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3 An asymmetry in the frequency and position of mitosis in the epiblast
4 precedes gastrulation and suggests a role for mitotic rounding in cell
5 delamination during primitive streak epithelial-mesenchymal transition.

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10 Evangéline Despin-Guitard^{1*}, Navrita Mathiah^{1*}, Matthew Stower², Wallis Nahaboo¹, Elif
11 Sema Eski¹, Sumeet Pal Singh¹, Shankar Srinivas², and Isabelle Migeotte^{1,3}

12
13 ¹Université Libre de Bruxelles, IRIBHM, Brussels B-1070, Belgium.

14
15 ²Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1
16 3QX, United Kingdom.

17
18 ³Correspondence should be addressed to I.M. (e-mail: imigeott@ulb.ac.be).

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20 *Equal contribution

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22

23 **ABSTRACT**

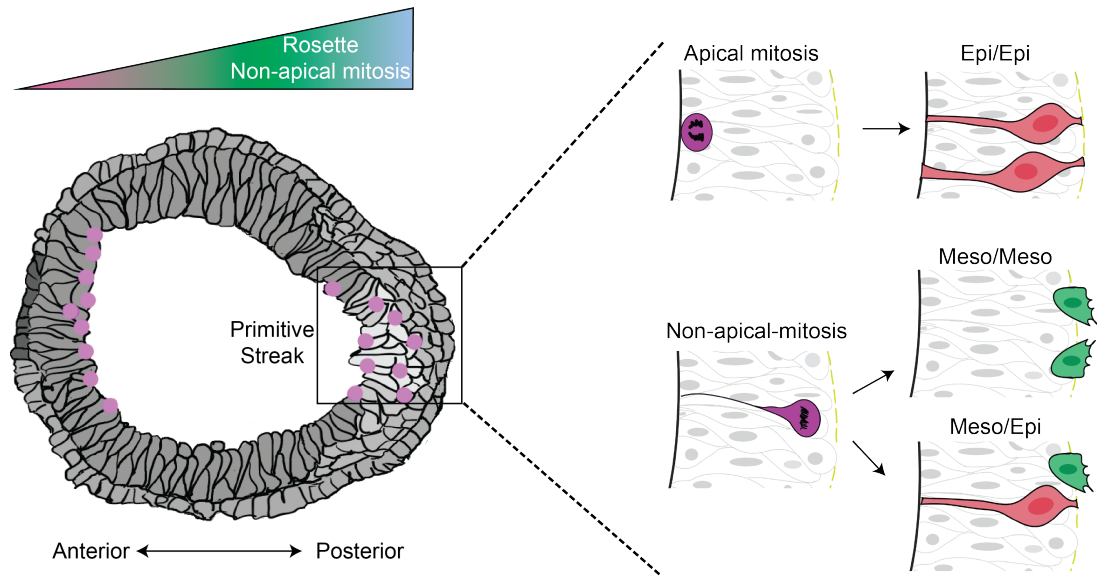
24 The epiblast, a pseudostratified epithelium, is the precursor for the three main germ
25 layers required for body shape and organogenesis: ectoderm, mesoderm, and
26 endoderm. At gastrulation, a subpopulation of epiblast cells constitutes a transient
27 posteriorly located structure called the primitive streak, where cells that undergo
28 epithelial-mesenchymal transition make up the mesoderm and endoderm lineages.

29
30 In order to observe the behavior of individual cells, epiblast cells were labeled
31 ubiquitously or in a mosaic fashion using fluorescent membrane reporters. The cell
32 shapes of individual cells and the packing and behaviour of neighbouring cells during
33 primitive streak formation were recorded through live time-lapse imaging. Posterior
34 epiblast displayed a higher frequency of rosettes, a signature of cell rearrangements,
35 prior to primitive streak initiation. A third of rosettes were associated with a central cell
36 undergoing mitosis. Interestingly, cells at the primitive streak, in particular delaminating
37 cells, underwent mitosis twice more frequently than other epiblast cells, suggesting a
38 role for cell division in epithelial-mesenchymal transition. Pseudostratified epithelia are
39 characterized by interkinetic nuclear migration, where mitosis occurs at the apical side of
40 the epithelium. However, we found that exclusively on the posterior side of the epiblast,
41 mitosis was not restricted to the apical side. Non-apical mitosis was apparent as early as
42 E5.75, just after the establishment of the anterior-posterior axis, and prior to initiation of
43 epithelial-mesenchymal transition. Non-apical mitosis was associated with primitive
44 streak morphogenesis, as it occurred specifically in the streak even when ectopically
45 located. Most non-apical mitosis resulted in one or two daughter cells leaving the
46 epiblast layer to become mesoderm. Furthermore, in contrast to what has been
47 described in other pseudostratified epithelia such as neuroepithelium, the majority of
48 cells dividing apically detached completely from the basal pole in the epiblast.

49
50 Cell rearrangement associated with mitotic cell rounding in the posterior epiblast during
51 gastrulation, in particular when it occurs on the basal side, might thus facilitate cell
52 ingression through the PS and transition to a mesenchymal phenotype.

53 **GRAPHICAL ABSTRACT**

54



55

56

57 INTRODUCTION

58 Gastrulation is an essential morphogenetic event that establishes the three layers of the
59 animal body plan (ectoderm, mesoderm, and endoderm) through an epithelial-
60 mesenchymal transition (EMT) that occurs at an organizing center called the primitive
61 streak (PS) in amniotes (S. J. Arnold & Robertson, 2009; Ramkumar & Anderson, 2011).
62 In mouse, the combination of multiple signaling gradients coming from embryonic
63 epiblast, as well as extraembryonic ectoderm and visceral endoderm, creates a
64 permissive zone in the posterior epiblast for PS establishment. The first marker of
65 anterior-posterior asymmetry is expression of *Wnt3* in a tiny region of VE at the
66 embryonic-extraembryonic border at embryonic day (E) 5.5 (Rivera-Pérez & Magnuson,
67 2005). Shortly after, between E5.5 and E5.75, a group of visceral endoderm cells at the
68 distal end of the embryo migrates towards the embryonic-extraembryonic border in the
69 direction opposite to *Wnt3* expression. This organizer, called the Anterior Visceral
70 Endoderm, secretes inhibitors of Nodal and Wnt signaling that consolidate anterior-
71 posterior axis specification (Stower & Srinivas, 2014). PS initiates in the proximal
72 posterior epiblast at the embryonic-extraembryonic boundary, and extends distally
73 towards the tip of the embryo. Contrary to other species, there seems to be no global
74 epiblast movement towards the posterior side in mouse, but rather a local change of fate
75 (Rivera-Pérez & Magnuson, 2005; Williams, Burdsal, Periasamy, Lewandoski, &
76 Sutherland, 2012). The streak can be identified through expression of markers in pre
77 streak (*Wnt3*), and early streak (*Eomesodermin*, and *Brachyury*) embryos at around E6
78 (Rivera-Perez 2005). It can be identified through morphology alone from the mid streak
79 stage (E6.25-E6.5).

80
81 EMT allows epithelial cells to become motile through loss of cell-cell adhesion and
82 change of polarity from apical-basal to front-rear, via cytoskeleton reorganization and
83 acquisition of migratory and matrix remodelling capacities (Nieto, Huang, Jackson, &
84 Thiery, 2016). Live imaging of mid and late streak embryos showed that epiblast cells in
85 the PS region delaminate through apical constriction (leading to the so-called “bottle
86 shape”) followed by retraction of the apical process and extrusion on the basal side
87 (Ramkumar et al., 2016; Williams et al., 2012). Analyses of mouse mutant lines

88 displaying an accumulation of cells within the primitive streak converge on a major role
89 for actin-myosin cytoskeleton regulation to allow delamination at the PS (Lee, Silva-
90 Gagliardi, Tepass, McGlade, & Anderson, 2007; Ramkumar et al., 2015, 2016). Control
91 of cell ingression is defined at the cellular level by the complementary pattern of
92 expression of *Crumbs2* (in delaminating cells) and Myosin IIB (in their neighbors) on the
93 apical side of epiblast cells (Ramkumar et al., 2016). *Crumbs2* mutant cells fail to detach
94 from their basal attachment, suggesting cellular anisotropy might help extrusion from the
95 epithelium. Interestingly, in *Crumbs2* mutant embryos, initiation of gastrulation occurs
96 normally, and only a proportion of cells fail to ingress, leading to progressive expansion
97 of the PS, while the rest of post-EMT cells join the mesodermal wings, suggesting that
98 several mechanisms may control PS cellular extrusion.

99
100 An alternative delamination mechanism based on asymmetric division has been
101 described in chick neural crest EMT from the neuroepithelium (Ahlstrom & Erickson,
102 2009). Similar to neuroepithelium, epiblast is a pseudostratified epithelium, and live
103 imaging experiments have shown apical-basal nuclear movement, suggesting it
104 undergoes interkinetic nuclear migration (IKNM) (Ichikawa et al., 2013). Classically, in
105 IKNM, cells retain apical and basal connections, while the nucleus migrates along their
106 apical-basal length according to cell cycle stage, and division occurs on the apical side
107 (P J Strzyz, Matejcic, & Norden, 2016).

108
109 In order to define the cell shape changes and cellular rearrangements taking place at
110 the prospective PS and explore alternative delamination mechanisms as gastrulation
111 proceeds, we performed confocal and lightsheet imaging of mouse embryos expressing
112 fluorescent reporters ubiquitously or mosaically. We found that posterior epiblast cells
113 form rosettes with higher frequency than anterior or lateral epiblast. Dynamic and static
114 observation of dividing cells revealed a higher frequency of mitosis in the primitive
115 streak, and non-apical mitosis were found specifically on the posterior side of the
116 epiblast, which resulted in ingression of one or two mesoderm cells per event.

117

118

119 **RESULTS**

120 *Posterior epiblast displays higher frequency of rosettes*

121 Lightsheet imaging, in which a thin bi-concave sheet of excitation laser optically sections
122 an imaged sample that can be rotated and imaged at successive angles, has emerged
123 as the method of choice for embryo live imaging, as it provides high time and space
124 resolution tridimensional images with minimal phototoxicity (Amat & Keller, 2013).
125 Recently, several culture methods have been developed to allow growing mouse
126 embryos within a lightsheet microscope (Ichikawa et al., 2013; McDole et al., 2018;
127 Udan, Piazza, Hsu, Hadjantonakis, & Dickinson, 2014). In order to record epiblast
128 dynamics prior to, and during PS establishment, we performed lightsheet imaging at
129 E5.75 (stage at which the anterior-posterior axis is defined) of CAG-TAG embryos
130 bearing a membrane Tomato reporter and a nuclear (H2B) GFP reporter (Trichas,
131 Begbie, & Srinivas, 2008), as well as *Hex*-GFP, a cytoplasmic reporter expressed in the
132 Anterior Visceral Endoderm (to aid anterior-posterior orientation) (Srinivas, Rodriguez,
133 Clements, Smith, & Beddington, 2004) (Fig. 1a and a', Sup. Fig. 1a-c, and Sup. Fig. 2a).
134 Embryos were mounted in a glass tube containing a hollow agarose cylinder. Samples
135 were rotated in order to obtain high quality images from 4 sides, and the corresponding
136 Z-stacks were fused to obtain a complete 3D representation. A striking feature was the
137 abundance of rosettes, transient epithelial multicellular structures in which five or more
138 cells interface at a central point, in the epiblast layer (Fig. 1a, Video 1). Rosettes were
139 quantified systematically on the anterior, lateral and posterior sides on Z sections
140 located 5 μ M from the epiblast basal side. Rosette frequency was calculated through
141 normalization according to the surface of the region of interest (in which clear cell
142 contours could be delineated) of each section as well as total time of acquisition. We
143 found that rosettes were twice more frequent on the posterior side (Fig. 1a and a" and
144 Sup. Fig. 1).

