SUPPLEMENTARY INFORMATION
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Sequential formation and resolution of multiple rosettes drive embryo remodelling after implantation

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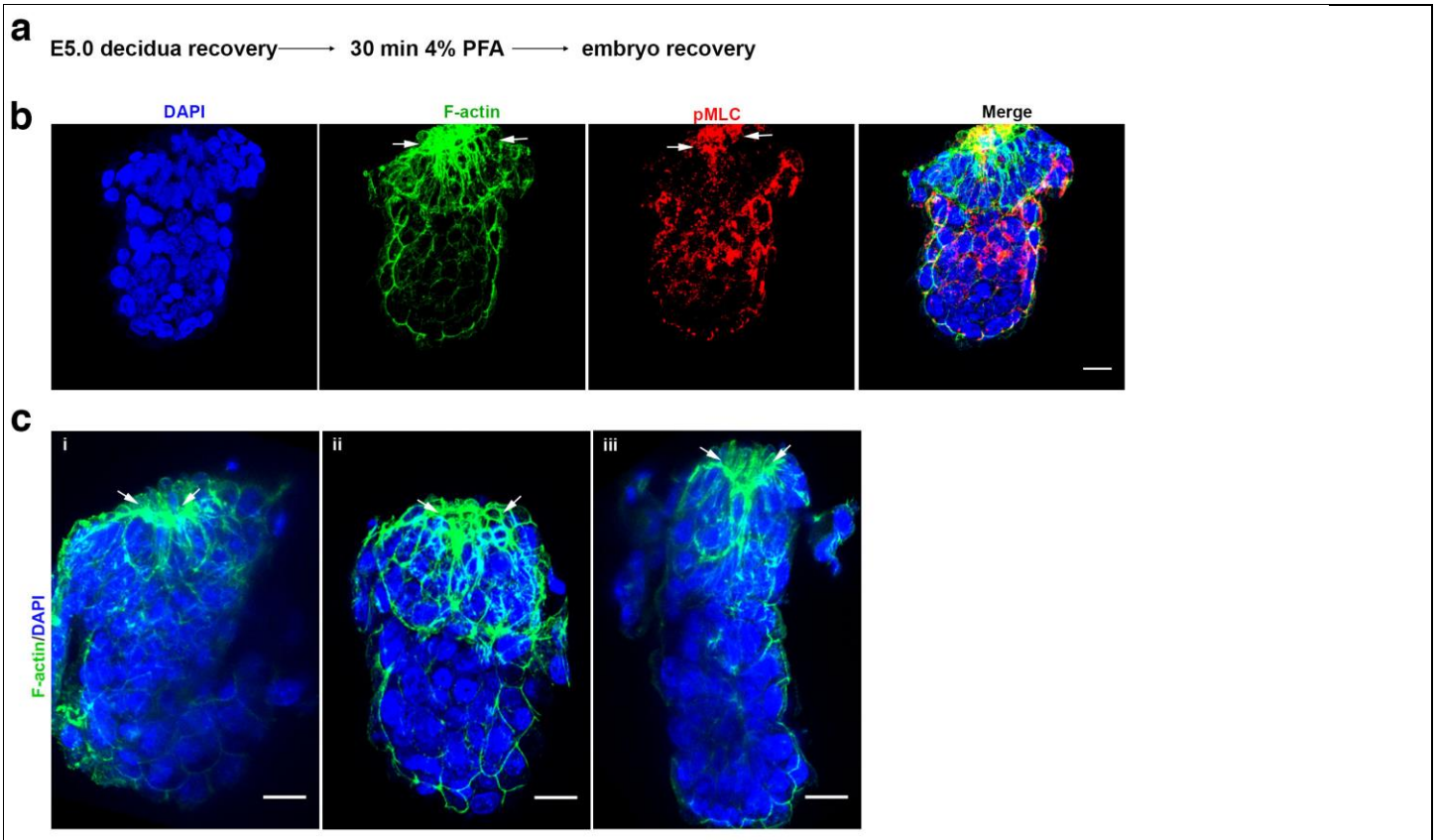
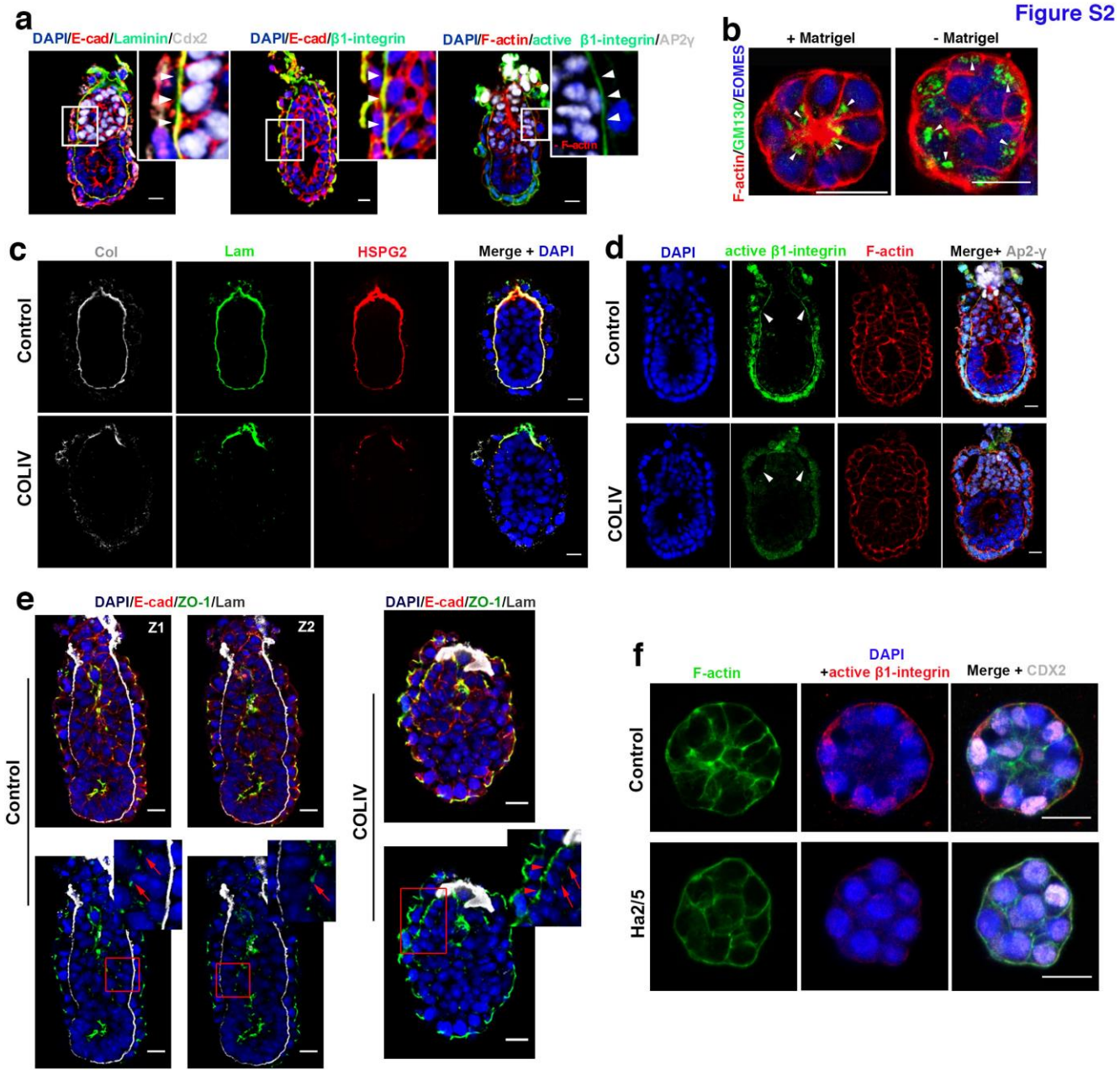

