

## RESEARCH ARTICLE SUMMARY

## HEART DEVELOPMENT

# Characterization of a common progenitor pool of the epicardium and myocardium

Richard C. V. Tyser\*, Ximena Ibarra-Soria\*, Katie McDole, Satish Arcot Jayaram, Jonathan Godwin, Teun A. H. van den Brand, Antonio M. A. Miranda, Antonio Scialdone, Philipp J. Keller, John C. Marioni†, Shankar Srinivas†

**INTRODUCTION:** The vertebrate heart is composed of diverse cell types, all essential for normal cardiac function. In the mouse, the earliest mesodermal progenitors of cardiomyocytes, formed during gastrulation, migrate rostrally from the primitive streak to form the cardiac crescent and initiate contractile activity. The cardiac crescent subsequently undergoes remodeling to form the linear heart tube. There are at least two distinct groups of mesodermal cardiac progenitors, the first and second heart fields (FHF and SHF, respectively), defined broadly on the basis of marker genes expressed in different but overlapping regions of the early embryo. Cells from outside these heart fields can also contribute to the heart. One such structure, the proepicardium, gives rise to the epicardium, the outermost layer of cells of the vertebrate heart. The epicardium provides important paracrine signals and can also generate several cardiac cell types, including cardiomyocytes, vascular smooth muscle, and fibroblasts.

**RATIONALE:** Our current understanding of when and how different cardiac cell types arise during early development is limited. Single-cell transcriptomics offers a powerful approach to

characterize the various cell types of the embryonic heart and generate hypotheses about their origin and fate. We therefore combined single-cell RNA sequencing with high-resolution volume imaging and time-lapse microscopy to precisely characterize the cells of the mouse embryonic heart at single-cell resolution. This powerful combinatorial approach provides a unified transcriptional and anatomical definition of cardiac progenitor types and their differentiation trajectories toward cardiomyocytes.

**RESULTS:** We used manual microdissection to isolate the cardiac region of mouse embryos, from early cardiac crescent to linear heart tube stages, and performed single-cell RNA sequencing. This enabled us to transcriptionally define the cardiac progenitor populations in this region, including FHF and SHF. As a user-friendly community resource, we created a web interface to investigate these data, accessible at <https://marionilab.cruk.cam.ac.uk/heartAtlas/>. To identify the anatomical locations of these cell populations at single-cell resolution, we used whole-mount immunohistochemistry, multiplexed fluorescence in situ hybridization, and high-resolution volume imaging of com-

binations of markers. This comprehensive imaging analysis revealed the discrete locations of these transcriptional clusters and highlighted the spatially ordered maturation of cardiomyocytes. It also identified a population of progenitor cells located rostral to the cardiac crescent, at the confluence of the embryonic and extraembryonic mesoderm, which we call the juxta-cardiac field (JCF). Using single-cell resolution time-lapse imaging and genetic lineage labeling, we established that the JCF can contribute to both cardiomyocytes and the proepicardium. The JCF therefore represents the earliest known progenitors of the epicardium, the outermost layer of the vertebrate heart.

**CONCLUSION:** This study provides a detailed characterization of the transcriptional states and anatomical locations of cardiac progenitors, as well as their transition states during differentiation toward cardiomyocytes, thereby representing a valuable community resource. Furthermore, it provides fresh insights into the formation of the heart. By identifying the juxta-cardiac field, our work widens the cardiac progenitor region and identifies the earliest progenitors of the proepicardium. This study will contribute to a better understanding of the origin of congenital cardiac defects and provide basic insights for informing the development of regenerative approaches to treat heart disease. ■

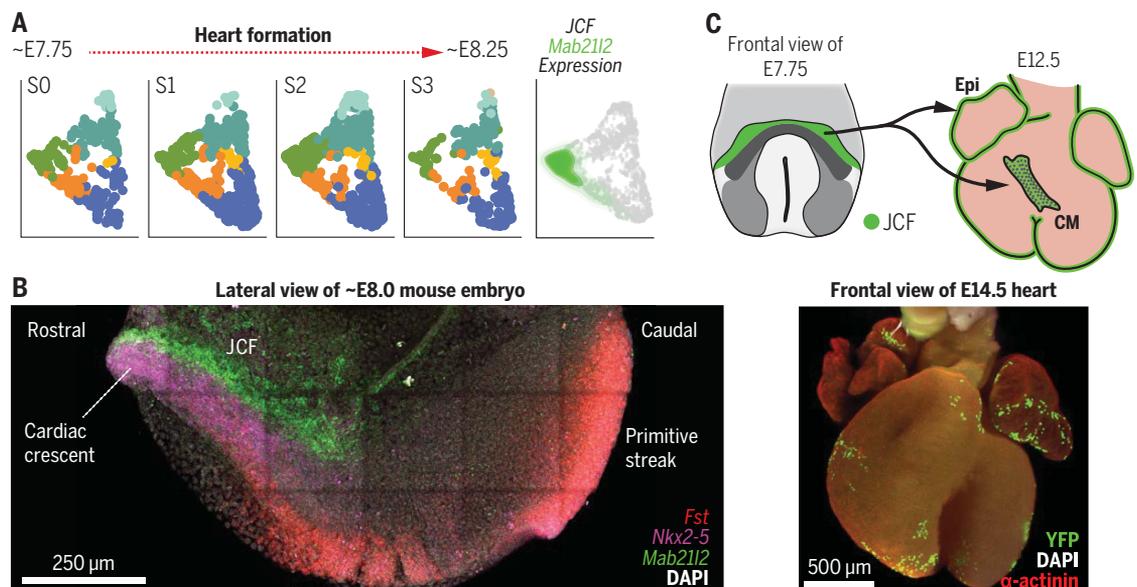
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## Identification of a progenitor pool for cardiomyocytes and epicardium.

(A) Single-cell RNA sequencing of the developing heart identified a cell population characterized by *Mab21l2* expression. E, embryonic day. (B) These cells lie rostral to the cardiac crescent and represent a juxta-cardiac field (JCF). DAPI, 4',6-diamidino-2-phenylindole. (C) This progenitor pool gives rise to both cardiomyocytes (CM) and epicardium (Epi). Yellow fluorescent protein (YFP) cells (bottom panel) are JCF-derived epicardial cells and cardiomyocytes.



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# Characterization of a common progenitor pool of the epicardium and myocardium

Richard C. V. Tyser<sup>1\*</sup>, Ximena Ibarra-Soria<sup>2\*,†</sup>, Katie McDole<sup>3</sup>, Satish Arcot Jayaram<sup>1</sup>, Jonathan Godwin<sup>1</sup>, Teun A. H. van den Brand<sup>4</sup>, Antonio M. A. Miranda<sup>1,†</sup>, Antonio Scialdone<sup>5,6,7</sup>, Philipp J. Keller<sup>3</sup>, John C. Marioni<sup>2,4,8,§</sup>, Shankar Srinivas<sup>1,§</sup>

The mammalian heart is derived from multiple cell lineages; however, our understanding of when and how the diverse cardiac cell types arise is limited. We mapped the origin of the embryonic mouse heart at single-cell resolution using a combination of transcriptomic, imaging, and genetic lineage labeling approaches. This mapping provided a transcriptional and anatomic definition of cardiac progenitor types. Furthermore, it revealed a cardiac progenitor pool that is anatomically and transcriptionally distinct from currently known cardiac progenitors. Besides contributing to cardiomyocytes, these cells also represent the earliest progenitor of the epicardium, a source of trophic factors and cells during cardiac development and injury. This study provides detailed insights into the formation of early cardiac cell types, with particular relevance to the development of cell-based cardiac regenerative therapies.

