Supplementary Materials and Methods

Maintenance and cardiac differentiation of hESCs

The cardiac differentiation protocol was adapted from Lian et al. [9]. In brief, ES03 hESCs were maintained in Essential 8 (E8) Flex Medium (Thermo Fisher Scientific, USA) on Matrigel (Corning, Germany) coated 6-well plates and passaged in Versene (Thermo Fisher Scientific). Cells were regularly tested for mycoplasma. Chromosome analysis of the ES03 cell line indicated normal 46, XX female karyotype (Fig. S1A). Upon differentiation on day -2, one confluent 6-well plate was split into one Matrigel coated 12-well plate, at a density of about 1 million cells per well, in E8 Flex with 5μM Y-27632 (Sigma, Germany). The medium was changed to E8 Flex without Y-27632 on the following day. On day 0 of differentiation, the media was replaced with 12 μM CHIR99021 (Sigma) in RPMI media (Thermo Fisher Scientific) supplemented with B-27 minus insulin (Thermo Fisher Scientific). The medium was replaced with RPMI/B-27 minus insulin exactly 24h later. On day 3 of differentiation, combined medium was prepared by mixing medium from the 12-well plate with fresh RPMI/B-27 minus insulin in 1:1 ratio and adding 5 μM IWP2. Medium was replaced with RPMI/B-27 minus insulin on day 5 of differentiation, and to RPMI supplemented with B-27 (Thermo Fisher Scientific) on day 7 and every three days thereafter.

Single cell library preparation and sequencing

Cells undergoing cardiac differentiation were collected for single cell RNA sequencing at eight time points, day 3, 4, 5, 6, 7, 8, 9 and 15 from two batches of differentiation. One well per time point and differentiation batch was dissociated into single cells using Trypsin-EDTA (0.25%) (Thermo Fisher Scientific), and 64 cells were manually picked from each batch using a hand-pulled capillary needle. Single cells were transferred into 8-tube PCR strips containing Smart-Seq2 lysis buffer with 1:10 000 ERCC RNA spike-in mix (Ambion, ThermoFisher

Scientific), snap frozen on dry ice, and thereafter kept at -20 ºC. Each single cell was processed into a cDNA library by following the Smart-Seq2 protocol [18, 19]. Fragment sizes were examined by Agilent 2100 Bioanalyzer (Agilent, USA) and the concentrations of the cDNA libraries were measured using Qubit (Thermo Fisher Scientific) according to the manufacturers' instructions. Barcoded single-cell Illumina libraries were prepared using Illumina Nextera XT DNA Sample preparation kit (Illumina, USA) with dual indexes. The libraries were pooled into batches of 128 cells and sequencing was performed on Illumina HiSeq 2500 for 50 bp single-end reads.

Single cell RNA sequencing data analysis

Raw sequencing reads were de-multiplexed using bcl2fastq (Illumina) and mapped to the human hg38 genome using TopHat. In total 1 792 transcriptomes were sequenced. Data from two hESC cardiac differentiation batches was combined to create one single-cell dataset with Seurat R package. Filtering, normalization and subsequent analysis including calculating principal components and t-SNE and UMAP dimensionality reduction were performed as described in the Seurat 3.0 R package tutorial (http://satijalab.org/seurat/). Briefly, cells with low levels of detected transcripts or expressing less than 200 genes, as well as genes that are expressed in less than 3 cells, were excluded from downstream analysis. We proceeded with the Seurat 3.0 package in R [21-23] for downstream analysis. To identify variable genes, the average expression level and dispersion for each gene were calculated, and the genes were divided into 20 bins based on their average expression and Z-scores were calculated for each bin. Following the Seurat 3.0 pipeline, we selected the 2000 genes with the highest standardized variance for downstream analyses.

The RunTSNE function in Seurat was used to compute a t-SNE dimensional reduction and clustering. The FindNeighbors considering nine first principal components and FindClusters function with resolution 0.5 in the Seurat package were used for clustering.

Identification of marker genes

The FindMarkers function in Seurat was used to identify positive marker genes for each cluster, and the gene list was filtered for genes having a significant adjusted p value (< 0.05). Differential gene expression analysis between wild-type and ISL1 knockout cells was also performed using FindMarkers function.

