1 # Supplementary Information: Focal adhesion-generated cues in
2 extracellular matrix regulate cell migration by local induction of
3 clathrin-coated plaques

4 ***Supplementary Movies***

5 **Supplementary Movie 1: Switch from FAs to clathrin-coated plaques during cell**
6 **migration.** Live-cell confocal spinning disc microscopy of U373 stably expressing AP2-
7 eGFP (green) and transiently expressing mCherry-zyxin (red). Scale bar: 10µm. Movie time
8 is displayed in the top left corner.

9 **Supplementary Movie 2: Disassembly of clathrin-coated plaques by integrin**
10 **inhibition.** Live-cell confocal spinning disc microscopy of U373 stably expressing AP2-eGFP
11 treated with Cilengitide (10 µM) for 20 minutes. Scale bar: 10µm. Movie time is displayed in
12 the top left corner.

13

14 **Supplementary Movie 3: Switch from FAs to clathrin-coated plaques at local gelatin**
15 **digestions during cell migration.** Live-cell confocal spinning disc microscopy of U373
16 stably expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red) on
17 coverslips coated with Alexa Fluor 647-labelled gelatin (blue). Scale bar: 10µm. Movie time
18 is displayed in the top left corner.

19

20 **Supplementary Movie 4: Clathrin-coated plaque formation at local gelatin digestions.**
21 Live-cell confocal spinning disc microscopy of U373 stably expressing AP2-eGFP (green) on
22 coverslips coated with Alexa Fluor 647-labelled gelatin (blue). Scale bar: 10µm. Movie time
23 is displayed in the top left corner.

24

25 **Supplementary Movie 5: Formation of clathrin-coated plaques in two migrating cells**
26 **at the same local gelatin digestions.** Live-cell confocal spinning disc microscopy of two
27 U373 stably expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red)
28 on coverslips coated with Alexa Fluor 647-labelled gelatin (blue). Scale bar: 10µm. Movie
29 time is displayed in the top left corner.

30

31 **Supplementary Movie 6: Switch from FAs to clathrin-coated plaques during ROCK**
32 **inhibition.** Live-cell confocal spinning disc microscopy of representative U373 cell stably
33 expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red) treated with
34 Y-27632 (10 µM) for 30 minutes. Scale bar: 10µm. Movie time is displayed in the top left
35 corner.

36

37 **Supplementary Movie 7: Switch from FAs to clathrin-coated plaques during myosin-II**
38 **inhibition.** Live-cell confocal spinning disc microscopy of representative U373 cell stably
39 expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red) treated with
40 Blebbistatin (20 µM) for 30 minutes. Scale bar: 10µm. Movie time is displayed in the top left
41 corner.

42

43 **Supplementary Movie 8: Clathrin-coated plaques form at topographical cues.** Spinning
44 disc confocal microscopy of U373 stably expressing AP2-eGFP (right) growing on optically
45 clear 3D-micropatterns (DIC, left). Scale bar: 10µm. Movie time is displayed in the top left
46 corner.

47

48 **Supplementary Movie 9: FAs disassembly in U373 shEPS15/R during ROCK inhibition.**
49 Live-cell confocal spinning disc microscopy of representative U373 shEPS15/R cell stably
50 expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red) treated with
51 Y-27632 (10 μM) for 30 minutes. Scale bar: 10μm. Movie time is displayed in the top left
52 corner.

53

Supplementary Materials and Methods

EM and CLEM of clathrin structures using metal replicas of unroofed membrane sheets

EM and CLEM of clathrin structures were performed as previously described[1]. Cells were seeded on PDL-coated coverslips (25 mm). Unroofing was performed 16 hours after seeding. The cells were washed three times with stabilisation buffer (30 mM HEPES pH 7.4, 70 mM KCl, 5 mM $MgCl_2$). Unroofing was performed in 2% paraformaldehyde (PFA) in stabilisation buffer using two short sonication pulses. Samples were immediately put into fresh 2 % PFA solution and fixed for 20 minutes at room temperature.

For CLEM, widefield epifluorescence microscopy of unroofed plasma membranes from AP2-eGFP expressing cells was performed with a Nikon N-STORM microscope with a 100x oil immersion objective and an EMCCD camera (Andor Ixon Ultra DU-897). To cover an area of 1 mm^2 a montage of 15 x 15 images with an overlap of 15% for stitching was taken. The imaged area was marked with a circle (4 mm in diameter) around the centre of the imaged area using an objective diamond scriber. The immersion oil was carefully removed from the bottom of the glass coverslip and the sample was prepared for EM.

Coverslips with unroofed membranes were fixed with 2% glutaraldehyde in PBS overnight. Samples were incubated with 0.1% tannic acid for 20 minutes at room temperature. After four washes with water, the samples were incubated with 0.1% uranyl acetate for 20 minutes at room temperature. After two washes with water, samples were dehydrated with a series of ethanol solutions (15% - 100%). Samples were placed in each ethanol solution for 5 minutes. After replacing the 100% ethanol solution, the samples where dried in a critical point dryer. The samples were then put under vacuum until they were coated. The samples were coated with JFDV JOEL Freeze Fracture Equipment with a first layer of platinum with an angle of 17 while rotating and with a second layer of carbon with an angle of 90° while rotating. For better orientation, the marked area of the coated samples were imaged with a phase contrast microscope. The samples were then cut to fit on EM grids (TED PELLA, 75 Mesh Copper, Support Films Formvar/Carbon). 5% hydrofluoric acid was used to remove the glass from the metal replica. The floating metal replica was extensively washed with water and then carefully placed on a glow discharged EM grid.

84 Samples were dried on filter paper and again imaged with a phase contrast microscope.
85 TEM imaging was performed using a JEOL 1400 equipped with SerialEM freeware for
86 montaging. Montages of large membrane sheets at 12,000 magnification (1.82 nm per pixel)
87 with 10% overlap were imaged.

88 ### *Transformation of images for CLEM*

89 Transformation of images for CLEM was performed as previously described[1]. The FM image
90 and the EM montage of the same membrane sheet were first manually and roughly overlaid
91 using Photoshop. MATLAB was used to transform the fluorescence image according to the
92 EM montage using three manually identified clathrin-coated structures. For the
93 transformation, the centre of the clathrin structure in the electron microscope montage and
94 the centre of the fluorescence signal determined by a Gaussian fit were used as landmarks.

95 ### *TEM and CLEM analysis of clathrin coats*

96 Analysis of TEM and CLEM data was performed as previously described[1]. For the TEM
97 analysis, clathrin-coated structures were manually identified. The projected area was
98 measured by outlining their surrounding using ImageJ/Fiji (https://fiji.sc). Classification
99 into flat, dome, pit and flat with budding dome/pit structures was done by visual evaluation
100 of their curvature. Flat structures could be distinguished from curved structures. Within the
101 curved coats, pit structures were discriminated from dome structures by the existence of a
102 highly contrasted surrounding ring, which represents a constricted neck. The circularity of
103 the outlines of clathrin-coated structures was measured using the shape descriptors tool in
104 ImageJ/Fiji. For the CLEM analysis, the fluorescent intensity was correlated to clathrin-
105 coated structures identified in TEM.

106 ### *Live-cell microscopy of CME at bottom and top plasma membrane*

107 For live-cell microscopy of CME at the bottom and top plasma membrane, cells stably
108 expressing AP2-eGFP were seeded on PDL-coated coverslips. Cleaned coverslips were
109 sterilized with ethanol and washed three times with PBS. Coverslips were inverted on a
110 drop of PDL hydrobromide (mol wt 70,000 - 15,000, 0.01% (w/v) in H_2O; Sigma, P6407)
111 and incubated for 5 minutes at room temperature followed by three washes with H_2O. CME
112 dynamics at bottom and top plasma membrane of the same cell were imaged with live-cell

113 spinning disc confocal microscopy 12 hours post-seeding. Therefore, first AP2-eGFP
114 dynamics were monitored for 10 minutes with a frame rate of 5 seconds at the bottom
115 plasma membrane. Afterwards CME dynamics of the top plasma membrane were imaged by
116 taking a z-stack of five images covering 2 μm in height of the very top of the cell with a rate
117 of 5 seconds between the z-stacks. Maximum projections of each z-stack were generated
118 and further used for tracking of CME at the top plasma membrane.

