

1 **Focal adhesion-generated cues in extracellular matrix regulate**
2 **cell migration by local induction of clathrin-coated plaques**

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17

18 **Abstract**

19 Clathrin is a unique scaffold protein, which forms polyhedral lattices with flat and curved
20 morphology. The function of curved clathrin-coated pits in forming endocytic structures is
21 well studied. On the contrary, the role of large flat clathrin arrays, called clathrin-coated
22 plaques, remains ambiguous. Previous studies suggested an involvement of plaques in cell
23 adhesion. However, the molecular origin leading to their formation and their precise
24 functions remain to be determined. Here, we study the origin and function of clathrin-
25 coated plaques during cell migration. We revealed that plaque formation is intimately
26 linked to extracellular matrix (ECM) modification by focal adhesions (FAs). We show that in
27 migrating cells, FAs digest the ECM creating extracellular topographical cues that dictate the
28 future location of clathrin-coated plaques. We identify Eps15 and Eps15R as key regulators
29 for the formation of clathrin-coated plaques at locally remodelled ECM sites. Using a genetic
30 silencing approach to abrogate plaque formation and 3D-micropatterns to spatially control
31 the location of clathrin-coated plaques, we could directly correlate cell migration
32 directionality with the formation of clathrin-coated plaques and their ability to recognize
33 extracellular topographical cues. We here define the molecular mechanism regulating the
34 functional interplay between FAs and plaques and propose that clathrin-coated plaques act
35 as regulators of cell migration promoting contact guidance-mediated collective migration in
36 a cell-to-cell contact independent manner.

37 .

38 Introduction

39 Each cell is surrounded by a plasma membrane composed of lipids that separates the
40 intracellular milieu from the extracellular space. To sense and interact with the
41 extracellular environment, cells exploit both integral and peripheral membrane proteins
42 and establish defined domains at the plasma membrane to mediate processes like
43 signalling, endocytosis and mechanotransduction.

44 The main component of the environment surrounding cells is the extracellular matrix
45 (ECM), a complex network of secreted macromolecules such as collagen, laminin and
46 fibronectin^{1,2}. Specific binding sites on these proteins serve as ligands for cellular
47 receptors³⁻⁵. The most important of adhesion receptors are integrins, which, together with
48 adaptors, scaffold and signalling proteins, assemble into the multi-layered adhesive unit
49 referred to as focal adhesions (FAs)^{3,6,7}. FAs mediate adhesion and signalling between the
50 inside and the outside of the cell^{7,8}. They are also viewed as mechanotransducing units
51 coupling biophysical properties of the ECM to intracellular processes⁸. Indeed, not only the
52 chemical but also the topographical and mechanical properties of the ECM influence cellular
53 functions such as guiding cell migration^{9,10} and maintaining stem cell niches^{11,12}. The ECM is
54 not a static scaffold; it is a highly dynamic mesh constantly renewed and remodelled by
55 cells, which in return react to the new properties of their surrounding matrix^{13,14}. ECM
56 remodelling is achieved both through cellular-mediated physical forces¹⁵ as well as through
57 enzymatic digestions by specific enzymes like the matrix metalloproteinases (MMPs)¹³.

58 Beside FA, other plasma membrane-associated supramolecular complexes have been
59 described to have mechanotransduction properties e.g. podosomes¹⁶ and cadherin-
60 mediated cell-cell junctions¹⁷. Recently, plasma membrane-associated clathrin structures
61 have been shown to assemble at specific plasma membrane sites in response to unique
62 topographical profiles and mechanical properties of the extracellular environment¹⁸⁻²¹.
63 Together with numerous adaptors and accessory proteins, clathrin molecules assemble at
64 the plasma membrane to form a highly dynamic array^{22,23}. During clathrin-mediated
65 endocytosis (CME), small transient clathrin coats can form initially as curved or flat arrays
66 which will rearrange to form clathrin-coated pits (CCPs) with a typical diameter of 100-150
67 nm²⁴⁻²⁶. Distinct from these small endocytic structures, flat long-lived larger clathrin coats,
68 known as clathrin-coated plaques, are frequently observed²⁷. These clathrin-coated plaques

69 appear to have pleiotropic functions. They can facilitate endocytosis and signalling *via*
70 clustering of plasma membrane receptors and nucleating endocytic events²⁸⁻³⁰. The putative
71 role of larger clathrin arrays in cell adhesion and migration has long been discussed^{31,32}. It
72 has recently been readdressed with the observation that specialized clathrin arrays named
73 tubular clathrin/AP2 lattices (TCALs) are responsible for binding collagen fibres in a 3D-
74 environment¹⁹ and that association of clathrin-coated plaques with the ECM is integrin
75 dependent^{27,33,34}.

76 To date, the mechanisms that lead to clathrin-coated plaque formation and stabilization at
77 the plasma membrane and the cellular and extracellular determinants that dictate whether
78 a clathrin-coated plaque displays an endocytic or a non-endocytic function are still unclear.
79 Similarly, the molecular and/or physical determinants that drive clathrin-coated plaque
80 formation remain poorly understood. There is evidence that some clathrin-coated plaques
81 are resulting from frustrated endocytic events³³. However, it remains unclear whether cells
82 can generate specific cellular and/or extracellular signals that lead to a local induction of
83 clathrin-coated plaques which in turn will provide the cell with a specific function.

84 In this work, we demonstrate that disassembling FAs, at the leading edge of the cell, are
85 replaced by clathrin-coated plaques during cell migration, in an integrin-dependent process,
86 that we term “switch from FAs to clathrin-coated plaques”. We could trace the signals
87 leading to this switch back to the digestion of the ECM and the generation of extracellular
88 topographical cues by FAs. We identified Eps15 and Eps15R as important players in the
89 formation of clathrin-coated plaques. Migration assays of wild type and Eps15/R depleted
90 cells, allowed us to correlate directionality of cell migration with the formation of clathrin-
91 coated plaques at topographical cues. This work reveals a novel function of clathrin-coated
92 plaques and demonstrates that flat clathrin arrays act as plasma membrane-associated
93 supramolecular complexes which sense extracellular topographical cues to influence cell
94 behaviour.

95

96 Results

97 *Dynamics and ultrastructural characterization of clathrin-coated plaques*

98 The presence of clathrin-coated plaques at the plasma membrane has been long known^{31,35},
99 but their function is still poorly understood. It was shown that their presence was cell type
100 specific and that they were limited to the ventral plasma membrane³⁶. This side-specific
101 localization and the fact that clathrin-coated plaques are in close contact with the
102 substrate^{31,32} as well as associated with the ECM receptors integrins^{27,32-34} strongly suggest
103 a function in cell adhesion. Aiming at characterizing this function, we exploited the human
104 glioblastoma U373 cell line previously reported to display clathrin-coated plaques^{36,37}. Live
105 fluorescence microscopy of U373 stably expressing the sigma subunit of the clathrin
106 adaptor AP2 fused to eGFP (AP2-eGFP) showed clathrin structures with high fluorescence
107 intensity and a long lifetime as well as non-terminated events (Supplementary Fig. 1a). In
108 contrast, transient endocytic clathrin structures had both a lower fluorescent intensity and
109 lifetime. Transmission electron microscopy (TEM) of metal replicas confirmed the presence
110 of flat clathrin-coated plaques with or without budding CCPs at the rim, displaying a surface
111 area of up to 100,000 nm². In comparison, the endocytic invaginated spherical CCPs display
112 an average projected area of 15,000 nm² (Supplementary Fig. 1b-e). Correlative light and
113 electron microscopy (CLEM) of these U373 cells expressing AP2-eGFP confirmed that the
114 high fluorescence long-lived clathrin-coated structures (Supplementary Fig. 1a) were
115 indeed clathrin-coated plaques (Supplementary Fig. 1f-g). As such, in this work, we will
116 define clathrin-coated plaques as long-lived clathrin coats using live-cell fluorescence
117 microscopy. We could confirm that these structures are exclusively found at the ventral side
118 of cells (Supplementary Fig. 2a-b) and furthermore, show that this side specific localization
119 of clathrin-coated plaques correlates with the interaction of the cell with the extracellular
120 environment. Cells grown on adhesive micropatterned substrates display both clathrin-
121 coated plaques and CCPs on the adhesive parts whereas only CCPs are observed on the non-
122 adhesive sections of the substrate (Supplementary Fig. 2c-e). In summary, in agreement
123 with previous studies^{36,37}, our results showed that U373 cells form flat clathrin-coated
124 plaques with a long lifetime exclusively at attached plasma membrane parts.

125 *Clathrin-coated plaques are formed after FA disassembly*

126 It has been proposed that the clathrin machinery is involved in recycling of FAs³⁸⁻⁴⁰. To
127 address whether clathrin-coated plaques might be involved in this process, FAs were
128 labelled in U373 cells by expressing the markers of FAs, zyxin, vinculin, focal adhesion
129 kinase (FAK) or paxillin fused to the fluorescent protein mCherry. Mature FAs and smaller
130 focal complexes did not colocalize with clathrin structures of any size, suggesting that
131 neither CCPs nor clathrin-coated plaques have a physical correlation with FAs (Fig. 1a). To
132 rule out that the lack of colocalization between clathrin-coated plaques and FAs was due to
133 the transient nature of their interaction during the disassembly process, we performed live-
134 cell fluorescence microscopy of migrating U373 cells expressing AP2-eGFP as a marker for
135 clathrin structures and mCherry-zyxin, as a marker for FAs. Interestingly, we observed that
136 during cell migration, clathrin-coated plaques frequently formed at the position where FAs
137 were disassembled (Fig. 1b and e, Supplementary Movie 1). In other words, following
138 disassembly of FAs, clathrin structures are actively assembled at the same locations (Fig.
139 1b). During this switch from FAs to clathrin-coated plaques, the clathrin structures and the
140 FA did not colocalize and were mutually exclusive overtime at a given position (Fig. 1c-d).
141 The formation of clathrin-coated plaques was a process specific to the locations of
142 disassembled of FAs whereas the appearance of clathrin-coated plaques at other locations
143 was a rare event (Fig. 1e). This process was not specific to zyxin as similar mutual
144 exclusions were observed using the FA markers vinculin, FAK, and paxillin (Supplementary
145 Fig. 3). Importantly, this phenomenon was not cell type specific as we could observe a
146 similar switch when using other cell types also known to display clathrin-coated plaques
147 (HT1080 and U2OS, Supplementary Fig. 4). These results report a so far undescribed switch
148 from FAs to clathrin-coated plaques and suggest that clathrin-coated plaques have a
149 molecular origin locally imprinted by FAs.

150 ***Clathrin-coated plaques are stabilized by integrins***

151 In the context of integrin internalization during FA recycling, the above-described switch
152 from FAs to clathrin-coated plaques could generate an endocytic hot spot for the
153 internalization of integrins during FA disassembly. Most importantly, very recent reports
154 have demonstrated the importance of integrins in stabilizing clathrin-coated plaques^{33,34}. By
155 performing immunostaining of endogenous integrins, we could show that integrins $\alpha\beta5$
156 and $\beta1$ were enriched at clathrin-coated plaques in U373 cells (Fig. 2a-b and Supplementary
157 Fig. 5a, respectively). This suggests that integrins are left behind at the position where FAs

158 are disassembled and could recruit or stabilize the clathrin machinery to locally promote
159 the formation of long-lived clathrin-coated plaques. To challenge the possible function of
160 integrins in stabilizing clathrin-coated plaques that are formed after FA disassembly, we
161 used the cyclic RGD peptide Cilengitide, which acts as an integrin antagonist and inhibits
162 integrin binding to their ECM ligands^{41,42}. We observed that Cilengitide treatment induced
163 the rapid dissociation of clathrin-coated plaques (Fig. 2c-e, Supplementary Movie 2). During
164 Cilengitide treatment, clathrin-coated plaques disassembled by the formation of several
165 smaller transient structures resembling endocytic hot spots (Fig. 2d and Supplementary
166 Movie 2). Together these results indicate that clathrin-coated plaques replace
167 disassembling FAs and are recruited and/or stabilized by integrins.

