Figure S1 (related to Figure 1)

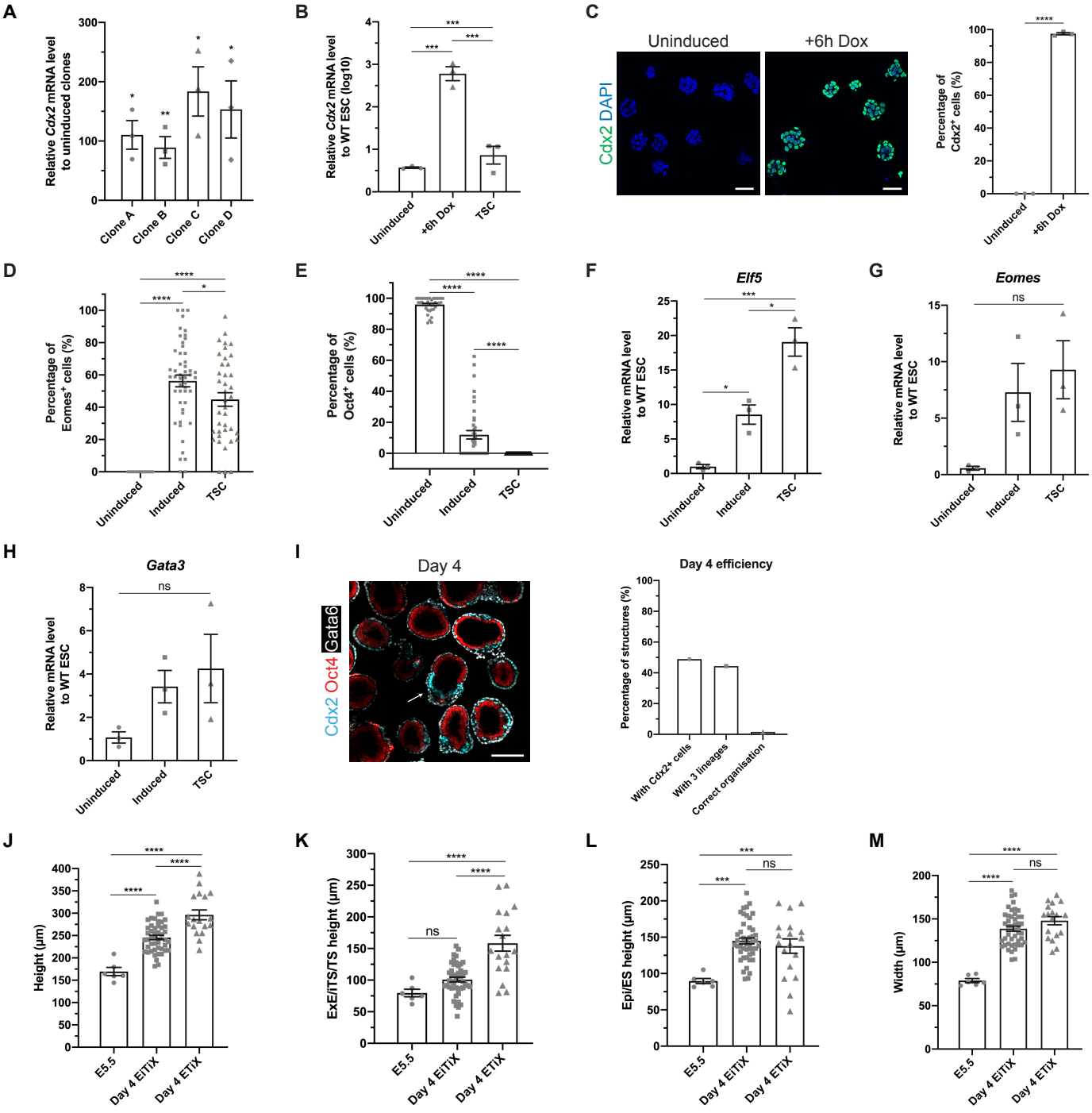


Figure S1. Characterisation of *Cdx2* inducible cells and Day 4 EiTiX-embryoids, related to Figure 1.

