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MOLECULAR CONTROL OF MUSCLE PATTERNING, DEVELOPMENT AND STABILITY IN DROSOPHILA

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STABILITY IN *DROSOPHILA*

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B.S., Biology, University of New Mexico
B.S., Anthropology, University of New Mexico

DISSERTATION

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DEDICATION

To Wayne and Lynne LaBeau, Holly and Shannon LaBeau BlueEyes and Kayley LaBeau, Thank you for your support, love and stewardship. I know it has been tough, but whether it was a hug or a home cooked meal, you always knew what I needed. You are the best family I could have ever asked for. I love you all.

To Mark DiMenna, my love, you willingly sacrificed years of evenings and weekends, picked up the slack around the house and acted as an emotional pillar so that I could pursue an advanced degree in science. I sincerely wish to thank you for all of your patience and support during this stressful time of our lives. I know it wasn't easy, and that the path that we have chosen for our lives may never be, but you have exemplified by your love, preserving actions and unwavering commitment to our family during this time the great measure the man that I have chosen to marry. You are my best friend, soul mate and greatest romance.

To Madeleine, my little baby girl, you are my sprite and my inspiration. Let nothing stand between you and the realization of your dreams. I can't wait to see what wonders you will encounter, create and achieve as your life unfolds. I love you with all of my being and from depths of my heart.

ACKNOWLEDGMENTS

COLLABORATION

Collaboration is key to the generation of quality science. Whether it be through discourse, experimental design, teaching, technical generation of data or simply contributing a unique piece of the story, each of the individual of the collaborative team is essential to the coherent and successful outcome of the joint research effort.

Learning to successfully collaborate with my senior advisors, peers and trainees has been a large and important component of my graduate training. Learning to collaborate as a graduate student meant understanding that the responsibility for outcomes and interpretations rests equally between collaborators as should the recognition of their individual efforts.

In recognition of these facts that the first two chapters of this manuscript are presented as previously published with all collaborators recognized on the chapter title page. The last chapter recognizes the individuals who have contributed thus far to the work.

MOLECULAR CONTROL OF MUSCLE PATTERNING, DEVELOPMENT AND STABILITY IN *DROSOPHILA*

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ABSTRACT

Muscle is a highly conserved and specialized tissue in animals. Failure of proper muscle patterning, maintenance and stability in animals can have variety of consequences for the individual. Mutations in loci that control muscle can result in death during embyogenesis, dysmorphism, and/or reduced lifespan after progressive muscle degeneration (Emery 2002, Mathews and Moore 2003, Amato and Griggs 2011). Muscle defects, such as these, have origins ranging from improper transcriptional control to direct sequence disruptions in proteins that play a wide variety of roles in the cell (Emery 2002, Mathews and Moore 2003, Amato and Griggs 2011). Thus, out of this disorder, comes the opportunity to elucidate the role(s) of individual players and interactions required to build a molecular portrait of normal, functional muscle. Here in, are presented three studies carried out in the *Drosophila* model in an effort to explore some of these required genetic elements of muscle and the role that their resultant expression cooperates in order to build and sustain normal, healthy muscle: 1) Bithorax complex control of alary (cardiac support) muscle patterning during embryogenesis 2) a novel structural support function identified for a common ubiquitin ligase (*thin/Trim32*

ortholog) acting in myofibril stability 3) developmental delay and sex-lethality found during re-evaluation of a *vinculin* (cell adhesion/migration/myofibril stability complex component) resulting from a chromosomal inversion mutant.

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INTRODUCTION

Muscle is constantly under the stress and strain of its own powerful force and torsion. Secure and proper patterning of muscle, along with a reliable maintenance/repair mechanisms, have been key to the evolutionary conservation of this cell type in animals. Muscle development and maintenance has long been modeled using the genetically tractable *Drosophila* system.

During embryogenesis in *Drosophila,* the mesoderm migrates dorsally along the ectoderm from the ventral furrow to its final orientation (Kam *et al.* 1991, Leptin *et al.* 1992). Invagination of precursor mesodermal cells, during gastrulation, initiates the formation of the ventral furrow (Kam *et al.* 1991, Lilly *et al.* 1994). During this stage Mef2 and Twist, transcription factors responsible for myogenesis, (Gossett *et al.* 1989, Nguyen *et al.*1994, Baylies *et al.*1996) increase in abundance (Gravely *et al.* 2011) as the mesoderm begin their migration dorsally (Kam *et al.* 1991, Leptin *et al.* 1992). The mesoderm matures and begins to divide into both the visceral and skeletal muscle groups (Kam *et al.* 1991, Leptin *et al.* 1992). As the germ band retracts the dorsal crest appears and cardiac fate specifies with the accumulation of Tinman (Bodmer 1993, Evans *et al.* 1995, reviewed Cripps and Olsen 2002). The hemisegments of the cardiac tube rest at the apex of the dorsal ridge. These rows of cardiac cells are the leading edge if the migrating mesoderm. As the migrating cardiac hemisegments meet at the dorsal midline they fuse together thus forming the dorsal vessel (Rugendorff *et al.*1994).

Maturation of muscle precursors involves the development of myotubes. Myotubes are generated via the fusion of newly fated founder cells and fusion competent myoblasts into elongated multinucleated cells (Bate 1990, Broadie and Bate 1993, review Haralalka *et al.* 2010). As syncytial muscle fibers begin to form, filapodia extend to tendon cells that, in turn, attach to the ectoderm (Broadie and Bate 1993, review, Schweitzer *et al.* 2010).

Muscle patterning organizes and diversifies along an anteroposterior (AP) axis in *Drosophila* and is controlled by the expression of members of the Hox cluster of transcription factors (review in Lewis 1982, Azpiazu *et al.*1996). Muscle patterning is controlled, borders established, and myoblast orientation and number is segmentally specific as is dictated by the local Hox factors (Enriquez *et al.*2010).Cardiac diversification of the heart and aorta regions, during the later stages of embryogeneisis, is specifically controlled by members of the Bithorax complex to form the heart and aorta regions of the dorsal vessel (Lovato *et al.* 2002; Ponzielli *et al.* 2002;Lo *et al.*, 2002; Perrin *et al.*, 2004; Ryan *et al.*, 2005). Patterning of many of the myotendonous juntions form in the developing embryo concentrate at the segmental borders and are focal points of attachment to tendon cells. (Lewis 1982, Volk 1999) These myotendonous junctions at the segment borders are rich in attachment complexes, such as adherens junctions and the Integrin/Talin/Vinculin complex, which also work to attach and stabilize myofibrils at the z-line of the sarcomere.

Sarcomeres are the most basic contractile unit of muscle. Sarcomeres function by the calcium dependant, ATP coupling of Myosin as it binds to and

shifts Actin in a 'power stroke' (Kamisago *et al*. 2000; Lange *et al*. 2006). Each

sarcomere produces a remarkably strong and swift cellular force. Sarcomeres

are repeated structures and are interlinked at the z-line of muscle (Kamisago *et*

al. 2000; Lange *et al*. 2006). This linear organization of multiple sarcomeres,

which extends the length of the muscle cell is known as the myofibril. Each

muscle cell has numerous myofibrils which are stabilized by structural-functional

complexes, such as the costamere (Pardo *et al*. 1983; review Ervasti 2003).

The costamere stabilizes and organizes large-scale myofibril bundles

along the exterior of the z-line of the collected sarcomeres. Costameres are

thought to be essential to myofibril stability by keeping sarcomeres in register

during contraction (Pardo *et al*. 1983; review Ervasti 2003). Costameres are also

thought to possibly act as force stabilizers during contraction, thus mitigating

damage that would otherwise be inflicted on the non-contractile components of

muscle cells, such as the sarcolemma and nuclei. Costameres provide a force-

coupling bridge between the myofibrils to the sarcolemma and are able to

interact directly with the extracellular matrix via β-Integrin (Pardo *et al*. 1983;

review Ervasti 2003). Individual components of the costamere have been shown

to bind myosin and ubiquitinate or simply bind to actin thus indicating that

filament processing during muscle reloading may depend on components of the

costamere (Kudryashova *et al*. 2005; review Ervasti 2003) Other components are

involved, not only in this myofibril stability complex, but act to bind the

cytoskeleton to the extracellular matrix through focal adhesions at myotendonous

junctions (Critchley 2000; review Ervasti 2003). This may suggest an evolved,

modulated duplication of function (as opposed to duplication and sequence evolution for novel function) of costamere complex components in multiple modes of muscle stability and inter/intracellular adhesion.

Here in, three distinct studies are presented using the *Drosophila* model that further clarify the role of human orthologs: Ultrabithorax, proteins either controlling or contributing to the normal patterning and stability of muscle.

Observations of muscle development, maintenance and stability were carried out in the *Drosophila system* and presented in the following three discrete studies. The first investigation explores the impact of the Bithorax complex on the ability of the animal to properly form, attach and pattern alary muscles along the cardiac tube during embryonic development. The second study shifts examine the novel structural role of a known ubiquitin ligase (*thin/Trim32* ortholog) as it acts in the costamere to secure myofibrils during muscle motion. The final inquiry observes the overall phenotypic effects coinciding with a loss of function in a known actin binding costamere component, Vinculin during the latter developmental stages.

CHAPTER 1

BITHORAX COMPLEX GENES CONTROL ALARY MUSCLE PATTERNING

ALONG THE CARDIAC TUBE OF *DROSOPHILA*

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ABSTRACT

Cardiac specification models are widely utilized to provide insight into the expression and function of homologous genes and structures in humans. In *Drosophila*, contractions of the alary muscles control hemolymph inflow and support the cardiac tube, however embryonic development of these muscles remain largely understudied. We found that alary muscles in *Drosophila* embryos appear as segmental pairs, attaching dorsally at the *seven-up* (*svp*) expressing pericardial cells along the cardiac dorsal vessel, and laterally to the body wall. Normal patterning of alary muscles along the dorsal vessel was found to be a function of the Bithorax Complex genes *abdominal-A* (*abd-A*) and *Ultrabithorax* (*Ubx*) but not of the orphan nuclear receptor gene *svp*. Ectopic expression of either *abd-A* or *Ubx* resulted in an increase in the number of alary muscle pairs from seven to 10, and also produced a general elongation of the dorsal vessel. A single knockout of *Ubx* resulted in a reduced number of alary muscles. Double knockouts of both *Ubx* and *abd-A* prevented alary muscles from developing normally and from attaching to the dorsal vessel. These studies demonstrate an additional facet of muscle development that depends upon the Hox genes, and define for the first time mechanisms that impact development of this important subset of muscles.

INTRODUCTION

The cardiac system in *Drosophila* is an excellent model system for the analysis of cardiac development in higher animals. The insect cardiac tube, or

dorsal vessel, is comprised of a number of distinct cell types that arise through complex processes of cell specification in response to signals both from within the mesoderm and from the ectoderm (reviewed in Cripps and Olson 2002 and Tao and Schulz 2007). The mature dorsal vessel is comprised of several distinct cell types: muscular cardial cells, which mediate pumping of hemolymph (Bodmer and Frasch 1999); pericardial cells, which play diverse roles in homeostasis, cardiac physiology, and formation of the adult circulatory system (Fujioka *et al*. 2005, Miller 1950 and Togel *et al*. 2008); and alary muscles, that are thought to support the dorsal vessel during locomotion, to help modulate the flow of hemolymph into the cardiac tube (Bate 1993 and Rizki 1978), and to serve as scaffolding for neurons at later developmental stages (Dulcis and Levine 2003).

Within each of these components of the cardiac tube, there is also diversity along the anteroposterior (AP) axis. For example, hemolymph enters the muscular cardiac tube in a region termed the heart that spans approximately three posterior segments. Located in these heart segments are sets of specialized cells forming valve-like structures that express the *seven-up* (*svp*) orphan nuclear receptor gene (Molina and Cripps 2001 and Ponzielli *et al*. 2002). Hemolymph then is pumped anteriorly from the heart through the aorta, and ejected near the brain, prior to percolating back through the body cavity. A series of studies have demonstrated that the AP diversity in the cardiac tube arises from the actions of the homeotic selector genes, predominantly those of the Bithorax

Complex (Lovato *et al.* 2002, Ponzielli *et al.* 2002, Lo *et al.* 2002, Perrin *et al.* 2004 and Ryan *et al.* 2005).

While some of the factors that control patterning of many cell types in the dorsal mesoderm are understood, relatively little is known about the formation of alary muscles in *Drosophila*. The alary muscles are located symmetrically along the dorsal vessel in the mature embryo, attaching dorsally close to the cardiac tube and laterally to the body wall (Bate 1993). However a detailed description of their organization, and the identification of genes which impact their patterning, has yet to be presented. In this manuscript, we have studied the development of the alary muscles in wild-type and mutant embryos. We show that the alary muscles attach dorsally to the *svp* expressing pericardial cells adjacent to the cardiac tube, and laterally to one of two distinct locations on the body wall. In addition, we show that not all segments form alary muscles attaching to the cardiac tube. Importantly, genes of the Bithorax Complex, whose function is required for normal alary muscle development, control this process. These studies, for the first time, describe important characteristics of the alary muscles in *Drosophila*, and provide further insight into the roles of the Hox genes in muscle development.

MATERIALS AND METHODS

Drosophila stocks

Drosophila stocks were maintained at 25 °C on Carpenter's medium (Carpenter 1950) in plastic bottles or vials. The strain w^{1118} was used as the non-

mutant control. Knockout stocks were: *TM1/Ubx*[109] for *abd-A* plus *Ubx*

; *TM1/Ubx*[9.22] for *Ubx*; *TM1/abdA*[MX1] for *abd-A*; *TM3/svp*[1] and *TM3/svp*[3] for *svp*.

These stocks were obtained from the Bloomington *Drosophila* Stock Center,

except the *svp*[1] stock which was from James Skeath (Washington University, St.

Louis, MO). Expression assays were carried out utilizing the GAL4-UAS system

(Brand and Perrimon 1993): *UAS-Ubx* and the *24B-Gal4* driver line were

obtained from the Bloomington Stock Center. *UAS-abd-A* was obtained from

Alan Michelson (Harvard University, MA). The SCE *svp–lacZ* strain was

described in Ryan *et al*. (2007).

Immunohistochemistry

Antibody staining of embryos was performed in accordance with Patel

(1994). Larval fillets were prepared according to Molina and Cripps (2001). The

primary antibodies and concentrations were: Mouse anti-Pericardin at 1:4

(Chartier *et al*. 2002), rabbit anti-Tinman at 1:250 (Yin *et al*. 1997), mouse anti-

UBX at 1:50 (White and Wilcox 1984), mouse anti-ABD-A at 1:000 (Macias *et al*.

1990), mouse anti-myosin heavy-chain (MHC) at 1:500 (Kiehart and Feghali,

1986), and rat anti-Tropomyosin (Abcam Inc., Cambridge, MA) at 1:1000.

Biotinylated secondary antibodies were anti-mouse and anti-rat, both used at

concentrations of 1:1000 (Vector Laboratories, Burlingame, CA). Antibody

detection was performed using the Vectastain Elite kit (Vector Laboratories,

Burlingame, CA) and DAB staining kit (Vector Laboratories, Burlingame, CA).

Fluorescent staining was generated using the anti-mouse or anti-rabbit

secondary antibodies Alexa-488 or Alexa-568 conjugated, at concentrations of

1:2000 (Molecular Probes, Seattle, WA). Non-fluorescent double-stains were carried out by staining embryos sequentially with antibodies and developing the first antibody using DAB staining reagent with $NiCl_2$ added; followed by the second antibody detected with DAB staining solution lacking $NiCl_2$.

Imaging

Microscopy was performed on an Olympus BX51 microscope using DIC or fluorescent optics. Stained embryos used for dissection were initially submerged in 100 μL of 80% (v/v) glycerol/1XPBS, and dissected as described in Lovato *et al.* (2002). Filleted preparations were viewed at 600× magnification using an oil immersion lens and DIC optics. Representative samples were photographed using 35 mm slide film, and the resulting slides were scanned and assembled using Adobe Photoshop. For the filleted preparations, the figures presented here are representative montages of individual photographs taken at slightly different focal planes in order to fully document the dorsal vessel and alary muscles.

RESULTS

One obstacle in the characterization of alary muscles has been the paucity of molecular markers that specifically identify these cells. Therefore, to determine the best method for observing the general physical structure of the alary muscles, wild-type *Drosophila melanogaster* embryos were stained with antibodies for targets prevalent in either cardiac or cardiac and skeletal muscle tissue.

In a first series of experiments, we utilized an antibody to Pericardin (anti-Prc), an extracellular matrix protein secreted by the pericardial cells (Chartier *et al*. 2002; Fig. 1A and C), and anti-Tinman (anti-Tin), which marks the majority of cardial cells and a subset of pericardial cells (Bodmer 1993 and Azpiazu and Frasch 1993; Fig. 1B and C). Localization of these primary antibodies was visualized by immunofluorescence in whole-mount embryos. We found that, whereas it was possible to find preparations that showed Prc accumulation along the alary muscles, the signal in this region was faint and difficult to observe in all samples.

As an alternative, we stained embryos with antibodies to muscle structural genes, such as anti-myosin heavy-chain (anti-MHC; Kiehart and Feghali 1986) or anti-Tropomyosin (anti-Tm; Peckham *et al*. 1992). Localization of these antibodies was accomplished using immunohistochemistry. Stage 16 embryos were filleted and the dorsal vessel and associated tissues were viewed from the ventral side of the body wall. While more labor-intensive, this preparation gave richer structural images of alary muscles, presenting a much higher level of detail.

Using these preparations, we verified that there are seven pairs of alary muscles that attach medially and dorsally along the dorsal vessel (Fig. 1D). These muscles were usually paired, although we occasionally observed a muscle attached in a slightly disordered manner, even in wild-type (arrowhead in Fig. 1D). For the lateral attachment sites, the most anterior five alary muscle pairs extended past muscle DA2 and terminated at the cuticle slightly posterior to the

dorsal insertion of skeletal muscle DT1 (Bate 1993, Fig. 1E and F). By contrast, the two most posterior alary muscle pairs extended further ventrally past DA2 and DA3, and inserted within the attachments of a cluster of muscles (Fig. 1G). This pattern of alary muscles attachment was retained through the end of the larval stage (Fig. 1F and G are preparations of third instar larvae). Clearly, the formation of the alary muscles is controlled by a complex series of processes that ensure that the correct number of fibers are generated and attached appropriately within the body.

In order to more directly characterize the dorsal attachment sites of the alary muscles, we studied the development of the alary cells relative to cardiac expression of *svp*. In wild-type embryos, *svp* is expressed continuously in seven groups of cardial cells along the AP axis (Bodmer and Frasch 1998), and transiently in an equivalent number of pericardial cells (Lo and Frasch, 2001). To compare the arrangement of the alary muscles relative to the Svp cells, we double-stained embryos with antibodies to MHC (brown in Fig. 2) and β-galactosidase expressed from a *svp–lacZ* fusion gene (black in Fig. 2A–C; Ryan *et al.*, 2005).

At stage 14, it was possible to identify all of the alary muscles as extensions running between the dorsal vessel and the lateral ectoderm (Fig. 2A). The dorsal ends of the alary muscles consistently attached close to the Svp expressing pericardial cells. In particular, the alary muscles extended processes in either direction along the AP axis where they contacted the *svp* pericardial cells. This observation was also apparent at stage 16 (Fig. 2B). At higher

magnifications, details of the alary muscles could be clearly observed, including the multinucleate nature of the cells, and the processes that extend specifically towards the Svp pericardial cells (Fig. 2C). These studies identified the alary muscles as skeletal muscles (based upon their syncytial nature), and suggested a mechanism for how they might be oriented towards the dorsal vessel.

