

1 **Mechanisms of integrin α V β 5 clustering in flat clathrin lattices**

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16 Key words: clathrin; integrin; keratinocytes; vitronectin

17

18 **Summary statement**

19 This article highlights several molecular mechanisms that result in the assembly of integrin α V β 5-
20 containing flat clathrin lattices in human keratinocytes.

21

22 **Abstract**

23 The family of integrin transmembrane receptors is essential for the normal function of multicellular
24 organisms by facilitating cell-extracellular matrix adhesion. The vitronectin-binding integrin α V β 5
25 localizes to focal adhesions (FAs) as well as poorly characterized flat clathrin lattices (FCLs). Here we
26 show that in human keratinocytes α V β 5 is predominant found in FCLs and that formation of the α V β 5-
27 containing FCLs requires the presence of vitronectin as ligand, calcium, and the clathrin adaptor proteins
28 ARH, Numb, and EPS15/EPS15L1. Integrin chimeras, containing the extracellular and transmembrane
29 domains of β 5 and the cytoplasmic domains of β 1 or β 3, almost exclusively localize in FAs. Interestingly,
30 lowering actomyosin-mediated contractility promotes integrin redistribution to FLCs in an integrin tail-
31 dependent manner, while increasing cellular tension favors α V β 5 clustering in FAs. Our findings strongly
32 indicate that clustering of integrin α V β 5 in FCLs is dictated by the β 5 subunit cytoplasmic domain,
33 cellular tension, and recruitment of specific adaptor proteins to the β 5 subunit cytoplasmic domains.

34 **Introduction**

35 Cell adhesion to the surrounding extracellular matrix (ECM) is a basic requirement in multicellular
36 organisms. Integrins are a major family of cell adhesion transmembrane receptors that are formed
37 through heterodimerization of α and β subunits (Hynes, 1987). Integrins can assemble different types of
38 cell-matrix adhesions, for instance by clustering in focal adhesions (FAs) and forming a mechanical link
39 between the ECM and intracellular actin bundles (Geiger and Yamada, 2011; Jansen et al., 2017) or by
40 connecting the ECM to the intracellular intermediate filament system in hemidesmosomes (de Pereda et
41 al., 2009; Litjens et al., 2006). Other integrin-containing complexes include tetraspanin-enriched
42 microdomains (Charrin et al., 2014; Hemler, 2005), fibrillar adhesions (FBs) (Geiger and Yamada, 2011),
43 podosomes (Geiger and Yamada, 2011; Linder and Wiesner, 2016) and flat clathrin lattices (FCLs) (Grove
44 et al., 2014; Lampe et al., 2016).

45 Depending on the combination of α and β subunits integrins bind different ECM components, including
46 laminins, collagens, and fibronectin (Barczyk et al., 2010; Hynes, 2002). The RGD-binding integrins form
47 a subset of integrins that recognize an arginine (R), glycine (G), aspartic acid (D) tri-peptide sequence
48 present in ECM proteins such as fibronectin and vitronectin (Bodary and McLean, 1990; Charo et al.,
49 1990; Cheresh et al., 1989; Cheresh and Spiro, 1987; Smith and Cheresh, 1990). Several RGD-binding
50 integrins can bind the same ligands, but despite this, RGD-binding integrins can have distinct subcellular
51 localization patterns. For example, in many cell types the integrin $\alpha V\beta 3$ localizes exclusively in FAs, while
52 $\alpha 5\beta 1$ is present in both FAs and FBs. Additionally, in some cell types, these integrins are concentrated in
53 podosomes. The integrin $\alpha V\beta 5$ can be found in both FAs and clathrin-coated membrane domains (De
54 Deyne et al., 1998; Leyton-Puig et al., 2017; Wayner et al., 1991).

55 The clathrin structures in which integrin $\alpha V\beta 5$ clusters reside, have in later years been described as FCLs
56 or clathrin “plaques” (Akisaka et al., 2008; Grove et al., 2014; Lampe et al., 2016; Leyton-Puig et al.,
57 2017; Maupin and Pollard, 1983; Saffarian et al., 2009). These structures comprise large assemblies of
58 clathrin that are distinct from the more dynamic clathrin-coated pits that play an active role in the
59 selective internalization of membrane-bound proteins through a process known as clathrin-mediated
60 endocytosis (CME) (Kaksonen and Roux, 2018; Lampe et al., 2016). The physiological relevance of FCLs is
61 not completely understood. They could be involved in CME by providing stable platforms for the
62 recruitment of cargo (Grove et al., 2014) and/or play a role in cell adhesion (Batchelder and Yarar, 2010;
63 Elkhatib et al., 2017; Saffarian et al., 2009).

64 Since RGD-binding integrins are mainly found to reside in FAs and associate with the actin cytoskeleton,
65 we wondered which processes drive the clustering of integrins in other cell adhesion structures. In the

66 present study, we unravel different mechanisms that lead to integrin α V β 5 clustering in FCLs in human
67 keratinocytes. These epidermal cells are able to assemble different types of cell-matrix adhesions,
68 including FAs and FCLs, and constantly need to adapt to changing conditions, such as coping with diverse
69 mechanical forces. We report that binding of integrin α V β 5 to its ligand vitronectin in the presence of
70 calcium is essential for the assembly of integrin α V β 5-containing FCLs. The clustering of integrin α V β 5 in
71 FCLs is mediated by the clathrin adaptor proteins ARH and Numb that bind to the membrane proximal
72 NPxY-motif in the integrin β 5 cytoplasmic domain. Alternatively, Numb can also link integrin β 5 and
73 clathrin via an NPxY-motif independent mechanism through its interaction with EPS15/EPS15L1. Besides
74 these mechanisms, we show that the localization of integrins in distinct cell-matrix adhesion complexes
75 is controlled by actomyosin-driven cellular tension.

76

77 **Results**

78 **Integrin α V β 5 localizes in FCLs in human keratinocytes**

79 Here we analyze the distribution of integrin β 5 in PA-JEB/ β 4 patient-derived immortalized keratinocytes
80 in which the stable expression of integrin β 4 was restored by cDNA transfection (Schaapveld et al.,
81 1998). Using super-resolution microscopy (Fig. 1A) we show that integrin β 5 is often found outside of
82 FAs in circular structures. The morphology of these structures reminded us of the assembly of clathrin
83 proteins, shown by EM as the irregular and triskelion structures that are located away from the cell
84 periphery (Fig. 1B). By immunofluorescence and confocal microscopy imaging we confirmed that
85 integrin β 5 outside of FAs is concentrated on the ventral surface of the cells in clathrin-coated
86 structures (Fig. 1C,D and 1SA). Morphometric analysis on integrin β 5 clusters that are found outside FAs
87 indicates that the shape and size of these clusters is very similar to that of FCLs (Fig. S1B), as previously
88 described (Grove et al., 2014). These results were confirmed in HaCaT immortalized human
89 keratinocytes, in which a similar subcellular distribution of integrin β 5 can be observed (Fig. S1C).
90 Integrin α V follows the distribution pattern of the β 5 subunit (Fig. 1D), indicating the presence of
91 heterodimerized α V β 5 in clathrin lattices. We do not find other integrin subunits associated with FCLs,
92 as demonstrated for the RGD-binding integrin β 1 (Fig. S1E). Occasionally, we can find integrin β 5 in FCLs
93 associated with actin (Fig. S1F), as has been previously described (Leyton-Puig et al., 2017).

94 **Clustering of integrin β 5 requires vitronectin and calcium**

95 The distribution pattern of integrin β 5 as presented in Figure 1 could only be observed when the serum-
96 free keratinocyte culture medium (KGM, containing epidermal growth factor and pituitary extract) had

97 been replaced by DMEM supplemented with 10% fetal calf serum (FCS). To investigate whether
98 vitronectin in the fetal calf serum-containing culture medium is critical for the formation of integrin β 5-
99 containing FCLs, we cultured keratinocytes on vitronectin-coated or uncoated coverslips in the presence
100 of low or high calcium concentrations (KGM or DMEM culture medium, respectively). We observed that
101 integrin β 5-containing FCLs are only efficiently formed if both vitronectin and calcium are present (Fig.
102 2A), indicating that ligand binding, promoted by calcium (Asch and Podack, 1990), plays a role in integrin
103 clustering. Similar results were obtained by culturing keratinocytes in calcium-depleted DMEM culture
104 medium supplemented with low (0.09 mM) or high (1.8 mM) concentrations of calcium (Fig. S1G), which
105 correspond with the calcium concentrations found in KGM or DMEM culture medium, respectively.
106 Although the presence of vitronectin and calcium promotes the clustering of integrin β 5 in FCLs, it does
107 not alter its surface expression (Fig. 2B). Culturing keratinocytes on fibronectin-coated coverslips in the
108 presence of a high calcium concentration did not lead to clustering of integrin β 5 in FCLs (Fig. 2C).
109 Integrin β 5 clustering in the presence of vitronectin and calcium is needed for cell adhesion to
110 vitronectin. Indeed, adhesion of keratinocytes to vitronectin in high calcium is significantly decreased
111 upon treatment with cilengitide (Fig. 2D), an integrin α V β 3 and α V β 5 antagonist. Since like other normal
112 keratinocytes, PA-JEB/ β 4 cells do not express the integrin β 3 subunit (data not shown, Adams and Watt,
113 1991; Kubo et al., 2001; Larjava et al., 1993), cilengitide could be considered as a specific inhibitor of
114 integrin β 5. Next, we studied whether integrin β 5-containing FCLs behave as static or dynamic structures
115 by preventing binding of integrin β 5 to vitronectin in keratinocytes in which integrin β 5 clusters had
116 already been formed. Cilengitide acts as a competitive ligand-mimetic inhibitor but is not able to disrupt
117 pre-existing integrin-ligand interactions (Mould et al., 2014), thus it can only prevent the formation of
118 new integrin β 5 clusters. We observed by immunofluorescence analysis that after 10 minutes of
119 cilengitide treatment the integrin β 5 clusters began to dissociate and after 30-90 minutes of treatment,
120 only very few and small integrin β 5 clusters remained visible at the basal membrane (Fig. 2E,F, Fig.
121 S2A,C,E). However, cilengitide treatment did not decrease the surface expression of integrin β 5 (Fig.
122 S2D). Furthermore, cilengitide treatment did not result in a dramatic decrease of clustering of clathrin or
123 of the clathrin adaptor protein Numb near the cell membrane (Fig. 2G, Fig. S2B,C,F), although the
124 treatment resulted in slightly smaller clusters.

125 **BioID reveals proximity interactors of integrin β 5 clusters**

126 To investigate what other proteins are associated with integrin β 5-containing FCLs and might play a role
127 in the recruitment of integrin β 5 in clathrin lattices, we made use of the proximity biotinylation assay
128 BioID (Roux et al., 2012). To this end, we deleted the integrin β 5 subunit in PA-JEB/ β 4 keratinocytes

129 using CRISPR/Cas9 and introduced an integrin $\beta 5$ -BirA* fusion protein, allowing the biotinylation of
130 proteins in close proximity of the integrin $\beta 5$ subunit. We performed BioID experiments in the presence
131 or absence of calcium and vitronectin to detect proteins that are found associated with $\alpha V\beta 5$ when it is
132 concentrated in clathrin lattices. Identification of biotinylated proteins by mass spectrometry revealed
133 multiple proteins involved in CME as proximity interactors of integrin $\alpha V\beta 5$ (Fig. 3A, Table S1), including
134 the adaptor protein complex 2 (AP2) subunits $\alpha 1$, $\beta 1$, and $\mu 1$, epidermal growth factor receptor
135 substrate15-like 1 (EPS15L1), intersectin-1 (ITSN1), and low density lipoprotein receptor adapter protein
136 1 (LDLRAP1 or ARH), and the E3 ligases Itch and NEDD4L. The presence of the proteins involved in
137 endocytosis in the proximity of integrin $\beta 5$ clusters was confirmed by immunofluorescence and
138 calculation of Pearson's correlation coefficient in PA-JEB/ $\beta 4$ and HaCaT keratinocytes (Fig. 3B-D, Fig.
139 S3A). The cytoskeletal linker protein talin (TLN1) was also found by BioID, though immunofluorescence
140 analysis shows that this protein is mainly present in FAs near the cell periphery and does not colocalize
141 with integrin $\beta 5$ -containing FCLs (Fig. S3B).

142

143 **ARH/Numb interact with the integrin $\beta 5$ MP-NPxY motif**

144 We wondered how the proximity interactors identified in the BioID screen interact with integrin $\beta 5$ to
145 regulate its localization in FCLs. The BioID assay does not distinguish between indirect and direct
146 interactors of integrin $\beta 5$. Since the latter ones are more likely to regulate the assembly of integrin $\beta 5$ -
147 containing FCLs, we first analyzed which clathrin adaptor proteins can bind directly to the cytoplasmic
148 domain of integrin $\beta 5$. Integrin $\beta 5$ has a short (<60 residues) cytoplasmic domain that contains a
149 membrane-proximal (MP)-NPLY and membrane-distal (MD)-NKSX motif, two domains from which the
150 NPXY and NXXY sequences are highly conserved among different integrin β subunits. These so called
151 NPXY/NXXY motifs are canonical signals for clathrin-mediated endocytosis and serve as docking sites for
152 PTB containing adaptor proteins (Calderwood et al., 2003; Traub and Bonifacino, 2013). The clathrin
153 adaptor proteins ARH, protein disabled homolog 2 (Dab2), and Numb all contain a PTB domain (Mishra
154 et al., 2002; Morris and Cooper, 2001; Santolini et al., 2000). PA-JEB/ $\beta 4$ keratinocytes express ARH and
155 NUMB, but not Dab2 (Fig. S3C). ARH was identified in the BioID screen (Fig. 3A) and the presence of ARH
156 in integrin $\beta 5$ -containing FCLs was confirmed by immunofluorescence analysis (Fig. 3B). We also found
157 Numb associated with integrin $\beta 5$ -containing FCLs (Fig. S2C), although it had a lower significance in our
158 BioID screen. To study the role of the NPXY motifs on clustering of integrin $\beta 5$ in FCLs, we created
159 mutants (Y>A) of the NPXY and NXXY motifs for integrin $\beta 5$ -BirA* in PA-JEB/ $\beta 4$ keratinocytes (Fig. 4A,B,
160 Fig. S4A,B). In agreement with the notion that talin binds to the MP-NPxY motif and plays a key role in

161 the formation of FAs, mutation of this motif but not that of the MD-NxxY motif, abrogated the
162 localization of integrin β 5 in FAs (Fig. 4A). The localization of integrin β 5 in FCLs is not affected when
163 either one or both NPxY and NxxY motifs have been mutated (Fig. 4B), indicating that the assembly of
164 integrin β 5-containing FCLs does not require the prior presence of the protein in FAs. Although integrin
165 β 5-containing FCLs were still able to form after mutating the NPxY and NxxY motifs, mutation of the MP-
166 NPxY motif resulted in the formation of fewer and smaller, and more circular-shaped clusters (Fig. 4C-E).
167 Next, we studied whether the mutations in the NPxY and NxxY motifs affected the proximity interaction
168 between integrin β 5 and ARH or Numb by using the BioID assay and analysis by western blot (Fig. 4F, Fig.
169 S4C,D,E). Additionally, we studied colocalization of integrin β 5 and ARH or Numb in FCLs by
170 immunofluorescence and found that the MP-NPxY motif was required for the interaction of integrin β 5
171 with ARH (Fig. 4G,H). A dispersed distribution of ARH was seen when binding of ARH to β 5 was
172 abrogated, yet ARH could still be observed in clathrin-coated structures (Fig. S4F). The MD-NxxY motif is
173 not needed for the interaction between integrin β 5 and ARH. Similar results were obtained for Numb
174 (Fig. S5A,B), although the effect of the MP-NPxY mutation on the interaction between integrin β 5 and
175 Numb was less pronounced than that of integrin β 5 and ARH. Finally, knock down of ARH reduced the
176 number and size of integrin β 5 clusters (Fig. 4I-K), indicating the role of this clathrin adaptor protein in
177 regulating the localization of integrin β 5 in FCLs.