a
E5.0 decidua recovery
30 min 4% PFA
embryo recovery
b
DAPI
F-actin
pMLC
Merge
c
i
ii
iii
F-actin/DAPI

Supplementary Figure 1

Analysis of extra-embryonic ectoderm tissue folding in embryos derived after decidua fixation

(a) Experimental strategy for post-fixation embryo recovery. (b) Representative E5.0 recovered from a fixed decidua. Accumulation of actin and phosphorylated myosin (arrows) is evident at the proximal tip of extra-embryonic ectoderm cells in recovered embryos post-fixation. This indicates that apical actin accumulation and apical constriction tissue folding (see Fig. 1) is an active morphogenetic process and not a wound healing response stemming from the recovery of embryos from deciduae. (c) 3 representative examples of E5.0 embryos recovered from fixed deciduae. Arrows indicate actin apical accumulation at extra-embryonic ectoderm most proximal cells. Image represents 3 biological replicates. Scale bars= 20um.

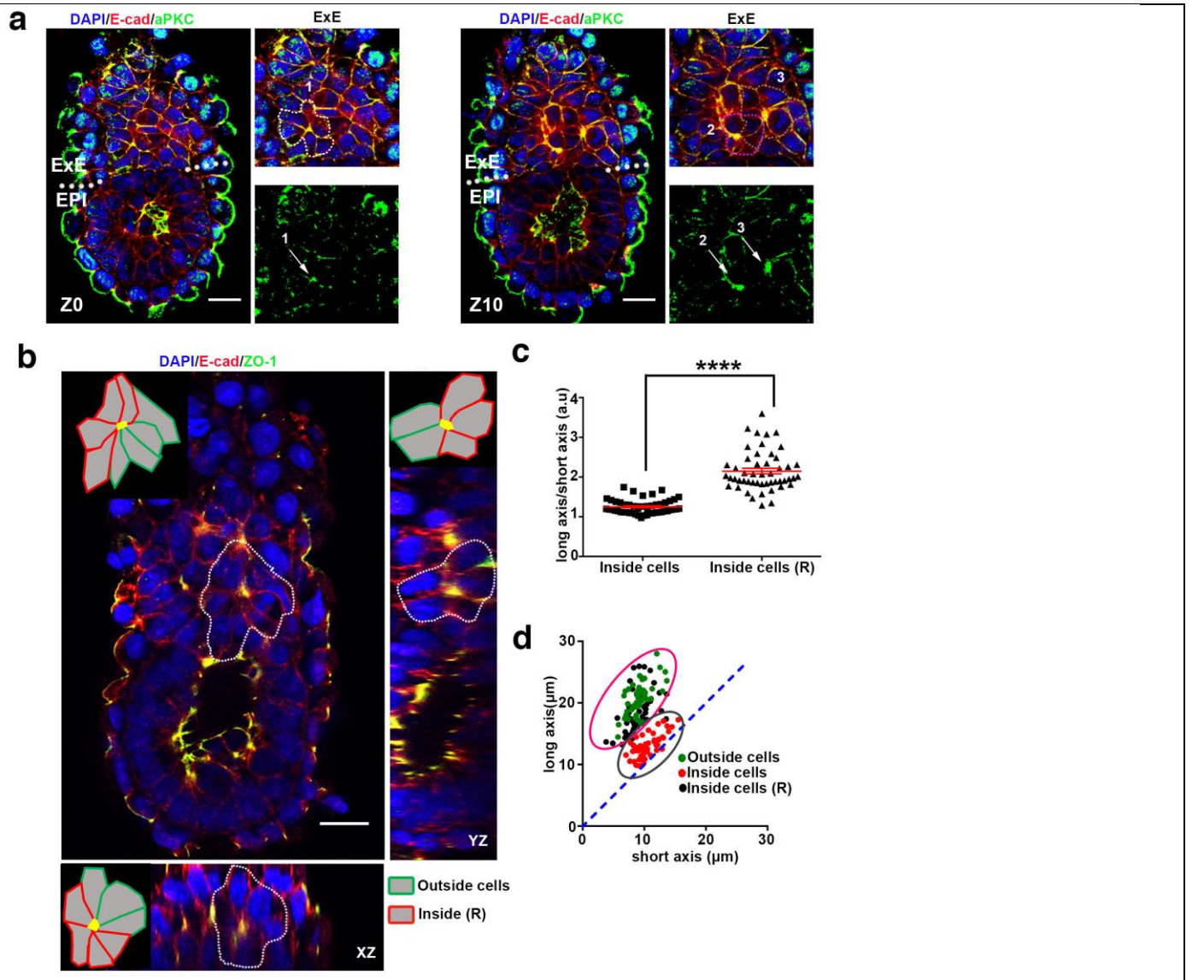
Figure S2


a
DAPI/E-cad/Laminin/Cdx2
DAPI/E-cad/β1-integrin
DAPI/F-actin/active β1-integrin/AP2γ
- F-actin
b
+ Matrigel
- Matrigel
F-actin/GM130/EOMES
c
Col
Lam
HSPG2
Merge + DAPI
Control
COLIV
d
DAPI
active β1-integrin
F-actin
Merge+ Ap2-γ
Control
COLIV
e
DAPI/E-cad/ZO-1/Lam
Z1
Z2
Control
DAPI/E-cad/ZO-1/Lam
COLIV
f
F-actin
DAPI
+active β1-integrin
Merge + CDX2
Control
Ha2/5

Supplementary Figure 2

ECM/ β1-integrin during extra-embryonic ectoderm and TSCs polarization

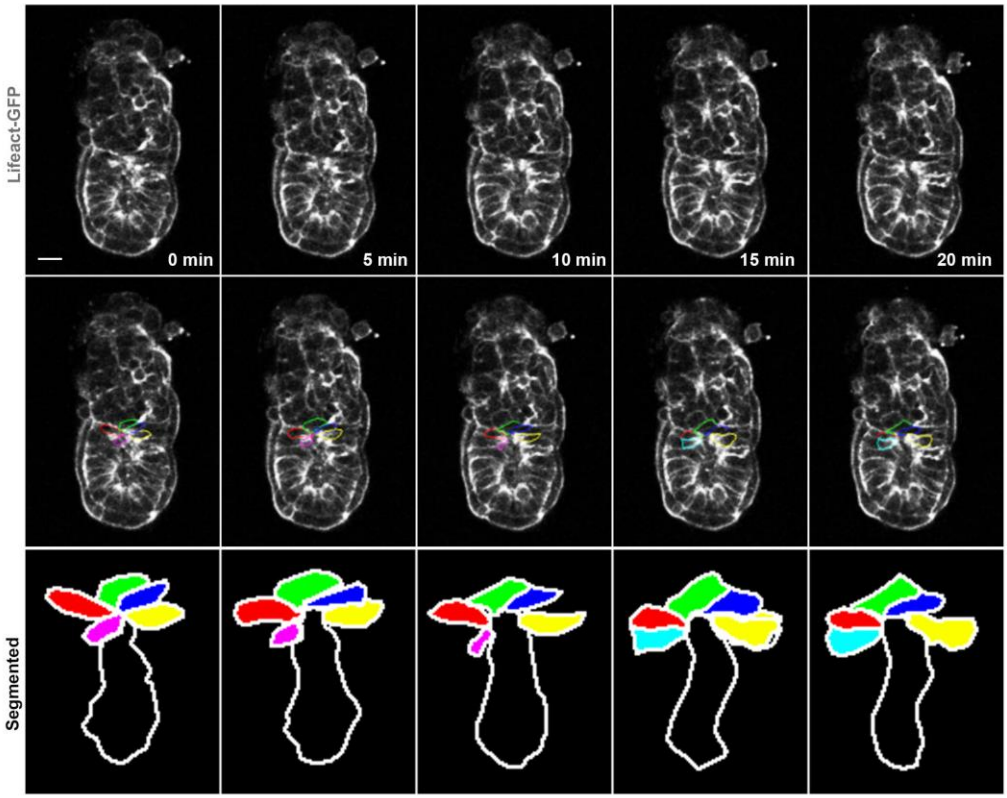
