Online Methods

Animal work

Mesp1-Cre[1], Tbx1-Cre[2], Islet1-Cre[3], Hcn4-GFP[4,5], Wntless loxp (stock no. 012888, Jackson Laboratory[6]), and Ai9 (stock no. 007909, Jackson Laboratory[7]) mouse strains were utilized in *in vivo* experiments. Embryos were harvested from embryonic day 8.0-9.5 for further analysis. For evaluating the phenotype of each Cre driver we performed dissections in at least 3 litters to determine penetrance. No embryos were excluded from analysis, however yolk sacs without embryos were discarded. Each embryo was genotyped using tissue from individual yolk sacs for Wls loxp and relevant Cre driver. Embryos intended for cryo-sectioning and immunofluorescence were cleaned, fixed in 4% PFA for two hours, and stored in 30% sucrose overnight. Embryos were then embedded in OCT and flash frozen before sectioning. Tbx5 (Atlas Antibodies; HPA008786; 1:250), cTnt (Thermo Fisher; MS-295-P1; 1:500), CD31 (BD Biosciences; 553371; 1:200), Islet1 (DSHB; 39.3F7; 1:200), Nkx2-5 (SCBT; sc-8697; 1:250), PHH3 (Millipore Sigma; 06-570; 1:1000, Thermo Fisher; MA5-15220;1:300), RFP (ChromoTek; 5F8; 1:200). We have verified these antibodies in previous studies[8,9]. Stained sections were imaged on a Keyence BZ-X710 and ACCM Leica SP8 Confocal microscope. For proliferation analysis, proliferating cells on stained sections were quantified using ImageJ where we quantified $Mesp1^{+/}$ $Tbx5^{-}$/$Phh3^{+}$ cells in the SHF domain. Two sample t-test was used to establish significance.

Embryos intended for transcriptomic analysis were dissected, staged, and stored on ice in cold PBS -/-. Yolk sacs were saved for genotyping. Embryos were dissociated using TrypLe and barcoded using MULTI-seq anchor and primers[10], before cell sorting was completed for $Mesp1^{+}$/$tdTomato^{+}$ cells using Sony SH800. Cells were then captured for 10x library prep and single cell RNA sequencing. All protocols involving animals followed U.S NIH guidelines and were approved by the ACUC of JHMI.

Hybridization Chain Reaction whole mount in situ

E8.5 NIH/Swiss embryos were collected and fixed overnight in 4% paraformaldehyde then dehydrated in methanol. HCR was performed as previously described[11,12]. V3.0 HCR probes were purchased from Molecular Instruments, Inc. HCR labeled embryos were cleared in Ce3D++[11,13] and imaged on a Nikon A1R confocal microscope using a 10x objective NA = 0.4.

Single cell RNA-sequencing

We multiplexed samples using the MULTI-seq protocol[10]. Anchor and co-anchor LMOs were kindly provided to us by the Gartner lab. Samples were prepared and sequenced using the 10x Chromium 3' v3 workflow, and sequenced on either a Next-seq5000 (runs 1 and 2) or NovaSeq6000 (run 3). Demultiplexing and barcode identification was performed using the deMULTIplex package (1.0.2)[10]. We performed integration of the runs using the SCTransform workflow implemented in Seurat (3.1.4)[14–16]. To identify clusters, we overclustered by using a resolution of 2.0, and subsequently merged clusters based on expression of marker genes. Differential gene expression testing was performed using the Wilcoxon Rank Sum test as implemented in Seurat, with differentially expressed genes identified with Bonferonni-correction-adjusted p-values < 0.05. Gene Ontology analysis was done using the Gene Ontology Resource online tool[17,18], and visualization was performed with REViGO[19]. Trajectory reconstruction was performed in Monocle 2[20] (2.12.0). We generated trajectories using the list of genes differentially expressed between control and knockout cells at e9.5, though we obtained similar results using dpFeature. Differential expression analysis across the trajectories was performed with tradeSeq[21] (1.3.15). We focused on genes with Benjamini-Hochberg-corrected p-values < 0.05 and $\log_2$(Fold Change) >= 0.8. We clustered gene trends using complete-linkage hierarchical clustering as implemented in the pheatmap package (1.0.12). All of the code used for analysis in this manuscript can be found on Github at: https://github.com/skannan4/wls.

scRNA-seq Library Preparation and Sequencing

We multiplexed samples for sequencing using the MULTI-seq protocol[10]. We adapted the protocol described below from the instructions provided to us by the Gartner lab. The following primers/adapters were used:

- Anchor LMO: 5'-TGGAATTCTCGGGTGCCAAGGgtaacgatccagctgtcact-{Lipid}-3'
- Co-Anchor LMO: 5'-{Lipid}-AGTGACAGCTGGATCGTTAC-3'
- Barcode Oligo: 5'-CCTTGGCACCCGAGAATTCCA**NNNNNNNN**A_{30}-3'
- MULTI-seq Primer: 5'-CTTGGCACCCGAGAATTCC-3'
- TruSeq RPIX: 5'-CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGT
- TCCTTGGCACCCGAGAATTCCA-3'

- Universal I5: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

The anchor and co-anchor LMOs were kindly provided to us by the Gartner lab; the barcode oligos, MULTI-seq primer, and TruSeq RPIX were ordered from Integrated DNA Technologies using barcodes provided by the Gartner lab; and the Universal i5 was part of the 10x Chromium 3' v3 Reagent kit.