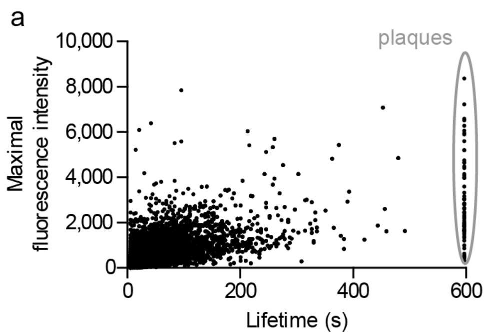
119 ***Generation of adhesive micropatterns by photo-patterning***

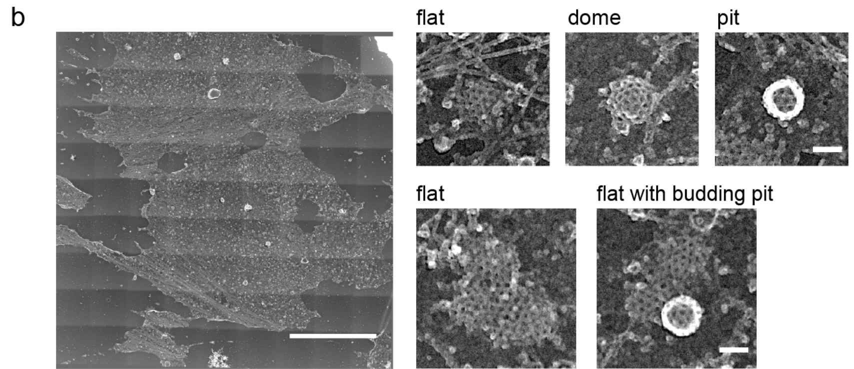
120 Adhesive micropatterns have been generated by photo-patterning as previously described[2].
121 In brief, an UVO-cleaner (Model 342, Jelight) was used to illuminate samples with deep UV.
122 A synthetic quartz mask with customized features was purchased from Toppan. Coverslips
123 were activated by illuminating with deep UV for 10 minutes. For passivation, the coverslips
124 were inverted on a drop of 0.1 mg/ml Poly(L-lysine)-graft-poly(ethylene glycol) co-polymer
125 (PLL-g-PEG) (SuSoS Surface Technology, PLL(20)-g[3.5]-PEG(2)) in 10 mM HEPES (pH 7.4)
126 (50 μl for 12 mm diameter coverslips; 100 μl for 24 mm diameter coverslips) and incubated
127 for 30 minutes. To visualize passivated areas, a 1:1 mix of unlabelled and Atto633-labelled
128 PLL-g-PEG (SuSoS Surface Technology, PLL(20)-g[3.5]-PEG(2)-Atto633) was used.
129 Passivated coverslips were washed with H_2O for 10 minutes. Before usage the quartz mask
130 was washed with acetone and isopropanol and dried. The quartz mask was activated by
131 illuminating with deep UV for 5 minutes on the metal bearing side to clean the surface and
132 make it hydrophilic. A drop of 4 μl of millipore H_2O was put on the quartz mask and the
133 coverslip was placed on the drop with the pegylated side facing down. Air bubbles were
134 removed by pressing the coverslip against the mask. Deep UV was illuminated for 3 minutes
135 through the mask with the quartz side facing the light source. After illumination, an excess
136 of H_2O was used to remove coverslips from the quartz mask by floating. The coverslips were
137 washed with PBS and directly used for experiments.

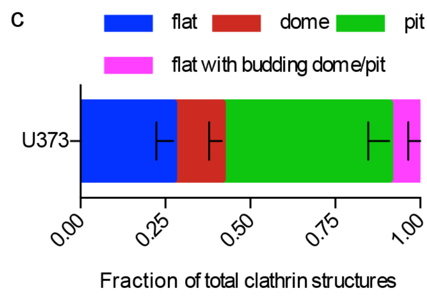
138 ***Surface topography measurements using AFM***

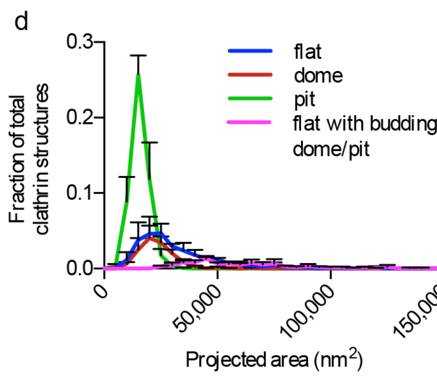
139 AFM measurements were performed on a MultiMode 8 (Bruker). For topography
140 measurements, an area of 12 μm x 12 μm of the surface was scanned in peak force tapping
141 mode with a scanasyst-air cantilever (resonant frequency 70 kHz, spring constant 0.4 N/m,
142 tip radius 5-10 nm; Bruker). AFM images were processed using gwyddion

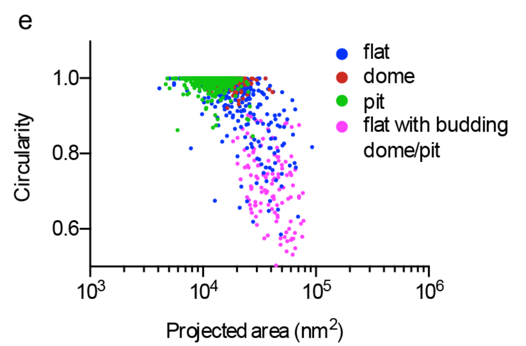
143 (http://gwyddion.net). Line profiles were computed by averaging profiles of 1-3 μm along
144 the gratings.

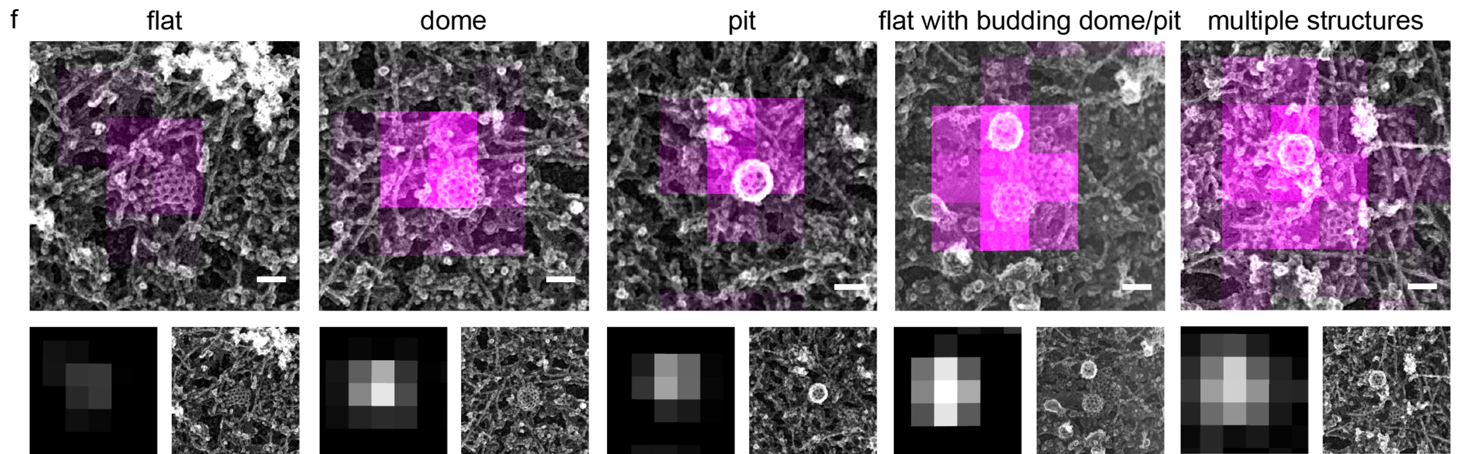

a
plaques
Maximal fluorescence intensity
10,000
8,000
6,000
4,000
2,000
0
200
400
600
Lifetime (s)

