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SUPPLEMENTARY INFORMATION

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Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures

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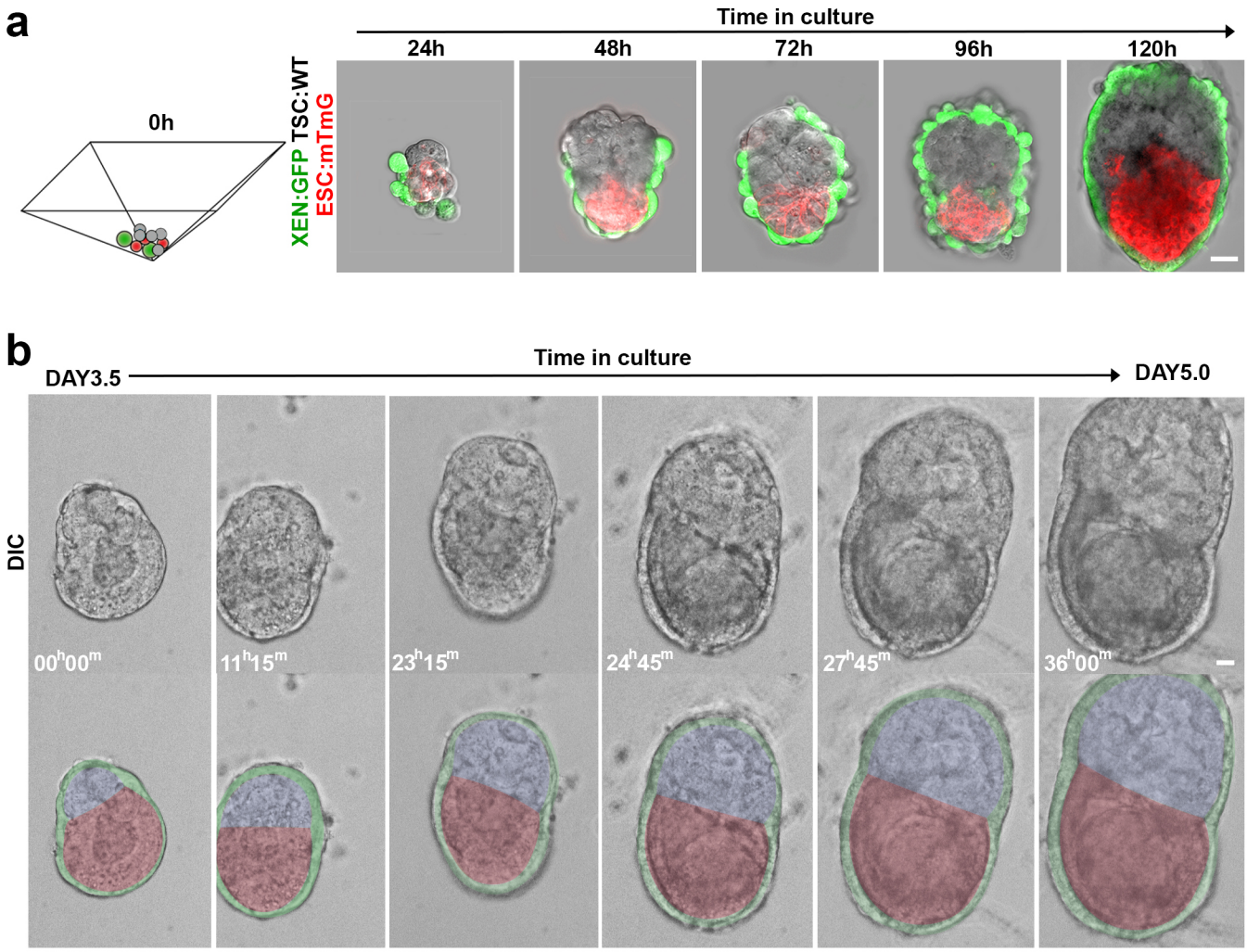
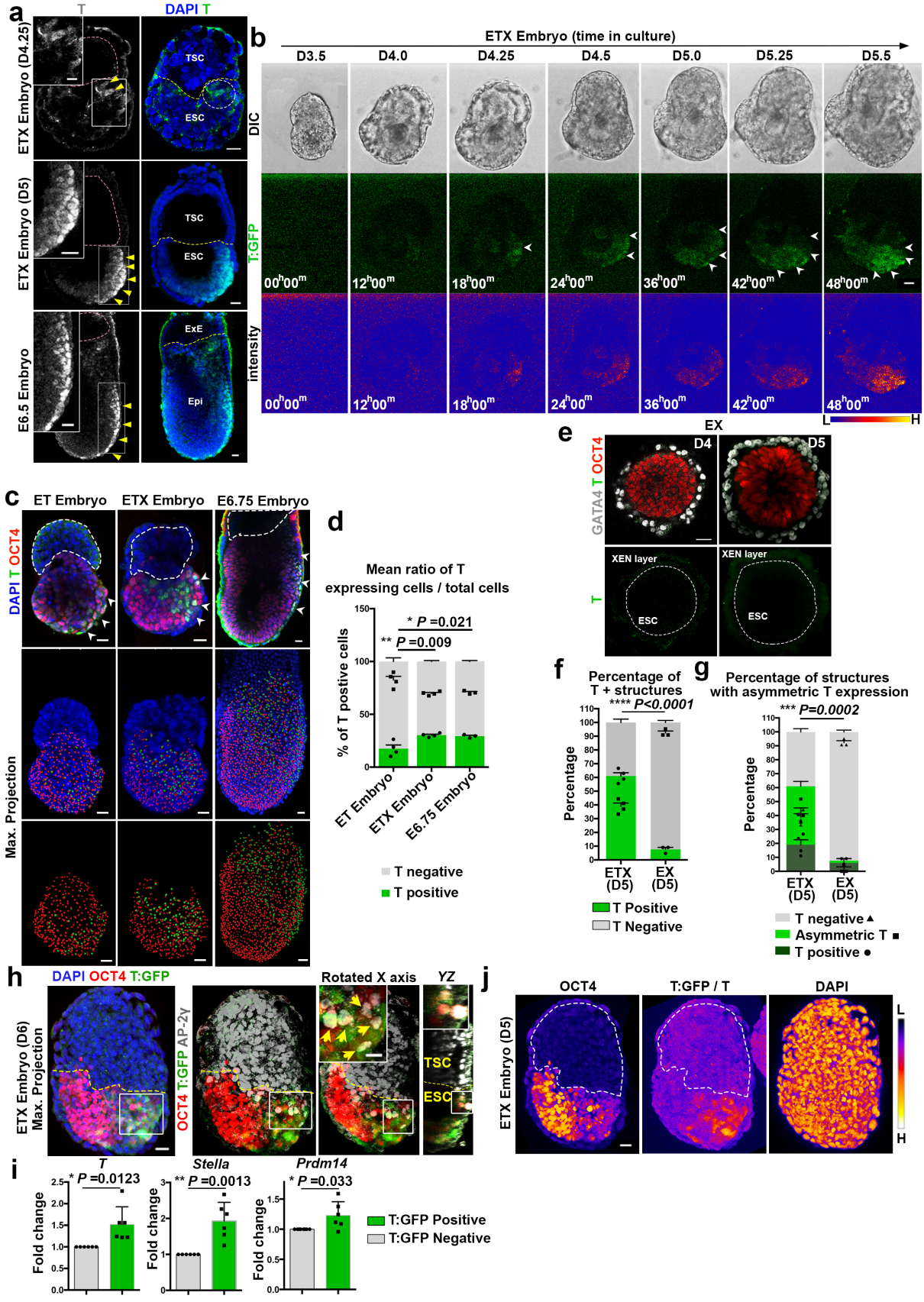