(a) ECM/ β1-integrin localization in E5.5 embryo revealed by antibodies for laminin, β1-integrin and active β1-integrin (9EG7). Image representative of 10 embryos (b) Representative examples of TSC clumps culture in suspension in the presence or absence of Matrigel. In the absence of Matrigel TSCs fail to polarize. Specifically the cells fail to acquire columnar morphology (indicated by F-actin staining) and they don't display apical Golgi polarization (GM130 staining; arrowheads) compared with TSCs cultured in the presence of Matrigel. Image representative of 3 biological replicates (c) The effect of collagenase IV (COLIV) treatment on the surrounding basement membrane (assessed by laminin, collagen and perlecan staining) of E5.5 embryos cultured for 5h in the presence or absence of COLIV. Image representative of 5 biological replicates (d) β1-integrin activation status (indicated by 9EG7 antibody staining) in control and COLIV treated embryos. White arrowheads indicate basal site of ExE outside cells. Image representative of 2 biological replicates (e) The effect of COLIV treatment on the localization of the tight junction's localisation assessed by ZO-1 staining in E5.5 embryos (two different z slices in control embryo; Z1 and Z2). Arrows point to apical site while arrowheads point towards the basal site. Image representative of 10 embryos (f) β1-integrin activation status (indicated by 9EG7 antibody staining) in control and β1-integrin blocking antibody (Ha2/5) treated TSCs in 3D culture; Image representative of 2 biological replicates. Scale bars=20um


a
DAPI/E-cad/aPKC
ExE
DAPI/E-cad/aPKC
ExE
ExE
EPI
Z0
ExE
EPI
Z10
b
DAPI/E-cad/ZO-1
YZ
XZ
Outside cells
Inside (R)
c
****
long axis/short axis (a.u)
Inside cells
Inside cells (R)
d
long axis(μm)
short axis (μm)
Outside cells
Inside cells
Inside cells (R)

