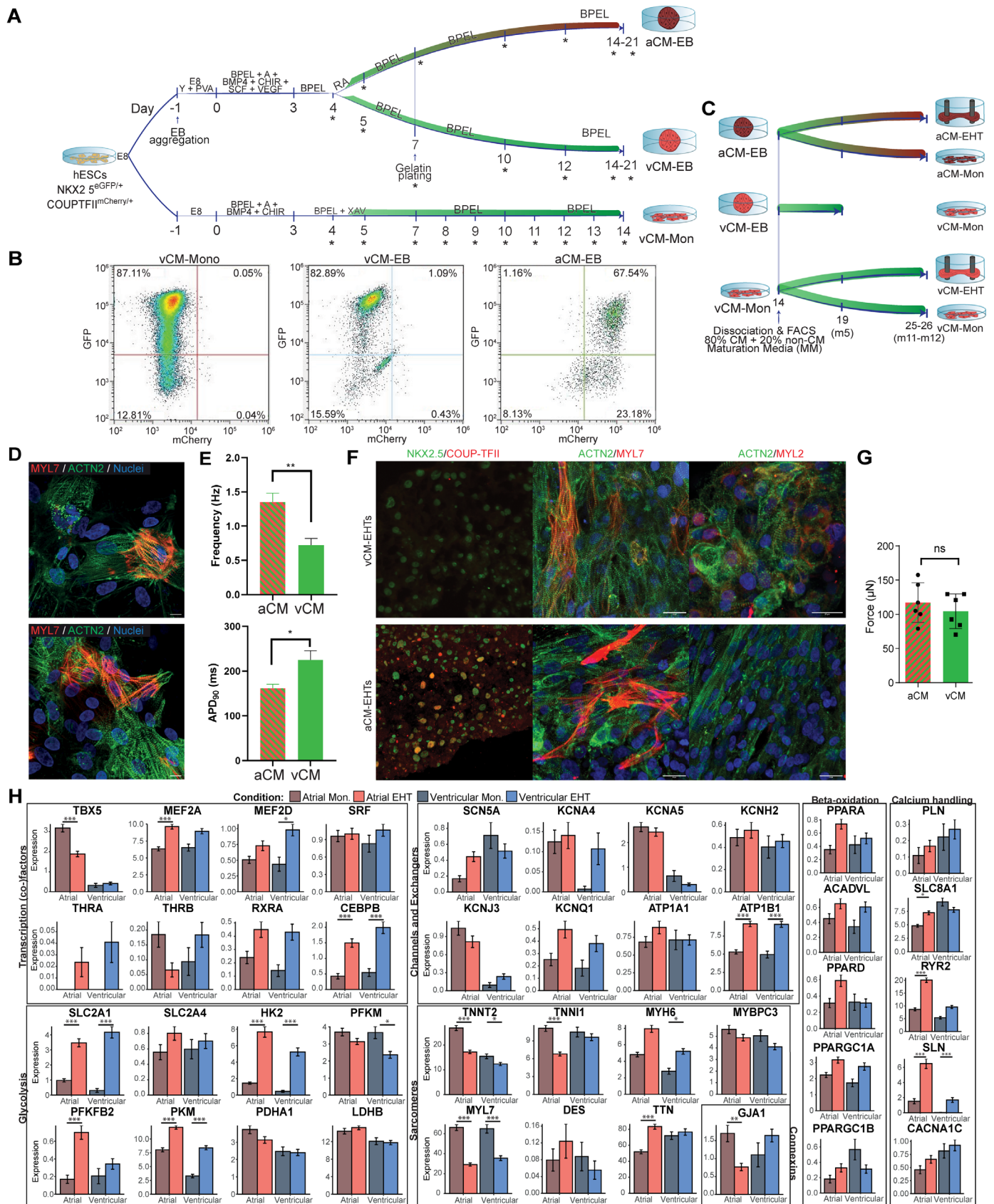


Stem Cell Reports, Volume 20

Supplemental Information

Cardiac differentiation roadmap for analysis of plasticity and balanced lineage commitment

Rebecca R. Snabel, Carla Cofiño-Fabrés, Marijke Baltissen, Verena Schwach, Robert Passier, and Gert Jan C. Veenstra



Supplemental Figure S1. Atrial and ventricular differentiation protocols and CM characterization.

(A) Overview of differentiation protocols from hPSC-derived atrial or ventricular embryonic bodies (aCM-EBs, vCM-EBs, respectively) or ventricular monolayers (vCM-Mon). Stars indicate time points of harvest for single-cell RNA-sequencing. E8 = Essential 8 media; BPEL = Bovine Serum Albumin Polyvinylalcohol Essential Lipids media; A = Activin A; BMP4 = Bone Morphogenetic protein 4; CHIR = Chir99021; SCF = Stem Cell Factor; VEGF = Vascular Endothelial Growth Factor; RA = retinoic acid analog; XAV = xav939; m = day in maturation medium. Each protocol (Mon, EB, EHT) and culture condition (+/- RA) was sampled in at least two independent experiments.

(B) Representative flow cytometry plots depict differentiation efficiency of CMs at day 14 from each differentiation protocol used in (A), using NKX-2.5^{eGFP+/-} and COUP-TFII^{mCherry+/-} reporters. Atrial CMs are both GFP- and mCherry-positive, ventricular CMs only GFP positive.

(C) Schematic overview of the protocol used at day 14 to generate EHTs and monolayer controls per culture type. Note from vCM-EB, only monolayers were generated since EHTs from vCM-Mon were more efficient to generate. All indicated time points were harvested for single-cell RNA-sequencing. m5-m12 = day 5-12 in maturation medium (day 14-26; EHT and monolayer only).

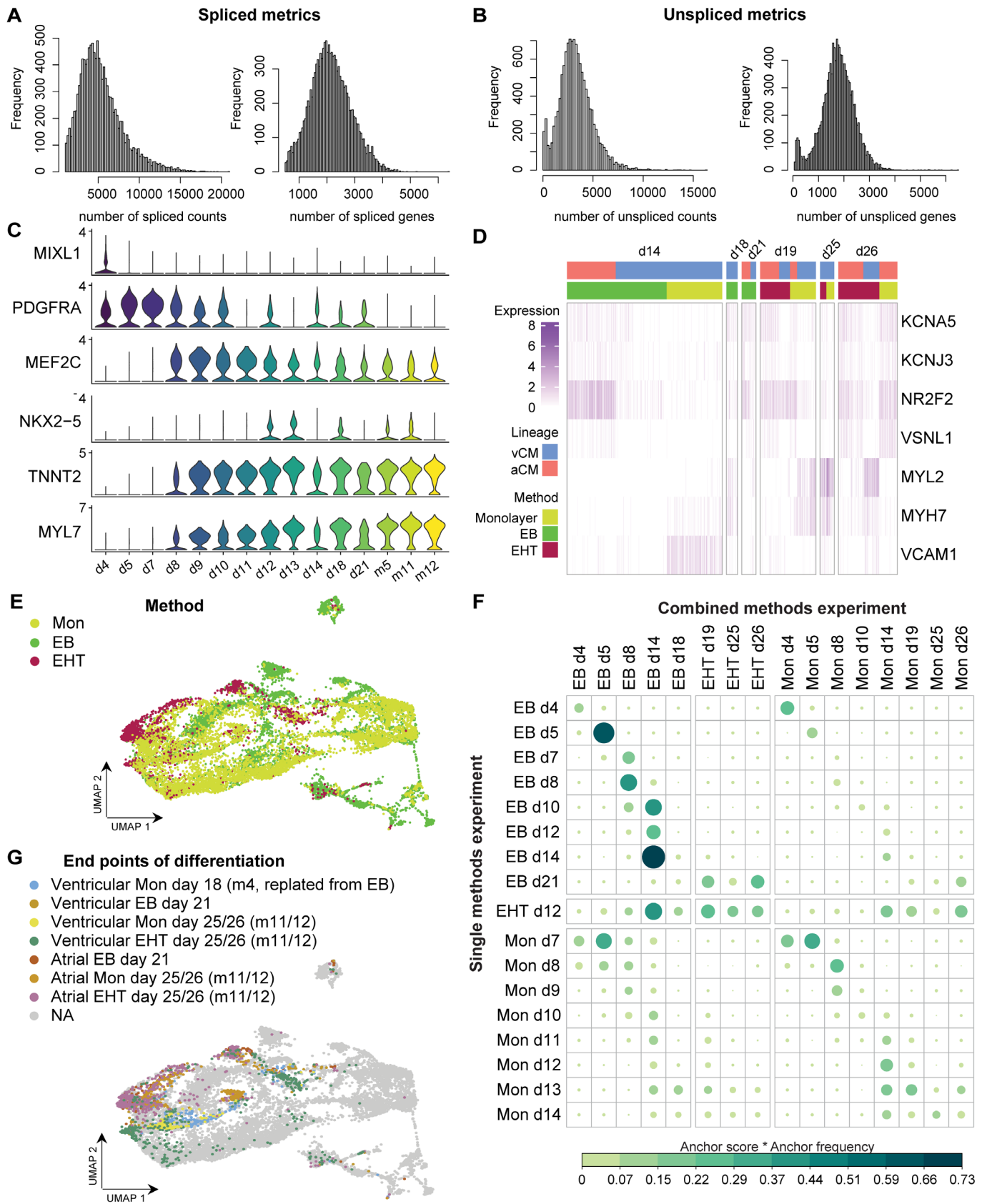
(D) Representative immunohistochemistry of general cardiomyocyte markers MYL7 and ACTN2 in day 21 vCM-EBs (top) and aCM-EBs (bottom). Scale bar = 20 μ m.

(E) Frequency (top) and action potential duration at 90% of repolarization (APD90) (bottom) of aCMs and vCMs EBs at day 14, determined by FluoVolt voltage sensitive dye. Data are mean $\pm$ s.e.m. Two-tailed Student's t-test. *p < 0.05; **p < 0.01. (n = 6 EBs from 3 differentiation batches).

(F) Confocal images of ventricular and atrial EHTs (aCM-EHTs, vCM-EHTs, respectively) stained for NKX-2.5, COUP-TFII, ACTN2, MYL7 and MYL2 confirming their lineage identity. Scale bar = 20 μ m.

(G) Contraction forces measured in μ N for the atrial and ventricular EHTs using n = 6 per lineage, cultured as indicated in (A) and (C). Bar represents average force, error bars indicate standard deviations and significance was determined with a two-tailed Student's t-test. ns = non-significant.

