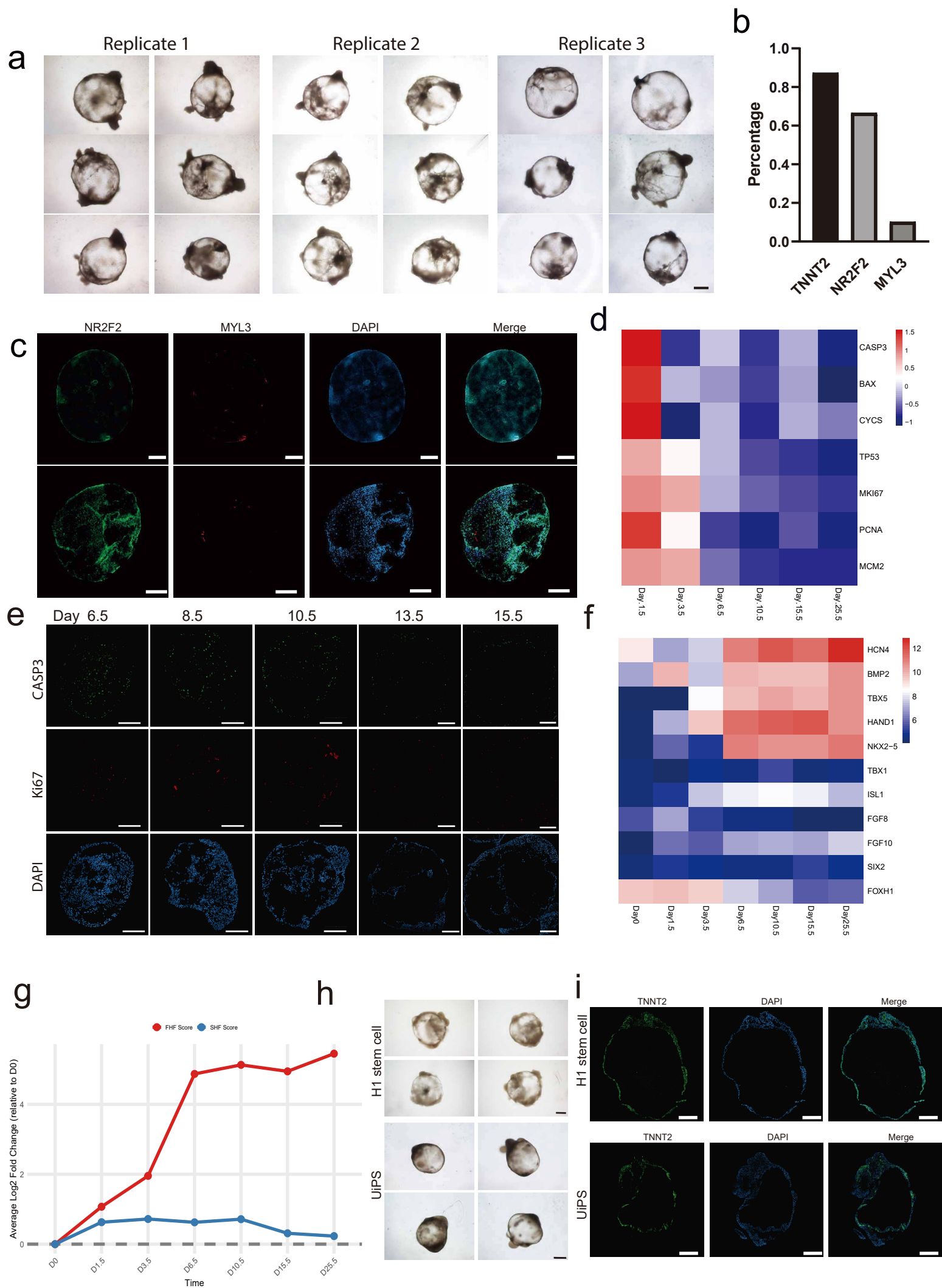
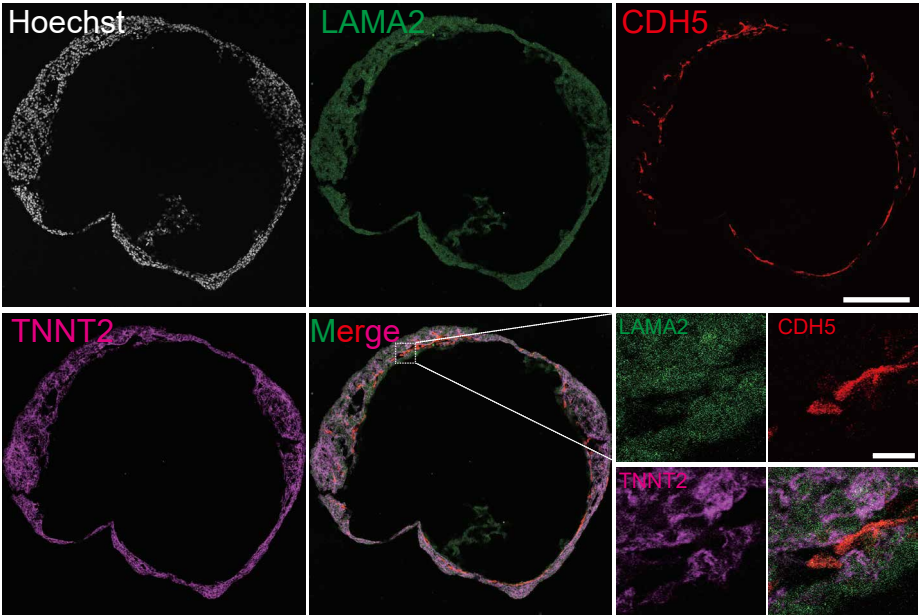




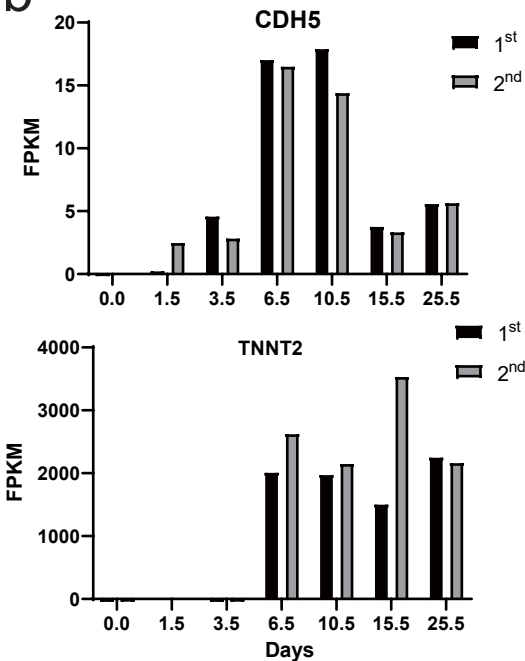
Supplementary Figure 1. Chamber formation of chamber-like cardiac organoids (CCOs) deprived from pluripotent stem cells.

a Representative brightfield images from day 15.5, demonstrating the consistent emergence of cavity-containing structures across three separate biological replicates. Scale bar, 500 μm . **b** Cardiomyocyte, atrial, and ventricular cell proportions in CCOs through the single cell sequencing data. **c** Immunofluorescence images of CCOs at day 53, illustrating the distributions of atrial and ventricular cells using the ventricular marker MYL3 and the atrial marker NR2F2. Scale bar: 200 μm . **d** Heatmap of proliferation related genes (*MCM2*, *PCNA*, *MKI67*, *TP53*) and apoptosis related genes (*CYCS*, *BAX*, *CASP3*) during CCO differentiation. **e** Representative immunofluorescence staining for Ki67 and cleaved CASP3 in CCOs during chamber formation. Scale bar, 200 μm . **f** Heatmap of FHF (*HCN4*, *BMP2*, *TBX5*, *HAND1*, *NKX2-5*) and SHF (*TBX1*, *ISL1*, *FGF8*, *FGF10*, *SIX2*, *FOXH1*) marker expression during CCO differentiation. **g** Gene set-based scores for first heart field (FHF) and second heart field (SHF) calculated as mean log2 fold changes relative to Day 0 across differentiation. **h** Representative brightfield images of CCOs derived from H1 and UiPS cell lines on day 15.5. UiPS, urine-induced pluripotent stem cells. Scale bar, 500 μm . **i** Cryosections of CCOs on day 16.5 showing the expression of the cardiomyocyte marker TNNT2 derived from H1 and UiPS cell lines. Scale bar, 200 μm . Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.