Given the close association of alary muscle development with the Svp pericardial cells, *svp* null embryos were evaluated for alary muscle patterning (Fig. 2D and E). Despite the lack of differentiation of dorsal vessel cardial cells into Svp cells in these mutants, alary muscles still attached to the dorsal vessel and pericardial cells at the same locations (Fig. 2D). While occasional patterning defects were observed, these were not significantly more prevalent than in wild-type animals (see for example Fig. 1A).

To determine if the alary muscles were still extending towards the Svp pericardial cells in *svp* mutants, we studied alary muscle attachment in homozygous mutants for a *svp* enhancer trap line, *svp*[3]. In these mutants, the Svp pericardial cells can still be visualized based upon residual β-galactosidase accumulation (black in Fig. 2E); nevertheless, the alary muscles were still observed to orient towards these marked pericardial cells (Fig. 2E). These data indicated that alary muscle patterning along the dorsal vessel is not dictated by the presence or absence of *svp* expression. One interpretation of these data is that there is some form of molecular signature in the Svp pericardial cells, presumably produced independently of *svp* function, that is required for normal alary muscle attachment. A more simple explanation is that the alary muscles

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attach to the dorsal vessel in the location immediately above where they are specified, and that this location just happens to be in the vicinity of the Svp pericardial cells.

Since the alary muscles attaching to the dorsal vessel only formed in a subset of embryonic segments, and given the role of Hox genes in controlling AP pattern in the cardiac tube in *Drosophila*, we next investigated a potential influence of the Hox genes upon the patterning and development of the alary muscles. Presence or absence of Hox expression in the alary muscles was evaluated using wild-type embryos immunohistochemically stained for the products of the two Hox genes *abd-A* and *Ubx*. The presence of Hox gene products is indicated by a stained black nucleus. Absence of Hox gene expression is signified by a clear or unstained muscle. Representative images of stained alary muscles are shown in Fig. 3A–D. We found that *Ubx* and *abd-A* expression in the alary muscles was generally consistent with the known pattern of segmental expression of these genes. The nuclei of the three most anterior alary pairs were positive for *Ubx* expression and negative for *abd-A*. Posterior to the three pairs, the expression pattern in the nuclei of the alary muscles switched to positive for *abd-A* and negative for *Ubx* (Fig. 3A–D). These studies suggested that individual Hox genes might be responsible for the development of different subsets of alary muscles. A summary of our descriptive studies of the alary muscles is presented in Fig. 3E.

Based on our findings of Hox gene expression in the nuclei of the alary muscles, we sought to determine whether patterning changes in the alary muscles resulted from knockout or over-expression for each of *Ubx*and *abd-A*.

Loss-of-function experiments provided evidence that the patterning of alary muscles was under the direct control of Hox genes (Fig. 4). When compared to the wild-type (Fig. 4A), mutants unable to express *Ubx*failed to develop the 2–3 most anterior alary pairs, consistent with those muscles which we had identified as accumulating Ubx protein in wild-type (Fig. 4B). By contrast, animals that were unable to produce *abd-A* still formed seven pairs of alary muscles along the cardiac tube (Fig. 4C). This apparently wild-type phenotype presumably arises from the ability of *Ubx* to specify alary muscles in posterior segments in the absence of*abd-A* function. Double knockout mutants for both *Ubx* and *abd-A* produced individuals with a drastically reduced number of alary muscles. Of the few muscles that did develop, all were randomly positioned and consisted of no true alary muscle pairs (Fig. 4D).

To complement the loss-of-function data, we also ectopically expressed either *Ubx* or *abd-A* throughout the mesoderm (Fig. 5). In both cases, this expansion of Hox gene expression produced embryos with elongated cardiac tubes and an increase in the number of normally developed alary muscle pairs (Fig. 5). For ectopic expression of *Ubx*, we observed 9.75 ± 0.16 ($n = 8$) muscles on each side of the dorsal vessel (Fig. 5A), significantly more than the seven observed in wild-type. Similarly, ectopic *abd-A* produced on average 9.50 ± 0.23 ($n = 12$) alary muscles on each side of the dorsal vessel (Fig. 5B). These

supernumerary muscles were usually paired, and in many cases the most anterior pair of muscles was angled acutely to ensure contact with the dorsal vessel (arrowheads in Fig. 5). This latter observation probably arises from the fact that the ventrolateral attachment site on the cuticle is still located in an anterior segment, whereas even under conditions of Hox gene over-expression the dorsal vessel ultimately shortens to occupy slightly more posterior sections.

Consistent with the requirements for *Ubx* or *abd-A* (in the absence of *Ubx*) to specify alary muscles in loss-of-function assays, both *Ubx* and *abd-A* were equally effective at generating supernumerary alary muscles. Taken together, these studies identify the Hox genes as being critical to alary muscle development, and further expand the cadre of tissues types which are subject to Hox gene influence.

DISCUSSION

In *Drosophila*, the seven pairs of embryonic alary muscles attach to Svp pericardial cells along the dorsal vessel as it migrates dorsally towards its final location. The alary muscles persist throughout larval development, playing what are thought to be important roles in stabilizing the location of the heart in the body cavity. In addition, modified alary muscles are also found in the adult (Molina and Cripps 2001), and there is evidence from some insects that contraction of these adult muscles is concordant with heart beating (Dulcis and Levine 2003). These published data suggest important functions for the alary muscles throughout the life cycle.

Attachment of the alary muscles to the cardiac tube occurs in the vicinity of the Svp pericardial cells. Our data clearly identify processes emanating from the alary muscles towards the pericardial cells. It is reasonable to propose that the Svp pericardial cells produce or present some molecule(s) to which the alary muscles attach, although the nature of this molecule has yet to be defined. This suggestion is consistent with the observations of Curtis *et al.* (1999), who noted that in the developing pupa the pericardial cells and alary muscles are connected by significant amounts of connective tissue. In addition, the expression of this unknown molecule must be independent of *svp* function, since in *svp* mutants the patterning of the alary muscles appears largely normal. The cardiac tube expresses several secreted molecules which are known to function in cell attraction (Tao and Schulz 2007). However enrichment for any of these in the Svp pericardial cells has not been reported.

Our data also demonstrate that normal alary muscle patterning is under the direct control of the Hox genes *Ubx* and *abd-A.* This finding is consistent with previous research on the role of the Bithorax Complex in patterning of cardiac and skeletal muscle within the mesoderm (Lovato *et al.* 2002, Michelson 1994, Lo *et al.* 2002 and Ponzielli *et al.* 2002), and the more general function of the Hox genes in controlling AP diversity. We note that the domains of Hox gene function in alary muscles bear a closer resemblance to their expression in developing skeletal muscles rather than Hox gene expression in the cardiac tube (compare the conclusions of Michelson (1994) with those of Lovato *et al.* 2002 and Lo *et al.* 2002, and Ponzielli *et al.* 2002). This observation is consistent

with the conclusion that the alary muscles are skeletal muscle derivatives based upon their multinucleate nature.

Previous research further illustrates the requirement of the Hox genes *abd-A* and *Ubx* in the normal development and patterning of Svp cardial and pericardial cells (Perrin *et al.* 2004 and Ryan *et al.* 2005). These studies also conducted experiments whereby both *abd-A* and *Ubx* were manipulated in knockout as well as over-expression conditions, and the nature of our results are in general consistent with previous findings for the cardiac Svp cells: over-expression of *abd-A* and *Ubx* produced three additional sets of Svp expressing cardial cells; and knockout conditions produced either no change in Svp cardial cell number (for *abd-A* mutants), loss of anterior Svp cardial cells (for *Ubx* mutants), or almost no sets of Svp cardial cells (for *Ubx abd-A* mutants).

Orthologs of *Drosophila* Hox genes are detected in the developing human heart, as well as being widely expressed in the neighboring viscera such as the lungs, spleen, liver, pancreas and epidermis (Hwang *et al.* 2006). In other vertebrates, Hox genes have been generally (although not specifically) implicated in cardiac development (reviewed in Lo and Frasch 2003). A recent genome-scale study of skeletal muscle development also established an AP pattern of Hox gene expression in the developing embryonic skeletal myoblasts (Biressi *et al.* 2007). Together, our studies, and those cited here, further support a general role for Hox gene patterning of muscle derivatives broadly across the Animal Kingdom.

What are the embryonic origins of the alary muscles? As indicated previously, this question is difficult to answer absent a specific marker for the alary muscles early in embryonic development, and it must be noted that our analyses are therefore by necessity end-point assays carried out at stage 16. Nevertheless given the syncytial nature of the alary muscles, they likely arise from specific founders cells specified at particular locations in the somatic mesoderm. Furthermore, since there is only one alary muscle which arises in each hemisegment, it can be proposed that the founder for this muscle arises from an asymmetrical cell division. Experiments were carried out to test this hypothesis using mutants for *sanpodo* and *numb,* which are genes in the asymmetric cell division pathway (reviewed in Roegiers and Jan 2004). While *sanpodo* mutants produced individuals with a partial loss of alary muscles, the *numb* mutants were so disrupted at the level of the whole organism that we were unable to discern any sign of the forming alary muscles if present. This issue might be addressed in the future via analysis of alary muscle precursor formation in these mutants, once suitable markers become available. Alternatively, a strategy for following cell lineages in founder cells might prove useful.

Are there mammalian versions of the alary muscles that we see in *Drosophila*? While in some cases it is difficult to assign directly homologous structures between insects and mammals, there are a number of ligaments known to stabilize the location of the heart in mammals. These include in particular the ligaments which attach the outer pericardial layer to the diaphragm

and spinal column, as well as the sternopericardiac ligaments which connect the pericardium to the sternum (Standring 2005). Since the requirement for structures to stabilize the heart within the body cavity appears to be conserved, the molecular mechanisms responsible for their development might also bear some resemblances to each other.

CHAPTER 2

***Thin*, A *Trim32* ORTHOLOG, IS ESSENTIAL FOR MYOFIBRIL STABILITY**

AND IS REQUIRED FOR THE INTEGRITY OF THE COSTAMERE IN

DROSOPHILA

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ABSTRACT

Myofibril stability is required for normal muscle function and maintenance. Mutations that disrupt myofibril stability result in individuals who develop progressive muscle wasting, or muscular dystrophy, and premature mortality. Here we present our investigations of the *Drosophila* l(2)thin [*l(2)tn*] mutant. The "thin" phenotype exhibits features of the human muscular disease phenotype in that *tn* mutant larvae show progressive muscular degeneration. Loss-of-function and rescue experiments determined that *l(2)tn* is allelic to the *tn* locus [previously annotated as both CG15105 and another b-box affiliate (abba)]. *tn* encodes a TRIM (tripartite motif) containing protein highly expressed in skeletal muscle and is orthologous to the human limb-girdle muscular dystrophy type 2H disease gene Trim32. Thin protein is localized at the Z-disk in muscle, but *l(2)tn* mutants showed no genetic interaction with mutants affecting the Z-line–associated protein muscle LIM protein 84B. *l(2)tn* , along with loss-of-function mutants generated for *tn* , showed no relative mislocalization of the Z-disk proteins α-Actinin and muscle LIM protein 84B. In contrast, *tn* mutants had significant disorganization of the costameric orthologs β-integrin, Spectrin, Talin, and Vinculin, and we present the initial description for the costamere, a key muscle stability complex, in *Drosophila*. Our studies demonstrate that myofibrils progressively unbundle in flies that lack Thin function through progressive costamere breakdown. Due to the high conservation of these structures in animals, we demonstrate a previously unknown role for TRIM32 proteins in myofibril stability.

INTRODUCTION

Degenerative muscle disease results from mutations in genes that are diverse in function (Emery 2002, Mathews and Moore 2003 and Amato and Griggs 2011). Muscle degeneration in the cell can result from the force-dependent disruption of subcellular membranes and/or internal cellular architecture, which unbundles normal myofibril organization and prevents proper function of the myofibrils (Emery 2002, Mathews and Moore 2003). Progressive degenerative muscle disease, such as muscular dystrophy, worsens over time because myofibril elements are disrupted and are unable to be properly processed or replaced in muscle (Emery 2002, Mathews and Moore 2003). Widespread muscle degeneration impacts other organ systems, such as the respiratory and circulatory circuits, that rely on healthy muscle for proper function and support. Failure of these systems in individuals with degenerative muscle disorders often results in reduced life span after a protracted disease progression, limited mobility, and decreased quality of life (Emery 2002, Mathews and Moore 2003).

Many genes that are now known to be required in muscle tissue were originally discovered to be mutated in individuals with degenerative muscle disorders (Emery 2002, Mathews and Moore 2003). Investigations of such genes in both human and model systems have enabled researchers to build models of the conserved elements of muscle structure and function (Emery 2002, Mathews and Moore 2003, Amato and Griggs 2011, Sandona and Betto 2009, Carrie *et al.* 1997, Bonnemann *et al.* 1995, Noguchi *et al.* 1995, Nigro *et al.* 1996, Bonne *et al*

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1999, Nagano *et al.* 1996, Hoffmann *et al.* 1987, Hoffmann *et al.* 1988, Watkins *et al.* 1988). Disruptions in sarcoglycan genes (*α-*, *β-*, *γ-*, or *δ-sarcoglycan*) prevent the proper production of these sarcolemmal transmembrane proteins (Sandona and Betto 2009, Carrie *et al.* 1997, Bonnemann *et al.* 1995, Noguchi *et al.* 1995, Nigro *et al.* 1996). Consequent aberrant function (sarcoglycanopathy) in humans results in multiple forms of limb-girdle muscular dystrophy (LGMD), a heterogeneous disease characterized by progressive muscle wasting of the limb, trunk, shoulder, and pelvic muscles (Bonne *et al.* 1999). Emery–Dreifuss muscular dystrophy (EMD) can result from mutations in either *emerin* or the gene *LMNA* (lamin A/C), which are proteins that interact with the nucleoskeleton and/or nuclear components to maintain nuclear envelope architecture (Nagano *et al.* 1996). Mutations in *Dystrophin*, a protein central to muscle cell stability that functions as a linkage between actin filaments to the cell membrane, result in individuals with Duchenne or Becker muscular dystrophy (Hoffmann *et al.* 1987, Hoffmann *et al.* 1988, Watkins *et al.* 1988). Identification of these disease genes, as well as an understanding of the roles that they play in normal healthy muscle, is central to diagnosis and the development of more effective therapies.

Mutations in the E3 ubiquitin ligase TRIM32 result in individuals with Bardet–Biedl syndrome, a pleiotropic human disease, or limb-girdle muscular dystrophy 2H (LGMD2H) (Frosk *et al.* 2002, Watkins *et al.* 1988, Hoffman *et al.* 1987). The mouse model was used to confirm that TRIM32 is highly expressed in muscle and accumulates during muscle remodeling (Kudryashova *et al.* 2005, Kudryashova *et al.* 2009). Disruptions in murine TRIM32 were able to

recapitulate the myopathies observed in human LGMD2H (Kudryashova *et al*. 2005, Kudryashova *et al.* 2009, Kudryashova *et al.* 2011). Muscle studies conducted using the mouse model have shown the murine ortholog of *Trim32* to be diverse in function as it has the ability to bind myosin and ubiquitinate actin and to interact directly with the dystrophin-associated complex member, dysbindin (Kudryashova *et al.* 2005, Kudryashova *et al.* 2009, Kudryashova *et al.* 2011). Nevertheless, there is still much to learn concerning a mechanistic understanding of the nature of cellular-level breakdown involving the specific mutations in *Trim32* that lead to myofibril disorganization and muscular dystrophy.

The genetically tractable *Drosophila* model has established itself as an efficient system for understanding human disease (Chartier *et al.* 2006, Feany *et al.* 2000, Crowther *et al.* 2005). In this report, we describe our analysis of a muscle degenerative mutant in *Drosophila* for which the affected gene is orthologous to TRIM32. Our analyses demonstrate that costamere structure is lost in the *Drosophila* mutants and suggest that compromised costamere function could contribute to the mechanism(s) of LGMD2H in humans.

MATERIALS AND METHODS

Drosophila stocks and genetics

Drosophila melanogaster stocks were maintained at 25°C under standard laboratory conditions, unless otherwise indicated in the text. w^{1118} (BL-3605) was used as the WT control. The GAL4/UAS system was utilized for all RNAi and

rescue studies using the *24B-Gal4* (BL-1767) driver. The original *l(2)tn* allele was generously provided by John Sparrow, University of York, UK. The *tn*$^{\Delta A}$ and *tn*$^{\Delta B}$ deletion alleles were generated by imprecise excision of the Minos transposable element *Mi{ET1}abba*^{MB03490} (BL-24804) located in the first intron of *tn* using standard techniques. All *tn* alleles were maintained over *CyO-GFP* (BL-6662) or *CyO-WgLacZ* balancers. The generation of transgenic flies was achieved using established procedures. *UAS-abba* (analogous to *UAS-tn*) was generated using the GH06739 cDNA clone (*Drosophila* Genomics Resource Center) as a template. The RNAi line for *abba/tn* (Transformant ID:19290) was obtained from the Vienna *Drosophila* RNAi Center. The *mlp84B*^{p8} and *sls*^{j1D7} alleles used for genetic interaction analysis were described in Clark *et al*. 2007. Lethal-phase analysis of the *tn* mutants was performed after allowing GFP-balanced *tn* stocks to lay embryos on apple juice plates. GFP-negative embryos were selected using a dissecting fluorescent scope and gridded 100 to a new apple juice plate (10 by 10). After 24 hours, unhatched embryos were counted. All larvae were transferred to new plates or vials and analyzed every 24 hours for lethality. Totals are represented as percentages.

Quantiation of pupal axial ratios

Axial ratios were determined by measuring pupal length and then dividing by pupal width. Average axial ratios from at least 12 individuals were calculated.

Generation of anti-Thin antibodies

Two different anti-Thin antibodies were generated. The first was a peptide antibody against residues 998-1013 (TGDDSDSRYGTGRSR) that was injected into rabbits (CHI Scientific Inc., Maynard, MA). The second antibody was generated against purified protein. By standard RT-PCR techniques, gene-specific primers (Forward primer: 5'-TAACGCGTCGACAATGGAGCAATTCGAGCAGCTGTTGACG; Reverse primer: 5'-AATAAGAATGCGGCCGCAGTGACGACTAAGAAAACGACGACGGC), were used to amplify the region of *tn* corresponding to the first 498 AA using the GH06730 cDNA as a template. The forward and reverse primers were engineered to contain SalI And NotI restriction sites, respectively. This cDNA fragment was cloned into the pT7HMT expression vector and soluble protein was purified as described (Geisbrecht *et al.* 2006). This soluble protein was sent to Pocono Rabbit Farm and Laboratory Inc. for injection into guinea pigs. Both antibodies gave similar staining results.

In Situ Hybridization and Immunostaining

The digoxigenin-labeled *tn* antisense probe was generated by Sp6 polymerase transcription of linearized pOT2-GH06739 (Roche). Embryo collection and probe hybridization were carried out as described (28). Labeled samples were stained using the Alkaline Phosphatase BCIP/NBT substrate (Vector Laboratories, Burlingame, CA). Multiple representative samples were selected, mounted in an 80% (v/v) glycerol solution and photographed.