(H) Expression of maturation markers at end points of differentiation in EHT versus monolayer. Shown are averaged expression levels across single cells as pseudobulk, harvested at maturation day 11/12 for atrial monolayer (n = 333) and ventricular monolayer (n = 146) from one differentiation experiment, and atrial EHT (n = 465) and ventricular EHT (n = 418) from two independent differentiations, as indicated in (C). Black boxes represent genes related to the same biological process and represent subset from (Harlaar et al., 2022). Note that while most genes are expected to increase upon maturity, *TNNI1* expression levels are expected to decrease in maturing CMs. Data are mean $\pm$ s.e.m. and statistical test performed with unpaired Wilcoxon rank-sum test and corrected for multiple testing with the Benjamini and Hochberg method. *p < 0.05, **p < 0.01 and ***p < 0.001.



Supplemental Figure S2. Single-cell RNA-sequencing dataset.

(A-B) Histogram shows frequency of cells counted in the dataset after quality filtering with (A) spliced (exonic) or (B) unspliced (intronic) reads, summarized as read counts (left) and detected genes (right) per cell (x-axis).

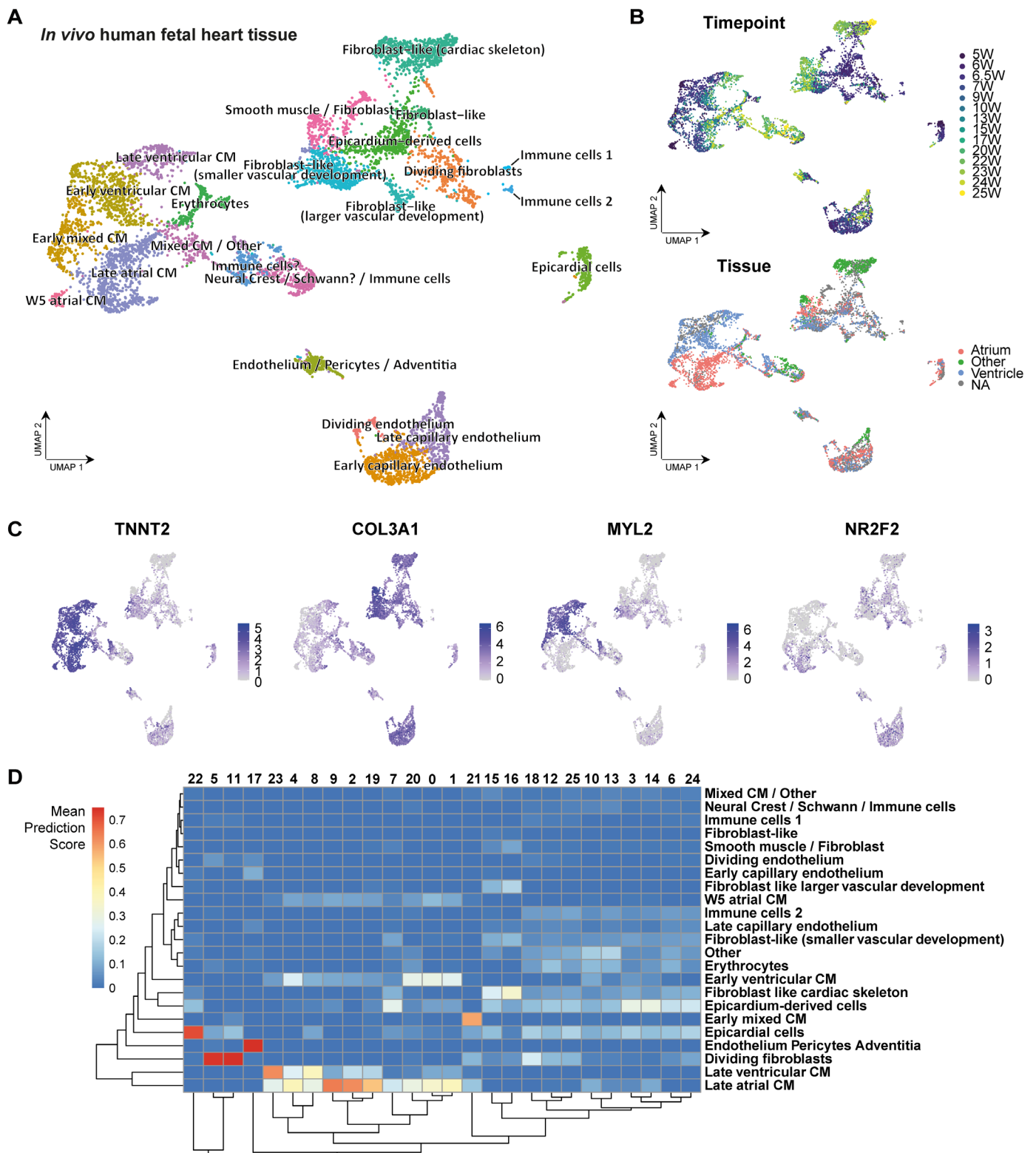
(C) Combined expression of mesoderm (*MIXL1*, *PDGFRA*), cardiac progenitor (*MEF2C*, *NKX2-5*) and cardiomyocyte (*MYL7*, *TNNT2*) marker genes across the full dataset per time point.

(D) Heatmap representation of aCM and vCM markers for evaluation of lineage identity in atrial (salmon-red) and ventricular (blue) cultures from day 14 to day 26. Yellow-green, green and carmine-red indicate monolayer, embryoid body and engineered heart tissue respectively.

(E) UMAP representation of the integrated dataset as shown in Fig. 1C, cells labeled per method.

(F) Dotplot visualizing frequency and score of mutual nearest neighbors (integration anchors) between the culture experiments, columns show samples of one experiment where all methods were cultured in parallel, the rows indicate separate experiments for each method. Size and color of the dots represent the product of anchor score and anchor frequency.

(G) UMAP representation of the integrated dataset as shown in Fig. 1C, cells labeled for the end points of differentiation for each method (full protocol of differentiation is shown in Fig. 1A, Supplementary Fig. S1A,C). m4-m11-m12 = day 4-11-12 in maturation medium (day 18-25-26; EHT and monolayer only).



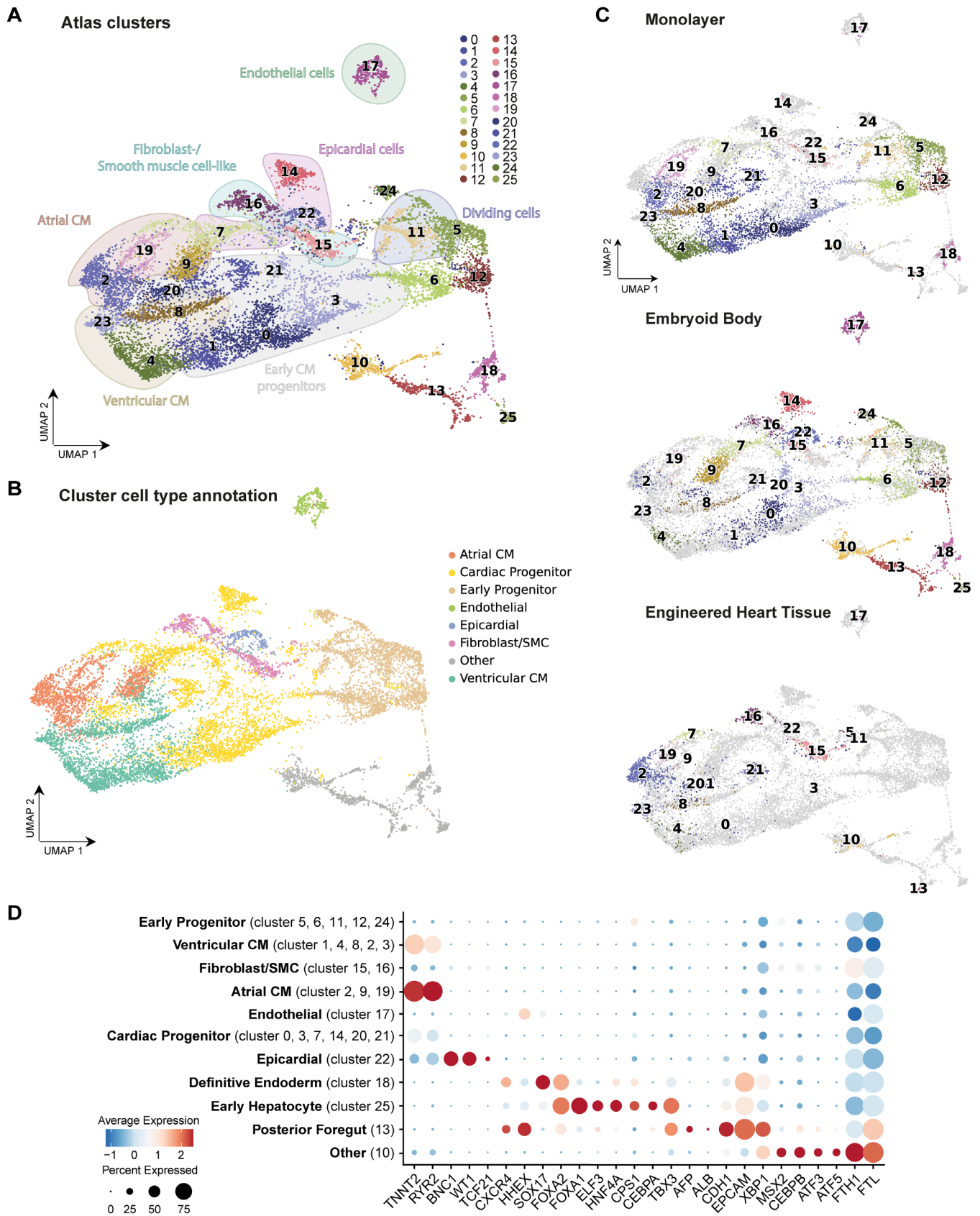
Supplemental Figure S3. Cell types of the human fetal heart, and a comparison with *in vitro* atlas clusters.

(A) Integrated scRNA-seq datasets of human fetal heart tissue from Cui et al. (2019) and Asp et al. (2018), labeled with extended annotation based on the cell types annotated in the Asp et al. dataset.

(B) UMAP representation as shown in (B) labeled with time point (top) and tissue (bottom) of harvest where this information was provided (w, weeks after conception).

(C) Gene expression of known markers *TNNT2* (general cardiac muscle marker), *MYL2* (ventricular cardiomyocyte marker), *NR2F2* (atrial cardiomyocyte marker), and *COL3A1*.