Supplementary Figure 1

Self-assembly of ESCs, TSCs, and XEN cells into ETX embryos.

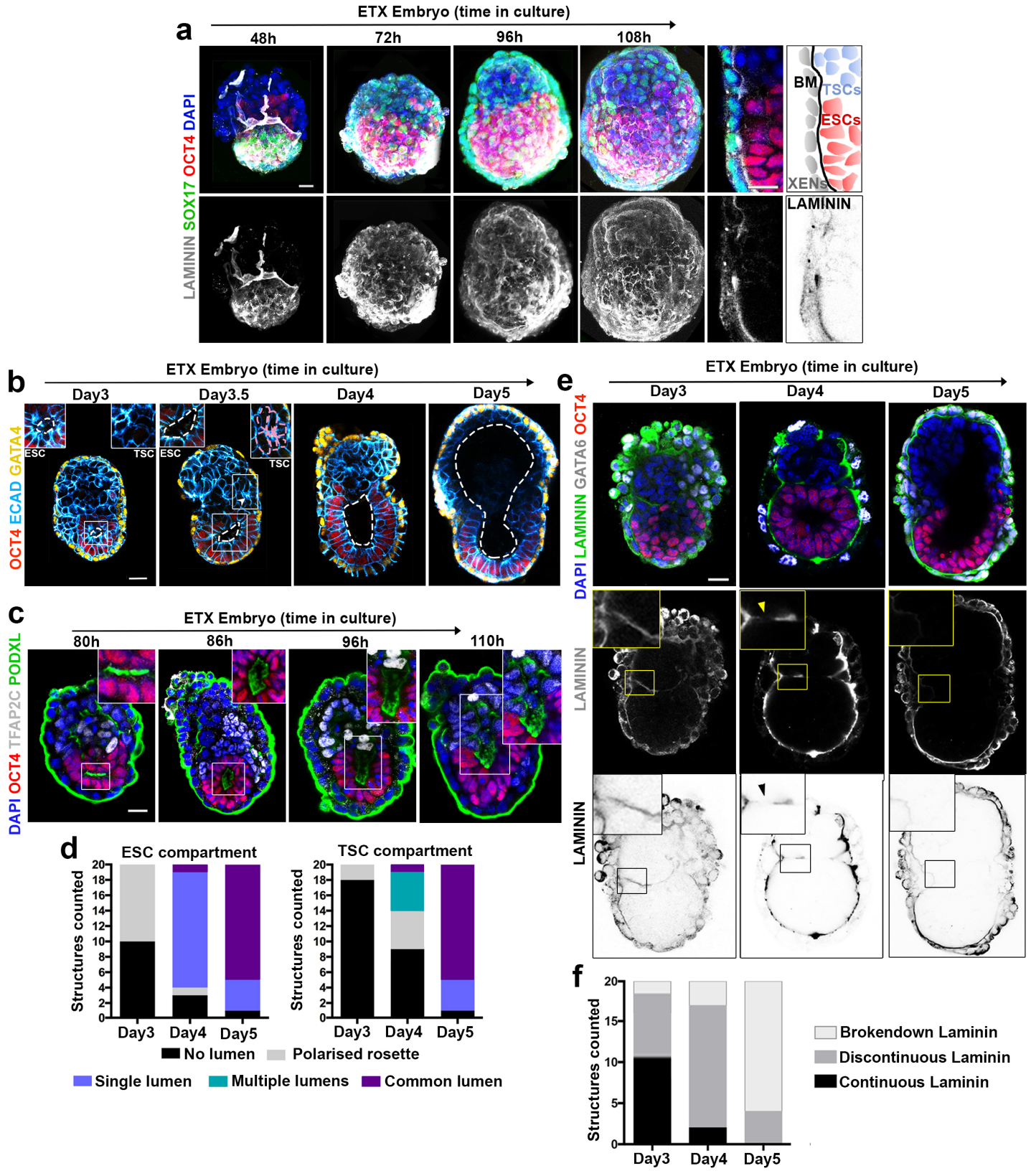
a. Confocal images of ETX embryos developed from fluorescence reporter cell lines at indicated time intervals: EGFP XEN cells, green; mTmG ES cells, red; wild-type TS cells. At day 2 of a typical experiment around 75% (23/31) of ETX embryos have an outer layer of XEN cells on ES cell side. Each image represents a different ETX embryo. Bar=20μm. **b.** Still images of time-lapse recording during development of a single ETX embryo. Lineages were pseudocoloured below to help visualisation. XEN layer, green; ES cells, red; TS cells, blue. Time-lapse images captured at 45 min intervals. Representative of 3 separate time-lapse movies of 10 ETX embryos. Bar=20μm.


