1 **Sequential formation and resolution of multiple rosettes drives embryo remodeling after**

2 **implantation**

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Summary

The dramatic morphogenetic remodeling of embryo architecture after implantation culminates in pro-amniotic cavity formation. Despite its key importance, how this transformation occurs remains unknown. Here, we apply high-resolution imaging of embryos developing *in vivo* and *in vitro,* spatial RNA sequencing and 3D trophoblast-stem-cell models to determine the sequence and mechanisms of these remodeling events. We show that cavitation of the embryonic tissue is followed by folding of extra-embryonic tissue to mediate formation of a second, extra-embryonic cavity. Concomitantly, at the boundary between embryonic and extra-embryonic tissues, a hybrid 3D rosette forms. Resolution of this rosette enables the embryonic cavity to invade the extra-embryonic tissue. Subsequently, $\beta 1$-integrin signalling mediates formation of multiple extra-embryonic 3D rosettes. Podocalyxin exocytosis leads to their polarized resolution permitting extension of embryonic and extra-embryonic cavities and their fusion into a unified pro-amniotic cavity. These morphogenetic transformations of embryogenesis bring a novel mechanism for lumen expansion and fusion.

Introduction

Embryogenesis involves a gradual increase in complexity of embryo architecture as cells and tissues take shape. These events require intricate coordination and in mammals involve close cooperation between embryonic and extra-embryonic tissues that become established by the time of implantation[1-4]. The embryonic tissue, the epiblast, will give rise to the new organism, while one of the two extra-embryonic tissues, the trophectoderm, will generate the extra-embryonic ectoderm giving rise to the placenta and the other, the primitive endoderm, will generate the visceral endoderm and finally the yolk sac. The critical interaction between these tissues during mouse implantation development enables the transformation of the blastocyst into the egg cylinder, a more complex structure with an entirely different architecture.

The major morphogenetic event in the blastocyst-to-egg cylinder transition involves formation of the pro-amniotic cavity that spans the whole length of the egg cylinder. We have shown that this morphogenesis is initiated by polarization and lumenogenesis of the embryonic compartment that is triggered by β1-integrin signalling[5,6] and coordinated with a transition in stem cell potential[5,6]. However, how the lumen becomes established in the trophectoderm-derived extra-embryonic compartment and how then the embryonic and extra-embryonic lumens unify to form single cavity have remained unknown. Here, we use mouse embryos developing *in vivo* and *in vitro* together with 3D stem cell models, high resolution time-lapse microscopy and spatio-temporal transcriptome analyses to determine the sequence of events and mechanisms underlying coordination between the embryonic and extra-embryonic tissues in driving embryo remodeling and pro-amniotic cavity formation.

Results

Morphogenetic steps of pro-amniotic cavity formation

To understand how the pro-amniotic cavity forms, we first sought to determine the sequence of morphogenetic events involved. Based on detailed observations of 142 embryos freshly recovered during implantation stages, we could distinguish five distinct stages of global tissue rearrangements culminating in pro-amniotic cavity formation (Fig. 1a-e). During stage I (E4.75-5.0 embryos), epiblast cells became polarized into a rosette-like structure with a lumen opening at its centre, as previously described[5], while trophectoderm cells proliferated with the most proximal cells undergoing apical constriction. In stage II (E5.25-5.5 embryos), a thin elongated cavity formed at the proximal end of the extra-embryonic ectoderm as a result of tissue folding (Fig. 1f-h; Fig. S1, Movie 1-3). During stage III (E5.5 embryos), the

73 epiblast rosette re-organized into a cup-shaped epithelium while extra-embryonic ectoderm

74 cells at the embryonic/extra-embryonic boundary became polarized with their apical sides

75 flanking the epiblast cavity. At the same time, the apico-basal polarity axis of the epiblast at

76 the embryonic/extra-embryonic boundary changed from parallel to perpendicular, relative to

77 the proximo-distal axis of the embryo (Fig. 1b,e). During stage IV (E5.5-E5.75 embryos), the

78 embryonic cavity extended into the extra-embryonic compartment (Fig. 1b). Finally, during

79 stage V (E5.75 embryos), the two cavities fused into a unified single cavity spanning the

80 whole embryo. These observations allowed us to determine the sequence of cellular re-

81 organization that remodels the embryonic and extra-embryonic tissues leading to pro-

82 amniotic cavity formation.

83 Extra-cellular matrix is required for morphogenesis of extra-embryonic compartment

84 As extra-cellular matrix (ECM) signalling directs the formation of a cellular rosette that

85 undergoes lumenogenesis in the embryonic compartment[5], we wondered whether a similar

86 mechanism operates in the extra-embryonic compartment. High resolution analysis of tissue

87 remodeling on the cellular level revealed that immediately upon implantation, the extra-

88 embryonic compartment is comprised of two cell populations: one on the outside in direct

89 contact with the basement membrane (BM), and a second on the inside (n=35 embryos, Fig.

90 2a). While the outside cells were apico-basally polarized, as revealed by the distribution of

91 Golgi apparatus and cell shape, the inside cells were not (Fig. 2a-c). Since laminin is a ligand

92 for integrin receptors[7], we hypothesized that such a polarization mechanism could act via the

93 ECM surrounding the extra-embryonic compartment. Analysis of the activation status of β1-

94 integrin, the main ECM signalling mediator[8] on the basal side of the outside extra-embryonic

95 cells (Fig. S2a), supported this hypothesis.

96 To determine whether the ECM is sufficient to provide the signalling cues required for

97 polarization of the extra-embryonic ectoderm, we established a trophoblast stem cell (TSC)

98 3D model to mimic the development of the extra-embryonic compartment *in vitro*. This

99 entailed the suspension of small clumps of TSCs in 3D Matrigel (as ECM substitute). We

100 found that provision of ECM components led TSCs to arrange into spherical aggregates

101 resembling the extra-embryonic compartment (n=20, Fig. 2d). Whereas the TSCs in contact

102 with the ECM acquired columnar polarized morphology, TSCs not in contact with the ECM

103 retained an apolar character (Fig. 2d-e), supporting a role for the ECM in the polarization of

104   extra-embryonic cells. TSCs clumps cultured in the absence of the ECM failed to polarize

105   confirming that the ECM is necessary for TSCs polarization (Fig. S2b).

106   To determine whether provision of the ECM is indeed required for polarization of extra-

107   embryonic ectoderm *in vivo*, we disrupted the ECM by treating embryos with collagenase IV

108   (COLIV)[9]. The COLIV treatment led to BM disruption and defective β1-integrin activation

109   (Fig. S2c-d): whereas outside cells in control embryos polarized, the outside cells of COLIV-

110   treated embryos did not (n=12 and 13 respectively; Fig. 2f-g; Fig. S2e). These results indicate

111   that ECM-mediated signalling is necessary for extra-embryonic ectoderm organization.

