projected cell area. Bar plots, mean, SEM.



892
 893 **Supplementary Figure S3. Protein density calibration using fluorescence in bulk**
 894 **and on SLBs (Methods).**

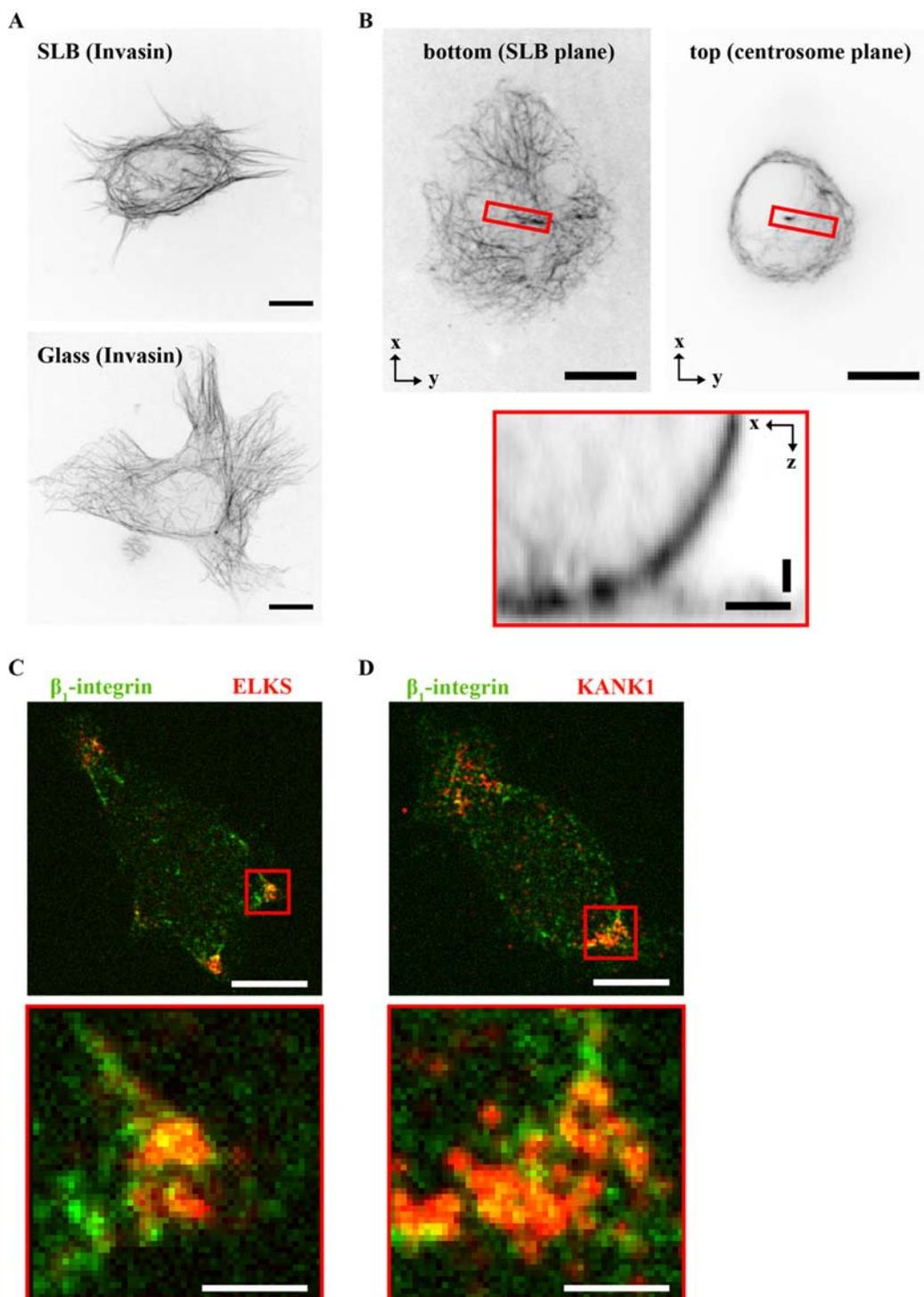
895
 896 A) Illumination field (left) and profile along the black line (right) revealed by imaging
 897 AlexaFluor 488 dye in bulk solution;
 898 B-D) AlexaFluor488 fluorescence calibration:
 899 B) Fluorescence intensity – concentration curve for AlexaFluor488 in bulk;

- 900 C) Fluorescence intensity – concentration curve for BodipyFL in bulk;
- 901 D) Concentration – fluorescence intensity curve for BodipyFL on SLB;
- 902 E-G) JF549 fluorescence calibration:
- 903 E) Fluorescence intensity – concentration curve for JF549 in bulk;
- 904 F) Fluorescence intensity – concentration curve for TexasRed in bulk;
- 905 G) Concentration – fluorescence intensity curve for TexasRed on SLB.
- 906



907
908 **Supplementary Figure S4. Correlation between the maximal β_1 -integrin density**
909 **in the clusters (ρ) and the projected area and the circularity of Mn^{2+} -treated**
910 **MEF cells on Invasin-SLBs between 0 – 1h after seeding to chambers.**

911
912 Data from 157 cells studied in at least 3 independent biological experiments ($N_{\text{exp}}=3$) for ρ
913 >500 ; 61 cells studied in at least $N_{\text{exp}}=3$ for $300 < \rho < 500$ and 87 cells studied in at least
914 $N_{\text{exp}}=3$ for $\rho < 300$.
915



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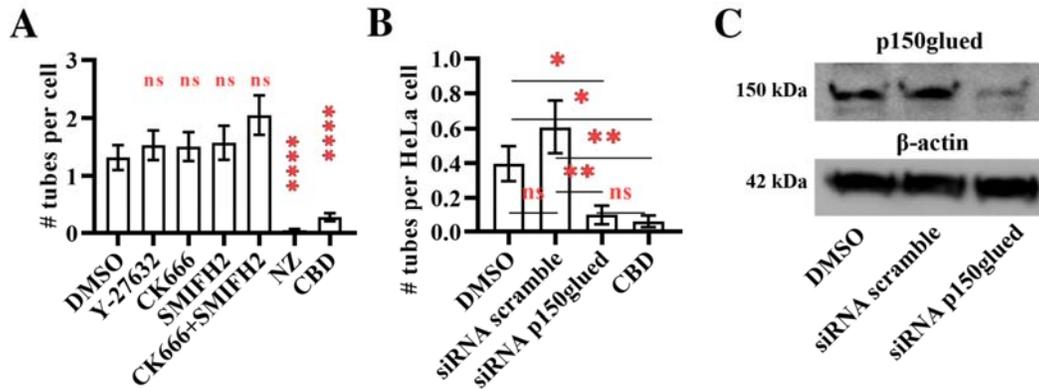
918 **Supplementary Figure S5. Microtubule organization and colocalization of**
919 **microtubule adaptor proteins with β_1 -integrin clusters in MEF cells.**

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A-B) Microtubules are stained with SiR-tubulin.