178

179 **Numb/EPS15L1 bind β 5 independently of the NPxY motif**

180 Since mutating the NPxY motif does not completely abrogate integrin β 5 clustering in FCLs, we
181 investigated whether Numb could also mediate integrin β 5 clustering in FCLs by a mechanism different
182 from that of binding to the NPxY motif. Previous studies have shown that Numb binds EH-domain-
183 containing proteins (Salcini et al., 1997), such as ITSN1 and EPS15L1, two scaffold proteins that are
184 important for endocytosis by binding to clathrin and the AP-2 complex (Coda et al., 1998; Drake et al.,
185 2000; Pechstein et al., 2010; Shih et al., 2002; Teckchandani et al., 2012; Wang et al., 2008; Yamabhai et
186 al., 1998). Both proteins were identified as integrin β 5 proximity interactors in our BioID screen (Fig. 3
187 and Table S1). EPS15L1 contains two ubiquitin-interacting motifs that are required for endocytosis of
188 ubiquitinated cargo (Hofmann and Falquet, 2001), suggesting that this scaffold protein may form a link
189 between clathrin and integrin β 5 by binding to ubiquitin-modified β 5. Indeed, integrin β 5 is
190 ubiquitinated, and its ubiquitination can be enhanced by inhibiting its lysosomal degradation or by
191 preventing degradation of ubiquitinating enzymes by proteasomal inhibition (Fig. 5A). To test the
192 hypothesis that an EPS15L1-Numb complex binds both ubiquitinated β 5 and clathrin, we analyzed the

193 effect of knock down of Numb and EPS15/EPS15L1 on integrin $\beta 5$ clustering in FCLs. Depletion of Numb
194 resulted in a significant decrease of $\beta 5$ clustering in PA-JEB/ $\beta 4$ keratinocytes (Fig. 5B-D). Moreover, if
195 Numb was depleted in keratinocytes expressing the $\beta 5$ subunit carrying mutations in the NPxY and NxxY
196 motifs, clustering of integrin $\beta 5$ in FCLs could almost completely be prevented (Fig. 5B-D). Similar results
197 were obtained for knock down of EPS15/EPS15L1 (Fig. 5E-G), providing evidence for the role of
198 EPS15/EPS15L1 and Numb in localizing integrin $\beta 5$ in clathrin lattices.

199

200 **Integrin $\beta 5$ intracellular domain dictates clustering in FCLs**

201 With the exception of integrin $\beta 5$, RGD-binding integrins mainly cluster in FAs or FBs and associate with
202 the actin cytoskeleton through an interaction with cytoskeletal linker proteins, such as talin. Integrin $\beta 5$
203 and $\beta 3$ are both RGD-binding integrins that share homologous regions (e.g. NPxY and NxxY motifs) and
204 can both heterodimerize with the integrin αV subunit. Although PA-JEB/ $\beta 4$ keratinocytes do not
205 endogenously express integrin $\beta 3$, this integrin localizes in FAs and not in FCLs once exogenously
206 introduced in these cells (Fig. S5C). Integrin $\beta 5$ and $\beta 3$ are both vitronectin receptors, however, integrin
207 $\beta 3$ binds many more ligands, including fibronectin, von Willebrand factor, thrombospondin,
208 osteopontin, and fibrinogen (Humphries et al., 2006). We wondered whether differences in ligand
209 binding are responsible for the localization of integrins in FCLs versus FAs and decided to create a
210 system in which integrins possess equal ligand-binding properties and that enables us to study the
211 contribution of the integrin $\beta 5$ cytoplasmic domain on its localization in FCLs. To this end, we
212 constructed different integrin chimeras that contain the extracellular and transmembrane domains of
213 the $\beta 5$ subunit and the intracellular domain of the $\beta 1$ ($\beta 5^{\text{ex}}/\beta 1^{\text{in}}$) or $\beta 3$ ($\beta 5^{\text{ex}}/\beta 3^{\text{in}}$) subunits and visualized
214 their clustering by immunofluorescence (Fig. 6A,B). Like its full-length $\beta 3$ counterpart, the integrin
215 $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ chimera predominantly clustered in FAs (Fig. 6A,C) and less in FCLs (Fig. 6B,D). Similar results
216 were obtained for the integrin $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$ chimera, which is also found predominantly in FAs (Fig. 6). The
217 distribution pattern of this chimera differs from that of its full-length counterpart (Fig. S1E), since the
218 $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$ chimera exclusively binds vitronectin, while the full-length $\beta 1$ subunit can heterodimerize with
219 12 different α subunits and is thus able to reside in many different adhesion structures, including
220 tetraspanin-enriched microdomains (Charrin et al., 2014; Hemler, 2005). A unique feature of the integrin
221 $\beta 5$ intracellular domain is the 8-amino acid stretch between the NPxY and NxxY motifs. However,
222 deletion of this sequence did not prevent clustering of integrin $\beta 5$ in FCLs (Fig. S5D,E). In summary, our
223 results suggest that the intracellular domain of integrin $\beta 5$ is important for its assembly in clathrin
224 lattices.

225

226 **Cellular tension regulates integrin localization**

227 Although we did not find an association between exogenously expressed integrin $\beta 3$ or the $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$
228 chimera and clathrin, others have shown that the clathrin adaptor Dab2 binds to integrin $\beta 3$ clusters
229 when force generation is inhibited (Yu et al., 2015). In addition, the collagen-binding integrin $\alpha 2\beta 1$ can
230 be found in clathrin lattices when cells interact with collagen fibers in a soft 3D environment (Elkhatib et
231 al., 2017). We hypothesized that the integrin subcellular distribution patterns might also be regulated by
232 traction force generation. To this end, we treated keratinocytes with the myosin inhibitor blebbistatin in
233 order to reduce cellular tension and observed that this treatment practically abolished clustering of
234 integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ in FAs, as marked by vinculin staining (Fig. 7A,B) and favored their
235 clustering in FCLs (Fig. 7C,D). A similar trend, though less dramatic, was observed by analyzing the
236 colocalization of $\beta 5$ and the different integrin $\beta 5$ chimeras with the focal adhesion component talin and
237 the clathrin adaptor protein Numb (Fig. S6).

238 Alternatively, we transfected PA-JEB/ $\beta 4$ keratinocytes with constitutively active RhoA to increase
239 actomyosin contractility and cellular tension. We observed that in these keratinocytes the assembly of
240 integrin $\beta 5$ -containing FCLs was decreased and that integrin $\beta 5$ localized primarily in large FAs (Fig. 8).
241 These findings were confirmed in HaCaT keratinocytes, in which integrin $\beta 5$ colocalization with clathrin
242 was decreased and colocalization with vinculin was increased after stimulation with LPA (Fig. S7).
243 Clustering of integrin $\beta 5$, either in clathrin lattices or focal adhesions, thus seems a dynamic and
244 mechanosensitive process.

245

246 **Discussion**

247 We investigated how the clustering of integrin $\alpha V\beta 5$ in FCLs is regulated. Integrin $\alpha V\beta 5$ is the only RGD-
248 binding integrin that has been clearly shown to localize in these enigmatic clathrin structures, yet the
249 reason of this distinct distribution pattern was so far unexplored. Here, we report that integrin $\alpha V\beta 5$
250 clusters in FAs, but predominantly in FCLs, in human keratinocytes that are cultured in the presence of
251 vitronectin and high levels of calcium (e.g. in serum-rich culture medium). The clustering of integrin
252 $\alpha V\beta 5$ in FCLs is a dynamic process. Blocking integrin-ligand interactions or increasing actomyosin-
253 mediated cellular tension, results in reduced integrin $\alpha V\beta 5$ clustering in FCLs.

254

255 The formation of FCLs in keratinocytes may be the result of “frustrated endocytosis”, triggered by
256 integrin $\alpha V\beta 5$ binding to vitronectin and the inability to internalize this serum protein because of its

257 strong binding to the solid substratum. A recent study by Elkhatib *et al.* describes how the collagen-
258 binding integrin $\alpha 2\beta 1$ associates with clathrin lattices to promote cell migration in soft 3D collagen gels
259 (Elkhatib et al., 2017). Since fragments of collagen, generated by MMP cleavage, but not intact collagen
260 fibers can be internalized by cells, the formation of integrin $\alpha 2\beta 1$ -containing clathrin lattices on collagen
261 fibers can also be regarded as a form of frustrated endocytosis. This frustrated endocytosis of adhesion
262 receptors might also lead to the “flat” organization of the clathrin lattices by preventing invagination of
263 the membrane. FCLs may contribute to cell migration by providing additional sites of adhesion. In re-
264 epithelializing wounds, the binding of $\alpha V\beta 5$ to vitronectin within the provisional matrix in the early
265 wound bed may ensure that migrating keratinocytes remain associated with the substratum when
266 exposed to mechanical forces. In line with the described findings that FCLs act as dynamic structures
267 controlled by actin-based mechanisms (Leyton-Puig et al., 2017), our results demonstrate that integrin
268 $\beta 5$ -containing FCLs rearrange in response to environmental changes, like the availability of ligand and/or
269 the magnitude of force exerted on integrin $\alpha V\beta 5$.

270 Noticeably, FCLs do not have to form as a result of frustrated endocytosis of integrin ligands per se.
271 Large clathrin lattices can also be formed in the absence of adhesion molecules in skeletal muscles
272 (Vassilopoulos et al., 2014) or at non-adherent cell surfaces (Grove et al., 2014). We propose that FCLs
273 can be formed under different conditions and might be required for several biological processes.
274 However, when integrins localize in these structures they act as alternative sites for cell-matrix
275 adhesion.

276
277 Cell adhesion to vitronectin plays a major role in diverse biological processes. In normal keratinocytes,
278 adhesion to vitronectin is solely mediated by integrin $\alpha V\beta 5$ (Adams and Watt, 1991; Kubo et al., 2001;
279 Larjava et al., 1993) and regulates keratinocyte migration and wound healing (Clark et al., 1996).
280 Moreover, vitronectin synthesis and integrin $\beta 5$ expression on tumor cells is correlated with disease
281 progression of different tumor types, including melanoma (Desch et al., 2012; Vogetseder et al., 2013)
282 and neural (Gladson et al., 1997; Uhm et al., 1999), breast (Bianchi-Smiraglia et al., 2013; Vogetseder et
283 al., 2013) and non-small cell lung cancer (Bai et al., 2015; Vogetseder et al., 2013). Similar to
284 keratinocytes, integrin $\beta 5$ displays a punctate distribution over the ventral cell surface outside of FAs in
285 human melanoma and lung carcinoma cells (Wayner et al., 1991). Integrin $\beta 5$ interacts with different
286 adaptor proteins in FCLs than in FAs (i.e. clathrin adaptor proteins versus cytoskeletal linker proteins).
287 Therefore, localization of integrins in distinct adhesion structures might have important consequences
288 for their downstream signaling and contribution to cellular processes that promote tumor progression.

289 Furthermore, vitronectin regulates bacterial pathogenesis by serving as a cross-linker between bacteria
290 and host cells and stimulating bacterial uptake through interaction with the host cell's integrin receptors
291 (Singh Molecular Microbiology 2010). The presence of integrin α V β 5 in FCLs might promote the uptake
292 of pathogens and ECM components, due to the close proximity of this vitronectin receptor to the
293 endocytic machinery and the notion that CME actively takes place at the edge of clathrin lattices (Lampe
294 et al., 2016).

295
296 For the formation of integrin β 5-containing FCLs, the intracellular domain of integrin β 5 is crucial, as has
297 been demonstrated previously (De Deyne et al., 1998; Pasqualini and Hemler, 1994) and shown in our
298 studies with integrin chimeras. By making use of proximity-biotinylation assays (BioID), we were able to
299 conduct unbiased screens to identify proximity interactors of integrin β 5 in FCLs. We demonstrate that
300 the clathrin adaptor proteins ARH and Numb interact with the MP-NPXY motif on the integrin β 5
301 cytoplasmic domain. Moreover, an EPS15L1-Numb complex can interact with integrin β 5 independently
302 of the MP-NPXY motif and provides an additional mechanism for clathrin association, which is in line
303 with other studies (Kang et al., 2013; Teckchandani et al., 2012). ARH does not contain the NPF motif
304 that is needed for binding to EPS15L1, which could explain why the association between ARH and
305 integrin β 5 is lost after mutating the β 5 MP-NPXY motif, while this mutation has a less dramatic effect on
306 the association between integrin β 5 and Numb. This observation is supported by the fact that knock
307 down of Numb and EPS15/EPS15L1 has a larger effect on reducing the clustering of integrin β 5 subunits
308 carrying mutations in the NPXY and NxxY motifs than of wild type integrin β 5, which can still interact
309 with ARH. The interaction between EPS15L1-Numb and integrin β 5 is likely to be mediated by binding of
310 the ubiquitin-interacting motifs of EPS15L1 to ubiquitinated residues in the integrin β 5 cytoplasmic
311 domain. This interaction could possibly be enhanced by Numb, since this clathrin adaptor protein is able
312 to further promote receptor ubiquitination by recruiting the E3 ligase Itch (Di Marcotullio et al., 2006; Di
313 Marcotullio et al., 2011; McGill and McGlade, 2003). The clathrin adaptor Dab2 (not expressed in our
314 cell lines) might have a similar role as Numb since it contains very similar domains (NPF) to bind EH-
315 domain-containing proteins, like members of the epsin protein family.