112   **ECM/β1-integrin signalling is required for extra-embryonic compartment cavitation**

113   As β1-integrin signalling plays a central role in cell polarization in many different systems

114   [5,10], we therefore hypothesized that it might be required for polarization of the extra-

115   embryonic compartment. Since β1-integrin knock-out embryos die during the implantation

116   period[11], we tested our hypothesis using the TSC 3D system and a β1-integrin blocking

117   antibody[12]. TSCs cultured in the presence of the β1-integrin blocking antibody became

118   disorganized and apolar, in contrast to the organized and polarized control aggregates (n=20

119   for each group, Fig. 2h-j; Fig. S2f). Therefore, blocking β1-integrin function caused failure of

120   TSC polarization that resembled the effects of ECM disruption and loss of β1-integrin

121   activity upon extra-embryonic ectoderm polarization.

122   Since the above results indicated that extra-embryonic ectoderm polarizes in response to an

123   ECM/β1-integrin-dependent mechanism, we next assessed whether ECM/β1-integrin-

124   mediated polarization is essential for pro-amniotic cavity morphogenesis. To this end we

125   recovered embryos just after implantation and cultured them for 24 hrs in the absence or

126   presence of COLIV. Whereas in the great majority of cultured control embryos the

127   embryonic and extra-embryonic cavities fused, this process failed in most COLIV-treated

128   embryos (Fig. 2k-l). Importantly, COLIV-treated embryos in which the formation of the pro-

129   amniotic cavity was unaffected (3/27 embryos) had a residual BM (Fig. 2k right panel).

130   These results indicate that ECM/β1-integrin-mediated polarization of the extra-embryonic

131   ectoderm is essential for pro-amniotic cavity morphogenesis.

132   **ECM/β1-integrin signalling drives the formation of extra-embryonic rosettes**

133   To determine the cellular dynamics as the extra-embryonic compartment forms, we analyzed

134   82 freshly collected embryos prior to the fusion of the cavities. We found that in contrast to

the embryonic compartment, the extra-embryonic compartment of nearly all E5.5 embryos examined (93%, n=82) had multiple rosette structures (Fig. 3a; Fig. S3a). These extra-embryonic rosettes comprised 6 to 10 cells (n=60 rosettes, 40 E5.5 embryos) and had all the features of epithelial rosettes described thus far[13,14]: the apical localization of polarity markers (aPKC, Par6, ZO-1) and actomyosin, indicated that extra-embryonic ectoderm cells were apically polarized towards the rosette's centre (Fig. 3b). Strikingly, the number of extra-embryonic rosettes depended on the developmental stage: they were present more frequently in stages II and III; their frequency was significantly reduced in stage IV and were completely absent in stage V (so after cavities fusion) (Fig. 3c).

The majority of epithelial rosettes described so far are 2D structures forming within single-layered epithelia[15]. 3D cell segmentation analyses revealed that extra-embryonic rosettes are 3D structures (Fig. 3d-e; Movie 4). However, in contrast to other 3D rosettes that participate in lumen formation[5,16], the extra-embryonic rosettes do not form lumens (n=60 rosettes, 40 E5.5 embryos) and, unusually, two adjacent rosettes could share cells that are bipolar contributing to both a rosette and the cavity (Fig. 3d-f; Movie 5).

To gain insight into the role of rosettes during extra-embryonic morphogenesis, we examined their formation. Analyses of 142 embryos revealed that as many as 95% of the rosettes only contacted the BM on one side (with their outer cells) and had a second group of inner cells that, although polarized with their apical side towards the rosette's centre, were not in contact with the BM (Fig. S3b-d). This led us to hypothesize that inner cell polarization might be dependent upon outer cells becoming polarized through ECM/β1-integrin signalling. To test this hypothesis, we analyzed the organization of rosettes in COLIV-treated embryos in which polarization of outside cells was prevented. In contrast to all control embryos, rosette formation was defective in the presence of COLIV (n=12 and n=12 control and experimental embryos respectively) (Fig. 3g-h). Together, these results suggest that ECM-β1-integrin signalling is required for polarization of outside cells as well as subsequent polarization of inside cells and rosette formation (Fig. 3i).

Resolution of extra-embryonic rosettes drives pro-amniotic cavity formation

The defective pro-amniotic cavity formation seen in the absence of extra-embryonic rosettes suggested an involvement of these rosettes in cavity formation. To address this possibility, we analyzed the timing of events leading to the appearance of rosettes in relation to the five stages of pro-amniotic cavity formation we observed here (Fig. 1a-b). We found that just

167 before the re-organization of the epiblast from a sphere to a cup-shaped epithelium, a rosette
168 consisting of embryonic and extra-embryonic cells was established at the boundary between
169 the compartments (Fig. 4a). The formation of this hybrid rosette was preceded by the
170 breakdown of the BM separating the two compartments at stage I (E4.75; n=20 embryos, Fig.
171 4b). Importantly, resolution of this hybrid rosette was concomitant with epiblast remodeling
172 from a sphere to a cup-shaped epithelium (E5.5, n=10 embryos, Fig. 4c).

173 To establish the exact sequence of these events, we filmed the development of embryos
174 expressing LifeAct-GFP transgene[17]. This revealed a strong apical enrichment of actin at the
175 centre of the hybrid rosette. This actin enrichment was lost just before resolution of the
176 hybrid rosette, allowing the cavity to progress into the extra-embryonic compartment (Fig.
177 4d; Fig. S4, Movie 6,7). We confirmed that epiblast cells contributing to a hybrid rosette re-
178 oriented their polarity so that a tract of cells with common polarization connected the centre
179 of the rosette with the cavity (Fig. 4e). To determine if loss of cell-cell adhesion along this
180 track drives cavity extension, we laser ablated epiblast cells at the adhesion sites connecting
181 the epiblast cavity with the centre of the hybrid rosette. This laser ablation led to hybrid
182 rosette resolution and cavity progression (Fig. 4f; Movie 8), confirming that loss of adhesion
183 at specific sites of epiblast cells drives the cavity progression.

184 After hybrid rosette resolution, a new rosette became established near the embryonic/extra-
185 embryonic boundary in proximity to the expanding embryonic cavity (Fig. 4d). This rosette
186 comprised only extra-embryonic cells and was detected only in stage II and III embryos
187 (n=20 embryos; Fig. S5a). The main morphological change during the transition from stage
188 III to IV was the extension of the embryonic cavity. We found that at stage III, embryos
189 typically displayed a tract of the polarized apical parts of cells extending from the tip of the
190 embryonic cavity to the centre of an adjacent extra-embryonic rosette (Fig. 5a; Movie 9).
191 When embryos reached stage IV, extra-embryonic cells facing the embryonic cavity acquired
192 an open rosette conformation (Fig. 5b; Fig. S5b), suggesting that resolution of the rosettes
193 drives the extension of the cavity during the transition from stage III to IV.

