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Ripply1 and Gsc collectively suppress anterior endoderm differentiation from prechordal plate progenitors

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eLife Assessment

This study provides a **useful** analysis of the changes in chromatin organization and gene expression that occur during the differentiation of two cell types (anterior endoderm and prechordal plate) from a common progenitor in zebrafish, together with investigations into the molecular factors involved. Although the findings are consistent with previous work, the evidence presented appears to be **incomplete** and would benefit from more rigorous quantification of live imaging and Cre-Lox experiments, a stronger rationale and controls for experiments manipulating chromatin remodeling factors, and a strong justification for the explant model especially given differences between explant and whole embryo data. This work may be of interest to zebrafish developmental biologists investigating the mechanisms underlying specification.

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Abstract

During gastrulation, the mesendoderm is firstly specified by morphogens such as Nodal, and then segregates into endoderm and mesoderm in a Nodal concentration-dependent manner. However, the mechanism underlying the segregation and crosstalk of different sub-groups within the meso- and endoderm lineages remains unclear. Here, taking zebrafish prechordal plate (PP) and anterior endoderm (Endo) as research model, using single-cell multi-omics and live imaging analyses, we show that anterior Endo progenitors originate directly from PP progenitors. A single-cell transcriptomic trajectory analysis of wild-type, *ndr1* knockdown and *lft1* knockout Nodal explants confirms the diversification of anterior Endo fate from PP progenitors. Gene Ontology (GO) enrichment analysis identifies that the change of chromatin organization potentiates the segregation of anterior endodermal cell fate from PP progenitors. Single-cell ATAC & RNA sequencing further reveals that two transcriptional regulators, *gsc* and *ripply1*, exhibit varied activation patterns in PP and anterior Endo cell trajectories at both the chromatin and RNA expression levels. We further demonstrate that Ripply1 functions coordinately with Gsc to repress anterior endodermal cell fate by directly binding to the *cis*-elements of *sox32*. Modulating the expression levels of these regulators tilts the cell fate decision between the PP and anterior Endo.

Introduction

During gastrulation, instructed by genetic and epigenetic signals, naïve cells from blastula progressively acquire distinct cell fates and are allocated to specific domains within the embryo[1,2]. Nodal, a member of the transforming growth factor β (TGF-β) superfamily, acts as a morphogen[1,3–5] and plays highly conserved roles in mesendoderm induction and patterning[6–8].

It is widely accepted that Nodal induces endodermal and mesodermal cell fates via its concentration gradient[8–10]. Higher Nodal signaling activity bias cells toward endoderm, while lower levels are thought to promote mesoderm induction[11,12]. Mesodermal and endodermal common progenitors are induced by Nodal and mixed together at the onset of gastrulation[1], while it remains elusive how the common progenitors of mesendoderm took on distinct lineages. Several recent studies reported that multiple important signaling pathways interacted with Nodal to drive mesendoderm separation[2,13–15]. Notably, lateral endoderm specification may comply with a stochastic cell fate switching model in zebrafish[16].

The specification of dorsal mesendoderm requires the highest level of Nodal signaling, as evidenced by that this cell population was first to be depleted when Nodal signaling was gradually inhibited[11]. Both the PP and anterior Endo originate from anterior mesendoderm (derived from the dorsal mesendoderm), and the commitment of these cell fates is regulated by similar levels of Nodal signaling. However, the mechanisms governing the separation of cell fates within the anterior mesendoderm remain unclear. Previous studies show that Nodal signaling enhances the duration of cell-cell contact in PP cells, creating a positive feedback loop between cell-cell contact duration and cell fate specification[17]. However, what factors downstream of Nodal pathway play major roles in segregating these two cell fates, when and how PP and anterior Endo cells can be distinguished transcriptionally are the questions remaining to be investigated.

Previous research has shown that Gsc, whose expression can be induced by Nodal, acts as a repressor to suppress anterior endoderm specification by binding to the promoter region of *sox17*[18]. However, investigations into loss-of-function of Gsc in zebrafish have not yielded conclusive evidence supporting its significant roles in mesoderm and/or endoderm specification[19,20]. These results imply that there might be additional, unidentified transcriptional repressors that work in collaboration with Gsc and possess a redundant function in suppressing the specification of anterior endoderm.

In this study, we constructed a single-cell transcriptional trajectory to delineate the cell state segregation between PP and anterior Endo in zebrafish embryos and Nodal-injected zebrafish explants[21,22]. Single-cell transcriptional trajectory and live imaging analyses demonstrated that anterior Endo cells originated and were specified from PP progenitors. Interestingly, this cell trajectory separation can be traced back to as early as the onset of gastrulation. To achieve a more comprehensive understanding, we integrated single-cell datasets from wild-type, *ndr1* (Nodal ligand) knockdown and *lft1* (Nodal inhibitor) knockout Nodal explants at shield stage to construct an extended single-cell trajectory, which fully captured the branching event between PP and anterior Endo fates and unveiled a potential involvement of epigenetic regulation in this process. This finding was further supported by the experimental evidence that perturbation of the chromatin remodeler *srcap* could affect the cell specification of PP and anterior Endo. Furthermore, combining the multi-omic analysis and gain-/loss-of-function experiments, we found that a transcriptional repressor, Ripply1, together with Gsc, collectively suppresses anterior endoderm specification by directly binding to the regulatory elements of *sox32*.

Results

Anterior Endo originates from PP progenitors in zebrafish

Both anterior Endo and PP are derived from embryonic shield and require high Nodal signaling to be specified[11,18,23]. To investigate how these two cell lineages are further segregated, we collected the single-cell RNA-seq datasets from the published studies[24,25], identified the subpopulations representing the anterior Endo and PP cells, and reconstructed their trajectory branching tree (Figures 1A-1C and S1A). We found that these two trajectories started to be separated at shield stage, and their common progenitors highly expressed PP marker genes, like *gsc*, *frzb*, etc. (Figures

1C and S1A). Then, we further explored the dynamic molecular signatures of these two trajectories during their fate determination (Figures S1B-S1E). Pseudotime analysis revealed that PP and anterior Endo exhibited distinct differentiation states by the end of gastrulation (Figure S1B). Of note, compared to *sox32*, *gsc* displayed earlier transcriptional differences in these two cell trajectories (Figure S1C). The cell trajectory prediction analysis[26] also revealed that the progenitor cells resembled more of the PP fate (Figures 1D, 1E and S1F), and similar result was obtained using another single-cell RNA-seq dataset (Figures S1G-S1I). Therefore, our analysis suggest that anterior Endo and PP originate from the same progenitor with transcriptome profile more similar to PP fate. Anterior Endo becomes transcriptionally distinguishable from PP as early as at the shield stage.

Cells from hatching gland (derived from PP) and pharyngeal pouch (derived from anterior Endo) are distant on the transcriptome-based phylogenetic tree of zebrafish embryos[25]. Thus, the resolution of this dataset may not be sufficient to identify the exact trajectory separation point between PP and anterior Endo. To investigate the key molecules that regulate the PP and anterior Endo separation, we employed our previously published Nodal induced embryonic explant system (referred to Nodal explant hereafter), which encompasses the axial mesendoderm, along with the PP and anterior Endo fates[22]. Notably, the single-cell trajectory tree of Nodal explants showed a closer transcriptomic relationship between the PP and anterior Endo (Figure 1F). Thus, we reconciled the single-cell transcriptome dynamics to look for the branching point of PP and anterior Endo in the Nodal explants (Figures 1G, 1H, S2A and S2B). Consistent with the observations in wild-type embryos, the common progenitors of these two cell trajectories highly expressed the PP marker gene (*gsc*), and the segregation between PP and anterior Endo had already occurred by 6 hpf (hours post fertilization) (Figures 1G, 1H and S2B). Cell trajectory prediction analysis on the Nodal explant datasets further indicated that the common progenitor cells were transcriptionally similar to PP (Figures 1I and S2I), which aligned with our previous observations in wild-type embryos (Figures 1D, 1E, S1H and S1I).

To explore the molecular signatures during the specification of PP and anterior Endo, we performed differential expression analysis and the gene module identification analysis for these two cell trajectories in both wild-type embryos and Nodal explants[27]. These genes enriched GO terms[28] were highly correlated with the developmental processes and functions of these two cell types (Figures S2C-S2H).

To further validate this observation and investigate the dynamics of cell fate segregation between anterior Endo and PP during development, we performed live imaging analysis on embryos of Tg(*gsc*:EGFP;*sox17*:DsRed) (Figures 2A and 2B). Notably, we observed a gradual emergence of *sox17*-positive cells from a subset of the *gsc*-positive cells (Figures 2A-2D, S3A-S3D and Movies S2, S3). And this cell fate transition was also evident in Nodal explants constructed from Tg(*gsc*:EGFP;*sox17*:DsRed) embryos (Figures S3E-S3H and Movie S1). Furthermore, we generated *sox17*:Cre and *gsc*:loxP-STOP-loxP-mCherry constructs (Figure S4D) and co-injected them with transposase mRNA into one-cell stage embryos (Figure S4A). Interestingly, distinct mCherry-positive cells were observed in the injected embryos, providing clear evidence that *gsc*-positive cells can give rise to *sox17*-positive cells (Figures S4B and S4C).

We co-stained *gsc* and *sox17* in embryos and in Nodal explants respectively at different time points during gastrulation (Figures 2E and S3I). By assessing the expression levels of *sox17* and *gsc* in *sox17*-positive anterior mesendoderm cells, we observed a positive correlation between *sox17* and *gsc* expression at the onset of gastrulation. However, with advancing development, *sox17* and *gsc* expression exhibited a negative correlation in both embryos and Nodal explants (Figures 2F and S3J). This suggests that the increase of *sox17* expression during anterior Endo differentiation is accompanied by the simultaneous reduction of *gsc* expression. These findings align with our live imaging experiments (Figures 2A, S3B and S3F). In summary, combing single-cell transcriptomic trajectory analyses and live imaging experiments using embryos and explants, we demonstrated that anterior Endo cells were derived from PP progenitor cells in zebrafish.

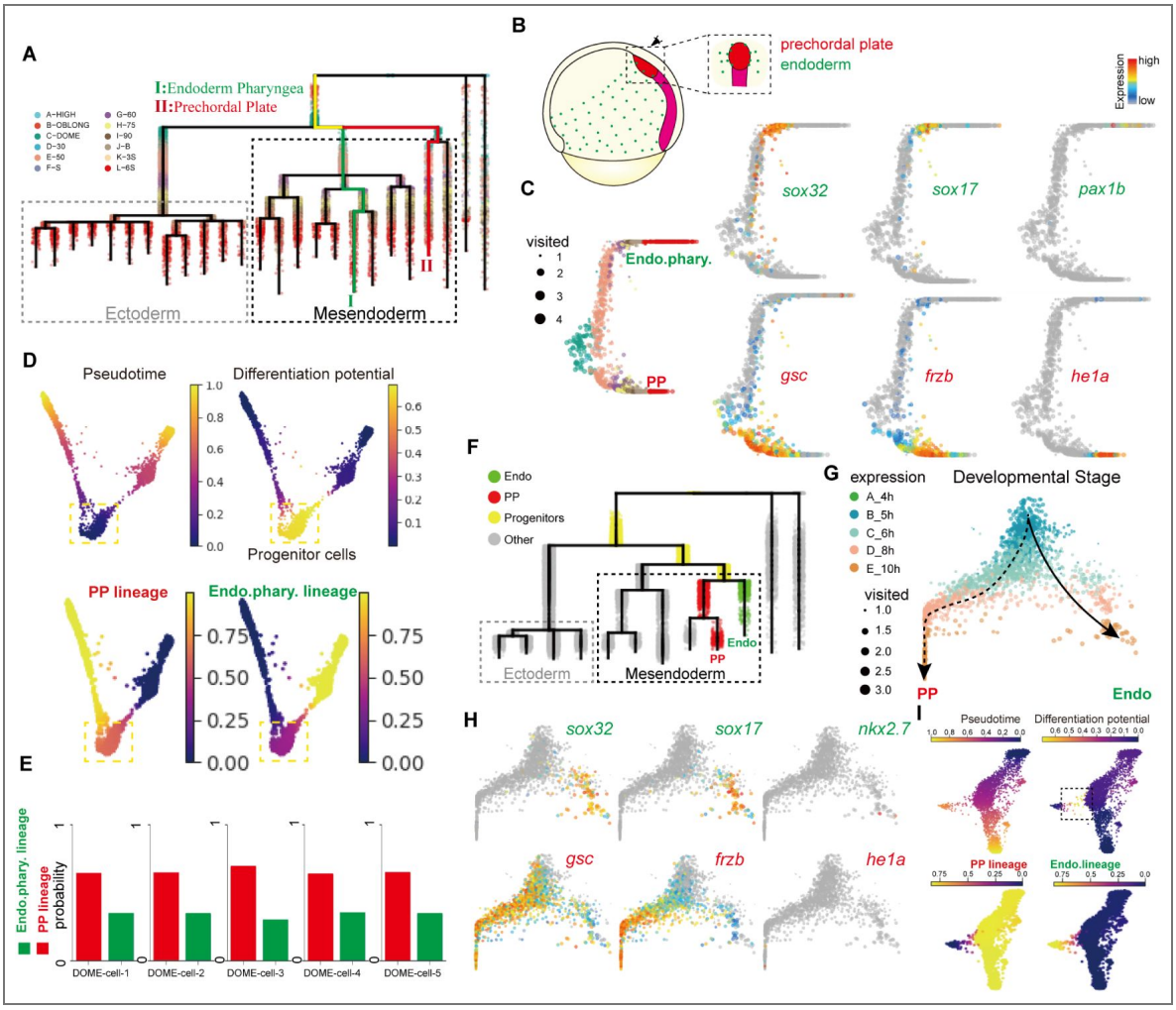

Figure 1. Single-cell trajectory analysis for studying PP and anterior Endo specification.

(A) URD differentiation tree of zebrafish embryos from blastula stage to somitogenesis stage[73]. Cells are colored according to developmental stages. Gray dotted frame indicates the ectoderm trajectories and black dotted frame indicates the mesendoderm trajectories. Green line shows the pharyngeal endoderm trajectory (anterior endoderm, roman number I), red line shows the prechordal plate trajectory (roman number II) and yellow line indicates their progenitors. (B) Simplified schematic showing the patterns of anterior endoderm and axial mesoderm of zebrafish embryos at 75% epiboly. Dotted frame indicates the enlarged view of dorsal anterior region, showing that anterior Endo sporadically locates near PP. (C) URD branchpoint plots of the PP and anterior Endo development from scRNA-seq data of zebrafish embryos[73], showing pseudotime (x-axis) and random walk visitation preference from pharyngeal endoderm to prechordal plate domains (y-axis). Cells are colored by developmental stages (left) and expression of anterior Endo markers (*sox32*, *sox17* and *pax1b*; right top) and PP markers (*gsc*, *frzb* and *he1a*; right bottom). (D) Force-directed layout of PP and anterior Endo cells of zebrafish embryos from blastula stage to somitogenesis stage. Cells are colored by Palantir[26] pseudotime (top left), differentiation potential (top right) and branch probabilities of PP (bottom left) and anterior Endo (bottom right). (E) Branch probabilities of five cells randomly selected from progenitor cells. Bars are colored by cell types. (F) URD differentiation tree of Nodal explants from blastula stage to the end of gastrulation. Gray dotted frame indicates the ectoderm trajectories and black dotted frame indicates the mesendoderm trajectories. The green, red and yellow dots indicate the Endo cells (anterior Endo), PP cells and the progenitor cells respectively, and the gray dots indicate other cells. (G-H) URD branchpoint plots of the PP and anterior Endo development from scRNA-seq data of Nodal explants showing pseudotime (y-axis) and random walk visitation preference from PP to anterior Endo domains (x-axis). Cells are colored by developmental stages (G) and expression of anterior Endo markers (*sox32*, *sox17* and *nkx2.7*; top) and PP markers (*gsc*, *frzb* and *he1a*; bottom) (H). (I) Force-directed layout of PP and anterior Endo cells of Nodal explants from blastula stage to the end of gastrulation. Cells are colored by the levels of Palantir[26] pseudotime (top left), differentiation potential (top right) and branch probabilities of PP (bottom left) and anterior Endo (bottom right).

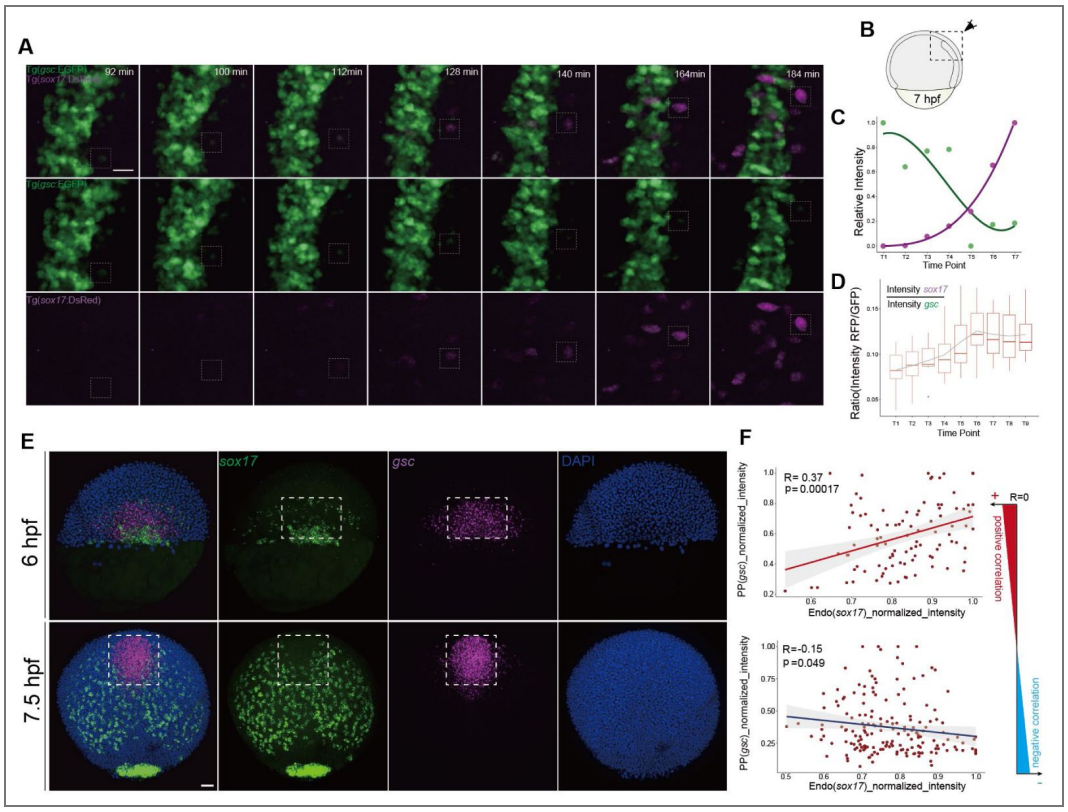

Figure 2. Live imaging analyses for Tg(*gsc*:EGFP;*sox17*:DsRed) embryos.

(A) Time series of 3D reconstruction of a representative Tg(*gsc*:EGFP;*sox17*:DsRed) embryo. (B) Schematic diagram indicating the focused region in the Tg(*gsc*:EGFP;*sox17*:DsRed) embryo. (C) Temporal profiles of relative intensity of *sox17* (RFP) & *gsc* (GFP) in the highlighted cell from (A). (D) Temporal profiles of the ratio between RFP intensity and GFP intensity in multiple tracked cells. (E) Double-color RNA-fluorescence *in situ* hybridization (FISH) of *sox17* and *gsc* in wild-type embryos at 6 hpf (top) and 7.5 hpf (bottom). (n = 5/5, 6 hpf; n = 5/5, 7.5 hpf; n: embryos were imaged, expression observed/total imaged) (F) Scatter plots and inferred linear regression comparing the relative intensities of *sox17* and *gsc* in wild-type embryos at 6 hpf (top) and 7.5 hpf (bottom). Each FISH experiment was performed for at least 3 independent replicates (technical replicates); more than 30 embryos were analyzed. Scale bar: 30 μm (A) and 50 μm (E).

