

Supplementary Information

Time Space and Single-Cell Resolved Tissue Lineage Trajectories and Laterality of Body Plan at Gastrulation

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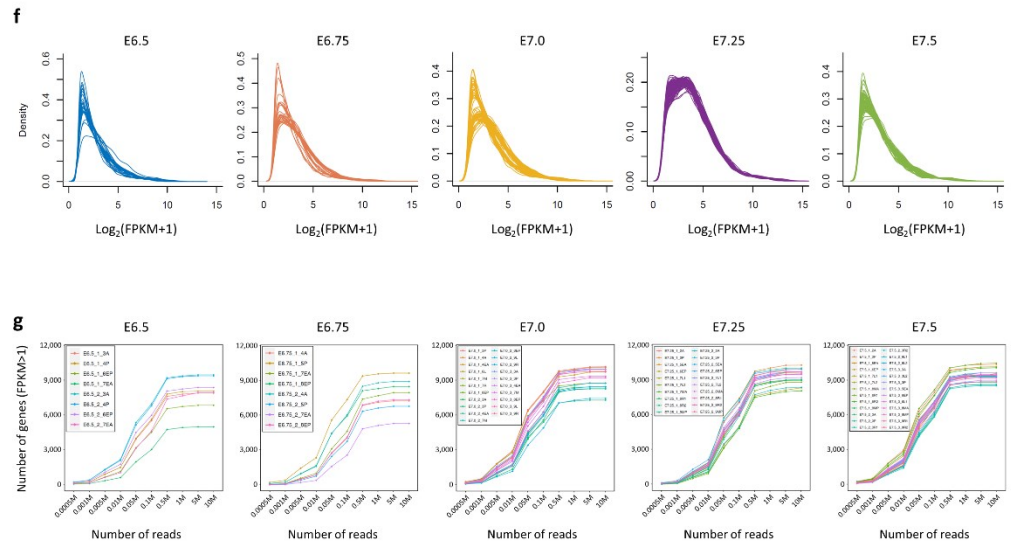
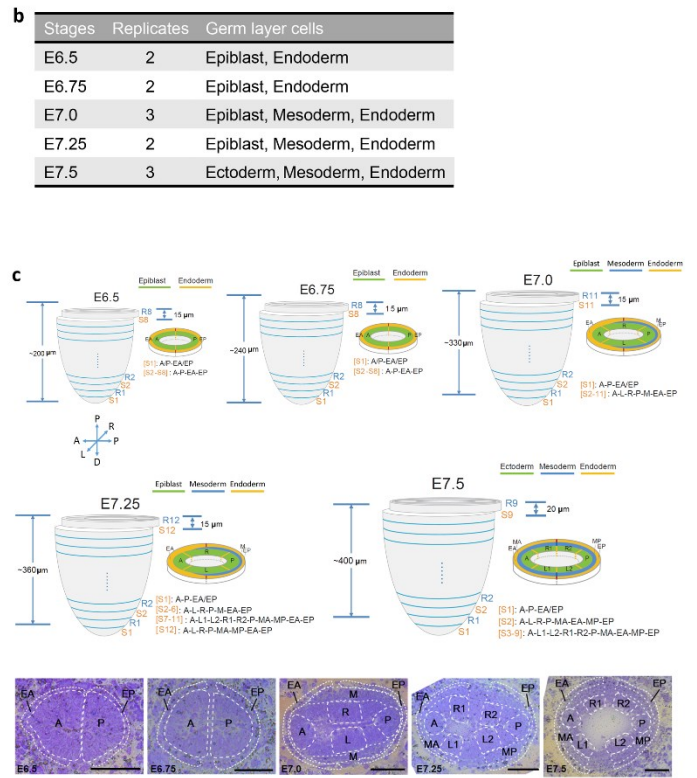
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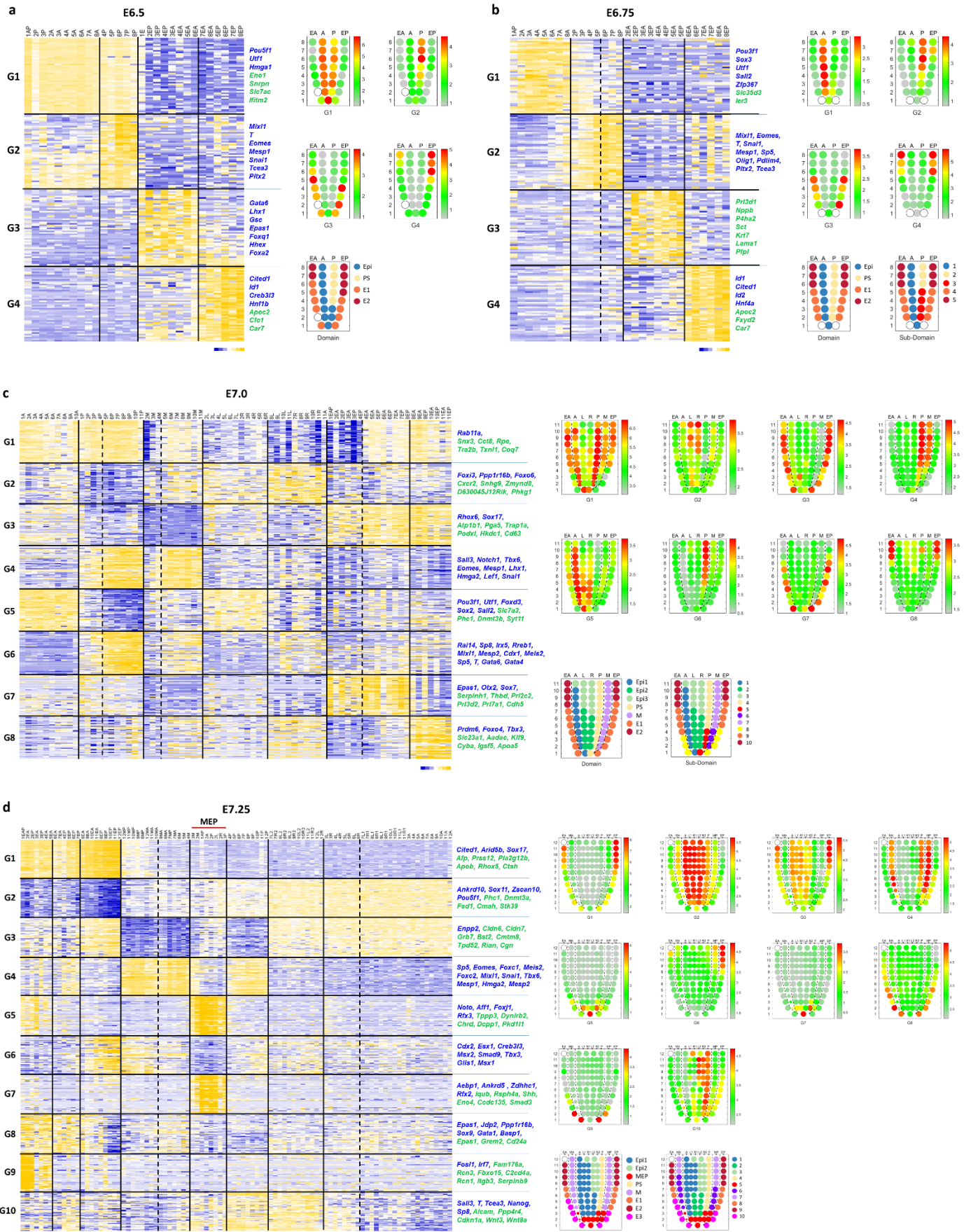
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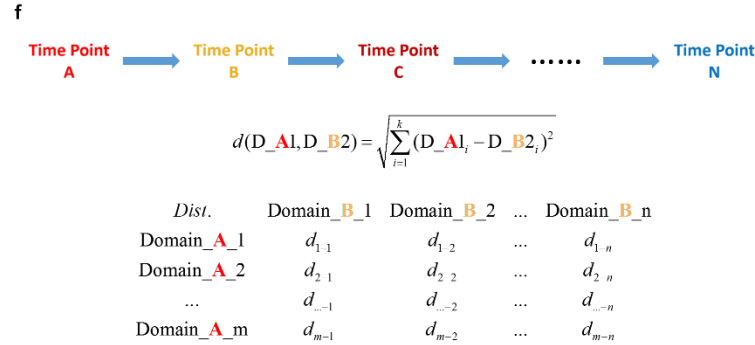
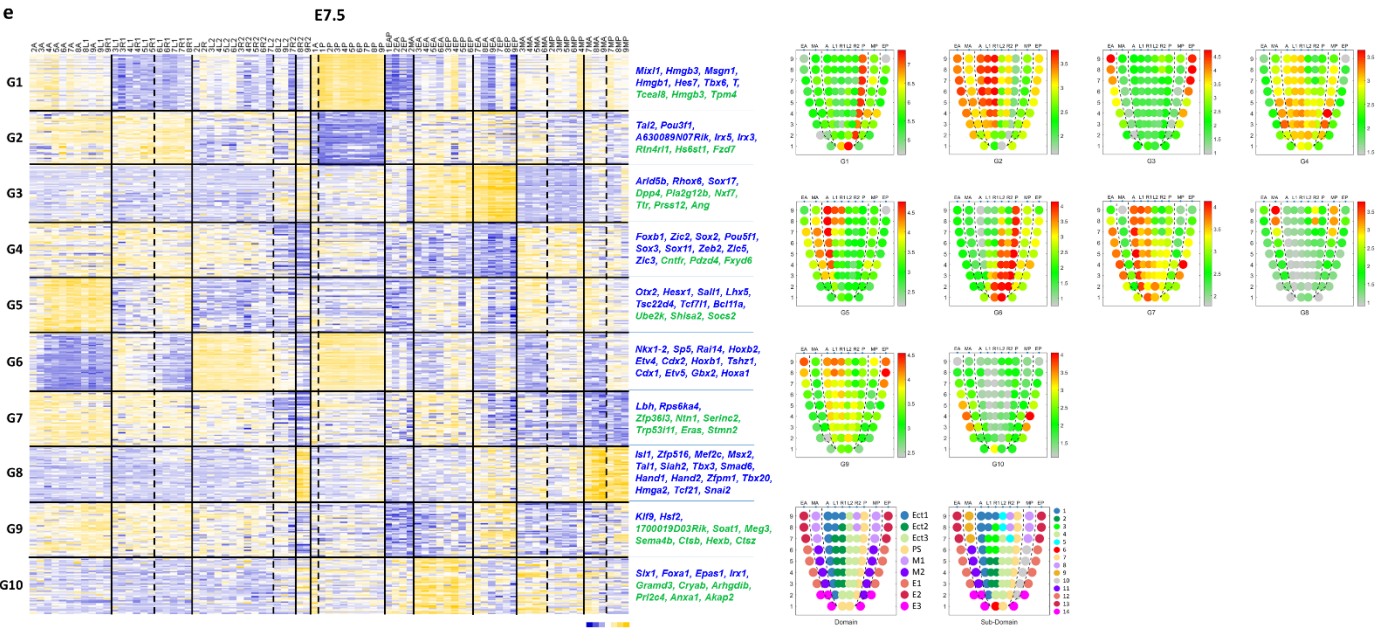
Supplementary Figure 1. Geo-seq analysis.

- a. Schematics of laser capture microdissection of cell samples in E6.5-E7.5 embryos. A, anterior; P, posterior; L, left lateral; R, right lateral; L1, anterior left lateral; R1, anterior right lateral; L2, posterior left lateral; R2, posterior right lateral; M, mesoderm; MA, anterior mesoderm; MP, posterior mesoderm; EA, anterior endoderm; EP, posterior endoderm.
- b. Samples for Geo-seq: embryonic stages, biological replicates and the germ layer cells. Stages: E6.5-E6.75, early-streak stage; E7.0-E7.25, mid- to late-streak stage; E7.5, late-streak to no-bud stage.
- c. The strategy of sampling of cell populations in the epiblast/ectoderm and endoderm in E6.5 to E7.5 embryos and mesoderm in E7.0 to E7.5 embryos (areas of sampling shown in histology images). Samples were designated in ascending order of serial sections (1 = the most distal section) and the regions in the section (R, reference section; S, sample section). Embryonic axes: anterior – posterior, A ↔ P; proximal – distal, P ↔ D; left – right, L ↔ R. Scale bar, 50 μ m.
- d. Corn plots showing the spatio-temporal pattern of expression of *T* and *Otx2*. *T* expression domain marks the length of the primitive streak for staging the development of the embryo; *Otx2* is a representative ectoderm marker.
- e. Box plot showing the number of detected genes (FPKM > 0) in samples of E6.5-E7.5 embryos (1 biological replicate for E6.5, E6.75 and E7.25 embryos; 2 biological replicates for E7.0 and E7.5 embryos). The center line marks the median and box edges represent 25th and 75th percentiles. The median of genes detected in reference embryo per stage is 11,033 (E6.5), 10,150 (E6.75), 12,340 (E7.0), 12,549 (E7.25) and 14,246 (E7.5).
- f. Gene expression density plot of Geo-seq data of samples of E6.5-E7.5 embryos. The X-axis of the density plots is harmonized to the same scale.
- g. Saturation analysis for reads for samples from each development stage. Different numbers of reads were selected, and the number of detected genes was plotted.

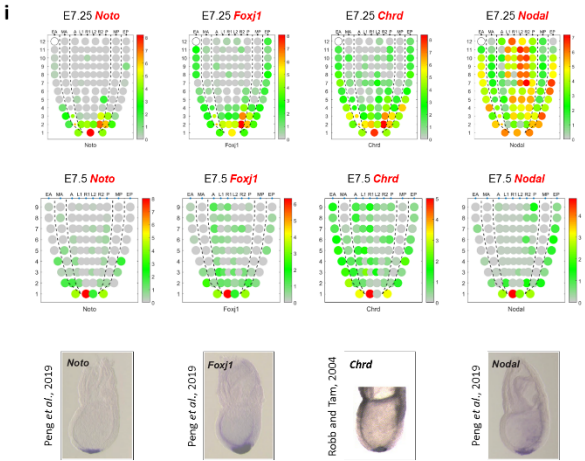
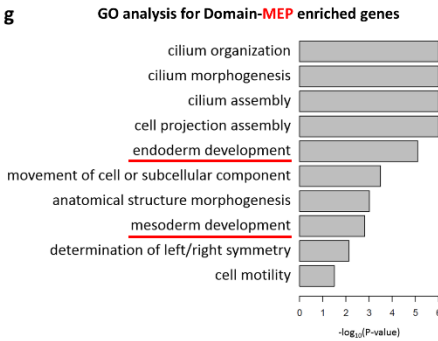
Panel 1



Panel 2



Consecutive operations to identify the downstream developmental domains.



