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## Single-cell Resolution of Temporal Gene Expression during Heart Development

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### Abstract

Activation of complex molecular programs in specific cell lineages governs mammalian heart development, from a primordial linear tube to a four-chamber organ. To characterize lineage-specific, temporal-spatial developmental programs, we performed single-cell RNA sequencing of >1200 murine cells isolated at seven time points spanning E9.5 (primordial heart tube) to P21 (mature heart). Using unbiased transcriptional data we classified cardiomyocytes (CM), endothelial cells (EC), and fibroblast-enriched cells, thus identifying markers for temporal and

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#### Author Contributions

Conceptualization, Methodology and Writing – Original Draft, D.M.D., A.G.B., J.G.S., and C.E.S.; Investigation, D.M.D., A.G.B., H.W., and J.M.G.; Software, Formal Analysis and Visualization, D.M.D. and A.G.B.; Resources, D.M., I.K., J.T.H., J.G.; Review & Editing, H.W., D.M., I.K., J.T.H., J.H., J.G., W.P., B.G.B. C.E.S.; Supervision and Funding Acquisition, J.G., W.P., B.G.B., J.G.S., C.E.S.

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chamber-specific developmental programs. Harnessing these datasets, we defined developmental ages of human and mouse pluripotent stem cell-derived CMs and characterized lineage-specific maturation defects in hearts of mice with heterozygous mutations in *Nkx2.5* that cause human heart malformations. This spatial-temporal transcriptome analysis of heart development reveals lineage-specific gene programs underlying normal cardiac development and congenital heart disease.

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## Introduction

Formation and maturation of the mammalian heart is governed by signaling interactions among distinct cell lineages during development that transform a linear heart tube into four-chamber heart. Transcriptional profiles of human, mouse, and chick hearts have identified dynamic changes in RNA expression throughout cardiogenesis Jensen et al. (2013); (Wagner and Siddiqui, 2007). Understanding how these relate to cellular differentiation and maturation remains incompletely understood, in part because of the inability of conventional transcriptome analysis experiments to de-convolute the multiple cell populations within the heart. Recent advances in single-cell transcription profiling approaches enable rapid capture and profiling of hundreds of cells (Buettner et al., 2015; Streets and Huang, 2014; Trapnell et al., 2014; Treutlein et al., 2014) that allow separation and characterization of lineages and cell lineage subtypes, independent of preexisting cell markers. Longitudinal assessments of transcriptional maps provide information that can be assembled into a transcriptional atlas across development or in response to disease.

To investigate cellular heterogeneity during cardiogenesis, we characterized the developmental temporal and spatial transcriptomes of heart development at single cell resolution. Wildtype (WT) murine cells isolated from the left atria (LA), primordial ventricle, and subsequently left (LV) and right (RV) ventricles at time points spanning embryonic to post-natal cardiac development were studied using single-cell RNA sequencing (RNA-seq). Cells were classified using unbiased transcriptome-wide analysis without *a priori* selection of cell-specific markers. We identified transcripts that delineated atrial and ventricular CMs, and ECs and fibroblast-enriched cell lineages. We also characterized CMs derived from stem cells and CMs and ECs derived from a congenital heart disease mouse model with haploinsufficiency of *Nkx2.5*, a critical transcription factor that is expressed early in cardiogenesis (Ashraf et al., 2014; Biben et al., 2000; Bruneau et al., 2001).

Single cell transcriptional data of distinct cardiac lineages provided insights into lineage specific markers and subpopulations that contribute to normal and aberrant cardiac development and provided a robust benchmark to assess the maturity of stem cell-derived CMs.

## Results

We captured mouse (SV129) cardiac cells during important embryonic (E) developmental milestones (**Fig 1A**): E9.5 during heart looping, when the atria and ventricle become morphologically distinct; E11.5 when left and right cardiac chambers form, E14.5 when

septation occurs with development of compact and trabecular layers of myocardium; E18.5 when the heart undergoes metabolic remodeling. Cells were also captured at birth (P0) when the left atria and ventricle experience hemodynamic changes due to higher systemic pressures, and at P3, P7, and P21, when the heart transitions from proliferative growth (P3) to hypertrophic growth (P21).

The common atria and ventricle (E.9.5) were carefully dissected away from the adjacent atrioventricular canal or outflow tract (OFT), and at E14.5-P21, the free wall was dissected from the LV and RV to exclude any valve or septal tissue. Tissues were digested into single cell suspensions and isolated in a microfluidic device, after which single-cell RNA-seq analyses were performed. To assess technical variation and the dynamic range of transcript data we compared and observed good correlation of data derived from pooled single cell RNA-seq and whole LV RNA-seq. (**Fig S1**). Consistent with studies of single cells from other tissues, we found high single-transcript sensitivity and detected transcripts in a broad dynamic range ( $\sim 10^5$ ) between the most and least abundant transcripts.

### Unbiased transcriptome-wide identification of cell populations

We studied over 1200 single cells isolated at different stages and chambers of murine heart development (**Fig 1A-B**, **Table S1**). To demonstrate that single cell RNA-seq could distinguish distinct cell lineages in the heart, we employed Principal Component Analysis (PCA) of all single cells from LV, RV, and LA tissues captured at E14.5 (261 cells) when the four-chambered heart is formed. Informative PCA components (**Table S2**) clustered cells into three groups (**Fig 1C**). Examining transcripts that were enriched in each of the three groups revealed that this unbiased clustering method faithfully identified previous markers known to be expressed in CMs (red), ECs (purple), or fibroblast-enriched cells (black, e.g., non-CM, non-ECs). For example, among the top 30 genes in PCA component 1 that explain the largest proportion of the variation (**Table S2**; see Methods), CMs contained 11 sarcomere proteins genes (e.g. *Myh6*, *Myh7*, *Ttn*), ECs contained 14 genes encoding cell surface markers (e.g. *Pecam1*, *Cd93*), and fibroblast-enriched cells contained nine genes encoding extracellular matrix components (e.g. *Col1a2*, *Col1a1*).

To independently confirm cell classifications, unsupervised clustering was undertaken using the Single-Cell Consensus Clustering (SC3) tool (Kiselev VY, 2016). SC3 uses a consensus clustering approach that integrates several methods for clustering of single-cell RNA-seq data to generate user-defined numbers of clusters (k). Assessments of variable numbers of clusters demonstrated that k=3 and k=6 maximized the silhouette width metric, which quantifies the ratio of each cell's transcriptional similarities with other co-clustered cells to the transcriptional differences with cells outside of its cluster (Rousseeuw, 1987). This independent approach yielded cell clusters that closely mirrored PCA method (**Fig 1B**) with enriched expression of genes associated with CMs, endothelial cells, or fibroblast-enriched cells (**Fig 1D**).

We undertook “perfect marker” gene analysis of PCA-separated cells (Treutlein et al., 2014) to identify markers for CMs, ECs, and fibroblast-enriched cells (**Table S3**). This approach identifies genes with high expression in specified cells and low expression in all other cells.

“Perfect marker” gene analyses of PCA-separated CMs identified eight sarcomere protein genes (**Table S3**, bolded) and *Sh3bgr*, which encodes a SH3 domain binding glutamate-rich protein encoded within the trisomy chromosome 21 region that contributes to congenital heart defects. Morpholino-mediated knockdown of *Sh3bgr* in *Xenopus*, results in disrupted segmentation of somites and heart malformations (Jang et al., 2015). Eight “perfect marker” genes for ECs encoded prototypic molecules (*Pecam1*, *Cdh5*) and two additional genes: *Klhl4* and *Gpr116* (**Table S3**, italicized). *Klhl4* encodes an actin-binding kelch domain protein with unknown functions that has been implicated in developmental anomalies (Braybrook et al., 2001; Cheroki et al., 2008). Notably, an automated *in situ* hybridization screen (Gene Expression Database (Diez-Roux et al., 2011; Smith et al., 2014) of the E14.5 heart also identified *Klhl4* in the endocardium. *Gpr116* encodes a highly conserved G-protein coupled adhesion receptor that is widely expressed in vascular beds, that when depleted from ECs produced vascular leakage (Niaudet et al., 2015; Yang et al., 2013). Six of ten “perfect” marker genes identified in fibroblast-enriched cells encoded ECM proteins (**Table S3**, underlined).