Supplementary Figure 3

Characterization of extra-embryonic ectoderm cells

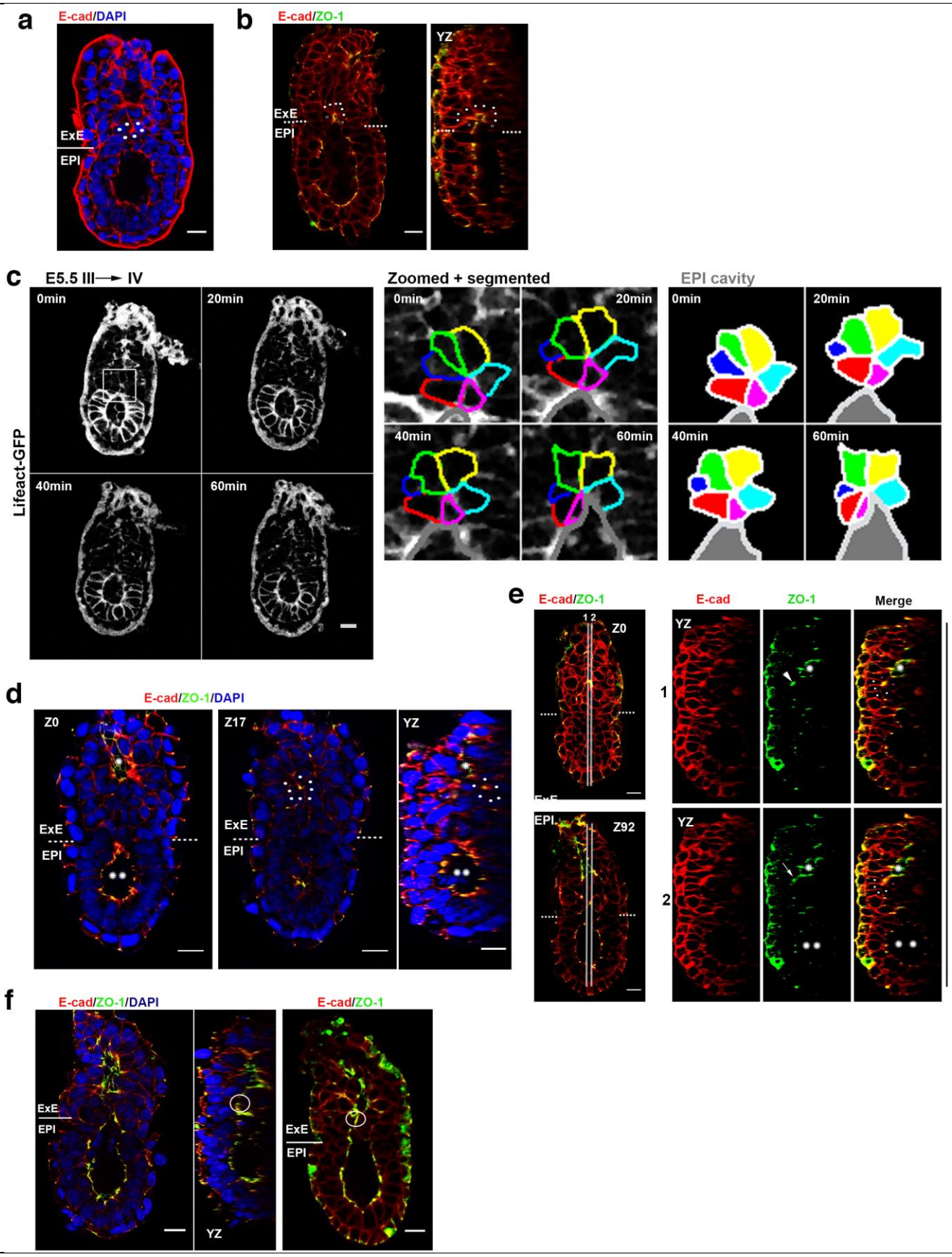
(a) Images of different Z slices (0.8 um) from a representative E5.5 (Stage III) embryo. Extra-embryonic ectoderm (ExE) rosettes are outlined with a dashed line. Arrows denote rosettes' centre. Image representative of 20 embryos (b) Representative example of an ExE rosette (dashed outline) found in E5.5 embryo as revealed by cell morphology (E-cadherin) and polarization (ZO-1). Orthogonal views (XZ, YZ) of the embryo are displayed on the right and the bottom of the image with the segmented rosette as viewed in the respective orthoslice. Image representative of 20 embryos. (c) Cell aspect ratio comparison of outside, inside vs inside cells that contribute to rosettes (Inside cells, R). Two sided unpaired student t-test;****$p<0.0001$;mean± SEM; n=50 inside cells and n=47 inside rosette cells. (d) Plot of long and short axis. Two separate clusters are indicated (magenta and grey outlines). Cluster shown with magenta consists of outside cells and inside cells contributing to rosettes (inside cells R). Cluster shown with grey consists of inside cells that do not contribute to rosettes. Blue dashed line: y=x. n=50 outside and inside cells and n=47 inside rosette cells. Scale bars=20um