a
T
DAPI T
ETX Embryo (D4.25)
ETX Embryo (D5)
E6.5 Embryo
TSC
ESC
ExE
Epi
b
ETX Embryo (time in culture)
D3.5
D4.0
D4.25
D4.5
D5.0
D5.25
D5.5
DIC
T:GFP
intensity
00h00m
12h00m
18h00m
24h00m
36h00m
42h00m
48h00m
L
H
c
ET Embryo
ETX Embryo
E6.75 Embryo
DAPI T OCT4
Max. Projection
d
Mean ratio of T expressing cells / total cells
* P =0.021
** P =0.009
% of T postive cells
ET Embryo
ETX Embryo
E6.75 Embryo
T negative
T positive
e
EX
D4
D5
GATA4 T OCT4
T
XEN layer
ESC
f
Percentage of T + structures
**** P<0.0001
Percentage
ETX (D5)
EX (D5)
T Positive
T Negative
g
Percentage of structures with asymmetric T expression
*** P=0.0002
Percentage
ETX (D5)
EX (D5)
T negative ▲
Asymmetric T ■
T positive ●
h
DAPI OCT4 T:GFP
ETX Embryo (D6) Max. Projection
OCT4 T:GFP AP-2γ
Rotated X axis
YZ
TSC
ESC
i
T
Stella
Prdm14
* P =0.0123
** P =0.0013
* P =0.033
Fold change
T:GFP Positive
T:GFP Negative
j
OCT4
T:GFP / T
DAPI
ETX Embryo (D5)
L
H