(D) Heatmap with predicted fetal cell types from (A) in rows, for each of the atlas clusters from Fig. 1C and S4A (columns). Multiple late time point clusters (day 14 and older) from the ventricular and atrial lineage, show most similarity to *in vivo* vCM and aCM clusters, respectively. Cluster 22 matched best with epicardial and cluster 17 with endothelial cells. Early clusters were similar to proliferating fibroblasts (cluster 5 and 11).



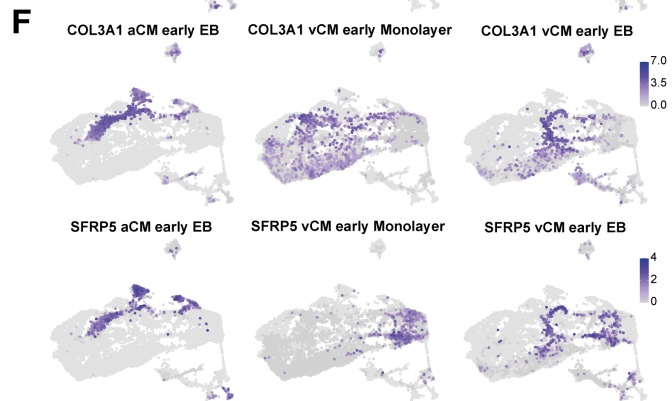
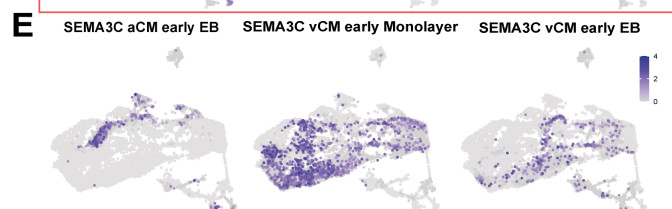
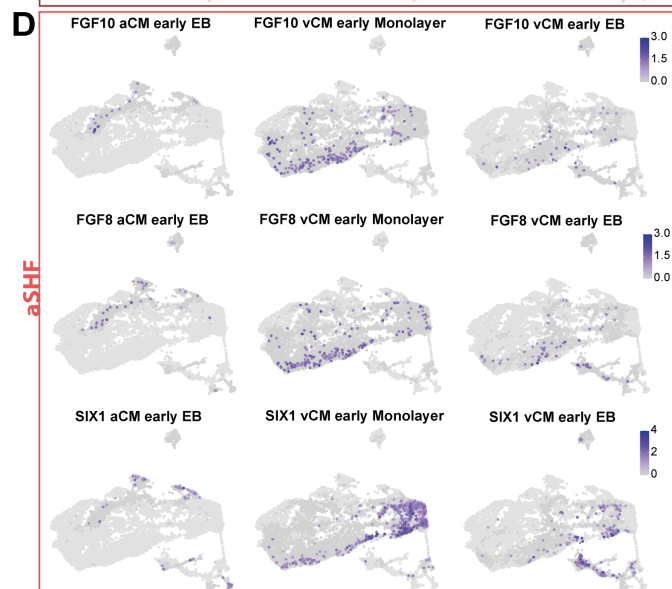
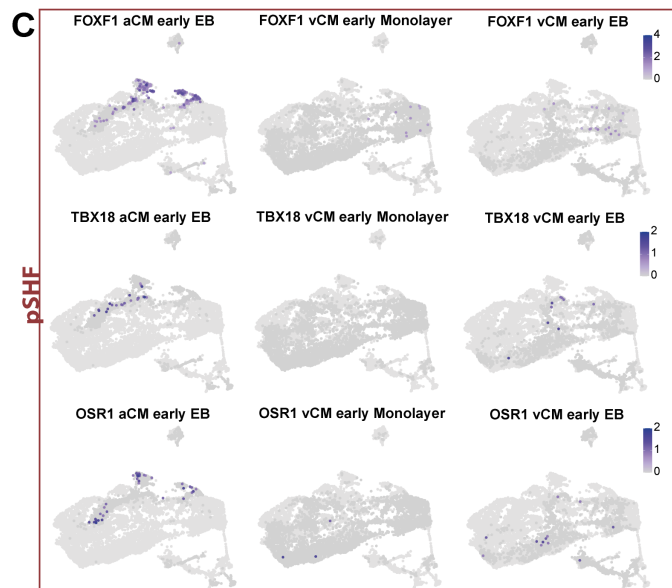
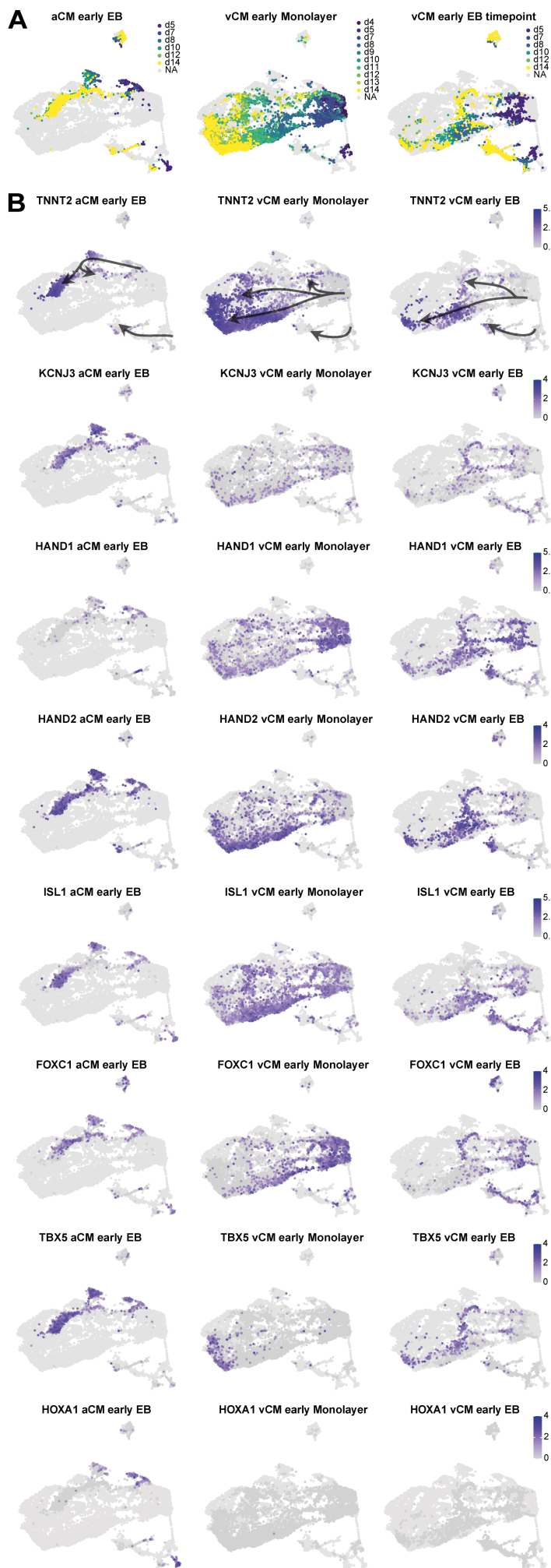
Supplemental Figure S4. Annotation of temporal single-cell atlas clusters.

(A) UMAP representation of the atlas with cluster labeling, colored stains show the annotation process based on the scoring in S3D.

(B) UMAP representation of the atlas with final cell state annotation.

(C) UMAP representation of full atlas with only cells labeled for each culture method, with monolayer cells highlighted in the upper, cells harvested from EB method in the middle and EHT cells indicated in the bottom UMAP representation. The colors indicate the cluster a cell belongs to, using clustering as shown in (A). Cells in light grey fall outside of the culture method of focus.

(D) Dot plot showing expression levels per cluster for a selection of marker genes. Includes general cardiomyocyte markers *TNNT2*, *RYR2*; epicardial *BNX1*, *WT1*, *TCF21*; and markers for definitive endoderm (*SOX17*, *CXCR4* (or *CD184*), *FOXA1*, *FOXA2*), early hepatocyte (*FOXA1*, *ELF3*, *HNF4A*, *CPS1*, *CEBPA*, *TBX3*, *AFP*, *ALB*), (posterior) foregut (*ELF3*, *HHEX*, *CDH1*, *EPCAM*), and marker genes found in the Other cluster (*MSX2*, *CEBPB*, *ATF3*, *ATF5*, *FTH1*, *FTL*) (Ang et al., 2018; Scheibner et al., 2021).



Supplemental Figure S5. Early trajectory subsets of the roadmap labeled with expression levels of developmental cardiac markers.

(A) Atlas UMAP representation with cells from early trajectories (cultured from d4 until d14) labeled according to their time point of harvest separated per culture protocol, n = 2 for EB protocols at day 4, 5, 8 and 14, and for vCM early monolayer day 8, 10 and 14. Atrial EB cells are highlighted in the left, ventricular monolayer in the middle and ventricular EB in the right column. Cells that do not belong to the protocol of focus, are indicated in the roadmap with light grey (NA).