Supplementary Figure 2. The spatial domains in the germ layers of E6.5-E7.5 embryos delimited by the expression profile of zipcode genes.

- a-e.** Heat maps and corn plots of zipcode groups in E6.5 (**a**), E6.75 (**b**), E7.0 (**c**), E7.25 (**d**) and E7.5 (**e**) embryos. Transcription factors (blue marked) and top DEGs (green marked) of each gene group were listed on the right side of heat map. G, group.
- f.** Population Tracing algorithm. The Euclidean distance of any two domains in embryos of successive stages were computed, following by delineating the mutual nearest neighbors of each domain from one stage against the domains of embryo at the successive stage. D_A1 denotes domain 1 at time point A, D_B2 denotes domain 2 at time point B.
- g.** Functional ontology of enriched genes associated with the mesendoderm progenitors (MEP).
- h.** Corn plots showing representative genes associated with the MEP.
- i.** Expression pattern of MEP- and node-related genes in E7.25 and E7.5 embryo. The spatial transcriptome (corn plot) data of *Noto*, *Foxj1*, *Chrd* and *Nodal* were verified with reference to whole mount in situ hybridization (WISH) data. WISH images of *Noto*, *Foxj1* and *Nodal* from Peng *et al.* (2019), and *Chrd* from Robb and Tam (2004).