The heart is formed from several lineages, which give rise to the various cardiac cell types. The earliest mesodermal progenitors of cardiomyocytes emerge from the primitive streak, starting at about embryonic day 6.5 (E6.5) in the mouse (1–3). These cells migrate laterally across the egg cylinder to occupy an anterior position, initially forming the cardiac crescent before subsequently forming the linear heart tube (LHT) (4) (Fig. 1A). There are at least two distinct types of mesodermal cardiac progenitors, the first and second heart fields, defined broadly on the basis of marker genes expressed in different but overlapping anatomical locations (5, 6). First heart field (FHF) cells occupy a cranio-lateral region and, together with early cardiomyocytes, form the cardiac crescent. The second heart field (SHF) represents a wider domain, dorsal and medial to the cardiac crescent. FHF cells show limited proliferation, instead rapidly differentiating into cardiomyocytes in order to lay

down a primitive contractile apparatus (7) (Fig. 1A). In contrast, SHF cells are more proliferative, contributing to the growth of the heart before the cells differentiate. Given the overlap in expression of FHF and SHF markers, particularly with SHF cells up-regulating FHF markers as they differentiate into cardiomyocytes, there is some debate about whether FHF and SHF correspond to fundamentally distinct cell types, or whether they represent different states along a shared differentiation trajectory from a common progenitor toward cardiomyocytes.

Retrospective clonal analyses support the idea that there are two separate lineages that contribute to the cardiomyocytes of the heart. The “first lineage” contributes primarily to the atria and left ventricles, whereas the “second lineage” contributes primarily to the atria, right ventricle, and outflow tract (5, 8). These two lineages are assumed to correspond to the FHF and SHF, respectively. However, it is unclear whether the difference in fate of these two lineages is due to fundamental differences in their potential or due only to differences in their embryonic location during early cardiac morphogenesis.

Cells from outside the traditional heart fields also contribute to the heart. Notably, the proepicardium gives rise to the epicardium, the outer layer of cells of the vertebrate heart. The epicardium generates several cardiac cell types, including vascular smooth muscle cells and fibroblasts, in addition to providing important paracrine signals. It has attracted considerable attention as a possible source of cells and trophic factors in the context of repair after injury (9, 10). The proepicardium is initially situated at the inflow end of the looping heart tube at ~E9.0, but its embryonic origin remains unclear (11–13).

Single-cell transcriptomics offers a powerful approach to characterize the various cell types of the embryonic heart and generate hypotheses about their lineal origins and fates. Several recent studies have used this approach to characterize cardiogenesis, focusing on subpopulations of cells isolated using predefined genes (14–17). In this study, we perform a spatially resolved transcriptomic analysis of a microdissected region of the anterior embryo, collecting cells from the region corresponding to the embryonic heart over the time course of cardiac crescent formation and LHT development.

## Single-cell analysis of heart formation

We sampled cells, using manual microdissection to isolate the anterior cardiac region of mouse embryos, from early crescent to LHT stages and performed single-cell RNA sequencing (scRNA-seq) (Fig. 1, A and B; fig. S1A; and movie S1). Our collection was highly time-resolved, covering six defined stages spanning ~12 hours of development (roughly E7.75 to E8.25; fig. S1A) (18). After quality control, the transcriptomes of 3105 cells were retained for further analysis (fig. S2A; see methods). We identified genes with highly variable expression and performed unsupervised clustering to determine the different cell types within our dataset. This revealed 12 distinct clusters (Fig. 1, C and D), representing mesodermal cell types [including cardiac-related (Me3 to Me8), which represented a majority (66.1%) of the cells; endothelial (Me2); and blood (Me1)], overlying endoderm [including definitive (En1) and yolk sac (En2)], and adjacent ectoderm [surface/amnion (Ec1) and neuroectoderm (Ec2)] (19–21). We experimentally validated cluster identity using whole-mount immunofluorescence for cluster-specific combinations of marker genes and single-cell resolution imaging (Fig. 1, E and F; figs. S3 and S4; and movie S2). As a user-friendly community resource, we have created a web interface to investigate these data, accessible at <https://marionilab.cruk.cam.ac.uk/heartAtlas/>.

Me3 represented the most-differentiated cardiomyocytes in our dataset, showing elevated expression of contractile-related genes *Myh6*, *Actn2*, and *Ttn* (Fig. 1D). Me3 started to emerge between stages 0 to 1 and increased over time (fig. S1B), consistent with the formation of cardiomyocytes and onset of contractile activity at stage 1 (7). Me3 also showed reduced proliferative capacity in comparison to other clusters, with a higher proportion of cells in G1 (Fig. 1G and fig. S1C).

Me4 and Me6 were characterized by strong expression of cardiac progenitor markers but intermediate-to-low levels of contractile genes (Fig. 1D) and therefore represented differentiating cardiac progenitors. Me5, Me7, and Me8 represented the least-differentiated populations, on the basis of the low expression of

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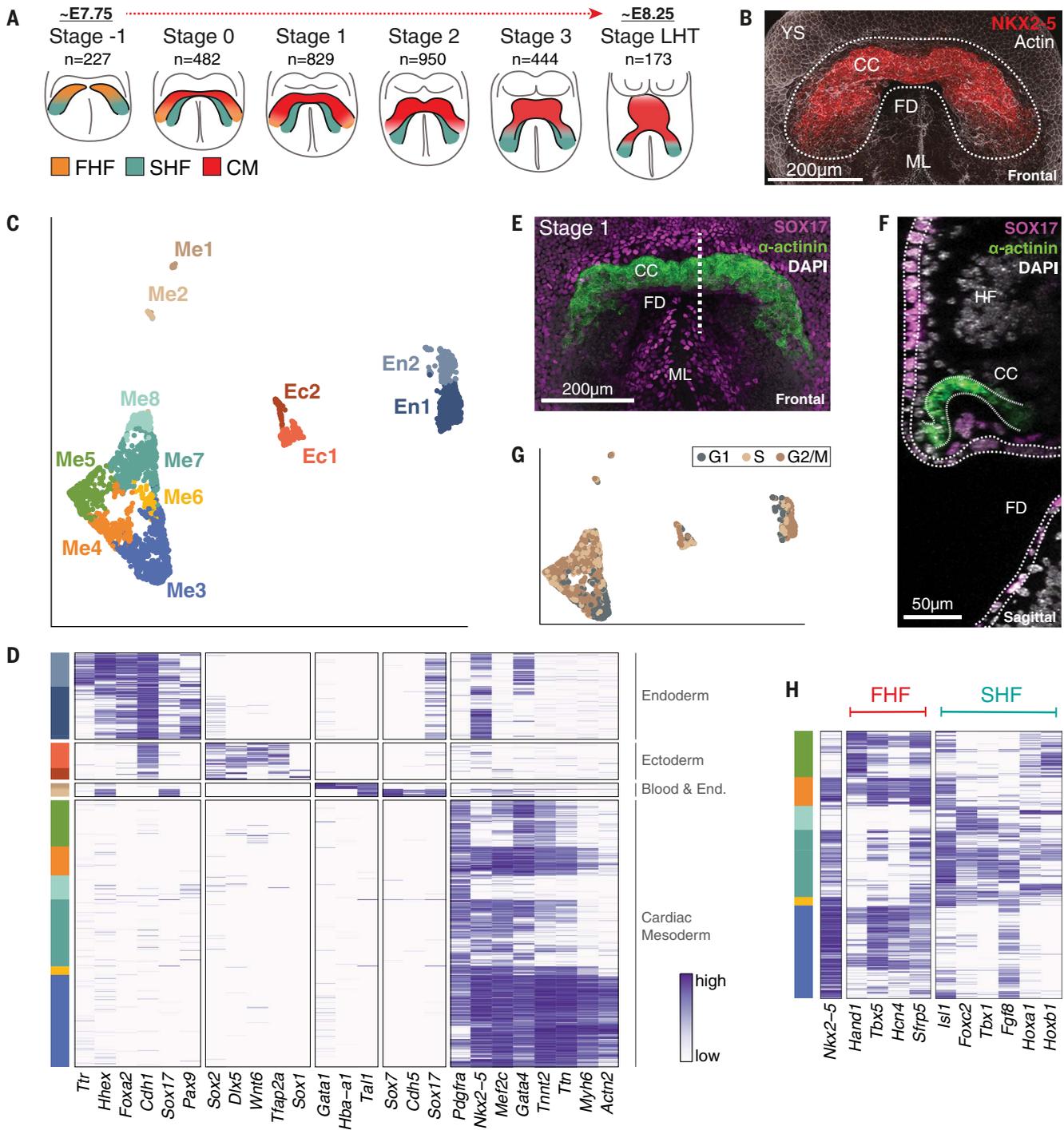
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**Fig. 1. Single-cell resolution spatiotemporal analysis of the forming murine heart.** (A) Schematic of crescent and linear heart tube stages collected for scRNA-seq.  $n$  = number of single cells that passed quality control. FHF, first heart field; SHF, second heart field; CM, cardiomyocytes. (B) Maximum intensity projection (MIP) of a stage 1 cardiac crescent region, marked by NKX2-5 protein expression (movie S1). The dotted region highlights the area microdissected for collecting cells. CC, cardiac crescent; FD, foregut diverticulum; YS, yolk sac; ML, midline. (C) Uniform manifold approximation and projection (UMAP) plot of all cells that passed quality control ( $n = 3105$ ) computed from highly variable genes. Cells with similar transcriptional profiles were clustered into 12 different groups, as indicated by the different colors. (D) Heatmap showing the expression of well-