We then evaluated the distribution and maturation of CMs, ECs, and fibroblast-enriched cells throughout development. At E9.5, no fibroblast-enriched cells were identified in atria, ventricle or outflow tract, but subsequently, the proportion of CMs in these region decreased, while fibroblast-enriched cells increased (**Fig 1B**). At P0 approximately, 30% of all LV cells and 45% of RV cells were fibroblast-enriched cells, a finding that is consistent with prior reports that cardiac fibroblasts appear by E12.5 and increase in number through P1 (Ieda et al., 2009). The proportion of ECs remained relatively constant from E9.5 (10%) to P0 (15%). At P21, the larger chip size used to capture CMs excluded many smaller non-CMs (**Table S1**), including hematopoietic-derived cells that comprise 5-10% of non-CM cells in the adult mouse heart (Pinto et al., 2016). These cells were also not captured in embryonic hearts, likely due to their low abundance.

Among developing ventricular CMs, RNAseq profiles were indicative of chamber myocardium and included two distinct sub-populations. One sub-population, (identified through consensus clustering using SC3 k=6), expressed transcripts associated with cell division (**Fig S2A**). Using hierarchical clustering of the expression of cell cycle genes (e.g., *Prc1*, *Ccna2*, *Cdca8*, *Cdca3*, *Top2a*, *Ccnb2*, *Mki67*, *Ccnb1*) we identified the proportion of CMs with proliferative capacity during development (**Fig S2B**). Approximately 60% of E9.5 and E14.5 CMs, but only 20% of P0 and P7 CMs, expressed these cell cycle transcripts (**Fig S2B**). At P21, this subpopulation was absent.

A second CM subpopulation was identified by hierarchical clustering (**Fig 2A**) of marker genes for CMs and fibroblast-enriched cells (identified in **Fig 1C** and listed in **Table S2**). This CM subpopulation expressed transcripts encoding extracellular matrix (ECM) proteins (denoted CME+) that are typically associated with cardiac fibroblasts (e.g. *Colla1*, *Col3a1*, *Dcn*). RaceID, which identifies rare cell populations in complex data, (Grun et al., 2015) also defined cluster of cells that expressed both sarcomere components and ECM (**Fig S2C**: cluster 7). To determine if these cells were more similar to either CMs or fibroblast-enriched cells, gene expressions for all CMs, fibroblast-enriched cells, or CME+ were averaged at E14.5, E18.5, or P0 (**Fig S1C**). Comparisons of the mean gene expression values and

Pearson correlations indicated that the average transcriptome of CME+ cells were more similar to CMs (E14.5,  $r=0.77$ ; E18.5,  $r=0.96$ ; P0,  $r=0.88$ ) than fibroblast-enriched cells (E14.5,  $r=0.61$ ; E18.5,  $r=0.66$ ; P0,  $r=0.43$ ). To independently confirm cell identities, preparations of single cell suspensions from P0 and P3 LV tissues were studied by immunohistochemistry (**Fig 2B**, **Fig S2D**) to detect the sarcomere protein troponin I (Tnni3), and collagen (Col3a1). Cell diameters (long axis) were compared ( $n=145$ ) from CM and non-CMs expressing Tnni3 and/or Col3a1. In the P3 LV, 47% of CMs expressed Col3a1, similar to the proportion observed in RNA-seq data (36%). CMs and CME+ cells had a similar mean length ( $17.8\pm 3.7\ \mu\text{M}$  vs  $17.5\pm 3.3\ \mu\text{M}$ ) compared to all non-CMs ( $10.0\pm 3.2\ \mu\text{M}$ ) and non-CM that expressed Col3a1 ( $8.2\pm 2.3\ \mu\text{M}$ ) (**Fig S2E**). Based on the comparable cell sizes, organized sarcomeres, and expression of Col3a1 and other ECM proteins, we deduced that CME+ cells are a subpopulation of CMs, and not fibroblasts that express sarcomere components, such as myofibroblasts (Mayer and Leinwand, 1997). Throughout LV development, CEM+ cells comprised variable proportions of all CMs (**Fig 2C**): 9% at E14.5, 22% at E18.5, 29% at P0, 36% at P3, and 1% at P21.

### Developmental transcription profiles of non-CM cells

Despite increased numbers of fibroblast-enriched cells throughout cardiac development, we observed minimal changes in the transcriptional profiles of non-CMs from E9.5 to P21 (**Fig S3A**) possibly due to limited numbers of captured cells (**Fig 1B**). By contrast, the transcriptional profiles of ventricular ECs (**Fig S3B**) showed a distinct temporal progression with three stages of evolution. During heart looping and chamber formation (E9.5, E11.5), unsupervised clustering using SC3 followed by gene-enrichment analysis (**Fig S3C**) identified high expression levels of *Hapln1* (hyaluronan and proteoglycan link protein 1), and *Hmga1* (high mobility group AT-hook protein). *Hapln1* is an important regulator of developmental extracellular matrix interactions (DeLaughter et al., 2013) while *Hmga1* regulates chromatin organization and promote endothelial-mesenchymal transformation (Benecke and Eilebrecht, 2015). Expression of these genes during chamber formation is consistent with matrix remodeling that participates in early ventricular trabeculation. Later in development (E14.5, E18.5), ECs showed increased expression of *Fabp4* (fatty acid binding protein-4), a molecule that contributes to cardiac metabolic shifts from glycolysis to oxidative phosphorylation (Vander Heiden et al., 2010). From P0 through to P21 the EC transcriptional program remained unchanged.

### Developmental transcriptional profiles of CMs

E9.5 CMs isolated from the common atria or the primordial ventricle had differential expression of 563 genes (**Table S4**) and PCA analyses identified gene subsets that reliably discriminated atria from ventricular CMs (**Fig S4A**, **Table S2**). For example, E9.5 ventricular CMs expressed high levels of *Hey2* and *Pln* while E9.5 atrial CMs expressed high levels of *COUP-TFII* (**Fig S4B**). As these profiles remained distinct over time, we characterized the individual temporal transcriptional programs governing the maturation of atrial or ventricular CMs separately.

## Atrial CMs

Maturation of atrial CMs resulted in both decreased and increased expression of genes from E9.5 to E14.5 and from E14.5 to P0 (**Table S4**). PCA suggested progressive transcriptional states (**Fig 3A, Table S2**) that we characterized by hierarchical clustering of the genes associated with each state. Three distinct gene expression patterns were identified (**Fig 3B, C**). Pattern 1 consisted of genes with binary expression (i.e., on or off) in an increasingly larger proportion of cells. This pattern included genes that regulate many aspects of adult atrial CM physiology, including cell-cell adhesion (*Gja1*) (Desplantez et al., 2012; Luo et al., 2007), calcium buffering (*Csq2*, encoding *calsequestrin*), and electrophysiologic conductance (*Kcnk3*; (Liang et al., 2014). Pattern 2 identified genes that were expressed early and globally in atrial development but were silent at E14.5 and thereafter. These genes included *Wnt2*, a regulator of early atrial development (Tian et al., 2010), genetic markers of immature CMs (*Acta2*), and a regulator of chromatin remodeling that may promote early CM differentiation (*Hmga2*; (Monzen et al., 2008). Pattern 3 genes included globally expressed genes with increased expression as development progressed. This group includes genes with fundamental CM function including sarcomere proteins (*Tnni3*).