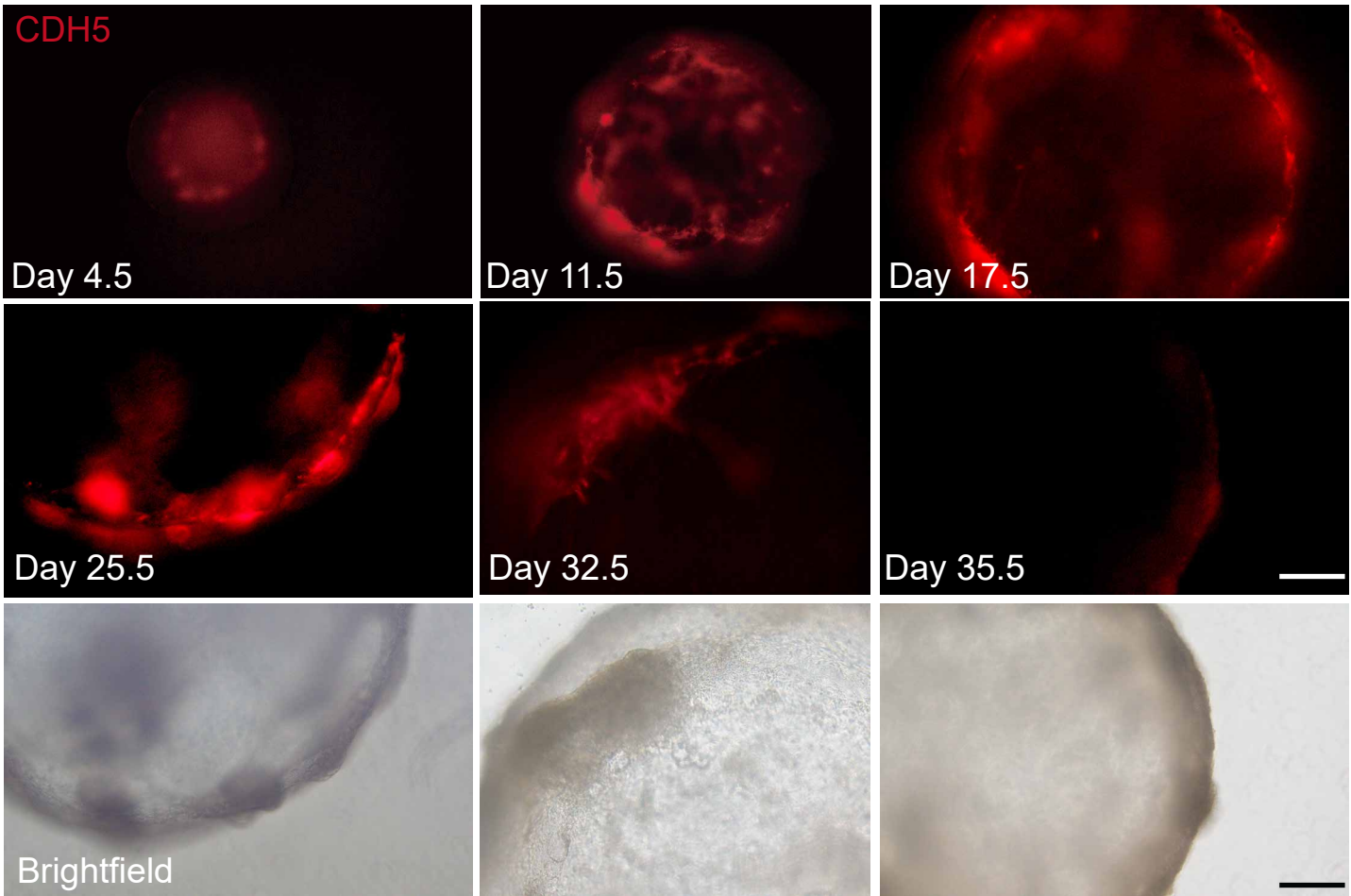
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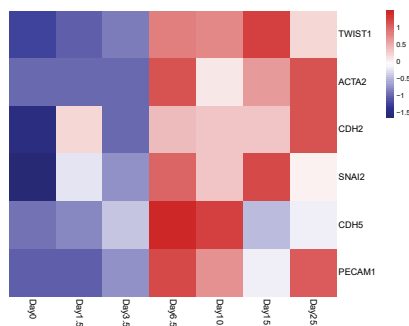
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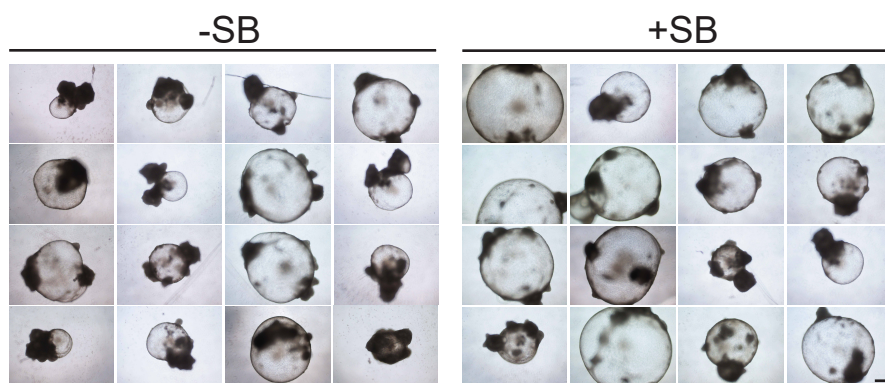
Supplementary Fig. 2. Endothelial dynamics and laminin expression in CCOs.

a Immunofluorescence of LAMA2 in CCOs shows localization to the TNNT2⁺ myocardial layer, consistent with single-cell transcriptomic predictions (D22.5). Scale bars: 200 μm (full image), 20 μm (magnified area). **b** Time-course bulk RNA-seq analysis of *CDH5* (top) and *TNNT2* (bottom) expression during CCO differentiation. Bar graphs represent FPKM values from two independent experiments (N=2). **c** Live imaging of CDH5-P2A-Scarlet showing the transient presence of CDH5⁺ endothelial cells within CCOs during differentiation. Scale bar, 200 μm . Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.

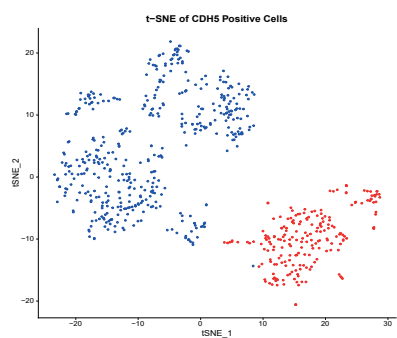
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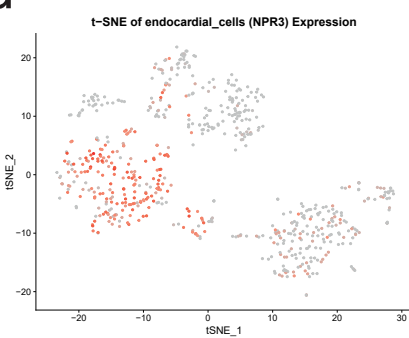
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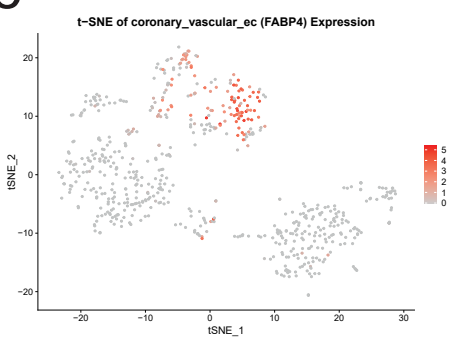
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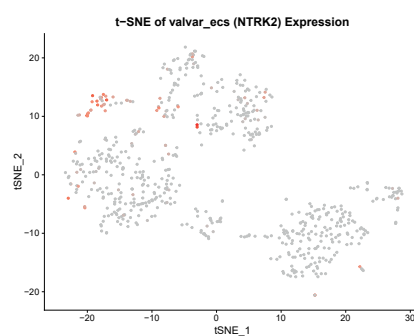
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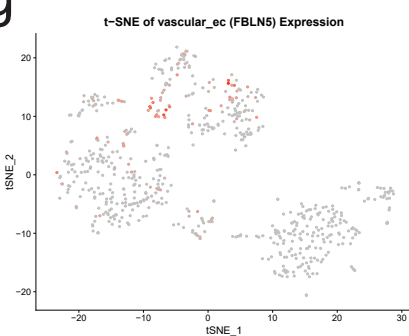
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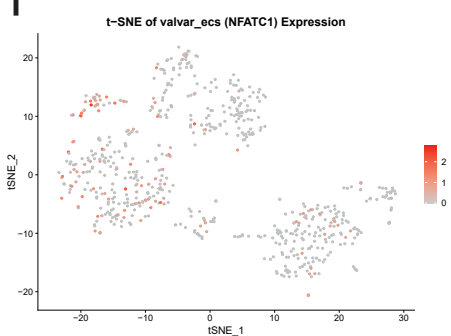
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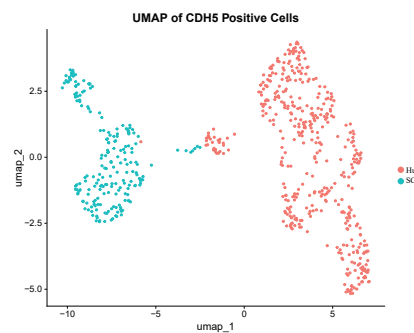
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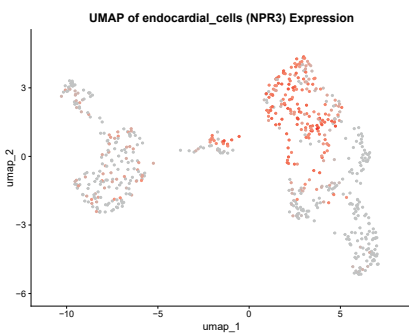
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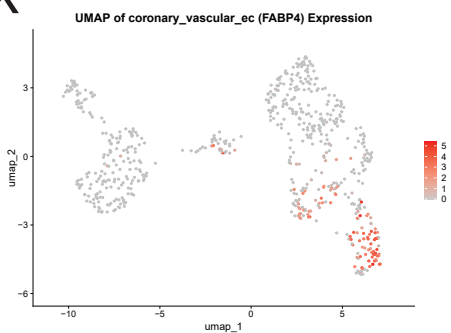
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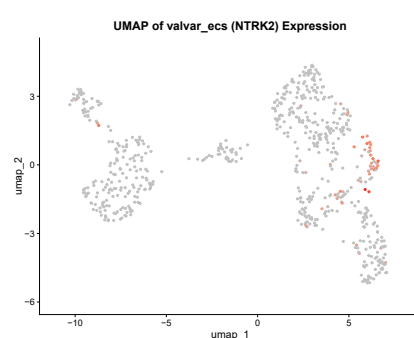
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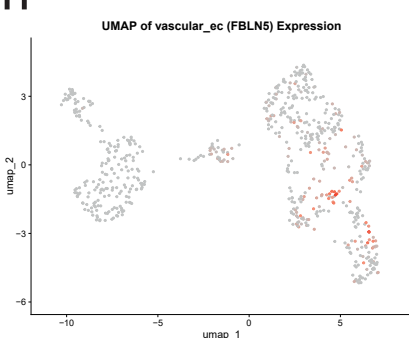
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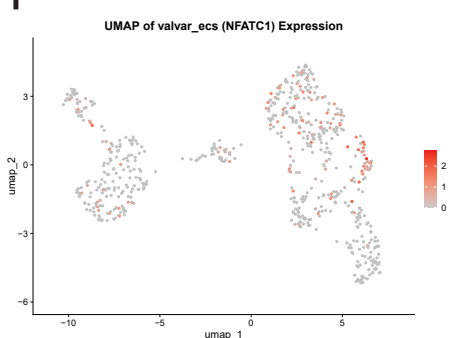
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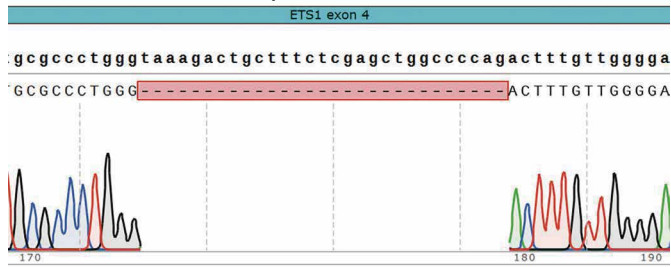
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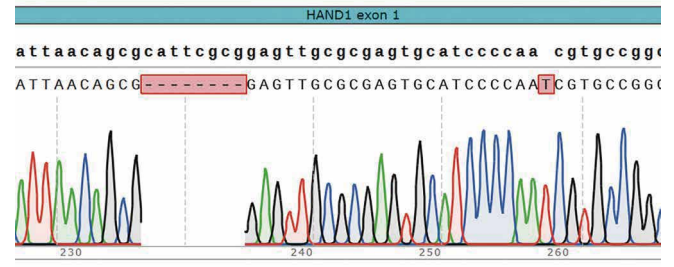
Supplementary Figure 3. Characterization of Endocardial-like Endothelial Cells in CCOs and Their Comparison to Fetal Heart Endothelial Subtypes.

a Heatmap displaying gene expression of markers associated with endothelial-to-mesenchymal transition (EndoMT) during CCOs development from day 0 to day 25. **b** Representative brightfield images of CCOs treated with or without 0.5 μ M SB431542 for 20 days, captured on day 48.5. Scale bar, 500 μ m. **c** t-SNE plot showing the clustering of CDH5-positive endothelial cells derived from CCOs and human fetal hearts. Endothelial cells from CCOs and fetal hearts form two distinct, yet adjacent, populations. **d-g** Feature plots of representative marker gene expression for each endothelial subtype on the t-SNE map: *NPR3* (endocardial), *FABP4* (coronary vascular ECs), *FBLN5* (vascular ECs), and *NTRK2* (valvar ECs). **h** Expression of *NFATC1*, highly expressed in endocardial cushions and valvar ECs but not in free wall endocardium. **i** UMAP plot showing the clustering of CDH5-positive endothelial cells derived from CCOs and human fetal hearts. **j-n** Feature plots of the same subtype marker genes as in panels of d-h on the UMAP projection, confirming that CCO-derived endothelial cells predominantly express *NPR3*, but not markers of coronary, vascular, or valvar endothelial subtypes. All images are representative of at least 3 independent experiments with similar results.