168 ***Extracellular signals drive the formation of clathrin-coated plaques***

169 To address whether a specific extracellular signal created by FAs was left after FA
170 disassembly and was in turn responsible for the integrin-dependent recruitment of clathrin-
171 coated plaques, we monitored the switch from FAs to clathrin-coated plaques in migrating
172 cells. We found that the location of clathrin-coated plaques is somehow defined by the
173 extracellular environment. Indeed, we observed that clathrin-coated plaques often appear
174 at the same locations when the same migrating cell revisits a position twice (Fig. 3a, first
175 cell at t=0 and first cell at t=4.4h). More strikingly, if a different cell migrates over these
176 plaque-forming areas, clathrin-coated plaques are also observed in the new cell at the same
177 positions (Fig. 3a, merge between second cell t=7.7 h and first cell t=0 and t=4.4 h). To
178 unambiguously demonstrate that an extracellular signal was responsible for clathrin-coated
179 plaque formation at the same location, we correlated the location of clathrin-coated plaques
180 on gridded coverslips when cells were seeded sequentially in multiple rounds (Fig. 3b). In
181 this experiment, we seeded first U373 expressing AP2-eGFP on gridded coverslips and
182 imaged clathrin dynamics to identify the locations of clathrin-coated plaques. Following
183 EDTA-mediated removal of the first cells, a second seeding of cells was performed on the
184 same grid and both clathrin dynamics and plaque locations were again recorded. Results
185 show that clathrin-coated plaques formed at the same position in the first and the second
186 round of cell seeding (Fig 3c-d). These results demonstrate that specific extracellular signals
187 are responsible for the local induction of clathrin-coated plaques. Trypsin and KOH-based
188 cleaning of the gridded coverslips between both rounds of cell seeding resulted in a strong

189 reduction of the number of clathrin-coated plaques forming at the same position (Fig. 3d),
190 suggesting that these extracellular signals are of proteinaceous nature

191 ***Remodelled ECM represents an extracellular cue that promotes clathrin-coated plaque***
192 ***formation***

193 FAs are supramolecular complexes not only involved in ECM binding, but also responsible
194 for ECM remodelling^{13,15}. One of the important remodelling processes driven by FAs is the
195 enzymatic digestion of the ECM⁴³. To directly correlate digestion of ECM and recruitment of
196 clathrin-coated plaques, we employed fluorescently labelled gelatin as a substitute for ECM
197 and monitored live the digestion of the extracellular environment together with the
198 dynamics of clathrin-coated structures. During cell migration, we could observe that FAs
199 located at the leading edge of the cell actively digested the fluorescent gelatin, as can be
200 seen by the appearance of dark non-fluorescent areas (Supplementary movie 3).
201 Interestingly, by monitoring clathrin dynamics on fluorescently labelled gelatin coatings, we
202 unravelled that the locations of long-lived clathrin-coated plaques correlated with the
203 locations of the digested fluorescent gelatin (Fig. 4a-d and Supplementary movie 4, black
204 area). To temporally correlate gelatin digestions by FAs and clathrin-coated plaque
205 formation, U373 cells expressing AP2-eGFP and mCherry-zyxin were seeded on fluorescent
206 gelatin and the dynamics of the switch from FAs to clathrin-coated plaques was observed
207 using live fluorescence confocal microscopy. Analyses revealed that during cell migration,
208 FAs dynamically formed and locally digested the gelatin coat, which was followed by FA
209 disassembly and finally clathrin-coated plaque formation (Fig. 4e-g, Supplementary Movie
210 3). Importantly, in line with the above results showing that two different migrating cells
211 form their clathrin-coated plaques at similar locations (Fig. 3a), our fluorescent gelatin
212 assay reveals that the extracellular cues that lead to clathrin-coated plaques formation
213 correspond to spots of digested ECM (Fig. 4h, Supplementary movie 5). Together, these
214 results strongly suggest a model where FAs digest and modify the ECM creating
215 extracellular domains responsible for the integrin-dependent recruitment of clathrin-coated
216 plaques.

217 ***Pharmacologically induced FA disassembly reveals that digestion of the ECM directly***
218 ***correlates with clathrin-coated plaque recruitment***

219 To study the switch from FAs to clathrin-coated plaques and its link to FA disassembly
220 and/or creation of extracellular cues, we exploited a pharmacological approach to induce
221 FA disassembly. We used the ROCK inhibitor Y-27632 previously shown to induce FA
222 disassembly^{44,45}. Treatment of U373 cells with Y-27632 induced the disassembly of actin
223 stress fibres and FAs within 20 minutes (Supplementary Fig. 6a-c). By monitoring the
224 dynamics of both the clathrin machinery and of FAs during drug treatment, we could
225 demonstrate that FAs are disassembled and are efficiently replaced by clathrin-coated
226 plaques during the drug-induced FA disassembly (Fig 5a-b, Supplementary Movie 6). This
227 replacement was similar to the process seen during cell migration as FA and clathrin-coated
228 structures were located at the same site but temporally mutually exclusive (Fig. 5b,
229 Supplementary Fig. 6g). Lifetime analyses of the newly formed clathrin-coated structures
230 revealed that they were clathrin-coated plaques (Fig. 5c). Quantification of the efficiency of
231 the drug-induced switch from FAs to clathrin-coated plaques revealed that approximately
232 40% of all FAs were replaced by clathrin-coated plaques (Fig. 5d). Similar to Y-27632-
233 induced FA disassembly, treatment of cells with the myosin-II inhibitor Blebbistatin also
234 induced FAs to switch to clathrin-coated plaques (Supplementary Fig. 6d-f and h,
235 Supplementary Movie 7). By performing immunostaining of integrins in cells treated with
236 Y-27632, we observed that integrins were enriched at clathrin-coated plaques located at the
237 cell periphery and the overall colocalization of integrins with clathrin structures increased
238 (Fig. 5e-f and Supplementary Fig. 5b). These structures corresponded to the FAs which
239 disassembled during drug treatment, leaving behind integrins which in turn promoted the
240 recruitment of the clathrin coat. By performing the drug-induced FA disassembly assay on
241 labelled gelatin coatings, we could show that clathrin-coated plaques were recruited to
242 former FA sites, which actively digested the ECM (Fig. 5g). Quantification of the frequency of
243 drug-induced switch from FAs to clathrin-coated plaques revealed that most of the FAs
244 which at the time of Y-27632 treatment had actively digested the fluorescent gelatin were
245 replaced by clathrin-coated plaques, in comparison to FAs without visible gelatin digestion
246 (Fig. 5h). These results show that in this experimental set-up, gelatin digestion is an efficient
247 signal for clathrin-coated plaque recruitment after FA disassembly. To further correlate
248 recruitment of clathrin-coated plaques with ECM digestion, we knocked down or
249 overexpressed the matrix metalloproteinase MMP14 in U373 cells (Supplementary Fig. 7).
250 MMP14 is a transmembrane metalloproteinase that plays a key role in digesting the ECM
251 and activating other metalloproteinase and thereby regulates FAs turnover and

252 subsequently cell migration^{43,46-50}. Upon MMP14 knock-down, we observed that digestion of
253 the fluorescent gelatin was significantly reduced (Fig. 5i-j, Supplementary Fig. 7) and this
254 was associated with a significant reduction of the number of FAs switching to clathrin-
255 coated plaques (Fig. 5k). Interestingly, overexpression of MMP14, which led to global
256 digestion of gelatin coats instead of locally defined digestions as seen for WT cells (Fig. 5i-j,
257 Supplementary Fig. 7), is also associated with a strong reduction of the drug-induced switch
258 (Fig. 5k). Altogether, these results demonstrate that the switch from FAs to clathrin-coated
259 plaques is tightly associated with the FA-mediated digestion of the ECM.

260 ***Extracellular topographical cues induce the formation of clathrin-coated plaques***

261 Cellular processes, e.g. protein secretion, fibrillogenesis or enzymatic digestion, which occur
262 at cell/extracellular environment contact sites, can rearrange the ultrastructural
263 organization of the ECM^{1,13}. In this way not only the protein content of the ECM can be
264 changed, but also its physical properties^{9,10,51,52}. Therefore, the above observed correlation
265 between the location of clathrin-coated plaques and the site of gelatin digestion could be the
266 result of a change in the topographical organization of the ECM which in turn will induce
267 local plaque formation. To directly investigate whether local topographical cues can induce
268 clathrin-coated plaque formation, we generated 3D-micropatterns suitable for microscopy
269 made of an optically clear glue (Norland Optical Adhesive (NOA)) using a soft lithography
270 approach. These 3D-micropatterns were made from optical gratings and displayed peaks of
271 200 nm height with a periodicity of 2 μm (Supplementary Fig. 8a-c). Analysis of the 3D-
272 micropatterns using atomic force microscopy (AFM) revealed that they precisely
273 reproduced the peak height and periodicity of the optical gratings (Supplementary Fig. 8b-
274 f). U373 cells growing on such 3D-micropatterns align both their FAs and actin stress fibres
275 parallel to the pattern as previously reported with different cell lines (data not shown
276 and⁵³⁻⁵⁷). Interestingly, most clathrin structures were also found aligned parallel to the
277 grating with the same periodicity of 2 μm (Fig. 6a-c). Strikingly, analyses of the clathrin
278 structure dynamics of cells seeded on such 3D-micropatterns revealed that the long-lived
279 clathrin-coated plaques only formed on top of the gratings whereas transient CCPs formed
280 independently of the topographical features (Fig. 6d and e). This local induction of clathrin-
281 coated plaque formation by substrates displaying 3D topographical micropatterns again
282 highlights the relation between clathrin-coated plaques and extracellular physical cues.

283 ***Clathrin-coated plaques are promoted by specific CME initiator proteins***

284 Formation of clathrin-coated structures is achieved through multiple adaptor and accessory
285 proteins, which are regulated in space and time to precisely coordinate coat assembly at the
286 ultrastructural level^{22,23}. Most studies that have looked at the functions of different proteins
287 in clathrin coat formation have focused on their function during endocytosis. On the
288 contrary, very little is known about proteins specifically regulating clathrin-coated plaque
289 formation and function²⁷. Previously, it was suggested that proteins of the clathrin pioneer
290 module that initiate CME and lead to the recruitment of clathrin to the plasma membrane,
291 e.g. FCH01/2 and Eps15/R, stabilize the clathrin lattice early during coat formation and
292 help to establish the hexagonal organization of flat clathrin structures^{34,58-60}. Therefore, we
293 investigated if early clathrin-related proteins of the pioneer module are important for
294 clathrin-coated plaque formation. We found that depletion of Eps15 and Eps15R in U373
295 through shRNA-mediated knock-down (shEPS15/R) resulted in a dramatic reduction in the
296 number of long-lived clathrin-coated plaques (Fig. 7a-b, Supplementary Fig. 9a). In line with
297 their function as a pioneer module, knock-down of Eps15/R was also associated with a
298 reduction in the number of CCP initiation events (Fig. 7a). Importantly, the switch from FAs
299 to clathrin-coated plaques was severely impaired in cell knocked down for Eps15/R, both
300 during cell migration (data not shown) and during Y-27632-induced FA disassembly (Fig.
301 7c-e, Supplementary Fig. 9b). While drug treatment was efficiently inducing disassembly of
302 FAs, only transient CCPs formed occasionally at the sites of disassembled FAs (Fig. 7d-e). Of
303 note, individual knock-down of Eps15 and Eps15R, also resulted in reduction of switch from
304 FAs to clathrin-coated plaques, but to a lesser extent compared to the dual depletion of both
305 Eps15 and Eps15R (Supplementary Fig. 9b). These results show that, in addition to their
306 role in the initiation of CCP formation as part of their pioneer module function⁶⁰⁻⁶², Eps15/R
307 also play a role in regulating plaque formation and FA switch to clathrin-coated plaques.

308 ***Cell migration is influenced by substrate topography through clathrin-coated plaque*** 309 ***formation***

310 Topographical cues of the ECM have been shown to influence the direction of cell
311 migration^{10,57,63}. This phenotype called contact guidance plays an important role during
312 development⁶⁴⁻⁶⁶ as a well as tumour metastasis^{67,68}. Our findings that clathrin-coated
313 plaques can form after FA disassembly and the fact that this recruitment appears to be

314 linked to the creation of extracellular cues in the ECM, led us to hypothesize that clathrin-
315 coated plaques might participate in contact guidance-mediated directionality of cell
316 migration by recognizing the extracellular cues generated by FAs in the ECM. To study the
317 influence of clathrin-coated plaques on the directionality of cell migration, we followed
318 U373 cells on 3D-micropatterns as well as on flat substrates made of the same material and
319 we characterized the contact guidance-mediated directionality of cell migration. As
320 described above, WT U373 displayed clathrin-coated plaques aligned with the 3D-
321 micropatterns (Fig. 8a) and we found that migration of these cells was directed parallel to
322 the grating following the grid lines (Fig. 8c, e (right panel) and g). In comparison, WT U373
323 cells display random cell migration on flat surfaces made of the same optically clear glue
324 (Fig. 8e and g). As expected, Eps15/R knock-down cells seeded on the 3D-micropattern
325 failed to display clathrin-coated plaques (Fig. 8b). Importantly, cell migration analysis of
326 Eps15/R knock-down cells on 3D-micropatterns revealed that cells depleted of clathrin-
327 coated plaques do not show directional migration along the grating orientation (Fig. 8b, d, f
328 and g). Altogether, these results indicate that clathrin-coated plaques participate in contact
329 guidance-mediated directionality of cell migration.

330 **Discussion**

331 Here, we describe how modifications of the ECM can lead to recruitment of clathrin-coated
332 plaques and correlate their spatio-temporal formation with the regulation of cell migration.
333 We demonstrate that clathrin-coated plaque formation is associated with the modification
334 of the extracellular environment by FAs. Precisely, by combining live monitoring of ECM
335 digestion/modifications and dynamics of clathrin-coated plaque formation, we show that,
336 during cell migration, metalloproteinases located in FAs, digest the ECM creating specific
337 extracellular cues which in turn drive the formation of flat clathrin arrays. Localization of
338 clathrin-coated plaques and FAs at these specific sites reveal that these structures are
339 temporally mutually exclusive. We name this process “FA switch to clathrin-coated
340 plaques”. By exploiting 3D-micropatterns, we show that these extracellular signals
341 represent topographical cues, which are recognized by the clathrin machinery inducing the
342 local formation of plaques. We identify Eps15 and Eps15R as key regulators of plaque
343 formation. Finally, we show that by recognizing the extracellular topographical cues,
344 clathrin-coated plaques regulate the directionality during cell migration.