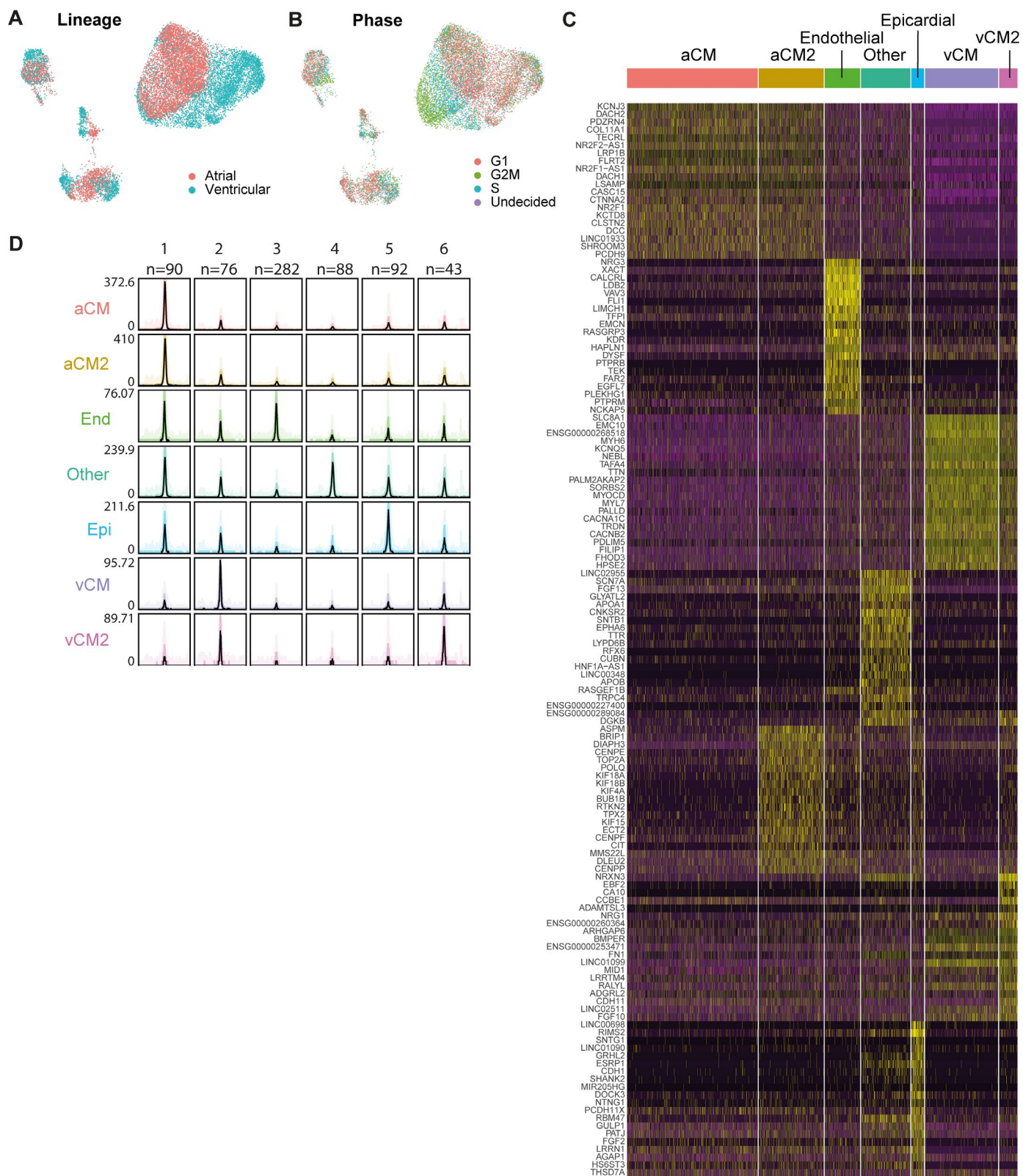
(B) Expression levels of genetic markers for CMs (*TNNT2*), aCM (*KCNJ3*), first heart field (*HAND1*), second heart field (*HAND2*, *ISL1*, *FOXC1*, *TBX5*).

(C) Expression levels of genetic markers for the posterior second heart field (pSHF).

(D) Expression levels of genetic markers for the anterior second heart field (aSHF).

(E) Expression levels of *SFRP5* and *COL3A1* show elevated co-expression in the non-CM branches of the UMAP, where *SFRP5* was downregulated and to a lower extent *COL3A1* in vCM branch (middle and right), whereas both remain upregulated in aCM differentiation (left).

(F) Expression levels of *SEMA3C* marker of aSHF-derived vCMs.

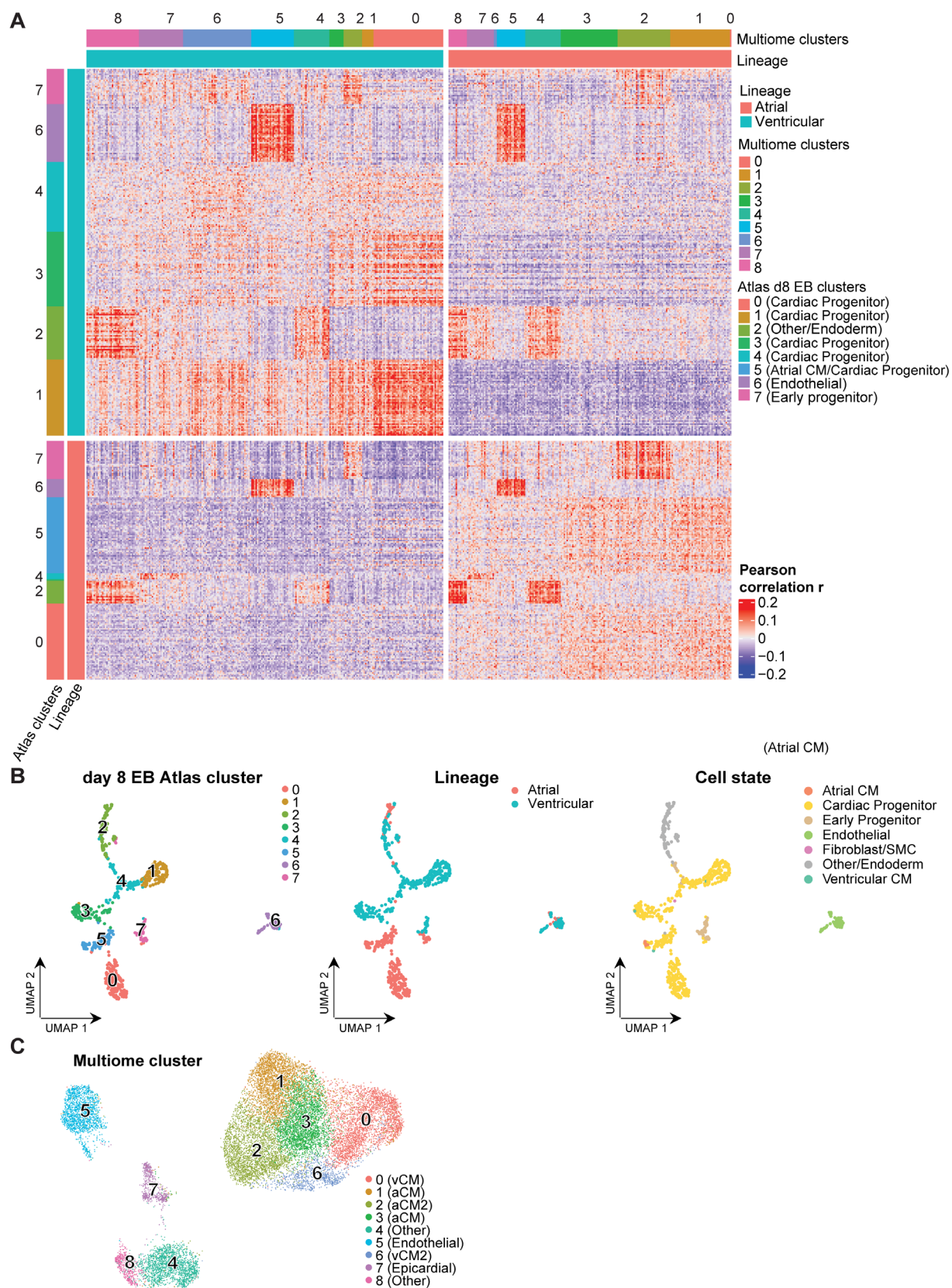


Supplemental Figure S6. Cluster identification and patterns of gene expression and chromatin accessibility in the multiome dataset.

(A-B) UMAP representation from Fig. 4A with labels of the lineage they were cultured towards (A) and predicted cell cycle phase (B).

(C) Heatmap visualizing expression levels of top 20 genetic markers, as called per cell type.

(D) Bandplot overview of differentially accessible peaks per cell type and separated into six clusters as found in the chromatin accessibility assay of the day 8 multiome dataset of both lineages. Differential peaks (Supplementary Table S1) were used for motif analysis (Supplementary Table S2).

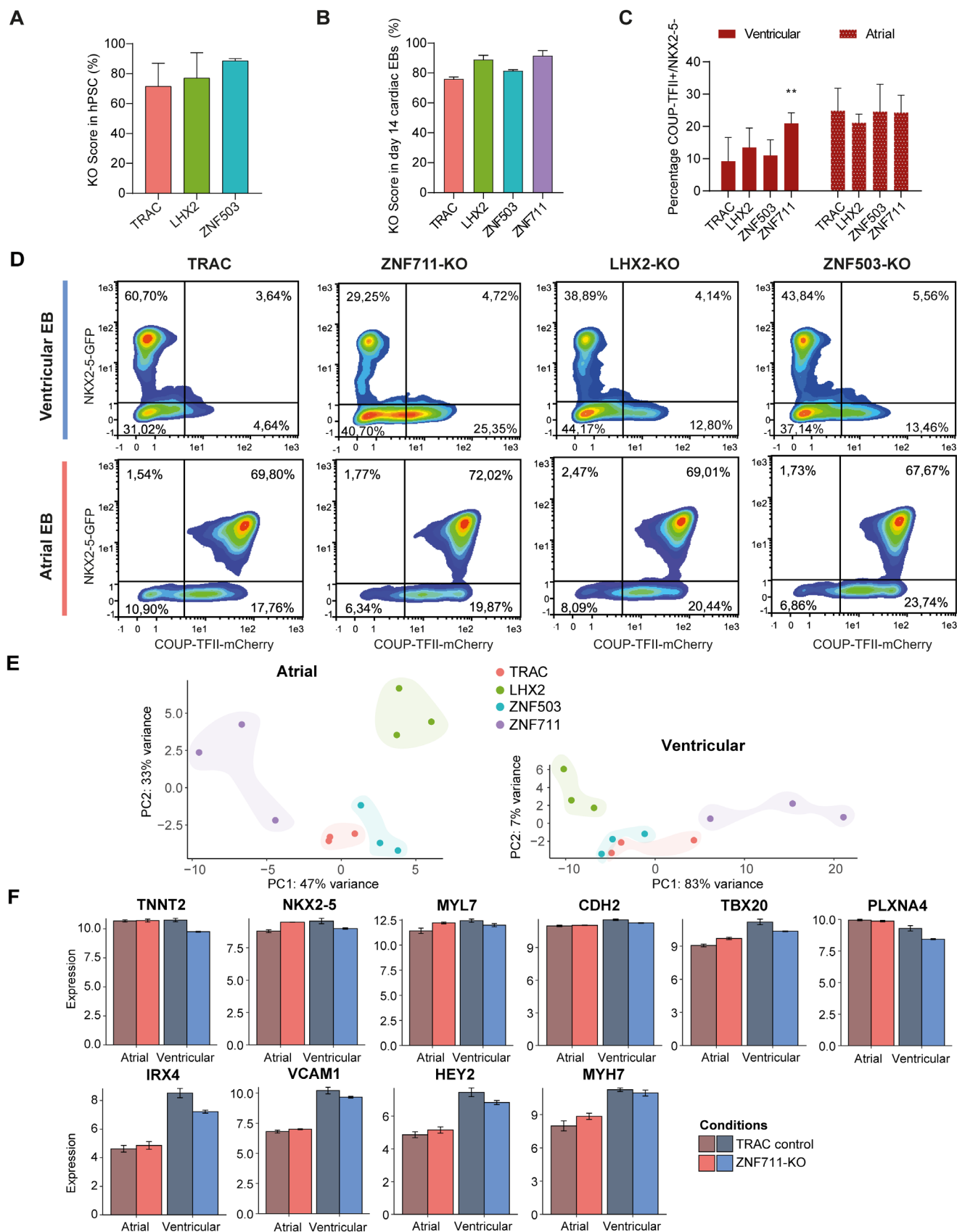


Supplemental Figure S7. Correlations between day 8 EB subset of the temporal Atlas and day 8 multiome data.