Lifeact-GFP
0 min
5 min
10 min
15 min
20 min
Segmented

Supplementary Figure 4

Epiblast remodelling upon hybrid rosette resolution

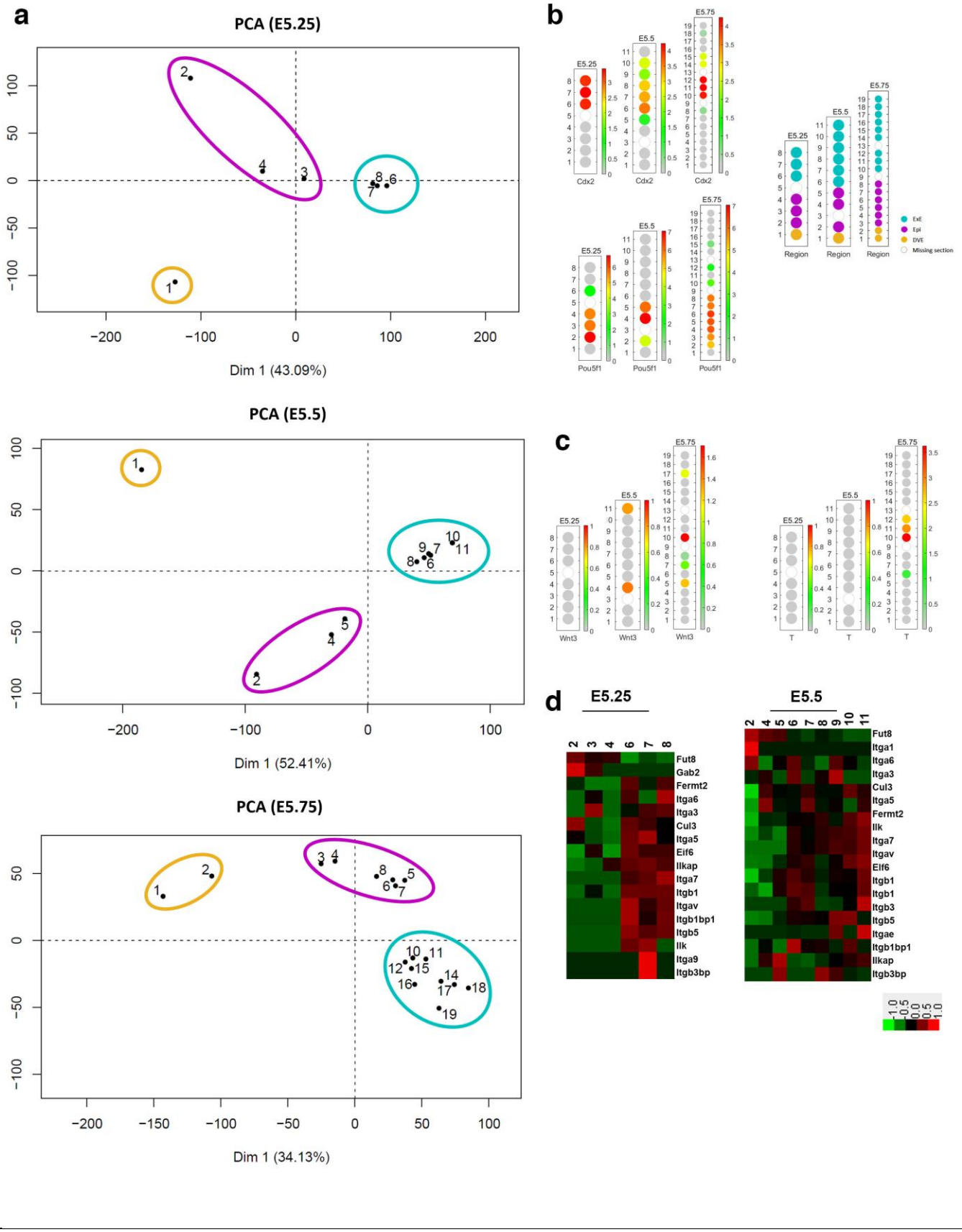
Stills from a time lapse movie of a LifeAct-GFP embryo showing epiblast EPI remodelling after hybrid rosette resolution. Cells are arbitrarily colour-coded for tracking. EPI: purple, yellow and cyan cells; ExE: blue, red and green cells. Image representative of 3 embryos. Scale bar=20um


a
E-cad/DAPI
ExE
EPI
b
E-cad/ZO-1
YZ
ExE
EPI
c
E5.5 III→ IV
Lifeact-GFP
0min
20min
40min
60min
Zoomed + segmented
EPI cavity
d
E-cad/ZO-1/DAPI
Z0
Z17
YZ
e
E-cad/ZO-1
E-cad
ZO-1
Merge
Z92
f
E-cad/ZO-1/DAPI
E-cad/ZO-1