characterized marker genes. Cells (rows) are ordered first by cluster and then by hierarchical clustering. Each gene's normalized log expression levels are regularized to be within [0,1]. (E) MIP of representative embryo showing SOX17 and sarcomeric  $\alpha$ -actinin protein (movie S2). This serves as an example of the approach used to anatomically localize in the intact embryo the cell types identified in the scRNA-seq dataset. The dotted line represents the location of the section shown in (F). DAPI, 4',6-diamidino-2-phenylindole. (F) Sagittal section of the embryo in (E). Dotted lines outline the endoderm (SOX17<sup>+</sup>) and cardiac crescent ( $\alpha$ -actinin<sup>+</sup>). See also fig. S3. HF, headfold. (G) UMAP plot as in (C), but colored according to the inferred cell cycle stage of each cell. (H) Heatmap of cardiac-related clusters showing the expression of genes associated with the first (FHF) and second (SHF) heart fields.

contractile- and cardiac physiology-related genes (Fig. 1D).

### Transcriptional and spatial identification of cardiac progenitor subtypes

To characterize these cardiac-related clusters (Me3 to Me8) in more detail, we analyzed the expression of common FHF and SHF markers (Fig. 1H). The cardiac progenitor marker *Nkx2-5* was expressed in all these clusters except Me5 and Me8. Me4 had the highest combined expression of canonical FHF markers, such as *Tbx5*, *Nkx2-5*, *Hcn4*, and *Sfrp5*, although some of these were also found in Me3, Me5, and, to a lesser extent, Me7. Me6 and Me7 showed a distinct SHF-like signature (Fig. 1H), although again, some SHF markers were also seen in Me3 and Me5.

The FHF and SHF are defined not only on the basis of markers but also on their anatomical location. Hence, we next confirmed our assignment of clusters to a FHF or SHF identity by generating a spatially resolved scRNA-seq reference dataset from four anatomically defined regions of mesoderm in stage 3 crescent embryos (~E8.0). We manually microdissected and sequenced cells from the ventral mesoderm containing the cardiac crescent (VM), abutting dorsal mesoderm (DM), cranial mesoderm (CrM), and paraxial mesoderm (PM) (Fig. 2A). We generated an average of 70 high-quality single-cell transcriptomes for each of these reference regions, which together clustered into five different subpopulations (clusters 1 to 5) that were dispersed to varying degrees across the four regions (Fig. 2B and fig. S5A). Cluster 1 expressed the CrM marker *Cyp26c1* (22) (Fig. 2C) and was almost exclusively derived from this reference region. Cluster 2 represented PM (*Tcf15* and *Meox1*) (23, 24) and was composed of cells from this region as well as from the DM. Clusters 3, 4, and 5 expressed cardiac markers such as *Tnn* and *Gata5* (25) and were made up predominantly of cells from the VM and DM (Fig. 2, B and C). VM cells could be categorized as either more mature, differentiated cardiac cells (cluster 5) or less mature “cardiac-like” cells (clusters 3 and 4) (Fig. 2B and fig. S5A). Similarly, DM cells were split across cardiac-like and “PM-like” (clusters 3 and 2, respectively; Fig. 2B). *Hoxa1*, which marks cells from the DM, highlights the pronounced transcriptional heterogeneity within this region, being split across clusters 2 and 3 (Fig. 2, B and C).

To use these reference cells to guide spatial localization of the cells assigned to clusters Me3 to Me8, we trained a random forest classifier using the combined information from the dissection labels and clustering analysis (see methods; Fig. 2B and fig. S5, A and B). Upon application of this classifier to the main dataset, most of the cells in clusters Me3 to Me6 were assigned to the VM (Fig. 2, D and E), which corresponds broadly to the

cardiac crescent. Consistent with the differences in maturity observed between Me5 and Me3/4/6, the former matched cardiac-like VM cells, while the latter mapped to the more-mature cardiac VM (Fig. 2, D and E). Cells from Me7 predominantly mapped to cardiac-like DM reference cells, whereas Me8 primarily mapped to CrM but also PM-like DM (Fig. 2, D and E). In particular, although Me7 and Me8 were both characterized by high *Tbx1* and *Foxc2*, they could be distinguished by expression of *Nkx2-5* (high in Me7, low in Me8) and *Fst* (low in Me7, high in Me8) (Fig. 2, F and G; figs. S6 to S8; and movies S3 to S5 and S7). Together, our spatially defined scRNA-seq reference regions and combinatorial marker gene expression analyses allowed us to localize Me7 to areas of mesoderm medial and dorsal to the crescent, consistent with it representing cells from the SHF.

### Intermediate states in cardiomyocyte differentiation are spatially localized across development

We next used a diffusion map approach to infer differentiation trajectories among clusters Me3 to Me7, excluding Me8 owing to its CrM- and PM-like identity. This analysis revealed two distinct trajectories, both leading to the differentiated cardiomyocytes of Me3 (Fig. 2H and fig. S9). One trajectory connected Me7 to Me3 via two distinct intermediates, Me6 and Me4. The second trajectory linked Me5 to Me3 via Me4 (Fig. 2H and fig. S9, A to C).

The trajectory linking Me5, Me4, and Me3 is consistent with a FHF-like differentiation trajectory. Although Me4 had a broad FHF transcriptional signature, Me5 did not fit with straightforward categorization, as it expressed some FHF markers (*Hand1* and *Tbx5*) but substantially lower levels of the canonical cardiac progenitor marker *Nkx2-5* (Fig. 1, D and H). Therefore, to further characterize these clusters, we determined their precise embryonic locations in three dimensions at different stages using the combinatorial expression of multiple markers (fig. S10) and single-cell resolution volume imaging.

Me5 cells are *Hand1*- and *Snail*-positive but also *Nkx2-5*-negative. Using these and other markers, such as *Smadcd3* and *Tbx5*, we were able to locate Me5 at the rostral border of the cardiac crescent, as a discrete group of cells at the confluence of extraembryonic and splanchnic mesoderm, corresponding anatomically with the primitive septum transversum (26) (Fig. 3, A to C, and fig. S11). The splanchnic mesoderm here is a monolayer continuous with the extraembryonic mesoderm of the yolk sac. Within this sheet, at stage 1 and earlier, Me5 was rostrally positioned with respect to the more caudal NKX2-5- and TITIN-positive cardiac progenitors (Fig. 3B). Later, at stage 2, rostral folding causes this mesoderm to buckle, such that Me5 is now sandwiched between the