145
146 The high time (7 minutes intervals) and space (1 μ m Z slice) resolution allowed
147 reconstruction of rosettes along the entire height of the epithelium, as well as follow up
148 of individual cells fate. Around 30% of rosettes had a central cell with apical rounding,
149 usually preceding cell division (Sup. Fig. 2b and c). However, not all apical round cells

150 were associated with a rosette, and the frequency of apical rounding was similar on all
151 sides (Sup. Fig. 1). Lifespan of rosettes associated with apical rounding and mitosis was
152 longer than that of rosettes without rounding (Sup. Fig. 2c).

153
154 In order to increase sample number and to cover a longer period of development
155 through concomitant imaging of several embryos, we performed live confocal imaging of
156 embryos dissected at E5.75 or E6.25 (when the primitive streak is specified and cells
157 start delaminating), and placed in optically compatible conical wells with either the
158 anterior, posterior or lateral side facing the objective. Cell membranes were labeled
159 ubiquitously using Rosa26::membrane dtTomato/membrane GFP (mTmG) (Muzumdar,
160 Tasic, Miyamichi, Li, & Luo, 2007), and the *Hex*-GFP transgene was used for
161 orientation (Fig.1b' and Sup. Fig. 2a'). Z-stacks at 3 μm interval encompassing roughly
162 half of the embryo were recorded with time intervals of 25 minutes. Epiblast cell
163 contours were segmented, and rosettes were quantified as described above (Fig. 1b
164 and c, Video 2). Due to the longer intervals between time points, normalization was
165 performed according to surface alone. Confocal live imaging confirmed a higher
166 frequency of rosettes on the posterior, compared to anterior and lateral epiblast (Fig. 1b"
167 and c'), at E5.75 and E6.25.

168
169 The higher frequency of rosettes on the posterior side prior to the onset of gastrulation is
170 compatible with a dynamic epithelium primed for epithelial-mesenchymal transition.

171
172 *Primitive streak cells use multiple mechanisms to delaminate*

173 We hypothesized that the excess of rosettes on the posterior side might arise due to cell
174 shape changes related to EMT. However, the pseudostratified and packed arrangement
175 of the epiblast made it quite challenging to follow individual cells shape changes. OH-
176 tamoxifen-induced recombination of mTmG through Sox2Cre-ERT2 (K. Arnold et al.,
177 2013) activation allowed mosaic labeling of the epiblast (Fig. 2a, a', Videos 3-6). At that
178 stage of development, anterior-posterior axis can be determined through morphology
179 alone. Due to toxicity of tamoxifen prior to implantation, we could not observe the early
180 events of gastrulation. However, when the injection was given at E6.25, litter size and

181 embryo morphology were normal. Live imaging was performed at E7.25, when PS
182 elongation is complete, which offered a higher probability to observe numerous
183 delamination events within the observation window. Embryos were placed on the lateral
184 side in conical wells, to examine anterior and posterior epiblast. Two-photon imaging
185 allowed recording the whole width of the embryo, with Z-intervals of 3 μm and time
186 intervals of 25 minutes, which permitted 3D reconstruction over time. Cells acquiring
187 either a round or a bottle shape were segmented and tracked to identify their destiny
188 (within the epiblast or mesoderm layers) as well as that of their progeny when division
189 occurred.

190
191 So-called "bottle-shaped" cells, with a round basal cell body and an apical extension,
192 were present on both sides, but more frequent on the posterior side, and cell
193 delamination only took place on the posterior side. Cells maintained an apical
194 attachment until their basally located cell body had begun crossing the basal membrane,
195 and only fully detached after delamination (Fig. 2b, b').

196
197 In most cells dividing apically, a connection to the basal membrane could not be
198 identified (Fig. 2c-c"), even when performing imaging at higher spatial resolution and
199 laser intensity. Although we cannot exclude the possibility of a very thin cell process
200 missed by the imaging technique, those observations are compatible with a profound
201 reorganization in which dividing cells detached basally upon apical mitotic rounding.
202 Daughter cells then elongated and reinserted separately in the epithelium, resulting in
203 cell dispersion (as described in (Abe, Kutsuna, Kiyonari, Furuta, & Fujimori, 2018)).
204 Those findings suggest that although epiblast is a pseudostratified epithelium, and
205 undergoes IKNM, cells shape and attachment dynamics during mitosis are distinct to
206 what is known in neuroepithelia.

207
208 For systematic quantification, epiblast regions were defined as anterior or posterior by
209 tracing a line joining the distal pole to the middle of the embryo at the
210 embryonic/extraembryonic border, and cells undergoing rounding were followed
211 overtime (Fig. 3a-c). Although the frequency of cell division was similar in anterior and

212 posterior epiblast, there was a trend towards a higher division rate specifically in cells
213 undergoing delamination (Fig. 3d). Surprisingly, and exclusively on the posterior side,
214 cell rounding and division happened all along the apical-basal length of the cell, so that
215 non-apical division represented 30 % of all divisions in posterior epiblast (Fig. 3e, f). It
216 was preferentially associated with EMT, as it resulted in formation of one or two
217 mesoderm cells (Fig. 3g), suggesting mitotic rounding could be an alternative
218 mechanism for cell delamination.

219
220 *Higher frequency of mitosis, including non-apical mitosis, in primitive streak and pre-*
221 *gastrulation posterior epiblast*

222 In order to document the proportion and position of mitotic nuclei all along the
223 gastrulation period, we performed immunostaining for phospho-histone H3 on sections
224 or whole-mount embryos from E5.75 to E7 using lightsheet and confocal imaging (Fig. 4
225 and 5). Samples were counterstained for nuclei (DAPI) for cell count and F-actin
226 (Phalloidin) for cell shape, and we quantified mitotic index, apical-basal position of
227 positive nuclei, as well as, when possible, orientation of mitotic plate relative to apical-
228 basal axis.

229
230 We first focused on early to mid streak embryos (E6.5, E6.75, and E7). Based on
231 anatomy and localization of Collagen IV, a basal membrane marker, we defined anterior
232 (anterior half), posterior (posterior half except PS area), and PS region (where basal
233 membrane is perforated) on transverse sections (Fig. 4a and Sup. Fig. 3a). Remarkably,
234 mitosis was around 2 times more frequent in the PS region compared to the rest of the
235 epiblast (Fig. 4b). In addition, over a third of mitotic nuclei were located away from the
236 apical surface in PS cells (Fig. 4c). Contrary to apical mitosis, the majority of phospho-
237 histone H3 positive cells on the basal side retained a connection with the apical side
238 (Sup. Fig. 3b). We found no preferential mitotic plate orientation (Sup. Fig. 3c). At the
239 PS, the mitotic index and the proportion of non-apical mitosis were stable overtime (Sup.
240 Fig. 3d-f). The higher frequency of mitosis in the streak was corroborated by examining
241 signatures of cell cycle phases in single cell transcriptomes from the Mouse Gastrulation
242 2018 atlas (Pijuan-Sala et al., 2019). We selected cells annotated as PS and mesoderm

243 at E6.5, E7, and E7.25. At each time point, cells with high *Brachyury* expression, which
244 likely represent cells at the PS since *Brachyury* is progressively down-regulated in
245 nascent mesoderm, had a higher probability to display a G2/M signature (Fig.4d and
246 Sup. Fig. 4).

247
248 For earlier embryos, anterior-posterior orientation was determined either through *Hex*-
249 GFP (Anterior Visceral Endoderm, Fig. 5a) or immunostaining for Eomesodermin (PS,
250 Fig. 5b), and embryos were examined in whole-mount in a sagittal orientation. The
251 proportion of non-apical mitosis was significantly higher on the posterior side, even
252 before the primitive streak could be identified morphologically (Fig. 5a', a'', b', b'').

253
254 *Non-apical mitosis is associated with primitive streak morphogenesis*
255 PS specification occurs at the region of the embryo opposite the Anterior Visceral
256 Endoderm: when the Anterior Visceral Endoderm remains distal, PS forms a ring at the
257 embryonic/extraembryonic boundary, and when it migrates partially, PS is skewed to the
258 posterior side but fails to elongate (Rakeman & Anderson, 2006). To assess whether
259 non-apical mitosis is a feature systematically associated with the PS, we examined
260 embryos displaying defective Anterior Visceral Endoderm migration resulting in an
261 ectopic primitive streak. E6.25 *Rac1*^{KO} (Migeotte, Omelchenko, Hall, & Anderson, 2010)
262 or *RhoA*^{VE-deleted} (Christodoulou et al., in press) embryos were immunostained for
263 Cerberus1 (an Anterior Visceral Endoderm marker), phospho-histone H3, and
264 counterstained for nuclei (DAPI) and F-actin (Phalloidin). In both models, Anterior
265 Visceral Endoderm migration was impaired (Fig. 6a-d and Sup. Fig. 5a-d). Non-apical
266 mitosis was significantly and consistently more frequent in the region of the embryo
267 most distant from the Anterior Visceral Endoderm (Fig. 6b, c, e and Sup. Fig. 5b, c, e),
268 showing it is specifically associated with PS morphogenesis, rather than with a
269 geographic position in the embryo.

270
271 **DISCUSSION**
272 Anterior-Posterior axis specification of the mouse embryo precedes detection of PS
273 markers by half a day, and initiation of EMT by a day. Through lightsheet and confocal

274 live imaging of embryos that had completed Anterior Visceral Endoderm migration, we
275 observed a high frequency of rosettes in the posterior epiblast, prior to and during PS
276 morphogenesis. Rosettes are intermediate stages of epithelial reorganization observed
277 in multiple morphogenetic events in diverse organisms (Harding, McGraw, &
278 Nechiporuk, 2014). There are two main classes of rosettes, characterized by their
279 mechanisms of formation and resolution. Rosettes arising through planar polarized
280 constriction are usually short lived, and often contribute to tissue elongation. On the
281 other hand, rosettes arising through apical constriction persist for longer periods of time,
282 and do not resolve in a stereotyped fashion but rather remodel to participate to the
283 formation of a structure or organ, often through generation of a fold or lumen. Nodal-
284 dependent rosettes with actin-rich centers have been observed within developing PS of
285 the chick embryo (Wagstaff, Bellett, Mogensen, & Münsterberg, 2008), and they are
286 more frequent as development progresses (Yanagawa, Sakabe, Sakata, Yamagishi, &
287 Nakajima, 2011). However, their mode of formation is unclear. In view of the complex
288 pseudostratified organization of the epiblast, we could not identify rosettes mode of
289 formation with precision. A proportion of rosettes related to collapse of the basal process
290 during apical mitotic rounding resulting in bridging of the surrounding cells. In later
291 stages, rosettes were likely formed in a similar fashion (although with an inverse apical-
292 basal polarity) through apical constriction of delaminating bottle-shaped cells. However,
293 since we observed rosettes prior to initiation of EMT, it is likely that a proportion of those
294 arise through a yet to discover mechanism, possibly linked to the need for increased
295 epithelium fluidity during PS morphogenesis. The recent advances of mouse embryo
296 lightsheet imaging (McDole et al., 2018), including dynamic 3D reconstruction of
297 multicolor embryos, will likely allow to resolve the different modes of rosette formation in
298 the PS along gastrulation.