## Right versus Left Ventricular CMs

Before considering the developmental programs in maturing ventricular CMs, we used PCA to evaluate the transcriptomes of E11.5 CMs within the developing right and left ventricular chambers (**Fig S4A, Table S2**). We identified 279 genes significantly differentially expressed ( $p < 1 \times 10^{-7}$ ) between LV and RV CMs using Mann-Whitney-Wilcoxon rank sum test (**Table S5**). Included in this subset are genes previously described as regulators of ventricular chamber formation (**Fig S4C**), including *Hand1* and *Tbx5*, which had restricted expression in LV CMs (Liberatore et al., 2000; Thomas et al., 1998). By contrast, *Tbx5*-repressed genes had higher expression in RV CMs, findings that support prior studies indicating that *Tbx5* is critical for the early establishment of chamber identity (Bruneau et al., 2001). Genes enriched in RV CMs also included proteins associated with the ECM, microfibrillar-associated protein 2 (*Mfap2*) which binds active Tgf $\beta$ -1 Tgf $\beta$ -2 and Bmp2 (Combs et al., 2013), and a cell adhesion, melanoma cell adhesion molecule (*Mcam*) that marks neural crest-derived lineages (Medic and Ziman, 2010; Pujades et al., 2002).

Gene ontology and KEGG pathway analyses (**Fig S4D**) of 138 genes that were significantly enriched in RV CMs identified extracellular matrix organization ( $n=11$ ,  $p=2 \times 10^{-7}$ ), cell adhesion ( $n=19$ ,  $p=1.2 \times 10^{-6}$ ), and glycolysis ( $n=7$ ,  $p=1.9 \times 10^{-6}$ ) or enrichment in pathways involved in dilated cardiomyopathy ( $n=7$ ,  $p=7 \times 10^{-5}$ ) and arrhythmogenic right ventricular cardiomyopathy ( $n=7$ ,  $p=1.8 \times 10^{-4}$ ). Although parallel analyses of 141 genes enriched in the LV CMs (**Table S4**) were less informative of specific pathways, we observed several genes that participate in processes implicated in congenital heart disease (e.g., retinoic acid signaling (Nash et al., 2015); histone modification in response to Nodal signaling (Dahle et al., 2010; Homsy et al., 2015). Notably, these right/left differences were not observed when comparing ECs or fibroblast-enriched cells. Thus, we suggest that developmental pathways that regulate left and right chamber ventricular formation are expressed primarily in CMs rather than in non-myocytes cells.



Between E14.5 and P0, LV and RV CMs became transcriptionally more similar to one another (**Fig S4 E-G, Table S2**) and expressed genes that characterize CM maturation (e.g. *Myh6, Myl3*). Taken together we propose that ventricular CMs exhibit distinct transcriptional programs underlying LV and RV chamber morphogenesis after which transcriptional convergence promotes CM maturation.

### Developmental Transcriptional Programs in Ventricular CMs

Similar to atrial myocytes, we observed a temporal evolution of ventricular CM gene expression (**Fig 4A**). PCA analyses of CMs isolated from four time points (E9.5, E14.5, P0, P21) were undertaken using 997 genes expressed in at least 75% of CMs (**Table S6**). PCA component A2 (**Fig 4A, Table S2**) separated cells in overlapping, stepwise clusters ordered by age that were visualized using a density plot (**Fig 4B**). Among the genes that demarcated the transition from least to most mature ventricular CMs, 11 genes are involved in mitochondrial fatty acid beta-oxidation (e.g. *Fabp3, Fabp4, Uqcrl1, Cox6c*) and accounts for the developmental metabolic switch from embryonic glycolysis to mitochondrial fatty acid beta-oxidation of adult CMs (Vander Heiden et al., 2010). Indeed, ventricular CMs expressed reciprocally increased and decreased transcript levels for *Fabp3* and *Fabp4, Myh7* and *Myh6*, and prenatal *Cox8a* but post-natal *Cox8b* provided evidence of progressive maturation (**Fig 4C**). To examine whether CMs remained this distinct between all stages of cardiogenesis, the PCA was expanded to include 3 intermediate time points (E11.5, E18.5, P3). From E9.5 to P21 (**Fig 4B**) ventricular CMs transcriptomes exhibited a continuum of maturation, but also demarcation from the prior and subsequent stage. This resulted in a progressive wave of ventricular maturation, a developmental pattern that correlated with progressive cell cycle exit (**Fig S2A**).

Within the developmental waves, density plots revealed three stages of CMs maturation between E9.5 and P21 (**Fig 4B**). CMs on each extreme of this developmental axis are derived primarily from E9.5/E11.5 cells and from P21 cells. Between these extremes we observed a heterogeneous population of CMs comprised of cells isolated from E14.5-P3. This same clustering was also observed when unsupervised clustering using the SC3 tool was performed on ventricular myocytes (**Fig S5A**). To examine how gene expression changes between these stages of CM maturation, hierarchical clustering was performed on the 50 genes that explained most of the component A2 variance in **Fig 4A**. Cells ordered by their expression of component A2 (**Fig 4D**) also yielded three clusters with distinct patterns of gene expression. Parallel analyses of 400 genes confirmed these patterns (**Fig S5B and Table S7**). Cluster 1 consisted of genes whose expression began on or before E9.5 and increased steadily until P21, such as *Myl2, Myh6, Fabp3, and Cox6c* (**Table S7**). The second cluster was defined by genes whose expression decreased as development proceeded, either at E14.5 (*Cdk1b, Pgam1* encoding phosphoglycerate mutase 1) or at P21 (*Myl7 and Hmgn2* (encoding high mobility group nucleosomal binding domain 2)). Cluster 3 genes were lowly expressed early (E9.5-E11.5) but had increasing expression at E14.5 (*Oxct1* encoding succinyl-CoA:3-ketoacid-coenzyme A transferase 1, *Ryr2* encoding ryanodine receptor 2) or at p21 (*S100a2* encoding S100 calcium binding protein A2). Thus, the genes observed in these three clusters implicate different biological processes critical in heart maturation;



sarcomere structure and fatty acid oxidation in cluster 1; glycolysis and proliferation in cluster 2; mitochondrial and calcium handling in cluster 3.

The heterogeneity in maturity of individual CMs, isolated at the same time point, raised the question of whether whole tissue samples harvested at specified distinct embryonic times could be distinguished based on gene expression. To assess this, we processed whole LV free wall at E14.5 and E18.5 for RNA-seq similar to single cell methods. Analyses were restricted to 997 genes expressed at  $\geq 1$  FPKM (fragments per kilobase of transcript per million mapped reads) in 75% of single CMs harvested at E9.5, E11.5, E14.5, E18.5, P0, P3 and P21 (**Table S6**). A density plot was generated from the most informative temporal component (A2, **Fig 5A**). LV free wall tissue from E14.5 and E18.5 mice mapped to the middle of the distribution of single cells, confirming that the maturation profiles from many single cells accurately captured *in vivo* developmental transcriptional profiles and also confirmed that whole tissue RNA-seq data averages the expression of CMs with different maturation stages.

## Developmental Ages of Stem Cell-derived CMs

Applying the method described above (**Fig 4B**), we used published RNA-seq datasets that were processed through our pipeline to determine the developmental ages of mouse and human ES cells. Two replicate RNA-seq datasets from cardiac progenitors (mES-CP) and CMs (mES-CM) derived from murine ES-cells (Wamstad et al., 2012) showed high concordance. Expression data from mES-CP corresponded to E9.5 single-cell CMs, while data from mES-CM cells corresponded to E14.5 cells (**Fig 5A**).

Similar analyses of human ES cell-derived (hES) CMs were performed using 847 genes with human orthologs (Bick et al., 2012). RNA-seq datasets from hES-derived CMs cultured for 20 days (hES-D20) or approximately one (13.5 months) year (hES-Y1) (Kuppusamy et al., 2015) showed high concordance. The hES-D20 cells corresponded to E14.5 ventricular CMs while hES-Y1 cells corresponded to E18.5 ventricular CMs. To confirm that these conclusions did not reflect differences in mouse and human development, we evaluated RNA-seq data from fetal and neonatal human tissues, including 2 LV tissues from 8 and 12 weeks of gestation and 5 tissue samples from post-natal infant hearts. Human fetal heart samples corresponded to late E18.5, while neonatal samples corresponded to P0 to P3 (**Fig 5B**). Together these findings support the conclusion by Kuppusamy and colleagues that prolonged culturing increases CMs maturation, but also indicated that hES-Y1 CMs remain more immature than P21 cells (**Fig 5B**).