The following antibodies were used: rabbit anti-Thin (1:400, this study); mouse anti-α-actinin (1:10, donated by Judith Saide, Harvard), mouse anti-β-integrin (CF.6G11, Developmental Studies Hybridoma Bank, DSHB), mouse anti-MHC (NA4, DSHB), mouse anti-Talin (1:5 dilution of 1:1 ratio of clones A22A and E16B, DSHB), rabbit anti-MLP84B (1:500, Clark *et al.*, 2007(17)), rat anti-TM (1:1000, Abcam Inc, Cambridge, MA), mouse anti-Spectrin (1:5, DSHB), goat anti-Vinculin (1:200, N-19, Santa Cruz Biotechnology Inc.). For immunohistochemistry, biotinylated secondary antibodies were used at 1:1000 and detection using a Vectastatin Elite kit by a standard DAB reaction (Vector Laboratories, Burlingame, CA). For immunofluorescence, the secondary antibodies used were AlexaFluor-488 or AlexaFluor-568-conjugated, at 1:2000 (Molecular Probes, Seattle, WA). Preparation and antibody staining of larval muscle and embryos were performed according to described methods (Molina and Cripps 2001, Patel 1994, respectively).

Imaging

Digital images of pupae and embryos were captured using an Olympus BX-51 stereomicroscope. Confocal images were captured using an Olympus Fluoview 300 and Nikon Diaphot MRC600. All images were assembled into figures using Adobe Photoshop.

RESULTS

thin Is a Human *Trim32* Ortholog. The original *l(2)tn* mutation was described by Ball *et al*. 1985 as a muscle degenerative mutant in *Drosophila*. In order to analyze the 'thin' phenotype and further map the mutation, we crossed the *l(2)tn* allele to deficiency stocks either within or spanning the original mapped region of *l(2)tn* (2R: 55D-56B). We found that the *l(2)tn* chromosome was lethal in combination with *Df(2R)Exel6068* but not with *Df(2R)Exel6067* or *Df(2R)BSC349*, enabling us to narrow the location of the *l(2)tn* locus to a small section of Chromosome II. This molecularly-defined genomic region contained only eleven candidate genes (Fig. 1A). Among these genes was *CG15105/abba*, an ortholog of human *Trim32,* a gene that is required for muscle maintenance and stability and that is mutated in patients with LGMD2H.

Compared to wild type (WT) (Fig. 1*B*), *l(2)tn /Df* animals produced pupa that were thin and long (Fig. 1*C*). In L3 larval muscles, in contrast to WT (Fig. 1*D*), 'thin' mutant skeletal muscles were narrower, round in cross-section, and relatively loose at the attachment sites (Fig. 1*E*). At higher magnification, WT muscles were oval in cross-section and showed aligned sarcomeres within the tightly bundled myofibrils (Fig. 1*F, H*). WT muscles also had evenly spaced, linearly organized, nuclei aligned along the midline of each muscle (Fig. 7*A*). Gaps between the myofibrils were apparent in *tn* mutants, as the fibers appeared unbundled at higher magnification (Fig. 1G, I). There was also irregular clustering of nuclei within the mutant muscle cells (Fig. 7B). The elongated pupal case

results from the inability of the musculature to compact the animal at the onset of pupariation (Emery 2002)

tn is allelic to *abba* and the encoded protein is localized to the Z-disk in muscle. We next sought to determine if *l(2)tn* is allelic to the annotated *abba* gene. UAS-RNAi lines specific for *abba* were crossed to the muscle driver *24B-Gal4* to generate knockdown animals (*24B>abbaRNAi*). We also created *abba* deletion alleles (*tn $^{\Delta A}$* and *tn $^{\Delta B}$*) by standard *Minos* mediated excision techniques (33) as well as a rescue allele (*UAS-abba*) for use in a double mutant background (*UAS-abba Df/tn $^{\Delta A}$;24B*). Both *24B>abbaRNAi* and *tn $^{\Delta A}$/Df* animals recapitulated the 'thin' pupal phenotype (Fig. 2A, B), whereas the rescue animals had partial to full restoration of the WT pupal phenotype (Fig. 2C). Whole mounts of L3 larval skeletal muscle for the *tn $^{\Delta A}$/Df* and RNAi knockdown animals phenocopied the thinner, looser musculature observed in *l(2)tn /Df* animals (Fig. 2D, E). *UAS-abba Df/tn $^{\Delta A}$;24B* animals generally showed a WT myofibrillar organization (Fig. 2F). High resolution images of muscle from both *24B>abbaRNAi* and *tn $^{\Delta A}$/Df* animals (Fig. *2G, J-K*) revealed the same myofiber instability and unbundling as seen in *l(2)tn /Df* homozygotes (Fig. 1G, I), while rescue muscles were relatively more stable and had sarcomeres appearing in register (Fig. *2L).*

We further characterized the 'thin' phenotype quantitatively by measuring the axial ratios between groups of mutants and WT pupae (Fig. 2M). The length/width ratios of pupae between *l(2)tn /Df*, *l(2)tn /tn $^{\Delta A}$, l(2)tn /tn $^{\Delta B}$* and *24B>abbaRNAi* groups were substantially higher and displayed a greater degree

of variability around the mean compared to WT and rescue (*UAS-abba Df/tn* $^{\Delta A}$*;24B)* values. Lethal phase determination of *l(2)tn* allelic combinations

revealed that mutant animals died at two stage-specific time points in the

Drosophila life cycle. A large proportion of *l(2)tn /Df, l(2)tn /tn* $^{\Delta A}$*, l(2)tn /tn* $^{\Delta B}$ and

24B>abbaRNAi animals died in embryogenesis. Remarkably, very low numbers

or no mutant individuals were lost during the larval stages, but were pupal lethal.

The rescue animals mostly eclosed as adults. Our lethality data suggested that

the *l(2)tn* allele may be a hypomorph as more animals survived until the pupal

stage. These data, together with the degenerative phenotypes observed in the

muscles of all mutant L3 larvae, provide strong evidence that the original *l(2)tn*

mutation affects the CG15105/abba gene, which we name *tn* .

Given that *tn* gene function is essential for myofiber stability, we next

sought to determine the spatial and temporal accumulation of Thin protein during

development. In embryos, *tn* mRNA and protein were expressed in the skeletal

muscle precursors (Fig. 3 A *and* D) and remained high in a muscle-specific

pattern until the completion of embryonic muscle development (Fig*.*

3 B *and* E*).* *l(2)tn* /Df animals showed a marked reduction in accumulation

of *tn* mRNA, but the signal remained muscle specific (Fig. 3*C*). This muscle-

specific, yet reduced, accumulation was also observed in *l(2)tn* /Df mutant

embryos immunostained for Thin protein expression (Fig. 3*F*) and supports the

idea that the *l(2)tn* allele is a hypomorphic allele. Through sequencing of cDNA

for *tn* we did not observe any amino acid changes, suggesting that the

original *l(2)tn* mutation might affect the regulatory regions of this gene.

Thin protein expression (Fig. 3F) and supports the idea that the *l(2)tn*

allele is an hypomorphic allele of the gene we identified. Thin protein

accumulation in WT animals was also detected in the mature muscles of L3

larvae and localized in a striated pattern across the muscle (Fig. 3G).

Immunolocalization with the Z-disk–associated protein muscle LIM protein 84B

(MLP84B) (Fig. 3H) revealed overlap between Thin and MLP84B at the Z-disks

(Fig. 3I*). Because there was a small amount of Thin accumulation in the *l(2)tn*

/Df mutants, we examined *24B > abbaRNAi* progeny to test the sensitivity and

specificity of our anti-Thin antibodies (Fig. 3 J–L*). Relative to WT, Thin

accumulation was depleted at the Z-lines of the mutant muscles, and no other

staining in muscle was present (Fig. 3 J *and* L). MLP84B remained present and

localized within the disrupted myofibrils of *24B > abbaRNAi* animals (Fig.

3 K *and* L). Together, these data demonstrate that the Thin protein is expressed

within muscle tissue and that the encoded protein is closely associated with the

muscle sarcomere, consistent with our demonstrated role for this protein in

muscle maintenance.

tn Mutants Reveal No Genetic Interaction with *mlp84B* and *sls* The

localization of Thin to the Z-disk raised the possibility that Thin associates with Z-

line proteins already known to have roles in myofiber stability. To test this

hypothesis, we performed genetic interaction analyses to determine if the *l(2)tn*

allele enhanced the thin pupal phenotypes of mutations in *mlp84B* or the titin

gene *sallimus* (Table 1). Mutants for *tn* , *mlp84B*, and *salimus/D-titin* (*sls*) alone

each produced pupa with a "thin-like" phenotype, observable as higher axial

ratios compared with WT (Fig. 4) (Clark 2007). Both *mlp84B* and *sls* mutants

exhibited a lesser thin phenotype compared with *tn* mutants (Fig. 4). Removal of

one copy of *mlp84B* or *sls* in a *l(2)tn /tn*$^{\Delta A}$ background did not enhance the thin

phenotype over *l(2)tn /tn*$^{\Delta A}$ individuals alone (Fig. 2*M*). An increased axial ratio

was observed upon removal of one copy of *sls* in *mlp84B* homozygotes,

consistent with the published genetic interactions between these two genes

(Clark 2007). Taken together, these results suggest that either there is no

interaction between *l(2)tn* and *sls* and/or *mlp84B*, or the full thin phenotype

observed in *l(2)tn /tn*$^{\Delta A}$ homozygotes and *sls/mlp84B* interaction crosses is the

most extreme incarnation of the "thin" phenotype, leaving any genetic interaction

unobservable in this phenotype.

α-Actinin, Actin, and MLP84B Remain Localized in *tn* Mutants, Whereas

Myosin Heavy Chain and Tropomyosin Are Mislocalized. Vertebrate TRIM32 can

bind to the thick filament protein myosin and ubiquitinates actin (Kudryashova

2005). To determine if these and other sarcomeric components are altered upon

loss of *tn* , we analyzed the expression and localization of key muscle proteins in

our mutants. WT animals stained for α-Actinin or MLP84B showed specific

localization to the Z-disk in well-organized sarcomeres (Fig. 5 A and B). Also,

myosin heavy chain (MHC) and tropomyosin (TM) were found organized as

cross-striations within the tightly bundled myofibrils of normal muscle (Fig.

5 C and D). α-Actinin, MLP84B, MHC, and TM were all present in both *l(2)tn*

/Df and *tn* Δ*A/Df* muscle. The key differences were in their relative subcellular

localization within the muscle tissue compared with WT. Despite the widespread

degeneration of myofibrillar organization in both *l(2)tn /Df* and *tn ΔA/Df* animals,

α-Actinin (Fig. 5 E and I) and MLP84B (Fig. 5 F and J*)* remained relatively well

localized within the sarcomeres of the mutant muscle, with significant areas of

cross-striations still observed. By contrast, MHC (Fig. 5 G and K) and TM (Fig.

5 H and L*)* were present, but mislocalized, in the mutants. Rather than retaining a

periodic localization within the sarcomeres, both MHC and TM accumulated

along the sarcolemmal membrane in globular structures. These results,

combined with the observation that both MHC and TM accumulate normally in

embryos mutant for *tn*, suggested that myofibril destabilization occurs in actively

contracting muscles upon loss of Thin

The degenerative muscle phenotype was further examined by comparing

the relative state(s) of myofibrillar unbundling in confocal sections through

the *z* axis in WT and mutant muscle preparations of the genotype *tn* ^{ΔA}*/Df* (Fig.

5 M and N). Myofibrils of muscle cells became unbundled directionally: the

muscle unbundling phenotype was more severe toward the visceral surface of

the muscle (Fig. 5M), but appeared to retain a more WT appearance toward the

most lateral (i.e., closest to the cuticle) region of the cell (Fig. 5N). We

hypothesize that the loss of Thin in actively crawling larvae could be temporarily

compensated for by a steady-pressure force directed from the cuticle. Muscle in

older individuals appeared to have a greater degree of degeneration than that of

younger individuals. This is consistent with the observations of Ball *et al.* 1985

that suggest that the loss of *tn* results in progressive muscle degeneration that

manifests in the unbundling of myofibrils as well as the dislodging of specific

sarcomeric proteins. This evidence also supports the role of Thin as a stabilizing protein in the structural maintenance of muscle.

In vertebrates, the costamere is positioned at the Z-disk of the sarcomere and links the myofibrils to the overlying sarcolemma to provide sustained stability of the myofibrillar apparatus with the sarcolemma during muscle contraction. Although implied, the existence of costameres has not yet been demonstrated in flies (Ribeiro *et al* 2011, Sparrow and Schöck 2009). *Drosophila* orthologs of the costameric proteins β-Integrin, Talin, Spectrin, and Vinculin exist and could play conserved roles in muscle stability and function (Ervasti 2003). Given the nature of the physical breakdowns in the musculature of *tn* mutants, we next sought to determine the existence of the costamere in larval skeletal muscles.

In WT larvae, β-integrin, Talin, Spectrin, and Vinculin were present in cross-striations overlying the Z-disk (Fig. 6). To confirm that these proteins were localized to a position in fly muscles similar to the position of the costamere in vertebrate muscles, confocal sections through the *z* axis were collected. These micrographs clearly showed that the components of the Vinculin–Talin–Integrin complex, along with Spectrin, exist in flies (Fig. 6). Furthermore, these proteins remain conserved as Z-line–associated structures between the myofibrils and the overlying sarcolemma. Scans through the center of the bundle of myofibrils showed that β-integrin, Talin, Spectrin, and Vinculin do not appear at the internal Z-lines (Fig. 6), as also seen for these proteins in vertebrate muscles. Thus, proteins that compose the vertebrate costamere have orthologs that accumulate in *Drosophila* larval muscles in a pattern highly reminiscent of the costamere. We

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conclude that costameres exist in *Drosophila* larval body-wall muscles and that the structure of the costamere shares many similarities with vertebrate costameres.

We next tested if a loss of Thin has an impact on the stability of costamere-associated proteins in muscle tissue. The spatial organization of β-integrin, Talin, Spectrin, and Vinculin was examined in tn $^{\Delta A}$/Df animals. The distribution and accumulation of β-integrin was normal in embryonic development in both mutant and WT embryos. (Fig. 9). However, in mutant larvae, we observed a similar phenotype for β-integrin, Talin, Spectrin, and Vinculin (Fig. 6), where these proteins no longer retained localization at the Z-disk and were randomly distributed along the membrane of the sarcolemma. In addition, noncostameric, trans-sarcolemmal proteins such as δ-sarcoglycan (Fig. 10) also showed widespread mislocalization due to *tn* loss of function. From this evidence, we conclude that Thin is required for maintaining proper costamere stability in muscle and that loss of Thin ultimately results in the degenerative muscle phenotype characteristic of *tn* mutants

DISCUSSION

This work demonstrates that Thin is a key structural protein in maintaining myofibrillar stability. Myofibrils in *tn* mutants progressively unbundled during development, most likely due to a loss of costamere integrity. Over time, this results in progressive muscle wasting. Gaps in the myofibrils of *tn* mutants may be analogous to gaps in the myofibrils of diseased muscle tissue from individuals

with muscle myopathy that also displays myofibrillar disorganization and abnormal vacuole position (Emery 2002). The myofibril defects that we observe in *Drosophila* are more extensive than those observed for TRIM32 deficits in mammals, suggesting that Thin has a greater functional role in muscle than do the vertebrate orthologs. Because it is likely that multiple mechanisms cooperate to produce the muscle breakdown that occurs in humans suffering from LGMD2H, the role of Thin in maintaining myofiber stability could be one contributing factor.

Antibodies generated during this study showed that Thin is expressed in muscle and localizes at the Z-line. This is consistent with the Z-line localization observed for murine TRIM32 (Kudryashova 2009). Given the high level of conservation from sequence to muscle phenotypes of both *tn* and *Trim32* mutants, further studies using *tn* mutants will serve as a good model for understanding the molecular mechanisms underlying LGMD2H. Moreover, the apparent single Trim32 ortholog in flies allows for the direct examination of Thin function in myofibril stability without the potential complication of redundancy with other TRIM domain proteins.

We also illustrate in detail in *Drosophila* a detailed characterization of the muscle stability complex known in vertebrates as the costamere. Our observations place the costameric proteins β-integrin, Talin, Spectrin, and Vinculin at the Z-line on the periphery of the myofibers in flies. Loss of *tn* in flies leads to the disintegration of these core components and the overall breakdown of the costamere. Our phenotypic studies indicate that stability of the costameres

is central to keeping sarcomeres in register and to preventing myofibrillar unbundling. Secondarily, muscle proteins such as MHC and TM are dependent on this complex for proper localization or stability.

Studies here assign a molecular role for a mutant first described over 20 y ago and named *l(2)tn* for its elongated, thin pupal phenotype (Ball *et al.* 1985). Our analysis of the *l(2)tn* allele shows that there is some residual transcript expression in the embryonic musculature compared with WT. Also, a substantially larger proportion of *l(2)tn /Df* mutants survive until the pupal stages compared with the earlier lethality observed in the *tn* deletion alleles or *24B > abbaRNAi* knockdown. This indicates that *l(2)tn* is likely a hypomorphic allele and that there is a minimal requirement for Thin accumulation to maintain normal muscle stability. Interestingly, muscles form normally in *tn* knockout mutants, indicating that Thin is required for myofibril stability, but not required for initial myofibril formation.

Our data position Thin in close proximity to the costamere complex, and reveal it is associated with the Z-disk in more central muscle areas. Based on the relative positions of stable versus mislocalized Z-line–associated proteins in *tn* mutants, Thin function is likely to be critical to sustaining costameric associations between MLP84B and Vinculin or Spectrin, and it must also bind and stabilize/costabilize other proteins in the sarcomere. The Z-line proteins α-Actinin and MLP84B, along with actin, remained associated with the Z-disk in *tn* mutants. This observation indicates that their localization, other than general

myofibril disassociation, was unaffected by the loss of Thin. Dislodged and disorganized accumulations of MHC and TM along the surface of the sarcolemma suggested that Thin could be a possible key anchor point of the myofibril architecture, and future studies aimed at defining binding partners for Thin will be informative in uncovering its molecular function.

The costameres form rings, positioned at the Z-lines of the outermost sarcomeres of myofibril bundles, just below the membrane (Sparrow and Schöck 2009, Ervasti 2003). These costamere rings provide physical support and stability as the muscle moves and contracts. Although the existence of these structures has been suggested in *Drosophila* muscle (Ribeiro 2011, Sparrow and Schöck 2009), they had yet to be characterized. Loose, destabilized myofibrils in *tn* mutants are consistent with a phenotype of muscles that suffer from a lack of stabilizing pressure provided from intact and functional costameres.

How a loss of TRIM32 at the cellular level results in progressive muscle wasting in individuals with LGMD2H is unknown. Insight into TRIM32 is further complicated by its widespread expression and pleiotropic functions as observed in satellite cells as well as in non-muscle tissues (Reymond *et al.* 2001, Kudryashova 2012, Nicklas 2012). Although separable from a specific role in muscle cells, loss of TRIM32 prevents satellite cells from proper maturation and proliferation, thus preventing muscle cell replacement and repair (Kudryashova 2012, Nicklas 2012). In vitro, the RING domain of TRIM32 has been shown to interact with myosin (Kudryashova *et al.* 2005). This may explain why a loss of function in the fly ortholog results in such a severe phenotype for MHC

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accumulation, although it does not correlate with the general Z-disk localization of Thin protein. Consistent with the canonical function of RING finger motifs, TRIM32 possesses E3 ubiquitin ligase activity, where both actin and dysbindin are in vitro targets (Kudryashova *et al.* 2005, Meroni and Diez-Roux 2005, Locke *et al.* 2009). Mutations that result in LGMD2H affect the C-terminal NHL repeats of TRIM32. Modeling of these repeats suggests that they fold into a β-propeller structure that may mediate protein–protein interactions or TRIM protein dimerization (Saccone *et al.* 2008). Recapitulation of the causal mutations found in the NHL domains of LGMD2H patients demonstrate that TRIM32 can become destabilized and therefore lose its relative abundance in the cell (Kudryashova *et al.* 2011, Saccone *et al.* 2008). TRIM32 participates in multiple cellular interactions and likely has a variety of roles in the cell.