194 To visualize the dynamics of cavity extension, we filmed the development of embryos
195 expressing membrane-Tomato[18] or LifeAct-GFP. This revealed that the resolution of a rosette
196 near the embryonic/extra-embryonic boundary preceded the extension of the epiblast cavity
197 (Fig. 5c; Fig. S5c; Movie 10-11). Specifically, rosette cells lost adhesion at cell interfaces
198 connecting the epiblast cavity with the centre of the rosette (blue and purple cells Fig. 5c,

199 magenta and cyan cells Fig. S5c) in agreement with our observations of embryos developing
200 *in vivo*.

201 Finally, we found that the centres of rosettes appearing near the extra-embryonic cavity were
202 linked with the tip of the extra-embryonic cavity through polarized tracts, priming the
203 cavity's expansion during stage IV (Fig. 5d; Fig. S5d-e; Movie 12). These observations
204 suggest that progressive expansion of embryonic and extra-embryonic cavities upon
205 resolution of rosettes results in their fusion (Fig. S5f, Movie13). Together, the observations of
206 tissue remodeling of embryos developing *in vivo* and *in vitro* indicate that rosettes play a
207 central role in pro-amniotic cavity formation.

208 Polarized Podocalyxin exocytosis during rosette resolution and cavities expansion

209 To gain insight into the molecular mechanisms of polarized rosette resolution and fusion of
210 cavities, we generated a spatial transcriptome map[19] of post-implantation embryos by
211 generating sequential sections of embryos along their proximo-distal axis at successive
212 developmental stages and performing RNA sequencing (Fig. 6a; Table S1). These analyses
213 revealed three distinct cell populations corresponding to distal visceral endoderm, epiblast
214 and extra-embryonic ectoderm. The identity of these tissues was confirmed by examining the
215 spatial expression pattern of epiblast (Pou5f1) and extra-embryonic ectoderm (Cdx2) markers
216 (Fig. S6a-b). Additionally, Wnt3 transcripts were detected in the distal extra-embryonic
217 ectoderm and proximal epiblast and T/Brachyury transcripts were detected in the distal extra-
218 embryonic ectoderm of E5.75 embryos (Fig. S6c) as described previously[20], highlighting the
219 sensitivity of this method.

220 Analysis of differential gene expression between embryonic and extra-embryonic regions
221 revealed the upregulation of 764 genes in the E5.25 embryo extra-embryonic compartment;
222 1119 genes in the E5.5 embryo extra-embryonic compartment; and 306 genes in the E5.75
223 embryo extra-embryonic compartment (Table S1, Fig.S7a). Importantly, functional
224 enrichment analysis revealed that several genes involved in integrin-mediated signalling were
225 specifically upregulated in the extra-embryonic compartment during stages II-III (Fig. S6d,
226 Table S1). This is in accord with our results showing that ECM/integrin signalling drives
227 extra-embryonic tissue polarization and rosette formation.

228 In accord with the requirement for Rab11-mediated exocytosis and the reorganization of
229 exocytotic vesicles in lumen formation [6,21-23], we found several regulators of exocytosis and
230 vesicle organization to be upregulated in the extra-embryonic compartment during pro-

231   amniotic cavity formation (Fig. 6b, Table S1). Specifically, expression of Rab11A, the main

232   mediator of exocytosis during lumen formation, was enriched in the extra-embryonic

233   compartment, suggesting that Rab11-mediated apical exocytosis is involved in this process.

234   In MDCK cells, lumenogenesis requires Par/aPKC mediated cell polarization and the

235   concerted activity of Rab GTPases, to direct the polarized exocytosis of Podocalyxin[16].

236   Examination of expression dynamics of genes involved in the regulation of Podocalyxin

237   exocytosis revealed that they were upregulated in the extra-embryonic compartment either at

238   one or both stages of tissue remodeling (II, E5.25 or III E5.5), during which time the

239   transcriptional profile of the extra-embryonic lineage differs from stage IV-V(E5.75) (Fig.6c;

240   Fig.7a; Fig.S7b). Together this suggests that Podocalyxin exocytosis plays a role during pro-

241   amniotic cavity formation.

242   In agreement with the transcriptome analysis, we found Podocalyxin positive exocytotic

243   vesicles in all cells of the extra-embryonic compartment at E5.5, whereas in the embryonic

244   compartment Podocalyxin was present only at the apical side of cells (Fig.7b). These

245   Podocalyxin vesicles showed a polarized pattern of secretion, along the borders of polarized

246   tracts connecting rosettes with cavities, in both hybrid and extra-embryonic rosettes (Fig. 7c-

247   d). After fusion of the cavities, the extra-embryonic ectoderm cells not contributing to the

248   cavity still contained Podocalyxin exocytotic vesicles (Fig.7e). Polarized cell intercalation

249   resulted in the formation of a pseudostratified epithelium in the extra-embryonic

250   compartment in which all the extra-embryonic ectoderm cells faced the cavity (Fig.8a,

251   Fig.S8, Movie 14). At this point all the cells facing the cavity were negative for exocytotic

252   vesicles (Fig.8b). These results indicate that whereas polarized rosette resolution drives the

253   initial fusion of cavities, further tissue remodeling driven by intercalation completes the

254   process during the final step of pro-amniotic cavity formation.

255

256   **Discussion**

257   Upon implantation, the blastocyst transforms into the egg cylinder, a structure of an entirely

258   different architecture. We show that this drastic remodeling is driven by a series of major

259   morphogenetic events that we break down into five stages: stage I) polarization and

260   lumenogenesis of the embryonic tissue and generation of the extra-embryonic tissue; stage II)

261   the folding of the extra-embryonic tissue leading to its lumenogenesis and formation of a

262   hybrid rosette on the embryonic/extra-embryonic boundary; stage III) remodeling of the

263   embryonic tissue and formation of multiple 3D rosettes within the extra-embryonic tissue

264   mediated by ECM-β1-integrin signalling; stage IV) the polarized resolution of extra-

265   embryonic rosettes mediating expansion of embryonic and extra-embryonic cavities mediated

266   by Rab11/Podocalyxin vesicles exocytosis; stage V) the fusion of embryonic and extra-

267   embryonic cavities and polarized cell intercalation resulting in the formation of a unified pro-

268   amniotic cavity (Fig. 8c).

269   Two-dimensional rosettes have been described to contribute to tissue re-arrangements during

270   morphogenesis in many model systems[15]. Three-dimensional rosettes on the other hand, were

271   shown to contribute to tissue shaping by generating self-contained central lumens rather than

272   by rearranging cells[5,16]. Our detailed analysis of embryos developing *in vivo* as well as time-

273   lapse imaging of embryos developing *in vitro* indicate that whereas extra-embryonic

274   ectoderm rosettes have the characteristics of 3D epithelial rosettes, they do not form self-

275   contained lumens but, instead, form and resolve in a polarized manner to permit extension of

276   cavities through the compact extra-embryonic tissue. The spatial and temporal profile of

277   morphogenetic reorganization accords with progressive resolution of rosettes from the

278   embryonic to the extra-embryonic lumen to form the pro-amniotic cavity.

279   Our results indicate that resolution of extra-embryonic ectoderm rosettes is preceded by re-

280   orientation of cell polarity and expansion of the apical domain in a subset of cells. This

281   creates polarized tracts along cell interfaces connecting lumens with the centers of rosettes.

