Drosophila heart cell movement to the midline occurs through both cell autonomous migration and dorsal closure

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Short title: Heart cell migration

Keywords: Dorsal vessel, heart, *Drosophila*, *wunen*, amnioserosa, dorsal closure, organogenesis

Abstract

The *Drosophila* heart is a linear organ formed by the movement of bilaterally specified progenitor cells to the midline and adherence of contralateral heart cells. This movement occurs through the attachment of heart cells to the overlying ectoderm which is undergoing dorsal closure. Therefore heart cells are thought to move to the midline passively. Through live imaging experiments and analysis of mutants that affect the speed of dorsal closure we show that heart cells in *Drosophila* are autonomously migratory and part of their movement to the midline is independent of the ectoderm. This means that heart formation in flies is more similar to that in vertebrates than previously thought. We also show that defects in dorsal closure can result in failure of the amnioserosa to properly degenerate, which can physically hinder joining of contralateral heart cells leading to a broken heart phenotype.

Introduction

The *Drosophila* heart (or dorsal vessel) is a linear structure composed of 2 inner rows of contractile cardioblasts flanked on either side by a row of pericardial cells (Rugendorff and Hartenstein, 1994). Although much simpler than a vertebrate heart, many genes that are expressed and required for heart development in *Drosophila* are also required in vertebrates (such as the transcription factor Tinman and its vertebrate homolog Nkx2.5) suggesting that these two organs are homologous (reviewed in Bodmer and Venkatesh, 1998; Hartenstein and Mandal, 2006). The *Drosophila* heart is therefore an excellent genetic system to study the mechanisms of heart formation.

Common to many organisms is the early requirement to move bilaterally positioned heart precursors medially, concurrent with underlying morphogenetic movements. In zebrafish the myocardial precursors move medially, either individually or as a collective, in concert with the medial movements of the anterior lateral plate mesoderm in which they are embedded (reviewed in Glickman and Yelon, 2002). In chick and mouse cardiac progenitors migrate away from the

primitive streak to occupy bilateral positions and must migrate back to midline to form the heart tube (Brand, 2003; Moorman and Christoffels, 2003). Failure of this migration or the ability of heart cells to join with one another leads to *cardia bifida*, one type of congenital heart defect, which collectively form the largest class of human birth defects and are a leading cause of mortality of newborns.

In *Drosophila,* heart cells are specified in bilateral rows in the lateral mesoderm (Poulson 1950). The movement of these cells medially is dependent on dorsal closure, the movement of lateral ectodermal cells towards the dorsal midline thereby sealing the hole left following germ band retraction. This hole is covered by an extra-embryonic tissue, the amnioserosa, that involutes and eventually degenerates to partly provide the force needed to bring the leading edges of the ectodermal cells together. During dorsal closure the cardioblasts appear stretched and make dorsally directed protrusions whilst the pericardial cells remain rounded (Rugendorff and Hartenstein, 1994). As the ectodermal leading edge cells meet with their contralateral counterparts, the underlying cardioblasts also meet and adhere, firstly with their dorsal most edges, then with their ventral most edges, with the medial portion remaining unattached and forming the dorsal vessel lumen (Medioni et al., 2008; Santiago-Martínez et al., 2008). The heart becomes contractile at the end of embryogenesis, beating with increasing regularity.

Although the movement of the heart cells to the midline occurs concurrent with dorsal closure, it remains surprisingly ambiguous if the heart cells are capable of migration independent of the ectoderm or whether dorsal closure can fully account for the movement of the heart cells.

At mid dorsal closure the heart cells are 1 or 2 cells behind the leading edge cells of the ectoderm and are not in contact with the amnioserosa (Chartier et al., 2002; MacMullin and Jacobs, 2006; Rugendorff and Hartenstein, 1994). However at the end of dorsal closure the heart cells lie directly below the leading edge cells and abut the amnioserosa. To effect this change in position the heart cells could alter their attachment to the ectoderm or changes in ectodermal cell shape could bring the heart cells closer to the leading edge.

We became interested in the migratory properties of heart cells whilst

investigating the role of the lipid phosphate phosphatases, Wunen (Wun) and Wunen2 (Wun2), during embryogenesis. We noticed that embryos mutant for *wun* and *wun2* display defects in the heart, as has also been reported in a screen by others (Tao et al., 2007). Wunens control germ cell migration and survival (Renault et al., 2004; Starz-Gaiano et al., 2001; Zhang et al., 1997) but also have non-migratory roles such as promoting septate junction function in tracheal development (Ile and Renault, 2013; Ile et al., 2012). Therefore we investigated whether Wunens were affecting the heart via migratory or other mechanisms.

In this paper we performed live imaging of heart cells to show that cardioblast cells are highly protrusive during dorsal closure, similar to those exhibited by leading edge cells. However the mechanisms that promote filopodia in leading edge cells are not required for actin rich extensions of heart cells. Using multi-colour live imaging we show that heart cells are able to move independently of the ectoderm in wild type. We show that the primary defect in *wun wun2* mutants is not a failure to migrate but actually due to dorsal closure being slower. Under such circumstances the heart cells reach the amnioserosa much earlier than in wild-type. The defect in dorsal closure causes frequent failures of cardioblasts to make contact with their contralateral partners leading to breaks in the cardiac lumen. These become amplified as the embryo attempts to hatch leading to a 'broken-hearted' phenotype. Screens have identified many *Drosophila* mutants with similar 'broken-hearted' phenotypes (Yi et al., 2006; 2008). Therefore understanding whether the underlying cause is the same for these cases, will be useful for interpreting the functions of the affected genes.

Methods

Fly stocks

The following *Drosophila* lines were from the Bloomington stock center: *enGal4*, *UAS act5cGFP*, *ubi>shgGFP, UASmoesinT559D* and *UAS FP4mito*. *tinGal4* was from Achim Paululut (Osnabrück University), *UAS enaGFP* was from Mark Peifer (University of North Carolina) and *UAS wun2myc* is described in Starz-Gaiano et al.,

(2001). *wun wun2* maternal (M) zygotic (Z) mutant embryos were made using a *FRT wun[49] wun2[EP2650ex34]* chromosome (Renault et al., 2004) to make germ-line clones using the domain female sterile technique. For phenotypic analyses (Fig. 6,7) the resulting females were mated to *Df(2R)wun[GL] / CyO Dfd>YFP* or *Df(2R)wun[GL] ubi>shgGFP/ CyO Dfd>YFP* males. Embryos inheriting the *Df(2R)wun[GL]* chromosome are *wun wun2* M-Z- whilst those inheriting the labeled balancer *CyO Dfd>YFP* chromosome will be *wun wun2* M-Z+ and were used as controls. For the rescue analysis (Fig. 8F) the resulting females were mated to males *wun[CE] UASwun2myc / CyO Dfd>YFP* with and without *handGal4, 69B Gal4* or both drivers on the third chromosome. *wun[CE]* disrupts both *wun* and *wun2* expression (Starz-Gaiano et al., 2001).

The *hand>moe-GFP* and *hand>moe-mCherry* constructs were made by replacing the D-Six4 enhancer in the pD-Six4III Colourless pelican vector (Clark et al., 2007) with the 513bp *hand* enhancer (Han and Olson, 2005) using BglII and BamHI sites. moe-EGFP and moe-mcherry coding sequences were excised from *pKS moe EGFP* and *pUASp mcherry-moe* (Tom Millard, University of Manchester) plasmids and inserted into the *hand* enhancer vector using KpnI and NotI sites. Transgenic flies were made by P-element mediated transformation.

Immunohistochemistry and live imaging

Embryos were laid at room temperature, dechorionated in 50% bleach for 3 minutes and chemically fixed for 20 minutes in 4% formaldehyde/heptane or heat fixed by boiling in 60mM NaCl, 0.03% Triton-X. For chemical fixation the formaldehyde was removed and methanol added and the embryos shaken to remove the vitelline membrane. For the heat fixation the salt solution was removed, heptane added followed by methanol. In both cases, the embryos were washed in methanol and stored frozen until needed. Embryos were rehydrated in PBST, stained using standard protocols and mounted in 70% glycerol, 2.5% DABCO. The following antibodies were used at the indicated concentrations: mouse anti-Armadillo (β-catenin) 1:100 from the DSHB (N2781); rabbit anti-Dystroglycan, 1:300 courtesy of Wu-Min Deng (Deng et al., 2003); chicken anti-GFP, 1:1000 from

Abcam (ab13970); rabbit anti-Neurexin IV, 1:2000 courtesy of Hugo Bellen (Baumgartner et al., 1996). Cy3, Cy5 (Jackson Immuno Research) and Alexa488 (Invitrogen) coupled secondary antibodies were used at 1:500. For live imaging, embryos were dechorionated and mounted in halocarbon oil (10S Voltalef, VWR). Images were taken using a UPlanSApo 60x water objective (NA=1.2) on an Olympus FV1000 confocal microscope. Ablations were carried out with a 355nm pulsed laser using 2 - 3 repetitions of 2 secs each. Maximum intensity projections were made in ImageJ 1.45 (National Institutes of Health, USA) and 3D reconstructions were made using Imaris 7.4.0 (Bitplane). Tracking was carried using the manual tracking plugin of ImageJ. Live imaging of figure S1 was carried out using a Zeiss Z1 widefield fluorescence microscope using a GFP filterset. For figure S2 and supplemental movie 10, images were captured using an UltraVIEW spinning disc confocal (PerkinElmer).