A) Distributions of microtubules in the adhesion plane of MEF cells on the SLB (top) and glass (bottom) coated with Invasin. Scale bars: 10 μ m.

924 B) Main panels: distributions of microtubules at the SLB plane (top left) and centrosome
925 plane (top right) adhesion plane of MEF cells on an Invasin-SLB. Scale bars: 10 μm .
926 The zoom panel corresponding to the red frames in the main panels (bottom) containing a
927 centrosomal microtubule is shown in the orthogonal to the SLB xz plane. Scale bars: 2 μm
928 (zoomed panel xy); 1 μm (zoomed panel xz).
929 C-D) ELKS (C) and KANK1 (D) (both red) colocalize with β_1 -integrin clusters (green) in
930 MEF on Invasin-SLBs. Main panels (top) and zoomed panels (bottom) corresponding to the
931 red frames in the main panels are shown. Scale bars: 10 μm (main panels); 2 μm (zoomed
932 panels).
933



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935

Supplementary Figure S6. Number of tubes per cell (MEF and HeLa) under different treatments (drug inhibitors, siRNA) on Invasin-SLBs.

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937

A-B) Number of MEF (A) and HeLa (B) cells treated with drug inhibitors and siRNA in the presence of Mn^{2+} between 0 – 1h after seeding on Invasin-SLBs. Bar plots: mean, SEM.

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939

940

A) Data from 78 cells, $N_{exp}=4$ with DMSO; 104 cells, $N_{exp}=4$ with CK666; 71 cells, $N_{exp}=3$ with SMIFH2; 58 cells, $N_{exp}=3$ with CK666+SMIFH2; 72 cells, $N_{exp}=3$ with Y27632; 86 cells, $N_{exp}=3$ with Nocodazole (NZ); 69 cells, $N_{exp}=4$ with Ciliobrevin D (CBD).

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B) Data from 58 cells, $N_{exp}=3$ for DMSO; 56 cells, $N_{exp}=3$ for siRNA scramble; 61 cells, $N_{exp}=4$ for siRNA p150glued and 50 cells, $N_{exp}=4$ for CBD.

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C) Representative Western Blots showing a 77% reduction in the expression of p150glued subunit of dynactin in HeLa cells due to siRNA knockdown. Lysates of HeLa cells were prepared 48h after transfection with siRNA. p150glued (top) and β -actin (bottom) protein expression levels were analyzed by Western blotting using antibodies recognizing these proteins.

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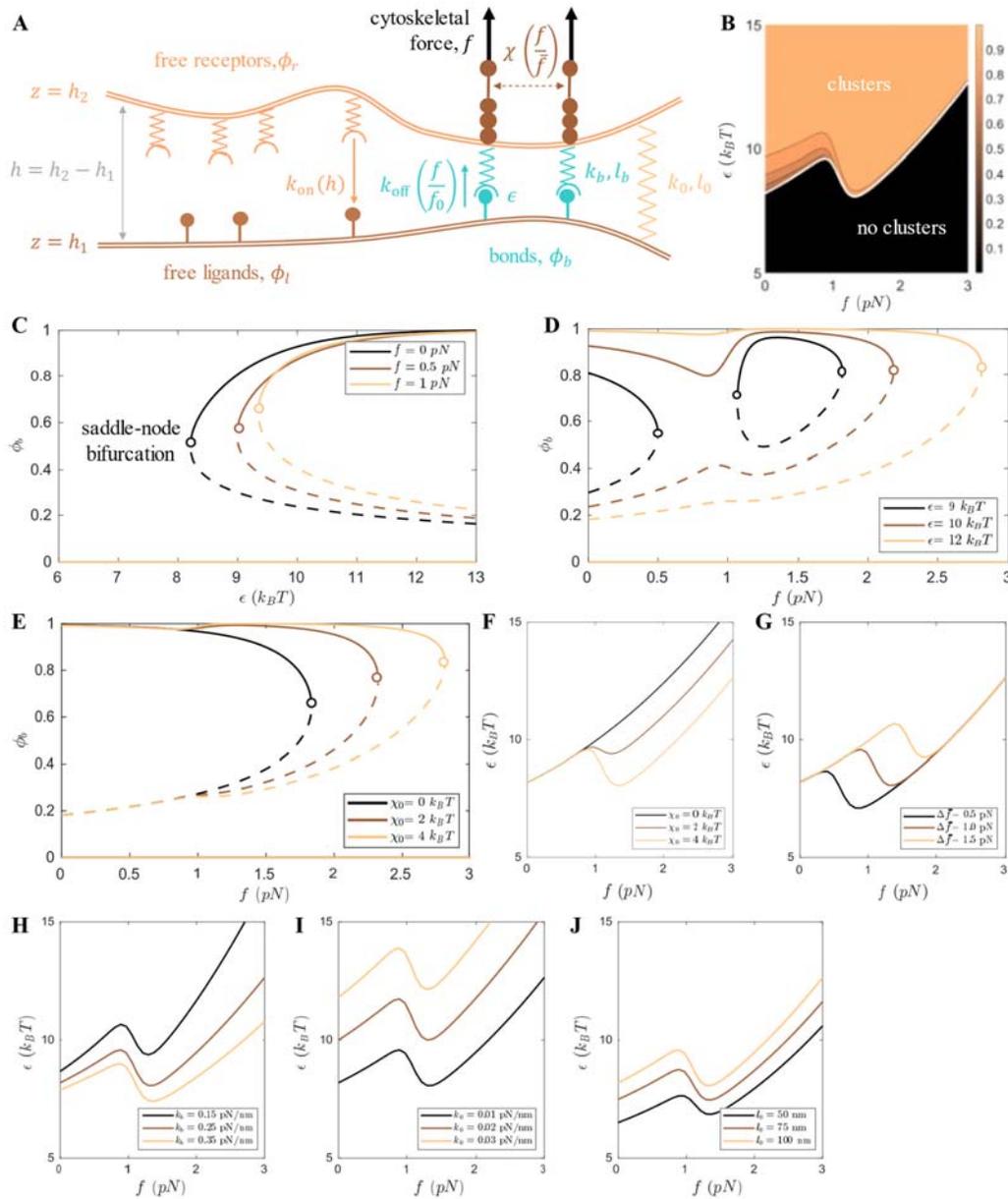
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Supplementary Figure S7. Theoretical predictions of integrin clustering as phase separation.