282   This re-orientation of cell polarity is followed by polarized exocytosis and lumen extension,

283   as Rab11/Podocalyxin vesicles become targeted to the rosettes' polarized tracts. Podocalyxin

284   is a negatively charged sialomucin causing membrane repulsion[24] and therefore its secretion

285   at these sites could indeed result in rosette resolution. Since we observe that each rosette is

286   exposed to the cavity before its resolution, it is possible that osmotic pressure from the cavity

287   fluid contributes to membrane separation during rosette resolution. Thus, a model proposing

288   that rosette resolution driven by exocytosis mediates expansion of the cavities is more in line

289   with our data than mechanisms described for lumen expansion in other systems[25,26].

290   In summary, the detailed developmental, cellular and molecular characterization of the

291   morphogenetic steps involved in the reorganization of the blastocyst into the egg cylinder

292   structure leads us to propose a novel mechanism for cavity fusion that is mediated by tissue

293   rearrangements governed by the formation and polarized resolution of multiple multicellular

294   3D rosettes. Our model suggests that polarized resolution of these cell arrangements mediates

295 the remodeling of the whole embryo leading to fusion of embryonic and extra-embryonic

296 cavities. Reorientation of apico-basal polarity, cell rearrangement and the expansion and

297 fusion of cavities have been described during development of different organisms[27-30] and

298 therefore it is likely that the morphogenetic processes leading to cavity formation we describe

299 here might represent a common molecular and cellular mechanism utilized in processes other

300 than mammalian embryogenesis.

301

Author Contributions

303 N.C and C.K designed and carried out the experiments and data analysis. A.W contributed to

304 embryo live imaging. G.C and G.P performed the embryo cryosection, laser microdissection

305 and library construction experiments for RNA-seq. R.W carried out the RNA sequencing

306 analysis. N.J supervised the work related to spatial trancriptome analysis. M.Z.G conceived,

307 supervised the study and wrote the manuscript with the help of N.C and C.K.

308

317

Methods

319 **Embryo recovery and culture:** Mice were kept in the animal house in accordance with

320 national and international guidelines. All experiments have been regulated by the Animals

321 (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review

322 by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

323 Experiments were approved by the Home Office (Licence number: 70/8864). Animals

324 were inspected daily and those that showed health concerns were culled by cervical

325 dislocation. Implantation stage embryos were recovered from wild type F1, MF1 or CD1

326 females mated with mTmG [18], LifeAct-GFP[17] and wild type males. Pre-implantation embryos

327 were flushed out from the uteri as previously[5]. Post-implantation embryos were dissected out

328 of the deciduae and transferred into IVC2[31]. After recovery, the embryos were transferred

329 into drops of IVC2 (1 embryo/drop) covered with mineral oil and cultured according to the

330 experimental designed. For the enzymatic removal of the BM, Collagenase IV (500ug/ml)

331 was used for 3-5 hrs in IVC2 and then replaced with 50ug/ml for overnight culture. Embryos

332 were cultured at 37°C in 5% CO_2.

333 **Immunostaining:** Embryos were fixed in 4% paraformaldehyde (PFA) for 20 min at room

334 temperature. Post-implantation embryos were permeabilized for ~12-20 min in 0.3% Triton

335 X-100 / 0.1M Glycin in PBS. The primary antibodies were then added to the blocking buffer

336 (0.1% Tween-20 / 10% filtered FCS in PBS) and embryos incubated overnight at 4°C.

337 Embryos were then incubated for 3-4 hrs at room temperature with secondary antibodies in

338 blocking buffer. All washes were done in filtered PBS + 0.05% Tween-20 (PBST). Pre-

339 implantation embryos were permeabilized in 0.5% Triton X-100 in PBS for 20 min at room

340 temperature. Primary antibodies were added in the blocking buffer (3% BSA in PBS + 0.1%

341 Tween-20) and embryos incubated overnight at 4°C. For visualizing exocytotic vesicles

342 through immunofluorescence permeabilization was performed with 0.1% (w/v) Saponin

343 (Sigma S7900) + 0.2% (w/v) gelatin (Sigma G7765) + 5mg/ml BSA for 30 min at room

344 temperature. Primary and secondary antibodies were prepared in 0.2% (w/v) gelatin + 0.01%

345 (w/v) Saponin. Primary antibodies were added to the sample overnight at 4°C while

346 secondaries were added for 2 hrs at room temperature after washing 3 times in PBS.

347 Primary antibodies used: ZO-1 (1:200; Thermofisher Scientific, 33-9100), E-cadherin (1:300;

348 Thermofisher Scientific, 13-1900), aPKC (1:100; Santa Cruz Biotechnologies, sc-216), Oct4

349 (1:400; Santa Cruz Biotechnologies, sc-5279), GM130 (1:200; BD, 610822), Laminin (1:400;

350 Sigma, L9393), Cdx2 (1:200; Biogenex, MU392A-UC), β1-integrin (1:50; Ha2/5; BD,

351 561796), active β1-integrin (1:50; 9EG7; BD, 553715), Ap2γ (1:300; Santa Cruz

352 Biotechnologies, sc-8977), Eomes (1:400; Abcam, ab23345), Elf5 (1:400; Santa Cruz

353 Biotechnologies, sc-9645), Podocalyxin (1:300; R&D systems, MAB1556), Pard6b (Santa

354 Cruz Biotechnologies, sc-67393), pMLC (1:100; Cell Signalling Technologies, 3671P),

355 Collagen IV (1:100; Millipore, AB769), HSPG2 (1:100; Millipore, MAB1948P), Rab11a

356 (1:100; Cell Signalling Technologies, 2413S) Anxa2 (1:700; Abcam, ab41803). Secondary

357 antibodies in pre-implantation blocking buffer were then added to the embryos for ~2 hrs at

358 room temperature. Secondary antibodies used: Alexa 647 donkey anti-rabbit (1:500;

359 Thermofisher Scientific, A31573), Alexa 594 donkey anti-rat (1:500; Thermofisher

Scientific, A21209), Alexa 568 donkey anti-mouse (1:500; Thermofisher Scientific, A10037), Alexa 488 donkey anti-mouse (1:500; Thermofisher Scientific, A21202), Alexa 488 donkey anti-goat (1:500; Thermofisher Scientific, A11055), Phalloidin 488 (1:500; Thermofisher Scientific, A12379). All washes were done in PBS + 0.1% Tween-20. Nuclear staining: minimum 15 min incubation in DAPI + PBS (5mg/ml).

Imaging: Freshly recovered and fixed embryos were imaged on a Leica SP5 or SP8 confocal microscope. For live imaging a multiphoton Leica SP8 was used. The wavelengths used for 2-photon excitation fluorescence (2PEF) when imaging mTmG (unconverted; Tomato) and LifeAct (GFP) were 1040nm and 910nm respectively.

