UNIVERSITY OF CALIFORNIA SAN DIEGO

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

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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Professor Deborah Yelon

2021

The thesis of Bill Kin Hing Hum is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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ABSTRACT OF THE THESIS

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With congenital heart disease as the most common birth defect in the world, investigating the genetic basis of heart development is an important endeavor that can be greatly advanced by studying the model organism *Drosophila melanogaster*. At the end of embryonic fly heart development, there are three types of pericardial cells (PCs) that are distinguished by their

gene expression profiles. Among these PCs, those that specifically express the cardiac master regulator *tinman* (*tin*) are not well characterized (Tin-PCs). In this study, we use single-cell RNA sequencing, fluorescent staining, and mutational analysis to characterize the role of Tin-PCs in embryonic fly heart development. We have identified and confirmed the expression of several genes—*CrzR*, *Neurotactin*, *Lachesin*, *coracle*, *sinuous*, *Wnt4*, and *cut*—that were either distinctly expressed or highly enriched in Tin-PCs, but not in other types of PCs. Here, we present that knockdown of *sinuous* delayed dorsal closure of the heart and epidermis, knockdown of *tin* reduced expression of the cell adhesion protein Neurotactin, and loss of *cut* caused abnormal closure of Tin-PCs while ectopic Cut expression in cardioblasts reduced expression of the PC marker *even-skipped*. From these findings, we conclude that Tin-PCs play some role in dorsal closure, cardiac cell adhesion, and *even-skipped* expression in PCs of the *Drosophila* embryo. By elucidating the role of Tin-PCs in *Drosophila* embryogenesis, we hope to highlight the great potential of single-cell RNA sequencing in identifying novel cell types and provide further understanding of the cellular and genetic mechanisms that underlie *Drosophila* heart development.

Introduction

Congenital Heart Disease (CHD) describes structural and functional anomalies that occur during embryonic and fetal heart development [1]. CHD is the most common developmental abnormality in the world, with an estimated heart defect in 8 out of 1,000 live births [1]. Some of the most common CHDs include atrioventricular and ventricular septal defects (incomplete separation of the heart chambers), patent ductus arteriosus (connection between the pulmonary artery and aorta that remains open instead of closed), and pulmonary stenosis (largely inhibited blood flow into the pulmonary artery) [1]. Although recent advancements in medicine improved the survival rates of CHD for infants in their first year of life to more than 75%, this has contributed to a growing population of adults living with the effects of CHD, such as heart failure, arrhythmias, and pulmonary hypertension later in life [2], [3]. Given the high frequency of heart defects at birth and the long-term complications of CHD, research on elucidating the genetic basis for CHD is an important endeavor in identifying the short and long-term genetic risk for CHD as well as for treating CHD patients.

While the genetic basis of CHD can be studied in humans, the genetic model organism *Drosophila melanogaster*, commonly known as the fruit fly, can act as an excellent experimental model for studying human disease. This is because of their small size, short generation time, abundance and frequency of eggs laid, and the plethora of tools available to manipulate the timing and spatial location of gene expression. More importantly, the fruit fly shares 75% of the genes associated with human diseases, including heart disease [4]. The high similarity in genetic mechanisms that underlie heart development and disease between flies and humans has paved the way for extensive research on the genetic basis of cardiogenesis. For example, previous studies identified the fly gene *tinman* (*tin*) as the transcription factor responsible for the

specification, proliferation, and differentiation of cardiac progenitor cells in many cardiac gene pathways; without sufficient *tin* expression, the embryonic fly heart will not form (the name refers to the Tin Man, who does not have a heart, in the film *The Wizard of Oz*) [5]. Similarly, the human homolog of *tin*, called *NKX2-5*, is a key transcription factor in human heart development [6]. The role of *tin/NKX2-5* in embryonic heart development is further supported in previous research that identified numerous mutations in *NKX2-5* that led to CHD [7]. In another study, the fly gene *H15* (also known as *neuromancer1/2*) was identified as transcription factors necessary for fly heart function; likewise, the human homolog of *H15*, known as *TBX20*, was found to be mutated in patients with CHDs [8].

While studying individual cardiac transcription factors such as *tin* and *H15* can provide mechanistic insight into monogenic CHDs (caused by variations in a single gene), learning how these genes interact with each other in a network can elucidate the mechanisms of oligogenic CHDs (diseases caused by the effects of multiple genes, such as hypoplastic left heart syndrome) [9], [10]. The mechanisms of these genetic interactions within a common pathway can be tested efficiently due to the simple genetic architecture of the *Drosophila* genome: in the fly genome, most genes have no paralogs and therefore lack genetic redundancy. To date, there are many genetic interactions characterized in signaling pathways by *Drosophila* enhancer and suppressor screens, such as the genes of the Notch, EGFR, and Wnt pathways, all of which play a role in both *Drosophila* and human heart development [6].

As previous studies elucidated the roles of individual cardiac genes and their genetic interactions in flies and humans, *Drosophila* has also been used to develop specific fly cardiac disease models and identify new cardiac risk factors. For example, dilated cardiomyopathy—a disease where the heart muscle is weakened and cannot pump blood well—was modeled in

Drosophila hearts through the CCR4-Not pathway: flies with a knockdown of *not3*, and other components of this pathway, demonstrated heart impairments that were subsequently also found in patients with dilated cardiomyopathies [11]. Ultimately, the genetic architecture of the fly genome and its similarity to humans highlight the usefulness and strength of the *Drosophila* model system for basic and disease-related research.

To better understand the genetic basis of how defects can occur in human heart development through the fly model system, it is necessary to understand how the *Drosophila* embryonic heart is formed. During fly embryogenesis, many signaling pathways that are also highly conserved and active in humans—such as the ones mentioned previously (e.g. Notch, EGFR, Wnt)—contribute to the specification of cardiomyocyte and pericardial precursor cells [6], [12], [13]. In the final stages of embryonic development, the precursor cell for cardiomyocytes, called cardioblasts (CBs), migrate towards the dorsal midline from two lateral regions of the mesoderm to fuse together to form the linear heart tube and lumen [12], [13] **(Figure 1)**. During this fusion process at the dorsal midline, the CBs are arranged in a specific, repetitive fashion depending on its gene expression: four CBs that express the gene *tin* followed by two CBs that express the gene *seven-up* [14]. The *tin*-positive CBs and *seven-up*-positive CBs will eventually differentiate into the contractile cardiomyocytes and the ostial cells that form the inflow tract, respectively [15]. Following the dorsal midline closure of the CBs, the newly formed heart becomes attached to the alary muscle cells and the pericardial cells [15]. The resulting heart is a linear tube that consists of contractile cardiomyocytes and associated non-contractile cells, the pericardial cells [16].

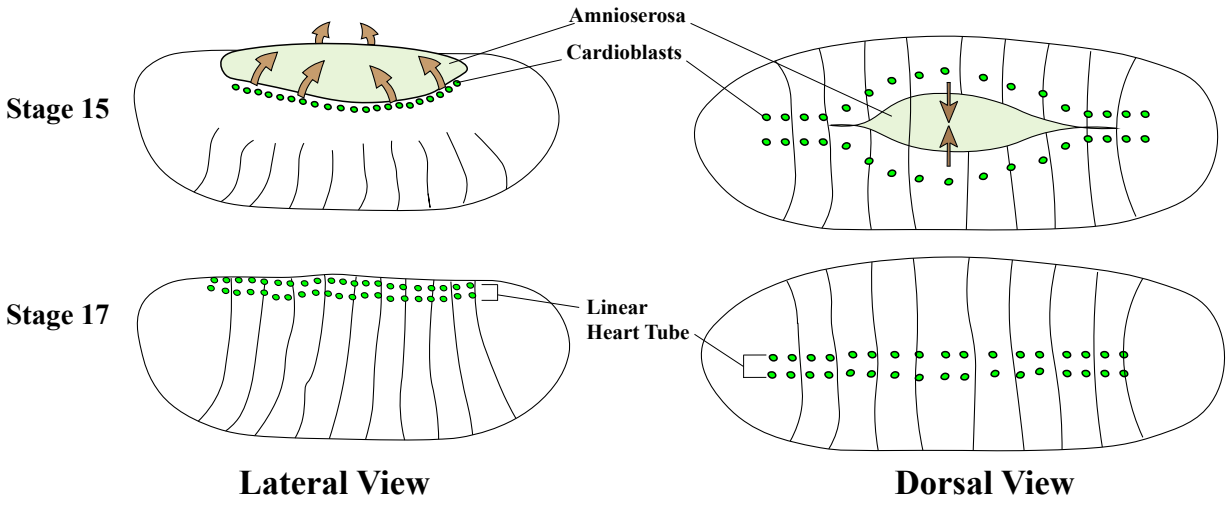

Figure 1. Schematic of *Drosophila* heart morphogenesis.
In the final stages of embryonic development, cardioblasts (green dots) migrate towards the dorsal midline to form a linear heart tube. At stage 17, all cardioblasts align and enclose a central heart lumen. The left side of the embryo is anterior; the right side of the embryo is posterior.

Just as the cardiomyocyte precursor cells play a role in fly cardiogenesis, the embryonic pericardial cells also contribute to heart development, depending on what type of pericardial cell it is. The three types of embryonic pericardial cells can be distinguished by the expression of the genes *even-skipped* (*eve*), *odd-skipped* (*odd*), and *tinman* (*tin)*, respectively [17]. Previous studies have suggested that a lack of *eve*-positive pericardial cells contributes to a significantly lower heart rate and higher susceptibility to heart failure in the adult fly [18]. Additionally, it was also found that the *odd* gene is present in many mesodermal and ectodermal cells of the embryo, suggesting an important role in embryonic development [19].

Currently, there is not much known about the specific role of *tin*-positive pericardial cells (Tin-PCs) in embryonic development, unlike the *tin*-positive cardioblasts discussed earlier. With recent advancements in single-cell RNA sequencing technology, it is now possible to identify and further study various cell types, such as Tin-PCs, in the *Drosophila* embryo in greater detail. This is because single-cell RNA sequencing can provide a comprehensive view of the transcriptome in individual cells. With this technology, we performed single-cell sequencing on

embryonic cardiac cells and were able to distinguish between the *odd* and *tin* expressing types of embryonic pericardial cells based on their gene expression profiles **(Figure 2)**. Furthermore, we identified new marker genes that were expressed in the Tin-PCs, but not in *odd*-positive pericardial cells [20]. These marker genes include *Corazonin receptor* (*CrzR*), *Lachesin* (*lac)*, *Sinuous* (*sinu*), *Coracle* (*cora*), *Neurotactin* (*Nrt*), and *cut* (*ct*).

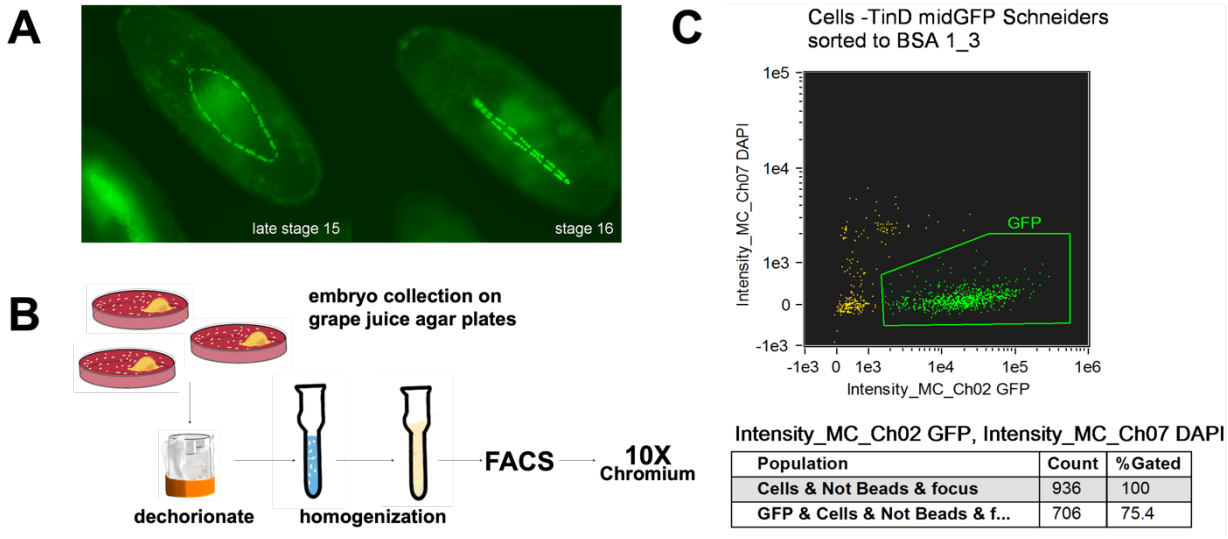

Figure 2. Schematic of single-cell RNA sequencing.
(A) Representative stage 15 and 16 embryos homozygous for *mid*^{E19}::GFP. GFP is strongly expressed in cardioblasts and pericardial cells.
(B) Schematic for collecting embryos for single-cell RNA sequencing. Embryos were homogenized and the resulting cells were isolated and sorted for strong GFP expression using fluorescence-activated cell sorting (FACS) Aria II before being loaded on a 10X Genomic Chromium chip for sequencing.
(C) Plot of individual cells collected from FACS.