299
300 Through mosaic labeling of the epiblast, we followed cell shape changes in mid to late
301 streak embryos. Cells delaminating through apical constriction retained an apical
302 connection until their cell body had exited the epiblast layer. Interestingly, cells leaving
303 the PS appeared to have a higher frequency of division. This was confirmed by a
304 systematic quantification of mitotic nuclei in anterior, posterior, and primitive streak

305 epiblast, showing higher mitotic index in cells adjacent to the perforated segment of the
306 basement membrane. Data mining from single cell transcriptomes (Pijuan-Sala et al.,
307 2019) highlighted a higher probability to be in G2/M phase for PS and mesoderm cells
308 expressing high level of Brachyury. Similarly, it has been shown in rat embryos that cells
309 in the PS region cycled more rapidly than the rest of the epiblast (Mac Auley, Werb, &
310 Mirkes, 1993). Together, those observations point towards a role for mitosis in EMT at
311 the PS. A potential mechanistic explanation comes from recent work in *Drosophila*
312 showing that pseudostratified epithelial cells down-regulate E-cadherin as they round up
313 for mitosis (Aguilar-Aragon, Bonello, Bell, Fletcher, & Thompson, 2020).

314
315 In addition, we found difference in localization of mitotic rounding and cell division
316 depending on anterior-posterior localization in the epiblast. In anterior epiblast, mitotic
317 rounding was universally apical. Different to other pseudostratified epithelia, in the
318 majority of dividing cells we could not distinguish a basal attachment, suggesting cells
319 detach from the basal membrane prior to division. In posterior epiblast, and particularly
320 in the streak area, mitotic rounding was not exclusively apical, but could occur all along
321 apical-basal cell length. In mutant embryos where primitive streak was ectopic due to
322 Anterior Visceral Endoderm migration failure, non-apical division was detectable in the
323 region of the epiblast further away from the Anterior Visceral Endoderm, hence
324 associated to PS morphogenesis. Remarkably, non-apical division resulted in extrusion
325 of one or two daughter cells. Non-apical mitosis has been described in chick dorsal
326 neural tube, where neural crest delamination occurs (Ahlstrom & Erickson, 2009): live
327 imaging showed that a proportion of rounded mitotic cells detached from the lumen to
328 undergo mitosis, and that daughter cells from those non-apical mitosis all became
329 neural crest. In the zebrafish retina, perturbation of CDK1-driven apical IKNM resulted in
330 non-apical mitosis and disrupted integrity of the pseudostratified neuroepithelium
331 because of aberrant cell delamination (Paulina J. Strzyz et al., 2015). In *Drosophila*
332 tracheal placode, mitotic rounding is necessary to accelerate epithelial invagination
333 (Kondo & Hayashi, 2013). Similarly, cell division is critical for chick embryo gastrulation
334 via its role in epithelial rearrangements through regulation of cortical actomyosin
335 (Firmino, Rocancourt, Saadaoui, Moreau, & Gros, 2016).

336
337 In light of those results, we propose that increase in epithelial fluidity, redistribution of
338 cell contacts through acquisition of the round morphology associated with mitosis, as
339 well as, possibly, mechanical pressure on the basal membrane from non-apical dividing
340 cells, could facilitate transition to a mesenchymal phenotype at the PS.

341
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352
353 **METHODS**
354 **Mouse strains and genotyping**
355 The mTmG (Muzumdar et al., 2007) and the Sox2-Cre-ERT2 (K. Arnold et al., 2013)
356 lines were obtained from the Jackson Laboratory, the CAG-TAG (Trichas et al., 2008)
357 and the *Hex*-GFP (Srinivas et al., 2004) lines from Shankar Srinivas. Mice were kept on
358 a CD1 background. Mice colonies were maintained in a certified animal facility in
359 accordance with European guidelines. The local ethics committee (CEBEA) approved all
360 experiments. OH-tamoxifen (Sigma) was suspended at 100 mg/ml in ethanol 100%, and
361 diluted in sesame oil (Sigma) to a final concentration of 10 mg/ml. Females were
362 injected intraperitoneally with 2 mg OH-tamoxifen at E6.25 and E6.75. Embryos were
363 collected at E7.25.

364

365 Mouse genomic DNA was isolated from ear biopsies following overnight digestion at
366 55°C with 1.5% Proteinase K (Quiagen) diluted in Lysis reagent (DirectPCR, Viagen),
367 followed by heat inactivation.

368

369 **Embryo culture and live imaging**

370 ***Confocal Imaging***

371 Embryos were dissected in Dulbecco's modified Eagles medium (DMEM) F-12 (Gibco)
372 supplemented with 10% FBS and 1% Penicillin-Streptomycin and L-glutamine and 15
373 mM HEPES. They were then cultured in 50% DMEM-F12 with L-glutamine without
374 phenol red, 50% rat serum (Janvier), at 37°C and 5% CO₂. Embryos were observed in
375 suspension in individual conical wells (Ibidi) to limit drift, under a Zeiss LSM 780
376 microscope equipped with Plan Aplanachromat 25x/0.8, C Aplanachromat 32x/0.85, and LD C
377 Aplanachromat 40x/1.1 objectives. Stacks were acquired every 25 minutes with 3 μm Z
378 intervals for up to 10 hours. Embryos were cultured for an additional 6 to 12 hours after
379 imaging to check for fitness.

380

381 ***Lightsheet Imaging***

382 20 μL glass capillaries (Brand, 701904) with plungers were used to create a 2% agarose
383 cylinder, in which a copper wire of 150 μm (for E5.75 embryo) or 195 μm (for E6.5
384 embryos) was inserted. Once agarose was solidified, the wire was removed to leave a
385 tunnel in the agarose cylinder, in which the embryo was placed.

386

387 Embryos were dissected in dissection medium, and allowed to recover in equilibrated
388 culture medium (50% CMRL Medium, 50% KO Serum, and 0,02% glutamine) in an
389 incubator (37°C, 5% CO₂) for 1 h. Embryos were transferred to a Petri dish filled with
390 culture medium in which the agarose cylinders were lying flat, and were gently moved
391 into the hollow cylinder. Once the cylinders were mounted into glass capillaries, those
392 were placed into a syringe adapted with tips on both ends to secure the capillary.

393

394 The lightsheet microscope (Zeiss Lightsheet Z.1) chamber was filled with culture
395 medium, and left to equilibrate with the sample holder at 37°C and 5% CO₂ prior to

396 imaging for 1h. Imaging was performed using a 20x, NA1.0 Plan-Apochromat water
397 immersion objective, and dual side illumination. For CAG-TAG embryos, 1 to 2% of laser
398 power (488 nm, 543 nm) was applied with an exposure time of 29 ms. Embryos were
399 imaged on 4 sides (anterior, posterior, 2 laterals) with an interval of 90°. Stack Images
400 were taken in dual illumination. Images were captured at a 1024x1024 resolution, with Z
401 intervals of 1 μ m for maximum 200 μ m, and time intervals of 7 minutes. Embryos were
402 cultured for up to 12 hours.

403

404 **Antibodies**

405 Antibodies were: rat anti-Ph3 (Abcam; 1/500), rabbit anti-Tbr2/Eomes (Abcam; 1/100),
406 goat anti-Brachyury (R&D Systems; 1/20), Goat anti-collagenIV (Millipore, 1/500), Goat
407 anti-Cerberus1 (R&D, 1/500), Rabbit anti-Phh3 (Sigma, 1/500). F-actin was visualized
408 using 1.5 U/ml TRITC-Phalloidin (Invitrogen; 1/100), and nuclei using DAPI (Sigma;
409 1/1000). Secondary antibodies were: anti goat Alexa Fluor 647, anti-goat Alexa Fluor
410 488, anti-rabbit Alexa Fluor 488, anti-rat Alexa Fluor 543, anti-rat Alexa Fluor 647, all at
411 1/500 (Jackson).

412

413 **Embryo Analysis**

414 For immunofluorescence, embryos were fixed in PBS containing 4% paraformaldehyde
415 (PFA) for 2 hours at 4°C, cryopreserved in 30% sucrose, embedded in OCT and
416 cryosectioned at 7-10 μ M. Staining was performed in PBS containing 0.5% Triton X-100,
417 0,1% BSA and 5% heat-inactivated horse serum. Sections and whole-mount embryos
418 were imaged on a Zeiss LSM 780 or Lightsheet Z.1 microscope.

419

420 **Image analysis**

421 Lightsheet Z-stacks from 4 sides were fused using Zeiss plugin for Lightsheet Imaging.
422 Images were then processed using Arivis Vision4D v2.12.3 (Arivis, Germany). Embryo
423 contours were segmented manually on each Z-slice and time point. Embryo rotation was
424 adjusted manually if necessary. For 3D reconstruction, cells were manually segmented
425 on each Z-slice and time point by highlighting cellular membranes using Wacom's Cintiq
426 13HD. For quantification, rosettes were manually annotated and counted on Z sections

427 located 5 μm from the basal side of the epiblast. Presence of apical rounding on the
428 corresponding apical side was assessed for each rosette. For Phospho-histone H3
429 quantifications, sections were chosen at least 10 μm apart to ensure that each cell was
430 only counted once, and counting was performed using the Icy software
431 (<http://icy.bioimageanalysis.org>). Videos were generated using the Arivis Vision4D
432 software.

433
434 For each population, normality was assessed using a Shapiro-Wilk test. According to the
435 results of the precedent test, samples were compared using a non-parametric Mann-
436 Whitney test or an unpaired t-test. Ns: non-significant, *: P-value \leq 0.05, **: P-value \leq 0.01
437 and ***: P-value \leq 0.001.

438
439 **Single-cell RNA-seq Analysis**

440 To mine the Mouse Gastrulation Atlas (Pijuan-Sala et al., 2019), the scRNA-seq data
441 was downloaded using the 'MouseGastrulationData' package (Griffiths & Lun, 2019).
442 The data include raw gene counts and annotations for each cell (cell-type, stage and
443 UMAP-coordinates). To plot the data, we utilized the UMAP-coordinates, cell-type and
444 stage annotations provided by the authors. We subset the data by selecting cells
445 annotated as primitive streak, nascent mesoderm, extraembryonic mesoderm, mixed
446 mesoderm and intermediate mesoderm from E6.5, E7.0 and E7.25 stages. Raw data
447 was normalized for library size and mitochondrial counts and scaled using the
448 'SCTransform' function from Seurat 3.1 (Hafemeister & Satija, 2019). Cell cycle score
449 was calculated from scaled data using 'CellCycleScoring' function in Seurat package
450 (Butler, Hoffman, Smibert, Papalexi, & Satija, 2018). This function predicts the cell
451 phase for each cell using G2/M and S phase markers provided in the package, and
452 assigns each cell a quantitative G2/M and S score. These scores and predicted phase
453 of each cell are stored in Seurat object meta data. Further, after assigning cells as high-
454 T or low-T based on the expression level of *Brachyury* (cut-off on scaled expression:
455 >0.5), UMAP and violin plots of G2M scores were plotted in R. Statistical analysis was
456 performed in R (Student's t-test).

457

458 **VIDEOS LEGENDS**

459 **Supplementary Video 1: Rosette formation in epiblast.** Z sections from lightsheet
460 microscopy live imaging of a E5.75 embryo expressing *Hex*-GFP (green) and membrane
461 Tomato (grey) acquired from the anterior (left), lateral (middle) and posterior (right)
462 sides. Rosettes are highlighted in color. Interval time: 7 min and scale bar: 25 μm .

463
464 **Supplementary Video 2: Rosette dynamics in posterior epiblast.** Z sections from
465 confocal microscopy live imaging of a E5.75 embryo expressing *Hex*-GFP (green) and
466 membrane Tomato (grey) placed on its posterior side. Rosettes are highlighted in color.
467 Interval time: 20 min and scale bar: 50 μm .