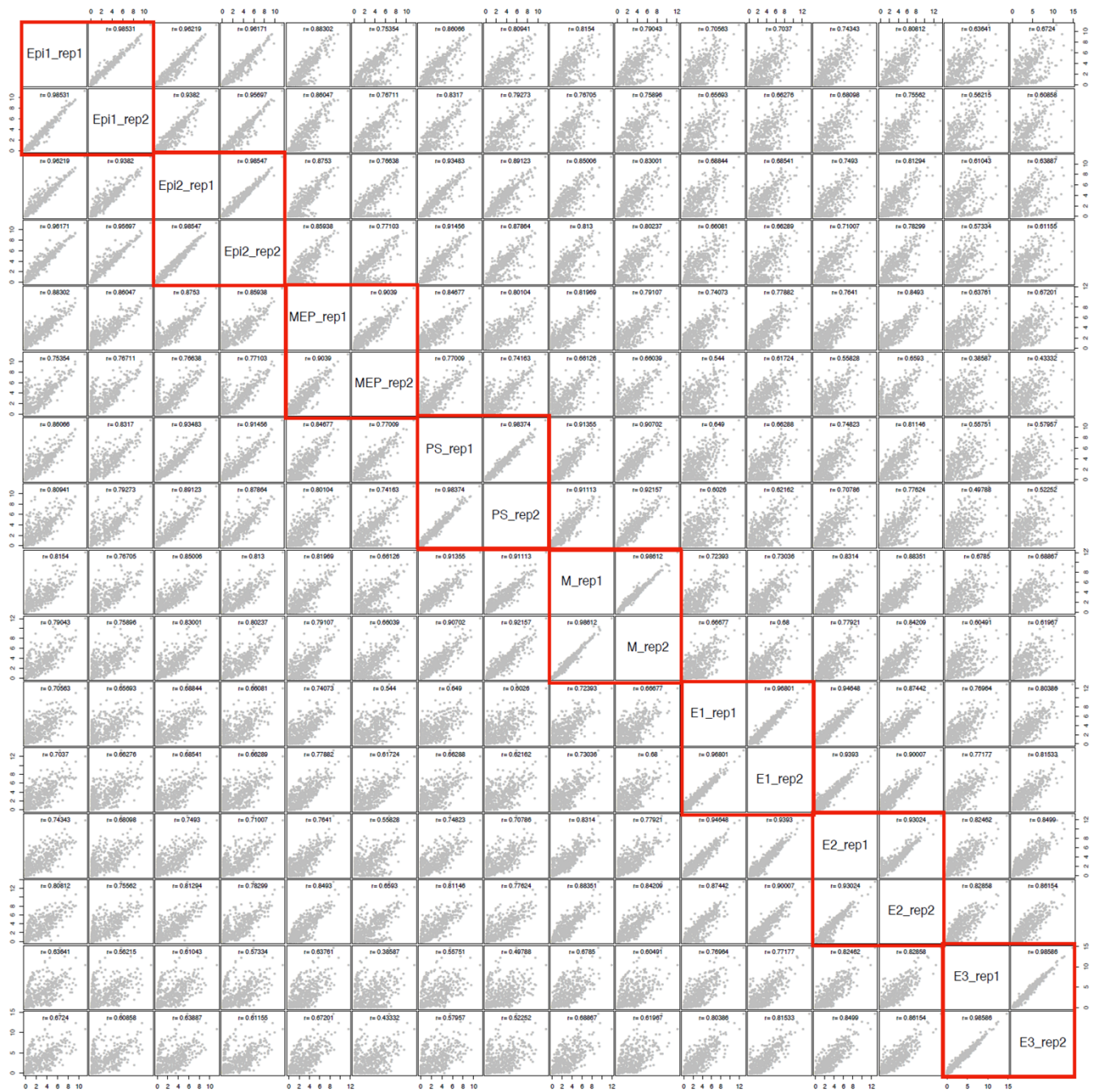
Panel 1



Panel 2

d

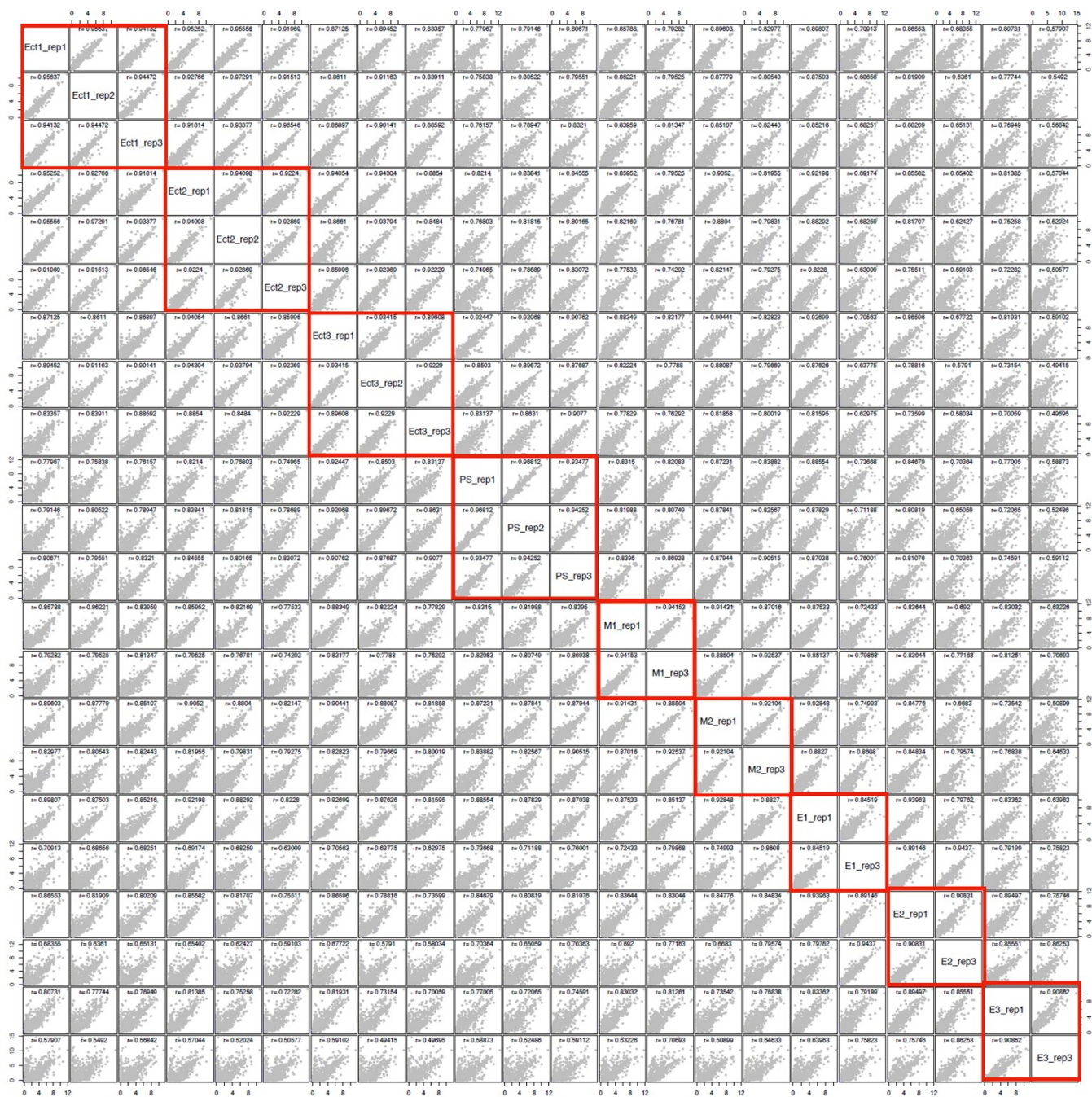
E7.25



Panel 3

e

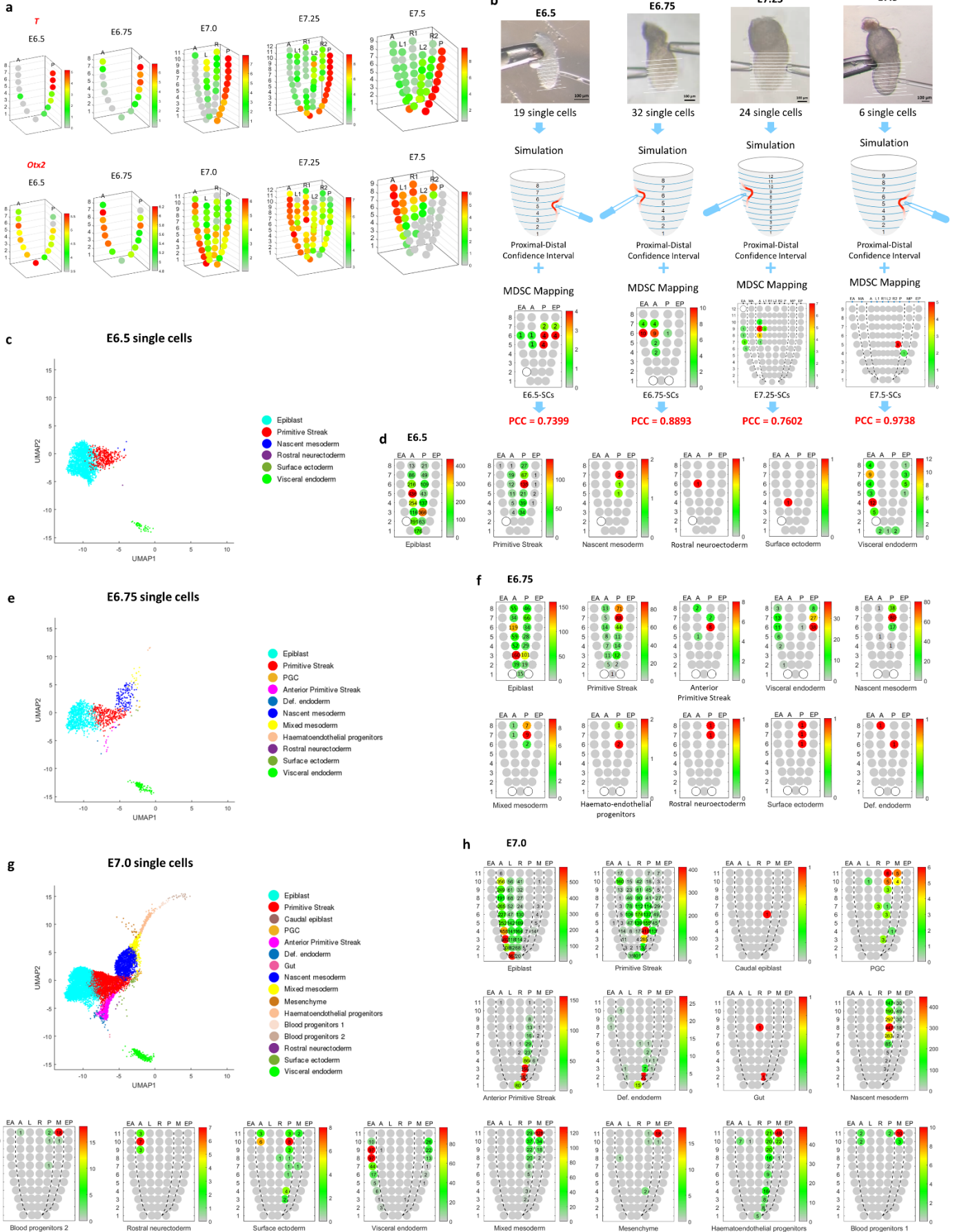
E7.5



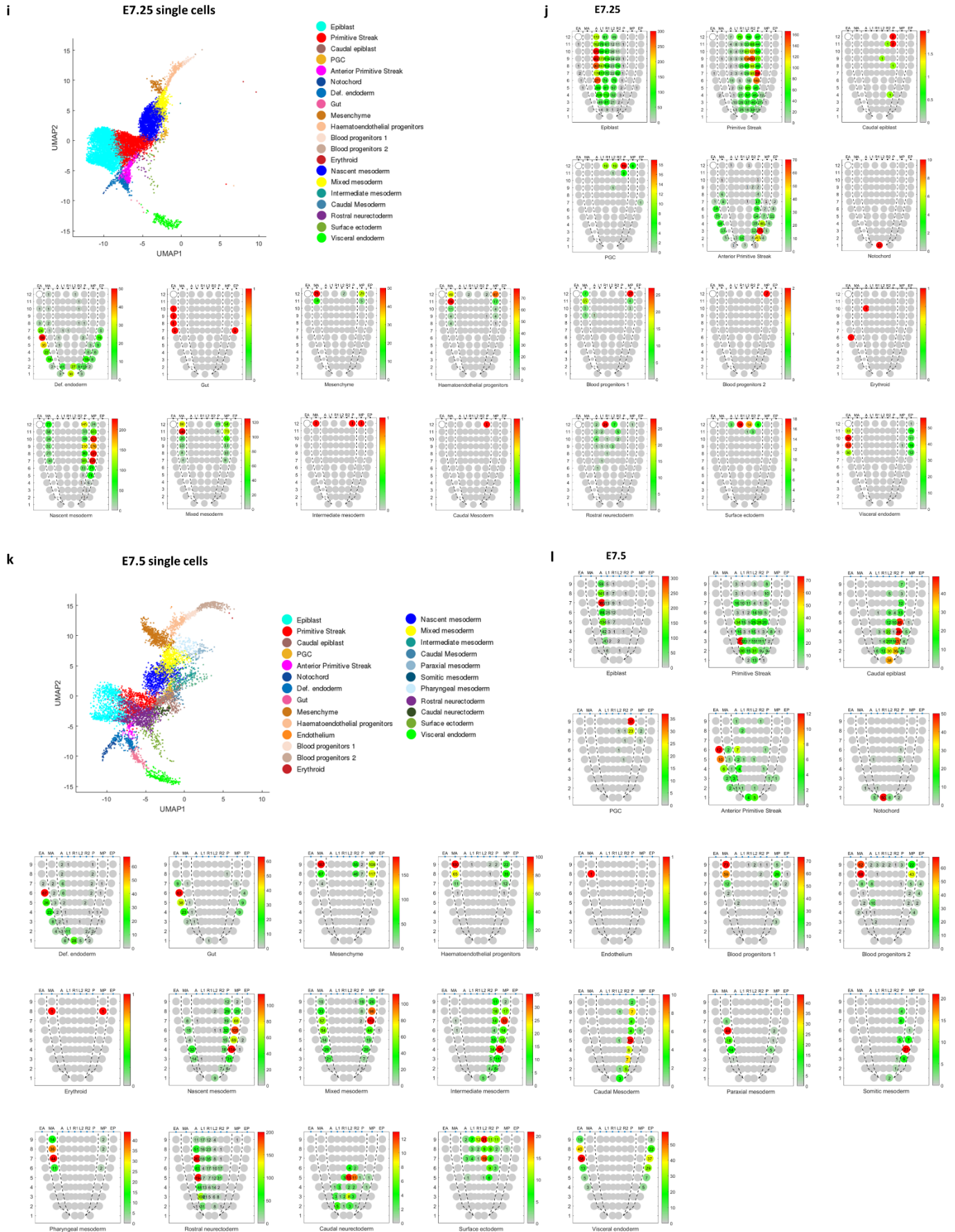
Supplementary Figure 3. Inter-embryo correlation of spatial transcriptome data.

a-e. The Pearson Correlation Coefficient (PCC) of spatial domains between biological replicates at E6.5 (**a**), E6.75 (**b**), E7.0 (**c**), E7.25 (**d**) and E7.5 (**e**). For E7.0 replicate 2 and E7.5 replicate 2, only epiblast/ectoderm domains were assessed.

Panel 1



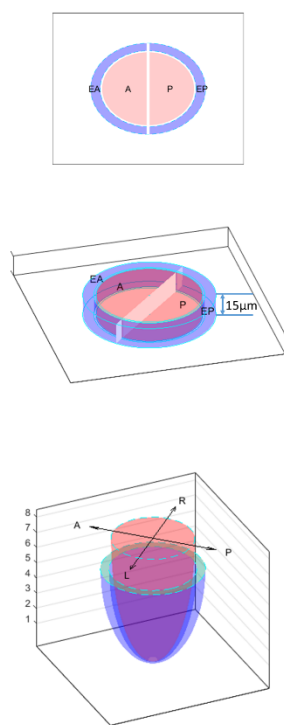
Panel 2



Supplementary Figure 4. 3D model for displaying transcriptome data and the verification of MDSC Mapping results.

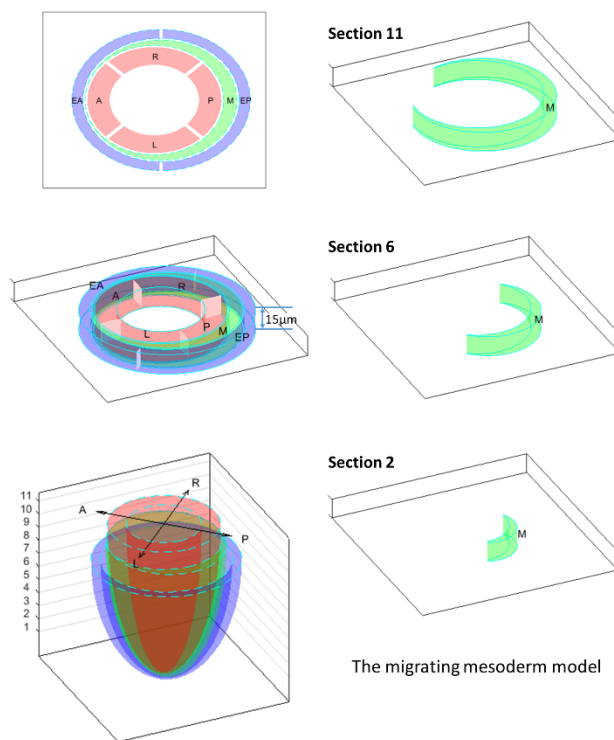
- a.** 3D corn plots showing the spatio-temporal pattern of expression of *T* and *Otx2* in epiblast/ectoderm layer of E6.5-E7.5 embryos.
- b.** Verification of the results of MDSC Mapping of single cells isolated from known positions of E6.5, E6.75, E7.25 and E7.5 embryos. The number on each corn indicates the number of cells mapped to the specific position in the germ layers. PCC values and confidence intervals shown in the simulation.
- c-l.** Uniform manifold approximation and projection (UMAP) plots (**c, e, g, i, k**) showing the data structure of single cells identified in the ‘Gastrulation Atlas’ and MDSC Mapping results (**d, f, h, j, l**) for E6.5 (**c, d**), E6.75 (**e, f**), E7.0 (**g, h**), E7.25 (**i, j**) and E7.5 (**k, l**) embryos. Cell types are annotated (legend of UMAP) and the spatial distribution of each annotated cell types is displayed in corn plots, with the number of cells mapped to specific Geo-seq position (number in the corn) shown.

a E6.5/E6.75 Spatial Model



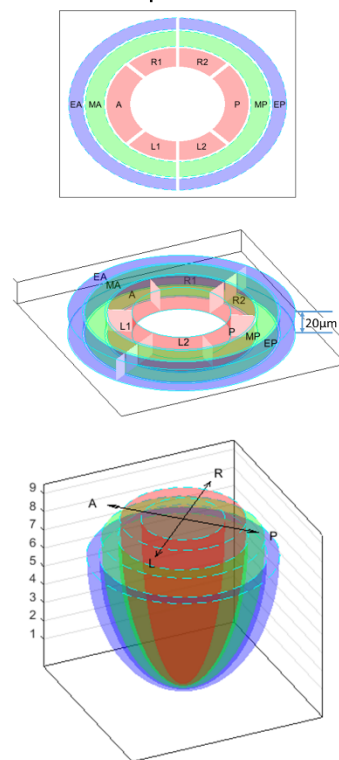
Epiblast
VE

b E7.0/E7.25 Spatial Model

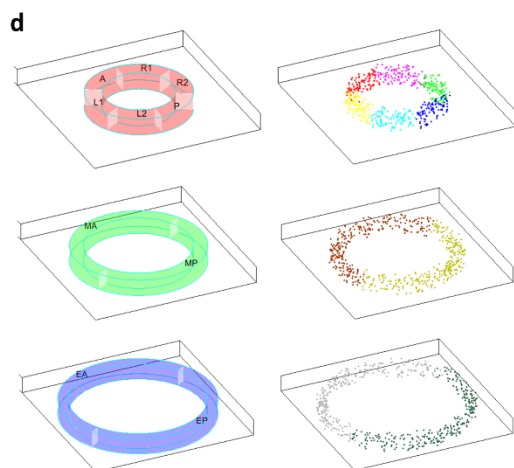


Epiblast
Mesoderm
Endoderm

c E7.5 Spatial Model



Ectoderm
Mesoderm
Endoderm

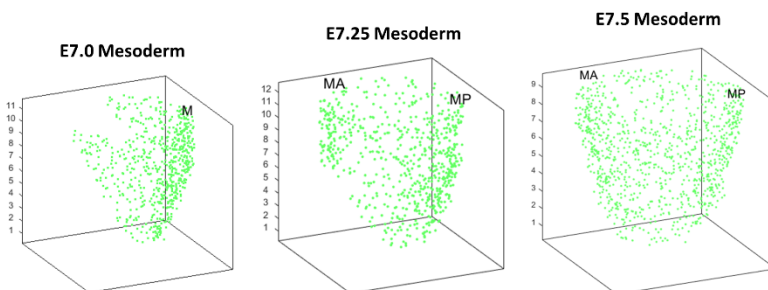


Ect
• A SCs
• P SCs
• L1 SCs
• R1 SCs
• L2 SCs
• R2 SCs

Mes
• MA SCs
• MP SCs
End
• EA SCs
• EP SCs

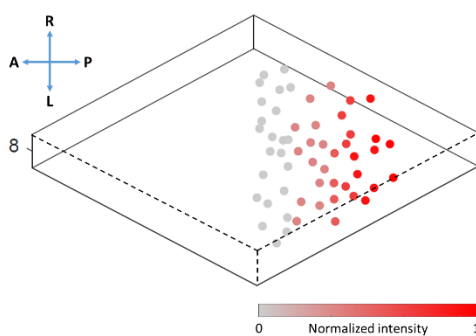
Ectoderm
Mesoderm
Endoderm

e



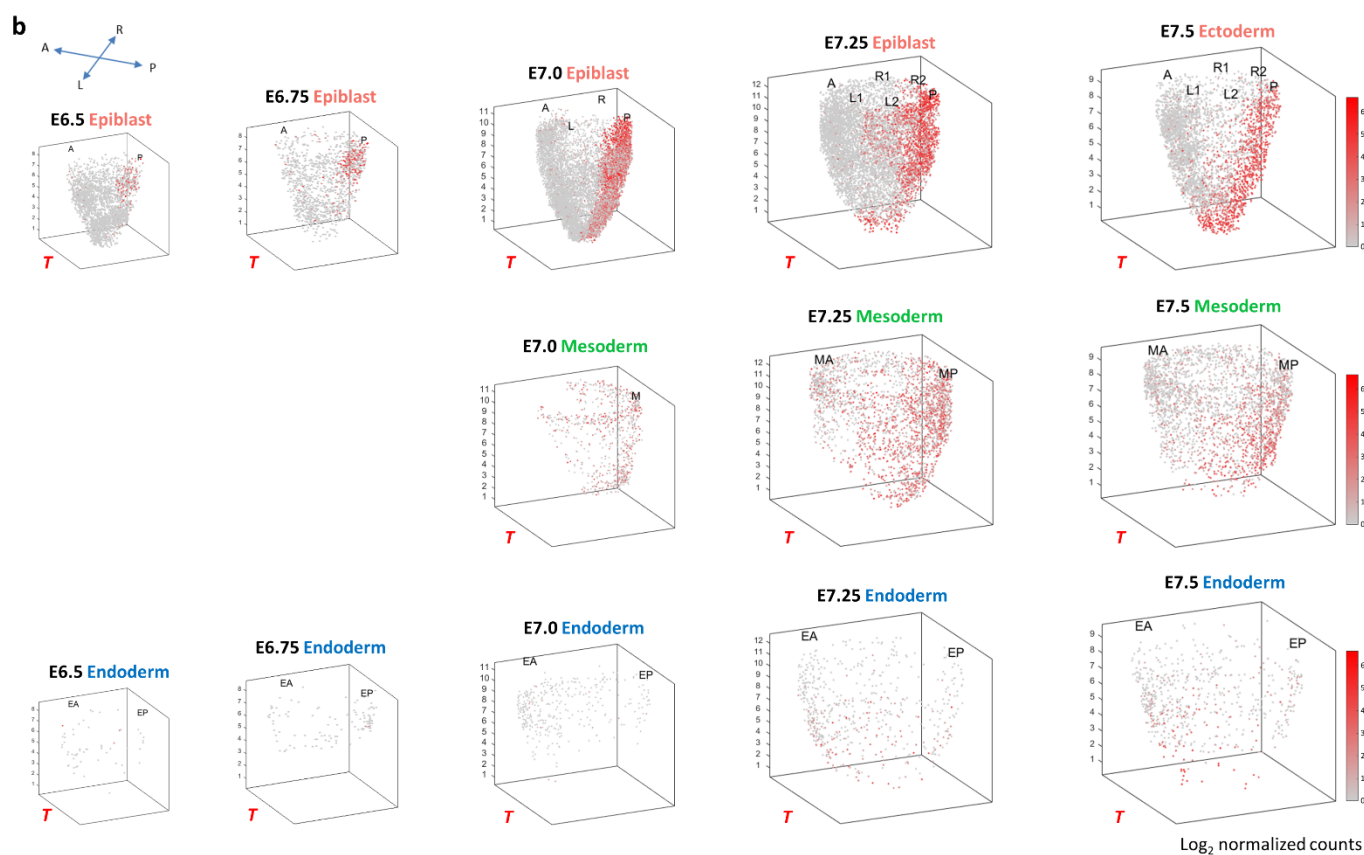
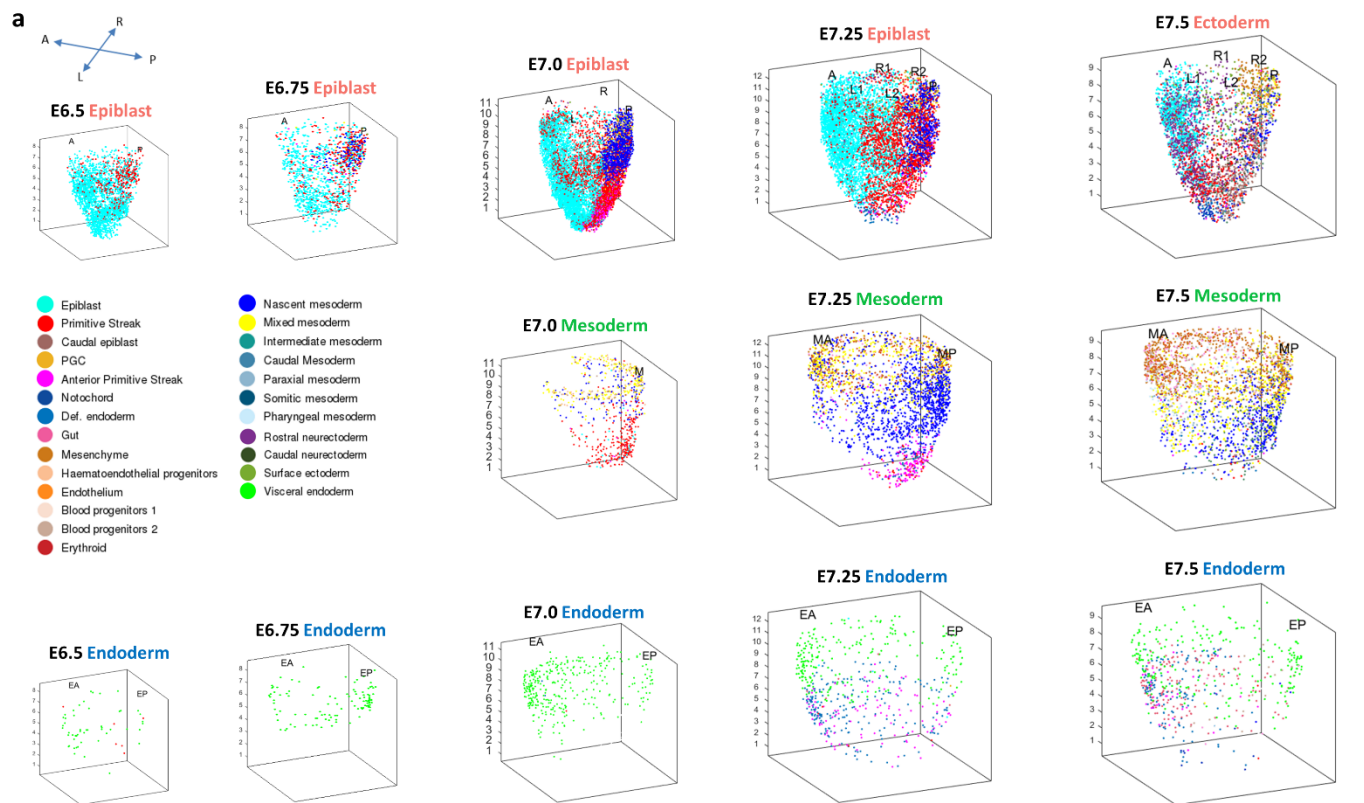
f