Results

Wunen mutants show heart defects

Wun and Wun2 are lipid phosphate phosphatases (LPPs) important for regulating both migratory and non-migratory events during embryogenesis: They act redundantly in controlling directionality for germ cell migration and are required later in development for septate junction function in the tracheal system. Whilst investigating these roles, we noted that hearts in *wun wun2* mutant embryos are defective. To image heart cells more easily we created a transgene driving the actin binding domain of Moesin under the control of the *hand* driver, which expresses in both cardioblasts and pericardial cells. We noted that in particular the cardioblasts are often not attached their contralateral neighbour which can occur at both anterior and posterior positions (Fig. 1A-C). These regions of non-attached cardioblasts initially can appear quite small but become enlarged as the embryo muscles contract in preparation for hatching (Fig. S1; supplemental movies 1,2). In addition, the pericardial cells show a much looser association with the cardioblasts once dorsal closure has completed compared to wild-type (Fig 1B-C). This latter

'broken-hearted' phenotype is observed in a number of mutants (Yi et al., 2006; 2008) indicating a possible conserved underlying mechanism.

Cardioblasts are highly protrusive during their migration

In wild type embryos, during the movement of the heart cells to the midline, the cardioblasts make actin rich protrusions towards the midline (Fig. 2A; supplemental movie 3), as has been reported for fixed tissue (Rugendorff and Hartenstein, 1994). These protrusions were highly dynamic with an average length of 1.6 um (59 protrusions measured from 4 embryos). From three-dimensional reconstructions, these protrusions emanate solely from the dorsal side of the cardioblasts (Fig. 2B) and are therefore projecting onto the basal side of the overlying ectodermal cells.

To determine whether these protrusions are unidirectional, the *tin* driver was used to express *actinGFP* in cardioblasts and pericardial cells. Using this labelling technique, not all heart cells were highly labelled. By focusing on labelled cardioblasts which had an unlabelled neighbouring pericardial cell, the cardioblast projections were directed mostly towards the midline with some lateral projections also evident (Fig. 2C). Projections however were not observed in the direction of the associated pericardial cells (Fig. 2C).

Cardioblasts and leading edge cells contain actin-rich protrusions formed through different molecular mechanisms

During dorsal closure the leading edge cells which are overlying the heart cells, make protrusions towards the midline which are required to align the segmental boundaries as the ectoderm is sealed (Jacinto et al., 2000; Millard and Martin, 2008). These protrusions are driven by the actin regulator Ena which labels the tips of these filopodia (Gates et al., 2007), and drives actin filament elongation by preventing binding of actin capping proteins (Bear et al., 2000). To examine whether the cardioblast protrusions were driven by similar actin regulators we expressed EnaGFP in cardioblasts or leading edge cells using the *tin* and *en* drivers respectively. When EnaGFP was expressed in leading edge cells, GFP foci were

present beyond the medial edge of these cells, showing that Ena is localized to the tips of these filopodia as has previously been reported (Fig. 3A; Gates et al., 2007). In the cardioblasts, on the other hand, GFP foci occurred only intracellularly, and no foci could be visualized beyond the medial edge of these cells (Fig. 3B). We conclude that cardioblast protrusions do not contain Ena accumulations at their tips.

To see if a severe reduction in Ena levels in cardioblasts would abolish their projections we made use of an Ena mitochondrial targeting protein, FP4mitoGFP that blocks Ena function (Gates et al., 2007). Expression of this protein in leading edge cells abolished the projections of these cells (Fig. 3C, E) as was previously reported (Gates et al., 2007). However expression in the heart cells, did not abolish cardioblast projections (Fig. 3F) and did not affect heart closure (data not shown).

The projections from leading edge cells also require the activity of the actin binding protein Moesin (Karagiosis and Ready, 2004). In agreement with data previously reported, expression of a constitutively active form of Moesin (MoeT559D) in leading edge cells blocks the formation of filopodia and instead only wide membrane ruffles are observed (Fig. 3G, arrowheads). Expression of this form of Moesin in heart cells however, did not abolish cardioblast projections (Fig. 3H) and did not affect heart closure (data not shown). Taken together we conclude that leading edge cells and cardioblasts produce actin-rich protrusions through different molecular mechanisms.

Cardioblasts can move independently of the ectoderm

Given that cardioblasts are highly protrusive we asked whether these cells were migratory *in vivo*. The movement of the heart cells to the midline is concurrent with the medial movements of the ectoderm during dorsal closure. Therefore to examine the relative contribution of the ectodermal versus autonomous movement the heart cells were labelled using a *hand>moe-mcherry* construct and all embryonic cells co-labelled with an *ubi>shgGFP* construct, which is especially prominent in the amnioserosa and ectoderm. Live imaging shows that at mid-dorsal closure the heart cells lie 1-2 cell diameters behind the leading edge-amnioserosa boundary (Fig. 4A, 0'). During the following 60 minutes the

amnioserosa contracts, and the leading edge cells and heart cells approach the midline. The heart cells however gradually approach the leading edge - amnioserosa boundary.

To verify if this change in relative position of heart and ectodermal cells represented heart cell autonomous movement, ectodermal cell junctions were used as landmarks and followed over-time and compared to the movement of the heart cells (Fig. 4A; supplemental movie 4). Ectodermal cell junctions that at time point zero were more dorsal (ie. closer to the dorsal midline) than the heart were tracked in cyan, that overlaid the cardioblasts and pericardial cells in yellow and white respectively, and that were more ventral (further from the dorsal midline) to the heart in blue. As dorsal closure proceeded the ectodermal landmarks move dorsally, however by 45 minutes the cyan landmarks were no longer dorsal to the cardioblasts and the yellow landmarks, that overlaid the cardioblasts originally, now overlaid the pericardial cells. Similar results were obtained in three further movies (data not shown). We conclude that heart cells move faster dorsally than the ectodermal cells.

At time point 52.5', ectodermal junctions start to move anteriorly due to head involution (Peralta et al., 2007) which continues until dorsal closure is completed (Fig. 4A). However heart cells move only very slightly anteriorly (compare position of cardioblasts at 52.5' and 82.5'). This uncoupling of the anterior movement would indicate that the heart cells are not firmly attached to the ectodermal cells at this stage.

To verify these results, the same movie was analysed in a second way. In this case paired landmarks were tracked, with one landmark in the ectoderm (Fig. 4B, white tracks; supplemental movie 5) that overlaid a second landmark at the boundary of two heart cells (between a highly and weakly labelled heart cell) (Fig. 4B, magenta tracks, supplemental movie 5). For each pair, the heart cells moved further dorsally than the ectodermal cells and the anterior movement was much more pronounced in the ectodermal tracks than in the heart tracks. We conclude that heart cells can move autonomously.

The linkage of heart cells to the ectoderm is dynamic

If heart cells are migratory then their attachment to the ectoderm should be more labile than if their movement was entirely dependent on dorsal closure. To test this hypothesis we ablated cell junctions in the ectoderm at the midline in embryos in which dorsal closure had just finished and monitored the extent of lateral movement of both the ectoderm and heart cells. Upon ablation the ectodermal cells moved ventrally away from the dorsal midline, due to tension in the ectoderm, as has been previously reported (Kiehart et al., 2000). In embryos in which heart cells were still several cell diameters from the midline, the heart cells moved ventrally in line with the movement of the ectodermal cells (Fig. 5A, supplemental movie 6, representative of 4 movies made). This indicates a strong attachment of heart cells to the ectoderm at this stage.