We envision two additional roles for Thin/TRIM32 in muscle cells, which are not mutually exclusive. First, Thin may serve as a critical link to stabilize the structure of costameric proteins. The individual NHL domains that comprise the β-propeller structure could form a bridge by binding to Vinculin and/or Spectrin near the sarcolemma membrane on one side and to MLP84B anchored at the myofibril Z-line. In this way, Thin/TRIM32 would function to link costameric proteins, thus stabilizing the structure at a crucial point of intersection between the membrane and myofiber. TRIM32 has been shown to homodimerize (Kudryashova *et al.* 2011). It may be that complexes of homodimers interact to form Thin/TRIM32 multimers to fulfill this role. Second, Thin/TRIM32 may also function to ubiquitinate sarcomeric proteins, such as Actin, Dysbindin, and

possibly MHC (Ball *et al .* 1985, Locke *et al.* 2009). The costamere may be a critical junction for this process, allowing for Thin/TRIM32 to process sarcomeric components as they are either damaged (polyubiquitination) or possibly reintegrated during reloading (monoubiquitination). Future experiments will be needed to elucidate which (or whether both) of these mechanisms predominate in muscle tissue.

CHAPTER 3

DEVELOPMENTAL DELAY AND FEMALE SEX-LETHALITY DURING THE

PUPAL STAGE ARISE IN THE *DROSOPHILA VINCULIN* MUTANT :

In(1LR)pn2a, *^{Vinc1}*

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ABSTRACT

Proper developmental processes and timing rely on the stability and flexibility of cytoskeletal binding complexes that interact via the plasma membrane with the extracellular matrix (ECM). (Zambonin-Zallone *et al.* 1989, Ezzell *et al.* 1997, Imanaka-Yoshida *et al.* 1998, Ruediger *et al.* 1998, Mandai *et al.* 1999, Critchley *et al.* 2004) Disruptions that interfere with the normal function of these complexes and proteins often result in developmental delay, stunted growth and fragile or under developed physical structures (Shiraishi *et al.* 1997, Xu *et al.* 1998, Zemljic-Harpf *et al.* 2004 and 2007, Marg *et al.* 2010). The cytoskeletal protein Vinculin binds with both Talin and Actin and participates in complexes which anchor cells to the ECM (Burridge *et al.* 1984, Marchisio *et al.* 1988, Zambonin-Zallone *et al.* 1989, Turner *et al.* 1990, Gilmore *et al.* 1996, Yoshida, *et al.* 1998, Anastasi *et al.* 2003, Izard *et al.* 2003, Critchley *et al.* 2004). Severe repercussions for loss of *Vinculin* in mammals include developmental delay, death during the embryonic stage as well as cardiomyopathy for loss of function in the vertebrate specific splice variant *metavinculin* (Maeda *et al,*1997, Olson *et al.* 2002, Vasile *et al.* 2006 and 2006). Previous studies of a *Drosophila* loss of function mutant*, ln(1LR)pn2a,Vinc[1]*, determined that *Vinculin* was expressed, but not essential for viability in flies (Alatortsev *et al.*, 1997). Fifteen years later, our studies continuing the evaluation of the *ln(1LR)pn2a,Vinc[1]* mutant, observed developmental delay, a sex-linked lethality presumably during the pupal stage, yet normal organization of sarcomeres and costameres in muscle.

INTRODUCTION

Vinculin is a highly conserved protein in animals and has been shown to be key for proper organismal development as well as adult tissue and cellular stability (Burridge *et al.* 1984, Marchisio *et al.* 1988, Zambonin-Zallone *et al.* 1989, Turner *et al.* 1990, Gilmore *et al.* 1996, Xu *et al.,* 1998, Imanaka-Yoshida *et al.* 1998, Anastasi *et al.* 2003, Izard *et al.* 2003, Critchley *et al.* 2004, Zemljic-Harpf *et al.,* 2004 and 2007, Shen *et al.* 2011). Vertebrates possess a second splice form, *metavinculin*, which carries an extra exon and is cardiac specific (Maeda *et al.,* 1997, Olson *et al.* 2002, Vasile *et al.* 2006 and 2006). Homozygosity for mutant alleles of *vinculin* has yet to be detected in humans (Maeda *et al.,* 1997, Olson *et al.* 2002, Vasile *et al.* 2006 and 2006). Although rarely observed, humans heterozygous for alleles of *Vinculin,* with mutations in the *metavinculin* specific exon 19, have been observed to develop cardiomyopathies (Maeda *et al.,* 1997, Vasile *et al.* 2006 and 2006).

Vinculin localizes at the costamere in striated muscle tissue and plays roles in cell signaling and motility (Imanaka-Yoshida *et al.* 1998, Anastasi *et al.,* 2003, Fernández *et al.,* 2005, LaBeau-DiMenna *et al.* 2012) Vinculin shares high homology with Catenin, a proposed actin adapter molecule (Herrenknecht *et al.*1991, Ruediger *et al.,*1998, Weiss *et al.* 1998) As part of complexes involving Integrins and Cadherins, the Vinculin N-terminus directly associates with Talin and Actinin and the Vinculin C-terminus with Actin and Paxillin (Burridge *et al.* 1984, Marchisio *et al.* 1988, Zambonin-Zallone *et al.* 1989, Turner *et al.* 1990,

Gilmore *et al.* 1996, Yoshida, *et al.* 1998, Anastasi *et al.* 2003, Izard *et al.* 2003, Critchley *et al.* 2004).

Vinculin function in vivo has been studied in a number of model systems. Loss of function for *Vinculin* in mice resulted in death during embryogenesis (Xu *et al.* 1998, Shiraishi *et al.* 1997, Izard *et al.* 2003). During the developmental process preceding death, numerous defects were observed. These included heart development arrest at day E9.5, sparse and fragile tissue formation, as well as abnormally high cell motility rates presumably due to constitutive binding activity (Xu *et al.,* 1998, Marg *et al.*, 2010). Mutants for *Vinculin* were also shown to mature only to 30-40% of the wild type body size expected relative to stage at time of death (Xu *et al.,* 1998, Marg *et al.*, 2010). Later studies in mice of *Vinculin* mutant heterozygotes showed that the mice recapitulated cardiomyopathies observed in heterozygote humans (Vasile *et al.* 2006 and 2006. Homozygote mice were reported to have a 49% sudden death rate but, upon dissection, surprisingly displayed preserved contractile function (Shiraishi *et al.* 1994, Zemljic-Harpf *et al.,* 2004 and 2007). Interestingly, *Vinculin* loss of function in murine embryonic carcinoma cells shows reduced spreading and motility due to a decreased ability to attach and translocate via the ECM (Ezzell *et al.,* 1997). These experiments concluded that Vinculin is essential for the stabilization of focal adhesions and force management required by the cytoskeleton during cellular motility (Ezzell *et al.,* 1997).

MATERIALS AND METHODS

Drosophila stocks and genetics

   Drosophila melanogaster stocks were maintained at 25°C under standard laboratory conditions on Carpenter's medium (Carpenter, 1950). w^{1118} (BL-3605) was used as the wild type (WT), non-mutant control. The original *In(1LR)pn2a,Vinc^1 / FM7c/Dp(1;Y)B^S* stock was obtained from Bloomington Stock Center. The *In(1LR)pn2a,Vinc$^{1(2012)}$* line was rebalanced to *FM7i, GFP.*

Western blots

   Standard Western blot protocol was performed using SDS-PAGE 4-20% Mini-PROTEAN TGX precast gels from BioRad. Protein transfer to nitrocellulose membrane occurred at 200 mA for 2.5 hours. Membranes were incubated with a three antibody cocktail: goat anti-Vinculin (N-19)(1:500; Santa Cruz Biotechnology), mouse anti-Vinculin MH24 (1:2; Developmental Studies Hybridoma Bank) and mouse anti-Vinculin, Metavinculin VN 3-24 (1:2; Developmental Studies Hybridoma Bank). Rabbit anti-Pan-actin 4968 (1:500; Cell Signaling) was used as the control. Horseradish peroxidase conjugated secondary antibodies were incubated at 1:3000.

Immunohistochemisty

   L3 larva were collected fixed and mounted according to standard methods (Morriss *et al.,* 2012) mouse anti-Talin (1:5 dilution of 1:1 ratio of clones A22A

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and E16B; Developmental Studies Hybridoma Bank); rat anti-TM (1:1,000;

Abcam Inc); goat anti-Vinculin (1:200, N-19; Santa Cruz Biotechnology Inc.). For

immunohistochemistry, biotinylated secondary antibodies were used at 1:1,000

and immunofluorescence, the secondary antibodies used were AlexaFluor-488 or

AlexaFluor-568 conjugated at 1:2,000 (Molecular Probes).

Imaging

Images were digitally captured using an Olympus BX-51

stereomicroscope. Figures were collected and processed using Adobe

Photoshop.

RESULTS

Vinculin showed a conserved localization pattern in the muscles of

Drosophila L3 larvae (Fig. 1 A,B). Antibodies specific for the conserved N-

terminus of human Vinculin were used both *in situ* and for Western blotting of the

fly ortholog on late stage larvae (Fig 1A-C). Vinculin was found to be widely

expressed in the cardiac tube, alary muscles and the skeletal muscles (Fig. 1A).

Within the cardiac tube, Vinculin was observed to have two very different

patterns of expression: striated and ubiquitous (Fig. 1A). While the muscle walls

of the dorsal vessel possessed a striated pattern, the pericardial cells had a

ubiquitous, or possibly membrane bound, accumulation (Fig. 1A). Skeletal

muscle had a definitively striated pattern (Fig. 2). Skeletal muscles also had a unique second arrangement of Vinculin that surrounds the nuclei in a 'nest-like' perinuclear accumulation (Fig. 1B)

Mutants were confirmed using western blot analysis for the loss of accumulation of Vinculin. In 1997, Alatortsev *et al.* evaluated a chromosomal inversion mutant on X which had a breakpoint in *Vinculin* and effectively knocked out transcription of the locus. With the intention of using this mutant for our current study, we acquired a balanced stock from Bloomington Stock Center carrying the *In(1LR)pn2a,Vinc^1* (termed *In(1LR)pn2a,Vinc$^{1(2012)}$* for our study) mutation. Western blotting was carried out for Vinculin (~105 kDa) and pan-Actin (control, ~45 kDa) using late stage larvae (Fig. 1C). WT controls showed high accumulations of Vinculin at this stage. Vinculin presence was also observed for siblings carrying the balancer chromosome (*Vinculin* genotype either being *In(1LR)pn2a,Vinc$^{1(2012)}$/+, +/+* or *+/Y*). Mutant animals (*In(1LR)pn2a,Vinc$^{1(2012)}$/ In(1LR)pn2a,Vinc$^{1(2012)}$* or *In(1LR)pn2a,Vinc$^{1(2012)}$/y*). were observed to have no Vinculin accumulation even at doubled loading concentration of cell extract during the SDS-PAGE relative to the WT and balancer/+ siblings (Fig. 1C).

Costameres and sarcomeres of *In(1LR)pn2a,Vinc $^{1(2012)}$* L3 larva remain intact. Vinculin is known to bind to both sarcomere and costamere components. Vinculin is localized to the Z-line of muscle at the costamere, which stabilizes muscle fibers and surrounds the myofibril bundle under the sarcolemma (2,12-16,23, 24). We then sought to determine if a loss of *Vinculin* would impact the stability of muscle by studying the accumulation and distribution of components

of the costamere (Talin and Integrin) as well as the sarcomere (Actin and Tropomyosin) in the skeletal muscle of mutant, late stage larva.

L3 WT and mutant animals were dissected, fixed and immunoflourecently stained for both sarcomere and costamere components (Fig. 2). Phalloidin was used to mark the striated Actin filaments of the myofibrils (Fig. 2A,B). Vinculin is also observed to be present and striated along the length of the skeletal muscle (Fig. 2C,D). Consistent with the description of the costamere, Vinculin accumulates along the surface exterior of the myofibril bundle in flies (Fig. 2C,E). Both WT and mutant larvae showed a normal arrangement for the sarcomere component Tropomyosin (Fig. 2G,H, merge with Talin K,L). A known Vinculin binding partner, Talin, remained enriched at both the Z-lines as well as muscle attachment site in both WT and mutant animals (Fig. 2I, J, merge with Tropomyosin K,L). Vinculin binding partner, Actin, remained undisrupted in the mutant animals when compared to WT (Fig. 2M, N, merge with Integrin Q,R) as did the terminal costamere transmembrane protein Integrin (Fig. 2 O,P merge with Actin Q,R).

Vinculin is present in the costamere but based upon the above observations was determined not to be essential to maintain sarcomere, costamere or myofibril stability in L3 *Drosophila* larvae. The interaction partners of Vinculin, Talin and Actin, continued to remain properly localized in muscle despite this loss. In light of its highly conserved localization, it is yet still unclear as to what function Vinculin may serve in the costamere of the developing *Drosophila*.

Vinculin mutants are smaller compared to WT and siblings at four days past egg lay (Fig. 3). Mutant animals at four days past egg lay, presented here were, averaged 0.25 mm x 2-2.50 mm for width and length. The WT and balancer carrying siblings were 0.50 mm x 3.50 mm and were much larger in size compared to the mutant animals.

 In vials of collected mutant larva, animals appeared to be unable to efficiently burrow into the food media as opposed to WT and sibling animals of the same developmental stage that were collected at the same time. WT and siblings were often able to burrow extensively, or even able to reduce the food-media to a semisolid state.

Many of the homozygotes are highly susceptible to injury of death from trauma sustained during the normal collection. These larvae often died during the transfer using paintbrushes or probes normally used to safely collect wild type animals and siblings.

Although *Vinculin* mutants do eventually develop into normal sized pupae, they grow at a reduced rate and are smaller than WT and siblings by four days past egg lay. Mutant animals injure easily during transfer and may be less efficient at feeding. It is unclear at this point if this developmental delay results from a mutation in *Vinculin* or a novel mutation on the *In(1LR)pn2a,Vinc[1]* chromosome.

Mutants for *Vinculin* are sex-lethal and the surviving males that do eclose do so later than siblings. Unlike Alatortsev *et al.* 1997, we were unable to collect

homozygous mutant females from the balanced stocks. In order to detect mutant animals, we balanced *In(1LR)pn2a,Vinc* [1(2012)] to FM7i, GFP on the X chromosome. Using this method, we confirmed that mutant animals are present as larvae (Figs. 1-3) with developmental delay also present during the larval stages (Fig. 3). We next asked if a delay during pupal development was also observable.

First, we separated newly pupariated animals into two groups: GFP-positive non-mutants, and GFP-negative Vinculin mutants. Next, pupa from each group were separated into vials of ten each. This process was repeated four times, per group.

On average, only about half of the GFP-negative pupae eclosed (Fig. 3), all of which were male. These males were then crossed to WT virgin females in order to test for fertility. Although offspring were not quantified, progeny were produced from these crosses.

The surviving males eclosed approximately two days after their siblings. Siblings eclosed between 2-3 days after pupariaton and mutants after 4-5 days. We conclude from these findings that a developmental delay exists in *Vinculin* mutant *Drosophila* as larva as well as pupa and that mutant females die prior to eclosing.

DISCUSSION

Vinculin was found to be conserved in a muscle specific pattern during development in *Drosophila*. The cardiac tube, alary muscles and skeletal muscle all expressed Vinculin in striated, ubiquitous or perinuclear arrangements. Vinculin has been shown to accumulate in the costamere (LaBeau-DiMenna *et al.* 2012). Loss of function mutants for *Vinculin* showed no costamere or sarcomere defects as late stage larvae. Vinculin binding partners, Talin and Actin, also remained undisrupted despite a loss of Vinculin expression. Developmental delay was observed at 4 days past egg lay in mutants. These animals were smaller in body size when compared to siblings and WT as well as were fragile during animal transfers between vials using standard methods. Only about half of the *Vinculin* mutants eclosed. All of the eclosed flies were fertile males.

L3 is the last larval stage before pupariation. The majority of female fatalities are hypothesized to occur during the pupal stage in mutant animals. Since both the sarcomeres and costameres of late stage larvae are stable in mutants, it may be that Vinculin is not required for muscle stability in order to survive until pupariation in female *Drosophila*. It remains unclear as to whether or not this stability is then compromised during adult muscle formation as a pupa. Vinculin's role in cell motility may be more crucial for the development of adult musculature during the pupal stage. It is uncertain why female loss of function animals are unable to survive into adulthood.

The Vinculin/Talin/Integrin complex is a conserved costamere component from flies to humans (Anastasi *et al.* 2003). Loss of *Vinculin* in flies does not appear to impact sarcomere stability or disrupt the relative positioning of Talin or Integrin by the L3 larval stage (Fig. 2). Previous work by our group has shown that in the absence and/or mislocalization of key costamere stability components, the L3 larval muscle can generate enough torsion and force to induce myofibril instability (LaBeau-DiMenna *et al,* 2012). It is yet unclear as to how or why conservation of both sequence and costamere localization of *Vinculin* has remained in flies since it does not appear to be required in larval muscle. Mutants for *Vinculin* fail to reach developmental milestones as quickly as WT or their heterozygous siblings. Vinculin has been shown to act in a variety of complexes which are related to differentiation and cell motility. Unphosphorylated Paxillin, a Vinculin binding partner, have been shown to have an impact on cell motility during differentiation . Vinculin has also been shown to link Actin/Actinin dimers with Catenin/Cadherens (Burridge *et al.* 1984, Wachsstock *et al.* 1987, Zambonin-Zallone *et al.* 1989, Turner *et al.* 1990, Gilmore *et al.* 1996, Weiss *et al.* 1998, le Duc *et al.,* 2010, Shen *et al.,* 2011). Cadherns have been shown to play instrumental roles in cell adhesion and tissue separation during development (Weiss *et al.,* 1998, le Duc *et al.,* 2010). Recently, knockdown of *Vinculin* in murine chondrocytes reduced the expression of both Aggrecan (cartilage formation, ECM) and Col2al (collagen formation) thus impairing cell growth in culture (Koshimizu *et al.* 2012). Vinculin expression itself has been induced in the presence of cell growth factors in culture (Ben-Ze'ev *et al.,* 1990).

It may be that Vinculin is required by the growing larvae to induce cell growth or motility in order to simply increase in size at a normal rate. Vinculin may also play a role in cell motility and adhesion during tissue remodeling at the pupal stage.

The exact nature of the sex linked lethality remains unclear. Female *Drosophila* had been shown to require 50% more food than males in order to reach peak life span over males (Magwere *et al.,* 2004). Female *Drosophila,* typically larger than males, may require a greater degree of cell motility or differentiation in order to construct female specific structures or produce their characteristically larger size. Perhaps this inability for females to successfully develop these structures occurs to a critical point thus preventing female flies from reaching maturity. This may have been coupled with the fact that time spent as a pupa was much longer that of sibling and WT flies (Fig. 4). It may also be that fat/energy reserves were simply not sufficient enough to sustain the females during the longer (mutant) period of pupariation. The uneclosed mutant females undergoing the longer developmental stage may have died due to starvation as pupa.

Slow development of the mutants, due to starvation and/or poor cell motility during remodeling, may have resulted from recent accumulations of new mutations on the *In(1LR)pn2a,Vinc* [1(2012)] chromosome. These mutations may have rendered the animals auxotrophic when feeding from the current food/media. It has been observed, in flies, that sex-linked auxotrophic mutations exist and impact growth as well as viability (Falk *et al.,*1974). Recent mutation accumulations may simply produce impairments to the normal function(s) of

other growth regulating complexes, such as those involving the Insulin, ecdysone or dFOXO (Colombani *et al.* 2005).