Single cells within position-8P at E6.5: **Wnt** signaling intensity



Supplementary Figure 5. 3D modeling for single-cell resolution embryo map.

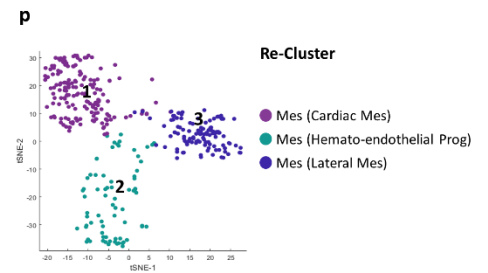
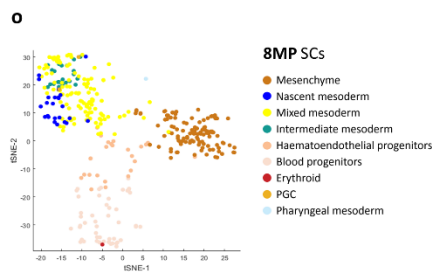
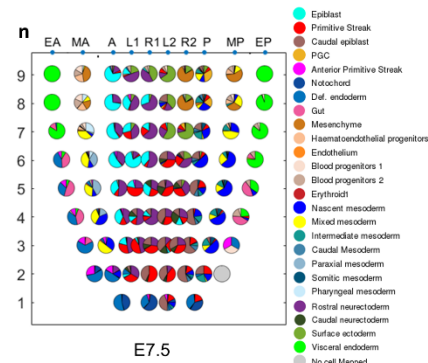
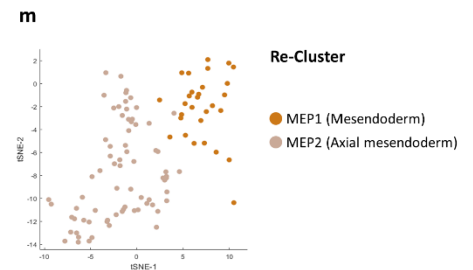
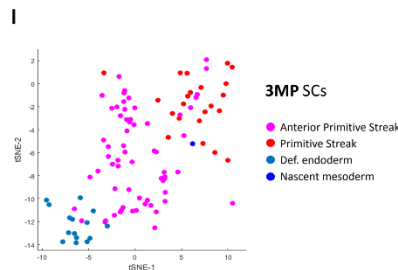
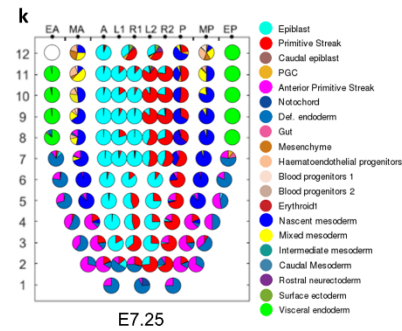
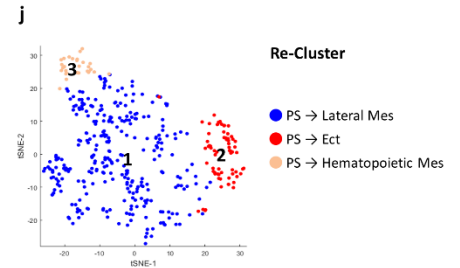
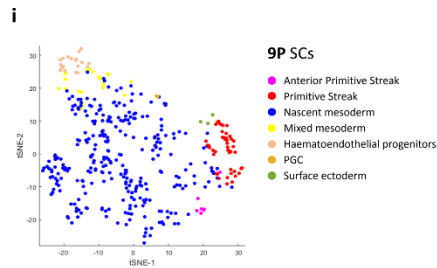
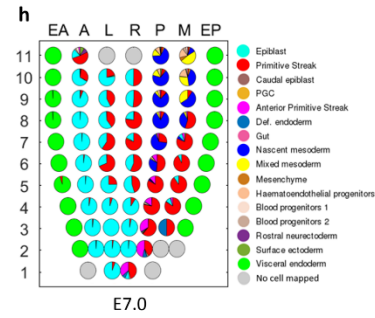
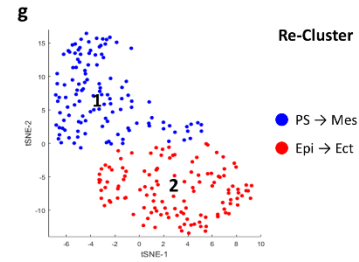
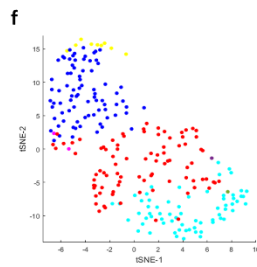
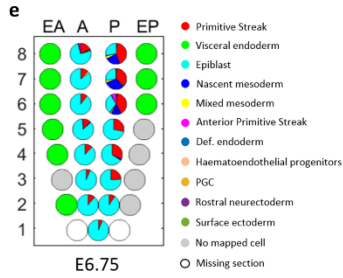
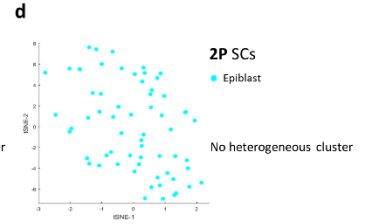
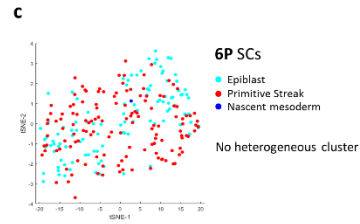
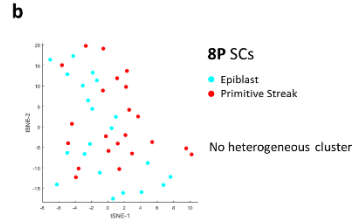
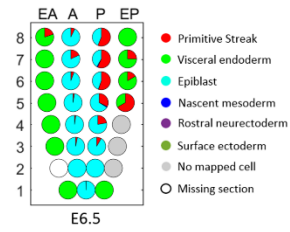
- a.** The 3D model of E6.5 and E6.75 embryos. Based on Geo-seq sampling strategy, semicircular pattern for the epiblast and Annulus Model for the endoderm were devised to display the spatial distribution of single cells. Section thickness, 15 μm .
- b.** The Annulus Model of the epiblast and endoderm of E7.0 and E7.25 embryos. At E7.0-E7.25, the Migration Model mirrors the mesoderm layer spanning from posterior to anterior of the embryo. Section thickness, 15 μm .
- c.** The Annulus Model of E7.5 embryo. Concentric annuli represent the ectoderm, mesoderm and endoderm from the inside outward. Section thickness, 20 μm .
- d.** The display of single cells in the three germ layers of E7.5 embryo. Single cells that mapped to a Geo-seq position were distributed uniformly across the corresponding interior space of each domain in the annulus section.
- e.** The display of single cells in the Migration Model of the mesoderm of E7.0-E7.5 embryo.
- f.** Bubble Sort algorithm re-ordered the distribution pattern of single cells in E6.5 position-8P by graded WNT activity in the anterior-posterior axis. The color legend indicates the normalized expression level determined by averaged transcript counts.



Supplementary Figure 6. The spatio-temporal distribution of single cells in E6.5-E7.5 mouse embryos.

- a.** The spatio-temporal distribution of all the single cells identified in the ‘Gastrulation Atlas’ in the epiblast/ectoderm, mesoderm and endoderm of E6.5-E7.5 mouse embryos.
- b.** The spatio-temporal distribution of *T*-expressing cells in E6.5-E7.5 embryos. The color legend indicates the level of expression determined by transcript counts.

a Annotated by 'Gastrulation Atlas'



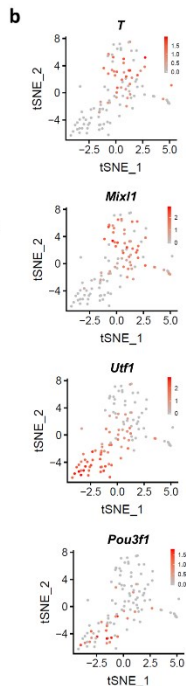
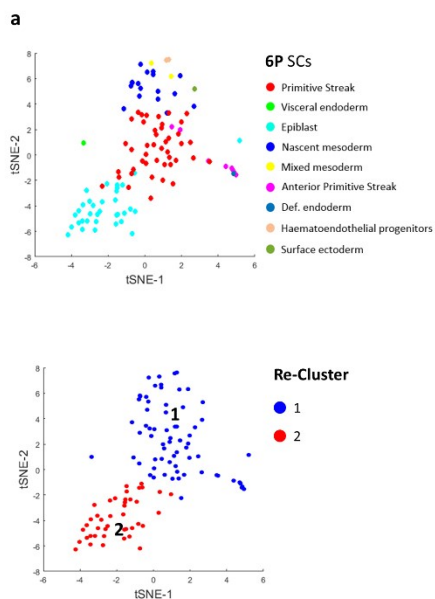
Supplementary Figure 7. Analysis of heterogeneity of single cell population.

a-p. Heterogeneity Map of single cell types annotated by ‘Gastrulation Atlas’ displayed as pie charts in corn plots (**a, e, h, k, n**) and *t*-SNE plots of single cells (**b, c, d, f, g, i, j, l, m, o, p**) showing examples of heterogeneity of cell types (with revised annotation according to **Supplementary Fig. 8**) in specified Geo-seq positions of E6.5 (**a-d**), E6.75 (**e-g**), E7.0 (**h-j**), E7.25 (**k-m**) and E7.5 (**n-p**) embryos.