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A) Schematic illustration of the model. Free receptors and ligands form bound complexes (bonds) at a rate k_{on} which depends on the distance between the cell membrane and the SLB, $h = h_2 - h_1$. The binding process is characterised by a gain in binding energy, ϵ . Once formed, a bond dissociates at a rate k_{off} that increases when the bond is under force. The cytoskeleton applies a constant force, f , to each bond, which can stretch the adaptor proteins (force scale \bar{f}) or even break the bond (force scale $f_0 > \bar{f}$). The interaction strength, χ , describes the effective attractive interaction between bonds. The bonds are modelled as harmonic springs with rest length l_b and spring constant k_b , while the short-range interaction between cell membrane and SLB is also modelled as a spring of rest length l_0 and spring constant k_0 .

967

B-C) Clustering as phase separation.

968

969 B) Phase diagram of the system against the cytoskeletal force per bond, f , and the binding
970 energy, ϵ . Above a critical binding energy (white line), the system can sustain two distinct
971 bond concentrations at steady state, indicating that clusters can form. The color indicates the
972 highest sustainable bond density.
973 C) Bifurcation diagram of the steady state bond concentration against the binding energy, ϵ .
974 The dilute phase (solid line, $\phi_b \approx 0$) always exists, but a dense phase (solid line, $\phi_b \rightarrow 1$)
975 appears above a critical binding energy through a saddle-node bifurcation. The middle
976 solution (dashed line) is always unstable.
977
978 D-E) Clustering under force. The clusters can sustain larger forces (i.e. the dense phase
979 persists until a larger cytoskeletal force per bond) for (D) higher affinity ligands (increasing
980 ϵ) or (E) stronger attractive interaction through the adaptor proteins (increasing χ_0). In (E),
981 the value of the binding energy is $\epsilon = 12 k_B T$.
982
983 F-J) Dependence of phase diagram on model parameters. Each subfigure depicts the critical
984 binding energy for clustering (white line in B) for several values of (F) interaction strength of
985 activated bonds, χ_0 , (G) force required to activate the bonds, $\Delta \bar{f}$, (H) spring constant of the
986 receptors/bonds, k_b , (I) spring constant of the glycocalyx, k_0 , and (J) size of the glycocalyx,
987 l_0 .
988

989 Supplementary video V1 :
990 Brightfield time-lapse imaging of a trembling MEF cell on Invasin-SLB. Frames were
991 acquired at an interval of 10 sec and video playback speed is at 7 fps. Time in min:sec. Scale
992 bar: 5 μ m.
993
994 Supplementary video V2 :
995 Brightfield time-lapse imaging of an adherent MEF cell on Invasin-SLB. Frames were
996 acquired at an interval of 10 sec and video playback speed is at 7 fps. Time in min:sec. Scale
997 bar: 5 μ m.
998

1 **Methods**

2 **Cell chamber assembly**

3 For the bilayer to be always hydrated it is formed in a sample flow chamber that always has buffer
4 inside. The sample chamber for the cell adhesion experiment is a custom-made flow chamber that
5 consists of a 26 mm x 76 mm coverglass slide and a 1.5 thickness coverslip from VWR glued together
6 by two melted stripes of parafilm. Prior to chamber assembly the coverslip is cleaned and activated in
7 a series of sonication steps of 30 min in the water bath sonicator (Elmasonic S 10 (H)): in distilled
8 water, 2% Hellmanex III, distilled water, 1M KOH, distilled water. The KOH has a dual role of
9 cleaning and glass activation. At the end of the cleaning steps the coverslip is dried under nitrogen
10 flow and assembled to form the microscopy chamber. Coverglass slides are simply rinsed with EtOH,
11 H₂O, EtOH and blow dried with nitrogen.

12 The distance between the two glass slides (the height of the channel) is about 250 μ m. The chamber
13 volume is about 50 μ l. The assembled chambers can be stored in closed Petri dishes (protected from
14 dust) at room temperature for about 3 days.

15

16 **Small Unilamellar Vesicles (SUVs) preparation**

17 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-
18 carboxypentyl)iminodiacetic acid)succinyl] nickel salt (DGS-NTA(Ni)), 1,2-dioleoyl-*sn*-glycero-3-
19 phosphoethanolamine-N-[4-(p-(cysarginylglycylaspartate-maleimidomethyl)cyclohexane-
20 carboxamide] sodium salt (DOPE-RGD) were purchased from Avanti Polar Lipids (Alabaster, AL,
21 USA). Invitrogen™ Marina Blue™ 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Marina
22 Blue™ DHPE) was purchased from Invitrogen (Waltham, MA, USA). SLBs were formed by fusion of
23 Small Unilamellar Vesicles (SUVs) on the coverslip. We used two lipid compositions to prepare
24 SUVs and consequently SLBs: 1) DOPC/DGS-NTA(Ni)/DHPE-Marina Blue (94/2/4, mol/mol); 2)
25 DOPC/DOPE-RGD/DHPE-Marina Blue (94/2/4). Similar lipid compositions were successfully used
26 by other groups to prepare SLBs¹ (Nye and Groves [2008]). The SUVs were prepared using the
27 following protocol:

- 28 1. Lipids solubilized in chloroform are mixed together in a glass vial at 1 mg/ml, blow dried with
29 a nitrogen flow, placed in vacuum desiccator for 1 hour, then rehydrated with distilled water
30 for 15 min at room temperature, to a final lipid concentration of 1 mg/ml;
- 31 2. After rehydration, the glass vial is vortexed to detach the liposomes;
- 32 3. The solution is sonicated for 30 min in the water bath sonicator (Elmasonic S 10 (H));
- 33 4. The solution is then centrifuged at 20k RCF for 1 hour;
- 34 5. The solution is filtered through a 200 nm filter (Millipore) with a syringe;
- 35 6. The sample is dissolved in the SUV fusion buffer at the final composition: 10 mM Tris pH
36 7.3; 120 mM NaCl.

37 Lipids preparations are closed under with argon at all stages of SUV preparation to minimize their
38 degradation by oxidation. The prepared SUVs were not stored but used immediately to prepare SLBs.

39

40 **Invasin preparation**

41 While RGD-ligands bind to a larger repertoire of integrins ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$ and
42 $\alpha_{IIb}\beta_3$)² (Humphries2006), Invasin binds to a subset of β_1 -integrins ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$), including
43 the fibronectin integrin $\alpha_5\beta_1$, with which Invasin has a dissociation constant (K_d) two orders of
44 magnitude lower than that of RGD peptide³ (Van Nhieu1991).

45 - Plasmid preparation

46 The plasmid for invasin expression was constructed in two steps. First, a DNA sequence coding for a
47 TEV cleavage site followed by a 6xHis tag followed by the sequence coding for the last 474 amino
48 acids of Invasin, inv474 (PDB: 1CWV) (in 5' to 3' direction) and flanked by SacI and HindIII restriction
49 sites was synthesized and subcloned by Thermo Fisher Scientific. Second, the insert of interest was
50 cloned into a pMal p5x expression vector by digesting with SacI and HindIII enzymes and ligation. As
51 a result, we have obtained the plasmid that expresses a periplasmic maltose binding protein (MBP)
52 fused to Invasin in *E. coli* periplasm under IPTG inducible P_{tac} promoter (pOM3474).