SUMMARY

High conservation between modes of muscle development, maintenance and stability exist from humans to flies. In alignment with this condition, and the efficiency of the *Drosophila* system, flies have proven to be a good model for which to observe muscle.

Alary muscle development and patterning was determined to be dependent on the relative physical presence of members of the Bithorax complex. Alary muscles were observed to normally develop segmentally in pairs. Alary muscles were found to follow the migrating dorsal vessel to its final position as opposed to a subsequent attachment after dorsal closure in wild type animals. The number of alary muscles that developed and/or attached to the dorsal vessel was found to be dependent on the level, or absence, of expression of Bithorax complex members *Ultrabithorax* (*Ubx*) or *abdominal-A* (*abd-A*). In loss-of-function experiments, some of the alary muscles that were observed to form were unable to, or unable to maintain attachment at the *seven-up* expressing cells of the heart and aorta. In contrast, over expression of *ubx* and *abd-A* resulted in an average increase from 7-10 pairs of muscles. Bithorax complex members *ubx* and *abd-A* were shown to influence both the number as well as ability of the alary muscles to attach at the dorsal vessel. These results provide further insight into not only

the segmental patterning of structures but into the specific point during development required for normal progression of development.

Drosophila l(2)thin (tn) locus was identified as CG15105, an ortholog of human muscular dystrophy disease gene *Trim32*. *tn* mutant larvae develop progressive muscular degeneration. The Thin protein was found to be localized at the Z-disk in muscle. Mutants for *tn* develop unbundling of the myofibrils. The nature of the myofibular disorganization in *tn* mutants, in addition to the mislocalization of costameric orthologs of β-integrin, Spectrin, Talin, and Vinculin, indicate that Thin acts in the costamere of muscle. Rescue experiments proved successful but did not restore the exact wild type phenotype. This may suggest that *tn* dosage may be selectively controlled. Based on our findings in flies, we speculate that the, as yet undetermined, cause of type 2H Limb-girdle muscular dystrophy in humans may result from a similar structural breakdown.

Vinculin is present in the costamere and is required in vertebrates for proper cell migration and muscle attachment during embryogenesis. We reconfirmed that the *In(1LR)pn2a,Vinc[1]* inversion carries a knockout allele for *vinculin.* Vinculin was shown to retain a conserved costameric position in flies. Binding partners Talin and Actin along with additional components of the costamere (Integrin) and sarcomere (Tropomyosin) were also observed in flies. All of these components remained stable in *vinculin* mutants. Despite having normal costameres and sarcomeres in skeletal muscle during development, mutants were observed to experience developmental delays and a sex-linked lethality. Mutant larvae are substantially smaller at 4 days past egg lay than their

control siblings but do eventually reach proper size prior to pupariation. After

pupariation, mutant flies take between 2-3 days more to eclose. Only ~50% of

mutant flies eclose, all of which are male. We conclude that Vinculin is required

by females but is not essential in male *Drosophila.* In addition, loss of Vinculin

results in longer juvenile periods in flies.

The observations contained herein seek to shed light on the

developmental process and players of muscle as well as to expand the

knowledge base and tool set in the fly model. Methods of muscle development,

stability and maintenance are conserved. Inferences can be made on the

inherited basis of evolution and human disease using the *Drosophila* system for

comparison. By understanding how conserved mechanisms of muscle unfold in

model systems, we are further able to understand how complex cell types have

emerged, evolve and function in the animal kingdom.

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Legends

CHAPTER 1

Figure 1: **Physical structure of the alary muscles relative to the dorsal vessel.** (A–C) A stage 16 embryo stained for accumulation of Pericardin (A and C) and Tinman (B and C) to visualize the alary muscles. Dorsal views are shown, with anterior to left and dorsal midline along the center of the panel. While the muscles were apparent in some preparations (white arrows), they were not consistently observed even in wild-type. (D–G) Developing organisms were stained for accumulation of muscle structural proteins. Filleted preparations were used to image alary muscles (am) and their attachments, viewed with the dorsal midline either along the center of the panel (D) or to the top of the panel (E–G). (D) A stage 16 embryo fillet stained immunohistochemically for myosin heavy-chain (MHC) viewed from the inside of the body wall. Alary muscles appeared as seven pairs of muscles extending perpendicular to the dorsal vessel (arrows point to one member of each pair), and a single eighth muscle pair at the posterior apex (asterisk). Occasionally, alary muscles were mis-aligned even in wild-type (arrowhead). (E–G) Lateral attachments of the alary muscles are located approximately half way down the interior of the cuticle. For the five anterior-most muscles, the attachment lies just posterior to DT1 (E), and just ventral to DA2 (labeled 2 in panel F and G). For the sixth and seventh alary muscle pairs, the lateral attachment is further ventral, since the alary muscle passes internally over DA3 (labeled 3 in panels F and G). Panel E is an exterior

lateral view of a stage 16 embryo stained immunohistochemically for Tropomyosin (Tm). Panels F and G are ventral views of third instar larval fillets stained by immunofluorescence for the accumulation of Tm. dv, dorsal vessel; 1, indicates muscle DA1. Bar, 100 μm for A–D; 50 μm for E, 125 μm for F and G.

Figure 2: **Alary muscle patterning relative to** *svp* **expression in the dorsal vessel.** (A–C) Embryos were stained for accumulation of MHC (brown) and β-galactosidase produced from a *svp–lacZ* transgenic construct (black). (D) *svp*^{1} mutant embryo stained for accumulation of Tm (brown). (E) *svp*^{3} mutant embryo stained for accumulation of Tm (brown) and β-galactosidase (black). All preparations are ventral views of filleted samples. For A, C–E the dorsal midline is to the top of the panel. For B, the dorsal midline is in the center of the panel. Anterior is to left. (A) Stage 14 *SCE–lacZ* embryo showing alary muscles (arrows) attached to the Svp pericardial cells (spc) prior to dorsal closure of the cardiac tube. Note that the Svp pericardial cells stain more faintly for β-galactosidase accumulation than the Svp cardial cells (scc), since they lose endogenous *svp* expression earlier in embryogenesis (Lo and Frasch, 2001). Arrowhead indicates the terminal muscle that is positive for *svp–lacZ* expression. (B) Alary muscles (am) in their final orientation at stage 16 still associated with the Svp expressing pericardial cells (only the posterior region of the dorsal vessel is shown, dv). (C) Close-up of dorsal terminations of the alary muscles as observed attaching to Svp pericardial cells. The terminations bifurcate between two neighboring sets of Svp pericardial cells. The nuclei of the muscles are also

clearly apparent (n). (D) svp^1 mutants produced the normal number of fully developed and attaching alary muscles. Although occasional patterning defects were observed, this was not more prevalent in *svp* mutants than in controls. (E) Close-up of the dorsal terminations of the alary muscles of a svp^3*lacZ* enhancer trap mutant, with β-galactosidase identifying the Svp cardial and pericardial cells. The dorsal terminal attachments are unaffected by the loss of *svp* expression. Expression of *lacZ* in this mutant is weaker than the *SCE–lacZ* transgene. Bar, 100 μm for A; 50 μm for B, D; 25 μm for C, E.

Figure 3: **Hox gene expression in the alary muscles**. (A–D) Embryos were stained with antibodies for the Hox proteins Ubx or ABD-A, and the accumulation of each protein was assessed in filleted samples. Representative alary muscles which were positive and negative for each Hox gene product are presented. Dorsal midline is to the top, anterior is to the left. The nuclei of the three most anterior alary muscle pairs were positive for the presence of Ubx (A), whereas more posterior alary muscles did not accumulate Ubx (B). Conversely, the anterior three alary pairs were negative for ABD-A (C), yet the posterior four alary pairs were positive for ABD-A (D). (E) Summary of Hox gene expression and patterning in the alary muscles. Bar, 12.5 μm.

Figure 4**: Loss-of-function experiments demonstrate requirements for *Ubx* and a*bd-A* function in alary muscle development.** Samples were stained for accumulation of MHC, and dissected as described above. Ventral

views of filleted samples are shown. (A) Wild-type, showing the normal pattern of seven pairs of alary muscles (arrows). (B) *Ubx* mutants showed lack of a full complement of normally developed alary muscles, with the 2–3 most anterior pairs not observed. (C) *abd-A* mutants showed a normal complement of alary muscles. (D) *Ubx* and *abd-A* double knockout mutants showed a large reduction in alary muscle number, with only occasional muscles observed (arrowhead). Bar, 100 μm.

Figure 5: **Ectopic expression of Hox genes expands the alary muscle field**. Stage 16 embryos ectopically expressing either *Ubx* (A) or *abd-A* (B) throughout the mesoderm were stained for accumulation of MHC and examined. In both cases, mutants showed an increased number of alary muscle pairs, from the seven normally observed in wild-type, to approximately ten (see text for details). The additional muscles were located in the more anterior region (arrowheads). Bar, 100 μm.

CHAPTER 2

Figure 1: **The *l(2)tn* mutation is located on the second chromosome and results in muscle degeneration**. (A) The *l(2)tn* mutation was localized—by complementation analysis using deletions containing molecularly defined breakpoints—to the boxed area. This area contains 11 annotated genes. (B and C) Representative pupae from WT (*w^1118*) and thin mutants (*l(2)tn* /Df).

Compared with WT (B), *l(2)tn* mutants have narrower and elongated pupal cases (C). (D–I) Immunofluorescent stains of L3 larval pelts stained with anti-TM (green) and phalloidin to visualize F-actin (red). The basic patterning of the WT (D) larval musculature is similar in the *l(2)tn* mutants (E), but muscles appear more robust in WT animals. (F–I) High-magnification images of muscles show highly organized sarcomeres in WT (F and H) compared with *l(2)tn* mutants (G and I), which show thinner, looser muscles with a loss of cross-striation and irregular sarcolemma. Arrows indicate sarcomeres that have remained in register; arrowheads indicate gaps between myofibrils. (Scale bars, 1.3 mm for B and C; 300 μm for D and E; 50 μm for F and G; 12 μm for H and I.)

Figure 2: **Knockdown of Thin function by RNAi or genetic deletion mirrors the degenerative muscle phenotypes** observed inthin mutants. Representative pupal cases from 24B > abbaRNAi (A) or *tn*/Df (B) individuals have the same elongated pupa phenotype observed in the original *l(2)tn* mutant. (C) (UAS-abba,Df/*tn* ΔA;24B) are able to partially rescue the WT phenotype in a mutant background. (D–L) Immunofluorescent antibody stains of L3 muscle pelts stained with anti-TM (green) and phalloidin to visualize F-actin (red). Knockdown (D) and knockout (E) animals mimic the original *l(2)tn* phenotype by displaying thinner, looser muscles that appeared less robust than partial rescue (F) and WT animals. (G–L) Higher-magnification images of the individual myofibers in knockdown (G and J) and knockout (H and K) larvae show a general loss of sarcomere organization and cross-striation, whereas myofibrils of partial rescue

animals are relatively well organized (I and L) and appear more stable than in the mutant. (M) Axial ratios for pupae of the indicated genotypes. The median value is indicated by the line through the box bounded by the upper quartile and lower quartile. Error bars indicate the upper and lower limits of the data points. The distribution of axial ratios revealed a consistently larger length/width ratio for *tn* mutants compared with w^{1118} and rescue animals. (N) Lethal-phase data indicate that the majority of the *tn* mutant offspring are embryonic and pupal lethal. Although some rescue animals were embryonic lethal, the majority of hatched larvae eclosed as adults. (Scale bars, 1.3 mm for A–C; 300 µm for D–F; 50 µm forG–I; 16 µm for J–M.)

Figure 3: **Thin is expressed in developing and mature muscle.** (A–C) In situ hybridizations showing *tn* mRNA expression. *tn* mRNA is robustly expressed in stage 13 (A) and stage 16 (B) WT embryos. Reduction of *tn* levels is apparent in the *l(2)tn* /Df mutants (C). (D–L) Thin protein expression in developing muscle (D and E) and mature muscle (G and I). Thin protein accumulated in a muscle-specific pattern in stage 13 (D) and stage 16 (E) WT embryos, but was markedly reduced upon loss of *tn* function (F). (G) A WT preparation shows that Thin is present in L3 larval muscle (green) in a repeated pattern within the sarcomeres. Costaining for the Z-line protein MLP84B (H, red) reveals that Thin also is found at the Z-disk of the sarcomere (I). Knockdown of *tn* using RNAi results in a reduction of Thin accumulation at the Z-lines and a general decrease in muscle expression levels (J) compared with MLP84B, which is retained at the Z-lines

(K and L). Black arrows (A–F) point to corresponding muscle segments in the

developing embryos. White arrows (G–I) illustrate the corresponding Z-lines in

the muscle of the L3 larva preparations. (Scale bars, 140 µm for A–F; 15 µm

for G–L.)

Figure 4: ***tn* does not genetically interact with mutations affecting the Z-disk components** MLP84B or D-Titin. D-Titin, also known as *sallimus* (*sls*), is

the fly ortholog of mammalian Titin. Box plots of the indicated genotypes show

the distribution of axial ratios in mutant pupae. Pink shading represents the span

of the box-plot ratios for control (w^{1118}). Blue shading indicates the range of box-

plot ratios for the original *l(2)tn* mutant and removal of mlp84B or *sls* in

a *tn* mutant background. *Df(3R)dsx2M* is a deletion that removes Mlp84B.

Figure 5: Loss of *tn* selectively affects the localization of known sarcomere

proteins within the myofiber. (A–N) Confocal micrographs of the L3 larval

musculature in WT (A–D) and *tn* mutants (E–N) stained with the indicated

antibodies (green) and phalloidin to observe F-actin (red). In WT muscle, α-

Actinin (A) and MLP84B (B) are Z-line associated. These proteins remain

properly localized at the sarcomeres despite the loss of *tn* and disorganization of

the myofibrils in *l(2)tn* /Df (E and F) or *tn* ΔA/Df (I and J) mutants. WT stains

exemplify the highly organized localization of the sarcomeric proteins MHC (C)

and TM (D) in the myofiber. In the genotypes *l(2)tn* /Df and *tn* ΔA/Df, MHC

(G and K) and TM (H and L) are mislocalized, and in some preparations, the

disassociated protein accumulates along the surface of the sarcolemma and occasionally forms globular structures. Visceral (M) and cuticle (N) images of muscle stained for MLP84B (green) and actin (red) show the directional disorganization of myofibrils. Muscle along the cuticle maintains a relatively well-organized cross-striated pattern (N) compared with the unbundling myofibrils along the visceral (M) side of the muscle. (Scale bars: full size, 30 µm; Inset, 12 µm.)

Figure 6: **The costameric proteins Integrin, Talin, Spectrin, Vinculin are consistently mislocalized in mutants**. Both WT and *tn* ΔA/Df late-stage larvae were dissected and immunofluorescently labeled for the antibody (green) and for F-actin (red). Images were captured by confocal microscopy. Merged Z-stack scans show the surface of the muscles. A middle slice from these scans is also presented to illustrate the sarcolemmal accumulation of some proteins. WT animals share two localization features of costameric proteins: localization at the Z-disk and localization around the surface of the muscle, but not in the center. In *tn* ΔA/Df mutants, these proteins are mislocalized from the Z-disk and often appear as clumps near the surface of the muscle. Arrowheads indicate the localization of costamere proteins along the surface of muscle. (Scale bars, 30 µm.)

Figure 7: **Clustalw amino acid sequence alignments of the murine, human, andfly orthologs of Trim32/Thin show levels of conservation between the**

Conserved Domain Database (CDD) identified domains. Clustalw alignments

of best matched, conserved domains show high levels of sequence identity that

have been preserved in the RING (A) and NHL domains, of which only the first

NHL domain is shown (C). Homology to the BBOX (B) region was detected, but

exhibits a much lower level of conservation between the mammalian orthologs

and flies. (D) Table of pairwise alignment scores (calculated by the number of

identities in the best-match alignment divided by the length of the compared

residues, not including gap spaces and expressed as a percentage) as well as

the E-values generated by the CDD that were used to identify the domains within

each ortholog

Figure 8: **Nuclear position in *tn* mutants is disrupted**. Larval fillets of WT

animals show nuclei organized and evenly spaced in rows along the length of the

skeletal muscle (A). *tn* mutants have muscles with nuclei that are mislocalized

and unevenly spaced (B). White arrows indicate regular nuclei position in WT

and irregular position in *l(2)tn* /Df animals. (Scale bar, 18 μm.)

Figure 9: **The sarcomeric proteins MHC and tropomyosin (Tm) accumulate**

normally in *tn* mutants during embryonic myogenesis. Organized muscles in

latestage WT embryos show expression of both tropomyosin (A, in red) and MHC

(B, in green). In addition, WT muscle normally expresses β-integrin (Int) at

muscle attachment sites (A, in green). Late-stage embryos of the genotype *l(2)tn*

/Df or *tn ΔA* /Df stained for Tm and β-integrin (C and E) as well as for MHC (D

and F) accumulate Tm (red) and β-integrin (green) to equivalent levels and in comparable patterns to that of WT embryos. This indicates that the strong reduction in β-integrin staining in the mutant larval muscles arises from postembryonic defects. (Scale bar, 55 μm.)

Figure 10: **Delta sarcoglycan is normally positioned along the sarcolemma in muscle in a striated pattern but this patterning is disrupted in animals with *tn* loss of function.** Delta sarcoglycan (A and C) accumulates normally along with F-actin (B and C) in a striated pattern along the surface of muscle in WT animals. *l(2)tn* / Df animals show a loss of both delta sarcoglycan (E and G) and F-actin (F and G) normal patterning similar to the phenotype observed for disrupted costameric

proteins Z-stacks confirm Delta sarcoglycan positioning along the sarcolemma in the WT (D) and a loss of this organization in *tn* mutants (H). (Scale

bars: 15 μm and 3.5 μm.)

Table 1: *Drosophila* stocks used in this study

CHAPTER 3

Figure 1: **Expression pattern of Vinculin in *Drosophila* muscle.** (A) Vinculin expression in L3 larva dissections show robust accumulation in both the dorsal vessel (orange arrow) and skeletal muscle (pink arrows). Vinculin is also

specifically visible in the alary muscles (yellow arrows) as well as the pericardial cells (white arrows). (B) Higher magnification images of Vinculin in the skeletal muscle of L3 larva show that Vinculin is both striated at the sarcomeres (white arrows) and 'nested' around the nuclei. (F) Western blots for Vinculin and pan-Actin (control) show that wild type (WT) expression of Vinculin is present and robust. Homogenates of siblings ($vinc^{1(2012)}$/+ and +/+ (balanced stock)) known to be carrying at least one vinc + (indicated in figure as +) allele (balancer + = vinc +) also successfully express Vinculin. Knockout animals ($vinc^{1(2012)}$/$vinc^{1(2012)}$) show a complete loss of Vinculin. The expression on the blot was loaded with a 2x concentration of cellular extract in order to underscore this point.

Figure 2: **Expression of Vinculin in L3 *Drosophila* larvae occurs in the costameres.** Muscle is stable and known orthoogus binding partners of Vinculin in the costamere, sarcomere and muscle attachment sites remain intact in mutants of *Vinculin.* WT stains for Vinculin show normal localization in three dimentions relative to sarcomere marker and Vinculin binding partner Actin (A-F). (A) WT XZ confocal scan for Phallodin (Actin) shows the inner layer of muscle at the sarcomeres. (B) XY confocal scan illustrates the normal striation of the sarcomeres in muscle. (C) WT XZ confocal image of Vinculin localization. (D) XY confocal image of striations in muscle resulting from Vinculin (green) accumulation. (E) Vinculin is observed above (green arrows) the surface of Actin (red) in the sarcomeres (red arrows) consistent with the position of costamere in vertebrates. (F) Vinculin is shown to remain in relative register with the

sarcomeres in WT muscle. Tropomyosin (Tm) is observed to remain present and

localized in the sarcomeres of WT (G) and mutant (H) muscle. Vinculin binding

partner remains enriched in both WT (I) and mutant (J) costameres and muscle

attachment sites. Merge images for Tm (red) and Talin (green) in WT (K) and

mutant (L) muscle. Vinculin binding partner Actin was stained with Phalloidin in

WT (M) and mutant (N) muscle showed no loss of cross striation between

sarcomeres for a loss of Vinculin. Proper localization of Integrin in the WT (O) is

mirrored in the mutant (P) with accumulation at both the striations of the

costamere and the muscle attachment sites. Merge images for Actin (red) and

Integrin (green) in WT (K) and mutant (L) muscle.