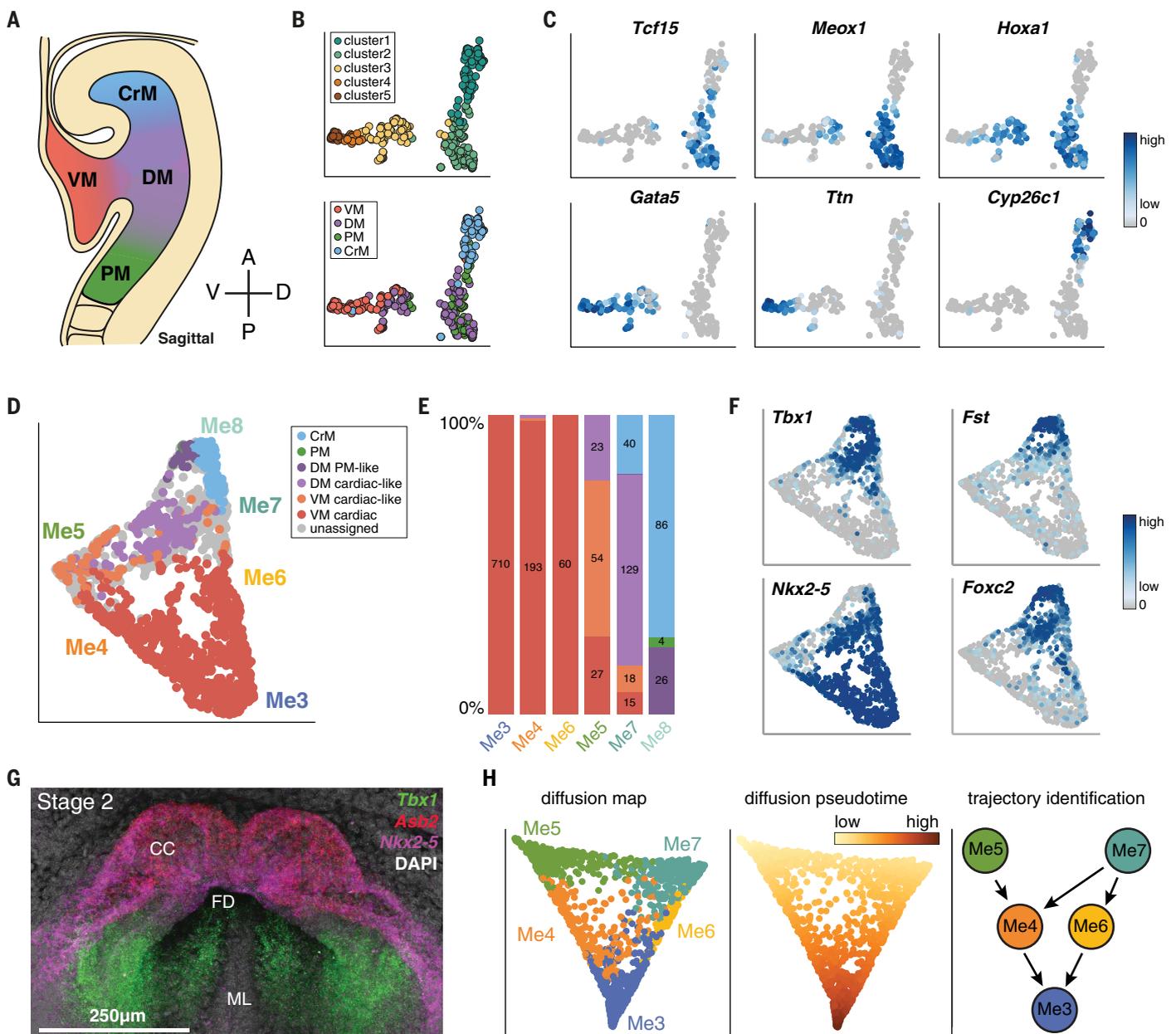
endoderm and the more dorsal layer of NKX2-5-positive cardiac progenitors, while maintaining its relative position within the plane of the mesodermal sheet (Fig. 3, C and D). Using our scRNA-seq data, we identified *Mab21l2* as a marker of Me5 (Fig. 3, E and F; figs. S8B and S12; and movies S6 and S7). Expression of *Mab21l2* confirmed the location of Me5 at the rostral border of the crescent prior to rostral folding, and between the endoderm and crescent during folding (Fig. 3E and fig. S12).

Me6, characterized by overlapping expression of *Asb2* and *Tbx1*, represented a small population of cells restricted to the medial-dorsal interface between Me7 and the cardiac crescent (Fig. 4, A and F; fig. S13; and movie S3), consistent with it representing a transitional state during the differentiation of SHF progenitors (Me7) into cardiomyocytes (Me3).

Me4, identified by expression of *Vsnll* and absence of *Fsd2*, localized at stage 1 to a narrow arc within the cardiac crescent, at the interface between Me5 and Me3 (Fig. 4, B, C, and F; figs. S14 and S15; and movies S8 to S10). At stage -1, before formation of cardiomyocytes (Me3), Me4 formed the earliest arcs of the cardiac crescent, just caudal to Me5 (Fig. 4, D and F; figs. S16 and S17; and movie S11), consistent with it representing the FHF. The discrete anatomical locations of these transcriptional clusters highlight the spatially graded maturation of cardiomyocytes in this region. The Me4 state could be detected as late as the LHT stage (fig. S14C).

Our diffusion analysis indicated that Me7 (SHF) could contribute to Me3 (cardiomyocytes) via not only Me6, a transient transcriptional state specific to the SHF trajectory, but also via Me4, which captures the FHF transcriptional state. Consistent with trajectories from both Me5 and Me7 to Me3 via Me4, we detected Me4 cells not only at the interface between Me5 and Me3 (above) but also at the interface of Me7 and Me3 (Fig. 4, E and F, and figs. S16C and S17, C and D). In addition, Me3, defined by the expression of *Fsd2*, could be separated into two subgroups on the basis of the presence or absence of the Me4 marker *Vsnll*. Imaging revealed that the *Vsnll*-positive cardiomyocytes of Me3 were located in the ventral crescent, adjacent to Me4, whereas the *Vsnll*-negative cardiomyocytes were located more medial-dorsally, adjacent to Me7 and Me6 (Fig. 4C and fig. S14, D and E). Given the different anatomical locations and transcriptional profiles of SHF intermediates, the branched trajectory of SHF progenitors possibly reflects the early separation of anterior and posterior SHF.

The consensus FHF transcriptional signature and anatomical location of Me4 within the crescent indicate that it represents the FHF. In contrast, the *Nkx2-5*-negative Me5 corresponds to progenitors of the FHF. *Hand1* has



**Fig. 2. Classification of cells from clusters Me3 to Me8 based on an anatomically resolved mesoderm reference dataset.** (A) Schematic indicating the four regions of mesoderm microdissected to generate the reference dataset. Single cells from each region were isolated and used for scRNA-seq. (B) UMAP plot of reference cells that pass quality control, colored by their anatomical origin (top) or clustering based on their transcriptional profiles (bottom). These cells were used to build a random forest classifier. (C) Expression of genes that are important for the random forest, distinguishing the different types of cells. (D) UMAP plot of clusters Me3 to Me8, as in Fig. 1C, but colored according to the class assigned by the random forest classifier. Cells in gray cannot be confidently assigned to a single class. (E) Barplot indicating the proportion of