345 The presence of clathrin-coated plaques at the ventral plasma membrane has been long
346 known³⁶. Recently, although their origin and function remained enigmatic, several studies
347 have reported that the presence of clathrin-coated plaques at the plasma membrane was
348 dependent on integrins^{33,34}. These findings described that not only are integrins enriched at
349 clathrin-coated plaques, but also describes that they are mandatory to stabilize flat clathrin
350 arrays^{27,33,34}. This is consistent with our findings that interference with integrin binding to
351 the ECM proteins impacts clathrin-coated plaque stability and induces their disassembly.
352 The cyclic RGD peptide Cilengitide was recently shown to be a competitive antagonist which
353 exerts its activity only on integrins which are not bound to ECM RGD motifs⁶⁹. As such the
354 observation that Cilengitide can induce plaque disassembly (this work and³³) suggests that
355 although plaques are stable structures at the plasma membrane, their integrin-dependent
356 binding to the ECM is highly dynamic. Importantly, our work also suggests that the presence
357 of integrin clusters is necessary, but not sufficient for clathrin-coated plaques. A great
358 number of integrin clusters can be found at the plasma membrane, but not associated with
359 the formation of clathrin-coated plaques (Fig. 2, Supplementary Fig. 5). Recent work by
360 Baschieri et al. highlighted the connection of clathrin-coated plaque formation and physical
361 properties of the substrate suggesting a mechanosensing function of plaques. They showed
362 that stiff ECM substrates favour the formation of long-lived clathrin arrays by preventing
363 receptors (e.g. integrins) from being internalized³³. In light of our findings, FAs-mediated
364 remodelling might change the physical properties of the ECM leading to the induction of
365 clathrin-coated plaques. We could show that topographical cues of the substrate (i.e. gelatin
366 digestions and 3D-micropatterns) are potent signals for plaque formation in an integrin-
367 dependent manner. Furthermore, ECM remodelling can change the mechanical properties
368 of the substrate that could be sensed by clathrin-coated plaques⁷⁰⁻⁷².

369 The presence of extracellular 3D nanostructures has been previously described to be able to
370 induce the formation of clathrin-coated structures¹⁸⁻²¹. Endocytically active CCPs are found
371 to be induced both by artificial nanostructures^{18,20} and by virus particles which impose local
372 membrane curvature²¹. Recruitment and/or induction of clathrin lattice assembly has
373 previously been proposed to be linked with the specific recruitment of membrane curvature
374 sensing proteins like N-BAR proteins¹⁸. Extracellular collagen fibres have also been
375 reported to induce the formation of a unique clathrin coat called tubular clathrin/AP2
376 lattices (TCALs)¹⁹. Although TCALs seem to have some similarities to clathrin-coated

377 plaques, it is not clear if they describe the same phenomenon in a different context. Both of
378 these structures contain integrin receptors and form at locally defined ECM structures. One
379 important difference between TCALs and clathrin-coated plaques emanating from a FA
380 switch is that TCALs only form in a complex 3D environment and are of a more transient
381 nature. On the contrary, clathrin-coated plaques are stable for hours and form in classical
382 2D cell culture but are also induced by extracellular 3D features.

383 We have identified Eps15 and Eps15R as key players for clathrin-coated plaque formation
384 and for the switch from FAs clathrin-coated plaques. Additional to their function in
385 initiation of CCPs as part of the pioneer module⁶⁰⁻⁶², a recent study describes that together
386 with the clathrin adaptor Numb, Eps15/R contribute to integrin β 5 localization in flat
387 clathrin lattices³⁴. By binding to the integrin β 5 cytoplasmic domain, the Numb-Eps15/R
388 complex links integrins to the clathrin coat and helps to establish integrin-containing
389 clathrin-coated plaques. This mechanism could explain why cells depleted from Eps15/R
390 fail to form clathrin-coated plaques after FA disassembly.

391 During migration, we show that cells actively remodel their surrounding and generate
392 topographical cues. Importantly, formation of these cues correlates with the integrin-
393 dependent formation of clathrin-coated plaques after FA disassembly. Additionally, using
394 our 3D-micropatterns, we could demonstrate that topographical cues can directly promote
395 the formation of clathrin-coated plaques. As such, it tempting to propose that during cell
396 migration, the FA-mediated topographical cues represent the signal leading to plaque
397 formation. This model is supported by the fact that removing these topographical cues
398 indeed leads to a loss of clathrin-coated plaque formation. Specifically, we could show that
399 when cells are either depleted of or overexpressing the matrix metalloproteinase MMP14,
400 the ECM is not digested or fully digested, respectively (Fig. 5i-j). On these surfaces which are
401 lacking topographical cues (either because there is a lack of local digestion or because there
402 is a complete digestion of the entire surface), switch from FAs to clathrin-coated plaques
403 was severely impaired (Fig. 5k). Similarly, in our cell seeding experiments (Fig. 3b-c), when
404 the ECM was removed by treatment of the surfaces using either KOH or trypsin, a loss of
405 spatial colocalization of plaque between the two rounds of seeded cells was observed (Fig.
406 3d). As such we propose that FAs generate extracellular guidance patterns which will be

407 recognized by clathrin-coated plaques ultimately influencing cell migration and establishing
408 directionality along these topographical cues.

409 The exact mechanisms by which plaques can influence cell migration remains to be
410 determined. Several studies report clustering and recruitment of transmembrane receptors
411 and signalling molecules at clathrin-coated plaques^{29,30,33}. Their stable nature would provide
412 the cells with a long-lasting signalling platform which could influence fundamental
413 processes regulating cell migration. However, in such a model, it is hard to explain how the
414 presence of plaques can influence migration directionality. In the case of FA assembly and
415 turnover, it has been proposed that internalizing integrin at the rear and their transport to
416 the front of migrating cells might contribute to the establishment of directionality^{73,74}. As
417 such, it is possible that a similar gradient of integrin interaction with and/or integrin
418 recycling by clathrin-coated plaques participates in directional cellular migration.

419 The regulation of clathrin-coated plaques and the formation of cell type-specific clathrin
420 structures might serve as topography sensing protein complexes and influence cellular
421 behaviour due to ECM features. In the context of a population of cells, our results support a
422 model where a leader cell will create extracellular cues through FA switch to clathrin-
423 coated plaques, which will then be recognized by the following cells influencing its
424 migration. The best characterized mechanisms leading to directed cell migration is
425 chemotaxis where cells have the ability of sensing external gradient of chemotactic
426 factors⁵⁵. In the complex environment of tissue in a whole organism, chemotactic gradients
427 are further shaped by ECM (diffusion and immobilization) and neighbouring cells (secretion
428 and sequestering)⁷⁵. Importantly, cells not only respond to chemical gradients but also have
429 been described to respond to mechanical gradients within the extracellular space
430 (durotaxis)⁷⁵. As such it is possible that clathrin-coated plaque might participate in
431 regulating a unique type of collective migration, where cell-to-cell contact is dispensable
432 and where the collective behaviour comes from sensing extracellular topographical cues left
433 by the preceding cells. The formation of clathrin-coated plaques and its impact on guiding
434 cell migration during contact guidance as well as collective cell migration might be a highly
435 important process in development as well as tumour migration.

436 **Materials and Methods**

437 ***Cell culture***

438 U373, HT1080, and U2OS cells were maintained in DMEM (Gibco, 41965-039) and Hek293T
439 cells for Lentivirus production were maintained in IMDM (Gibco, 21980-032) both
440 supplemented with 10% FBS (Biochrom, S0615) and Penicillin/Streptomycin (Gibco,
441 15140) at 37°C and 5% CO₂. Sf9 were maintained in Sf-900 III SFM (Gibco, 12658019) at
442 27°C.

443 ***Plasmids, shRNA and antibodies***

444 Plasmids for mammalian expression of mCherry-FAK (55044), mCherry-paxillin (55114),
445 and mCherry-vinculin (55160) were purchased from Addgene. Mammalian expression
446 plasmid for mCherry-zyxin used for the cloning was a gift from Benjamin Geiger. The
447 gateway vector pDONR 221 (12536017) and BacMam pCMV-DEST (A24223) were
448 purchased from Invitrogen and were used for cloning of BacMam pCMV mCherry-zyxin to
449 generate recombinant bacmid DNA for BacMam production. Mammalian expression
450 plasmid containing the rat AP2 subunit sigma2 C-terminally fused to eGFP⁷⁶ was used for
451 the generation of stable cell lines. The lentiviral backbones pLKO.1 puro was a gift from Dr.
452 Björn Tews and pLKO.1 blast (26655) was purchased from Addgene and were used for
453 cloning of shRNA expression vectors for lentivirus production. The gateway entry vector
454 pENTR MMP14 was obtained from the DKFZ Vector and Clone Repository and the gateway
455 destination vector pWPI puro (Invitrogen) was used for cloning of MMP14 expression
456 vector for lentivirus production. The VSV-G envelope expressing plasmid pMDG.2 (12259)
457 and the lentiviral packaging plasmid psPAX (12260) were purchased from Addgene were
458 used for lentivirus production.

459 The target sequences shscrambled (shscr) (5' CAACAAGATGAAGAGCACCAA 3'), shMMP14
460 (5' CATTGCATCTTCCCTAGATAG 3'), shEPS15 (5' CCCAGAATGGATTGGAAGTTT 3'), and
461 shEPS15R (5' GAAGTTACCTTGAGCAATC 3') were used for the shRNA-mediated protein
462 knock down.

463 Monoclonal antibody against α v β 5 integrin (ab177004; used 1:100 for IF), MMP14
464 (ab51074; used 1:100 for IF and 1:5,000 for WB) and Eps15R (EP1146Y; used 1:5,000 for
465 WB) were purchased from Abcam. Monoclonal antibody against β actin (A5441; used
466 1:5,000 for WB) and vinculin (V9131; used 1:800 for IF) and polyclonal antibody against
467 Eps15 (HPA008451; used 1:5,000 for WB) were purchased from Sigma. Monoclonal
468 antibody against β 1 integrin (552828; used 1:1,000 for IF) was purchased from BD. The
469 secondary antibodies anti-mouse IgG HRP (NA931; used 1:5,000 for WB) and anti-rabbit
470 IgG HRP (NA934; used 1:5,000 for WB) were purchased from GE Healthcare. The secondary
471 antibodies anti-mouse Alexa Fluor 568 (A-11004), anti-mouse Alexa Fluor 647 (A-21235),
472 and anti-rat Alexa Fluor 647 (A-21247) were purchased from Invitrogen.

473 **Western blot (WB)**

474 For Western blot (WB) analysis, protein samples from cell lysates were produced. Cells
475 were detached and pelleted (300 x g, 3 minutes). After one time washing with PBS, cells
476 were pelleted again and the cell pellet was lysed with appropriate volume of NP-40 buffer
477 (50 mM Tris pH 8.0, 150 mM sodium chloride, 1% (v/v) NP-40, protease inhibitor (Roche,
478 11873580001)) for 30 minutes on ice. The cell lysate was spun down for 20 minutes at
479 12,000 x g and the supernatant was transferred to a fresh tube.

480 To measure the protein concentration of protein samples, DC (detergent compatible)
481 Protein Assay Kit II (BioRad, #5000112) was used and compared to a bovine serum
482 albumin (BSA) protein standard. To compare amounts of proteins of interest in different
483 samples, the concentration of the samples was adjusted by diluting higher concentrated
484 samples with NP-40 buffer.

485 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were
486 prepared with appropriate concentrations of the separating gel. Protein samples were
487 mixed with 4x Laemmli buffer (0.2 M Tris-HCl pH 6.8, 0.05 M EDTA, 40% (v/v) glycerol, 8%
488 (w/v) SDS, 4% (v/v) β -mercaptoethanol, 0.03% (w/v) bromophenol blue), heated for
489 10 minutes at 95°C and spun down for 1 minute at 12,000 x g before loading. As molecular
490 weight marker 10 μ l Precision Plus Protein Dual Color Standards (BioRad, 1610374) was
491 separated together with the protein samples in SDS-Tris-Glycine buffer (25 mM Tris-base,
492 200 mM Glycine, 1% (w/v) SDS). SDS-PAGE was first performed at 80 V until samples

493 moved through the stacking gel and then turn up to 100 V until loading dye run out of the
494 gel. Proteins were transferred to a nitrocellulose blotting membrane (0.45 μm ; GE
495 Healthcare, #10600003) by wet blot using transfer buffer (20 mM Tri-base, 160 mM
496 Glycine, 20% methanol) at 100 V for 1 hour. Then membranes were blocked with 5 % milk
497 in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween) for 1 hour at room
498 temperature. Incubation with primary antibody diluted in TBST with 5% milk was
499 performed overnight at 4°C. After four washes for 5 minutes with TBST, membranes were
500 incubated with secondary antibody diluted in TBST with 5% milk for 1 hour at room
501 temperature. The membranes were washed four times for 5 minutes with TBST and
502 developed with ECL Western Blotting Detection Reagents (GE Healthcare, RPN2106) or
503 Western Bright Chemilumineszenz Substrate Sirius (Biozym, 541021) using high
504 performance chemiluminescence films (GE Healthcare, #28906837).

505 ***Cloning of short hairpin RNA (shRNA) encoding vector for lentivirus production***

506 Forward and reverse oligonucleotides containing short hairpin RNA (shRNA) sequence
507 were designed according to RNA interference (RNAi) consortium (TRC)
508 ([https://www.broadinstitute.org/scientific-community/science/projects/rnai-](https://www.broadinstitute.org/scientific-community/science/projects/rnai-consortium/rnai-consortium)
509 [consortium/rnai-consortium](https://www.broadinstitute.org/scientific-community/science/projects/rnai-consortium/rnai-consortium)) and ordered from Eurofins. Complementary oligonucleotides
510 were designed to generate 5' and 3' overhangs after annealing complementary to AgeI and
511 EcoRI restriction sites, respectively, for direct ligation into linearized vectors. While the
512 EcoRI restriction site was kept intact by the oligonucleotide insertion, the AgeI restriction
513 site was destroyed, which was used for test digestions as described later. Oligonucleotides
514 were diluted in H₂O at a concentration of 1 $\mu\text{g}/\mu\text{l}$. 2.5 μl of forward and reverse
515 oligonucleotides were mixed with 5 μl NEB Buffer 2 (NEB, B7002S) and 40 μl H₂O. The
516 oligonucleotide mix was heated at 95°C for 5 minutes and slowly cooled down to room
517 temperature for annealing of both strands. For the ligation reaction, 150 ng of pLKO.1
518 backbone, which has been sequentially digested with EcoRI (R0101S) and AgeI-HF
519 (R3552S) purchased from NEB, and 2 μl annealed oligonucleotides were used. A 20 μl
520 ligation reaction was set up using T4 Ligase (NEB, M0202S) and incubated for 1 hour at
521 room temperature. The whole ligation mix was used for transformation into DH5 α
522 (Invitrogen, 18265017).