Stage	Gastrulation Atlas (Pijuan-Sala, B. <i>et al.</i> , 2019)	Geo-seq (Figure 1a)	Heterogeneity Map (Figure 3d, e)	Cell label
E6.5	Epiblast	Epi	Epi	Epiblast
	Primitive Streak	PS	PSLC	Primitive streak like cells
	Visceral endoderm	E1	E1 (Non-proximal Ve)	Non-proximal visceral endoderm
		E2	E2 (Proximal Ve)	Proximal visceral endoderm
E6.75	Epiblast	Epi	Epi	Epiblast
	Primitive streak	PS	Epi → Ect	Posterior epiblast: ectoderm progenitor
	PSLC		Primitive streak like cells	
	Nascent mesoderm	PS → Mes	Primitive streak: mesoderm progenitor	
	Visceral endoderm	E1	E1 (Non-proximal Ve)	Non-proximal visceral endoderm
		E2	E2 (Proximal Ve)	Proximal visceral endoderm
E7.0	Epiblast	Epi1	Epi → Neuroectoderm	Epiblast: neuroectoderm progenitor
		Epi2	Epi → Ect	Epiblast: ectoderm progenitor
		Epi3	Epi → SEct/AmEct	Epiblast: surface ectoderm, amnion ectoderm progenitor
	Primitive streak	PS	PS → Ect	Primitive streak: ectoderm progenitor
			PS → Lateral Mes	Primitive streak: lateral mesoderm progenitor
			PS → Hematopoietic Mes	Primitive streak: hematopoietic mesoderm progenitor
	PS → Paraxial Mes Prog		Primitive streak: paraxial mesoderm progenitor	
	PS (Anterior PS)		Primitive streak: anterior-streak like cells	
	PS → Endoderm		Primitive streak: endoderm progenitor	
	Nascent mesoderm	M	Mes (Mesoderm Prog)	Mesoderm progenitor
	Mes → Axial mesendoderm		Axial mesendoderm progenitor	
	Mixed mesoderm		Mes (Cardiogenic Mes)	Cardiogenic mesoderm
	Haematoendothelial progenitors		Mes (Paraxial Mes)	Paraxial mesoderm
		Mes (Vasculogenic Mes)	Vasculogenic Mesoderm	
Visceral endoderm	E1	E1 (Non-proximal Ve)	Non-proximal visceral endoderm	
	E2	E2 (Proximal Ve)	Proximal visceral endoderm	
E7.25	Epiblast	Epi1	Epi (Neuroectoderm Prog)	Epiblast: neuroectoderm progenitor
			Epi (Ectoderm Prog)	Epiblast: ectoderm progenitor
		Epi2	Epi (Posterior epiblast)	Epiblast: posterior epiblast
			Epi (Surface Ect Prog)	Epiblast: surface ectoderm progenitor
	Primitive streak	PS	PS → ExE Mes	Primitive streak: extraembryonic mesoderm progenitor
			PS → Mesoderm Prog	Primitive streak: mesoderm progenitor
			PS (PSLC)	Primitive streak: primitive streak like cells
			PS → Mesendoderm Prog	Primitive streak: mesendoderm progenitor
	Anterior primitive streak	MEP	MEP1 (Mesendoderm)	MEP1: mesendoderm
			MEP2 (Axial mesendoderm)	MEP2: axial mesendoderm
	Nascent mesoderm	M	Mes (Nascent Mes)	Nascent mesoderm
	Mixed mesoderm		Mes (Paraxial Mes)	Paraxial mesoderm
	Haematoendothelial progenitors		Mes (Hematopoietic Mes)	Hematopoietic mesoderm
	Mesenchyme		Mes (Cardiac Mes)	Cardiac mesoderm
	Visceral endoderm	E1	E1 (Non-proximal Ve)	Non-proximal visceral endoderm
		E2	E2 (Proximal Ve)	Proximal visceral endoderm
Def. endoderm	E3	E3 (Distal endoderm)	Distal endoderm	
E7.5	Rostral neuroectoderm	Ect1	Ect (Neuroectoderm)	Ectoderm: neuroectoderm progenitor
	Epiblast	Ect2	Ect (Epiblast)	Ectoderm: epiblast
	Caudal epiblast	Ect3	Ect (PSLC)	Ectoderm: primitive streak like cells
	Surface ectoderm		Ect (Surface Ect)	Ectoderm: surface ectoderm progenitor
	Mesenchyme		Ect (Lateral Mes Prog)	Ectoderm: lateral mesoderm progenitor
	Primitive streak	PS	PS → Lateral Mes	Primitive streak: lateral mesoderm progenitor
			PS → Paraxial Mes	Primitive streak: paraxial mesoderm progenitor
			PS → Mesoderm Prog	Primitive streak: mesoderm progenitor
			PS → Anterior Mes	Primitive streak: anterior mesoderm progenitor
			PS → Upper trunk Mes	Primitive streak: upper trunk mesoderm progenitor
	Node1 (Def endoderm)		Definitive endoderm	
	Notochord	Node2 (Axial mesendoderm)	Axial mesendoderm	
	Anterior primitive streak	M1	Mes (Hemato-endothelial Prog)	Hemato-endothelial progenitor
	Mes (Cardiac Mes)		Cardiac mesoderm	
	Haematoendothelial/Blood progenitors	M2	Mes (Lateral Mes)	Lateral mesoderm
	Mixed mesoderm		Mes (Paraxial Mes)	Paraxial mesoderm
	Intermediate mesoderm		Mes (Nascent Mes)	Nascent mesoderm
	Nascent mesoderm	E1	E1 (Non-proximal Ve)	Non-proximal visceral endoderm
	Visceral endoderm		E2 (Proximal Ve)	Proximal visceral endoderm
Def. endoderm	E3	E3 (Distal endoderm)	Distal endoderm	

Supplementary Figure 8. Re-annotation of cell types.

Revised annotation of single cell types mapped to the Geo-seq position in the epiblast/ectoderm, primitive streak, mesoderm and endoderm. Annotation of cell types in 'Gastrulation Atlas' is shown for comparison. The nomenclature 'X→Y' and 'X(Y)' represent different cell states. X represents the germ layer information of cell population. 'X→Y' indicates these cells are representing a transitional cell state from X to Y. And 'X(Y)' represents the precursor of a specified cell type (Y) in the germ layer X.



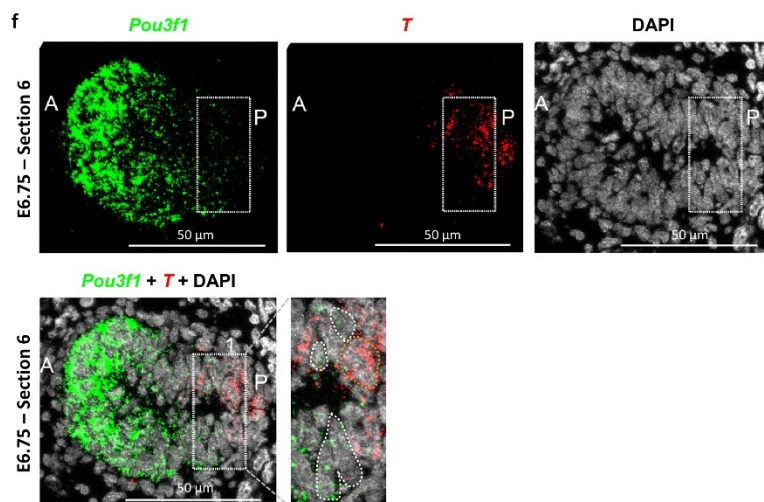
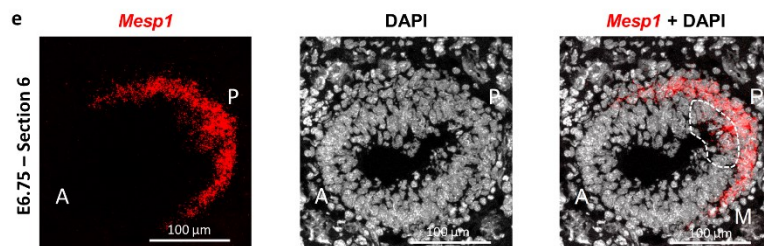
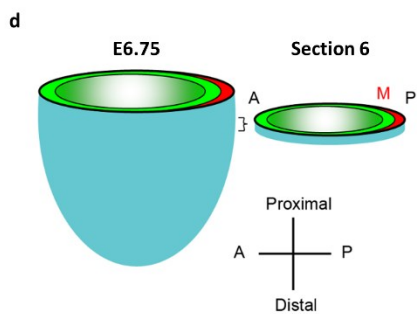
GO Terms

signal transduction involved in gene expression
multicellular organism development
gastrulation
anterior/posterior pattern specification
heart development
regulation of transcription
positive regulation of cell proliferation
mesoderm formation
cell differentiation
mesodermal cell migration

PS → Mes

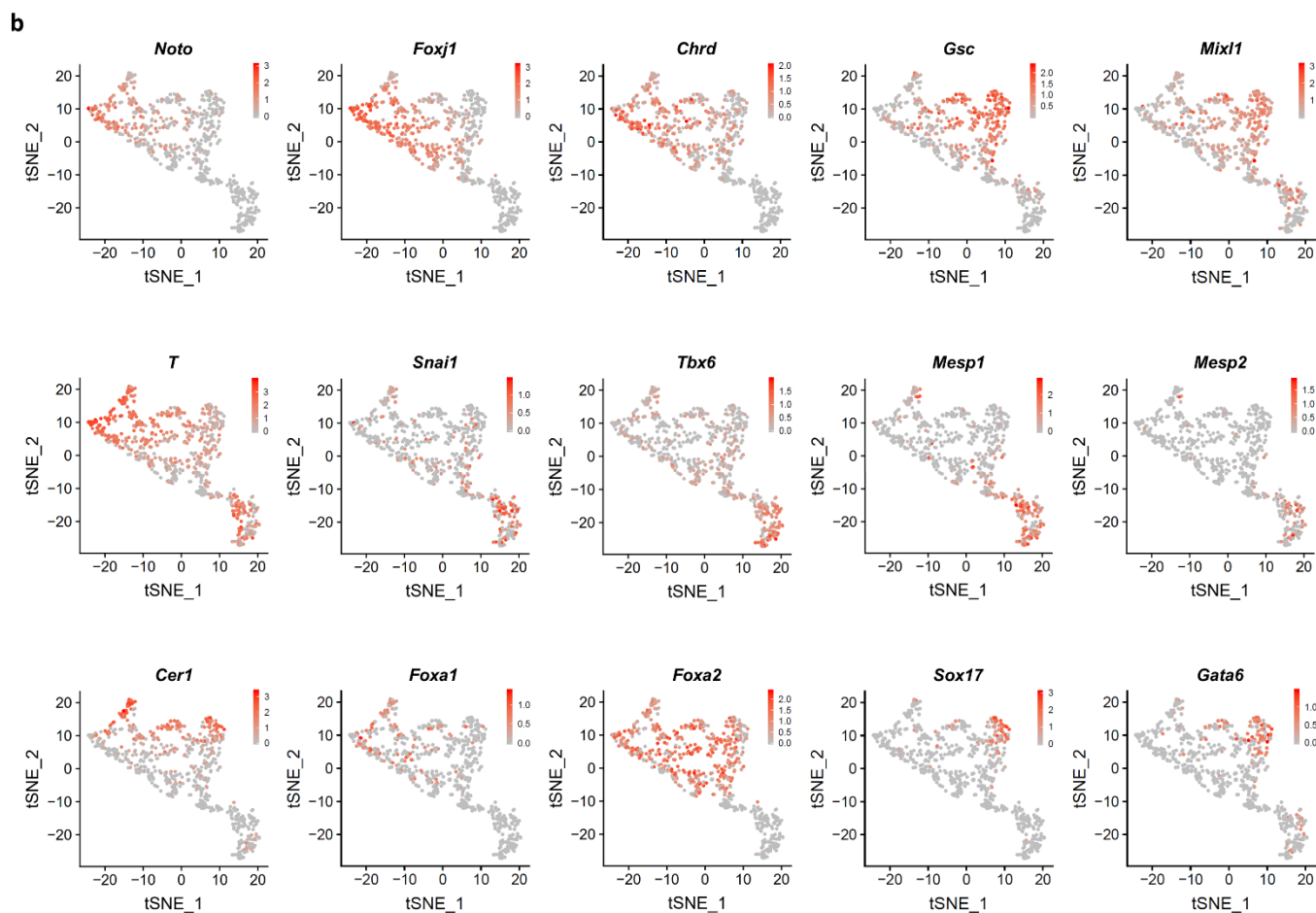
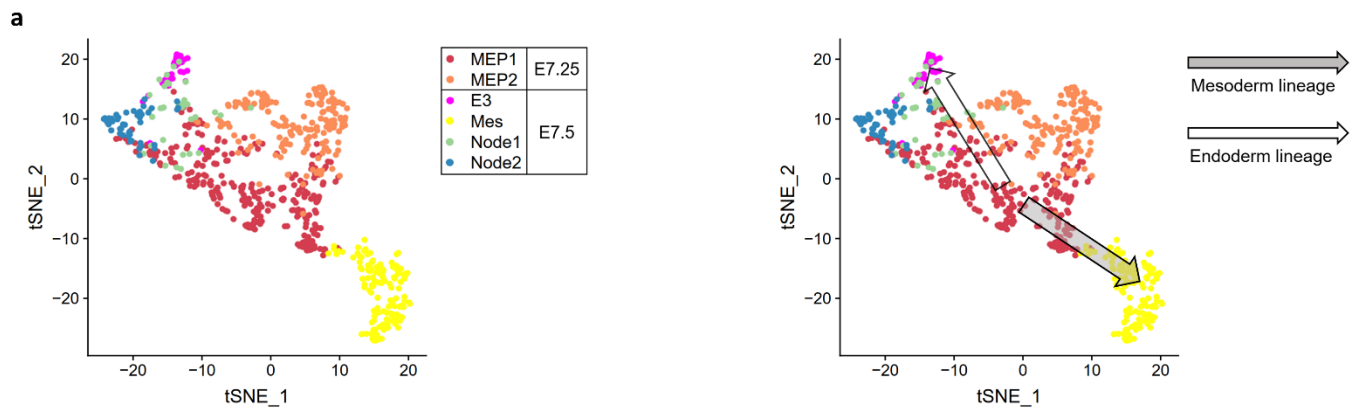
DNA methylation
DNA methylation during embryonic development
chromatin silencing
DNA modification
regulation of transcription

Epi → Ect



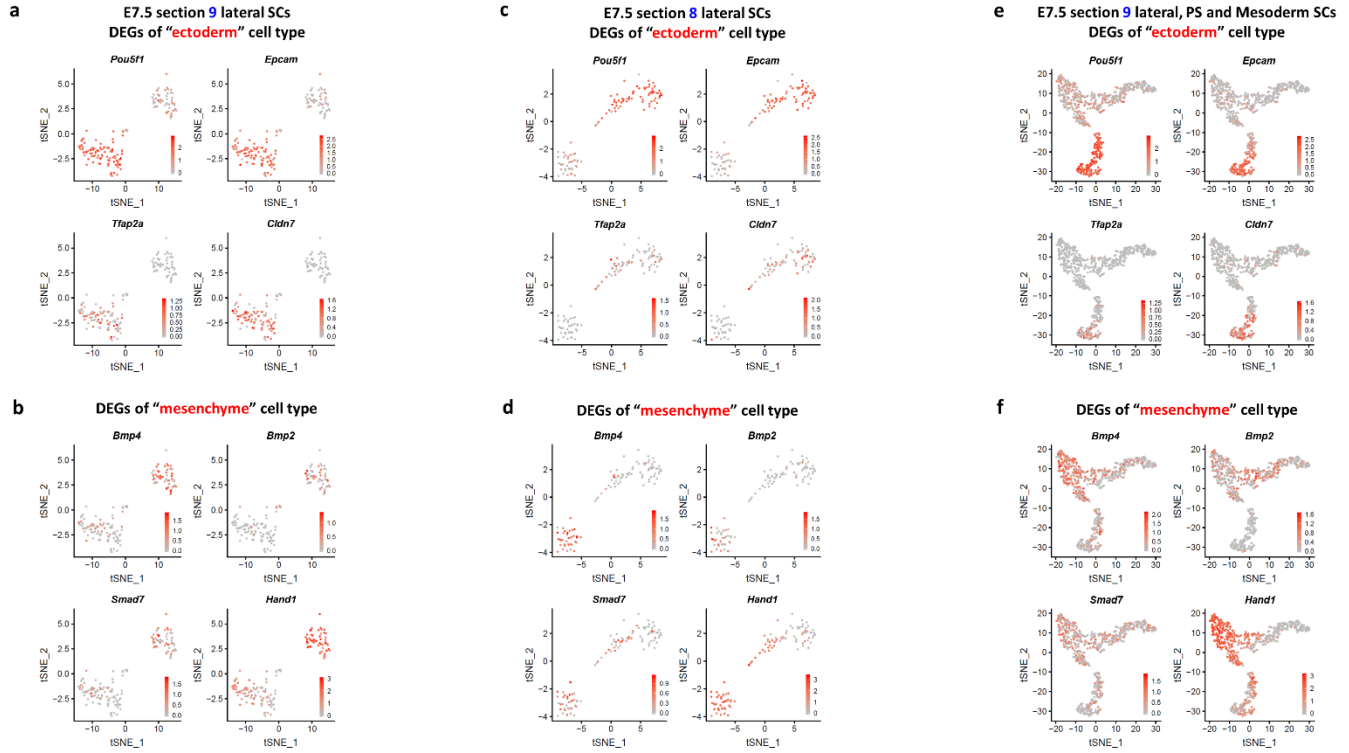
Supplementary Figure 9. Heterogeneity analysis and validation of marker genes.