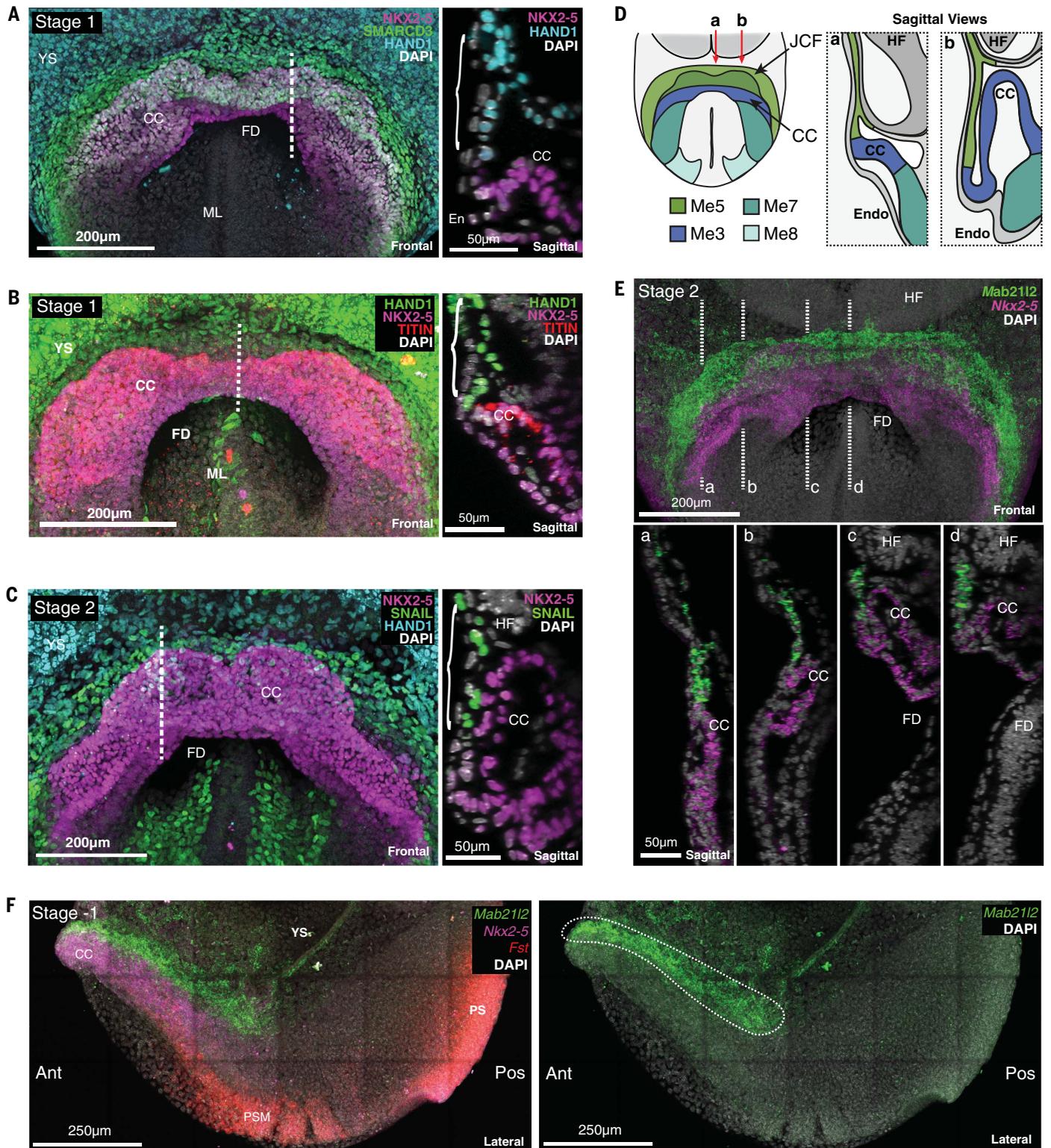
cells in each cluster assigned to the different classes. Numbers indicate the number of cells. (F) UMAP plots showing the expression of genes used to identify clusters Me7 and Me8. (G) MIP of a stage 2 crescent showing expression of *Nkx2-5* and *Tbx1* using hybridization chain reaction (movie S3). (H) Diffusion map of cells from the cardiac mesoderm meta-cluster (Me3 to Me7,  $n = 1772$ ); the first two diffusion components are shown. On the left, cells are colored by their cluster annotation, as in Fig. 1C. In the middle, the color gradient indicates diffusion pseudotime measurements for each cell. On the right, schematic diagram summarizing the trajectories identified, linking the progenitor clusters Me5 and Me7 to the mature cardiomyocytes of Me3 (see also fig. S9). Color scales in (C) and (F) represent log<sub>2</sub> normalized counts.

been considered a FHF marker owing to in situ hybridization using chromogenic detection that appears to label the cardiac crescent (27). However, our single-cell resolution imaging shows that *HAND1* in fact marks Me5, which is distinct from the FHF and

cardiac crescent (Fig. 3, A to C); because of Me5's ventral position overlying the crescent, volume imaging is required to distinguish the two. Given the proximity of Me5 to the cardiac crescent, we named it the juxta-cardiac field (JCF).

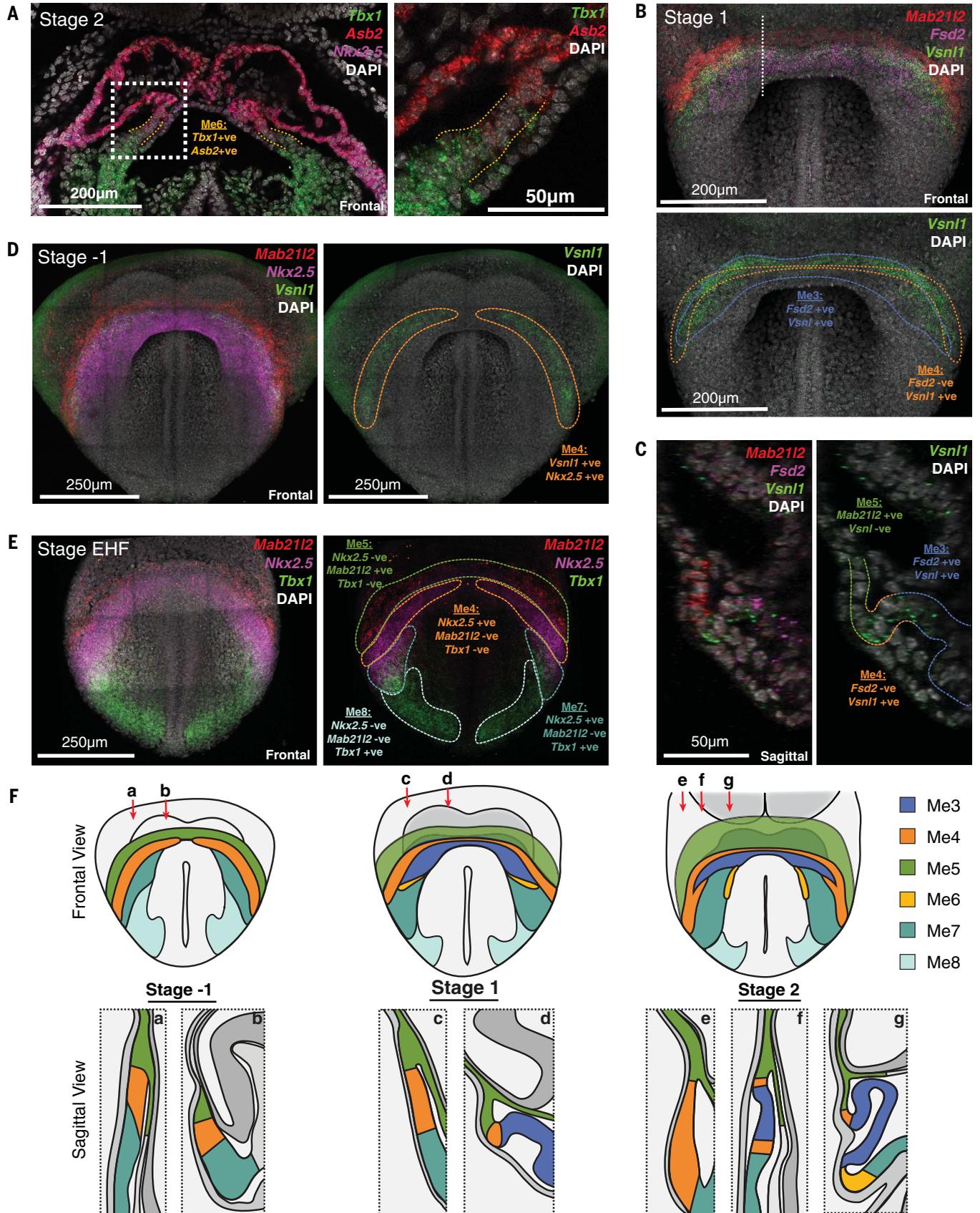
#### The JCF represents a cardiac progenitor population

To determine whether the JCF, similar to other cardiomyocyte progenitors, is derived from cells that expressed *Mesp1*, we used a *Mesp1-Cre* (3) driver line. This verified that



**Fig. 3. Identification of a transcriptionally and anatomically distinct cell type with cardiac progenitor-like properties.** (A to C) MIP of immunostained embryos revealing anatomical localization of Me5 (brackets). Dashed line indicates the location of the sagittal section shown at right in each panel. En, endoderm. (D) Schematic highlighting the localization of Me5 [juxta-cardiac field (JCF)], Me7, and Me8, relative to the myocardium of the cardiac crescent (Me3). (E) MIP of hybridization chain reaction (HCR) staining of an embryo at

stage 2 revealing the distinct anatomical localization of JCF cells marked by *Mab2112* (movie S6). Dashed lines (labeled a to d) correspond to the sagittal sections shown below. (F) (Left) MIP of lateral view of HCR on a stage -1 embryo showing distinct expression domains of *Mab2112*, *Nkx2-5*, and *Fst* (movie S7). Ant, anterior; Pos, posterior; PS, primitive streak. Right panel shows only *Mab2112* expression highlighting location of Me5 cells (white dotted region).