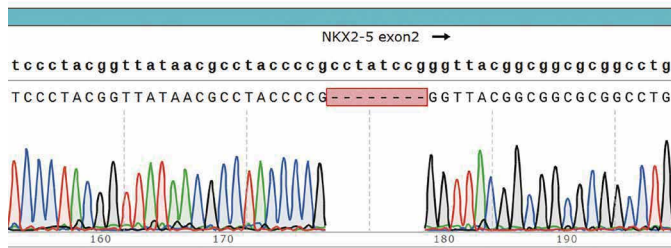
a ETS1 KO exon4 29bp deletion



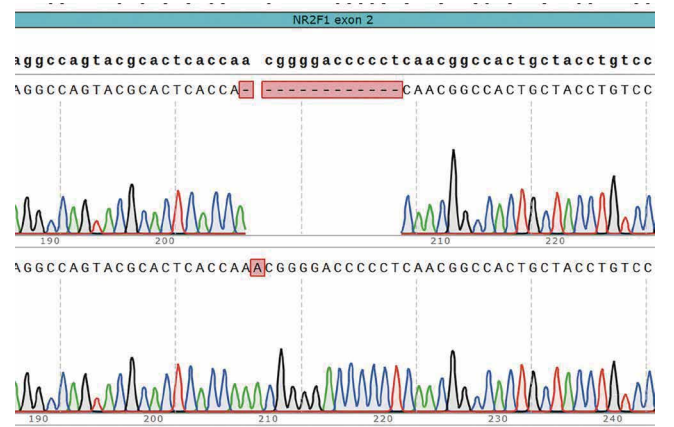
b HAND1 KO exon1 8bp deletion+1bp insertion



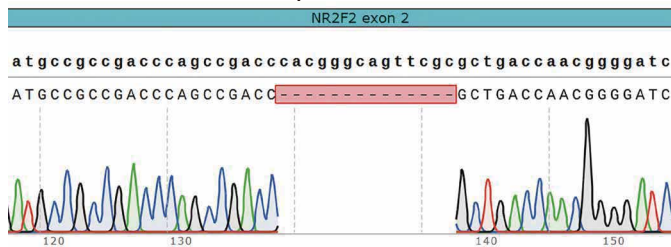
c NKX2-5 KO exon2 8bp deletion



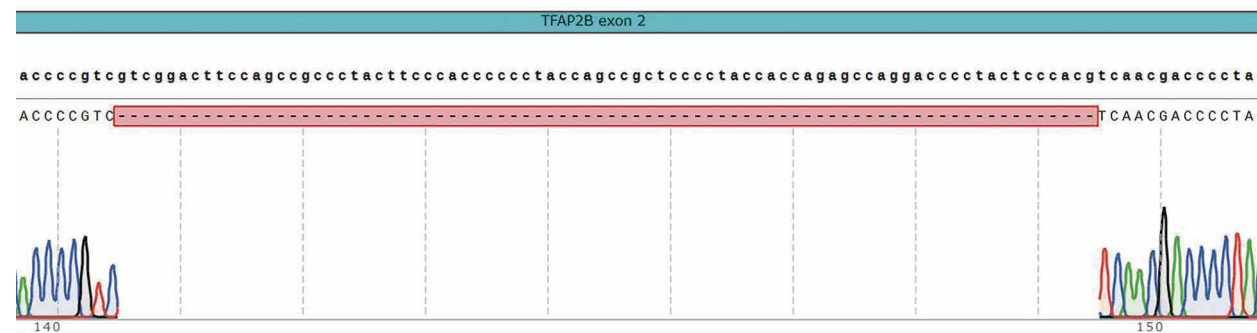
d NR2F1 KO exon2 13bp deletion/1bp insertion heterozygote



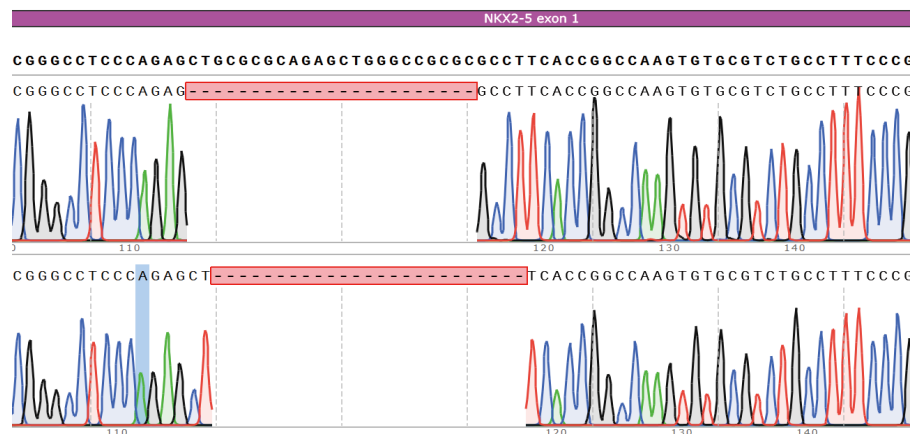
e NR2F2 KO exon2 14bp deletion



f TFAP2B KO exon2 80bp deletion

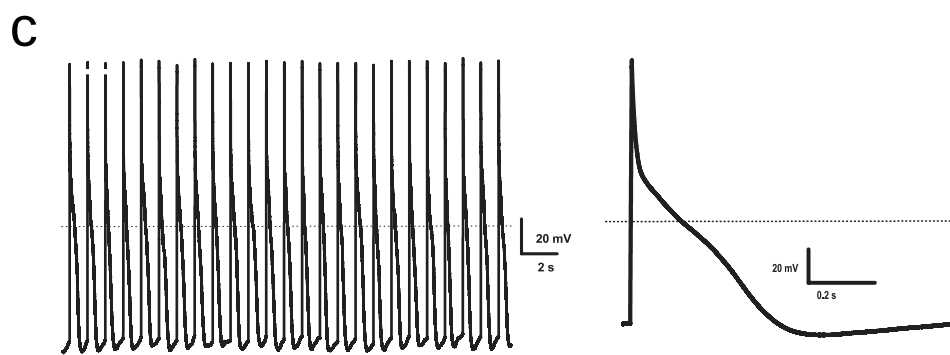
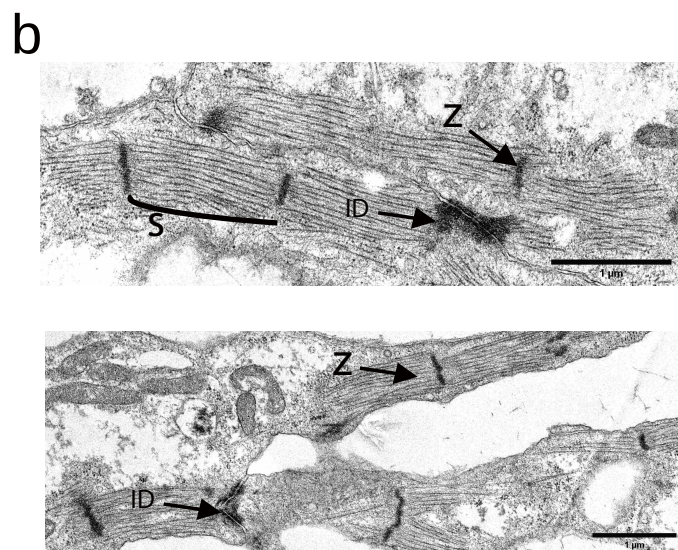
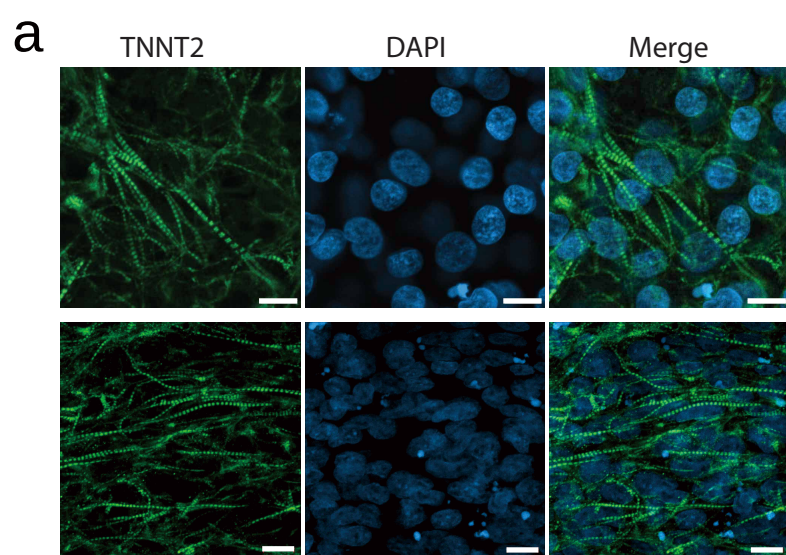