Supplementary Figure 5

Extra-embryonic ectoderm rosettes facilitate cavities extension and fusion

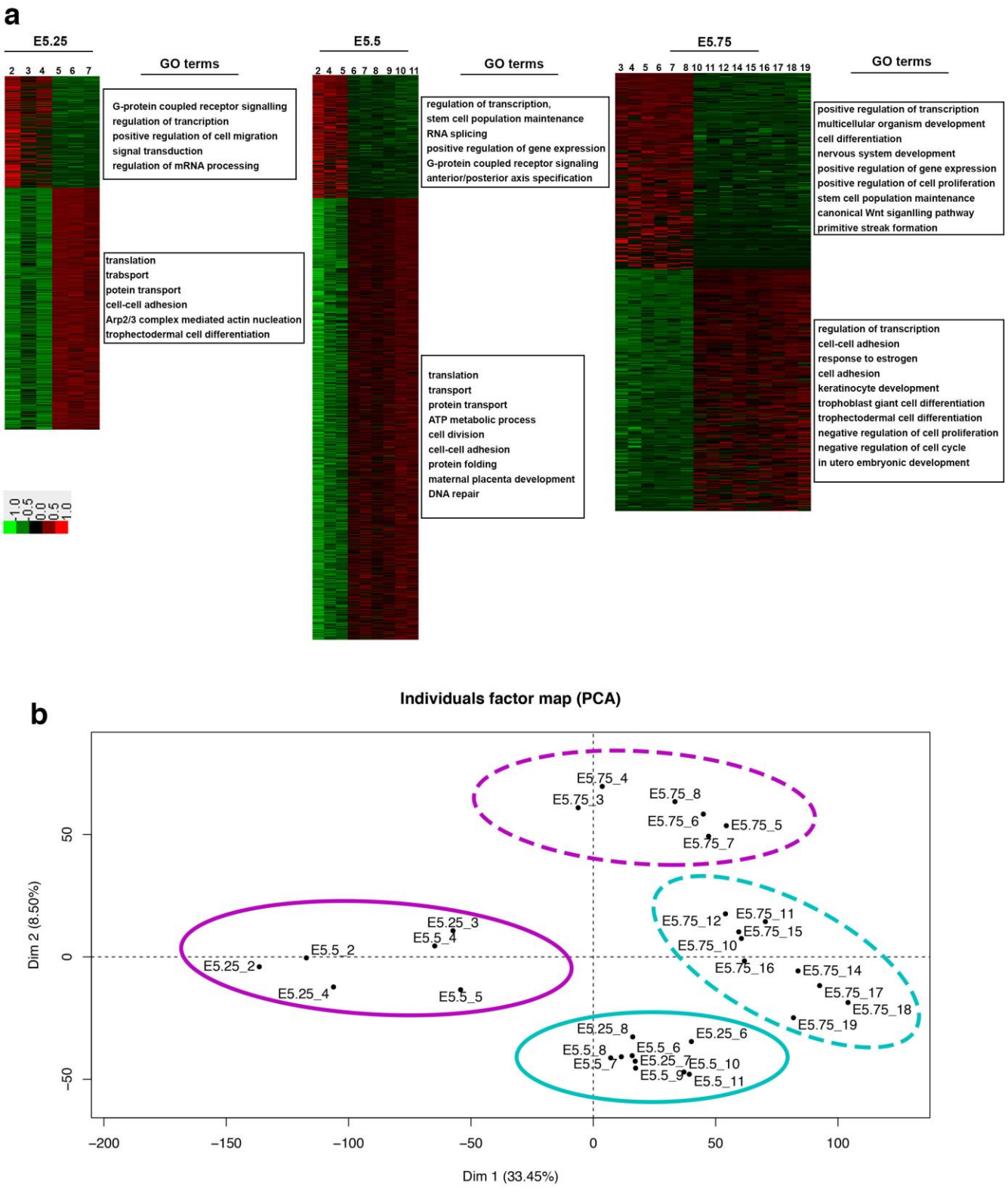
(a) Representative example of E5.5 (stage III) embryo stained for E-Cadherin. White dots label the cells contributing to the epiblast (EPI) cavity's proximal rosette (adjacent to EPI-ExE boundary). Image representative of 15 embryos. (b) Example of a resolving EPI cavity's proximal rosette (dots show cells contributing to the rosette) through loss of cell-cell contacts (cells marked with hollow dots) of ExE cells at the boundary. YZ projection is displayed on the right of the image. Image representative of 10 embryos (c) Stills from a time lapse movie of an E5.5 LifeAct-GFP embryo showing EPI cavity expansion after EPI cavity's proximal rosette resolution. Cells are arbitrarily color-coded for tracking. Image representative of 3 embryos (d)E5.5 (stage III) embryo stained for E-Cadherin and tight junction protein ZO-1. Two different z slices are presented (Z0 and Z17, Z step=0.8 um) one showing the ExE cavity and the other the overlaying rosette (white dots). YZ projection of rosette (white dots showing cells contributing to rosette) and ExE cavities shown on the far right of the panel. *=ExE cavity, **=EPI cavity. Image representative of 10 embryos. (e) Representative example of ExE cavity (asterisk in YZ projections, right panel) connected with the centre of an ExE cavity proximal rosette (arrowhead points to the centre of this rosette; magenta dots indicate cells contributing to both rosette and ExE cavity where white dots indicate the rest of rosette's cells) through a tract of ZO-1 (arrow). White dotted line = EPI-ExE boundary. *=ExE cavity, **=EPI cavity. Image representative of 10 embryos. (f) Two representative examples of an invading EPI cavity and an extended ExE cavity connected with a polarized tract of ZO-1 (white circle). Image representative of 6 embryos. Scale bars = 20um


a
PCA (E5.25)
Dim 1 (43.09%)
PCA (E5.5)
Dim 1 (52.41%)
PCA (E5.75)
Dim 1 (34.13%)
b
E5.25
E5.5
E5.75
Cdx2
Pou5f1
Region
ExE
Epi
DVE
Missing section
c
Wnt3
T
d
E5.25
E5.5
Fut8
Gab2
Fermt2
Itga6
Itga3
Cul3
Itga5
Eif6
Ilkap
Itga7
Itgb1
Itgav
Itgb1bp1
Itgb5
Ilk
Itga9
Itgb3bp
Itga1
Itga6
Itga3
Cul3
Itga5
Fermt2
Ilk
Itga7
Itgav
Elf6
Itgb1
Itgb1
Itgb3
Itgb5
Itgae
Itgb1bp1
Ilkap
Itgb3bp