Briefly, after sorting, cells were incubated with a 10x anchor:barcode solution for 5 minutes on ice, followed by incubation with 10x co-anchor solution for 5 minutes. We subsequently added 1% BSA in PBS and washed 2-3 times. We used the 10x Chromium 3' v3 workflow to encapsulate and capture cells, reverse transcribe mRNA, and purify cDNA. To capture the barcode sequences, we performed the 10x cDNA amplification reaction, but with addition of the MULTI-seq primer. Cleanup with 0.6x SPRI beads enabled separation of endogenous transcript cDNA and barcodes – endogenous transcripts remained bound to the beads while barcodes were eluted in the supernatant. We then separately processed the endogenous cDNA and barcodes using the 10x workflow. For the experiments sequenced on 10/21/19, sequencing of the endogenous transcripts was done on a NextSeq500 high output lane using a 28/8/91 bp design for R1/i7/R2. Sequencing of the barcodes was done a NextSeq500 mid output lane using a 28/8/8 design for R1/i7/R2. For the experiments sequenced on 03/04/20, sequencing for both endogenous and barcode transcripts was done on a NovaSeq6000 S1 flow cell, using a 28/8/91 bp design; the barcode transcripts were subsequently trimmed using the BBMap/BBTools suite[22].

scRNA-seq Analysis

All of the code used for analysis in this manuscript can be found on Github at https://github.com/skannan4/wls.

Mapping and Demultiplexing

Endogenous cDNA reads were mapped using Kallisto|Bustools (0.46.1)[23]. We removed poor quality barcodes and demultiplexed the samples using deMULTIplex (1.0.2)[10], with minor modifications as described in our code. We removed all cells classified as "Negative" or "Doublet". Our final analysis yielded the following samples:

- Run 1 (10/21/19): 1 e8.5 KO, 2 e8.5 WT, 1 e9.5 KO, 2 e9.5 WT
- Run 2 (10/21/19): 1 e7.5 WT, 2 e9.5 KO, 2 e9.5 WT
- Run 3 (03/04/20): 3 e8.0 KO, 3 e8.0 WT, 3 e9.5 KO, 2 e9.5 WT

We performed further quality control by removing low quality cells and putative doublets using the following selection criteria – 2500 < genes < 9000; mitochondrial percentage < 22%; total UMIs < 62500.

Dataset Integration, Dimensionality Reduction, and Clustering

Integration of the three runs was performed using the SCTransform workflow in the Seurat package (3.1.4)[16] as described by the vignette provided by the authors. In particular, we selected 3000 features for integration. Following integration, we found little evidence of batch-specific clustering, suggesting successful integration of the three runs. Dimensionality reduction via UMAP and clustering (using the Louvain algorithm) were performed in Seurat. We selected 15 principal components for clustering, as an elbow plot indicated that these were sufficient for capturing the majority of variation in the dataset; moreover, we found that increasing the number of principal components led to abiological clustering. As discussed below, we overclustered by using a resolution of 2.0 and subsequently merging similar clusters.

Clustering and Annotation Strategy

There is no established method for identifying the optimal number or size of clusters for an scRNA-seq experiment, though various metrics and strategies have been proposed[24]. Indeed, the appropriate clustering approach will vary from experiment to experiment based on the goals and desired resolution. Further, clustering multiple groups of transcriptomically similar progenitor cell populations also complicates this process. Our strategy here was to purposely overcluster (e.g. generate more clusters than biologically expected) and then manually merge and label clusters based on expression of marker genes of interest. Here, we discuss the markers we selected for identification of clusters: We utilized a set of known canonical markers to identify each cluster of cells. Clusters that were labeled as the same cell identity were merged. Our initial clustering (after filtering of obvious RBCs) resulted in 34 clusters of cells. First, we determined somitic mesoderm (sclerotome and dermomyotome), through high expression of the gene Meox1[25]. We were able to differentiate between sclerotome and dermomyotome through expression of Pax1[26–29] (Sclerotome; clusters 9, 12, 21) and Pax3[27,30,31] (Dermomyotome; clusters 8,23). Next, we utilized a recent very thorough single cell analysis of heart development[32], as well as other studies, to