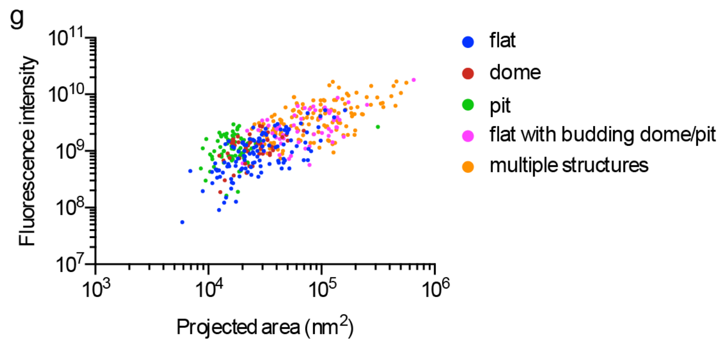

b
flat
dome
pit
flat
flat with budding pit


c
flat
dome
pit
flat with budding dome/pit
U373
0.00
0.25
0.50
0.75
1.00
Fraction of total clathrin structures


d
Fraction of total clathrin structures
0.3
0.2
0.1
0.0
flat
dome
pit
flat with budding dome/pit
0
50,000
100,000
150,000
Projected area (nm2)

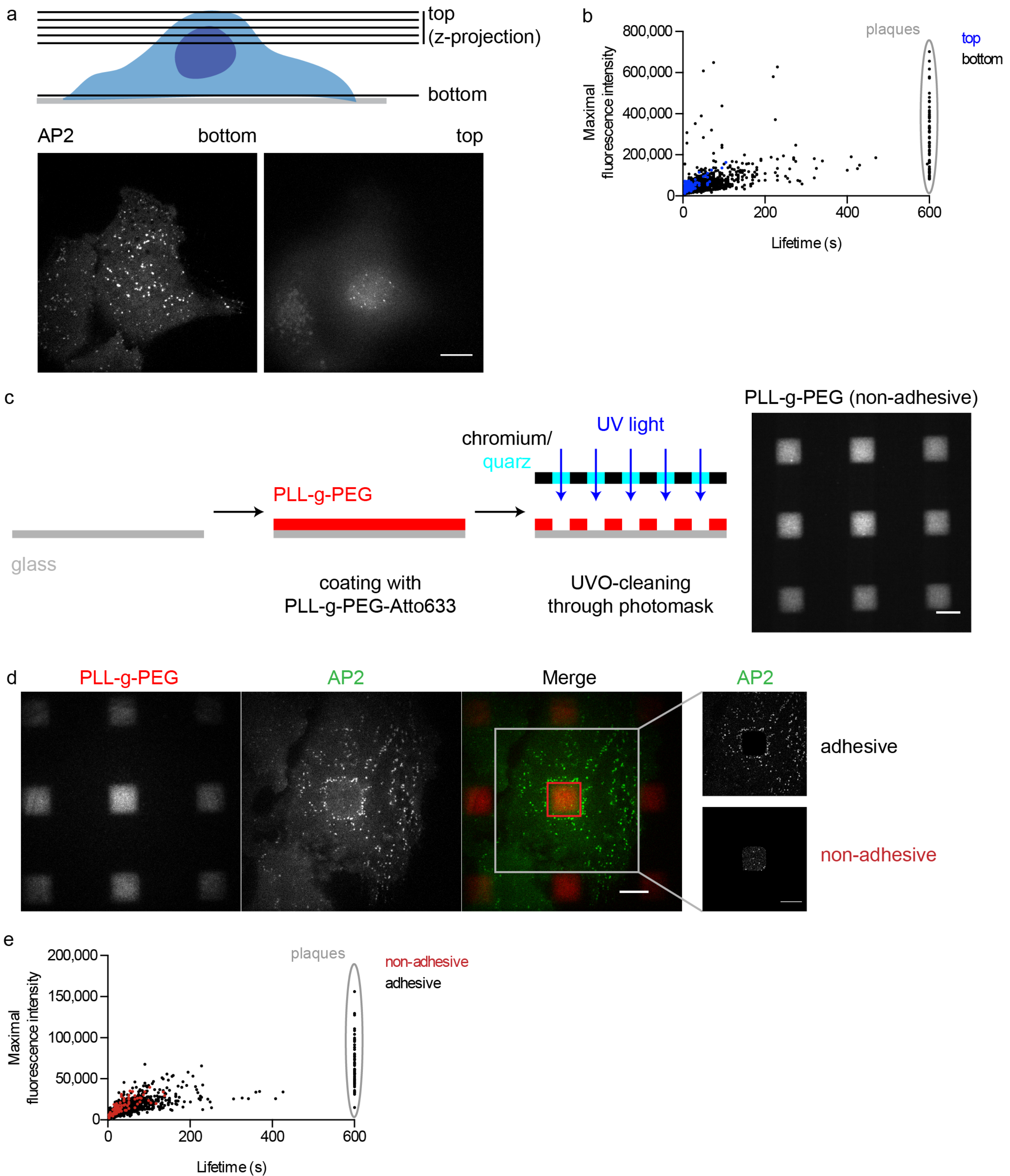

e
Circularity
1.0
0.8
0.6
flat
dome
pit
flat with budding dome/pit
Projected area (nm2)


f
flat
dome
pit
flat with budding dome/pit
multiple structures


g
Fluorescence intensity
flat
dome
pit
flat with budding dome/pit
multiple structures
Projected area (nm2)

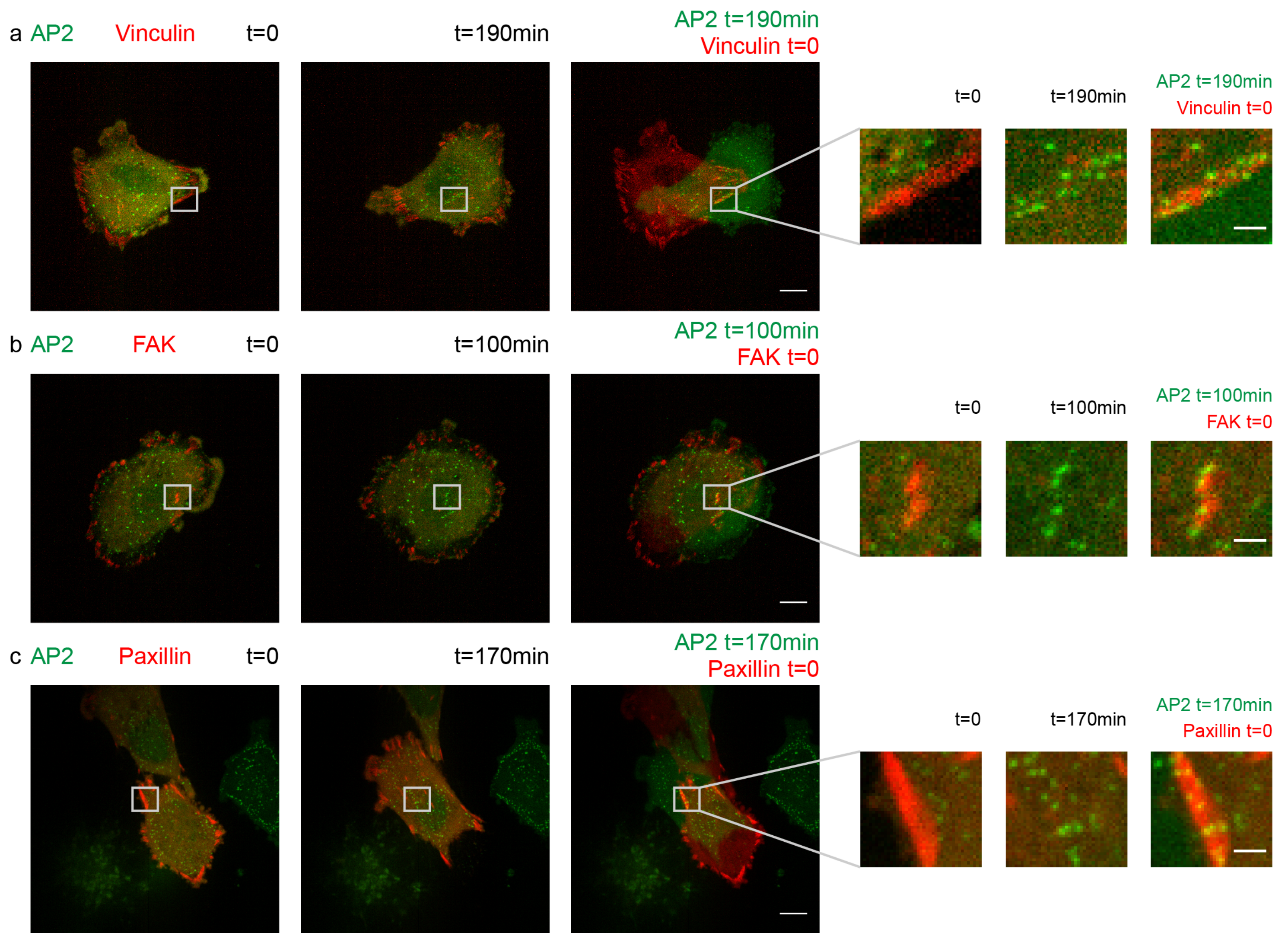
146 **Supplementary Figure 1: Dynamical and morphological characterization of clathrin**
147 **structures in U373 cells.**