Laser ablation was carried out on two-photon microscope (LaVision BioTec TriM Scope II). A multiphoton Insight DeepSee dual-line laser tuned to 920 nm was used to perform ablations. A region of interest was set to cell-cell interface and scanned at 3 different Z planes (1um) with the multiphoton laser set at 70% transmission with a pixel dwell time of 9.1μs. Images were taken immediately before, after and every 1 min following ablation.

Image processing and analysis: Fiji image processing software was used while 3D reconstructions of cells were carried out on 3D Slicer software using manually segmented images from Fiji.

Cell culture: Unconverted Confetti TSCs derived in defined conditions on fibronectin were used for TSCs aggregate experiments. TSCs were maintained and passaged under defined conditions on fibronectin-coated wells as described previously[32]. The cells were passaged when they reached ~70% confluency (normally once every three days) and medium was changed the day after passaging and then every other day. The cells were kept at $37^{o}C$ in 5% CO_2.

Trophoblast stem cell aggregates: TSCs were incubated for 2 min at $37^{o}C$ in 0.05% trypsin-EDTA (Invitrogen) when they reached confluency. Trypsinization was stopped by adding TS medium (RPMI 1640 (Sigma), 20% FCS (GIBCO), penicillin/streptomycin (50um/ml) (GIBCO), Sodium pyruvate (1Mm) (GIBCO), β-mercaptoethanol (100uM), L-glutamine (2mM) (GIBCO)). The cell suspension was collected and spun down (1000 rpm for 5 min). The supernatant was discarded, and the pellet re-suspended as clumps in defined culture medium prior to plating in Matrigel (BD, 356230). Using a haemocytometer the cell density was worked out and then appropriate volume of cell suspension was aspirated to suspend ~20000 cells/Matrigel drop. The cell suspension was spun down (1000 rpm for 5 min) and

392   supernatant removed. The TSCs in the pellet were gently re-suspended as clumps in a volume

393   of Matrigel corresponding to 20ul / drop before plating each 20ul drop in one well of an

394   ibiTreat microscopy plastic μ plate (Ibidi). After 2 min of incubation at 37°C to allow

395   polymerization of the Matrigel, the media were added according to the designed experiment.

396   For cells grown in the absence of Matrigel, cells were plated on a non-adherent suspension

397   culture plate (CELLSTAR, 662 102). The cells were kept at 37°C in 5% CO_2.

398   **Embryo laser capture microdissection and RNA isolation**: The spatial transcriptome of

399   embryos was obtained according to the Geo-seq methods [33]. In brief, E5.25, E5.5, E5.75 MF1

400   embryos in deciduas were embedded in OCT compound and cryo-sectioned serially at 15 μm

401   along the proximodistal embryo axis. Serial sections were mounted on polyethylene

402   terephthalate-coated slides. Frozen sections were allowed to thaw at room temperature and

403   then dehydrated in ice-cold 100% ethanol. Fixation was performed in 75% ethanol, then the

404   slides were stained with 1% cresyl violet acetate solution (Sigma-Aldrich, prepared in 75%

405   ethanol), dehydrated in a series of 75%, 95%, 100% ethanol (30 secs for each step), and

406   finally subjected to LCM on an MMI Cellcut Plus system (MMI, Zurich, Switzerland).

407   Approximately 20 cells in each section were harvested by LCM. Cell samples were lysed in

408   50 μl of 4 M guanidine isothiocyanate solution (GuSCN; Invitrogen, 15577-018) at 42°C for

409   10 min. The volume of the lysate was adjusted to 200 μl by nuclease-free water, and was

410   further concentrated by ethanol precipitation in the presence of 1/10 volume of acetate

411   sodium (pH 5.7, 3 M; Ambion) and 2 μl of carrier glycogen (20 mg/ml; Roche). Total RNA

412   pellets were dissolved in lysis solution and used as a template for low-cell number RNA-seq.

413   **RNA-Seq data pre-processing**: Raw reads were evaluated the quality with the FASTQC.

414   Density distribution of gene expression for all samples were also plotted to assess whether

415   there are inconsistent samples. Raw reads were mapped to mm10 version of mouse genome

416   using Tophat2 v2.0.4 program[34]. We calculated fragment per kilobase per million (FPKM) as

417   expression level using Cufflinks v2.0.2 with default parameters[35]. Genes with the FPKM >

418   1.0 in at least one sample across all samples were retained for further analysis. Finally, the

419   expression levels were transformed to logarithmic space by using the log_2(FPKM+1).

420   **Hierarchical clustering** and **PCA** are based on all the expressed genes as described in the

421   RNA-Seq data pre-processing.

Differentially expressed genes (DEGs) analysis between epiblast (EPI) and extra-embryonic ectoderm (ExE) were identified using RankProd [36] with P value **<** 0.05 and fold change > 1.5.

Functional enrichment analysis of gene sets with different expression patterns was performed using the Database for Annotation, Visualization and Integrated Discovery v6.8 (DAVID v6.8)[37].

Gene expression visualization: to visualize the genes expression patterns in each embryo section, we wrote a program for visualization in MATLAB (version: 2015a). To analyze the genes expression dynamics during development (E5.25, E5.5, E5.75), we normalized the three sequencing batches to the same standard using ComBat [38], then ran the MATLAB program.

Statistics and Reproducibility

Statistical analyses were performed using GraphPad Prism 6.0 software. Embryos were randomly allocated to control and experimental groups. Sample size was determined based on previous experimental experience. Investigators were not blinded to group allocation. Quantitative data are presented as mean ± s.e.m. Quantitative data were analysed using a two-sided unpaired Student's *t*-test and qualitative data were analysed with χ^2 test. Unless otherwise noted, each experiment was performed at least three times

Data availability:

RNA–seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE110808. Source data for Fig. 1b, 2c,2e,2g,2i,2j,2l,3c,3h and Supplementary Fig. 3c,3d have been provided as Supplementary Table 2. Source data for Fig.1g,h; Fig 3e,f; Fig. 4d,f; Fig. 5c,d and Fig. S3c are provided as supplementary movies. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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524