53 - Invasin expression

54 For Invasin expression we followed the previously described protocol⁴ and expressed Invasin in the *E.*
55 *Coli* periplasm to insure the oxidizing environment for the proper disulfide bond formation. We used
56 an *E. coli* strain lacking *DegP* protease⁴. Proteins were expressed in 2YT medium + selection
57 antibiotics (ampicillin for the plasmid and kanamycin for the *E. coli* strain at final concentrations 100
58 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$, respectively) + 0.2% glucose. Glucose is needed to inhibit amylase expression
59 that can later perturb MBP-Invasin purification. Bacterial culture was inoculated from a single colony
60 and incubated at 30 °C while shaking until the $\text{OD}_{600} = 0.5\text{--}0.6$. Then cells were induced with 0.5 mM
61 IPTG for 4 hours. MBP-Invasin is well expressed at the right molecular size (approximately 96 kDa),
62 thus we can proceed with the protein production, purification and labelling at the bigger scale.

63 - Invasin purification

64 Invasin purification and labelling was performed in several steps:

- 65 1. Affinity purification using an amylose resin (New England Biolabs);
- 66 2. MBP cleavage by TEV protease;
- 67 3. Protein labeling with a succinimidyl ester (SE, Tocris) reactive dye (optional);
- 68 4. Purification by size exclusion chromatography using a Superdex 75 column.

69 The more detailed protocol illustrates the purification process from 2 liters of bacterial culture that
70 expressed MBP-Invasin:

71 Cell lysis

- 72 1. Resuspend cell pellets in approximately 80 mL Lysis Buffer: 25 mM HEPES pH 7.3, 500 mM
73 NaCl, 1 mM EDTA;
- 74 2. Add a final of 1x EDTA Free cOmplete protease inhibitor tablet (Roche), 1 mM PMSF, 10 $\mu\text{g/ml}$
75 DNase and 10 $\mu\text{g/ml}$ lysozyme;
- 76 3. Cells were lysed via sonication (misonix sonicator ultrasonic processor XL, large probe at 20%
77 intensity) on ice at 70% intensity: 3 sec - On, 3 Sec Off for 5 minutes;
- 78 4. Sample was centrifuged at 20,000 RCF for 60 Min, 4°C and supernatants collected; Affinity
79 purification against the MBP;
- 80 5. 10 mL Amylose Resin were pre-washed in water then Lysis buffer;
- 81 6. Cleared extracts were applied to Amylose Resin, and allowed to bind using a tube roller for 2h, 4°C;
- 82 7. Samples were washed 2x 40mL using batch method with Wash Buffer 1 (+EDTA);
- 83 8. Amylose beads were applied to a column and washed a further 10x CV with the same buffer;

84 9. Proteins were eluted in 15 mL of Elution Buffer: 25 mM HEPES pH7.3, 500 mM NaCl, 10 mM
85 maltose (the steps of the affinity purification against the MBP were repeated);

86 10. Protein concentration was estimated by measuring the absorbance spectrum at the Cary Eclipse
87 Fluorescence spectrophotometer; the samples were analyzed by protein electrophoresis on SDS-PAGE
88 gels.

89 **TEV cleavage and labelling with JF549 SE dye**

90 11. Add TEV protease at about 1:100 (TEV/Invasin, mol/mol);

91 12. Add JF549 SE dye (Tocris) at about 1:2 (Invasin/dye, mol/mol);

92 13. Incubate at 4°C in aluminum foil on the rolling table o/n; Size exclusion purification;

93 14. Labelled samples were concentrated down to approximately 500 µL and purified further by size
94 exclusion chromatography using a Superdex 75 column. Buffer: 25 mM HEPES pH 7.3, 120 mM
95 NaCl. The elution fractions were analyzed by protein electrophoresis on SDS-PAGE gels.

96 The fractions corresponding to Invasin were collected and its absorbance spectrum was by measured at
97 the Cary Eclipse Fluorescence spectrophotometer. Invasin concentration C and labelling ratio n^* (the
98 labeling molar ratio of the fluorescence species) were estimated from the sample absorbance
99 measurements A_{280} and A_{\max} (absorbances at $\lambda = 280$ nm and λ corresponding to the fluorescence of
100 the dye which for the JF549 is 549 nm), the extinction coefficients of JF549 dye ϵ_{dye} (from tocris.com)
101 and Invasin ϵ_{prot} (expasy.org/protparam) 101000 [$\text{M}^{-1} \text{cm}^{-1}$] and 57995 [$\text{M}^{-1} \text{cm}^{-1}$], respectively, and
102 the CF correction coefficient that is equal to 0.169 (from tocris.com):

$$103 C = (A_{280} - A_{\max} \cdot CF) / \epsilon_{\text{dye}};$$

$$104 n^* = A_{280} / \epsilon_{\text{prot}} \cdot C.$$

105

106 **Supported lipid bilayer (SLB) preparation**

107 The protocol is inspired by the protocol developed in the group of Jay Groves^{1,5}.

108 The SUV solution is incubated in the sample chamber for 30 min to allow the SUV fusion on the
109 substrate. Then the unfused vesicles are washed away with 10 x chamber volumes of the SUV fusion
110 buffer. Next, the SLBs are washed with 10 x chamber volumes of the cell buffer: 25 mM HEPES pH
111 7.3; 120 mM NaCl; 7 mM KCl; 1.8 mM CaCl_2 ; 0.8 mM MgCl_2 ; 5 mM glucose. Any remaining defect
112 on the surface is then passivated by incubation of the chamber in a "blocking" solution made of the
113 cell buffer complemented with 0.1 mg/ml β -casein for 15 min. Then the SLBs is washed again with 10
114 x chamber volumes of the cell buffer to remove the excess of the blocking solution.

115 To prepare Invasin-coated SLBs, bilayers containing Ni-lipids are functionalized with 6xHis-tagged
116 Invasin following the protocol¹. SLBs are incubated with 400 nM Invasin in the cell buffer for 1 hour
117 at room temperature. Then the unbound proteins are washed away with 10 x chamber volumes of the
118 cell buffer in two steps spaced in time by 30 min.