Nodal-Lefty regulatory loop is needed for PP and anterior Endo fate specification

In zebrafish, the PP and anterior Endo lineages are physically in close proximity and direct contact[17]. During development, interactions through cell-cell contacts between neighboring cell types often play a crucial role in promoting cell fate segregation[17,29]. We hypothesized that communications between PP and anterior Endo cells may influence each other's cell fate specification. We employed LIANA[30], a ligand-receptor analysis framework, to investigate the molecular signals involved in these interactions using single-cell RNA-seq datasets. Our analysis revealed strong interactions between PP and anterior Endo cells in both wild-type embryos and Nodal explants (Figures S5C-S5F). Interestingly, key molecular factors in the Nodal-Lefty regulatory loops (Figure 3D), such as *ndr1*, *lft1* and *lft2*, were significantly enriched in the interaction between PP and anterior Endo cells (Figures 3A, 3B, S5A, S5B and S5G-S5I).

The results presented above suggest that differences in the Nodal-Lefty signaling may exist between PP and anterior Endo cells. It is widely accepted that Nodal-Lefty functions as reaction-diffusion system to facilitate Nodal gradient formation and cell fate determination[32–36] (Figure 3D). As zebrafish possess redundant Nodal (Ndr1, Ndr2 and Ndr3) and Lefty (Lefty1 and Lefty2) paralogs, we firstly investigated the perturbation effects of individual Nodal or Lefty paralog on Nodal activity and its gradient. We generated an ectopic Nodal gradient in zebrafish animal pole cells by injection of *ndr2* mRNA at 128-cell stage in wild-type embryos, *ndr1* morphants and *lft1* mutants (Figures 3E, S6A-S6F and S6H-S6M). By immunostaining and quantification of the pSmad2 signaling, we observed a significant enhancement of Nodal activity in *lft1* mutants, while its level decreased upon *ndr1* knockdown (Figures 3E, 3F and S6A-S6M).

To further determine how Nodal-Lefty is involved in the regulation of PP and anterior Endo separation, we performed single-cell transcriptomic sequencing for Nodal explants constructed from *ndr1* morphants and *lft1* mutants at shield stage (Figure 3G). We integrated these two single-cell RNA-seq datasets with the dataset of wild-type Nodal explants[22]. After applying unsupervised clustering, we identified a total of 9 different cell clusters (Figures 3H, S7A and S7B). Among these clusters, two mesodermal clusters named mesoderm_PP and mesoderm_NT (Figure 3H) were obtained. Mesoderm_PP cells transcriptionally resembled the progenitors of PP evidenced by the high expression of *gsc* and *chrd*, while mesoderm_NT cells were the progenitors of notochord characterized by the high expression of *noto* and *tbxta* (Figures S7A and S7B). We observed that a small cluster of *sox32* and *sox17* positive anterior Endo cells was close to the PP cells on the UMAP (Figure 3H). We then calculated the proportions of PP and anterior Endo cells in the three datasets, and found that the proportions of both cell types increased in *lft1*-mutant explants, while decreased in *ndr1*-morphant explants compared to wild-type explants (Figures 3I and S7C).

To better estimate Nodal activity during PP and anterior Endo cell fate segregation, we defined a Nodal score by regressing out the expression level of all the Nodal downstream targets, which were differentially expressed upon Nodal treatment[22] and also possessed pSmad2 binding peaks near their genetic loci[22,37] (**see Methods**). A total of 29 Nodal downstream targets, whose expression exhibited a high correlation with Nodal activity, were used to calculate Nodal score (Figure 3J and S7L). We found Nodal score was dramatically decreased in *ndr1* knockdown samples and slightly increased in *lft1* mutant samples compared to that in wild-type samples (Figures 3K and S7D), which is consistent with the results of pSmad2 immunostaining (Figures 3E and 3F). We then examined the levels of Nodal score across all cell types in Nodal explants at 6 hpf, and found that axial mesendodermal cells, including PP, notochord and anterior Endo, displayed the highest Nodal scores (Figure S7E), which was consistent with previous knowledge that Nodal activity was the highest in the dorsal margin of zebrafish embryos at the onset of gastrulation[1,38,39]. A further comparison of Nodel scores between PP and anterior Endo revealed that PP had a sightly higher Nodal score than anterior Endo (Figure S7E). These analyses underscored the importance of Nodal signaling levels in PP and anterior Endo cell fate specification.

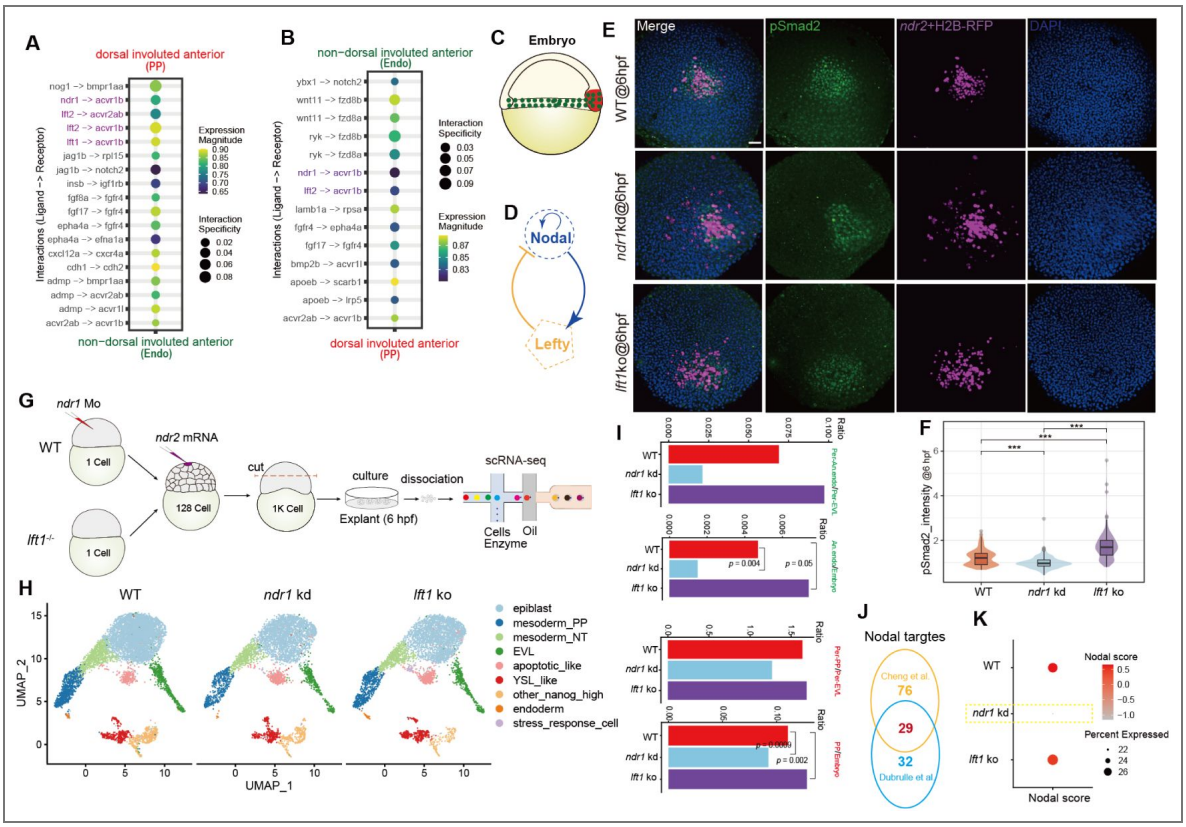

Figure 3. Nodal-Lefty regulatory loops are involved in cell fate separation between anterior Endo and PP.

(A and B) Dot plots visualizing ligand-receptor interactions between PP and anterior Endo. The interactions from PP (ligand) to anterior Endo (receptor) and from anterior Endo (ligand) to PP (receptor) are plotted respectively in A and B. (C) Schematic diagram illustrating PP (red) and anterior Endo (green) in zebrafish embryos at 6 hpf. (D) Simplified schematic showing the interaction network between Nodal and Lefty. (E) 3D reconstruction of representative confocal-scanned images showing pSmad2 levels in zebrafish embryonic animal pole stimulated by Nodal injection at 128-cell stage (WT, n = 3/4; *ndr1*kd, n = 3/4; *lft1*ko, n = 4/4; n: embryos were imaged, observed/total imaged). (F) Comparisons of pSmad2 levels in wild-type embryos, *lft1* mutants and *ndr1* morphants at 6 hpf (quantified from E). Statistical differences between two samples were evaluated by Student's t-test. *indicates P-value < 0.05, **indicates P-value < 0.01 and ***indicates P-value < 0.001. (G) Schematic diagram showing the experimental workflow of single-cell RNA sequencing of Nodal explants constructed from *lft1* mutants and *ndr1* morphants at 6 hpf. (H) Overall UMAP plot of integrated single-cell datasets of Nodal explants generated from wild-type embryos (left), *ndr1* morphants (middle) and *lft1* mutants (right). (I) Histograms showing the ratios of the cell proportions of anterior Endo against EVL or embryo (top) and prechordal plate (bottom) against EVL or embryo. Per indicates percentage. As EVL cells can be specified spontaneously and are Nodal-independent[74], here the proportions of prechordal plate and anterior Endo were adjusted by using the proportions of EVL cells as an internal control. (J) Venn plot showing the selection of Nodal direct target genes used for defining Nodal score (**see Methods**). (K) Dot plot visualizing Nodal score in wild-type, *ndr1*-morphant and *lft1*-mutant Nodal explants. Dot size indicates the percentage of cells within the cell group; the color indicates the average Nodal score levels. Each immunostaining experiment was performed for at least 3 independent replicates (technical replicates); more than 10 embryos were analyzed. Scale bar: 50 μm (E).

A meticulous single-cell trajectory tree reveals the role of chromatin remodeling in PP and anterior Endo segregation

To thoroughly investigate the dynamic transcriptome changes during PP and anterior Endo separation, we reconstructed a single-cell trajectory tree using all PP and anterior Endo cells from three Nodal explant datasets at 6 hpf which was a crucial time point previously identified for the separation of these two cell trajectories (Figure 4A). This trajectory tree captured a continuous cell-state-transition process from PP progenitors to anterior Endo, and a key branching point in this process was identified (Figures 4A and S7F). To elucidate the molecular dynamics in this branching process, we performed differential expression analysis for these two cell trajectories around the branching point (Figure 4B). Three clusters of genes were identified, which were highly expressed in pre-branch (progenitors), left-branch (anterior Endo) and right-branch (PP) respectively. GO enrichment analysis using these three gene sets identified terms related to mesendodermal cell fate specification (Figure 4B). Interestingly, "chromatin organization" was significantly enriched in the pre-branch cells, and maintained high enrichment during PP specification, but was down-regulated upon endodermal fate specification (Figure 4B). Furthermore, the GO term "chromatin organization" was also enriched in the differentially expressed genes of the PP cells when comparing *lft1* mutant or *ndr1* knockdown samples to wild-type samples (Figures 4C-4G and S7G).

These analyses unveiled a potential role of chromatin regulators in the regulation of PP and anterior Endo segregation, prompting us to identify the specific factors involved in this process. Among the candidates within GO term of "chromatin organization" (Figure 4B-4E), we identified a key member of SWI/SNF complex, *srcap*, whose expression is slightly higher in the PP than in the anterior endoderm cells in both embryos and Nodal explants (Figures 4J, 4K and S7H). Previous studies have demonstrated that the SWI/SNF nucleosome remodeling complex is necessary for TGF-β-induced transcription of numerous target genes[40,41]. To investigate the role of the SWI/SNF complex in the separation of PP and anterior Endo cell fates, we treated embryos with AU15330 (Figure 4H), a known degrader of SWI/SNF ATPase components[42]. Subsequently, we assessed the cell fate specification of PP and anterior Endo by HCR co-staining for *gsc*, *frzb* and *sox32*. We found that AU15330 treatment significantly increased the number of anterior Endo cells while decreasing the number of PP cells compared to DMSO-treated control embryos (Figures 4H, 4I and S7J). Similar effects were observed in *srcap* morphants, where the cell number of anterior Endo increased compared to wild-type embryos (Figures 4L, 4M, S7I and S7K).

Collectively, these findings underscore the importance of SWI/SNF complexes in ensuring accurate cell fate segregation within the anterior mesendoderm.

Integrative analysis of single-nucleus RNA-Seq and ATAC-Seq reveals distinct chromatin states underlying differential expression profiling in PP and Endo cells

To delve deeper into the involvement of chromatin states in the separation of PP and Endo, we conducted single-cell assays for transposase-accessible chromatin (ATAC) and nuclear RNA sequencing in zebrafish embryos at 6 hpf (Figure 5A). Recognizing that accurate cell clustering cannot solely rely on chromatin accessibility dataset, we integrated this dataset with RNA expression data to achieve precise cell type identification. The resulting cells were clustered into 10 different groups on the UMAP (Figures 5B, S8A, and S8B).

Using the marker gene *gsc*, we identified PP progenitor cells within the axis mesoderm, and then Endo and PP progenitor cells were selected for downstream analysis (Figure 5C). A small cluster of cells expressing *sox32*, *sox17* and *foxj1a*, which were located distantly from PP and Endo, were classified as dorsal forerunner cells (DFCs) and excluded from further analysis (Figures 5C and S8C).

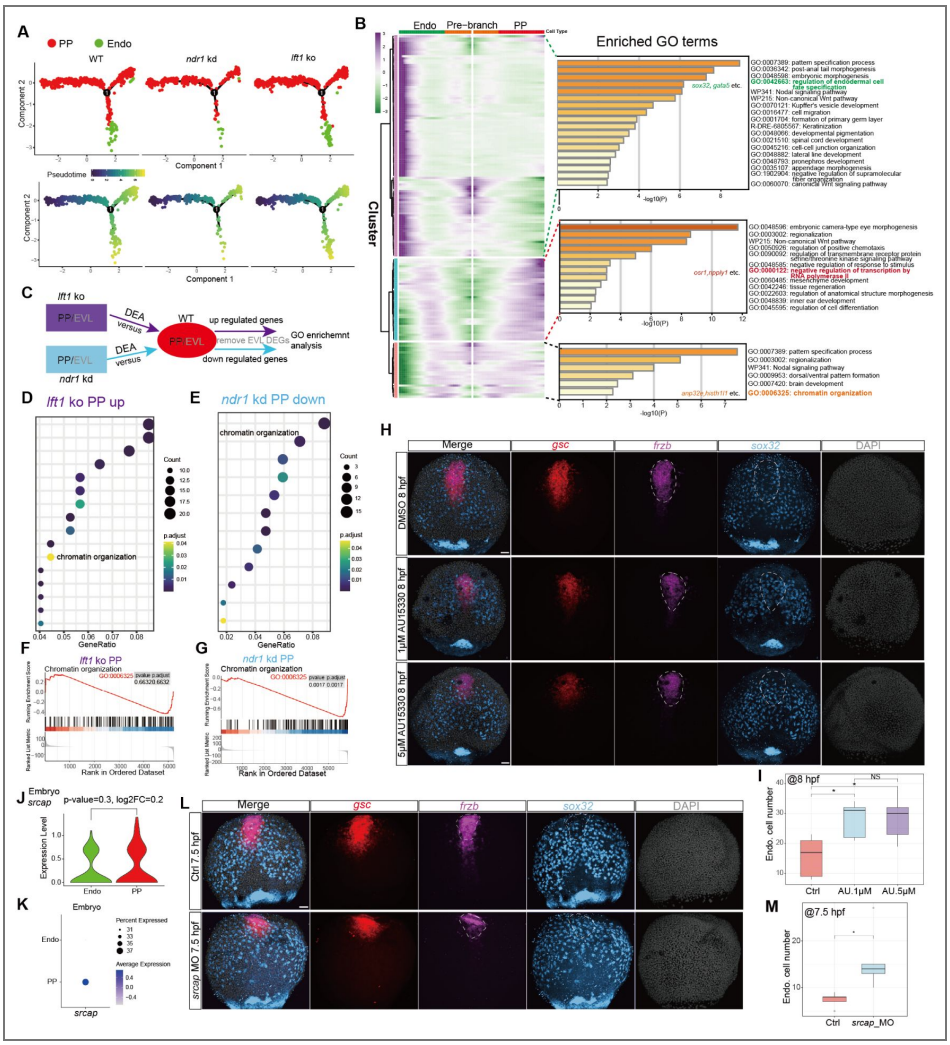

Figure 4. Single-cell trajectory tree analyses of PP and anterior Endo reveal that chromatin organization is involved in the separation of these two cell trajectories.

(A) Pseudotime trajectory analysis of PP and anterior Endo using integrated single-cell datasets of wild-type (left), *ndr1*-morphant (middle) and *lft1*-mutant (right) Nodal explants. Cells are colored by cell types (top) and pseudotime levels (bottom). (B) Heatmaps representing clusters of genes that co-vary along the pseudotime during the separation of PP and anterior Endo from common progenitors. Top GO terms enriched by the genes in each cluster are listed with their corresponding adjusted P-values. Representative GO terms of each cluster are highlighted in different colors. (C) Diagram of gene set selection for GO analysis. As a control of batch effects, EVL differentially expressed (DE) genes are removed from mesoderm PP DE genes. (D and E) GO enrichment analysis of mesoderm PP up-regulated genes in *lft1* mutants (D) and down-regulated genes in *ndr1* morphants (E). Redundancy of enriched GO terms are removed, and each GO term contains at least 10 transcripts. Note that only the top 16 enriched GO terms identified in *lft1* mutant are plotted for a visualization purpose. (F and G) GSEA of "chromatin organization" term on PP cells from *lft1* mutants (F) and *ndr1* morphants (G) respectively. P-adjust indicates Benjamini-Hochberg adjusted p-value. (H-M) SWI/SNF complexes involved in regulating cell fate separation between the PP and anterior Endo. (H) HCR co-staining of *gsc*, *frzb* and *sox32* in wild-type embryos (top, n = 5/5, n: embryos were imaged, expression observed/total imaged), as well as those treated with 1 μM (middle, n = 4/5, n: embryos were imaged, expression observed/total imaged) and 5 μM (bottom, n = 5/6, expression observed/total imaged) AU15330. Regions framed by dotted white lines were used to quantify the cell number of anterior Endo. (I) Box plot showing the cell numbers of anterior Endo in (H). (J and K) Volin plot (J) and dot plot (K) indicating the expression levels of *srcap* in PP and anterior Endo cells in zebrafish embryos. (L) HCR co-staining of *gsc*, *frzb* and *sox32* in wild-type embryos and embryos with *srcap* knockdown. Embryos at 7.5 hpf (Ctrl, n = 6/6; *srcap* MO, n = 4/5; n: embryos were imaged, expression observed/total imaged) were evaluated. (M) Box plot showing the cell numbers of anterior Endo in (L). Statistical differences between two samples were evaluated by Student's t-test (I and M). * indicates P-value < 0.05; NS indicates P-value >=0.05. Each HCR experiment was performed for at least 3 independent replicates (technical replicates); more than 40 (H) and 30 (L) embryos were analyzed. Scale bar: 50 μm (H and L).