Figure legends

Figure 1: Stages of embryo remodeling and pro-amniotic cavity formation

(a) Morphological staging of early post-implantation embryos based on the development of a pro-amniotic cavity; Arrowhead points to apical side of extra-embryonic ectoderm (ExE) cells at the boundary and arrows point to the ExE cells facing the epiblast (EPI) cavity; circled portion of stage III embryo highlights the invading EPI cavity into the ExE. n=142 embryos. (b) Quantification of the distance between the EPI and ExE cavities during pro-amniotic cavity formation. Two sided unpaired student's t-test; ****P<0.0001; mean±SEM; n= 21 stage II, 28 stage III and 20 stage IV embryos. (c) Percentages of embryos found in specific pro-amniotic cavity stages according to embryonic day staging systemn; =73 E5.5 + 31 E5.75 embryos.(d) Schematic representation of the five major stages of pro-amniotic cavity formation as identified through analysis of freshly recovered embryos. VE, visceral endoderm; ECM, extracellular matrix; EPC, ectoplacental cone. (e) Magnification of the area at the EPI-ExE boundary from d showing the reorientation of EPI boundary cells (red arrows). (f) Extra-embryonic cavity formation. Each stage of extra-embryonic cavity (cyan

asterisks) formation was characterized by localization of tight junction protein ZO-1 (white arrowheads) and enrichment of E-cadherin (yellow arrowheads) as well as cell shape changes accompanied with bending of the tissue (white arrows). Yellow asterisk indicates embryonic cavity. White dots mark ExE proximal cells acquiring columnar morphology. Red dots: cell apical site; P: proximal; D: distal. n=25 embryos. (g) Stills from a time-lapse movie showing apical constriction driven tissue bending during extra-embryonic cavity formation. Red arrowheads mark apical actin enrichment during apical cell constriction which leads to cell ingression and tissue bending. Dotted line: cavity. Segmented panel: Red outline marks cells undergoing apical constriction. Yellow line: tissue outline. Note that these cells ingress inducing tissue bending and cavity formation. Green outline: lateral cells. n=5 embryos. (h) Stills from single cells in (g); Cells show apical surface area reduction before ingression. Enrichment of apical actin (red arrowheads) coincides with apical constriction. Scale bars=20um.

Figure 2: Extra-cellular matrix/β1-integrin signalling is essential for extra-embryonic ectoderm polarization and morphogenesis

(a) Polarity pattern assessment in stage II E5.5 extra-embryonic ectoderm (ExE) based on Golgi position(GM130); Schematic: position of Golgi in manually segmented and position-based colour coded ExE. White dots: Golgi. n=10 embryos. (b) Cell morphology of outside (i) and inside (i') cells as shown by 3D cell segmentation. (c) Cell aspect ratio of outside vs inside ExE cells. Two sided unpaired student's t-test; ****P<0.0001;mean± SEM; n=50 cells, 6 E5.5 embryos. (d) TSCs grown as aggregates in Matrigel for 48 hrs in the presence of N2B27 and FGF2, stained for tight junction marker ZO-1 or golgi marker GM130, F-actin and the TSC marker Eomes. n=3 biological replicates. (e) Cell aspect ratio of outside (n=31) vs inside cells(n=36) in TSCs .Two sided unpaired student's t-test; ****P<0.0001; mean±SEM. (f) Cell morphology of outside ExE cells in control and COLIV treated embryos. Embryos in each group are shown at two different z slices and a zoomed image of outside cells is displayed on right of each image. n=3 biological replicates. (g) Comparison of cell aspect ratio of outside cells in control and COLIV-treated embryos. Long axis = axis perpendicular to basement membrane; short axis = axis perpendicular to long axis at half length; Two sided unpaired student's t-test; ****P<0.0001; mean±SEM; n=60 cells, 6 Control and 10 COLIV treated embryos. (h) TSC aggregates cultured in Matrigel in the presence of N2B27 and FGF2 for 48 hrs. β1-integrin function blocking antibody (Ha2/5) was added at 24hrs. n=3 biological replicates. (i) Quantification of apical ZO-1 subcellular

573 localization (apical ZO-1 intensity/basolateral ZO-1 intensity) in control (n=40 cells) and β1-
574 integrin function-blocking antibody-treated TSC aggregates (n=40 cells); Two sided unpaired
575 student's t-test; ****P<0.0001; mean±SEM. (j) Comparison of cell aspect ratio of outside
576 cells in control (n=51 cells) and β1-integrin function-blocking antibody-treated TSC
577 aggregates (n=32 cells) Two sided unpaired student's t-test; ****P<0.0001; mean±SEM. (k)
578 Basement membrane disruption after treatment with COLIV leads to failure of pro-amniotic
579 cavity formation. (l) Quantification of pro-amniotic cavity formation efficiency in control and
580 COLIV-treated embryos; χ^2 test ; ****P<0.0001. For (k) and (l) n= 27 control and 24
581 COLIV-treated embryos. n=3 biological replicates. Scale bars=20um.

Figure 3: Extracellular matrix-dependent 3D rosette formation throughout the extra-embryonic ectoderm

584 (a) Representative examples of E5.5 embryos showing formation of extra-embryonic
585 ectoderm (ExE) rosettes as identified by cell morphology. n=20 embryos. (b) E5.5 embryos
586 stained to reveal tight junctions (ZO-1), polarity (aPKC, Par6) and actomyosin markers (F-
587 actin, pMLC). Magnified images centered on ExE rosettes (outlined by dashed line). Arrows:
588 rosette center. n=20 embryos. (c) Average number of ExE rosettes observed per stage of pro-
589 amniotic cavity development (17 stage II, 21 stage III, 23 stage IV, 10 stage V embryos; Two
590 sided unpaired student's t-test; ***P=0.0002; mean±SEM. (d) Representative image of an
591 E5.5, stage III embryo. Orthogonal projections are displayed on the right and bottom of the
592 image. Asterisks label bipolar cells in the rosette either having a second apical side in the
593 ExE cavity or the epiblast cavity. Rosette schematics are displayed on the bottom right with
594 red dots indicating bipolar cells. Inset shows magnified image of the rosette. n=10 embryos.
595 (e) Rosettes identified and segmented at different z optical sections from a single E5.5, stage
596 III embryo. Segmented panel: Segmented cells for each rosette are differentially and
597 arbitrarily colour-coded for 3D rotation clarity. 3D Rendering panel: Each cell is also coded
598 with a roman numeral (rosette 1: i – vi; rosette 2: i' – vi'). Rotation and magnification of
599 rosette centres indicated by the dashed circle demonstrate absence of self-contained lumen as
600 apical surface of cells meet at rosette centre. (f) Combined 3D rendered rosettes in the same
601 spatial frame to assess cell sharing. Shared cells are highlighted. Both colour and number
602 coding is retained from (e) and displayed in this panel. Magnification of rosette centres
603 indicated on top right of each box. (g) ExE rosette formation in control and COLIV treated
604 embryos. Orthogonal slices are projected on the right and bottom of the images. n=12
605 embryos;3 biological replicates. (h) Percentage of embryos with or without identified ExE

606   rosettes in control and COL IV-treated groups. χ^2 test ; ****P<0.000; n= 12 control and 12

607   COLIV treated embryos; 3 biological replicates. (i) Model of ECM/β1-integrin-mediated

608   signalling mechanism for polarity establishment and rosette formation in ExE. Scale

609   bars=20um.

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611

612   **Figure 4: Epiblast-extra-embryonic ectoderm hybrid rosette polarized resolution drives**

613   **epiblast reorganization**

614   (a) Two representative examples of hybrid rosette forming at the EPI (filled dots)/extra-

615   embryonic ectoderm (ExE) (hollow dots) boundary. Arrowhead: pMLC enrichment. Insets

616   show EPI/ExE boundary. Dashed lines in magnified images indicate ExE cells where solid

617   lines indicate EPI cells. Dashed outline in example II points to an ExE rosette. YZ orthoslice

618   is projected on the right of example II. n=10 embryos. (b) Peri- and early post-implantation

619   embryos stained for laminin to assess integrity of basement membrane between EPI and polar

620   trophectoderm (pTE)/ExE (yellow arrowheads). White dots indicate pTE cells. n=15

621   embryos. (c) Freshly recovered embryos at different stages of hybrid rosette development.

