Coordination of muscle maintenance and innate immunity through integrated tissue physiology in *Drosophila*

by

Nicole Marie Green

B.S., Southern Illinois University Edwardsville, 2012

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Biochemistry & Molecular Biophysics Graduate Group

KANSAS STATE UNIVERSITY
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Abstract

Maintenance of muscle tissue during development is greatly dependent upon the extracellular matrix (ECM) to stabilize, sense, and compensate for changes in the local environment. Muscle has a particularly high demand for a dynamic ECM to allow for contraction and to transmit forces necessary for generating movement. Inefficient contraction and/or detachment can lead to muscle tissue damage and the release of damage-associated molecular patterns (DAMPs), which overactivate immune responses and drive the progression of muscle diseases. Our lab uses the *Drosophila* muscle attachment site (MAS) as a model to characterize novel genes and mechanisms involved in muscle maintenance. Initially, we were focused on characterizing a novel ECM protein, Fondue (Fon), which had previously been shown as a critical mediator of ECM stability in the hemolymph clot. Mutations in *fon* and the knockdown of *fon* through RNAi causes body wall muscles to detach and also creates large gaps between muscle hemisegments. TEM analysis of *fon* mutant MASs revealed a loss of ECM integrity and important support features including disruption of cuticle and tendon architectures, a lack of muscle-tendon interdigitation, and a loss of electron-dense matrix accumulation. More interestingly, a sensitized background screen revealed a subset of coagulation proteins, *fon*, *Tiggrin,* and *Lsp1*γ, that were necessary for stabilizing muscle attachment sites.

Further investigation into gene expression profiles of mutants experiencing hypercontraction-induced muscle tissue stress indicated a clear trend of innate immune activation, suggesting a broader connection between muscle development and innate immunity. In *fon* mutants with muscle detachment, we also observe abnormal melanin accumulation as melanotic tumors or along the larval MASs, activation of Toll signaling in

the fat body, and constitutive expression of the antimicrobial peptide (AMP), *drosomycin.* In a *fon*-sensitized background assay, we identified genetic interactions between *fon* and Toll pathway members, including the NFκB inhibitor/IκB, *cactus*. At the local level, *fon*-mediated muscle detachment and muscle hypercontraction mutants, Mhc^{S1} and $Brkd^{J29}$, cause JAK/STAT activation within muscle tissue. We propose a model where muscle tissue stress caused by disruptions to muscle homeostasis progresses muscle disease through overactivation of the innate immune system. Understanding the mechanisms by which these two biological processes are intertwined will advance our knowledge of how tissue stresses can be sensed and elicit multi-tissue responses.

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And to the flies: thank you for your sacrifice in the pursuit of scientific knowledge.

Dedication

I dediate this thesis to every non-scientist in my life. For those of you on your way towards science (to my students); for those of you that choose to love science on my behalf (to my friends and family); and for those of you that teach me new ways to be a better scientist through your passion (to my very valuable non-scientific mentors). To every unexpected teacher---family, students, and acquaintances whose questions challenge me to be more intentional and insightful about my own work---thank you for continually shifting and re-focusing my perspective. I am the sum of our time together and books I have read by your suggestion.

> *"If you trust in Nature, in the small Things that hardly anyone sees and that can so suddenly become huge, immeasurable; if you have this love for what is humble and try very simply, as someone who serves, to win the confidence of what seems poor: then everything will become easier for you, more coherent and somehow more reconciling, not in your conscious mind perhaps, which stays behind, astonished, but in your innermost awareness, awakeness, and knowledge. You are so young, so much before all beginning, and I would like to beg you, dear Sir, as well as I can, to have patience with everything unresolved in your heart and to try to love the questions themselves as if they were locked rooms or books written in a very foreign language. Don't search for the answers, which could not be given to you now, because you would not be able to live them. And the point is, to live everything. Live the questions now. Perhaps then, someday far in the future, you will gradually, without even noticing it, live your way into the answer."*
>
> -Rainer Maria Rilke, *Letters to a Young Poet*

Preface

Like many *Drosophila* projects, this story is driven by information gained during a genetic screen and the goal to comprehensively describe the cohort of genes relevant to muscle development. In the culmination of this thesis project, we have uncovered extensive overlap between two important biological processes: muscle development and innate immunity. While the components of the innate immune system are normally activated only upon immune challenge, we show that secreted proteins forming the clot matrix are regularly incorporated into larval muscle attachment sites (MASs). Moreover, the essential nature of this suite of proteins is observed by muscle detachment occurring as a result of the loss of these proteins.

In characterizing the pleiotropic roles of these secreted proteins, we noted additional immune phenotypes which suggested that the overlap between muscle and immune tissues extended beyond structural parallels in the clot and MAS. Following disruptions if muscle maintenance, local and systemic immune signaling is activated in *Drosophila* larvae. We show not only a new mechanism for sterile induction of the insect immune response, but also evidence for pathological consequences to muscle tissue upon excessive levels of immune signaling. In Chapter 1, we provide a general overview of muscle development in *Drosophila melanogaster* and the immune responses which aid in insect survival. Furthermore, we review emerging models of immune activation during the invasion of pathogens and sterile tissue damage.

In Chapter 2, we characterize the function of a novel muscle gene, *fondue* (*fon*), in *Drosophila* muscle attachment. Observations from literature describing *Drosophila*

muscle mutants and the work of previous members in our lab indicated that abnormal pupal morphology could be used as a novel marker for the identification of new genes involved in muscle development. In collaboration with the Bloomington *Drosophila* Stock Center (BDSC), members from the Erika Geisbrecht and Mitchell Dushay labs visually screened thousands of mutant stocks for the presence of elongated or abnormally shaped pupae.

It was in this screen that Fon, a protein previously characterized for its role in coagulation, was discovered to play a crucial role in maintaining muscle attachments. Transmission electron microscopy (TEM) images revealed widespread loss of MAS cellular architecture upon loss of Fon, suggesting that Fon acts as a critical organizer of MAS extracellular matrix (ECM), unlike many *Drosophila* ECM proteins which produce mild or undiscernible mutant phenotypes. In an effort to classify genetic interactors of *fon*, we analyzed secreted candidates present in *Drosophila* hemolymph, the larval clot, and MAS-associated proteins characterized over several decades by fellow fly geneticists. To our surprise, we identified two unconventional proteins essential for muscle attachment (Fon, Lsp1γ) functioning in concert with a third protein, Tig, to form a specific set of secreted hemolymph proteins with overlapping roles in maintaining the structural integrity of the *Drosophila* clot and MAS.

In Chapter 3, we extend our understanding of an expected series of immune phenotypes induced in *fon* mutants to expose a complex tissue communication network underlying tissue maintenance. Using *fon* as a tool, we dissected the extent of humoral and cellular

immune responses activated upon *fon*-mediated muscle detachment and discovered that overactivation of systemic Toll signaling has the capacity to cause muscle detachment.

While searching through invertebrate and vertebrate literature, we discovered a strong trend between muscle tissue damage and innate immunity. By mining existing gene expression profiles of disease models and tissue biopsies from muscular dystrophy patients, we noted the increased expression of immune-responsive genes and genes central to immune signaling pathways, particularly Toll signaling during muscle damage. Relying on well-defined immune assays and reporters, we screened major immune pathways and various mutant alleles known to elicit tissue stress capable of immune activation following a disruption of muscle homeostasis. Furthermore, we show a causal link between Toll and JAK/STAT within our muscle maintenance model revealing reciprocal signaling pathways in regulating organismal physiology. Compiling knowledge from cases of sterile inflammation, infection, and our own data in muscle tissue, we continue to test and refine a model of tissue coordination between muscle and fat body with hemocytes acting as a mobile source of secreted molecules.

In Chapter 4, we address two major themes from our work and potential future directions of this thesis project: 1) the pleiotropic role of proteins across life stages; and 2) the emerging complexity of multi-tissue communication and physiology required to aid and preserve tissue morphology and integrity. We also address unexplained phenomena encountered in our work and avenues for future exploration.

Chapter 1 - Introduction

Tissue formation occurs early in organismal development and encompasses a variety of cellular activities including cell fate specification, migration, and the formation of cellular attachments or adhesions. Despite being defined early, these tissues must be able to adapt to the growth and movement of organisms during their lifespan. Homeostatic mechanisms which help maintain the 'status quo' balance tissue integrity and internal physiology. Muscle tissue presents particularly unique challenges to tissue maintenance. It is a dynamic tissue which demands high energy currency and high stress resistance to perform its primary function of contraction.

We are interested in exploring new molecular mechanisms to address the challenges of muscle morphogenesis and the processes required to maintain these tissues throughout the developmental profile of an organism. Particularly in the context of aging and disease, loss of tissue integrity and degradation occur as a result of normal maintenance processes breaking down. For many decades, clinicians have provided anecdotal and descriptive evidence of immune activation in muscular dystrophies and myopathies [1]. We have combined the rich history of *Drosophila* investigations in both muscle biology and innate immunity to form an integrative model for these two processes. In this chapter, the intersection between muscle maintenance and innate immunity with an emphasis on utilizing the genetics and simplicity of the *Drosophila* model is explored through theories and data found in published literature.

Overview of *Drosophila* muscle development

Drosophila melanogaster is a holometabolous insect with a developmental profile consisting of a brief embryonic period followed by three larval instar stages and pupariation, for a life cycle of 10-11 days before adult eclosion. Muscle development begins in stage 10 of embryogenesis (5 hours after egg laying (AEL)) with muscle cell specification and proceeds through stage 16 (15 hours AEL) [2]. *Drosophila* muscle development alters muscle morphology to adapt to changing body plans and new functions of muscle groups required in each stage. This review of muscle development will be restricted to major groups of skeletal muscle involved in locomotion (crawling and flight), although it should be noted that *Drosophila* contains many specialized muscles involved in behavioral-based movements such as feeding and reproduction.