523 Colonies were tested by digestion with AgeI-HF and BamHI (NEB, R0136S). Insertion of
524 oligonucleotides containing shRNA led to the loss of AgeI restriction site. Therefore
525 plasmids with successful insertion showed only one linearized vector band whereas re-
526 ligated plasmids were recognized by a double band. Plasmids were further controlled by
527 sequencing with U6 primer.

528 ***Lentivirus production***

529 For lentivirus production, Hek293T cells were cultured in 10 cm dishes until 80%
530 confluence. The cells were transfected with pMDG.2, psPAX, and the shRNA encoding
531 pLKO.1 vector or the pWPI vector containing the gene of interest using PEI. 50 µl of PEI
532 (1 mg/ml) was diluted in 200 µl Opti-MEM (Gibco, 31985062) and mixed well. 4 µg pMDG.2,
533 4 µg psPAX, and 8 µg pLKO.1 or pWPI were mixed with Opti-MEM to a final volume of 250 µl
534 and mixed well. After 5 minutes, both solutions were mixed, vortexed and incubated for
535 20 minutes at room temperature. Then the transfection mix was added dropwise to cells.
536 Culture medium was exchanged the next day. After another two days, the supernatant was
537 harvested, centrifuged (10 minutes, 4,000 x g) and filtered through a syringe filter (Millex-
538 HA, 0.45 µm, Millipore, SLHA033SS). For short time storage, the lentivirus containing
539 supernatant was kept at 4°C; for long time storage, the supernatant was aliquoted and
540 stored at -80°C.

541 ***Gateway Cloning***

542 The Gateway Technology (Invitrogen) was used for generating entry and expression
543 plasmids. This highly efficient cloning system is based on site-specific recombination
544 reactions using recombination sequences (*att*-sites) and enzymes, which catalyse
545 recombination reactions (Clonases). This system uses a set of donor and destination vectors
546 to quickly move DNA sequences between multiple vectors. Donor and destination vectors
547 contain a cassette flanked by *att*-sites, which hold the *ccdB* gene for negative selection after
548 recombination reaction and a chloramphenicol resistance gene (CM^R) for counterselection
549 during propagation. Therefore, donor and destination vectors need to be propagated in
550 *E.coli* strains resistant to CcdB effects. This cassette is removed by the recombination
551 reactions and replaced by the gene of interest. More details on the system and the
552 procedure can be found in the manufacturer's manual.

553 To generate entry vectors Gateway BP Clonase II Enzyme Mix (Invitrogen, 11789) was used.
554 In brief, BP-recombination reactions between PCR products containing *attB*-sites and
555 pDONR 221 containing *attP*-sites were set up by mixing 150 ng of PCR product with 150 ng
556 of pDONR 221. TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) was added to reach a final
557 volume of 8 μ l. Then, 2 μ l of BP Clonase II Enzyme Mix were added and incubated for 1-
558 3 hours at 25°C. 1 μ l of Proteinase K (Invitrogen, AM2548) was added to the reaction mix
559 and incubated for 10 minutes at 37°C. The reaction mix was transformed into DH5 α .
560 Plasmid DNA was isolated and sequenced with M13 forward and reverse primers.

561 To generate expression vectors Gateway LR Clonase II Enzyme Mix (Invitrogen, 11791) was
562 used. In brief, LR-recombination reactions between Gateway entry vectors containing *attL*-
563 sites and Gateway destination plasmids containing *attR*-sites were set up by mixing 150 ng
564 of the entry vector with 150 ng of the destination vector. TE buffer was added to reach a
565 final volume of 8 μ l. Then, 2 μ l of LR Clonase II Enzyme Mix were added and incubated for 1-
566 3 hours at 25°C. 1 μ l of Proteinase K was added to the reaction mix and incubated for
567 10 minutes at 37°C. The reaction mix was transformed into DH5 α .

568 ***BacMam production***

569 ViraPower BacMam Expression System (Life Technologies, A34227) was used to design and
570 produce BacMam mCherry-zyxin for transduction of mammalian cell lines following the
571 user guide.

572 In brief, the Gateway cloning system was used to generate the Gateway expression plasmid
573 BacMam pCMV mCherry-zyxin. First, a Gateway entry plasmid containing the mCherry-
574 zyxin sequence (pENTR mCherry-zyxin) was generated. The sequence was amplified from
575 the mammalian expression plasmid mCherry-zyxin using the primers mCherry-zyxin
576 forward (5'
577 GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACCATGGTGAGCAAGGGCGAGGAGGATA 3') and
578 reverse (5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACGTCTGGGCTCTAGCAGTGTG 3')
579 to flank the sequence with *attB*-sites by PCR. The PCR product was used to generate the
580 pENTR mCherry-zyxin by BP-recombination reaction. pENTR mCherry-zyxin was then used
581 to generate the BacMAM pCMV-DEST mCherry-zyxin expression plasmid by LR-
582 recombination reaction.

583 For transposition of the mCherry-zyxin gene into the bacmid, DH10Bac (Invitrogen,
584 10361012) were transformed and incubated for two days at 37°C. Using a blue/white
585 screening, positive colonies were selected which contained the recombinant bacmid. The
586 bacmid DNA was isolated following the protocol described in the user guide.

587 To produce BacMam mCherry-zyxin, insect cells were transfected with the bacmid
588 mCherry-zyxin using Cellfectin II reagent (Invitrogen, 10362100) to generate a P1 viral
589 stock. This P1 baculoviral stock was used to amplify BacMam mCherry-zyxin by infection of
590 insect cells. This time, the infected insect cells were culture in a spinner flask to produce a
591 high viral titre. Two rounds of amplification were performed to produce a P2 and a P3
592 baculoviral stock. 3% FBS was added to the P3 baculoviral stock of BacMam mCherry-zyxin
593 and stored in aliquots at -80°C.

594 ***Transfection and viral transduction***

595 Transfection of cells was done using Lipofectamine 2000 (Invitrogene, 11668027) if not
596 stated otherwise. Cells were plated in 6-well plates one day before transfection. The next
597 day, cells were transfected at 70-80% confluence. 2 µg DNA and 4-8 µl Lipofectamine 2000
598 were separately mixed with 100 µl OptiMEM. The two solutions were mixed together. After
599 incubation for 20 minutes at room temperature, the transfection mix was added drop-wise
600 onto the cells. For generation of stable cell lines, the growth medium was exchanged for
601 fresh growth medium after 8 hours. The cells were put under selection two days after
602 transfection, selected for 2 weeks and sorted using FACS. For live-cell imaging of cells
603 transiently expressing fluorescently tagged FA proteins, the growth medium was exchange
604 for fresh growth medium after 8 hours. The transfected cells were seeded 24 hours after
605 transfection and imaged 6-8 hours after seeding.

606 For transient expression of mCherry-zyxin, cells were transduced with BacMam mCherry-
607 zyxin. Cells were seeded with BacMam mCherry-zyxin in appropriate dishes and were used
608 for experiments 1 day after seeding. 3.5 µl of the P3 stock was used per 10,000 cells. For
609 lentiviral transduction for generation of stable cell lines, 10,000 cells per well were seeded
610 into 6-well plates together with 500 µl of lentivirus containing supernatant. After 2 to
611 3 days, growth medium was exchange to selection medium with appropriate antibiotics.
612 Cells were sub-cultured in selection medium for 2 weeks and then used for experiments.

613 ***Widefield epifluorescence microscopy***

614 If not stated otherwise, widefield epifluorescence microscopy was performed with an
615 inverted Ti microscope (Nikon) with a 20x (0.75 numerical aperture, Plan Apo λ , Nikon) dry
616 objective or a 40x (1.3 numerical aperture, Plan Fluor, Nikon) oil immersion objective and a
617 digital camera (DS-Qi1MC, Nikon) equipped with LED (Lumencor-Sola) as light source.

618 ***Spinning disc confocal microscopy***

619 Confocal live-cell imaging was performed with an inverted spinning disc confocal
620 microscope (Nikon, PerkinElmer) with a 60x (1.42 numerical aperture, Apo TIRF, Nikon) or
621 100x (1.4 numerical aperture, Plan Apo VC, Nikon) oil immersion objective and a CMOS
622 camera (Hamamatsu Ocra Flash 4) or an EMCCD camera (Hamamatsu C9100-23B). An
623 environment control chamber was attached to the microscope to maintain cells at 37°C and
624 5% CO₂. If not stated otherwise, live-cell imaging was performed in DMEM without phenol
625 red (Gibco, 21063-029) containing 10% FBS.

626 ***Total internal reflection fluorescence (TIRF) microscopy***

627 TIRF microscopy was performed with an inverted Ti microscope (Nikon) with objective
628 TIRF illumination, with a 60x (1.49 numerical aperture, Apo TIRF, Nikon) oil immersion
629 objective and an EMCCD camera (Andor iXon Ultra DU-897U).

630 ***Quantification of FA replacement by clathrin-coated plaques during cell migration***

631 To quantify the specific formation of clathrin-coated plaques at the position of former FAs
632 during cell migration, live-cell confocal spinning disc was performed on U373 cells stably
633 expressing AP2-eGFP and transiently expressing mCherry-zyxin. The position of all FAs at a
634 given time point was used to check for replacement by clathrin-coated plaques within the
635 following 2 hours. Only FAs that were at a position still covered by the cell after this time
636 were analysed. Each FA was categorized into either replaced by clathrin-coated plaques or
637 not. The formation of long-lived (>10min) clathrin-coated plaques was manually verified. As
638 a control, random positions with the same size, which did not show any zyxin signal were
639 analysed in the same way.

640 ***Immunofluorescence (IF)***

641 Cells growing on glass coverslips (#1.5, diameter 12 mm purchased from Thermo Scientific
642 or 24 mm purchased from Marienfeld) or glassbottom dishes (ibidi, 80827) for TIRF were
643 washed once with PBS and fixed with PFA, formaldehyde, or methanol (only for $\alpha\beta5$
644 integrin staining). Precisely, cells were incubated either with 2% PFA or 4% formaldehyde
645 in PBS for 20 minutes at room temperature or overnight at 4°C or with ice cooled methanol
646 for 10 minutes at -20°C. All following steps were performed at room temperature. After
647 three washes with PBS, cells were permeabilized with either 0.5% TritonX in PBS for
648 15 minutes or 0.05% saponin (only for $\alpha\beta5$ integrin staining) in PBS for 10 minutes
649 followed by a blocking step with 1% BSA in PBS for 1 hour. Samples were incubated with
650 appropriate primary antibody dilution in 1% BSA in PBS for 1 hour. After three washes with
651 PBS, samples were stained with appropriate secondary antibody and/or Phalloidin labelled
652 with Alexa Fluor 647 (Invitrogen, A22287) for 45 minutes followed by four washes with
653 PBS. For normal microscopy, samples were washed with H₂O and mounted with ProLong
654 Gold antifade reagent with DAPI (Invitrogen, P36931). For TIRF microscopy, samples were
655 fixed with 4% formaldehyde solution for 20 minutes, washed three times with PBS and kept
656 in PBS.

657 ***Inhibitor-induced disassembly of FAs***

658 For inhibitor-induced disassembly of FAs either the ROCK inhibitor Y-37632
659 dihydrochloride (Enzo, ALX-270-333) or Blebbistatin (Sigma, B0560) were used with a final
660 concentration of 10 μ M or 20 μ M, respectively. The inhibitors were diluted in medium with
661 10% FBS. To check the effect of these inhibitors on FAs and the actin cytoskeleton, cells
662 were incubated with pre-warmed inhibitor-containing medium and fixed with
663 formaldehyde. FAs and the actin cytoskeleton were stained using an antibody against
664 vinculin and phalloidin, respectively. Widefield epifluorescence microscopy was used to
665 quantify the presence of FAs and actin stress fibres in each cell. To analyse the lifetime of
666 clathrin structures forming after inhibitor-induced FA disassembly, each FAs visible before
667 the application of the drug was followed over 30 minutes of treatment and categorized
668 according to the formation of clathrin structures at its position after FA disassembly. The
669 lifetime of clathrin structures appearing during that time were analysed manually and
670 differentiated between transient pits (<10min) and long-lived plaques (>10min).

671 To quantify the inhibitor-induced switch from FAs to clathrin-coated plaques, cells
672 expressing AP2-eGFP as well as mCherry-zyxin were used. 10 cells per sample were imaged
673 before and after treatment with pre-warmed inhibitor-containing medium. To correct for
674 lateral shift (pixels shifts) of the samples during the exchange of medium, the images before
675 and after the inhibitor treatment were re-aligned using clathrin-coated plaques or other
676 immotile fluorescent spots as reference landmarks. An automated image analysis workflow
677 by KNIME (<https://www.knime.com>) was used to perform object-based colocalization
678 analysis of FAs and clathrin structures to quantify the switch. A binary mask for the
679 mCherry-zyxin signal before and the AP2-eGFP signal after the inhibitor treatment was used
680 to check for overlapping objects. Only FAs that were at a position still covered by the cell
681 after the treatment were analysed. As a control for thresholding, the number of overlapping
682 objects of the mCherry-zyxin and the AP2-eGFP signal before the inhibitor treatment was
683 determined and subtracted for normalization.