g NKX2-5 KO exon1 23bp and 25bp deletion in TNNT2-P2A-GFP



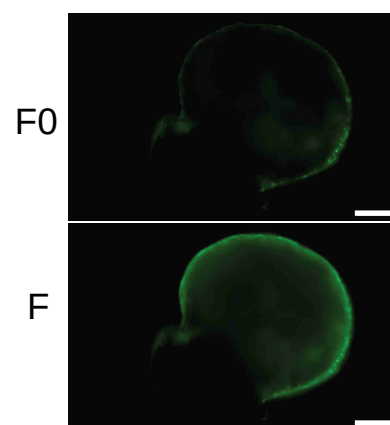
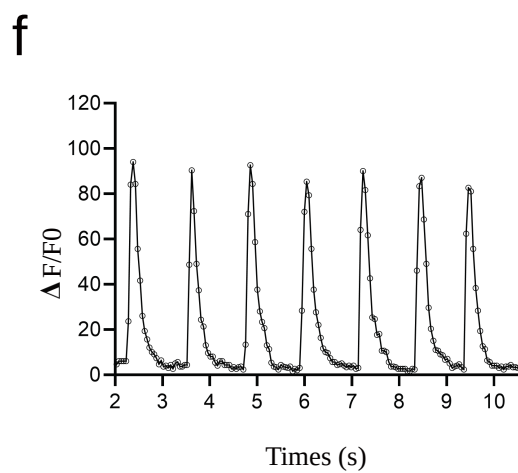
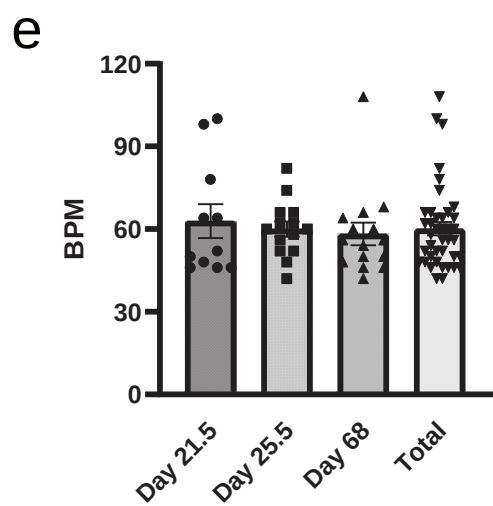
Supplementary Figure 4. Validation of Gene Knockouts in CCO Models.

a-f Sanger sequencing confirmation of knockout lines used in this study. **a** *ETSI* knockout with a homozygous 29 bp deletion in exon 4. **b** *HAND1* knockout with a homozygous 8 bp deletion and a 1 bp insertion in exon 1. **c** *NKX2-5* knockout with a homozygous 8 bp deletion in exon 2. **d** *NR2F1* knockout with compound heterozygous frameshift mutations: a 13 bp deletion in one allele and a 1 bp insertion at the same site in the other allele of exon 2. **e** *NR2F2* knockout with a homozygous 14 bp deletion in exon 2. **f** *TFAP2B* knockout with a homozygous 80 bp deletion in exon 2. Each panel shows the targeted exon region and the corresponding mutation type, confirming successful genome editing for subsequent functional studies in CCOs. **g** *NKX2-5* knockout in TNNT2-P2A-GFP cell line with compound heterozygous deletion mutations: a 23 bp deletion in one allele and a 25 bp deletion at the same site in the other allele of exon 1.



d

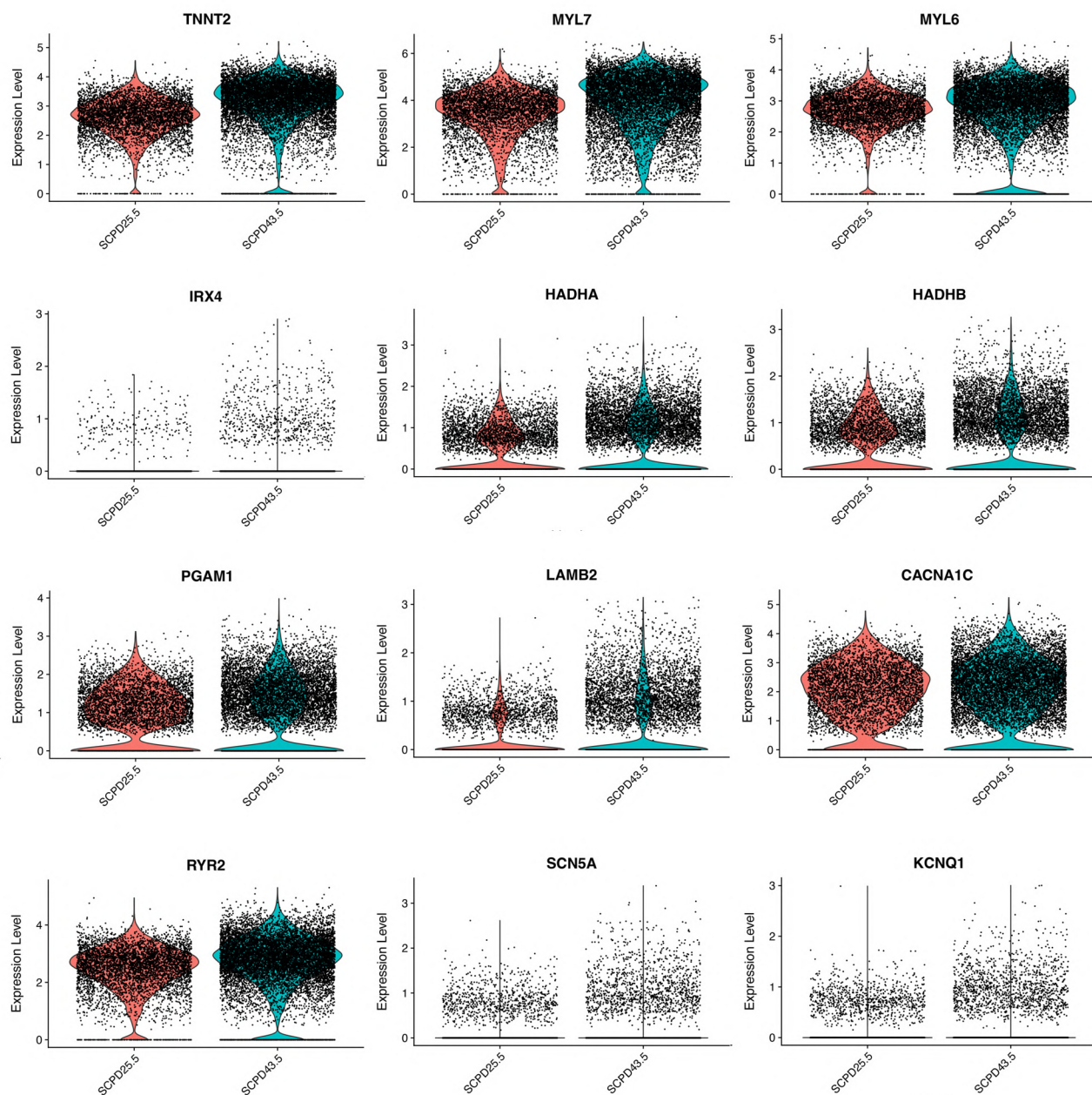
RMP (mV)	-60.99 ± 4.91
APA (mV)	119.05 ± 8.69
APD20 (ms)	11.15 ± 2.29
APD50 (ms)	88.57 ± 25.65
APD90 (ms)	316.97 ± 47.89



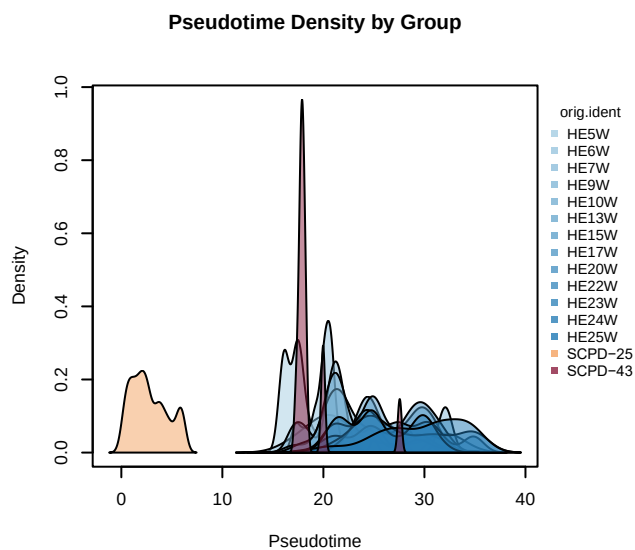
Supplementary Figure 5. Functional characterization of CCOs.

a Confocal images showing the sarcomeric organization within cardiomyocytes of CCOs on day 22 (down) or 62 (top). Scale bar: 10 μm . **b** Electron micrographs of CCOs on day 90.5, highlighting key structures: S, sarcomere; ID, intercalated disc; Z, Z-line. Scale bar: 1 μm **c** Representative traces and average action potential recordings of CCOs on day 80. **d** Characteristics of action potentials observed in the data presented in **c** on day 26 and day 80. RMP, rest membrane potential; APD, action potential duration; APA, action potential amplitude. Mean $\pm$ SEM (N=3, n=6). **e** Beating rate of cardiac organoids on different days. BMP, beats per minute. All bar graphs show mean $\pm$ SEM (N=3, n=40). **f** Calcium imaging analysis involved loading CCOs with Fluo-4-AM and monitoring fluorescence intensity over time. F/F₀ represents fluorescence intensity relative to background levels. Scale bar, 500 μm . Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.

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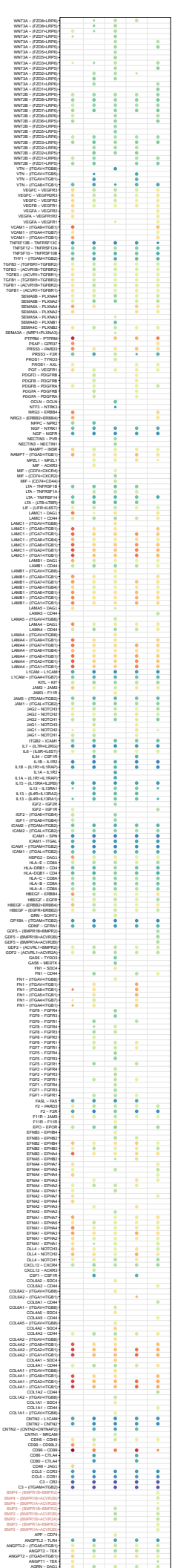
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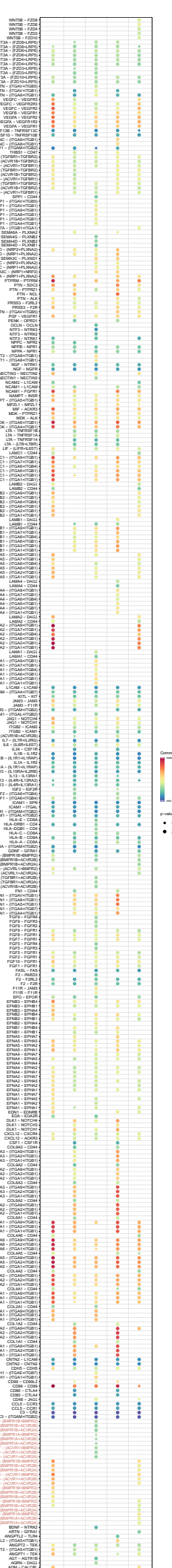
Supplementary Figure 6. Maturation Analysis of Cardiomyocytes in Cardiac Organoids from Day 25.5 to Day 43.5.

