Materials and Methods

Embryo recovery and culture

Mice were kept in the animal house in accordance with national and international guidelines. All experiments have been regulated by the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). Experiments were approved by the Home Office. Animals were inspected daily and those that showed health concerns were culled by cervical dislocation.

Six-week-old female CD-1 mice were naturally mated and sacrificed after 5, 6 or 7 days post coitum. The uterus was recovered and embryos dissected from deciduae in M2 medium. Blastocysts were recovered at E4.5 by uterine flushing, their mural trophectoderm dissected away and cultured in IVC1 and IVC2 media (Cell Guidance Systems) as previously[28].

Cell culture

ES cells and TS cells were cultured at 37°C and 5% CO_2 on mitotically inactivated CF1 or NMRI mouse embryonic fibroblasts (MEFs) and passaged once they reached 80% confluency. Cells were cultured in ETX-medium consisting of Dulbecco's modified essential medium (DMEM, Gibco cat: 21969) with 12.5% FBS, 2 mM GlutaMax (Gibco cat: 35050-038), 0.1mM 2-mercaptoethanol (2-ME, Gibco cat: 31350-010), 0.1 mM nonessential amino acids (Gibco cat: 11140-035), 1 mM sodium pyruvate (Gibco cat: 11360-039), 0.02 M HEPES (Gibco cat: 15630080) and 1% penicillin-streptomycin (Gibco cat: 15140122), ESC media was supplemented with PD0325901 (1mM), CHIR99021 (3 mM) (2i), and leukemia inhibitory factor (0.1 mM, LIF) (ETX-2iLIF). TSC media was supplemented with FGF2 (25 ng/mL; Peprotech), FGF4 (25ng/mL; Peprotech) and heparin (1μg/ml; Sigma) (ETX-F42H). XEN cells were cultured on gelatinized tissue-culture-grade plates, in 70% MEF-conditioned ETX-medium (C-ETX), without any additional supplements.

Cell lines

Experiments were performed using mouse wild-type ES cells, Nodal HBE-YFP ES cells[18], T:GFP ES cells[29], CAG:GFP ES cells[30], wild-type TS cells, wild-type XEN cells and EGFP XEN cells. Wild-type TS cells were a gift from Dr. Jenny Nichols (University of Cambridge, UK) and EGFP XENs were a gift from Dr. Peter Rugg-Gunn (Babraham Institute, Cambridge).

Preparing and Plating Cell Suspensions for 'AggreWell' Aggregation Experiments

The full protocol was repeated independently in the University of Cambridge, Charité University and MaxPlanck Institute in Berlin, and is available on Protocol Exchange[31].

For the AggreWell plate preparation, the manufacturer's protocol was followed. Briefly, wells were rinsed with the rinsing solution (Stem cell technologies), centrifuged for 5 min at 2000*g* and incubated at room temperature in the tissue culture hood for 20 min. After incubation, the wells were washed with 2mL of 1X PBS. After PBS removal, 500 μL of filtered C-ETX medium was added to each well and the plate was spun for 5 min at 2000*g* and then placed at 37°C and 5% CO_2 until ready to use.

ES and TS cell colonies were dissociated to single cells by incubation with 0.05% trypsin-EDTA at 37°C for 3 min. Cells were pelleted by centrifugation for 4 min at 1000 rpm and resuspended in MEF-conditioned ETX-medium (C-ETX, as above). Cell suspension was pre-incubated at 37°C and 5% CO_2 on gelatinized tissue-culture-grade plates for 30 min to

remove MEFs until the re-collection of ES and TS cells suspensions. Meanwhile, XEN cells were dissociated to single cells by incubation with 0.05% trypsin-EDTA at 37°C for 3 min, and pelleted by centrifugation for 4 min/1000 rpm and re-suspended in C-ETX medium. 7200 ES cells/per well, 19200 TS cells/per well and 5400 XEN cells/per well were counted using a haemocytometer. Then ES, TS, and XEN cells suspensions were mixed and re-pelleted. The cell mixture was re-suspended in complete embryoid-medium (C-ETX medium) consisting of 70% MEF-conditioned DMEM with 12.5% FBS, 2 mM GlutaMax, 0.1mM 2-mercaptoethanol (2-ME), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.02 M HEPES, plus ROCK inhibitor (Y27632 (Stemgent, 5 nM), and added dropwise to the well. The AggreWell plate was centrifuged for 3 min at 100*g*, outside wells were filled with PBS to prevent evaporation and plates were placed at 37°C and 5% CO_2. On the following day (day 1), 1 mL of media from each well was slowly removed and replaced with 1 mL of fresh medium without ROCK inhibitor. This step was performed a second time to fully remove the ROCK inhibitor. On day 2, C-ETX medium was replaced with fresh medium. On day 3, the media was changed to IVC1 (Cell Guidance Systems). At day 4, IVC1 was replaced with IVC2 (Cell Guidance Systems). On day5 and day6 IVC2 was replaced with fresh IVC2.

Immunofluorescence staining

ETX and natural embryos were fixed in 4% paraformaldehyde for 20 min at room temperature, washed twice in PBT [phosphate-buffered saline (PBS) plus 0.05% Tween-20] and permeabilized for 30 min at room temperature in 0.3% Triton-X-100, 0.1% glycin. Primary antibody incubation was performed overnight at 4°C in blocking buffer [PBS plus 10% fetal bovine serum (FBS), 1% Tween-20]. The next day, embryos were washed twice in PBT, then incubated overnight with secondary antibody (1:500) in blocking buffer at 4°C. On day 3, embryos were washed twice in PBT and incubated for 1 hour at room temperature in 4′,6-diamidino-2-phenylindole (DAPI) plus PBT (5mg/ml). Embryos were transferred to PBT drops in oil-filled optical plates before confocal imaging. For antibodies used see supplementary table 1.