148 (a) Lifetime versus maximal fluorescence intensity of AP2-eGFP of CME in U373 cells.
149 Persistent clathrin-coated plaques are marked by a grey circle. Shown is a plot of a
150 representative cell. Each dot represents one tracked CME event of a 10 minute long movie.
151 (b) TEM of metal replicas of unroofed plasma membrane sheets from of U373. Left:
152 Overview of whole membrane. Right: Zooms on representative examples of morphological
153 categories found in the U373 cells: Flat, dome, pit, and flat with budding dome/pit. Scale
154 bar: 10 µm (overview); 100 nm (zooms). (c) Fraction of flat (blue), dome (red), pit (green),
155 and flat with budding dome/pit (magenta) clathrin-coated structures on plasma membrane
156 sheets. Results are calculated from four different membranes. Total number of clathrin-
157 coated structures: 3,627. Means with SD are shown. (d) Projected area distribution of the
158 different clathrin morphologies found on plasma membrane sheets of U373. Means with SD
159 are shown. (e) Circularity and size of clathrin-coated structures with different
160 morphologies. Each dot represents one clathrin coat. Shown are the results of one
161 representative plasma membrane sheet. (f) Examples of flat, dome, pit, flat with budding
162 dome/pit, and multiple structures, which cannot be distinguished by FM, observed with
163 CLEM. Top panel: CLEM, lower left: fluorescence microscopy, lower right: TEM. Scale bar:
164 100 nm. This panel has been adapted from Bucher *et al.*, 2018[1]. Published by Springer
165 Nature Limited under a Creative Commons Attribution 4.0 International Licence
166 (http://creativecommons.org/licenses/by/4.0/). (g) Correlation of size and AP2-eGFP
167 fluorescence intensity of all clathrin structures categorized by their morphologies
168 (flat (blue), dome (red), pit (green), flat with budding dome/pit (magenta), and multiple
169 structures (orange)). Graph shows representative CLEM analysis of one plasma membrane
170 sheet of U373. Total number of clathrin structures: 427.



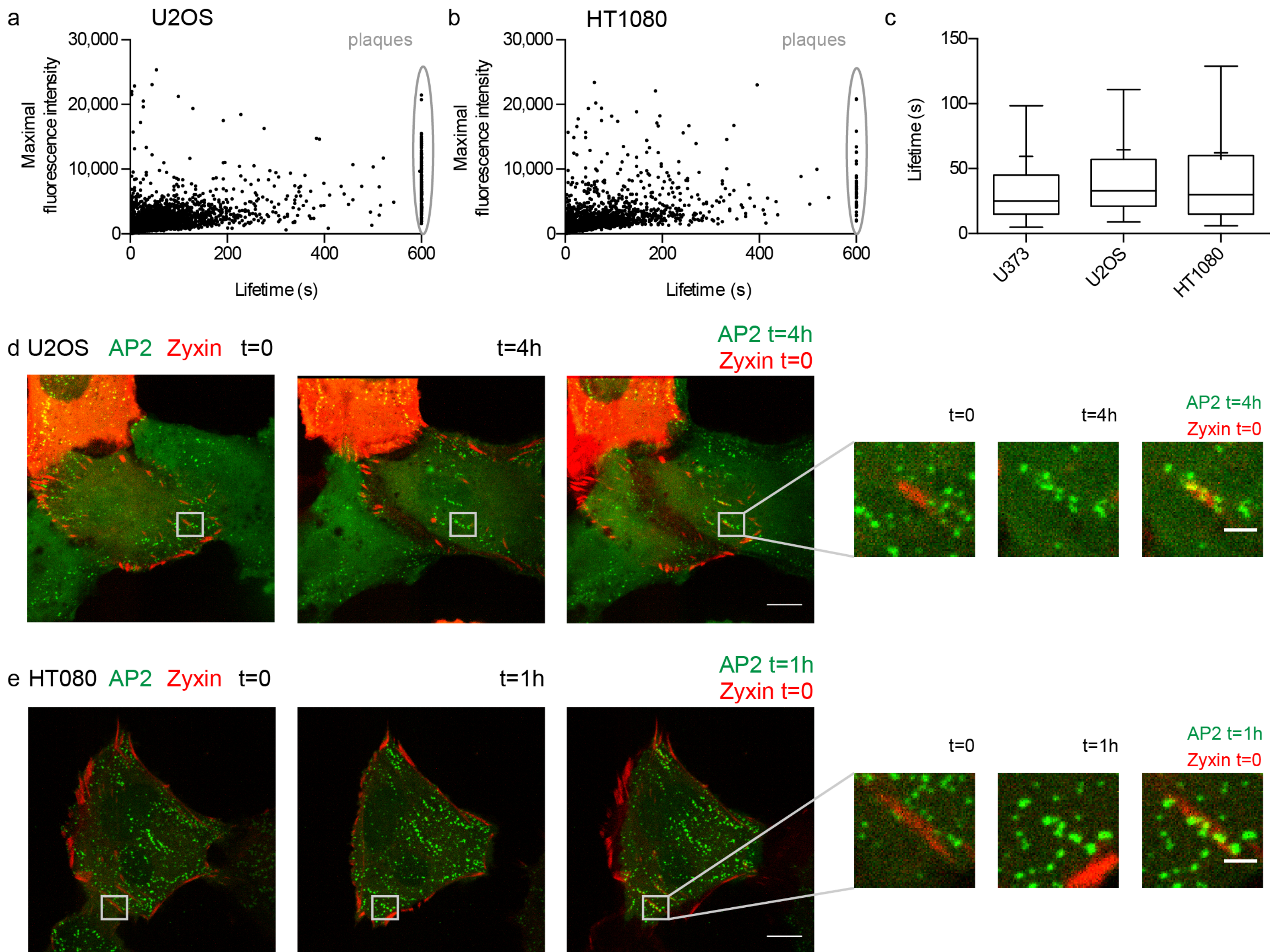
171 **Supplementary Figure 2: Clathrin-coated plaques form exclusively at attached plasma**
172 **membrane parts.**