Figure 3: **Homozygotes for *In(1LR)pn2a,Vinc $^{1(2012)}$* are consistently smaller**

and more fragile than wild type and their heterozygote siblings. Larva are

pictured here at 4 days since egg lay. Lab strain wild type (far right of figure,

w^{1118}) are consistently comparable in size to the heterozygote siblings (center,

FM7iGFP/ *In(1LR)pn2a,Vinc $^{1(2012)}$*). Mutant homozygotes (four smaller larva left

side of figure) in addition to being smaller than wild types and siblings, are

physically more fragile . Scale bar: 3.5mm

Figure 4: **Mutant pupa eclose approximately 2 full days after their siblings.**

Four cohorts of 10 pupa each were collected and assessed for (first graph) GFP+

animals (wild type for *Vinculin* and heterozygotes) as well as for (second graph)

Vinculin null animals. Of the *Vinculin* null flies that did eclose, 100% of them were males. Adult female mutant flies were never recovered.

Drosophila provides a unique insight into the role of *Vinculin* in muscle. It remains unclear as to why Vinculin remains enriched at the costamere in flies. Unlike humans, flies are able to live past embryogenesis despite a loss of *Vinculin.* These animals compel us to ask if the more vital role of Vinculin is not in muscle, but in regulating the timing or execution of development. The slower rate of development may be contributed to the accumulation of novel mutations on the *In(1LR)pn2a,Vinc* $^{1(2012)}$ chromosome. These aberrations may act alone, or in concert, with the known loss of Vinculin. As a possible result of disruptions in cell motility during remodeling, female lethality may result either from an inability to complete the maturation process or simply from starvation due to a protracted pupal developmental stage.

FIGURES

CHAPTER 1

Figure 1: Physical structure of the alary muscles relative to the dorsal vessel.
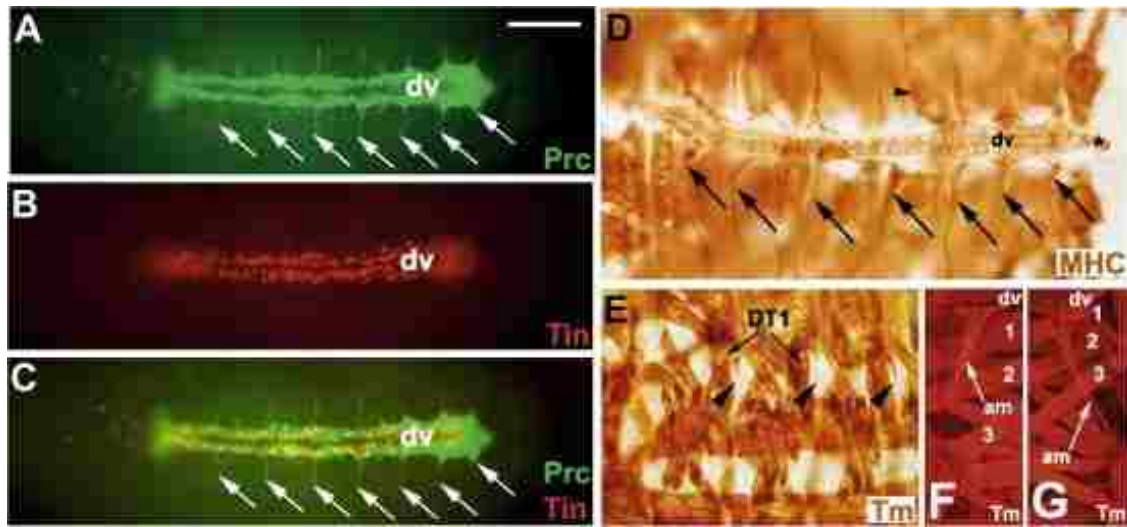
CHAPTER 1

Figure 2: Alary muscle patterning relative to *svp* expression in the dorsal vessel.
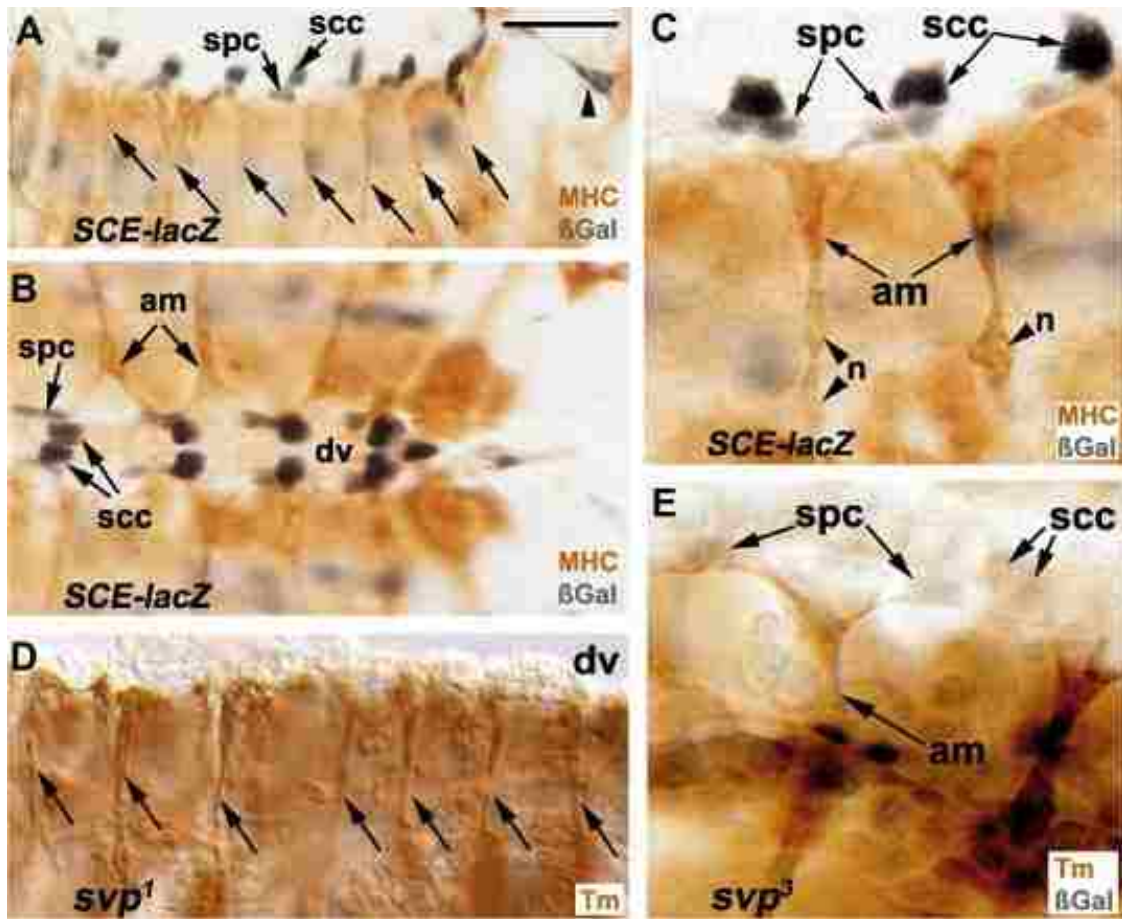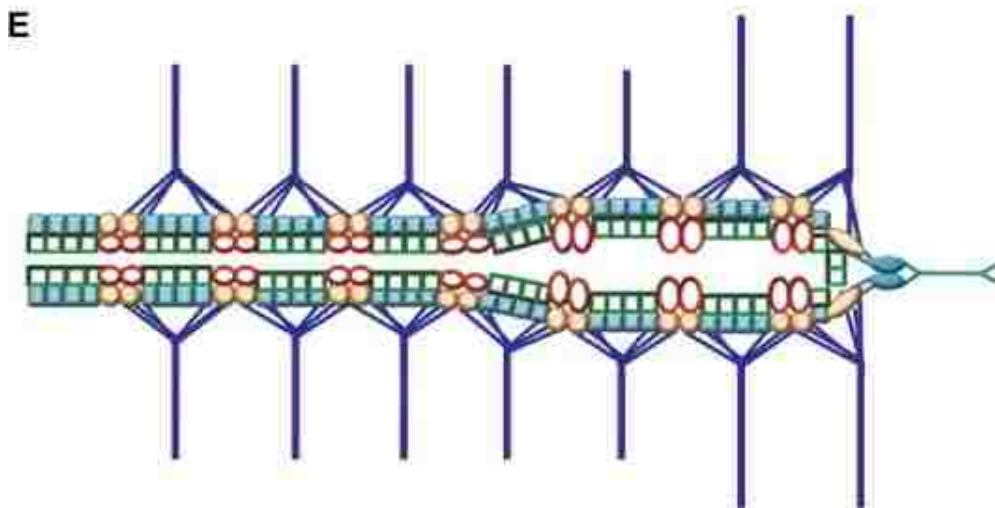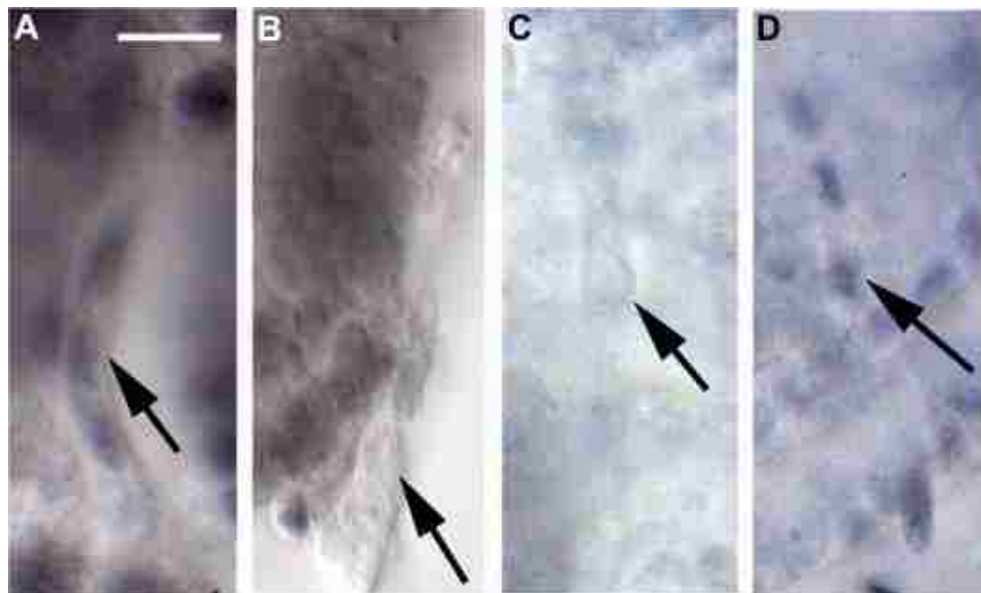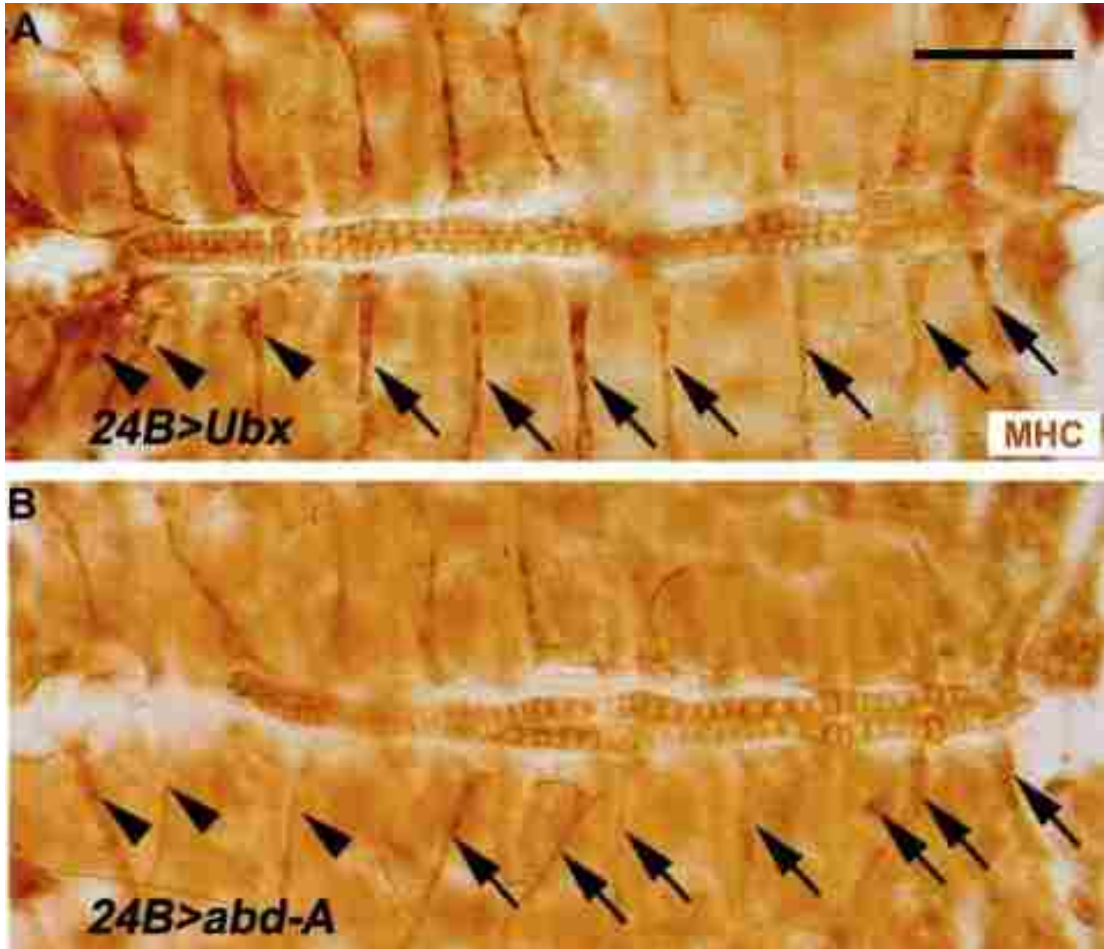
Figure 3: Hox gene expression in the alary muscles

CHAPTER 1

Figure 4: :   Loss-of-function experiments demonstrate requirements for *Ubx* and
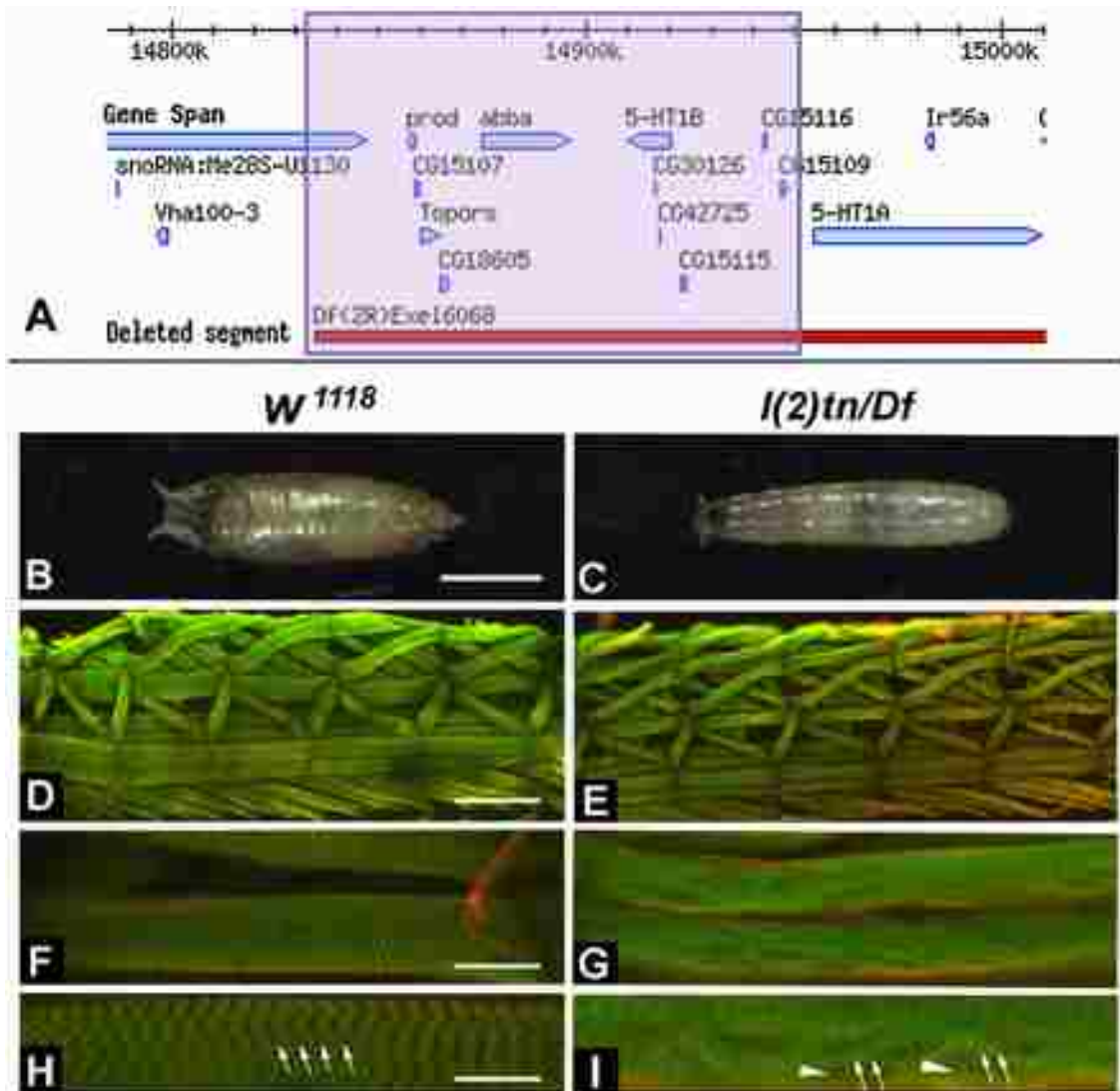
a*bd-A* function in alary muscle development.
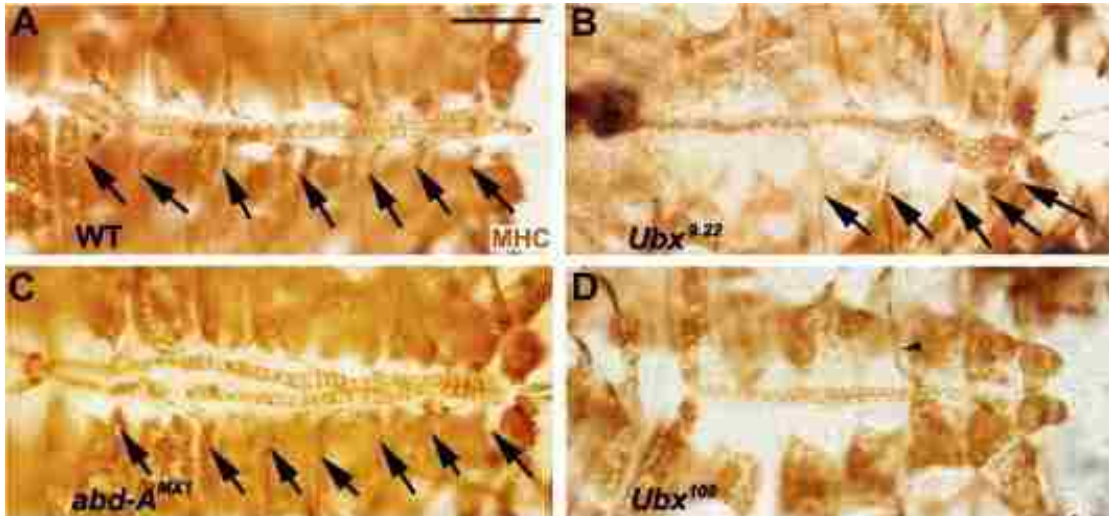
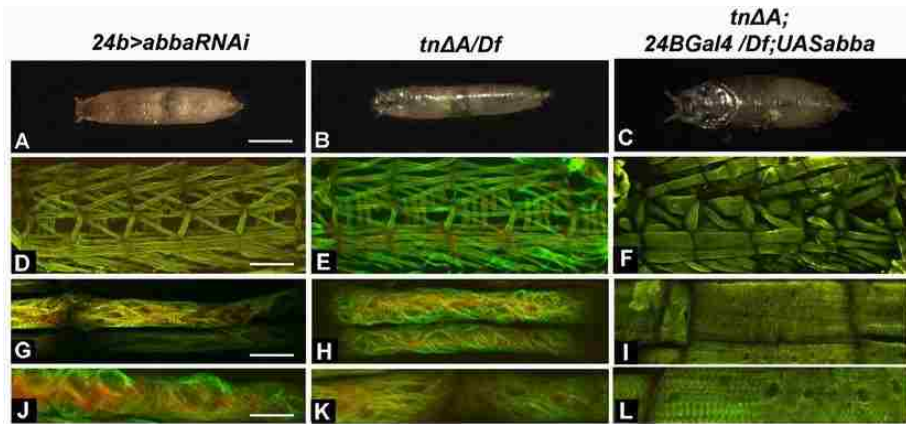Figure 5: Ectopic expression of Hox genes expands the alary muscle field