(**A**) Fold changes of *Cdx2* mRNA level in four different clones of i*Cdx2* ESCs 6 hours after Dox induction, determined by qRT-PCR. Student's t-test compares fold change with respect to uninduced counterparts. (**B**) Relative *Cdx2* mRNA levels in uninduced i*Cdx2* ESCs, 6-hour induced CAG iCdx2 ESCs, and TSCs as compared to unmodified ESCs. (**C**) Immunofluorescence images of Cdx2 expression in uninduced and 6-hour induced iCdx2 ESCs. Percentage of $Cdx2^+$ cells is shown in righthand graph. n = 3 experiments, 5-10 random fields imaged for each condition in each experiment. Scale bar, 50μm. (**D-E**) Percentage of $Eomes^+$ (D) and $Oct4^+$ (E) cells in Day 3 aggregates of uninduced, induced i*Cdx2* ESCs and TSCs. (D) n = 43 uninduced aggregates, 50 induced aggregates and 39 TSC aggregates. (E) n = 40 uninduced aggregates, 40 induced aggregates and 43 TSC aggregates. (**F-H**) Relative mRNA levels of TSC markers *Elf5* (F), *Eomes* (G) and *Gata3* (H) in Day 3 aggregates, normalised to mRNA levels in unmodified ESC aggregates. (**I**) Day 4 structures generated using the ETiX embryo protocol, stained to reveal Cdx2 (cyan), Oct4 (red) and Gata6 (white). Arrow indicates structure with correct organisation. Scale bar, 150μm. Graph shows percentages of structures with Cdx2-positive cells, with cells from all three lineages and with correct organisation. Scale bar, 150μm. (**J-M**) Size comparison of E5.5 mouse embryos, Day 4 EiTiX-embryoids and Day 4 ETiX embryos in terms of height (J), ExE/iTS/TS height (K), Epi/ES height (L) and width (M). n = 6 E5.5 mouse embryos, 46 Day 4 EiTiX-embryoids and 15 Day 4 ETiX embryos. All experiments were performed minimum 3 times with the exception of (I). Statistics: (A and C) Student's t-test. (B, D-H, J-M) One-way ANOVA followed by Bonferroni's multiple comparisons test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, non-significant.

Figure S2 (related to Figure 2)