173 (a) Schematic of imaging of CME at bottom and top plasma membrane. AP2-eGFP was
174 imaged at the bottom plasma membrane in a single focal plane, for 10 minutes with a frame
175 rate of 5 seconds. The top plasma membrane was imaged by taking a z-stack of 5 images
176 with a spacing of 0.5 µm at the very top of the same cell. For tracking of CME on the top
177 plasma membrane, a maximal intensity z-projection was used. Scale bar: 10 µm. (b) Lifetime
178 versus maximal fluorescence intensity of AP2-eGFP of CME tracks from the top (blue) and
179 the bottom (black) plasma membrane of a representative U373 cell. Each dot represents
180 one tracked CME event of a 10 minute long movie. Persistent clathrin-coated plaques are
181 marked by a grey circle. Number of tracks at bottom PM: 1,874. Number of tracks at top PM:
182 118. (c) Schematic illustrates the production of adhesive micropatterns using photo-
183 patterning. First, glass coverslips were passivated by coating with Atto633-labelled PLL-g-
184 PEG. Spatially controlled UVO-cleaning through a photomask was performed to generate a
185 micropattern of adhesive and non-adhesive regions. Right: Image of the adhesive
186 micropattern used in this experiment consisting of 10 µm x 10 µm squares of PLL-g-PEG
187 with a spacing of 10 µm. Scale bar: 10 µm. (d) Example of a U373 cell stably expressing AP2-
188 eGFP (green) growing on an adhesive micropattern of Atto633-labelled PLL-g-PEG (red).
189 Adhesive (black) and non-adhesive (red) areas separated by the PLL-g-PEG signal for
190 further analysis of CME dynamics. Scale bars: 10 µm. (e) Lifetime versus maximal
191 fluorescence intensity of AP2-eGFP of CME tracks from adhesive (black) and non-
192 adhesive (black) areas of a representative cell. Each dot represents one tracked CME event
193 of a 10 minute long movie. Persistent clathrin-coated plaques are marked by a grey circle.
194 Number of tracks at the attached plasma membrane part: 2,849, at the non-attached plasma
195 membrane part: 87.



196 **Supplementary Figure 3: Different FA markers show switch from FAs to clathrin-**
197 **coated plaques during cell migration.**

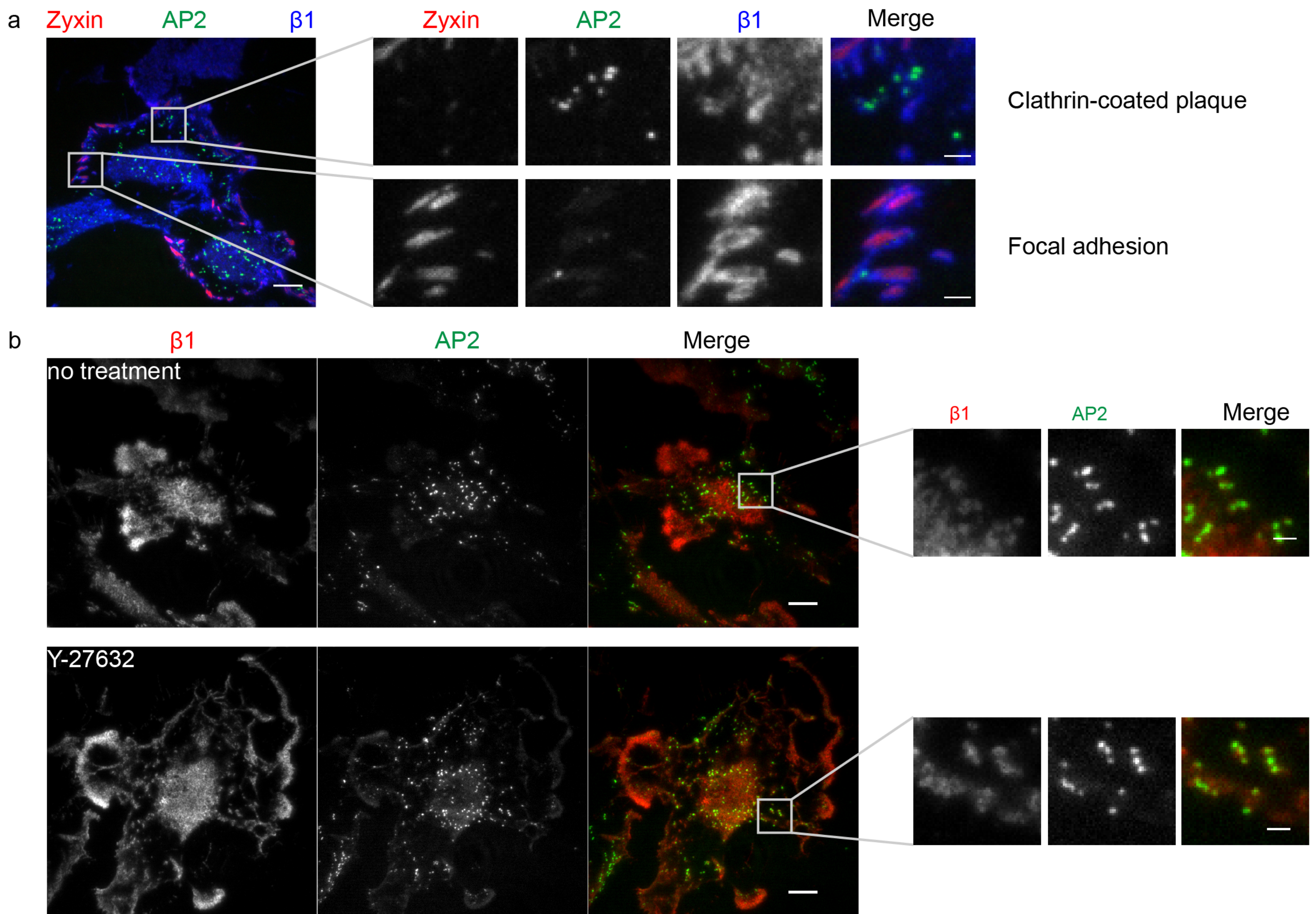
198 Live-cell confocal spinning disc microscopy of U373 stably expressing AP2-eGFP (green)
199 and transiently expressing FA-associated proteins fused to the fluorescent protein mCherry
200 (red): mCherry-vinculin (a), mCherry-FAK (b), or mCherry-paxillin (c). Left: Overview of a
201 representative migrating cell at time 0 and a later time point (190, 100, or 170 minutes,
202 respectively). Merged images of the FA protein signal at time 0 (red) and the AP2-eGFP
203 signal at the later time point (green). Right: Zoom on FAs that switch to clathrin-coated
204 plaques. Scale bars: 10 µm (overview), 2 µm (zoom).