Somatic muscles are arranged in stereotyped patterns of 30 muscles within hemisegments organized along the body wall of embryos [3, 4]. Muscles are formed in late embryogenesis and begin their first contractions at the end of embryogenesis to aid in the emergence of larvae from the eggshell. These muscles persist through three instar phases of larval development, growing rapidly to accommodate a dramatic increase in body size and the frequent muscle contractions necessary for movement and feeding. During pupariation, a few larval muscles are preserved to act as scaffolds for adult muscle formation, while the vast majority are histolyzed to prepare for adult muscle reorganizations [5]. Adult muscle is formed from an arrested pool of muscle precursors formed during embryogenesis and retained in the imaginal disc. Each stage of muscle development exhibits unique organizations making muscle an excellent model for uncovering fundamental cellular and developmental processes.

Two waves of myogenesis occur during the *Drosophila* life cycle—the first in embryogenesis and a second synthesis of muscle tissue during metamorphosis. Embryonic muscle forms from the fusion of cells specified through gene expression programs early in development out of the mesodermal lineage. During specification, cells are fated to become either founder cells (FCs) or fusion-competent myoblasts (FCMs) [6]. Both cell types possess unique immunoglobulin domain proteins on their membranes, with FCs expressing the myoblast attractant Dumbfounded (Duf), and FCMs with the marker Sticks and stones (Sns) [7, 8]. During embryogenesis, a set of precursor myoblasts are formed and set aside for adult myogenesis [9]. Muscle fiber, or myofiber, formation occurs when an FC fuses with a number of neighboring FCMs [10, 11]. FC cells are specified with a unique transcriptional profile of identity genes which alters the gene expression FCMs to match FCs upon fusion and to regulate muscle features such as morphology and patterning [12, 13]. Myoblast fusion gives rise to a multinucleate muscle fiber whose size is related to the number of fusion events [12, 13]. These iterative and combinatorial mechanisms have been extensively studied and reviewed in [14] for their role in creating the diversity of muscle seen in *Drosophila* development.

The major challenge of larval development is tissue maintenance. *Drosophila* larvae undergo body mass increases of up to 200x in comparison to their embryonic counterparts [15]. Muscle attachments (described below) formed during embryogenesis are tested as muscles begin to contract and force is transmitted through the muscle to the anchoring tendon cell and cuticle at the myotendinous junction (MTJ). Late in larval development the majority of muscles are histolyzed along with other organs to make way for new adult structures. Adult myoblast precursors proliferate within the imaginal disc

and migrate to larval scaffolds during the pupal phase and then mimic the cellular processes of myoblast fusion first observed in embryogenesis [14, 16]. Many of the molecular cues of embryonic myogenesis are recycled in adult muscle development, including the use of identity genes to give adult muscle precursors positional information [17, 18]. However, the process of adult muscle development is far less studied than embryonic myogenesis and there are certainly shared and novel mechanisms left to be discovered. Adult *Drosophila* muscles must endure much longer lifespans than the embryonic musculature, while facing similar challenges of stress resistance and structural integrity alongside natural aging. These features have made adult muscle a valuable model for the study of molecular mechanisms regulating tissue degeneration and models of muscle diseases such as muscular dystrophy, atrophy, muscle wasting, and Parkinson's Disease [3, 19].

Drosophila **muscle structure and attachment**

Drosophila muscles use the well-conserved sarcomere as a basic unit of internal muscle organization (Figure 1.1A,B). Sarcomeres are organized towards the end of muscle development [4]. Sarcomeres are considered conserved mechanical units comprised of the same basic components through vertebrates and invertebrates: thin actin filaments and thick myosin filaments which contract and relax using a sliding filament model and protein complexes that form the M-line and Z-disk to anchor muscle to the sarcolemma and basement membrane [4]. Analogies between overall musculoskeletal organization and sarcomeric design are visualized through shared color schemes in Figure 1.1A and 1.1B. The first set of muscles used by embryos and larvae consist of a single fiber, in contrast to adult muscles which are bundles of several myofibers more similar to

vertebrate muscle (Figure 1.1A). During muscle growth, new sarcomeres are added to maintain sarcomeric length but adapt to increases in overall muscle length [4, 20]. It is the conservation of developmental strategies and fundamental architecture at the molecular and organizational level that has led to the many insights on vertebrate muscle biology from *Drosophila* research.

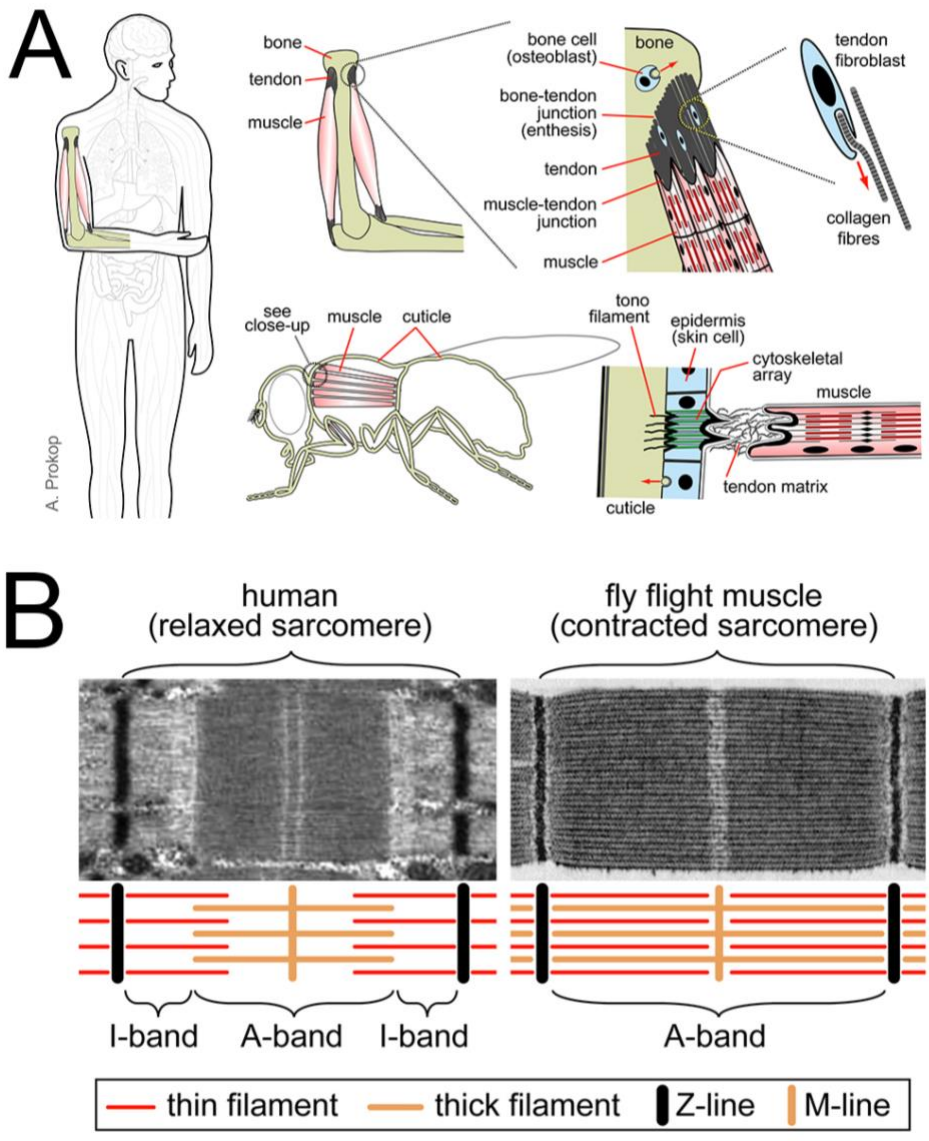

Figure 1.A Comparison of *Drosophila* and vertebrate muscle structure.

(A) Schematic comparison of vertebrate and *Drosophila* muscle morphology. The overall organization of muscle (red) is conserved between invertebrates and vertebrates. In both

groups, muscle contains internal structural components organized into sarcomeric units. Muscle tissue anchors to a hard surface such as bone or exoskeleton/cuticle (beige) in humans or *Drosophila*, respectively through connective tissues such as tendons (light blue) or ECM (grey meshwork). Coloration indicates analogous structures in human and fly muscle architecture. (B) Electron micrographs of human and *Drosophila* flight muscle sarcomeres with accompanying sarcomeric representations below. Flies and humans share basic sarcomere organization such as sliding filaments, actin and myosin, to form distinct banding features (note presence of I-band or A-band in each organism), though *Drosophila* uses a mix of insect-specific and conserved proteins to build the sarcomere. Reused with permission from Andreas Prokop; original image at https://droso4schools.wordpress.com/organs/.

The formation of a muscle requires not only the cooperation of myoblasts to form a myotube, but also the participation of tendons and a meshwork of cellular 'glue' to attach cells to one another. The following questions can be asked about the construction and maintenance of MASs: *1) How are developing myotubes targeted to specific tendon cells?; 2) How are attachment sites formed?; 3) How are MASs maintained during development?; and 4) What protein complexes form the ECM of the MAS?*

Embryonic and larval body wall muscles are organized into hemisegments of thirty muscles each (Figure 1.2A,B). Tendon cell specification from the epidermal layer occurs in parallel to muscle development through *stripe* expression [21, 22] (Figure 1.2B). Tendon and muscle cells participate in reciprocal signaling to direct muscle cells to extend and form attachment sites analogous to the vertebrate myotendinous junction (MTJ) [5]. As muscles near their tendon cells, muscles respond by extending finger-like filopodia to sense and guide myotubes [10, 23]. Failure of this pathfinding process results in muscles that attach to incorrect tendons often forming unstable attachments or muscles that are degraded when they fail to attach. Some of the proteins involved in migration include Slit-Robo interactions (also used during neural pathfinding) [24, 25], D-Grip and Kon-

tiki/Perdido which associate in a complex at the tip of extending myotubes [26-28] and Echinoid which forms a similar D-Grip interaction complex [29].

