Elucidating the Role of Uncharacterized Tinman-Positive Pericardial Cells in *Drosophila* Heart Development

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Abstract

Drosophila is an excellent model system for studying developmental biology because of its small size, short generation time, the abundance of eggs laid, and the plethora of genetic tools for the manipulation of gene expression in time and space. In addition, many genes that cause human heart diseases are found in *Drosophila* and play similar roles in heart function and development. We are studying heart development at high spatio-temporal resolution in the fruit fly by single cell RNA sequencing of cardiac cells and whole mount in situ hybridization. Specifically, we are focusing on novel genes involved in heart development in fly embryos to learn what stages of development these genes are expressed and, using mutational analysis, to what extent they are required for the formation and differentiation of cardiac tissue.

We have found new marker genes for a group of uncharacterized embryonic pericardial cells that express the cardiac master regulator Tinman (tin-positive pericardial cells, TPCs), but not Even- or Odd-skipped, the other markers for pericardial cells. These TPCs co-express a specific set of genes, including *cut, Wnt4, Nrt, Scb, CrzR,* and *DOR*. We are conducting immunofluorescent staining and imaging of fly embryos to visualize the protein and mRNA expression patterns of these marker genes in the entire embryo, the heart, and heart-associated tissues. In addition, we are characterizing these cells to learn about their cell-specific roles during heart development. For the purpose of studying these roles in heart development, we are knocking down or overexpressing them in the embryonic heart using heart-specific Gal4 driver lines and examining trans-heterozygous combinations of loss of function alleles for these genes.

By determining the gene regulatory program of the TPCs and characterizing in-depth the development and function of these pericardial cells, we hope to elucidate their role in Drosophila heart development.

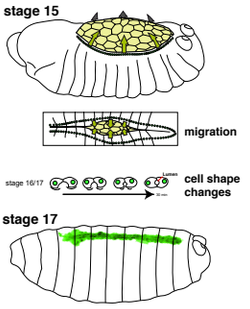
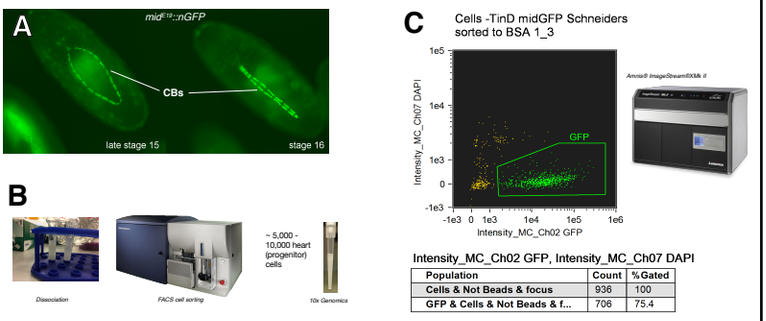


Figure 1. *Drosophila* heart morphogenesis. During final stages of embryonic development, cardioblasts (CBs; green dots) migrate towards the dorsal midline to form a linear heart tube. At stage 17 all CBs are aligned, and enclose a central heart lumen.

Single - Cell Sequencing of *Drosophila* Cardiac Cells



Population	Count	%Gated
Cells & Not Beads & focus	936	100
GFP & Cells & Not Beads & f...	706	75.4

Figure 2 Single-cell RNA sequencing of GFP-labeled cardiac cells. (**A**) The fluorescent *mid*^{E19}-GFP-reporter was used for cardiac cell isolation. GFP is strongly expressed in cardioblasts (CBs), as well as occasionally in pericardial cells (PCs). Note that weak expression is also found in muscle cells. (**B**) Embryos are dissociated, and cells are sorted using a FACS Aria II using optimized settings. About 10,000 GFP-positive cells undergo droplet-based single cells sequencing (using 10xGenomics pipeline). (**C**) FACS optimization included confirmation of high enrichment of intact GFP-positive cells using ImageStream.