Among these Tin-PC marker genes, *lac*, *cora*, and *sinu* are septate junction proteins that play a role in mediating adhesion between pericardial cells and cardiomyocytes [21]. Without sufficient expression of these genes, the embryonic heart loses its structural integrity, aptly giving rise to the "*broken heart*" phenotype [21]. Similarly, previous research suggested that *Neurotactin* (*Nrt*) is a cell adhesion receptor that is strongly expressed during central nervous system development, yet interestingly we also found high *Nrt* expression levels in Tin-PCs from

our single cell sequencing data [22]. The homeodomain transcription factor Cut (*ct)*, important for cell specification of the peripheral nervous system and the eye, was also found to be highly and specifically expressed in Tin-PCs throughout cardiogenesis [23], [24]. Additionally, we found expression of the mouse ortholog of *Gnrhr*, called *Corazonin Receptor* (*CrzR*), in Tin-PCs. *Gnrhr* is highly expressed in mouse cardiac fibroblasts, a type of cell that is involved in extracellular matrix and connective tissue formation [25]. Similarly, the fly gene *M-spondin* (*mspo*), which is an ortholog of the cardiac fibroblast mouse gene *Spon2*, was also found to be highly expressed in Tin-PCs **(Figure 3)**. While there is research done on these genes in development, it is still unclear as to what their specific roles are in Tin-PCs.

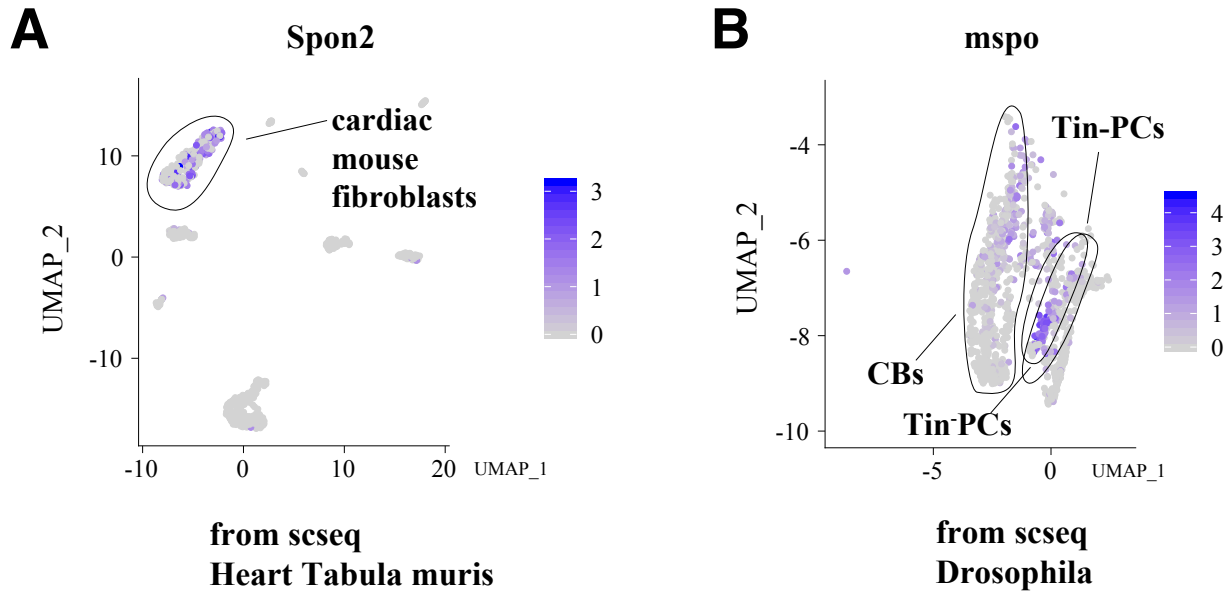

Figure 3. Expression of cardiac mouse fibroblast gene *Spon2* and its *Drosophil*a ortholog *mspo.*
(A) Public single-cell sequencing data of mouse heart tissue from Tabula muris with cardiac fibroblast cluster highlighted (circle). This cluster expresses the cell adhesion and ECM protein Spon2.
(B) The *Drosophila* ortholog of Spon2, mspo, is strongly expressed in Tin-PCs.

In this study, we aim to elucidate the cell specific roles of the Tin-PC marker genes during embryonic heart development. To do so, we utilized tools such as RNAi fly lines and the

UAS-Gal4 system—which allowed us to express genes in tissues of interest—to conduct mutational analysis of Tin-PC marker genes [26]. Given the current knowledge of Tin-PCs and their marker genes, we hypothesize that they may have an important role for the formation and integrity of the developing heart, maybe similar to that of cardiac fibroblasts in vertebrate heart development. By investigating these genes in the context of Tin-PCs, we hope to provide further understanding of how Tin-PCs fit into the cellular and genetic mechanisms that underlie *Drosophila* embryonic heart development.

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Materials and Methods

A. *Drosophila* Stocks and Maintenance

All fly stocks were maintained at 25°C in vials containing standard fly food mixture (corn meal, malt, molasses, salt, yeast, agar, and water).

Table 1. List of *Drosophila* Stocks

Simplified Genotype	Full Genotype	Library	Stock #/RRID	Source
GD Control	*w*^{1118}	N/A	60000	VDRC
tinD::Gal4; midE19::GFP	*tinD::Gal4; midE19::GFP*	N/A	N/A	R. Bodmer
lac^{Df}	*w[1118]; Df(2R)BSC305/CyO*	N/A	BDSC_23688	BDSC
lac^{Bg}	*w[1118]; P{w[+mGT]=GT1}Lac[BG01462]/CyO*	N/A	BDSC_14577	BDSC
cora^{2}	*y[1] w[*]; P{y[+t7.7]=y.FRT.GAL4}52B cora[2]/CyO*	N/A	BDSC_58805	BDSC
cora^{5}	*w[*]; P{ry[+t7.2]=neoFRT}43D cora[5]/CyO*	N/A	BDSC_52233	BDSC
twi-Gal4	*P{GAL4-twi.G}108.4*	N/A	BDSC_914	BDSC
UAS-*tin*-RNAi^{GDv12656}	UAS-*tin*-RNAi^{GDv12656}	GD	12656	VDRC
UAS-*tin*-RNAi^{GDv32510}	UAS-*tin*-RNAi^{GDv32510}	GD	32510	VDRC
UAS-*tin*-RNAi^{KKv101825}	UAS-*tin*-RNAi^{KKv101825}	KK	101825	VDRC
ct^{db7}	y1 w* ctdb7/FM7c, P{ftz-lacC}YH1	N/A	BDSC_78562	BDSC
ct^{c145}	y[1] w[1] ct[C145]/FM3/Dp(1;Y)ct[+]y[+]	N/A	BDSC_6946	BDSC
Wnt4$^{[EMS23]}$	Wnt4[EMS23] bw[1]/CyO, P{ry[+t7.2]=HB-lacZ}GS1	N/A	BDSC_6650	BDSC

Table 1. List of *Drosophila* Stocks, Continued

Simplified Genotype	Full Genotype	Library	Stock #/RRID	Source
UAS-*stinger GFP*	w[1118]; P{w[+mC]=UAS-Stinger}2	N/A	BDSC_84277	BDSC
UAS-*cut*	UAS-*cut*	N/A	F004505	FlyORF
Mef2-Gal4	P{GAL4-Mef2.R}3	N/A	BDSC_27390	BDSC

B. Embryo Collection and Fixation

Flies were kept in cages containing grape juice agar plates with yeast paste at 25°C. To obtain appropriately aged embryos (up to early stage 17), plates were changed at 5:00 PM and collected again the next morning at 10:00 AM. The embryos were transferred into a collection basket and then dechorionated in bleach (6.25%) for three minutes. Afterwards, embryos were transferred into a fixative solution (0.5 mL heptane, 0.25 mL 10% formaldehyde, 0.25 mL 2X PBS) on a shaker for 20 minutes. Afterwards, the fixative layer was aspirated and 0.5 mL of methanol was added. The collection tube was then vortexed for 30 seconds to remove the vitelline membrane. Finally, the solution was aspirated and rinsed twice with methanol prior to a one-hour wash in methanol on a shaker.

C. Single-Cell Sequencing

The single-cell sequencing protocol was carried out as described in "Single-cell sequencing of the Drosophila embryonic heart and muscle cells during differentiation and maturation" [20]. In short, embryos collected from grape juice agar plates were homogenized to obtain a cell suspension. The cells underwent FAC sorting and processed on a Chromium 10X Chip for transcript labelling. Library preparation and sequencing were done using the manufacturer's protocol (10x Genomics).

D. In Situ Hybridization Chain Reaction (HCR)

HCR protocol was optimized based on the protocol described by Choi et al [27]. Fixed embryos were rinsed with ethanol four times in a 1.5 mL tube before washing in 0.5 mL ethanol and 0.75 mL xylenes on a shaker for an hour. Next, embryos underwent a 3 x 5 minute wash of ethanol, 2 x 5 minute wash of methanol, 1 x 5 minute wash of one part methanol and one part PBST (0.1% Tween 20 in 1X PBS), 1 x 10 minute wash of PBST, 2 x 5 minute wash of PBST, 1 x 7 minute incubation in 4 µg/mL proteinase K solution, 2 x 5 minute wash of PBST, 1 x 25 minute post-fixation in 4% formaldehyde solution, and a 5 x 5 minute wash of PBST. The embryos were then hybridized in a probe solution at 37°C overnight (16-24h). The next day, embryos underwent 4 x 15 minute washes with probe wash buffer at 37°C and 2 x 5 minute washes of 5X SSCT (0.1% Tween 20 in 5X SSC). The embryos were then incubated in a fluorescent hairpin solution in the dark at room temperature overnight (16-24h). On the third day, embryos were washed with 5X SSCT (2 x 5 minute, 2 x 30 minute, 1 x 5 minute) then stored in 1X PBS at 4°C away from light. All probes and hairpins were ordered from Molecular Instruments, Inc.

Table 2. List of HCR Probes

HCR Probes	**Probe Type**
Puratrophin-1-Like	B2
tinman	B1
CG6415	B2
CrzR	B2
lachesin	B2
d2eGFP	B3

Table 3. List of HCR Fluorescent Hairpins

Hairpin Fluorophore	Target
488	B1
594	B1
647	B1
594	B2
647	B2
488	B3

E. Immunohistochemistry

Fixed embryos were washed in 0.3% Triton in 1X PBS three times for 15 minutes followed by a 1h wash on a shaker. Afterwards, the wash solution was aspirated, and the primary antibody solution was added to the embryos in a tube. The embryos were then left on a shaker for 16-24 hours overnight at 4°C. The next day, the primary antibody solution was aspirated and underwent 3 x 40 minute washes with 0.3% Triton in 1X PBS on a shaker. Afterwards, the wash solution was aspirated and the secondary antibody solution was added to the embryos in a tube for 2 hours on a shaker protected from light. Finally, the secondary antibody solution was aspirated and the embryos underwent 3 x 40 minute washes with 0.3% Triton in 1X on a shaker and protected from light. Embryos were then stored in 1X PBS at 4°C prior to mounting and imaging. Primary antibody concentration varied depending on the antibody. All secondary antibody concentrations were 1:500 and ordered from Jackson ImmunoResearch Labs, Inc.

Table 4. List of Primary Antibodies

Primary Antibodies	Host Animal	Concentration	Source
Mef2	Mouse	1:1000	B. Paterson
Zfh1	Rabbit	1:500	R. Lehmann
Neurotactin (BP 106)	Mouse	1:50	DSHB
Tinman	Rabbit	1:1500	M. Frasch
Cut (2B10)	Mouse	1:10	DSHB
H15	Guinea Pig	1:2000	J. Skeath
Even-skipped	Rabbit	1:3000	M. Frasch
GFP-1020	Chicken	1:400	Aves Labs

Table 5. List of Secondary Antibodies

Secondary Antibodies	Animal Target	Host Animal	Concentration
Alexa 594	Mouse	Goat	1:500
Alexa 594	Rabbit	Goat	1:500
647	Mouse	Goat	1:500
647	Rabbit	Goat	1:500
647	Guinea Pig	Goat	1:500
488	Mouse	Goat	1:500
488	Chicken	Goat	1:500

F. Embryo Mounting & Imaging

Embryos were mounted onto microscope slides with the dorsal side of the embryo facing up for the view on the embryonic heart. Embryos were mounted in Prolong Gold and cured overnight prior to imaging. Images were taken using confocal microscopy and analyzed using FIJI/ImageJ.

G. Quantification and Statistics

Experiments involved with cell counting was counted on FIJI/ImageJ. To categorize differently observed phenotypes, images of embryos were blinded and sorted on FIJI/ImageJ. Statistical analyses were performed on Prism 8.

Results

A. Single Cell Sequencing and Confirmation

Embryos homozygous for *mid*^{E19}::GFP had GFP strongly expressed in the cardiac cells, and showed low expression in other, non-cardiac cell types **(Figure 2A)**. These embryos were used in the single-cell sequencing protocol, and their cells were isolated and sorted based on GFP signal. All of the single cell transcriptomic data were then plotted on a UMAP **(Figure 4A)**. Each point on this plot represents a single cell, and every point is clustered in a way such that the closer two points are to each other, the more similar their transcriptomes are. As such, each grouping of cells can be considered as a cell type. The cardiac cell types were identified based on which cell clusters strongly expressed the genes *tin* and *Hand*, two important cardiac transcription factors necessary for heart development **(Figure 4B)** [12], [28]. Among the cardiac cell type cluster, the pericardial cells were identified based on how strong the cardiac cells expressed *pericardin*, a marker gene of pericardial cells [29]. The clusters for pericardial cells appear to be split into two groups, with one of these groups specifically expressing *tin*. This suggests that the cluster that specifically express *pericardin* and *tin* represent the Tin-PCs.