Criteria for ETX embryo inclusion and comparison to natural embryos

Upon completion of any given aggregation experiment, ETX embryos were fixed (see above) and those clearly displaying an outer layer of cells and two-inner, well-defined compartments, were manually selected for further analysis. Identity of the compartments was verified by immunofluorescence using Oct4 to mark the ES cell-derived embryonic compartment, Tfap2c or Eomes for the TS cell-derived and Gata4, Gata6 or Sox17 for the XEN cell-derived extra-embryonic compartments. This was not necessary in instances where cell lines with appropriate fluorescent reporters were used.

ETX embryos were staged relative to natural embryos based on their common morphological features and developmental milestones. In a typical experiment, lumenogenesis was initiated at day 3 with only a single cavity present in the ES-derived embryonic compartment. This stage is comparable to E5.0/E5.25. By day 4, one or more small additional cavities developed within the TS-derived extra-embryonic compartment; this stage is comparable to an E5.5 stage natural embryo. By day 5, the cavities in the ES- and TS-compartments combined into a single large cavity, T/Brachyury was expressed and cell migratory events took place; this stage is comparable to an E6.0/E6.75 natural embryo. Finally, definitive endoderm and axial mesoderm formation in ETX embryos could be observed from day 6 onwards; this stage was comparable to a natural E6.75/E7.5 embryo.

Cryosectioning and immunostaining
ETX embryos and natural embryos were fixed for 20 min in 4% PFA at room temperature and washed twice in PBS. Samples were infiltrated in ice cold 20% sucrose (in PBS) until each sample dropped to the bottom of a plate, embedded in 20% sucrose/7.5% gelatin (in PBS), frozen in liquid nitrogen. Blocks were serially sectioned in the transverse plane at 15 µm thickness. Sections on slides were stored at -20°C overnight. For the immunostaining, sections were rehydrated for 10 min in PBS, permeabilised for 15 min in 0.1M Glycin/0.3% Triton X-100, washed twice in PBS, blocked for at least 1 hour and then incubated with primary antibodies overnight at $4C^0$. Following, sections were washed twice in 0.5% Tween/PBS, incubated with secondary antibodies for 1 hour at room temperature, washed twice in 0.5% Tween-20/PBS and mounted for confocal microscopy with ProLong® Gold Antifade Mountant with DAPI. For antibodies used see supplementary table 1.

Image data acquisition, processing, and quantification
Images were acquired with an inverted Leica SP8 confocal microscope (Leica Microsystems), using a Leica Fluotar VISIR 0.95 NA 25x objective. Fluorophores were excited with a 405-nm diode laser (DAPI), a 488-nm argon laser (GFP), a 543-nm HeNe laser (Alexa Fluor-543/555) and a 633-nm HeNe laser (Alexa Fluor-633/647). Images were acquired with 0.5–1.2µm *z*-separation. Raw data were processed using open-source image analysis software "Fiji" or "Imaris" software (Bitplane) and assembled in Photoshop CC 2017 (Adobe). Digital quantifications and immunofluorescence signal intensity graphs were obtained using Fiji software, quantification of the cell number was performed in Imaris software.

Immunofluorescence intensity measurements of gene expression
Nodal-YFP asymmetry: Analysis was performed using Fiji software. A line from the proximal-distal axis, defining the 'midline' of ETX embryos, was drawn to determine the left and right sides of the structure. Secondly, the shape of each nucleus in embryonic compartment (Oct4 positive) was traced and added as a region of interest (ROI) to the ROI manager tool. The corresponding Nodal-YFP channel was then selected and all the previously defined ROIs were used to measure a mean intensity value in the Nodal-YFP channel for each cell nucleus. This procedure was repeated 5 times for other Z-planes. All the values for the left side were then plotted against all the values for the right side and tested with Student's t-test. ETX embryos were considered to express Nodal-YFP asymmetrically where statistically significant differences were shown between ''left and right'.

Lefty1 asymmetry: Analysis was performed using Fiji software. A line from the proximal distal axis, defining the 'midline' of ETX embryos, was drawn to determine the left and right sides of the structure. For each selected Z-plane, a line was drawn from left to right of ETX embryos, following the curvature of the XEN layer (XEN/VE markers). The Grey value (intensities) for Lefty1 were calculated using the "plot-profile" function. This measurement was performed on the right and left side of at least 6 z-planes (see figure legends). If average Lefty1 intensity was statistically significantly different between left and right sides (Student's t-test), Lefty1 was considered asymmetric.

Reconstruction of the embryonic compartment of ETX embryos and natural embryos
Reconstruction was performed using Imaris software (Bitplane). Every single cell expressing Oct4 in the embryonic compartment and every T/Brachyury expressing cell was plotted upon

a projection of all cells in the structure. Cells expressing T/Brachyury were represented as green dots and every other cell in the embryonic compartment was represented as a red dot.

Mitosis in mesoderm regions in ETX embryos and E6.75 embryos
Cells positive for H3S10-P protein expression were counted in the mesoderm region and outside this region. This procedure was repeated for 3 chosen Z-planes. Z-planes were selected from the top (1/3 of total), middle (2/3 of the total) or end (3/3 of the total) region of each ETX embryo or natural embryo, and the values obtained were averaged. Student's t-test was performed.

Time-Lapse Imaging
Confocal time-lapse imaging during ETX embryo culture was performed using spinning-disc microscope (3i), using a Zeiss EC Plan-NEOFLUAR 20x/0.5 objective. The embryos were imaged every 45 min in 100 μm image stacks of 4 μm z-planes. Images were processed using Slidebook 5.0 (3i). Cell tracking time-lapse images were captured using an inverted SP8 confocal microscope (Leica Microsystems), using a Leica Fluotar VISIR 0.95 NA 25x objective. Images were acquired in 6 min intervals with a z-step of 1 μm. ETX embryos were imaged in a humidified chamber with 37 $^{\circ}$C, 5% CO_2.