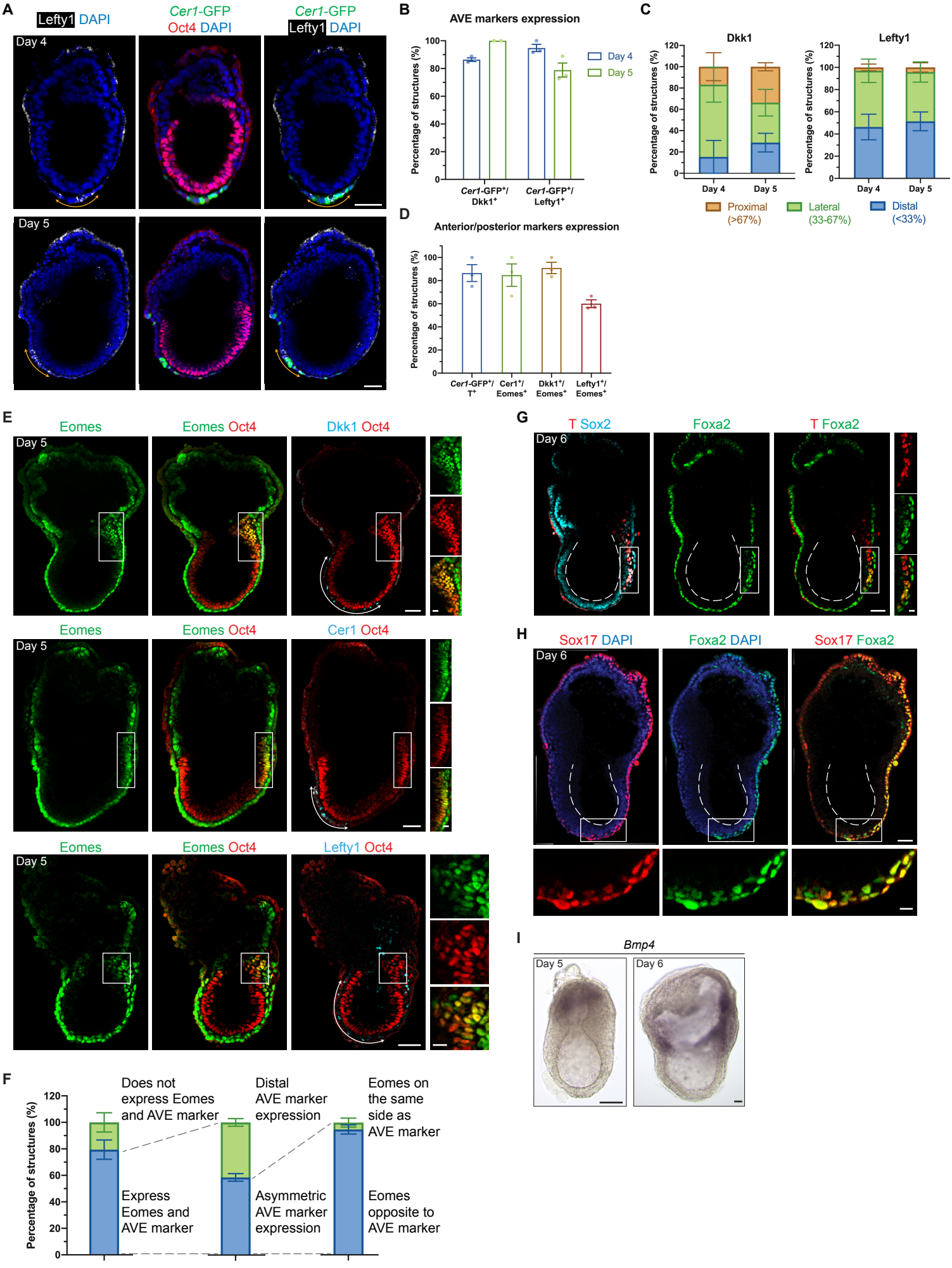