identify cardio-pharyngeal clusters through published marker genes. We determined our pharyngeal mesoderm (PhM; clusters 1, 4, 19, 20, 26) through co-expression of Fst, Ebf1 and Tbx1[32]. We determined anterior SHF identity through co-expression of Fgf8/10, Isl1, Tbx1, and Sema3c (aSHF; clusters 0, 29)[32]. The posterior SHF (pSHF) cluster comprised the pSHF and its close derivatives. It was identified through upregulation of Osr1, Hoxb1, and Foxf1, with mild Islet1 expression (pSHF; clusters 5, 7, 27, 33)[32]. The FHF cluster comprised the FHF and its close derivatives. This cluster was mostly present at e8.0, and expressed FHF markers Hcn4, Sfrp5, Tbx5, and mild expression of the cardiomyocyte markers Tnnt2 and Nkx2-5 (FHF; cluster 24)[32]. Cardiomyocytes were identified by cardiomyocyte markers including Tnnt2, Nkx2-5, Myh6 (CM; cluster 17)[32]. OT CMs were identified specifically by co-expression of CM markers and Fgf8 and Isl1 (OT CM; cluster 15). The proepicardium was identified through Wt1 and Tbx18 co-expression[33,34] (proepicardium; clusters 6, 13). Mesenchymal cells were identified through co-expression of Postn[35], Vim [36], Pitx1, Twist, and Tek (Mesenchyme; cluster 22). Forelimb identity was established through co-expression of Fgf10, Tbx5[37,38], Tshz2 and Lmx1b[39] (Forelimb; clusters 2, 11, 14, 18, 29, 32). We differentiated between forelimb and hindlimb through Tbx4 and Tbx5 expression (forelimb expressing Tbx5 and hindlimb expressing Tbx4)[40], in addition to expression of Pitx1[41], Hoxb8[42], and Hoxc6[43] (Hindlimb; clusters 10, 25, 32). We identified the endocardium/endothelial identity through co-expression of Kdr and Tek (EC; cluster 3)[44]. Remaining cells which clustered separately from other cell types and showed no discernable gene expression patterns were identified as extraembryonic in origin (Extraembryonic, clusters 16, 34).

Differential Gene Expression Testing and Analysis

Differential expression testing (as in Fig. 3F was performed by comparing control to knockout cells in each population at each timepoint using the Wilcoxon rank sum test as implemented as Seurat. For a comparison to be performed, we required at least 10 cells to be present in both the control and knockout. A minimum expression of 25% of cells was used as a cutoff for testing genes, and a minimum log fold change of .25 was used for reporting results. We subsequently selected genes with Bonferroni correction-adjusted p-value < 0.05. We performed Gene Ontology Overrepresentation Analysis by inputting differentially expressed genes into the online Gene Ontology Resource[17,18]. Summary and visualization of GO terms was done by selecting the top 150 GO terms by fold enrichment and inputting to REViGO[19]. For Fig. 3G, we selected genes that were differentially expressed in only one tissue at e9.5, while for Fig. S6F, we selected genes that were differentially expressed in at least 7 tissues.

Ligand-receptor Interaction Analysis

We used the CellTalkDB mouse ligand-receptor database as our source of ligand-receptor interactions. We narrowed down our analysis using the following criteria: 1) the receptor should be differentially expressed in aSHF cells, based on our tradeSeq analysis using diffEndTest; and 2) the corresponding ligand should be expressed (at any level) in at least 25% of FHF cells. Receptors were then visualized as branched heatmaps.

Trajectory Reconstruction and Analysis

Trajectory reconstruction was performed using Monocle 2[20](2.12.0); while we initially tested Monocle 3 and Slingshot, we found that Monocle 2 offered the most flexibility and yielded trajectories that most matched the expected biology. We used a semi-supervised approach to generating trajectories. We first selected the 8000 most variable genes; these genes were provided as input to differential gene expression testing between the control and knockout cells at e9.5. We subsequently selected the 3000 most differentially expressed genes to construct the trajectory. This approach potentially biases to differences between control and knockout at the expense of other biological variation. We favored this approach because our specific goal was to understand the deviation in the states of the control and knockout cells. However, we found that an unbiased approach (dpFeature, as implemented in Monocle 2) yielded similar trajectories, suggesting that the control vs knockout differences are the most notable biological variation in our tested cells. We performed differential gene expression testing across the branches by using tradeSeq (1.3.15)[21]. As the pseudotimes from Monocle 2 may be somewhat arbitrary across branches, we stretch branches such that they had the same overall length and end pseudotime. We additionally pruned small side branches that were likely artefactual. We fit generalized additive models to genes expressed in at least 20% of cells across the branches, and identified genes differentially expressed across the end states using the diffEndTest function in tradeSeq. The p-values reported by tradeSeq are to be interpreted with some caution; however, simply to specify a threshold for further analysis, we focused on genes with Benjamini-Hochberg-corrected p-values < 0.05 and $\log_2$(Fold Change) >= 0.8. We clustered gene trends using complete-linkage hierarchical clustering as implemented in the pheatmap package (1.0.12).

Precardiac organoid experiments

Organoid experiments were performed as previously described[8,45]. Briefly, we treated differentiation day 4 precardiac organoids with IWP-2 (to block Wnt secretion) +/- recombinant Wnt2 protein. We analyzed GFP/RFP percentage on differentiation day 7 using Sony SH800

(Sony Biotechnologies). Analysis was performed using FlowJo Software. Samples with less than 30,000 events were discarded from analysis.

Data Availability:

Full data set and code are available on Synapse and GitHub:

Data: https://www.synapse.org/#!Synapse:syn24200678/files/

Code: https://github.com/skannan4/wls

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