Whole mount *in situ* hybridization (WMISH)
WMISH was performed exactly as previously described[32], with the only difference that the 10X DIG-blocking solution was prepared by dissolving 1 g of blocking reagent in 10 mL of malate buffer. Samples were digested with Proteinase K for 3 minutes at room temperature with rocking. The probe for Cerl was kindly provided by Prof. E. De Robertis, the probe for Nodal was kindly provided by Prof. H. Hamada, the probes for Wnt3 and Bmp4 were kindly provided by Prof. J. Rivera, and the probes for Cripto and T were kindly provided by Prof. T. Rodriguez.

Cell isolation and qRT–PCR
After 5 or 6 days of culture ETX embryos were collected. Asymmetric T expression was confirmed under a fluorescent microscope, and the TS-compartment was dissected away. The ES compartment was cut through a line corresponding to the long axis, equivalent to the 'midline' of an ETX embryo (perpendicular to the embryonic-extraembryonic boundary). *T*:GFP positive and negative domains were collected separately and transferred into lysis buffer (Life Technologies). Total RNA was extracted with the Arcturus Pico Pure RNA Isolation Kit, and qRT-PCR was performed with the Power SYBR Green RNA-to-CT 1-Step Kit (Life Technologies) and a Step One Plus Real-time PCR machine (Applied Biosystems). The amounts of mRNA were measured with SYBR Green PCR Master Mix (Ambion). Relative levels of transcript expression were assessed by the ΔΔCt method, with Gapdh as an endogenous control. For qPCR primers used, see Supplementary Table2.

Sample collection and RNA isolation
To collect RNA for RNA-Seq, ETX embryos generated using a *T/Brachyury*-GFP reporter ES line were collected and TS compartments were dissected away. The ES compartment was further dissected in half on the long side and each half was analysed separately under a fluorescent microscope to identify GFP+ve (prospective posterior) and GFP-ve (prospective anterior) cells. Samples were collected in lysis buffer (2.3μl of 0.2 % Triton X-100 (Sigma) supplemented with 1U/μl RNAsIN (Ambion)) and stored at -80^{0}C until library preparation.

Library preparation, RNA-sequencing and mapping of reads

Low-input RNA-seq was performed using the Smart-seq2 protocol[33,34], using 18 cycles of amplification in the PCR-preamplification step. A pool of indexed libraries was sequenced on a HiSeq 2500 in rapid run mode. Reads were mapped to the *M. musculus* genome (Ensembl version 38.77) and quantified using the HTSeq-count[35].

Quality assessment and pre-processing of RNA-Sequencing data

The quality of the reads was evaluated using the FASTQC tool[36]. Density distribution of gene expression for all samples was also plotted to assess sample consistency. Raw reads were mapped to mm10 version of the mouse genome using the Tophat2 v2.0.4 program[37]. Samples with raw sequencing reads count > 10 million and mapping ratio > 50% were retained for further analysis. We calculated fragment per kilobase per million (FPKM) as expression level using Cufflinks v2.0.2 with default parameters [38]. Genes with the FPKM value > 1.0 in at least one sample across all samples were retained for further analysis. Finally, the expression levels were transformed to logarithmic space by using $\log_2$(FPKM+1). With these criteria, 3 ETX embryos were retained for further analysis (ETX-A, ETX-B and ETX-C) and since they were cut in half, each side was arbitrarily called side 1 or side 2.

Principal Component Analysis (PCA)

PCA was based on all the expressed genes as described in the RNA-Seq data preprocessing and was performed using the FactoMineR package in R.

Differentially expressed genes (DEGs) analysis

DEGs between ETX embryo side1 and ETX embryo side2 were identified using RankProd[39] with P value <0.05 and fold change >1.5. The heatmaps were generated using Cluster 3.0 and JavaTreeView[40].

Functional enrichment analysis

Functional enrichment of gene sets identified as differentially expressed between samples was performed using the Database for Annotation, Visualization and Integrated Discovery v6.8 (DAVID v6.8)[41].

Zip Code Mapping and corn-plot analysis to compare side 1 and side 2 of ETX embryos to regions of the epiblast

The transcriptome data from each side of ETX embryo was compared to embryo samples at three different stages (E6.5: 13 laser capture microdissection (LCM) samples, E7.0: 42 LCM samples, E7.5: 46 LCM samples). The reference embryo samples were subdivided into different epiblast regions as previously described[27]. To assess the similarity between ETX embryos and natural embryo samples, a Pearson Correlation Coefficient (PCC) was calculated as a measure of correlation between gene expression of reference embryo samples (E6.5: 13 samples, E7.0: 42 samples, E7.5: 46 samples) and transcriptome data from cells isolated from each side of ETX embryos. A PCC of 1 indicated that the gene expression pattern of a sample taken from the ETX embryo and a region of the epiblast was identical, and therefore the two were perfectly correlated. Conversely, a PCC of 0 would indicate no correlation. Therefore, a high PCC value reflects closely matching gene expression patterns between ETX embryo samples and post-implantation reference samples, whereas a low PCC reflects no match. This PCC was calculated based on the expression of 'zip code' genes identified as robust markers of different regions of the epiblast of the post-implantation embryo [27]. The PCC values for each side of the ETX embryo sample compared to each region of the epiblast for each reference embryo was depicted in the form of a 'corn plot' using MATLAB (version 2015a) [27].