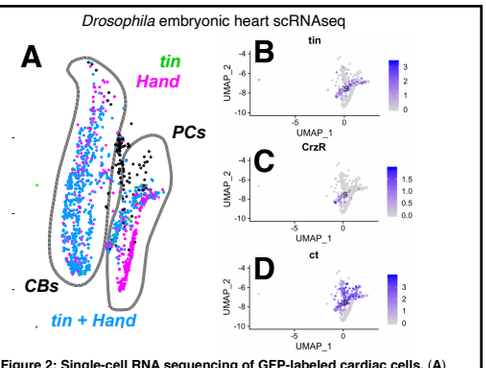


Figure 2: Single-cell RNA sequencing of GFP-labeled cardiac cells. (**A**) UMAP representation of two scSeq clusters from the entire data set, which represent Hand-positive cells subdivided into CBs and PCs. Note that one set of PCs is negative for the transcription factor Tinman. (**B-D**) Selection of markers that are expressed in the Tin-positive set of PCs. This data indicates that the G-protein coupled receptor for Corazonin (CrzR) and the transcription factor Cut are expressed in this subset of PCs.

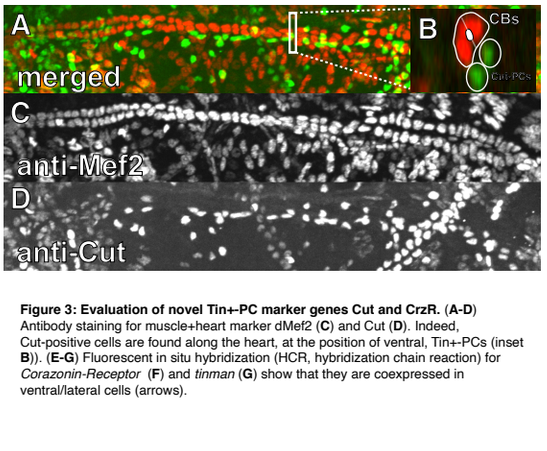
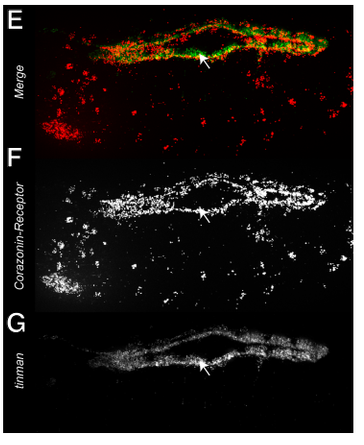
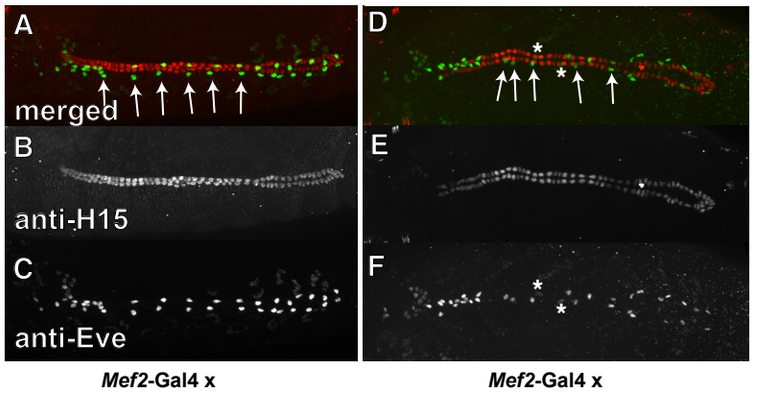


Figure 3: Evaluation of novel Tin+-PC marker genes Cut and CrzR. (**A-D**) Antibody staining for muscle+heart marker dMef2 (**C**) and Cut (**D**). Indeed, Cut-positive cells are found along the heart, at the position of ventral, Tin+-PCs (inset **B**)). (**E-G**) Fluorescent in situ hybridization (HCR, hybridization chain reaction) for *Corazonin-Receptor* (**F**) and *tinman* (**G**) show that they are coexpressed in ventral/lateral cells (arrows).