- a. *t*-SNE plot showing the single cells mapped to position-6P at E6.75 (top panel). Re-clustering revealed the presence of two distinct clusters (bottom panel).
- b. *t*-SNE plot showing the expression pattern of *T* and *Mixl1* for cluster-1, and *Utf1* and *Pou3f1* for cluster-2.
- c. Heat map showing the differentially expressed genes of the two cell clusters ($p < 0.01$, fold change > 1.5). The enriched gene ontology (GO) terms ($p < 0.05$) provided additional information for annotating Cluster-1 as 'PS→Mes' and Cluster-2 as 'PS→Ect'.
- d. RNAscope analysis for E6.75 embryo.
- e. RNAscope analysis validated the expression of *Mesp1* in the posterior epiblast of E6.75 embryo.
- f. RNAscope analysis validated the co-localization of *T* and *Pou3f1* at the posterior epiblast of E6.75 embryo.



Supplementary Figure 10. Imputation of molecular trajectories of MEP.

- a.** *t*-SNE plots showing the E7.25 putative MEPs and the inferred E7.5 derivatives.
Left panel: annotated cell types, right panel: developmental trajectory.
- b.** *t*-SNE plots showing the expression pattern of marker genes of node (top panels), mesoderm (middle panels) and endoderm (bottom panels). The color legend indicates the level of expression determined by transcript counts.



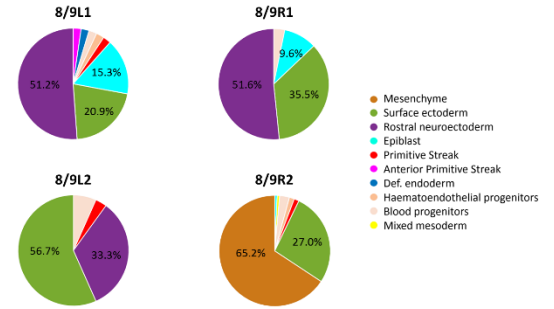
g E7.5 8/9R2 SCs E7.75 downstream SCs E8.0 downstream SCs E8.25 downstream SCs E8.5 downstream SCs

$$d(\text{Cell}_m, \text{Cell}_n) = \sqrt{\sum_{i=1}^n (\text{Cell}_m - \text{Cell}_n)^2}$$

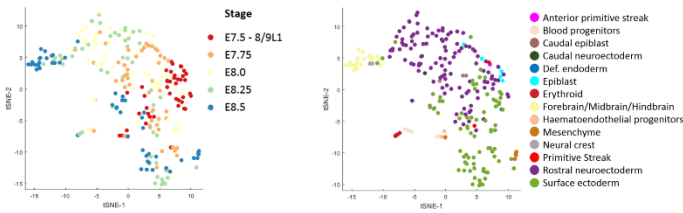
Dist.	E7.75_Cell_1	E7.75_Cell_2	...	E7.75_Cell_n
E7.5_8/9R2_Cell_1	d_{1-1}	d_{1-2}	...	d_{1-n}
E7.5_8/9R2_Cell_2	d_{2-1}	d_{2-2}	...	d_{2-n}
...	d_{m-1}	d_{m-2}	...	d_{m-n}
E7.5_8/9R2_Cell_m	d_{m-1}	d_{m-2}	...	d_{m-n}

Consecutive operations to identify the developmental descendants in the following stages.

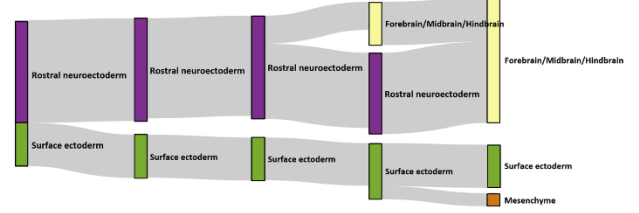
h Fraction of cell type



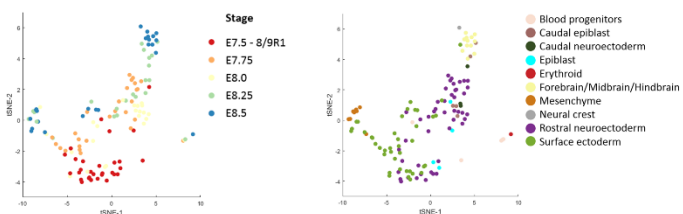
i E7.5 8/9 L1 single cells and lineage descendants



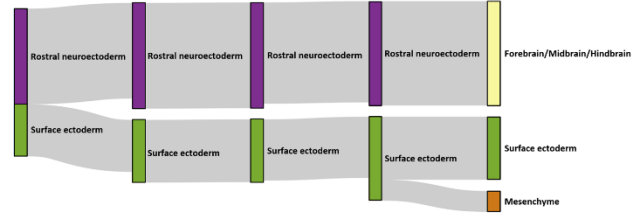
j E7.5 (8/9L1) E7.75 E8.0 E8.25 E8.5



k E7.5 8/9 R1 single cells and lineage descendants



l E7.5 (8/9R1) E7.75 E8.0 E8.25 E8.5



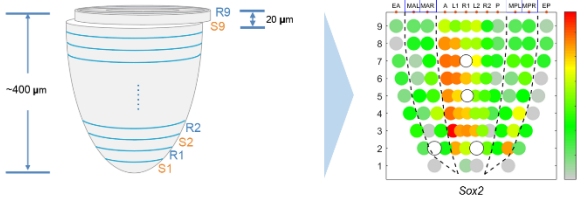
Supplementary Figure 11. Imputation of molecular trajectories of proximal-lateral ectoderm.

- a-f.** *t*-SNE plots showing the differentially expressed genes of “ectoderm” cell type (**a, c, e**) and “mesenchyme” cell type (**b, d, f**) in position-8R2/9R2 (corresponding to **Fig. 4a-f**). Panel **a-d** showed the single cells of proximal-lateral ectoderm positions in section-9 (**a, b**) and -8 (**c, d**). Panel **e, f** showed the single cells of proximal-lateral ectoderm, primitive streak and mesoderm positions in section-9.
- g.** Schematics of Population Tracing algorithm for single cells of E7.5-E8.5 embryos.
- h.** Pie charts showing the fraction of cell types at position-8L1/9L1, 8R1/9R1, 8L2/9L2 and 8R2/9R2.
- i-l.** *t*-SNE plots (**i, k**) and the molecular trajectories (**j, l**) of single cells (imputed using the Population Tracing algorithm) at position-8L1/9L1 (**i, j**) and position-8R1/9R1 (**k, l**) of E7.5 embryo and cells in E7.75-E8.5 embryos. Developmental timepoints (stage) and cell types (see legend of panel **i, k**) are indicated in the *t*-SNE plots. Cell types in E7.75-E8.5 embryos are annotated according to the ‘Gastrulation Atlas’.

a

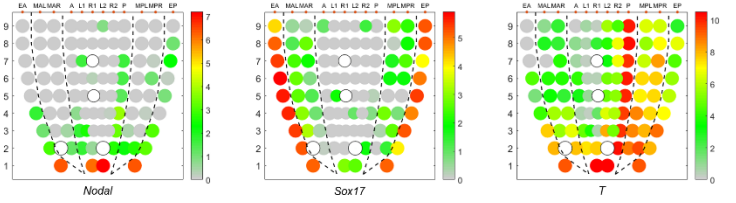
E7.5 Mes L/R LCM Strategy

[S1]: AP-EA/EP
 [S2]: A-L-R-P-MAL-MPL-MAR-MPR-EA-EP
 [S3-9]: A-L1-L2-R1-R2-P-MAL-MPL-MAR-MPR-EA-EP



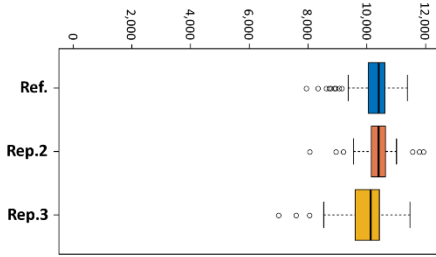
b

Representative Markers

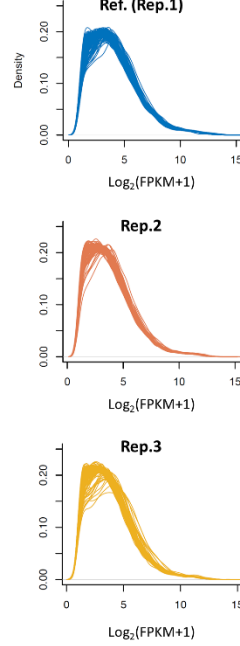


c

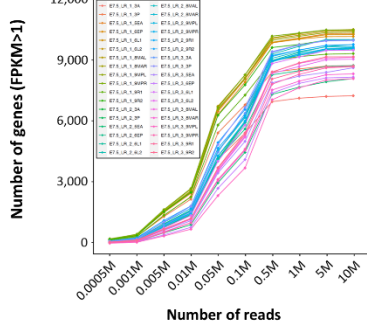
Number of gene (FPKM>1)



d

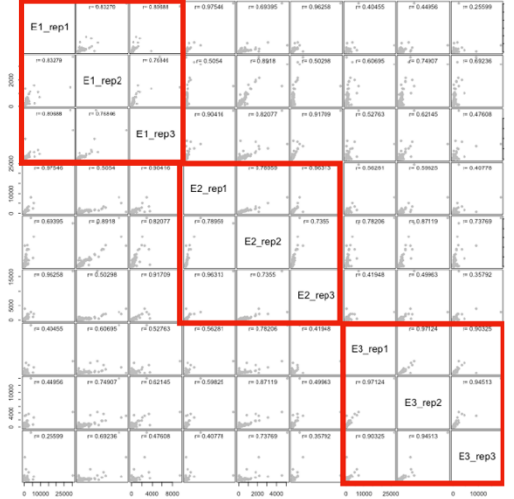


e



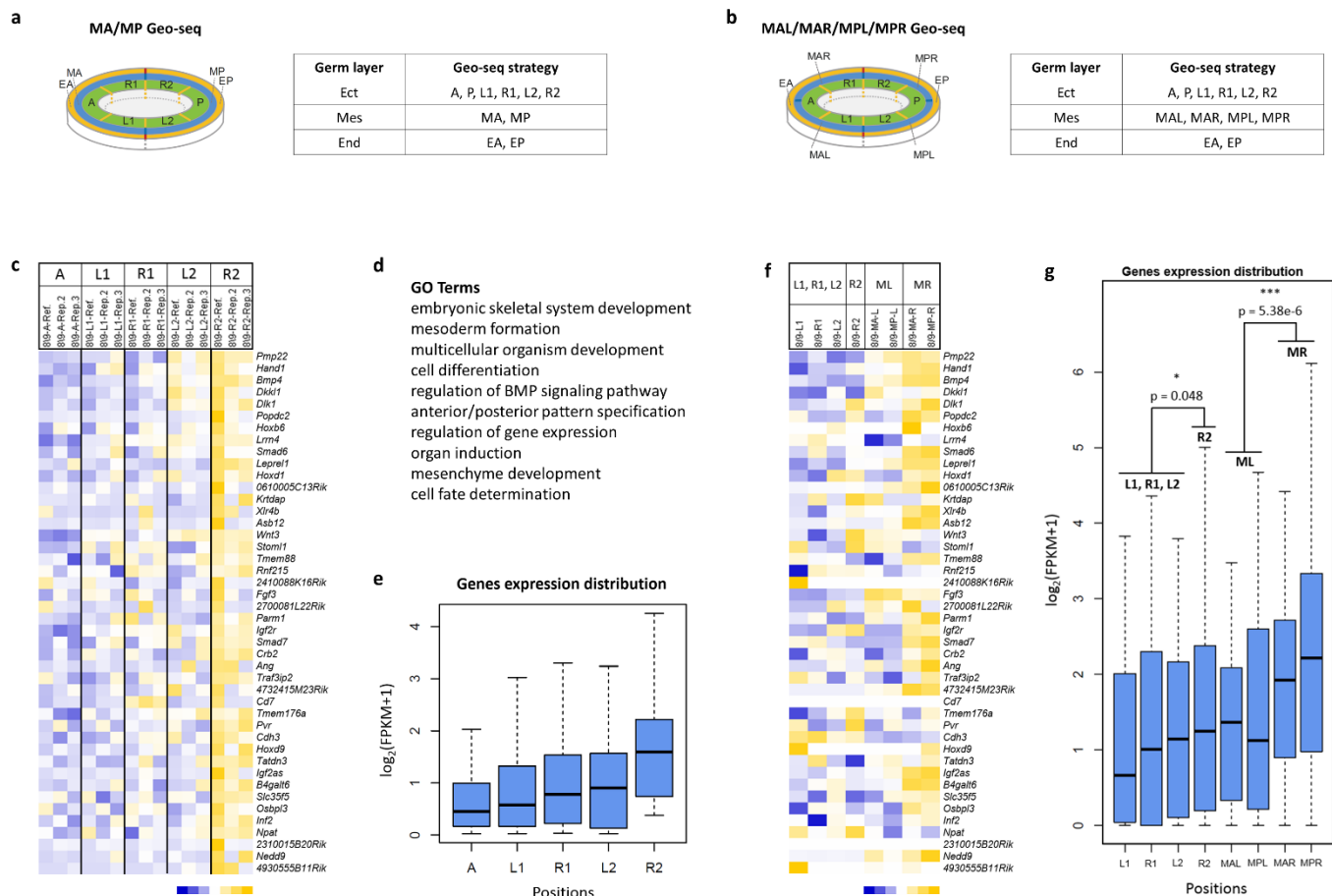
Replicate	Germ layer cells	Geo-seq strategy for mesoderm	Sequencing depth
Ref. (Rep.1)	Ect, Mes, End	MAL, MAR, MPL, MPR	~15 M reads per sample
Rep.2	Ect, Mes, End	MAL, MAR, MPL, MPR	~20 M reads per sample
Rep.3	Ect, Mes, End	MAL, MAR, MPL, MPR	~20 M reads per sample