a Violin plots showing expression of maturation-related genes in cardiomyocytes from cardiac organoids at day 25.5 versus day 43.5. **b** Slingshot-based pseudotime density plot of cardiac organoid cells at day 25.5 (SCPD-25) and day 43.5 (SCPD-43) compared with human fetal heart reference samples (HE). The x-axis indicates inferred pseudotime along the developmental trajectory, and the y-axis indicates the normalized cell density for each sample group. SCPD-25 cells are concentrated at earlier pseudotime values, whereas SCPD-43 cells are shifted toward later pseudotime positions and partially overlap with HE reference samples occupying higher pseudotime ranges.

a



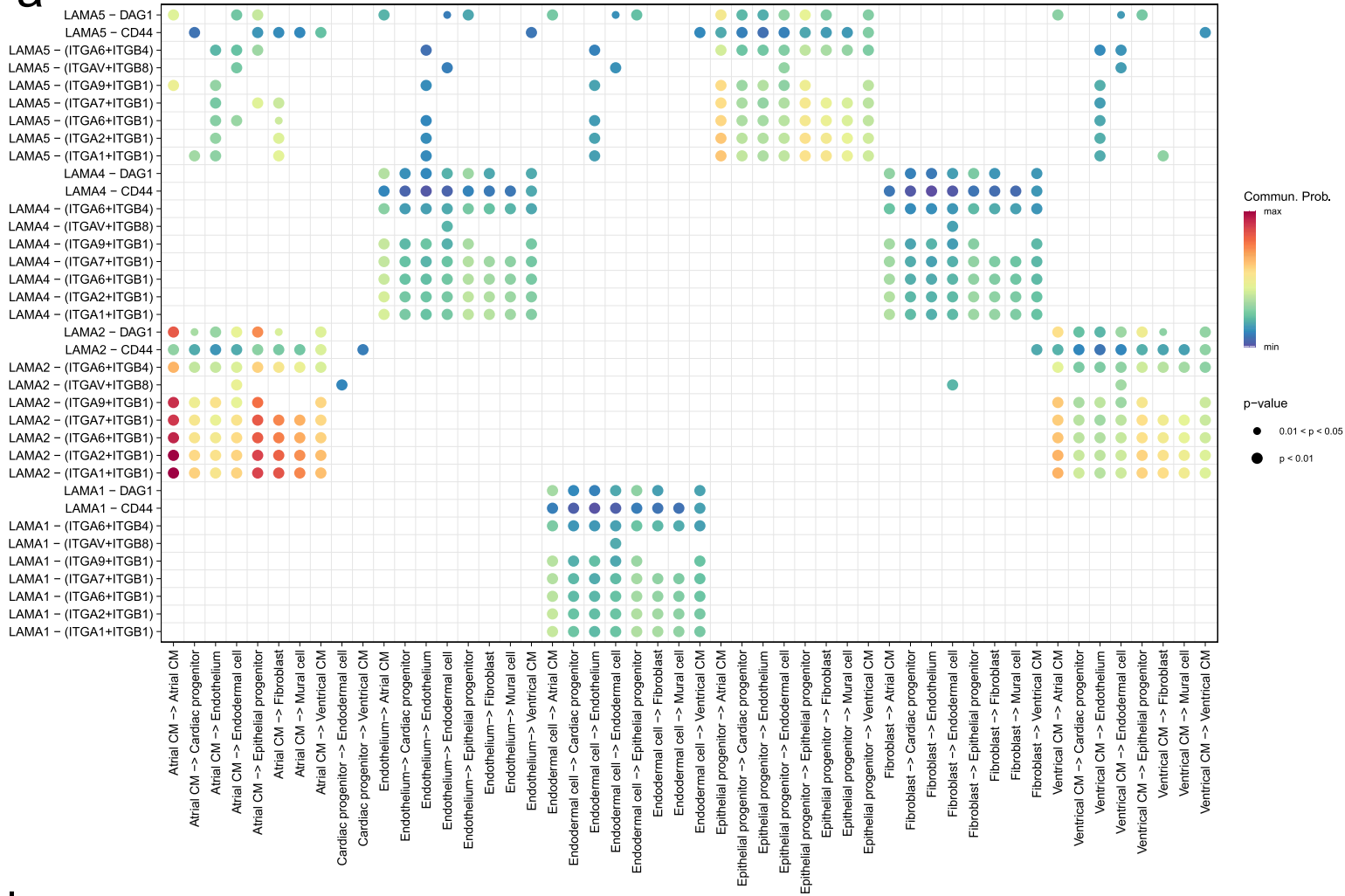
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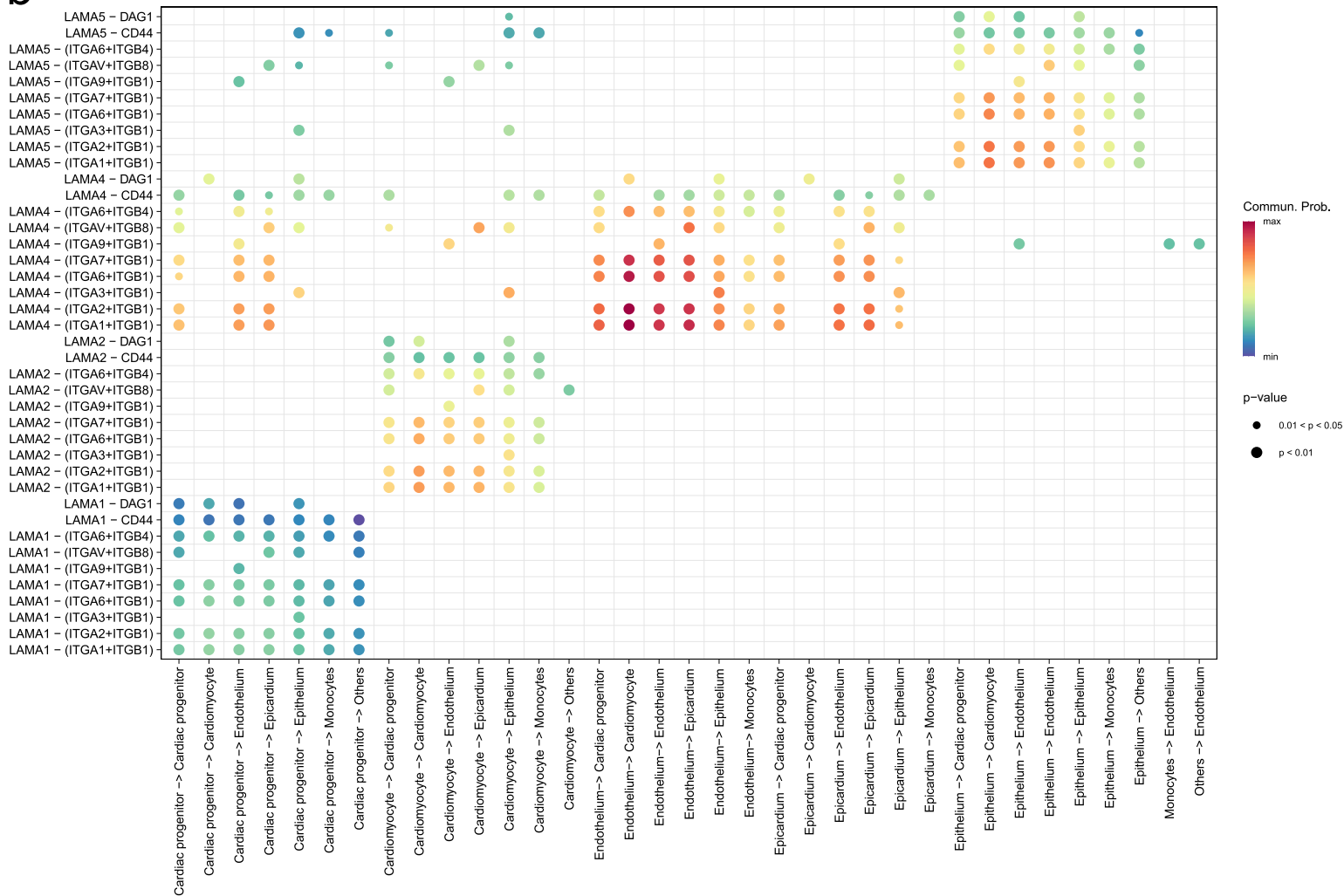
Supplementary Figure 7. Interaction of signaling pathways in the cell communication network.

a Signals from the endothelium: Visualization of signaling pathways originating from the endothelium to other cell types, with colors indicating communication probabilities and circle sizes representing pathway significance. **b** Signals to the endothelium: Visualization of signaling pathways directed toward the endothelium from other cell types, with the same representation of probabilities and significance.