205 **Supplementary Figure 4: Switch from FAs to clathrin-coated plaques in U2OS and**
206 **HT1080 cells.**

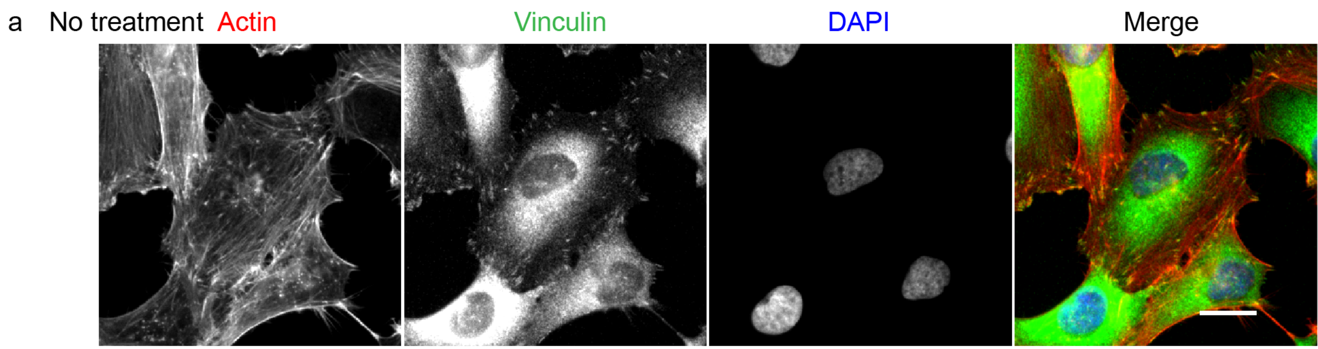
207 Lifetime versus maximal fluorescence intensity of AP2-eGFP of CME tracks for U2OS (a) and
208 HT1080 (b). Shown are plots of a representative cell. Each dot represents one CME event in
209 a 10 minute long movie. Number of tracks: 5,492 (U2OS) and 3,287 (HT1080). (c) Box and
210 whiskers plot showing the lifetime distributions of CME in U373, U2OS, and HT1080
211 computed from three to four cells for each cell line. Whiskers represent 10-90 percentile,
212 box represents second and third quartile, the line marks the median, and the cross marks
213 the mean. Number of tracks: 4,472 (U373), 19,366 (U2OS), and 7,144 (HT1080). Live-cell
214 confocal spinning disc microscopy of U2OS (d) and HT1080 (e) stably expressing AP2-
215 eGFP (green) and transiently expressing mCherry-zyxin (red). Left: Overview of a

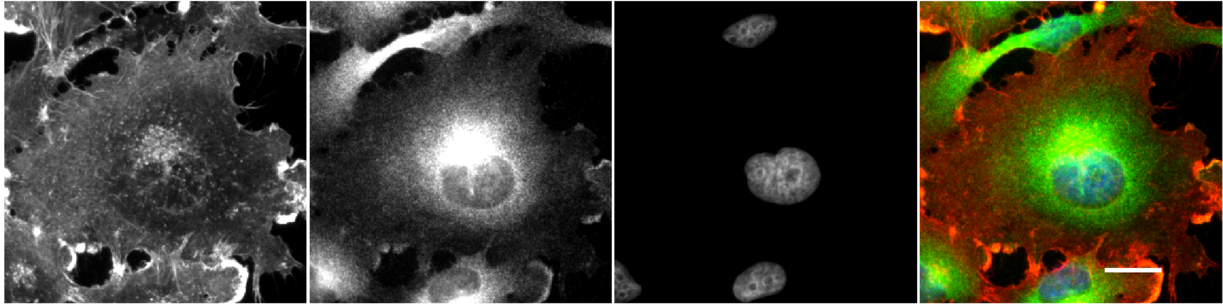
216 representative migrating cell at time 0 and a later time point (4 hours and 1 hour for U2OS
217 and HT1080, respectively). Merged image of the mCherry-zyxin signal at time 0 (red) and
218 the AP2-eGFP signal at the later time point (green). Right: Zoom on FAs that switch to
219 clathrin-coated plaques. Scale bars: 10 µm (overview), 2 µm (zoom).

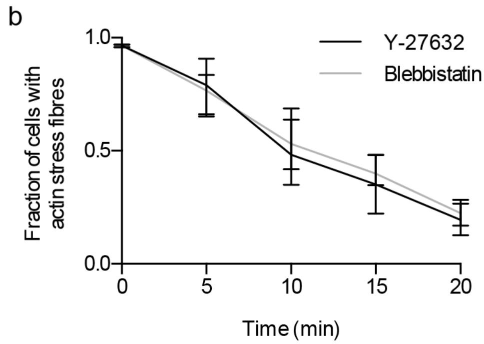


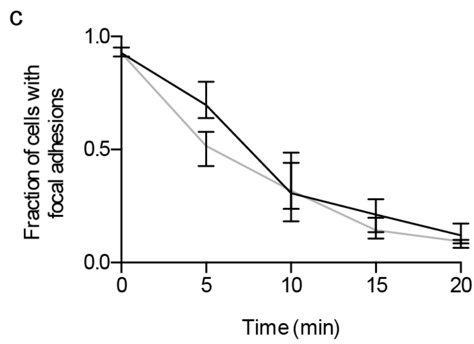
220 **Supplementary Figure 5: Enrichment of β1 integrin at clathrin-coated plaques.**

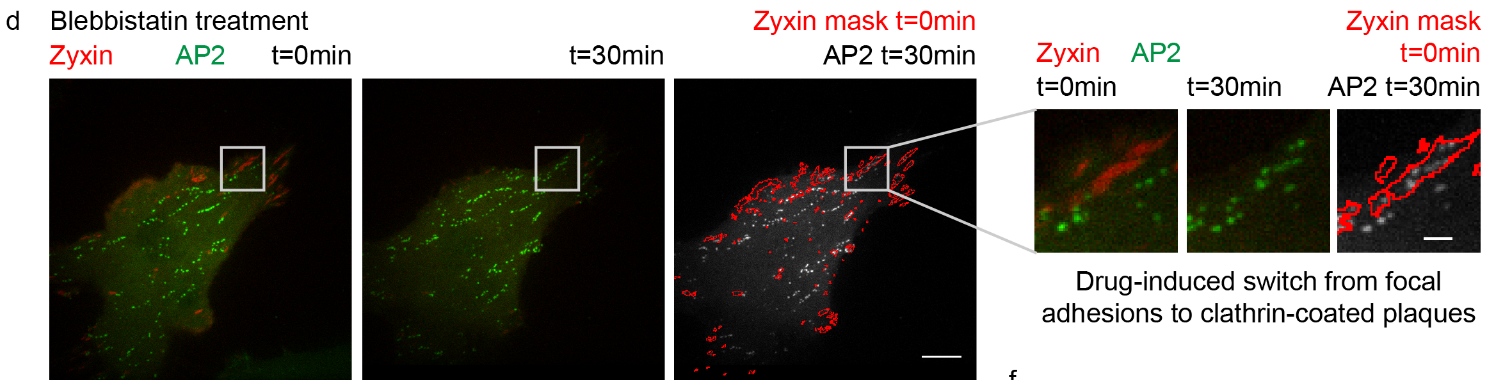
221 (a) Representative image from total internal reflection fluorescence (TIRF) microscopy of
222 U373 stably expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red)
223 immunostained for β1 integrin (blue). Right: Zoom on clathrin-coated plaques marked by
224 bright AP2-eGFP signal (upper panel) and on FAs marked by mCherry-zyxin (lower panel).
225 (b) Representative image from TIRF microscopy of U373 stably expressing AP2-eGFP
226 (green) immunostained for integrin β1 (red) untreated (upper panel) or treated with
227 Y25632 (10 µM) for 30 minutes (lower panel). Right: Zoom on clathrin-coated plaques.
228 Scale bars: 10 µm (overview), 2 µm (zoom).