119

120 **SLB quality and fluidity control**

121 We prepared RGD-SLB and SLB(Ni), functionalized SLB(Ni) with fluorescently labelled Invasin or
122 EGFP (as a control). To assess the quality of SLBs (absence of defaults) they were inspected visually
123 under the microscope. We observed fluorophore distributions in both DHPE-Marina-Blue and
124 fluorescent proteins. If no defaults were detected, we proceeded further and tested the SLBs on

125 fluidity. The fluidity of the prepared SLBs were checked by using the fluorescence recovery after
126 photobleaching (FRAP) technique (Figure S1A). We can deduce the diffusion coefficients of
127 fluorescent lipids or proteins attached to the bilayer by analyzing the fluorescence recovery curves
128 after photobleaching of a small area of the SLB⁶. For each sample we have measured 10 FRAP curves
129 in 2 experiments from a photobleached circular zone of approximately 20 μm^2 . A typical normalized
130 fluorescence recovery curves for lipids (DHPE-Marina Blue) of the SLB(Ni) in presence of Invasin
131 and Invasin on the SLB(Ni) are shown in the Figure S1B. They were fitted with the following
132 function:

$$133 \quad f(t) = f_0 + (f_{\text{plateau}} - f_0)(1 - e^{-kt}),$$

134 from which we can find the immobile fraction of fluorophores as $1 - f_{\text{plateau}}$. The interaction of the lipid
135 bilayer with the solid surface is limited since the immobile fraction (Figure S1C) for fluorescent lipids
136 is lower than 7% for SLB-RGD, and even lower for SLB-Ni. Lipids remain mobile when Invasin or
137 EGFP is bound to the SLB-Ni. We also see that the immobile fraction of Invasin on the SLB is of the
138 order of 11%, and 5% for EGFP. The diffusion coefficient D , the radius of the bleached area r and the
139 half-recovery time $t_{1/2}$ are link by the following expression: $D = 0.224 \cdot r^2/t_{1/2}$.

140 The diffusion coefficients for lipids are approximately 75% lower in SLB(Ni) than in SLB(RGD),
141 maybe due to electrostatic interactions between the glass and the Ni lipids (0.8 versus 1.4 $\mu\text{m}^2/\text{s}$,
142 respectively) (Figure S1D). These values are in fairly good agreement with the previous studies of
143 lipid diffusion in lipid bilayer (SLBs or liposomes)^{7,8}. Binding of Invasin or EGFP to the bilayer does
144 not change the lipid diffusion coefficient, showing that the bilayer is fluid. As expected, since proteins
145 are bigger than lipids, the protein diffusion coefficients are lower, more than two times lower than that
146 of the lipids (0.4 and 0.3 $\mu\text{m}^2/\text{s}$ for Invasin and EGFP respectively). These results show that the RGD-
147 lipids and Invasin-lipids complexes in our experiments are mobile in the bilayer.

148

149 **Fluorescence calibration of SLBs**

150 The aim of the calibration is to quantify the density of fluorescence species in the cell adhesion plane
151 by using supported lipid bilayers as fluorescence standards. We adapted the original protocol² to
152 perform the calibration. Within a certain range of fluorophore densities, we can relate the density of
153 the fluorescence species n_{fl} [μm^{-2}] and the intensity I_{fl} [AU] of the fluorescence image of the cell
154 surface in the adhesive contact by the following:

$$155 \quad N_{\text{fl}} = A_{\text{fl}} \cdot I_{\text{fl}};$$

156 where A_{fl} is the proportionality factor that depends on the fluorescent molecule and imaging
157 conditions. This linear relationship holds for concentrations below the critical concentration at which
158 fluorophores start to self-quench⁹. The range of densities of fluorescence species where this linear
159 relationship holds is verified during the calibration process.

160 Standards have to be used to calibrate the fluorescence in membranes, in illumination conditions
161 similar to the experiments. In practice, fluorescent lipids are convenient since they can be incorporated
162 in the bilayer at controlled concentration, thus their density is known and the corresponding
163 fluorescence can be measured as a function of their density. Here, SLBs are particularly suitable if the
164 fluorescence intensity is measured in the exact same conditions as in the experiments where the
165 contact zone between cells and SLBs are imaged. But, to account for differences in fluorescence yield
166 between the fluorescent markers on the lipid and the protein, the fluorescence of the lipids and the
167 proteins at known concentrations must be compared; for this, measurements in bulk are well-suited.

168 The proportionality factor A_{fl} is related to the proportionality factor of the calibration standard A_{st} by
169 the following relation:

170 $A_{fl} = A_{st} / (F \cdot n^*)$;

171 where the correction factor $F = I_{fl}/I_{st}$ is the ratio between the intensities of the fluorescence species I_{fl}
172 and the standard I_{st} at a given concentration in solution and n^* is the labeling molar ratio of the
173 fluorescence species. $n^* = 1$ for lipids, but might be not equal to 1 for proteins, depending on the
174 efficiency of the labeling protocol. n^* must be measured for each preparation of proteins with a
175 spectrophotometer.

176 F considers the optical properties of the microscope and spectral differences between the fluorophore
177 and the standard. In order to determine A_{fl} for a given fluorophore, we need to do the following:

178 1. Calibrate our imaging system with a standard fluorophore and measure the proportionality factor A_{st}
179 in SLB with fluorescent lipids;

180 2. Measure the correction factor F with experiments in solution.

181 More precisely, in the first step, we prepare a series of SLBs with fluorescence lipids (standard)
182 incorporated at known densities. Then we image these SLBs with exactly the same imaging conditions
183 (laser powers, exposure times and fluorescence channels, or sets of fluorescence filters) as we do for
184 the fluorophore of interest. We have taken at least 10 images in different areas of the SLB for every
185 density of every studied fluorophore. In the second step, we measure the correction factor F , the
186 dimensionless factor that represents the efficiency of the fluorophore versus the standard. Practically,
187 to calculate F , we measure the ratio of intensities between the fluorophore and the standard at a given
188 concentration in solution (in bulk), or more precisely, the ratio of the slope of the plots Intensity versus
189 bulk concentration, for both fluorophores, α_{st} and β_{fl} . This measurement must be done directly at the
190 microscope with exactly the same imaging conditions as for the first step. We did 2 calibrations (in
191 green and red channel) for the following molecules: AlexaFluor488 and JF549.

192 We used Bodipy FL DHPE (Molecular probes, referred to as BodipyFL in the following) and Texas
193 Red DHPE (Invitrogen, referred to as TexasRed in the following) lipidated dyes as standards for the
194 green and the red calibrations, respectively.

195 **Fluorophore preparation for calibration**

196 All fluorophores, except the lipidated ones, were diluted in the working cell buffer (25 mM HEPES
197 pH 7.3; 120 mM NaCl; 7 mM KCl; 1.8 mM CaCl₂; 0.8 mM MgCl₂; 5 mM glucose). As lipids would
198 aggregate in aqueous solution, we have solubilized them in detergent. Lipid solutions of BodipyFL or
199 TexasRed were blow-dried under argon and vacuum for 30 minutes, and re-solubilized in the working
200 cell buffer with 2.25 mM n-Dodecyl β -D-maltoside (DDM, Sigma Aldrich).