468
469 **Supplementary Videos 3-6: Cell shape and cell division tracking in mosaically**
470 **labelled epiblast.** 3D reconstructions from two-photon microscopy live imaging of
471 mTmG; *Sox2*Cre-ERT2 E7.25 embryos where epiblast cells are mosaically labelled
472 through OH-tamoxifen injection at E6.25. Interval time: 20 min. Scale is indicated by a
473 grid in μm at the beginning of the videos.

474

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FIGURES

Figure 1: Epiblast cells organize as rosettes at the onset of primitive streak morphogenesis. (a) Manually segmented rosettes on 3D reconstructions from Z-stacks of 25 μm of an E5.75 embryo imaged using lightsheet microscopy. AVE cells are in green, and all cell membranes are labelled in grey. Left: anterior view; Middle: lateral view (anterior to the left); Right: posterior view. Scale bars: 20 μm . (a') Mouse line strategy used to perform live imaging. (a'') Left: Quantification of the number of rosettes observed per frame. Right: Frequency of rosettes, normalized by the surface of the epiblast region in focus and the time of observation. Mean \pm SEM, n=3. (b, c) Rosettes outlines on optical slices from E5.75 (b) and E6.25 (c) embryos imaged by confocal microscopy. AVE cells are in green, and all cell membranes are labelled in grey. Left: anterior view; Middle: lateral view (anterior to the left); Right: posterior view. Scale bars: 20 μm . (b') Mouse lines strategy used to perform live imaging. (b'', c') Left: Quantification of the number of rosettes observed per frame. Right: Rosettes density normalized by the surface of the epiblast region in focus. The posterior region includes the PS region, as it could not be precisely discriminated. Mean \pm SEM. (b'') E5.75: n anterior=47 frames from 4 embryos, n lateral=77 frames from 9 embryos, n posterior=67 frames from 5 embryos. (c') E6.25: n anterior=20 frames from 2 embryos, n lateral=16 frames from 1 embryo and n posterior=69 frames from 7 embryos.

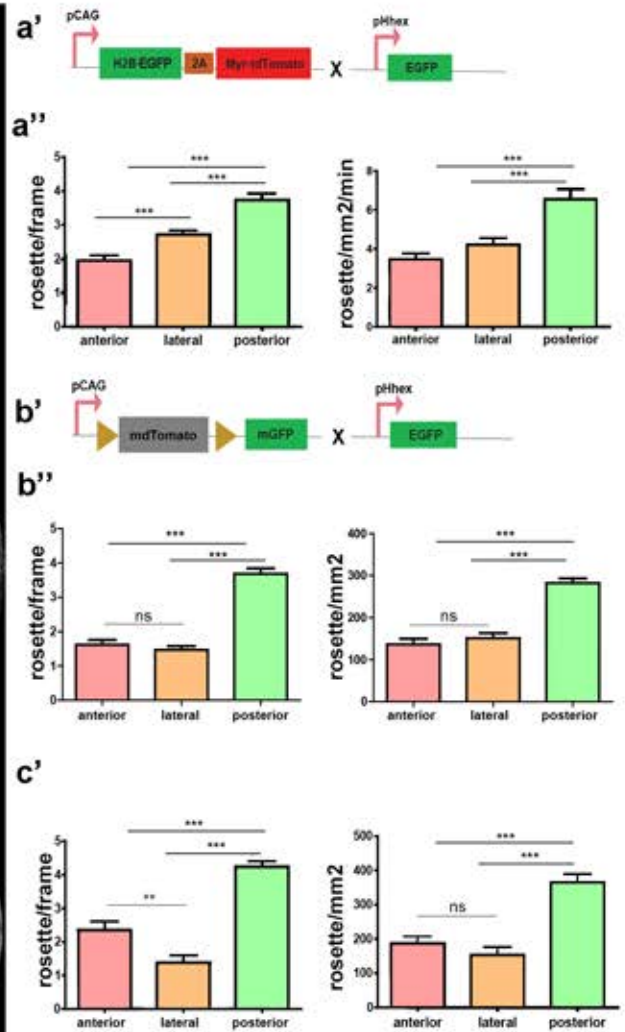
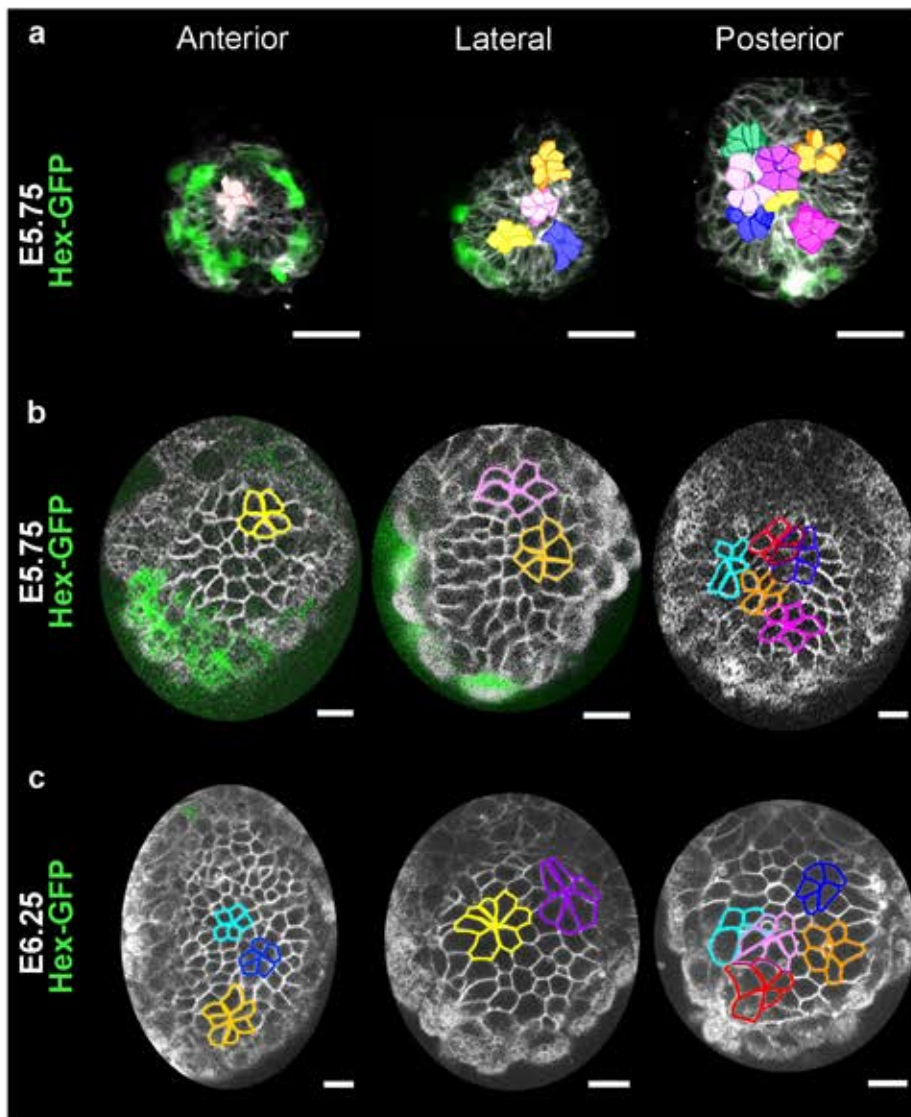


Figure 2: Mosaic epiblast labelling allows tracking of bottle-shaped cells and cell division. (a) Z-projection of an E7.25 embryo mosaically labelled through OH-tamoxifen injection and imaged by two-photon microscopy. Scale bar: 50 μm . n=63 embryos. (a') Mouse line strategy used to produce embryos mosaic for membrane dtTomato (grey) and membrane GFP (green) in the epiblast. (b) Z-projections of stills from live imaging recording of a bottle-shaped cell that delaminates through the basal membrane. Scale bar: 50 μm . (b') Quantification of bottle shape and delamination events. Mean \pm SEM, n=54. Z-projection (c) and 3D reconstruction (c') of stills from live imaging recording showing an apical division without basal contact, followed by daughter cells dispersion. Scale bar: 20 μm . (c'') Quantification of apical divisions with or without persistence of a basal contact, and their outcome in term of daughter cells dispersion. The posterior region includes the PS region, as it could not be precisely discriminated. Mean \pm SEM when applicable, n=52.