a

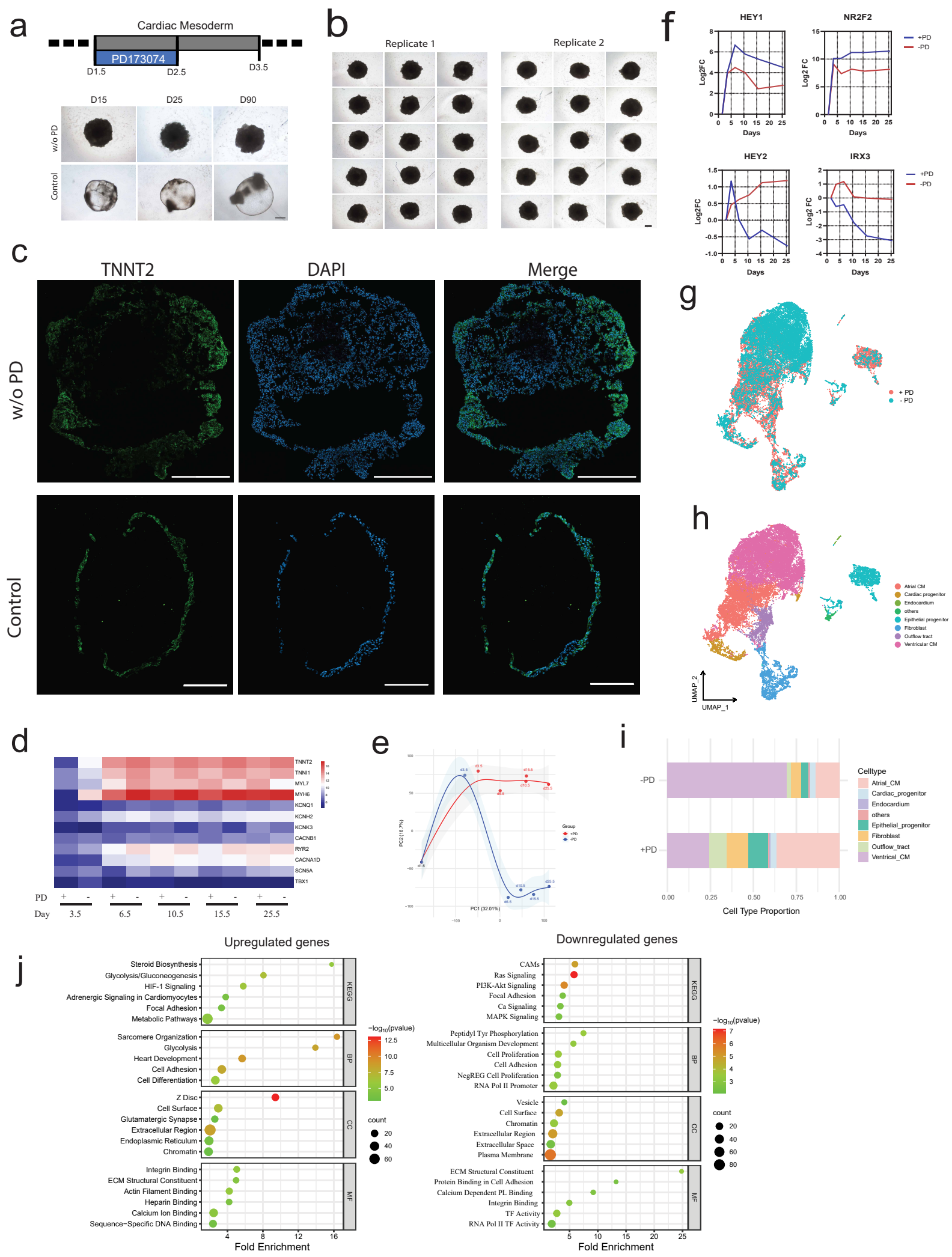


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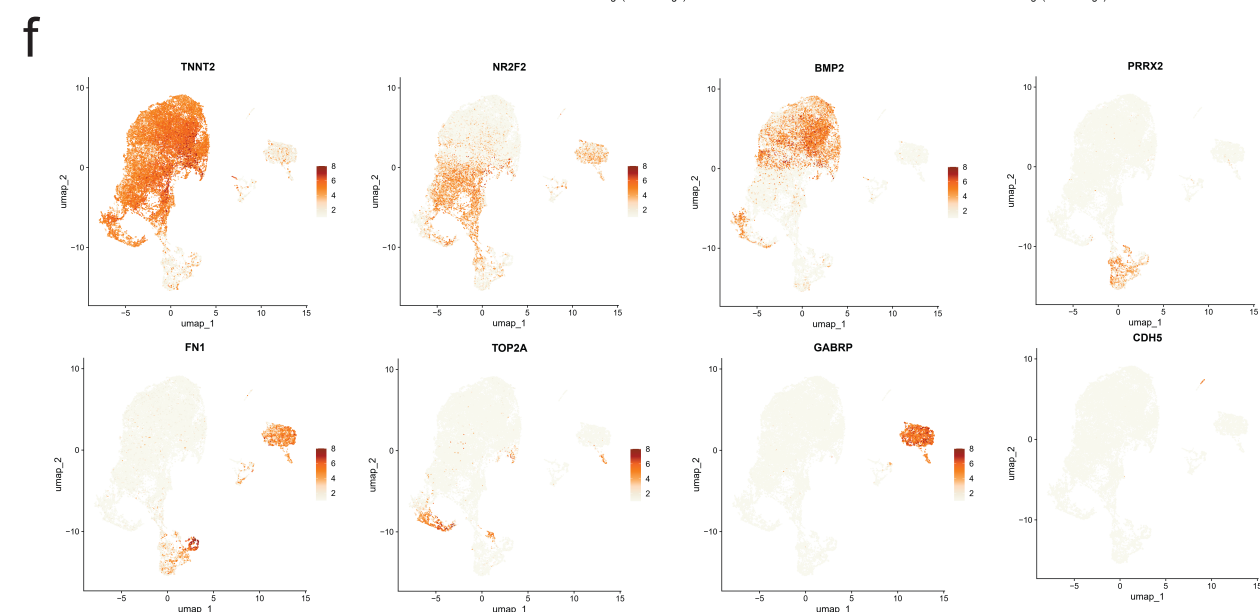
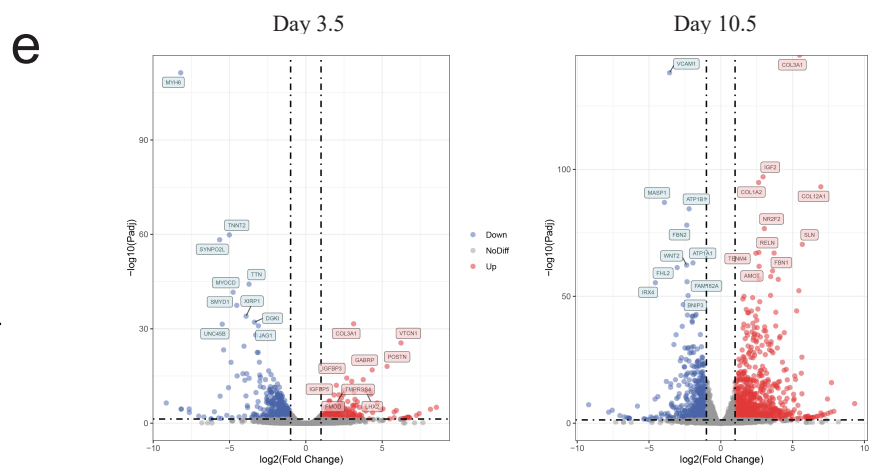
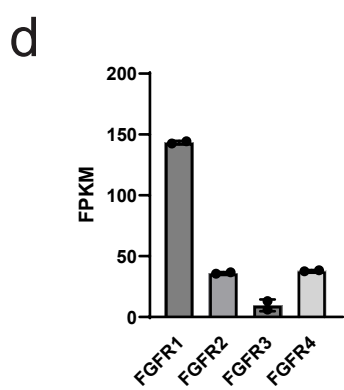
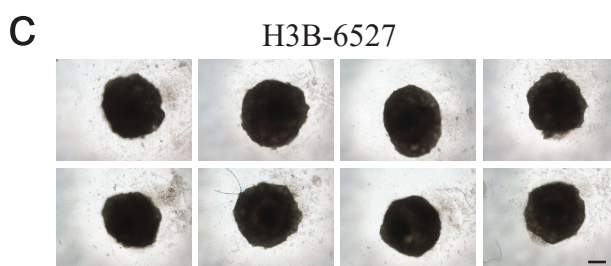
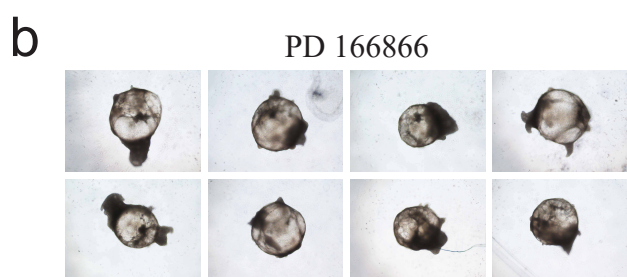
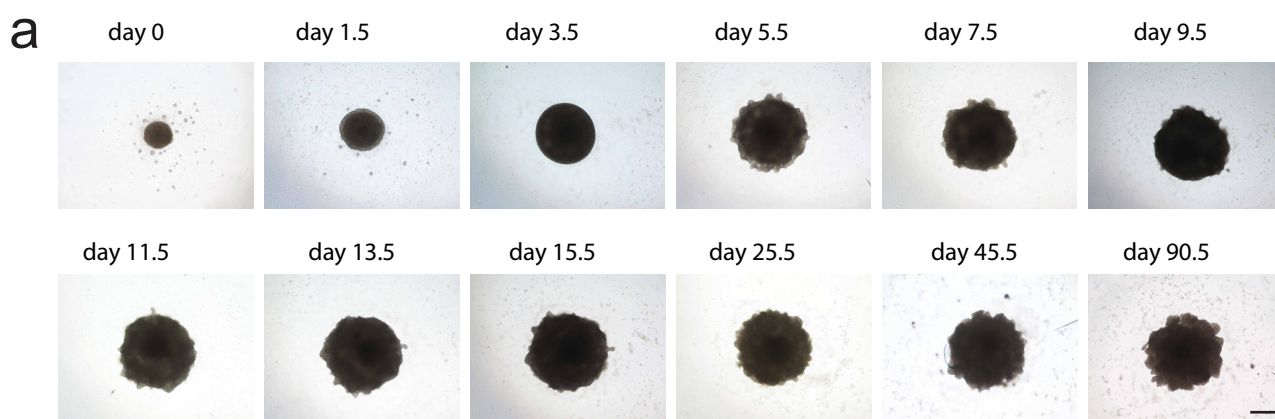
Supplementary Figure 8. Heatmap of interaction probabilities for LAMA signaling pathways within the cell-cell communication network.

The heatmap displays interaction probabilities of LAMA signaling pathways among various cell types in the CCO model a and 5- to 6-week human fetal hearts b. This analysis reveals the spatially specific communication patterns of individual LAMA family members.



Supplementary Figure 9. Inhibition of FGF is crucial for CCOs formation during cardiac mesoderm induction stage.