Supplementary Figure 2

Posterior patterning in ETX embryos

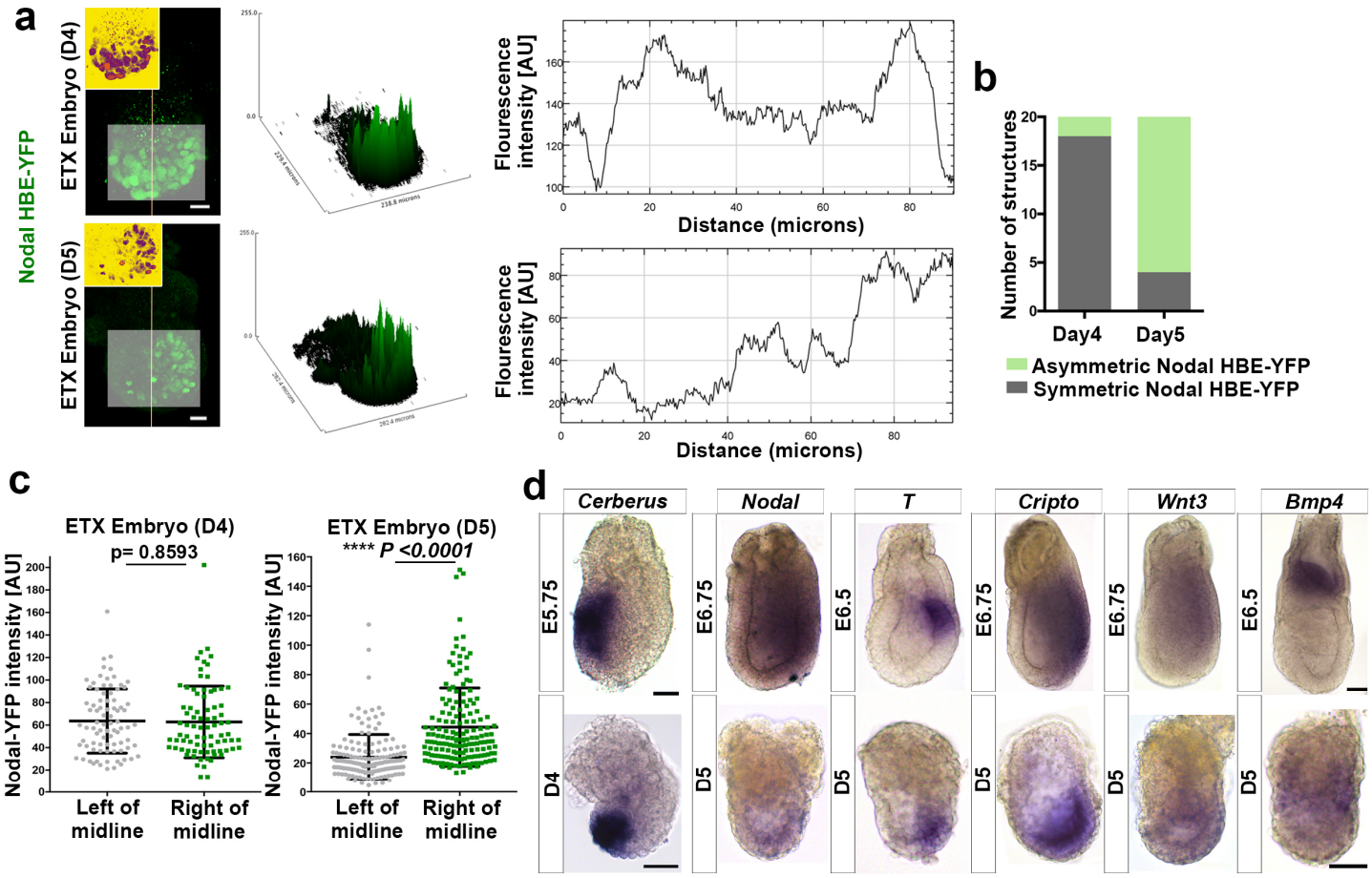
a. Induction of *T/Brachyury* in a representative ETX embryo at day 4.25 (top); localised *T/Brachyury* in a representative ETX embryo at day 5 (middle) and in E6.5 embryo (bottom). Yellow arrowheads indicate induction (top) or asymmetric *T/Brachyury* expression throughout posterior (middle and bottom). White boxes indicate zoomed inset. Purple dashed lines outline TS-derived extra-embryonic compartment or ExE for clarity. Yellow dashed line outline embryonic/extra-embryonic boundary. Non-nuclear anti-*T/Brachyury* VE fluorescence is non-specific staining. Representative of 43 ETX embryos, 4 experiments; 20 E6.5 embryos, 2 experiments. Bar=20μm. **b.** Still images from time-lapse recording of development of ETX embryo derived from *T*:GFP reporter ES cells. Time-lapse images captured at 45 min intervals. Representative of 3 separate time-lapse movies of 3 ETX embryos. Bar=20μm. Bottom row shows intensity gradient for *T*:GFP signal. L, low; H, high. **c.** Single planes of ET embryo (top left) and ETX embryo (top middle) 8 hours after onset of *T/Brachyury* expression, and E6.75 natural embryo (top right). Arrowheads indicate side of *T/Brachyury* expression. White dashed lines outline TS-derived extra-embryonic compartment or ExE for clarity. Middle row shows reconstruction of the embryonic compartment overlaid over DAPI; red dots indicate Oct4-positive and green dots indicate Oct4 and *T/Brachyury* double positive cells. Bottom panels show reconstruction of embryonic compartment alone. Representative of 4 ET embryos, 4 ETX embryos and 3 natural embryos; each from separate experiments. Bar=20μm. **d.** Mean ratio of *T/Brachyury* expressing cells to total cells in embryonic compartment. One-way ANOVA, n=4 ET embryos, n=4 ETX embryos and n=3 natural embryos. Columns are means ± SEM. Non-nuclear anti-*T/Brachyury*/Oct4 VE fluorescence is non-specific staining. **e.** Majority of EX (ES + XEN cells only) structures (92.3%) at day 4 and 5 under the same culture conditions did not express *T/Brachyury;* remaining (7.6%) displayed non-regionalised expression of *T/Brachyury*. Representative of 20 ETX embryos per time point; 3 experiments. Bar=20μm. **f.** Proportion of ETX embryos expressing *T/Brachyury* at day 5 is significantly higher than EX structures at day 5. Two-sided Student's t-test, n=4 experiments for ETX embryos, n=3 experiments for EX structures. Each dot represents the percentage of positive or negative structures for *T*:GFP expression scored from each separate independent experiment. The number of structures scored in each independent experiment to calculate the percentage are reported in supplementary table 3 (101 ETX embryos and 43 EX structures scored in total). Columns are means ± SEM. **g.** Proportion of *T/Brachyury* expressing ETX embryos or EX structures with asymmetric *T/Brachyury* expression. Two-sided Student's t-test. Columns are means ± SEM. n=4 experiments for ETX embryos, n=3 experiments for EX structures. The number of structures scored in each independent experiment to calculate the percentage are reported in supplementary table 3 (101 ETX embryos and 43 EX structures scored in total). **h.** ETX embryo at day 6. Boxed area on maximum projected images shows ROI with subset of triple positive cells for *T/Brachyury,* Oct4 and AP-2γ next to embryonic/extra-embryonic boundary (yellow dashed lines). Single orthogonal YZ plane and rotated X axis views provided to visualise expression of PGC markers on the boundary. 38%, 10/26 ETX embryos, 2 experiments **i.** RT-qPCR analysis of candidate PGC specification genes performed on *T:*GFP positive and negative cells from day 6 ETX embryo. Two-sided Student's t-test, *n*=7 biological replicates. Columns are means ± SD. **j.** Day 5 ETX embryo revealing reciprocal gradients of Oct4 and *T/Brachyury,* both pseudocoloured with “fire” lookup table in Fiji to show expression levels. White dashed lines outline the TS-derived extra-embryonic compartment for clarity. L, low; H, high. Representative of 6 ETX embryos, 3 experiments. Bar=20μm.