In slightly older embryos where the heart cells were almost at the midline, the ectoderm retracted ventrally as previously however without pulling the underlying heart cells with them (Fig. 5B, supplemental movie 7, representative out of 3 movies made). This shows that the heart cells are not as strongly attached to the ectoderm at this late stage and we hypothesize that this is because they are autonomously moving to the midline at this time.

Heart cells are polarized normally but mispositioned in wunen mutants

Given this ability of heart cells to migrate in *Drosophila* we examined whether mis-migration was the cause of the defects in *wun wun2* mutant embryos. We examined the position of the heart cells relative to the ectoderm and amnioserosa in both wild type and mutant embryos. We found that at mid dorsal closure, in wild type, the heart cells remained 1-2 cell diameters behind the leading edge cells (Fig. 6A,C,I) and did not contact the amnioserosa (Fig. 6E; supplemental movie 8). The latter only occurs very late during dorsal closure at the point where the amnioserosa is very narrow, such that individual amnioserosa cells are not distinguishable (Fig. 6G; supplemental movie 8).

In *wun wun2* mutant embryos however, heart cells moved underneath the ectodermal leading edge cells much earlier, already during mid dorsal closure (Fig.

6B,J). The cardioblasts also made contact with the amnioserosa much earlier than in wild-type, whilst individual amnioserosa cells were still clearly distinguishable (Fig. 6F,J; supplemental movie 9).

In wild type, cardioblasts become polarized during dorsal closure in preparation for joining to their contralateral counterpart. We used the localization of Dysotroglycan (Dg) to examine whether heart cells were correctly polarized during their migration. We found that similar to wild type, *wun wun2* mutant cardioblasts contained domains enriched in Dg (Fig 6K, L) and are therefore polarized.

Upon completion of dorsal closure opposing cardioblasts bind their dorsal and ventral surfaces to create a dorsal and ventral junctional domain (Medioni et al., 2008) (both enriched for Arm, Fig. 6O, arrows). Between these 2 domains lies a luminal domain (Medioni et al., 2008) (enriched in Dg, Fig. 6O arrowhead) that will not bind but instead form the wall of the heart lumen. We checked to see what occurs at sites of failure of cardioblast adhesion in *wun wun2* mutant hearts. We found that in contrast to wild-type in which there is a single lumen (Fig. 6O, arrowhead) in *wun wun2* mutant hearts there are multiple lumens (Fig. 6P, arrowheads) and dense Arm staining from the remnants of the amnioserosa (Fig. 6P, arrow) which is likely to interfere with heart closure.

Wunen mutants show a delay in dorsal closure

The mispositioned heart cells in *wun wun2* mutants could result from premature migration of heart cells to the leading edge or a delay in dorsal closure. To test for the latter we examined the time taken for dorsal closure to proceed as measured by the reduction in width of the amnioserosa over time. We observed that dorsal closure was indeed slower in *wun wun2* embryos (Fig. 7A-C). In addition the amnioserosa cells had much longer and wave-like apical edges compared to wild-type (Fig. 7A,B). We conclude that this delay in dorsal closure leads to a mis-positioning of heart cells, too far towards the dorsal midline, resulting from their continued autonomous migration in the face of delayed dorsal closure.

wun and wun2 are expressed and required in both the ectoderm and heart cells.

To test in which tissues Wunens are required for heart formation we examined the *in situ* hybridization patterns of *wun* and *wun2* in late stage embryos (see also Renault et al., 2002). We observed both genes to be expressed in a segmental fashion in patches of the lateral ectoderm (Fig. 8A,B) and in heart cells (Fig. 8C,D). *wun2* is also expressed in the posterior 4 pairs (of a total of 7 pairs) of alary muscles (arrows in Fig. 8D). To verify which heart cells are expressing Wunens we used fluorescent detection and examined stage 16 embryos in which the heart cells had met at the midline. We observed expression of *wun2* in just the outer rows of cells of the heart, the pericardial cells (Fig. 8E).

To verify whether the ectodermal or pericardial expression of Wunens is critical for heart formation we attempted to rescue the defects of *wun wun2* embryos with an inducible tagged version of Wun2 (*UAS wun2myc*) using drivers that express in the heart (*handGal4*) or ectoderm (*69BGal4*). We found that expression using each driver alone was able to slightly reduce the percentage of embryos showing either regions where the cardioblasts did not join with the contralateral counterpart at the midline ('unjoined cardioblasts' in Fig. 8F) or pericardial cells that are only loosely associated with the cardioblasts ('loose pericardial cells' in Fig. 8F). However using both drivers together we were able to rescue both aspects of the defective hearts (Fig. 8F). We conclude that Wunens are required in both the ectoderm and pericardial cells for proper heart formation.

Discussion

The movement of the heart progenitor cells to the midline has long been established to be dependent on dorsal closure (Poulson 1950; Campos-Ortega and Hartenstein, 1985). The cardioblasts were noted as being several cell diameters away from the dorsal edge of the epidermal primordium and moving relatively little compared to the ectoderm (Rugendorff and Hartenstein, 1994). In this paper we have determined that heart cell movement to the midline in *Drosophila* occurs

through additional autonomous heart cell migration. Several lines of evidence support this conclusion:

Firstly live imaging of heart cells and ectodermal cells during dorsal closure shows uncoupling of the movement of heart and ectodermal cells. Secondly cardioblasts make extensive protrusions during dorsal closure. We speculate that these protrusions are required for motility, but they could be used for attachment to contralateral cardioblasts. In support of the former hypothesis, the protrusions occur from the onset of dorsal closure several hours before heart cells meet and are not suppressed through genetic mechanisms that are sufficient to suppress protrusions of ectodermal leading edge cells. The latter are generally longer (up to 10um; Jacinto et al., 2000) than cardioblast protrusions (below 2um) and are required for attachment to contralateral leading edges cells (see below). Thirdly the strength of adhesion of heart cells to the ectoderm, as judged by the ablation experiments, is reduced as the heart cells approach the midline. We speculate this is because the heart cells are moving partly autonomously at this time. Finally, when dorsal closure is delayed, as occurs in *wun wun2* mutants, the heart cells migrate up to the amnioserosa before dorsal closure has completed (Figure 9). This phenotype appears to be a general feature when dorsal closure is delayed as it is reported to occur in other mutants (MacMullin and Jacobs, 2006).

Several molecular players are implicated in linking heart cells to the ectoderm: Spot adherens junctions (AJ) have been reported between cardioblasts and ectodermal cells (Rugendorff and Hartenstein, 1994). The AJ component DE-Cadherin (encoded by *shotgun*, *shg*) is highly expressed in heart cells and *shg* mutants display defects in cardioblasts reaching the midline as well as lumen formation (Haag 1999). The extracellular collagen-like protein Pericardin (Prc) is expressed by pericardial cells and surrounds both them and cardioblasts (Chartier et al., 2002). Reduction in Prc levels causes interruptions in the cardioblasts lines, which appears to result from a loss of interaction with the ectoderm (Chartier et al., 2002). Finally, disruption of integrin complexes, which are receptors for extracellular matrix proteins, using *scab* or *mys* mutants (encoding integrin alpha and beta subunits respectively) causes mislocalisation of pericardial cells (Stark et

al., 1997). If and how and these complexes and proteins are regulated to allow the heart cells to dynamically attach to the ectoderm remains an open question.

How might Wun and Wun2 be working mechanistically to promote heart formation and dorsal closure? The forces for dorsal closure arise from three sources (Kiehart et al., 2000). Firstly, actin rich filopodia from leading edge cells make contact with contralateral partners at the anterior and posterior most ends (canthi) and act in a zippering fashion (Jacinto et al., 2000). These filopodia are also important for correct alignment of the ectoderm with respect to parasegmental boundaries (Jacinto et al., 2000; Millard and Martin, 2008). Secondly, an actin-myosin-rich cable at the leading edge acts as a supracellular purse-string (Edwards et al., 1997). Finally contractility and coordinated internalization of amnioserosa cells pulls the leading edges towards the midline (Gorfinkiel et al., 2009; Toyama et al., 2008).

We find in *wun wun2* mutants that the actin cable and leading edge filopodia are present, and we see internalization of amnioserosa cells (data not shown). What we do observe is that amnioserosa cells have highly wavy edges, that are normally only observed in much earlier embryos, during germ band retraction. Therefore we favour the idea that there are defects in tension in the ectoderm in *wun wun2* mutants. *wun* and *wun2* are expressed in the ectoderm and ectodermal *wun2* expression is needed (along with heart cell expression) to rescue the heart defects of *wun wun2* mutants (Fig. 8). This loss of tension would also explain why the pericardial cells often lie away from the cardioblasts in *wun wun2* mutants. In wild type the pericardial cells are strongly associated with the cardioblasts during dorsal closure. However, by the time the embryo is ready to hatch these two cell types are not tightly attached as can be seen during a heartbeat when the pericardial cells are thrust laterally and normally immediately rebound (Fig. S2, supplemental movie 10). We speculate that if tension is lost then this rebound is weak leading to displacement of the pericardial cells.