**Fig. 4. Anatomical identification of transition states during cardiomyocyte differentiation.** (A) Coronal section of a HCR stained stage 2 embryo, showing the anatomical location of Me6 (dotted orange lines) marked by coexpression of *Tbx1* and *Asb2*. Dashed white box represents region of higher magnification at right. (B) MIP of HCR on stage 1 embryo showing expression of *Mab21l2*, *Vsnl1*, and *Fsd2* to identify cluster Me4. Colored dotted lines correspond to Me3 and Me4 (movie S8). White dot-dash line shows location of section in (C). (C) Sagittal section of embryo in (B) highlighting locations of

Me3, Me4, and Me5. (D) MIP of HCR on stage -1 embryo showing *Mab21l2*, *Vsnl1*, and *Nkx2-5*, to identify cluster Me4 (orange dotted line) (movie S11). (E) MIP of an early headfold (EHF) stage embryo, prior to crescent formation, showing expression of *Mab21l2*, *Tbx1*, and *Nkx2-5*, to identify clusters Me4, Me5, Me7, and Me8. Colored dotted lines outline corresponding regions. (F) Schematic highlighting the anatomical location of all mesoderm transcriptional states identified at multiple stages of heart formation. Red arrows represent the location of sagittal sectional views.

the JCF arises from *Mesp1*-positive mesoderm (Fig. 5A). Similarly, using an *Nkx2-5*-Cre (27) driver line showed that JCF cells had never previously expressed *Nkx2-5* (Fig. 5A).

Supporting a differentiation trajectory from the JCF to cardiomyocytes, the cardiac progenitor gene *Nkx2-5* increased in expression along pseudotime as cells transitioned from JCF to Me3 (Fig. 5B). We therefore tracked JCF cells in embryos where expression of *Nkx2-5* could be monitored using nuclear-enhanced green fluorescent protein (eGFP) fluorescence (28, 29) using single-cell resolution light-sheet microscopy (30). Confirming the diffusion map trajectory, we found that JCF cells activated expression of *Nkx2-5*:GFP as they moved toward the cardiac crescent during rostral folding (Fig. 5, C to E, and two representative cells in movies S12 and S13).

The Me5 marker *Mab21l2* is expressed rostral to the cardiac crescent and is down-regulated during cardiomyocyte differentiation (Fig. 5F). Before crescent formation, at early headfold stages (31), *Mab21l2* was detected rostral to an *Nkx2-5*-positive domain, at the boundary between embryonic and extraembryonic mesoderm (Fig. 5F, fig. S18B, and movie S14). At early preheadfold stages, we could not detect *Mab21l2* but found *Nkx2-5* expression in the anterior mesoderm (fig. S18C). This suggests that the Me5 population forms after other cardiac progenitor populations.

Our transcriptomic data showed that Me8, Me4, and a small subset of Me5 expressed *Tbx18* (Fig. 5G). A *Tbx18*-positive (and *Nkx2-5*, *Isl1*-double negative) population of caudal-lateral mesoderm can give rise to sinus venosus myocardium (32). To test whether the JCF and sinus venosus precursors might overlap, we assayed for *Tbx18* and *Mab21l2* coexpression by in situ hybridization chain reaction (Fig. 5G and fig. S19). In contrast to *Mab21l2*, *Tbx18* was not detected until stage 1, with weak expression in two separate domains, a region of the lateral crescent and medial paraxial mesoderm, corresponding with Me8 (Fig. 5G). In contrast, *Mab21l2* was expressed earlier, at early headfold stages, rostrally and in a significantly larger region covering both medial and lateral regions of mesoderm (fig. S19B). *Tbx18* was never detected in the rostral-medial JCF, indicating that the JCF is a spa-

tially and transcriptionally distinct progenitor population from the sinus venosus progenitors (Fig. 5G).

#### JCF progenitors contribute to cardiomyocytes and epicardium

To test our diffusion trajectory results indicating that the JCF gives rise to cardiomyocytes, we knocked CreERT2 into the *Mab21l2* locus in a homozygous R26R-YFP (yellow fluorescent protein) reporter background (33). At E8.0, nuclear localized CreERT2 was found only in the JCF and was not detected in cardiomyocytes (fig. S20B).

To specifically label the JCF, we administered a single low dose of tamoxifen to pregnant dams at 6.5 days post coitum and then assessed the fate of labeled cells at several subsequent time points (Fig. 6A). The early dose of tamoxifen at E6.5 ensured that tamoxifen was available when *Mab21l2* first begins to mark the JCF around E7.5, while minimizing the possibility of labeling cells outside the JCF or at subsequent stages. This protocol allowed us to stringently assess the fate of the JCF but likely underrepresents the contribution of this progenitor pool owing to the early, limiting dose of tamoxifen.

We first tested specificity of labeling by looking in E8.0 embryos. Fewer than half of *Mab21l2*-CreERT2 transgenic embryos showed any labeled cells (19 of 45 embryos), consistent with the limiting dose of tamoxifen. YFP-positive cells could be found only in the JCF at early crescent stages and did not express markers of maturing FHF cells, such as sarcomeric  $\alpha$ -actinin or cardiac troponin T (cTnT) (Fig. 6B and fig. S20, B to D). On average, five YFP-positive cells could be detected per embryo between stage 1 and LHT (fig. S20A), highlighting the low level of recombination. These data confirmed that the new *Mab21l2*-iCreERT2 transgenic line specifically targeted JCF cells and would allow us to investigate their fate.