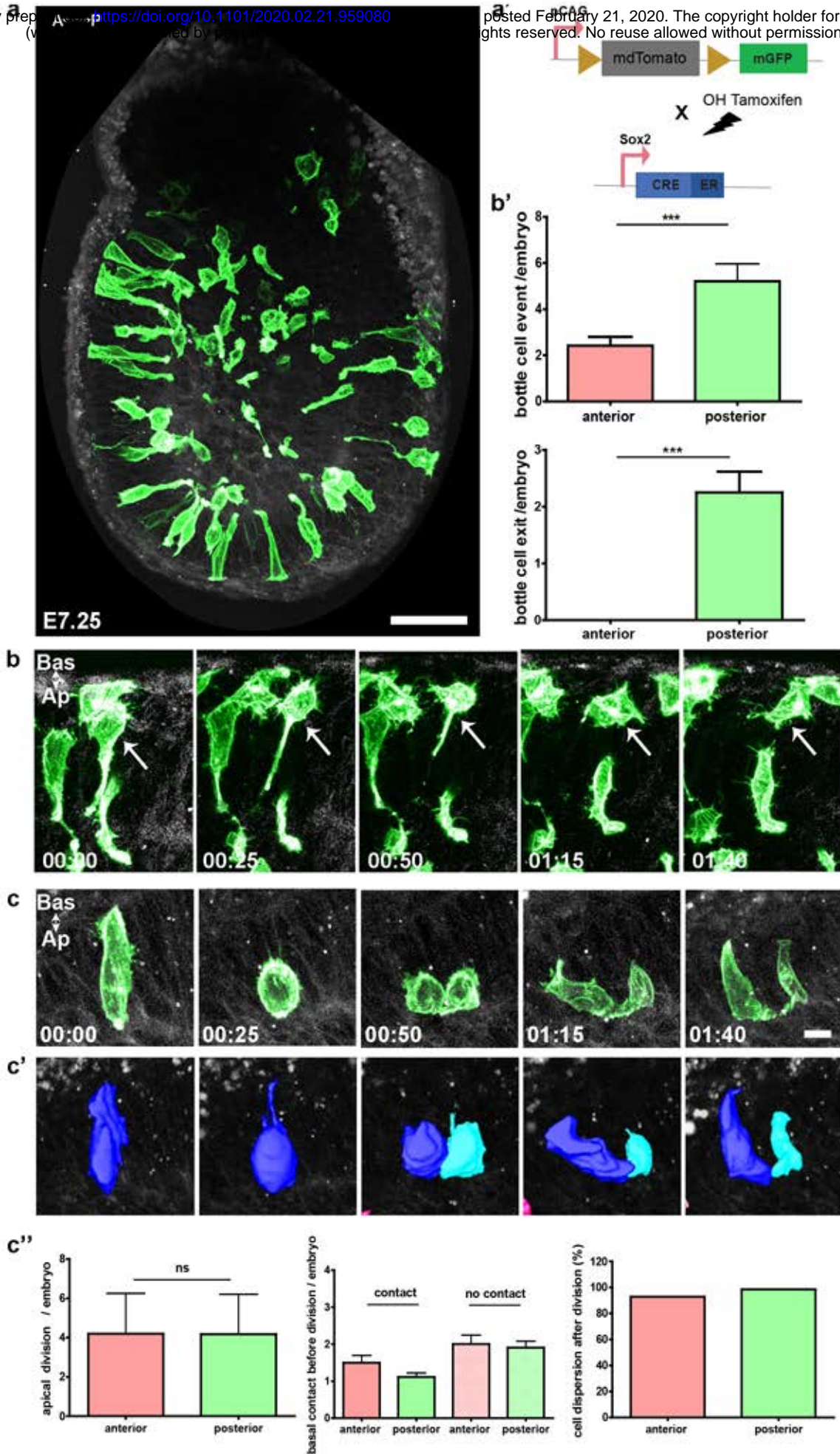


Figure 3: Non-apical division occurs in the posterior epiblast and can give rise to one or two mesoderm cells. Z-projection (a) and 3D cell reconstruction (a') of stills from two-photon live imaging of an E7.25 embryo mosaically labelled for epiblast cells showing a division giving rise to an epiblast and a mesoderm cell that exits from the epiblast layer. Scale bar represents 20 μm . (b) (c) Z-projection of stills from two-photon live imaging of an E7.25 embryo mosaically labelled for epiblast cells. Non-apical division can be observed in the posterior epiblast only, without (b) or with (c) attachment to the apical side. Scale bar: 20 μm (d) Mitotic index (green dividing cells/ total number of green cells) in the anterior and posterior region, as well as among cells exiting the epiblast at the primitive streak. The posterior region includes the PS region, as it could not be precisely discriminated. (e) Ratio of non-apical rounding (non-apical rounding/ total rounding) normalized by the number of green cells and expressed in percentage for the anterior and posterior regions. (f) Ratio of non-apical division (non-apical division/ total division) normalized by the number of green cells and expressed in percentage for the anterior and posterior region of the embryo. (g) Quantification of posterior cell divisions outcomes: either 2 epiblast daughter cells (epi/epi), one epiblast and one mesoderm daughter cells (epi/meso) or 2 mesoderm daughter cells (meso/meso). Data are normalized by the number of green cells. n=43 embryos.

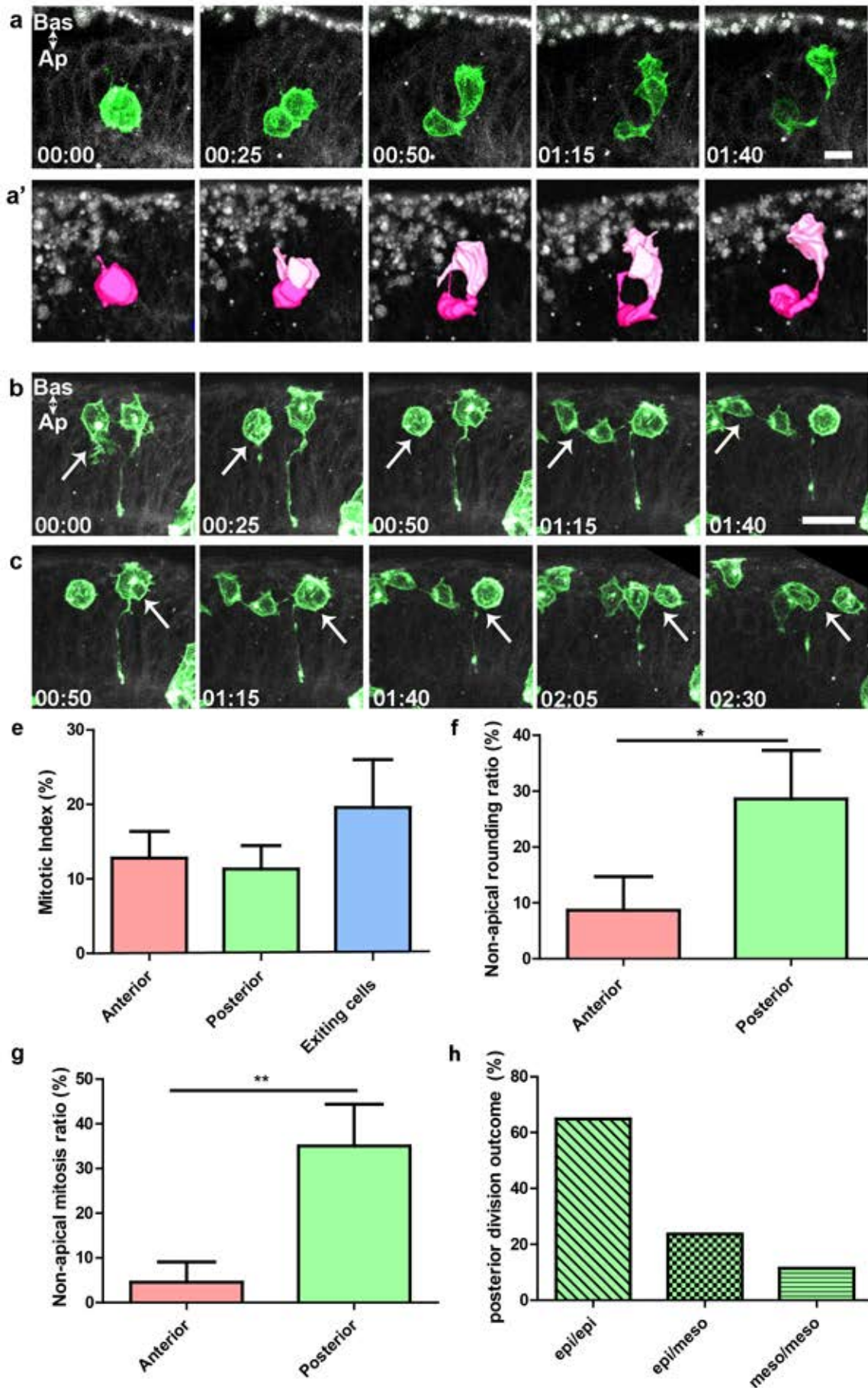


Figure 4: Mitosis is more frequent at the primitive streak, and primitive streak mitotic rounding is not always apical.

(a) Z-projections of transversal sections from E6.5 (top), E6.75 (mid) and E7 (bottom) embryos. Samples were stained for F-actin (Phalloidin, grey) and mitosis (Phh3, magenta) on the left and nuclei (DAPI, cyan) and basal membrane (collagen IV, yellow) on the right. Yellow arrowheads show non-apical mitosis. Scale bar: 25 μ m. (b) Mitotic index (Phh3/DAPI) in percentage at the anterior, posterior, and primitive streak (PS) regions of E6.5 (top), E6.75 (mid) and E7 (bottom) embryos. (c) Ratio of non-apical mitosis (non-apical Phh3/ Phh3, normalized by number of nuclei) in percentage at the anterior, posterior, and PS regions of E6.5 (top), E6.75 (mid) and E7 (bottom) embryos. The PS region is defined by the area where the basal membrane (yellow) is degraded, and the posterior region quantification excludes counts from the PS region. Values are shown as Mean \pm SEM. E6.5: n=20 frames from 6 embryos, E6.75: n=13 frames from 5 embryos, E7: n=20 frames from 6 embryos. (d) Violin plots representing the distribution of cells in G2/M phase in cells with high, compared to low, *Brachyury* expression among cells annotated as primitive streak and mesoderm harvested from E6.5, E7, and E7.25 embryos. A: anterior, P: posterior, PS: primitive streak

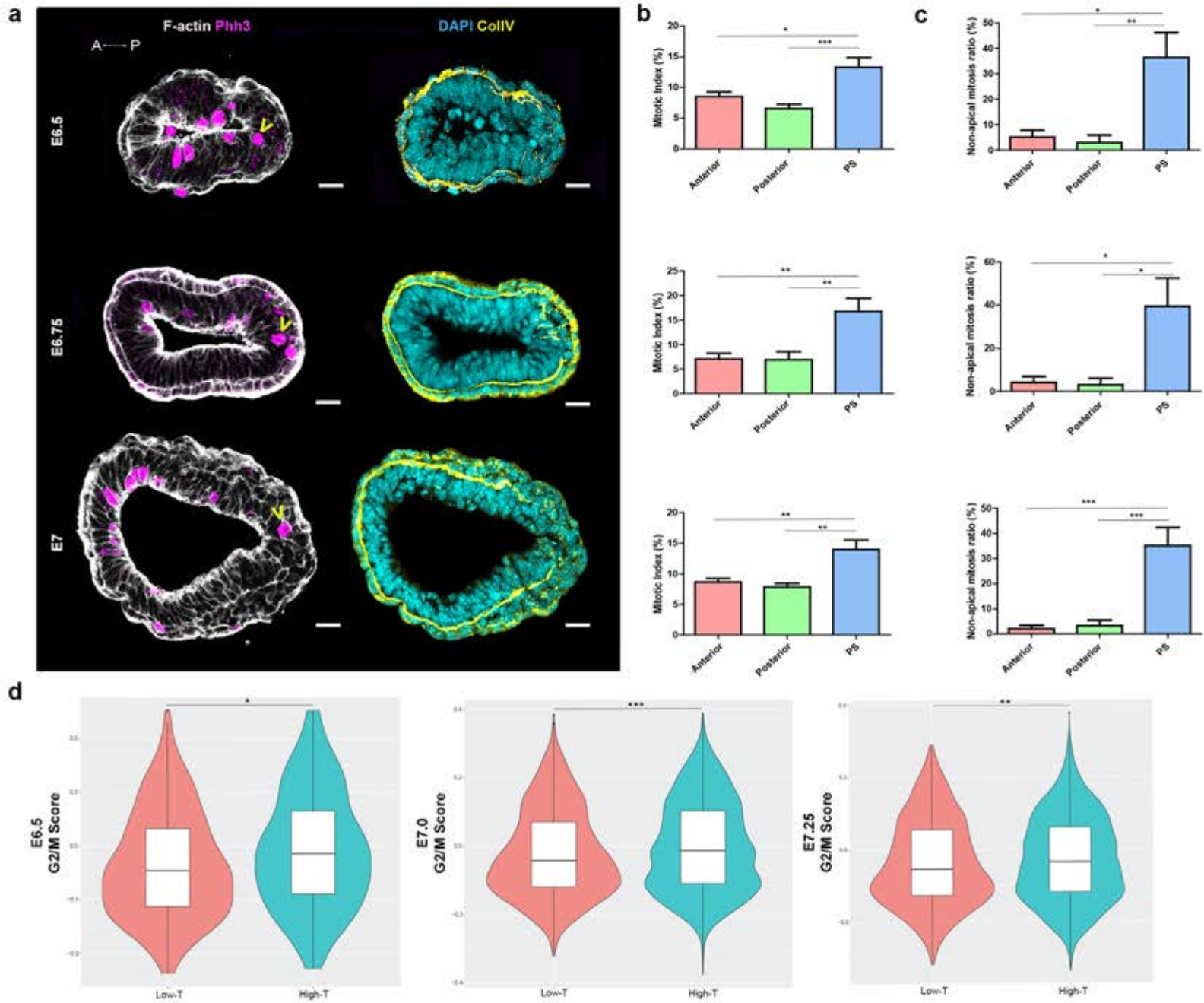


Figure 5: Non-apical divisions occur in the posterior epiblast before and at the onset of gastrulation. (a) 3D reconstruction of wholemount lightsheet imaging of an E5.75 *Hex*-GFP (green, anterior marker) embryo, stained for nuclei (DAPI, grey) on the left, mitosis (Phh3, magenta) and F-actin (Phalloidin, grey) on the right. (b) Z-slice (left) and 3D reconstruction (right) of wholemount lightsheet imaging of an E6.25 embryo stained for a posterior marker (Eomesodermin, cyan), mitosis (Phh3, magenta) and F-actin (Phalloidin, grey). Scale bar: 50 μ m. (a', b') Mitotic index (Phh3/DAPI) in percentage at the anterior and posterior regions of E5.75 (a') and (b') E6.25 embryos. (a'', b'') Ratio of non-apical mitosis (non-apical Phh3/Phh3, normalized by number of nuclei) in percentage in the anterior and posterior region of E5.75 (a'') and E6.25 (b'') embryos. Posterior region includes the PS region, as it could not be precisely discriminated. Values are shown as Mean \pm SEM. E5.75: n=11 embryos, E6.75: n=6 embryos.