684 ***Inhibition of integrins***

685 For inhibition of integrins, the cyclic pentapeptide Cilengitide (Selleckchem, S7077) was
686 used. The inhibitor was diluted in medium containing 10% FBS at a final concentration of
687 10 μ M. Live-cell spinning disc confocal microscopy of CME dynamics of the same cell was
688 performed before and after drug treatment with a frame rate of 3 seconds to follow
689 clathrin-coated plaque disassembly. To calculate the numbers of clathrin-coated plaques,
690 CME was tracked during 30 minutes of Cilengitide treatment. The movie was separated into
691 5 minute long sections and the number of clathrin structures that stayed for the whole
692 5 minutes was calculated for each section. This numbers were normalized to the first
693 section directly after applying the drug.

694 ***Quantification of colocalization between integrins and AP2***

695 TIRF microscopy images of cells expressing AP2-eGFP and immunostained for α v β 5 were
696 analysed using the ImageJ/Fiji plugin Coloc2. As indicated, the Pearson correlation
697 coefficient was calculated from background corrected images either for the whole cell or
698 areas covering FAs or clathrin-coated plaques with the same size.

699 ***ECM degradation assay***

700 ECM degradation assays were performed as previously described⁷⁷. To prepare labelled
701 gelatin, a solution of 0.2% gelatin (from porcine skin; sigma (G-2500)) in PBS was prepared.
702 To dissolve the gelatin, the solution was heated up to 37°C for 30 minutes. For sterilization,
703 the solution was filtered using a syringe filter membrane (Millex-GS, 0.22 µm, Millipore,
704 SLGS033SB). To label the gelatin with Alexa Fluor 647, 500 µl of 0.2% gelatin solution was
705 preheated to 37°C for 30 minutes. 5 µl of Alexa Fluor 647 NHS Ester (10 mg/ml, Invitrogen,
706 A37573) was added and incubated for 1 hour at room temperature protected from light. To
707 remove the free dye, the labelling mix was dialyzed with a Slide-A-Lyzer MINI Dialysis
708 Device (20 kMWCO, 0.5 ml; Thermo Scientific (88402)) against PBS for 2 hours at room
709 temperature. After changing the PBS, the mix was dialyzed overnight at 4°C. During dialysis,
710 the labelled gelatin was protected from light.

711 To coat coverslips with labelled gelatin, washed coverslips were first coated with poly-L-
712 lysine (PLL Sigma, P8920), using a 50 µg/ml solution in PBS for 20 minutes at room
713 temperature. The coverslips were washed three times with PBS. Then the PLL coat was
714 fixed with 0.5% glutaraldehyde (Sigma, G5882) in PBS for 15 minutes at room temperature.
715 The coverslips were washed three times with PBS. Mixture of 0.2% labelled and unlabelled
716 gelatin in a ratio of 8:1 was preheated to 37°C for 30 minutes. Coverslips were inverted on a
717 drop of gelatin mix (80 µl for 12 mm diameter coverslips; 120 µl for 25 mm diameter
718 coverslips) and incubated for 10 minutes at room temperature. Coverslips were washed
719 three times with PBS. The coated coverslips were directly used for experiments.

720 To seed cells on coverslips coated with labelled gelatin, detached cells were resuspended in
721 DMEM containing 10% FBS. The cells were pelleted by centrifugation (300 x g, 3 minutes)
722 and washed with PBS to remove residual trypsin. Cells were pelleted again and
723 resuspended in DMEM containing 10% FBS and plated on coverslips with fluorescent
724 gelatin matrix (20,000 cells for 12 mm diameter coverslips; 80,000 cells for 25 mm
725 diameter coverslips). Cells were cultured for one day on the gelatin matrix and then used
726 for experiments.

727 To analyse the degree of gelatin digestion of different cell lines, widefield microscopy
728 images were used. The labelled gelatin coat underneath each cell was manually evaluated
729 into no, low or high digestion dependent on the loss of fluorescent signal compared to the
730 undigested coat. For drug-induced disassembly of FAs on labelled gelatin matrix, the

731 quantification of the switch from FAs to clathrin-coated plaques was performed as
732 described before. Additionally, each FA was evaluated manually for gelatin digestion.

733 ***Production of 3D-micropatterns by soft lithography***

734 To prepare 3D-micropatterns suitable for microscopy, soft lithography with a PDMS master
735 and optically clear glue was performed. The protocol for PDMS master production was
736 adapted from Lückner *et al.*, 2014⁷⁸ and the protocol for 3D-micropattern production with
737 optically clear glue from Ray *et al.*, 2017⁷⁹.

738 To produce the PDMS master, diffraction gratings (Edmund Optics, #54-510) with a groove
739 density of 500 grooves per mm were glued into a 10 cm petri dish using double-faced
740 adhesive tape. One clip-pack PDMS (SYLGARD 184, 10 g clip-pack; Sigma, #761036) was
741 mixed, poured in the petri dish, and de-gased for 1 hour. The PDMS was cured at 50°C
742 overnight. The PDMS on the diffraction gratings was cut into small squares (approximately
743 1 x 1cm) and peeled off.

744 These PDMS masters were used to generate 3D-micropatterns made of Norland optical
745 adhesive (Norland Products, NOA73). A drop of NOA 73 was put on a coverslip and the
746 PDMS master was placed on top of this drop. NOA 73 was cured by UV light (UV Stratalinker
747 1800, Stratagene) for 15 minutes and the PDMS master was peeled from the coverslip
748 leaving behind the micropatterned optically clear NOA 73 film. The 3D-micropatterns were
749 illuminated with UV light a second time for 15 minutes to ensure completeness of curing.
750 These 3D-micropatterns were sterilized with ethanol before usage.

751 ***Sequential seeding of cells on gridded coverslips***

752 80,000 cells were seeded in μ -Dish 35 mm (high Grid-50 Glass Bottom; ibidi, 81148).
753 12 hours after seeding, cells on grids were imaged by live-cell confocal microscopy.
754 Afterwards, cells were removed with 15 mM EDTA in PBS for 10 minutes. After the removal
755 of cells, the gridded coverslips were either left untreated, treated with 0.05% Trypsin/EDTA
756 for 20 minutes at 37°C or cleaned by sequential sonification in 1 M KOH, acetone, ethanol,
757 and H₂O for 10 minutes. Another round of cells was seeded as described above. Live-cell
758 confocal imaging was performed 12 hours after seeding at the positions imaged in the first
759 round of cells. To evaluate the amount of clathrin structures formed at the same position in

760 both rounds of cells, the images were aligned with ImageJ/Fiji using the landmarks plugin.
761 Three landmarks from the grid were selected in both pictures and the second image was
762 registered to the first image with the Best Rigid Registration method. An automated image
763 analysis workflow by KNIME was used to perform object-based colocalization analysis of
764 clathrin structures for quantification. A binary mask for the AP2-eGFP signal in both images
765 was used to check for overlapping objects and to quantify the percentage from all clathrin
766 structures found in the first round of cells. Only clathrin structures at regions covered by
767 cells in both images were analysed.

768 ***Tracking of clathrin structures***

769 For the tracking of CME events, we used ilastik (<http://ilastik.org>). First the images were
770 segmented using the pixel classification and object classification workflow. For tracking, the
771 automatic tracking workflow was used. The maximal distance was put to 5 to avoid merging
772 of close tracks. Tracking results were further analysed by automated workflows using
773 KNIME to calculate features (lifetime, maximal fluorescence intensity and average position)
774 of each CME event.

775 ***Tracking of cell migration***

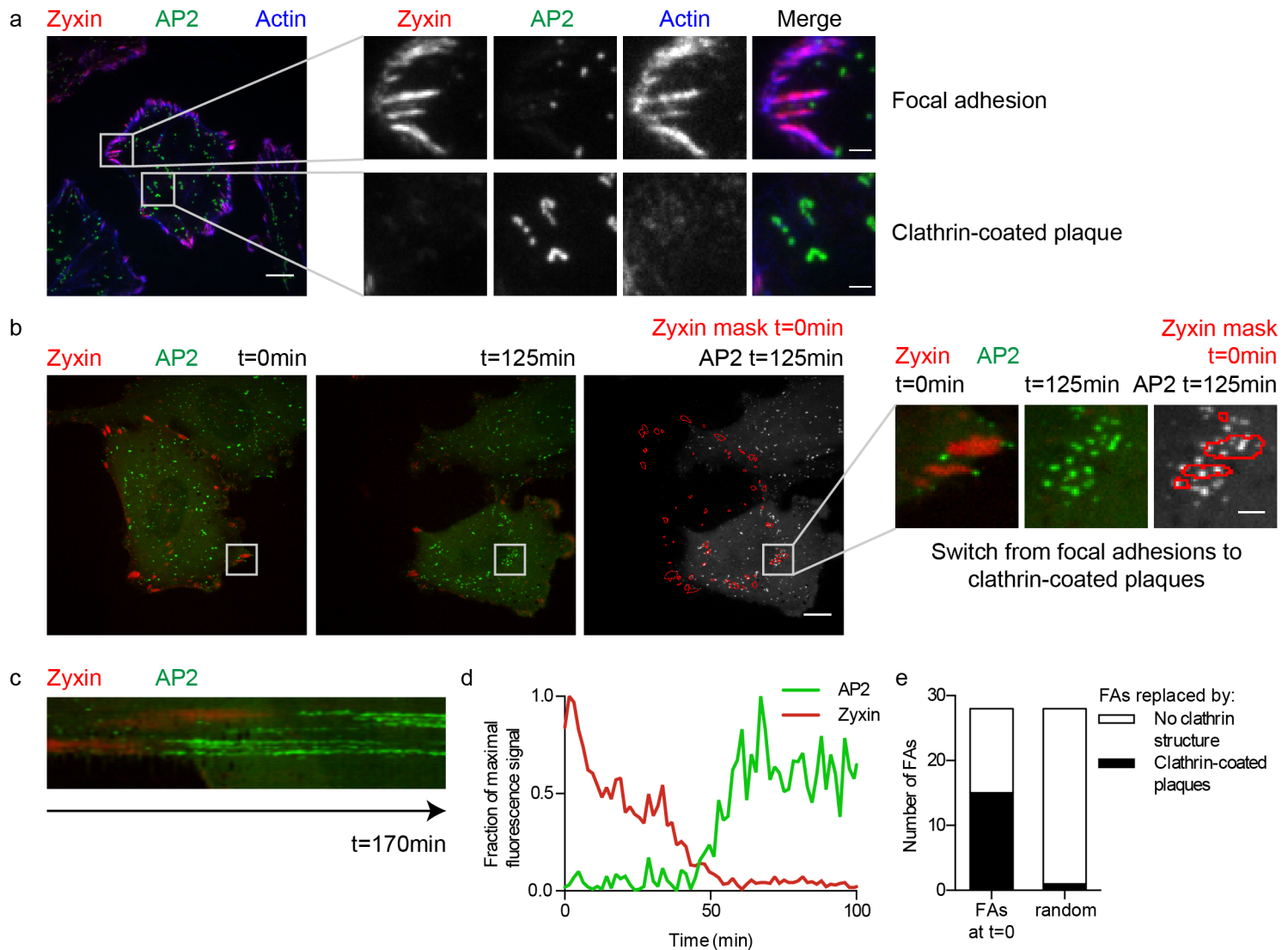
776 For the tracking of cell migration, we used the ImageJ/Fiji plugin Manual Tracking. The
777 position of a cell was manually defined by the centre of its cell body for each time point. The
778 tracking results were further analysed by automated workflows using KNIME to calculate
779 the directionality of cell migration. The directionality was defined as the angle between two
780 sequential positions of a cell.

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794 use of equipment.