Building Neighbour-joining (NJ) tree
For combined ETX embryo and natural embryo samples, we first removed batch effects with ComBat (package in R) if individual samples were from different batches. Then, the Euclidean distance of any two samples was calculated based on differentially expressed genes, and then fed into MEGA (version: 7.0) software to build the NJ tree (under the Phylogeny menu).

Statistics and Reproducibility Statistical tests were performed on GraphPad Prism 7.0 software for Windows. Data were checked for normal distribution and equal variances before each parametric statistical test was performed. Qualitative data are presented as a contingency table and were analysed with Fisher's exact test. Where appropriate, Student's t-tests (two groups) or ANOVA (multiple groups) were performed with Welch's correction if variance between groups was not equal. Error bars represent SEM or SD as specified. Figure legends indicate the number of independent experiments performed in each analysis. 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 GSE110105. LCM sequencing data is available under accession number GSE65924.

Source Data for RT–qPCR experiments (Fig. 4c, Supplementary Fig. 2i) and quantifications of the immunofluorescence data (Fig. 1f, Fig. 2a-b-c, Fig. 3e, Supplementary Fig. 2d-f-g, Supplementary Fig. 4c, Supplementary Fig. 5d, h) and the differentially expressed gene list (Fig. 7b) have been provided in Supplementary Table 3.

All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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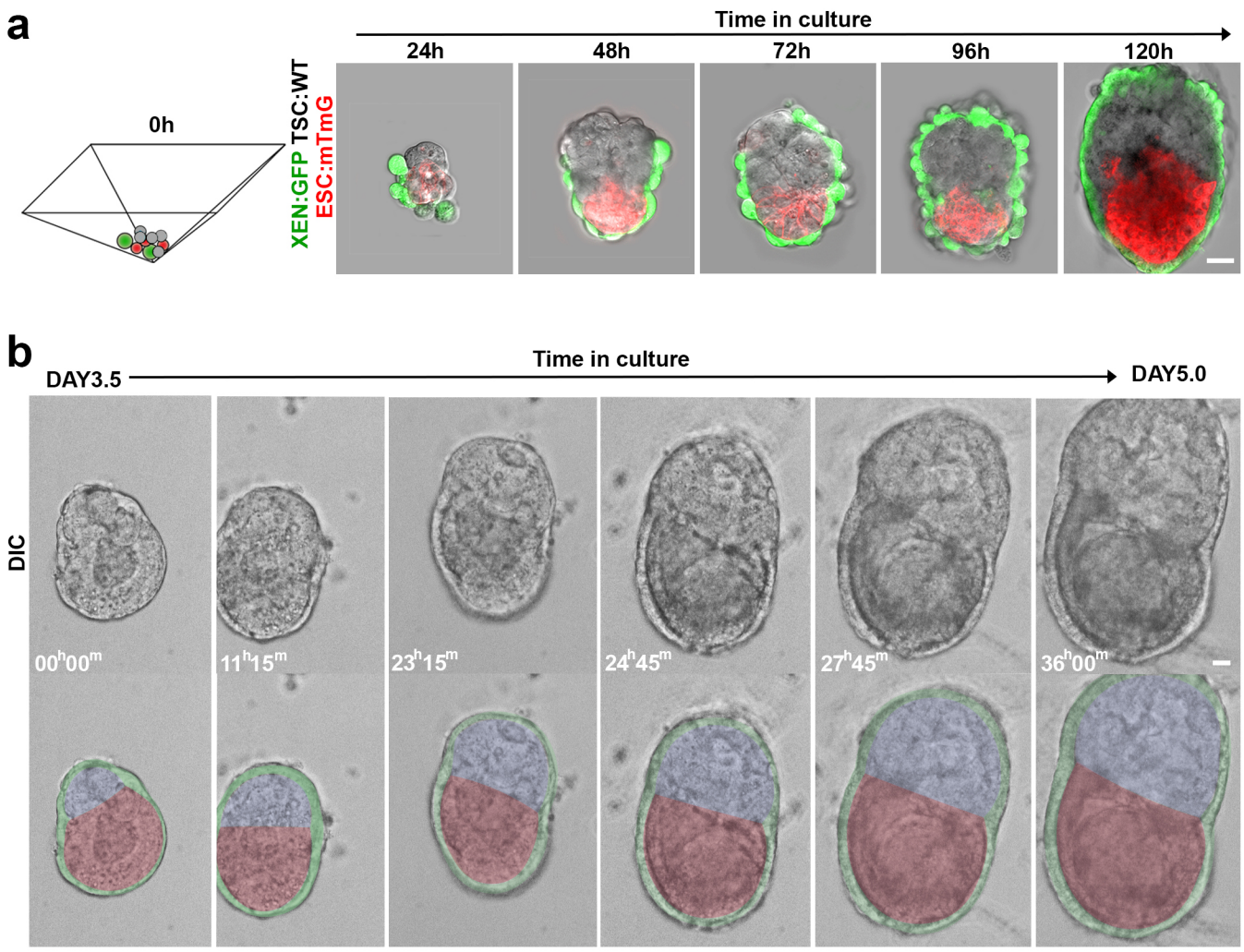
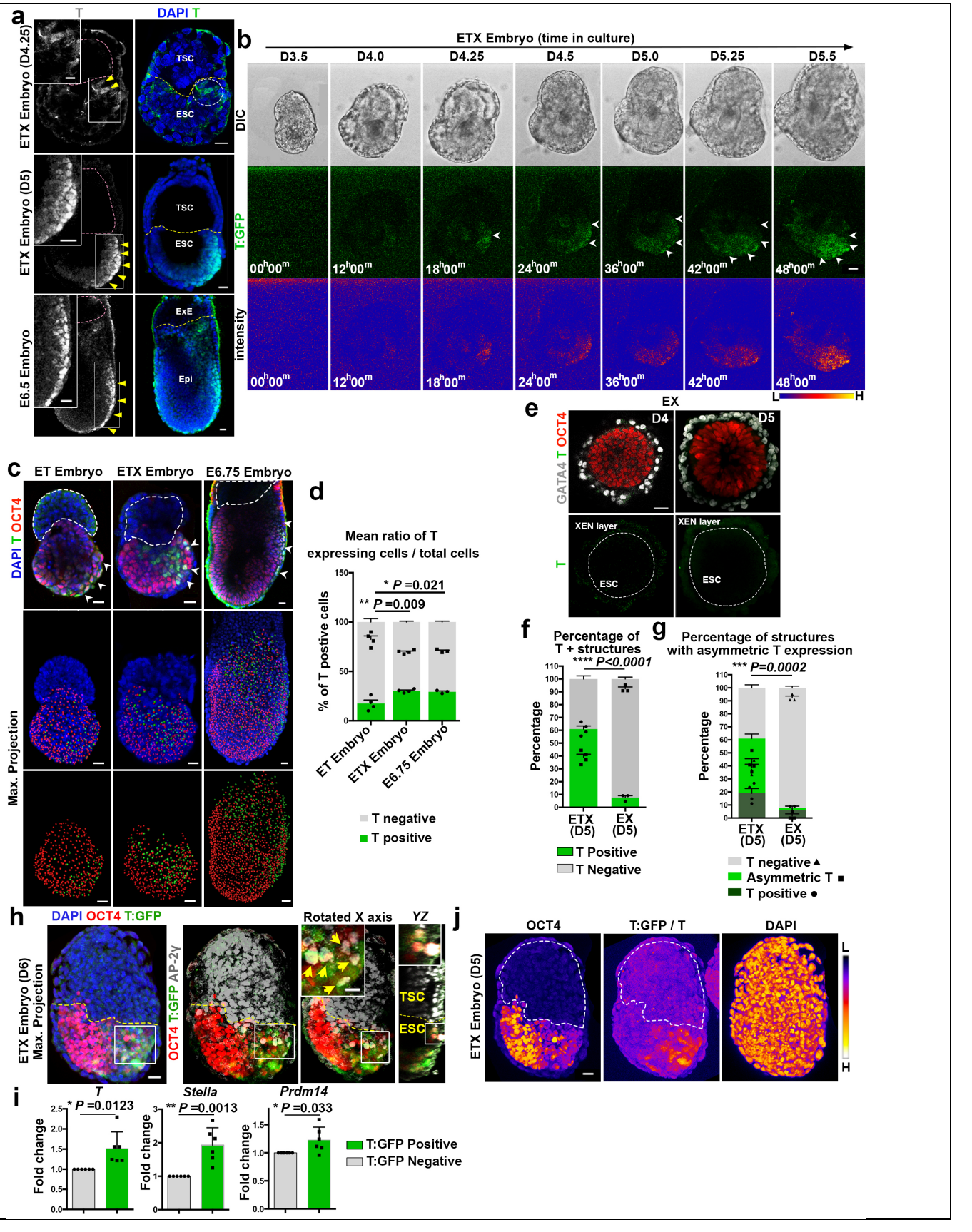