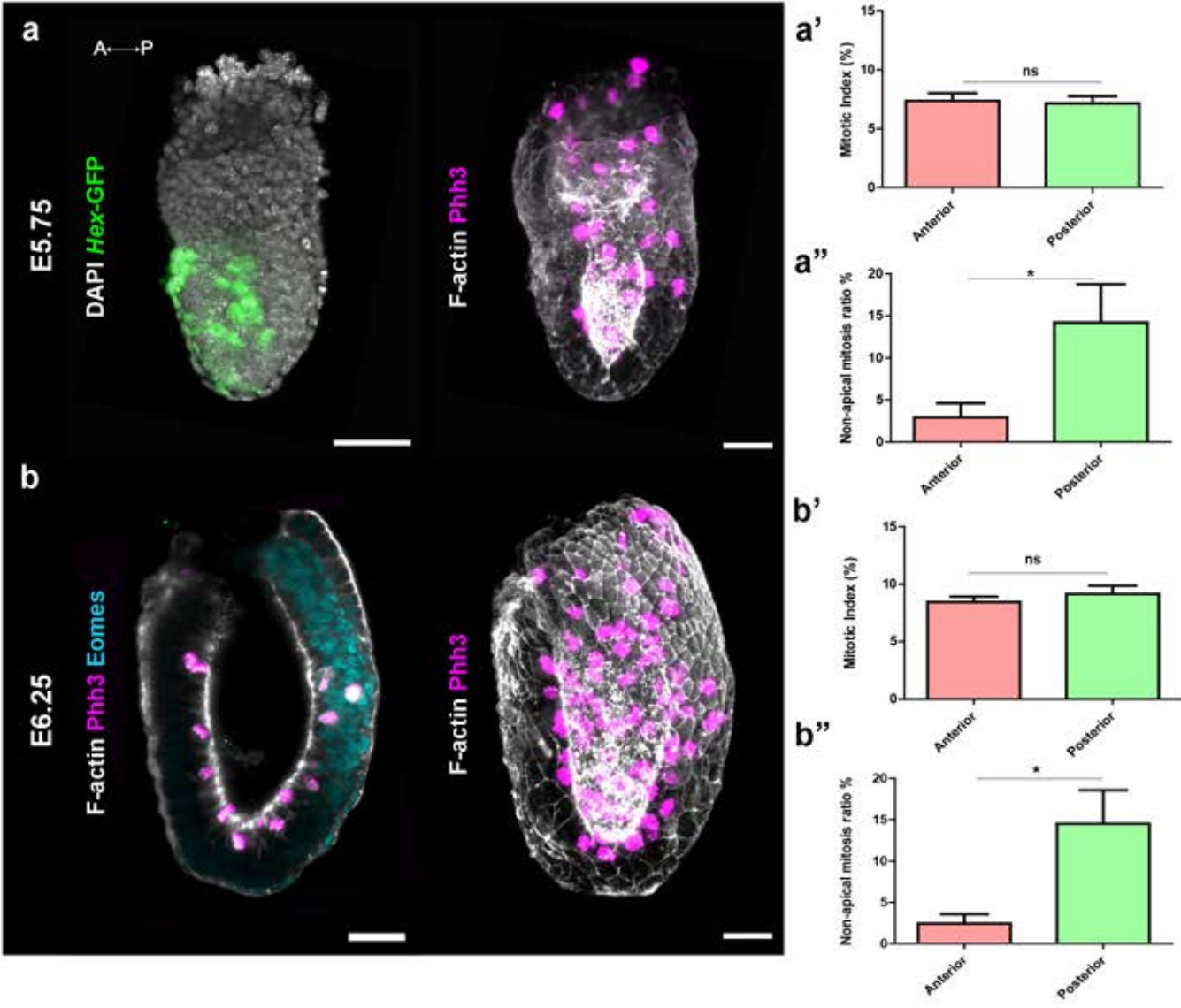
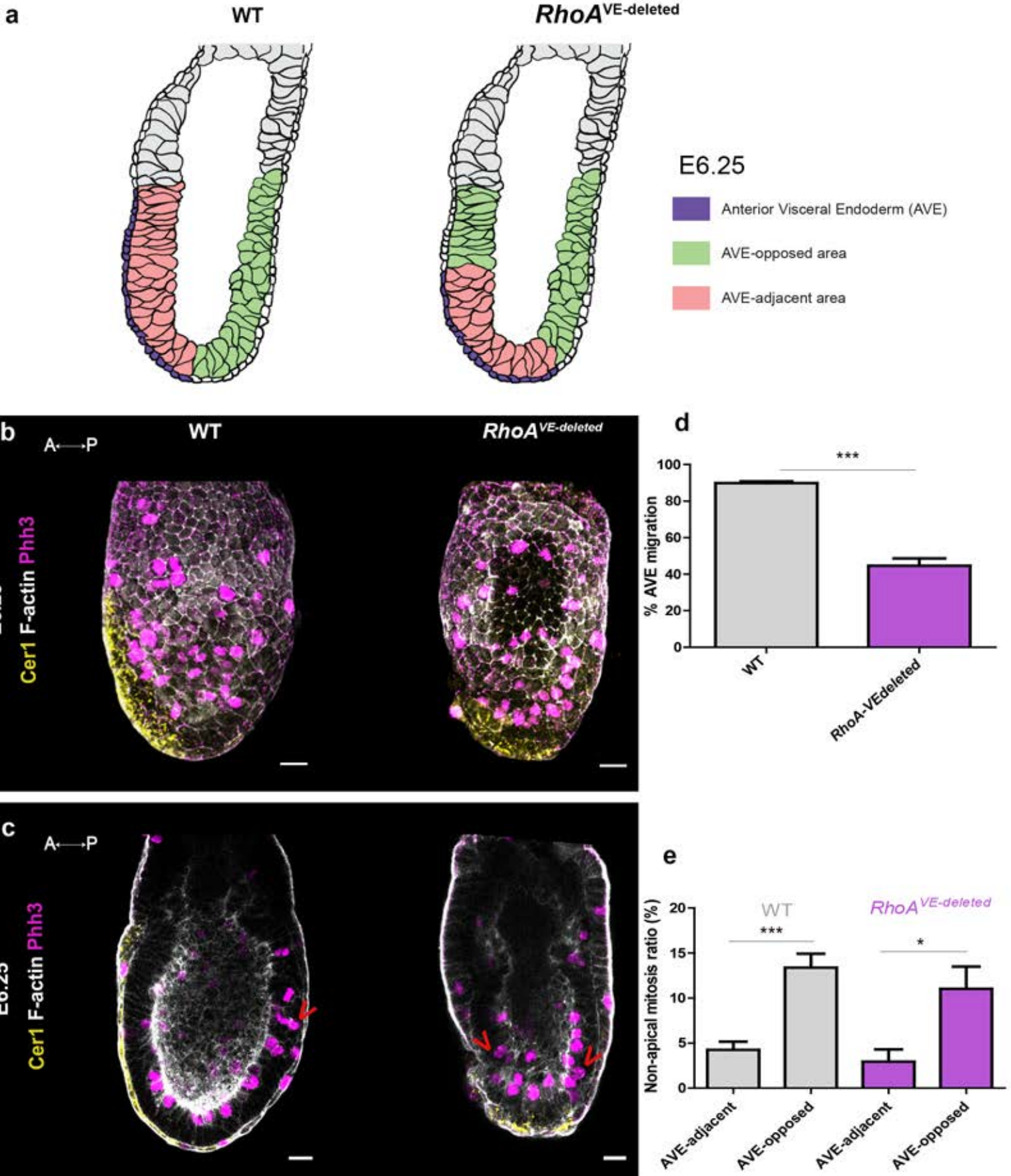


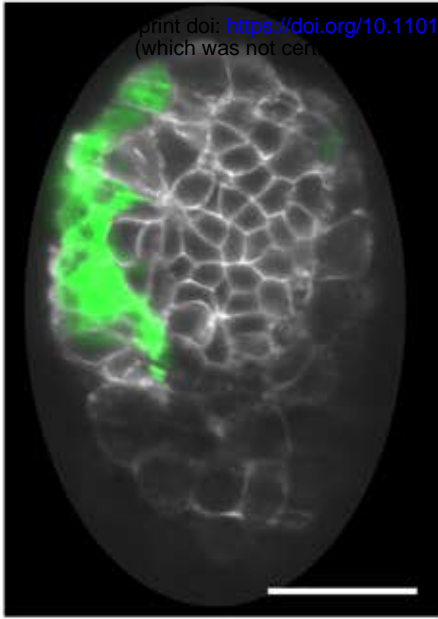
Figure 6: Non-apical mitosis is a feature associated with the primitive streak. (a) Representation of the position of the AVE-opposed area (green) and the AVE-adjacent area (pink) in WT (left) and mutants (right) embryos. (b, c) 3D reconstruction (b) and confocal Z-slice (c) of E6.25 WT (left) and *RhoA*^{VE-deleted} (right) embryos stained for a AVE marker (Cerberus 1, yellow), F-actin (Phalloidin, grey) and mitosis (Phh3, magenta). Scale bar: 25 μm . Red arrowheads point to non-apical mitosis. (d) Percentage of AVE migration in WT and *RhoA*^{VE-deleted} mutants. (e) Ratio of non-apical mitosis (non-apical Phh3/Phh3, normalised by the volume in μm^3) in percentage, in AVE-adjacent and AVE-opposed regions from WT and *RhoA*^{VE-deleted} embryos. Values are shown as Mean \pm SEM. WT: n=31; *RhoA*^{VE-deleted}: n=17.



Supplementary Figure 1: Quantification of rosette events in three pre-gastrulation embryos using lightsheet imaging. (a, b, c) Single optical slices from 3 embryos at E5.75. All embryos are in lateral orientation, with the anterior side to the left as defined by the position of AVE cells (Hex-GFP, in green). Membranes are marked by dtTomato (in grey) (a') (b') (c') Upper row: Quantification of the number of rosettes observed per frame; Mid row: Frequency of rosettes, normalized by the surface or the epiblast region in focus and the time of observation; Bottom row: Frequency of apical cell rounding, normalized by the surface or the epiblast region in focus and the time of observation. Data show consistently a higher number of rosettes in the posterior side compared to the anterior and lateral side, both in absolute and normalized values. Time interval between acquisition is 7 min, and interval between Z-sections is 1 μm . Values are shown as Mean \pm SEM, and number of time points analysed per embryo are $n(a')=12$, $n(b')=19$ and $n(c')=10$. Scale bars represent 20 μm in (a) and (c), and 50 μm in (b).