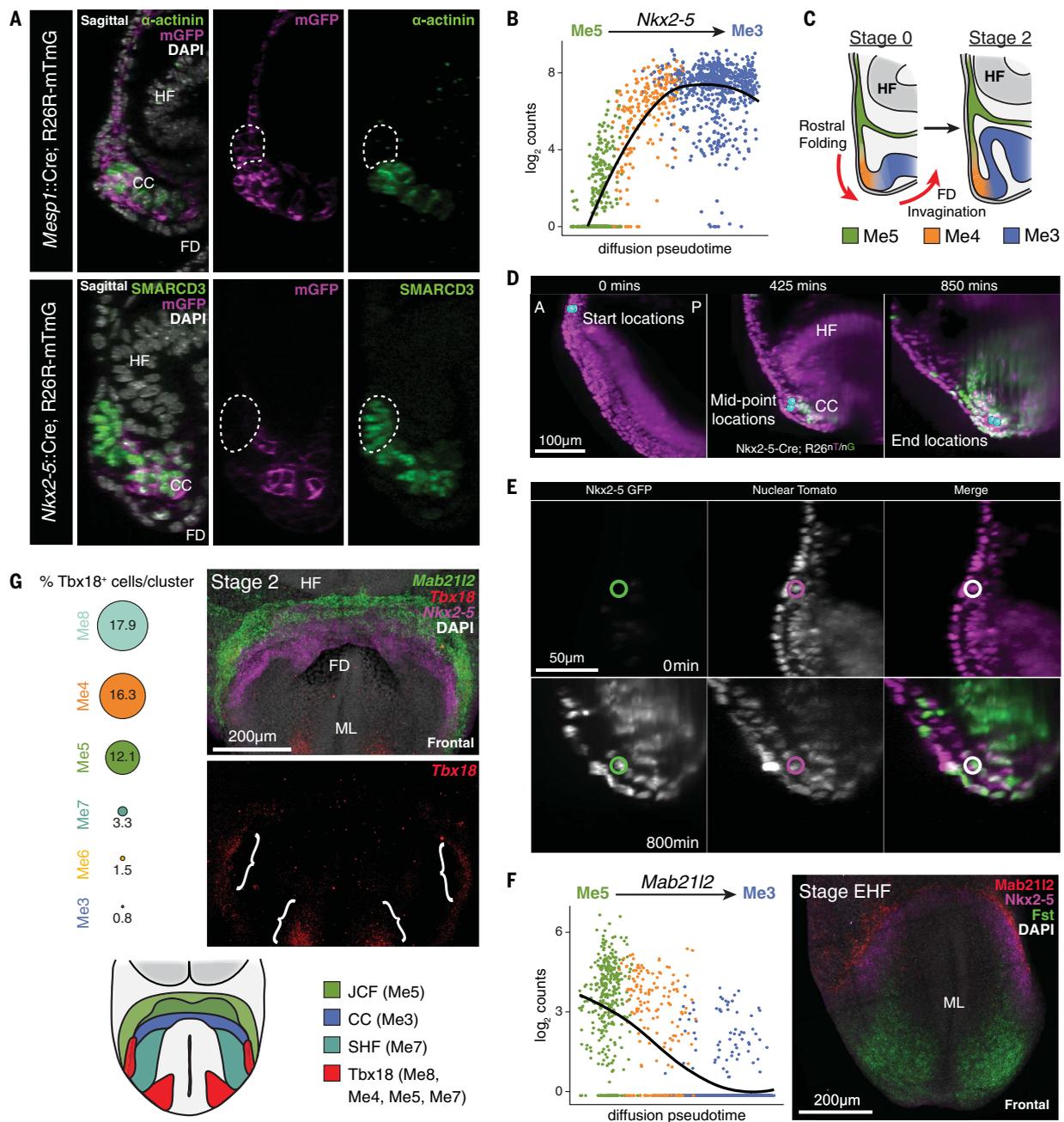
We next analyzed the early looping heart tube at E8.5. Consistent with time-lapse results (Fig. 5D), this analysis revealed that JCF cells contribute to cardiomyocytes within the linear heart tube, as verified by sarcomeric striations of  $\alpha$ -actinin in YFP-positive cells (Fig. 6C and table S1). Additionally, the JCF was maintained throughout these stages as YFP-positive non-cardiomyocyte cells were still present in the

JCF domain, which by this stage was positioned by rostral folding between the cardiac tube and endoderm (Fig. 6C).

JCF cells, in addition to forming cardiomyocytes, also contributed to the proepicardium at E9.5. Whole-mount immunofluorescence for WT1, a proepicardium and epicardium marker, revealed colocalization with YFP cells (Fig. 6D and table S2). Consistent with this, at E10.5 we found YFP-positive cells in both the epicardium and myocardium. The latter cells were found in the left ventricle, atrioventricular canal, and atria but never in the right ventricle or outflow tract (Fig. 6, E and F, and tables S3 and S4). The direct contribution of the JCF to myocardium, as opposed to via differentiation of later-formed epicardial cells, is evident from the JCF-derived myocardial cells observed already at E8.5, before the formation of the epicardium (Fig. 6C and tables S1 and S2). These data together establish the JCF as a progenitor pool of cardiomyocytes and the proepicardium.

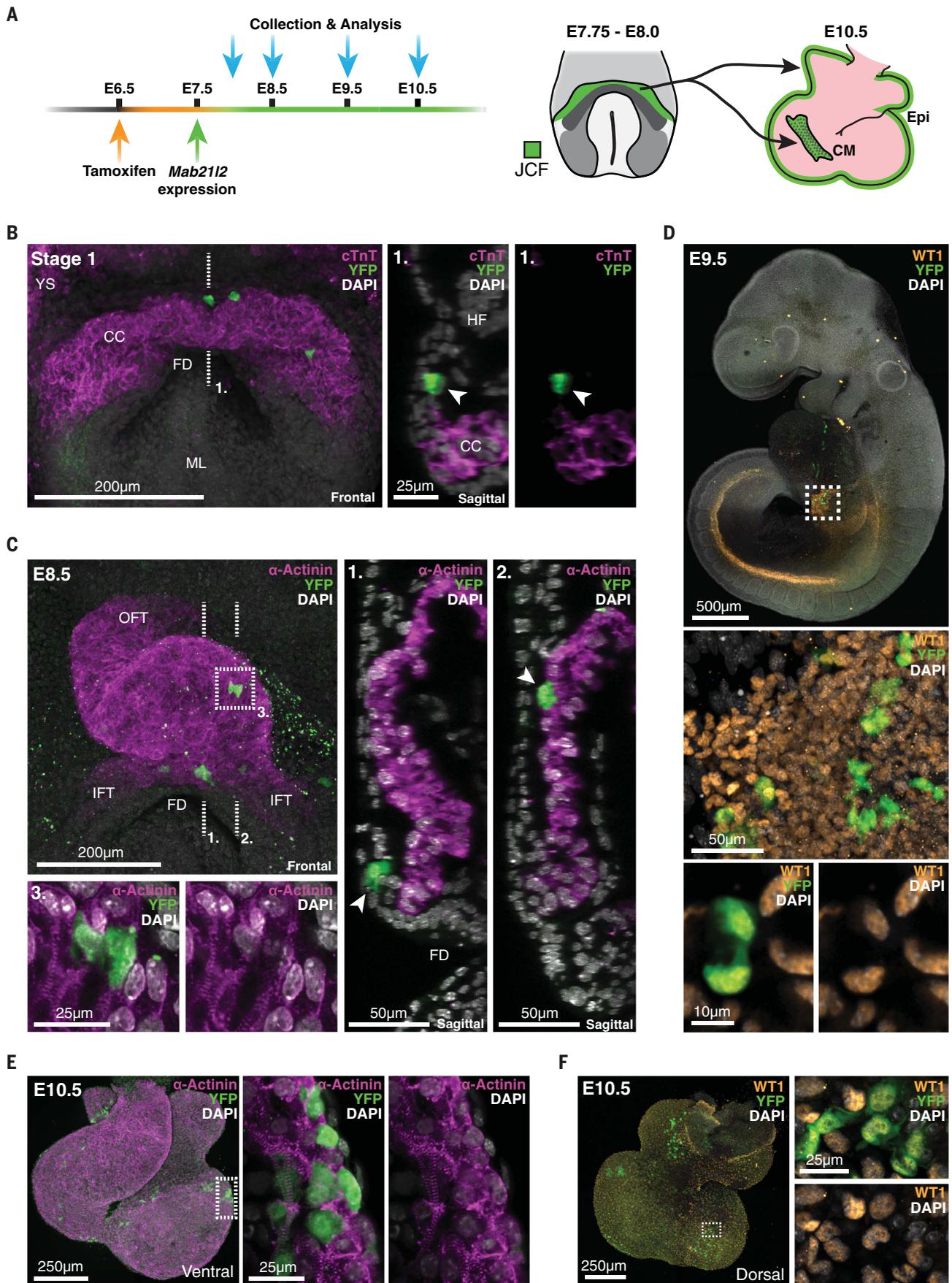
Our spatially and temporally resolved single-cell anatomical and transcriptional analysis provides a multifaceted resource for understanding how the heart forms. It also provides a spatial and transcriptomic reference definition of the FHF, SHF, and intermediate states to cardiomyocytes. Given that the transcriptional state of a cell is labile but that scRNA-seq offers only a snapshot of its transcriptional state, caution must be exercised when using “marker” genes to make inferences regarding lineage (past history, often with the implicit assumption that a shared transcriptional state implies a shared history) and fate (the future descendants). This is particularly evident with Me4 that represents a FHF transcriptional state, through which cells of two different origins (JCF or SHF) can transition to form cardiomyocytes. This potential for the convergence of transcriptional states highlights the importance of using independent approaches such as genetic fate mapping to determine the fate of cells.