316
317 Our data indicate that the integrin β 5 localization pattern is regulated by the amount of cellular tension.
318 When tension is increased, integrin β 5 is localized predominantly in FAs. Alternatively, we show that
319 integrin chimeras switch from clustering in FAs to FCLs when cellular tension is reduced. It is intriguing
320 that under normal culture conditions the integrin β 1, β 3, and β 5 subunits show a distinct subcellular

321 distribution pattern, despite the presence of highly conserved domains. Since the $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$ and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$
322 chimeras are able to cluster in FCLs under low cellular tension, it seems likely that the differences in
323 integrin localization arise from the abilities of their β subunits to recruit specific adaptor proteins
324 (Calderwood et al., 2003; Sun et al., 2016) and mediate traction forces. Indeed, it has been shown in pull
325 down experiments that the $\beta 1$, $\beta 3$, and $\beta 5$ subunit differ in their ability to bind kindlin-2 (Sun et al.,
326 2016). We have confirmed these results and find that the $\beta 5$ cytoplasmic domain is unable to bind
327 kindlin-2 and kindlin-1 (unpublished). Hence, we surmise that on a stiff substratum the $\beta 5$ integrin can
328 mediate less traction forces than the $\beta 1$ and $\beta 3$ integrins, as it is unable to establish additional linkages
329 to the actin cytoskeleton via kindlin and the ILK-PINCH-Parvin complex (Bledzka et al., 2016; Wickstrom
330 et al., 2010). In apparent contrast to this view is that in cells subjected to high tension, the integrin $\beta 5$
331 appeared to be primarily localized in large FAs rather than in FCLs. This pool of integrin $\beta 5$, however,
332 may not necessarily have to be involved in force generation but has become localized in FAs because of
333 binding to the excessive amounts of talin available at these sites. In line with the fact that the $\beta 1$ and $\beta 3$
334 integrins by binding of their cytoplasmic domains to both talin and kindlin can mediate high traction
335 forces on the substratum, we observed that the localization of the $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$ and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ chimeras in
336 FAs is dramatically decreased when cellular tension is lowered. This is seen most clearly when FAs are
337 marked by vinculin (Carisey et al., 2013; del Rio et al., 2009; Grashoff et al., 2010). When FAs were
338 stained for talin, the decrease was less dramatic. This observation can be explained by the fact that talin,
339 in contrast to vinculin, can reside in FAs under variable and low tension (Atherton et al., 2015; Kumar et
340 al., 2016).

341 In agreement with our findings, integrin $\alpha V\beta 3$ and $\alpha 2\beta 1$ colocalize with clathrin in lattices in soft
342 environments (Elkhatib et al., 2017; Yu et al., 2015). In general, it seems that integrin clustering in FCLs
343 might give rise to additional sites of cell adhesion that are only formed when cells experience low
344 tension. Yet, translocation to FAs occurs in response to increased actomyosin-based forces, which could
345 be relevant in pathological conditions like cancer progression, in which vitronectin deposition and ECM
346 stiffness are often increased.

347

348 In summary, we show how integrin $\alpha V\beta 5$ clustering in FCLs is regulated by multiple mechanisms in the
349 presence of vitronectin, high calcium, and low cellular tension. Our results provide novel insights into
350 the regulation of integrin clustering in different adhesion structures. Additionally, these findings may
351 shed light on the properties and function of the poorly characterized FCLs.

352 **Materials and Methods**

353 **Antibodies**

354 Primary antibodies used are listed in Table S2. Secondary antibodies were as follows: goat anti-rabbit
355 Alexa Fluor 488, goat anti-mouse Alexa Fluor 488, goat anti-mouse Texas Red, goat anti-mouse Alexa
356 Fluor 568, donkey anti-rabbit Alexa Fluor 594, goat anti-rabbit Alexa Fluor 647, and goat anti-mouse
357 Alexa Fluor 647 (Invitrogen), PE-conjugated donkey anti-rabbit antibody (Biolegend #406421), stabilized
358 goat anti-mouse HRP-conjugated and stabilized goat anti-rabbit HRP-conjugated (Pierce).

359

360 **Cell lines**

361 Immortalized keratinocytes were isolated from a patient with Pyloric Atresia associated with Junctional
362 Epidermolysis Bullosa (PA-JEB), as published elsewhere (Schaapveld et al., 1998). The derivation of this
363 cell line was done for diagnostic purposes, thus the research conducted using these cells was exempt of
364 the requirement for ethical approval. PA-JEB/ β 4 keratinocytes stably expressing integrin β 4 were
365 generated by retroviral transduction, as described previously (Sterk et al., 2000). Cells were maintained
366 in serum-free keratinocyte medium (KGM; Invitrogen) supplemented with 50 μ g ml⁻¹ bovine pituitary
367 gland extract, 5 ng ml⁻¹ EGF, and antibiotics (100 units ml⁻¹ streptomycin and 100 units ml⁻¹ penicillin).
368 HaCaT keratinocytes, obtained from the American Type Culture Collection were cultured in Dulbecco's
369 modified Eagle's medium (DMEM) containing 10% heat-inactivated FCS and antibiotics. All cells were
370 cultured at 37°C in a humidified, 5% CO₂ atmosphere.

371

372 **Transient transfection**

373 Human LDLRAP1 (M-013025-03-0020), EPS15 (M-004005-01-0020), EPS15L1 (M-004006-00-0020) and
374 NUMB (M-015902-01-0020) siGENOME SMARTpool siRNAs were purchased from Dharmacon. The
375 cDNAs encoding constitutively active (LZRS-IRES-GFP-RhoA(V14)) and dominant negative RhoA (LZRS-
376 IRES-GFP-RhoA(N19)) were kindly provided by Jacques Neefjes. PA-JEB/ β 4 keratinocytes were
377 transiently transfected with siRNAs or cDNAs using lipofectamine® 2000 (Invitrogen). Lipofectamine (20
378 μ l ml⁻¹) and cDNA (6.5 μ g ml⁻¹) or siRNA (1 μ M) solutions in Opti-MEM were mixed (1:1) and incubated
379 for 20 min at room temperature. Cells were incubated with the transfection solution overnight.

380 **Generation of integrin β 5-deficient keratinocytes**

381 The target sgRNA against *ITGB5* (exon3; 5'-ACGGTCCATCACCTCTCGGT-3') was cloned into pX330-U6-
382 Chimeric_BB-CBh-hSpCas9 (a kind gift from Feng Zhang (Cong et al., 2013); Addgene plasmid #42230).

383 PA-JEB/ β 4 keratinocytes were transfected with this vector in combination with a blasticidin cassette, as
384 previously described (Blomen et al., 2015). Integrin β 5-deficient cells were selected by supplementing
385 the culture medium with 4 μ g ml⁻¹ blasticidin (Sigma) for four days following transfection.

386

387 **Stable cellular transduction**

388 For the generation of the integrin β 5-BirA* fusion proteins, pcDNA3- β 5-BirA*(R118G) was obtained by
389 inserting the coding sequence for β 5, derived from pLenti-III-HA-ITGB5-mCherry (kindly provided by
390 Dean Sheppard) together with the coding sequence of BirA* (R118G) into the *EcoRI/XhoI* sites of
391 pcDNA3. The pcDNA3.1 MCS-BirA*(R118G)-HA plasmid was used as a template for the BirA* (a kind gift
392 from Kyle Roux (Roux et al., 2012); Addgene plasmid #30647). Point or deletion mutants of β 5 Y774
393 and/or Y794 and *del.8aa* were generated by site-directed mutagenesis with the PCR-based overlap
394 extension method using Pwo DNA polymerase (Roche), and fragments containing the different
395 mutations were exchanged with corresponding fragments in the β 5 pcDNA3 vector. For the generation
396 of the expression vectors encoding β 5^{ex}/ β 1ⁱⁿ and β 5^{ex}/ β 3ⁱⁿ chimeric integrin subunits, the codon
397 encoding L744 in β 5 was mutated from ctg to ctt, creating a *HindIII* site. Subsequently, this *HindIII* site
398 was used for exchanging β 1 or β 3 cytoplasmic domain in the β 5 pcDNA3 vector. Retroviral vectors
399 containing mutant β 5 cDNAs were generated by subcloning the mutant β 5 cDNAs into the *EcoRI* and
400 *XhoI* restriction sites of the LZRS-MS-IRES-Zeo vector. PA-JEB/ β 4 keratinocytes expressing different β 5
401 mutants were generated by retroviral transduction. HEK293 cells were transiently transfected with 10
402 μ g of cDNA, using the DEAE-dextran method.

403

404 **Super-resolution microscopy**

405 For immunofluorescent analysis, keratinocytes grown on glass coverslips were fixed with 4%
406 paraformaldehyde, permeabilized with 0.2% Triton-X-100, blocked in 5% BSA and incubated with
407 primary and secondary antibodies at room temperature with extensive washing steps in between.
408 Super-resolution microscopy was performed with a Leica SR GSD microscope (Leica Microsystems,
409 Wetzlar, Germany) mounted on a Sumo Stage (#11888963) for drift free imaging. Collection of images
410 was done with an EMCCD Andor iXon camera (Andor Technology, Belfast, UK) and a 160x oil immersion
411 objective (NA 1.47). To image, the samples have been immersed in the multi-color super-resolution
412 imaging buffer, OxEA (Nahidiazar et al., 2016). Laser characteristics were 405 nm/30 mW, 488 nm/300
413 mW and 647 nm/500 mW, with the 405 nm laser for back pumping. Ultra clean coverslips (cleaned and
414 washed with base and acid overnight) were used for imaging. The number of recorded frames was

415 variable between 10,000 to 50,000, with a frame rate of 100 Hz. The data sets were analyzed with the
416 Thunder Storm analysis module (Ovesny et al., 2014), and images were reconstructed with a detection
417 threshold of 70 photons, sub pixel localization of molecules and uncertainty correction, with a pixel size
418 of 10 nm.

419

420 **Electron Microscopy (EM)**

421 For EM, samples were fixed in Karnovsky's fixative. Postfixation was done with 1% Osmiumtetroxide in
422 0.1 M cacodylatebuffer, after washing the cells were stained and blocked with Ultrastain 1 (Leica,
423 Vienna, Austria), followed by ethanol dehydration series. Finally, the samples were embedded in a
424 mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, USA). This was all done in the tissue culture petri
425 dish. Sectioning was performed parallel to the growing plane from the basal cell membrane upwards.
426 Analysis was done with a Tecnai12G2 electron microscope (FEI, Eindhoven, The Netherlands).

427

428 **Flow cytometry**

429 Cells were treated as indicated, trypsinized, and washed twice in PBS containing 2% FCS, followed by
430 rabbit anti-human integrin $\beta 5$ antibody (EM09902; 1:200 dilution) incubation for 1 h at 4°C. Then, cells
431 were washed 3 times in PBS containing 2% FCS and incubated with PE-conjugated donkey anti-rabbit
432 antibody (Biolegend #406421; 1:200 dilution) for 1 h at 4°C. After subsequent washing steps, integrin $\beta 5$
433 expression was analyzed on a Becton Dickinson FACS Calibur analyser. For FACS sort, a PE-conjugated
434 anti-human integrin $\beta 5$ antibody (Biolegend #345203) was used and the desired cell populations were
435 isolated using a Becton Dickinson FACSAria IIu or Beckman Coulter Moflo Astrios cell sorter.

436

437 **Adhesion assay**

438 For adhesion assays, 96-well plates were coated with 10 $\mu\text{g ml}^{-1}$ fibronectin from bovine plasma (Sigma
439 #F1141), 5 $\mu\text{g ml}^{-1}$ vitronectin (Sigma #SRP3186) or 3.2 $\mu\text{g ml}^{-1}$ collagen 1 (Advanced Biomatrix #5005),
440 overnight at 37°C. Before use, plates were washed once with PBS and blocked with 2% BSA (Sigma) in
441 PBS for 1 h at 37°C. PA-JEB/ $\beta 4$ cells were trypsinized and resuspended in serum-free KGM or DMEM in
442 the presence or absence of cilengitide (a kind gift from Coert Margadant). The cells were seeded at a
443 density of 1×10^5 cells per well and incubated for 30 min at 37°C. Nonadherent cells were washed away
444 with PBS and the adherent cells were fixed with 4% paraformaldehyde for 10 min at room temperature,
445 washed twice with H₂O, stained with crystal violet for 10 min at room temperature and washed

446 extensively with H₂O. Cells were air-dried overnight and lysed in 2% SDS, after which absorbance was
447 measured at 490 nm on a microplate reader using MPM6 software.

448

449 **Immunofluorescence**

450 Unless mentioned otherwise, PA-JEB/ β 4 keratinocytes were seeded on glass coverslips and cultured in
451 complete KGM medium for 24 h, and then treated with high calcium and vitronectin (DMEM + 10% FCS)
452 for 24 h. HaCaT keratinocytes were seeded on glass coverslips and cultured in DMEM + 10% FCS. Cells
453 were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.2% Triton-X-100 for 5 min, and
454 blocked with PBS containing 2% BSA (Sigma) for at least 30 min. Next, cells were incubated with the
455 primary antibodies for 1 h at room temperature. Cells were washed three times before incubation with
456 the secondary antibodies for 1 h. Additionally, the nuclei were stained with DAPI and filamentous actin
457 was visualized using Alexa Fluor 488 or 647-conjugated phalloidin (Invitrogen). After three washing steps
458 with PBS, the coverslips were mounted onto glass slides in Mowiol. Images were obtained using a Leica
459 TCS SP5 confocal microscope with a 63x (NA 1.4) oil objective.

460 **Image analysis and statistical analysis**

461 Image analysis was performed using Fiji (ImageJ) (Schindelin et al., 2012; Schneider et al., 2012). For
462 analysis of the colocalization between integrin β 5 clusters and clathrin adaptor proteins, Pearson's
463 correlation coefficient was calculated (without threshold) using the JaCoP plug-in (Bolte and Cordelieres,
464 2006). Cluster size, amount, and circularity were calculated using the Analyze Particle function, after
465 drawing a region of interest (ROI) at the cell periphery (based on actin staining). The total cluster area
466 was divided by the total ROI area to define cluster density. To quantify integrin clustering in FAs (based
467 on vinculin or talin staining) versus FCLs (clathrin staining), background was subtracted in both channels
468 using a bilateral filter and the ROI was selected at the cell periphery. Colocalization of integrin clusters
469 and FAs or FCLs was determined using the Image Calculator (command "multiply") on both channels and
470 calculating the area of overlapping clusters as a percentage of the total integrin cluster area per cell
471 using the Analyze Particle function.

472 Mann-Whitney test (two-tailed P value) was performed using GraphPad Prism (version 7.0c). In figures,
473 statistically significant values are shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
474 Graphs were made in GraphPad Prism and show all data points represented as box-and-whisker plots, in
475 which the box extends the 25th to 75th percentiles, the middle line indicates the median, and whiskers go
476 down to the smallest value and up to the largest.