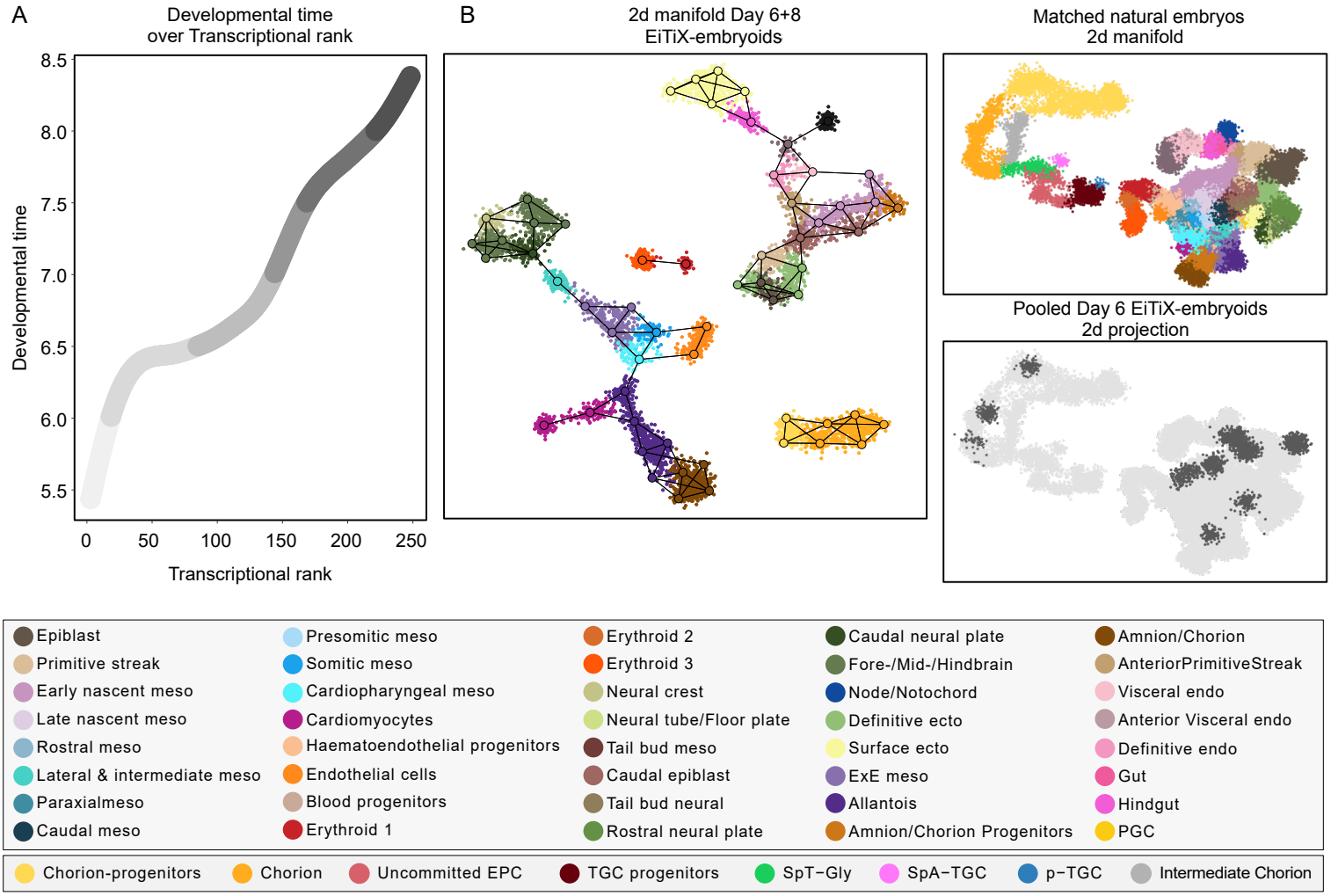