(A) Correlation heatmap with random subsets of 1,000 cells per cluster of multiome dataset (columns) and all cells per cluster of the atlas dataset (rows), additionally separated per lineage.

(B) UMAP representations of a subset of atlas data for cells cultured with the EB method and harvested at day 8 of each of the lineages, matching method and time point sampled with the multiome experiment (C). UMAP labeled for the clusters called on this subset (left), labeled for lineage from which the cells were harvested (middle) and labeled for cell types as annotated in the atlas (right).

(C) UMAP representation of the multiome dataset labeled with the cluster numbers.



Supplemental Figure S8. Transcription factor knockout experiment read-out.

(A) Knockout (KO) efficiency per target of hPSCs at start of differentiation, *TRAC* is used as a CRISPR control. Data are mean $\pm$ s.e.m; ordinary One-way ANOVA with Tukey's multiple comparisons test. ($n=2$ independent hPSC cultures per KO target). ZNF711-KO missing due to sample loss.

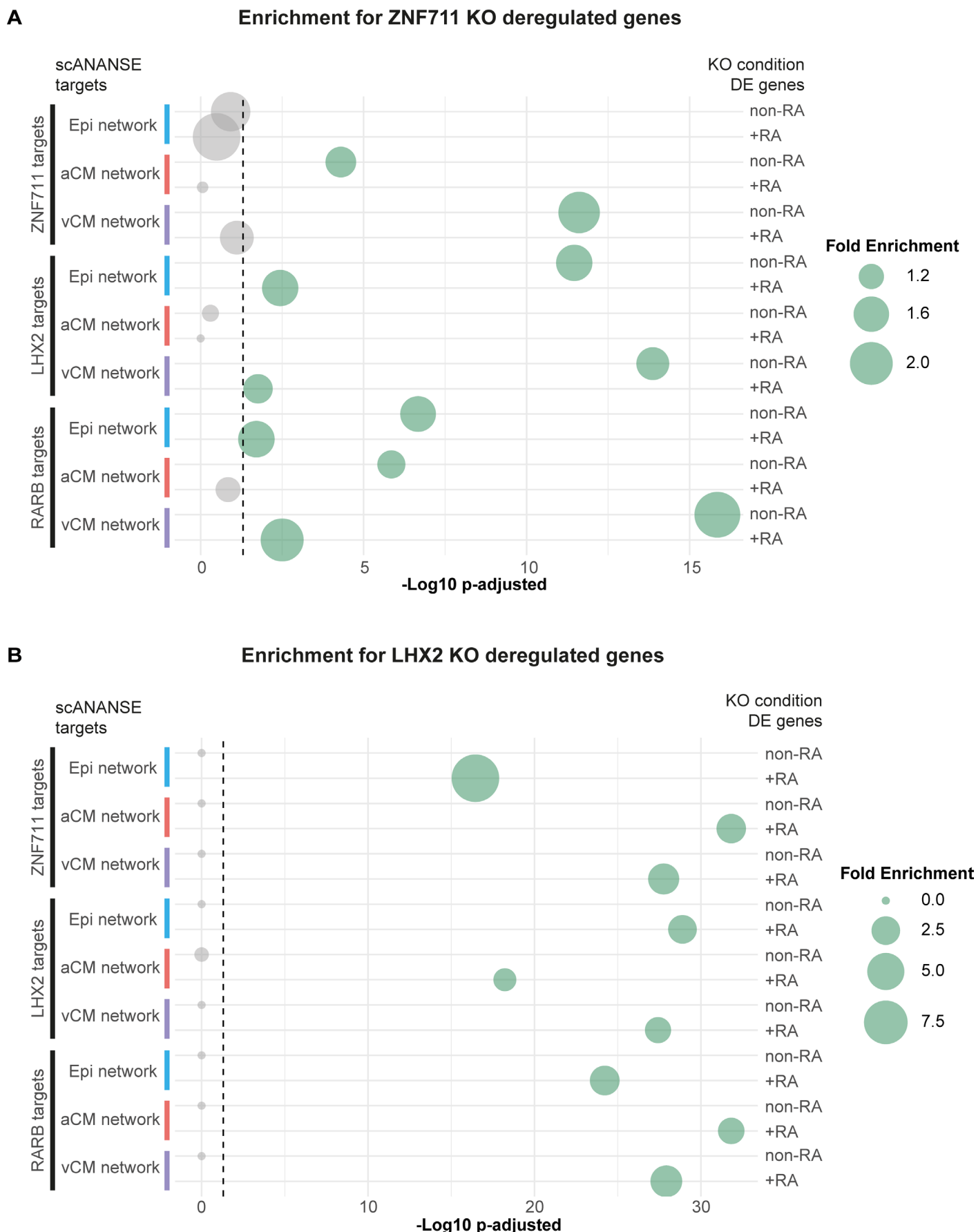
(B) KO efficiency per target after differentiation of each KO-hPSC culture to aCM or vCMs, measured at day 14 of EB culture. Data are mean $\pm$ s.e.m; ordinary One-way ANOVA with Tukey's multiple comparisons test ($n=4$ per target, resulting from two independent EB differentiations per lineage).

(C) Percentage of COUP-TFII (NR2F2)-positive and NKX2-5-negative cells per KO target measured by flow cytometry in non-RA-treated (ventricular, left red bars) and RA-treated (atrial, red, white-dotted bars) conditions. Data are mean $\pm$ s.e.m; ordinary Two-way ANOVA with Tukey's multiple comparisons test. **p < 0.001 (n=3 independent differentiations per culture type).

(D) Representative flow cytometry results from panel (C), showing on the y-axis signal measured for NKX2-5-GFP and on the x-axis signal measured for COUP-TFII(NR2F2)-mCherry, represented with a density plot for each of control condition (TRAC) and KO condition, cultured with ventricular (non-RA, top row) or atrial (+RA, bottom row) conditions. aCMs are both NKX2-5-GFP and COUP-TFII-mCherry positive while vCMs only GFP positive. Non-CMs are either NKX2-5-GFP negative or both NKX2-5-GFP negative and COUP-TFII-mCherry positive. In these plots an increase was observed of the non-CM (NKX2-5-negative) population with NR2F2 expression in the ventricular *ZNF711*-KO culture, indicative of a lineage shift under these conditions compared to control.

(E) PCA on RNA-seq of KO target samples obtained after 14 days of EB differentiation separate for atrial (plus RA; top) and ventricular lineage (no RA; bottom).

(F) Expression levels of general and ventricular cardiomyocyte markers upon knock-out of *ZNF711*-KO. *TNNT2*, *NKX2-5*, *MYL7*, *CDH2* and *TBX20* used as general and *PLXN4*, *IRX4*, *VCAM1*, *HEY2* and *MYH7* used as aSHF or ventricular marker. All markers were found differentially downregulated in the ventricular conditions upon *ZNF711*-KO (Supplementary Table S3). Of these genes, only *NKX2-5* and *MYL7* were found differentially expressed in the atrial conditions, showing upregulation upon *ZNF711*-KO (Supplementary Table S3). Expression values shown represent the averaged regularized log-transformed expression values across three replicates per condition. Error bars indicate standard error.



(A-B) Enrichment and significance (hypergeometric test, multiple testing-corrected) of ZNF711 KO targets (A) or LHX2 KO targets (B), overlapping with predicted scANANSE targets for ZNF711, LHX2 and RARB, for each cell type network. Indicated on the left of the bubble plot are regulators for which predicted targets were selected and from which cell type-specific scANANSE network. On the right of the plot, conditions are indicated from which ZNF711-KO differential genes were selected, non-RA (ventricular) or +RA (atrial). Dashed line indicates threshold for significant p-adjusted value of 0.05. Epi = epicardial cells, aCM = atrial cardiomyocyte, vCM = ventricular cardiomyocyte.

SUPPLEMENTAL METHODS

hPSC culture

Experiments were performed using the human embryonic stem cell (hESC) lines NKX2.5^{EGFP/+}COUP-TFII^{mCherry/+} (Schwach et al., 2017). hPSCs were maintained as undifferentiated colonies in Essential 8 medium (Thermo Fisher, A1517001) on vitronectin (Thermo Fisher, A31804)-coated 6-well plates (Greiner Bio-One, 657160) and passaged every 3 to 4 days by dissociating to single cells with EDTA and seeded at density between 8×10^3 to 15×10^3 cells/cm². Undifferentiated hPSCs were frozen in E8 medium containing 10% DMSO. To start hPSC culture, around 5×10^5 hPSC were thawed at 37°C in E8 and seeded into 1 well of a 6 well plate coated with vitronectin. For all experiments, hPSCs were used between passage 20 and 35. Authentication of the hESC line used in this manuscript as well as its genomic and pluripotency characterization (karyotyping and protein expression, respectively) was performed in previous work (Figure S2 from Schwach et al., 2017). Mycoplasma tests were performed on a six-months basis.

Differentiation of hPSCs into ventricular monolayers

Differentiation of hPSCs to VM monolayers was performed as previously described (Birket et al., 2015). Briefly, one day before starting the differentiation, hPSCs were seeded at a density of $20\text{--}25 \times 10^3$ cells per cm² on Matrigel (83 µg protein/mL) (Corning, 354230)-coated 6-well plates in Essential 8 medium. After 24 h (day 0), mesodermal differentiation was induced by addition of Activin-A (20 - 30 ng/mL, Miltenyi 130–115-010), BMP4 (20 -30 ng/mL, R&D systems 314-BP/CF) and WNT activator CHIR99021 (1.5 - 2.25 µmol/L, Axon Medchem 1386) in BPEL medium (Ng et al., 2008). At day 3, BPEL containing WNT inhibitor XAV939 (5 µmol/L, R&D Systems 3748) was used to refresh cells. On day 7, 10 and 14 cells were refreshed with plain BPEL.