CHAPTER 2

Figure 1: The *l(2)tn* mutation is located on the second chromosome and results in muscle degeneration

CHAPTER 2

Figure 2: Knockdown of Thin function by RNAi or genetic deletion mirrors the

degenerative muscle phenotypes

Box plots of axial ratios of pupal cases
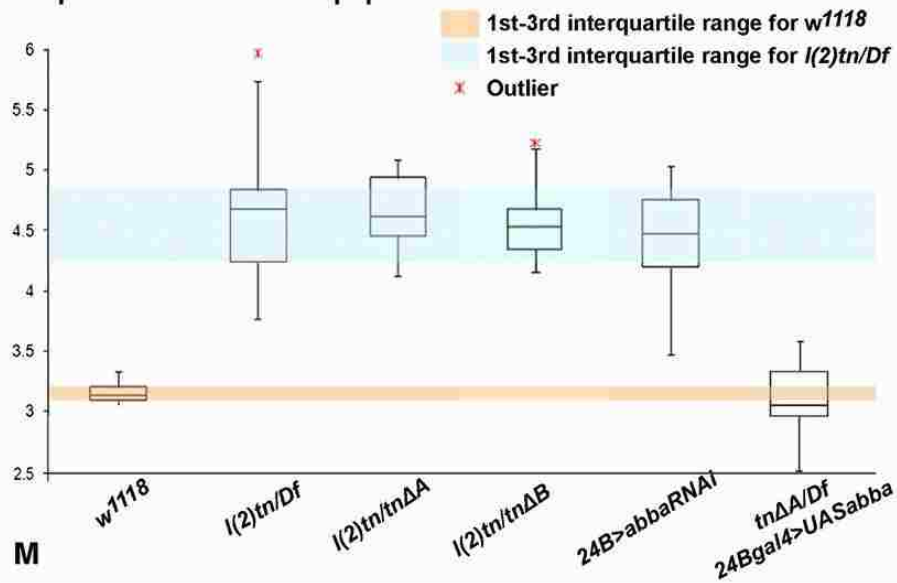
Developmental stage specific lethality data

	l(2)tn/Df	tnΔA/Df	tnΔB/Df	24B> abbaRNAi	UASabba,Df /tnΔA;24B
unhatched	39.0%	63.1%	68.8%	57.8%	26.5%
L1 larval lethal	0.6%	2.4%	2.1%	5.6%	0%
L2 larval lethal	0.0%	0.0%	0.0%	0.4%	0%
L3 larval lethal	0.0%	0.0%	0.0%	1.1%	0%
pupal lethal	60.3%	32.4%	28.9%	34.8%	2.1%
eclosed adults	0%	0%	0%	0%	61.6%
(n)	300	290	321	422	373

N
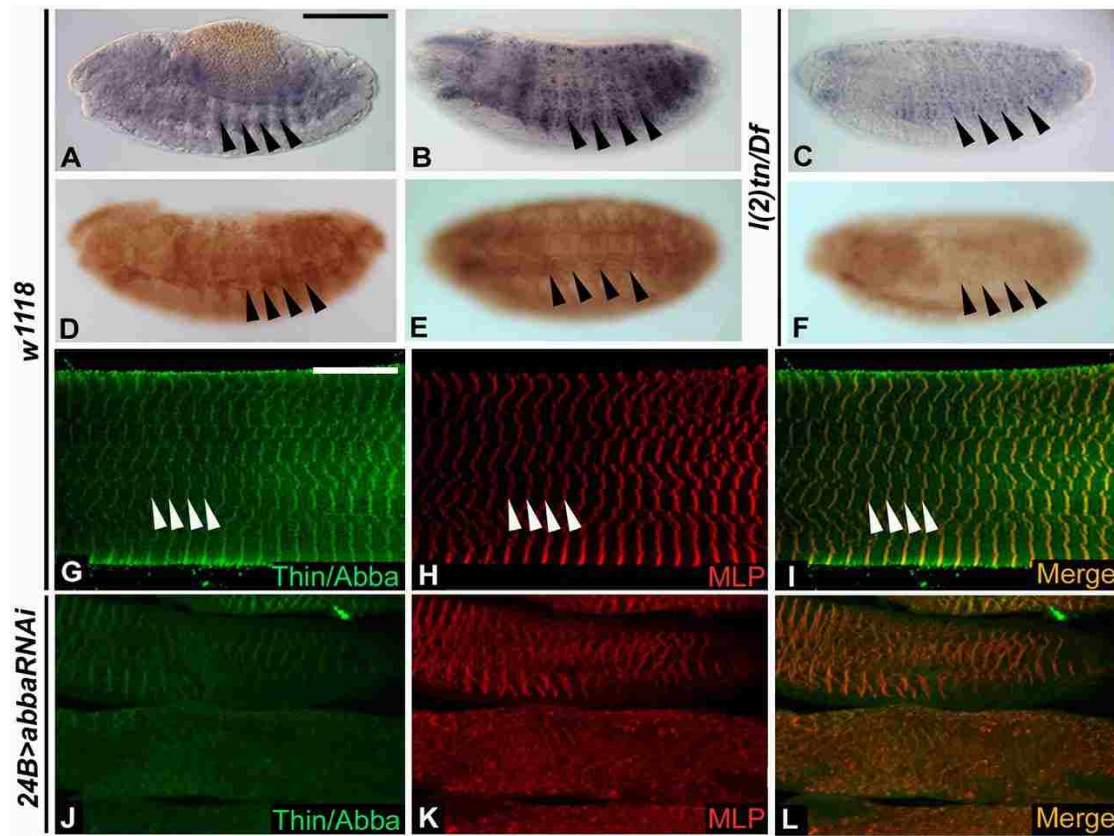
Figure 3: Thin is expressed in developing and mature muscle.

Figure 4: : *tn* does not genetically interact with mutations affecting the Z-disk
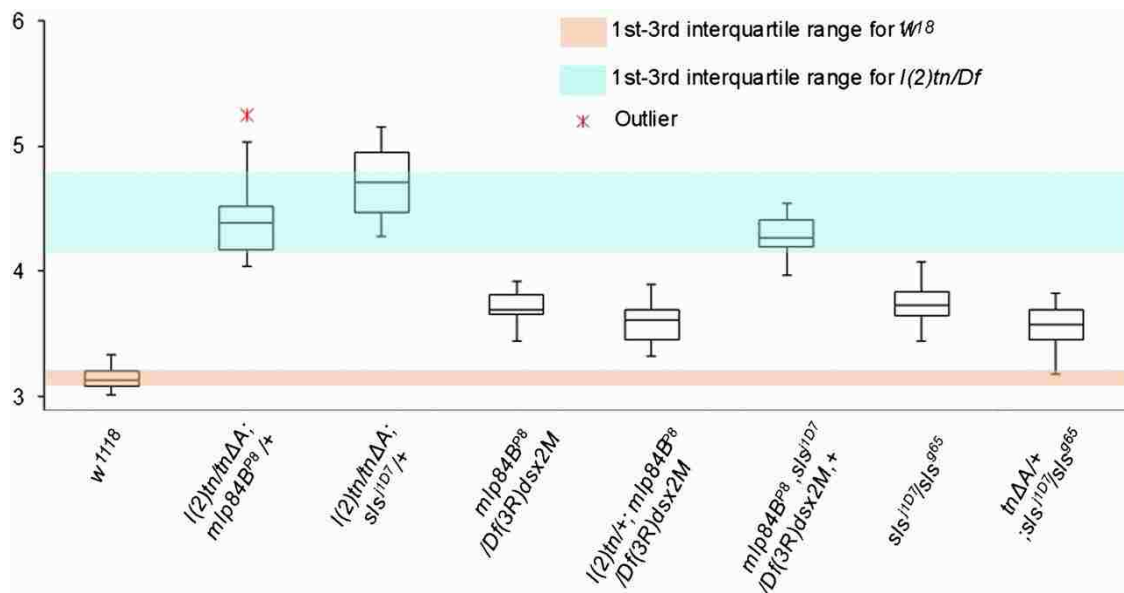
components

CHAPTER 2

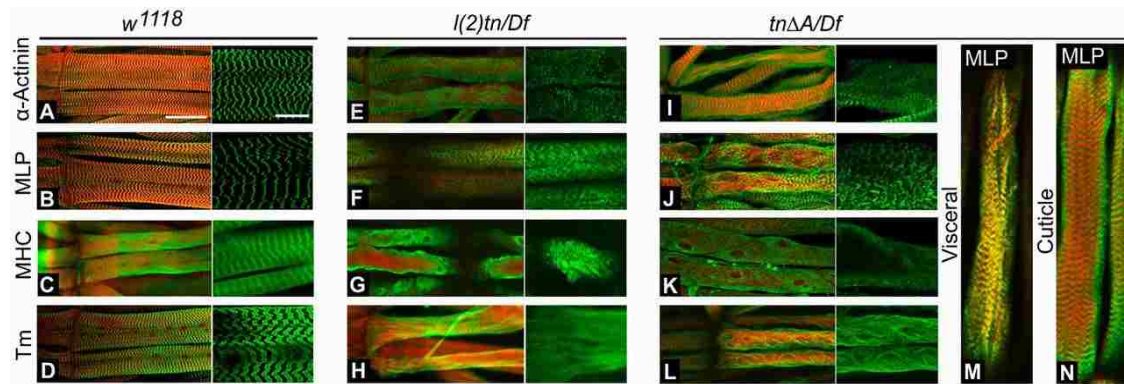Figure 5: The costameric proteins Integrin, Talin, Spectrin, Vinculin are consistently mislocalized in mutants

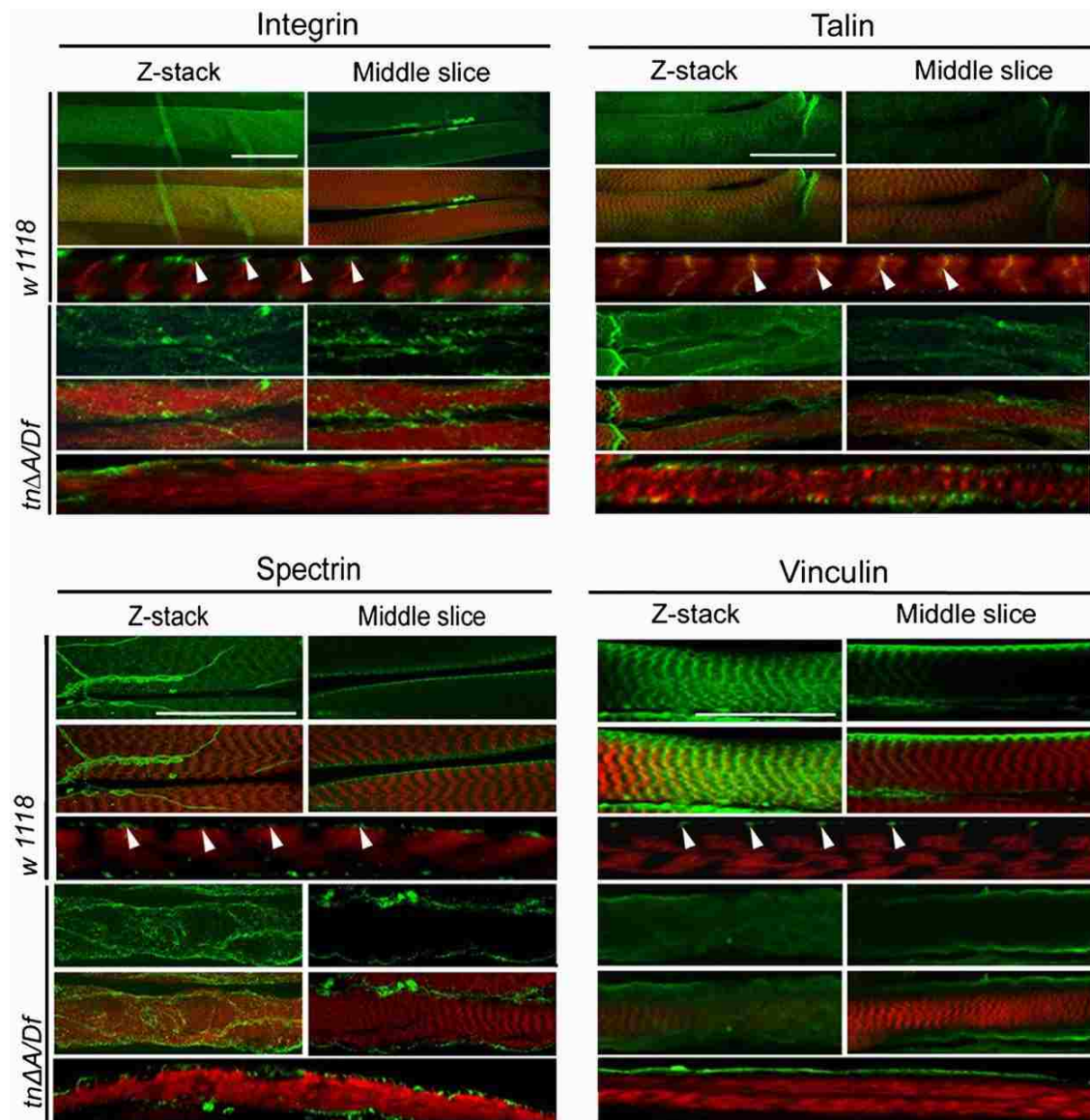Figure 6: The costameric proteins Integrin, Talin, Spectrin, Vinculin are consistently mislocalized
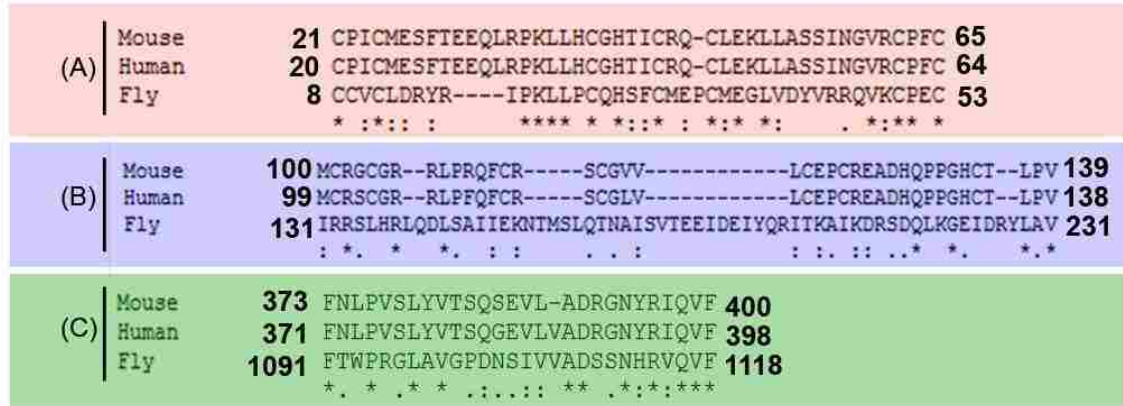

CHAPTER 2

Figure 7: Clustalw amino acid sequence alignments of the murine, human, andfly orthologs of Trim32/Thin show levels of conservation between the Conserved Domain Database (CDD) identified domains.


```
        Mouse     21 CPICMESFTEEQLRPKLLHCGHTICRQ-CLEKLLASSINGVRCPFC 65
(A)     Human     20 CPICMESFTEEQLRPKLLHCGHTICRQ-CLEKLLASSINGVRCPFC 64
        Fly        8 CCVCLDRYR----IPKLLPCQHSFCMEPCMEGLVDYVRRQVKCPEC 53
                      * :*:: :      **** * *::* : *:* *:    . *:** *
```

```
        Mouse    100 MCRGCGR--RLPRQFCR-----SCGVV-----------LCEPCREADHQPPGHCT--LPV 139
(B)     Human     99 MCRSCGR--RLPFQFCR-----SCGLV-----------LCEPCREADHQPPGHCT--LPV 138
        Fly      131 IRRSLHRLQDLSAIIEKNTMSLQTNAISVTEEIDEIYQRITKAIKDRSDQLKGEIDRYLAV 231
                      : *.   *    *.  : :        . . :         : :. :: ..* *.     *.*
```

```
        Mouse    373 FNLPVSLYVTSQSEVL-ADRGNYRIQVF 400
(C)     Human    371 FNLPVSLYVTSQGEVLVADRGNYRIQVF 398
        Fly     1091 FTWPRGLAVGPDNSIVVADSSNHRVQVF 1118
                      *. * .* * .:..:: ** .*:*:***
```