Supplementary Figure 1

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
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
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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
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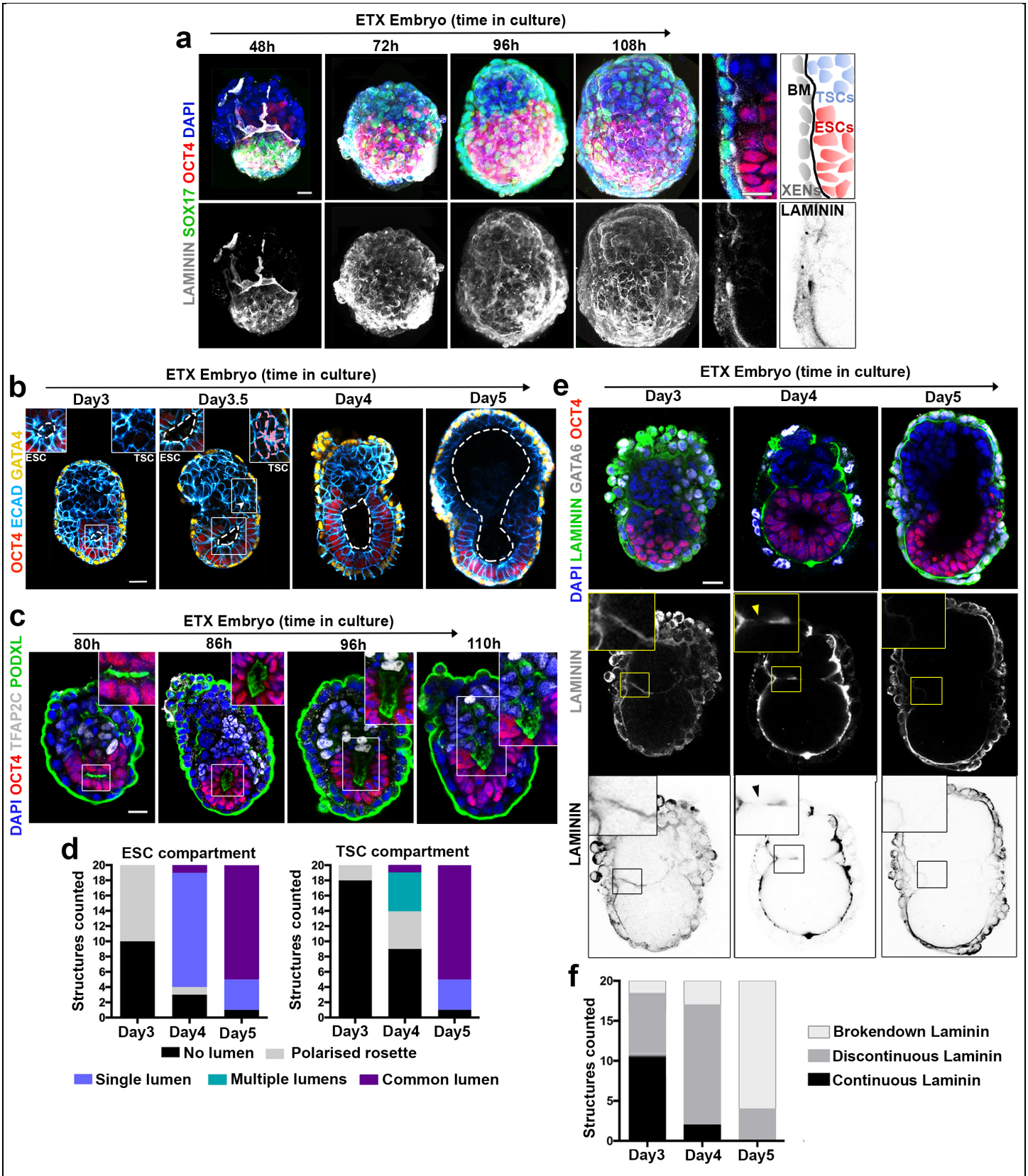
Supplementary Figure 2