Migration is quickly followed by MTJ formation which is mediated through integrin-based adhesion of muscle and tendon (Figure 1.2C). Integrins form heterodimers of α and β subunits, of which αPS1 associates with the single βPS subunit at the tendon face of the MTJ and the αPS2βPS integrin complex which is found on muscle cell membranes [30, 31]. Mutations in either of the subunits of the αPS2βPS complex result in muscle detachment upon contraction despite normal fusion, extension, and preliminary formation of MASs [32-35]. These integrin complexes link the internal actin cytoskeleton of cells to the ECM through integrin ligands which can be distinguished by the RGD or KGD motifs found in their primary sequences [36]. Several ECM components bind to specific integrin pairs in *Drosophila*, such as the binding of Thrombospondin (Tsp) to αPS2βPS [37, 38], Tiggrin (Tig) to αPS2βPS [39, 40], and Laminin to αPS1βPS [41, 42]. ZASP, Talin, Tensin, and Integrin-liked Kinase (ILK) bind intracellularly to integrin complexes to support integrin signaling and strengthen attachments between muscles and tendons [43, 44]. Loss of these proteins weakens muscle attachment and results in detachment of internal cytoskeletal components away from the membrane which can remain attached to tendon cells [36]. Integrin complexes are turned over during development based on mechanical force and the recycling of MTJ components is an essential maintenance process [45, 46].

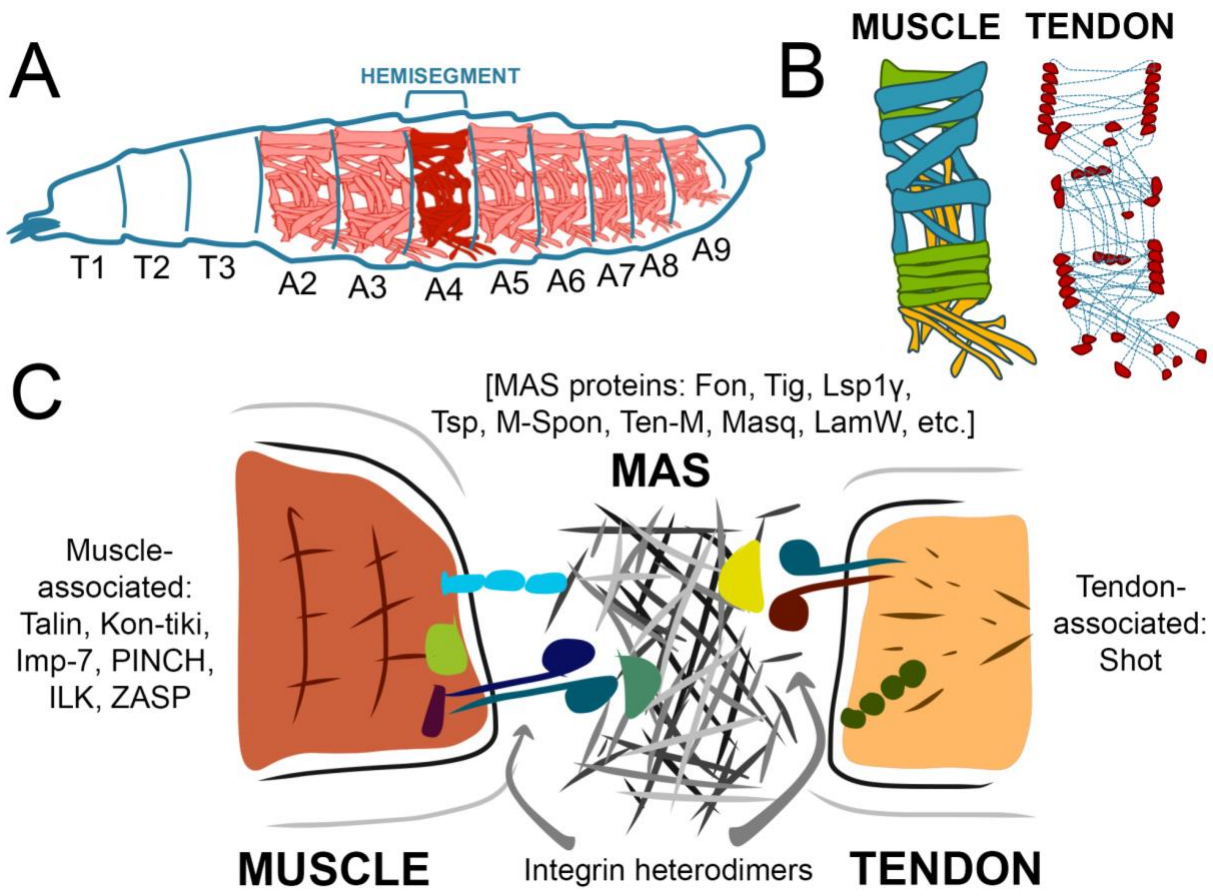

Figure 1.B *Drosophila* larval muscle structure and attachment.

(A) Schematic of larval somatic body musculature organized into hemisegments within segments A2-A9. (B) Closeup view of a hemisegment highlighting important features of muscle (left) or tendon (right). Important muscles are colorized within the hemisegment: muscles which attach indirectly across hemisegmental borders are teal, except for muscles in green which are featured in several analyses in Chapter 2. Yellow muscles represent direct muscle attachments at one or more end. Lateral transverse muscles in the middle of the hemisegment directly attach at both insertions and are commonly used as representative direct attachments. Removal of muscles (dotted outline) shows the location of tendon cells (red) which are specified from the epithelial layer to form MASs. (C) MAS architecture of *Drosophila* larvae. Proteins at the internal face of both muscle and tendon form complexes to link internal cytoskeletal components to the ECM. Integrin heterodimers and MAS adaptors bind to form complexes which attach to structural components of the ECM.

Overview of *Drosophila* innate immunity

Innate immunity is an ancient defense mechanism found throughout invertebrates and vertebrates. The *Drosophila* immune response consists of both cellular and humoral responses to foreign molecules. Insects utilize a mix of conserved and unique strategies to prevent pathogens from entering or persisting in the body. These include common defense mechanisms such as epithelial barriers, clot formation, the production of antimicrobial peptides (AMPs) (humoral response), and cellular activities to phagocytose or encapsulate larger foreign invaders (cellular response). The fruit fly presents a happy medium between complexity and simplicity as a model of innate immunity. Without the complications of an adaptive immune response, the genetic tools of *Drosophila* have allowed researchers to isolate novel components of innate immune response and the critical roles they play in organismal survival.

Cellular responses in insects include phagocytosis, nodule formation, and encapsulation, all of which involve hemocytes (Figure 1.3). Hemocytes are the *Drosophila* equivalent of vertebrate blood cells of which there are three types: macrophage-like cells known as plasmatocytes, crystal cells which contain prophenoloxidase for melanization, and lamellocytes which differentiate upon infection for the encapsulation of large objects [47]. Hemocytes are recruited to sites of infection and wounds to secrete ECM and cellular components for scab formation and epithelial repair and to produce ligands to propagate immune signaling [48]. When epithelial barriers are breeched, formation of the hemolymph clot prevents pathogens being introduced while various cellular and humoral activities are used to eliminate pathogens introduced through wounding. Coagulation

requires components originating from both hemocytes and fat body, such as Hemolectin (Hlm) and Fondue (Fon) which are essential for clot integrity [49-51].

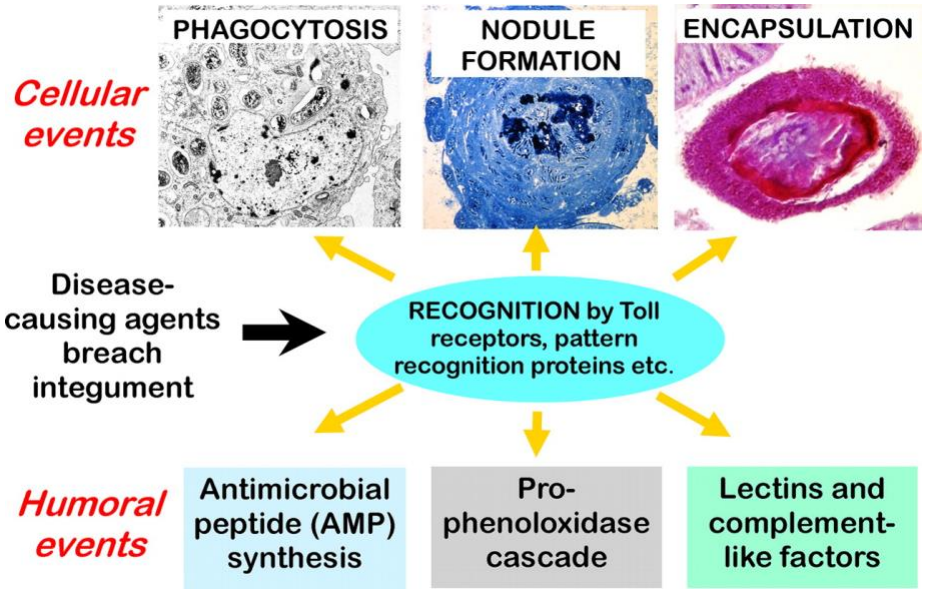

Figure 1.C Innate immune processes in Drosophila.

Insect innate immune responses consists of cellular and humoral events which can be mediated through interactions of hemocytes and tissues capable of immune signaling. Cellular activities such as phagocytosis and melanization-related strategies such as encapsulation as mediated by hemocytes similar to macrophages. Humoral events lead to the activation of proteolytic cascades and immune-responsive gene expression of AMPs, cytokines, and complement-like factors. Reused with permission from AAI and the *Journal of Immunology* [52].