Supplementary Figure 6

Spatial transcriptome analysis of E5.25-E5.75 embryos

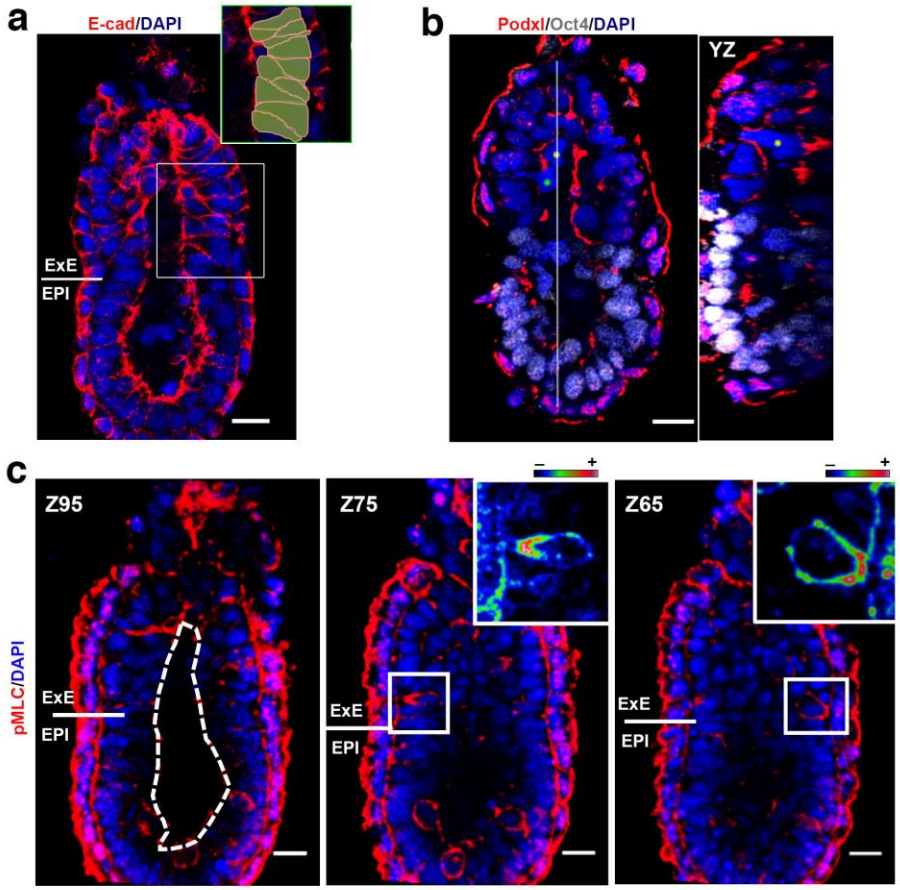
(a) Principal component analysis (PCA) of sections along the proximo-distal embryo axis from at E5.25, E5.5 and E5.75. Yellow outline: VE, Purple outline: epiblast (EPI), Cyan outline: extra-embryonic ectoderm (ExE). (b) Gene expression pattern for EPI (Pou5f1) and ExE (CDX2) markers from E5.25-E5.75. (c) Gene expression pattern for Wnt3 and T from E5.25-E5.75. (d) Differential gene expression analysis heat maps for GO term: integrin mediated signalling. E5.25: sections 2-4 EPI, sections 6-8 ExE ; E5.5: sections 2,4,5 EPI, sections 6-11 ExE.


a
E5.25
2 3 4 5 6 7
GO terms
G-protein coupled receptor signalling
regulation of trancription
positive regulation of cell migration
signal transduction
regulation of mRNA processing
translation
trabsport
potein transport
cell-cell adhesion
Arp2/3 complex mediated actin nucleation
trophectodermal cell differentiation
-1.0
-0.5
0.0
0.5
1.0
E5.5
2 4 5 6 7 8 9 10 11
GO terms
regulation of transcription,
stem cell population maintenance
RNA splicing
positive regulation of gene expression
G-protein coupled receptor signaling
anterior/posterior axis specification
translation
transport
protein transport
ATP metabolic process
cell division
cell-cell adhesion
protein folding
maternal placenta development
DNA repair
E5.75
3 4 5 6 7 8 10 11 12 14 15 16 17 18 19
GO terms
positive regulation of transcription
multicellular organism development
cell differentiation
nervous system development
positive regulation of gene expression
positive regulation of cell proliferation
stem cell population maintenance
canonical Wnt siganlling pathway
primitive streak formation
regulation of transcription
cell-cell adhesion
response to estrogen
cell adhesion
keratinocyte development
trophoblast giant cell differentiation
trophectodermal cell differentiation
negative regulation of cell proliferation
negative regulation of cell cycle
in utero embryonic development
b
Individuals factor map (PCA)
E5.75_4
E5.75_3
E5.75_8
E5.75_6
E5.75_5
E5.75_7
E5.25_3
E5.5_4
E5.5_2
E5.25_2
E5.25_4
E5.5_5
E5.75_12
E5.75_11
E5.75_15
E5.75_10
E5.75_16
E5.75_14
E5.75_17
E5.75_18
E5.75_19
E5.25_8
E5.25_6
E5.5_8
E5.5_6
E5.5_7
E5.25_7
E5.5_10
E5.5_9
E5.5_11
Dim 2 (8.50%)
50
0
−50
−200
−150
−100
−50
0
50
100
Dim 1 (33.45%)

Supplementary Figure 7

Differential gene expression analysis of embryonic and extra-embryonic compartments

(a)Differential gene expression analysis heat maps between epiblast (EPI) and extra-embryonic ectoderm (ExE) in E5.25, E5.5 and E5.75 embryos. Representative gene ontology (G0) terms enriched in each tissue are shown on the right of each heat map. E5.25: sections 2-4 EPI, sections 6-8 ExE; E5.5: sections 2,4,5 EPI, sections 6-11 ExE; E5.75 sections 3-8 EPI, sections 9-19 ExE. n=1 E5.25, n=1 E5.5 and n=1 E5.75 embryos **(**b) Combined principal component analysis (PCA) for embryonic and extra-embryonic sections from E5.25-E5.75. Purple outline: E5.25-E5.5 EPI; Cyan outline: E5.25-E5.5 ExE; Dashed purple outline: E5.75 EPI; Dashed cyan outline: E5.75 ExE. n=1 E5.25, n=1 E5.5 and n=1 E5.75 embryos


a
E-cad/DAPI
ExE
EPI
b
Podxl/Oct4/DAPI
YZ
c
pMLC/DAPI
Z95
Z75
Z65
ExE
EPI
ExE
EPI
ExE
EPI
– +
– +