f

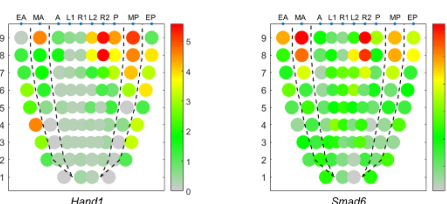


Supplementary Figure 12. Refined Geo-seq analysis of the mesoderm cell layer of the E7.5 embryo.

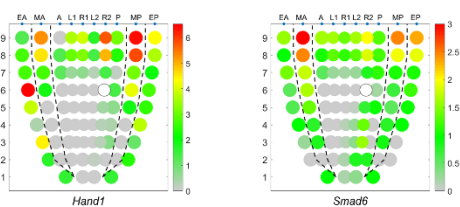
- a.** The strategy of sampling cell populations in the E7.5 embryo and the presentation of the spatial pattern of gene expression in the corn plot. MAL, anterior left mesoderm; MAR, anterior right mesoderm; MPL, posterior left mesoderm; MPR, posterior right mesoderm.
- b.** Corn plots showing the spatial pattern of expression of representative marker genes: *Nodal*, *Sox17*, *T*. Hollow circles indicate missing samples. Table: Geo-seq strategy and sequencing depth for biological replicates.
- c.** Box plot showing the number of detected genes (FPKM > 1) in the 3 biological replicates. The center line marks the median and box edges represent 25th and 75th percentiles. The median genes detected per replicate is 10,358 (Ref.), 10,389 (Rep. 2) and 10,042 (Rep. 3).
- d.** Gene expression density plot of Geo-seq data of the 3 biological replicates.
- e.** Saturation analysis. Different numbers of reads were selected, and the number of detected genes was plotted.
- f.** Inter-embryo correlation of spatial transcriptome data. The Pearson Correlation Coefficient (PCC) of spatial domains between biological replicates were calculated and showed high inter-embryo consistencies.



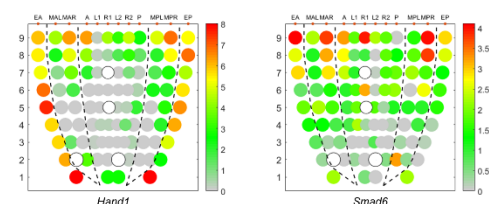
h E7.5 MA/MP Geo-seq: Ref.



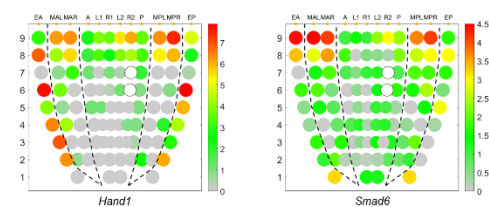
E7.5 MA/MP Geo-seq: Rep.2



i E7.5 MAL/MAR/MPL/MPR Geo-seq: Ref.

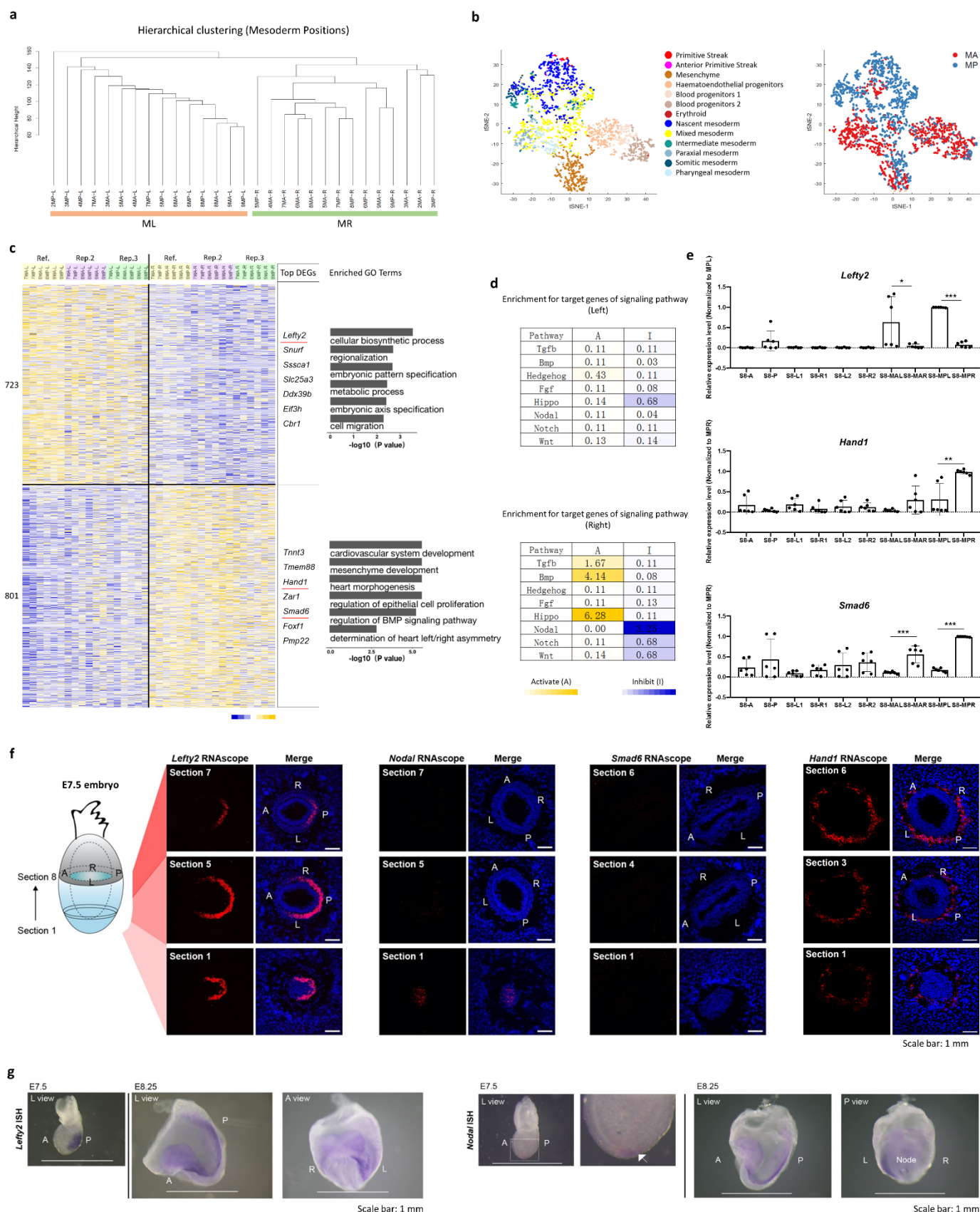


E7.5 MAL/MAR/MPL/MPR Geo-seq: Rep.2



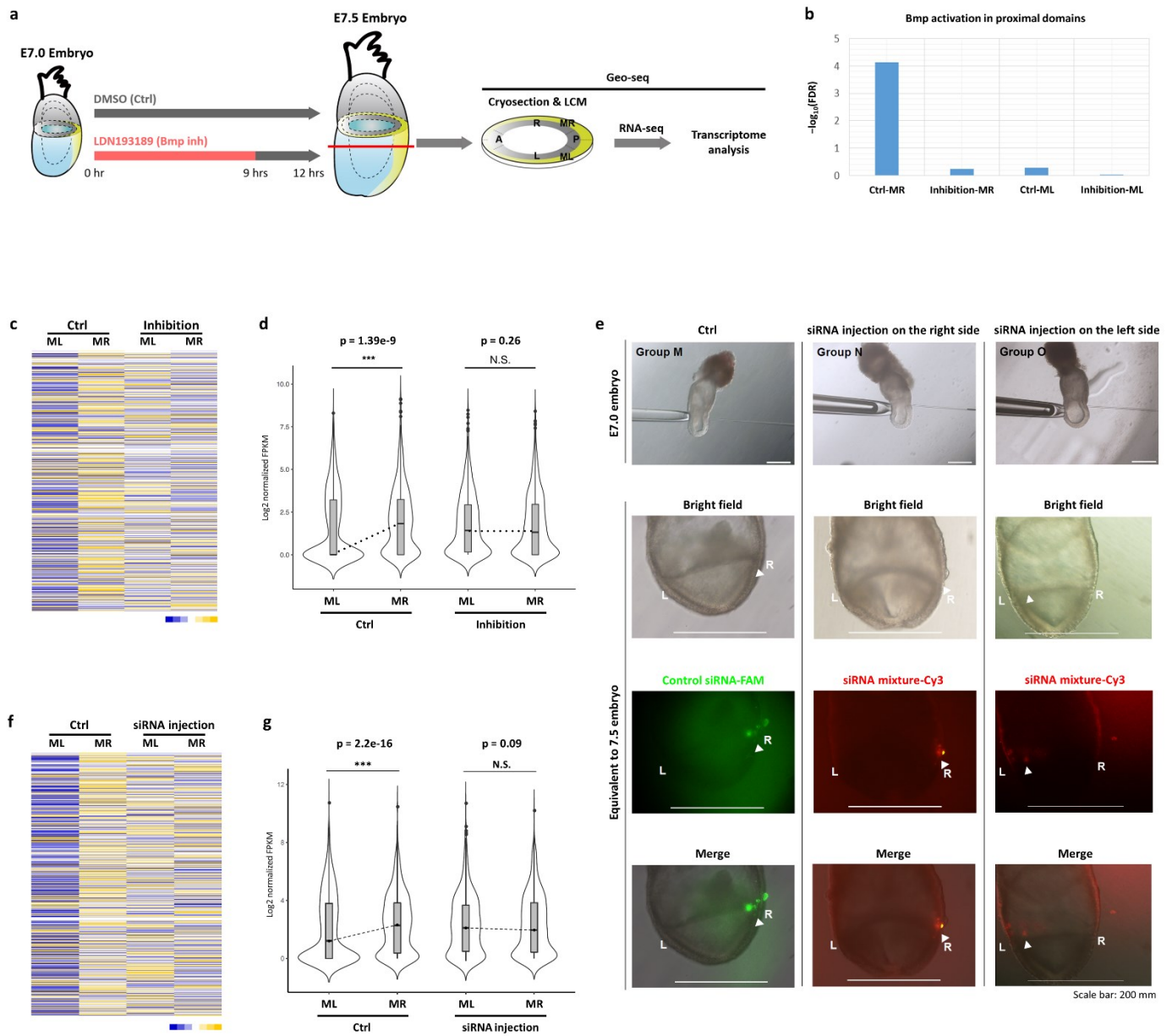
Supplementary Figure 13. The right-side DEGs of the proximal-lateral ectoderm showed significantly different expression between the proximal-lateral mesoderm on contralateral sides.

- a. The Geo-seq strategy of sampling cells from MA/MP populations in the E7.5 embryo. MA, anterior mesoderm; MP, posterior mesoderm.
- b. The Geo-seq strategy of sampling cells from MAL/MAR//MPL/MPR populations in the E7.5 embryo. MAL, anterior left mesoderm; MAR, anterior right mesoderm; MPL, posterior left mesoderm; MPR, posterior right mesoderm.
- c. Heatmap showing the differentially expressed genes (DEGs) in 8/9R2 regions compared with other lateral regions (8/9L1, 8/9L2, and 8/9R1) and anterior (A) region in the 3 Geo-seq replicates (Original MA/MP Geo-seq strategy), $p < 0.1$, fold change > 1.5 .
- d. The enriched gene ontology (GO) terms for the 8/9R2 specific DEGs.
- e. Box plot showing the distribution of expression level (Log2 normalized FPKM) of 8/9R2 specific DEGs in different ectoderm regions.
- f. Heatmap showing the expression pattern of 8/9R2 specific DEGs (identified in c) in different regions of the MAL/MAR/MPL/MPR Geo-seq embryo.
- g. Box plot showing the distribution of expression level (Log2 normalized FPKM) of 8/9R2 specific DEGs in different regions of the MAL/MAR/MPL/MPR Geo-seq embryo. T-test p-values were labelled on the box plot. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
- h. Corn plots showing the spatial expression pattern of *Hand1* and *Smad6* in the MA/MP Geo-seq embryos. Both reference and replicate embryos show L-R difference in the proximal ectoderm.
- i. Corn plots showing the spatial expression pattern of *Hand1* and *Smad6* in the MAL/MAR/MPL/MPR Geo-seq embryos. Both reference and replicate embryos show more significant difference in the right-side proximal lateral mesoderm.