## Nkx2.5 Haploinsufficiency Impairs Maturation of Distinct Cardiac Cell Lineages

Dominant mutations in *NKX2.5* cause human cardiac septation defects (Elliott et al., 2003; Schott et al., 1998; Yuan et al., 2015). *Nkx2.5*<sup>+/-</sup> mice recapitulate many of these cardiac phenotypes albeit with incomplete penetrance (Ashraf et al., 2014; Biben et al., 2000; Bruneau et al., 2001). We hypothesized that haploinsufficiency of transcription factors *Nkx2.5* cause heart malformations by altering developmental transcription in specific

cardiac lineages. To test this model we isolated cells from the LV at E14.5, P0, P7, and P21 in *Nkx2.5<sup>+/-</sup>* mice and compared PCA of single-cell RNA-seq data to that from WT mice (**Table S4**).

*Nkx2.5<sup>+/-</sup>* CMs expressed lower levels of *Myh7* (-5.0 fold,  $p=2.3 \times 10^{-11}$  compared to WT) implying a potential defect in CM maturation. We therefore compared the maturity of *Nkx2.5<sup>+/-</sup>* cardiac cell lineages using single cell transcriptomes from mutant and WT CMs isolated at E14.5, P0, P7 and P21 (**Fig 6A**). To better visualize maturation differences we compared density plots of cell distributions in mutant (solid waves) and WT (dashed waves) mice derived from PCA components A2 (**Fig 6B**). *Nkx2.5<sup>+/-</sup>* and WT CMs isolated at p0 or p21 had similar developmental distributions but E14.5 *Nkx2.5<sup>+/-</sup>* CMs were skewed towards more immature CM populations. Comparing the mean FPKM (Student's T-test), 420 genes were differentially expressed between *Nkx2.5<sup>+/-</sup>* and WT CMs of which several were associated with myocyte maturation including *Ttn* and *Myh6* (**Table S4**).

To more precisely quantitate the maturity defect in *Nkx2.5<sup>+/-</sup>* CMs, we defined the top 20 genes contained in PCA component A2 that distinguished E14.5 mutant from WT CMs (**Fig 6A**; **Table S2**) and used these genes for hierarchical clustering of CMs. Two populations of WT CMs were identified: cells that were more mature (52%, 10/19) or less mature (48%, 9/19) based on gene expression (*Myl2*, *Myl3*, *Fabp3*; **Fig 6B**). At P7 (60%; 12/20) and P21 (97%; 62/64) increasing numbers of WT CMs were more mature.

In contrast, P0 *Nkx2.5<sup>+/-</sup>* CMs clustered predominantly as less mature (87%; 13/15) rather than more (2/15) mature cells (compared to WT,  $p=0.012$ ; Fisher exact test). P7 gene expression profiles of *Nkx2.5<sup>+/-</sup>* CMs were more comparable to P0 WT CMs and only 20% of CMs exhibited mature gene expression patterns, in comparison to 60% of WT CMs ( $p=0.01$ ) (**Fig 6B**, **Fig S5C**). At P21 most (78%, 21/27) of *Nkx2.5<sup>+/-</sup>* CMs were mature, albeit a significantly less proportion than in WT LV (97% 62/64,  $p=0.0071$ , Fisher exact) (**Fig 6B**).

Prior studies demonstrate *Nkx2.5* expression principally in CMs (Kasahara et al., 1998), which we confirmed by single cells analyses (**Fig S5D**). As recent evidence indicates developmental cross-talk between CMs and ECs lineages (Palencia-Desai et al., 2015), we also assessed the maturation of ECs from *Nkx2.5<sup>+/-</sup>* mice (**Fig S3D**). Transcriptional profiles of *Nkx2.5<sup>+/-</sup>* ECs were comparable to WT ECs at E14.5, but significantly delayed at P0 (*Nkx2.5<sup>+/-</sup>* 12/14 immature ECs versus WT 4/19;  $p=0.038$ , Fisher's exact test).

Together these data indicate that that *Nkx2.5<sup>+/-</sup>* haploinsufficiency delays maturation in both CMs and ECs and provides further support that the myocardium is crucial for endocardial differentiation.

## Discussion

Using single cell RNA-seq we performed a high-resolution time course analysis of cardiac development. Our efforts leveraged unbiased, single-cell analyses to separate and characterize over 1200 cardiac cells from three distinct lineages. In addition to characterizing longitudinal developmental programs we identified distinct transcriptional

profiles for CM subpopulations and chamber-specific profiles. Leveraging these analyses, we established developmental ages of CMs derived from stem cells, and characterized lineage-specific consequences of haploinsufficiency of the transcription factor *Nkx2-5*.

We identified considerable transcriptional heterogeneity among CMs. Among E14.5 ventricular CMs, a subpopulation (CME+) expressed extracellular matrix proteins that are emblematic of fibroblasts, even though their overall transcriptome typified CMs. The proportion of this CM subpopulation increased until P3, after which CME+ cells were rarely identified. We speculate that CME+ cells provide a developmental scaffold for the formation and maintenance of heart architecture and may be disease-responsive, therein accounting for increased cardiomyocyte expression of extracellular matrix proteins (Ambrosino et al., 2006) due to increased proportions of CME+ cells rather than global transcriptional changes in all CMs.

Another CM subpopulation was distinguished by the expression of cell cycle genes, which we presumed implies active proliferation. The transcriptional profiles of these cells were not suggestive of stem cells nor recently described cardiomyoblasts (Jain et al., 2015); earlier developmental time points are likely needed to capture these cells. Instead, cell cycle gene expression was found in 60% of E9.5 CMs. With developmental progression, these transcripts rapidly declined. At P21 no CMs expressed cell cycle genes.

Notably, the transcriptomes of proliferating and non-proliferating CMs were remarkably similar, indicating that these cells had comparable developmental maturity. Consistent with this, genes that demarcated distinct waves of development by CMs did not include cell cycle genes. We suggest that throughout embryogenesis newly born CMs ‘kept pace’ with differentiating cells, perhaps due to a rich milieu of intrinsic and secreted factors that promoted maturation. Identification of these molecules could be important to accelerate *in vitro* maturation of stem-cell derived CMs.

Ventricular CMs exhibited overlapping, stepwise developmental patterns that we grouped into three transcriptional stages of maturation. At E9.5-E11.5 CM expressed genes involved in proliferation (e.g., cell cycle genes, **Fig. S2A**) and morphogenesis (e.g., *Sall4*, a *Tbx5*-regulated transcription factor that promotes ventricular morphogenesis and chamber maturation (Koshihara-Takeuchi et al., 2006). CMs subsequently matured through sequential overlapping waves of gene expression (**Fig 4B**) between E14.5-P3. These transitional CM expressed genes involved in cellular growth (e.g., *Tcea1*, encoding transcription elongation factor), CM differentiation (*Cbx3* an epigenetic silencer that promotes pluripotent cells differentiation; *Casq2*, calsequestrin 2; *Ckm*, creatinine kinase) and the metabolic switch from glycolysis (reduced *Pgam1* expression) to fatty acid oxidation (increased *Fabp3*, *Fabp4*, encoding fatty acid binding protein; **Fig 4C**) that characterizes mature CMs (Vander Heiden et al., 2010). Further maturation occurred by P21 when there was decreased expression of calmodulin-interacting proteins, *Bex1* and *Bex4*, that promote muscle regeneration (Koo et al., 2007).

Using these maturation stages we characterized stem-cell derived CMs, which are advancing understanding of human heart disease and propelling the development of new therapeutics,

despite the limitation that these cells fail to recapitulate adult CM gene expression even after a year in culture (Kuppusamy et al., 2015). While a few markers have been previously used to gauge the maturation of stem-cell derived CMs (Li et al., 2015), our spatio-temporal CM transcriptional profile of single cells and *in vivo* mouse and human tissues precisely defined the developmental “age” of stem cells and derived CMs (Fig 5). We observed remarkably homogeneity and consistent immaturity (E18.5) in both mouse and human CMs derived from stem cells. Knowledge of the repertoire of genes expressed through development should advance chemical or genomic strategies to improve both the maturation and subpopulation diversity of stem-cell derived CMs and cardiac lineages.