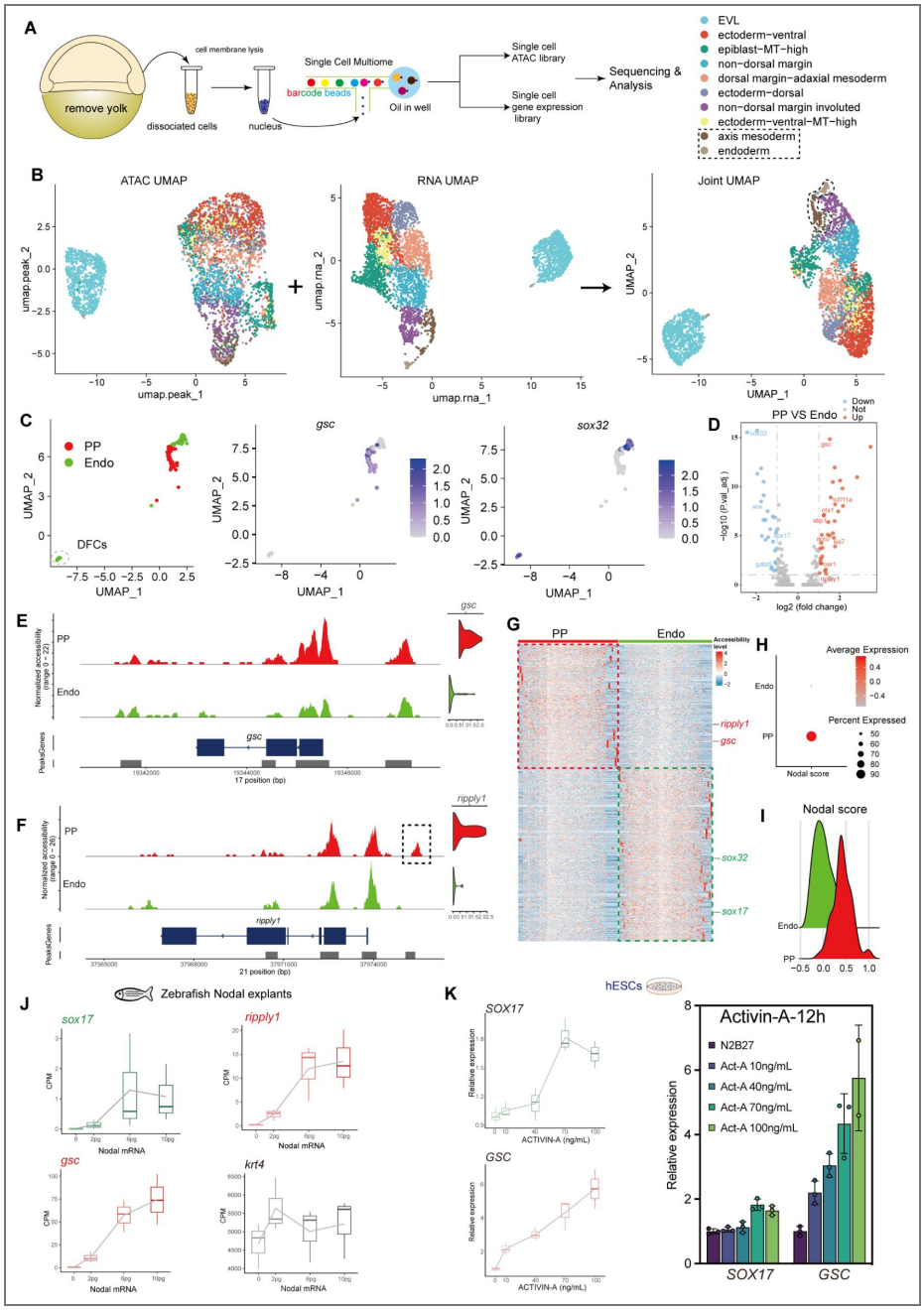

Figure 5. Investigating the mechanisms of PP and anterior Endo separation through integrative single-cell RNA-seq and ATAC-seq analysis.

(A) Schematic diagram showing the experimental workflow of single-cell multi-omics of 6 hpf zebrafish embryos. (B) UMAPs based on scATAC-Seq (left), scRNA-Seq (middle) and co-embedding scRNA-seq and scATAC-seq (right) datasets. Cells are colored by different cell types. (C) UMAP plot of the PP and Endo cells. Cells are colored by cell types (left) and the expression of *gsc* (middle) and *sox32* (right). (D) Volcano plot showing the differentially expressed genes in PP and Endo based on RNA levels of single-cell multi-omics datasets. (E-F) Track plot showing chromatin accessibility of each cell type on the gene locus of *gsc* (E) and *ripply1* (F). (G) Heatmap showing the chromatin accessibility level of genes with differential chromatin accessibility in PP and Endo. Red and green box indicates genes with higher chromatin accessibility levels in PP and Endo cells respectively. (H and I) Dot plot (H) and ridge plot (I) showing the level of Nodal score in PP and Endo. (J) Box plots showing the expression profiles of *sox17* (Endo), *gsc* (PP), *ripply1* (PP) and *krt4* (EVL) in our previous bulk RNA-seq datasets of Nodal explants. (K) Box plots (left) and bar plot (right) showing the expression levels of *SOX17* and *GSC* in the human embryonic stem cells (hESCs) treated with different dosages of Activin protein (**see Methods**). The values were calculated as the mean ± SEM (K).

Our data unveiled differential chromatin accessibility between PP and Endo cells (Figures 5G, S8K, and S8L). Correlation analysis revealed a strong correspondence between chromatin accessibility and RNA expression levels for marker genes in these two cell types (Figure S8D). To identify master regulators of PP and Endo separation, we performed differential analysis for both chromatin accessibility and gene expression (Figures 5D and S8E). A total of 170 up-regulated genes in PP, including *gsc* and *ripply1*, and 261 up-regulated genes in Endo, such as *sox32* and *sox17*, were identified. Most of these genes also exhibited differential chromatin opening states in PP and Endo fates (Figures 5E-5G and S8F-S8I).

Previous study reported that Nodal signaling can boost chromatin accessibility both *in vivo* and *ex vivo*[43,44]. And we observed that PP displayed a higher Nodal level compared to Endo cells (Figures S2J and S7E). In this ATAC and RNA-seq dataset, we also observed a higher Nodal score in PP cells (Figures 5H and 5I). Thus, it is possible that the differences in Nodal activity may lead to differential chromatin accessibility states and gene expression level in PP and Endo.

To further determine how Nodal signaling levels may differentially influence the gene expression dynamics, we first explored the expression profiles of key markers of anterior mesendoderm in the bulk RNA-seq data of Nodal explants injected with different dosages of Nodal mRNA[22]. We observed that the highest Nodal dosages did not result in further elevation of the expression of the endodermal marker *sox17* (Figure 5J), while higher concentrations of Nodal promoted the expression of *gsc* and *ripply1* (Figure 5J). Importantly, these patterns of differential marker expression in response to Nodal/Activin concentration were not only observed in our Nodal explant model but also validated in a human embryonic stem cell system (Figure 5K). These findings suggest that Nodal signaling levels play a conserved role in determining the cell fate specification, which influences the differentiation towards either Endo or PP fates. To further validate this finding, we injected different dosages of Nodal mRNA into one zebrafish animal pole blastomere at the 128-cell stage, and performed HCR co-staining for *sox32* and *frzb* at 6 hpf (Figure S9A). By quantifying the cell numbers of Endo and the area sizes of PP cluster, we found that moderate concentration of Nodal levels promoted Endo cell specification (Figures S9B-S9E).

Based on our aforementioned findings, it becomes evident that subtle variation in Nodal signaling levels may regulate PP and Endo gene expression. This variation is likely regulated by Nodal-Lefty regulatory loops, and contributes to the establishment of distinct chromatin states and transcriptional response of key transcriptional regulators within these two cell fates.

Gsc and Ripply1 collaborate to suppress the specification of anterior Endo in zebrafish

It has been reported that endoderm differentiation could be suppressed by upregulation of *gsc*[18], while the role of *ripply1* in endoderm development remains unknown, though it has been characterized as a transcriptional repressor [45,46]. Moreover, both Gsc and Ripply1 are capable of inducing a secondary axis formation when overexpressed in the ventral side of zebrafish embryos[19,22]. Combining these findings from literature with our observation of high expression of *ripply1* in PP of Nodal explants and zebrafish embryos at 6 hpf (Figures 5D and S8I), we hypothesized that Ripply1 may also play a role in suppressing Endo specification. To test this hypothesis, we generated mutant lines for *gsc* and *ripply1* in zebrafish (Figure 6B). In order to comprehensively analyze the regulatory role of Ripply1 and Gsc in the specification of cell fates between PP and anterior Endo cells, we conducted a self-cross of the Tg($gsc^{+/-}$;$ripply1^{+/-}$) lines (Figure 6A). Subsequently, we collected the descendant embryos to perform HCR co-staining for *frzb* and *sox32*, along with genotyping (Figure 6A). From the descendant embryos, we obtained a total of 9 genotypes with different phenotypes displaying variable defects in PP and anterior Endo separation (Figures 6C and S10A). Interestingly, we observed an increase in the number of anterior Endo cells with the loss of wild-type alleles of *gsc* and *ripply1* (Figures 6D, S10B, and S10C). To further investigate the mechanisms of Ripply1 suppressing anterior Endo specification in zebrafish, we injected *ripply1* mRNA at 1-cell stage in zebrafish embryos and found that Endo cell number was dramatically decreased as shown by whole-mount *in situ* hybridization **(**WISH) of *sox32* (Figure 6F). Additionally, we constructed a plasmid that contained *ripply1* cDNA

downstream of the *sox17* promoter. Injection of this plasmid at 1-cell stage also resulted in a decrease in Endo specification shown by EGFP expression (Figure 6E). Taken together, these results demonstrated that Ripply1 acted as an Endo repressor in zebrafish.

It is well established that Gsc functions as an Endo suppressor by directly binding to the promoter region of *sox17*[18]. However, the mechanism by which Ripply1 suppresses Endo specification remains unknown. To address this question, we injected HA-tagged *ripply1* mRNA into zebrafish embryos for conducting CUT&Tag experiments (Figures 6G, S11A and S11B). The CUT&Tag sequencing dataset was analysed following the standard process (Figures S11C-S11I), and 72,683 peaks were enriched in HA-*ripply1* injected samples (Figure 6H). Genes nearby the differentially enriched peaks were used to perform GO enrichment analysis (Figure 6I). Notably, we observed significant enrichment of terms related to "muscle structure development", "skeletal system development" and "connective tissue development" (Figure 6I), which were consistent with the well-established functions of Ripply1 reported in the published studies[45–47]. Additionally, we also observed significant enrichment of GO terms such as "regulation of cell differentiation", "negative regulation of signaling" and "negative regulation of transcription by RNA polymerase II" (Figure 6I), further supporting the transcriptional repressive function of Ripply1. More interestingly, Ripply1 binding peaks were enriched directly upstream of two key genes: *mespbb*, a known Ripply1 target, and *sox32*, a master regulator of endodermal specification (Figures 6J and 6K), suggesting a direct function of Ripply1 in the transcriptional regulation of the endodermal marker.

In summary, our data proposes a model within PP and anterior Endo cell fate separation. Variations of Nodal activities promote the establishment of distinct chromatin states in PP and anterior Endo fates, which is facilitated by SWI/SNF complexes. These differential chromatin accessibility patterns, in turn, drive the differential expression of key regulators such as *gsc* and *ripply1* in PP and anterior Endo. Collectively, these regulators collaborate to finely regulate the segregation of mesendoderm cell fates (Figure 7).

Discussion

In this study, we delved into the intricacies of specifying and segregating PP and anterior Endo cell fates in zebrafish through single-cell multi-omics and live imaging analyses. Our findings revealed the origin of anterior Endo from PP progenitors and unveiled a correlation between subtle differences of Nodal signaling levels and fate commitment. These signaling variations were identified as potential regulators influencing the expression of cell-fate-specific genes at both epigenetic and transcriptional levels, with the collaboration of SWI/SNF complexes. Through gain-/loss-of-function studies, we demonstrated the collaborative suppression of Gsc and Ripply1 in anterior Endo specification.

While the induction of mesendodermal cell fates has been extensively studied[9,12,48,49], the mechanisms underlying the separation of meso- and endo-fates from common progenitors remain a subject of ongoing research. The widely accepted notion posits that high Nodal signaling levels promote endoderm specification, while lower levels induce mesoderm[11–13]. These quantitative effects of Nodal signaling lead to differential activation of transcriptional targets which have differential sensitivity to Nodal activation[2,37]. However, recent studies challenge this perspective, proposing that Nodal signal level may not strictly determine cell fate separation of mesendoderm. Instead, Nodal signaling provides competency for mesendodermal progenitors to stochastically switch and commit to lineage-specific fates in zebrafish[16]. Moreover, the duration of Nodal signaling has been implicated in specifying different cell fates of mesendoderm, with prolonged signaling promoting prechordal plate specification and suppressing endoderm induction[18]. Besides, other important signaling pathways in early embryonic development also interact with Nodal signaling to regulate cell fate separation[13,31]. It has been well established that Fgf signaling plays a crucial role in regulating cell fate switching between endoderm and mesoderm in lateral margin of zebrafish embryo. In our current work, we preliminarily explored whether Fgf signaling plays roles in regulating cell fate segregation between PP and anterior Endo cells (Figure S12). We observed that several genes related to Fgf signaling were highly enriched in PP and anterior Endo cells (Figure S12B), and inhibition of Fgf signaling could obviously increase the cell

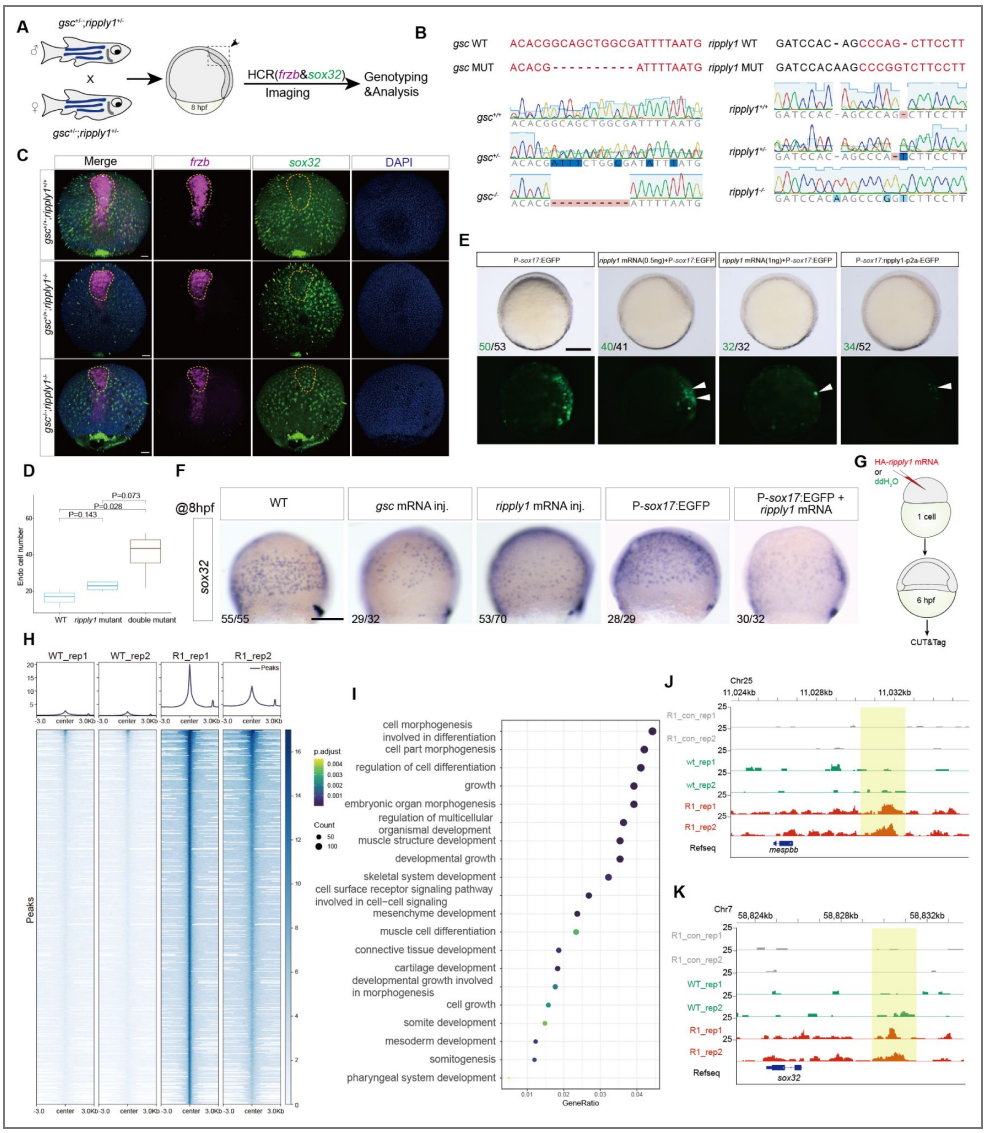

Figure 6. Exploring the mechanisms underlying the role of Ripply1 in the regulation of cell fate separation between PP and anterior Endo.

(A) Schematic diagram illustrating the workflow for investigating the role of *gsc* and *ripply1* in mesendoderm cell fate separation through loss-of-function studies. (B) Schematic presentation of *gsc* and *ripply1* mutations and the sequencing results of each genotype. The mutations are a 10-bp deletion and a 1-bp insertion in *gsc* and *ripply1* coding sequences respectively. Texts in red indicate coding sequences. Sanger sequencing results display at the bottom, showcasing wild-type (top), heterozygous mutant (middle) and homozygous mutant (bottom) embryos of *gsc* and *ripply1* mutations. (C) HCR co-staining of *sox32* and *frzb* in the embryos of Tg($gsc^{+/+}$;$ripply1^{+/+}$) (WT), Tg($gsc^{+/+}$;$ripply1^{-/-}$) (*ripply1* mutant) and Tg($gsc^{-/-}$;$ripply1^{-/-}$) (double mutant) at 8 hpf (Here embryos of three different genotypes are shown. Other genetypes are shown in Figure S10A.) [Tg($gsc^{+/+}$;$ripply1^{+/+}$), n = 4/5; Tg($gsc^{+/+}$;$ripply1^{-/-}$), n = 4/6; Tg($gsc^{-/-}$;$ripply1^{-/-}$), n = 5/5; expression observed/total imaged]. (D) Box plot showing the quantification of the numbers of anterior Endo cells identified in (C). (E and F) Assessing the role of *ripply1* in anterior Endo specification. EGFP driven by *sox17* promotor (E) and WISH of *sox32* (F) are used to indicate the Endo cells. Embryos with *gsc* overexpression are used as a positive control. The statistics of observed/total sampled embryos were shown in bottom left of the panels (E and F). (G) Schematic diagram showing the strategy of conducting CUT&Tag experiment for *ripply1*. (H) Metaplots and heatmaps for CUT&Tag signals over R1_rep1 binding peaks (n=72,683) in wild-type and HA-*ripply1*-injected embryos. Two replicates were performed. (I) Dot plot showing the enriched GO terms using the genes that annotated from the differentially enriched peaks. (J and K) Screenshot of R1_con (technical control, without primary antibody, **see Methods**), WT (ddH_2O-injected) and R1 (HA-*ripply1*-injected) CUT&Tag peaks around *mespbb* (J, positive control) and *sox32* (K) loci. Each HCR and ISH experiment was performed for at least 3 independent replicates (technical replicates). Statistical differences between two samples were evaluated by Student's t-test (D). Scale bar: 50 μm (C) and 200 μm (E and F).