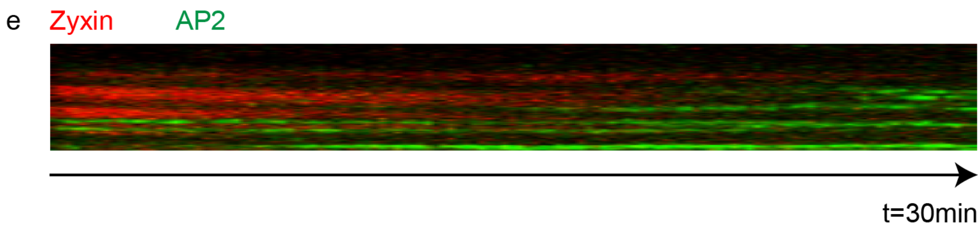

a
No treatment
Actin
Vinculin
DAPI
Merge

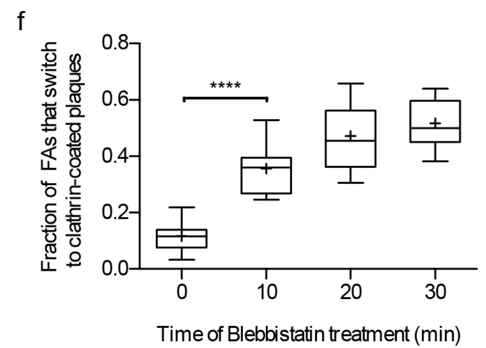

Inhibitor treatement

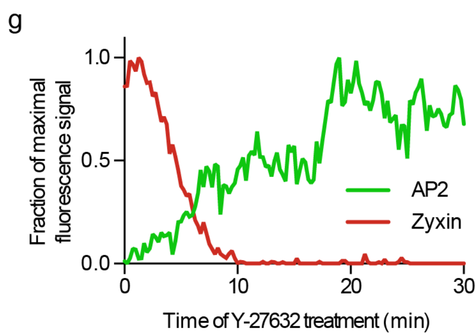

b
Fraction of cells with actin stress fibres
Time (min)
Y-27632
Blebbistatin

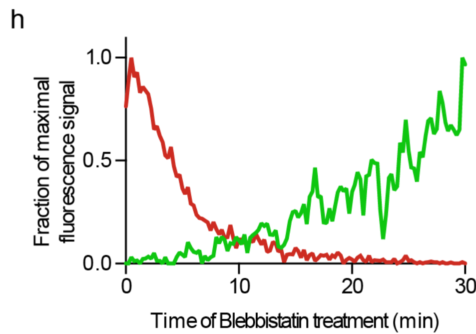

c
Fraction of cells with focal adhesions
Time (min)


d
Blebbistatin treatment
Zyxin
AP2
t=0min
t=30min
Zyxin mask t=0min
AP2 t=30min
Drug-induced switch from focal adhesions to clathrin-coated plaques


e
Zyxin
AP2
t=30min

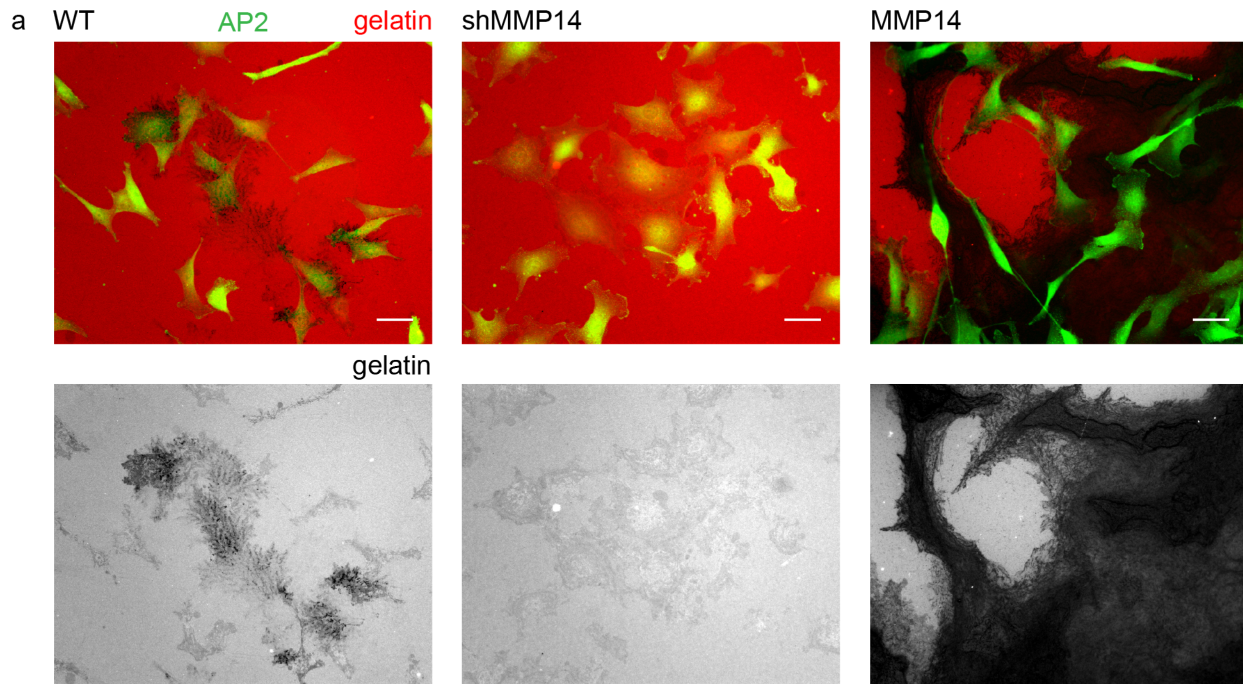

f
Fraction of FAs that switch to clathrin-coated plaques
****
Time of Blebbistatin treatment (min)

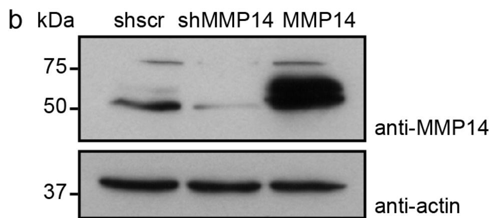

g
Fraction of maximal fluorescence signal
AP2
Zyxin
Time of Y-27632 treatment (min)


h
Fraction of maximal fluorescence signal
Time of Blebbistatin treatment (min)

230 **Supplementary Figure 6: Drug-induced FA disassembly leads to switch to clathrin-**
231 **coated plaques.**