Overall this work clarifies the relative contributions of autonomous cell migration and hitchhiking on the ectoderm of *Drosophila* heart cells in their journey to the dorsal midline. The dual contribution of cell autonomous migration and

movement of surrounding cells in *Drosophila* parallels that seen in a diverse array of other species including *Ciona intestinalis* (Davidson et al., 2005), chick (Münsterberg and Yue, 2008), mouse (Buckingham et al., 2005), and zebrafish (Trinh and Stainier, 2004). Further work will hopefully elucidate the extent to which the mechanisms that regulate the relative contributions of these processes are also conserved.

Acknowledgements

We thank Hugo Bellen, Tom Millard, Mark Peifer, Achim Paululut, Wu-Min Deng, and Bloomington stock centre at Indiana University for flies and reagents. We also acknowledge the Flybase consortium for maintenance of the stock and genomic databases. We thank members of the lab especially Michaela Spirk for production of transgenic lines. This work was funded by the Max Planck Society and by a Marie Curie international reintegration grant, IRG224942, to A.R.

Figure 1: *wun wun2* mutants show heart defects

(A-C) Dorsal view of stage 16 *Drosophila* embryos carrying a *hand>moeGFP* transgene to label the heart (anterior to left) including cardioblasts (c) and pericardial cells (pc). Wild type (A) and *wun wun2* M-Z- (B-C) embryos showing a break between cardioblasts at the posterior (B) or anterior (C) (arrows) and loose attachment of the pericardial cells (arrowheads).

Figure 2: Heart cells show protrusive activity as they move to midline

(A) Live imaging of a *Drosophila* embryo with *hand>moeGFP* transgene to label the actin in cardioblasts (arrows) and pericardial cells (arrowheads) during dorsal closure (dorsal view, stages 14-15). Images were recorded every 1.5 mins, and every 5th time point is shown. Scale bar = 10 um. Full movie is supplemental movie 3.

(B) Longitudinal view of a 5 um cross-section of the central region of the embryo from a 3D-reconstruction showing that the protrusions emanate only from the dorsal side of the cardioblasts. Scale bar = 10 um.

(C) Live imaging of a single row of cardioblasts (c) and pericardial cells (pc) moving towards the embryo midline (downward) during dorsal closure (dorsal view, stages 14-15) using *tin* driven *act5cGFP* which is expressing in a subset of heart cells. Images were recorded every 2 mins, and every 2nd time point is shown. Cardioblast projections occurred mostly towards the midline (arrowhead) but some lateral protrusions were also evident (arrow). Scale bar = 10 um.

Figure 3: Protrusions in cardioblasts and leading edge cells are differently affected by actin regulators

(A-F) Dorsal views of living stage 14-15 embryos. Scale bars are 10um.

(A) Embryo expressing EnaGFP in the ectoderm using the segmental *en* driver. The ectoderm is in the process of closing and its limits are indicated by the dashed lines. Medially directed EnaGFP positive filopodia are observed (arrows).

(B) Embryo expressing EnaGFP in the heart using the *tin* driver. The heart cells are about to meet at the dorsal midline. Although GFP foci are visible in cardioblasts (arrows) and pericardial cells (arrowheads) these are not located beyond the medial edge of the cardioblasts (asterisks) and therefore not present on the medially projecting protrusions.

(C) Embryo expressing ActinGFP in the ectoderm using the segmental *en* driver. Medially directed ActinGFP filopodia are observed.

(D) Embryo expressing moeGFP in the heart using the *hand* promoter (taken from the movie in figure 2) showing medially projecting cardioblast protrusions.

(E,G) Embryo expressing ActinGFP (to visualise protrusions) and FP4mitoGFP (to sequester Ena to the mitochondria) (E) or MoeT559D (G) using the *en* driver. Medially directed ActinGFP filopodia are not observed. Arrowheads in (G) show wider lamellipodia like projections.

(F, H) Embryo expressing MoeGFP in the heart using the *hand* promoter and FP4mitoGFP (F) or MoeT559D (H) under the *tin* driver. Medially projecting cardioblast protrusions are still observed. Note the GFP from the FP4mito construct is visible in other deeper mesodermal cells.

Figure 4: The heart can move independently of the ectoderm

(A-B) Maximum intensity Z-projection of a dorsal view of a wild type living stage 14 embryo with ectodermal and amnioserosa cells labeled using a *ubi>shgGFP* (green) construct and cardioblasts (c) and pericardial cells (pc) with *hand>moe-mCherry* (red) construct. Images were taken every 2.5 minutes and representative time points are shown. Edges of amnioserosa are shown with white arrows. Cell junctions serving as landmarks have been tracked in 2 ways:

(A) Ectodermal cell junctions have been colour coded according to their position relative to the underlying heart cells. At *t=0*, those junctions that are closer to the dorsal midline than heart cells are marked in cyan, those overlying the cardioblasts in yellow, those overlying the pericardial cells in white, those ventral to both cell types in blue. By 30 mins the heart cells have moved more dorsally than the overlying ectodermal landmarks such that the cyan tracked points are now overlying the cardioblasts and the yellow tracked points are overlying the pericardial cells in many cases. By 60 min ectodermal landmarks move also in the anterior-posterior direction without corresponding movement of the heart cells. Full movie is supplemental movie 4.

(B) Pairs of landmarks, one ectodermal cell junction (white) and its nearest underlying heart cell junction (magenta), that are in the same *x y* position at *t=0* have been tracked. After 45 minutes, the heart cell landmarks have clearly moved more dorsally than their paired ectodermal landmarks. After 90 minutes, cardioblasts have met at the dorsal midline and there has been extensive anterior-posterior movement of the ectodermal cells with only minor anterior-posterior movement of the heart cells. Full movie is supplemental movie 5.

Figure 5: Ablation of ectodermal cell junctions at the midline causes different effects on the heart cells depending on their position.

(A-B) Maximum intensity Z-projection of a dorsal view of a wild type living stage 15 embryos with ectodermal and amnioserosa cells labeled using a *ubi>shgGFP* (green) construct and cardioblasts (c) and pericardial cells (pc) with *hand>moe-mCherry* (red) construct. Ablation was carried out at *t=0* along the dotted line to break cell

junctions of the ectoderm along the midline and images recorded continuously (every 10-20 seconds depending on number of Z slices) and representative images shown. Arrows indicate particular ectodermal cell junctions as they move laterally following ablation. (A) Ablation when the heart cells are still one cell diameter away from the midline, causes both ectoderm and heart cells to move laterally. Heart cells stay beneath marked junctions (arrows).

(B) Ablation in slightly older embryos at the point when heart cells are just about to meet at the midline causes lateral movement of the ectoderm without movement of the heart. Heart cells remain in place and do not follow lateral movement of marked junctions (arrows).

Figure 6: Mispositioning of hearts cells relative to ectoderm in *wun wun2* mutants

Dorsal view (A-B) and reconstructed sagittal view from position of dashed line (C-D) of control (A,C) and *wun wun2* mutant (B,D) stage 14 embryos containing a *hand>moeGFP* construct to label the heart stained with antibodies against GFP (green), and the septate junction component NrxIV (red) to label the ectodermal cells and DAPI to label nuclei. In control embryos, the leading edge cells of the ectoderm (white arrows in A,B and red arrows in C,D) were more medial then the underlying heart cells (green arrows in C,D) whilst in *wun wun2* embryos the heart cells and leading edge cells occupied the same medial position.

Dorsal view (E-H) and reconstructed sagittal views (insets) from position of dashed lines of GFP fluorescence from living stage 14 embryos containing a *ubi>shgGFP* construct to label the amnioserosa (as) and ectoderm and *hand>moeGFP* construct to label the heart. In control (E,G) the heart cells do not make contact with the amnioserosa until the end of dorsal closure (G). Full movie is supplemental movie 6. In *wun wun2* mutants (F,H) the cardioblasts (c) make contact with the amnioserosa during mid dorsal closure (F). Full movie is supplemental movie 7.