Single cell-derived transcriptome maps also uncovered lineage-specific perturbations due to haploinsufficiency of the cardiac transcription factor *Nkx2.5*<sup>+/-</sup>, a cause of human congenital heart disease. Our data extends earlier transcriptional analyses of *Nkx2.5*<sup>+/-</sup> mouse heart tissues (Bouveret et al., 2015) by delineating lineage-specific and temporal changes that persisted late into development. In comparison to WT cells, we demonstrate that developmental immaturity of E14.5 *Nkx2.5*<sup>+/-</sup> LV CMs and P0 ECs (Fig 6, Fig S3D). As *Nkx2.5* is not expressed outside of CMs, we suggest that the dysregulated EC gene expression in *Nkx2.5*<sup>+/-</sup> hearts indicates non-autonomous signals that are evoked by haploinsufficiency of this transcription factor.

In conclusion, we demonstrate that single cell RNA expression analyses improved delineation of lineage-specific and spatio-temporal developmental transcriptional programs. Expansion of these analyses to include earlier developmental time points and additional lineages is expected to expand insights into molecular pathways that are critical for heart development.

## Methods

### Mice and Dissection

Pregnant female 129SV mice (Taconic) were used for tissue dissection. The construction of *Nkx2.5*-del (Tanaka et al., 1999) mice was previously described. *Nkx2.5* heterozygous males from a mixed background were crossed with 129SV females. Embryonic age was confirmed by ascertaining the Thielier stage of each pup. Tissue derived from one pup was used for each individual chip. Hearts were excised into PBS and incubated with agitation at 37° for 20-60 minutes, depending on stage, in digestion buffer consisting of PBS, 0.70% weight/volume % collagenase B, and 0.75% weight/volume % collagenase D (Roche).

### Cell Capture

Fluidigm integrated fluidic circuits (IFC) were used to capture single cells. After capture, the IFC lysed individual cells, reverse transcribed RNAs and amplified complementary DNAs. The resulting cDNAs from individual cells were barcoded, indexed and sequenced to an average depth of 1.5 million reads per library. IFCs were selected to capture all major cell populations from all cell size ranges observed using IFCs which capture cells of different sizes: 5-10uM (embryonic), 10-17uM (embryonic, neonatal), 17-25uM (3 weeks of age). No batch effects were observed between chips of the same size. Captured cells were imaged on

chip to confirm the number of cells per site and the viability of the cell was confirmed using a LIVE/DEAD cell assay (Life Technologies). Only single, viable cells were used for further analysis. Onboard cell lyses, *in vitro* transcription and cDNA synthesis were performed using IFC RNA-seq reagent kit (Fluidigm) and SMARTer Ultra Low RNA kit (Clontech) reagents following the manufacturer's protocol.

### Library Construction and DNA Sequencing

Libraries were constructed from cDNA yielded from IFCs in 96 well plates using Nextera XT DNA sample preparation kit (Illumina). cDNA concentrations before and after library prep was confirmed using High-Sensitivity DNA Tapes (Agilent) run on an Agilent Tape Station. For whole tissue experiments, RNA was extracted from whole tissue using phenol-chloroform. Reverse transcription, initial cDNA synthesis and cDNA library preparation were performed using the same reagents as for single cells. cDNA size distribution of single-cell, whole tissue and pooled libraries was performed using High-Sensitivity DNA Tapes (Agilent) on an Agilent Tape Station. Up to 96 single-cell libraries were pooled together for 50 bp, paired-end DNA sequencing on a single lane of an Illumina HiSeq2500 following the manufacturer's standard protocol. Single-cell libraries with >400,000 reads were used for further analysis. Reads were aligned to the mouse genome (mm10) using Tophat (Trapnell et al., 2009).

### Statistical Analysis of Single Cell Transcriptomes

PCA, Mann-Whitney-Wilcoxon rank sum test, hierarchical clustering, and Student's t-tests were performed using custom scripts in R. PCA was performed on  $\text{Log}_{10}$  FPKM. Principal components with eigenvalues greater than 1 were evaluated to cluster cells. Genes identified by these components were used to perform hierarchical clustering. Euclidean distances were calculated from  $\text{Log}_{10}$  FPKM and clustered by the complete linkage method. Consensus clustering was performed using Single-Cell Consensus Clustering (SC3) package (Kiselev VY, 2016). Only cells expressing >3000 genes were used for clustering. The k and distance metrics were chosen to maximize the silhouette width. Dendrograms were derived from consensus clustering and overlaid over heatmaps depicting marker genes expression also calculated with the SC3 tool. RaceID was performed as described (Grun et al., 2015). Student's t-test were 2 tailed and assuming unequal variance.

Gene ontology and KEGG pathway enrichment analysis were performed using DAVID software (Huang et al., 2007). "Perfect marker" gene analysis was performed as described (Treutlein et al., 2014). Briefly, a "perfect marker" gene was defined for each cell lineage such that its expression was set at  $\text{Log}_{10}(\text{FPKM})=10$  in all cells of the desired lineage (i.e. myocytes) and 0 in all other cells. A pairwise Pearson's correlation was calculated comparing the expression profile across all cells for a given gene to that of the perfect marker gene.

### Stem-cell derived CM analysis

We derived a gene expression axis correlating with developmental time from the set of 997 genes expressed in 75% of ventricular myocytes at E9.5, E11.5, E14.5, E18.5, P0, P3 and P21. For comparisons to human cells, we limited this list to the 847 genes with human

orthologs (Bick et al., 2012). Sequence from short-read files were downloaded from NCBI GEO databank (GEO ID GSE47948 and GSE62913 respectively) and rerun through our RNA-Seq pipeline (Li et al., 2015; Wamstad et al., 2012). Human whole tissue isolation, cDNA library construction and mRNA-seq analysis were performed as previously described in (McKean et al., 2016).

### Immunohistochemistry

Cells isolated from p0 or p3 LVs were plated onto laminin coated chamber slides and incubated at 37°C for 1 hour. Cells were fixed with 4% paraformaldehyde for 20 minutes and washed with phosphate buffered saline (PBS) subsequent to permeabilization with 0.1% (vol/vol) Triton-X100 in PBS. 10% (vol/vol) donkey serum was used to block nonspecific binding. Cells were incubated with two primary antibodies, rabbit anti-Tnni3 (Abcam-ab56357), or goat anti-Col3a1 (Abcam-ab7778) diluted 1:250 in blocking solution for 1 hour at room temperature. Cells were washed in PBS and fluorophore-conjugated secondary antibody (Molecular Probes) diluted 1:1000 in PBS for one hour. Slides were washed and then mounted and counterstained using VECTASHIELD antifade mounting medium with DAPI (Vector Laboratories).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