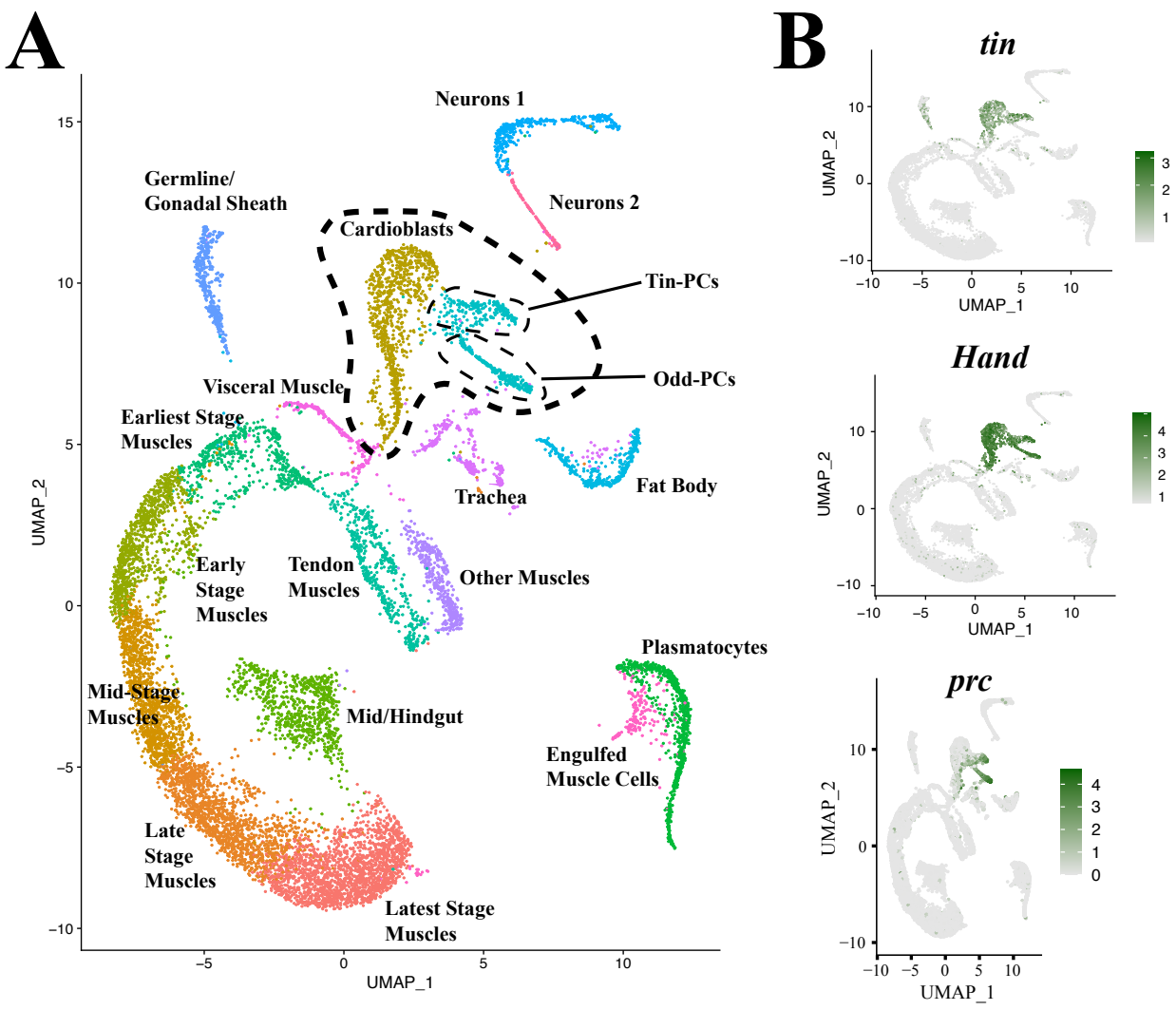

Figure 4. Single-cell sequencing UMAP plot of *mid*^{E19}::GFP cells.
(A) UMAP Plot of the gene expression profiles from sorted GFP-positive cells. Circled groups include the cardiac cells that highly expressed GFP, including the cardioblast and pericardial cells.
(B) UMAP Plot of the gene expression patterns used to identify the cardioblast and pericardial cell clusters (*tin*, *Hand*, *prc*).

To confirm if the transcriptomic data obtained from single-cell sequencing reflects what is truly expressed in each cell cluster, the genes *Puratrophin-1-like* (*Pura*), *CG6415*, and *CrzR* were analyzed in wildtype embryos through *in-situ* hybridization chain reaction [27]. Based on the single-cell sequencing data, *Pura* is expressed specifically in the cardioblasts and odd-PCs **(Figure 5A)**, *CG6415* is specifically expressed in fat body cells **(Figure 5B)**, and *CrzR* is

specifically expressed in Tin-PCs **(Figure 6A)**. Based on the stained embryo images, it appears that *Pura* is indeed specifically expressed in Odd-PCs due to its position bilateral to the closing heart tube **(Figure 5A')**. Similarly, *CG6415* can be seen expressed in the fat body of the embryo **(Figure 5B')** and *CrzR* in Tin-PCs **(Figure 6A')**, highlighting the consistency of the single-cell sequencing transcriptomic data with various *Drosophila* cell types.

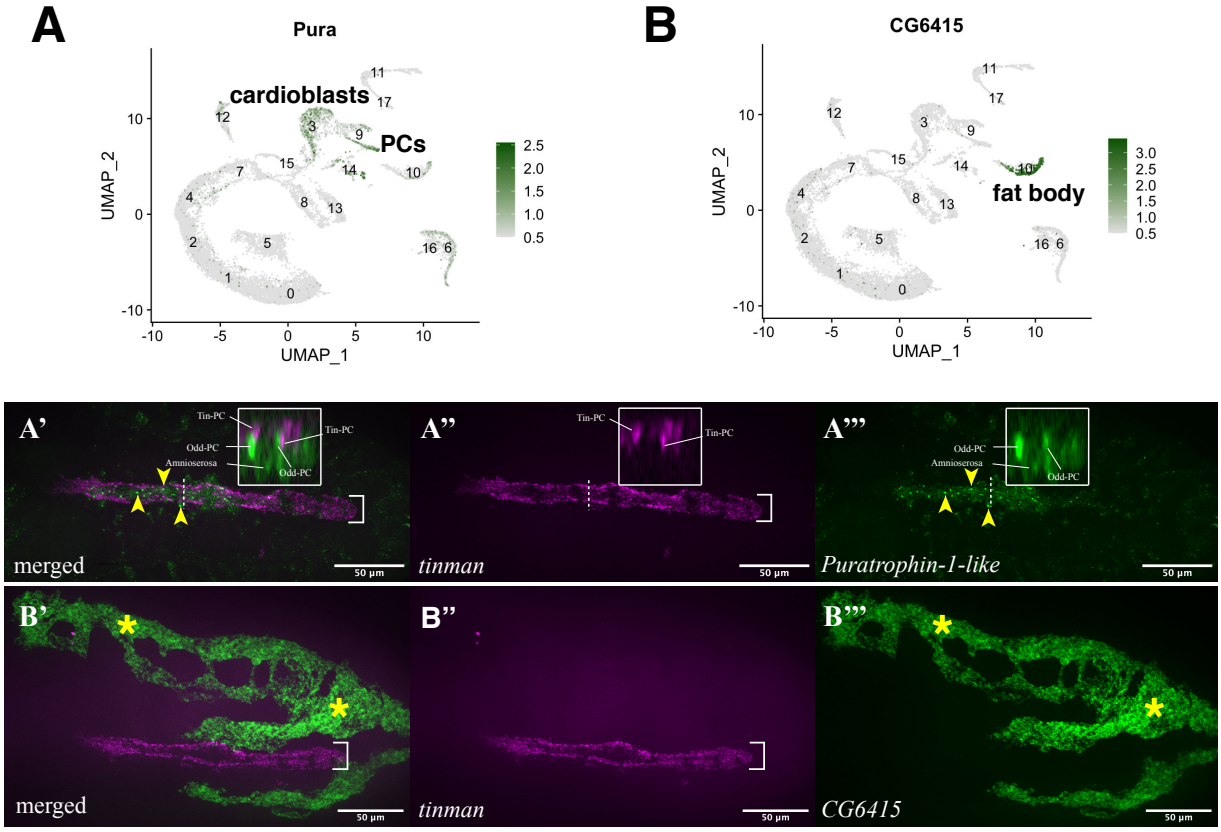

Figure 5. Confirmation of single-cell sequencing analysis for marker genes of cell clusters.
(**A)** Single-cell sequencing analysis revealed the gene *Puratrophin-1-like* (*Pura*) to be specifically expressed in various cell clusters, including Odd-PCs.
(A'-A''') In-situ hybridization chain reaction fluorescently labeling *tinman* (bracket) and *Pura* mRNA (arrowheads) in wildtype embryos. Box provides a cross-sectional view of a slice at the dashed line.
(B) Single-cell sequencing analysis revealed the gene *CG6415* to be specifically expressed in the fat body cell cluster.
(B'-B''') In-situ hybridization chain reaction fluorescently labeling *tinman* (bracket) and *CG6415* mRNA (arrowheads) in wildtype embryos. Asterisks indicates fat body.

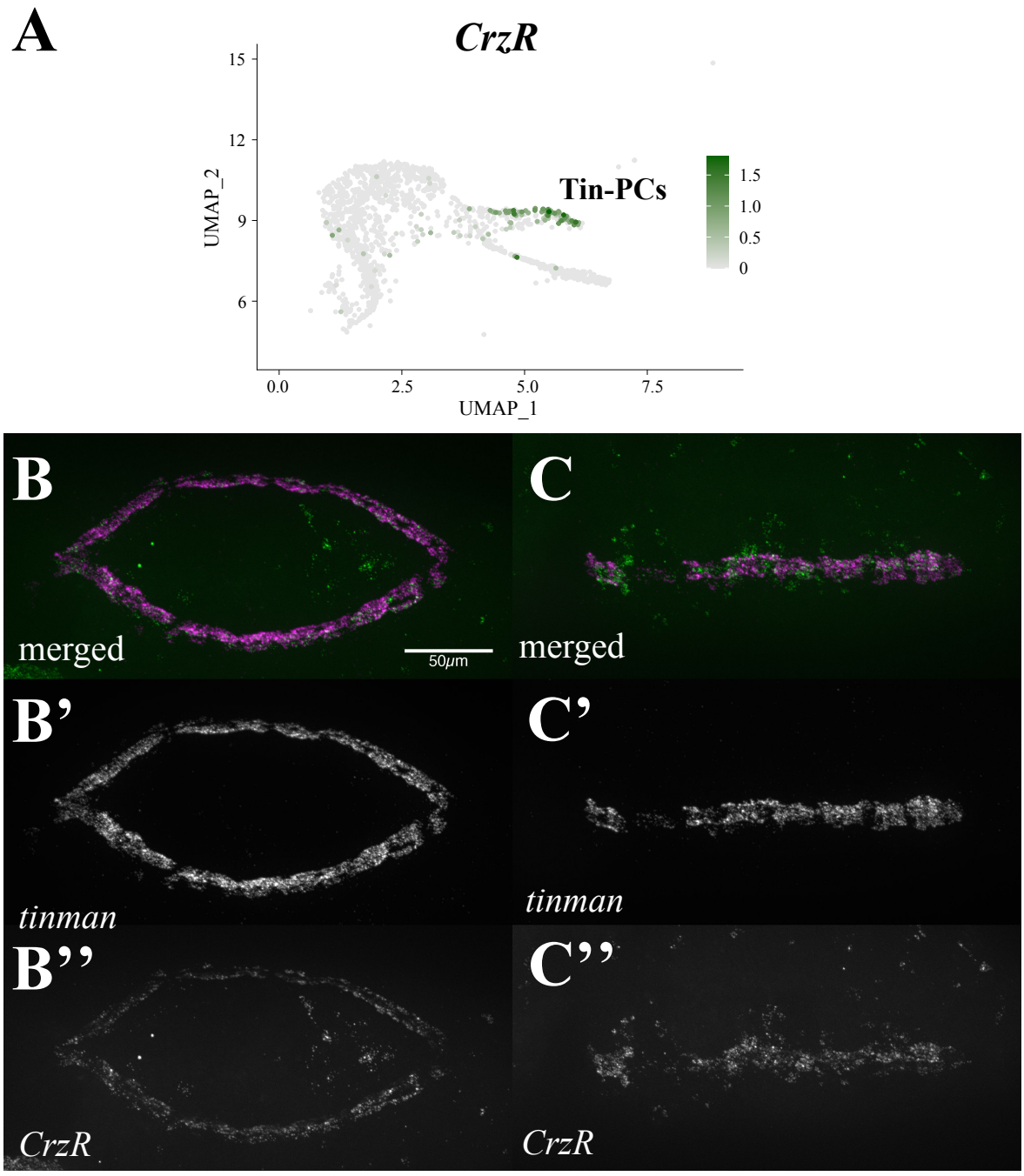

Figure 6. Confirmation of single-cell sequencing analysis for the Tin-PC marker gene *CrzR*. **(A)** UMAP plot of the gene expression profile for CrzR shows that *CrzR* is a marker gene for Tin-PCs. In-situ hybridization chain reaction fluorescently labeled *tinman* (magenta) and *CrzR* (green) mRNA in stage 15 **(B-B'')** and late stage 16 **(C-C'')** embryos.

B. Investigating the Role of Septate Junction Proteins in Tin-PCs

To understand how Tin-PCs are localized during embryogenesis, immunostainings were conducted to visualize the proteins Mef2, Zfh1, and Nrt **(Figure 7, 8)**. With Mef2 labeling cardioblasts and somatic muscle nuclei and Zfh1 labeling pericardial cell nuclei, a combined single sliced Z-stack image was produced to provide a cross-sectional view of the relative positions of each cardiac cell to each other **(Figure 7B-B')**. Given that Nrt expression is highly enriched in Tin-PCs, the Tin-PCs were distinguished from Odd-PCs based on where Nrt was expressed. As a result, the imaging suggests that the Tin-PCs are localized ventral to the closing heart tube. Additionally, Nrt is not localized in the nuclei of Tin-PCs; instead, it is seen surrounding pericardial cells throughout the heart tube, which is consistent with Nrt's role as a cell adhesion receptor in nervous system development **(Figure 8)** [22]. We therefore conclude that Nrt appears to play a role in adhesion between pericardial cells as well.