Supplementary Figure 3

Basement membrane and pro-amniotic cavity formation in ETX embryos.

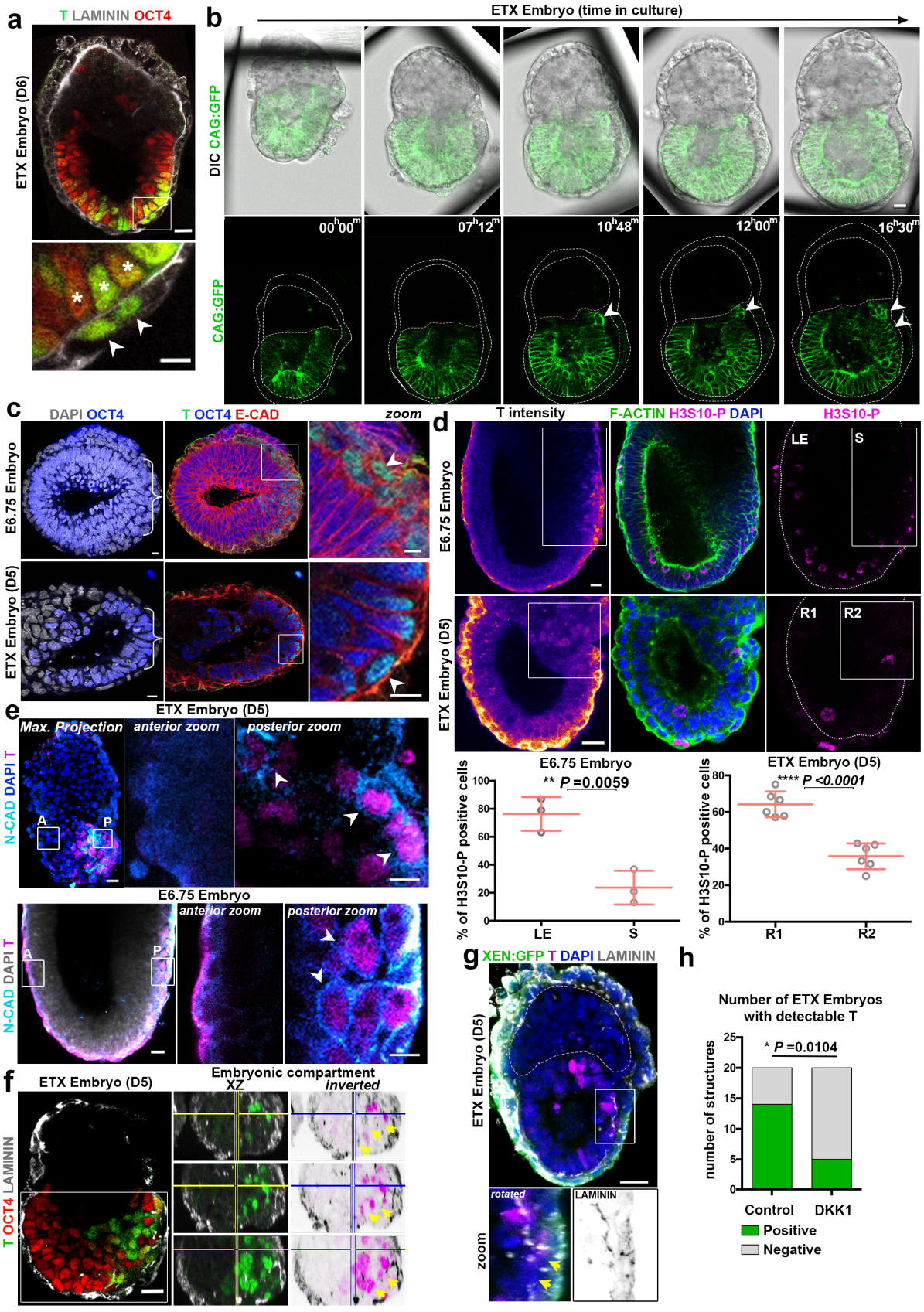
a. Maximum projections from time points indicated showing formation of basement membrane during ETX embryo development. Right-most single-plane images: magnified middle plane of ETX embryo at 108h. Bar=20μm; Representative of 6 ETX embryos for each time point. **b.** ETX embryo after 3, 3.5, 4 and 5 days showing progression of cavitation. White dashed lines outline the cavity; purple dashed lines outline rosette; boxes, region of magnified inset. Bar=20μm; Representative of 20 ETX embryos for each time point, 4 experiments. **c.** Progressive formation of the pro-amniotic cavity during ETX embryo development. White boxes indicate magnified inset showing polarised Podxl. Bar=20μm; Representative of 6 ETX embryos for each time point, 2 experiments. **d.** Quantification of cavities in respective ES and TS-compartments of ETX embryos at days 3, 4 and 5. *n*=20 ETX embryos per time point. **e.** ETX embryos during cavitation showing laminin break-down between embryonic and extra-embryonic compartments. Yellow boxes, region of magnified inset. Yellow or black arrowheads indicate break in laminin. Lower panels have laminin staining inverted for better contrast. Black boxes indicate region of zoomed inset. Representative of 20 ETX embryos, 2 experiments. Bar=20μm. **f.** Quantification of laminin break-down at different developmental time points of ETX embryo embryogenesis. *n*=20 ETX embryos per time point, 2 experiments.