(D)	RING	BBOX	NHL
Mouse vs. Human	100	100	96
Human vs. Fly	38	5	42
Mouse vs. Fly	38	5	33
Mouse (NCBI/CDD)	1.54e-08	2.77e-07	4.14e-04
Human (NCBI/CDD)	1.36e-08	6.18e-06	6.54e-06
Fly (NCBI/CDD)	6.84e-09	1.05e-03	8.81e-08

pairwise score

E-value

CHAPTER 2

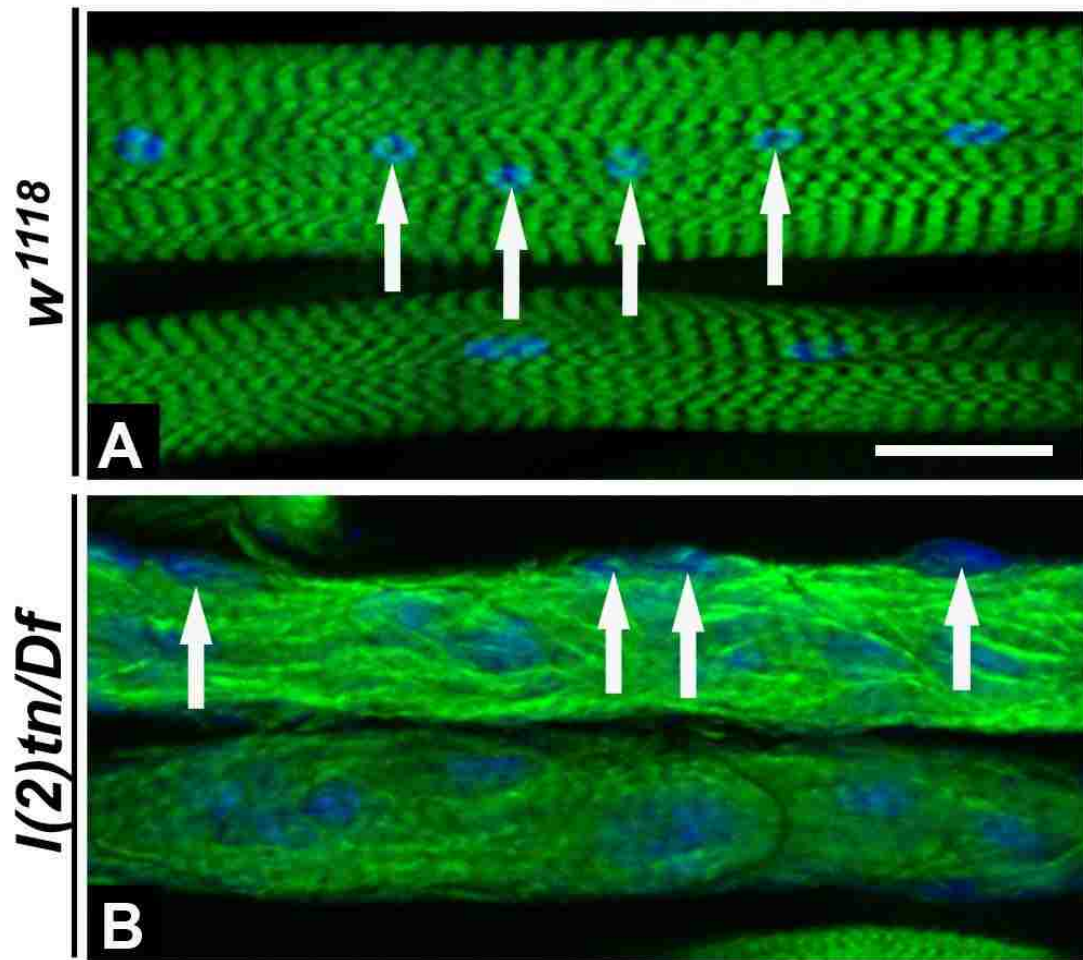Figure 8: Nuclear position in *tn* mutants is disrupted

Figure 9: The sarcomeric proteins MHC and tropomyosin (Tm) accumulate normally in *tn* mutants during embryonic myogenesis
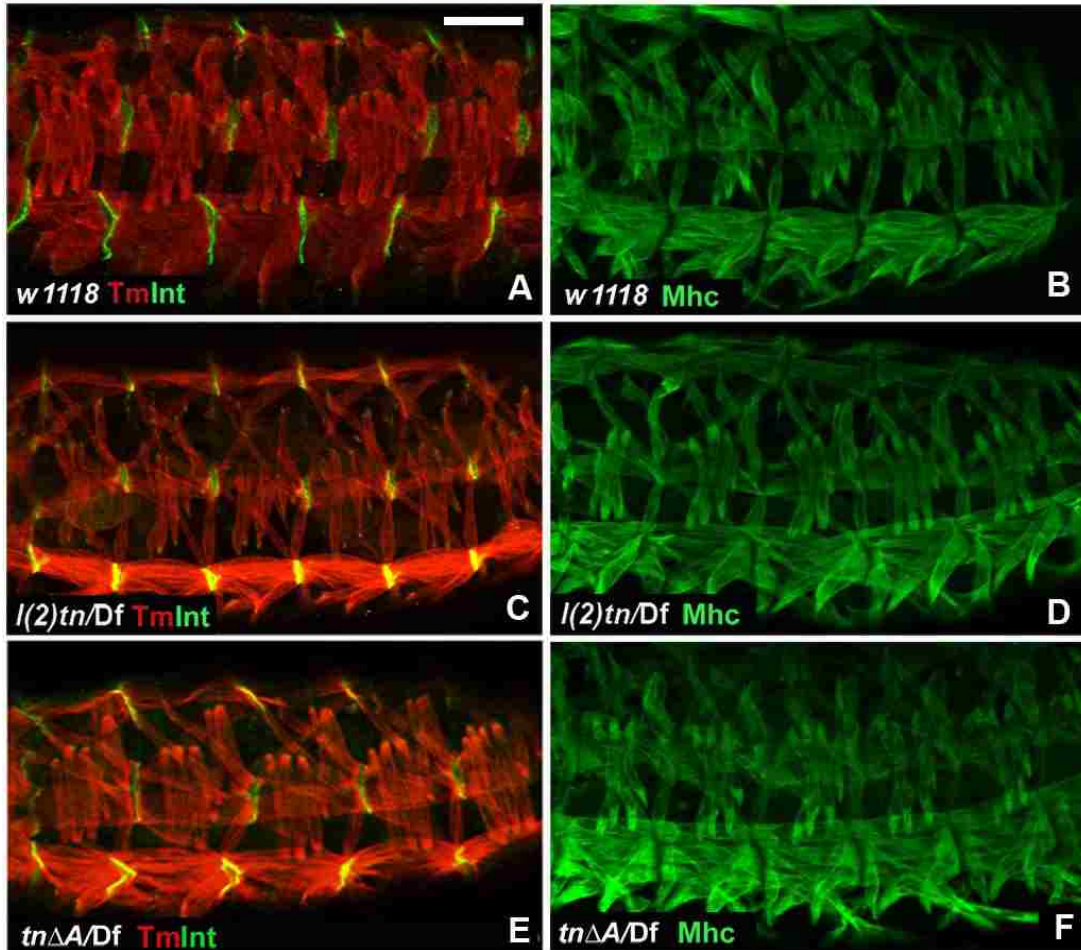
Figure 10: Delta sarcoglycan is normally positioned along the sarcolemma in muscle in a striated pattern but this patterning is disrupted in animals with *tn* loss of function.
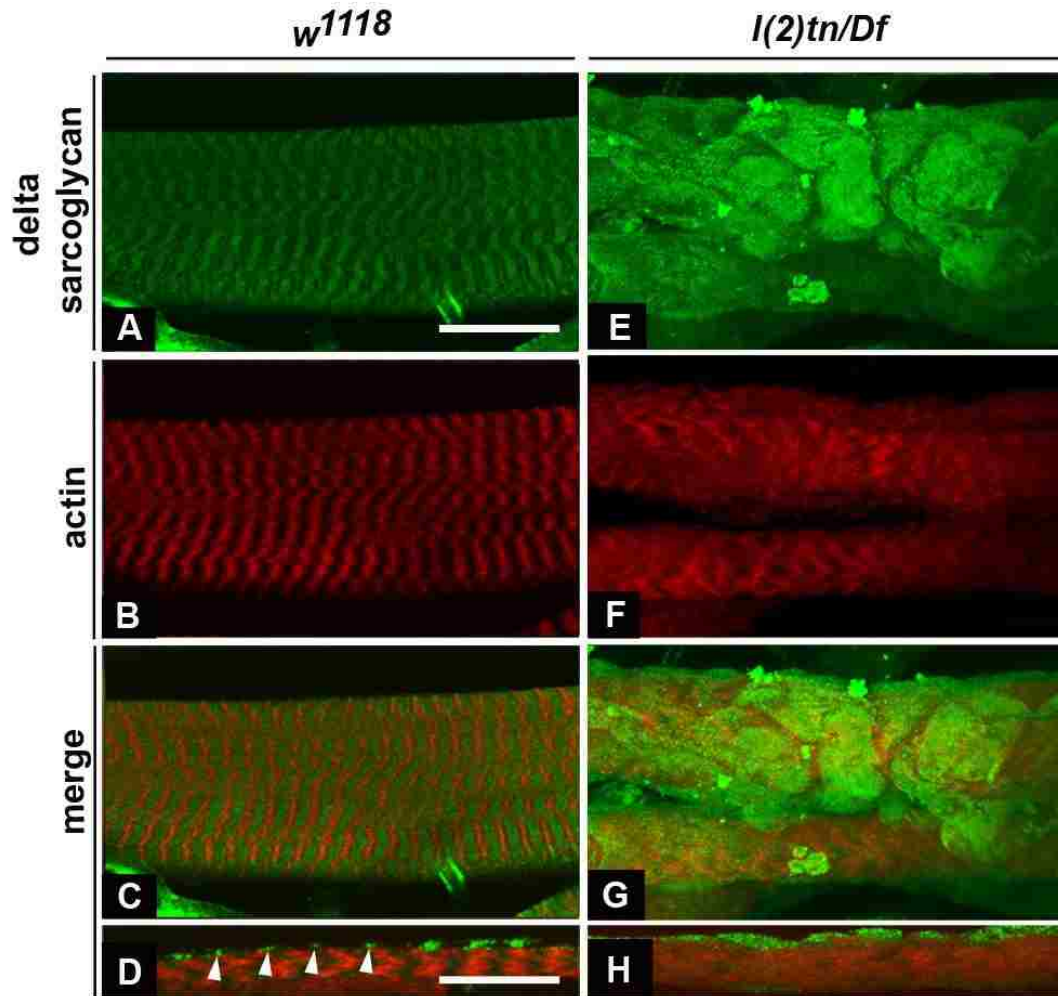

CHAPTER 2

Table 1: *Drosophila* stocks used in this study

Table 1. *Drosophila* stocks used in this study

Genotype	Description
w^{1118}	Wild type
l(2)tn	Spontaneous *abba* mutant (Ball et al. 1985)
tn$^{\Delta a}$	P-element mediated knockout for *abba* (null)
RNAiabba	RNAi knockdown for abba (VDRC stock 19290)
UAS·tn	Controlled expression construct (CEC) for *abba*
P[w+] mlp84B^{P8}	P-element mediated knockout for *mlp84B* (null)
P[w+] sls ^{j1D7}	Strong loss-of-function *sls* allele
Df(3R) dsx2M	Deficiency that removes *mlp84B*
Df(2R) 6068	Deficiency that removes *abba*

CHAPTER 3

Figure 1: Expression pattern of Vinculin in *Drosophila* muscle.
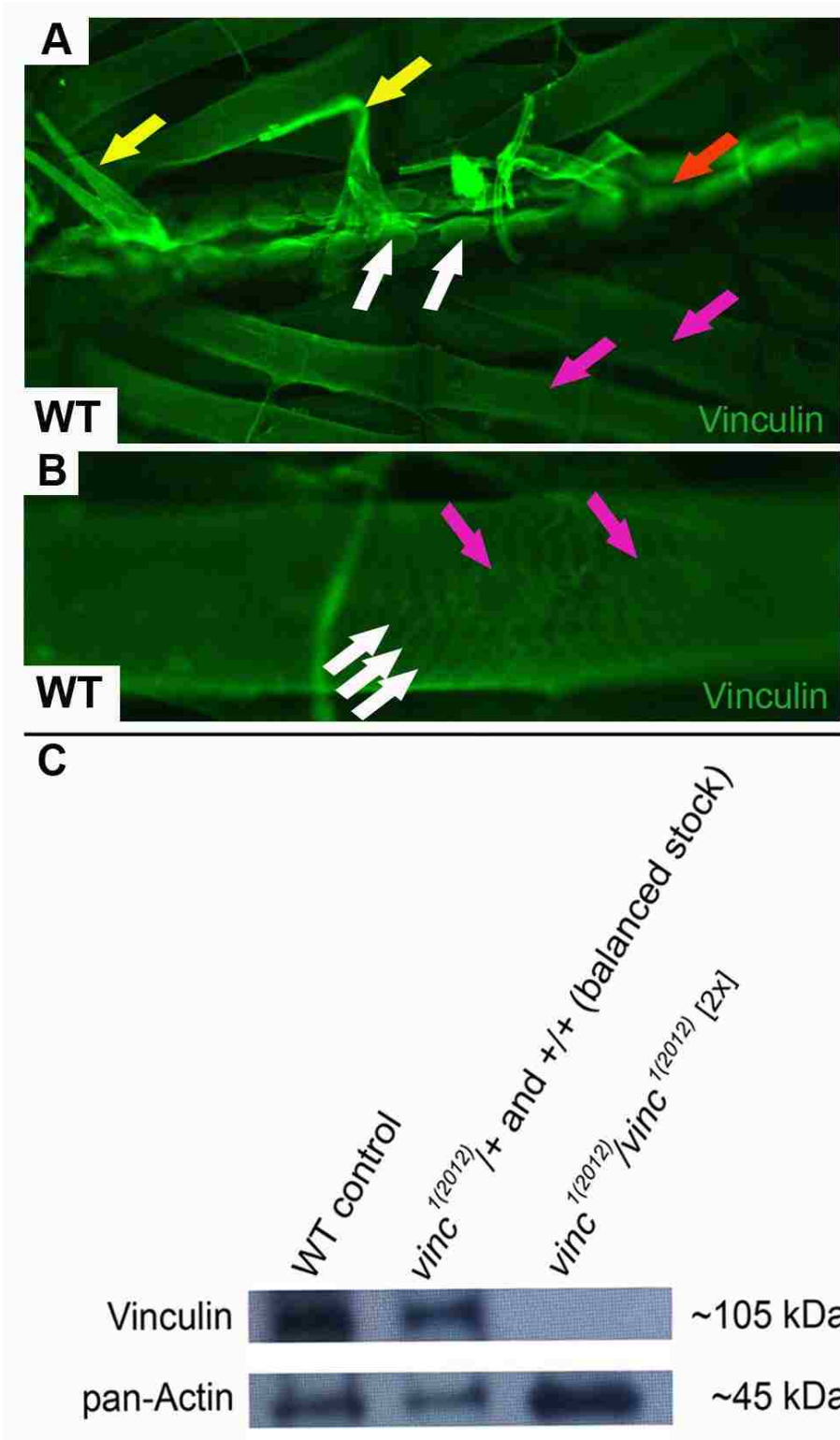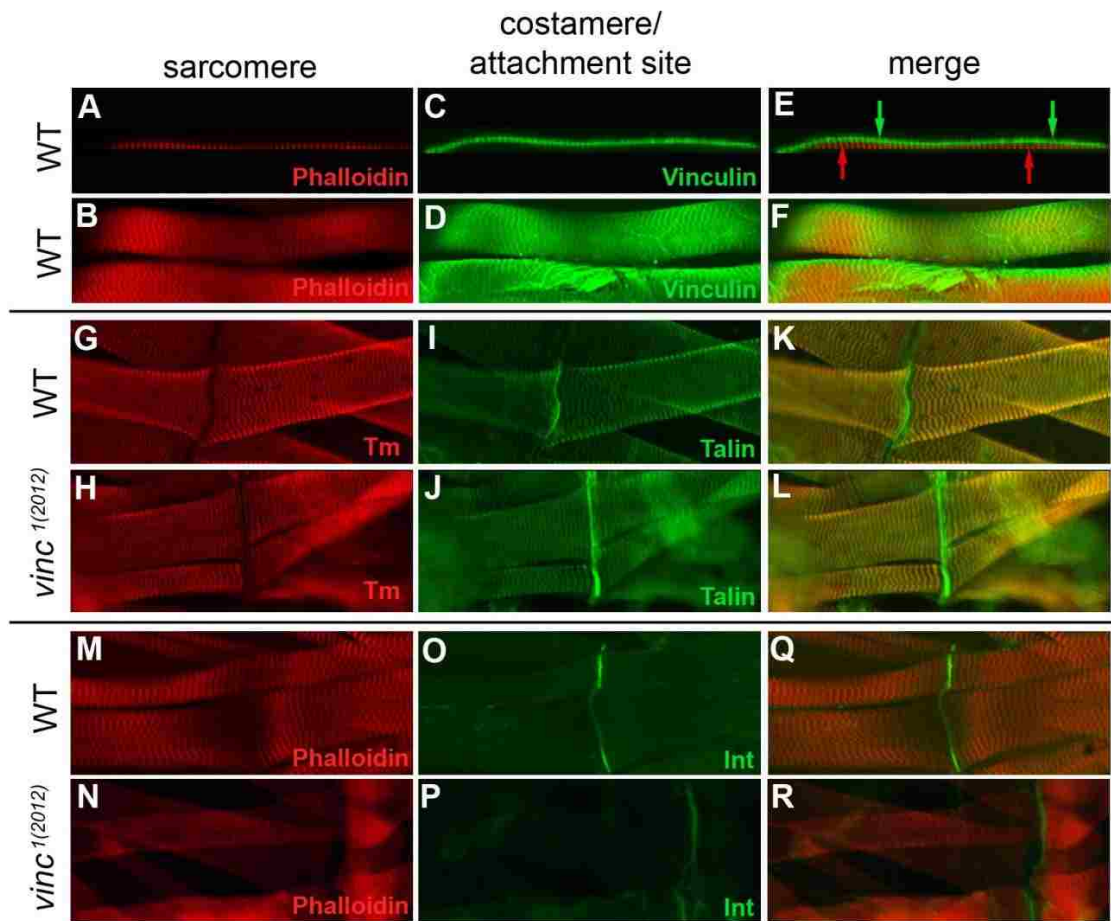
Figure 2: Expression of Vinculin in L3 *Drosophila* larvae occurs in the costameres.

Figure 3: Homozygotes for *In(1LR)pn2a,Vinc [1(2012)]* are consistently smaller and more fragile than wild type and their heterozygote siblings.
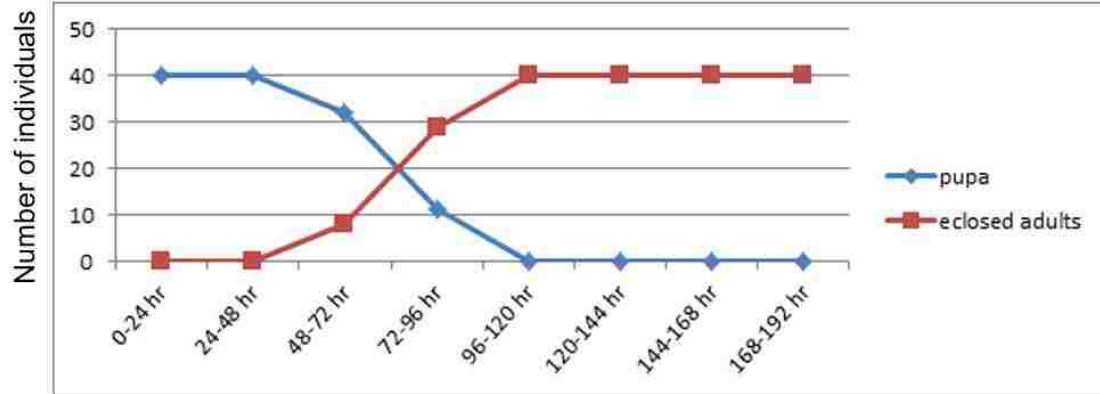
Figure 4: Mutant pupa eclose approximately 2 full days after their siblings.

Time from pupariation to eclose

GFP+,*vinc*+ siblings
10 pupa to a vial, repeated for 4 vials

GFP-,*vinc*- mutants
10 pupa to a vial, repeated for 4 vials