Posterior patterning in ETX embryos

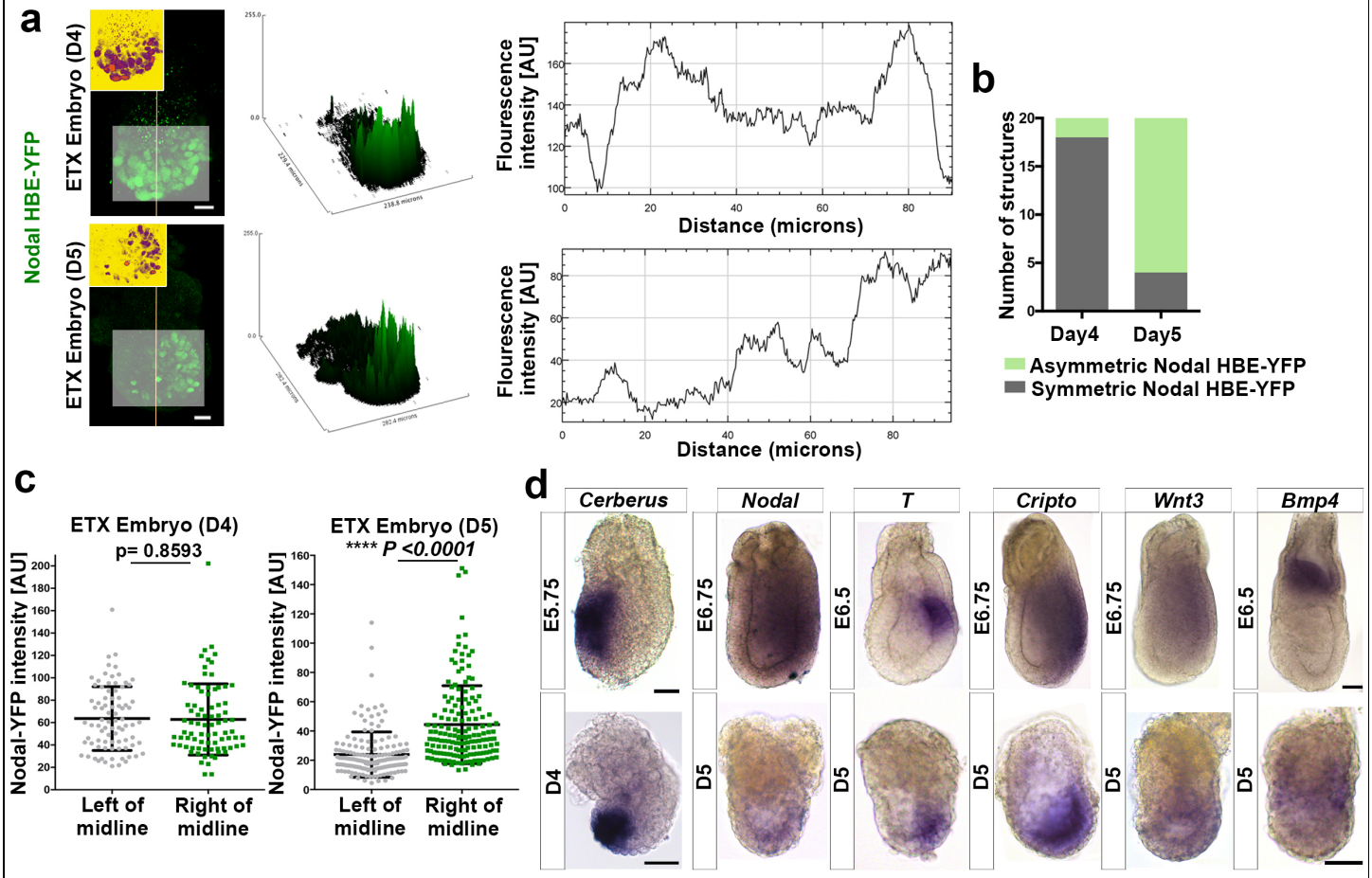
a. Induction of *T/Brachyury* in a representative ETX embryo at day 4.25 (top); localised *T/Brachyury* in a representative ETX embryo at day 5 (middle) and in E6.5 embryo (bottom). Yellow arrowheads indicate induction (top) or asymmetric *T/Brachyury* expression throughout posterior (middle and bottom). 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.