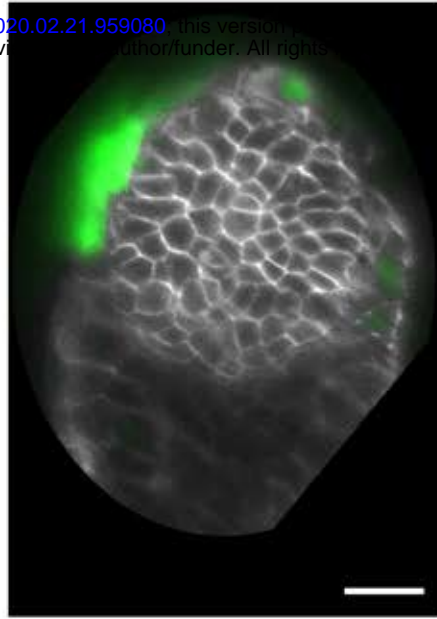
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embryo 1



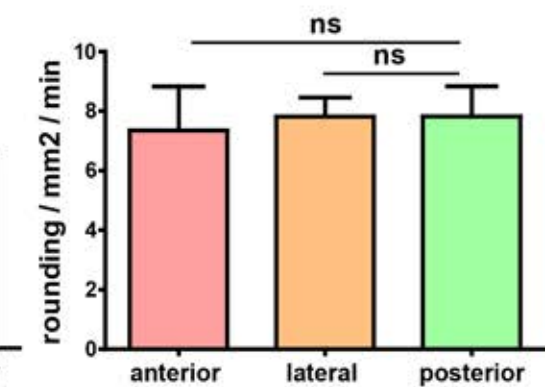
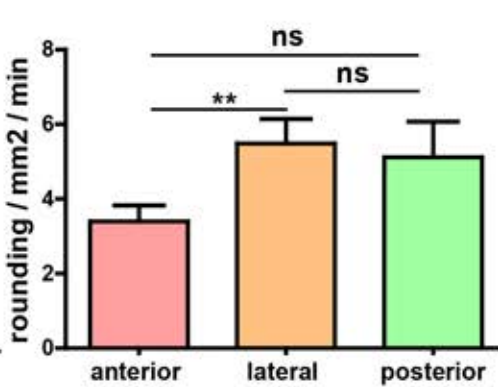
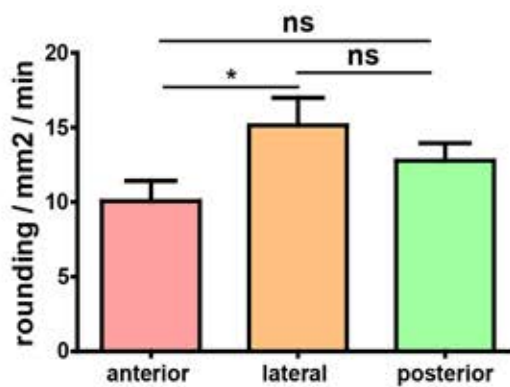
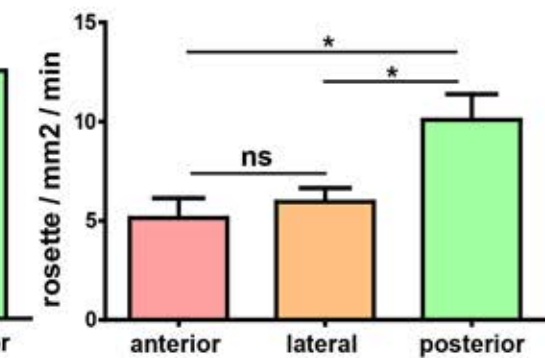
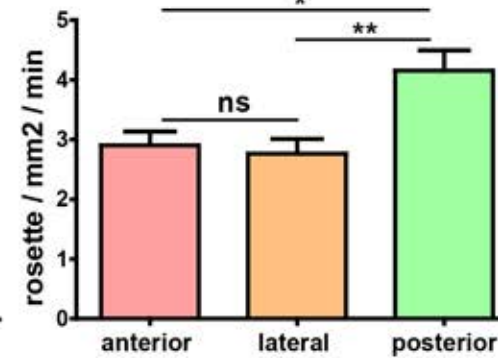
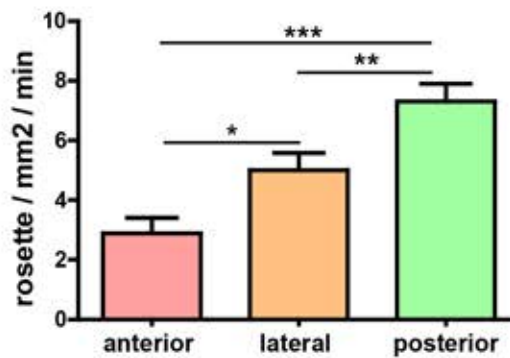
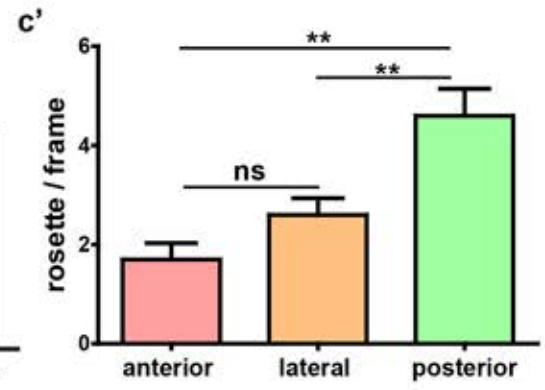
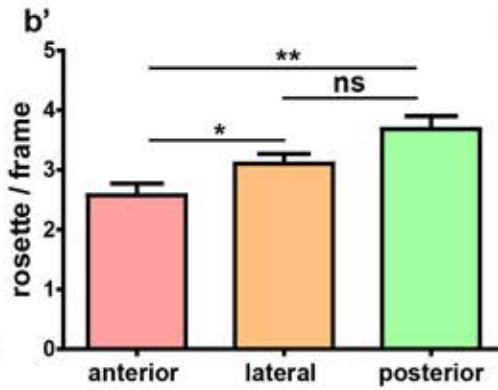
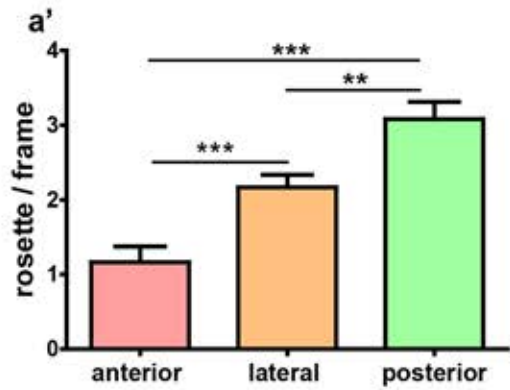
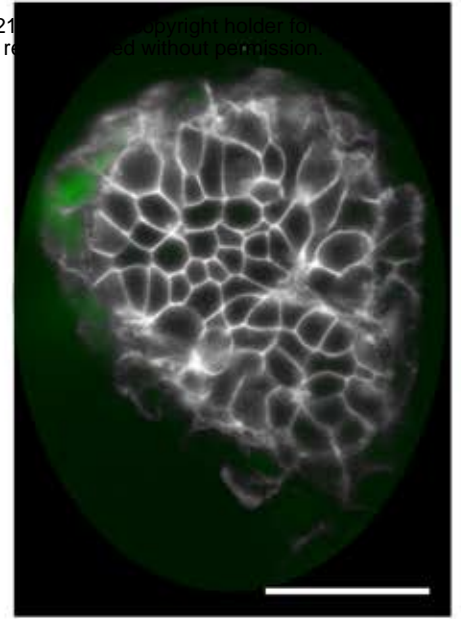
b