477

478 **BioID assay**

479 PA-JEB/ β 4 expressing β 5-BirA* cells grown on 145 mm plates were cultured in complete KGM or DMEM
480 and treated with 50 μ M biotin (Sigma #B4501) for 24 h. Cells were washed in cold PBS, lysed in RIPA
481 buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM EDTA (pH 7.5), 1% NP-40, 0.1% SDS, 0.5% sodium
482 deoxycholate) supplemented with a protease inhibitor cocktail (Sigma), and cleared by centrifugation at
483 14.000 x g for 60 min at 4°C. Lysates were incubated with Streptavidin Sepharose High Performance
484 beads (GE Healthcare) overnight at 4°C. Beads were washed three times with NP40 buffer (20 mM Tris-
485 HCl (pH 7.5), 100 mM NaCl, 4 mM EDTA (pH 7.5), 1% NP-40) and twice with PBS and the isolated
486 biotinylated proteins were analyzed by mass spectrometry or western blotting.

487

488 **Mass spectrometry**

489 For mass spectrometry, samples were shortly separated on a 4-12% SDS-PAGE gel and stained with
490 Coomassie Blue. The lane was excised from the gel after which proteins were reduced with dithiothreitol
491 and alkylated with iodoacetamide. Proteins were digested with trypsin (mass spec grade, Promega)
492 overnight at 37°C and peptides were extracted with acetonitrile. Digests were dried in a vacuum
493 centrifuge and reconstituted in 10% formic acid for MS analysis. Peptide mixtures (33% of total digest)
494 were loaded directly on the analytical column and analyzed by nanoLC-MS/MS on an Orbitrap Fusion
495 Tribrid mass spectrometer equipped with a Proxeon nLC1000 system (Thermo Scientific). Solvent A was
496 0.1% formic acid/water and solvent B was 0.1% formic acid/80% acetonitrile. Peptides were eluted from
497 the analytical column at a constant flow of 250 nl min⁻¹ in a 140-min gradient, containing a 124-min
498 linear increase from 6% to 30% solvent B, followed by a 16 min wash at 80% solvent B.

499

500 **Mass spectrometry data analysis**

501 Raw data were analyzed by MaxQuant (version 1.5.8.3) (Cox et al., 2014) using standard settings for
502 label-free quantitation (LFQ). MS/MS data were searched against the human Swissprot database (20,183
503 entries, release 2017_03) complemented with a list of common contaminants and concatenated with
504 the reversed version of all sequences. Trypsin/P was chosen as cleavage specificity allowing two missed
505 cleavages. Carbamidomethylation (C) was set as a fixed modification, while oxidation (M) was used as
506 variable modification. LFQ intensities were Log₂-transformed in Perseus (version 1.5.5.3) (Tyanova et al.,
507 2016), after which proteins were filtered for at least two valid values (out of 3 total). Missing values
508 were replaced by imputation based a normal distribution using a width of 0.3 and a downshift of 1.8.

509 Differentially expressed proteins were determined using a t-test (threshold: $P \leq 0.05$) and $[x/y] > 0.3$ |
510 $[x/y] < -0.3$.

511

512 **Immunoprecipitation and western blotting**

513 Subconfluent PA-JEB/ β 4 cells cultured in 100 mm cell plates were treated with DMEM supplemented
514 with 10% FCS overnight and then additionally treated with 0.25 μ M Bafilomycin A1 (InvivoGen #tlr-
515 baf1) or 5 μ M MG132 (Sigma #M7449) for 3h at 37°C. Cells were washed in cold PBS and lysed in RIPA
516 buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM EDTA (pH 7.5), 1% NP-40, 0.1% SDS, 0.5% sodium
517 deoxycholate) supplemented with 1.5 mM Na_3VO_4 , 15 mM NaF (Cell Signaling), protease inhibitor
518 cocktail (Sigma), and 5 mM *N*-methylmaleimide (Sigma). Lysates were incubated on ice for 10 min and
519 cleared by centrifugation at 14.000 x *g* for 20 min at 4°C. Next, lysates were incubated with 1 μ g ml⁻¹
520 integrin β 5 antibody (EM09902) or control normal rabbit serum for 2 h at 4°C. Subsequently, lysates
521 were incubated for 2 h at 4°C with Protein G Sepharose 4 Fast Flow beads (GE Healthcare). Beads
522 carrying the immune complexes were washed 4 times in RIPA buffer supplemented with inhibitors,
523 eluted in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02%
524 bromophenol blue) containing a final concentration of 2% β -mercaptoethanol and denatured at 95°C for
525 10 min. Proteins were separated by electrophoresis using Bolt Novex 4–12% gradient Bis-Tris gels
526 (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp) and blocked for at least
527 30 min in 2% BSA in TBST buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 0.3% Tween-20). Primary
528 antibody (diluted 1:1000 in 2% BSA in TBST buffer) incubation took place overnight at 4°C. After washing
529 twice with TBST and twice with TBS buffer, blots were incubated for 1 h hour at room temperature with
530 horseradish peroxidase–conjugated goat anti-mouse IgG or goat anti-rabbit IgG (diluted 1:3.000 in 2%
531 BSA in TBST buffer). After subsequent washing steps, the bound antibodies were detected by enhanced
532 chemiluminescence using SuperSignal™ West Dura Extended Duration Substrate (ThermoFisher) or
533 Clarity™ Western ECL Substrate (Bio-Rad) as described by the manufacturer. Signal intensities were
534 quantified using ImageJ.

535 **Acknowledgements**

536 We thank Bram van den Broek (Netherlands Cancer Institute) for his help with data analysis, and Marc
537 Block, Simon Goodman, Marina Glukhova, Coert Margadant, and Jacques Neefjes for sharing reagents.

538

539 **Competing interests**

540 The authors declare no competing interests.

541

542 **Funding**

543 This work was supported by grants from the Netherlands Organization for Scientific Research (NWO;
544 project number 824.14.010) and Dutch Cancer Society (project number 2013-5971) and by a grant from
545 NWO as part of the National Roadmap Large-scale Research Facilities of the Netherlands,
546 Proteins@Work (project number 184.032.201).

547

548 **Data availability**

549 All relevant data are available from the authors on reasonable request.

550

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763

764 **Figure legends**

765 **Fig. 1. Integrin α V β 5 clusters are present in both focal adhesions and flat clathrin lattices in human**
766 **keratinocytes.**

767 **A)** Representative super-resolution microscopy images showing integrin β 5 (green) in and near focal
768 adhesions, visualized by vinculin staining (red), at the cell periphery (left image) and more centrally
769 located integrin β 5 (right image). Scale bar, 5 μ m.

770 **B)** Electron microscopy image showing the area near the cell periphery containing both FAs and
771 triskelion clathrin structures. Scale bar, 2 μ m.

772 **C)** Island of four keratinocytes showing integrin β 5 (green in merge), clathrin (red in merge), and DAPI
773 (blue). Scale bar, 20 μ m.

774 **D)** Intensity profiles of integrin β 5 (green) and clathrin (red) along the cyan line in panel C.

775

776 **Fig. 2. Binding to vitronectin in the presence of calcium is required for clustering of integrin β 5 in flat**
777 **clathrin lattices**

778 **A)** Keratinocytes were grown on vitronectin-coated or uncoated coverslips in the presence or absence of
779 high calcium levels. Images focus on the ventral cell surface. Left panel shows integrin β 5 (green in
780 merge), clathrin (red), and the cell nuclei (blue). Right panel shows vitronectin coating (red).

781 **B)** FACS plot showing the expression of integrin β 5 in keratinocytes grown in low calcium in KGM (blue)
782 or in high calcium in DMEM supplemented with 10% FCS (red). Staining with the PE-conjugated
783 secondary antibody only was used as a negative control (grey) (n=2).

784 **C)** Keratinocytes seeded on fibronectin-coated coverslips. Integrin β 5 (green in merge), fibronectin (red),
785 and the cell nuclei (blue) are shown.

786 **D)** Cells were treated with different concentrations of cilengitide (as indicated) in suspension, before a
787 short-term (30 min) adhesion assay was performed on fibronectin-, collagen-, or vitronectin-coated
788 substrates, in the presence (DMEM) or absence (KGM) of high calcium levels. Two-sided t-test was
789 performed to calculate statistical significance between the control and samples treated with 1 μ M
790 cilengitide. *, P < 0.05; **, P < 0.01; ns, not significant. Columns show mean values with s.d. of three
791 independent experiments.

792 **E)** PA-JEB/ β 4 keratinocytes were grown in 10% FCS-supplemented DMEM culture medium overnight to
793 induce integrin β 5 clustering in FCLs and then treated with 1 μ M cilengitide for the indicated times
794 before fixation. Merged images show integrin β 5 (green), clathrin (red), actin (blue) and the cell nuclei
795 (cyan).

796 **F,G** The amount of integrin (**F**) or clathrin (**G**) clustering is defined as the total area of clusters on the
797 cell membrane as a percentage of the total cell area. Data were obtained from three independent
798 experiments. In total between 104 and 125 cells were analyzed per condition. Mann-Whitney U test was
799 used to calculate statistical significance. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant.
800 Box plots range from the 25th to 75th percentile; central line indicates the median; whiskers show
801 smallest to largest value. Scale bar, 20 μm .

802

803 **Fig. 3. Clathrin adaptor proteins reside in close proximity of integrin $\beta 5$ clusters**

804 **A)** PA-JEB/ $\beta 4$ keratinocytes expressing integrin $\beta 5$ fused to the promiscuous biotin ligase BirA* were
805 used to perform proximity biotinylation assays with LC-MS/MS to determine the proximity interactors of
806 integrin $\beta 5$ subunits that are dispersed over the cell membrane (cell cultured in KGM) and of integrin $\beta 5$
807 clusters (cells treated with 10% FCS-supplemented DMEM). Results of three independent experiments
808 are shown in the volcano plot. The y-axis shows the negative log P values (dashed line at $y=1.3$ indicates
809 a P value of 0.05) and the x-axis the difference in expression between the two conditions. Proteins are
810 highlighted that are known to be associated with clathrin (red), FAs (orange), or play a role in protein
811 ubiquitination (blue). Statistics: two-sided t-test.

812 **B)** Representative confocal microscopy images ($n=2$) show the colocalization of integrin $\beta 5$ (green in
813 merge) and the clathrin adaptor proteins ARH, EPS15L1, and ITSN1 (red in merge). Nuclei are shown in
814 cyan and actin in blue. Scale bar, 20 μm .

815 **C)** Pearson's correlation analysis of integrin $\beta 5$ and ARH, EPS15L1, and ITSN1 in PA-JEB/ $\beta 4$ and in

816 **D)** HaCaT keratinocytes. At least 24 cells obtained from 2 experiments were analyzed per condition. Box
817 plots range from the 25th to 75th percentile; central line indicates the median; whiskers show smallest to
818 largest value.

819

820 **Fig. 4. The membrane-proximal NPxY motif on the integrin $\beta 5$ cytoplasmic domain interacts with the** 821 **clathrin adaptor proteins ARH and Numb**

822 **A)** Integrin $\beta 5$ (green) does not cluster in FAs, visualized by vinculin staining (red) after mutation (Y>A) of
823 the MP-NPxY motif (N1). See Fig. S4A for the sequence information.

824 **B)** N1 and N2 Y>A mutations do not prevent clustering of integrin $\beta 5$ (green) in clathrin structures (red).

825 **C-E)** The number of integrin $\beta 5$ clusters is reduced by N1 Y>A mutation and the average cluster size is
826 reduced. The circularity of the smaller clusters as a result of the N1 mutant is increased. The y axis
827 describes the shape ranging from 0 (irregular) to 1 (circle). At least 32 PA-JEB/ $\beta 4$ keratinocytes obtained

828 from 2 independent experiments were analyzed per condition. Box plots range from the 25th to 75th
829 percentile; central line indicates the median; whiskers show smallest to largest value. Scale bar, 20 μ m.
830 Mann-Whitney U test was performed to determine statistical significance. ****, $P < 0.0001$; ns, not
831 significant.

832 **F)** PA-JEB/ β 4 keratinocytes expressing integrin β 5 (containing Y>A mutations in the MP-NPXY (N1)
833 and/or MD-NxxY (N2) motif) fused to the promiscuous biotin ligase BirA* were used to perform
834 proximity biotinylation assays. One representative western blot is shown out of three independent
835 experiments. Quantifications of ARH/Numb signal intensities are shown in Fig. S4E.

836 **G,H)** Proximity interaction between integrin β 5 (green in merge) and ARH (red in merge) is reduced by
837 the N1 Y>A mutation. ARH appears more diffuse over the cell membrane and the Pearson's correlation
838 coefficient is decreased. Scale bar, 20 μ m.

839 **I-K)** Knock down of ARH was accomplished by treating PA-JEB/ β 4 ARH siRNA for 24 h, prior to 24 h
840 treatment with 10% FCS-supplemented DMEM culture medium to induce integrin β 5 clustering. Merged
841 images show integrin β 5 (green), ARH (red), actin (blue) and the cell nuclei (cyan). Analysis of integrin β 5
842 clustering shows a decrease of clustering after siRNA treatment. The amount of clustering is defined as
843 the total area of clusters on the cell membrane as a percentage of the total cell area.

844 Data were obtained from three independent experiments (approximately 100 cells in total). Mann-
845 Whitney U test was performed to determine statistical significance. ****, $P < 0.0001$. Box plots range
846 from the 25th to 75th percentile; central line indicates the median; whiskers show smallest to largest
847 value. Scale bar, 20 μ m.

848

849 **Fig. 5. Knock down of Numb and EPS15/EPS15L1 prevents clustering of integrin β 5 containing**
850 **mutations in the NPXY motifs.**

851 **A)** Integrin β 5 immunoprecipitation samples show (poly-) ubiquitination that is increased upon
852 lysosomal or proteasomal inhibition, by treatment with 0.25 μ M bafilomycin A1 and 5 μ M MG132,
853 respectively, for at least 3 h before cell lysis. Normal rabbit serum (IgG) is used as negative control for
854 the immunoprecipitation. A representative western blot is shown (n=3).

855 **B-G)** Knock down of Numb (**B)** or EPS15/EPS15L1 (**C)** was accomplished by treating PA-JEB/ β 4
856 keratinocytes (with Y>A mutations in the NPXY and NxxY motifs) with Numb or EPS15L1 and EPS15
857 siRNAs for 24 h, prior to 24 h treatment with 10% FCS-supplemented DMEM culture medium to induce
858 integrin β 5 clustering. Merged images show integrin β 5 (green), Numb/EPS15L1 (red), actin (blue) and
859 the cell nuclei (cyan). Analysis of integrin β 5 clustering shows a decrease of clustering after siRNA

860 treatment. The amount of clustering is defined as the total area of clusters on the cell membrane as a
861 percentage of the total cell area.

862 Data were obtained from three independent experiments (approximately 120 cells in total). Mann-
863 Whitney U test was performed to determine statistical significance. **, $P < 0.01$; ***, $P < 0.001$; ****, P
864 < 0.0001 ; ns, not significant. Box plots range from the 25th to 75th percentile; central line indicates the
865 median; whiskers show smallest to largest value. Scale bar, 20 μm .