Figure S2. EiTiX-embryoids establish an anterior-posterior axis and undergo gastrulation, related to Figure 2.

(**A**) Day 4 and Day 5 EiTiX-embryoids stained to reveal *Cer1*-GFP (green), Oct4 (red) and Lefty1 (white). Arrows indicate the Lefty1-positive domain. (**B**) Percentages of EiTiX-embryoids expressing different combinations of AVE markers. n = 29-32 Day 4 EiTiX-embryoids and 18-28 Day 5 EiTiX-embryoids. (**C**) Localisation of Dkk1 and Lefty1 in Day 4 and Day 5 EiTiX-embryoids. See Materials and Methods for quantification method. Dkk1: n = 29 Day 4 EiTiX-embryoids, 18 Day 5 EiTiX-embryoids; Lefty1: n = 30 Day 4 EiTiX-embryoids, 22 Day 5 EiTiX-embryoids. (**D**) Percentages of EiTiX-embryoids expressing different combinations of anterior/posterior markers. n = 42 Day 5 EiTiX-embryoids (*Cer1*-GFP$^+$/T$^+$), 39 Day 5 EiTiX-embryoids (Cer1$^+$/Eomes$^+$), 37 Day 5 EiTiX-embryoids (Dkk1$^+$/Eomes$^+$) and 38 Day 5 EiTiX-embryoids (Lefty1$^+$/Eomes$^+$). (**E**) Day 5 EiTiX-embryoids stained for Eomes (green), Oct4 (red) and Cer1 or Dkk1 or Lefty1 (cyan). Arrows indicate Dkk1- or Cer1- or Lefty1-positive domain while the box indicates Dkk1- or Cer1- or Lefty1- and Eomes- double positive domains. (**F**) Graph shows percentages of Day 5 EiTiX-embryoids with 1) AVE marker and Eomes expression, 2) asymmetric AVE marker expression, and 3) Eomes expression on the opposite side to AVE. n = 114 EiTiX-embryoids. (**G**) Day 6 EiTiX-embryoid stained to reveal T (red) and Foxa2 (green). Box indicates T- and Foxa2-double positive domain; white dotted lines, lumen of ES compartment. n = 15/16 EiTiX-embryoids with T- and Foxa2- double positive cells. (**H**) Day 6 EiTiX-embryoid stained to reveal Foxa2 (green) and Sox17 (red). Box, Foxa2- and Sox17- double positive domain; dotted lines, lumen of ES compartment. n = 15/17 EiTiX-embryoids with Foxa2- and Sox17-double positive cells. (**I**) Whole mount *in situ* hybridisation to reveal *Bmp4* in Day 5 and Day 6 EiTiX-embryoids. n = 9/10 Day 5 EiTiX-embryoids and 13/15 Day 6 EiTiX-embryoids. All experiments were performed minimum 2 times. Scale bars: 50μm, 15μm (zoomed).

Figure S3 (Related to Figure 3)



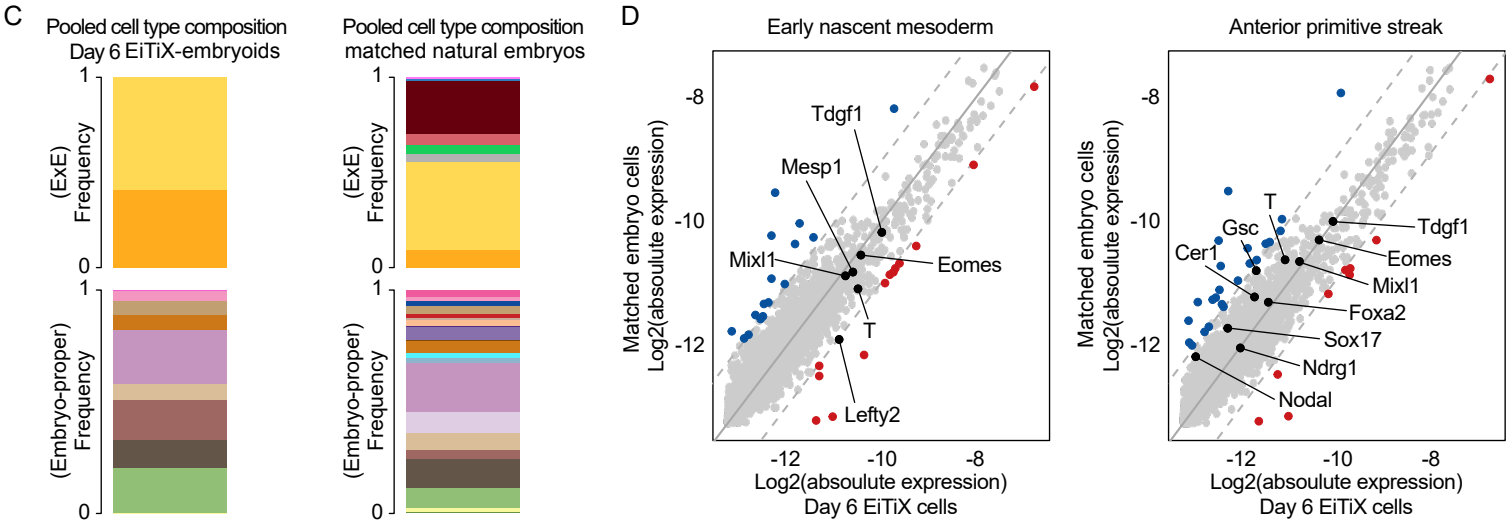


Figure S3. Day 6 EiTiX-embryoids capture major cell types of gastrulation, related to Figure 3.