795 **Author contributions**

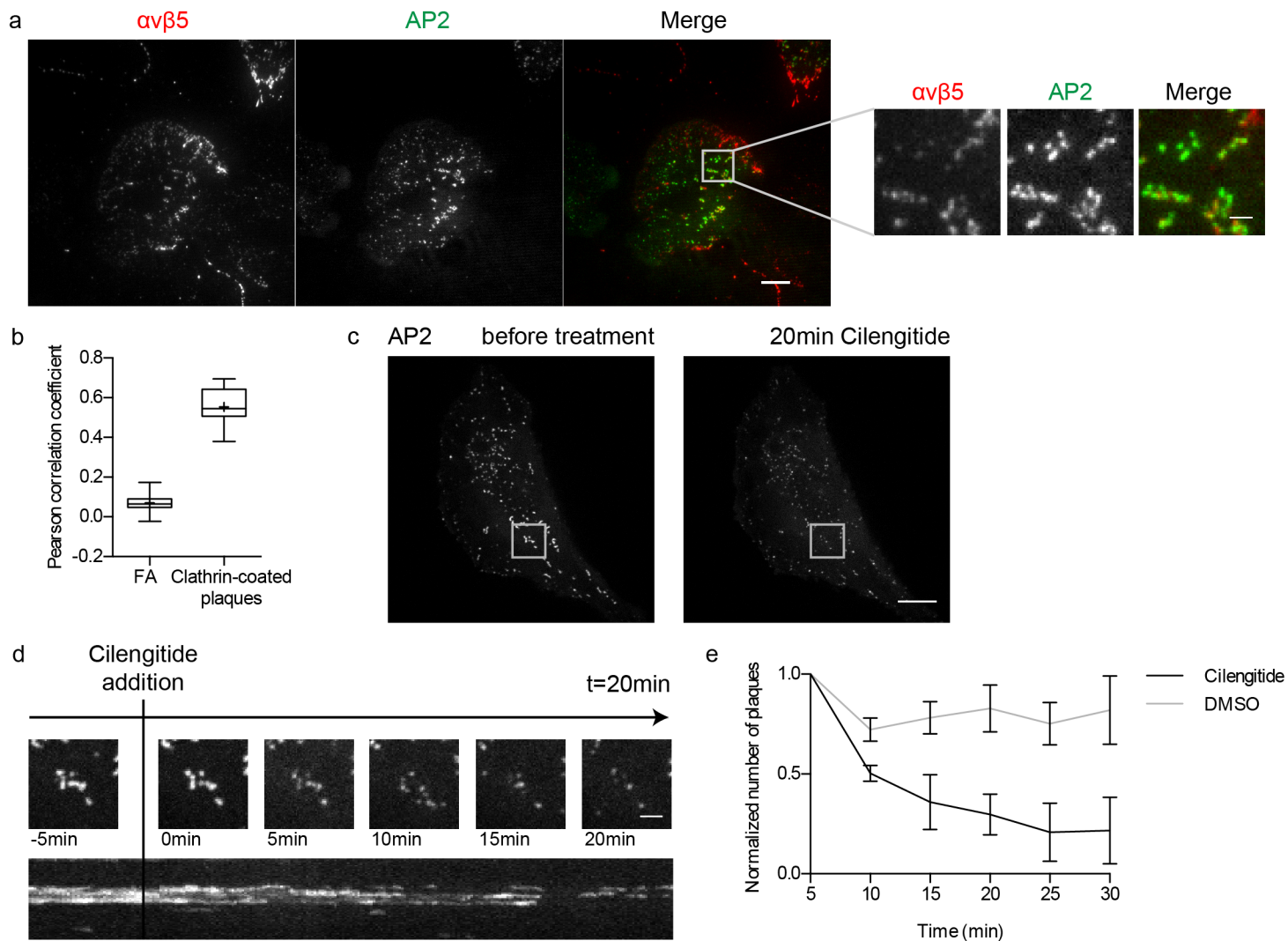
796 DB designed and performed experiments, analysed data and wrote manuscript. MM
797 designed and established BacMam mammalian expression system. VS performed drug-
798 induced FA disassembly assays and designed 3D-micropattern experiments. CH designed,
799 performed and analysed AFM measurements. CZ and EACA designed adhesive micropattern
800 experiments. KAS and JWT designed and performed TEM and CLEM experiments. SB
801 supervised the project, designed experiments, interpreted data and wrote manuscript. The
802 authors declare that they do not have competing financial interests.



803 **Figure 1: Switch from FAs to clathrin-coated plaques.**

804 (a) Representative image from total internal reflection fluorescence (TIRF) microscopy of
 805 U373 stably expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red)
 806 stained for actin with Alexa Fluor 647-labelled phalloidin (blue). Right: Zoom on FAs
 807 labelled by mCherry-zyxin (upper panel) and on clathrin-coated plaques labelled by AP2-
 808 eGFP (lower panel). (b) Live-cell confocal spinning disc microscopy of U373 stably
 809 expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red). Overview of
 810 a representative migrating cell at time point 0 (left) and 125 minutes later (middle).
 811 (Right) Merged images of a mask marking the mCherry-zyxin objects at time point 0 (red)
 812 and the AP2-eGFP signal 125 minutes later (green). Right: Zoom on FAs that switch to
 813 clathrin-coated plaques. (c) Kymograph of the switch from FAs to clathrin-coated plaques

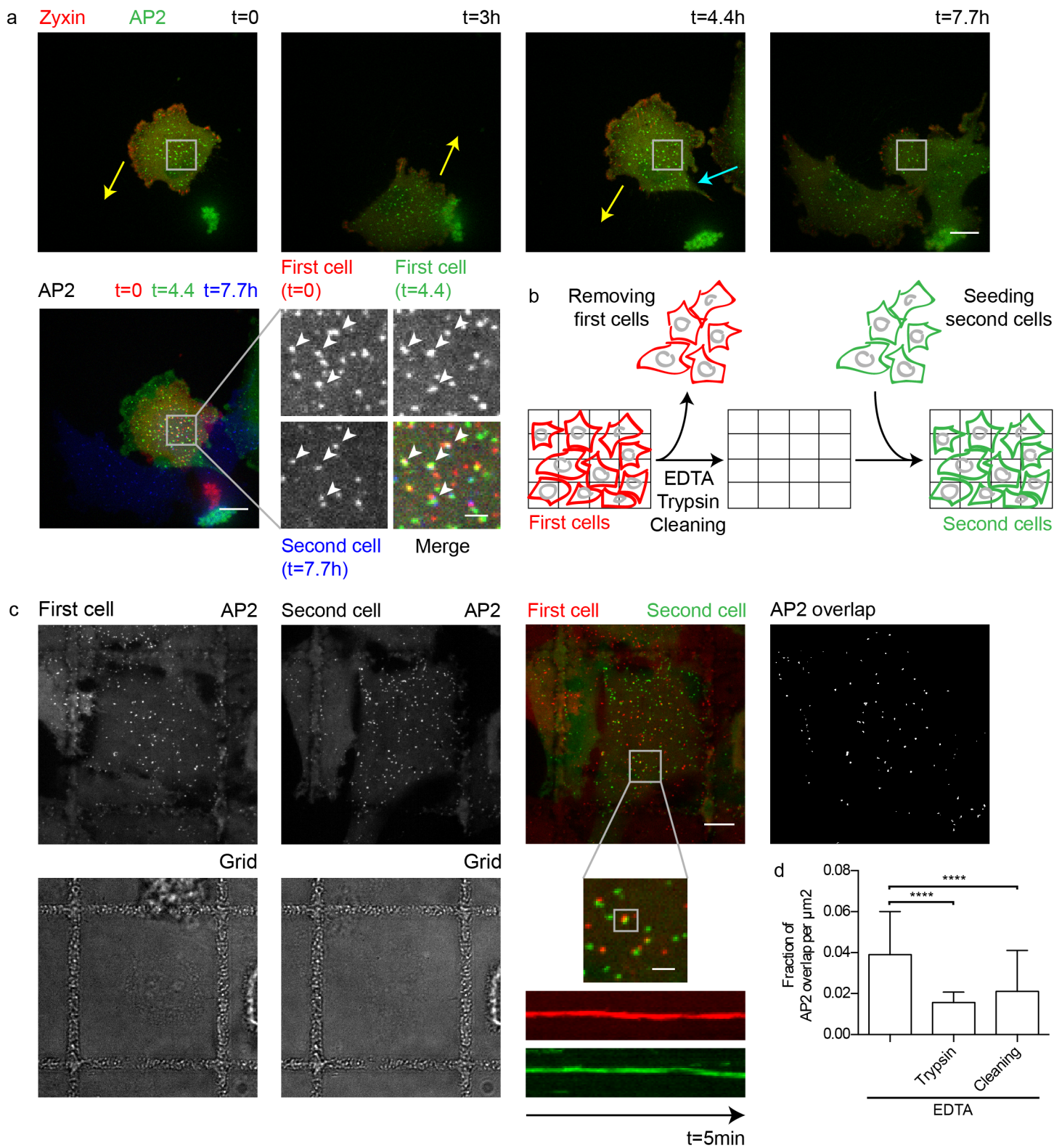
814 shown in b over 170 minutes. (d) Normalized fluorescence intensity profiles of AP2-eGFP
815 (green) and mCherry-zyxin (red) of the switch from FAs to clathrin-coated plaques shown
816 in b. (e) Quantification of the number of FAs being replaced by clathrin-coated plaques. Data
817 are from live experiment performed in b. FAs at time point 0 were followed over 2 hours to
818 quantify the number of FA replaced by clathrin-coated plaques (black) vs. the number of FA
819 not replaced by plaques (no clathrin structures (white)). As a control the appearance of
820 clathrin-coated plaques at randomly placed ROIs of the same size was analysed. Scale bars:
821 10 μm (overviews) or 2 μm (zooms).



822 **Figure 2: Clathrin-coated plaques are stabilized by integrins.**

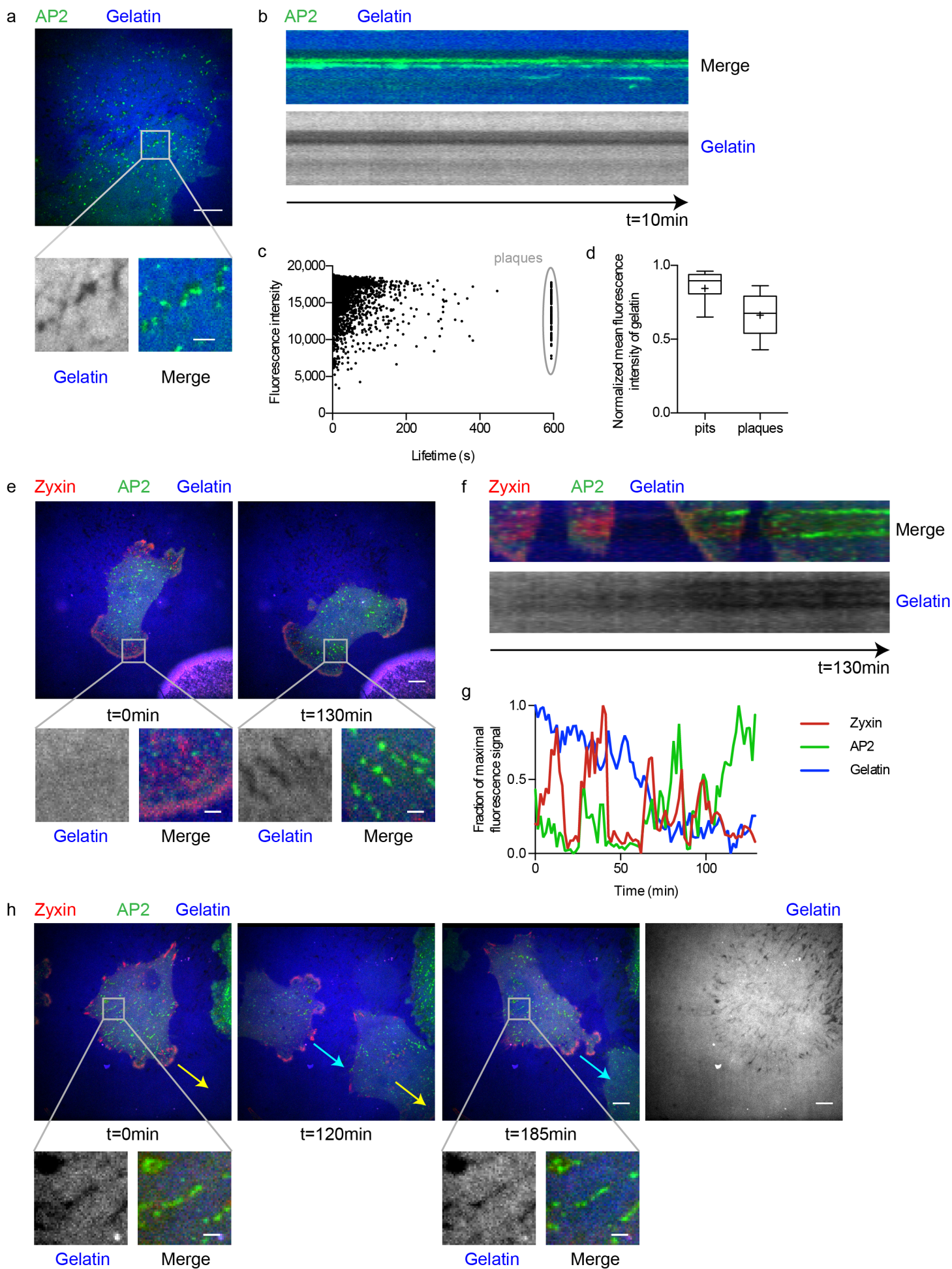
823 (a) Representative image from TIRF microscopy of U373 stably expressing AP2-eGFP
 824 (green) immunostained for integrin $\alpha\beta5$ (red). Right: Zoom on clathrin-coated plaques.
 825 (b) Quantification of the colocalization between $\alpha\beta5$ integrin and AP2-eGFP. The graph
 826 shows the distribution of the Pearson correlation coefficient between AP2-eGFP and
 827 integrin signals at either FAs or clathrin-coated plaques. Whiskers represent 10-90
 828 percentile, box represents second and third quartile, line marks the median and cross the
 829 mean. Results are computed from ten cells. (c) Live-cell spinning disc confocal microscopy
 830 of U373 stably expressing AP2-eGFP treated with Cilengitide (10 μM) for 20 minutes.
 831 Shown is a representative cell before (left) and after 20 minutes of treatment with
 832 Cilengitide (right). Clathrin-coated plaques were identified by monitoring the dynamic of
 833 the clathrin structures before treatment with Cilengitide. (d) Serial view of a clathrin-coated

834 plaque (ROI shown in b) (top panel) and corresponding kymograph (bottom).
835 (e) Quantification of the number of clathrin-coated plaques during Cilengitide (black) and
836 DMSO (grey) treatment. Number of plaques was normalized to the time at the beginning of
837 the treatment. Shown are the mean and SD calculated from four cells of each condition.
838 Scale bars: 10 μm (overviews) or 2 μm (zooms).