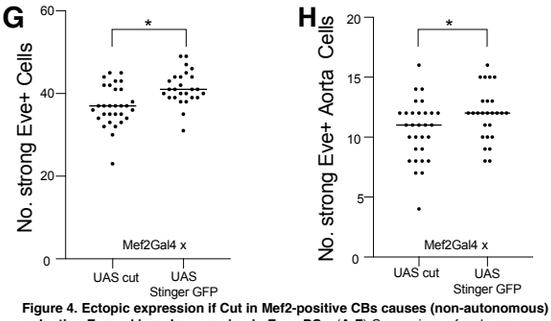


Figure 4. Ectopic expression if Cut in Mef2-positive CBs causes (non-autonomous) reduction Even-skipped expression in Eve+ PCs. (**A-F**) Comparison of embryos expressing nuclear GFP (StingerGFP) or Cut using the Mef2-Gal4 driver. Embryonic heart cells are immuno-labeled with anti-H15 (anti-Nmr1, labels CBs) and anti-Eve (arrows). Differences in strongly Eve-stained cells is indicated as asterisks (low Eve+ cells). (**G-H**) Cells from blinded genotypes were counted manually using ImageJ. Statical analysis of the number of Eve+ cells in the heart and specifically the aorta region shows significant differences in Eve+ cell number. P-values: (entire heart) 0.0007, (aorta) 0.0510; Mann-Whitney Test.

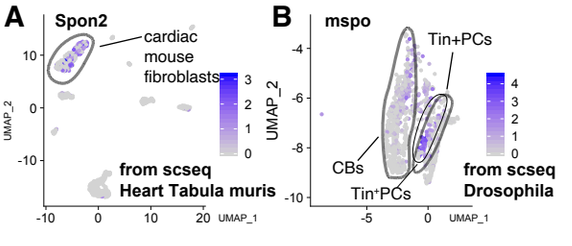


Figure 5. Cardiac mouse fibroblast gene *Spon2* and expression of its *Drosophila* ortholog *mspo* in Tin+-PCs. (**A**) Published single-cell sequencing data of mouse heart tissue (Tabula muris) with cardiac fibroblast cluster highlighted (circle). This cluster expresses the cell adhesion and ECM protein *Spon2*. (**B**) The *Drosophila* ortholog of *Spon2*, *mspo*, is strongly expressed in Tin+-PCs.

Discussion

The scSeq data revealed a cluster of pericardial cells that both expressed the cardiac transcription factors *tinman* and *Hand* (Figure 2a), and we hypothesize that these cells have a distinct role in heart development compared to other PCs, ie. Odd+PCs. Among these Tin+-PCs we found specific expression of the TF Cut and the GPCR CrzR (2B-D). We confirmed these results using in-situ hybridization and immunofluorescent staining (Figure 3) in wildtype embryos, highlighting the great potential of single-cell sequencing for the identification of novel cell and tissue markers.

As a first step towards identifying the role of these genes in heart development we overexpressed Cut in somatic and cardiac cells (using Mef2-Gal4) and found that there is a significant difference in the number of Eve-positive cells between the control line and the cut overexpression line (Figure 4). Specifically, there was a significant difference in the number of Eve-positive cells that line the aorta of the developing heart (Figure 4D). This leads to the hypothesis that Cut has the capacity to repress Eve-expression when expressed in Mef2+ cells, which is likely to be a non-autonomous effect that we will investigate further.

Comparison of Tin+-PC marker gene expression with published scRNAseq data from mouse cardiac cell types revealed that both express a number of ECM genes pointing towards a conserved function during heart formation. For example, the ECM genes *Spon2/mspo* are highly expressed in mouse cardiac FBs and fly TPCs (Figure 5). This leads us to hypothesize that TPCs are likely involved in cardiac fibroblast-related functions during heart formation and differentiation. Further experiments will be conducted to test the specific role of Tin+-PCs and their potential cardiac fibroblast-related functions, including analysis of mutants for Cut/CrzR.

References

Schaum, N., Karkanias, J., Neff, N.F. et al. Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. Nature 562, 367–372 (2018).

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