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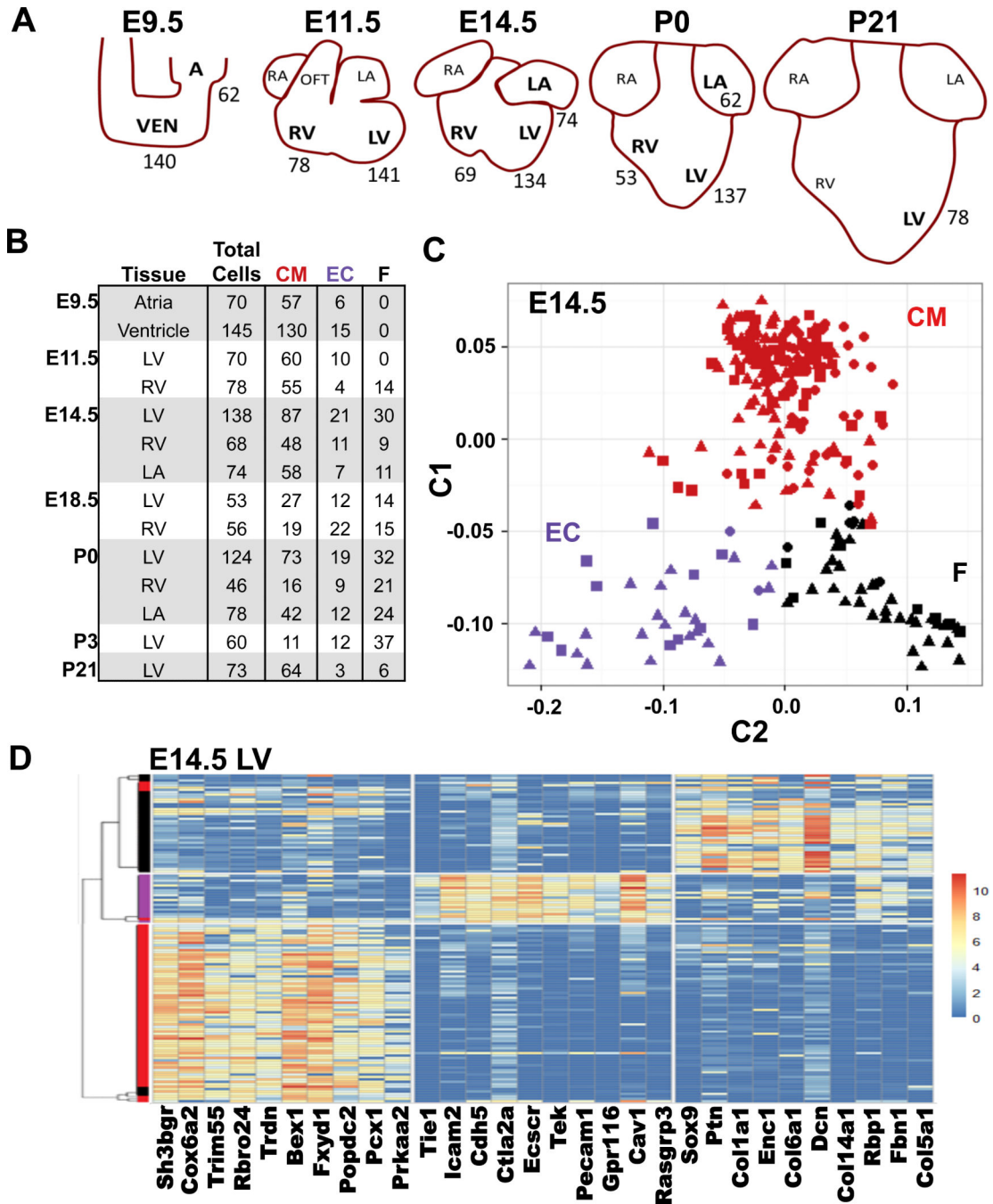


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**Highlights**

- Single-cell RNA-seq data characterizing >1200 murine cells from E9.5-P21 hearts
- Dynamic temporospatial gene expression defines distinct cardiomyocyte populations
- Human/mouse stem cell-derived cardiomyocytes are developmentally immature
- *Nkx2.5*<sup>+/-</sup> mice have lineage-specific maturation defects in cardiac cells



**Figure 1.** Single-cell RNA-seq of CMs, ECs, and fibroblast-enriched cells in the developing heart. **A)** Schematic representation of developing heart from which single cells were harvested (denoted by bold lettering). Numbers indicate numbers of captured wildtype cells. **B)** The number single cells captured per tissue and time point and their lineage as determined by PCA. CM, cardiomyocytes, EC, endothelial cells, F, fibroblast-enriched cells. **C)** PCA components C1 and C2 separates E14.5 cells into three subgroups, each that robustly expressed genes associated with CMs (red), ECs (E, purple), or Fs (black). The percent of

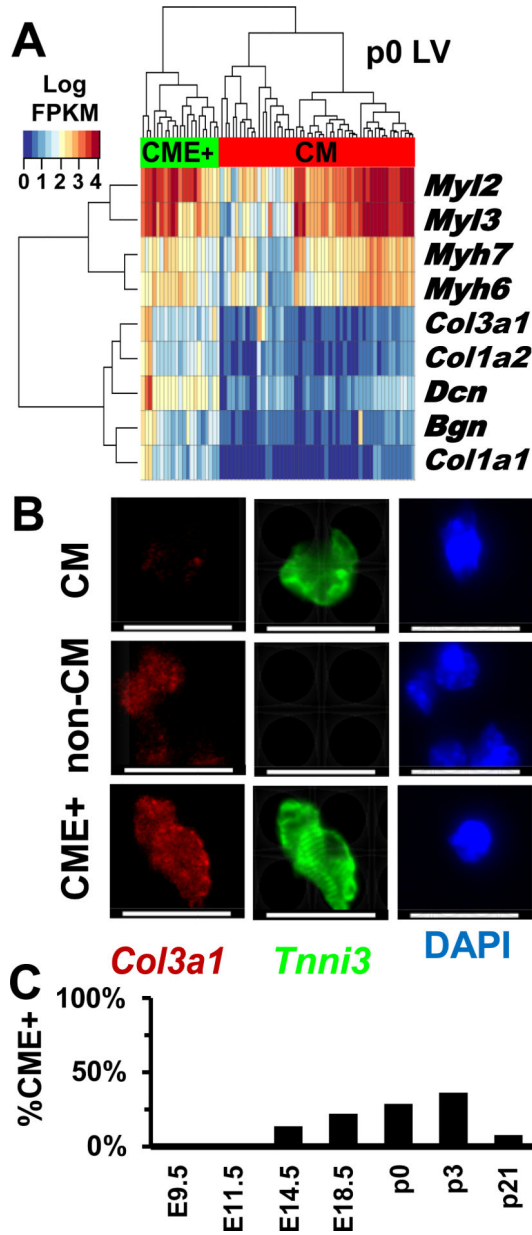
variance explained each principle component is provided in Table S2. **D)** Unsupervised clustering (SC3) of E14.5 cells separates CMs, ECs, and fibroblast-enriched cells. The dendrogram was generated using consensus clustering with  $k=3$ . The heatmap depicts the top ten most significant genes with enriched expression in each cluster. See also Fig S1, Fig S4, Table S1, Table S2, and Table S3.