(**A**) Developmental time (E_t) over embryo rank, annotated by age group (in $1E_t$ - $1.5E_t$ intervals, legend in Fig. 3C). (**B**) Day 6 and 8 EiTiX combined manifold, single cells (small dots) and Metacells (big dots) annotated by cell state (legend below). Right panels show manifold of matched natural embryos and projection of pooled Day 6 EiTiX-embryoids on the manifold. (**C**) Pooled ExE (top) and embryonic (bottom) cell-state frequencies of Day 6 EiTiX-embryoids (left panel) and time-matched natural embryos (right panel). (**C**) Bulk differential gene expression per cell state of Day 6 EiTiX cells against matched embryo cells; early nascent mesoderm (left) and anterior primitive streak (right). Dots represent individual genes. Colour annotated dots mark genes with a two-fold change in expression (blue – above two-fold decrease in Day 6 EiTiX cells, red – above two-fold increase in Day 6 EiTiX cells).

Figure S4 (related to Figure 4)

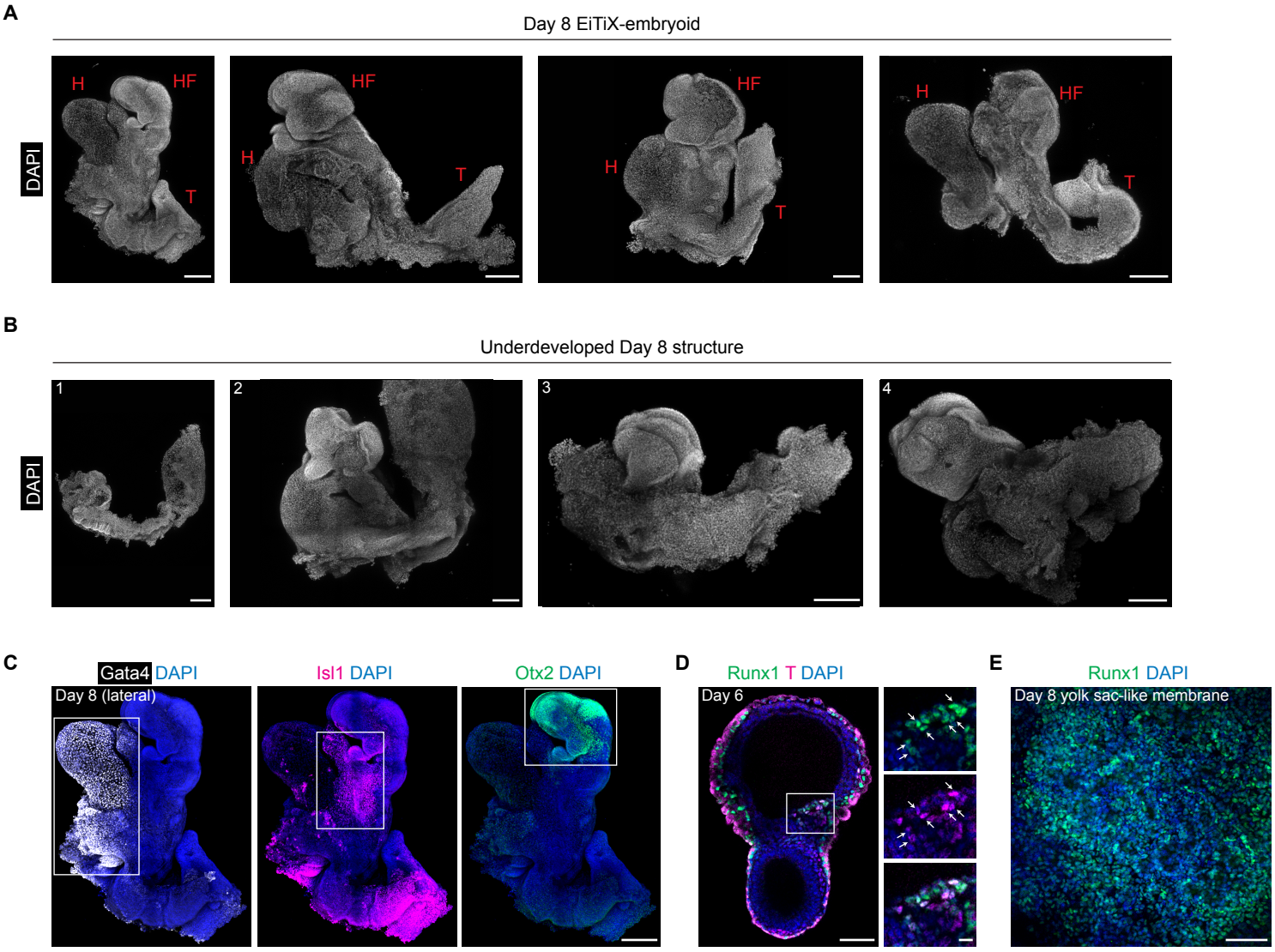


Figure S4. EiTiX-embryoids develop to late headfold stages with heart and chorion development, related to Figure 4.

Examples of DAPI stained Day 8 EiTIX-embryoids (**A**) and underdeveloped Day 8 structures (**B**). Underdeveloped Day 8 structures showed stunted overall development (1) or impaired axial elongation to generate posterior structures (2-4). H: heart, HF: headfolds, T: tail. Scale bar: 200μm. (**C**) Lateral view of Day 8 EiTiX-embryoid stained to reveal heart marker Gata4 (white), pharyngeal mesoderm marker Isl1 (magenta), and forebrain marker Otx2 (green). Scale bar, 200μm. (**D**) Day 6 EiTiX-embryoid stained to reveal Runx1 (green) and T (magenta). Arrow, Runx1- and T- double positive cells. n = 3/4 EiTiX-embryoids with Runx1- and T- double positive cells. Scale bar: 100μm, 20μm (zoomed). (**E**) Dissected yolk sac-like membrane from Day 8 EiTiX-embryoid stained to reveal Runx1 (green). n = 6/6 EiTiX-embryoids with Runx1 expression. Scale bar, 100μm.

Figure S5 (Related to Figure 5)