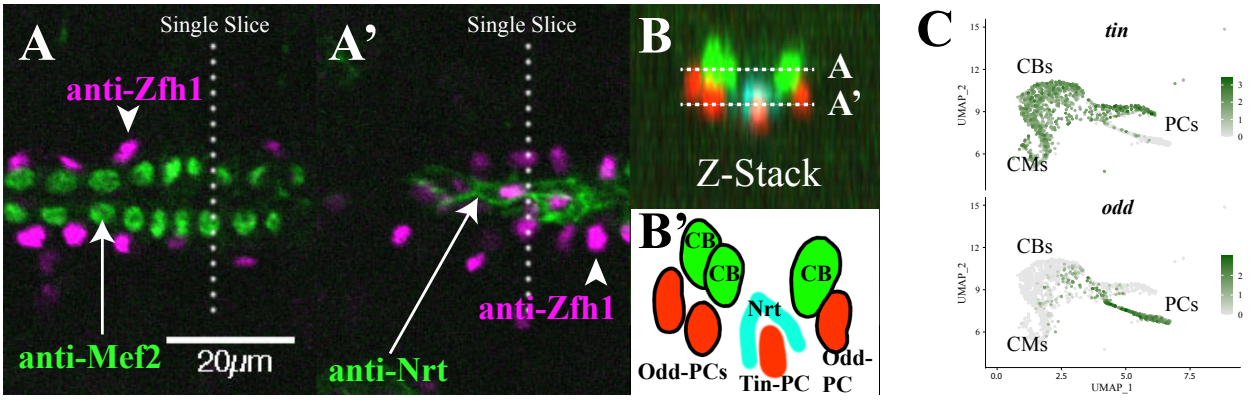

Figure 7. Tin-PCs localize ventrally to the closing heart tube
(A) Wildtype embryo staining of Mef2 (green; arrow) and Zfh1 (magenta; arrowhead) depict the localization of cardioblasts and pericardial cells, respectively.
(A') Wildtype embryo staining of Nrt (green; arrow) and Zfh1 (magenta; arrowhead) depict high expression of Nrt by the Tin-PCs.
(B) Combined Z-Stack view of Mef2, Zfh1, and Nrt staining reveals Tin-PCs localizing ventrally to the heart tube, whereas Odd-PCs localize laterally.
(B') Schematic drawing of the combined Z-Stack.
(C) UMAP plot depicting the gene expression of tin and odd in cardioblasts (CBs), cardiomyocytes (CMs), and pericardial cells (PCs). The cluster of PCs that express *tin* only are the Tin-PCs and the cluster of PCs that express *odd* only are the Odd-PCs.

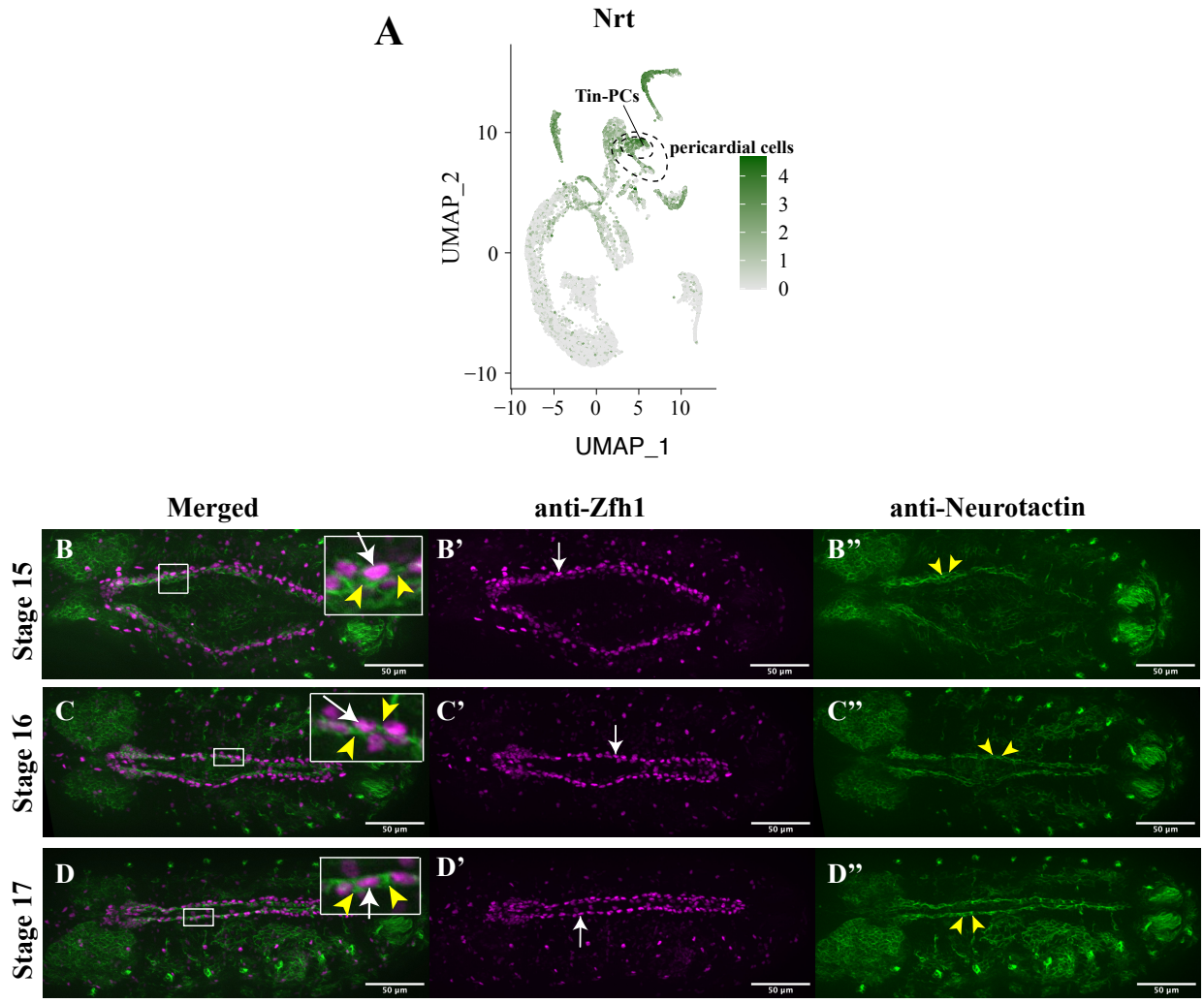

Figure 8. Wildtype description of Neurotactin expression in late-stage embryos. **(A)** UMAP plot depicting the gene expression of *Nrt* in multiple cell clusters including cardioblasts (CBs) and Tin-PCs. Stage 15 **(B-B'')**, Stage 16 **(C-C'')**, and Stage 17 **(D-D'')** wildtype embryos were immunostained with Zfh1 (magenta) and Neurotactin (green) antibodies. Arrows label nuclei of pericardial cells expressing Zfh1; arrowheads label Neurotactin protein in between these cardiac cells.

The Tin-PC markers *lachesin* and *coracle* were previously identified as septate junction proteins involved in the adhesion between cardiac cells and pericardial cells [21]. Because of this, we investigated whether these Tin-PC marker genes have any genetic interactions with Nrt given their similarities in cell adhesion function. First, the expression of *lac* in Tin-PCs was confirmed by in-situ hybridization chain reaction **(Figure 9A-A''')**. As seen in the image, there is expression of *lac* surrounding the developing heart. Next, two homozygous *lac* mutant lines

(*lac*^{Df} and *lac*^{Bg}), a heterozygous mutant of the deficiency mutated allele (*lac*$^{Df/+}$), and the trans-heterozygous mutant (*lac*$^{Df/Bg}$) were immunostained with Zfh1 to label pericardial cells and then Nrt **(Figure 9B-F'')**. Among every type of mutant, there was no visible differences in localization nor levels of Nrt compared to wildtype embryos: in each representative embryo, the Nrt protein is present between each pericardial cell of the embryonic heart. Furthermore, the structure of the embryonic heart does not appear compromised in any of the *lac* mutants. Similarly, *cora* also does not appear to present any visible phenotype in pericardial cell assembly in homozygous, (*cora*^{2} and *cora*^{5}), heterozygous (*cora*$^{2/+}$), nor trans-heterozygous mutant lines (*cora*$^{2/5}$) **(Figure 10B-F'')**. While previous research identified *lac* and *cora* to be important for maintaining the adhesion between pericardial cells and cardioblasts late in heart development, our results suggest that a deficiency in *lac* and *cora* expression may not affect the assembly of Tin-PCs during heart formation.

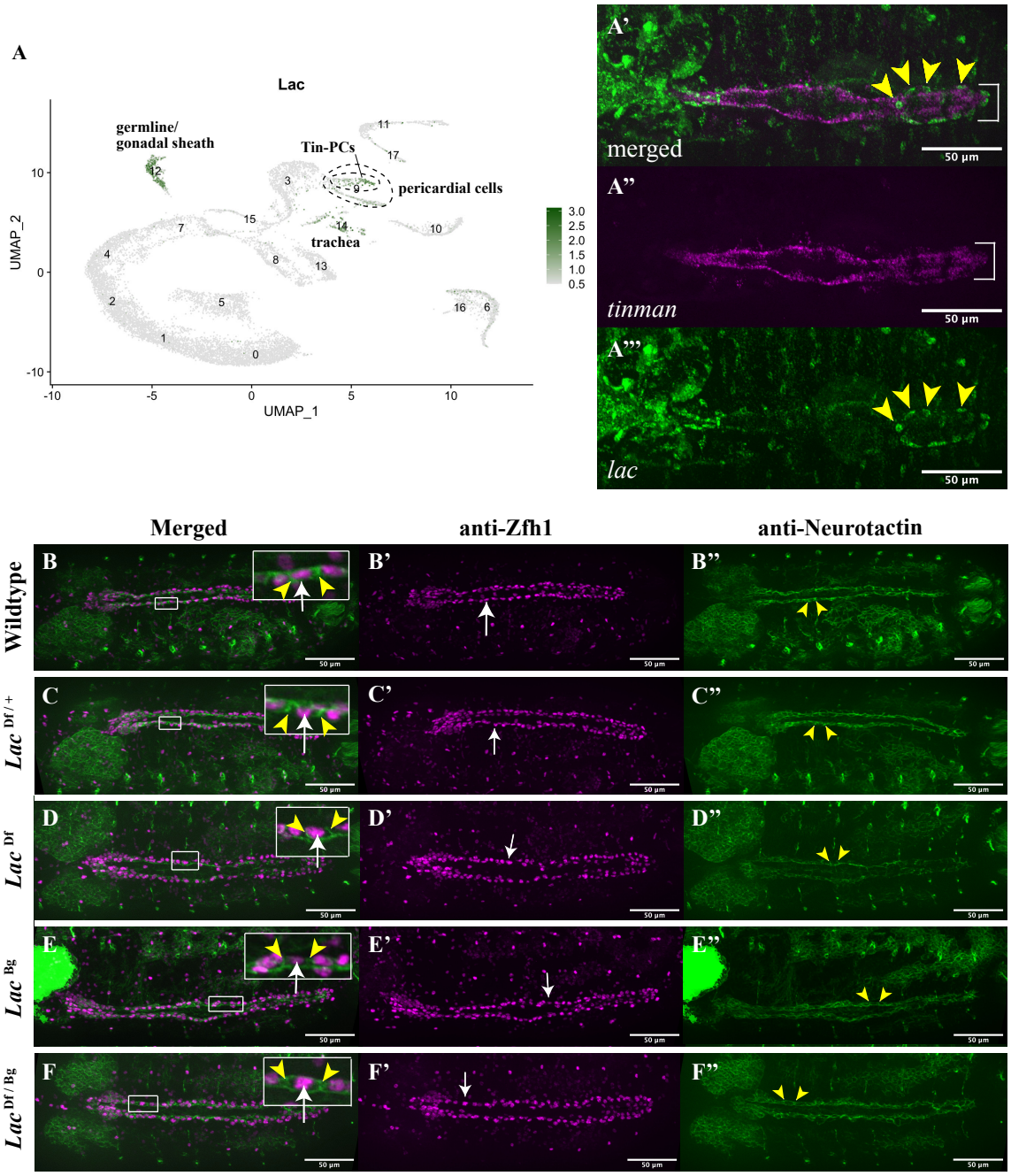

Figure 9. Lachesin is likely not required for the assembly of Tin-PCs during embryonic heart development.

(A) Single-cell sequencing analysis revealed the gene *lachesin* (*lac*) to be specifically expressed in several cell clusters, including Tin-PCs. **(A'-A''')** In-situ hybridization chain reaction fluorescently labeling *tinman* (magenta; bracket) and *lac* (green; arrowheads) mRNA in wildtype embryos. Wildtype **(B-B'')**, heterozygous **(C-C'')**, homozygous **(D-E'')**, and trans-heterozygous *lac* mutant **(F-F'')** embryos were immunostained with anti-Zfh1 (magenta) and anti-Neurotactin (green) antibodies. Arrows label nuclei of pericardial cells expressing Zfh1; arrowheads label Neurotactin protein in between these cardiac cells.