Functional enrichment analysis

50 top ranked marker genes for each cluster were selected for functional enrichment analyses. Gene ontology (GO) analysis was performed using STRING version 11.0 statistical overrepresentation test against *homo sapiens* genome [24]. PANTHER 14.1 statistical overrepresentation test against *homo sapiens* genome applying Fisher's exact test and calculation of Benjamin-Hochberg false discovery rate [35] was used to find GO terms for the integrated dataset, and PANTHER Pathways [36] were called for specific populations as indicated in the text.

Monocle pseudotime analysis

Monocle 2 package was used for pseudotime analysis of the cells belonging to the pluripotent and myogenic populations (clusters 0-5) following the online tutorial (http://cole-trapnell-lab.github.io/monocle-release/docs/) [25-27]. In addition, pseudotime values were determined for all hESC-derived cells, and the values were imported to the Seurat object and visualized as t-SNE, FDG and UMAP-based feature plots (Fig. S4B).

Force-directed graphing

We performed force-directed graphing using the igraph R package, and the nearest neighbour matrix was calculated using the method described by Kee et al. [20]. Briefly, we first performed t-SNE analysis on the entire dataset using the Seurat 3.0 package, supplemented by graphing analysis developed by Kee et al. to map out differentiation axes in pseudotime. We found that calculating the 14 nearest neighbors on the t-SNE produced a visually useful graph drawing (Fig. 3A). Calculating a range of number of nearest neighbors produced similar results (Fig. S5A-C).

Cell cycle analysis

In order to study the cell cycle heterogeneity between cell populations, we sought to analyze the overall cell cycle-related gene expression. Each cell was assigned a score for cell cycle phases S and G2/M using the CellCycleScoring function in Seurat and a published list of cell cycle genes, following the cell cycle regression tutorial (https://satijalab.org/seurat/v3.1/cell_cycle_vignette.html). The cells with positive scores for G2/M phases were considered as being in a proliferative state, whereas cells with negative cell cycle scores were likely not cycling. We visualized the cell cycle scores using UMAP dimensional reduction indicating differences between and within different clusters. We also ran an alternative UMAP analysis by regressing out cell cycle scores during data scaling using S.Score and G2M.score as input for vars.to.regress argument in ScaleData. We used WhichCells function in Seurat to calculate the percentage of cells with G2M.Score greater than 0 in each cluster to estimate the percentage of proliferative cells in each cluster.

RNA velocity analysis

RNA velocity was performed according to the Velocyto tutorial (http://velocyto.org/) [17]. In brief, a .loom file with counts divided in spliced/unspliced/ambiguous was produced in Python using the human hg38 genome annotation file (.gtf) and an expressed repeat annotation file from the UCSC genome browser. Subsequent RNA velocity analysis was performed in R according to the tutorial (http://pklab.med.harvard.edu/velocyto/notebooks/R/chromaffin2.nb.html). After filtering out reads with very low counts, we detected 8 911 genes that had both spliced and unspliced reads, of which 5 027 also had reads that spanned intron-exon junctions. Due to the relatively high number of genes detected with spanning reads, we used the spanning reads to produce an estimate of RNA velocity. We also compared several variants (with or without nearest cell pooling, and excluding spanning reads) of velocity estimates provided in Le Manno et al, and found very similar results. R scripts are available upon request.

Integration of single cell datasets

The RPKM (Reads Per Kilobase Million) read counts of the hESC-derived single cell dataset were converted to TPM (Transcripts Per Kilobase Million) to match with the dataset generated by Cui et al. The human fetal dataset had lower average number of detected genes per cell compared to the hESC-differentiation dataset (Fig. S2A).

We proceeded with Seurat version 3.0 workflow for integration and data transfer [21, 23], which essentially "anchors" diverse datasets together, enabling integration of datasets across scRNA-seq technologies and modalities. The workflow applies a correction vector for each individual cell in the query dataset, which is determined by a cell similarity score and an anchor score. For detailed workflow please see (https://satijalab.org/seurat/v3.1/integration.html).

Immunocytochemistry

For imaging with confocal microscope, ES03 cells were seeded on coverslips on 12-well plates for differentiation. Cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.5% Triton X-100 for 20 minutes and blocked with 3% BSA in PBS for 30 minutes at room temperature. Coverslips were incubated with primary antibodies (with simultaneous incubation of antibodies) in blocking buffer overnight at 4°C, followed by secondary antibody incubation for one hour at RT and nuclear counterstaining with DAPI. Coverslips were washed three times with PBST for 5 minutes between each step.