Dorsal view (I,J,M,N) and reconstructed sagittal views (K,L,O,P) from position of dashed lines from stage 14 (I,J) and 15 (M,N) embryos containing a *hand>moeGFP* construct to label the heart and stained with antibodies against Armadillo (red)

which labels the ectoderm (ec) and amnioserosa (as), Dystroglycan (cyan) and GFP (green). During dorsal closure in both wild type (I,K) and *wun wun2* mutants (J,L) cardioblasts are polarized as indicated by the prominent localization of Dg to medial membrane domains (arrows). After completion of dorsal closure wild type hearts (M,O) show a single lumen (arrowhead in O) whereas mutant hearts show multiple lumens (arrowheads in P) and an accumulation of Arm staining from remnants of the amnioserosa (arrow in P).

Figure 7: Dorsal closure is slower in *wun wun2* mutants

(A-B) Dorsal view of living stage 14 control (A) and *wun wun2* mutant (B) embryos containing a *ubi>shgGFP* construct to label the amnioserosa imaged every 5 minutes with every forth time point shown. At *t=0* the maximal width of amnioserosa is the same in both genotypes (just under 60um). After 100 minutes dorsal closure has finished in the wild type embryo whilst it is still proceeding in the mutant. Scale bar = 20 um.

(C) Graph showing rate of dorsal closure, as measured by the reduction in width of the amnioserosa at its widest point for control (n=7) and *wun wun2* mutant embryos (n=6). Dorsal closure was considered completed (*t=0*) when the maximal width of the amnioserosa first became smaller than 5um.

Figure 8: *wun* and *wun2* are expressed and required in ectodermal and heart cells

(A-E) In situ hybridisation patterns on stage 13-14 embryos of *wun* (A,C) and *wun2* (B,D,E) with a lateral view (A,B) and dorsal view (C,D) and a stage 16 embryo with a dorsal view in which the heart cells have met at the midline (E) using colorimetric (A-D) and fluorescent detection (E). Both *wun* and *wun2* are expressed in stripes in the ectoderm (arrowheads in A,B) and also in heart cells (arrowheads in C,D). For *wun2* these heart cells are pericardial cells (arrowheads in E) which sandwich the cardioblasts that do not express *wun2* (arrow in E). Scale bar is 20um for (E).

(F) Graph showing heart morphology as judged live using a *hand>moeGFP* construct in embryos laid by *wun wun2* germ line clone females mated to males carrying *wun[CE]*, which disrupts *wun* and *wun2*, a *UASwun2myc* construct in conjunction with drivers that express in heart cells (*handGal4*) or the ectoderm (*69BGal4*). *n* denotes number of embryos scored.

Figure 9: Schematic illustrating relationship between ectoderm and heart cells in wild type and *wun wun2* mutants.

Cross section schematic for dorsal side of wild type and *wun wun2* mutant embryos during (upper) and upon completion of dorsal closure (lower).

In wild type during mid dorsal closure, pericardial cells (pc) and cardioblasts (c) are initially more lateral to the overlying leading edge cells (le) of the ectoderm (e), do not make contact with the amnioserosa (a) and move to the midline using the movements of the ectoderm (grey arrows). During the final stages of dorsal closure the heart cells migrate autonomously to underneath the leading edge cells (green arrows).

In *wun wun2* mutants, dorsal closure is slower and before dorsal closure completes the heart cells have migrated to underneath the leading edge cells (green arrows) where they make contact with the amnioserosa. Upon completion of dorsal closure, amnioserosa remnants (blue) can disrupt cardioblasts from meeting at the midline.

Figure S1: Heart defects in *wun wun2* mutants become progressive worse

(A-B) Dorsal view of living stage 16 *Drosophila* embryos carrying a *hand>moeGFP* transgene to label the heart (anterior to left) viewed using widefield fluorescence microscopy. Images were taken every 2 minutes and every 30th time point is shown. *t=0* was defined when cardioblasts met at midline. In wild type (A) the remaining heart cells meet (arrowhead) and remain tightly aligned to the end of embryogenesis (full movie is supplemental movie 1). In *wun wun2* mutant embryos (B) initial defects in cardioblasts meeting at the midline (arrowheads) get progressively worse as the embryonic muscles begin to twitch (full movie is supplemental movie 2).

Figure S2: Pericardial cells loose their close association with cardioblasts at the end of embryogenesis

Dorsal view of living early (A) and late (B) stage 16 embryos carrying a *hand>moeGFP* transgene to label the heart (anterior to left) viewed using confocal microscopy showing the change in pericardial cell (pc) shape and loss of their close association to cardioblasts at the very end of embryogenesis. (C) A time series from the same embryo in (B), during a heart beat, showing the outward displacement of several pericardial cells (arrow in middle panel), followed by their recoil to again lie adjacent to the cardioblasts (arrow in lower panel).

Supplemental Movies

Supplemental movie 1: Movie relating to figure S1A (wild type embryo)

Supplemental movie 2: Movie relating to figure S1B (*wun wun2* mutant)

Supplemental movie 3: Movie relating to figure 2A

Supplemental movie 4: Movie relating to figure 4A

Supplemental movie 5: Movie relating to figure 4B

Supplemental movie 6: Movie relating to figure 5A, first image is prior to ablation, then every 12 secs post ablation.

Supplemental movie 7: Movie relating to figure 5B, first image is prior to ablation, then every 7 secs post ablation.

Supplemental movie 8: Movie relating to figure 6E,G (wild type embryo)

Supplemental movie 9: Movie relating to figure 6F,H (*wun wun2* mutant)

Supplemental movie 10: Movie relating to figure S2

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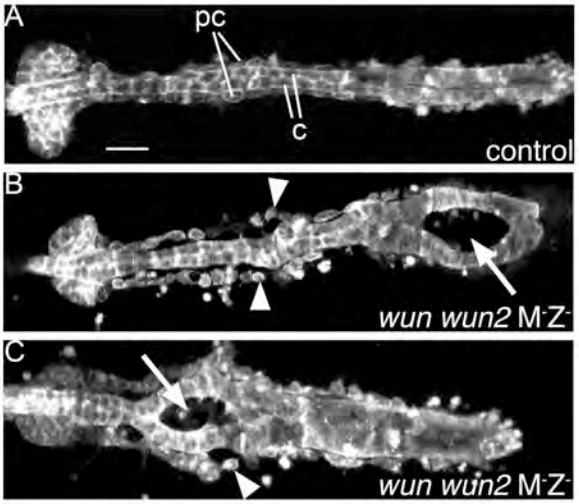
Figure 1



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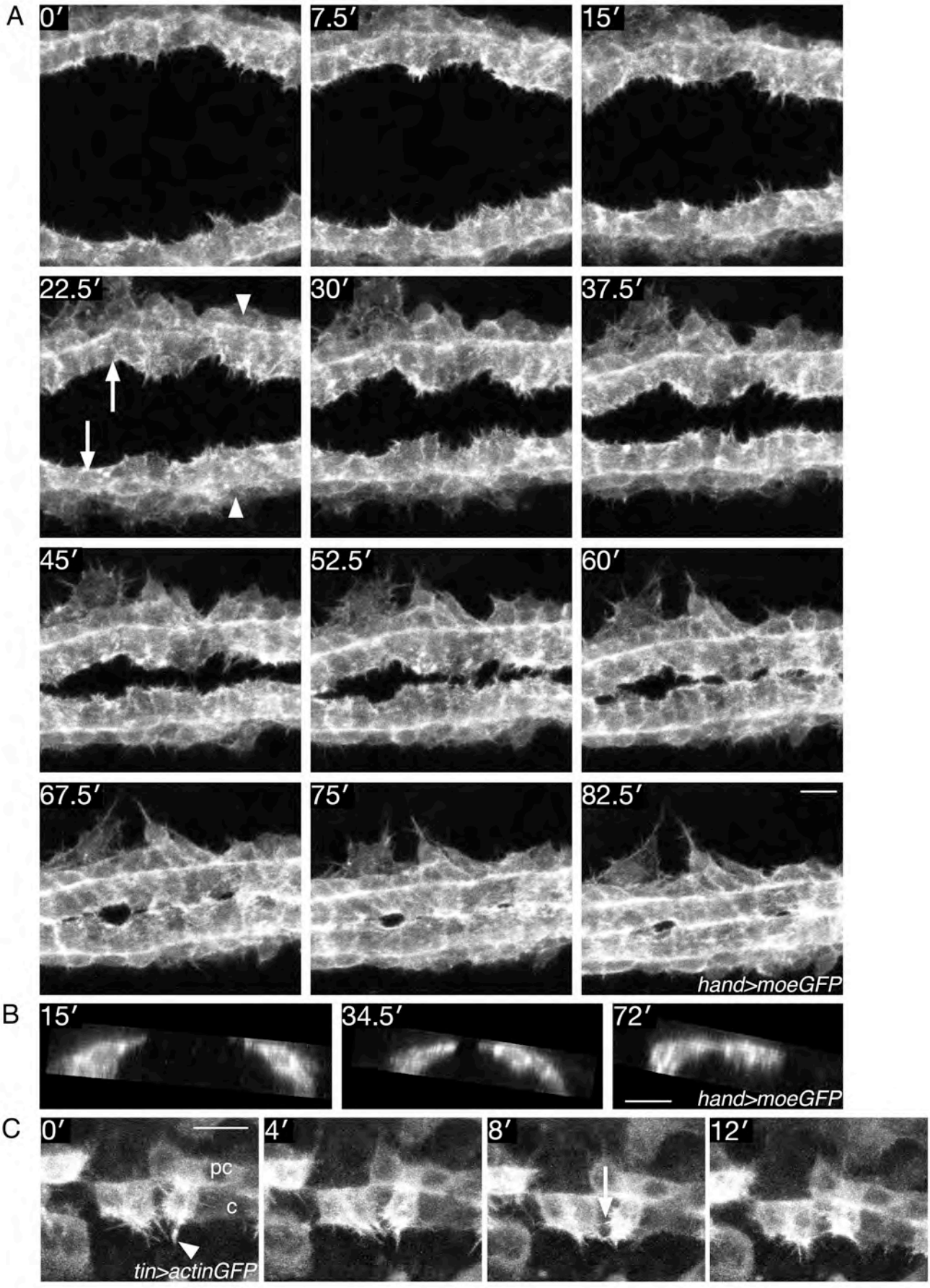
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Figure 3