embryo 2

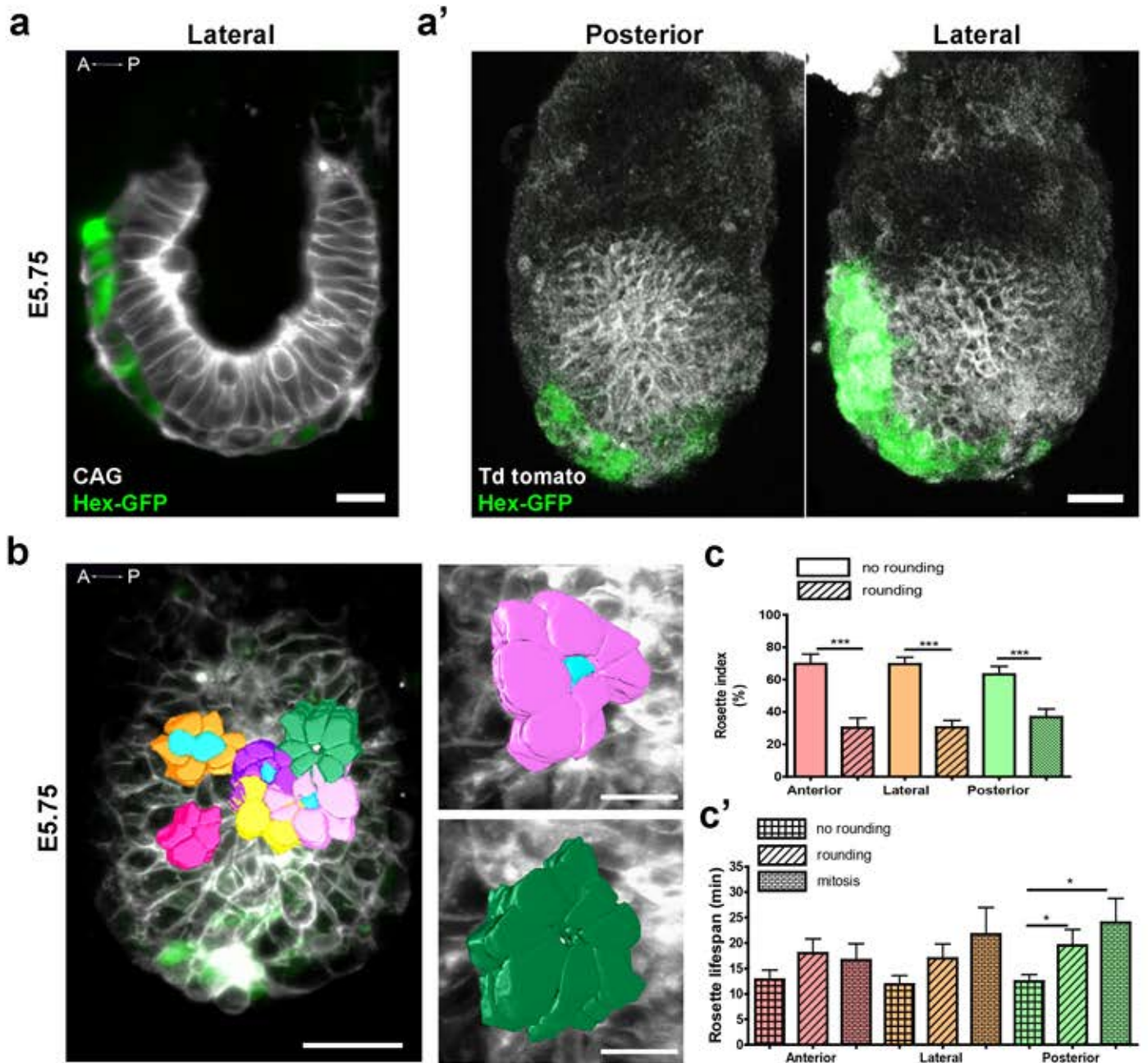


c

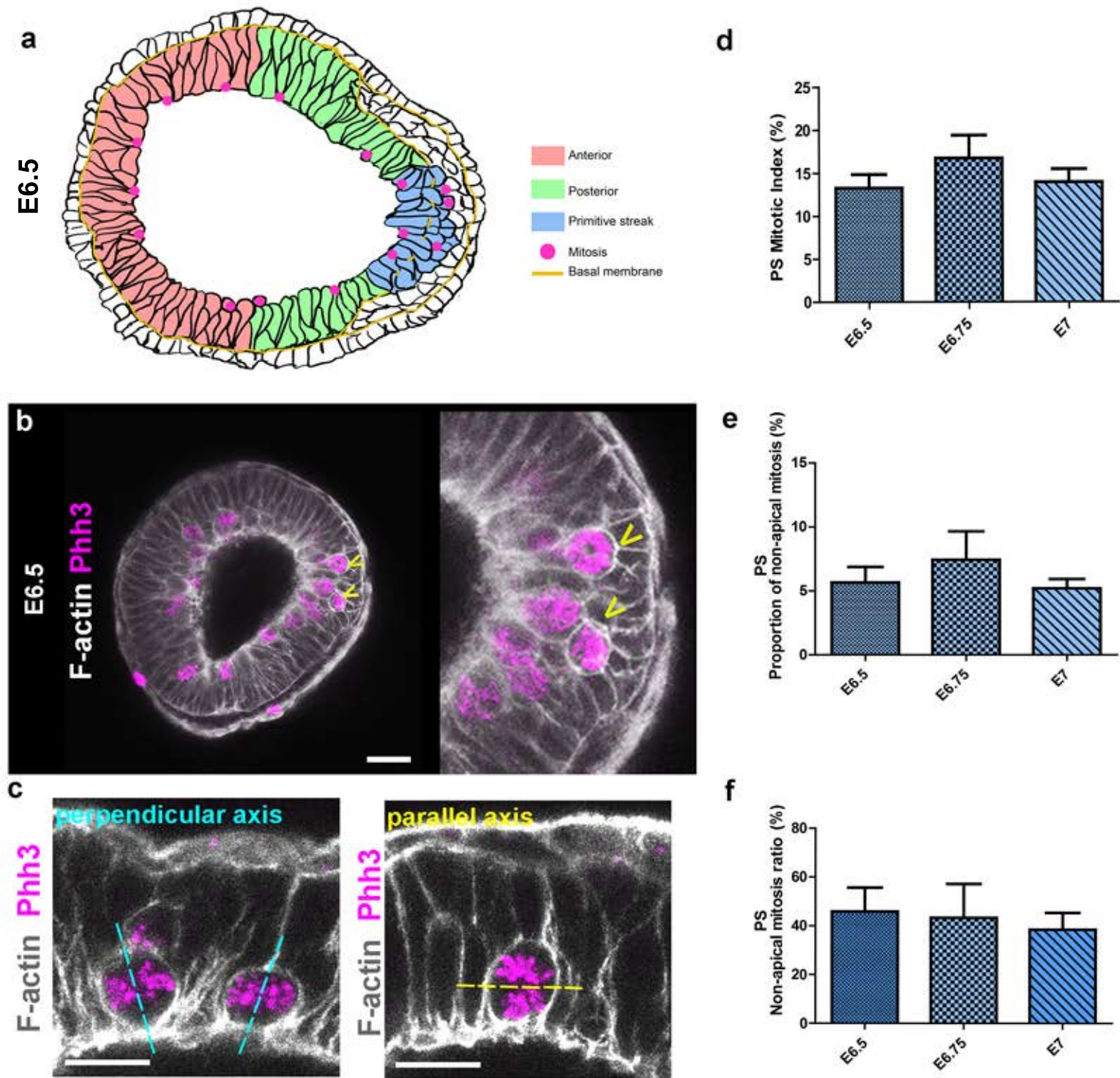
embryo 3



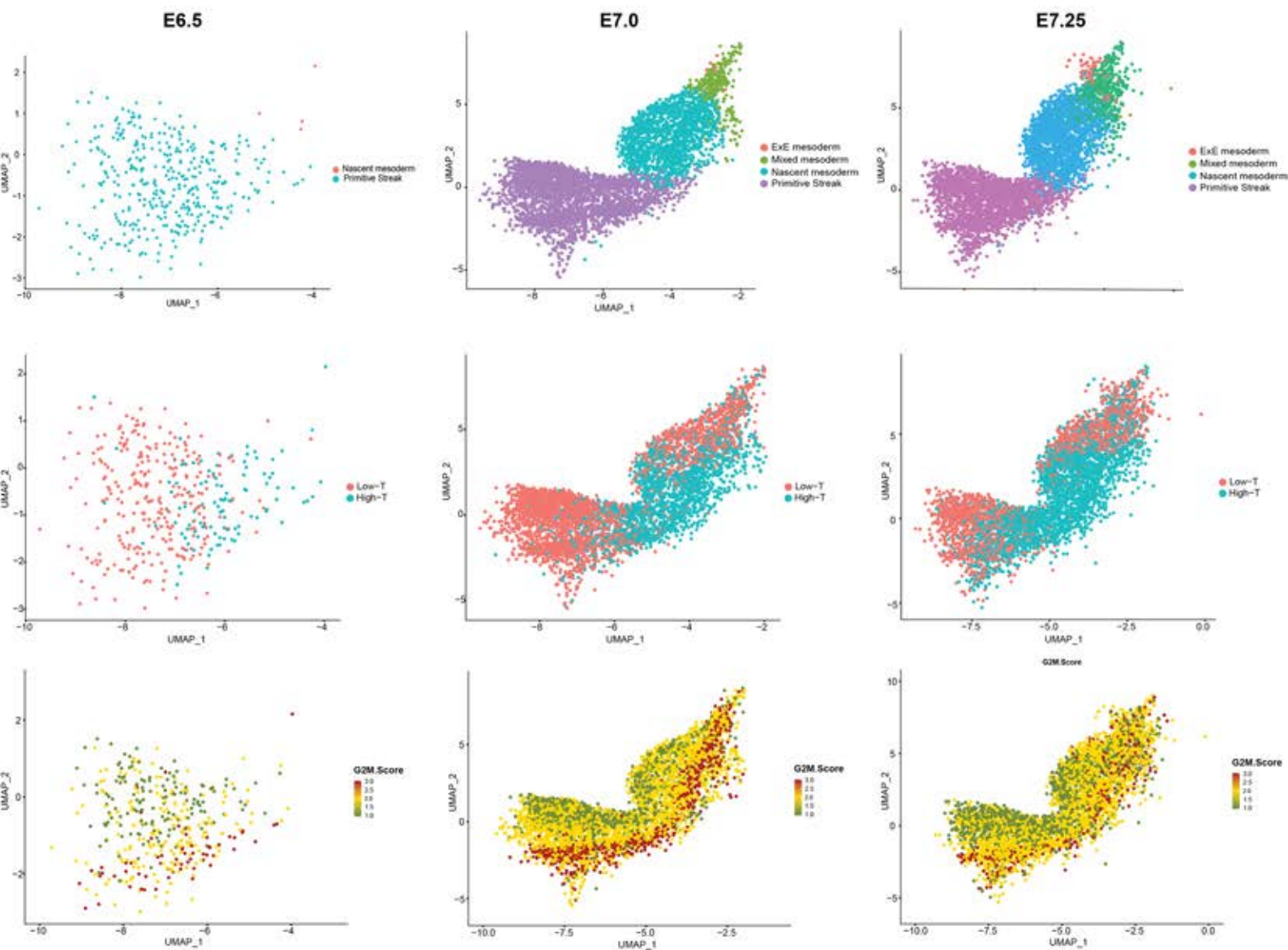
Supplementary Figure 2: Embryo orientation for lightsheet and confocal imaging, and quantification of rosettes linked to apical cell rounding (a) Z-projection of the middle section of an embryo imaged by lightsheet microscopy, shown in a lateral view with anterior to the left. Cell shape can be easily segmented. Scale bar: 10 μm . (a') 3D rendering of an embryo imaged by confocal microscopy, shown in posterior (left) and lateral (right) views. Scale bar represents 50 μm . In (a) and (a'), AVE cells are identified through the Hex-GFP reporter, allowing embryo antero-posterior orientation. (b) Left panel: 3D rendering of the apical side of manually segmented rosettes from an embryo imaged using lightsheet microscopy. Scale bar: 50 μm . Highlighted in light blue are rounded cells localised at the centre of the rosette. Right panels: zooms of a rosette linked to a rounded apical cell (top) and a rosette without an apically rounded cell (bottom). Scale bar: 20 μm . (c) Rosette rounding indexes: rosettes with or without central apical rounded cell /total rosettes in percentage and (c') Lifespan (min) of rosettes when bound to a central mitotic, central round or non-round cell in the anterior, lateral and posterior regions of the embryo. The posterior region includes the PS region. Values are shown as Mean \pm SEM.



Supplementary Figure 3: Non-apical division at the PS (a) Representation of sagittal section from an E6.5 embryo. The epiblast is divided in three distinct regions: anterior (red), posterior (green) and primitive streak (PS, blue). The PS region is defined by the area where the basal membrane (yellow) is degraded. A mitosis is considered non-apical when occurring away from the epiblast apical border at a minimum distance corresponding to the size of one nucleus ($\pm 10 \mu\text{m}$), in a cell that has not crossed the perforated basal membrane and does not display a mesenchymal phenotype. (b) Transversal optical section at streak level from a E6.5 embryo labelled for mitosis (Phh3, magenta) and F-actin (Phalloidin, grey). The apical cell attachment of non-apical Phh3-positive cells is highlighted in the zoom. Scale bar: $50 \mu\text{m}$. Arrowheads show non-apical mitoses. (c) Zoom of confocal imaging section of E6.25 embryos showing mitoses (Phh3, magenta) occurring with a parallel (left) or a perpendicular (right) axis at the apical pole. Scale bar: $30 \mu\text{m}$. (d, e, f) Proportion of mitosis and non-apical mitosis is stable through time at the PS: Graphs representing (d) mitotic index (Phh3/ DAPI) at the PS of E6.5, E6.75 and E7 embryos. (e) Proportion of non-apical mitosis (Non-apical Phh3/ DAPI, in percentage), and (f) Ratio of non-apical mitosis (Non-apical Phh3/ Phh3 normalized by the number of nuclei, in percentage). Values are shown as Mean \pm SEM. E6.5: n=20 frames from 6 embryos, E6.75: n=13 frames from 5 embryos, E7: n=20 frames from 6 embryos.



Supplementary Figure 4: Distribution of cell cycle phases among primitive streak and mesoderm cells. UMAP plots were generated from the Mouse Gastrulation 2018 atlas. Cells annotated as primitive streak and mesoderm were selected from E6.5, E7 and E7.25 samples. Cells are coloured based on annotation in (a), on level of *Brachyury* expression in (b), and on score for G2/M signature in (c).



Supplementary Figure 5: Non-apical mitosis is a feature associated with the primitive streak.

(a) Representation of the position of the AVE-opposed area (green) and the AVE-adjacent area (pink) in WT (left) and mutants (right) embryos. (b, c) 3D reconstruction (b) and confocal Z-slice (c) of E6.5 WT (left) and *Rac1*^{KO} (right) embryos stained for a AVE marker (Cerberus, yellow), F-actin (Phalloidin, grey) and mitosis (Phh3, magenta). Scale bar: 25 μ m. (d) Percentage of AVE migration in WT and *Rac1*^{KO} mutants. (e) Ratio of non-apical mitosis (non-apical Phh3/ Phh3 normalised by the volume) in percentage in WT and *Rac1*^{KO} mutants in AVE-adjacent compared to AVE-opposed regions. Values are shown as Mean \pm SEM. WT: n=30; *Rac1*^{KO}: n=7.