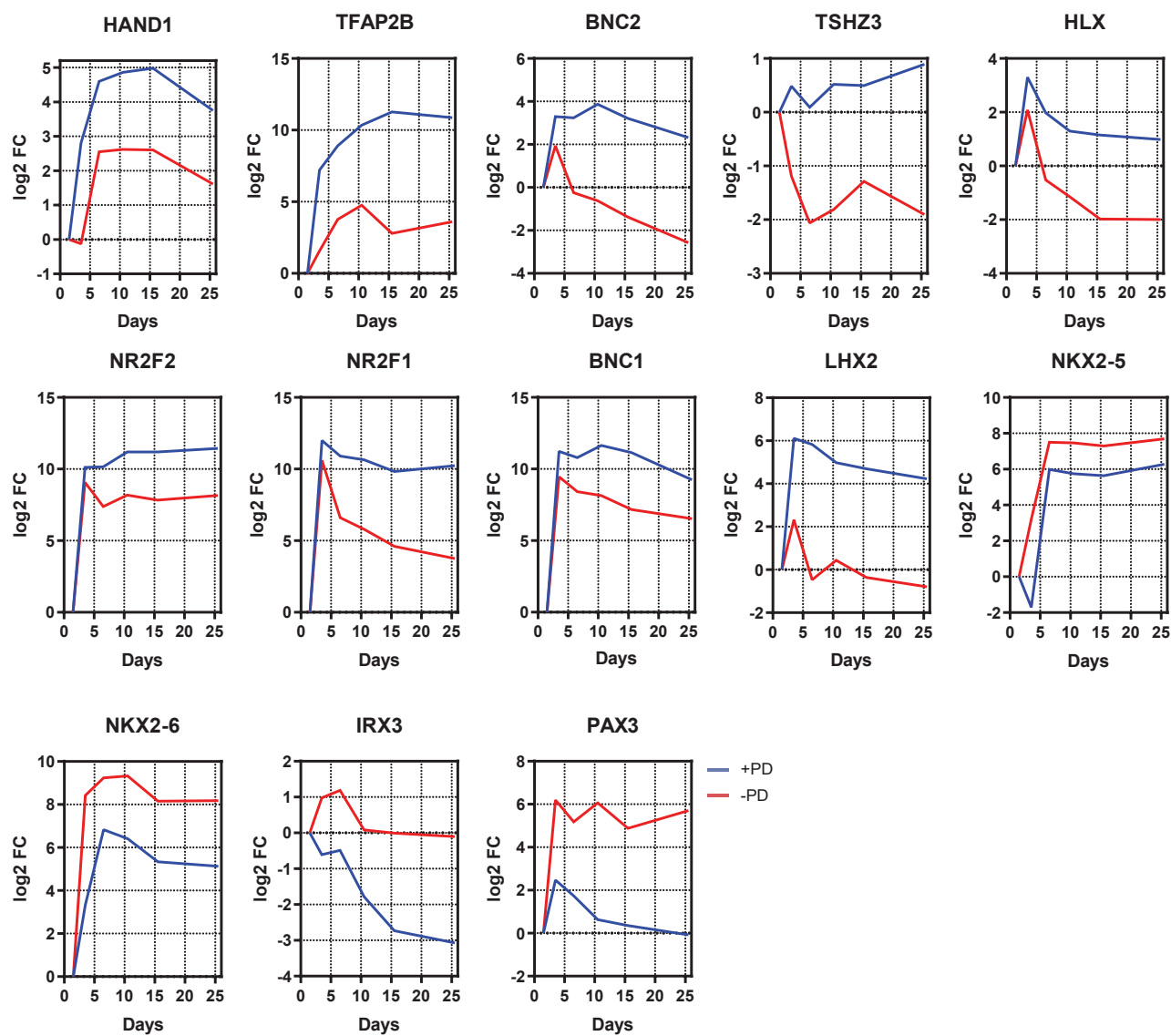
a Chamber formation of CCOs is dependent on PD173074 (PD). Scale bar, 500 μ m. **b** Brightfield images on day 16.5, showing solid cardiac organoids structures across three independent biological replicates without PD. Scale bar, 500 μ m. **c** Cryosections of CCOs on day 16.5 (with PD and without PD). Scale bar: 200 μ m. **d** Heatmap illustrating the expression patterns of key genes, including cardiomyocyte markers and ion channels, during cardiomyocyte differentiation with and without PD. VST, variance-stabilized transformed counts. **e** PCA plot illustrating distinct cell fates with and without PD. **f** Cardiac-specific cell type gene expression analysis (log₂ fold-change vs. day 1.5) in CCOs. **g** Data Distribution: Illustrates the cell distribution of CCOs with or without PD. Cells with PD are denoted in red, while those samples without PD are depicted in blue, facilitating visualization of cell distribution within each sample in the dataset. **h** Cell Type Annotation Visualization: Offers a UMAP visualization of cell type annotation using single-cell RNA-seq data from cultures, with colors representing different cell types. **i** Horizontal bar charts depicting the cell proportion of each cell cluster with or without PD groups. **j** GO and KEGG terms upregulated or downregulated in CCOs with PD as control on day 3.5. Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.



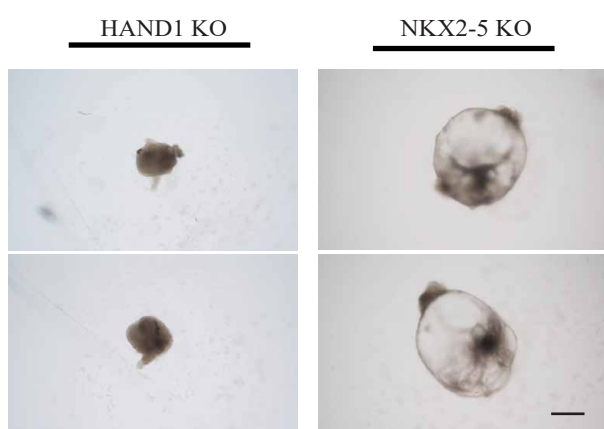
Supplementary Figure 10. Additional validation of FGF signaling roles in chamber formation.

a Time-lapse brightfield images illustrating the formation of cardiac organoids in the absence of the FGFR1 inhibitor PD173074. Scale bar, 500 μ m. **b** Representative brightfield images of CCOs induced with PD166866 on day 17.5. Scale bar, 500 μ m. **c** Representative brightfield images of cardiac organoids induced with H3B-6257 on day 17.5. Scale bar, 500 μ m. **d** Expression levels of *FGFR* family genes on day 0. FPKM, Fragments Per Kilobase of transcript per Million mapped reads. Mean $\pm$ SEM (N=2). **e** Volcano plot comparing gene expression profiles at the cardiac mesoderm stage on day 3.5 and the chamber formation stage on day 10.5, without PD treatment serving as the control. **f** Marker Gene Expression: A UMAP scatter heatmap illustrates the expression of marker genes for different cell types, with color gradients representing expression levels. Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.

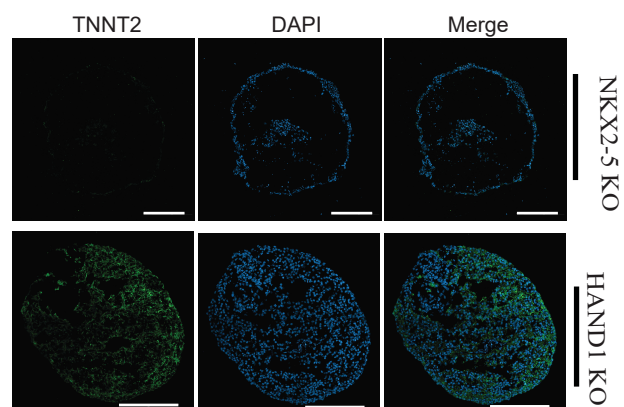
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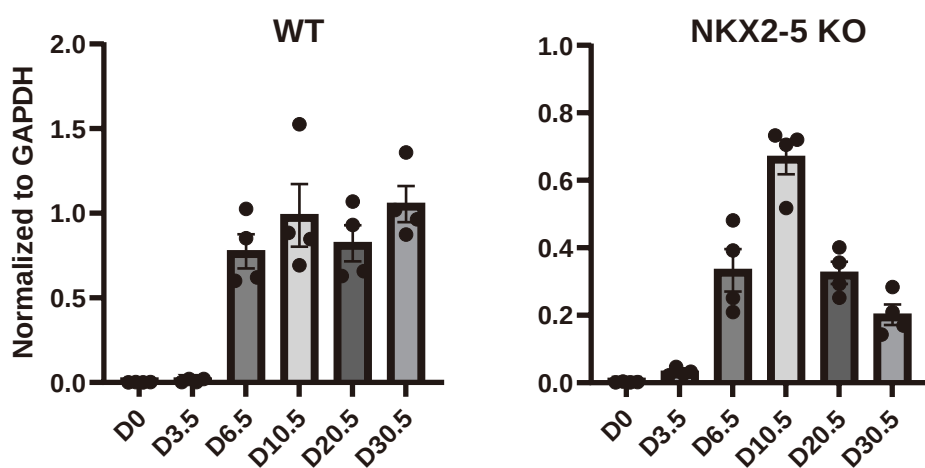
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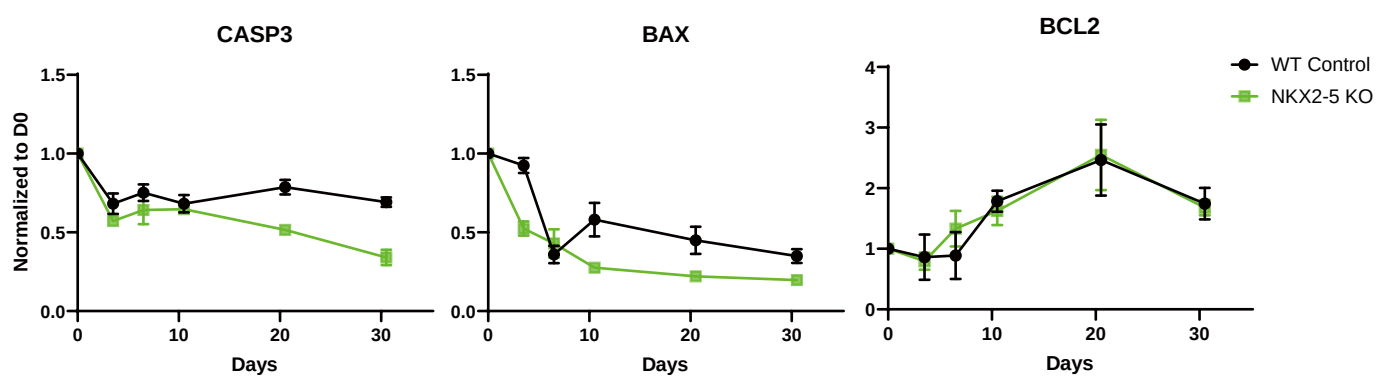
Supplementary Figure 11. *TFAP2B*, *NR2F2* and *HAND1* are crucial for the formation of CCOs.

a Gene expression analysis of 13 selected genes (log₂ fold-change vs. day 1.5) in cardiac organoids formation with or without PD173074. Blue line indicates with PD173074, while red line indicates without PD. To present the complete 13-gene panel, data for *TFAP2B*, *NR2F2*, and *HAND1* are integrated from Figure 5d, and data for *NR2F2* and *IRX3* are integrated from Supplementary Figure 9f. **b** Representative brightfield images of *HAND1* and *NKX2.5* KO cardiac organoids from day 15.5 to day 30. Scale bar, 500 μ m. **c** Confocal cryosection images of *HAND1* and *NKX2.5* knockout cardiac organoids stained with TNNT2. Scale bar, 200 μ m. Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.