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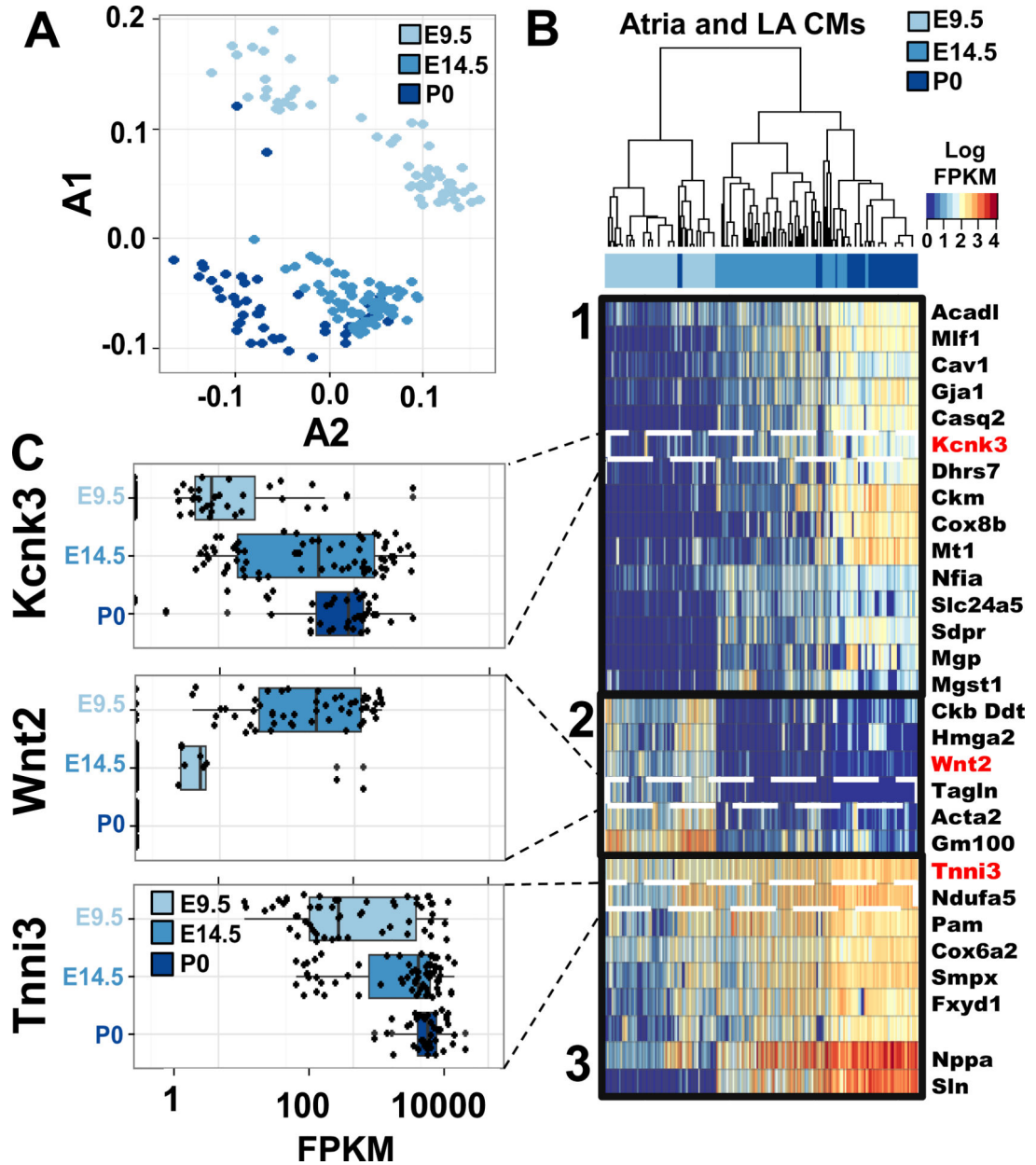
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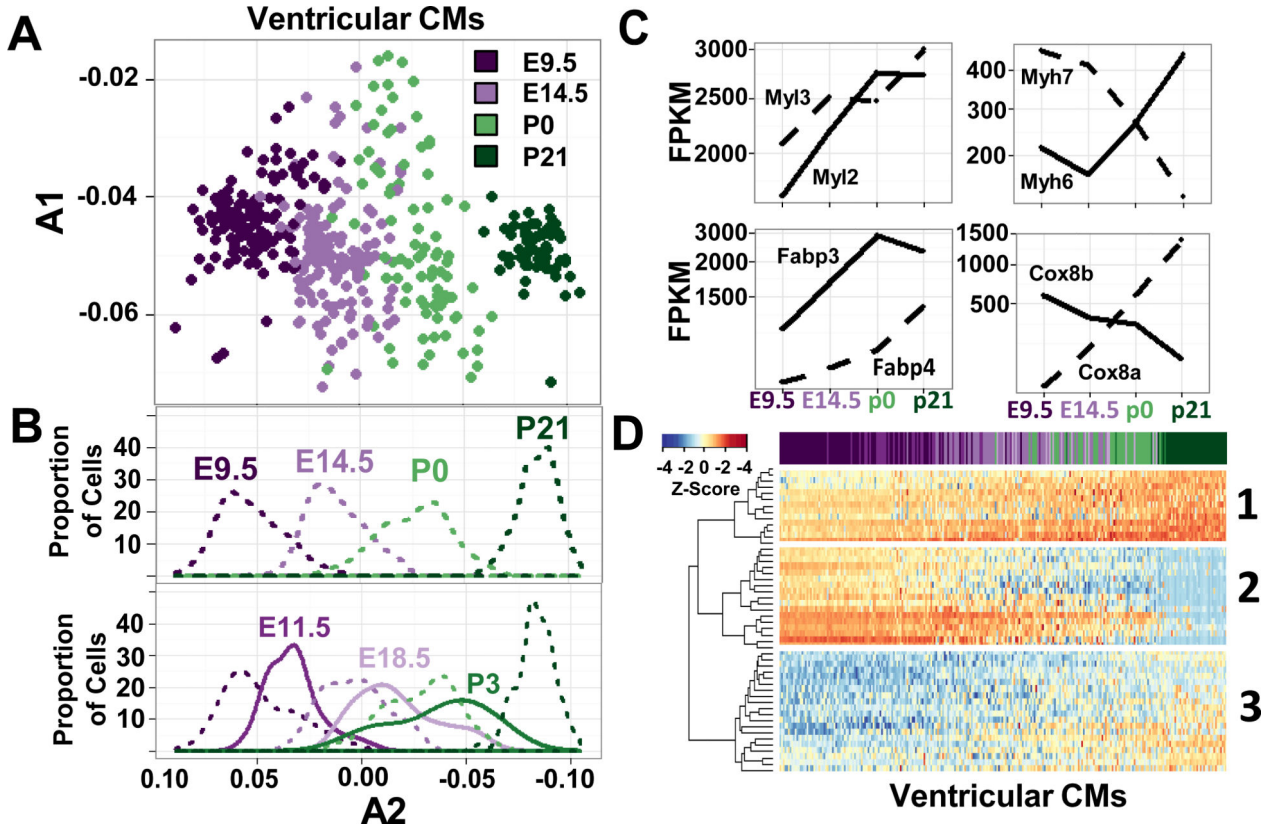
**Figure 2.** Subpopulations of CMs with distinct gene expression profiles. **A)** Hierarchical clustering distinguishes CMs (red) from CMs expressing extracellular matrix genes (CME+, green) isolated from the LV at p0. **B)** Micrographs of p3 LV cells stained with *Tnni3* (green), *Col3* (red), or DAPI (blue). Scale bar- 20µM. **C)** The proportion of LV CMs and CME+ cells varies during development. See also Fig S1 and Fig S2.



**Figure 3.**

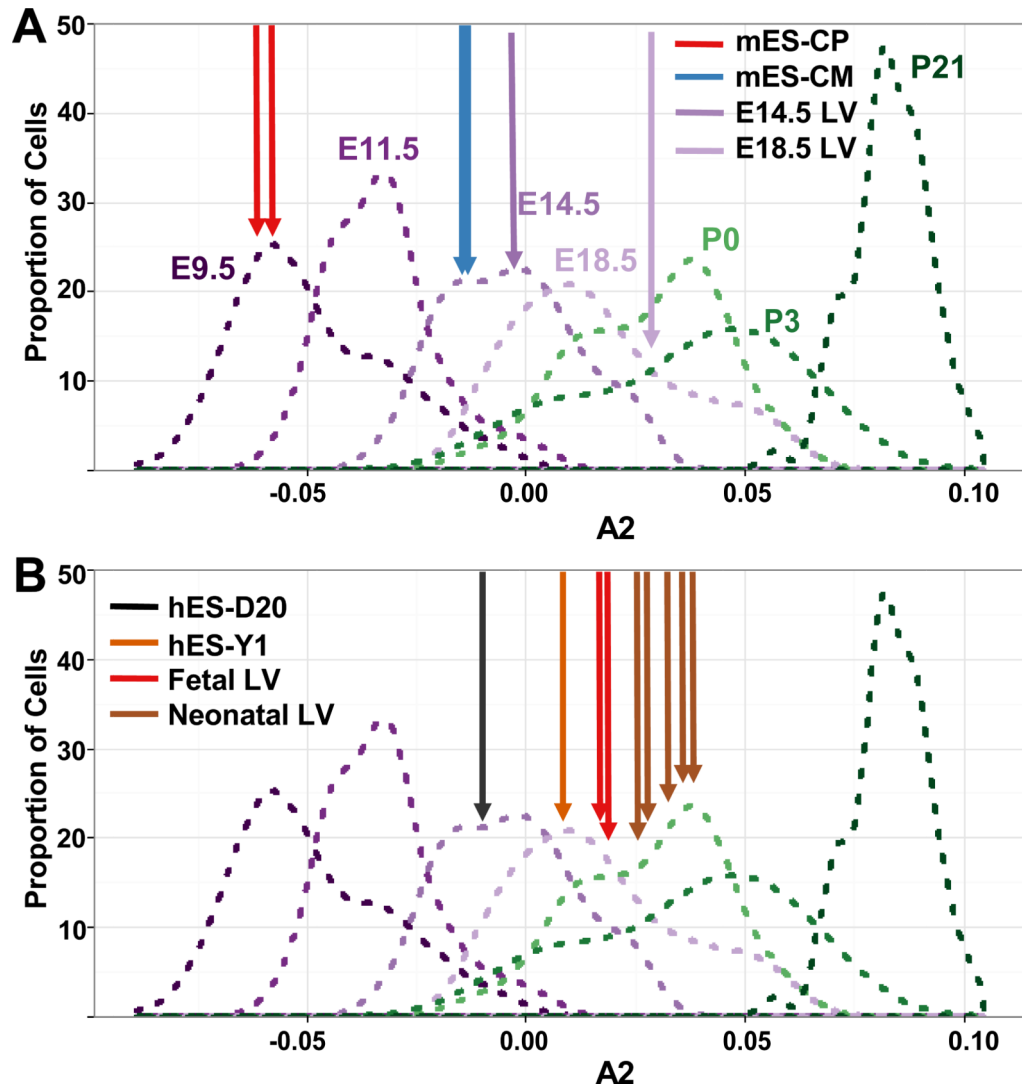
Distinct temporal and chamber gene expression patterns in CMs. **A)** PCA of gene expression in E9.5 common atria (light blue), E14.5 LA (blue), and P0 LA (dark blue) demonstrate that atrial CMs exhibited stepwise, temporal expression patterns. The percent of variance explained each principle component is provided in Table S2. **B)** Hierarchical clustering of E9.5 common atria (light blue), E14.5 LA (blue), and P0 LA (dark blue) CMs show three temporal gene expression programs. **C)** Representative genes depicting single cell gene expression (black dots) from each of the three temporal atrial CM gene expression programs. See also Fig S4, Table S2, and Table S5.