The major goal of the humoral arm of innate immunity is to activate signal transduction cascades resulting in the nuclear translocation of transcription factors and downstream gene expression [53]. In *Drosophila*, humoral immunity is regulated by the NF-κB pathways, Toll and Imd which are activated individually or in concert to produce the necessary effectors to clear an infection [54]. These two pathways are activated by specific classes of infections with Toll and Dif/Dorsal-mediated transcription responding to the presence of fungal and gram-positive bacterial components and Rel-mediated expression via Imd signaling acting in response to gram-negative bacteria (Figure 1.4)

[55-59]. A major class of gene expression includes the production of AMPs. *Drosophila* has seven classes of AMPs produced upon an immune challenge which are cationic or amphipathic to target and disrupt the cellular membranes of pathogens [60]. In addition to well-defined roles in cellular immunity, hemocytes act in humoral events after an immune challenge [61]. Activation of prophenoloxidase (proPO) released by crystal cells through a proteolytic cascade yields an active PO enzyme which acts on quinone derivatives, such as L-DOPA for the accumulation of melanin [62-64]. The signaling pathways mediating these processes are reviewed in more detail below.

Immune Signaling Pathways

For many years, Toll and Imd pathways have been described as the two arms of innate signaling. Roughly 80% of immune expression proceeds through these two major pathways [65]. The JAK/STAT pathway has been established as a secondary, but essential pathway involved in hematopoiesis, hemocyte proliferation, and viral infection [66]. Both overactivation of Toll or JAK/STAT signaling causes spontaneous melanization and melanotic tumors, strongly linking both in the melanization response [67]. More recently, other signaling pathways have been implicated in immunity such as c-Jun N-terminal kinase (JNK) signaling (tumor-based and stress-based immune activation), the Hippo pathway (negative regulation of immune signaling), and insulin signaling (immune signaling modulation and nutrient allocation). Many of these pathways were first identified for their role in development and have since been implicated in innate immunity.

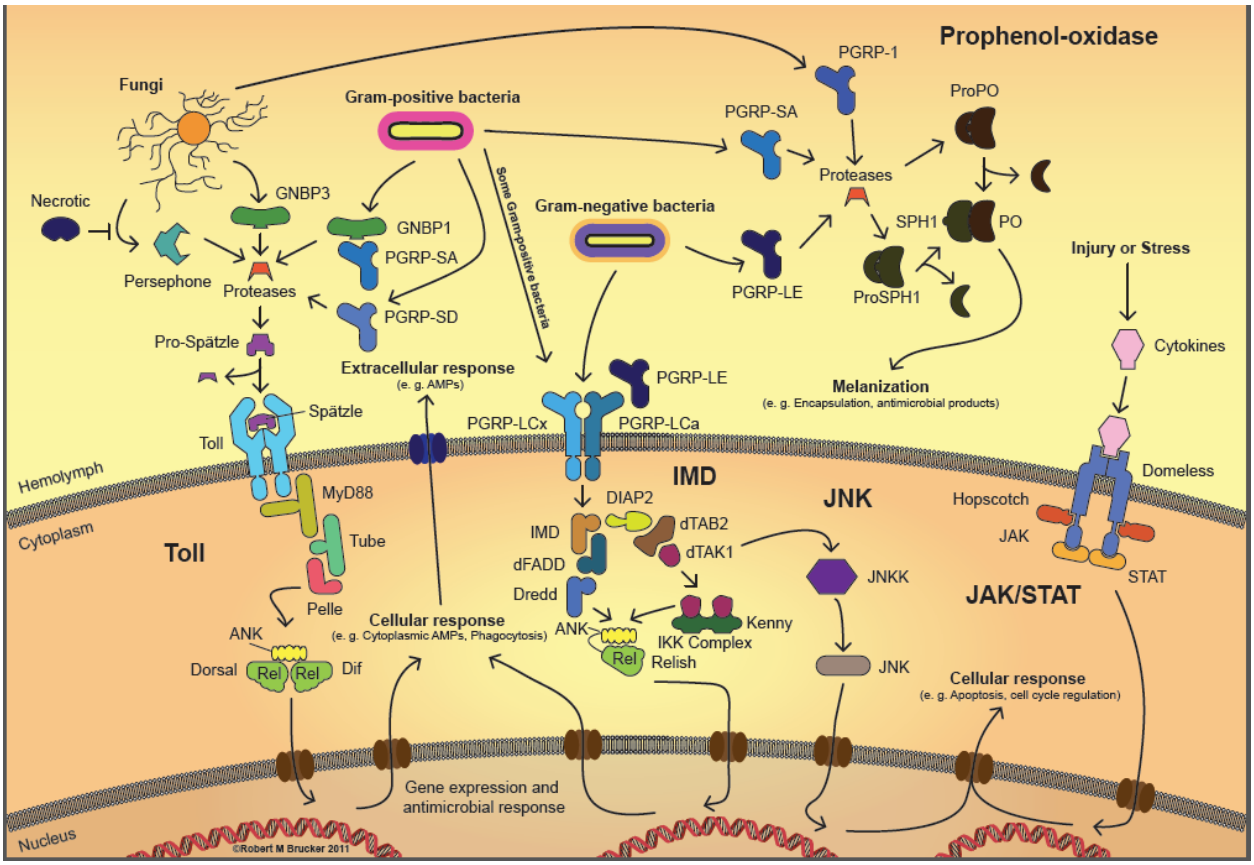

Figure 1.D Immune signaling and crosstalk in *Drosophila melanogaster.*

The major immune pathways regulating the humoral immune response for AMP production, immune gene expression, and activation of phenoloxidase (PO) through a proteolytic cascade for melanization. The NF-κB pathways, Toll and Imd respond to different subsets of infection (notated on figure), either through the indirect activation of Spz ligand or the direct binding and activation of Imd through pathogen-associated molecules (PAMPs). JAK/STAT and JNK pathways are activated upon stress and infection through crosstalk with over immune signaing pathways. Each signaling pathway leads to activation of a transcription factor and targeted gene expression. Originally produced by the Bordenstein Lab, NSF DEB-1046149, http://bordensteinlab.vanderbilt.edu/IIID/test/pathway.php.

NF-κB signaling: Toll and Imd pathways

Drosophila has three NF-κB family members encoded in its genome consisting of Dorsal and Dif, which are activated through Toll signaling, and Rel which participates in Imd signaling [68]. NF-κB components are transcription factors which have developmental roles such as dorsoventral patterning, embryonic muscle development, and

hematopoiesis [69-73] in addition to antimicrobial host defense [57, 74-76]. The goal of both NF-κB pathways is the translocation of transcription factors into the nucleus to induce gene expression, particularly of AMPs [65, 68, 77-79]. Toll signaling has also been implicated in hemocyte proliferation and melanization which is evidenced by the myeloproliferative phenotypes observed in mutants of the Toll pathway [80, 81].

There are nine Toll receptors in *Drosophila*, with only Toll-1/Toll involved in AMP production [82]. For the purpose of this review, we will limit our scope to canonical signaling through *Drosophila* Toll-1/Toll. Toll activation occurs through the activation of a single known ligand, Spatzle (Spz), in contrast to Imd signaling which proceeds through a direct pathogen recognition-receptor mechanism [83]. Upon infection or stress, damage signals to initiate a cleavage cascade leading to the activation of several proteases culminating in the activation of Spatzle-processing enzyme (SPE)[84]. SPE cleaves pro-Spatzle into its active form which is now capable of binding the Toll receptor [85]. Models of Spz binding to Toll propose either a 1:2 or 2:2 Spz to Toll interaction with both capable of inducing conformational changes in Toll for signal transduction [86, 87]. This leads to intracellular signaling through the death domain complex, dMyD88, Tube, and Pelle, to phosphorylate Cactus/IκB [55, 88]. Under normal conditions, Dif and Dorsal are sequestered in the cytoplasm by the inhibitor IκB/Cactus until Toll is activated [89, 90]. Phosphorylation of Cactus targets the inhibitor for degradation through ubiquitination leaving free Dif/Dorsal molecules to translocate into the nucleus and alter gene expression [91, 92].

The Imd pathway proceeds through the direct binding of pathogen-associated molecules to one of two transmembrane receptors known as Peptidoglycan Recogniation

Proteins (PGRPs) [93-96]. PGRP-LC has several splice isoforms for the recognition and binding specificity of bacterial peptidoglycan [97-99]. PGRP-LE is the only intracellular *Drosophila* PGRP that is capable of activating the Imd pathway through binding of pathogenic molecules and ectopic overexpression independent of PGRP-LC [98, 100-103]. Upon binding of a PGRP, Imd is recruited to the bound receptor along with FADD and Dredd to form an adaptor complex which activates TAK1 [104-106]. Activation of TAK1 continues the intracellular cascade of activation events with phosphorylation of the IKK signalosome (IKKβ and IKK γ/NEMO/Kenny). For the activation of Rel, the IKK complex phosphorylates Relish leading to cleavage through Dredd [107-110]. Cleaved Rel moves into the nucleus to bind and direct gene expression [58]. The Imd pathway is responsible for the bulk of AMP expression and has been shown to be indispensable in an effective immune response [56]. Despite the importance of this pathway, there are still many molecular events in this cascade that remain to be elucidated.

JAK/STAT pathway

The JAK/STAT pathway was first identified for its developmental signaling role in segmentation and cuticle patterning [111, 112] and has additional roles in sex determination, morphogenesis of tissues such as the hindgut, cell migration of primordial germ cells and border cells, and stem cell maintenance [113]. Since then, JAK/STAT signaling has been implicated in several aspects of immunity, mostly in relation to the activities of hemoctyes, including proliferation and differentiation [81, 114-118]. Flies with loss-of-function mutations in JAK/STAT signaling are able to combat bacterial and fungal infections but are susceptible to viral infections [119]. This signaling pathway plays a

crucial role in viral defense though further experiments show that JAK/STAT is not alone sufficient to fight viral infection.