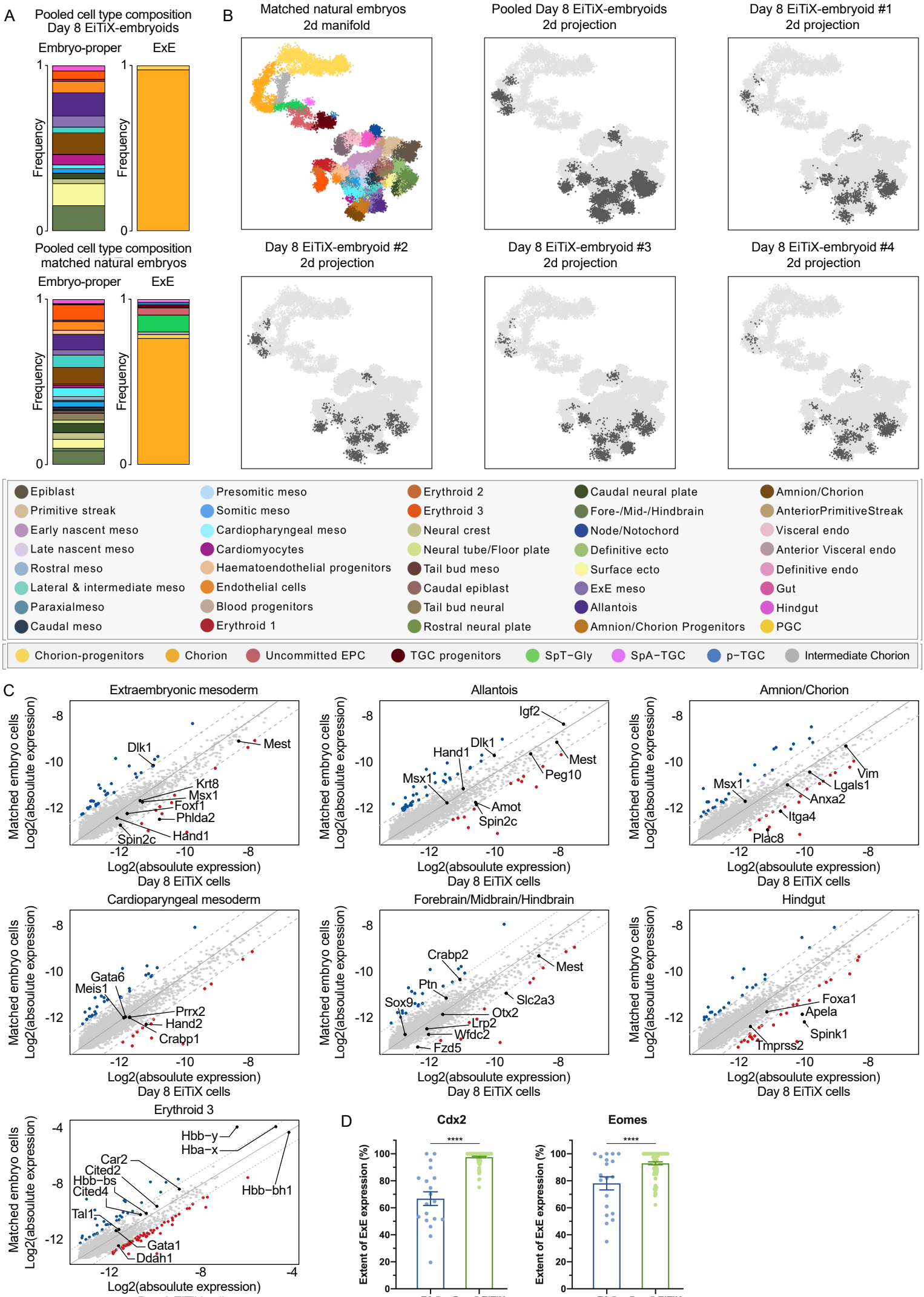


Figure S5. Cell state and composition analysis of neurulating embryoids using scRNA-seq, related to Figure 5.

(**A**) Pooled ExE (left bar) and embryonic (right bar) cell-state frequencies of Day 8 EiTiX structures (left panel) and time-matched natural embryos (right panel, annotated according to the legend below). (**B**) Manifold of matched natural embryos and projection of individual Day 8 EiTiX-embryoids on the manifold. (**C**) Bulk differential gene expression per cell state of Day 8 EiTiX cells against matched embryo cells (extraembryonic mesoderm, allantois, amnion/chorion, cardiopharyngeal mesoderm, forebrain/midbrain/hindbrain, hindgut and erythroid 3). Dots represent individual genes. Colour annotated dots mark genes with a two-fold change in expression (blue – above two-fold decrease in Day 8 EiTiX cells, red – above two-fold increase in Day 8 EiTiX cells). (**D**) Quantification of the extent of ExE expression Cdx2 and Eomes in E6.5 embryos and Day 5 EiTiX-embryoids. n = 19 E6.5 embryos from 2 experiments and 78 Day 5 EiTiX-embryoids from 3 experiments; ****$p < 0.0001$.