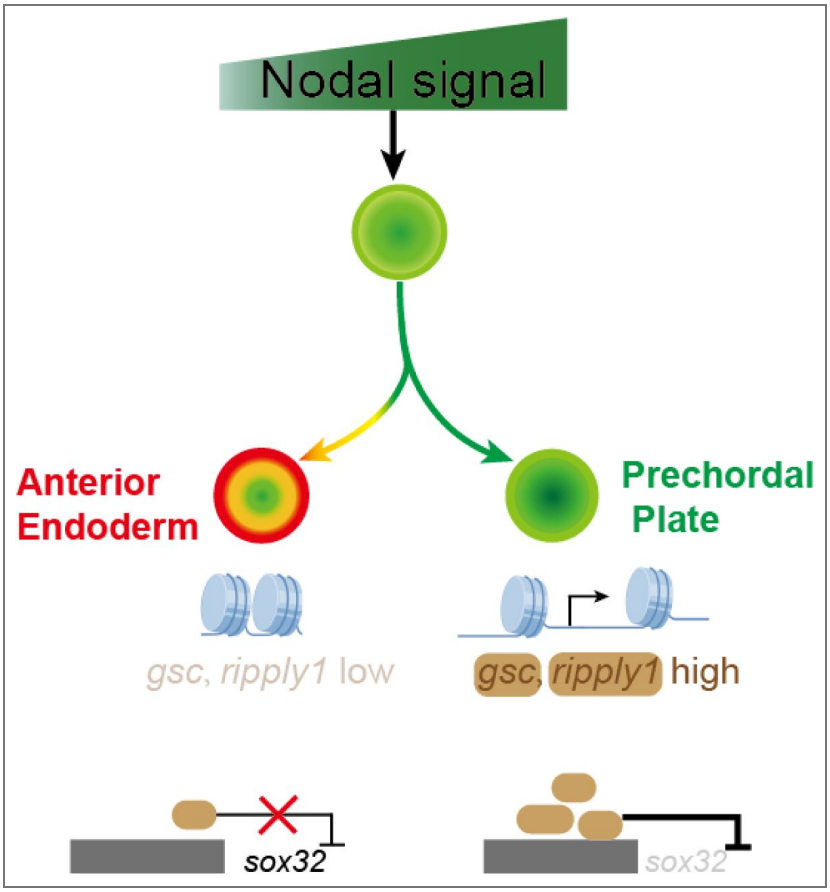

Figure 7. **Model underlying the separation of PP and anterior Endo.**

An outline depicts PP and anterior Endo specification. Nodal signaling drives differential chromatin states between PP and anterior Endo through epigenetic regulators. And then, key transcriptional factors, such as *gsc* and *ripply1*, are differentially expressed in these two cell trajectories and suppress anterior Endo specification by directly binding to the *cis*-elements of *sox32*.

number of anterior Endo (Figures S12A and S12C). The findings above suggest that Fgf signaling inhibits the specification of anterior Endo from PP progenitors in zebrafish, which is a similar process to the suppression observed during lateral endoderm specification. However, further investigation is required to understand the specific interactions between Fgf signaling[50] and factors such as Gsc, Ripply1, Sox32[51,52] and the SWI/SNF complexes, as well as their roles in regulating the fate diversification of PP and anterior Endo fates.

Our study contributed a new layer of understanding to the regulation of mesendodermal cell fate segregation by Nodal signaling. We proposed that Nodal signaling may influence chromatin remodeling factors, thereby affecting the chromatin accessibility states of key regulators such as *gsc* and *ripply1*. It is worth noting that *gsc* and *ripply1* have been identified as direct targets of Nodal signaling[22]. This suggests that the transcription of these genes may directly and sensitively respond to variations in Nodal signaling levels. Based on this understanding, we hypothesize that Nodal signaling can achieve more precise and robust control over the fate segregation of closely related cell types by introducing an additional layer of epigenetic regulatory mechanisms. Additionally, our findings underscored the pivotal role of chromatin openness in morphogen interpretation during development, emphasizing the need to consider chromatin structure when studying how morphogen gradients regulate patterning formation across different species.

In our work, combining single-cell multi-omics analyses with perturbation experiments, we revealed that chromatin remodeling together with Nodal morphogen promoted the PP and anterior Endo separation in zebrafish. However, which and how chromatin remodeling factors respond to Nodal signaling to facilitate fate diversification remains largely unknown. Our current study identified a key member of SWI/SNF complexes, *srcap*, whose expression was slightly higher in the PP than in the anterior endoderm cells. What triggers the transcriptional difference of *srcap* in these two cell types is still an open question. One of the hypotheses is that the transcription of *srcap* is very sensitive to Nodal signaling (Nodal concentration or Nodal duration), and a little variance in Nodal signaling can drive differential activation of *srcap*. Another speculation is that the activation of *srcap* needs the assistance of pioneer factors, like forkhead family[40,43], whose binding motifs are particularly accessible in PP cells compared to anterior Endo cells (Figures S8K and S8L). All these hypotheses need to be further determined in future works.

Despite these insights, several questions remain unanswered. Firstly, we discovered a potential role of Nodal activity levels in the separation of PP and anterior Endo cells. However, further investigation is required to understand how Nodal activity levels and signaling duration are integrated to regulate this process. Secondly, although our study determined the collective role of Gsc and Ripply1 in suppressing endoderm specification, the potential involvement of other unidentified transcriptional repressors, as suggested in previous work[18], warrants further investigation. Notably, even in double homozygous mutants of *gsc* and *ripply1*, the specification of PP clusters was still detected, and the enrichment of another transcriptional repressor, *osr1*, in PP cells (Figure S8J) suggested a complex network of collective endoderm repressors in preventing cells from differentiating into anterior Endo. Thus, it would be worth studying how Gsc, Ripply1, Osr1 and other potential factors collectively regulate PP and anterior Endo specification in the future. Lastly, the anterior endoderm and ventral-lateral endoderm were mixed together in our single-cell ATAC-seq dataset, which brought in interferences when we interpreted the mechanisms of the separation between PP and anterior Endo. More replicates of embryonic single-cell multi-omics or performing single-cell multi-omics on Nodal explants may help to address this issue in future studies.

Methods

Animal ethics

Zebrafish strains were conducted following standard procedures, and experimental procedures were approved by the Institutional Review Board of Zhejiang University. The protocol number is ZJU20220375.

Generation of Nodal explants

Nodal explant was generated as our previous work described[53]. 10 pg *ndr2* mRNA was injected into one blastomere of zebrafish embryonic animal pole at the 128-cell stage. About half of the animal pole region of the embryo was cut off from the blastula at the 1k-cell stage, and was cultured in a Petri dish coated with 1.5% agarose gel filled with Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) with 10 mM HEPES, 1x minimum essential medium (MEM) containing non-essential amino acids and supplemented with 7 mM $CaCl_2$, 1 mM sodium pyruvate, 50 μg/ml gentamycin, 100 μM 2-mercaptoethanol, 1× antibiotic-antimycotic (15240062, Thermo Fisher) and 10% serum replacement. For generating *ndr1*-morphant Nodal explants, *ndr1* morpholino (0.5 mM, 2 nL) was injected into yolk of wild-type zebrafish embryos at 1-cell stage; while for generating *lft1*-mutant Nodal explants, the embryos of *lft1* mutants were used to construct Nodal explants. The sequence of *ndr1* morpholino was 5' - ATGTCAAATCAAGGTAATAATC CAC - 3'.

Live imaging

Tg(*gsc*:EGFP;*sox17*:dsRed) embryos and Nodal explants generated from those embryos were used to perform live imaging analysis. 0.5% low-melt agarose was used to mount the embryos or Nodal explants in glass-bottom dishes (Cellvis, D35-20-0-N). The embryos or explants were live-imaged by confocal laser scanning microscopy (OLYMPUS FV12-IXCOV) using a 20X objective lens or a 40X oil immersion lens.

In situ hybridization chain reaction (HCR)

HCR co-staining of *frzb*, *sox32* and *gsc* was performed as previous studies described[21]. The probes of those genes were ordered and produced from Molecular Technologies (*http://www.moleculartechnologies.org/*). Embryos were fixed in DEPC-treated PBS with 4%(w/v) paraformaldehyde at 4°C overnight. The embryos were incubated in 500 μL 30% formamide probe hybridization with 2 pmol of each HCR probe set at 37°C overnight. Next day, 30% formamide probe wash buffer was used to stop hybridization by repeated washing of the embryos at 37°C. Fluorescent signals were generated and amplified by probes that bound to fluorescent HCR amplifiers in an amplification buffer overnight at room temperature. To halt this process, the samples were washed several times using 5× SSC with 0.001% Tween 20. The reaction buffers, fluorescent hairpins and probes were manufactured by Molecular Technologies. OLYMPUS FV12-IXCOV confocal microscope was used to photograph those HCR co-staining samples.

Double-color RNA-fluorescence *in situ* hybridization (FISH)

Double-color FISH of *gsc* and *sox17* was conducted according to the protocol from manufactory (*http://www.pinpoease.com/*, GD Pinpoease Biotech Co., Ltd.). Wild-type embryos or Nodal explants (6 hpf, 7 hpf and 8 hpf) were fixed in DEPC-treated PBS with 4%(w/v) paraformaldehyde at 4°C overnight. Pre-A solution was used to inhibit the peroxidase activity. Target RNA molecules were exposed through protease treatment, followed by hybridization with probes at 4°C for 2 hours. The fluorescent signal was amplified through sequential reactions 1, 2, and 3. Lastly, a tyramide fluorescent substrate (OpalTM520, Akoya Biosciences) was used to incubate the embryos, enabling fluorescent labeling of the target RNA through the Tyramide Signal Amplification (TSA) assay.

Immunostaining and quantification of pSmad2

Samples were fixed at 4% paraformaldehyde overnight for pSmad2/3 immunostaining. The experiment was performed as previously described[53]. Confocal laser scanning microscopy (OLYMPUS FV12-IXCOV) was used to scan the immunostained samples. Imaris (Version 9.7) software was used to quantify the signaling activity of pSmad2 (488 nm), RFP (561 nm) and DAPI (405 nm). Firstly, the raw image data was loaded in Imaris and visualized in a three-dimensional (3D) view. The Spot plugin from Imaris was used to detect spots with pSmad2 signal. The diameter of spot detection was set to 5-6 μm for GFP channel. The spots were automatically identified with default parameters. And then, as the identification of some spots was inaccurate, we manually added or removed some spots. Lastly, the coordinates and signal intensities of each spot were

exported for next-step analyses. To make all images comparable, we used DAPI signal as an internal control to normalize the signal intensity in other channels. When plotting a pSmad2 signal intensity gradient along a distance, the cell with the highest pSmad2 signal was assigned as the start point, and then the distance of other cells was calculated by comparing them with the start point cell. The fitting curve was plotted by ggplot2[54] in R (*https://www.r-project.org*).

Whole-mount *in situ* hybridization (WISH)

The processes of WISH and the construction of *sox32* probes were described in our previous work[53]. Embryos were fixed in DEPC-treated PBS with 4%(w/v) paraformaldehyde at 4°C overnight. To enable long-term storage, the embryos were dehydrated using 100% methanol. For WISH experiments, probes were labeled with digoxigenin-11-UTP (Roche Diagnostics), and the substrate was NBT/BCIP.

Activation of Activin/Nodal signaling in human embryonic stem cell system

Human embryonic stem cell (hESC) lines were used between passage 45-50. Cells were seeded equally on matrigel-coated plate and cultured in mTeSR medium. Before the experiment, each group was pretreated in N2B27 medium for 6 hours. Then the control group was maintained in N2B27, while the experiment groups were cultured in N2B27 medium supplemented with different concentrations of Activn-A (0, 10, 40, 70 and 100 ng/mL). The cellular RNA of each group was extracted with Trizol after 12 hours of treatment.

Overexpression of *gsc* and *ripply1* in zebrafish embryos

The mRNA of *gsc* and *ripply1* (synthesized *in vitro*) and *sox17* promotor-driven *ripply1* plasmid were co-injected into zebrafish embryos at 1-cell stage. The ddH_2O was injected as control. The embryos were imaged or fixed at 8 hpf. The *sox32* probe was used for WISH.

Generating heterozygous embryos of *gsc* and *ripply1*

The *ripply1* mutants were obtained from Dr. Ming Shao's lab at Shandong University. We knockout *gsc* in *ripply1*-mutant embryos by CRISPR-Cas9 system. F0 embryos were raised up, and then crossed with wild-type (AB). The descendant embryos (F1) were raised up and genotyped. The adult zebrafish of the target genotype were selected.

Quantifying the cell number of anterior Endo

Imaris (version 9.7) was used to identify the anterior Endo cells based on the HCR images. Briefly, the raw image data was imported into Imaris, and the object was visualized in a 3D view. The Spot plugin from Imaris was used to detect each cell. The diameter of spot detection was set to 10-12 μm for GFP channel (staining for *sox32* or *sox17*). The candidate spots were selected by setting an appropriate quality threshold. Finally, the coordinates and signal intensities of each spot were exported from Imaris and used for further analysis.

CUT&Tag sample preparation

HA-*ripply1* sequence with homologous sequences was generated through two PCR steps using two separate sets of primers (Table S1, reverse primer was common, *ripply1*-F1 primer: tcaaggcctctcgag cctctagaATGTACCCATACGATGTTCCAGATTACGCT; *ripply1*-F2 primer: ACGATGTTCCAGATTACGCTGG CAGCATGAATTCTGTGTGCTTTGCCACT; *ripply1*-R primer: TACGACTCACTATAGTTCTAGAtcagttgaaagc tgtgaagtga). The details of generating mRNA *in vitro* was described in our previous protocol[21]. Briefly, the HA-*ripply1* PCR products were inserted into pCS^{2+} plasmid through homologous recombination using ClonExpress II One Step Cloning Kit. The plasmid containing HA-*ripply1* was linearized using *Not1* cleavage, and HA-*ripply1* mRNA were generated through mRNA *in vitro* synthesis using mMESSAGE mMACHINETM SP6 transcription kit. Wild-type embryos were injected with 500 pg HA-*ripply1* mRNA or ddH_2O at one-cell stage and harvested at 6 hpf. Yolk was removed through pipetting, and the cells were collected. The cell viability was assessed by Trypan blue staining and the cell number was then counted.

CUT&Tag experiment

CUT&Tag was performed on samples of wildtype, HA-*ripply1*-injected and HA-ripply1-injected without primary antibody using Hyperactive Universal CUT&Tag Assay Kit for Illumina Pro (Vazyme, TD904), and each sample had two replicates. Briefly, around 100,000 cells were collected and immobilized on Concanavalin A-coat Magnetic Beads. Cells were then incubated with primary antibody (HA-Tag Rabbit mAb, Cell Signaling Technology, 3724S, 1:50 dilution) at 4°C for 12 h-16 h. Tethered cells were washed thoroughly to remove excessive primary antibody and incubated with secondary antibody (Goat Anti-Rabbit IgG H&L, Vazyme, Ab207-01, 1:100 dilution) for 60 minutes at room temperature. Then samples were washed thoroughly to remove unbound secondary antibody and incubated with pA/G-Tnp for 1 hour at room temperature. DNA was then fragmentated through Trueprep Tagment Buffer L (TTBL). Fragmented DNA was extracted using DNA extract beads and amplified through PCR. PCR products were purified using VAHTS DNA CLEAN Beads (Vazyme, N411) and then sent to Novogene Co., Ltd. (Beijing, China) for sequencing. Paired-end sequencing at 150 bp read length was performed on an NovaSeq 6000 System.

CUT&Tag analysis

Sequencing data of CUT&Tag samples were first treated by Cutadapt v4.5[55] to remove adapters and trim reads using the following parameters "*-m 18 -q 30,30 --max-n=0.05 -e 0.2 -n 2*". The trimmed reads were then aligned to zebrafish genome (danRer11) through Bowtie2 v2.5.2[56] using the following parameters "*--end-to-end --very-sensitive --no-mixed --no-discordant --phred33 -I 10 -X 700*". Samtools v1.18[57] and BEDtools v2.31.0[58] were then used for post-alignment processing to covert file formats in preparation for the peak calling and visualization. BigWig tracks were generated for visualization of chromatin landscapes in interested regions using the Integrative Genomics Viewer (IGV)[59]. To assess the reproducibility between replicates, the genome was spilt into 500 bp bins, and a Pearson correlation of the $\log_2$-transformed read counts in each bin was calculated between datasets.

SEACR[60] was used to call "non stringent" peaks for wild-type and HA-*ripply1*-injected samples using merged HA-*ripply1*-injected samples without primary antibody as control. Additionally, top 1% of enrich regions were selected as peaks by AUC without controls. Peaks were annotated by ChIPpeakAnno v3.34.1[61] using UCSC annotation from TxDb.Drerio.UCSC.danRer11.refGene v3.4.6[62]. Significantly enriched peaks in HA-*ripply1*-injected samples were identified using DESeq2 v1.40.2[63], and enriched GO terms were identified using clusterProfiler v4.9.0.2[64]. Signal intensity heatmaps were generated using deeptools v3.5.4[65]. The peaks of HA-*ripply1*-injected rep1 were used as the reference to generate computeMatrix using the *reference-point* model (*--skipZeros --afterRegionStartLength 3000 --beforeRegionStartLength 3000 --referencePoint center*), and *PlotHeatmap* function was used to plot signal intensity. To obtain more convincible Ripply1-binding targets, we annotated all peaks enriched in the treatment group and performed differential analysis, selecting genes with $\log_2$FoldChange > 3, padj < 0.5, and baseMean > 30 as candidate targets of Ripply1 binding.

Processing and analyzing single-cell RNA-seq data

Illumina sequencing reads were aligned to the reference genome (GRCz11) of zebrafish through 10× Genomics CellRanger pipeline (version 3.0.2) with default parameters. Nodal explants generated from *lft1* mutants and *ndr1* morphants yielded 5,419 and 5,195 cells respectively. The expression matrix of each sample was obtained via CellRanger pipeline analysis and used for further analysis. The single-cell RNA-seq data of wild-type Nodal explants was reused from our previous study[53].

Processing and analyzing single-cell multi-omics data

Illumina sequencing reads were aligned to the reference genome (GRCz11) of zebrafish using 10× Genomics CellRanger-arc pipeline (version 2.0.0) with default parameters. Expression matrix and fragments files were obtained after running the pipeline. 4,879 cells were obtained. Signac[66] was used for downstream analyses with default parameters. Low-quality cells were filtered out with nCount_ATAC < 100000 & nCount_RNA < 50000 & nCount_ATAC > 1000 & nCount_RNA > 1000 &

nucleosome_signal < 2 & TSS.enrichment > 1 & percent.mt < 4. And then, gene expression data and DNA accessibility data were processed respectively by Signac with default parameters. The RNA expression level was utilized to identify cluster markers for cell annotation. Gene activity matrix was obtained by assessing the chromatin accessibility associated with each gene through *GeneActivity* function.

Differential expression analysis of PP cells between wild-type, *lft1*-mutant and *ndr1*-morphant Nodal explants

The three sing-cell RNA-seq datasets of Nodal explants constructed from wild-type embryos, *lft1* mutants and *ndr1* morphants were integrated by Seurat[67]. To investigate the functional and regulatory effects of Nodal signals on cell differentiation, we compared the wild-type mesoderm PP cells to the mesoderm PP cells in *lft1* mutants and *ndr1* morphants respectively, and differentially-expressed (DE) genes were identified through Seurat v4.0.2 (*https://github.com/satijalab/seurat*) with a Bonferroni adjusted p-value < 0.05. As it is known that EVL is not affected by Nodal signaling[53,68], DE genes identified in EVL were regarded as background noises and were removed from the mesoderm PP DE genes. The mesoderm PP DE genes positively correlated with Nodal concentration were then selected, constructing two gene sets: upregulated DE genes in *lft1* mutants and downregulated DE genes in *ndr1* morphants. GO enrichment analysis was performed on these two gene sets respectively using clusterProfilter v4.2.2 (*https://github.com/YuLab-SMU/clusterProfiler*). The enriched GO terms in "biological process" subontology were identified using a Benjamini-Hochberg adjusted p-value < 0.05.

Cell-cell communication analysis by LIANA

R package, LIANA[30] v0.1.5 (*https://github.com/saezlab/liana/*), was used for the investigation of ligand-receptor interactions among different cell types. The ligand-receptor analysis was performed against the ligand-receptor reference achieved from Omnipath resource using 5 different methods implemented in LIANA, including Natmi, Connectome, SingleCellSingalR, iTALK and CellPhoneDBv2. The consensus rank aggregated from the results of these methods was used for the identification of enriched ligand-receptor interactions.