JAK/STAT signaling proceeds following ligand binding of one of three Unpaired (Upd) ligands [69, 120, 121]. Binding of an Upd ligand to the extracellular domain of the Domeless (Dome) dimerized receptor is predicted to induce a conformational change to allow for JAK activation [120, 122]. The intracellular domain of Dome contains a binding motif for JAK proteins. The *Drosophila* JAK named Hopscotch (Hop) has the ability to phosphorylate other JAKs as well as STAT transcription factor, Stat92E. Phosphorylation of Stat92E allows it to dimerize and move into the nucleus where the STAT binds to DNA to induce the expression of developmental or stress-related genes (Figure 1.4) [114, 123]. The main targets of STAT gene expression include Turandot (Tot) stress factors [124] and thioester-containing proteins (TEPs) which function as opsonins to aid phagocytic activities [125]. Negative regulators of JAK/STAT signaling belong to the Suppressors of Cytokine Signaling (SOCS) family of which Socs36E has demonstrated roles in *Drosophila* JAK/STAT suppression [126-128]. JAK/STAT integrates into transcriptional networks for stress-related gene expression including dependencies on Imd signaling for Tot expression [124, 129, 130] and Toll signaling in Tep expression [62, 131].

Immune-related pathways: JNK, Hippo, and Insulin Signaling

Recently, immune-related pathways have been identified which modulate the immune responsef and integrate the immune response to other aspects of organismal physiology. JNK signaling is primarily known for activation following cellular stresses such as wounding or tumor formation [132, 133]. Like Toll and JAK/STAT pathways, JNK activation has developmental roles in epithelial processes simulating wound repair, such

as embryonic dorsal closure [134-136]. JNK plays critical roles in apoptosis and has suggested functions in hemocyte activation [137], but can also be activated by bacterial infection through Imd crosstalk via TAK1 [123]. There is conflicting evidence for JNK playing a role in the regulation of AMPs with some reporting positive regulation through coordination of IMD and JNK signaling [138, 139] and others reporting negative regulation [140, 141]. Overall, JNK signaling is important for epithelial repair, melanization, and immune gene expression.

Hippo signaling is a developmental pathway which functions through its transcription factor, Yorkie (Yki) to regulate organ size. Upon loss of tight signaling control, dysregulation of Hippo signaling causes a variety of tumorigenic phenotypes in *Drosophila* and cancers in humans [142, 143]. In the context of immune signaling, Hippo signaling and Toll signaling form a regulatory network where Toll activation upon bacterial challenge activates Hippo signaling through the Pelle-mediated degradation of Cka [144]. Degradation of Cka decreases the amount of Yki in the nucleus, therefore decreasing transcript levels of a Hippo-target gene, Cactus, ultimately resulting in increased levels of *Drs* expression [144].

Finally, organisms require strategies for allocating energetic resources during infection, which involves insulin signaling during infection [145]. For example, activation of Toll signaling in the fat body negatively regulates insulin signaling in *Drosophila* establishing a communication network between innate immune, energy mobilization, and growth [145]. In patients and animal models of insulin resistance, inflammatory responses are upregulated, supporting this conclusion [146-150]. Activation of the JNK pathway through Imd signaling also leads to insulin resistance in mice and humans, but not

Drosophila, suggesting that organisms form communication networks for immune activation and nutrient allocation, although these networks may manifest differently [145, 149, 151]. Insulin signaling is also modulated in response to DNA damage in mice, presumably to limit proliferation of damaged cells and creates a pause for tissue repair mechanisms to occur [152-154]. Following DNA damage induced by UV radiation, endocrines are used to modulate innate immune signaling and insulin signaling in a spatiotemporal manner to best ensure organismal survival [155]. Finally, insulin signaling in muscles is necessary for the tissue-autonomous induction of JAK/STAT signaling which is required for both encapsulation and lamellocyte differentiation during wasp infection [156].

Innate immune responses are activated by tissue communication networks

The interconnectedness of the cellular immune response and humoral immune signaling have another level of complexity when considering that long-range tissue signaling. Two major tissue types have been associated with the systemic immune response: 1) fat body (analogous to the human liver) which is the source of immune signaling and AMP production; and 2) hemocytes which participate in both cellular and humoral immune responses through ligand secretion, phagocytosis, and the secretion of phenoloxidase (PO) to begin melanization [157]. However, a subset of peripheral tissues including epidermal layers, trachea, gut, and gonads can produce regional immune responses in the form of locally expressed AMPs, PO enzymes for melanization, and signaling molecules such as reactive oxygen species [158]. The expansion of tissues capable of inducing and/or producing immune responses suggests that our models of immune

activation may need to be re-evaluated to include tissues previously excluded from analysis. In this section, we present immune models derived from Danger Theory which is growing in popularity amongst immunological scholars with an emphasis on the hypothesis that muscle acts as an important axis in modulating the immune response of *Drosophila melanogaster*.

Danger Model vs. Damage Model of immune activation

Two major theories for immune activation have been proposed in the last century: the self-non-self theory (1950) and Danger Theory (1994) [159]. Many of us recognize the concept of immune activation by 'non-self' or foreign molecules that are found in organisms during infection [160]. First described by Polly Matzinger, the Danger Model rivals this theory, proposing that immune responses are activated through the release of endogenous 'danger signals' [161, 162]. One major problem with self-non-self theory is that it assumes an all-or-nothing immune response, where all foreign molecules elicit immune responses and the body never attacks its own tissues [163, 164]. We now know that activation of the immune response is far more complex, where we must allow for the presence of beneficial microflora and observe 'self' attacking 'self' in the case of autoimmunity and immune surveillance of tumors. In fact, many researchers are investing in ways to re-train and modify our existing immune system to combat diseases (ref).

While neither theory presents a perfect solution, Danger Theory does allow us to consider an emerging set of examples where sterile injury or internal tissue damage activates the immune system independent of pathogens. Literature today describes molecular activation through signals called either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). DAMPs,

sometimes also referred to as damage signals or danger signals, are capable of activating both hemocyte-based immune responses and immune signaling pathways for the production of AMPs [165]. Numerous DAMPs have been identified in vertebrate models including extracellular DNA, reactive oxygen species (ROSs), uric acid, cytokines, ATP, intracellular proteases, fragmented proteins in the ECM, and F-actin (Table 1). Many DAMPs can be categorized as intracellular components that become 'damage signals' when released into the extracellular environment. Some have called for a more restrictive definition of DAMPs which requires putative DAMPs to be highly active, to occur above specific thresholds in cases of damage, and and to be independent of pathogens [166]. More recently, the focus of Danger Theory has been extended to explain activation of the innate immune system although these observations are complicated by the co-activation of adaptive immune activities (REF). *Drosophila* is an important model for discovering novel and highly-detailed molecular descriptions of innate immune processes and lacks an adaptive immune response making this a prime organism for identifying novel DAMPs.

Table 1. DAMPs involved in sterile inflammation.

DAMP	Putative Receptor	Reference
HMGB1	TLR2, TLR4, TLR9, RAGE, and CD24	(Hori, O. et al., *J Biol Chem*, 1995; Andrassy, M., et al., *Circulation*, 2008; Yu, M, et al., *Shock*, 2006; Tian, J., et al., *Nature Immunol*, 2007)
Heat Shock Proteins	TLR2, TLR2, CD91, CD24, CD14, and CD40	(Quintana, F.J., et al., *J Immunol*, 2005; Vabulas, R.M., et al., *J Biol Chem*, 2001; Chen, G.Y., *Science*, 2009; Basu, S., et al., *Immunity*, 2001)
S100 proteins	RAGE	(Hofmann, M.A., et al., *Cell*, 1999)
SAP130	CLEC4E	(Yamasaki, S. et al., *Nat Immunol*, 2008)
Extracellular RNA	TLR3	(Cavassani, K.A., et al., *J Exp Med*, 2008; Kariko, K., et al., *J Biol Chem*, 2004)
Extracellular DNA, mitochondrial DNA, histones/nucleosomes/ chromatin	TLR9, AIM2, TLR2, TLR4, RIG-1, TLR3	(Imaeda, A.B., et al., *J Clin Invest*, 2009; Burckstummer, T., et al., *Nature Immunol*, 2009; Fernandes-Alnemri, T.,

		et al., *Nature*, 2009; Hornung, V., et al., *Nature*, 2009)
Uric acid	NLRP3	(Kono, H., et al., *J Clin Invest*, 2010; Martinon, F., et al., *Nature*, 2006)
Nucleotides (ATP)	NLRP3	(Jeter, C.R., et al., *Plant Cell*, 2004)
Hyaluronan	TLR2, TLR4, and CD44	(Jiang, D., et al., *Nature Med*, 2005; Scheibner, K.A., et al., *J Immunol*, 2006; Taylor, K.R. et al., *J Biol Chem*, 2007)
Versican	TLR2	(Kim, S., et al., *Nature*, 2009)
Heparan sulphate	TLR4	(Johnson, G.B., et al., *J Immunol*, 2002)
CPPD crystals	NLRP3	(Martinon, F., et al., *Nature*, 2006)
β-amyloid	NLRP3, CD36 and RAGE	(Halle, A., et al., *Nature Immunol*, 2008; Yan, S.D., et al., *Nature*, 1996; Stewart, C.R. et al., *Nature Immunol*, 2010)
Cholesterol crystals	NLRP3 and CD36	(Duewell, P., et al., *Nature*, 2010; Stewart, C.R., et al., *Nature Immunol*, 2010)
IL-1α	IL-1R	(Dinarello, C., *Annu Rev Immunl,* 2009)
IL-33	ST2	(Enoksson, M et al., *Blood*, 2013)
Adenosine	P1 receptors (A2A)	(Ohta, A. & Sitkovsky, M. *Nature*, 2001; Day,Y.J. et al., *Am J Physiol Gastrointest Liver Physiol*, 2004; Lukashev, D., et al., *J Immunol*, 2004; Burnstock, G. & Knight, G.E., *Int Rev Cytol*, 2004)
Actin filaments	DNGR-1	(Zhang, J.G. et al., *Immunity*, 2012; Ahrens, S. et al., *Immunity*, 2012)
Mitochondria *N*-formyl peptides	FPR1	(Fu, H., et al., *J Leukoc Biol*, 2006)
Peroxideroxin	TLR2 and TLR4	(Shichita, T. et al., *Nat Med,* 2012)

