**Supplementary Information**

**Supplementary figure and table contents:**

**Supplementary Figure 1, related to Figure 1.**

**Supplementary Figure 2, related to Figure 1.**

**Supplementary Figure 3, related to Figure 1.**

**Supplementary Figure 4, related to Figure 1.**

**Supplementary Figure 5, related to Figure 1.**

**Supplementary Figure 6, related to Figure 2.**

**Supplementary Figure 7, related to Figure 2.**

**Supplementary Figure 8, related to Figure 3.**

**Supplementary Table 1, related to Figure 1.**

**Supplementary Table 2, related to Figure 1.**

**Supplementary Table 3, related to Figure 2.**

**Supplementary Table 4, related to Figure 3.**

**Supplementary Table 5, related to Figure 3.**

**Supplementary Table 6, related to Figure 4.**

**Supplementary Table 7, related to Figure 5.**

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**Figure S1, related to Figure 1.** UMAP visualization of each transcriptomes from the embryos at indicated developmental stages. Colored by cell cluster.

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**Figure S2, related to Figure1.** Cell Lineage specification during amphioxus notochord development. **(A)** UMAP plot of notochord lineage separated from the total embryonic snRNA-seq data, colored by cluster.

**(B)** Coarse-grained graph from (A) by PAGA. **(C)** Box-plot of the predicted cell cluster pseudotime distributions during amphioxus notochord development. Note that cells in cluster N3\_13 has higher pseudotimes than that in cluster L0\_11.

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**Figure S3, related to Figure 1.** Marker gene expression associated with the formation of each mesoderm cell lineages during amphioxus embryo development. Eya, Six4/5 and Pax3/7 mark anterior pharyngeal mesoderm; Hox11 and Tbx6/16/24 mark posterior pharyngeal mesoderm and Cdx is a marker for posterior tailbud mesoderm.

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**Figure S4, related to Figure 1.** Marker genes expression associated with the formation of each endoderm cell lineages during Amphioxus embryo development. Six3/6 and Nkx2.1 mark anterior pharynx while Pax1/9, Ilp and Msxlx are markers for posterior gut lineage.

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**Figure S5, related to Figure 1.** Gene expression associated with the neural cell lineage specification during the late amphioxus development stages. The anterior neural precursor lineage show high expression of Pax4/6, Fezf, Otx, Six3/6 and Lhx2/9; the posterior neural precursor lineage shows high expression of Gbx, Wnt7b, Pax2/5/8, Cdx, Hox1, Hox3 and Msx while the expression of Hu\_Elav, Tlx, VaChT, VGlut, VGAT and CHAT enrich in the differentiated neuron lineage.

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**Figure S6, related to Figure 2.** Distribution of scATAC-seq peaks for each Amphioxus development stages.

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**Figure S7, related to Figure 2.** UMAP visualization of the combination of snRNA-seq and scATAC-seq data from amphioxus embryos at the indicated developmental stages, which is colored by the cell lineage transferred from the corresponding snRNA-seq data. The upper panel shows the distribution of each single cell trancriptome on the combined dataset while the lower panel shows the distribution of each single cell epigenome on the combined dataset.

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**Figure S8, related to Figure 3.** Whole mount *in situ* hybridization results showing the developmental expression pattern of the indicated genes.

**Supplementary Tables Legends:**

**Supplementary Table 1, related to Figure 1.** Data summary of single nuclear RNA-sequencing for amphioxus embryos at different developmental stages.

**Supplementary Table 2, related to Figure 1.** List of genes specifically expressed in the unassigned lineage G3.5.

**Supplementary Table 3, related to Figure 2.** Data summary of single cell ATAC-sequencing for amphioxus embryos at different developmental stages.

**Supplementary Table 4, related to Figure 3.** Top specific genes for each lineage identified by snRNA and scATAC data integration analysis.

**Supplementary Table 5, related to Figure 3.** Primers used to clone the new lineage-specific genes for whole mount *in situ* hybridization.

**Supplementary Table 6, related to Figure 4.** Stage specific new transcripts compared to the previous stage, based on snRNA-seq data for amphioxus developmental embryos.

**Supplementary Table 7, related to Figure 5.** Data summary of the single cell RNA-sequencing for adult amphioxus tissues.