Differentiation of hPSCs into ventricular and atrial EBs

Differentiation of hPSCs to ventricular or atrial EBs was performed as previously described (Schwach et al., 2022). Briefly, hPSC were seeded and aggregated in EB formation medium (Essential 8 containing 400 µg/mL Poly (vinil alcohol) (Sigma Aldrich, P8136) and 10 µM Y-27632 (Bio-Connect, S1049)) one day before starting the differentiation. After 24 h (day 0), mesodermal differentiation was induced by addition of Activin-A (30 ng/mL, Miltenyi 130–115-010), BMP4 (30 ng/mL, R&D systems 314-BP/CF), WNT activator CHIR99021 (1.5 µmol/L, Axon Medchem 1386), VEGF (30 ng/µL, Miltenyi Biotec, 130-109-386) and SCF (40 ng/µL, Stem Cell Technologies, 78062.1) in BPEL medium (Ng et al., 2008). On day 3 of differentiation, EBs were refreshed with plain BPEL. On day 4, atrial EBs were refreshed with either 1 µM of all trans RA (Sigma Aldrich, R2625) or 20 µM RARα selective agonist BMS-753 (Torcic Bioscience, 3505). Next day, atrial EBs treated with BMS-753 were refreshed with plain BPEL. EBs at day 7 were plated on 0.1% gelatin coated wells in BPEL medium. EBs were refreshed again with BPEL at day 10. Unless indicated, EBs at day 14 were switched to cardiomyocyte media containing 3',5-Triiodo-L-thyronine sodium salt (T3), Dexamethasone (D) and Long R3 IGF-I human (IGF, I) (CM-TDI) (Birket et al., 2015) and refreshed every 2-3 days until used at day 21.

Generation of engineered heart tissues

Ventricular monolayer, ventricular EBs and atrial EBs were sorted at day 14 to separate CMs positive cells (NKX2.5^{EGFP/+}-positive COUP-TFII^{mCherry/+}-negative for vCMs and NKX2.5^{EGFP/+}-positive COUP-TFII^{mCherry/+}-positive for aCMs) and non-CMs (NKX2.5^{EGFP/+}-negative). For each CM differentiation (ventricular mono and EBs, and atrial EBs), cells were mixed back at a specific ratio of 80% CMs + 20% non-CMs. EHTs from ventricular monolayers and atrial EBs were generated by following the protocol as described (Ribeiro et al., 2022). As a 2D control, monolayers from each CM differentiation were generated by seeding the cells with the same cell ratio (80% CMs + 20% non-CMs) in vitronectin coated 48 wells plates. EHTs and 2D controls were kept in CM-TDI media and refreshed every 2-3 days until use.

Immunohistochemistry and confocal imaging

For whole mount staining on intact EBs, EBs at day 7 were seeded on gelatine-coated glass bottom plates. Cells were fixed at day 21 with 4% Paraformaldehyde for 15 min at RT. Permeabilization was followed with 0.1% Triton X100 for 8 min at RT. After blocking for 1 h at RT with 5% Fetal Bovine Serum (FBS), cells were incubated at 4°C overnight with the primary antibodies (Table 1). After washing, cells were incubated for 1 h at RT with secondary antibodies (Table 1) and 5 min at RT with DAPI. EHTs at day 12 after tissue formation were fixed with 4% Paraformaldehyde for 1 h at RT. Whole tissue

immunostaining was performed as described (Ribeiro et al., 2022). Antibodies used are described in Table 1. Confocal images were captured using a Zeiss LSM 880 microscope.

Table 1. Antibodies used for immunohistochemistry on EBs and EHTs.

Antibody	Cat. Number	Dilution factor
Rabbit anti-NKX2.5	Cell Signaling, 8792	1:400
Mouse anti-COUP-TFII	Perseus Proteomics, 1:500	1:200
Mouse anti-ACTN2	Sigma, A7811	1:800
Rabbit anti-MYL7	R&D Systems, MAB90961-SP	1:100
Rabbit anti-MYL2	Sigma HPA019763	1:100
Mouse anti-cTnT	ThermoFisher MA512960	1:400
DAPI	Thermo Scientific, D1306	1:2000
Chicken anti Rabbit IgG (H+L) Alexa Fluor 488	Invitrogen, A-21441	1:500
Donkey anti Mouse IgG (H+L) Alexa Fluor 647	Invitrogen, A-31571	1:500

Optical membrane potential imaging

For optical membrane potential imaging, aCMs or vCMs at day 14 were stained for 30 min at RT with the voltage-sensitive dye FluoVolt™ according to manufacturer. For signal acquisition, the loaded sample was excited using a 488 nm laser and recorded for 10 seconds. Movies were acquired with a Nikon ECLIPSE Ti2 fluorescent microscope under temperature and humidity control (37°C and 5% CO₂). Videos were analyzed with BV Workbench, Brainvision Inc.

FACS and flow cytometry

Monolayers and EBs from day 3 to day 8 were dissociated to single cells with TrypLE 1X (Thermo Fisher, 12563029) for 5 minutes at 37°C. From day 8 onwards, monolayers and EBs were dissociated with TrypLE 10X (Thermo Fisher, A1217702) for 10 min at 37°C. EHTs were dissociated with collagenase type II (240U/ml) (Worthington Biochemical LS004176) for 20-30 min at 37°C followed by TrypLE 10X for 10-15 min at 37°C. Cells were transferred to FACS buffer (2mM EDTA, 0.5% BSA in DPBS) and filtered (VWR, 734-0001). For single cell RNA sequencing, cells were single cell sorted into 384-well primer plates. Sorting was performed with a Sony SH800. Flow cytometry was performed with a MACSQuant Analyzer 16 (Miltenyi). Flow cytometry data was analyzed with FlowJo software (Miltenyi).

CEL-Seq2 RNA-sequencing sample preparation

Cells were harvested from four independent differentiation experiments (Supplementary Table S5). One experiment covered all three culture methods cultured in parallel (Monolayer harvested on day 4, 5, 8, 10 and 14 and day 19, 25 and 26 in maturation medium; EB harvested on day 4, 5, 8, 14 and 18; EHT harvested on day 19, 25 and 26 in maturation medium), with two additional experiments performed for the EB (harvested on day 4, 5, 7, 8, 10, 12, 14 and 21) and EHT method (harvested on day 12), all cultured towards the ventricular (non-RA) and atrial (RA-treated from day 5) lineage. An additional monolayer differentiation experiment cultured for the ventricular fate was harvested on every day spanning day 7 until day 14. Libraries were prepared in 384-well plate format using a modified SORT-Seq, or CEL-Seq2, protocol (Hashimshony et al., 2016; Muraro et al., 2016). Primer plates were generated with CEL-Seq2 compatible oligo's containing the T7 promoter, a 5' Illumina adapter, unique molecular identifiers (UMIs; 8 bp), a unique cell barcode (8 bp) and oligo(dT)N for mRNA molecules tagging. The primers were designed reversed from classic CELSeq2 primers, leading to barcode and UMI priming in read 2, where the transcripts were sequenced from read 1, as previously described (Gerlach et al., 2019). After sorting, the plates were spun down and immediately stored at -80°C and thawed before the reverse transcription PCR (RT-PCR). ERCC RNA Spike-In Mix II (Invitrogen) was added in 1:50 x 10³ ratio to each of the wells administered in 150 nL volume by the micro-dispenser Nanodrop II (BioNex). The Nanodrop II was additionally used for dispensing the reverse transcription mix, consisting of Superscript II (Invitrogen) and RNasein Plus (Promega), as well as for the second strand synthesis with a mix of E. coli RNase H (Invitrogen), E. coli DNA polymerase and E. coli DNA ligase (New England Biolabs). After the second strand synthesis the cDNA of all single cells of a plate were pooled, before performing an overnight in vitro transcription reaction at 16°C using the MegaScript IVT kit (Invitrogen). Remaining primers were removed by using EXO1/rSAP-IT (Applied Biosystems) and the amplified RNA was chemically fragmented and cleaned up with AMPure XP beads (Beckman Coulter). Using random octamers the following RT was performed, after which the library amplification was one with Phusion High-Fidelity DNA Polymerase (New England Biolabs) during which the Illumina adapters were introduced, as well as the unique index sequence per library (NextFlex DNA barcodes,

PerkinElmer). After bead-purification, the library concentration and quality were assessed using the DeNovix and BioAnalyzer High Sensitivity DNA Kit (Agilent Technologies), respectively. Sequencing was performed with the NextSeq 500 (Illumina).

10X Multiome sample preparation

Nuclei from atrial and ventricular EBs at day 8 of differentiation were isolated with an ice-cold lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1% BSA, 0.1% Tween-20, 0.1% Nonidet P40 Substitute, 0.01% Digitonin, 1 mM DTT (Invitrogen, cat. no. 18064-014), 1 U/μL Protector RNase inhibitor (Roche, 3335399001) in Nuclease-free water. After three washes with ice-cold nuclease-free water containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1% BSA, 0.1% Tween-20, 1 mM DTT and 1 U/μL RNase inhibitor, isolated nuclei were kept on ice in PBS containing 0.04% BSA and 1 mM MgCl₂. The number of nuclei was counted with an hemacytometer. Droplet formation and library generation was performed as described using the 10X Genomics Next Gem Single Cell Multiome ATAC + Gene Expression Reagents kit.

Generation of CRISPR-Cas9 knockouts for LHX2, ZNF711 and ZNF503

hPSCs maintained in StemMACS™ PSC-Brew XF (Miltenyi Biotec, 130-127-865) were dissociated as the regular maintenance passaging procedure. Per knockout target, cell pellets of 200x10³ cells were resuspended with 20 μL of supplemented nucleofector solution P3 Primary Cell 4D-Nucleofector® S (Lonza, V4XP3032) with indicated amounts of multi-guide sgRNAs (Synthego) and SpCas9-2NLS Nuclease (Synthego) in a ratio of 3:1, and nucleofected with a 4D-Nucleofector Core and X unit (Lonza) using program CB-150. Human *TRAC* sgRNA was used as a positive control, as recommended by the supplier. After nucleofection, cells were seeded in 2 wells of a vitronectin-coated 24-well plate with StemMACS™ PSC-Brew XF supplemented with 1:200 RevitaCell (Thermo Fisher, A2644501). Cells were kept for 2-3 days. During passaging of each well, cells were expanded and lysed for PCR amplification and Sanger Sequencing. Two passages after nucleofection, differentiation towards atrial and ventricular lineages was initiated. After atrial and ventricular differentiation, cells were characterized and lysed for PCR amplification and Sanger sequencing. Knock-out efficiency was evaluated with the ICE CRISPR Analysis tool (Synthego).