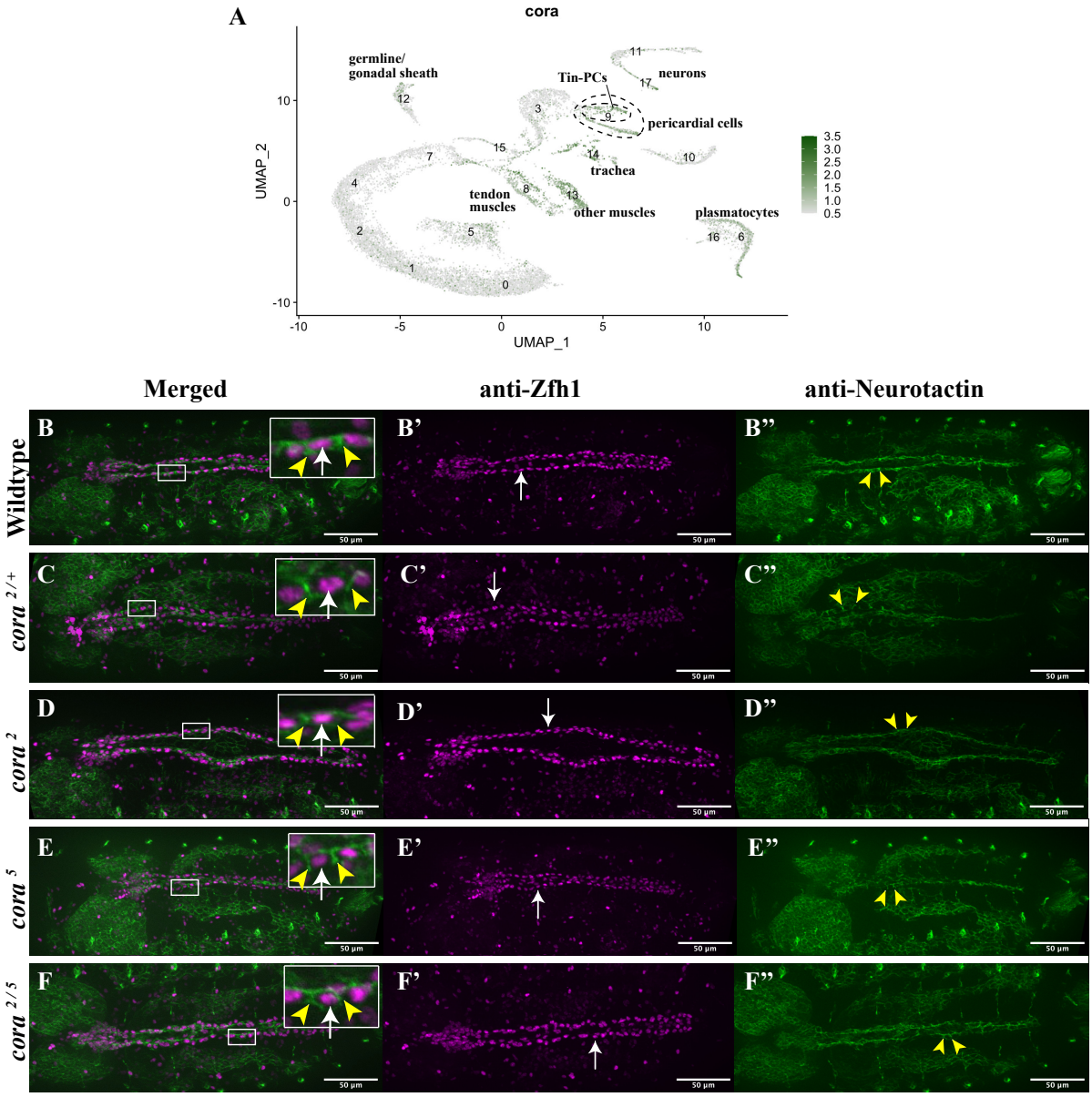

Figure 10. Coracle is likely not required for the assembly of Tin-PCs during embryonic heart development.

(A) Single-cell sequencing analysis revealed the gene *coracle* (*cora*) to be specifically expressed in several cell clusters, including Tin-PCs. Wildtype **(B-B'')**, heterozygous **(C-C'')**, homozygous **(D-D'', E-E'')**, and trans-heterozygous *cora* mutant **(F-F'')** embryos were immunostained with anti-Zfh1 (magenta) and anti-Neurotactin (green) antibodies. Arrows label nuclei of pericardial cells expressing Zfh1; arrowheads label neurotactin protein in between these cardiac cells.

In addition to the septate junction proteins Lachesin and Coracle, the septate junction protein Sinuous was also shown to contribute to the adhesion between cardiac cells and pericardial cells [21]. Just as *lac* and *cora* mutant embryos do not seem to visibly affect how Nrt is localized in the embryo, *sinuous* (*sinu*) mutant embryos do not as well. However, embryos that are homozygous and trans-heterozygous mutants for the Tin-PC marker *sinu* appear to have a delay in the dorsal closure of the heart **(Figure 11D-F)**. Based on the morphology of the hindgut and midgut (yellow outlined circles; **Figure 11D-E**), the embryonic heart should have enclosed a central heart tube at the dorsal midline at this developmental stage (as described in **Figure 1**). This phenotype was also seen in heterozygous mutant embryos ($sinu^{nwu7/+}$); however, there wasn't a significant enough of a difference when comparing these mutants to wildtype embryos **(Figure 11B-C, F)**.

Because the dorsal closure of the heart was delayed in homozygous and trans-heterozygous *sinu* mutants, we examined whether the dorsal closure of the epidermis was also delayed by staining for Armadillo, an epidermis marker **(Figure 12)**. All wildtype embryos had a proper dorsal closure of the epidermis, and there was no significant difference between the wildtype embryos and the heterozygous *sinu* mutants. However, just as the homozygous and trans-heterozygous *sinu* mutants were found to have a delay in the dorsal closure of the heart, a delay in the dorsal closure of the epidermis was observed in these mutants as well. These findings suggest that the Tin-PC marker gene *sinu* is important in the process of dorsal closure during embryogenesis.

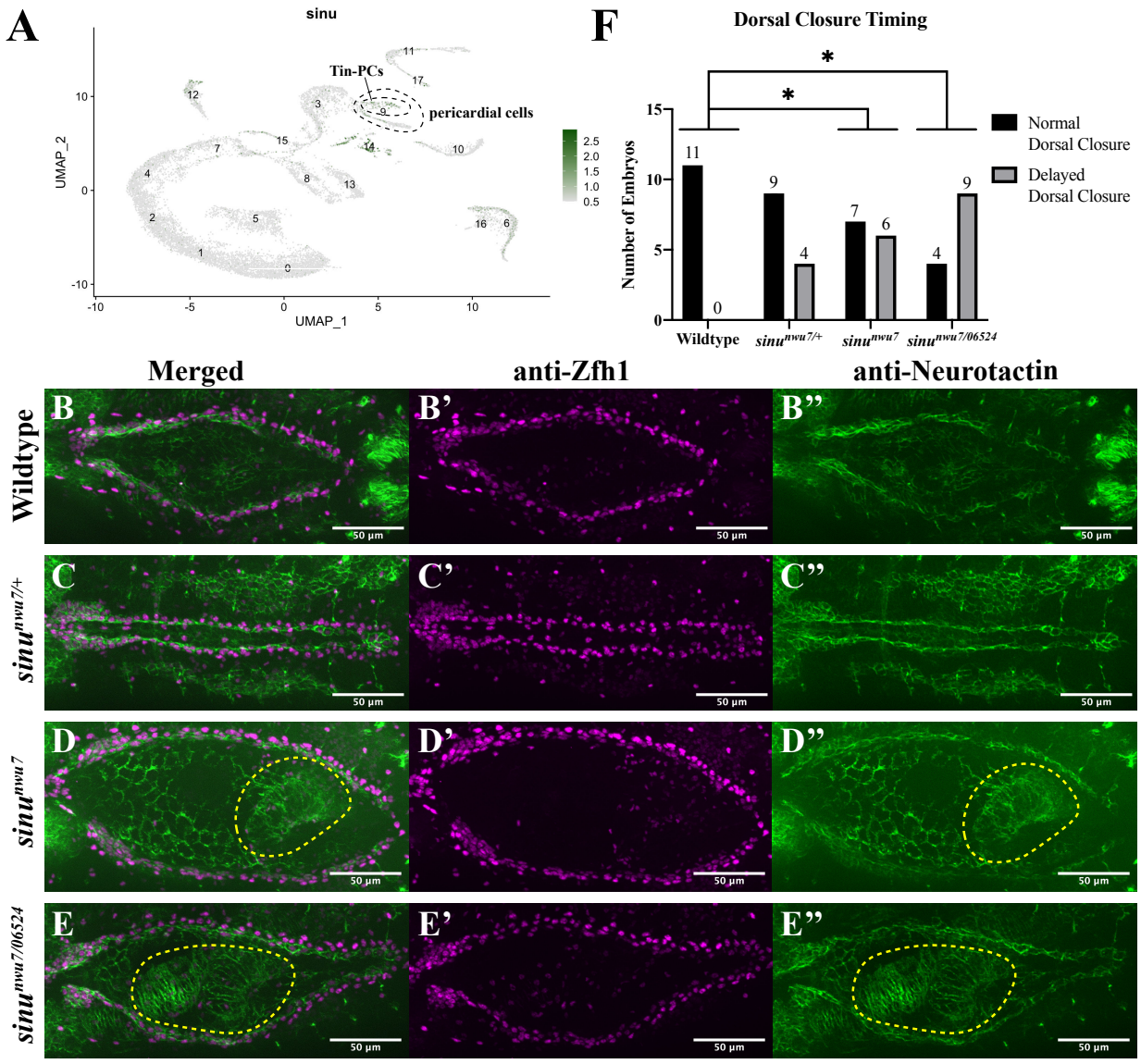

Figure 11. Sinuous is required for the dorsal closure of the embryonic heart.
(A) Single-cell sequencing analysis revealed the gene *sinuous* (*sinu*) to be specifically expressed in several cell clusters, including Tin-PCs. Wildtype **(B-B'')**, heterozygous **(C-C'')**, homozygous **(D-D'')**, and trans-heterozygous *sinu* mutant **(E-E'')** embryos were immunostained with anti-Zfh1 (magenta) and anti-Neurotactin (green) antibodies. Yellow dotted circles highlight the developed hind and midgut.
(F) Images were sorted into categories based on either normal dorsal closure or delayed dorsal closure. Statistical analysis shows a significant difference between the control group and the homozygous *sinu* mutants (p-value = 0.0162; Fisher's exact test) as well as the trans-heterozygous *sinu* mutants (p-value = 0.0006; Fisher's exact test). No significant difference was found between the control group and the heterozygous *sinu* mutants (p-value = 0.0983; Fisher's exact test).

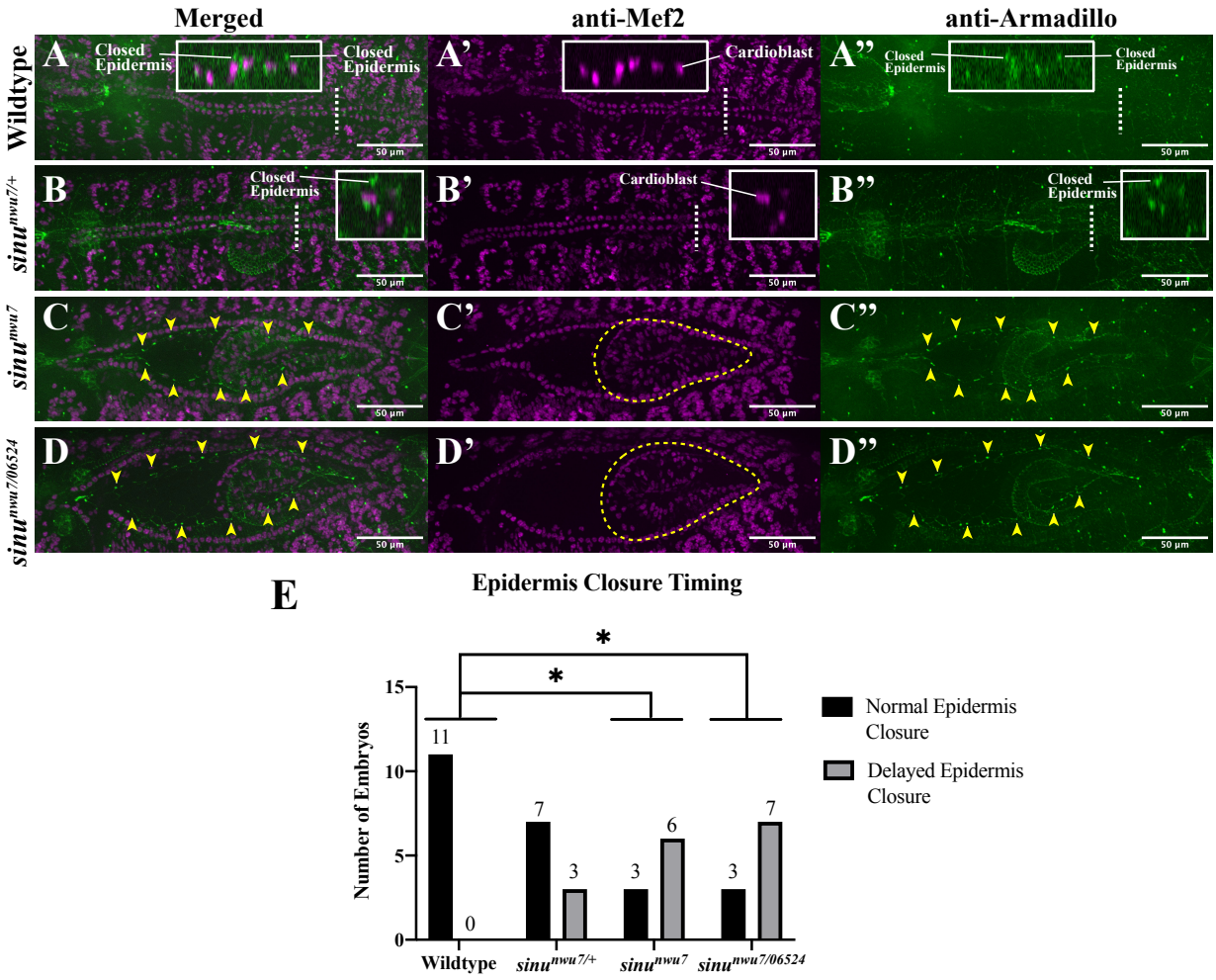

Figure 12. Sinuous is required for the dorsal closure of the epidermis.
Wildtype **(A-A'')**, heterozygous **(B-B'')**, homozygous **(C-C'')**, and trans-heterozygous **(D-D'')** *sinu* mutants were immunostained with anti-Mef2 (magenta; somatic and cardiac cell marker) and anti-Armadillo (green; epidermis marker). Box provides a cross sectional view of a slice at the dotted vertical line. Yellow arrowheads indicate the open epidermis in homozygous and trans-heterozygous *sinu* mutants. Dotted circles indicate the developed gut.
(E) Images were sorted into categories based on either normal or delayed epidermis closure. Statistical analysis showed a significant difference between the wildtype and homozygous *sinu* mutants (p-value = 0.0022; Fisher's exact test) and a significant difference between the wildtype and trans-heterozygous *sinu* mutants (p-value = 0.0010; Fisher's exact test). No significant difference was found between the wildtype and heterozygous *sinu* mutants (p-value = 0.0902; Fisher's exact test).