Inferring cell-cell communication by CellChat

CellChat[69] was employed to systematically analyze cell-cell communication based on prior known zebrafish ligand-receptor interaction database CellChatDB. As some known interactions were missing in the database, we manually added some interactions of interest into the database. The expression data of scRNA-seq was preprocessed to identify over-expressed ligands and receptors for each group. CellChat inferred communication probability between two interacting cell groups based on the average gene expression of a ligand in one cell group and the average gene expression of a receptor in another cell group. The communication probabilities of signaling pathways were then calculated by summarizing the communication probabilities of all ligands-receptors interactions associated with each pathway.

Gene set enrichment analysis

The wild-type mesoderm PP gene expression was compared to the mesoderm PP gene expression of *lft1* mutants and *ndr1* morphants respectively using Seurat v4.0.2 (*https://github.com/satijalab/seurat*). The genes expressed in more than 10% cells in either of the two compared populations were retained. A ranked list was formed on the retained genes using sign($\log_2$FC) * (-$\log_{10}$PValue) as the ranking statistic. The mapping between GO terms and zebrafish genes was achieved through org.Dr.eg.db v3.14.0 (*https://bioconductor.org/packages/org.Dr.eg.db/*). GSEA was performed on the selected GO terms by clusterProfiler v4.2.2 (*https://github.com/YuLab-SMU/clusterProfiler*), using fgsea (*https://github.com/ctlab/fgsea*) with 10^5 interactions. R package, enrichplot v1.14.2 (*https://github.com/YuLab-SMU/enrichplot*), was then used to visualize the GSEA results.

Single-cell RNA-seq trajectory analysis

Three different methods were used to perform single-cell RNA-seq trajectory analysis. The developmental trajectory tree of zebrafish embryos and Nodal explants was constructed by URD[70] with default parameters as described in previous studies[70]. The branchpoint preference plot of PP and anterior Endo was constructed by URD with default parameters. Firstly, *branchpointPreferenceLayout* function was used to define the preference layout for the branchpoint. And then stage, pseudotime information and gene expression were plotted on the branchpoint using *plotBranchpoint* function. The expression matrix of PP and anterior Endo cells was also used to construct trajectory tree by monocle2[27] and monocle3[71] with default parameters. And differential expression analysis was performed at the branching point of PP and anterior Endo separation on trajectory tree constructed by *BEAM* function in monocle2. Modules of co-regulated genes along PP or anterior Endo differentiation trajectory were identified by *find_gene_modules* function in monocle3 with default parameters. The cells contributed to the preference layout for the Endo and PP brachpoint from URD analysis were also selected as inputs for Palantir[26]. We used Harmony[72] to calculate the augmented affinity matrix for data of all time points. Data was visualized using force directed layouts. Palantir was used for downstream analysis, and a $nanog^{high}$cell was used as the start cell. A $frzb^{high}$ cell (PP) and a $sox17^{high}$ cell (Endo) were selected as the terminal cells. Anterior Endo and PP cell differential trajectory was detected by *palantir.core.run_palantir* function from Palantir. And finally, gene expression trend along Palantir inferred pseudotime was calculated by *palantir.presults.compute_gene_trends* function.

Calculating Nodal score in single-cell RNA-seq data

Nodal functions through activating a set of key downstream genes in a concentration-dependent manner. Our previous study identified 105 Nodal immediate target genes by analyzing bulk RNA-seq datasets of Nodal mRNA injected explants[22]. We overlapped these 105 genes with 61 Nodal direct targets, which were previously identified through ChIP-seq analysis of pSmad2[37]. This analysis allowed us to pinpoint 29 Nodal downstream genes whose expression was highly sensitive to Nodal activity. To quantify Nodal activity, we defined a Nodal score based on its downstream transcriptional response. This score was calculated by regressing out the expression levels of these 29 Nodal downstream genes. To accomplish this, the *AddModuleScore* function in Seurat was employed.

Data availability

The data generated in this study is deposited in the Gene Expression Omnibus (GEO). The single-cell RNA sequencing and single-cell multi-omics data is under accession number GSE223636, and the CUT&Tag data is under the accession number GSE249292. Any custom code and data are available from the authors upon request. All analyses are based on previously published code and software.

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Additional information

Author Contributions

PFX, TC and HQL conceived and designed the research; TC, XL, YD, YMT, YYX, CYC, CL, YFL, YH, DHZ, XX, XXF, ZXJ, JYL and JM performed research; TC and YD analyzed the single-cell RNA-seq and single-cell multi-omics data; YD performed the CUT&Tag experiment and analyzed the data; TC, YD, HQL and PFX wrote the manuscript; all authors reviewed and approved the manuscript. PFX and HQL supervised this study. Fundings for the study were provided by PFX, TC and YD.

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Additional files

Supplementary Information

Movie S1

Movie S2

Movie S3

Movie S4

Movie S5

Table S1

Data S1

Data S2

Data S3

Data S4

Data S5

Data S6

Data S7

Data S8

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74. **Sagerstrom C.G.,** Gammill L.S., Veale R., Sive H (2005) Specification of the enveloping layer and lack of autoneuralization in zebrafish embryonic explants. *Developmental dynamics : an official publication of the American Association of Anatomists* **232**:85-97 https://doi.org/10.1002/dvdy.20198

Peer reviews

Reviewer #1 (Public review):

Summary:

During vertebrate gastrulation, mesendoderm cells are initially specified by morphogens (e.g. Nodal) and segregate into endoderm and mesoderm in part based on Nodal concentrations. Using zebrafish genetics, live imaging, and single-cell multi-omics, the manuscript by Cheng et al presents evidence to support a claim that anterior endoderm progenitors derive primarily from prechordal plate progenitors, with transcriptional regulators goosecoid (gsc) and ripply1 playing key roles in this cell fate determination. Such a finding would represent a significant advance in our understanding of how anterior endoderm is specified in vertebrate embryos.

Strengths:

Live imaging based tracking of PP and endo reporters (Fig 2) are well executed and convincing, though a larger number of individual cell tracks will be needed. In the first round of review, only a single cell track (n=1) was quantified. Now, more tracks have been collected but these data are still not clearly reported in a way that warrants their evaluation.

Weaknesses:

(1) While the authors have made an effort to include a gsc:CRE lineage tracing component to their study, the experimental data now presented (Figure S4E and reviewer figures) could be much stronger and more thorough. In the new panel, authors show a single microscopy image containing both red and green fluorescent cells. The green signal, which seems to mark the PP, is presumably derived from Tg(gsc:EGFP). The red mCherry signal is presumably derived from the combined effects of a Tg(gsc:CRE) and Tg(sox17-lox-STOP-lox-mCherry), i.e., labeling the progeny of gsc+ progenitors which expressed CRE and underwent recombination to create a productive endoderm-specific Tg(sox17:mCherry) reporter. The result appears to be promising and in line with the authors' predictions. However, this result should be strengthened by performing the experiment in stable transgenic lines (not just freshly injected F0 embryos) and should be properly quantified. The authors state in the legend that "the experiment was performed on at least 3 independent replicates", but offer no further detail, explanations, or quantifications. This issue is reminiscent of concerns from the previous round of review, where live tracking data derived from examining just a single (n=1) cell were presented. These standards might be adequate for generating preliminary insights, but fall far below what we would have previously expected from an Elife publication.

(2) I found the authors' rebuttal to my concerns about URD-trajectory derived insights and gsc/sox17 expression timing confusing. The authors claim that they get different results regarding gsc expression prevalence in the hypothetical PP/endoderm progenitor cluster when comparing scRNAseq data from embryos vs explants. Then they seem to use this difference to justify the use of the explants over the embryos - presumably because the explants enriched for the behavior that they wanted to see? They conclude that "directly using embryonic data to dissect the mechanism of fate separation between PP and anterior endoderm might not yield highly accurate results." I strongly disagree with this. I would argue that the whole-embryo dataset is likely doing a better job of cleanly separating these trajectories from each other.

(3) My concern about the use of n=1 cell for live tracking has been partially but not fully addressed. The authors should plot data point from each individual cell in the revised Figure 2D, instead of just saying "multiple cells" they should report the total number of cells that are actually included now (n=?), and should provide representative movies for a few additional examples.

At present the authors' data, as presented, still only partially support their aims and conclusions.

https://doi.org/10.7554/eLife.100200.2.sa3

Reviewer #2 (Public review):

Summary:

During vertebrate gastrulation, the mesoderm and endoderm arise from a common population of precursor cells and are specified by similar signaling events, raising questions as to how these two germ layers are distinguished. Here, Cheng and colleagues use zebrafish gastrulation as a model for mesoderm and endoderm segregation. By reanalyzing published single cell sequencing data, they identify a common progenitor population for anterior endoderm and the mesodermal prechordal plate (PP). They find that expression levels of PP genes gsc and ripply are among the earliest differences between these populations, and that their increased expression suppresses the expression of endoderm markers. Further analysis of chromatin accessibility and Ripply CUT-and-TAG is consistent with direct repression of endoderm by this PP marker. This study demonstrates roles for Gsc and Ripply in suppressing anterior endoderm fate, but this role for Gsc was already known and the effect of Ripply is limited to a small population of anterior endoderm.

Strengths:

Integrated single cell ATAC- and RNA-seq convincingly demonstrate changes in chromatin accessibility that may underlie segregation of mesoderm and endoderm lineages, including gsc and ripply. Identification of Ripply-occupied genomic regions augments this analysis. The genetic mutants for both genes provide strong evidence for their function anterior mesendoderm development, although these phenotypes are subtle.

Weaknesses:

The use of zebrafish embryonic explants for cell fate trajectory analysis (rather than intact embryos) is not justified. Much of the work is focused on the role of Nodal in the mesoderm/endoderm fate decision, but the results largely confirm previous studies and again provide few new insights. The authors similarly confirm previous findings that FGF signaling likely plays a larger role in this fate decision, but these results are largely overlooked by the authors.

https://doi.org/10.7554/eLife.100200.2.sa2

Reviewer #3 (Public review):

Summary of work:

Cheng, Liu, Dong, et al. demonstrate that anterior endoderm cells can arise from prechordal plate progenitors, which is suggested by pseudotime reanalysis of published scRNAseq data, pseudotime analysis of new scRNAseq data generated from Nodal-stimulated explants, live imaging from sox17:DsRed and gsc:eGFP transgenics, fluorescent in situ hybridization, and a Cre/Lox system. Early fate mapping studies already suggested that progenitors at the dorsal margin give rise to both of these cell types (Warga), and live imaging from the Heisenberg lab (Sako 2016, Barone 2017) convincingly showed this previously. However, the data presented for this point are very nice and further cement this result. Though better demonstrated by previous work (Alexander 1999, Gritsman 1999, Gritsman 2000, Sako 2016, Rogers 2017, others), the manuscript presents confirmatory data that high Nodal signaling is required for both cell types. The manuscript generates new single-cell RNAseq data from Nodal-stimulated explants with increased (lft1 KO) or decreased (ndr1 KD) Nodal signaling and multi-omic ATAC+scRNAseq data from wild-type 6 hpf embryos, which can be used as a resource, though few new conclusions are drawn from it in this manuscript. Lastly, the manuscript presents suggests that SWI/SNF remodelers and Ripply1 may be involved in the anterior endoderm - prechordal plate decision, but these data are less convincing. The SWI/SNF remodeler experiments are unconvincing because the demonstration that these factors are differentially expressed or active between the two cell types are weak. The Ripply1 gain-of function experiments are unconvincing because they are based on incredibly high overexpression of ripply1 (500 pg or 1000 pg) that generates a phenotype that is not in line with previously demonstrated overexpression studies (with phenotypes from 10-20x lower expression). Similarly, the cut-and-tag data seems low quality and is based on high overexpression, so may not support direct binding of ripply1 to these loci.

During revision, the authors addressed some comments, including eliminating references to "lineage" when referring to pseudotime trajectories, eliminating conclusions drawn from locations of cells on UMAP plots, and reducing use of the term "cooperative" which may have been confusing in this context, as well as increasing the number of embryos analyzed for some experiments. The authors also point out that whole-embryo transcriptional trajectories typically do not associate endodermal cells with prechordal plate cells, despite classical evidence that they are related. This is most likely because endodermal cells arise from several different previous transcriptional states in different regions of the embryonic margin and are, as the authors point out, difficult to computationally sort into dorsal, lateral, and ventral populations. Thus, there is value in generating data to more specifically look at the relationship between dorsal mesodermal and endodermal populations. However, the decision to use an artificial Nodal-treated explant system, rather than isolating the relevant population from whole embryos (such as by dissection prior to dissociation) remains a weakness of the manuscript, since it is unclear whether endodermal specification has been altered in this system (there seem to be few endodermal cells produced and the system involves manipulating one of the signals under study in this work). Concerns about the rigor of experiments concerning ripply1 and SWI/SNF experiments remains. While the authors improved peak calling in their ripply1 cut-and-tag, it is still based on massive overexpression of ripply1 that may drive binding outside of its endogenous loci.

In the end, this study provides some additional details in the cell fate decision between the prechordal plate and anterior endoderm and generates new data that may be useful for reanalysis by other experts in the field. However, this work does not make clear how Nodal signaling, FGF signaling, and elements of the gene regulatory network (including gsc, possibly

ripply1, and other factors) interact to make the decision. I suggest that this manuscript is of interest to Nodal signaling or zebrafish germ layer patterning afficionados, but may not be of interest to a broad audience. While it provides new datasets and observations, it does not weave these into a convincing story that advances our understanding of the specification of these cell types.

https://doi.org/10.7554/eLife.100200.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***eLife Assessment***
>
> *This study provides a useful analysis of the changes in chromatin organization and gene expression that occur during the differentiation of two cell types (anterior endoderm and prechordal plate) from a common progenitor in zebrafish. Although the findings are consistent with previous work, the evidence presented in the study appears to be incomplete and would benefit from more rigorous interpretation of single-cell data, more in-depth lineage tracing, overexpression experiments with physiological levels of Ripply, and a clearer justification for using an explant system. With these modifications, this paper will be of interest to zebrafish developmental biologists investigating mechanisms underlying differentiation.*

We sincerely thank the editor and the reviewers for their valuable time and efforts. Their insightful comments were greatly appreciated and have been largely addressed in the revised manuscript. We are confident that these revisions have enhanced the overall quality and clarity of our paper.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *During vertebrate gastrulation, mesendoderm cells are initially specified by morphogens (e.g. Nodal) and segregate into endoderm and mesoderm in part based on Nodal concentrations. Using zebrafish genetics, live imaging, and single-cell multi-omics, the manuscript by Cheng et al presents evidence to support a claim that anterior endoderm progenitors derive primarily from prechordal plate progenitors, with transcriptional regulators goosecoid (Gsc) and ripply1 playing key roles in this cell fate determination. Such a finding would represent a significant advance in our understanding of how anterior endoderm is specified in vertebrate embryos.*

We would like to thank reviewer #1 for his/her comments and positive feedbacks about our manuscript.

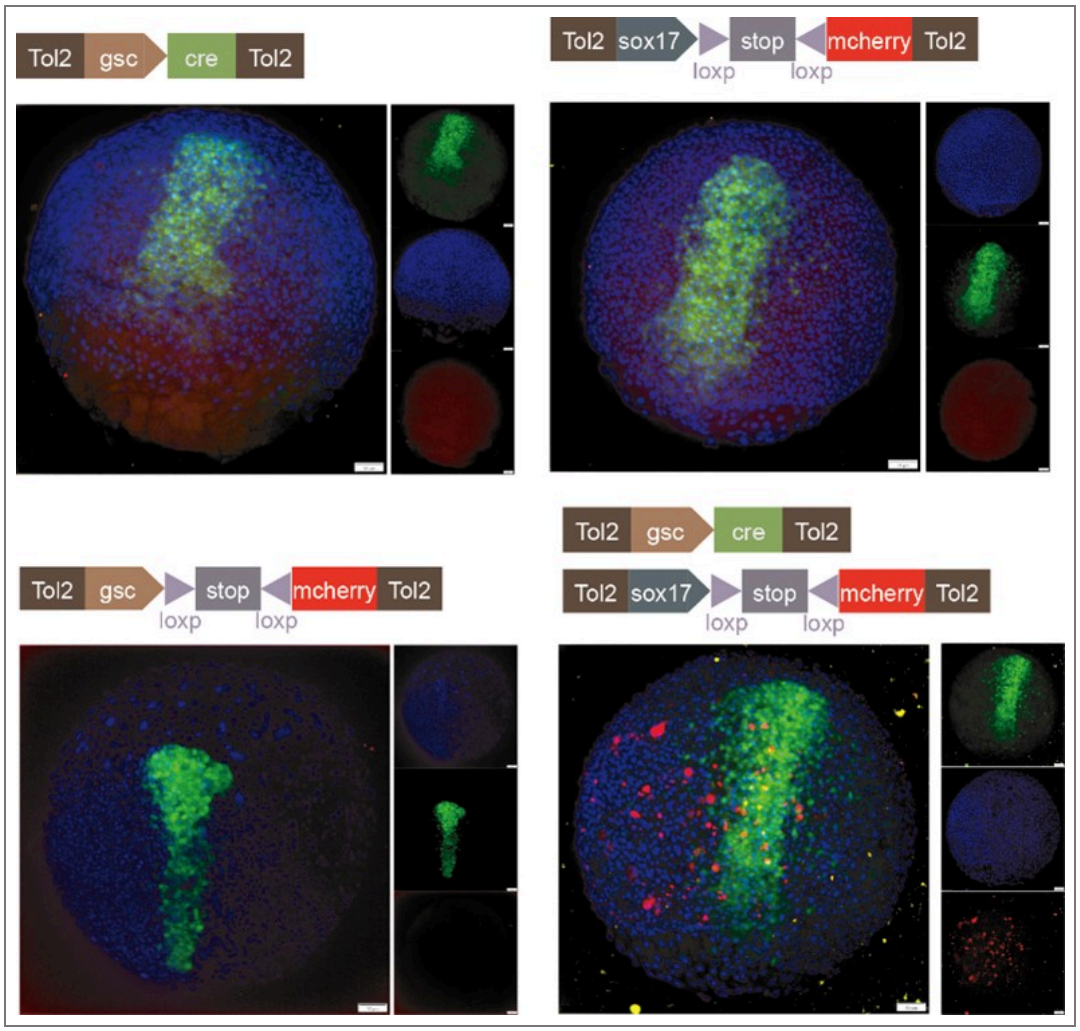
> *Strengths:*
>
> *Live imaging-based tracking of PP and endo reporters (Figure 2) is well executed and convincing, though a larger number of individual cell tracks will be needed. Currently, only a single cell track (n=1) is provided.*

We thank the reviewer for the positive comments and the valuable suggestion. As the reviewer suggested, we re-performed live imaging analyses on the embryos of Tg(*gsc*:EGFP;*sox17*:DsRed). We tracked dozens of cells during their transformation from *gsc*-positive to *sox17*-positive. Furthermore, we performed quantification of the RFP/GFP signal intensity ratio in these cells over the course of development (Please see the revised Figure 2D and MovieS4).

Weaknesses:

(1) The central claim of the paper - that the anterior endoderm progenitors arise directly from prechordal plate progenitors - is not adequately supported by the evidence presented. This is a claim about cell lineage, which the authors are attempting to support with data from single-cell profiling and genetic manipulations in embryos and explants. The construction of gene expression (pseudo-time) trajectories, while a modern and powerful approach for hypothesis generation, should not be used as a substitute for bona fide lineage tracing methods. If the authors' central hypothesis is correct, a CRE-based lineage tracing experiment (e.g. driving CRE using a PP marker such as Gsc) should be able to label PP progenitor cells that ultimately contribute to anterior endoderm-derived tissues. Such an experiment would also allow the authors to quantify the relative contribution of PP (vs non-PP) cells to the anterior endoderm, which is not possible to estimate from the indirect data currently provided. Note: while the present version of the manuscript does describe a sox17:CRE lineage tracing experiment, this actually goes in the opposite direction that would be informative (sox:17:CRE-marked descendants will be a mixture of PP-derived and non-PP derived cells, and the Gsc-based reporter does not allow for long-term tracking the fates of these cells).