Supplementary Figure 8
Cell intercalation after initial cavities fusion results in the formation of a pseudostratified epithelium
(a)ExE acquires a pseudostratified epithelium morphology shortly after unification of the cavities. Image representative of 10 embryos. (b)After cavities fusion ExE cells show intercalative behaviour (green and yellow asterisks). Image representative of 5 embryos. (c) 3 different optical Z slices (0.6um) of a representative example of an E5.75(stage IV) embryo. Dashed outline in left panel shows pro-amniotic cavity spanning through EPI and ExE. In different Z slices (middle and right panel) ExE cells intercalating towards the basement membrane can be identified. Polarized pMLC localization displays the polarized nature of this event. Insets in middle and right panel show magnified fluorescent intensity color coded images of ExE intercalating cells. Image representative of 5 embryos. Scale bars=20um

Supplementary Table 1: Processed RNA-seq data.

Supplementary Table 2: Statistics source data

Supplementary Movie 1. Tissue folding mediated extra-embryonic cavity formation

Time lapse movie (2D view) of a representative E5.0 Lifeact-GFP transgenic embryo showing tissue bending mediated extra-embryonic cavity formation. Representative of 5 separate time-lapse movies Time interval=5min.

Supplementary Movie 2. Tissue folding mediated extra-embryonic cavity formation in 3D

Top 3D view of Movie S1 showing apical constriction mediated cell ingression during extra-embryonic ectoderm cavity formation. Time interval=5min Representative of 5 separate time-lapse movies.

Supplementary Movie 3. Apical constriction during extra-embryonic ectoderm tissue folding

Time lapse movie showing two different single cells from movie S2. Note the increase of medioapical actin before apical cell constriction. Time interval=5 min.

Supplementary Movie 4. 3D segmentation of representative extra-embryonic ectoderm rosettes

3D segmentation of a representative extra-embryonic ectoderm rosette. Each rosette cell is represented with different colour. White circle highlights the rosette center.

Supplementary Movie 5. 3D segmentation of neighboring extra-embryonic ectoderm rosettes

3D segmentation of a representative extra-embryonic ectoderm rosettes sharing cells. Each cell is represented with different colour.

Supplementary Movie 6. Hybrid rosette resolution precedes epiblast remodelling(1).

Time lapse movie of a representative E5.5 Lifeact-GFP transgenic embryo showing epiblast remodelling after hybrid rosette resolution. Time interval=20 min. Representative of 3 separate time-lapse movies

Supplementary Movie 7. Hybrid rosette resolution precedes epiblast remodelling(2)

Time lapse movie of a representative E5.5 Lifeact-GFP transgenic embryo showing epiblast remodelling after hybrid rosette resolution. Time interval=5 min. Representative of 3 separate time-lapse movies

Supplementary Movie 8. Laser ablation mediated hybrid rosette resolution.

Time lapse movie of a representative E5.5 Lifeact-GFP (stage II) transgenic embryo. The cell-cell interface connecting the hybrid rosette centre with the epiblast cavity was ablated via laser ablation (red bar). After ablation, the epiblast cavity has extended towards the centre of hybrid rosette, Time interval=1 min. Representative of 2 biological replicates.

Supplementary Movie 9. Polarized tract connects the centre of a rosette with the embryonic cavity.

Representative images through Z (Z=0.9um) of the boundary between epiblast and extra-embryonic ectoderm of a representative E5.5 embryo. Polarized track extending from the EPI cavity towards the centre of an ExE rosette is evident. blue: DAPI, red:E-cad, green: ZO-1. White dots indicate ExE rosette cells. n=10 embryos

Supplementary Movie 10. ExE rosettes facilitate epiblast cavity extension(1).

Time lapse movie of a representative E5.5 mTmG transgenic embryo showing epiblast cavity expansion towards the ExE after ExE rosette resolution. Time interval=20 min. Representative of 3 separate time-lapse movies.

Supplementary Movie 11. ExE rosettes facilitate epiblast cavity extension(2).

Time lapse movie of a representative E5.5 LifeactGFP transgenic embryo showing epiblast cavity expansion towards the ExE after ExE rosette resolution. Time interval=20 min. Representative of 3 separate time-lapse movies

Supplementary Movie 12. ExE rosettes facilitate extra-embryonic cavity extension.

Time lapse movie of a representative E5.5 LifeactGFP transgenic embryo showing extra-embryonic ectoderm cavity extension towards the EPI after ExE rosette resolution. Time interval=5 min. Representative of 3 separate time-lapse movies.

Supplementary Movie 13. Rosette mediated embryonic and extra-embryonic cavities extension.

Time lapse movie of a representative E5.5 LifeactGFP transgenic embryo showing embryonic and extra-embryonic cavities extension after rosette resolution. Green outlines indicate embryonic (bottom) and extra-embryonic cavities (top). Yellow dots: cells contributing to resolving rosette during extra-embryonic cavity extension; Green dots: Yellow dots: cells contributing to resolving rosette during embryonic cavity extension; Time interval=5 min. Representative of 2 separate time-lapse movies**.**

Supplementary Movie 14. Extra-embryonic ectoderm cell intercalation upon initial cavities fusion.

Time lapse movie of a representative E5.75 mTmG (stage IV → V) transgenic embryo showing ExE cell intercalation after embryonic (bottom) and extra-embryonic(top) cavities fusion. magenta: cavities, green: Intercalating ExE cell. Time interval=20 min. Representative of 2 separate time-lapse movies.