201 The accuracy of the calibration greatly relies on the precise knowledge of the fluorophore
202 concentrations. The concentrations of all samples were checked on Cary Eclipse Fluorescence
203 Spectrophotometer using the following published extinction coefficients: AlexaFluor488 (73000 [M⁻¹cm⁻¹]),
204 BodipyFL (80000 [M⁻¹cm⁻¹]), JF549 (101000 [M⁻¹cm⁻¹]), TexasRed (116000 [M⁻¹cm⁻¹]),
205 mCherry (72000 [M⁻¹cm⁻¹]).

206 The images of the fluorophores in bulk were taken approximately 10 μ m above the coverslip. We
207 observed that the intensity distribution of the fluorescence signal of a dye in solution is not
208 homogenous in the imaging plane (Supplementary Fig. S3). This is related to the non-homogeneous
209 illumination. The illumination seems to be more or less homogeneous in the middle of the illumination
210 pattern “the plateau” (Supplementary Fig. S3A). For our analysis we either considered the intensity in
211 this region or corrected the inhomogeneity of the illumination by using the fluorescence signal of
212 bilayers. We have taken at least 10 images in different areas of the chamber for every concentration of
213 every studied fluorophore.

214 The standard SLBs (DOPC + BodipyFL) with different concentrations of BodipyFL were prepared
215 following the previously described protocol (“SLB preparation” in the Methods). We calculated the
216 surface densities of BodipyFL in the SLBs from their molar ratios and assuming the area of a DOPC
217 lipid projected on the SLB plane to be 0.72 nm^2 and, thus, the number of lipids per μm^2 to be $2 \cdot \mu\text{m}^2 /$
218 $0.72 \text{ nm}^2 = 2.8 \cdot 10^6$ (where the factor 2 is to take both bilayer leaflets into account)¹⁰. For each sample
219 30 images were taken and the intensities of the plateau region were averaged. We plotted the density-
220 intensity standard curves for both calibrations and also intensity-concentration curves for all
221 fluorophores in bulk (Supplementary Fig. S3). Therefore, we obtained the following calibration values
222 for our fluorescence species:

223 β_1 -integrin labelled with Alexa Fluor 488 ($A_{st} = 3.146 \mu\text{m}^{-2}$; $\alpha_{fl} = 3410 \mu\text{M}^{-1}$; $\beta_{st} = 1872$
224 μM^{-1} ; $F = 1.82$; $n^* = 1$; $A_{fl} = 1.73 \mu\text{m}^{-2}$);

225 JF549 labelled Invasin ($A_{st} = 3.857 \mu\text{m}^{-2}$; $\alpha_{fl} = 2366 \mu\text{M}^{-1}$; $\beta_{st} = 1167 \mu\text{M}^{-1}$; $F = 2.03$; $n^* =$
226 0.427 ; $A_{fl} = 4.45 \mu\text{m}^{-2}$).

227 Note that the calibration factors were measured from fluorescent dyes in solution and not from
228 fluorescent proteins. F was shown to be about 3 times higher with AlexaFluor488 bound to anti-biotin
229 than to Streptavidin⁵. But, weaker differences were observed in other cases. In practice, we should
230 purify the proteins of interest, label them and make the calibration, which is not possible. In addition,
231 it is possible that the dye fluorescence is different in the cell environment as compared to the buffer.
232 Thus, the absolute values of the protein densities in the adhesion structures that we deduce from our
233 experiments might be off by some systematic factor, however, it cannot be by an order of magnitude.

234 According to the fluorescence calibration we found that Invasin was distributed homogeneously on
235 SLBs and its density corresponded to approximately $600 \text{ Invasin}/\mu\text{m}^2$ (under our SLB preparation
236 protocol).

237

238 **Microscopy**

239 We have used a spinning disk microscope to image fluorescent lipid bilayers and cell adhesion on
240 them. The microscope consists of a CSU-X1 Yokogawa head mounted on an inverted Ti-E Nikon
241 microscope with a motorized XY stage (MadCity Lab®). Images were acquired with Metamorph
242 software (Molecular Devices®) through a 100x NA1.45 objective with a Photometrics 95B-sCMOS
243 camera. Live cell adhesion imaging was performed at 30°C using the stage top incubator (Tokai hit®).
244 Fixed cells were imaged at RT with the same microscope. The setup is equipped with 4 Cobolt lasers
245 from Hübner Photonics: 405 nm (100mW), 488 nm (100mW), 561 nm (50mW) and 633 nm
246 (100mW). They allow sample imaging in 4 fluorescence channels (405, GFP, Cy3 and Cy5). Samples
247 can also be imaged in wide field mode using the transmission light from LED source. The setup is also
248 equipped with a FRAP photoactivation module.

249 We have used the same imaging conditions in our microscopy experiments: 405 (laser power: 15%;
250 exposure time: 100ms), GFP (laser power: 30%; exposure time: 300ms), Cy3 (laser power: 30%;
251 exposure time: 300ms) and Cy5 (laser power: 30%; exposure time: 300ms).

252 For imaging cells above the bilayer Z-stack images of $3 \mu\text{m}$ height are taken. The stack is centered at
253 the SLB plane and its step is $0.3 \mu\text{m}$.

254 Diffraction limit (DL), or optical lateral (xy plane) resolution was calculated as follows:

$$255 \text{Res}_{xy} = 0.51 \cdot \lambda_{em} / \text{NA},$$

256 Where λ_{em} – emission wavelength of a fluorophore, NA – numerical aperture of the objective.

257

258 **Image analysis**

259 - **“trembling/adherent” cells**

260 Cells were qualitatively classified as “trembling” if their edges but not their centers of mass moved at
261 the time scale of 5 seconds. The edge movement was detected manually based on the bright field
262 images.

263 - **Cell contour detection and measurement of cell morphology parameters**

264 Cell contours were manually detected from bright field images by using a polygonal selection tool in
265 ImageJ. Then the “cell contour” selection was used to calculate the projected cell area A (“projected
266 area”) and the cell circularity index C (“circularity”) as follows:

267 $C = 4\pi * A/P^2$, where P is the perimeter of the “cell contour” selection.

268 - **Integrin cluster detection and quantification**

269 Before detecting integrin clusters, we made a correction on the illumination inhomogeneity. The
270 illumination is not homogeneous across the image (Supplementary Fig. S3A). Therefore, we
271 introduced the “illumination” map to correct this issue. It consists in normalizing the image by that of
272 a fluorescent supported lipid bilayer (SLB). The SLB has a homogeneous distribution of fluorescent
273 lipids in the image plane, so it is a perfect candidate for the illumination map. The intensities of the
274 illumination map range between 0 (the dimmest illumination) and 1 (the brightest illumination).
275 Therefore, the image correction for the illumination inhomogeneity is obtained by the division of
276 pixels intensities of the image by the intensities of the corresponding pixels of the illumination map.