866

867 **Fig. 6. Integrin chimeras containing the intracellular domain of integrin $\beta 1$ or $\beta 3$ cluster predominantly**
868 **in focal adhesions.**

869 **A)** Integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ (green in merge) colocalization with the FA marker talin (red).
870 Nuclei are shown in blue. Occasionally, aspecific nuclear staining is detected in the integrin $\beta 5$ channel.

871 **B)** Integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ (green in merge) and clathrin structures (red).

872 **C)** Integrin clustering in FAs defined by the area of integrin clusters overlapping with talin calculated as a
873 percentage of the total integrin area per cell.

874 **D)** Integrin clustering in FCLs defined by the area of integrin clusters overlapping with clathrin calculated
875 as a percentage of the total integrin area per cell.

876 Data were obtained from three independent experiments ($n=30$). Mann-Whitney U test was performed
877 to determine statistical significance. ****, $P < 0.0001$. Box plots range from the 25th to 75th percentile;
878 central line indicates the median; whiskers show smallest to largest value. Scale bar, 20 μm .

879

880 **Fig. 7. Integrin clustering in flat clathrin lattices versus focal adhesions is controlled by the amount of**
881 **cellular tension.**

882 **A)** Integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ (green in merge) colocalization with the FA marker vinculin (red)
883 is shown in response to treatment with the myosin inhibitor blebbistatin (20 μM) for 45 min prior to
884 fixation. Actin is shown in blue and the nuclei in cyan.

885 **B)** Integrin clustering in FAs is defined by the area of integrin clusters overlapping with vinculin
886 calculated as a percentage of the total integrin area per cell.

887 **C)** Colocalization of integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ (green in merge) with clathrin structures (red)
888 with and without blebbistatin treatment.

889 **D)** Integrin clustering in FCLs is defined by the area of integrin clusters overlapping with clathrin
890 calculated as a percentage of the total integrin area per cell.

891 Data were obtained from three biological replicates (60 cells total). Mann-Whitney U test was
892 performed to determine statistical significance. *, $P < 0.05$; ****, $P < 0.0001$. Box plots range from the
893 25th to 75th percentile; central line indicates the median; whiskers show smallest to largest value. Scale
894 bar, 20 μm .

895

896 **Fig 8. Increased cellular tension results in the clustering of integrin $\beta 5$ in focal adhesions.**

897 **A+C)** PA-JEB/ $\beta 4$ keratinocytes were transiently transfected with constitutively active RhoA (V14) or
898 dominant negative RhoA (N19) constructs. Transfected cells were selected based on the nuclear GFP
899 signal. Integrin $\beta 5$, vinculin/clathrin, and cell nuclei are shown in red, blue, and cyan, respectively, in the
900 merged images.

901 **B)** Integrin clustering in FAs defined by the area of integrin clusters overlapping with vinculin calculated
902 as a percentage of the total integrin area per cell.

903 **D)** Integrin clustering in FCLs defined by the area of integrin clusters overlapping with clathrin calculated
904 as a percentage of the total integrin area per cell.

905 Data were obtained from three independent experiments (60 cells total). Mann-Whitney U test was
906 performed to determine statistical significance. ****, $P < 0.0001$. Box plots range from the 25th to 75th
907 percentile; central line indicates the median; whiskers show smallest to largest value. Scale bar, 20 μm .

908

909 **Supplementary Figure legends**

910 **Supplementary Figure S1.**

911 **A)** Montage of image slices making up a z-stack showing PA-JEB/ $\beta 4$ keratinocytes with integrin $\beta 5$ (green
912 in merge), clathrin (red in merge), actin (blue), and nuclei (cyan). Distance in z between the image slices
913 is 1 μm . Scale bar, 20 μm .

914 **B)** Morphometric analysis of integrin $\beta 5$ clusters outside FAs (n=4). Circularity ranges from 0 (irregular)
915 to 1 (circle).

916 **C)** HaCaT keratinocytes showing integrin $\beta 5$ (green in merge), clathrin (red in merge), actin (blue), DAPI
917 (cyan). Scale bar, 20 μm .

918 **D)** PA-JEB/ $\beta 4$ keratinocytes showing integrin $\beta 5$ (green in merge), αV (red in merge), and DAPI (blue).

919 **E)** PA-JEB/ $\beta 4$ keratinocytes showing integrin $\beta 5$ (green in merge), $\beta 1$ (red in merge), and DAPI (blue).

920 **F)** Zoomed in regions of PA-JEB/ $\beta 4$ keratinocytes showing occasional overlap between integrin $\beta 5$
921 clusters (green) and actin (grey). Scale bar, 5 μm .

922 **G)** PA-JEB/ β 4 keratinocytes were grown in the presence of vitronectin and low or high calcium levels.
923 Different calcium concentrations were obtained by first depleting calcium from FCS-supplemented
924 DMEM culture medium using Chelex 100 resin, and then adding 0.09 mM (low) or 1.8 mM (high) CaCl_2 .
925 Merged images show integrin β 5 (green in merge), clathrin (red), actin (blue), and the cell nuclei (cyan).
926 Scale bar, 20 μm .

927

928 **Supplementary Figure S2.**

929 **A+E)** The number of integrin β 5 clusters is reduced by cilengitide treatment and the average cluster size
930 is reduced. The circularity of the smaller clusters as a result of cilengitide treatment is increased. The y
931 axis describes the shape ranging from 0 (irregular) to 1 (circle).

932 **B+F)** Number of clathrin (**B**) or Numb (**F**) clusters and their size and circularity.

933 **C)** PA-JEB/ β 4 keratinocytes were grown in 10% FCS-supplemented DMEM culture medium overnight to
934 induce integrin β 5 clustering in FCLs and then treated with 1 μM cilengitide for the indicated times
935 before fixation. Merged images show integrin β 5 (green), Numb (red), actin (blue) and the cell nuclei
936 (cyan). Scale bar, 20 μm .

937 **D)** FACS plot showing the expression of integrin β 5 in keratinocytes grown in DMEM supplemented with
938 10% FCS and treated with (red) or without (blue) 1 μM cilengitide for 90 min. Staining with the PE-
939 conjugated secondary antibody only was used as a negative control (grey).

940 Data were obtained from three independent experiments. In total between 104 and 125 cells were
941 analyzed per condition. Mann-Whitney U test was performed to determine statistical significance. *, $P <$
942 0.05; ***, $P <$ 0.001; ****, $P <$ 0.0001; ns, not significant. Box plots range from the 25th to 75th
943 percentile; central line indicates the median; whiskers show smallest to largest value.

944

945 **Supplementary Figure S3.**

946 **A)** Representative confocal microscopy images show the colocalization of integrin β 5 (green in merge)
947 and the clathrin adaptor proteins ARH, EPS15L1, and ITSN1 (red in merge) in HaCaT keratinocytes. Nuclei
948 are shown in cyan and actin in blue.

949 **B)** PA-JEB/ β 4 keratinocytes showing integrin β 5 (green in merge), talin (red in merge), actin (blue), and
950 DAPI (cyan). Scale bar, 20 μm .

951 **C)** Western blot showing the expression of the clathrin adaptor proteins Numb, ARH, and Dab2 in HaCaT
952 and PA-JEB/ β 4 keratinocytes. HeLa cells were used as a positive control for Dab2 expression. GAPDH is
953 used as loading control.

954

955 **Supplementary Figure S4.**

956 **A)** Amino acid sequences of the cytoplasmic domain of wild type integrin $\beta 5$ and the MP-NPxY and MD-
957 NxxY mutants. The Y>A mutations are marked in red.

958 **B)** Western blot showing the expression of integrin $\beta 5$ wild type and MP-NPxY and MD-NxxY mutants
959 fused to the BirA* biotin ligase (n=2). Fusion proteins were obtained by transfecting integrin $\beta 5$ -deficient
960 PA-JEB/ $\beta 4$ keratinocytes (gRNA 2) as described in methods.

961 **C,D)** Quantifications of signal intensities of ARH (**C**) and Numb (**D**) of three independent experiments.
962 Bars show mean with s.d.

963 **E)** Uncropped scans of the western blots shown in Fig. 4F.

964 **F)** ARH (green in merge) appears more diffuse over the ventral cell membrane when the integrin $\beta 5$ MP-
965 NPxY motif is mutated. Colocalization with clathrin (red) is quantified using Pearson's correlation
966 coefficient (R). Actin is shown in blue and the nuclei in cyan. Scale bar, 20 μm .

967

968 **Supplementary Figure S5.**

969 **A,B)** Proximity interaction between integrin $\beta 5$ (green in merge) and Numb (red in merge) is reduced by
970 the N1 Y>A mutation. Numb appears more diffuse over the cell membrane and the Pearson's correlation
971 coefficient is decreased. At least 32 PA-JEB/ $\beta 4$ keratinocytes obtained from 2 independent experiments
972 were analyzed per condition. Box plots range from the 25th to 75th percentile; central line indicates the
973 median; whiskers show smallest to largest value. Scale bar, 20 μm .

974 **C)** Exogenously expressed integrin $\beta 3$ (green in merge) colocalizes with talin (red) in integrin $\beta 5$ -deficient
975 PA-JEB/ $\beta 4$ keratinocytes. Actin is shown in blue and the nuclei in cyan. Scale bar, 20 μm .

976 **D)** Amino acid sequences showing the deletion of the 8-amino acid stretch in the cytoplasmic domain of
977 integrin $\beta 5$.

978 **E)** Deletion of the 8-amino acid stretch does not prevent clustering of integrin $\beta 5$ in FCLs. Merged image
979 shows integrin $\beta 5$ (green), clathrin (red), actin (blue) and the cell nuclei (cyan). Scale bar, 20 μm .

980

981 **Supplementary Figure S6.**

982 **A)** Integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ (green in merge) colocalization with the FA marker talin (red) is
983 shown in response to treatment with the myosin inhibitor blebbistatin (20 μM) for 45 min prior to
984 fixation. Actin is shown in blue and the nuclei in cyan.

985 **B)** Integrin clustering in FAs is defined by the area of integrin clusters overlapping with talin calculated as
986 a percentage of the total integrin area per cell.

987 **C)** Colocalization of integrin $\beta 5$, $\beta 5^{\text{ex}}/\beta 1^{\text{in}}$, and $\beta 5^{\text{ex}}/\beta 3^{\text{in}}$ (green in merge) with Numb structures (red)
988 with and without blebbistatin treatment.

989 **D)** Integrin clustering in FCLs is defined by the area of integrin clusters overlapping with Numb calculated
990 as a percentage of the total integrin area per cell.

991 Data were obtained from two independent experiments (30-50 cells total). Box plots range from the 25th
992 to 75th percentile; central line indicates the median; whiskers show smallest to largest value. Scale bar,
993 20 μm .

994

995 **Supplementary Figure S7.**

996 **A)** Integrin $\beta 5$ (green in merge) colocalization with the FA marker vinculin (red in merge) is shown in
997 response to treatment of HaCaT keratinocytes with 1 μM LPA for 60 min prior to fixation. Actin is shown
998 in blue and the nuclei in cyan.

999 **B)** Integrin clustering in FAs is defined by the area of integrin clusters overlapping with vinculin
1000 calculated as a percentage of the total integrin area per cell.

1001 **C)** Colocalization of integrin $\beta 5$ (green in merge) with clathrin structures (red) with and without LPA
1002 stimulation.

1003 **D)** Integrin clustering in FCLs is defined by the area of integrin clusters overlapping with clathrin
1004 calculated as a percentage of the total integrin area per cell.

1005 Data were obtained from three biological replicates (60 cells total). Mann-Whitney U test was
1006 performed to determine statistical significance. ****, $P < 0.0001$. Box plots range from the 25th to 75th
1007 percentile; central line indicates the median; whiskers show smallest to largest value. Scale bar, 20 μm .

1008

1009 **Supplementary Table 2: Primary antibody list**

Antibody	Clone	Obtained from	Host	Application
Integrin $\beta 5$	EM09902	Simon Goodman (Merck KGaA)	Rabbit	IF/FACS: 1:200 IP: $1\mu\text{g ml}^{-1}$
Integrin $\beta 5$	P1F6	Biologend (#920004)	Mouse	IF: 1:100
Integrin $\beta 5$	5HK2	Homemade	Rabbit	WB: 1:1000 SR: undiluted supernatant
Vinculin	VIIF9	Marina Glukhova	Mouse	IF: 1:5
Talin		Marc Block	Rabbit	IF: 1:100
ARH/LDLRAP1		AntibodyPlus (#A7093)	Rabbit	IF: 1:100 WB: 1:1000

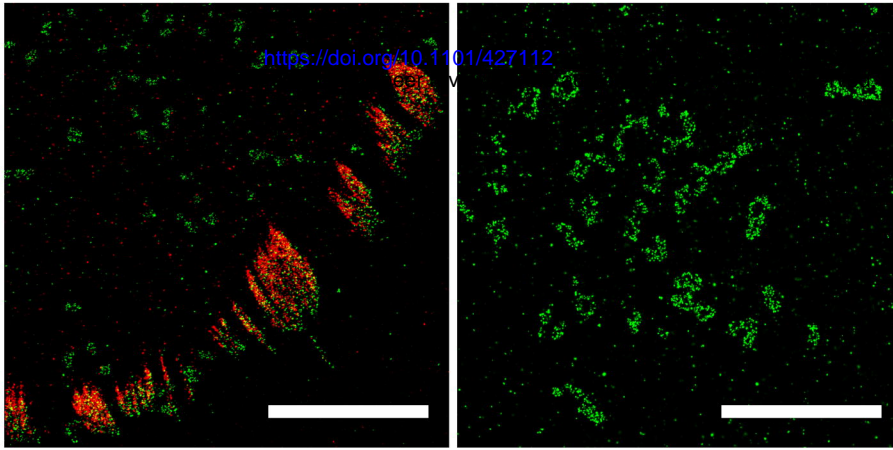
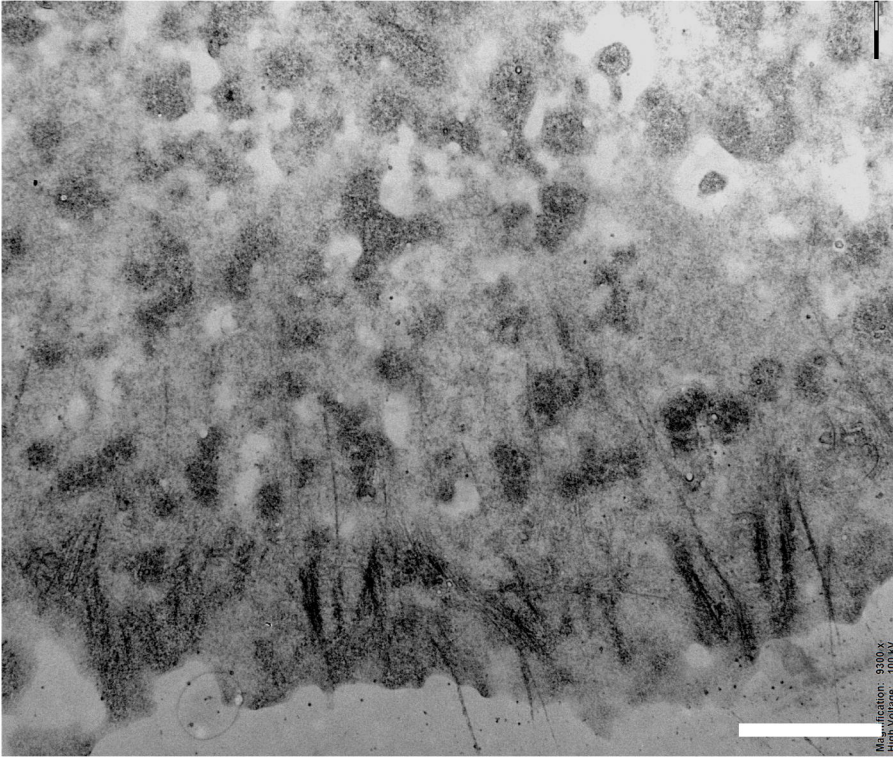
Numb	S.925.4	Invitrogen (#MA5-14897)	Rabbit	IF: 1:100 WB: 1:1000
Clathrin, Heavy chain	X22	Thermo Fisher (#MA1-065)	Mouse	IF: 1:400
ITSN1		Atlas antibodies (#HPA018007)	Rabbit	IF: 1:100
EPS15L1	EP1146Y	OriGene (#TA301237)	Rabbit	IF: 1:100
Ubiquitin	P4D1	Covance (#MMS-257P)	Mouse	WB: 1:1000
Fibronectin	FN15	Sigma (SAB4200760)	Mouse	IF: 1:1000
Vitronectin	342603	R&D Systems (#MAB2349)	Mouse	IF: 1:1000
Integrin β 5-PE	AST-3T	Biolegend (#345203)	Mouse	FACS