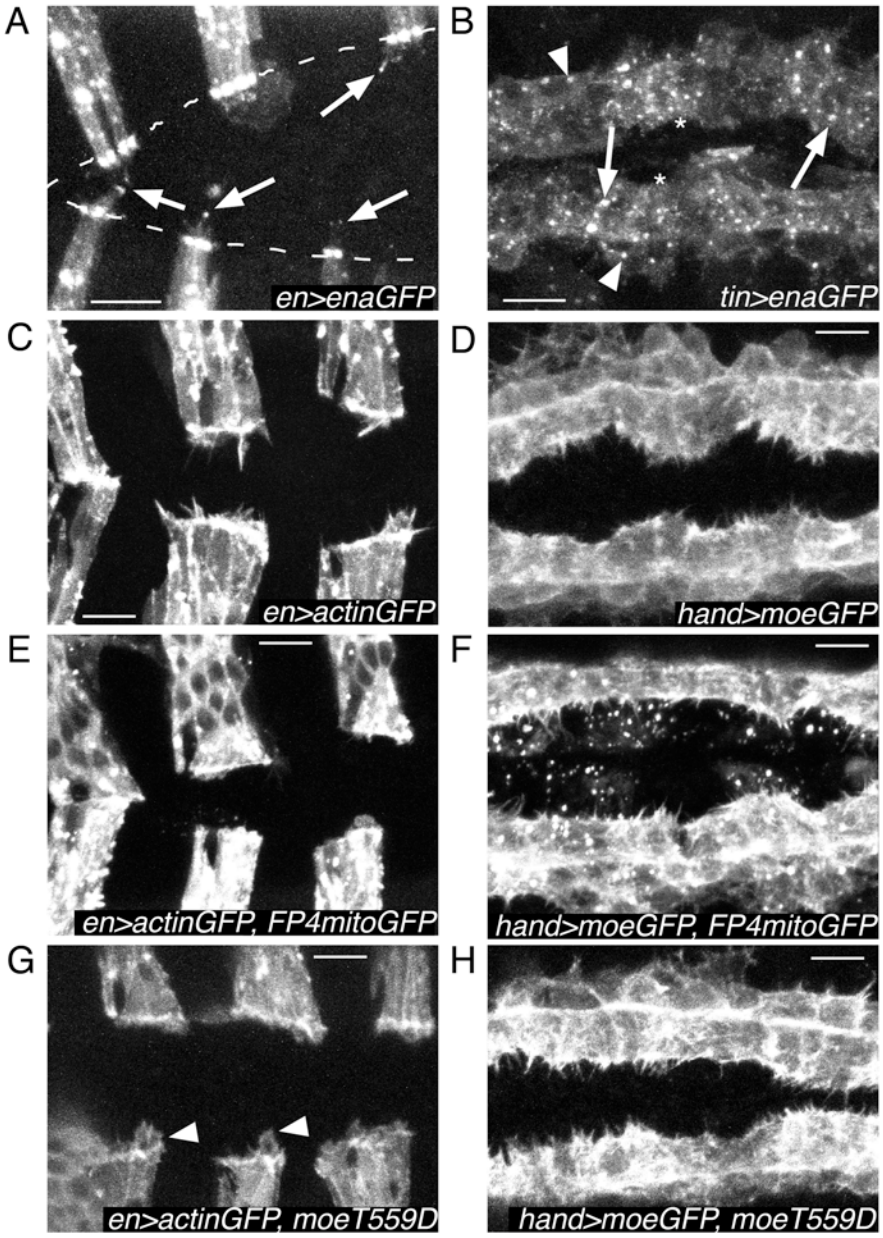
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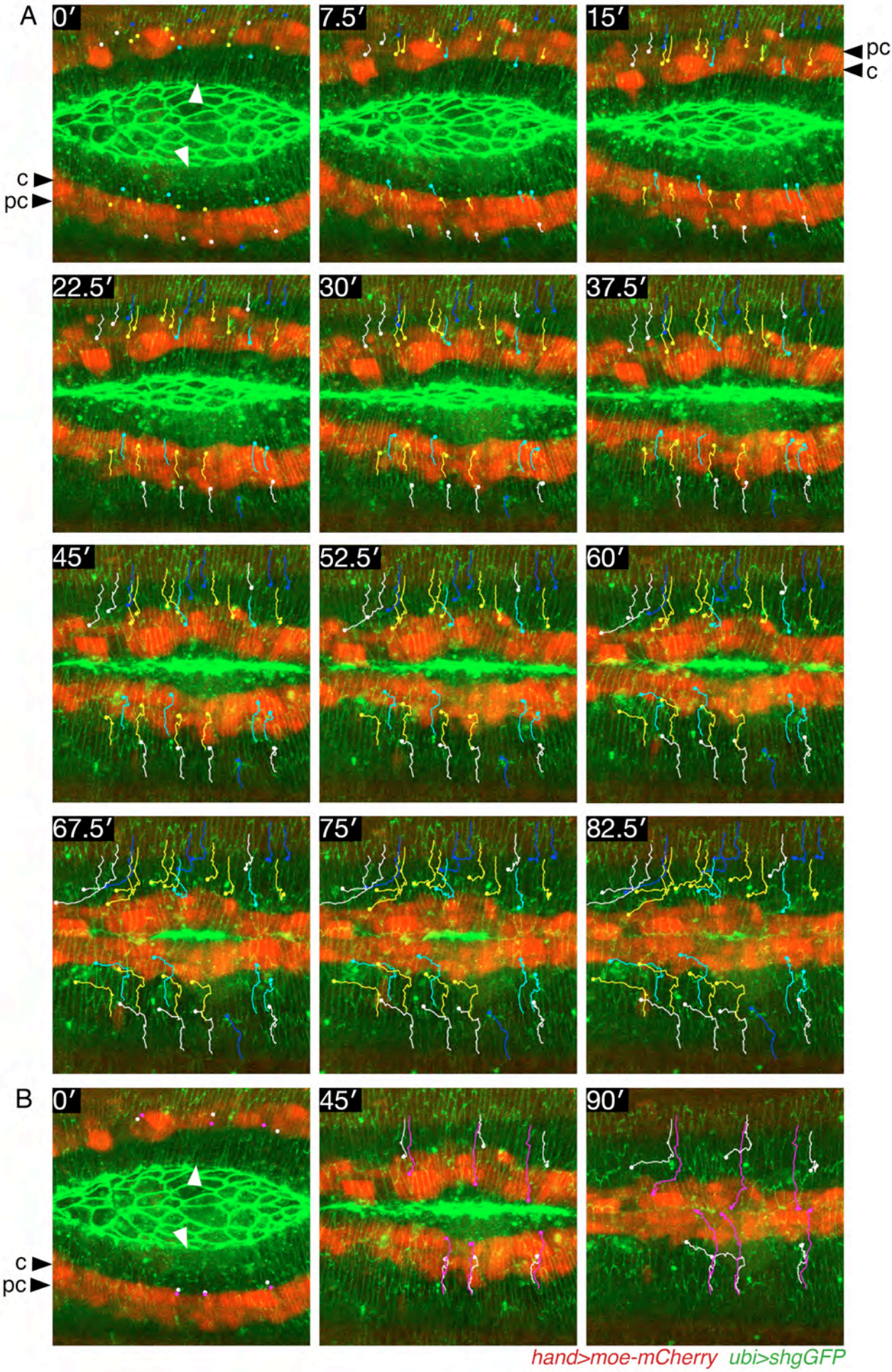
Figure 4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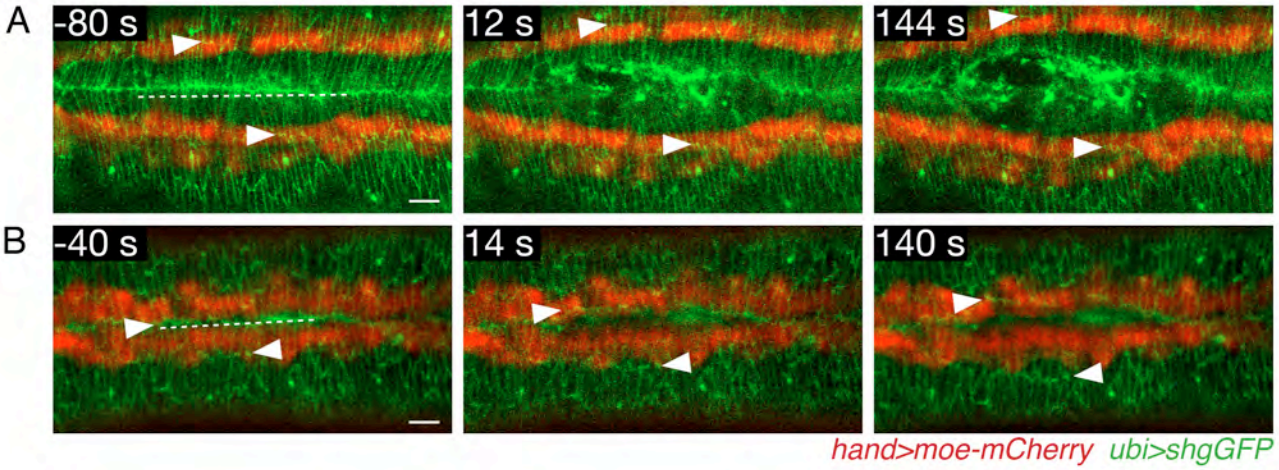