Supplementary Figure 4

Anterior-posterior patterning in ETX embryos.

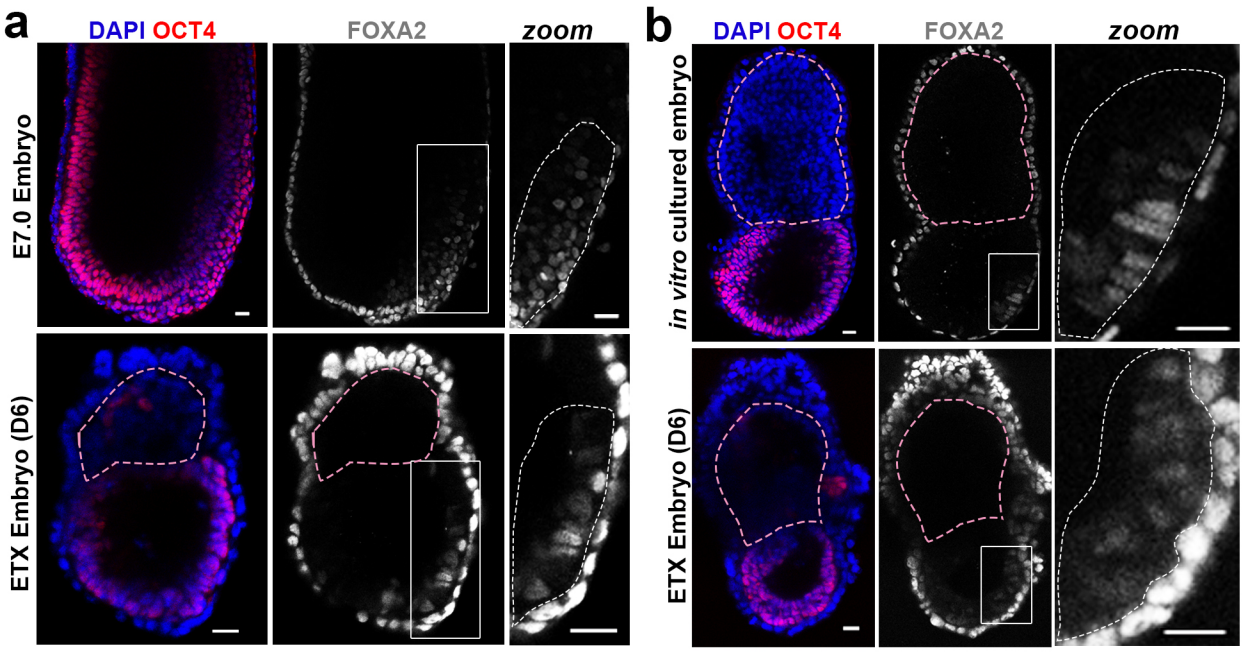
a. Images showing the same ETX embryos presented in Fig. 3a-b with intensity profiles for Nodal HBE-YFP fluorescence. Insets show intensity of YFP signal in embryonic compartment. White boxes indicate area selected for intensity measurement (right-most graphs). Orange line indicates midline of the structure. Surface plot graphs (middle) show intensity of YFP. Bar=20μm. **b.** Proportion of structures expressing either asymmetric or symmetric Nodal at day 4 versus day 5. *n*=20 structures per group, 3 experiments. **c.** Quantitative assessment of endogenous Nodal HBE-YFP asymmetric fluorescence intensity in representative ETX embryos at day 4 (left) and day 5 (right) presented in (a). Each dot represents a cell. Mean intensity was calculated for cells in the region left or right of the midline. Two-sided Student's t-test, *n*=83 (left of the midline), *n*=81 (right of the midline) cells in day 4 ETX embryos; *n*=153 (left of the midline), *n*=159 (right of the midline) cells in day 5 ETX embryos. Means ± SD. **d.** Whole mount *in situ* hybridization revealing *Cerberus* (3 embryos in 3 experiments; 8 ETX embryos in 3 experiments), *Nodal* (4 embryos in 2 experiments; 10 ETX embryos in 3 experiments), *T/Brachyury* (3 embryos in 2 experiments; 17 ETX embryos in 3 experiments), *Cripto* (7 embryos in 2 experiments; 13 ETX embryos in 2 experiments), *Wnt3* (9 embryos in 3 experiments; 10 ETX embryos in 3 experiments) and *Bmp4* (8 embryos in 2 experiments; 14 ETX embryos in 2 experiments) transcripts in natural embryos and ETX embryos at indicated time points. Bar=50μm.