Human fetal heart tissues

Human fetal hearts at 6.5 and 10 weeks of the gestation stages were obtained from authorized sources in Karolinska University Hospital with an approved ethical permission (Dnr 2015/1369-31/2) and appropriate informed consents. The fetal hearts were snap-frozen and cryosectioned at 10-μm thickness for immunostaining.

Immunofluorescence

Cardiac tissue sections were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, blocked (3% BSA, 3% normal donkey serum in PBS) and incubated with primary antibodies in blocking buffer overnight at 4°C. Sections were incubated with secondary antibodies in blocking buffer for one hour at RT and counterstained with DAPI.

Primary antibodies			Dilution
anti-HHEX	Atlas Antibodies	HPA051894	1:500
anti-ISL1	DSHB	39.4D5	1:100

anti-NKX2-5	Santa Cruz	sc-14033	1:1000
anti-TFAP2A	Abcam	ab108311	1:500
anti-TGFBI	Abcam	ab66957	1:100
anti-TNNT2	ThermoFisher	MS-295-P1	1:500
anti-Vimentin	Millipore	AB5733	1:2000

Genetic manipulation of hESCs

The genetic manipulation of hESCs was carried out as described in Xu et al. 2019. Briefly, two gRNAs targeting exon 4 of *ISL1*, with sequences CGATGTGATACACCTTGGAG and GGCGGCTGCAGGCCACACAG, were cloned into PB-CRISPR vector. Two million hESCs, 1μg pCyL43 and 4μg pPB-CRISPR-ISL1-gRNA DNA were mixed in 100μl nucleofection solution and nucleofection was performed with program B-16 of Nucleofector 2b device (Lonza). After two weeks of 0.5 μg/mL Puromycin selection, cells were singularized and sparsely seeded to generate single cell derived clones. Genomic DNA was extracted from putative *ISL1*mutated hESC clones using Quick-gRNA MiniPrep kit (Zymo Research, D3007). The targeted genome region was amplified using forward primer TCTGTGGTACAAGCTAGGTGTT and reverse primer ACACTACGACCACCCCTGTA. The Amplified PCR fragment was inserted into pCR4 TOPO vector via the TOPO-TA method (ThermoFisher Scientific). Plasmids from individual *E. coli* clones were Sanger sequenced using the T7 primer. One hESC clone with induced stop codons within the targeted exon was chosen for further analysis. ISL1 deletion was validated by quantitative PCR and western blot and immunostaining (Fig. S6B).

Quantitative PCR

Total RNA was purified using ZYMOGEN Direct-zol RNA MiniPrep kit using DNase I treatment to remove any genomic DNA contamination. 500 ng of total RNA was used for reverse transcription with SuperScript IV First- Strand Synthesis System (Invitrogen). Quantitative PCR was performed using the reagents and workflow as recommended by PowerUp SYBR Green (Applied Biosystems) guidelines. Gene expression levels were normalized to *GAPDH*. Results are represented as fold change compared to the wild-type ± SEM, and are averaged from four biological repeats, with three technical repeats each.

qPCR primers		
Target	Forward	Reverse
APOA2	GCAGCAACTGTGCTACTCCT	TCTCCGAACCAAAGCTCCTTC
COL3A1	GTTGCACGAAACACACTGGG	ACAGCCTTGCGTGTTCGATA
COL1A1	CCCCGAGGCTCTGAAGGTC	GGAGCACCATTGGCACCTTT
GAPDH	CGACAGTCAGCCGCATCTT	CCCAATACGACCAAATCCGTTG
HHEX	CTCAGCGAGAGACAGGTCAA	TCTCCTCCATTTAGCGCGTC
HNF4A	TGACGATGGGCAATGACACG	CTCGAGGCACCGTAGTGTTT
ISL1	TCAGTATTTGGACGAGAGCTG	CATTTGATCCCGTACAACCTGA
MYL2	AGGCTCCGGGTCCAATTAAC	AGGGTCCGCTCCCTTAAGTT
MYL7	AAGCCATCCTGAGTGCCTTC	AACATCTGCTCCACCTCAGC
NKX2-5	CAAGTGTGCGTCTGCCTTTC	CACAGCTCTTTCTTTTCGGCTC