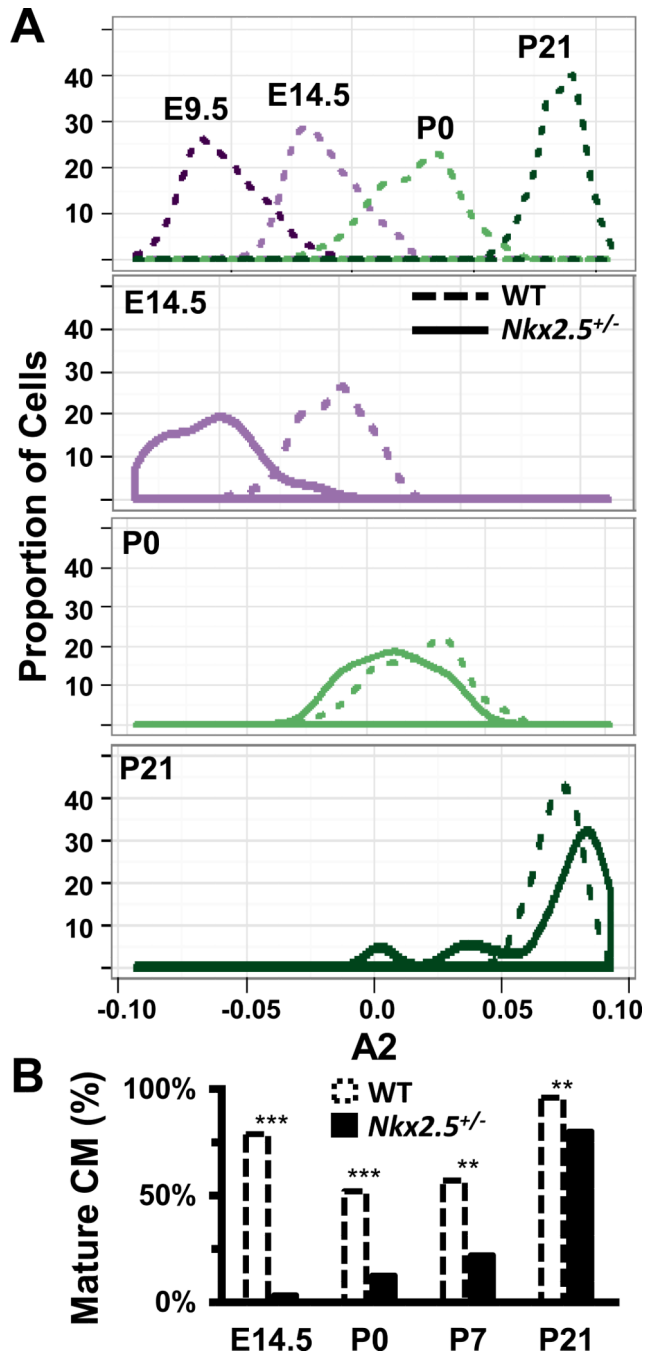
**Figure 4.**

Temporal gene expression profiles of ventricular CMs. **A**) PCA of ventricular CMs isolated at E9.5 (dark purple), E14.5 (light purple), P0 (light green), and P21 (dark green). The percent of variance explained each principle component is provided in Table S2. **B**) (Top panel) A density plot depicting the proportion of ventricular CMs isolated at four time points (E9.5, E14.5, P0, P21) on component A2. These time points were evenly distributed along A2. (Bottom panel) Density plot depicting the proportion of cells with a given value of component A2 from PCA of CMs isolated at seven time points (E9.5, E11.5, E14.5, E18.5, P0, P3, P21), show considerable overlap in temporal development from E14.5-P3. **C**) Box plots depict mean fragments per kilobase of transcript per million mapped reads (FPKM) of genes with notable isoform switches in CMs isolated at E9.5, E14.5, P0, or P21. **D**) Hierarchical clustering of the top 50 genes in component A2 from **B**. The genes clustered into three patterns (detailed in text). Columns represent individual single cells ordered by value of component A2 from **B** and colored by age at time of isolation. Z-score reflected normalization by column. See also Fig S4, Fig S6, Table S2, Table S6, and Table S7.



**Figure 5.**

Developmental ages of CMs derived from human and mouse stem cell and LV tissues (depicted using density plots as described in **Fig. 4B**). **A**) Murine whole tissue samples from E14.5 (purple arrow) and E18.5 LV (light purple arrow) have A2 values near the mean of the distribution of E14.5 and E18.5 LV single cells respectively. Murine embryonic stem cell-derived cardiac progenitors (mES-CP, red arrow) correspond to E9.5 CMs and murine embryonic stem cell-derived CM lines (mES-CM, blue arrow) correspond to E14.5 CMs. mES transcriptome data are from two independent clonal lines (Wamstad Cell 2012). **B**) Similar analyses of transcriptional analyses from pooled human embryonic stem cell-derived CMs (Kuppusamy, PNAS 2015) cultured for 20 days (hES-D20, black arrow) or one year (hES-Y1, orange arrow) indicate that these respectively correspond to mouse E14.5 and E18.5 CMs. The transcriptome of the free wall of human fetal LV (ages 8 and 12 weeks, red arrow, n=2) and human neonatal LV or RV (brown arrow; n=5) are shown for comparison. See also Table S6.



**Figure 6.** Maturation defects in *Nkx2.5*<sup>+/-</sup> CMs. **A)** Developmental profile of E9.5 VEN and E14.5, P0, and P21 LV CMs from WT (dashed line) and *Nkx2.5*<sup>+/-</sup> mice (solid line) depicted using density plots as described in **Fig. 4B**. At E14.5 (2<sup>nd</sup> panel), *Nkx2.5*<sup>+/-</sup> CMs are less mature than E14.5 WT cells. This immaturity decreases gradually from P0 (3<sup>rd</sup> panel) to P21 (4<sup>th</sup> panel). **B)** Using hierarchical clustering, the proportion *Nkx2.5*<sup>+/-</sup> CMs (CM) that cluster with similarly aged WT cells is significantly different at all time points (Fisher's exact p-

values:  $E_{14.5} = 2.1 \times 10^{-25}$ ,  $P_0 = 0.0012$ ,  $P_7 = 0.010$ ,  $P_{21} = 0.0071$ ). See also Fig S5 and Table S4.

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