In addition to probing for genetic interactions of *lac*, *cora*, and *sinu* with *Nrt*, the interactions between *tin* and *Nrt* was also tested by RNA interference. To do so, the UAS-Gal4

system was utilized to selectively knockdown *tin* expression in the cardiac mesoderm **(Figure 13A-D'')**. Among the three *tin*-RNAi lines used, only progeny from two of the crosses demonstrated a significant knockdown of *tin* expression in the cardiac mesoderm as determined by the anti-Tinman staining when compared to the control group **(Figure 13E)**. This suggests that each of these *tin*-RNAi fly lines have varying *tin* knockdown efficiencies. Furthermore, only progeny from one of the crosses demonstrated a significant decrease in *Nrt* expression in the cardiac tissue when compared to the control group **(Figure 13F)**. Given that the UAS-*tin*-RNAi^{GDv32510} line had both Tin and Nrt significantly knocked down, yet progeny from the UAS-*tin*-RNAi^{GDv12656} line only had Tin protein levels significantly reduced, it is possible that there is a genetic interaction between *tin* and *Nrt* where the transcription factor Tinman controls expression of *Nrt,* and that *tin* expression needs to be reduced beyond a certain threshold before *Nrt* expression is affected. Indeed, this is consistent with a chromatin immunoprecipitation study for the binding of Tinman where a ChIP peak was identified inside the first large intron of *Nrt*, which provides further evidence of the direct interaction between *tin* and *Nrt.* [30].

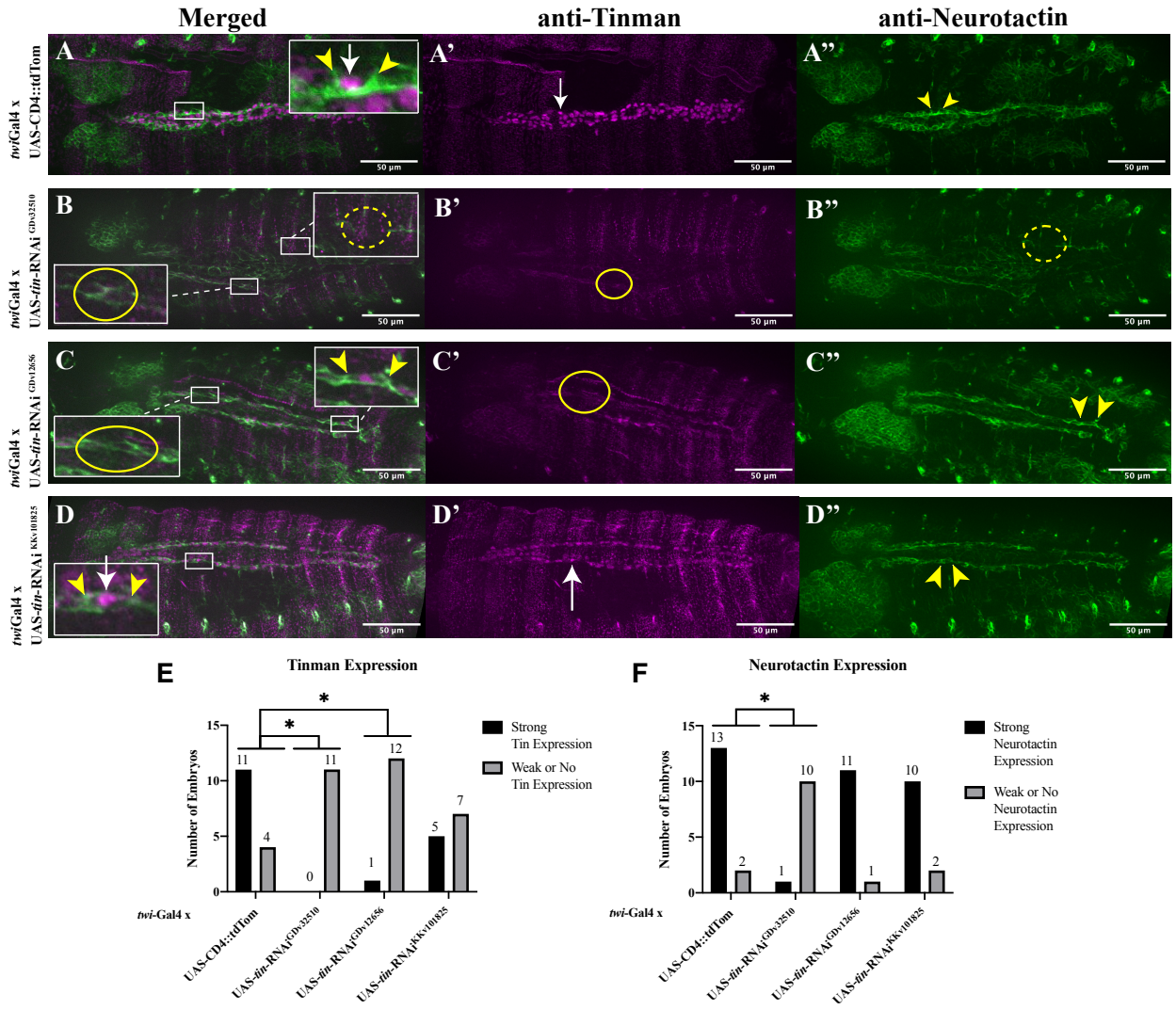

Figure 13. Tinman regulates the expression of the Tin-PC marker gene Neurotactin.
(A-D'') Immunostained representative images comparing the Tinman and Neurotactin expression between the control group **(A-A'')** and three different *tinman* RNAi fly lines **(B-B'', C-C'', D-D'')**. Embryonic heart cells are immuno-labeled with anti-Tinman antibodies (arrows) and the cell adhesion protein Neurotactin is labeled with anti-Neurotactin antibodies (arrowheads). Weak or no expression of Tinman and Neurotactin is indicated inside of circles and dashed circles, respectively.
(E-F) Number of embryos that show strong Tinman expression **(E)** and strong Neurotactin expression **(F)** was measured. Images were blinded and sorted into categories of either strong expression or weak/no expression. Statistical analysis shows a significant difference between the control group and the *twi*-Gal4 x UAS-*tin*-RNAi^{GDv32510} for Tinman and Neurotactin expression (p-value = 0.0002 for both; Fisher's exact test), a significant difference between the control group and *twi*-Gal4 x UAS-*tin*-RNAi^{GDv12656} for Tinman expression only (p-value = 0.0006; Fisher's exact test), and no significant difference between the control group and *twi*-Gal4 x UAS-*tin*-RNAi^{KKv101825}.

C. Investigating the Role of the Homeobox Transcription Factor Cut in Tin-PCs

The single-cell sequencing results showed the homeobox transcription factor *cut* to be highly expressed in Tin-PCs **(Figure 14A)**. To confirm this finding, immunostaining of Cut and Mef2 was conducted in the *tinD*::Gal4; *mid*^{E19}::GFP embryos **(Figure 14B-E)**. Additionally, another immunostaining of Cut and H15 (another marker of cardioblasts) in late staged wildtype embryos was conducted to provide further confirmation of *cut* expression in Tin-PCs **(Figure 15)**. Indeed, *cut* can be seen expressed in cells ventral to Mef2-labeled cardioblasts; given our finding that Tin-PCs are oriented ventral to the closing heart tube **(Figure 7)**, these images confirmed that the cells labeled by *cut* in the heart region are Tin-PCs, and that ultimately *cut* is a Tin-PC marker gene.

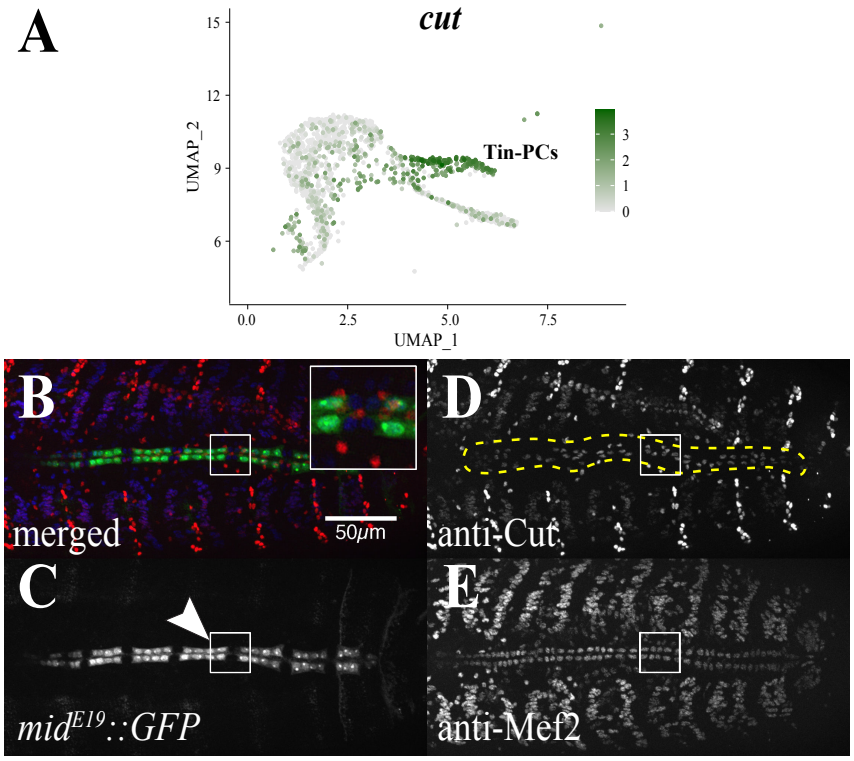

Figure 14. Confirmation of single-cell sequencing analysis for the Tin-PC marker gene *cut*.
(A) UMAP plot showed that *cut* is a marker gene for Tin-PCs.
(B-E) Immunohistochemistry staining for GFP (green), Cut (red), and Mef2 (blue) confirms cut expression in Tin-PCs. The *mid*^{E19}::GFP was expressed in cardiac cells (arrowhead) and Cut was expressed in Tin-PCs (circled) ventral to the Mef2-labeled cardioblasts.

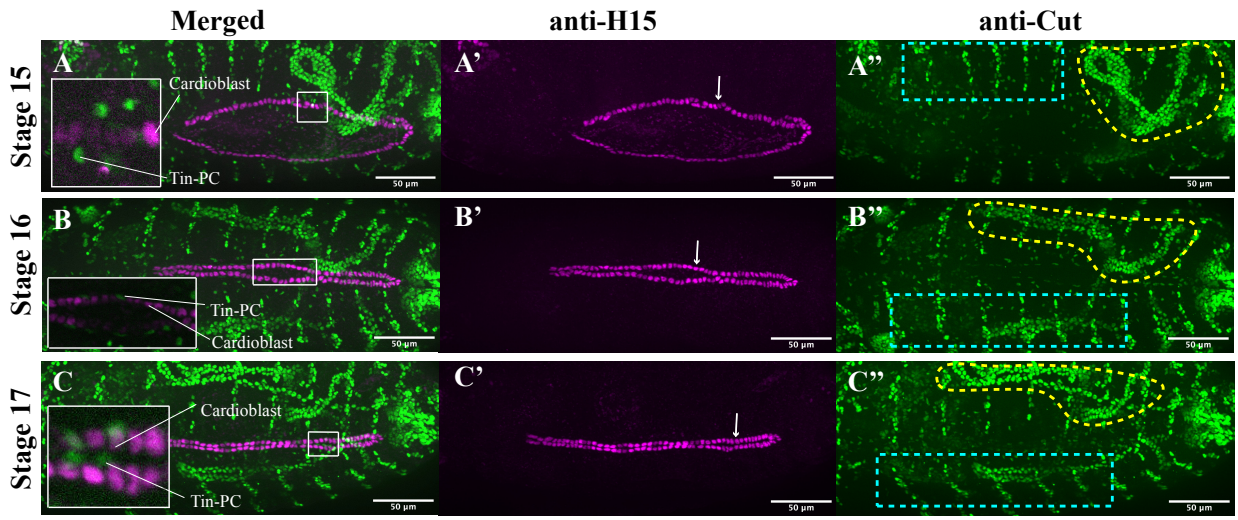

Figure 15. Wildtype description of Cut expression in late stage embryos.
Stage 15 **(A-A'')**, Stage 16 **(B-B'')**, and Stage 17 **(C-C'')** wildtype embryos were immunostained with H15 (magenta) and Cut (green) antibodies. Arrows label nuclei of cardioblasts expressing H15. Single-slice view in **A**, **B**, and **C** depict expression of Cut in Tin-PCs. Yellow dotted circles indicate Malpighian tubules and blue dotted rectangles indicate cells that will become part of the external sensory organs.