a
T LAMININ OCT4
ETX Embryo (D6)
b
ETX Embryo (time in culture)
DIC CAG:GFP
CAG:GFP
00h00m
07h12m
10h48m
12h00m
16h30m
c
DAPI OCT4
T OCT4 E-CAD
zoom
E6.75 Embryo
ETX Embryo (D5)
d
T intensity
F-ACTIN H3S10-P DAPI
H3S10-P
LE
S
R1
R2
E6.75 Embryo
ETX Embryo (D5)
% of H3S10-P positive cells
** P =0.0059
**** P <0.0001
e
ETX Embryo (D5)
Max. Projection
anterior zoom
posterior zoom
N-CAD DAPI T
A
P
E6.75 Embryo
f
ETX Embryo (D5)
Embryonic compartment
XZ
inverted
T OCT4 LAMININ
g
XEN:GFP T DAPI LAMININ
ETX Embryo (D5)
rotated
LAMININ
zoom
h
Number of ETX Embryos with detectable T
* P =0.0104
number of structures
Control
DKK1
Positive
Negative

Supplementary Figure 5

EMT events in ETX embryos.

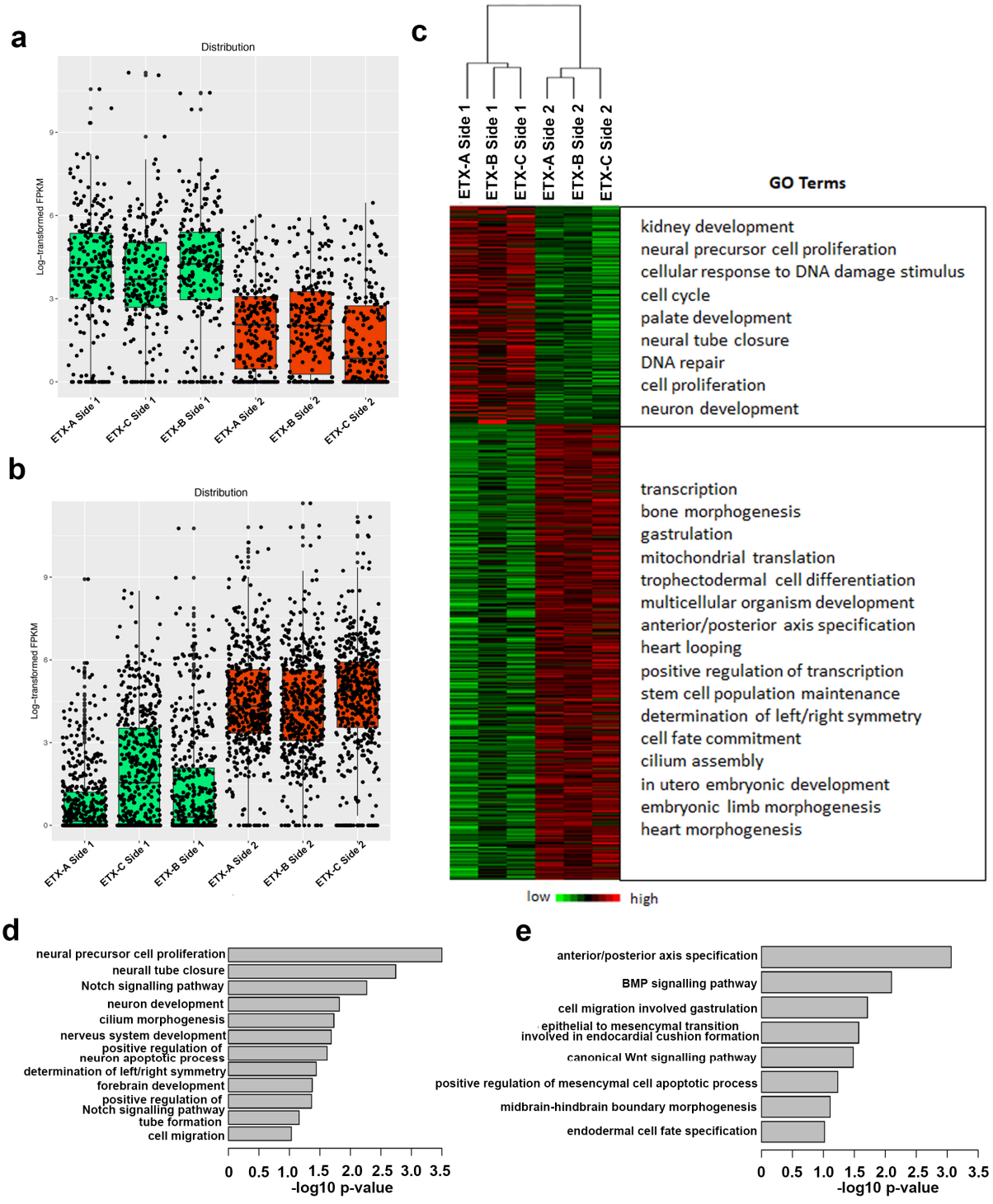
a. ETX embryos at day 5; white box indicates magnified field showing nuclei of *T/Brachyury*-positive cells changing from a plane perpendicular (asterisks) to parallel (arrowheads) to the basal membrane of the ES-derived compartment. Representative of 6 ETX embryos, 2 experiments. Bar=20μm. Bar in zoomed image=10μm. **b.** Still images from time-lapse movie of live ETX embryo built from CAG:GFP ES cells presented in Fig. 4d. White dashed lines outline XEN layer; purple dashed lines, embryonic/extra-embryonic boundary. White arrowhead indicates change in cell shape on the boundary of prospective posterior side. Representative of 3 separate time-lapse movies of 7 ETX embryos. **c.** Transverse sections from embryonic compartments of E6.75 embryo (top) (3 embryos) and day 5 ETX embryos (bottom) (3 ETX embryos). E-cadherin immunostaining reveals change in orientation of *T/Brachyury*-expressing mesenchymal cells (white arrowheads). 3 experiments. Bar=20μm. **d.** Immunostaining of E6.75 embryo (top) and day 5 ETX embryo (bottom) to reveal phosphorylated Histone 3 (H3S10-P) (magenta) – there is no increase in mitotic cells in the mesoderm, white boxes. ETX embryo presented in Fig. 4f re-stained for *T/Brachyury* in the same channel as GM130. Non-nuclear anti-*T/Brachyury* VE/XEN fluorescence is non-specific. Proportion of H3S10-P positive cells within and outside boxed region. LE, lateral epiblast; S, streak; R1, region 1; R2, region 2. *n*=3 E6.75 embryos; *n*=6 ETX embryos. Two-sided Student's t-test. Means ± SD. Bar=20μm **e.** Day 5 ETX embryo and E6.75 embryo immunostained for N-cadherin (cyan) and *T/Brachyury* (magenta). Magnified images below show up-regulated N-cadherin in re-oriented *T/Brachyury* expressing cells that identify mesoderm formation (white arrowheads). Non-nuclear anti-*T/Brachyury* VE fluorescence is non-specific. Representative of 4 E6.75 embryos; 3 ETX embryos. Bar on the zoomed images=5μm **f.** XZ sectioned orthogonal views from the ES-derived embryonic compartment of ETX embryo also presented in Fig.4h demonstrating laminin break-down on the T/Brachyury expressing side. Yellow arrows indicate break in laminin. **g.** Oblique section of an ETX embryo at day 5 showing break in laminin in *T/Brachyury* expressing posterior domain. Dashed lines outline the TS-derived extra-embryonic compartment. Representative of 3 ETX embryos, 2 experiments. **h.** Quantification of ETX embryos expressing *T/Brachyury* with and without DKK1 treatment (200ng/ml) for 24h presented as a bar chart. Contingency table used to perform statistical test. Two-sided Fisher's exact test, total of 20 structures scored per group from n=2 separate experiments. The number of structures scored in each independent experiment is reported in Supplementary Table 3.