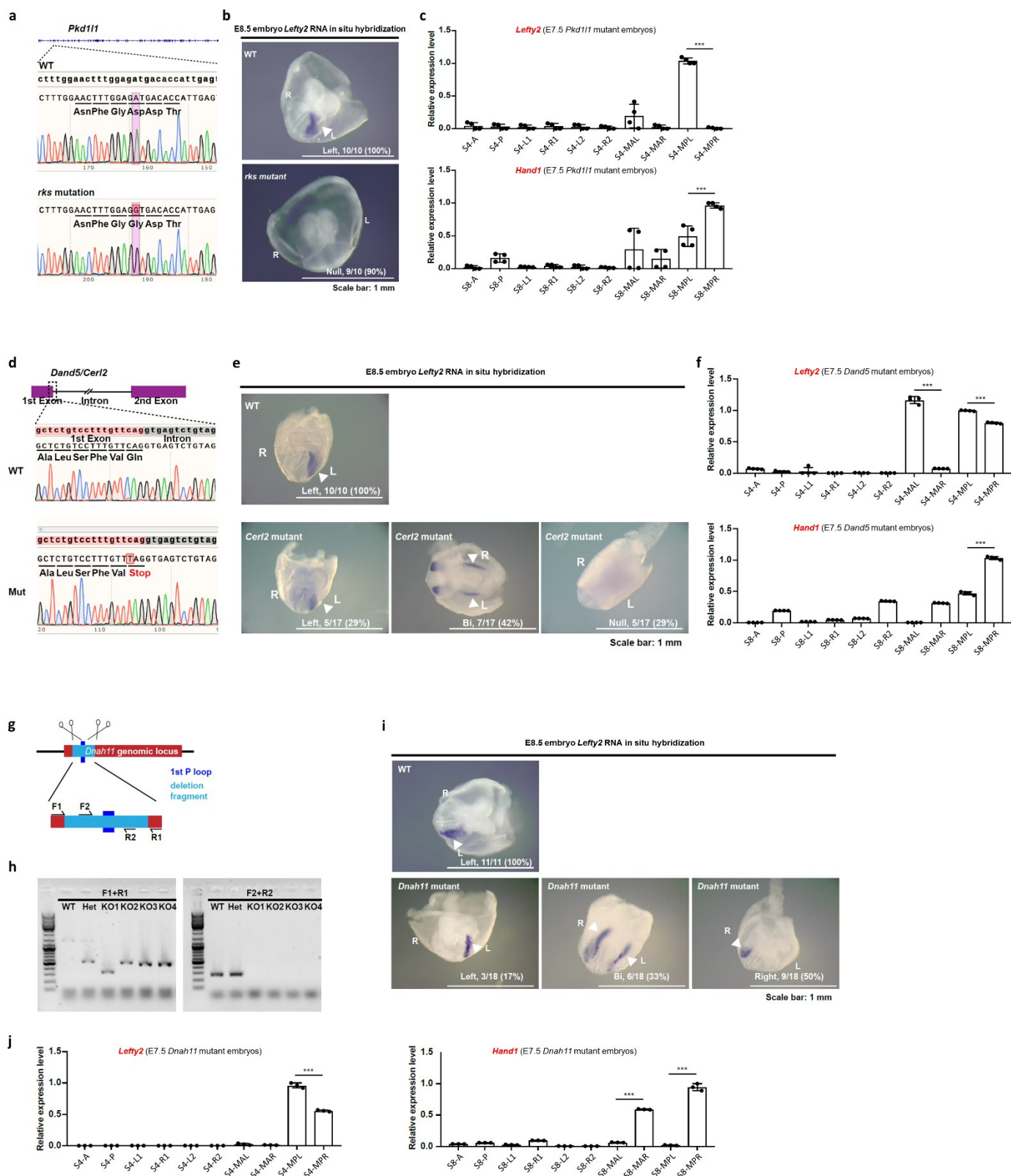
Supplementary Figure 14. Validation of the left-right asymmetric gene expression.

- a. Hierarchical clustering showed the difference between the mesoderm tissues on contralateral sides of the E7.5 embryo.
- b. *t*-SNE plot showing the data structure of single cells that are allocated to mesoderm positions at E7.5. Cells are colored by both their cell-type annotation according to ‘Gastrulation Atlas’ (left) and anterior/posterior location based on MDSC Mapping results (right).
- c. Heat map showing the differentially expressed genes (DEGs) of the proximal-left mesoderm (n = 723) and proximal-right mesoderm (n = 801) regions across the 3 Geo-seq replicates. The top DEGs and the enriched gene ontology (GO) terms for each group were listed on the right ($p < 0.01$). The genes with red underline were assayed by real-time PCR in e.
- d. The enrichment for target/response genes of development-related signaling pathways in the proximal-left and proximal-right mesoderm. Signaling activity: red, activating (A); green, inhibitory (I). The significance of $-\log_{10}(\text{FDR})$ value in each cell was calculated by one-sided Fisher’s exact test followed by Benjamini-Hochberg correction.
- e. Real-time PCR showing significant left/right differences of *Lefty2*, *Hand1* and *Smad6* expression in the proximal mesoderm (section 8) of E7.5 embryo. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
- f. RNAscope analyses showing the expression of *Lefty2*, *Nodal*, *Smad6* and *Hand1* in specified transverse sections (numbered) of E7.5 embryo. These RNAscope analyses serve as negative controls to the results in Fig. 5h. In these sections, there is no discernible difference between lateral sides, except for *Lefty2* in Section 5 and 7. A, anterior; P, posterior; L, left; R, right.
- g. Whole-mount in situ hybridization (WISH) showed the laterally asymmetric expression pattern of *Lefty2* (left panel) and *Nodal* (right panel, arrow) in E7.5 and E8.25 mouse embryos. A, anterior; P, posterior; L, left; R, right.



Supplementary Fig. 15. Geo-seq analyses of the *ex vivo* cultured embryos.

- a. Schematic showing the workflow of GEO-seq for *ex vivo* cultured embryos.
- b. The BMP signaling activity in the right-side lateral mesoderm region (MR) was curtailed by LDN193189 treatment.
- c. Heatmap showing the expression pattern of right-side specific genes of proximal lateral mesoderm (n = 801) in Control (Ctrl) and 9 hours BMP Inhibition embryos. A few right-side specific genes of proximal lateral mesoderm were not expressed in cultured embryos, these genes were then marked with white lines.
- d. Violin plot showing the expression level of right-side specific genes of proximal lateral mesoderm in control (Ctrl) and 9 hours BMP Inhibition embryos. The left-right differences were abrogated in the inhibition group. Rank sum test, ***, $p < 0.001$; N.S., no significant difference.
- e. Region-specific perturbation of BMP signaling by multiple siRNA knockdowns. Top panel: The experiment snapshot of siRNAs microinjection. Bottom panel: Region-specific transfection of corresponding siRNAs can be observed at embryos of equivalent E7.5 stage.
- f. Heatmap showing the expression pattern of right-side specific genes of proximal lateral mesoderm (n = 801) in Control (Ctrl) and siRNA knockdown embryos. A few right-side specific genes of proximal lateral mesoderm were not expressed in cultured embryos, these genes were then marked with white lines.
- g. Violin plot showing the expression level of right-side specific genes of proximal lateral mesoderm in control (Ctrl) and siRNA knockdown embryos. The left-right differences were abrogated in the Bmp siRNA microinjection group. Rank sum test, ***, $p < 0.001$; N.S., no significant difference.



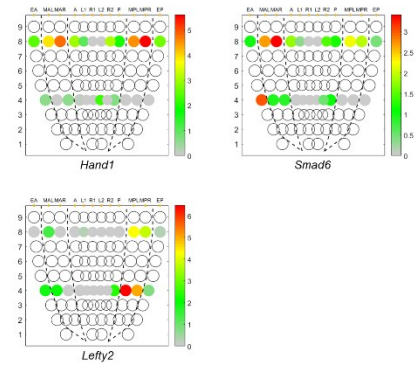
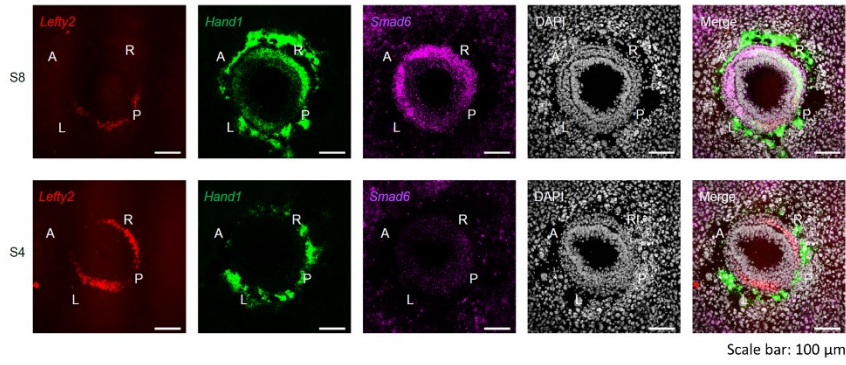
Supplementary Fig. 16. Asymmetric expression of *Lefty2* and *Hand1* persisted in *Pkd1l1*, *Cerl2* and *Dnah11* mutant embryos.

a-c. *Pkd1l1* mutant embryo: **(a)** Sequencing data of *Pkd1l1* mutant allele. **(b)** Whole-mount in situ hybridization of *Lefty2* in E8.5 mutant embryo. WT, wild type. L, left; R, right. Expression pattern: Null, no expression. **(c)** Real-time PCR showing the relative level of *Lefty2* and *Hand1* expression in E7.5 mutant embryos.

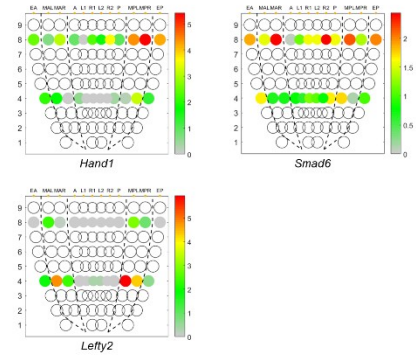
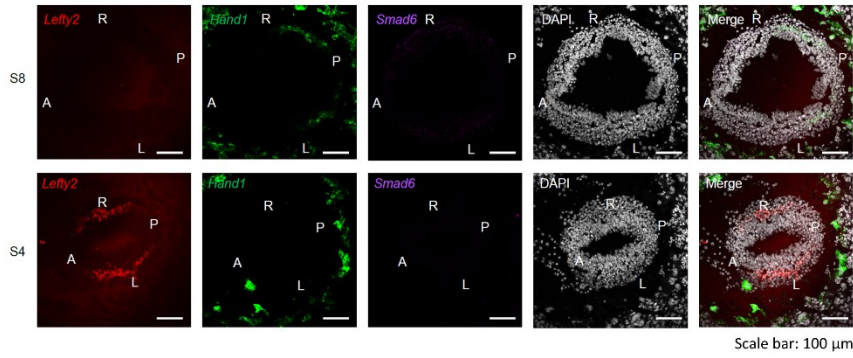
d-f. *Cerl2* (*Dand5*) mutant embryo: **(d)** Sequencing data of *Cerl2* mutant allele. **(e)** Whole-mount in situ hybridization of *Lefty2* in E8.5 mutant embryo. WT, wild type. L, left; R, right. Expression pattern: left; Bi, bilateral; Null, no expression. **(f)** Real-time PCR showing the relative level of *Lefty2* and *Hand1* expression in E7.5 mutant embryos.

g-j. *Dnah11* (*iv*) mutant embryo: **(g)** Schematic of the gene editing strategy. **(h)** Genotyping results of *Dnah11* mutant embryo. **(i)** Whole-mount in situ hybridization of *Lefty2* in E8.5 mutant embryo. WT, wild type. L, left; R, right. Expression pattern: left; Bi, bilateral; Null, no expression. **(j)** Real-time PCR showing the relative level of *Lefty2* and *Hand1* expression in E7.5 mutant embryos.

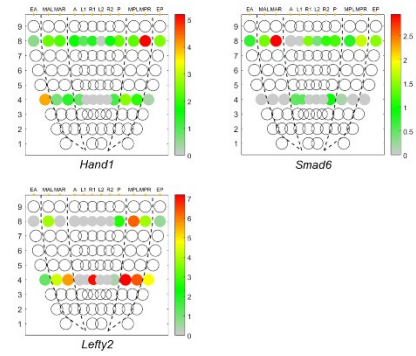
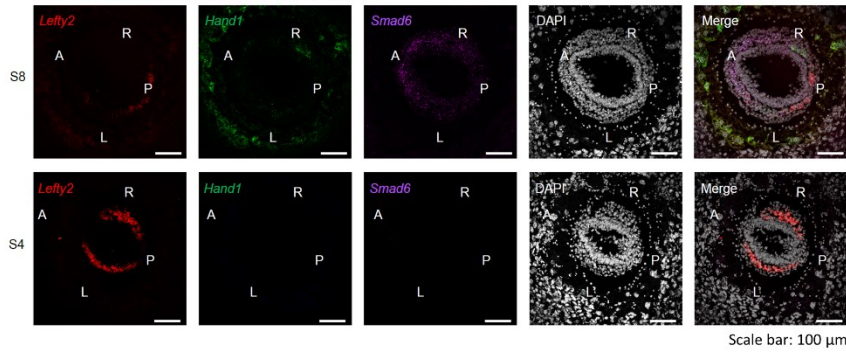
a E7.5 *Pkd1f1* mutation embryo (3/4 embryos)



b E7.5 *Cer1f2* mutation embryo (4/5 embryos)



c E7.5 *Dnah1f1* mutation embryo (3/4 embryos)



Supplementary Fig. 17. Left-right asymmetry persists in E7.5 mutant embryos.

a-c. Asymmetric pattern of expression of *Lefty2*, *Hand1* and *Smad6* in E7.5 *Pkd1l1* (**a**), *Cer12* (**b**) and *Dnah11* (**c**) mutant embryos revealed by RNAscope analysis (immunofluorescence images, A, anterior; P, posterior; L, left; R, right) and Geo-seq analysis (corn plots).