Abbreviations: AIM2, absent in melanoma 2; CLEC4E, C-type lectin 4E; CPPD, calcium pyrophosphate dihydrate; FPR1, formyl peptide receptor 1; HMGB1, high-mobility group box 1; IL, interleukin; MSU, monosodium urate; IL-1R, IL-1 receptor; NLRP3, NOD-, LRR- and pyrin domain-containing 3; RAGE, receptor for advanced glycation end products; SAP130, spliceosome-associated protein 130; TLR, Toll-like receptor. Adapated from Table 1 of [167] and Table 1 of [168].

Tissue coordination during infection

Traditionally, tissues such as the fat body, salivary glands, lymph glands, and hemocytes have been regarded as ‘immune tissues’. As described earlier, the involvement of many peripheral tissues have been implicated in the immune response. Muscle is quickly surfacing as a tissue which performs vital immune functions in addition to its other auxiliary roles as an endocrine energy-sensing organ. Early evidence of the immune-responsiveness of muscle materialized following gene profiling in *Drosophila* which showed an increase in muscle structural genes during infection [169]. Conversely, mutations to key muscle genes also impacts the ability of these individuals to fight

infection [170]. This relationship between muscle and the immune system is not a unique feature of *Drosophila*, but rather a conserved response that extends into vertebrates such as zebrafish and the fish species, *Paralichthys adpersus* [170, 171]. Therefore, muscle tissue physiology is tightly woven into the infection response and the survival of individuals following immune challenge.

Two well-defined *Drosophila* tissue communication networks for immune activation have been described in response to pathogens. Yang et al. present a tissue communication network incorporating Toll signaling (fat body), JAK/STAT signaling (muscle), and ligand secretion (hemocytes) in response to wasp parasitic wasp infections in *Drosophila* larvae [172, 173]. This paper established *Drosophila* muscle as a key component in a crucial cellular process for clearing the infection known as wasp encapsulation and the subsequent survival of these infections. Wasp infection led to the expression of *upd2* and *upd3* whose expression in hemocytes was necessary and sufficient for activation of JAK/STAT signaling in muscle and ultimately efficient wasp encapsulation [173]. Further work showed that effective wasp encapsulation in *Drosophila* larvae utilizes a positive feedback loop to indirectly link JAK/STAT and insulin signaling in the muscle [156]. One year later, Chatterjee et al. described the importance of the adult musculature, specifically the adult indirect flight muscles (IFMs) for survival of bacterial infection [170]. Mutations in various muscle structural genes caused decreased levels of AMPs that corresponded to the severity of muscle defects [170]. Not surprisingly, adult IFMs themselves are sources of both Toll- and Imd-induced AMPs upon infection. AMP production can be reduced by IFM-specific knockdown of Toll signaling via Dif RNAi or Imd signaling through Relish knockdown both of which are detriment to the organism's

survival [170]. These examples clearly illustrate that muscle can participate in the immune response through intrinsic activation of immune signaling and communication with systemic immune tissues in response to infection.

Tissue coordination to promote sterile inflammation

Danger Theory was designed to describe immune responses corresponding to the invasion of pathogens through tissue breaches and more accurately, biological scenarios including internal tissue damage such as: 1) injury induced in sterile environments, 2) crush-based injuries or trauma such as ischemia/reperfusion injury, 3) disease leading to tissue degeneration such as muscular dystrophy, and 4) loss of immune regulation such as autoimmune disorders or the weakening of immune regulation that occurs with aging, termed ‘inflammaging’ [168, 174]. While infection often includes molecular signals stemming from both pathogens (PAMPs) and the tissue damage that they cause during their infection cycle (DAMPs), isolating the contribution of each signal is difficult in these scenarios. However, systems of sterile inflammation mentioned above have lent credence to the Danger Theory and act as good models for understanding both shared and differential features of sterile immune induction.

Immune activation during sterile wounding. Several groups have identified methods to induce wounding without introducing pathogens. These include: 1) sterile puncture wounding via tungsten needle, 2) pinch wounding, which preserves the cuticle barrier while perturbing the epithelial layer, and 3) wounding through laser ablation [158, 175, 176]. It is important to note that while some differences in immune responses occur in the

absence of pathogens, the presence of foreign pathogens is not required to activate innate immunity [132]. For example, a lack of scab formation during pinch wounding leads to a hyperinduction of JNK and the process of wound closure is inhibited, unlike the mechanisms used in a sterile puncture injury [132].

Most sterile experiments have concentrated on cellular activities required for wound closure such as hemocyte recruitment [132, 176-179]. Like a septic injury, sterile wounding releases signals such as hydrogen peroxide that produce a rapid recruitment of hemocytes [179]. Furthermore, lamellocytes which differentiate from the hemocyte population during infection act similarly in response to sterile disruptions of the epithelial layer [178]. However, sterile wounding does not simply activate cellular responses, but humoral immune signaling can be observed as well. AMPs are activated in response to laser ablation, supporting the idea that internal DAMPs are sufficient for activating all areas of the innate immune response [180]. Stramer et al. and groups working in mice have performed microarray experiments on macrophage-deficient individuals to isolate genes and processes involved in phases of sterile wounding [180, 181]. Interestingly, these experiments allows for an aseptic injury to be broken down into cellular activities distinguished by genes induced by inflammation, those necessary for wound healing, and finally, the induction of immune-responsive genes such as AMPs [180, 181].

Immune activation following tissue damage and aging. Expression profiles of a *Drosophila* hypercontraction-induced myopathy model [182] and vertebrate tissues such as the *mdx* mouse model of muscular dystrophy or human muscle biopsies with dystrophies [183-185] have shown that the expression of immune-related genes increase upon tissue

damage induced in these samples. This is consistent with immune cell invasion seen in vertebrate muscle repair and regeneration [186]. Unfortunately, chronic activation of immune responses quickly moves from helpful to harmful in diseases such as muscular dystrophies and cardiomyopathies where muscle damage creates a cyclical loop of increased immune activation and the advancement of muscle damage (ref).

The major outputs of either Toll or Imd signaling are AMPs, therefore these molecules stand out as candidates that cause tissue damage observed in chronic inflammation. In both mammals and flies subjected to a traumatic brain injury (TBI) protocol, an increase in general innate immune gene expression, including AMPs, is observed in correlation with negative outcomes [187-189]. To differentiate between the comprehensive effects of immune signaling and direct contributions of AMPs on tissue toxicity, research groups have used both genetic tools and *in vitro* models. In *Drosophila* neurons, tissue-specific overexpression of either Toll or Imd-specific AMPs were sufficient to recapitulate neurodegenerative phenotypes observed in fly brains with bacterial infections [190]. This phenomenon is supported by fly brains showing that mutations in negative regulators of IMD caused neurodegeneration and locomotion deficits, but could be rescued through suppression of immune signaling [191]. Furthermore, *Drosophila* aging is characterized by an upregulation of the innate immune system with AMP levels correlating to increased neurological decline [191]. These experiments suggest that suppression of innate immune responses holds promise as a therapeutic approach for both muscle degeneration and neurodegeneration.

While debate continues regarding the exact molecular mechanisms and the contributions of newly classified immune tissues, it is clear that activation of the immune

system is much more elaborate and convoluted than anticipated. Researchers continue to expose new areas of crosstalk in both situational and tissue-specific contexts and are finding that 'immune-adjacent' pathways such as Hippo and insulin signaling can modulate the immune response. Based on these observations, it becomes increasingly important that we incorporate immune assays into research on stressed and damaged tissues and extend our focus to include broader tissue networks which may be involved in the regulation of immune responses.

Main Findings of This Study

A major goal of our lab is to identify new genes involved in fundamental biological processes related to muscle development through the genetic toolkit available in *Drosophila melanogaster*. We used genetic screens and mass spectrometry approaches to expand our understanding of protein composition and binding complexes at the MAS. Until we have a more comprehensive view of the complete protein set comprising the muscle ECM, understanding homeostatic mechanisms regulating tissue maintenance at the local (muscle) and global level (whole organism physiology) will be insufficient. The compilation of our work exposed a significant overlap between muscle development and innate immunity in *Drosophila* larvae for future study.

- **Characterizing novel proteins involved in muscle development.** Based on the results of a novel pupal lethal screen, we characterized the role of the secreted hemolymph protein Fon in larval muscle attachment. Through a combination of genetics and microscopy we showed that Fon plays an indispensable role in organizing the ECM at larval MASs.