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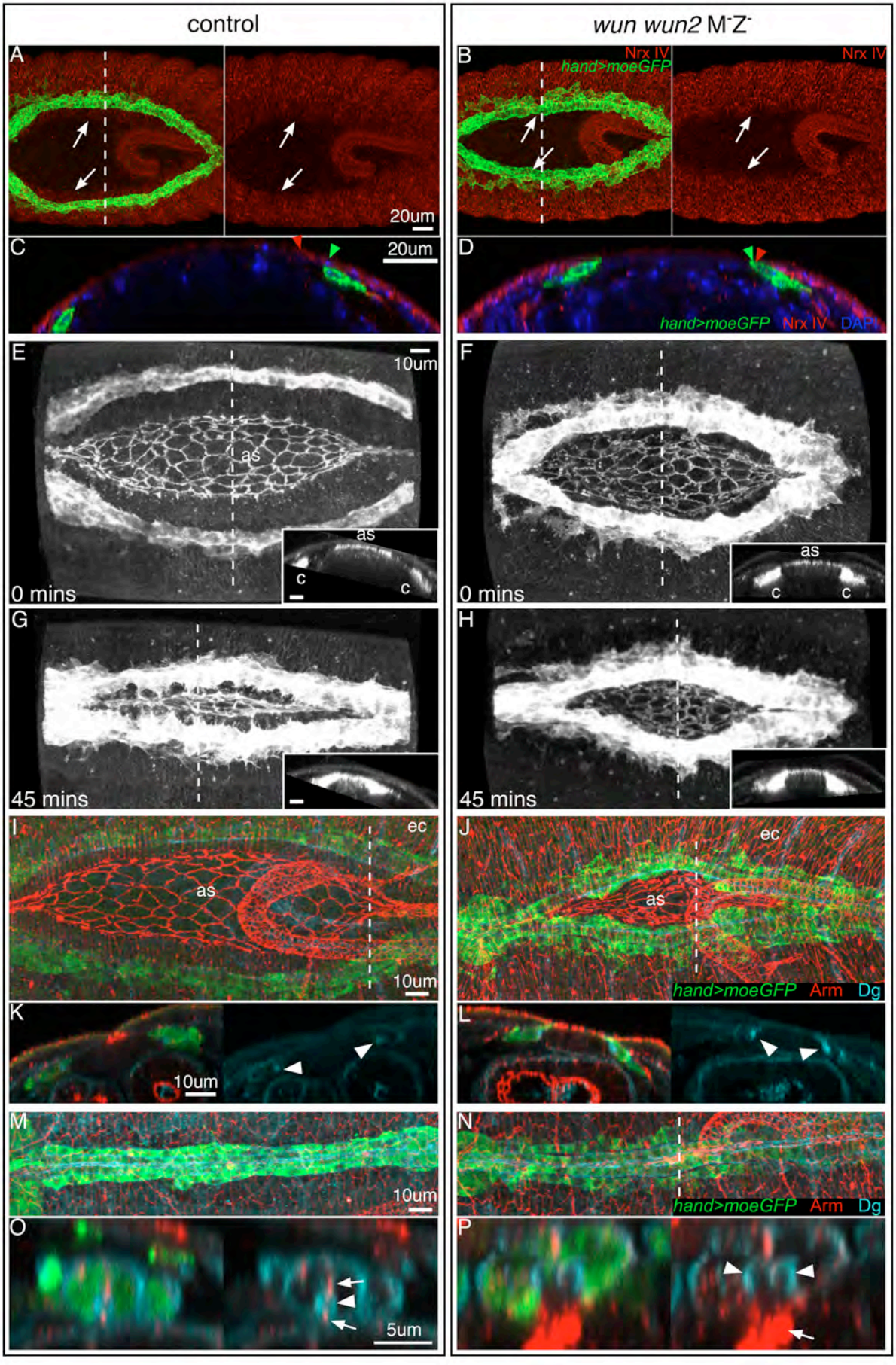
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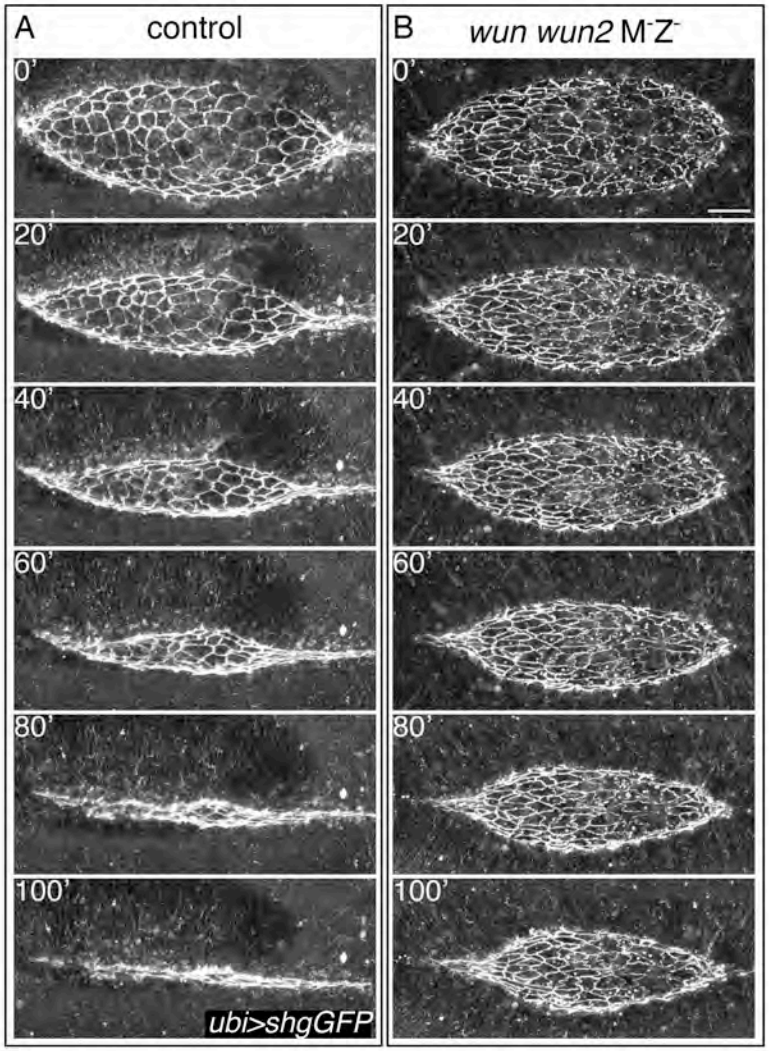
Figure 7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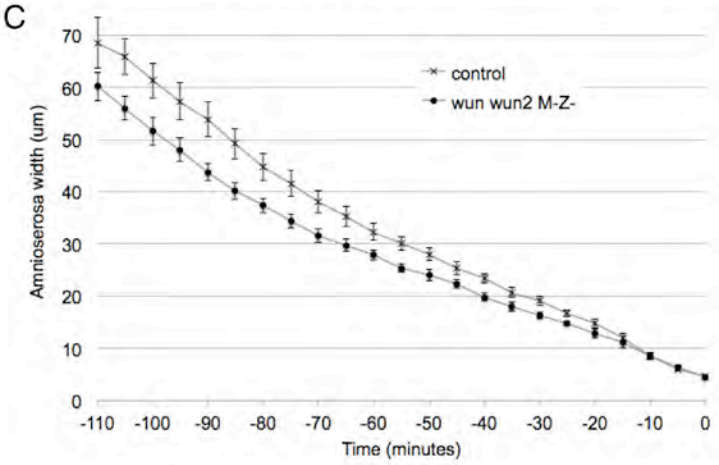
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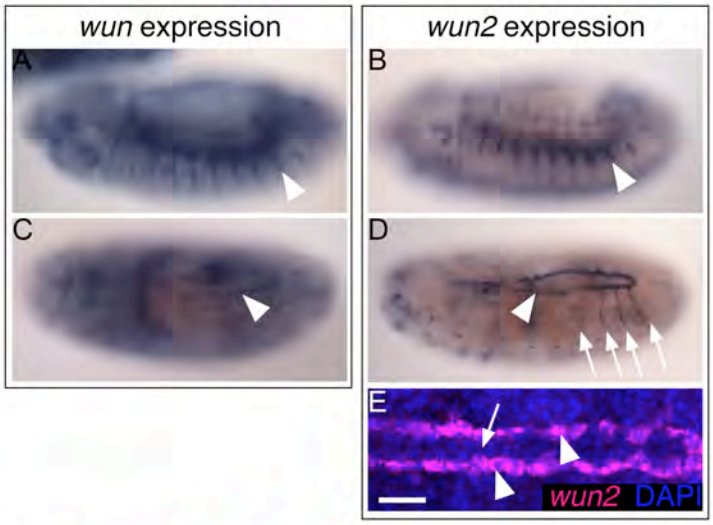
Figure 8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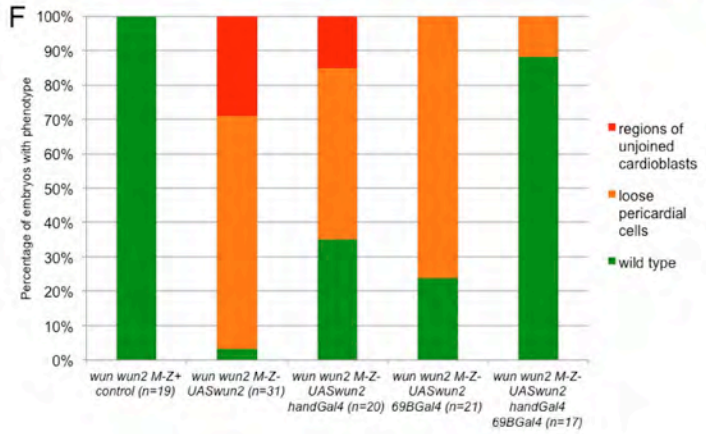
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Figure 9