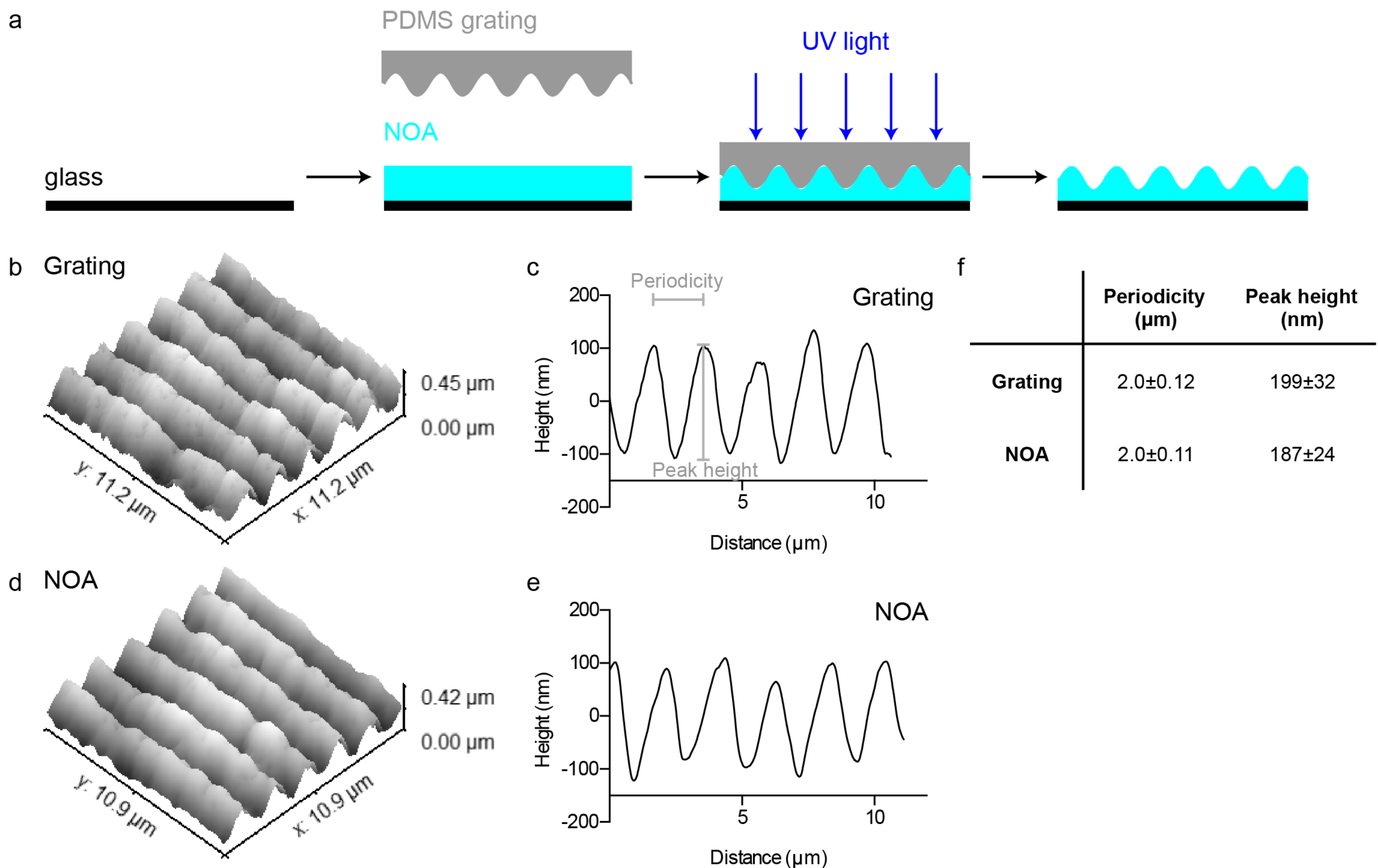
232 (a) Representative images of widefield microscopy of untreated (upper panel) and inhibitor
233 treated (lower panel) U373 cells stained for actin with Alexa Fluor 647-labelled
234 phalloidin (red), vinculin (green) and DNA with DAPI (blue). Scale bar: 10 µm. Kinetics of
235 stress fibre (b) and FA (c) disassembly by treatment with Y-27632 (10 µM, black) or
236 Blebbistatin (20 µM, grey). The graph shows the mean with SD of each time point. The
237 results are computed from three independent experiments with over a hundred cells per
238 experiment. (d) Live-cell confocal spinning disc microscopy of representative U373 cell
239 stably expressing AP2-eGFP (green) and transiently expressing mCherry-zyxin (red) treated
240 with Blebbistatin (20 µM). Cell before (left) and 30 minutes after drug treatment (middle).
241 (Right) Merged images of a mask marking the mCherry-zyxin objects before (red) and the
242 AP2-eGFP signal after Blebbistatin treatment (green). Right: Zoom on FA that switches to
243 clathrin-coated plaques during the treatment. (e) Kymograph of the switch from FAs to
244 clathrin-coated plaques shown in d over 30 minutes of Blebbistatin treatment.
245 (f) Quantification of the switch from FAs to clathrin-coated plaques during Blebbistatin
246 treatment. Whiskers represent 10-90 percentile, box represents second and third quartile,
247 line marks the median and cross the mean. Results are computed from three repetitions.
248 Statistical analysis: t test, $n=30$, $P<0.01$. Normalized fluorescence intensity profiles of AP2-
249 eGFP (green) and mCherry-zyxin (red) of a representative switch from FA to clathrin-coated
250 plaques during Y-27632 (g) and Blebbistatin (h) treatment.





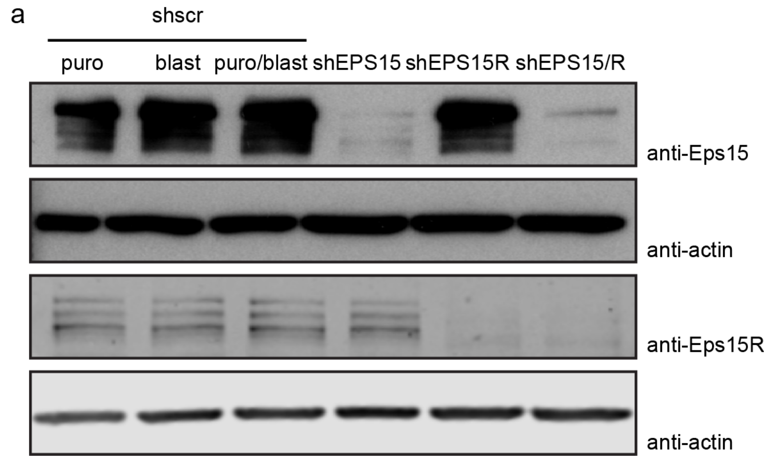
251 **Supplementary Figure 7: Gelatin digestion of U373 depends on MMP14.**

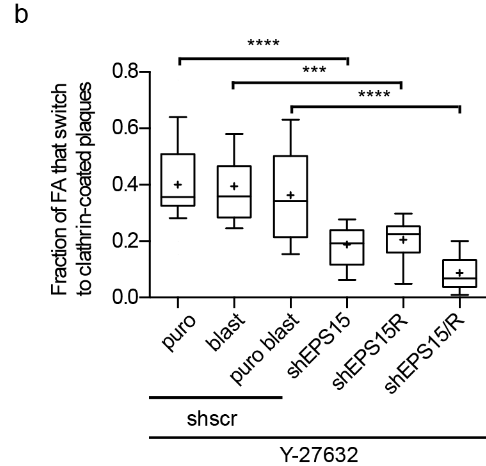
252 (a) Representative images of widefield microscopy of U373 stably expressing AP2-
253 eGFP (green) wild-type (WT), stable knock down for MMP14 (shMMP14), or stable
254 overexpression of MMP14 (MMP14) on coverslips coated with Alexa Fluor 647-labelled
255 gelatin (red). Scale bar: 10 µm. (b) Western blot showing the protein level of MMP14 of
256 U373 cell lines stably expressing shscrambled (shscr), shMMP14, and overexpressing
257 MMP14. The protein levels of actin were used as loading control.



258 **Supplementary Figure 8: Production of optically clear 3D-micropattern.**

259 (a) Schematic illustrates the production of optically clear 3D-micropatterns. First, a drop of
260 optically clear adhesive (NOA, turquois) was added on a glass coverslip and sandwiched by
261 a PDMS grating (grey) generated from a diffraction grating. The optically clear adhesive was
262 cured by UV-irradiation and finally, the PDMS grating was peeled from the optically clear
263 3D-micropatterns. Comparison of the topography of the diffraction gratings (b-c) and the
264 optically clear 3D-micropatterns (d-e) by atomic force microscopy (AFM). 3D-
265 representation of the topography of diffraction gratings (b) and optically clear 3D-
266 micropatterns (d). (c and e) Line profile of the topography shown in b and d. Periodicity and
267 peak height are marked. (f) Table lists the average periodicity and the peak height of
268 diffraction gratings and optically clear 3D-micropatterns. Shown are the mean with SD,
269 computed from three AFM measurements, with n=25 (periodicity) and n=28 (peak height).





270 **Supplementary Figure 9: Eps15/R depletion reduces the efficiency of the switch from**
271 **FAs to clathrin-coated plaques.**

272 (a) Western blot showing the protein level of Eps15 (top) and Eps15R (bottom) of U373 cell
273 lines stably expressing shscrambled (shscr), shEPS15, shEPS15R and shEPS15 together
274 with shEPS15R (shEPS15/R). The protein levels of actin were used as loading control. (b)
275 Normalized quantification of the switch from FAs to clathrin-coated plaques after 20
276 minutes of Y-27632 treatment of U373 shEPS15, shEPS15R and shEPS15/R. Data was
277 normalized to the mean of U373 stably expressing the corresponding shscr. Shown is the
278 mean with SD computed from three repetitions. Statistical analysis: t test, n=28 (shscr
279 puro), n=30 (shscr blast), n=24 (shscr puro blast), n=30 (shEPS15), n=25 (shEPS15R), n=29
280 (shEPS15/R), P<0.01.

281 ***Supplementary References***

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283 curved transition of the clathrin coat during endocytosis. *Nat. Commun.* **9,** 1109
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