Our analyses revealed a progenitor pool, the JCF, that can contribute to cardiomyocytes via a FHF transcriptional state, as well as to the proepicardium. The JCF is located at the rostral confluence of the embryonic and extraembryonic mesoderm and may represent a subset of the forming septum transversum. Although the JCF can contribute to the FHF, it is unlikely to be the sole progenitor. It is



**Fig. 5. The JCF represents a cardiac progenitor population.** (A) (Top) Sagittal section of *Mespl::Cre;R26R-mTmG* embryos immunostained for  $\alpha$ -actinin and GFP ( $n = 6$  embryos). (Bottom) Section of *Nkx2-5::Cre;R26R-mTmG* embryos immunostained for SMARCD3 and GFP ( $n = 4$  embryos). Dashed regions highlight the JCF, revealing that it is derived from *Mespl*- but not *Nkx2-5*-expressing cells. (B) *Nkx2-5* expression levels plotted against the diffusion pseudotime of all cells consistent with the trajectory from Me5 to Me3. Points are colored according to their assigned cluster. *Nkx2-5* is up-regulated during the transition from JCF toward cardiomyocytes. The line is the quadratic local linear fit of the expression levels as a function of pseudotime. (C) Schematic diagrams highlighting the relative positions of Me3, Me4, and Me5 during rostral folding and the transition from stage 0 to stage 2 cardiac crescents. (D and E) Frames from time-lapse movie of an E7.5 *Nkx2-5::Cre;R26R-mTmG* embryo imaged over a 16-hour period with light-sheet microscopy. (D) JCF cells migrated from a more rostral position toward the developing crescent (blue spheres indicate representative tracked cells). A, anterior; P, posterior. (E) *Nkx2-5* was up-regulated by JCF cells, as

shown by an increase in the nuclear expression of GFP. Circles indicate the same cell tracked across time, shown in different channels. Representative cells also shown in movies S12 and S13. (F) (Left) *Mab21l2* gene expression levels are plotted against the diffusion pseudotime of all cells consistent with the trajectory from Me5 to Me3. Points are colored according to their assigned cluster. *Mab21l2* expression is down-regulated during the transition from the JCF toward cardiomyocytes. (Right) MIP of HCR on an early headfold stage (EHF) embryo, prior to crescent formation, revealing the early rostral expression of *Mab21l2* in JCF progenitors proximal to *Nkx2-5* and *Fst* expression. Three-dimensional rendering of expression is shown in movie S14. (G) *Tbx18* is expressed in multiple cell clusters. The percentages of *Tbx18*-positive cells from each cluster are shown as proportionally sized circles. (Right) MIP of HCR for *Tbx18*, *Mab21l2*, and *Nkx2-5* in a stage 2 embryo, highlighting location of *Tbx18* expression (brackets) (movie S6). Schematic at bottom highlights *Tbx18* expression domains in the anterior crescent region of the developing embryo.



**Fig. 6. JCF progenitors contribute to cardiomyocytes and epicardium.**

(A) Schematic showing the experimental design of lineage labeling experiments using a *Mab21l2-iCreERT2* transgenic mouse line. (Right) A schematic highlighting that labeling of the JCF between E7.75 and E8.0 leads to labeling of both cardiomyocytes (CM) and the epicardium (Epi) at E10.5. (B) MIP of a stage 1 *Mab21l2-iCreERT2;R26R-YFP* embryo immunostained for cardiac troponin T (cTnT) and YFP. The dashed line represents the location of the sagittal section at right. YFP-positive cells (arrowheads) are located in the JCF and do not express cardiomyocyte marker cTnT. (C) MIP of a looping heart tube from an E8.5 *Mab21l2-iCreERT2;R26R-YFP* embryo immunostained for  $\alpha$ -actinin and YFP. Dashed lines show locations of sagittal sections at

right. Arrowheads highlight the location of recombined YFP cells in the myocardium (2.) and maintained in the JCF progenitor region (1.). The dashed box (3.) shows a zoomed-in view of JCF-derived cardiomyocytes with sarcomeric striations of  $\alpha$ -actinin. OFT, outflow tract; IFT, inflow tract. (D) MIP of an E9.5 embryo immunostained for WT1 and YFP. WT1 marks both the nephrogenic cord and proepicardium (boxed). Below are high magnification views of the proepicardium highlighting the coexpression of YFP and WT1. (E and F) Immunostaining of E10.5 hearts showing coexpression of  $\alpha$ -actinin (E) and WT1 (F) with YFP-positive cells, highlighting the dual potential of the JCF. Dashed boxes represent zoomed-in regions. Dorsal and ventral views are shown.

currently unclear whether the JCF is a pool of unipotent progenitors of cardiomyocytes and proepicardium or contains bipotent cells capable of giving rise to both. Future clonal analyses are required to address this question. The significance of the JCF is supported by a previous study which showed that loss of function of the JCF marker *Mab21l2* leads to early embryonic lethality owing to a reduction in myocardium and proepicardium (34). Our work further indicates there are likely multiple populations contributing to the proepicardium, the JCF representing an early population, and the *Tbx18*-positive population of paraxial mesoderm (11–13), a later population.

By identifying the JCF, our work widens the cardiac progenitor region, identifies the earliest progenitors of the proepicardium, and provides insights into the lineal origins of cardiac cell types. This, combined with the detailed characterization of these cell types at the transcriptomic level, will contribute to a better understanding of the origin of congenital cardiac defects as well as provide basic insights for informing regenerative approaches to treating heart disease.

**Materials and methods summary**

All animal experiments complied with the U.K. Animals (Scientific Procedures) Act 1986, were approved by the local Biological Services Ethical Review Process, and were performed under U.K. Home Office project licenses PPL 30/3420 and PCB8EF1B4. Anterior embryonic regions were dissected manually, dissociated to single cells, and processed to obtain single-cell transcriptomes. Single-cell analyses, whole-mount immunohistochemistry, in situ hybridization chain reaction, volume imaging, light-sheet live microscopy, two-cell homologous recombination–CRISPR based genetic engineering, and genetic lineage labeling are described in detail in the supplementary methods.

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S.A.J. and J.G. generated the *Mab2l2-iCreERT2* transgenic line. P.J.K., J.C.M., and S.S. supervised the study. All authors read and approved the final manuscript. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** The *Mab2l2-CreERT2* mouse line is available from S.S. under a material transfer agreement with the University of Oxford. Raw sequencing data has been deposited in the European Nucleotide Archive database, under study PRJEB14363 (sample accession identifiers provided in data S3), and ArrayExpress, under accession E-MTAB-7403. The raw count matrices for the unbiased and reference datasets are provided in data S1 and S2, respectively. Associated sample metadata are available in data S3 and S4, respectively. Raw and processed data are also available as R data objects from <https://content.cruk.cam.ac.uk/jmlab/mouseEmbryonicHeartAtlas/>. All code used for data analysis, figure generation, and the shiny app is available at [https://github.com/Marionilab/mouseHeart\\_2020](https://github.com/Marionilab/mouseHeart_2020).

#### SUPPLEMENTARY MATERIALS

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## Characterization of a common progenitor pool of the epicardium and myocardium

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### Forming the early heart

The heart is the first organ to form during development and is critical for the survival of the embryo. The precise molecular identities of the various cell types that make up the heart during these early stages remain poorly defined. Tyser *et al.* used a combination of transcriptomic, imaging, and genetic lineage-labeling approaches to profile the molecular identity and precise locations of cells involved in the formation of the mouse embryonic heart. This approach allowed them to identify the earliest known progenitor of the epicardium, the outermost layer of the heart, which is an important source of signals and cells during cardiac development and injury.

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