We sincerely thank the reviewer for the professional comments and the constructive suggestions. As the reviewer indicated, utilizing the single-cell transcriptomic trajectory analyses on zebrafish embryos and Nodal-injected explants system, along with the live imaging analyses on Tg(*gsc*:EGFP;*sox17*:DsRed) embryos, we revealed that anterior endoderm progenitors arise from prechordal plate progenitors. To further verify this observation, we conducted two sets of lineage-tracing assays. Initial evidence came from the results of co-injecting *sox17*:Cre and *gsc*:loxp-STOP-loxp-mcherry plasmids. We observed RFP-positive cells at 8 hpf, demonstrating the presence of cells that had expressed both genes. To explicitly follow the proposed lineage, we then implemented a reciprocal strategy, as suggested by the reviewer, that constructed and co-injected *sox17*:loxp-STOP-loxp-mcherry and *gsc*:Cre plasmids. The appearance of RFP-positive cells in the anterior dorsal region at 8 hpf provides direct evidence for a transition from *gsc*-positive to *sox17*-positive identity. These results are now included in the revised manuscript (Please see Author response image 1 and Figure S4E). However, in accordance with the reviewer's caution, we acknowledge that this does not prove this is the sole origin of anterior endoderm. Consequently, we have revised the text to clarify that our findings demonstrate that anterior endoderm can be specified from prechordal plate progenitors, without claiming that it is the only source.



Author response image 1. **Characterization of anterior endoderm lineage by Cre-Lox recombination system.**

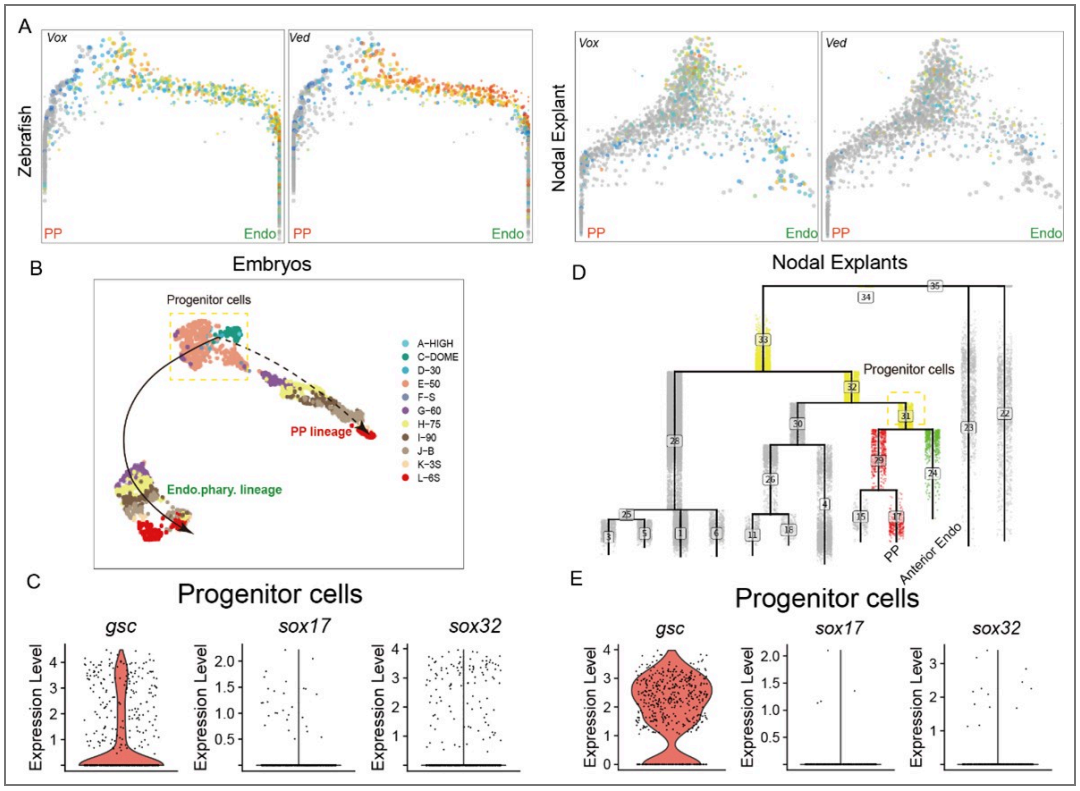
> *(2) The authors' descriptions of gene expression patterns in the single-cell trajectory analyses do not always match the data. For example, it is stated that goosecoid expression marks progenitor cells that exist prior to a PP vs endo fate bifurcation (e.g. lines 124-130). Yet, in Figure 1C it appears that in fact goosecoid expression largely does not precede (but actually follows) the split and is predominantly expressed in cells that have already been specified into the PP branch. Likewise, most of the cells in the endo branch (or prior) appear to never express Gsc. While these trends do indeed appear to be more muddled in the explant data (Figure 1H), it still seems quite far-fetched to claim that Gsc expression is a hallmark of endoderm-PP progenitors.*

We thank the reviewer for pointing out this issue. Our initial analysis proposed that the precursors of the prechordal plate (PP) and anterior endoderm (endo) more closely resemble a PP cell fate, as their progenitor populations highly express PP marker genes, such as *gsc*. The *gsc* gene is widely recognized as a PP marker[1]. The reviewer pointed out that in our analysis, these precursor cells do not initially exhibit high *gsc* expression; rather, *gsc* expression gradually increases as PP fate is specified.

The reason for this observation is as follows: First, for the *in vivo* data, we used the URD algorithm to trace back all possible progenitor cells for both the PP and anterior endo trajectory. As mentioned in the manuscript, the PP and anterior endo are relatively distant in the trajectory tree of the zebrafish embryonic data. Consequently, this approach likely included other, confounding progenitor cells that do not express *gsc* (like ventral epiblast,

Author response image 2). However, we further investigated the expression of *gsc* and *sox17* along these two trajectories. The conclusion remains that *gsc* expression is indeed higher than *sox17* in the progenitor cells common to both trajectories (Author response image 2). Combined with the live imaging analysis presented in this study, which shows that *gsc* expression increases progressively in the PP, this supports the notion that the progenitor cells for both PP and anterior endoderm initially bias towards a PP cell fate.

On the other hand, in our previously published work using the Nodal-injected explant system, which specifically induces anterior endo and PP, the cellular trajectory analysis also revealed that the specifications of PP and anterior endo follow very similar paths. Therefore, we proceeded to analyze the Nodal explant data. Similarly, when using URD to trace the differentiation trajectories of PP and anterior endo cells, a small number of other progenitor cells were also captured. This explains why a minority of cells do not express *gsc*—these are likely ventral epiblast cells (Author response image 2). However, based on the Nodal explant data, *gsc* is specifically highly expressed in the progenitor cells of the PP and anterior endo. Its expression remains high in the PP trajectory but gradually decreases in the endoderm trajectory (Figure 1H).



Author response image 2. (A) The expression of ventral epiblast markers in PP and anterior Endo URD trajectory. (B) The expression of *gsc*, *sox32* and *sox17* in the progenitors of PP and anterior endo in embryos and Nodal explants.

> *(3) The study seems to refer to "endoderm" and "anterior endoderm" somewhat interchangeably, and this is potentially problematic. Most single-cell-based analyses appearing in the study rely on global endoderm markers (sox17, sox32) which are expressed in endodermal precursors along the entire ventrolateral margin. Some of these cells are adjacent to the prechordal plate on the dorsal side of the gastrula, but many (most in fact) are quite some distance away. The microscopy-based evidence presented in Figure 2 and elsewhere, however, focuses on a small number of sox17-expressing cells that are directly adjacent to, or intermingled with, the prechordal plate.*

> *It, therefore, seems problematic for the authors to generalize potential overlaps with the PP lineage to the entire endoderm, which includes cells in ventral locations. It would be helpful if the authors could search for additional markers that might stratify and/or mark the anterior endoderm and perform their trajectory analysis specifically on these cells.*

We thank the reviewer for these comments and suggestions. We fully agree with the reviewer's point that the expression of *sox32* and *sox17* cannot be used to distinguish dorsal endoderm from ventral-lateral endoderm cells. However, during the gastrulation stage, all endodermal cells express *sox32* and *sox17*, and there are currently no specific marker genes available to distinguish between them.

After gastrulation ends, the dorsal endoderm (i.e., the anterior endoderm) begins to express pharyngeal endoderm marker genes, such as *pax1b*. Therefore, in the analysis of embryonic data *in vivo*, when studying the segregation of the anterior endoderm and PP trajectory, we specifically used the pharyngeal endoderm as the subject to trace its developmental trajectory.

In the case of Nodal explants, Nodal specifically induces the fate of the dorsal mesendoderm, which includes both the PP and pharyngeal endoderm (anterior endoderm). Precisely for this reason, we consider the Nodal explant system as a highly suitable model for investigating the mechanisms underlying the cell fate separation between anterior endoderm and PP. Thus, in the Nodal explant data, we included all endodermal cells for downstream analysis.

To avoid any potential confusion for readers, we have revised the term "endoderm" in the manuscript to "anterior endoderm" as suggested by the reviewer.

> *(4) It is not clear that the use of the nodal explant system is allowing for rigorous assessment of endoderm specification. Why are the numbers of endoderm cells so vanishingly few in the nodal explant experiments (Figure 1H, 3H), especially when compared to the embryo itself (e.g. Figures 1C-D)? It seems difficult to perform a rigorous analysis of endoderm specification using this particular model which seems inherently more biased towards PP vs. endoderm than the embryo itself. Why not simply perform nodal pathway manipulations in embryos?*

We sincerely thank the reviewer for raising this important question. In our study of the fate separation between the PP and anterior endoderm, we initially analyzed zebrafish embryonic data. However, when reconstructing the transcriptional lineage tree using URD, we observed that these two cell trajectories were positioned relatively far apart on the tree. Yet, existing studies have shown that the anterior endoderm and PP are not only spatially adjacent but also both originate from mesendodermal progenitor cells[2-4], and they share transcriptional similarities[5]. Therefore, as the reviewer pointed out, when tracing all progenitor cells of these two trajectories using the URD algorithm, it is easy to include other cell types, such as ventral epiblast cells (Author response image 2). For this reason, we concluded that directly using embryonic data to dissect the mechanism of fate separation between PP and anterior endoderm might not yield highly accurate results.

In contrast, our group's previous work, published in Cell Reports, demonstrated that the Nodal-induced explant system specifically enriches dorsal mesendodermal cells, including anterior endoderm, PP, and notochord[5]. Thus, we considered the Nodal explant system to be a highly suitable model for investigating the mechanism of fate separation between PP and anterior endoderm. Ultimately, by analyzing both *in vivo* embryonic data and Nodal explant data, we consistently found that the anterior endoderm likely originates from PP progenitor cells—a conclusion further validated by live imaging experiments.

Regarding the reviewer's concern about the relatively low number of endodermal cells in the

Nodal explant system, we speculate that this is because the explants predominantly induce anterior endoderm. Since endodermal cells constitute only a small proportion of cells during gastrulation, and anterior endoderm represents an even smaller subset, the absolute number is naturally limited. Nevertheless, the anterior endodermal cells captured in our Nodal explants were sufficient to support our analysis of the fate separation mechanism between anterior endoderm and PP. Finally, to further strengthen the findings from scRNA-seq analyses, we subsequently performed live imaging validation experiments using both zebrafish embryos and the explant system.

> *(5) The authors should not claim that proximity in UMAP space is an indication of transcriptional similarity (lines 207-208), especially for well-separated clusters. This is a serious misrepresentation of the proper usage of the UMAP algorithm. The authors make a similar claim later on (lines 272-274).*

We would like to extend our gratitude to the reviewer for their insightful comments. We have revised the descriptions regarding UMAP throughout the manuscript as suggested (Please see the main text in revised manuscript).

> ***Reviewer # 1 (Recommendations For The Authors):***
>
> *- Pseudotime trajectories constructed from single-cell snapshots are not true "lineage" measurements. Authors should refrain from referring to such data as lineage data (e.g. lines 99, 100, 103, 109, 112, 127, etc). Such models should be referred to as "trajectories", "hypothetical lineages", or something else.*

We are grateful to the reviewer for this comment. Following their recommendation, we have revised the terminology from "transcriptional lineage tree" to "trajectory" across the entire manuscript (Please see main text in revised manuscript).

> *- The live imaging data presented in Figure 2 (and supplemental figures) are compelling and do seem to show that some cells can switch between PP and endo states. However, the number of cells reported is still too low to be able to ascertain whether or not this is just a rare/edge-case phenomenon. Tracks for just a single cell are reported in Figure 2C-D. This is insufficient. Tracks for many more cells should be collected and reported alongside this current sole (n=1) example. The choice of time window for these live imaging experiments should also be better explained. These live imaging experiments are being performed at or after 6hpf, but authors claim in the text that "... the segregation between PP and Endo has already occurred by 6hpf." (lines 126-127). Why not perform these live imaging experiments earlier, when the initial fate decision between PP and endo is supposedly occurring?*

We sincerely appreciate the reviewer's insightful questions and constructive feedback. In response, we have made several important revisions. First, the reviewer noted that our original manuscript tracked only a single cell and suggested increasing the number of tracked cells. Following this recommendation, we repeated the live-imaging experiments and expanded the number of tracked endodermal cells (Please see the revised Movie S4 and Figure 2D). The experimental conditions were kept identical to the previous setup, and these cells consistently exhibited a gradual transition from a *gsc*+ fate to a *sox17*+ endodermal fate. In addition, the reviewer recommended performing live imaging at an earlier time point (Movie S5). Accordingly, we conducted additional experiments initiating live imaging at around 5.7 hours and observed the onset of a *sox17* expression in *gsc*+ cells at approximately 6 hpf, which is consistent with our single-cell transcriptomic analysis.

> *- The sections devoted to lengthy descriptions of GO terms (lines 131-146, 239-254) and receptor-ligand predictions (lines 170-185) are largely speculative. Consider streamlining.*

Thanks for the reviewer's comment. We have streamlined the content related to the GO analysis as suggested (Please see Lines 128-132, 157-167, 221-225).

> *- The use of a "Nodal Activity Score" (lines 212-226) is clever but might actually be less informative than showing contributions from individual nodal target genes. The combining of counts data from 29 predicted nodal targets means that the contribution (or lack of contribution) from each gene becomes masked. The authors should include supplementary dot plots that break down the score across all 29 genes, allowing the reader to assess overall contributions and/or sub-clusters of gene co-expression patterns, if present.*

Thank you very much for the reviewer's positive feedback on our use of the "Nodal Activity Score" and the valuable suggestions provided. Following the recommendation, we analyzed the expression of the 29 Nodal direct targets used in our study across the WT, *ndr1* knockdown (kd), and *lft1* knockout (ko) groups. We found that the known axial mesoderm genes, such as *chrd*, *tbxta*, *noto*, and *gsc*, contributed significantly to the Nodal score. The newly conducted analysis has been included in the Supplementary Information (Please see Figure S7L).

> *- The differential expression trends being reported for srcap (line 251) do not appear to be significant. Are details and P-values for these DEG tests reported somewhere in the manuscript?*

We thank the reviewer for raising this question. Based on the reviewer's comment, we performed statistical tests (Wilcoxon test) to compare the expression of *srcap* in PP and Endo. Our analysis revealed that while *srcap* expression is slightly higher in PP than in Endo, this difference is not statistically significant. The specific p-value and fold change have been indicated in the revised figure (Please see Figure 4J and S7H). Based on this analysis, we revised our description to state that *srcap* expression is slightly higher in the PP compared to in the anterior endoderm.

> *- Following the drug experiments with the drug AU15330 (lines 254-263), authors have only reported #s of endodermal cells, which seem to have increased, which the authors suggest indicates a fate switch from PP to endo. However, the authors have not reported whether the numbers of PP cells decreased or stayed the same in these embryos. This would be helpful information to include, as it is very difficult to discern quantitative trends from the images presented in Fig 4H and 4L.*

Thank the reviewer for his/her comments and suggestions. Following the reviewer's suggestions, we performed Imaris analysis on the HCR staining results from the DMSO (control), 1μM AU15330-treated, and 5μM AU15330-treated groups. Our analysis focused on the number of *frzb*-positive cells (PP), and the comparison revealed that treatment with AU15330 significantly reduces the PP cell number. These findings have been incorporated into the revised manuscript and supplementary information (Please see Figures S7J and S7K).

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *During vertebrate gastrulation, the mesoderm and endoderm arise from a common population of precursor cells and are specified by similar signaling events, raising questions as to how these two germ layers are distinguished. Here, Cheng and colleagues use zebrafish gastrulation as a model for mesoderm and endoderm segregation. By reanalyzing published single-cell sequencing data, they identify a common progenitor population for the anterior endoderm and the mesodermal prechordal plate (PP). They find that expression levels of PP genes Gsc and ripply are*

> *among the earliest differences between these populations and that their increased expression suppresses the expression of endoderm markers. Further analysis of chromatin accessibility and Ripply cut-and-tag is consistent with direct repression of endoderm by this PP marker. This study demonstrates the roles of Gsc and Ripply in suppressing anterior endoderm fate, but this role for Gsc was already known and the effect of Ripply is limited to a small population of anterior endoderm. The manuscript also focuses extensively on the function of Nodal in specifying and patterning the mesoderm and endoderm, a role that is already well known and to which the current analysis adds little new insight.*

We would like to thank the reviewer #2 for the constructive comments and positive feedback regarding our manuscript.

> *Strengths:*
>
> *Integrated single-cell ATAC- and RNA-seq convincingly demonstrate changes in chromatin accessibility that may underlie the segregation of mesoderm and endoderm lineages, including Gsc and ripply. Identification of Ripply-occupied genomic regions augments this analysis. The genetic mutants for both genes provide strong evidence for their function in anterior mesendoderm development, although these phenotypes are subtle.*

We thank the reviewer for recognizing our work, and we greatly appreciate the constructive suggestions from the reviewer.

> *Weaknesses:*
>
> *The use of zebrafish embryonic explants for cell fate trajectory analysis (rather than intact embryos) is not justified. In both transcriptomic comparisons between the two fate trajectories of interest and Ripply cut-and-tag analysis, the authors rely too heavily on gene ontology which adds little to our functional understanding. Much of the work is focused on the role of Nodal in the mesoderm/endoderm fate decision, but the results largely confirm previous studies and again provide few new insights. Some experiments were designed to test the relationship between the mesoderm and endoderm lineages and the role of epigenetic regulators therein, but these experiments were not properly controlled and therefore difficult to interpret.*

We sincerely thank the reviewer for the comments. As we previously answered, in our study of the fate differentiation between the PP and the anterior endoderm, we initially analyzed zebrafish embryonic data. However, when we used URD to reconstruct the transcriptional trajectory tree, we found that these two cell trajectories were distantly located on the tree. Existing studies have shown that the anterior endoderm and the PP are not only spatially adjacent but also both originate from mesendodermal progenitor cells and share transcriptional similarities[2-4]. Therefore, when tracing all progenitor cells of these two trajectories using the URD algorithm, it is easy to include other cell types, such as ventral mesendodermal cells (Please see Author response image 2A). Based on this, we believe that directly using embryonic data to decipher the mechanism of fate differentiation between the PP and the anterior endoderm may not yield sufficiently precise results. In contrast, our group's previous study published in Cell Reports demonstrated that the Nodal-induced explant system can specifically enrich dorsal mesendodermal cells, including the anterior endoderm, PP, and notochord[5]. Thus, we consider the Nodal explant system as an ideal model for studying the fate differentiation mechanism between the PP and the anterior endoderm. Ultimately, through comprehensive analysis of *in vivo* embryonic data and Nodal explant data, we consistently found that the anterior endoderm likely originates from PP progenitor cells—a conclusion further validated by live imaging experiments.