1010

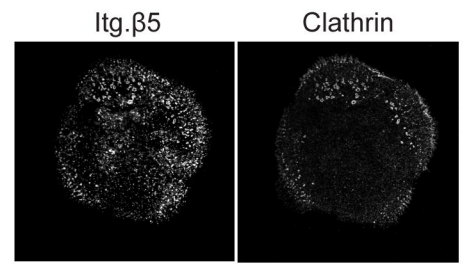
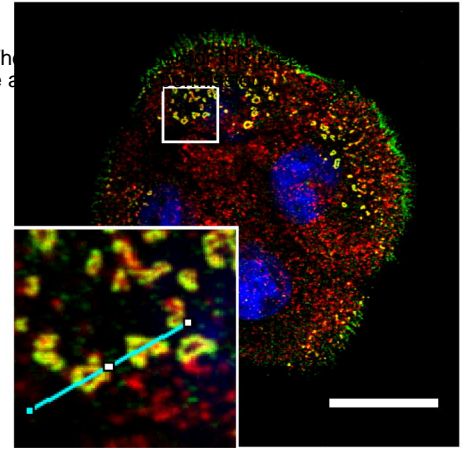
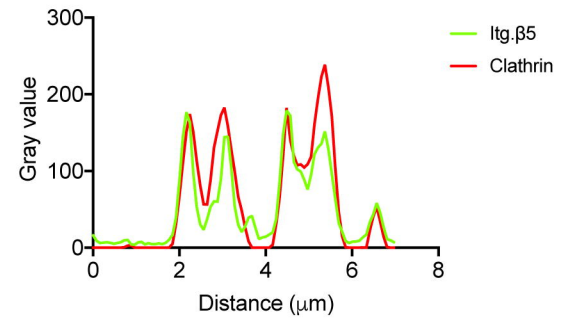
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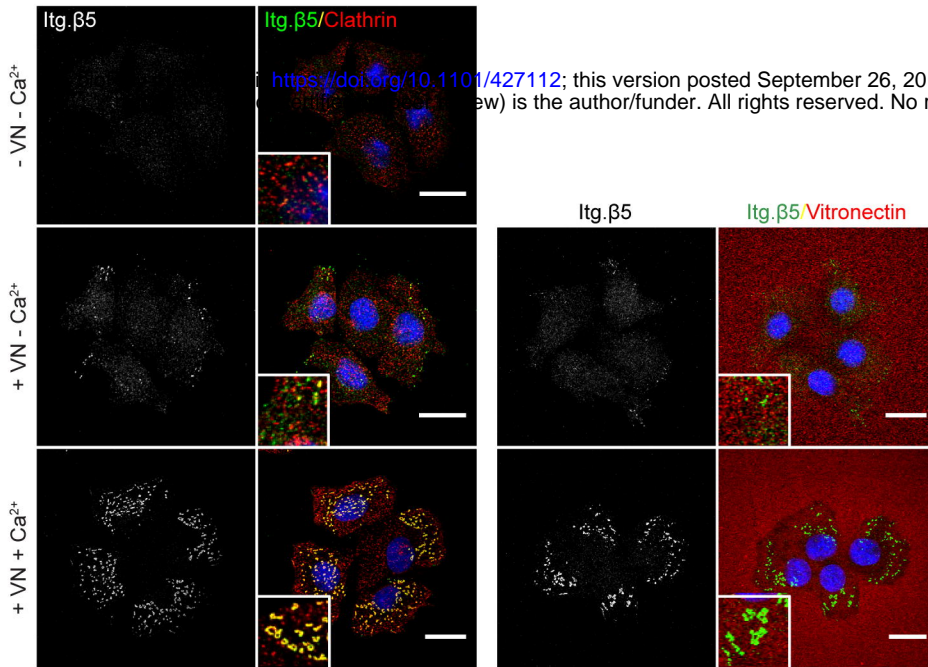
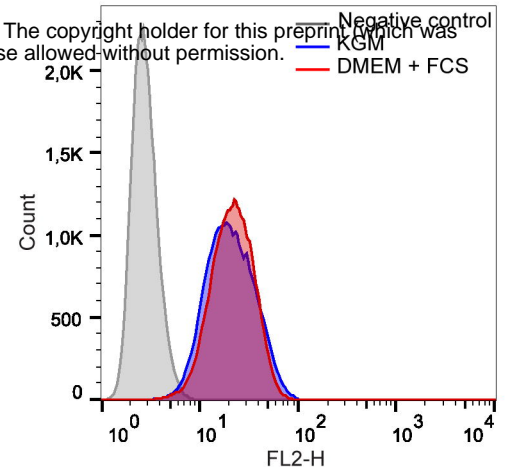
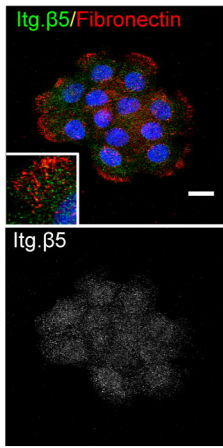
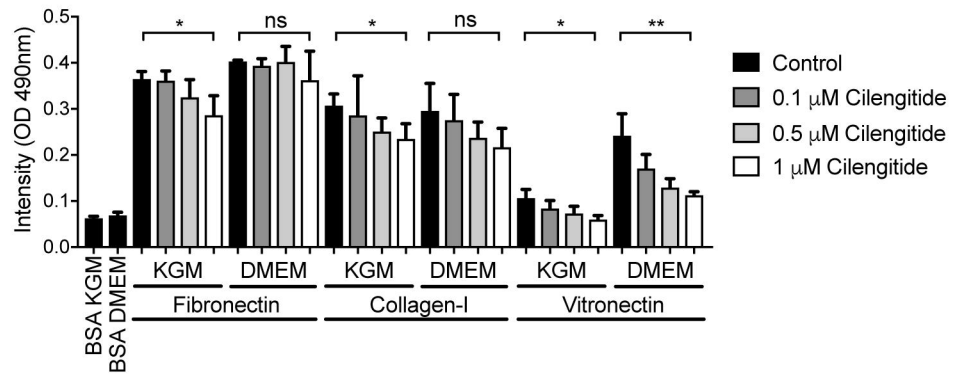
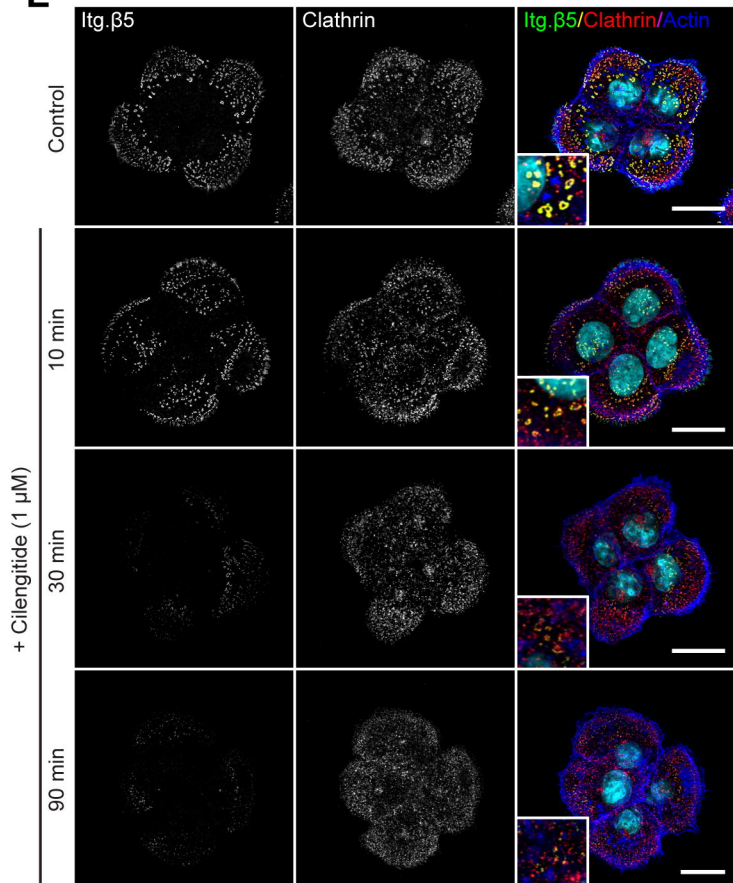
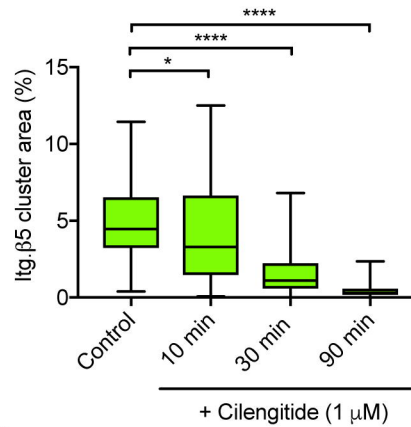
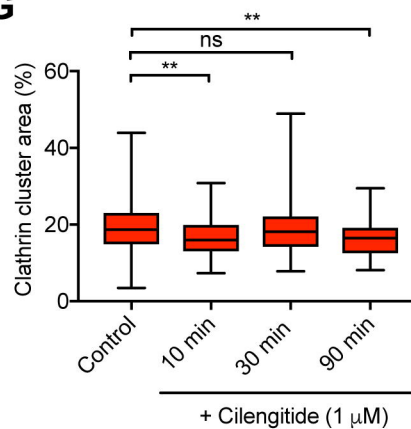
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Itg.β5