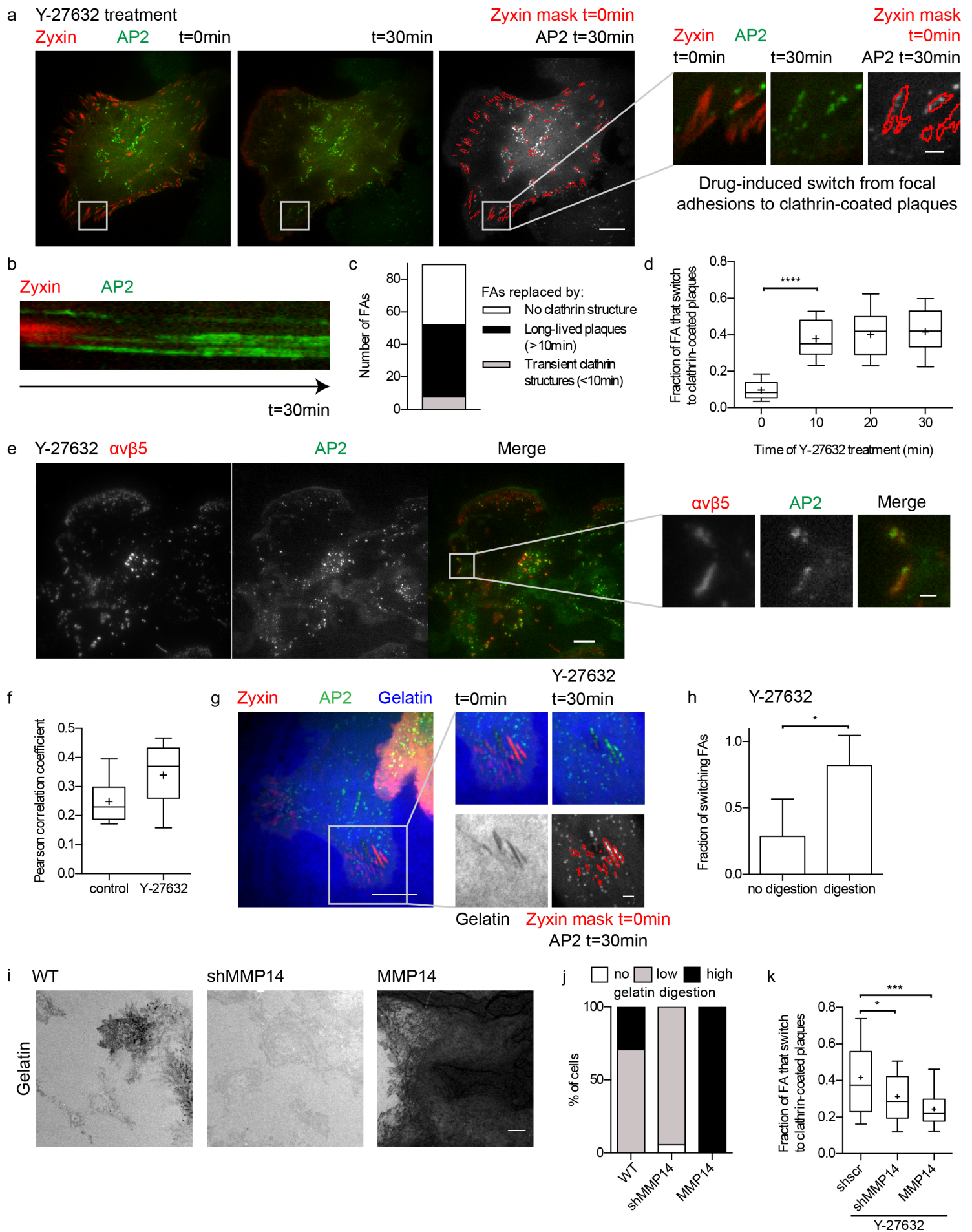
839 **Figure 3: Extracellular signal induces clathrin-coated plaque formation.**

840 (a) Live-cell confocal spinning disc microscopy of U373 stably expressing AP2-eGFP (green)
841 and transiently expressing mCherry-zyxin (red). (Top) Snapshots of a time series of two
842 representative cells moving over the same area. Arrows point in the direction of cell
843 migration (first cell: yellow, second cell: blue). (Bottom left) Merged images of the AP2-
844 eGFP signal at three time points (first cell at t=0: red, first cell at t=4.4h: green, second cell
845 at t=7.7h: blue). Zoom on clathrin coats found at the same positions in all three time points
846 marked by arrow heads. (b) Schematic illustrating the sequence of the experiment using
847 gridded coverslips. A first round of cells (red) was imaged to identify the position of
848 clathrin-coated plaques and cells were then removed by EDTA solution. The gridded
849 coverslip was either left untreated or treated with trypsin or cleaned with a sequence of
850 KOH, acetone, and ethanol to remove organic material. Afterwards a second round of
851 cells (green) was seeded on the same gridded coverslip and imaged to identify the position
852 of clathrin-coated plaques and to compare it with their position identified within the first
853 round of seeded cells. (c) Live-cell spinning disc confocal microscopy of two rounds of U373
854 stably expressing AP2-eGFP on the same position of a gridded coverslip with no treatment
855 before seeding the second round of cells. AP2-eGFP signal of first (top left) and second (top
856 middle left) round of cells. (Top middle right) Merged image of AP2-eGFP in first cell (red)
857 and second cell (green). Zoom in of representative overlapping clathrin coats from both
858 rounds of seeded cells and corresponding kymographs of 5 minute long movies of clathrin-
859 coated plaques from both rounds of seeded cells. (Top right) Binary mask of AP2-eGFP
860 overlap. (d) Quantification of overlapping clathrin coats in the first and second round of
861 cells using gridded coverslips. Shown are the mean with SD. Statistical analysis: t test,
862 number of analysed images: n=31 (untreated), n=16 (trypsin), and n=15 (cleaning), $P < 0.01$.
863 Scale bars: 10 μm (overviews), 2 μm (zoom).



865 **Figure 4: Remodelled ECM as signal for clathrin-coated plaque formation.**

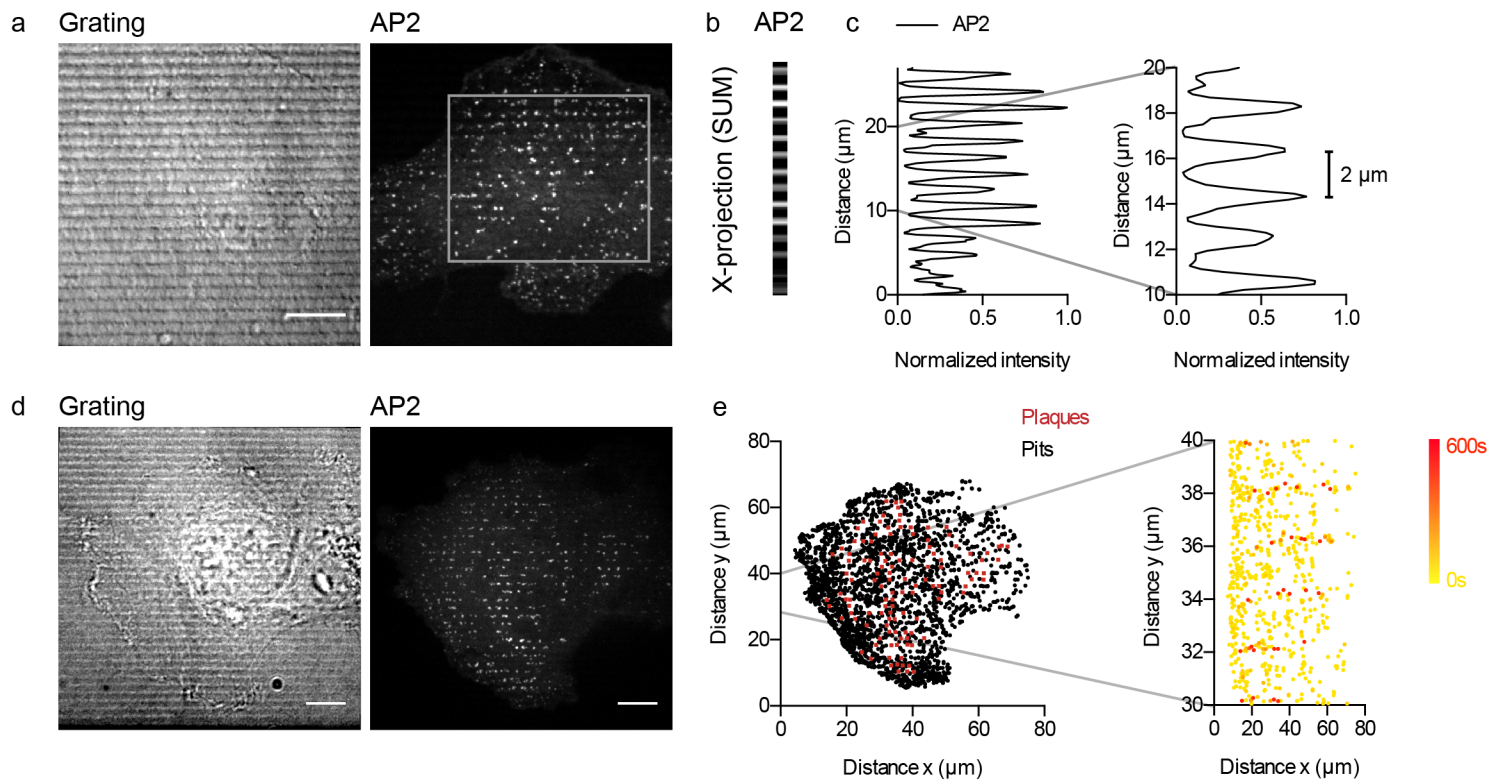
866 (a) Live-cell confocal spinning disc microscopy of U373 stably expressing AP2-eGFP (green)
867 on coverslips coated with Alexa Fluor 647-labelled gelatin (blue). Right: Zoom in of clathrin-
868 coated plaques at digested gelatin spots. (b) Kymograph of clathrin-coated plaques and
869 gelatin digestion shown in an over 10 minutes. (c) Lifetime vs. mean fluorescence intensity
870 of clathrin tracks in U373 cells seeded on Alexa Fluor 647-labelled gelatin. Clathrin-coated
871 plaques are marked by a grey circle. Shown is a plot of a representative cell. Each dot
872 represents one CME track from a 10 minute long movie. (d) Distribution of mean
873 fluorescence intensity of Alexa Fluor 647-labelled gelatin at transient pits and at long-lived
874 clathrin-coated plaques (>10min) shown in c. Whiskers represent 10-90 percentile, box
875 represents second and third quartile, line marks the median and cross the mean. Results are
876 computed from 3,731 pits and 75 plaques. (e) Long-term live-cell confocal spinning disc
877 microscopy of U373 stably expressing AP2-eGFP (green) and transiently expressing
878 mCherry-zyxin (red) on coverslips coated with Alexa Fluor 647-labelled gelatin
879 (blue). Overview of a representative migrating cell at time point 0 (left) and 130 minutes
880 later (right). Bottom: Zoom in of FAs that switch to clathrin-coated plaques with the
881 corresponding gelatin signal (grey). (f) Kymograph of the switch from FAs to clathrin-
882 coated plaques on coverslips coated with Alexa Fluor 647-labelled gelatin shown in c over
883 130 minutes. (g) Normalized fluorescence intensity profiles of AP2-eGFP (green), mCherry-
884 zyxin (red) and Alexa Fluor 647-labelled gelatin (blue) of the switch from FAs to clathrin-
885 coated plaques shown in c. (h) Two representative cells migrating over gelatin digestions.
886 Shown at time point 0 (left) and later time points (120 minutes (middle) and 185 minutes
887 (right)). Arrows point in the direction of migration (first cell: yellow; second cell: turquoise).
888 Right: Overview of the Alexa Fluor 647-labelled gelatin signal (grey). Bottom: Zoom in time
889 points 0 and 185 minutes showing clathrin-coated plaques at the same gelatin digestions in
890 both cells. Scale bars: 10 μm (overviews) or 2 μm (zooms).