277 To detect β_1 -integrin clusters we first used previously described fluorescence calibration transforming
278 raw intensity images of integrins (corrected for illumination inhomogeneity) to integrin concentration
279 maps. Second, we segmented these concentration maps by defining an integrin density threshold that
280 separates two distributions (clusters and background) on the density histogram. The integrin density
281 thresholds could be set manually or automatically by using an algorithm based on Renyi’s entropy
282 thresholding. This thresholding method was previously described¹¹ and is now one of the standard
283 threshold methods available in ImageJ. The method defines a threshold intensity value that maximizes
284 the informational entropy and entropic correlation of “cluster” and “background” distributions¹².

285 Custom-written code used to analyze the data in the current study is available from the corresponding
286 authors on reasonable request.

287 **FA protein, actin and microtubule enrichments in β_1 -integrin clusters**

288 Signal enrichment of the co-expressed fluorescent fusion proteins (FPs) for FA proteins, F-actin and
289 microtubule imaging at β_1 -integrin clusters was calculated as follows. Mean fluorescence intensity of
290 the FP was detected in the region of β_1 -integrin clusters and then was normalized by the mean intensity
291 of the FP in the cell. It was compared with “shuffled control enrichments”, enrichment calculated at
292 random pixels instead of β_1 -integrin clusters regions.

293 FA protein recruitment to integrin clusters experiments were done in fixed cells. Fluorescence
294 calibrations were not performed in these conditions. β_1 -integrin clusters were defined using the same
295 threshold (100 AU).

296 β_1 -integrin clusters were defined using the threshold of 100 integrins/ μm^2 for experiments of F-actin
297 and microtubule recruitment to integrin clusters.

298

299 Cell culture

300 We used the following cell lines in our experiments:

301 Mouse Embryonic fibroblasts (MEF) β_1 KO β_1 -Halotag and MEF β_1 KO β_1 -Halotag paxillin-mCherry
302 (both gifts from David Calderwood, Yale University). The former cell line was constructed by
303 lentiviral transfection of a β_1 KO MEF cell line with an ecto-tag construct based on pLENTI expression
304 vector and the latter as a consecutive lentiviral transfection with a paxillin-mCherry on pLENTI
305 expression vector. The Halotag sequence was inserted on an exposed loop (β_1 residues 91-114)¹³.

306 MEF β_1 KO β_1 -Halotag LifeAct-mScarlet that was constructed by lentiviral transfection of a β_1 KO β_1 -
307 Halotag MEF cell line with a LifeAct-mScarlet on pLVX expression vector.

308 MEF β_1 KO β_1 -Halotag EMTB-iRFP that was constructed by lentiviral transfection of a β_1 KO β_1 -
309 Halotag MEF cell line with an EMTB-iRFP on pLVX expression vector (gift from Simon De Beco,
310 Paris Diderot University).

311 HeLa WT and HeLa β_1 -Halotag that we constructed by lentiviral transfection of a HeLa WT cell line
312 with an ecto-tag construct based on pLENTI expression vector (gift from David Calderwood, Yale
313 University).

314 MEF and HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) high glucose +
315 GlutaMAX (Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (FBS; EuroBio)
316 and 1% penicillin-streptomycin (Thermo Fischer Scientific). Cells were cultured at 37°C in a
317 humidified 5% CO₂ atmosphere. Cells were routinely monitored for mycoplasma contamination
318 following a well-established PCR-based method (Young et al. [2010]) and found to be negative.

319 DNA plasmids

320 For transient cell transfections the following plasmids were used: talin-mCherry (Addgene plasmid #
321 55137), vinculin-mCherry¹⁴, VASP-mCherry (Addgene plasmid # 55151), kindlin-2-mCherry (gift
322 from Christof Hauck, Konstanz University), zyxin-mCherry (gift from Danijela Vignjevic, Institut
323 Curie).

324 For lentiviral transfections the following plasmids were used: β_1 -Halotag (gift from David
325 Calderwood, Yale University), pLVX EMTB-iRFP (gift from Simon De Beco, Paris Diderot
326 University) and pLVX LifeAct-mScarlet.

327 The pLVX LifeAct-mScarlet plasmid was prepared in two steps: a polymerase chain reaction using 5'-
328 TCTAGAGCTACTA ACTTCAGCCTGCTG-3' / 5'- CGGTGGATCCCCTTCTTCC-3' primers on
329 Ibidi USA 60101 LifeAct-GFPtag2 plasmid was cloned with In-Fusion HD enzyme kit (Takara) into
330 the pLVX vector (Clontech) digested with Not1 and BamH1 restriction enzymes (New England
331 Biolabs). It was followed by a second In-Fusion HD cloning of a polymerase chain reaction using 5'-
332 GAAGGGGATCCACCGATGGTGAGCAAGGGCGAGG-3' / 5'-
333 TTAGTAGCTCTAGACTTGTACAGCTCGTCCATGCC-3' (mScarlet insert) using BamHI and XbaI
334 (New England Biolabs).

335 Cell transfections (transient by electroporation and stable lentiviral)

336 Transient cell transfections were performed by electroporation following the protocol adapted from
337 Gautreau et al., 2000: trypsinized cells were resuspended at a concentration of 2.5×10^7 cells/ml in 15
338 mM Hepes, pH 7.4, buffered medium. 200 μ l of cell suspension was added to 50 μ l of a solution
339 containing 210 mM NaCl, 5 μ g of plasmid DNA, and 30 μ g of salmon sperm DNA carrier (Sigma
340 Aldrich). Then cells were electroporated with a BioRad Gene Pulser at 950 μ F and 240 V using 4-mm
341 width cuvettes. Transiently transfected cells were analyzed after 48 h of the expression of the plasmid
342 of interest.

343 Stable cell transfections were performed with lentiviral infections. Lentiviral particles (LVs) were
344 produced in HEK 293T cells cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher
345 Scientific), supplemented with 10% Fetal Bovine Serum (EuroBio) and 1% Pen/Strep + 1% Sodium
346 Pyruvate + 1% Non-Essential Amino Acids solution (Gibco) at 37°C in 5% CO₂ humidified
347 incubators. Cells were plated the day before transfection in T75 flasks (approx. 6 million) to achieve a
348 50–70% confluency the next day. Plasmids coding lentiviral components, pPAX2 (Gag-Pol-Hiv1) and
349 pMDG2 (VSV-G), and the plasmid of interest at a ratio of 4:1:4 (µg), respectively, were transfected
350 using PEI MAX 40k transfection reagent (Tebu-Bio) according to the manufacturer's protocol. After
351 48 hrs, LVs were concentrated using a 100k Amicon column (Merck Millipore) and the pellet was
352 resuspended up to 400 µL in PBS. MEF or HeLa cells were plated the day before infection in 6-well
353 plates (approx. 100,000 cells) to achieve a 50–70% confluency the next day. Cells were transduced
354 with 100 µL of the desired LVs, for 72 hrs. After transduction, expressing cells were treated with 2
355 µg/mL puromycin. Positive cells were sorted using a SH800 FACS Cell Sorter (Sony).