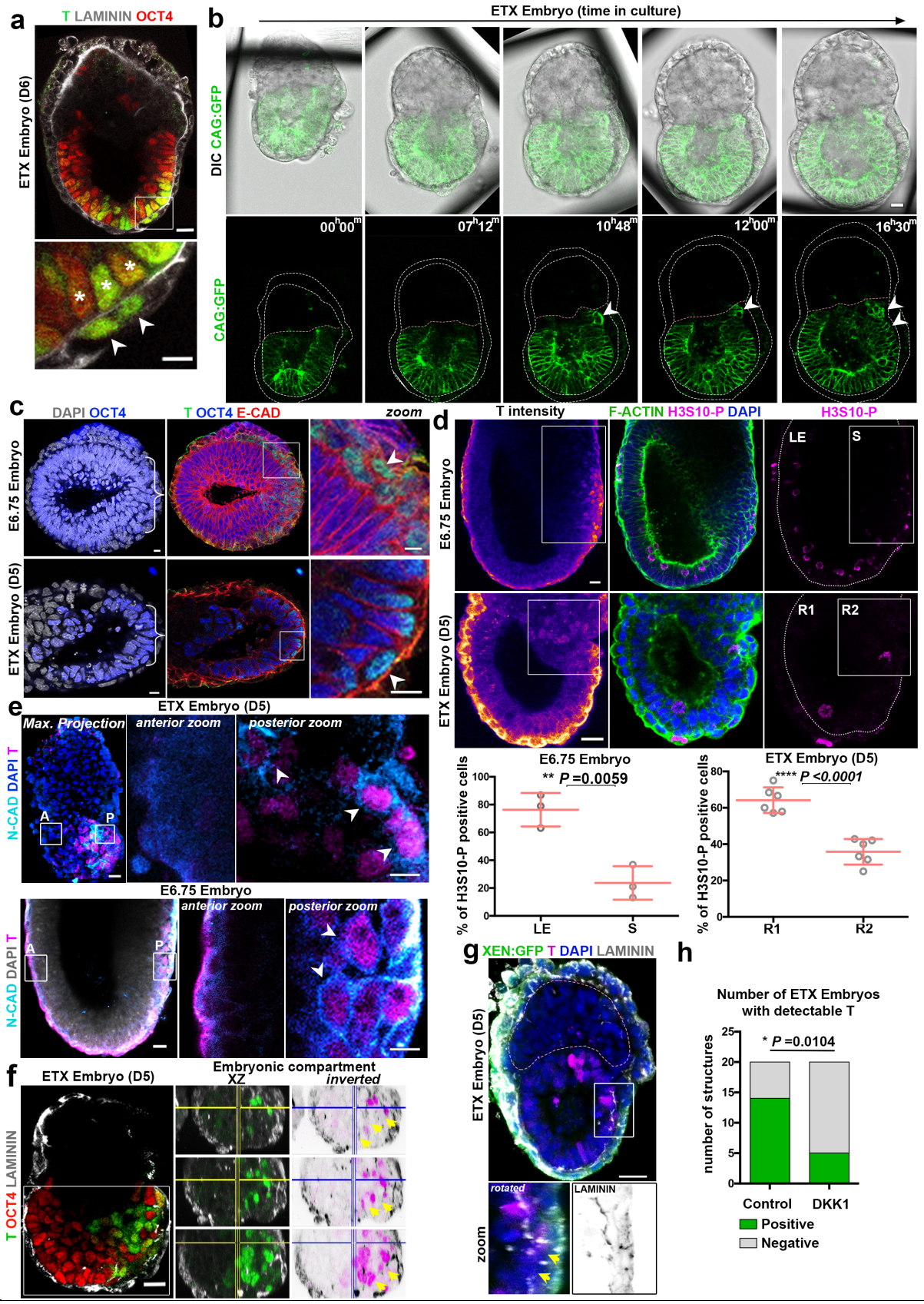
a. Maximum projections from time points indicated showing formation of basement membrane during ETX embryo development. Right-most single-plane images: magnified middle plane of ETX embryo at 108h. Bar=20μm; Representative of 6 ETX embryos for each time point. **b.** ETX embryo after 3, 3.5, 4 and 5 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.

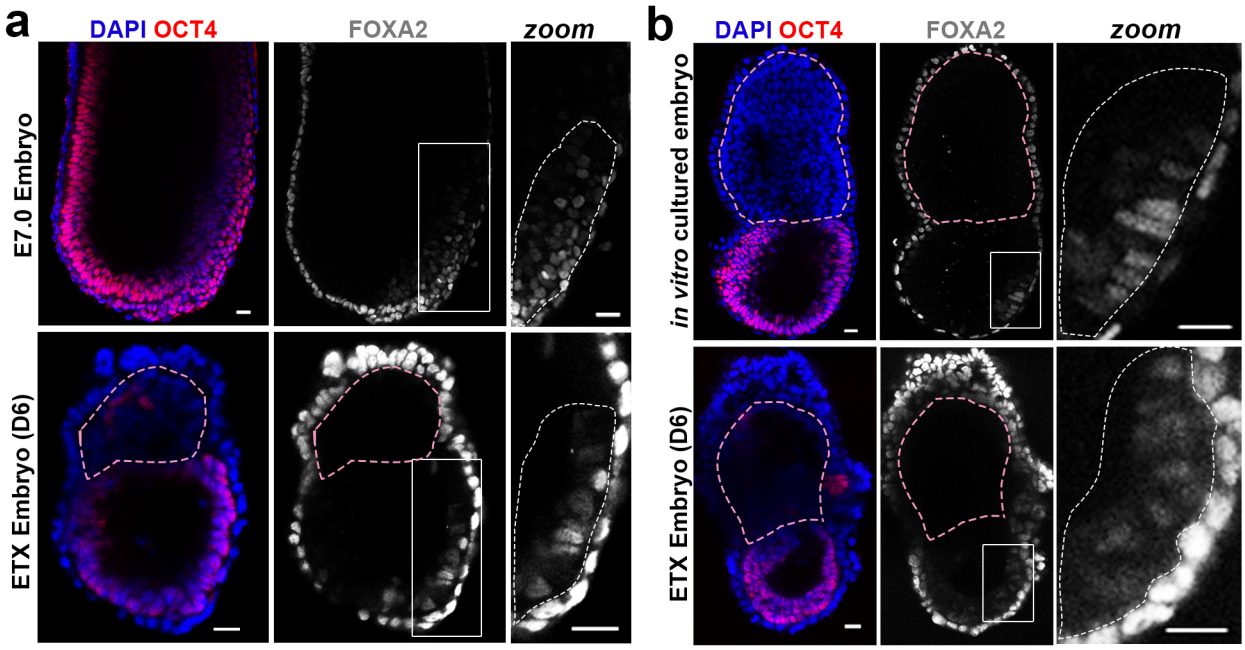
a. Images showing the same ETX embryos presented in Fig. 3a-b with intensity profiles for Nodal HBE-YFP fluorescence. Insets show intensity of YFP signal in embryonic compartment. White boxes indicate area selected for intensity measurement (right-most graphs). Orange line indicates midline of the structure. 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
** P =0.0059
ETX Embryo (D5)
**** P <0.0001
% of H3S10-P positive cells
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.