Supplementary Figure 6

Specification of axial mesoderm in ETX embryos.

Foxa2 expression within the axial mesoderm region of natural (*in vivo*; 10), *in vitro* cultured embryos (IVC; 3), or ETX embryos (10). E7.0 natural (top) and day 6 ETX embryo (bottom) shown in a; embryo cultured *in vitro* from E5.25 for 48h (top) and day 6 ETX embryo (bottom) shown in b. White boxes indicate magnified region to show Foxa2 positive cells. White dashed lines outline the axial mesoderm region; purple dashed lines, ExE or TS-derived extra-embryonic compartment, 3 experiments. Bar=20μm. Bar in zoomed images=10μm.



Supplementary Figure 7

Transcriptional profiling of ETX embryos reveals global similarity of anterior-posterior patterning to natural embryos.

a, b. Average expression level of differentially expressed genes in side 1 (prospective anterior) and side 2 (prospective posterior) of ETX embryos. Box plot elements represent differentially expressed genes (DEGs) between ETX-Side 1 and ETX-Side 2, n=499 DEGs for side 1 shown in a, n=239 DEGs for side 2 shown in b. Middle line shows the median value. DEGs were identified from 3 biological replicates divided into side1 and side2. DEGs were identified using RankProd statistical test with P value <0.05 and fold change >1.5.

c. Left: Gene expression heatmap from each side of the ETX embryos. Red, high gene expression; green, low gene expression. Right: Gene Ontology (GO) analysis on combined Side 1 samples (top box) and combined Side 2 samples (bottom box) illustrating significantly enriched terms. *n*=3 biological replicates, divided in side 1 and side 2. RankProd ($p < 0.05$, $fc > 1.5$) used to identify the differentially expressed genes. Functional enrichment of DEGs was performed using DAVID v6.8 (see method). **d, e.** Gene Ontology analysis highlighting developmental categories detected in side 1 and side 2 of ETX embryos. *n*=3 biological replicates, divided in side 1 and side 2. Functional enrichment of DEGs was performed using DAVID v6.8, then the p-values were transformed to logarithmic space by using -log10(p-value), then using R to draw the bar plot that indicates the significance of the biological processes (see method).

Supplementary Table 1. Antibodies used in this study.

Supplementary Table 2. qPCR primers used in this study.

Supplementary Table 3. Statistic source data.

Supplementary Movie 1. Time-lapse recording of an ETX embryo from day 3.5 to 5.0 in culture. Related to Supplementary Fig.1. Representative of 3 separate time-lapse movies of 10 ETX embryos.

Supplementary Movie 2. Cell tracking in an ETX embryo. Detailed cell tracking images of the same cell show step-by-step change in shape from bottle to mesenchymal between 16h 30m and 17h 00m time-points. Related to Fig.4e. Representative of 3 separate time-lapse movies of 7 ETX embryos.