Regarding the GO analysis, we have streamlined it as suggested by the reviewers. In the revised manuscript, we analyzed the expression of specific genes contributing to key GO functions. Additionally, in the revised version, we conducted more live imaging experiments and quantitative cell assays. We designed gRNA for *srcap* using the CRISPR CAS13 system to knock down *srcap*, which further corroborated the morpholino knockdown results, showing consistency with the morpholino data. We also performed Western blot validation of the SWI/SNF complex's response to the drug AU15330, confirming the drug's effectiveness. We hope these additional experiments adequately address the reviewers' concerns.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *(1) In the introduction, the authors state that mesendoderm segregates into mesoderm and endoderm in a Nodal-concentration dependent manner. While it is true that higher Nodal signaling levels are required for endoderm specification, A) this is also true for some mesoderm populations, and B) Work from Caroline Hill's lab has shown that Nodal activity alone is not determinative of endoderm fate. Although the authors cite this work, it is conclusions are not reflected in this over-simplified explanation of mesendoderm development. The authors also state that it is not clear when PP and endoderm can be distinguished transcriptionally, but this was also addressed in Economou et al, 2022, which found that they can be distinguished at 60% epiboly but not 50% epiboly.*

We sincerely thank the reviewer for raising this question and reminding us of the conclusions drawn from that excellent study. As the reviewer pointed out, Economou et al. demonstrated that Nodal signaling alone is insufficient to determine the cell fate segregation of mesendoderm[6]. However, their study primarily focused on the fate segregation of the ventral-lateral mesendoderm lineage. In contrast, we believe that the mechanisms underlying dorsal mesendoderm specification may differ.

First, it is well-studied that in zebrafish embryos, the most dorsal mesendoderm is initially specified by the activity of the dorsal organizer. Notably, the Nodal signaling ligands *ndr1* and *ndr2* begin to be expressed in the dorsal organizer as early as the sphere stage[7]. In our study, through single-cell transcriptomic trajectory analysis and live imaging analysis, we observed that the cell fate segregation of the dorsal mesendoderm can be traced back to the shield stage.

Second, the regulatory mechanisms governing dorsal mesendoderm fate differentiation may differ from those of the ventral-lateral mesendoderm. For instance, the *gsc* gene is exclusively expressed in the dorsal mesendoderm and is absent in the ventral-lateral mesendoderm. Given that *gsc* is a critical master gene, its overexpression in the ventral side can induce a complete secondary body axis. Similarly, *ripply1*, identified in our study, is also expressed early and specifically in the dorsal mesendoderm. Overexpression of *ripply1* in the ventral side similarly induces a secondary body axis, albeit with the absence of the forebrain[5]. In this study, we found that *gsc* and *ripply1* as the repressor, collectively inhibited dorsal (anterior) endoderm specified from PP progenitors.

In summary, our study focuses on the regulatory mechanisms of fate segregation in the dorsal (anterior) mesendoderm, which differs from the mechanisms of ventral-lateral mesendoderm lineage segregation reported by Economou et al. We believe that this distinction represents a key novelty of our work.

> *(2) As noted in the manuscript, Warga and Nusslein-Volhard determined long ago that PP and anterior endoderm share a common precursor. It is surprising that this close relationship is not apparent from the lineage trees in whole embryos but is apparent in lineage trees from explants. The authors speculate that the resolution of the whole embryo dataset is insufficient to detect this branch point and propose explants as the*

> *solution, but it is not clear why the explant dataset is higher resolution and/or more appropriate to address this question.*

We sincerely thank the reviewer for their thoughtful comments. As we mentioned previously, our investigation of fate differentiation between the PP and the anterior endoderm initially involved the analysis of zebrafish embryonic data. However, when we used URD to reconstruct the transcriptional trajectory tree, we observed that these two cell trajectories were located far apart. Previous elegant studies, as the reviewer mentioned, have shown that the anterior endoderm and the PP are not only spatially adjacent but also both originate from mesendodermal progenitor cells and share transcriptional similarities[2,3,8]. Consequently, when tracing all progenitor cells of these two trajectories using the URD algorithm, other cell types—such as ventral mesendodermal cells—are easily included. Based on this, we believe that directly using embryonic data to elucidate the mechanism of fate differentiation between the PP and the anterior endoderm may lack sufficient precision.

In contrast, our group's previous study published in Cell Reports demonstrated that the Nodal-induced explant system specifically enriches dorsal mesendodermal cells, including the anterior endoderm, PP, and notochord[5]. Therefore, we consider the Nodal explant system as an ideal model for studying the mechanism underlying fate differentiation between the PP and the anterior endoderm. Through comprehensive analyses of both *in vivo* embryonic and Nodal explant data, we consistently found that the anterior endoderm likely originates from PP progenitor cells—a conclusion further supported by live imaging experiments.

> *(3) Much of the analysis of DEGs between the lineages of interest is focused on GO term enrichment. But this logic is circular. The endoderm lineage is defined as such because it expresses endoderm-enriched genes, therefore the finding that the endoderm lineage is enriched for endoderm-related GO terms adds no new insights.*

We thank the reviewer for these comments. As the reviewers suggested, in the revised manuscript, we indicated specific genes associated with key GO terms (Please see Figure 4B). Additionally, we have streamlined the content related to the GO analysis as suggested.

> *(4) The authors describe the experiment in Figure S4 as key evidence that Gsc+ cells can give rise to endoderm, but no controls are presented. Only a few cells are shown that express mCherry upon injection of sox17:cre constructs. Is mCherry also expressed in the occasional cell injected with Gsc:lox-stop-lox-mCherry in the absence of cre? Although they report 3 independent replicates, it appears that only 2 individual embryos express mCherry. This very small number is not convincing, especially in the absence of appropriate controls.*

We thank the reviewer for raising this question. Following the reviewer's suggestion, we injected *gsc*:loxp-stop-loxp-mCherry into zebrafish embryos at the 1-cell stage as a control. After performing at least three independent replicates and analyzing no fewer than 100 embryos, we did not observe any mCherry-positive cells. Additionally, we co-injected *gsc*:loxp-stop-loxp-mCherry with *sox17*:cre and increased the sample size. Furthermore, we constructed plasmids of *sox17*:loxp-stop-loxp-mCherry and *gsc*:cre, and upon injection at the 1-cell stage, we observed RFP-positive cells at 8 hpf (Please see Author response image 1 and Figure S4E). Together with our live imaging data, these experiments collectively demonstrate that anterior endodermal cells can originate from PP progenitors.

> *(5) The authors spend a lot of effort demonstrating that PP and anterior endoderm are Nodal dependent. First, these data (especially Figures 3E and 3I) are not very convincing, as the differences shown are very small or not apparent. Second, this is already well-known and adds nothing to our understanding of mesoderm-endoderm segregation.*

We sincerely thank the reviewer for their insightful questions. First, the reviewer mentioned that in the initial version of our manuscript, the effects of *ndr1* knockdown and *lefty1* knockout on Nodal signaling and cell fate—particularly prechordal plate (PP) and anterior endoderm (endo)—in Nodal-induced explants were not very pronounced. We recognize that the negative feedback mechanism between Nodal and Lefty signaling may explain why Nodal acts as a morphogen, regulating pattern formation through a Turing-like model[9]. Therefore, knocking down a Nodal ligand gene, such as *ndr1* in this study, or knocking out a Nodal inhibitor, such as *lft1*, may only have a subtle impact on Nodal signaling[10].

Accordingly, in this study, we performed extensive pSmad2 immunofluorescence analysis and observed that although the overall intensity of Nodal activity did not change dramatically, there was a statistically significant difference. Importantly, this subtle variation in Nodal signaling strength is precisely what we intended to capture, since PP and anterior endoderm are highly sensitive to Nodal signaling[11], and even minor differences may bias their fate segregation.

This leads directly to the reviewer's second concern. While numerous studies suggest that the strength of Nodal signaling influences mesendodermal fate—with high Nodal promoting endoderm and lower concentrations inducing mesoderm—most of these studies focus on ventral-lateral mesendoderm development[4,6,10]. In contrast, the mechanisms underlying dorsal mesendoderm fate specification differ, which is a key innovation of our study.

Previous work by Bernard Thisse and colleagues demonstrated that even a slight reduction in Nodal signaling, achieved by overexpressing a Nodal inhibitor, is sufficient to cause defects in the specification of PP and endoderm[11]. This indicates that PP and endoderm require the highest levels of Nodal signaling for proper specification. Moreover, the most dorsal mesendoderm, PP and anterior endoderm are not only spatially adjacent but also share similar transcriptional states, making the regulation of their fate separation particularly challenging to study.

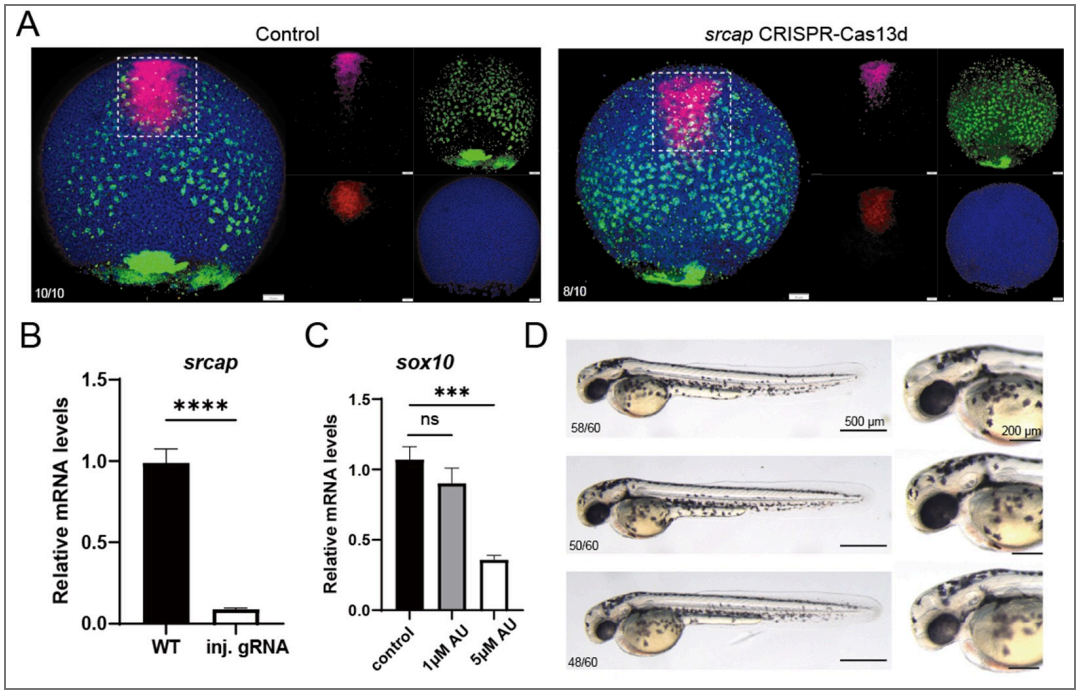
The Dr. C.P. lab made important contributions to this issue, showing that the duration of Nodal exposure is critical for segregating PP and anterior endoderm fates: prolonged Nodal signaling promotes expression of the transcriptional repressor Gsc, which directly suppresses the key endodermal transcription factor Sox17, thereby inhibiting anterior endoderm specification[3]. They also found that tight junctions among PP cells facilitate Nodal signal propagation[8]. However, their studies revealed that Gsc mutants do not exhibit endodermal phenotypes, suggesting that additional factors or mechanisms regulate PP versus anterior endoderm fate separation[3].

In our study, we first observed that subtle differences in Nodal concentration may bias the fate choice between PP and anterior endoderm. Given that *ndr1* knockdown and *lft1* knockout mildly reduce or enhance Nodal signaling, respectively, we reasoned that using these two perturbations in a Nodal-induced explant system combined with single-cell RNA sequencing could generate transcriptomic profiles under slightly reduced and enhanced Nodal signaling. This approach may help identify key decision points and transcriptional differences during PP and anterior endoderm segregation, ultimately uncovering the molecular mechanisms downstream of Nodal that govern their fate separation.

> *(6) The authors claim that scrap expression differs between the 2 lineages of interest, but this is not apparent from Figure 4J-K. Experiments testing the role of SWI/SNF and scrap also require additional controls. Can scrap MO phenotypes be rescued by scrap RNA? Is there validation that SWI/SNF components are degraded upon treatment with AU15330?*

We are very grateful for the reviewers' questions. Using single-cell data from zebrafish embryos and Nodal explants, we compared the expression of *srcap* in the PP and anterior Endo cell populations. We found that *srcap*

expression showed a slight increase in PP compared to anterior Endo, but the difference was not statistically significant (Please see Figure 4J and S7H). Therefore, we modified our description in the revised manuscript. However, we speculate that this slight difference might influence the distinct cell fate specification between PP and anterior endo. In the original version of the manuscript, we reported that either treatment with AU15330, an inhibitor of the SWI/SNF complex, or injection of morpholino targeting *srcap*—a key component of the SWI/SNF complex—enhanced anterior endo fate while reducing PP cell specification. During this round of revision, we initially attempted to follow the reviewer's suggestion to co-inject *srcap* mRNA along with *srcap* morpholino to rescue the phenotype. However, we found that the length of *srcap* mRNA exceeds 10,000 bp, and despite multiple attempts, we were unable to successfully obtain the *srcap* mRNA. Therefore, we were unable to perform the rescue experiment and instead adopted an alternative approach to validate the function of *srcap*. We aimed to use anthor knockdown approach (CRISPR/Cas system) to determine whether a phenotype similar to that observed with morpholino knockdown could be achieved. Using the CRISPR/Cas13 system, we designed gRNA targeting *srcap*, knocked down *srcap*, and examined the cell specification of PP and anterior endo. We found that, consistent with our previous results, knocking down *srcap* obviously reduced PP cell fate while increasing anterior endo cell fate (Author response image 3). Additionally, the reviewer raised the question of whether the SWI/SNF complex is degraded after AU15330 treatment. Following the reviewer's suggestion, we attempted to perform Western blot analysis on BRG1, one of the components of the SWI/SNF complex. However, despite multiple attempts, we were unable to achieve successful detection of the BRG1 protein by the antibody in zebrafish. Several studies have reported that knockdown or knockout of *brg1* leads to defects in neural crest cell specification in zebrafish[12,13]. Therefore, alternatively, we treated zebrafish embryos at the one-cell stage with 0 μM (DMSO), 1 μM, and 5 μM AU15330, and examined the expression of *sox10* and pigment development around 48 h. We found that treatment with 1 μM AU15330 reduced *sox10* expression and pigment production, though not significantly, whereas treatment with 5 μM AU15330 significantly disrupted neural crest cell development. Thus, this experiment demonstrates that AU15330 is functional in zebrafish. (Author response image 3).



Author response image 3. (A) Characterization of anterior endoderm and PP cells following CRISPR-Cas13d-mediated *srcap* knockdown. (B) Validation of *srcap* mRNA expression by RT-qPCR following CRISPR-Cas13d knockdown. (C) RT-qPCR shows the expression of *sox10* after treatment with increasing concentrations of

AU15300. (D) Morphology of zebrafish embryos at 48 hpf after treatment with increasing concentrations of AU15300.

> *(7) The authors conclude from their chromatin accessibility analysis that variations in Nodal signaling are responsible for expression levels of PP and endoderm genes, but they do not consider the alternative explanation that FGF signaling is playing this role. Such a function for FGF was established by Caroline Hill's lab, and the authors also show in Figure S5G that FGF signaling in enriched between these cell populations.*

Thank you very much for raising this issue. As the reviewer pointed out, Caroline Hill's lab has conducted elegant work demonstrating that FGF signaling plays a crucial role in the separation of ventral-lateral mesendoderm cell fates[4,6]. In contrast, our study primarily focuses on studying the mechanisms underlying the separation of dorsal mesendoderm cell fates. However, our research also reveals that FGF signaling significantly regulates the fate separation of the dorsal mesendoderm, as inhibiting FGF signaling suppresses PP cell specification while promoting anterior Endo fate. In our previously published work, we found that Nodal signaling can directly activate the expression of FGF ligand genes[5]. Therefore, we hypothesize that Nodal signaling, acting as a master regulator, activates various downstream target genes—including FGF—and how FGF signaling regulates the cell fate separation of the dorsal mesendoderm warrants further investigation in our further studies.

> *(8) When interpreting the results of their Ripply cut-and-run experiment, the authors again rely heavily on GO term analysis and claim that this supports a role for Ripply as a transcriptional repressor. GO term enrichment does not equal functional analysis. It would be more convincing to intersect DEGs between WT and ripply-/- embryos with Ripply-enriched loci.*

Thanks for raising this important issue and the constructive suggestion. In response to the reviewer's valid concern regarding the GO term analyses from our CUT&Tag data, we implemented a more stringent filtering strategy. We identified peaks enriched in the treatment group and applied differential analysis, selecting genes with a $\log_2$FoldChange > 3, padj < 0.05, and baseMean > 30 as high-confidence Ripply1 binding targets. A GO enrichment analysis of these genes revealed significant terms related to muscle development, consistent with Ripply1's established role in somite development, thereby validating our approach. We supplemented the related gene list in the revised manuscript. Moreover, within this refined analysis, we found that *sox32* met our binding threshold, while *sox17* did not. Furthermore, as suggested, we examined *mespbb*—a known Ripply1-repressed gene—which was present, and *gsc*, a Nodal target used as a negative control, which was absent. This confirms the specificity of our analysis (Figure 6 and Figure S11). Consequently, our revised analyses support a model in which Ripply1 directly binds the *sox32* promoter. Given that Sox32 is a known upstream regulator of *sox17*, this binding provides a plausible direct mechanism for the observed regulation of *sox17* expression. We have updated the figures and text accordingly. We attempted to generate *ripply1*$^{-/-}$ mutants but found that homozygous loss results in embryonic lethality.

> *(9) The way N's are reported is unconventional. N= number of embryos used in the experiment, n= number of embryos imaged. If an embryo was not imaged or analyzed in any way, it cannot be considered among the embryos in an experiment. If only 4 embryos were imaged, the N for that experiment is 4 regardless of how many embryos were stained. Authors should also report not only the number of embryos examined but also the number of independent trials performed for all experiments.*

Thank you very much for the reviewer's suggestion. As suggested, we have revised the description regarding the number of embryos and experimental replicates in the figure legends.

> *(10) The authors should avoid the use of red-green color schemes in figures to ensure accessibility for color-blind readers.*

Thanks for the suggestions. We have updated the figures in our revised manuscript and adjusted the color schemes to avoid red-green combinations.