- **Describing the overlap between coagulation and muscle development.** In an effort to understand the molecular interactions of Fon at the MAS, we identified two other proteins that function as a suite of secreted proteins necessary in maintaining MASs. Interestingly, these proteins have a dual function in forming the hemolymph clot exposing a previously unknown overlap between coagulation and muscle attachment.
- **Defining innate immune activation following muscle damage.** Further investigation of *fon* mutants revealed an activation of cellular and humoral innate immune responses following *fon*-mediated muscle detachment. Loss of *fon* produced specific activation of Toll-related immunity which was independent of pathogens. These observations led us to look more broadly at the intersection of muscle damage and the initiation of sterile inflammation in these individuals. Our experiments showed both local and systemic immune signaling activated in multiple tissues when muscle tissue homeostasis was disrupted.
- **Constructing a model of tissue communication initiated upon tissue stress.** Recently, examples of infection and tissue stress have revealed that immune-responsive muscle and traditional immune tissues such as fat body coordinate through signaling crosstalk to mount an effective immune response. Using genetic mutants, we were able to show that various muscles stresses activate immune signaling. We propose a model of tissue communication where localized stresses initiate JAK/STAT signaling which activates fat-body based Toll signaling in a reciprocal fashion. Future experiments will focus on

exploring immune activation in greater molecular detail and exploring the consequences of chronic immune activation on damaged muscle.

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Chapter 2 - "A Common Suite of Coagulation Proteins Function in *Drosophila* Muscle Attachment"

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Green, N., Odell, N., Zych, M., Clark, C., Wang, Z., Biersmith, B., Bajzek, C., Cook, K., Dushay, M., Geisbrecht, E.R. A common suite of coagulation proteins function in *Drosophila* muscle attachment.

Abstract

The organization and stability of higher order structures that form in the extracellular matrix (ECM) to mediate the attachment of muscles are poorly understood. We have made the surprising discovery that a subset of clotting factor proteins are also essential for muscle attachment in the model organism *Drosophila melanogaster*. One such coagulation protein, Fondue (Fon), was identified as a novel muscle mutant in a pupal lethal genetic screen. Fon accumulates at muscle attachment sites and removal of this protein results in decreased locomotor behavior and detached larval muscles. A sensitized genetic background assay reveals that *fon* functions with the known muscle attachment genes *Thrombospondin* (*Tsp*) and *Tiggrin (Tig*). Interestingly, Tig is also a component of the hemolymph clot. We further demonstrate that an additional clotting protein, Larval serum protein 1γ (Lsp1γ), is also required for muscle attachment stability and accumulates where muscles attach to tendons. While the local biomechanical and organizational properties of the ECM vary greatly depending on the tissue microenvironment, we propose that shared extracellular protein–protein interactions influence the strength and elasticity of ECM proteins in both coagulation and muscle attachment.

Introduction

Regulation of protein stability and remodeling in the extracellular environment is essential for the organization of higher order structures that comprise the extracellular matrix (ECM). The biochemical composition of the ECM can differ from one tissue to another. This heterogeneity has a dramatic effect on the strength and elasticity of cell–ECM interactions in development and tissue repair [1]. Despite the importance of the ECM in the development and physiology of multicellular organisms, a broad understanding of the shared physical properties among ECM substrates in diverse biological processes is unclear. To uncover mechanisms that underlie ECM biology, several groups including our own study muscle attachment in the *Drosophila* model.

Larval body wall muscles in *Drosophila* form in embryogenesis after repeated rounds of myoblast fusion, myofiber migration, and the subsequent attachment of muscles to their target tendon cells [2-4]. Detailed transmission electron micrographs (TEM) revealed two categories of muscle attachment sites (MASs), direct and indirect [5, 6]. Single muscles, such as the lateral transverse muscles, directly adhere to epidermally derived tendon cells at direct attachment sites (or muscle–tendon junctions) in closely associated conditions (30–40 μm) [5]. Indirect muscle attachments (or muscle–muscle junctions) occur at the hemisegmental borders where multiple muscles form attachments to adjoining muscles by connecting to an extended belt of ECM anchored to a cuticle-associated tendon cell. This nomenclature is analogous to vertebrate literature where direct attachments refer to tightly associated muscle–bone interactions or an indirect attachment site, which utilizes a rope-like extension of connective tissues to join muscle

to bone. At both direct and indirect muscle attachments, muscle and tendon cells form extensions and invaginations between the opposing plasma membranes of each cell type and connect to the ECM at a myotendinous junction (MTJ). This membrane interdigitation increases the muscle–tendon interface area to allow for increased resistance against forces generated during muscle contraction.

The direct or indirect attachment of muscles to other muscles or to tendon cells relies largely on the function of transmembrane integrin proteins. Individual integrin subunits form obligate heterodimer complexes on the surface of both muscle and tendon cells and link the internal actin cytoskeleton to proteins in the extracellular environment [7]. An αPS1βPS complex accumulates on tendon cell membranes, while αPS2βPS subunits are found on the surface of muscle cells. Mutations in *myospheroid* (*mys*), which encodes for the βPS subunit, causes embryonic muscles to detach from tendon cells following muscle contraction [8, 9]. Absence of the muscle-specific αPS2 (*inflated*, *if*) subunit leads to similar muscle detachment [10], while lack of αPS1 (*multiple edematous wings*, *mew*) on tendon cells shows no evidence of detachments [11]. This attachment role for integrins in muscle and tendon cell adhesion is conserved, as loss of the α (*pat-2*)- and β (*pat-3*)-integrin subunits alter muscle attachment in *C. elegans* [12]. Furthermore, mutations in mouse *integrin α7* lead to progressive muscular dystrophy resulting from impairment of MTJ function [13].

In the developing *Drosophila* musculature, the α-integrin subunits cannot substitute for one another [14], but rather impart extracellular ligand binding specificity. Laminins are trimeric ECM proteins that consist of α-, β-, and γ-chains. The α-chains are encoded by two genes, *Laminin A* (*LanA*) and *wing blister* (*wb*), which associate with the

αPS1βPS or αPS2βPS heterodimer complexes, respectively [15, 16]. Weak muscle detachment defects are present in *wb*, but not in *LanA* mutants [5, 17], suggesting that the muscle-specific αPS2 subunit is crucial for muscle attachment. However, it is also possible that functional redundancy of LanA precludes the observation of phenotypic consequences in *LanA* mutants.

Interactions between αPS2 and Laminin are mediated by the tripeptide RGD sequence present in the LanA α-chain [16]. The *Drosophila*-specific ECM protein Tiggrin (Tig) also possesses RGD integrin binding activity [18]. Tig is produced in fat body and hemocytes and accumulates at the site of muscle–muscle junctions [18]. Consistent with a role in integrin-mediated cell adhesion, *Tig* mutants exhibit a weak larval muscle detachment phenotype that appears after the onset of muscle contraction [19]. In screens aimed at identifying new muscle patterning genes, two groups identified *thrombospondin* (*Tsp*) as an additional αPS2 integrin ligand [20, 21]. Tsp contains the alternate KGD tripeptide motif and is secreted from the tendon cell into the extracellular space at the junctions between muscle and tendon contact zones. Additional secreted proteins, including M-spondin (Mspo) and Masquerade (Mas) also accumulate at *Drosophila* embryonic MASs [22, 23]. However, only mutations in *mas* exhibit loss of muscle attachment, once again suggesting that redundancy could account for the lack of somatic muscle defects observed in *mspo* mutants.

In an effort to identify new muscle mutants, we screened a collection of lethals for abnormal pupal morphology due to inefficient muscle contraction during the larval-to-pupal transition. One such mutant, named *fon*, was originally identified for its role in *Drosophila* hemolymph coagulation [24]. The muscle detachment phenotype

in *fon* mutants was remarkably similar to that observed in *Tig* mutants [19]. Moreover, Fon and Tig protein expression overlaps at MASs. Since Fon and Tig are also both components of the hemolymph clot [25, 26], we reasoned that there may exist other secreted coagulation proteins required for muscle attachment. Indeed, Larval serum protein 1γ (Lsp1γ) is found on the surface of larval tendon cells and *Lsp1γ* deficiency results in myofiber detachment. These data suggest that a specific subset of hemolymph proteins that participate in the larval clot coordinately function in the MAS matrix to mediate muscle attachment stability.

Materials and Methods

Fly genetics

Drosophila stocks were raised on cornmeal medium under standard laboratory conditions at 25° unless otherwise indicated. The lab control strain *yw* was used for detachment and gap distance experiments, while w^{1118} was used as a control in all other experiments. The following stocks were used to drive tissue-specific expression: *tubP(αTub84B.PL)*-Gal4 (BL-5138), *24B*-Gal4 (BL-1767), *sr*-Gal4 and *sr*-Gal4, UAS-*CD8-GFP* (gifts from T. Volk), *ppl*-Gal4 (a gift from L. Dobens), and *da*-Gal4 (originally BL-37291 outcrossed 10 times to w^{1118} to remove background lethals). The following *fondue* mutations were used: the null alleles $fon^{\Delta 24}$ and $fon^{\Delta 17}$ are deletions that remove only *fon* coding sequence [27]; hypomorphic allele w^{1118}; $Mi(ET1)fon^{MB11923}/SM6a$ (fon^{MB}; BL-29262) [27], *fon RNAi* [originally from R. Ueda; described in [24]], and w^{1118}; *P{UAS-fon.GFP}28e* [(*fon-GFP*; BL-43646) [28]]. Additional alleles and/or stocks analyzed are as follows: Tig^{A1} and Tig^{X} [19], $fon^{\Delta 24}$; *da*-

Gal4 [27]), *UAS-Tig RNAi* (BL-31570), UAS-*Tsp RNAi* (VDRC; v10072s), UAS-*Lsp1γ* RNAi (BL-55389), UAS-*Gelsolin RNAi* (BL-31205; BL-41704), and *P{PTT-un1}vkg*^{G00454} [29]. Deficiency (Df) stocks *Df(2L)Exel6043* and *Df(2L)BSC185* were used to remove *fon* and *Tig*, respectively. All *fon* and *Tig* mutant alleles and Dfs were maintained over a C*yo-Act-GFP* balancer. Non-GFP individuals were manually selected for mutant analysis. RNA interference (RNAi) experiments were performed at 29° except for crosses involving *Lsp1γ RNAi* (BL-55389), which were performed at 27° to minimize larval lethality.