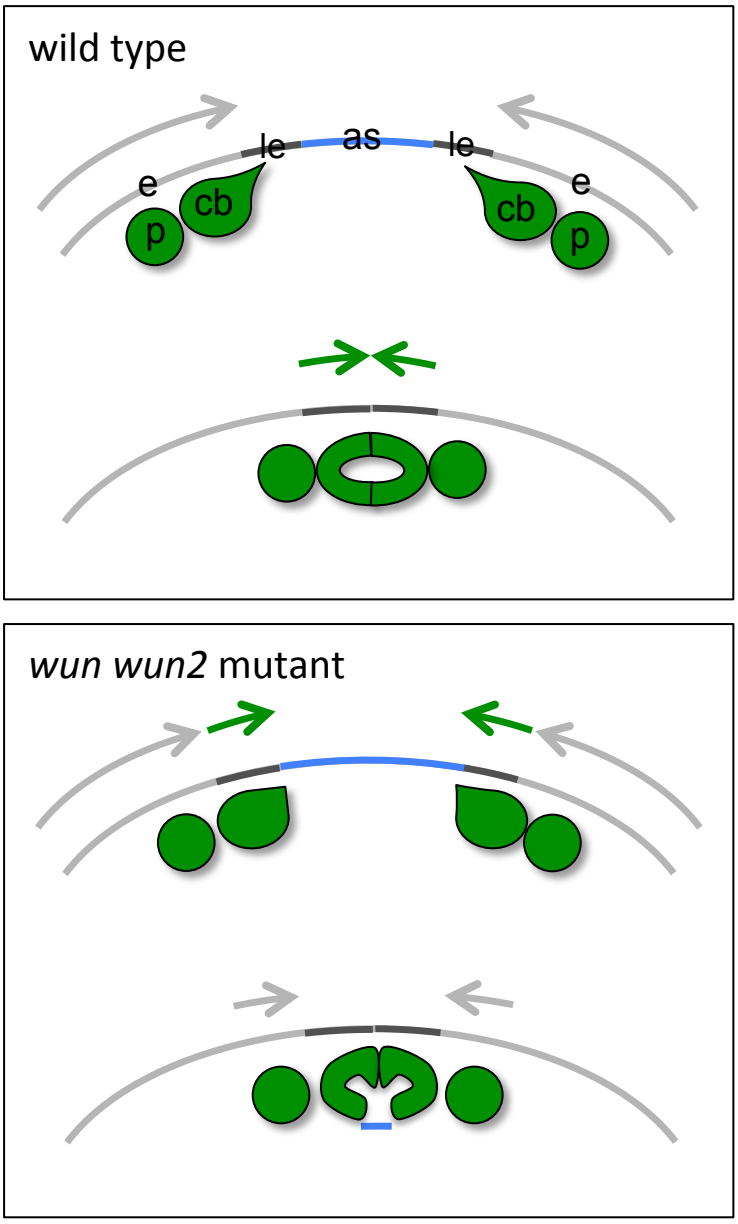

Figure S1

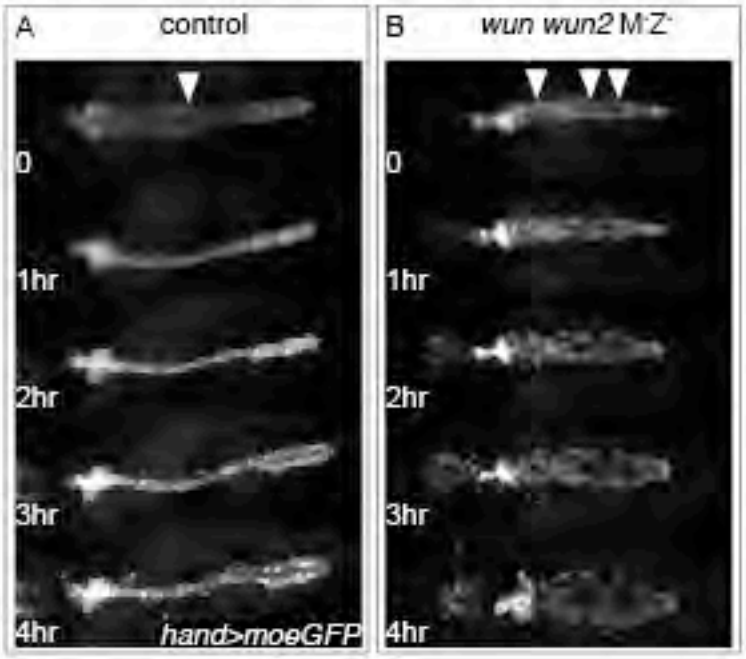
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Figure S2