Bulk RNA-sequencing sample preparation

For the transcription factor knockout validation experiments, RNA was harvested at day 14 of the protocols and purified using the Nucleo Spin RNA (Macherey-Nagel) kit according to the manufacturer's protocol. Libraries were generated with the RiboErase depletion RNA-sequencing protocol by KAPA (Roche, 8098131702) and sequenced on the Illumina Next Seq 2000. Per sample 200 ng of total RNA was used for library preparations with the KAPA RNA HyperPrep Kit with RiboErase (HMR) (Kapa Biosystems), including steps of the oligonucleotide hybridization, rRNA depletion, DNase digestion and RNA elution, were performed as instructed by the manufacturer. Fragmentation and priming were performed at 94°C for 6 min, followed by first-strand synthesis, second strand synthesis and A-tailing, which were performed according to protocol. Library amplification was performed with 10 cycles, followed by a 0.8X bead-based cleanup. Library concentration and quality were assessed using the DeNovix and BioAnalyzer High Sensitivity DNA Kit (Agilent Technologies). Sequencing was performed with the NextSeq 500 (Illumina).

Computational methods

Processing and integration of CEL-Seq2 scRNA-sequencing data

The FASTQs were trimmed and aligned with seq2science v0.4.0 (van der Sande et al., 2023). In short, kb-python v0.25.0 was used for quantifying (kb count with the setting '--technology 1,8,16:1,0,8:0,0,0') to map the reads against the GRCh38 genome and quantify the UMI counts per cell (Melsted et al., 2021). Fluorescence reporter genes and ERCC sequences were added to the genome before the index was generated with kb-python. The combined single-cell gene count matrix was filtered for good quality cells (UMI >= 1,000, detected genes >= 500, mitochondrial genes percentage < 50% or ERCC count < 20%), after which 12706 cells were left for downstream analysis. Scater v1.20.1 was used to perform quality control on the cells of the dataset and check confounding variables within the metadata (McCarthy et al., 2017). Since four culture experiments were combined, the object was split per experiment (Mon, EB or EHT experiment or one experiment combining all three), to individually normalize and select highly variable features (2,000 each) per experiment and finally perform integration anchor selection with Seurat (v4.0.4) (Hao et al., 2021), to lower batch effects and create one larger roadmap. Dimensionality reduction was performed with PCA and the first 50 principal components were used to generate the roadmap UMAP and compute the shared nearest neighbor graphs using Jaccard

index. Louvain clustering was performed with a resolution of 0.9 with the implementation FindClusters in Seurat. Optimal resolution settings for clustering, was evaluated with Clustree v0.4.3 (Zappia and Oshlack, 2018). For all gene expression visualizations onto the UMAP, the dots representing cells were plotted in the order of low to high expression.

Roadmap integration validation

A total of 33,108 anchors (cell-pairs) were found between the experiments (2 independent differentiations per culture type). Anchor pairs from the same time point and method were counted and their scores averaged, which resulted in a per time point and method pairs scoring across the experiments (with a total of 535 of combinations). The product of the mean score and frequency of the anchor ($\text{score} * (\text{frequency anchor} / \text{total number of anchors})$) was visualized with the R package pheatmap v1.0.12.

Comparison with *in vivo* human fetal scRNA-seq data

Two available human fetal scRNA-seq datasets from Cui et al. and Asp et al. were integrated by use of Seurat (Asp et al., 2019; Cui et al., 2019). The annotation of the Asp et al. dataset as established by the authors, was used to annotate the clusters of the integrated dataset. The first 20 principal components were used for running UMAP dimensionality reduction and neighborhood graph, and the Louvain clustering was performed with a resolution of 1. The resulting dataset was used for predicting the cell types of the *in vitro* roadmap clusters, by running the *TransferData* functionality of Seurat on the integrated assays with the *in vivo* dataset as the reference. The resulting prediction scores per cell of our *in vitro* roadmap (a calculation outlined in the paper of Stuart et al., 2019) were averaged to create a prediction score per roadmap cluster and visualized with pheatmap.

RNA velocity on early roadmap subsets

Subsets of the roadmap were generated by taking the time points of differentiation until day 14 (before transfer to maturation medium) and split per protocol, resulting in three early subsets: ventricular monolayer, atrial and ventricular EB cultures from day 4 to day 14. Dimensionality reduction was performed in Seurat and UMAPs were generated with the first 30 principal components and a minimum distance set to 0.75. The raw spliced, unspliced counts, roadmap cluster information and UMAP embeddings from the Seurat object, were transferred to an AnnData object for further analysis with Scanpy v1.8.2 in Python (Wolf et al., 2018). The genes selected for finding the integration anchors were taken along as HVG. RNA velocity was run with scVelo v0.2.4 (Bergen et al., 2020).

Embryoid body differential transcription factor analysis over time

For direct aCM and vCM comparison, a subset of only embryoid body time points was selected. Differential gene expression testing was performed on selected clusters, representing the progenitor stages contributing to aCM and vCM over time. Wilcoxon rank-sum test was used to find significant differentially expressed genes between the clusters 24 and 6, 14 and 0, 9 and 4 and 2 and 3 (atrial and ventricular clusters, respectively), expressed in at least 25% of the cells in one of the clusters in each comparison and with an adjusted p-value < 0.05. The resulting list of genes was filtered on genes considered to be transcription factors (Lambert et al., 2018). Hierarchical clustering of the factors was performed to separate specific expression patterns over the different time points across the lineages, and the results were visualized using Complex Heatmap v2.8.0 (Gu et al., 2016).

Retinoic acid-related gene sets

Differential expression with Wald's ranking test was performed for each of the time points between the lineages in the EB subset. The significantly upregulated genes in the atrial (the RA-induced condition) over ventricular (non-RA), were separated into three subsets relative to the time of RA administration. This was done in the following way: 1) Early RA genes were genes considered significantly upregulated at day 5, that were not upregulated at d4 or any of the time points after day 5 in aCM over vCM, 2) the Long Term RA genes were selected as not yet upregulated at day 4, but upregulated from day 5 onward and still upregulated at the later time points (d8 and d14), and 3) the Late RA genes were selected as genes the genes upregulated at d8 and not yet upregulated at day 4 or 5 in aCM over vCM.

Multiome data processing and integration

The multiome data was mapped with 10X Genomics Cell Ranger ARC v2.0.1 on the GRCh38 genome. To select droplets containing a quality cell, the cell-calling performed with running Cell Ranger ATAC on the ATAC data alone was combined with setting additional manual thresholds (only keeping droplets with a number of RNA counts > 1,000 and < 10,000, number of ATAC counts > 5,000 and < 20,000, nucleosome signal < 1, TSS enrichment score > 4 and percentage of mitochondrial reads < 20%). The

resulting dataset consisted of 17,027 cells and was analyzed using Signac v1.7.0 and Seurat v4.0.3 (Stuart et al., 2021). The EB day 8 samples, one from each lineage, were integrated using CCA on the scRNA-seq matrices and visualized using UMAP on the principal components of PCA analysis on the top 2,000 highly variable genes of the data. Clustering was also performed on the scRNA-seq matrices. Pearson correlations between the Multiome scRNA-seq clusters and the CEL-Seq2 derived clusters of d8 EB cells, were performed on subsampled (1,000 cells maximum per cluster) datasets, and the R2 values per cell-cell comparison was visualized with heatmap.

Quality control and motif analysis on scATAC-seq pseudobulk of the clusters

Per-cluster pseudobulk BED-files of the scATAC-seq modality of the multiome, were generated using the SplitFragments functionality in Signac. The BED-files were scaled and converted to BedGraph-files using *genomecov* from bedtools v2.30.0 (Quinlan and Hall, 2010), sorted, and processed to BigWig-files using the UCSC *BedGraphToBigWig* function (Kent et al., 2010). Visualization of the BigWig-files was performed in the Genome Browser (Kent et al., 2002), genes without gene name annotation were removed from this view. Peak calling was performed with the MACS2 implementation of Signac, for each of the clusters as well as the pseudobulk of all scATAC-seq reads in the dataset. The subsequent peak summits were merged in case they were at a distance of < 100 bp to one another, and replaced for a summit in the middle of those locations. To generate a merged peak set, all of the summits were extended with 100 bp up- and downstream of the summit. This peak set was used for differentially accessibility testing with the *FindMarkers* functionality from Signac using logistic regression, with the total number of fragments as latent variable. The resulting differential peak set was visualized in a bandplot using fluff (Georgiou and van Heeringen, 2016) and used as input for regression-based motif scanning analysis using the *maelstrom* function in Gimme Motifs (Bruse and van Heeringen, 2018). Motifs with in at least one of the clusters a z-score > 3, were selected and visualized with its potential binding transcription factors using an adjusted version of the *Motif2TF* function of scANANSE (Smits et al., 2023).

scANANSE analysis on Multiome data

The Seurat implementation of scANANSE (AnanseSeurat, v1.1.0) was used to obtain the important transcription factor regulators from the Multiome dataset (Smits et al., 2023; Xu et al., 2021). The resulting differential network for the aCM cluster over vCM cluster, was filtered on the top 20 transcription factors with the highest influence score. Interactions between the nodes with a score > 0.8 were selected from the full cell type-specific networks. Subsequent filtering with the three RA-induced gene sets (described in 7.4.18) from the EB analysis, was used to visualize the regulatory hierarchy within the GRN. The GRN visualizations were generated with Cytoscape v3.9.1 (Shannon et al., 2003). The *ZNF711* network was selected from the aCM full network, keeping only interactions with an interaction score > 0.8 and if they were found as factors of influence in this cell type or known cardiac markers.