892 **Figure 5: Local formation of clathrin-coated plaques by drug-induced FA disassembly**

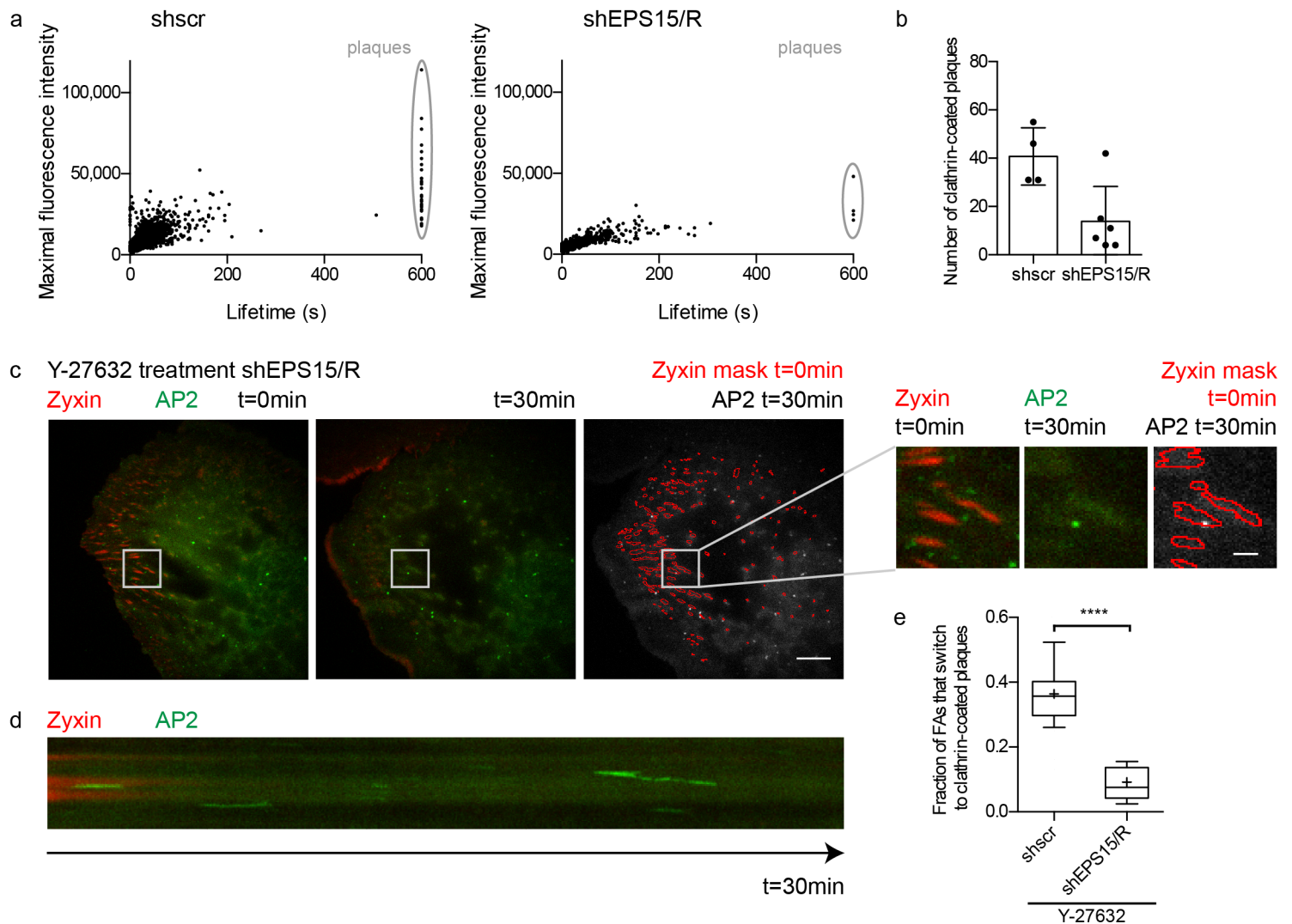
893 (a) Live-cell confocal spinning disc microscopy of representative U373 cell stably
894 expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red) treated with
895 Y-27632 (10 μ M). Cell before (left) and 30 minutes after drug treatment (middle).
896 (Right) Merged images of a mask marking the mCherry-zyxin objects before (red) and the
897 AP2-eGFP signal after Y-27632 treatment (green). Right: Zoom in of FAs that switch to
898 clathrin-coated plaques during the treatment. (b) Kymograph of the switch from FAs to
899 clathrin-coated plaques shown in b over 30 minutes of Y-27632 treatment.
900 (c) Quantification of FA replacement by clathrin structures during Y-27632 treatment
901 shown in a. Shown are the numbers of FAs that did not get replaced by clathrin structures
902 (white), that got replaced by transient (<10min) clathrin structures (grey) or long-lived
903 (>10min) clathrin-coated plaques (black). (d) Quantification of the switch from FAs to
904 clathrin-coated plaques during Y-27632 treatment. Whiskers represent 10-90 percentile,
905 box represents second and third quartile, line marks the median and cross the mean.
906 Results are computed from three repetitions. Statistical analysis: t test, n=28, P<0.01.
907 (e) Representative image from TIRF microscopy of U373 stably expressing AP2-eGFP
908 (green) immunostained for integrin $\alpha\beta$ 5 (red) treated with Y-27632 for 20 minutes. Right:
909 Zoom in of clathrin-coated plaques at the periphery. Scale bars: 10 μ m (overviews) or 2 μ m
910 (zoom). (f) Quantification of the colocalization between $\alpha\beta$ 5 integrin and AP2-eGFP. The
911 graph shows the distribution of the Pearson correlation coefficient between AP2-eGFP and
912 integrin signals in the whole cell either non treated (control) or treated with Y-27632 for 20
913 min. Whiskers represent 10-90 percentile, box represents second and third quartile, line
914 marks the median and cross the mean. Results are computed from ten cells. (g) Live-cell
915 confocal spinning disc microscopy of U373 cells stably expressing AP2-eGFP (green) and
916 transiently expressing mCherry-zyxin (red) on coverslips coated with Alexa Fluor 647-
917 labelled gelatin (grey) treated with Y-27632 (10 μ M). (Left) Overview of a representative
918 cell before treatment, (right) Zoom in of FAs that switch to clathrin-coated plaques during
919 the treatment on gelatin digestions. Scale bar: 10 μ m (overview), 2 μ m (zoom).
920 (h) Quantification of the percentage of switching FAs under Y-27632 treatment for FAs
921 without and with gelatin digestion. Shown is the mean with SD. Statistical analysis: paired t
922 test, n=7, P<0.05. (i) Images of widefield microscopy of coverslips coated with Alexa Fluor
923 647-labelled gelatin with representative digested areas generated by U373 wild-type (WT),

924 stable knock down for MMP14 (shMMP14), or stable overexpression of MMP14 (MMP14)
925 on (red). Scale bar: 10 μ m. (j) Analysis of the degree of gelatin digestion by U373 WT,
926 shMMP14 and MMP14. (k) Quantification of the switch from FAs to clathrin-coated plaques
927 after 20 minutes of Y-27632 treatment of U373 knock down for or overexpressing MMP14.
928 Whiskers represent 10-90 percentile, box represents second and third quartile, line marks
929 the median and cross the mean. Results are computed from three repetitions. Statistical
930 analysis: t test, n=28 (shscr), n=50 (shMMP14), and n=24 (MMP14), P<0.05.



931 **Figure 6: Induction of clathrin-coated plaques by topographical cues.**

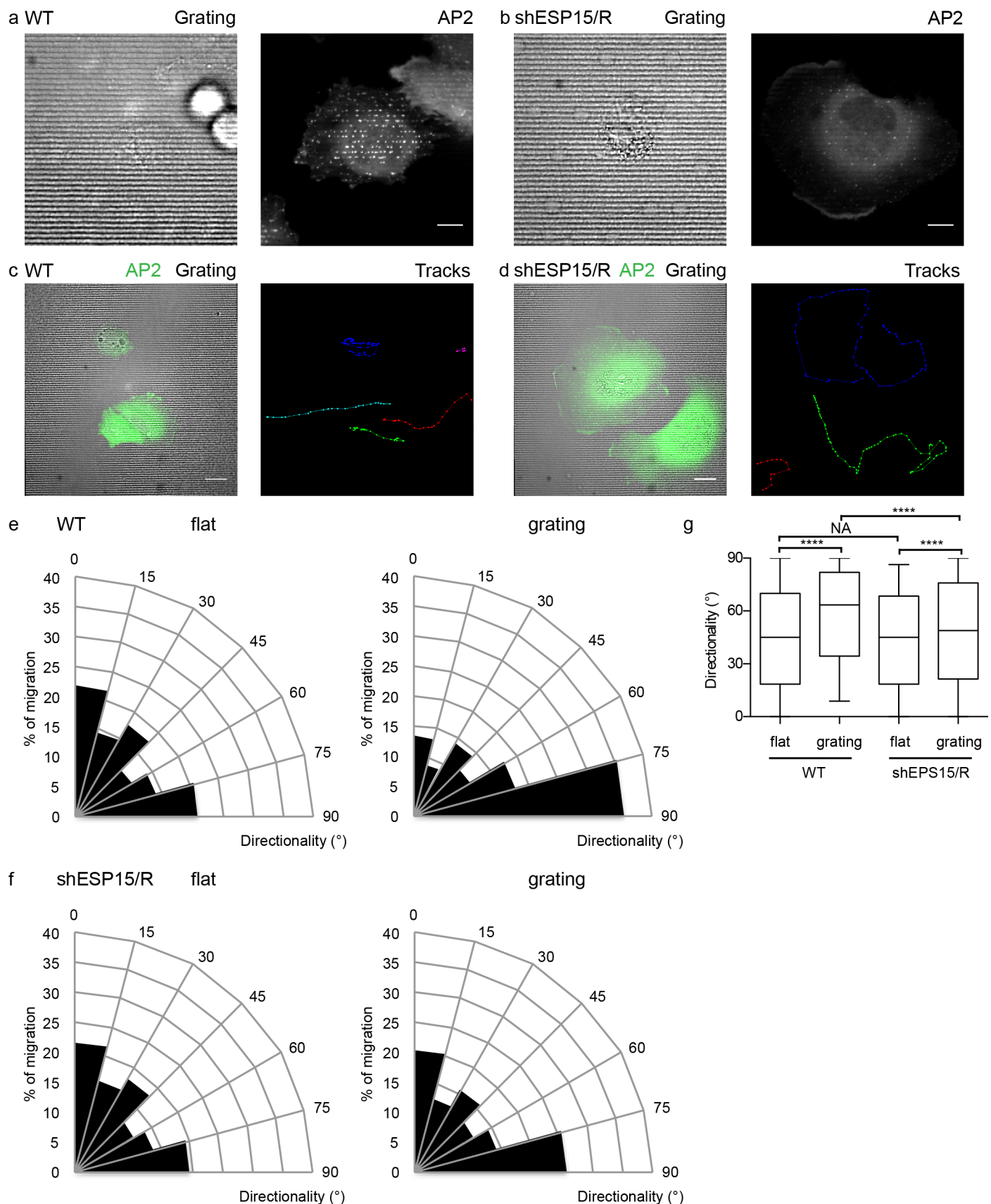
932 (a) Spinning disc confocal microscopy of U373 stably expressing AP2-eGFP (right) growing
933 on optically clear 3D-micropatterns (DIC, left). Box marks region used in b. (b) X-projection
934 of AP2-eGFP signal. (c) Left: Normalized intensity profile from the AP2-eGFP x-projection
935 shown in b. (d) Live-cell spinning disc confocal microscopy of U373 stably expressing AP2-
936 eGFP (middle) growing on optically clear 3D-micropatterns (DIC, left). (e) Left: Overview of
937 x/y-position of the tracking results from the cell shown in d. CME tracks were categories as
938 pits (transient, black dots) and plaques (persistent for > 10 minutes, red squares). CME
939 tracks were colour-coded according to their lifetime from yellow (0 s) to red (600 s). Scale
940 bars: 10 μm .



941 **Figure 7: Eps15/R are regulators of clathrin-coated plaques formation.**

942 (a) Lifetime versus maximal fluorescence intensity of AP2-eGFP of CME tracks for U373
 943 stably expressing scrambled shRNA shscr (left) and shEPS15/R (right). Persistent clathrin-
 944 coated plaques are marked by a grey circle. Shown are plots of a representative cell of each
 945 cell line. Each dot represents one tracked CME event of a 10 minute long movie. (b)
 946 Quantification of the number of clathrin-coated plaques in U373 stably expressing shscr and
 947 shEPS15/R cells. Shown is the mean with SD computed from the tracking results of at least
 948 four cells. Dots represent the results of each cell. (c) Live-cell confocal spinning disc
 949 microscopy of representative U373 shEPS15/R cell stably expressing AP2-eGFP (green) and
 950 transiently expressing mCherry-zyxin (red) treated with Y-27632 (10 μ M). Cell before (left)
 951 and 30 minutes after drug treatment (middle). (Right) Merged images of a mask marking

952 the mCherry-zyxin objects before (red) and the AP2-eGFP signal after Y-27632 treatment
953 (green). Right: Zoom on FAs that disassemble during the treatment. (d) Kymograph of the
954 disassembly of FAs shown in b over 30 minutes of Y-27632 treatment. (e) Quantification of
955 the switch from FAs to clathrin-coated plaques after 20 minutes of Y-27632 treatment of
956 U373 shEPS15/R. Whiskers represent 10-90 percentile, box represents second and third
957 quartile, line marks the median and cross the mean. Results are computed from three
958 repetitions. Statistical analysis: t test, n=38 (shscr), n= 29 (shEPS15/R), P<0.01. Scale bars:
959 10 μm (overviews) or 2 μm (zooms).



960 **Figure 8: Clathrin-coated plaques establish directionality during contact guidance-**

961 **mediated cell migration.**

962 U373 WT (a) and shEPS15/R (b) stably expressing AP2-eGFP seeded on optically clear 3D-
963 micropattern. Scale bar: 10 μm . Live-cell spinning disc confocal microscopy of U373 WT (c)
964 and shEPS15/R (d) stably expressing AP2-eGFP (green) on optically clear 3D-micropattern
965 (grey, DIC). Migration tracks of cells are shown by multi-coloured dots and lines. Scale bar:
966 20 μm . Angular histogram of the directionality of migration for WT (e) and shEPS15/R (f)
967 cells on flat substrate (left) or grating (right). (g) Distribution of directionality of cells
968 migrating on flat substrate or 3D-micropattern. Whiskers represent 10-90 percentile, box
969 represents second and third quartile, and the line marks the median. Statistical analysis:
970 nonparametric t test (Mann-Whitney test), $P < 0.01$. Directionality was calculated between
971 time frames with a rate of 10 to 13 minutes. Results are computed from three repetitions.
972 Number of tracks: WT: n=223 (flat), n=276 (grating), shEPS15/R: n=174 (flat),
973 n=155 (grating).

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