622   Rosette resolution is displayed in a temporal sequence from left to right. solid outlines: EPI

623   cells; dashed outlines: ExE cells; grey outline: EPI cavity. Arrow: pMLC puncta belonging to

624   the resolved rosette. Inset in right panel shows a different Z slice where the re-oriented EPI is

625   evident. n= 8 embryos. (d) Stills from a time-lapse movie of LifeAct-GFP E5.5 (late Stage II)

626   embryo showing EPI/ExE hybrid rosette resolution and EPI reorganisation. Green box

627   magnifications indicate the centre of hybrid rosette, made up of EPI (blue dots) and ExE

628   cells (red dots) as it loses the actin enrichment (yellow arrowheads) in preparation for

629   resolution. Green box at 80min time-point highlights an ExE rosette in proximity to EPI

630   cavity. Red dots indicate rosette cells. Right panel: Manually segmented version of the time-

631   lapse movie focusing on the resolving hybrid rosette and a forming EPI cavity proximal

632   rosette (green in second row).n= 3 embryos. (e) Representative E5.5 embryo with EPI/ExE

633   hybrid rosette formed at the EPI/ExE boundary. Arrowhead: rosette centre; Arrows: Polarized

634   tract; Hollow dots: ExE cells; Filled dots: EPI cells. n=6 embryos. (f) Left panel: Still images

635   from time lapse movie after laser ablation of adhesion sites connecting the EPI cavity with

636   hybrid rosette centre. Red line: ablation site. Right panel: Magnified images of the boundary

637   region before and after ablation. Purple region with green outline shows the EPI cavity.

638   Arrows show the ablated adhesion site and the progression of the cavity after ablation. n=2

639   biological replicates. Scale bars=20um.

640   **Figure 5: Rosette formation/resolution-mediated pro-amniotic cavity formation**

641   (a) Representative example of stage III E5.5 embryo. Magnified images show extra-

642   embryonic ectoderm (ExE) rosette in proximity to epiblast (EPI) cavity with segmented

643   counterparts. Arrows point to polarized tracts (assessed with tight junction marker ZO-1)

644   extending from EPI cavity to centre of rosette before EPI cavity progression. n=10 embryos.

645   (b) Representative examples (I & II) of stage IV E5.5 embryos. Magnified images show

646   resolved ExE rosette in proximity to EPI cavity with segmented counterparts. Arrows point to

647   expanded apical domain (assessed with polarity marker aPKC) of ExE cells after rosette

648   resolution and EPI cavity progression. Asterisks indicate cells which lost their connection

649   upon rosette resolution. n=10 embryos. (c) Left panel: Stills from a time-lapse movie of

650   mTmG stage III embryo transitioning to stage IV of pro-amniotic cavity development. Box

651   (i) focuses on the EPI-ExE boundary and segmentation analysis is shown in middle panel.

652   Cells are manually segmented and differentially and arbitrarily colour-coded for clarity.

653   Extraction of segmented-only data is displayed next in right panel for the period of 40–

654   100min where formation and resolution of rosette is observed. Arrows in right panel indicate

655   rosette resolution and EPI cavity progression. n= 3 embryos. (d) Stills from a time-lapse

656   movie of Lifeact-GFP embryo showing ExE cavity extension through polarized rosette

657   resolution. Note the loss of actin enrichment from polarized tract (arrows) connecting the

658   cavity with the rosette centre (arrowhead) before rosette resolution. Yellow filled area; ExE

659   cavity: Magenta dots: rosette cells; Green dots; cells that lost adhesion; n=3 embryos. Scale

660   bars=20um.

661   **Figure 6: Spatial transcriptome analysis of pro-amniotic cavity formation**

662   (a) Experimental strategy: cells were captured by laser capture microdissection along the

663   proximodistal embryo axis and analysed by RNA-seq (Methods). Purple: epiblast (EPI);

664   Cyan: extra-embryonic ectoderm (ExE); Yellow: VE. P: proximal; D: distal. (b) Differential

665   gene expression analysis heat maps for gene ontology terms: exocytosis, vesicle organization.

666   E5.25: sections 2-4 EPI, sections 6-8 ExE; E5.5: sections 2,4,5 EPI, sections 6-11 ExE; E5.75

667   sections 3-8 EPI, sections 10-19 ExE. Purple outlines highlight genes previously reported to

668   be involved in lumen formation. (c) Expression pattern of selected genes during post-

669   implantation development. Left: Schematic showing the position and tissue identity for each

670 sequenced section (numbers on the left of the plot) in E5.25, E5.5 and E5.75 embryos. Gene

671 expression is presented as colour coded corn plot. Colour coding represents $\log_2$ (FPKM+1)

672 values. FPKM: fragment per kilobase per million.

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674

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Figure 7: Polarized pattern of Podocalyxin exocytosis during rosette resolution and cavities expansion

678 (a) Annexin A2 localization in a representative E5.5 embryo. n=10 embryos. (b)

679 Rab11/Podocalyxin localization in E5.5 embryo. Arrows show Rab11/Podoxalyxin positive

680 exocytotic vesicles. n=10 embryos. (c) Representative E5.5 embryo with epiblast (EPI)/extra-

681 embryonic ectoderm (ExE) hybrid rosette (dots). Lower panel shows magnified images of

682 EPI/ExE boundary. Arrow indicated the polarized tract connecting the EPI cavity with the

683 rosette centre. Arrowheads show Rab11 and Podocalyxin positive vesicles docking cell/cell

684 interfaces forming the polarized tract. n=5 embryos. (d) Representative Z-stacks of an E5.5

685 embryo. Upper panel shows an ExE rosette being connected with the EPI cavity via polarized

686 tract (arrows). Rab11/Podocalyxin positive vesicles are docked (hollow arrowhead) or

687 polarized along the polarized tract (filled arrowhead). Lower panel shows ExE rosette being

688 connected with the ExE cavity via polarized tract (arrows). Rab11/Podocalyxin positive

689 vesicles are docked along the polarized tract (hollow arrowhead).n=5 embryos. (e) Maximum

690 intensity profile image of a representative E5.75 embryo just after cavities fusion. A unified

691 cavity (arrows) spans the embryonic and extra-embryonic compartment. Exocytotic vesicles

692 are present in extra-embryonic cells not directly in contact with the cavity (arrowheads). n=5

693 embryos Scale bars=20um.

Figure 8: Cell intercalation during the final step of pro-amniotic cavity formation

695 (a) Time-lapse movie extraction of an mTmG E5.5 (stage II) embryo with segmented cavities

696 (purple) showing a cell (green) intercalating towards the basement membrane. n=2 embryos.