Just as *lac*, *cora*, and *sinu* were investigated for any potential genetic interactions with *Nrt*, we examined whether *cut* (*ct*) had any effect on *Nrt* expression or localization by immunostaining two different *ct* mutants with anti-Zfh1 and anti-Nrt **(Figure 16)**. Like the results from our *sinu* analysis, there was no visible effect on Nrt expression or localization in either *ct* mutant. However, the embryos hemizygous for ct^{db7} demonstrated an abnormal dorsal closure of Tin-PCs **(Figure 16B-B'')**. As shown in **Figure 1**, both the anterior and posterior ends of a wildtype embryonic heart align at the dorsal midline first, followed by the middle region of the heart. However, ct^{db7} mutant embryos showed a significant difference in this pattern: in the representative mutant shown, the middle region of the heart appears to be closed first, but the anterior and posterior ends have not. While this phenotype was seen to be significantly different between wildtype and ct^{db7} mutants, mutants for ct^{c145} were not significantly different from wildtype embryos.

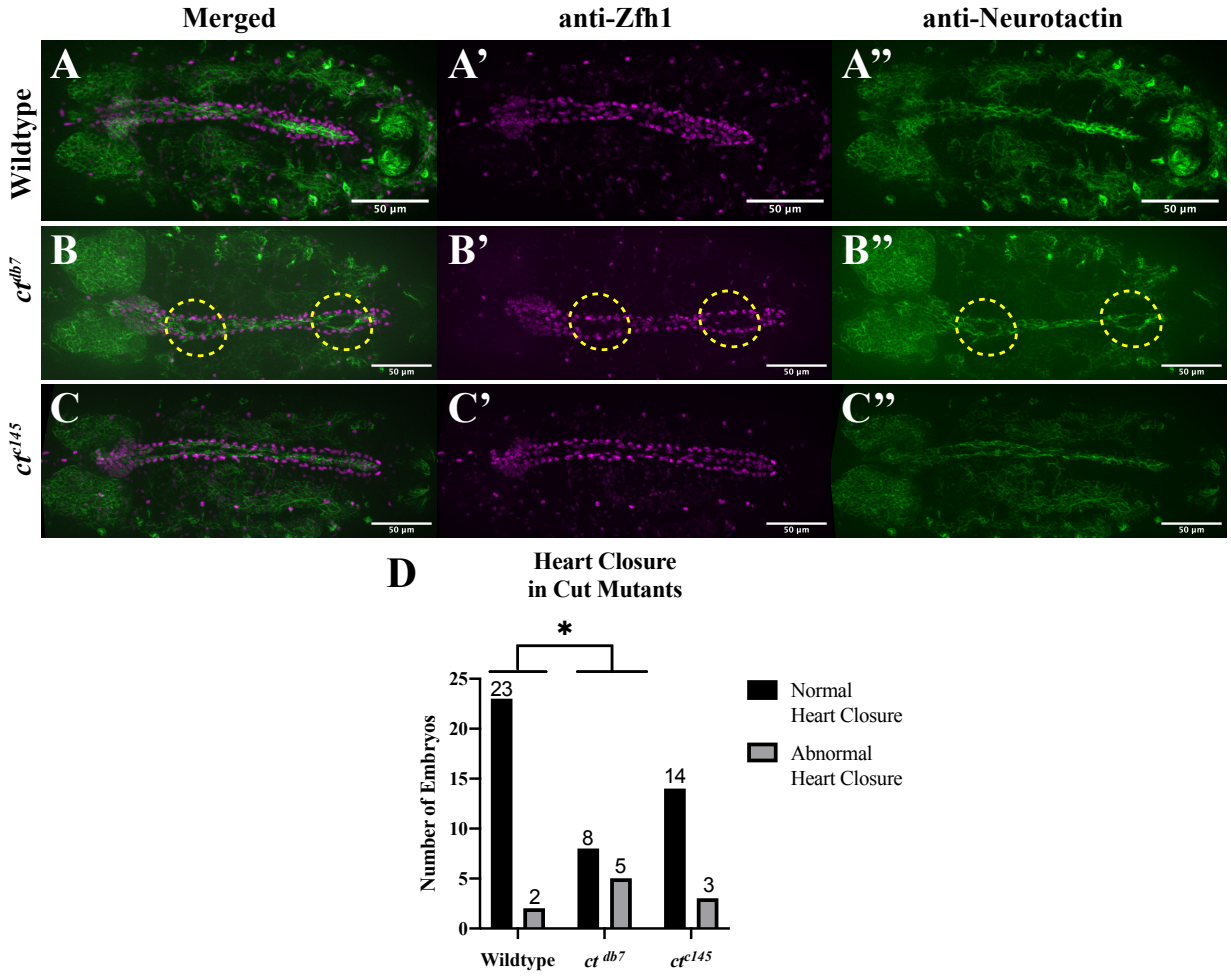

Figure 16. Cut regulates the dorsal closure of the heart.
Wildtype **(A-A'')** and two different hemizygous *cut* (*ct*) mutants **(B-B'', C-C'')** were immunostained with anti-Zfh1 (magenta) and anti-Neurotactin (green).
(D) Images were sorted into categories based on either normal or abnormal heart closure. Statistical analysis showed a significant difference between the wildtype and hemizygous ct^{db7} mutants (p-value = 0.0341; Fisher's exact test) but no significant difference between the wildtype and hemizygous ct^{c145} mutants (p-value = 0.3795; Fisher's exact test).

In addition to *cut*, the signaling pathway ligand *Wnt4* was also revealed to be highly expressed in Tin-PCs **(Figure 17A)**. Given *Wnt4*'s role in cardiac specification in the Wnt4 pathway, we investigated whether Wnt4 signaling occurred upstream of *cut.* To probe for any genetic interactions between *cut* and *Wnt4*, homozygous *Wnt4* mutant embryos were immunostained for the cardioblast marker H15 and Cut **(Figure 17B-C'')**. Through these images, the number of Cut-positive heart cells (i.e. Tin-PCs) were compared with wildtype

embryos; however, there was no significant difference in the number of Tin-PCs between wildtype and homozygous *Wnt4* mutant embryos **(Figure 17D)**, suggesting that *Wnt4* has no effect on *cut* expression.

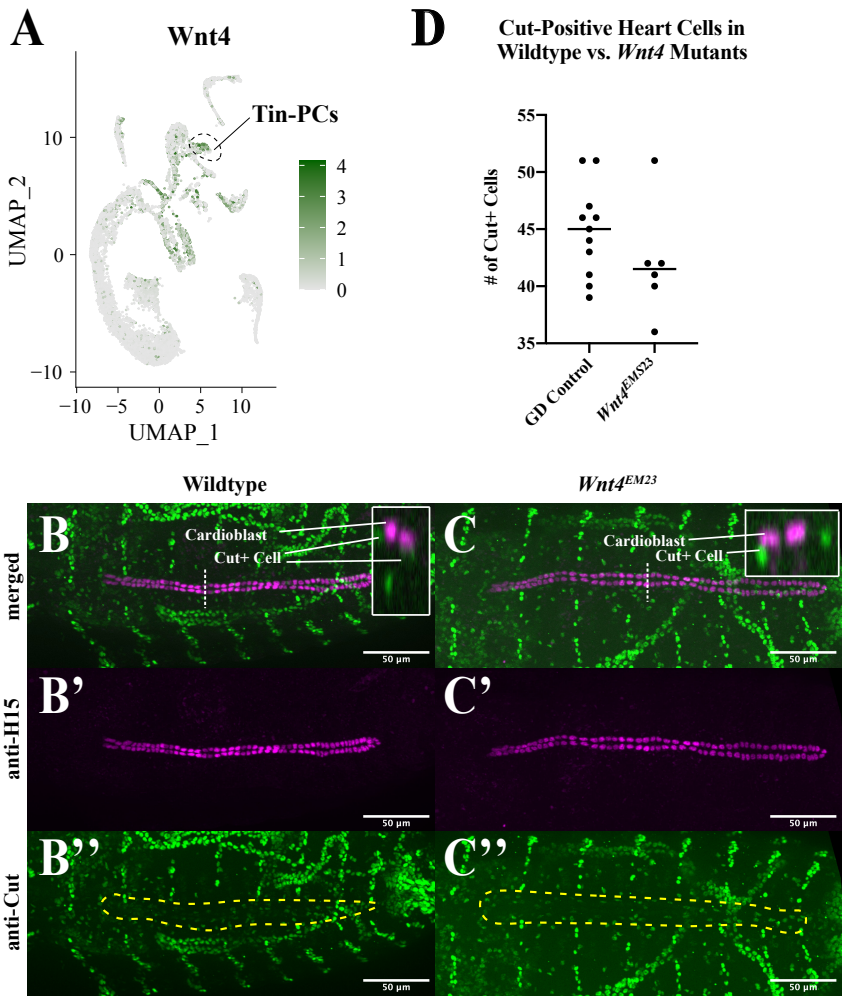

Figure 17. The Tin-PC marker gene *Wnt4* does not regulate *cut* expression in embryonic heart formation.
(A) Single-cell sequencing analysis revealed the gene *Wnt4* to be highly expressed in several cell clusters, including Tin-PCs.
(B-B'') Wildtype representative and *Wnt4* homozygous mutant representative **(C-C'')** immunostained for H15 (magenta) and Cut (green). **B** and **C** provide a single slice view of the localization of Cut-positive cells (i.e. Tin-PCs) lateral to the cardioblast. The outlined circle indicates the Cut-positive heart cells.
(D) The number of Cut-positive heart cells were counted manually via ImageJ for both wildtype and *Wnt4* homozygous mutant embryos. Statistical analysis showed no significant difference in the number of Cut-positive cells between the wildtype and mutant embryos (p-value = 0.2036; Mann-Whitney Test).

To further understand if *cut* could play a role in heart development, the UAS-Gal4 system was utilized to overexpress *cut* in cardioblasts through the Mef2-Gal4 driver **(Figure 18)**. These embryos, along with a control group that overexpressed exogenous GFP in these muscle cells, were immunostained to label one type of pericardial cell—the Eve-positive PCs (Eve-PCs)—and cardioblast marker H15. The number of Eve-PCs was compared between these two groups, and there was a significant difference in the number of Eve-PCs around the heart and more specifically in the aorta. This suggests that Cut can non-autonomously repress Eve expression in Eve-PCs when overexpressed in heart and muscle cells.

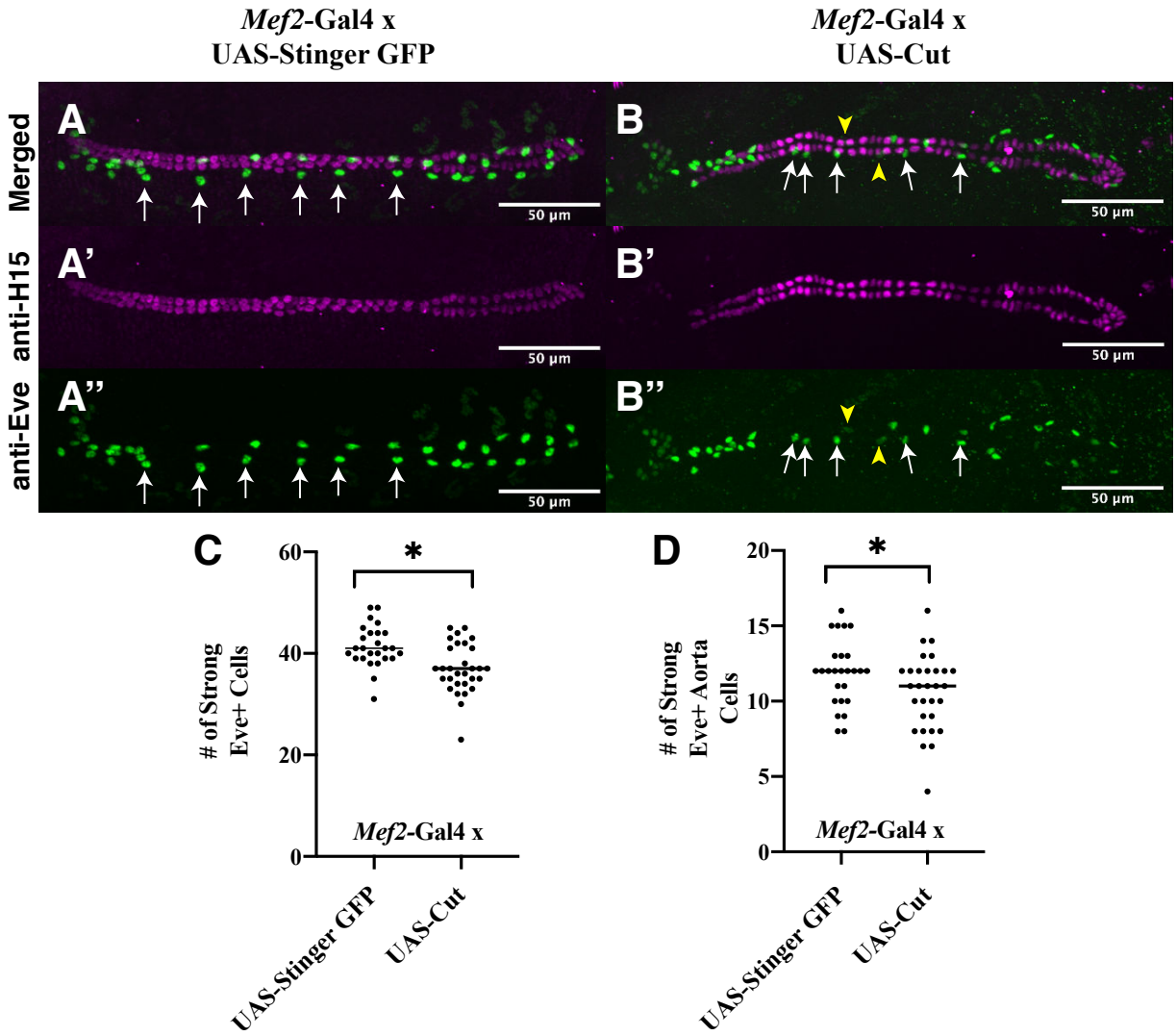

Figure 18. Ectopic expression of Cut in Mef2-positive cardioblasts caused non-autonomous reduction of Even-skipped expression in Eve-positive PCs.