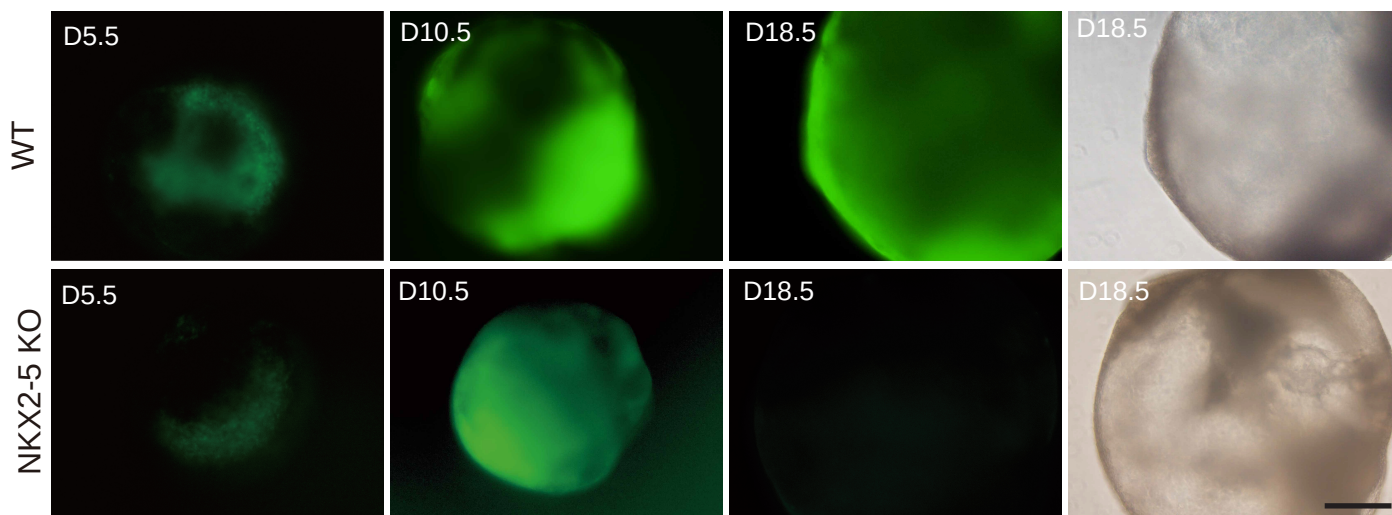
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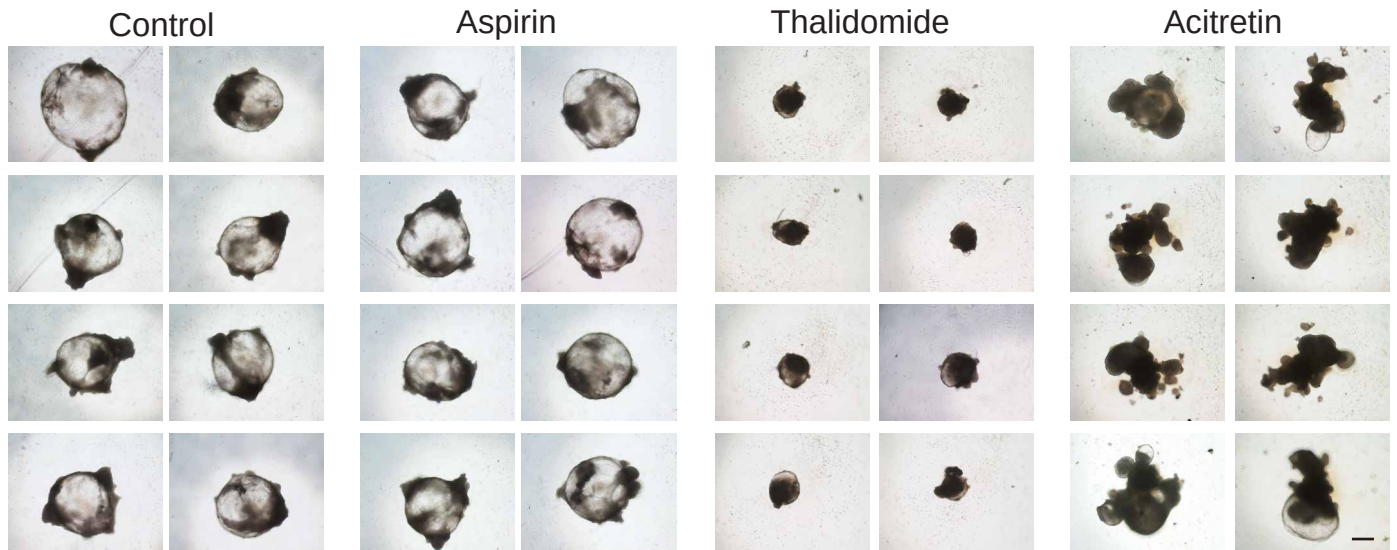
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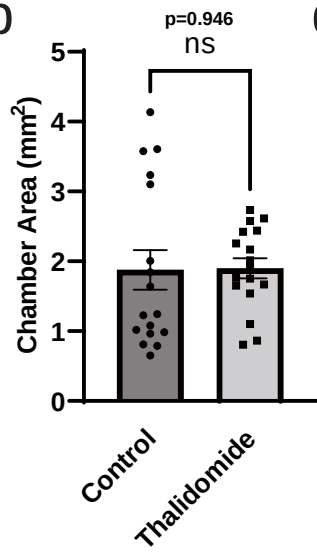
Supplementary Fig. 12 Time-course analysis of cardiomyocyte marker expression in WT and *NKX2-5* KO CCOs

a Time-course quantitative PCR analysis of *TNNT2* expression in WT and *NKX2-5* KO CCOs at indicated differentiation stages. Gene expression levels were normalized to *GAPDH* and presented relative to WT at each corresponding time point. Mean $\pm$ SEM (N=4). **b** Time-course qPCR analysis of apoptosis-related genes *CASP3*, *BAX*, and *BCL2* in WT and *NKX2-5* KO CCOs. Expression levels were normalized to Day 0 (D0) and plotted as fold changes over time. Mean $\pm$ SEM (N=4). **c** Representative live imaging of *TNNT2*-P2A-GFP reporter activity in WT and *NKX2-5* KO CCOs at indicated time points. N=3. Scale bars, 250 μ m. Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.

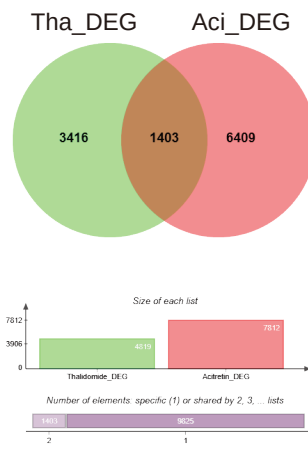
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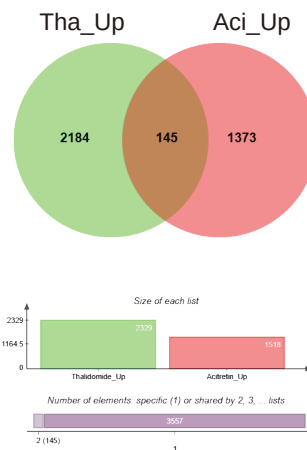
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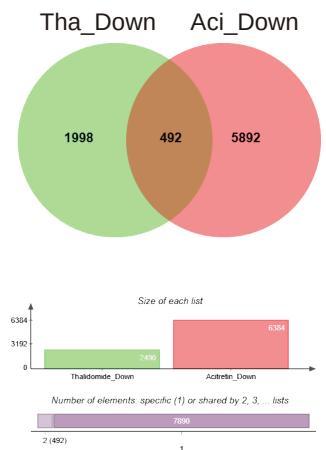
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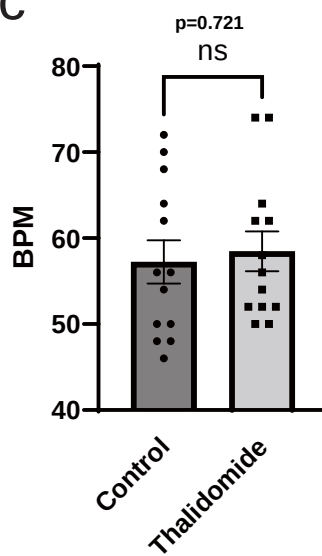
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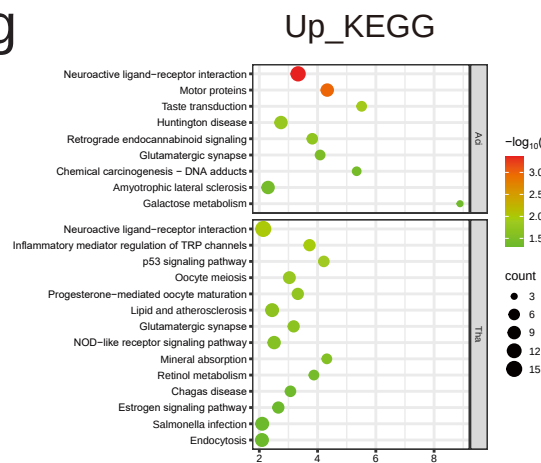
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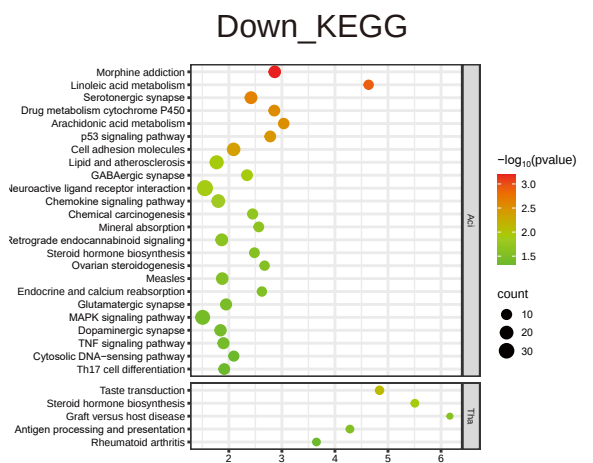
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Supplementary Figure 13. Teratogenicity Screening platform using CCOs.

a Representative brightfield images of CCOs treated with various drugs on day 20.5. Aspirin, 30 μ M; thalidomide, 10 μ g/ml; acitretin, 50 nM. Scale bar, 500 μ m. **b** Quantification of the chamber areas of CCOs captured on day 36.5, treated with Thalidomide from day 15.5. Mean $\pm$ SEM (N=3, n=34). Two-tailed unpaired t-test, $p = 0.946$. **c** Beats per minute of CCOs on day 36.5, treated with Thalidomide from day 15.5. Mean $\pm$ SEM (N=3, n=26). Two-tailed unpaired t-test, $p = 0.721$. (D-F) Venn diagrams showing DEG analysis from bulk RNA sequencing of CCOs treated with thalidomide (Tha) or acitretin (Aci). **d** Total DEGs, **e** upregulated DEGs, and **f** downregulated DEGs. **g** KEGG pathway enrichment analysis for upregulated DEGs. **h** KEGG pathway enrichment analysis for downregulated DEGs. Source data are provided as a Source Data file. All images are representative of at least 3 independent experiments with similar results.