697 (b) Representative example of an E6.25 embryo. At this stage all ExE cells contribute to the

698 unified pro-amniotic cavity and ExE is a pseudostratified epithelium. All ExE cells are

699 negative for intracellular exocytotic vesicles. n=10 embryos. (c)Model of pro-amniotic cavity

700 formation. During stage I, the epiblast cavity forms through hollowing and the polar

701 trophectoderm proliferates to form the extra-embryonic ectoderm (ExE). The ExE cells

702 undergo apical constriction, which induces the formation of the ExE cavity through tissue
703 folding. At stage II, in the presence of embryonic and ExE cavities, a hybrid rosette forms at
704 the embryonic/extra-embryonic boundary. Polarized resolution of this rosette results in
705 remodeling of embryonic compartment and progression to stage III. After, extra-embryonic
706 rosettes form and undergo polarized resolution, the embryonic and extra-embryonic cavities
707 extend. At stage IV the extended cavities merge. At stage V, a newly-formed unified pro-
708 amniotic cavity spans the egg cylinder. Scale bars=20um.

a Panel showing stages I–V with DAPI/F-actin and DAPI/E-cad/aPKC staining, with magnified insets below.

b proximity index(p.i) (a.u) plot comparing Stage II, Stage III, Stage IV. **** significance markers.

p.i = embryo length / cavities distance

c E5.5 and E5.75 pie charts. E5.5: 12.34%, 34.24%, 53.42%. E5.75: 32.25%, 67.75%. Legend: II, III, IV, V.

d Schematic of stages I–V. Legend: VE, ExE, EPI, Cavity, ECM, EPC.

e Schematics II and III.

f DAPI/ZO-1 and DAPI/E-cad panels for Stage I and Stage II. Lower right schematics: Polarization, Cell shape change, Tissue bending, Cavity formation.

g 2D view Lifeact, Segmented 2D view, 3D top view Lifeact at 0min, 45min, 65min, 100min.

h cell1 and cell2 at 0min, 5min, 10min, 15min, 20min.

a

E-cad | aPKC/GM130 | Merge + DAPI | Segmented ExE

Inside cells | Outside cells | Golgi

b

E-cad/GM130

ExE | EPI

Outside cell | Segmented | 3D rendering | Golgi

Inside cell | Segmented | 3D rendering | Golgi

c

long axis/short axis (a.u)

Outside cells | Inside cells

****

d

48h in N2B27 +FGF2

i — F-actin/GM130/Eomes

ii — F-actin/ZO-1/Eomes

e

long axis/short axis (a.u)

Outside cells | Inside cells

****

f

DAPI/Lam/E-cad/Elf5

Control | Zoomed | Segmented — Outside/Inside

COLIV | Zoomed | Segmented — Outside/Inside

g

long axis/short axis (a.u)

Control | COLIV

****

h

48h in N2B27 +FGF2

F-actin | ZO-1 | Merge + Eomes

Control

Ha2/5

FITC β1-integrin

i

ZO-1 apical localization (a.u)

Control | Ha2/5

****

j

long axis/short axis (a.u)

Control | Ha2/5

****

k

DAPI/Lam/Podxl/Cdx2

Control | COLIV

Lam

l

% embryos

Control | COLIV

+ pro-amniotic cavity | - pro-amniotic cavity

****

a

DAPI/E-cad — I

DAPI/E-cad — II

ExE / EPI

b

DAPI/E-cad/ZO-1

DAPI/F-actin/aPKC

DAPI/E-cad/Par6

DAPI/E-cad/pMLC

c

number of rosettes

***

stage II   stage III   stage IV   stage V

d

DAPI/F-actin/aPKC

YZ

ExE / EPI

XZ

XY / YZ / XZ

e

E5.5(III)

E-cad   Merge   z1   Segmented   Rosette 1

aPKC   z2   Rosette 2

3D rendering

Rosette 1 — i, ii, iii, iv, v, vi

Rosette 2 — i', ii', iii', iv', v', vi'

f

shared cells

Rosette 1 / Rosette 2

Rosette 1 + shared cells

Rosette 2 + shared cells

g

DAPI/ZO-1/E-cad/Lam — Control

YZ

DAPI/ZO-1/E-cad/Lam — COL IV

YZ

h

% embryos

− Rosettes
+ Rosettes

Control   COLIV

i

EPI/ExE/VE/Lumen

β1-Itg signalling
indirect polarity cues
tight junctions
apical markers

Outside
Inside
ECM

actomyosin
rosette cells

a

I · DAPI/E-Cad/pMLC
II · DAPI/Oct4/E-cad/pMLC · YZ

ExE
EPI

b

DAPI · E-cad · Lam · Merge

E4.75

E5.0
ExE
EPI

E5.25
ExE
EPI

c

DAPI/Oct4/E-cad/pMLC · E5.5(II)
Hybrid rosette
Segmented
ExE
EPI

DAPI//E-cad/Z0-1 · E5.5(III)
Resolving rosette
ExE
EPI
Segmented
ExE
EPI

DAPI/Oct4/E-cad/pMLC · E5.5(III)
XY · YZ · XZ
Resolved rosette · Zoom XZ
Segmented
YZ · Zoom
Segmented

d

E5.5 (II) ⟶ (III)

Lifeact
0 min · 20 min · 40 min
ExE
EPI
60min · 80 min · 100 min

Segmented
0 min · 20 min · 40 min
ExE
EPI
EPI
ExE
EPI-ExE apical sites
60 min · 80 min · 100 min
EPI
ExE
ExE rosette

e

DAPI/E-cad/aPKC
i
ii

f

Lifeact
pre-ablation · 0 min
1 min · 6 min
i · Pre
ii · Post

a

E5.5 (III)

E-cad | ZO-1 | Merge + DAPI | Zoom | Segmented

Rosette cells
Non-rosette cell
ExE
EPI

b

E5.5 (IV)

I

E-cad | aPKC | Merge + Oct4 + DAPI | Zoom | Rosette cells

ExE
EPI

II

Zoom | Rosette cells

ExE
EPI

c

mTmG

E5.5 (III) → (IV)

0 min | 20 min | 40 min
ExE
EPI
60 min | 80 min | 100 min

Zoom + segmented

0 min | 20 min | 40 min
60 min | 80 min | 100 min

EPI cavity

40 min | 60 min
ExE | ExE
EPI | EPI
80 min | 100 min
ExE | ExE
EPI | EPI

d

Lifeact

0 min | 5 min | 10 min | 15 min | 20 min

a

mTmG

0min 20min 40min
60min 80min 100min
120min 140min 160min

b

Merge + DAPI | F-actin | Podxl

c

EPI/ExE/VF

I
Cavities formation

II
Hybrid rosette formation
Polarised tract formation

III
EPI reorganisation
ExE rosette formation
Polarised tract formation

IV
EPI/ExE cavities expansion

V
Cavities fusion

ExE cavity formation
through folding of
apically polarized cells

Hybrid rosette resolution

ExE rosettes resolution

Polarised tract formation
Cell intercalation