Validation KOs RNA-seq, lineage marker enrichment and differential analysis

The Seq2Science v0.9.6 pipeline was used to map the RNA-seq reads to the GRCh38 genome (van der Sande et al., 2023). Regularized log transformation was performed with the R package DESeq2 v1.34.0 prior to Principal Component Analysis and heatmap visualization of the gene counts (Love et al., 2014). For each of the day 14 EB cell types from the roadmap, the top 200 marker genes were selected per cell type lineage. An average expression level per 200 markers was calculated for each of the bulk RNA-seq samples and corrected for the average expression of the *TRAC* control samples of both conditions, allowing direct comparison between the culture conditions. Significance was tested with a paired Wilcoxon signed-rank test for each of the replicates with its matching *TRAC* control sample, which was corrected for multiple testing using the Benjamini & Hochberg method and filtered for consistency between the replicates. The p-adjusted indicated in the heatmap are based on the highest p-adjusted value per set of replicates. Differential gene expression analysis between *TRAC* control and targeted conditions was performed with DESeq2. The differential gene list was filtered for a log2 fold change > 1 and p-adjusted value < 0.05, and visualized with heatmap v1.0.12 and EnhancedVolcano v1.12.0. Gene ontology analysis was performed with clusterProfiler v4.2.0 (Wu et al., 2021).

Hypergeometric tests on overlap scANANSE predicted targets

The hypergeometric test was performed with the *phyper* function in R. Per knockout and +RA or non-RA condition, the list of differentially expressed genes was used as *successes* and the overlapping genes between these lists and the scANANSE predicted targets of the *ZNF711*, *LHX2* or *RARB*, were considered the *sample successes* in the hypergeometric tests. All genes (16,232 in total) present in both the multiome scRNA-seq modality and the bulk RNA-seq samples, were considered the full population. For the comparison of *ZNF711* targets with the targets of all other regulators in the epicardial, aCM and

vCM networks predicted by scANANSE, all interactions per regulator with a probability of 0.8 were kept from the full network. Within each of the networks the Jaccard Index (the targets overlap divided by the union of the two target-sets minus the overlap) was used to calculate the amount of overlap between the predicted ZNF711 targets and the target sets of each other regulator in the network and enrichment was tested with a hypergeometric test, with all genes (26,128 in total) expressed in the multiome dataset as full population. The p-values from the hypergeometric tests were corrected for multiple testing using the Benjamini & Hochberg method. Calculated p-values smaller than $1e-100$ (including zero values) were reported as $p < 1e-100$.

REFERENCES

- Asp, M., Giacomello, S., Larsson, L., Wu, C., Fürth, D., Qian, X., Wärdell, E., Custodio, J., Reimegård, J., Salmén, F., et al. (2019). A Spatiotemporal Organ-Wide Gene Expression and Cell Atlas of the Developing Human Heart. *Cell* 179, 1647–1660.e19. <https://doi.org/10.1016/j.cell.2019.11.025>.
- Bergen, V., Lange, M., Peidli, S., Wolf, F.A., and Theis, F.J. (2020). Generalizing RNA velocity to transient cell states through dynamical modeling. *Nat. Biotechnol.* 38, 1408–1414. <https://doi.org/10.1038/s41587-020-0591-3>.
- Birket, M.J., Ribeiro, M.C., Kosmidis, G., Ward, D., Leitoguinho, A.R., van de Pol, V., Dambrot, C., Devalla, H.D., Davis, R.P., Mastroberardino, P.G., et al. (2015). Contractile Defect Caused by Mutation in MYBPC3 Revealed under Conditions Optimized for Human PSC-Cardiomyocyte Function. *Cell Rep.* 13, 733–745. <https://doi.org/10.1016/j.celrep.2015.09.025>.
- Bruse, N., and van Heeringen, S.J. (2018). GimmeMotifs: An analysis framework for transcription factor motif analysis. *BioRxiv* <https://doi.org/10.1101/474403>.
- Cui, Y., Zheng, Y., Liu, X., Yan, L., Fan, X., Yong, J., Hu, Y., Dong, J., Li, Q., Wu, X., et al. (2019). Single-Cell Transcriptome Analysis Maps the Developmental Track of the Human Heart. *Cell Rep.* 26, 1934–1950.e5. <https://doi.org/10.1016/j.celrep.2019.01.079>.
- Georgiou, G., and van Heeringen, S.J. (2016). fluff: exploratory analysis and visualization of high-throughput sequencing data. *PeerJ* 4, e2209. <https://doi.org/10.7717/peerj.2209>.
- Gerlach, J.P., van Buggenum, J.A.G., Tanis, S.E.J., Hogeweg, M., Heuts, B.M.H., Muraro, M.J., Elze, L., Rivello, F., Rakszewska, A., van Oudenaarden, A., et al. (2019). Combined quantification of intracellular (phospho-)proteins and transcriptomics from fixed single cells. *Sci. Rep.* 9. <https://doi.org/10.1038/S41598-018-37977-7>.
- Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*.
- Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W.M., Zheng, S., Butler, A., Lee, M.J., Wilk, A.J., Darby, C., Zager, M., et al. (2021). Integrated analysis of multimodal single-cell data. *Cell* 184, 3573–3587.e29. <https://doi.org/10.1016/J.CELL.2021.04.048>.
- Hashimshony, T., Senderovich, N., Avital, G., Klochender, A., de Leeuw, Y., Anavy, L., Gennert, D., Li, S., Livak, K.J., Rozenblatt-Rosen, O., et al. (2016). CEL-Seq2: Sensitive highly-multiplexed single-cell RNA-Seq. *Genome Biol.* <https://doi.org/10.1186/s13059-016-0938-8>.
- Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., and Haussler, and D. (2002). The human genome browser at UCSC. *Genome Res.* 12, 996–1006. <https://doi.org/10.1101/GR.229102>.
- Kent, W.J., Zweig, A.S., Barber, G., Hinrichs, A.S., and Karolchik, D. (2010). BigWig and BigBed: enabling browsing of large distributed datasets. *Bioinformatics* 26, 2204–2207. <https://doi.org/10.1093/BIOINFORMATICS/BTQ351>.
- Lambert, S.A., Jolma, A., Campitelli, L.F., Das, P.K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T.R., and Weirauch, M.T. (2018). The Human Transcription Factors. *Cell* 172, 650–665. <https://doi.org/10.1016/J.CELL.2018.01.029>.
- Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. <https://doi.org/10.1186/S13059-014-0550-8/FIGURES/9>.
- McCarthy, D.J., Campbell, K.R., Lun, A.T.L., and Wills, Q.F. (2017). Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R. *Bioinformatics* 33, btw777. <https://doi.org/10.1093/bioinformatics/btw777>.
- Melsted, P., Boeshaghi, A.S., Liu, L., Gao, F., Lu, L., Min, K.H. (Joseph), da Veiga Beltrame, E., Hjörleifsson, K.E., Gehring, J., and Pachter, L. (2021). Modular, efficient and constant-memory single-cell RNA-seq preprocessing. *Nat. Biotechnol.* 39, 813–818. <https://doi.org/10.1038/s41587-021-00870-2>.

- Muraro, M.J., Dharmadhikari, G., Grün, D., Groen, N., Dielen, T., Jansen, E., van Gurp, L., Engelse, M.A., Carlotti, F., de Koning, E.J.P., et al. (2016). A Single-Cell Transcriptome Atlas of the Human Pancreas. *Cell Syst.* 3, 385–394.e3. <https://doi.org/10.1016/j.cels.2016.09.002>.
- Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. *Nat. Protoc.* 3, 768–776. <https://doi.org/10.1038/nprot.2008.42>.
- Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. <https://doi.org/10.1093/BIOINFORMATICS/BTQ033>.
- Ribeiro, M.C., Rivera-Arbeláez, J.M., Cofiño-Fabres, C., Schwach, V., Slaats, R.H., Ten Den, S.A., Vermeul, K., van den Berg, A., Pérez-Pomares, J.M., Segerink, L.I., et al. (2022). A New Versatile Platform for Assessment of Improved Cardiac Performance in Human-Engineered Heart Tissues. *J. Pers. Med.* 12, 12. <https://doi.org/10.3390/JPM12020214/S1>.
- van der Sande, M., Frölich, S., Schäfers, T., Smits, J.G.A., Snabel, R.R., Rinzema, S., and van Heeringen, S.J. (2023). Seq2science: an end-to-end workflow for functional genomics analysis. *PeerJ* 11. <https://doi.org/10.7717/PEERJ.16380>.
- Schwach, V., Verkerk, A.O., Mol, M., Monshouwer-Kloots, J.J., Devalla, H.D., Orlova, V. V., Anastassiadis, K., Mummery, C.L., Davis, R.P., and Passier, R. (2017). A COUP-TFII Human Embryonic Stem Cell Reporter Line to Identify and Select Atrial Cardiomyocytes. *Stem Cell Reports* <https://doi.org/10.1016/j.stemcr.2017.10.024>.
- Schwach, V., Cofiño-Fabres, C., Ten Den, S.A., and Passier, R. (2022). Improved Atrial Differentiation of Human Pluripotent Stem Cells by Activation of Retinoic Acid Receptor Alpha (RAR α). *J. Pers. Med.* 12. <https://doi.org/10.3390/JPM12040628>.
- Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. .
- Smits, J.G.A., Arts, J.A., Frölich, S., Snabel, R.R., Heuts, B.M.H., Martens, J.H.A., van Heeringen, S.J., and Zhou, H. (2023). scANANSE gene regulatory network and motif analysis of single-cell clusters. *F1000Research* 2023 12243 12, 243. <https://doi.org/10.12688/f1000research.130530.1>.
- Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. *Cell* 177, 1888–1902.e21. <https://doi.org/10.1016/j.cell.2019.05.031>.
- Stuart, T., Srivastava, A., Madad, S., Lareau, C.A., and Satija, R. (2021). Single-cell chromatin state analysis with Signac. *Nat. Methods* 2021 1811 18, 1333–1341. <https://doi.org/10.1038/s41592-021-01282-5>.
- Wolf, F.A., Angerer, P., and Theis, F.J. (2018). SCANPY: Large-scale single-cell gene expression data analysis. *Genome Biol.* 19, 1–5. <https://doi.org/10.1186/S13059-017-1382-0/FIGURES/1>.
- Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innov. (Cambridge)* 2. <https://doi.org/10.1016/J.XINN.2021.100141>.
- Xu, Q., Georgiou, G., Frölich, S., Van Der Sande, M., Veenstra, G.J.C., Zhou, H., and Van Heeringen, S.J. (2021). ANANSE: an enhancer network-based computational approach for predicting key transcription factors in cell fate determination. *Nucleic Acids Res.* 49, 7966–7985. <https://doi.org/10.1093/NAR/GKAB598>.
- Zappia, L., and Oshlack, A. (2018). Clustering trees: a visualization for evaluating clusterings at multiple resolutions. *Gigascience* 7, 1–9. <https://doi.org/10.1093/GIGASCIENCE/GIY083>.