(A-B'') Comparison of embryos expressing nuclear GFP (Stinger GFP) or Cut using the *Mef2*-Gal4 driver. Cardioblasts were immunolabeled with anti-H15 and Eve-positive pericardial cells were immunolabeled with anti-Eve (arrows). Arrowheads indicate lowered expression of Eve-positive pericardial cells.

(C-D) Images were blinded and the number of Eve-positive heart cells were counted manually using ImageJ. Statistical analysis showed a significantly lower number of strongly expressing Eve-positive pericardial cells in the entire heart **(C)** and aorta **(D)** when Cut is ectopically expressed in cardioblasts (p-value = 0.0007 for entire heart; p-value = 0.0510 for aorta; Mann-Whitney Test).

Some of the figures in the results section contain material from a published preprint manuscript: G. Vogler, B. Hum, M. Tamayo, Y. Altman, and R. Bodmer, “Single-cell sequencing of the Drosophila embryonic heart and muscle cells during differentiation and maturation,” bioRxiv, 2021, doi: 10.1101/2021.01.15.426556. The thesis author was a co-author of this paper.

Some of the figures in the results section contain material from a published poster presentation: B. Hum, M. Tamayo, R. Bodmer, and G. Vogler, “Elucidating the Role of Uncharacterized Tin-Positive Pericardial Cells in Drosophila Heart Development”. TAGC 2020, 20-Apr-2020, doi: 10.6084/m9.figshare.12150615.v1. The thesis author was the first author and presenter of this poster.

Discussion

Heart morphogenesis is an intricate developmental process that involves the interaction of many genes, pathways, and cell types in vertebrates and invertebrates. Single-cell sequencing has dramatically increased the resolution of gene expression patterns to allow highly targeted studies of heart development by homing in on cell-specific genes and pathways. Here, single-cell sequencing of individual cells allowed us to identify distinctly clustered groups of cells using their individual transcriptional profiles **(Figure 4)**. Cardiac cells could be identified based on the marker genes expressed in each cluster: *tin* and *Hand* for cardiac cells, *prc* for pericardial cells, *Pura* for cardioblasts and odd-PCs, *CG6415* for the fat body, and *CrzR* for Tin-PCs **(Figure 5, 6)**. Embryos stained for these markers confirmed that the single-cell sequencing data indeed reflects what is expressed in various cell types. As a result, this technology highlights an approach in identifying and characterizing novel cell types, such as the Tin-PCs.

Neurotactin (*Nrt*) was found to be specifically enriched in Tin-positive pericardial cells based on the single-cell RNA sequencing of cardiac cells **(Figure 8)**. This was confirmed through the immunostaining of Nrt, where it was shown to be highly expressed in the region ventral to the closing heart tube, where the Tin-PCs are localized **(Figure 7, 8)**. To further understand how Nrt plays a role in embryonic heart development, other Tin-PC marker genes were knocked down to gauge whether or not they had an effect on Nrt expression or localization, as well as heart morphogenesis. The Tin-PC marker genes that were knocked down were *lac, cora*, and *sinu*, all of which were previously identified as septate junction proteins that were necessary for the assembly of the heart [21]. Despite knocking down *lac* and *cora* in different mutant genotypes (homozygous, heterozygous, and trans-heterozygous mutants), there was no

visible phenotype in the expression of Nrt when compared to the wildtype control **(Figure 9, 10)**. These results suggest that *lac* and *cora* do not affect Nrt expression nor localization.

Given that a previous study found *lac* and *cora* to contribute to the "broken heart" phenotype (where the adhesion between pericardial cells and cardioblasts is compromised) in mutant backgrounds, it was expected that the loss of these septate junction proteins would have an impact on the cell adhesion protein Nrt [21]. However, because there was no change in either Zfh1 expression (marker for pericardial cells) nor a visual difference in pericardial cell assembly when *lac* and *cora* were knocked down, it is possible that while these genes are indeed necessary for adhesion between cardiac cells, they may not be necessary for the assembly of Tin-PCs in the embryonic heart. Since their contributions to heart development were shown to occur later than the embryonic age that was examined, an impact on Nrt is still possible. As such, it may be worth probing for Nrt expression and localization in the "broken heart" phenotypes that occur at the very end of the last embryonic stage.

Mutants for *sinu*, another septate junction protein and Tin-PC marker gene, also did not demonstrate a Nrt phenotype; however, they demonstrated a delay in the dorsal closure of the heart tube **(Figure 11)**. Interestingly, this was not reported in the cardiac study on the septate junction proteins *lac*, *cora*, and *sinu* [21]. The delayed dorsal closure phenotype was indicated by the developmental disjoint between the hindgut and the closing heart tube. The closing heart tube in *sinu* mutants was still open and appear as embryonic developmental stage 15 **(Figure 1)**, yet the intestinal features were characteristic of a later embryonic developmental stage, such as stage 17. When looking at wildtype embryos at stage 15, the hindgut loop is not developed yet **(Figure 11B)** but can be clearly seen in *sinu* mutant embryos **(Figure 11D-E'')** without a formed, linear heart tube. This suggests that there is a delay in the process by which heart cells align at the

dorsal midline of the embryo. This finding provides evidence that the Tin-PC marker gene *sinu* is involved with the alignment of heart cells along the dorsal midline during development, and a knockdown of this gene can delay the timing of dorsal closure, both of which could be developmentally linked by interactions between the epidermal and cardiac tissues.

The epidermis of the developing *Drosophila* embryo also undergoes dorsal closure, and defects in epidermal closure typically affect the closure of the heart as well [31]. Because of this, we also investigated if there was also a delay in epidermis closure by visualizing the epidermis through a staining of the adherens junction marker, Armadillo. Indeed, a delayed closure of the epidermis was also observed in the homozygous and trans-heterozygous *sinu* mutants **(Figure 12C-D'')**. While both heart and epidermis dorsal closure delays were significantly different from controls in homozygous and trans-heterozygous mutants for *sinu*, it is worth noting that heterozygous *sinu* mutants also showed an epidermis closure and heart closure delay; however, this was not significant with the sample size. An increase in sample size of this study could potentially result in heterozygous *sinu* mutants and controls becoming statistically different, which would indicate that *sinu* could be haploinsufficient (yet viable) for the timing of dorsal closure. In addition, a rescue of *sinu* expression in either the heart or epidermis using the appropriate Gal4 drivers in a *sinu* mutant background should give a conclusive answer to whether the *sinu* heart defect is secondary to the epidermis defect.

While the three septate junction Tin-PC marker genes did not demonstrate a phenotype on Nrt expression, the knockdown of *tin* demonstrated a suppression in *Nrt* at varying levels **(Figure 13)**. In particular, the progeny from the UAS-*tin*-RNAi^{GDv12656} line had a significant *tin* knockdown and *Nrt* suppression, while the progeny from UAS-*tin*-RNAi^{GDv32510} only had a significant *tin* knockdown. Given that *tin* had previously been identified as the master cardiac

cell that is upstream of many signaling pathways necessary for heart development [5], these results suggest that *tin* may directly or indirectly regulate the expression of *Nrt*. Furthermore, the fact that only one of the significantly knocked down *tin* lines demonstrated a *Nrt* phenotype suggests that *tin* needs to be knocked down past a certain threshold before any effect can be seen on *Nrt*. With a previous chromatin immunoprecipitation study demonstrating that Tinman binds inside an intron of *Nrt* [30], our finding is consistent with a direct regulation of Nrt by Tinman. To further understand the genetic interaction between *tin* and *Nrt*, a future experiment to be conducted would be to analyze the pixel intensity of each stained heart in each image in order to quantify the protein levels of Tin and Nrt or use in-situ hybridization chain reaction to quantify transcript levels.

Our findings during the examination of the Tin-PC marker genes *sinu*, *tin*, and *Nrt* support our hypothesis that Tin-positive pericardial cells have a role in the structure and integrity of the developing heart during the embryonic stages 15 to 17 by contributing not only to the dorsal closure of the embryo, but also the assembly of heart cells and the cellular adhesion between pericardial cells. However, the results from the *lac* and *cora* experiments do not support this hypothesis at the embryonic stages that were investigated. As such, more studies need to be conducted on *lac* and *cora*, particularly in a later embryonic developmental stage, to further elucidate their roles in embryonic heart development in the context of Tin-PCs.

In addition to Nrt, the homeobox transcription factor gene *cut* was found to be highly expressed in Tin-PCs based on the single-cell sequencing data **(Figure 14)**. Co-immunostainings of Cut with Mef2 and H15 confirmed this finding in the embryo, thereby verifying another marker gene for Tin-PCs **(Figure 15)**. For a comprehensive analysis of genetic interactions between Nrt and many other Tin-PC marker genes, we investigated whether *cut* had an effect on

Nrt expression or localization **(Figure 16)**. As shown in the images, hemizygous ct^{db7} mutations of the X-chromosomal gene *ct* appeared to have a phenotype whereby the anterior and posterior ends of the heart did not align at the dorsal midline when compared to wildtype embryos. The other allele examined, ct^{c145}, did not differ significantly from the wildtype embryos. The difference between the two *ct* mutants is that ct^{db7} contains a 1 kb deletion in a region that encodes for the homeodomain of the Cut protein, whereas the ct^{c145} mutation is an X-ray induced mutation in the coding region [32]. A potential explanation for the abnormal heart dorsal closure in ct^{db7} mutants is that without the homeodomain, the Cut protein does not bind to DNA sequences to activate downstream genes but still might bind interacting proteins that interferes with heart closure. Again, tissue-specific expression of wildtype Cut in *ct* mutant backgrounds is necessary to ensure the phenotype is due to *ct,* and not a second-site mutation on the X-chromosome. Nevertheless, this finding on the potential role of the Tin-PC marker gene *cut* further provides support that Tin-PCs may play a role in regulating the dorsal closure of the heart in embryogenesis.

Given that Cut was previously identified as a transcription factor involved in cell specification in the nervous system, yet is highly expressed in Tin-PCs, we investigated whether *cut* had any genetic interactions with another Tin-PC marker gene, *Wnt4* [23]. *Wnt4* was chosen as a candidate to study due to its involvement in highly conserved signaling pathways that contribute to the specification of the cardiac mesoderm in *Drosophila* heart development [6]. To study the interactions between *cut* and *Wnt4*, homozygous *Wnt4* mutant embryos were immunostained with Cut and H15 to visualize the effect of *Wnt4* on *cut* expression **(Figure 17)**. However, when compared to the wildtype control embryos, there were no visible phenotypes with regards to Cut expression. Additionally, there was no significant difference in the number of

Cut-positive cells in the heart region (i.e. Tin-PCs) in the *Wnt4* mutants compared to the wildtype embryos, suggesting that Wnt4 does not regulate Cut expression in heart development. However, it is worth noting that a limitation of this finding is the relatively small sample size of mutants compared to wildtypes (six mutant embryos vs. eleven wildtype embryos). Given that the resulting p-value was 0.2036, it is worth repeating the experiment to obtain more embryos to further confirm if Wnt4 has no effect on Cut expression in Tin-PCs.

To further characterize how the Tin-PC marker gene *cut* plays an instructive role in embryonic heart development, we utilized the UAS-Gal4 system to ectopically express *cut* in cardioblasts **(Figure 18)**. This was done by having *cut* expression be driven by *Mef2*-Gal4. *Mef2* is a transcription factor that is highly expressed in cardioblasts [33]. To observe for any phenotypes in the developing embryonic heart, these embryos were immunostained for H15, a cardioblast marker, and Even-skipped (Eve), a marker for one type of pericardial cell [8, 17]. Unexpectedly, there was no visible phenotype in the cardioblasts, yet the expression of *eve* was significantly lowered in the pericardial cells in the experimental group compared to the control, suggesting that Cut can cause a non-autonomous reduction of Eve expression in Eve-positive pericardial cells. To further test the hypothesis that Cut has an influence on pericardial cell expression, a future experiment would be to examine the effects of ectopic Cut expression in cardioblasts on the other pericardial cell type, the Odd-PCs. Furthermore, *ct* mutant embryos could be analyzed using single-cell sequencing to identify differentially expressed genes that depend on Cut.

Conclusion

From his study, we can conclude that Tin-PCs could play a role in the dorsal closure of both the heart and the epidermis through the gene *sinuous* during embryogenesis. Furthermore, we found that the cardiac master regulator gene *tinman* regulates expression of the cell adhesion protein *Neurotactin*, implicating Tin-PC's role in cardiac cell adhesion during development. Finally, our investigation of the homeobox gene *cut* led us to conclude that Tin-PCs can potentially regulate the dorsal closure of the heart and influence the expression of *even-skipped* in Eve-positive pericardial cells during embryonic development. Despite all these findings, there are many more Tin-PC marker genes identified through single-cell RNA sequencing beyond those investigated in this study. As such, not only does this mean that there are more opportunities to further our understanding of the role of Tin-PCs in *Drosophila* heart development, but it also highlights the potential of single-cell RNA sequencing technology in characterizing novel cell types in developmental biology.

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