356 **Integrin labeling and cell seeding in imaging chambers**

357 Cultured cells were serum starved for 24 hours before experiment. Then cells were detached by
358 Versene solution (Sigma Aldrich) for 30 minutes at 37°C. Then they are incubated with an Alexa
359 Fluor488 Halotag® ligand (Promega) (400 nM per 1 ml containing approximately 1.5-2 million cells)
360 to label β₁-integrin-Halotag for 10-15 minutes at room temperature. Then cells are span down at low
361 centrifuge speed (1000 rpm in Megafuge 16R centrifuge from Thermo Scientific), resuspended in the
362 cell buffer (25 mM HEPES pH 7.3; 120 mM NaCl; 7 mM KCl; 1.8 mM CaCl₂; 0.8 mM MgCl₂; 5 mM
363 glucose) to remove the Versene and the excess of dye and filtered with Falcon 40 µm Cell Strainer
364 (Corning) to remove cell clumps. Cells then were gently flown into the imaging chamber containing
365 SLBs. The chamber is then sealed with mineral oil (M8410, Sigma Aldrich).

366 Since our aim was to study mechanosensitive aspects of cell adhesion, we avoided washing steps in
367 chambers once cells were seeded. Indeed, flows in the chamber exert shearing forces on adhering cells
368 that can reinforce cell adhesion¹⁵.

369

370 **Buffers, reagents, inhibitors**

371 The following cell buffer was used in imaging experiments: 25 mM HEPES pH 7.3; 120 mM NaCl; 7
372 mM KCl; 1.8 mM CaCl₂; 0.8 mM MgCl₂; 5 mM glucose.

373 SiR-tubulin (Cytoskeleton, Inc.) at the dilution suggested by the manufacturer was used to label
374 microtubules in live cells.

375 In the experiments with manganese-treated cells, MnCl₂ (Sigma Aldrich) was added (to the final
376 0.5mM concentration) just before putting cells into the imaging chamber.

377 We used the following concentrations of inhibitor drugs in this study: 50 µM for the Arp2/3 complex
378 inhibitor CK-666, 10 µM for the formin inhibitor SMIFH2 (Sigma Aldrich), 50 µM for the myosin
379 inhibitor *p*-nitro-Blebbistatin (Cayman Chemical), 50 µM for the ROCK inhibitor Y27631 (Sigma
380 Aldrich), 10 µM for Nocodazole (Sigma Aldrich) and 50 µM for the cytoplasmic dynein inhibitor
381 Ciliobrevin D (Sigma Aldrich). For the live cell imaging experiments drugs were added at the same
382 moment as cells were seeded to chambers. Control samples were treated with an equivalent amount of
383 DMSO which did not exceed 0.001% v/v.

384

385 **Immunofluorescence**

386 For endogenous ELKS and KANK1 labeling, MEF cells were fixed in 100% ice-cold methanol
387 (-20°C , 5 min) followed by incubation in PBS with 1 mg ml^{-1} BSA (blocking buffer (BB)) all along
388 the procedure. Fixed cells were washed in PBS and saturated in BB. Cells were incubated with the
389 primary antibody (rabbit anti-ELKS¹⁶, 1:200, or rabbit anti-KANK1, Atlas antibodies, HPA005539,
390 1:200) diluted in BB (45 min), washed three times in BB, and incubated with the anti-mouse
391 secondary antibody conjugated to Alexa Fluor 555 (Invitrogen) for 30 minutes. Cells were washed
392 twice in BB, once in PBS and once in dH₂O. Finally, coverslips were mounted in Abberior mounting
393 medium (Abberior) and examined under fluorescence microscope.

394

395 **siRNA interference**

396 The sequences of siRNA for p150Glued are obtained from Eurogentec:
397 GGUAUCUGACACGCUCCU and UAGGAGCGUGUCAGAUAC. Non-targeting siRNA ON-
398 TARGETplus (D-001810-10-05) (Dharmacon, GE Healthcare) served as the siRNA control (“siRNA
399 scramble”). siRNA transfection was performed using Lipofectamine RNAiMAX transfection reagent
400 (Thermo Fischer) at a final concentration of 40nM for 48 hrs prior to cell imaging. Protein knock
401 down efficiency was confirmed by real-time quantitative PCR and Western Blot analysis
402 (Supplementary Fig. S6C).

403

404 **Western Blot analysis**

405 Western blots were performed on protein extracts of siRNA silenced HeLa to estimate the degree of
406 knockdowns of Dynactin (p150glued) (Supplementary Fig. S6C). siRNA silenced Dynactin
407 (p150glued) HeLa cells were resuspended in RIPA Buffer (50 mM TRIS pH8.0, 150 mM NaCl, 1%
408 NP-40, 0,1% SDS) supplemented with cOmplete protease inhibitor cocktail (Roche). Sample was
409 centrifuged at $20,000 \times g$ for 1 min and protein supernatant were collected and concentrations checked
410 with Bradford assay. 100 μg of each sample was run on a 4-12% NuPAGE Gel, which was then
411 transferred onto a PVDF membrane using the BioRad Trans-Blot Turbo system. Membranes were
412 blocked in 5% Milk, TBST and treated with p150glued Antibody (Mouse, BD biosciences) or β -actin
413 (Mouse, Genetex) as the loading control at a 1:1000 dilution in TBS-T at 4°C overnight, then the
414 secondary Goat-anti-mouse HRP antibody (Sigma Aldrich) at 1:10 000 dilution in TBS-T, for 1h.
415 PierceTM ECL 2 Western Blotting Substrate (Thermo Scientific Kit) was added and blots developed
416 using the Amersham Imager AI 680. To assess the degree of siRNA silencing of p150glued we
417 quantified the relative change in the protein expression, using the Fiji “Measure” plugin and using the
418 same region of interest (ROI) across the bands in one blot normalized by the intensity of the
419 appropriate β -actin band (indicates the amount of loaded protein).

420

421 **Statistical analysis**

422 Statistical analysis was performed in GraphPad Prism. $P < 0.05$ was considered statistically significant.
423 At least three experiments were performed in order to conduct a statistical test (number of experiments
424 are indicated as *N*_{exp} in the figure captions). Data sets were tested with the D’Agostino and Pearson
425 normality tests. Normally distributed data sets were analyzed with Student’s *t* tests or with one-way
426 ANOVA Tukey tests if they contained two or more conditions respectively. Data sets with non-normal
427 distributions were analyzed with a Kruskal–Wallis test (multiple comparison) or Wilcoxon rank sum
428 test (two-sample comparison).

429

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