**B****C**

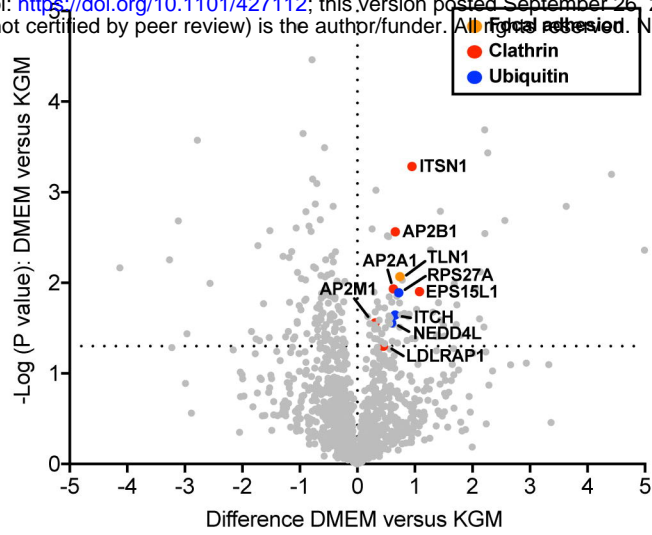
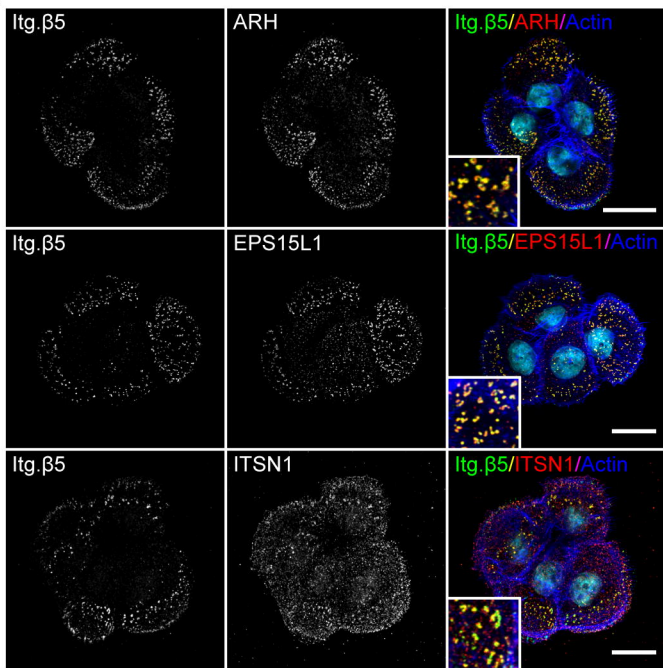
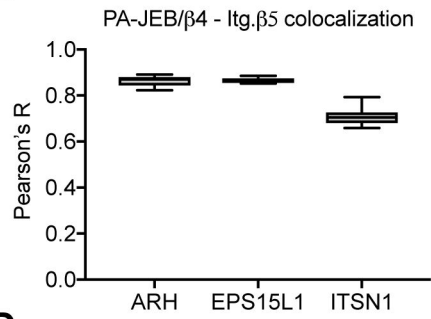
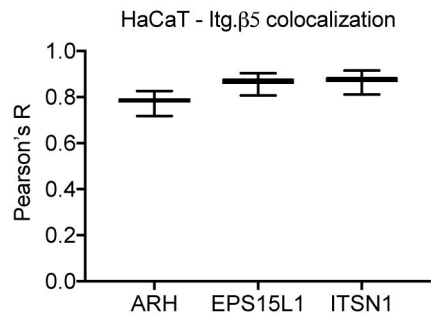
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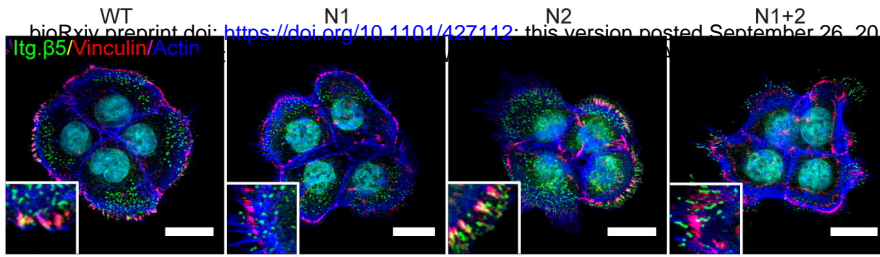
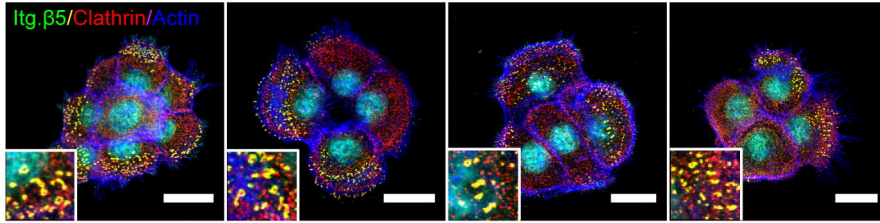
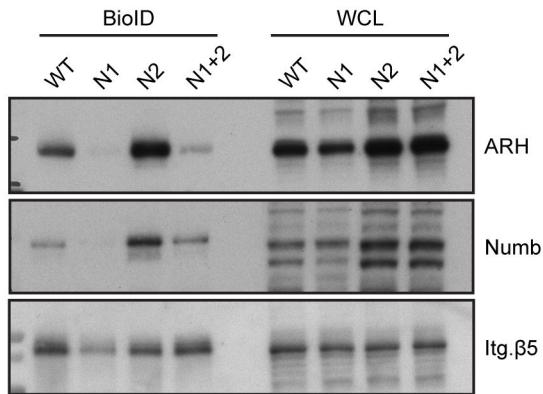
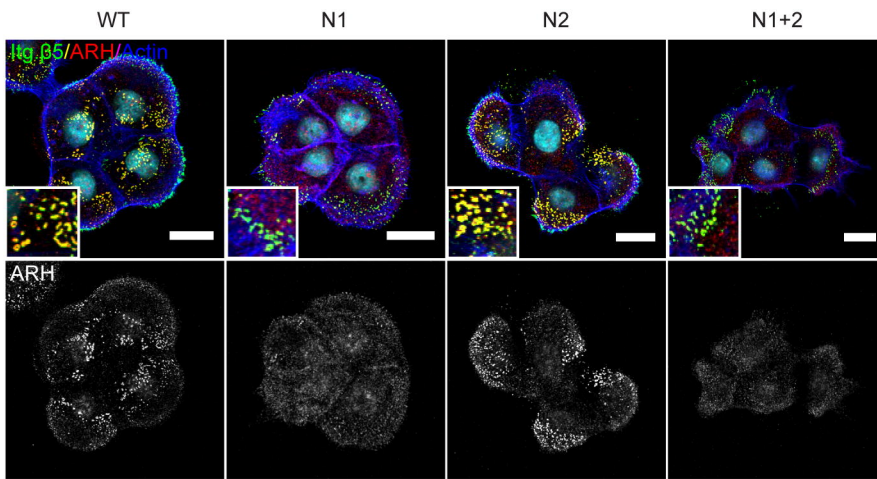
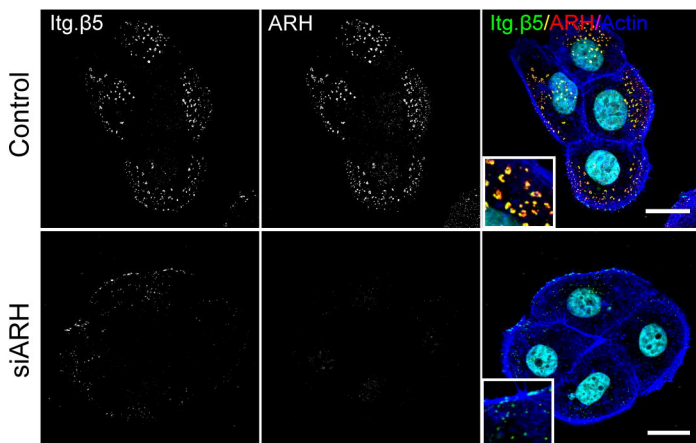
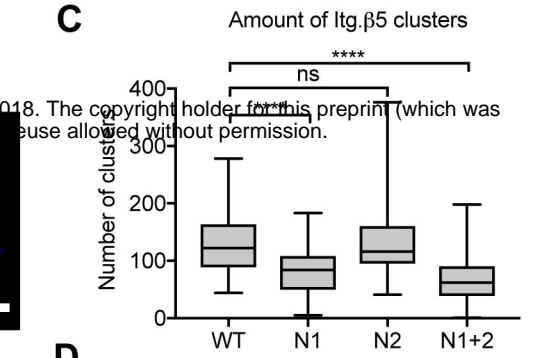
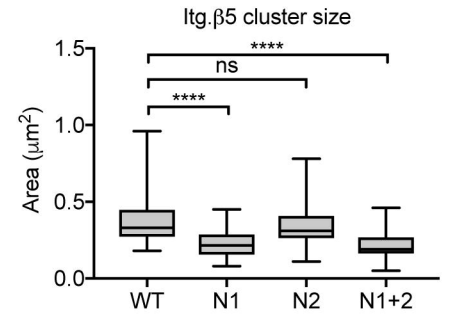
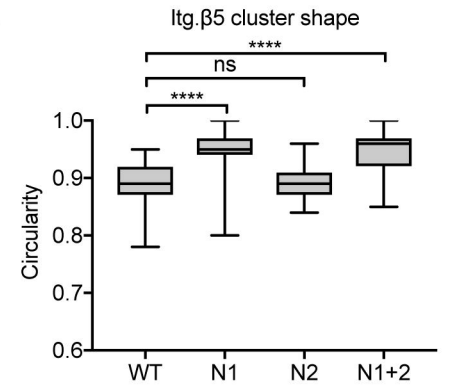
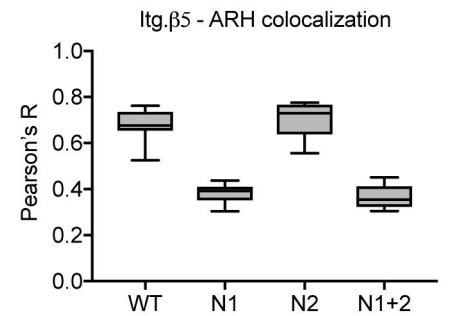
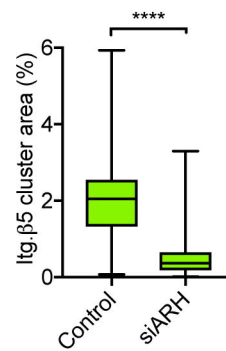
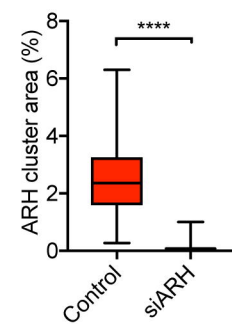
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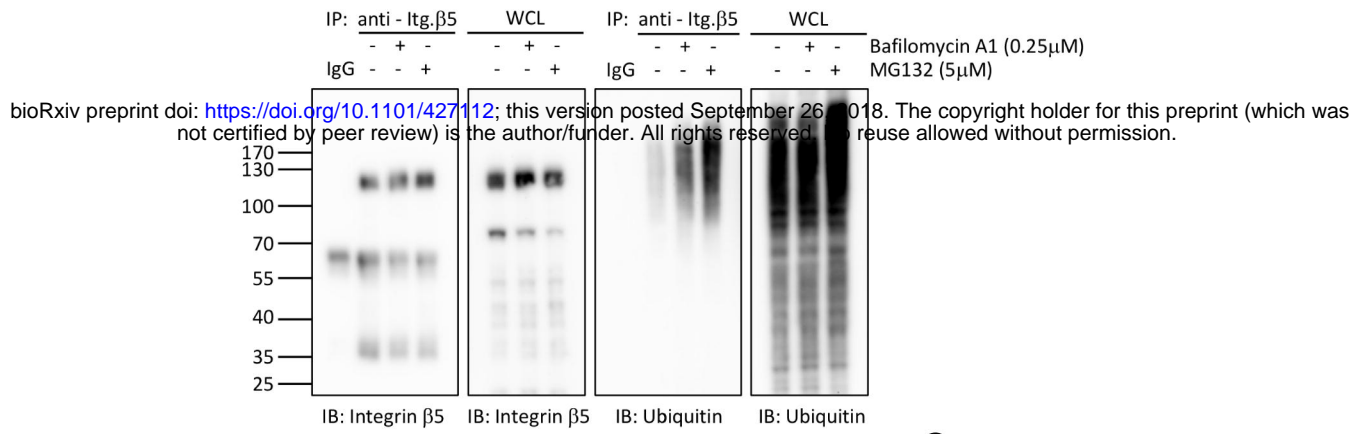
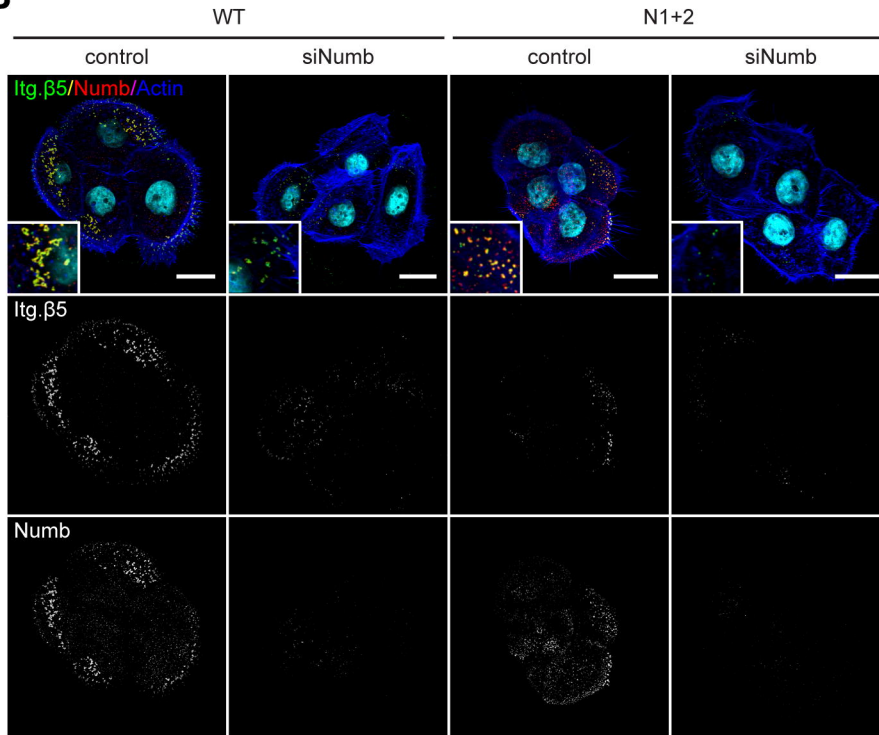
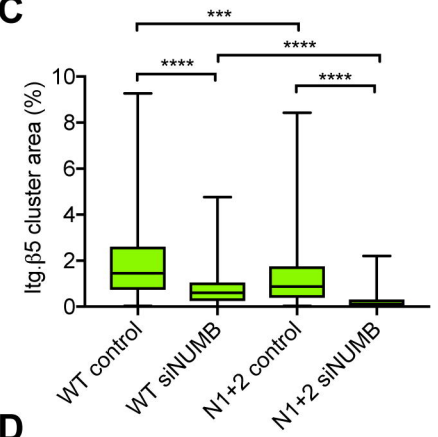
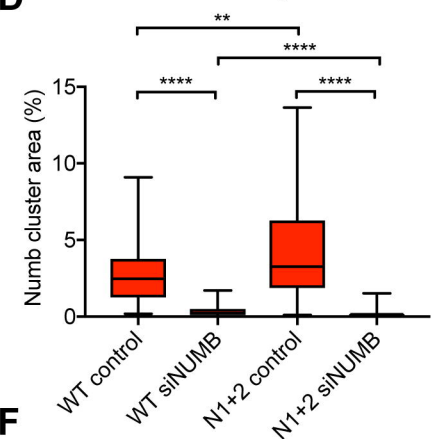
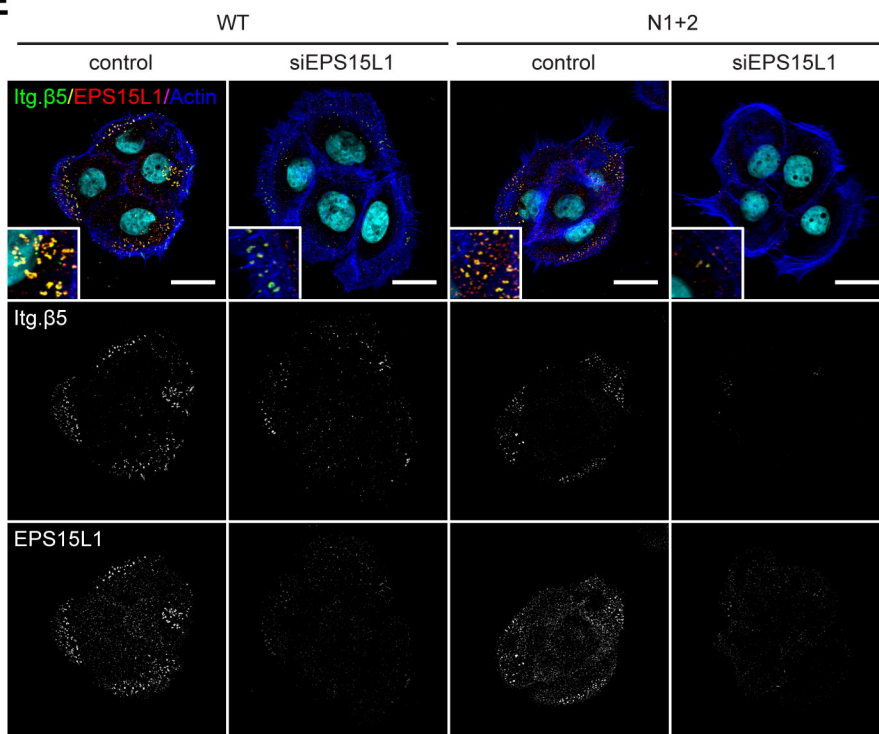
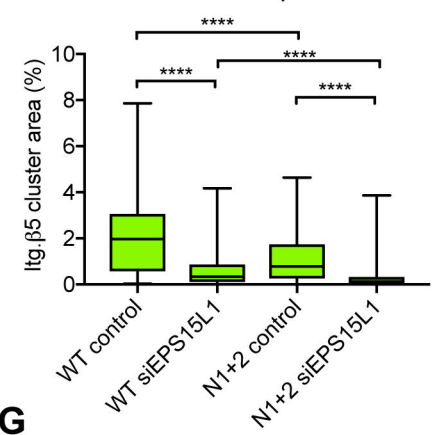
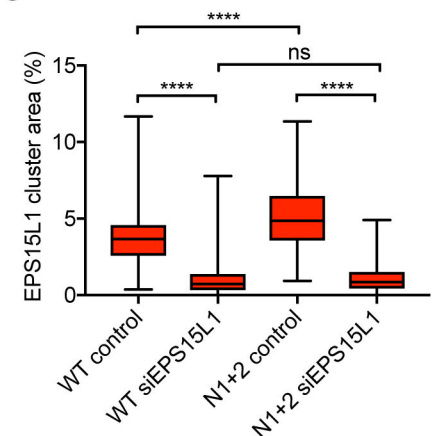
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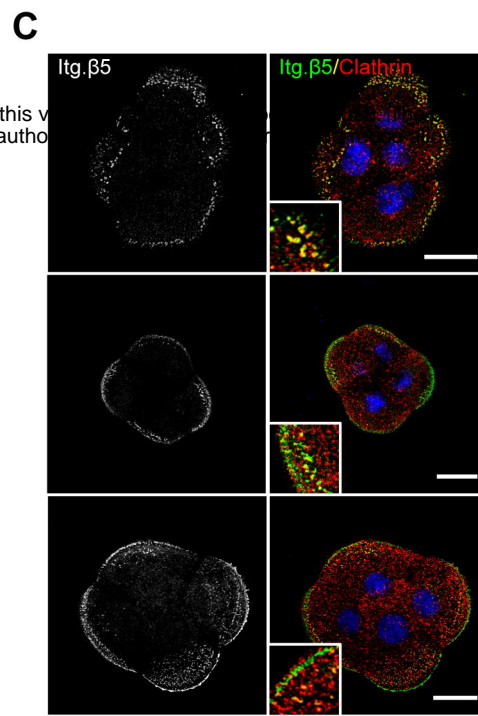
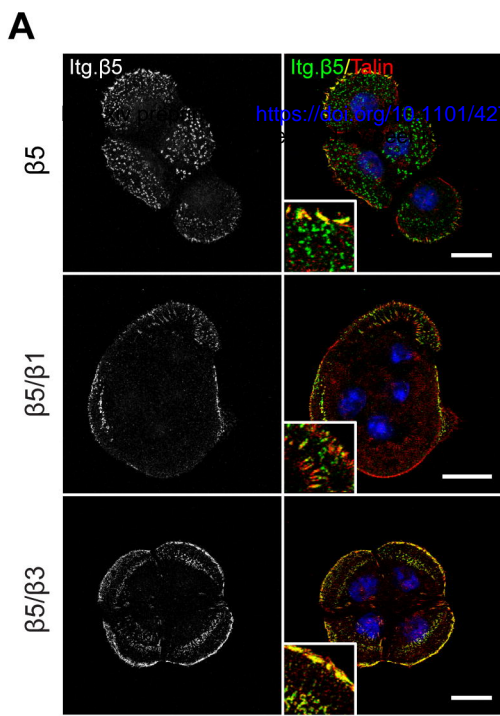
AIntegrin $\beta 5$ proximity interactors