> ***Reviewer #3 (Public Review):***
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> *Summary:*
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> *Cheng, Liu, Dong, et al. demonstrate that anterior endoderm cells can arise from prechordal plate progenitors, which is suggested by pseudo time reanalysis of published scRNAseq data, pseudo time analysis of new scRNAseq data generated from Nodal-stimulated explants, live imaging from sox17:DsRed and Gsc:eGFP transgenics, fluorescent in situ hybridization, and a Cre/Lox system. Early fate mapping studies already suggested that progenitors at the dorsal margin give rise to both of these cell types (Warga) and live imaging from the Heisenberg lab (Sako 2016, Barone 2017) also pretty convincingly showed this. However, the data presented for this point are very nice, and the additional experiments in this manuscript, however, further cement this result. Though better demonstrated by previous work (Alexander 1999, Gritsman 1999, Gritsman 2000, Sako 2016, Rogers 2017, others), the manuscript suggests that high Nodal signaling is required for both cell types, and shows preliminary data that suggests that FGF signaling may also be important in their segregation. The manuscript also presents new single-cell RNAseq data from Nodal-stimulated explants with increased (lft1 KO) or decreased (ndr1 KD) Nodal signaling and multi-omic ATAC+scRNAseq data from wild-type 6 hpf embryos but draws relatively few conclusions from these data. Lastly, the manuscript presents data that SWI/SNF remodelers and Ripply1 may be involved in the anterior endoderm - prechordal plate decision, but these data are less convincing. The SWI/SNF remodeler experiments are unconvincing because the demonstration that these factors are differentially expressed or active between the two cell types is weak. The Ripply1 gain-of-function experiments are unconvincing because they are based on incredibly high overexpression of ripply1 (500 pg or 1000 pg) that generates a phenotype that is not in line with previously demonstrated overexpression studies (with phenotypes from 10-20x lower expression). Similarly, the cut-and-tag data seems low quality and like it doesn't support direct binding of ripply1 to these loci.*
>
> *In the end, this study provides new details that are likely important in the cell fate decision between the prechordal plate and anterior endoderm; however, it is unclear how Nodal signaling, FGF signaling, and elements of the gene regulatory network (including Gsc, possibly ripply1, and other factors) interact to make the decision. I suggest that this manuscript is of most interest to Nodal signaling or zebrafish germ layer patterning afficionados. While it provides new datasets and observations, it does not weave these into a convincing story to provide a major advance in our understanding of the specification of these cell types.*

We sincerely thank the reviewer for their thorough and thoughtful assessment of our work. The reviewer acknowledged several strengths of our study, such as the use of multiple technical approaches to demonstrate that anterior endoderm differentiates from PP progenitor cells, and recognized the value of the newly added single-cell omics data. The reviewer also raised some concerns regarding the initial version of our work, including the SWI/SNF remodeler experiments and the Ripply1 gain-of-function experiment. In the revised manuscript, we have supplemented these parts with additional control experiments to better support our conclusions. We hope that our updated manuscript adequately addresses the points raised by the reviewer.

> *Major issues:*

> *(1) UMAPs: There are several instances in the manuscript where UMAPs are used incorrectly as support for statements about how transcriptionally similar two populations are. UMAP is a stochastic, non-linear projection for visualization - distances in UMAP cannot be used to determine how transcriptionally similar or dissimilar two groups are. In order to make conclusions about how transcriptionally similar two populations are requires performing calculations either in the gene expression space, or in a linear dimensional reduction space (e.g. PCA, keeping in mind that this will only consider the subset of genes used as input into the PCA). Please correct or remove these instances, which include (but are not limited to):*
>
> *p.4 107-110*
>
> *p.4 112*
>
> *p.8 207-208*
>
> *p.10 273-275*

We would like to thank the reviewer for raising this question. The descriptions of UMAP have been revised throughout the manuscript in accordance with the reviewer's suggestion (Please see the main text in the revised manuscript).

> *(2) Nodal and lefty manipulations: The section "Nodal-Lefty regulatory loop is needed for PP and anterior Endo fate specification" and Figure 3 do not draw any significant conclusions. This section presents a LIANA analysis to determine the signals that might be important between prechordal plate and endoderm, but despite the fact that it suggests that BMP, Nodal, FGF, and Wnt signaling might be important, the manuscript just concludes that Nodal signaling is important. Perhaps this is because the conclusion that Nodal signaling is required for the specification of these cell types has been demonstrated in zebrafish in several other studies with more convincing experiments (Alexander 1999, Gritsman 1999, Gritsman 2000, Rogers 2017, Sako 2016). While FGF has recently been demonstrated to be a key player in the stochastic decision to adopt endodermal fate in lateral endoderm (Economou 2022), the idea that FGF signaling may be a key player in the differentiation of these two cell types has strangely been relegated to the discussion and supplement. Lastly, the manuscript does not make clear the advantage of performing experiments to explore the PP-Endo decision in Nodal-stimulated explants compared to data from intact embryos. What would be learned from this and not from an embryo? Since Nodal signaling stimulates the expression of Wnts and FGFs, these data do not test Nodal signaling independent of the other pathways. It is unclear why this artificial system that has some disadvantages is used since the manuscript does not make clear any advantages that it might have had.*

We sincerely thank the reviewers for their valuable comments. As mentioned in our manuscript, although a substantial number of studies have reported on the mechanisms governing the segregation of mesendoderm fate in zebrafish embryos—including the Dr. Hill laboratory's work cited by the reviewers, which demonstrated the involvement of FGF signaling in the ventral mesendoderm fate specification—research on the regulatory mechanisms underlying anterior mesendoderm differentiation remains relatively limited. This is largely due to the challenges posed by the close physical proximity and similar transcriptional states of anterior mesendoderm cells, as well as their shared dependence on high levels of Nodal signaling for specification.

Several studies from the Dr. C.P. Heisenberg's laboratory have attempted to elucidate the fate segregation between anterior mesendoderm cells, namely the prechordal plate (PP) and anterior endoderm (endo) cells. They found that PP cells are tightly connected, facilitating the propagation of Nodal signaling[8]. Prolonged exposure to Nodal activates the expression of Gsc, which acts as a transcriptional repressor to inhibit *sox17* expression, thereby

suppressing endodermal fate[3]. However, they also noted that Gsc mutants do not exhibit endoderm developmental defects, suggesting the involvement of additional factors in this process.

The reviewer inquired about our rationale for using the Nodal-injected explant system. In our investigation of the fate separation between the PP and the anterior endo, we initially analyzed zebrafish embryonic data. Using URD to reconstruct the transcriptional lineage tree, we found that these two cell types were positioned distantly from each other. However, existing literature indicates that the anterior endoderm and PP are not only spatially adjacent but also derive from common mesendodermal progenitors and exhibit transcriptional similarities[2,8]. As the reviewer noted, when tracing all progenitor cells of these two lineages using URD, it is easy to inadvertently include other cell types—such as ventral epiblast cells—which may compromise the accuracy of the analysis. We therefore concluded that directly using embryonic data to dissect the mechanism of fate separation between PP and anterior endoderm might not yield highly precise results.

By contrast, our group's earlier study published in Cell Reports demonstrated that the Nodal-induced explant system specifically enriches dorsal mesendodermal cells, including anterior endo, PP, and notochord[5]. This makes the Nodal explant system a highly suitable model for studying the fate separation between PP and anterior endo. Ultimately, by analysing *in vivo* embryonic data and Nodal explant data, we consistently found that the anterior endoderm likely originates from PP progenitors—a conclusion further supported by live imaging experiments.

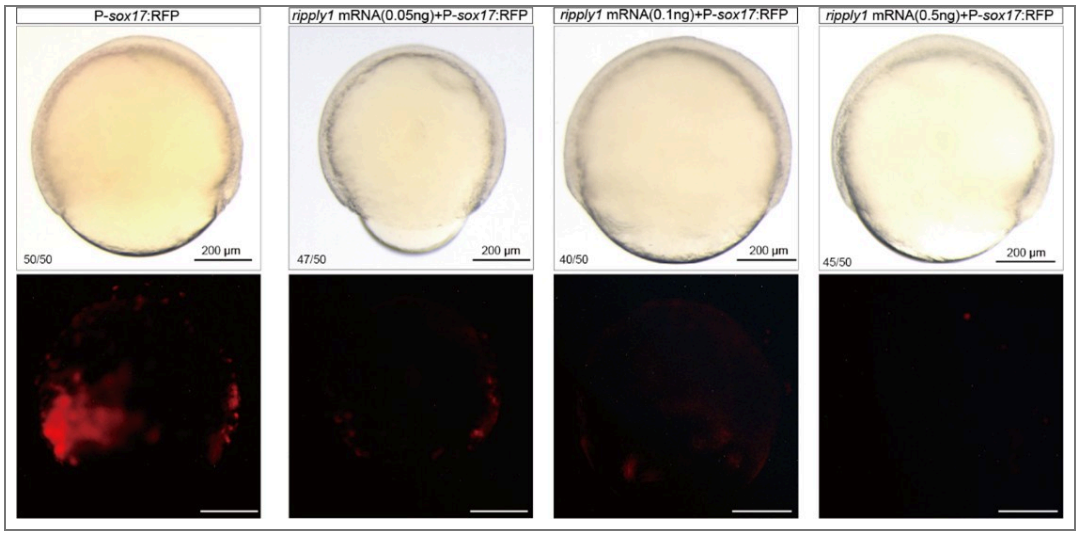
As we answered above, we first used the analyses of single-cell RNA sequencing and live imaging to demonstrate that anterior endoderm can originate from PP progenitor cells. Understanding the mechanism underlying the fate segregation between these two cell populations became a key focus of our research. We began by applying cell communication analysis to our single-cell data to identify signaling pathways that may be involved. This analysis specifically highlighted the Nodal-Lefty signaling pathway. Since Lefty acts as an inhibitor of Nodal signaling, we hypothesized that differences in Nodal signaling strength might regulate the fate of these two cell populations. By overexpressing different concentrations of Nodal mRNA and examining the fates of PP and anterior Endo cells, we confirmed this hypothesis.

Thus, we propose that even subtle differences in Nodal signaling levels may influence anterior mesendoderm fate decisions. To test this, we generated systems with slightly reduced Nodal signaling (via *ndr1* knockdown) and slightly elevated Nodal signaling (via *lft1* knockout). Using these models, we precisely captured the critical stage of fate segregation between PP and anterior endo cells and identified a novel transcriptional repressor, Ripply1, which works in concert with Gsc to suppress anterior endoderm differentiation.

> *(3) ripply1 mRNA injection phenotype inconsistent with previous literature: The phenotype presented in this manuscript from overexpressing ripply1 mRNA (Fig S11) is inconsistent with previous observations. This study shows a much more dramatic phenotype, suggesting that the overexpression may be to a non-physiological level that makes it difficult to interpret the gain-of-function experiments. For instance, Kawamura et al 2005 perform this experiment but do not trigger loss of head and eye structures or loss of tail structures. Similarly, Kawamura et al 2008 repeat the experiment, triggering a mildly more dramatic shortening of the tail and complete removal of the notochord, but again no disturbance of head structures as displayed here. These previous studies injected 25 - 100 pg of ripply1 mRNA with dramatic phenotypes, whereas this study uses 500 - 1000 pg. The phenotype is so much more dramatic than previously presented that it suggests that the level of ripply1 overexpression is sufficiently high that it may no longer be regulating only its endogenous targets, making the results drawn from ripply1 overexpression difficult to trust.*

We sincerely thank the reviewer for raising this question. First, we apologize for not providing a detailed description of the amount of HA-ripply1 mRNA injected in our previous manuscript. We injected 500 pg of HA-*ripply1* mRNA at the 1-cell stage and allowed the embryos to develop until 6 hpf for the CUT&Tag experiment. In the supplementary materials, we included a bright-field image of an 18 hpf-embryo injected with HA-ripply1 mRNA, which morphologically exhibited severe developmental abnormalities. The reviewer pointed out that the amount of *ripply1* mRNA we injected might be excessive, potentially leading to non-specific gain-of-function effects. The injection dose of 500 pg was determined based on conclusions from our previous study. In that study, injecting 24 pg of *ripply1* mRNA into one cell of zebrafish embryos at the 16–32 cell stage was sufficient to induce a secondary axis lacking the forebrain[5]. From this, we estimated that an injection concentration of approximately 500–1000 pg would be appropriate at the 1-cell stage, so that after several rounds of cell division, each cell gained 20-30 pg mRNA at 32 cell stage. Additionally, we conducted supplementary experiments injecting 100 pg, 250 pg, and 500 pg of *ripply1* mRNA, and observed 500 pg of *ripply1* mRNA led to a dramatic suppression of endoderm formation (Author response image 4).

Finally, our study focuses on the mechanism of cell fate segregation in the anterior mesendoderm, primarily during gastrulation. The embryos injected with *ripply1* mRNA underwent normal gastrulation, and our CUT&Tag experiment was performed at 6 hpf. Therefore, we believe that the amount of *ripply1* mRNA injected in this study is appropriate for addressing our research question.



Author response image 4. Different concentrations of *ripply1* mRNA were injected into zebrafish embryos at the one-cell stage, with RFP fluorescence labeling *sox17*-positive cells.

> *(4) Ripply1 binding to sox17 and sox32 regulatory regions not convincing: The Cut and Tag data presented in Fig 6J-K does not seem to be high quality and does not seem to provide strong support that Ripply 1 binds to the regulatory regions of these genes. The signal-to-noise ratio is very poor, and the 'binding' near sox17 that is identified seems to be even coverage over a 14 kb region, which is not consistent with site-specific recruitment of this factor, and the 'peaks' highlighted with yellow boxes do not appear to be peaks at all. To me, it seems this probably represents either: (1) overtagmentation of these samples or (2) an overexpression artifact from injection of too high concentration of ripply1-HA mRNA. In general, Cut and Tag is only recommended for histone modifications, and Cut and Run would be recommended for transcriptional regulators like these (see Epicypher's literature). Given this and the previous point about Ripply1 overexpression, I am not convinced that Ripply1 regulates endodermal genes. The existing data could be made somewhat more convincing by showing the tracks for other*

> *genes as positive and negative controls, given that Ripply1 has known muscle targets (how does its binding look at those targets in comparison) and there should be a number of Nodal target genes that Ripply1 does not bind to that could be used as negative controls. Overall this experiment doesn't seem to be of high enough quality to drive the conclusion that Ripply1 directly binds near sox17 and sox32 and from the data presented in the manuscript looks as if it failed technically.*

We sincerely thank the reviewer for raising this question. We apologize that the binding regions of *sox17* marked in our previous analysis were incorrect, and we have made the corresponding revisions in the latest version of the manuscript.

The reviewer noted that our CUT&Tag data contain considerable noise. To address this, we further refined our data processing: we annotated all peaks enriched in the treatment group and performed differential analysis, selecting genes with $\log_2$FoldChange > 3, padj < 0.5, and baseMean > 30 as candidate targets of Ripply1 binding. Subsequent GO enrichment analysis of these genes revealed significant enrichment of muscle development-related GO terms, which is consistent with previously reported roles of Ripply1 in regulating somite development. Therefore, we believe our filtering method effectively removes a large number of noise peaks and their associated genes.

Under these screening criteria, we found that *sox32* meets the threshold, while *sox17* does not. In addition, following the reviewer's suggestion, we examined *mespbb*—a known gene repressed by Ripply1—and *gsc*, a Nodal target gene, as a negative control.

Based on these new analyses, we have revised our figures and text accordingly. Our data now support the possibility that Ripply1 may directly bind to the promoter region of *sox32*. Since *sox32* acts as a direct upstream regulator of *sox17*, this binding could influence *sox17* expression (Figure 6 and Figure S11).

Finally, we would like to note that studies have reported Ripply1 as a transcriptional repressor, which may function by recruiting other co-factors, such as Groucho, to form a complex[14,15]. This might explain why our CUT&Tag data detected Ripply1 binding to a broad set of genes.

> *(5) "Cooperatively Gsc and ripply1 regulate": I suggest avoiding the term "cooperative," when describing the relationship between Ripply1 and Gsc regulation of PP and anterior endoderm - it evokes the concept of cooperative gene regulation, which implies that these factors interact with each biochemically in order to bind to the DNA. This is not supported by the data in this manuscript, and is especially confusing since Ripply1 is thought to require cooperative binding with a T-box family transcription factor to direct its binding to the DNA.*

We sincerely thank the reviewer for raising this important issue. The reviewer pointed out that the term "Cooperatively" may not be entirely appropriate in the context of our study. In accordance with the reviewer's suggestion, we have replaced "Cooperatively" with "Collectively" in the relevant sections.

> *(6) SWI/SNF: The differential expression of srcap doesn't seem very remarkable. The dot plots in the supplement S7H don't help - they seem to show no expression at all in the endoderm, which is clearly a distortion of the data, since from the violin plots it's obviously expressed and the dot-size scale only ranges from ~30-38%. Please add to the figure information about fold-change and p-value for the differential expression. Publicly available scRNAseq databases show scrap is expressed throughout the entire early embryo, suggesting that it would be surprising for it to have differential activity in these two cell types and thereby contribute to their separate specification during development. It seems equally possible that this just mildly influences the level of Nodal or FGF signaling, which would create this effect.*

Thank the Reviewer for this question. As suggested, we performed Wilcoxon tests to compare *srcap* expression between PP and Endo populations. The analysis shows that while *srcap*

expression is moderately elevated in PP compared to in Endo, this difference is not statistically significant. The corresponding p-value and fold change have now been included in the revised figure (Please see Figure 4J and S7H). Although the transcriptional level of *srcap* shows no significant difference between PP and anterior endoderm, our subsequent experiments—using AU15330 (an inhibitor of the SWI/SNF complex) and injecting morpholino targeting *srcap*, a key component of the SWI/SNF complex—demonstrated that its inhibition indeed promotes anterior endoderm fate while reducing PP cell specification. Therefore, we propose that subtle differences in the SWI/SNF complex may regulate the fate specification of PP and anterior endoderm through two mechanisms. First, as mentioned in our study, these chromatin remodelers modulate the expression of master regulators such as Gsc and Ripply1, thereby influencing cell fate decisions. Second, as noted by the reviewer, these chromatin remodelers may affect the interpretation of Nodal signaling, ultimately contributing to the divergence between PP and anterior endoderm fates.

> *The multiome data seems like a valuable data set for researchers interested in this stage of zebrafish development. However, the presentation of the data doesn't make many conclusions, aside from identifying an element adjacent to ripply1 whose chromatin is open in prechordal plate cells and not endodermal cells and showing that there are a number of loci with differential accessibility between these cell types. That seems fairly expected since both cell types have several differentially expressed transcriptional regulators (for instance, ripply1 has previously been demonstrated in multiple studies to be specific to the prechordal plate during blastula stages). The manuscript implies that SWI/SNF remodeling by Srcap is responsible for the chromatin accessibility differences between these cell types, but that has not actually been tested. It seems more likely that the differences in chromatin accessibility observed are a result of transcription factors binding downstream of Nodal signaling.*

We thank the reviewer for recognizing the value of our newly generated data. Through integrative analysis of single-cell data from wild-type, *ndr1* kd, and *lft1* ko groups of Nodal-injected explants at 6 hours post-fertilization (hpf), we identified a critical branching point in the fate segregation of the prechordal plate (PP) and anterior endoderm (Endo), where chromatin remodelers may play a significant role. Based on this finding, we performed single-cell RNA and ATAC sequencing on zebrafish embryos at 6 hpf. Analysis of this multi-omics dataset revealed that transcriptional repressors such as Gsc, Ripply1, and Osr1 exhibit differences in both transcriptional and chromatin accessibility levels between the PP and anterior Endo. Subsequent overexpression and loss-of-function experiments further demonstrated that Gsc and Ripply1 collaboratively suppress endodermal gene expression, thereby inhibiting endodermal cell fate. Previous studies have reported that for the activation of certain Nodal downstream target genes, the pSMAD2 protein of the Nodal signaling pathway recruits chromatin remodelers to facilitate chromatin opening and promote further transcription of target genes[16]. Therefore, our data provide chromatin accessibility profiles for Gsc and Ripply1, offering a valuable resource for future investigations into their pSMAD2 binding sites.

> *Minor issues:*
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> *Figure 2 E-F: It's not clear which cells from E are quantitated in F. For instance, the dorsal forerunner cells are likely to behave very differently from other endodermal progenitors in this assay. It would be helpful to indicate which cells are analyzed in Fig F with an outline or other indicator of some kind. Or - if both DFCs and endodermal cells are included in F, to perhaps use different colors for their points to help indicate if their fluorescence changes differently.*

Thank you for the reviewer's suggestion. In the revised version of the figure, we have outlined the regions of the analyzed cells.

> *Fig 3 J: Should the reference be Dubrulle et al 2015, rather than Julien et al?*

Thanks, we have corrected.

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