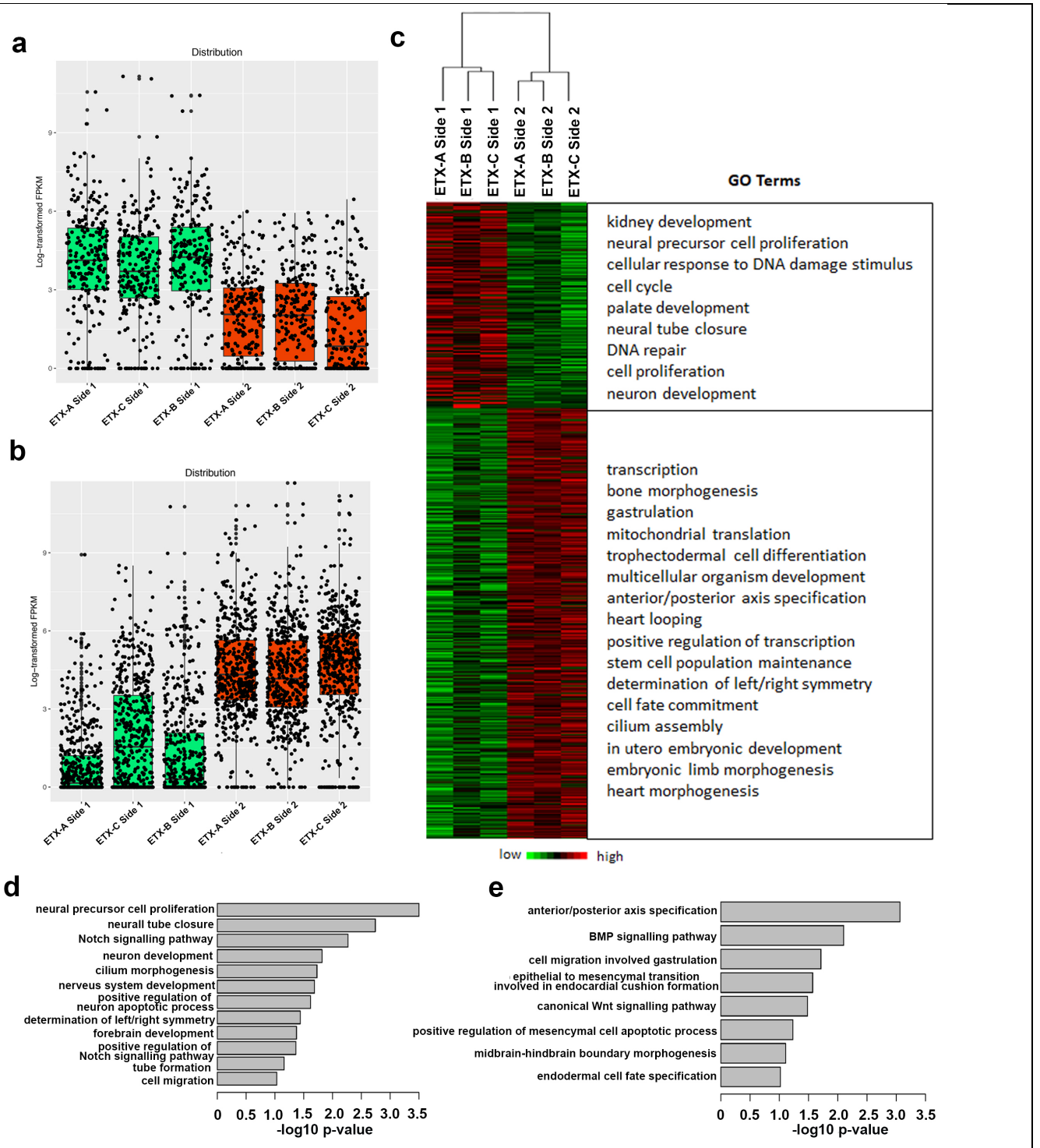
a. ETX embryos at day 5; white box indicates magnified field showing nuclei of *T/Brachyury*-positive cells changing from a plane perpendicular (asterisks) to parallel (arrowheads) to the basal membrane of the ES-derived compartment. Representative of 6 ETX embryos, 2 experiments. Bar=20μm. Bar in zoomed 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- 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. 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.