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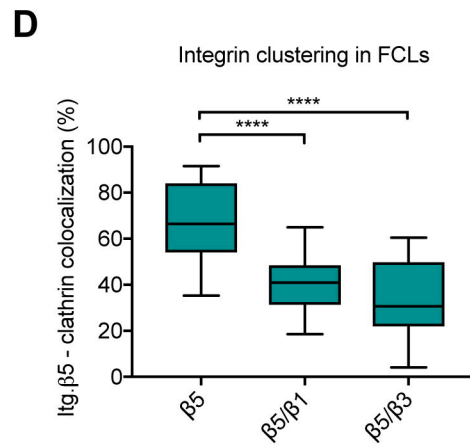
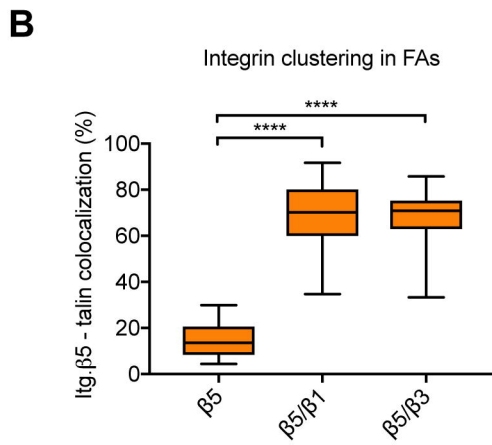
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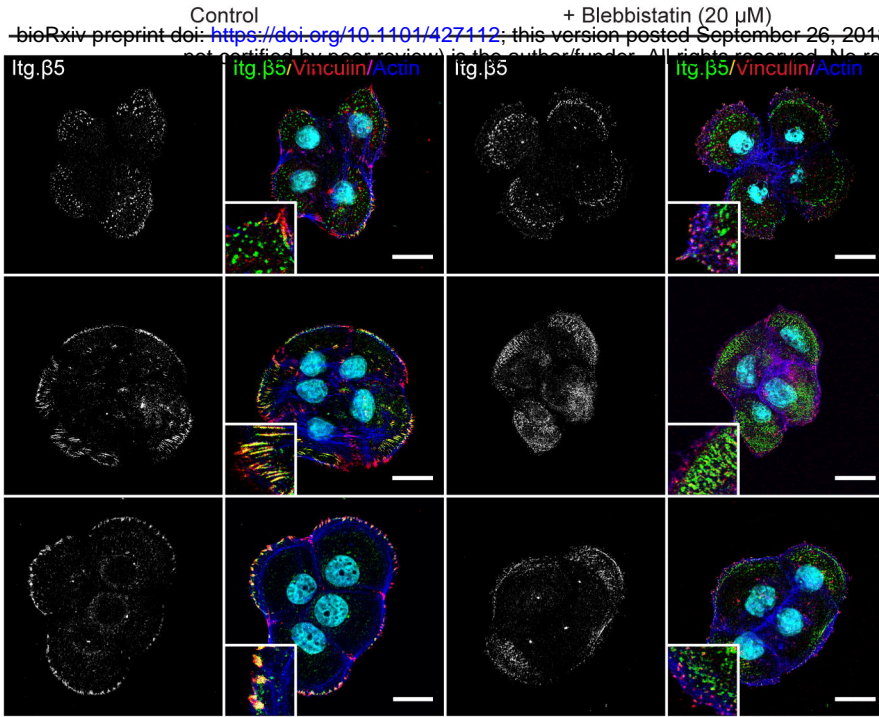
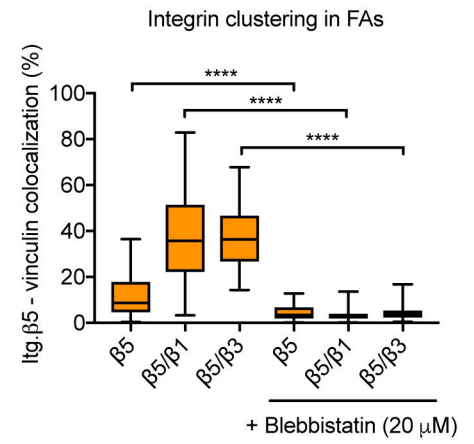
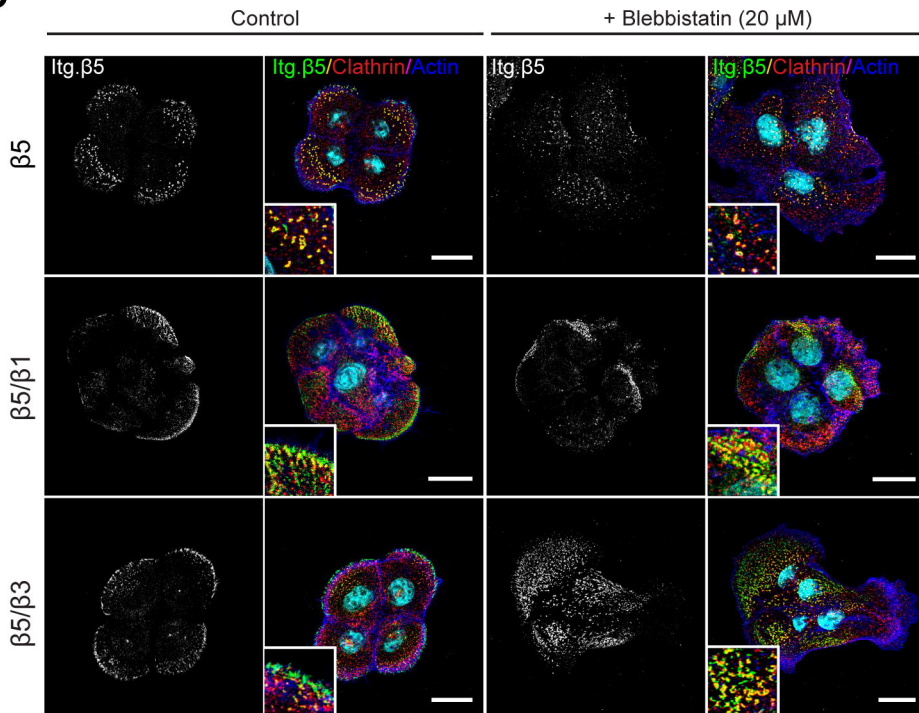
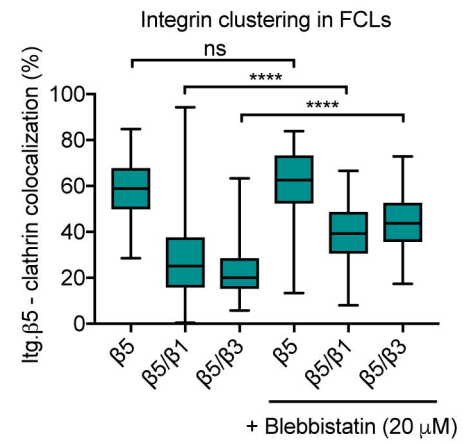
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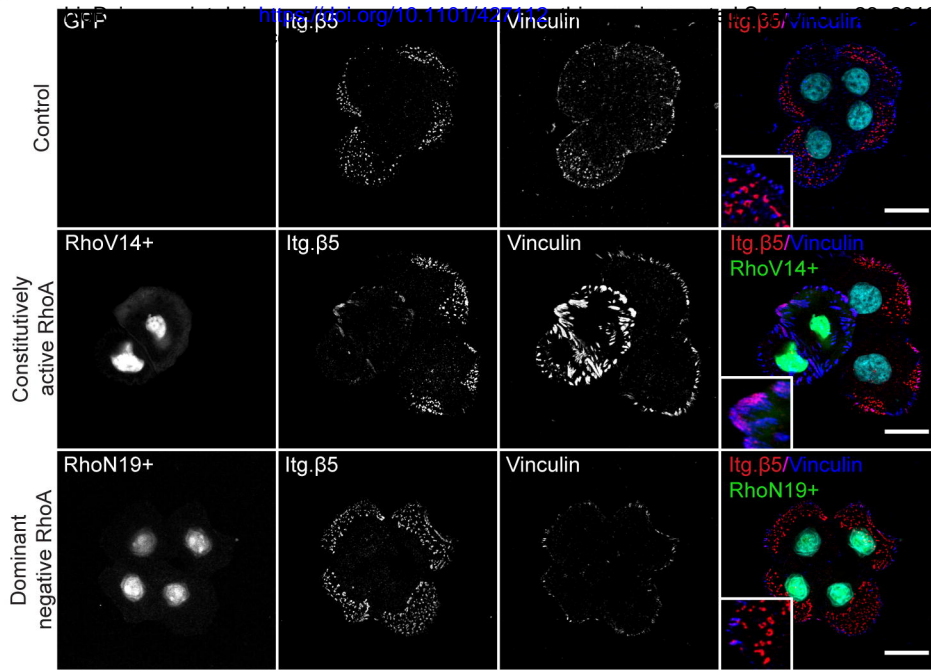
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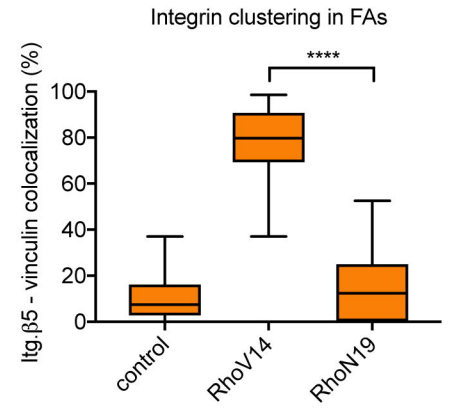
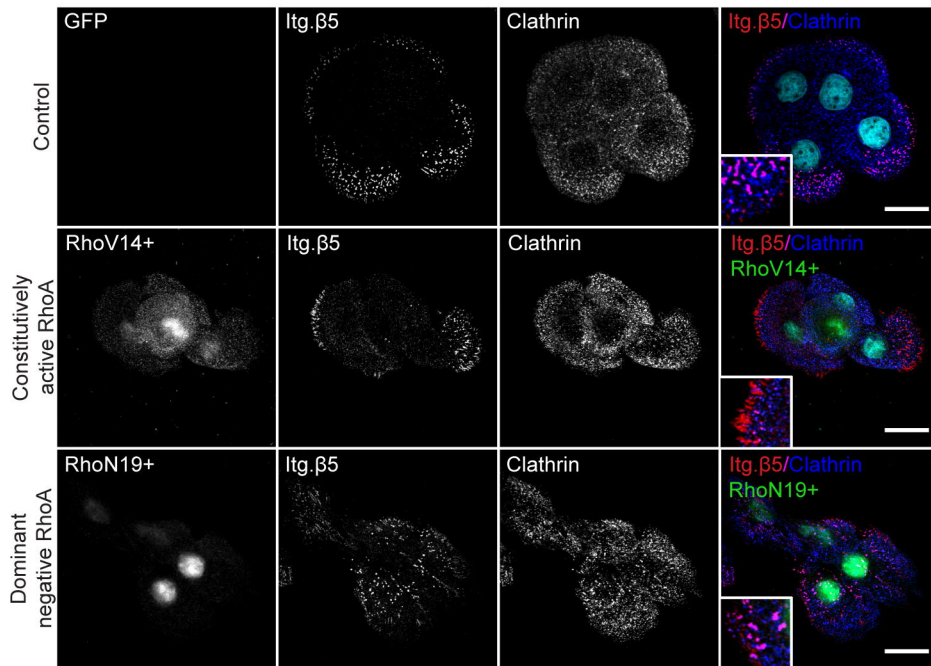
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**C****D**