Immunostaining and microscopy

L3 larvae were filleted and fixed with 4% formaldehyde prior to staining as previously described [30]. The following primary antibodies were used: mouse anti-βPS-integrin [1:50, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Tig [(1:1000) [18]], mouse anti-αPS2-integrin [1:10, (DSHB)], anti-Talin [1:10, (DSHB)], anti-Perlecan [(1:1000; [31]], anti-DLG [1:300, (DSHB)], and rabbit anti-GFP (1:1000, Medical and Biological Laboratories). The following secondary antibodies were used at 1:400 for fluorescent detection: Alexa Fluor anti-mouse 488, Alexa Fluor anti-rabbit 488, and Alexa Fluor anti-mouse 647 (Molecular Probes). Phalloidin 488, 594, and 647 were used for F-actin labeling (1:400; Molecular Probes, Eugene, OR). Fluorescent images were taken with a Zeiss 700 confocal microscope. Images were processed, analyzed, and compiled into figures using Zen Black (Zeiss), ImageJ (National Institutes of Health) software, and/or Adobe Photoshop Elements.

Brefeldin A treatment

Brefeldin A (BFA) treatment was modified from a published protocol developed for embryo analysis [32]. Briefly, larvae were dissected live in Schneider's Insect Medium buffer (Sigma Chemical, St. Louis, MO) and incubated in either a DMSO control solution or a BFA solution [20 μg/ml in DMSO (Cell Signaling Technology, Danvers, MA)] for 1.5 hr at 29°. Fillets were washed three times quickly with PBS and fixed with 4% formaldehyde. Fillets were then stained for F-actin and GFP and imaged as described above. To quantitatively assess Fon–GFP retention in the fat body, GFP fluorescence intensity was calculated using the measurement function in ImageJ. Briefly, the interior of individual fat body cells was selected for analysis in 20x images, with three cells measured across each image. A background measurement was collected outside of the frame of the fat body lobe. Corrected total fluorescence (CTF) was calculated using the following equation: CTF = integrated density − (area of selected cell × mean fluorescence of background) [33]. Raw data and statistical analysis for both BFA-treated and DMSO-treated fat bodies were compiled and analyzed using GraphPad Prism 6.0.

Transmission electron microscopy

Drosophila L3 larvae were filleted and fixed overnight in 1x Trump's fixative (4% formaldehyde/1% glutaraldehyde in phosphate buffer). Fillets were processed with osmium tetroxide and put through a graded alcohol dehydration series before embedding in Spurr resin. Ultrathin sections of the dissected fillets were taken in a parasagittal orientation starting at the dorsal edges of muscle hemisegments using uranyl acetate and lead citrate for contrast. Samples were observed and imaged with a FEI Tecnai 12 Bio-

Spirit Transmission electron microscope. Images were prepared using the Gatan Microscopy Suite software.

Quantitative PCR analysis

To assess the effectiveness of RNAi knockdown, RNA transcripts were collected from three wandering L3 larvae using the RNeasy Mini Kit (QIAGEN, Valencia, CA) for each line of interest. The driver line, *da*-Gal4, was used as a control genotype. Complementary DNA (cDNA) synthesis of 125 ng RNA was performed using the SuperScript II First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA). Dilutions of cDNA were optimized for primer pairs (Table 4) and combined with the SYBR Select Master Mix (Applied Biosystems, Foster City, CA) for quantitative measurement of transcripts on the CFX96 Touch Real-Time PCR Detection System with CFX Manager software (Bio-Rad, Hercules, CA). Both the housekeeping gene *rp49* and the gene of interest were measured for control and RNAi knockdown larvae. Results from three biological replicates and a minimum of three technical replicates were averaged to obtain Ct values. Fold expression change was calculated using the $2^{-\Delta\Delta Ct}$method, graphed using GraphPad Prism 6.0, and analyzed using the Kruskal–Wallis statistical test.

Phenotypic quantification and statistical analysis

Pupal axial ratio:

White pupae of the appropriate genotype were removed from vials, oriented dorsal side up, and attached to slides using a small drop of nail polish. Images were taken with a Leica M165 FC stereomicroscope. Length and width measurements of each pupae

were performed in ImageJ using the line and measure functions. Values were deposited into an Excel spreadsheet and the axial ratio (length/width) was calculated for each individual. The raw data were imported into Graphpad Prism 6.0 and graphed as a box and whiskers plot.

Gap distance:

Average gap distance quantifies the space between dorsal oblique 1 and 2 (external muscles 9 or 10) across the hemisegmental border using the “distance between two polylines” plug-in for ImageJ. Images were taken at 40x magnification and gap distances were calculated as an average of distances along the length of the muscle attachment surface for each genotype. Average distances were compiled in Excel and graphed as a dot plot using GraphPad Prism 6.0.

Locomotion:

Larval locomotion studies were performed on apple juice agar plates with a minimum of 15 individuals per genotype. Larvae crawling patterns were filmed for 1 min and analyzed using the “grid” plugin in ImageJ. Velocity was calculated from the distance the organism crawled (conversion of no. of squares crawled through to distance in centimeters) per second in Excel, graphed as average ± SD detachment. For all experiments, muscles were characterized as detached if: (1) muscles had rounded up following detachment or had clearly separated at an attachment site; (2) muscles were in the process of stripping away but were attached through muscle–muscle connections in another hemisegment; or (3) muscles were missing from the fillet. A detailed, individual muscle phenotyping was performed for *fon* alleles, tissue-specific *fon* knockdown, and to analyze *fon* and *Tig* genetic interactions. The following indirect and direct subsets were

scored within muscle (m.) hemisegments: dorsal acute [DA1-3 (m. 1–3)]; longitudinal lateral [LL1 (m. 4)]; lateral oblique [LO1 (m. 5)]; ventral longitudinal [VL1-4 (m. 6–7, 12–13)]; segment border muscle [SBM (m. 8)]; dorsal oblique [DO1-2 (m. 9–10)]; DO3-4 (m. 11,19); ventral oblique [VO4-6 (m. 15–17)]; lateral transverse [LT1-4 (m. 21–24)]. Direct muscle subsets were quantified as LT1-4 (m. 21–24), the medial attachments of VO4-6 (m. 15–17), and the dorsal attachment site of DO3-4 (m. 11, 19). All other muscles quantified were considered indirect attachments. Percent detachment was calculated as the number of individual detached muscles divided by the total number of muscles quantified per fillet. Individual detachment percentages were plotted and represented as a bar graph (average ± SD). For the *fon* sensitized background experiments, detachment was quantified as the percentage of hemisegments containing one or more detached muscles. Individual percentages were plotted per genotype and represented as a bar plot.

Statistical analysis:

All data points in each set of experiments/graphs were first analyzed for Gaussian distribution sampling. None of the data sets conformed to these parameters and were subjected to the Kruskal–Wallis test, a nonparametric test that compares three or more unmatched groups that do not conform to a Gaussian distribution. The Bliss independence test was used to determine the expected contribution of additive phenotypes [34]. Significance values are indicated in each figure legend.

Generating Lsp1γ transgenics

Total RNA was isolated from L3 larvae and reverse transcribed. The open reading frame for *Lsp1γ* was PCR amplified from this cDNA pool using the forward primer 5′-

CACCATGAAGTTGACCCTTGTTATATT-3′ and the reverse primer 5′-GTATTCAATGGAGTAGTCGAAGGTGC-3′, inserted into the Gateway pENTR/D-TOPO vector (Invitrogen), and recombined into the pTWV destination plasmid [(*Drosophila* Genomics Resource Center (DGRC)] using standard procedures to generate UAS–Lsp1γ–YFP (hereafter referred to as UAS–Lsp1γ–GFP). This construct was injected for the generation of transgenic flies by Rainbow Transgenic Flies.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Novel genetic screen to identify muscle mutants

We and others have identified mutants, including *thin(tn)/abba*, *sallamus* (*sls*), *Mlp84B*, and *Tiggrin* (*Tig*), that are defective in various aspects of larval muscle structure and/or function, including myofiber stability, sarcomere maintenance, and/or muscle attachment [19, 35-37]. A shared feature among these mutants is pupal lethality and an abnormally elongated, or curved, pupal morphology. We reasoned that this extended pupal case, caused by the inability of muscles to contract during the larval-to-pupal transition, could serve as the basis for a genetic screen to identify novel genes essential for larval muscle contraction.

We visually inspected 9323 lethal stocks at the Bloomington *Drosophila* Stock Center (BDSC) and identified 184 possible stocks (~1.9%) that exhibited an elongated

and/or curved pupal phenotype. We screened 15 of these candidates for muscle morphology defects in third instar (L3) larvae (Table 3). For one candidate, this abnormal pupal phenotype was caused by a Minos insertion into the *fondue* (*fon*) locus (*Mi{ET1}fon*^{MB11923} referred to hereafter as *fon*^{MB}). Interestingly, the Dushay group had previously reported that homozygous *fon* mutants exhibit longer or curved pupae that failed to eclose (Figure 2.1A) [24, 27]. To further characterize the pupal morphology phenotype, we measured the axial ratio (length/width) in *fon* alleles compared to wild-type (*WT*) control pupae. In *fon*^{MB}/*fon*^{MB} or deletion mutants (*fon*$^{\Delta 17}$ or *fon*$^{\Delta 24}$) that remove portions of *fon* coding region [27], pupae exhibited a greater axial ratio than *WT* individuals (Figure 2.1B). This lethality and associated pupal morphological changes seemed unlikely to result solely from the role of Fon in hemolymph coagulation, as this phenomenon occurred in unwounded individuals, and other more severe clotting mutants (*e.g.*, *hemolectin*) did not exhibit this same phenotype [38].

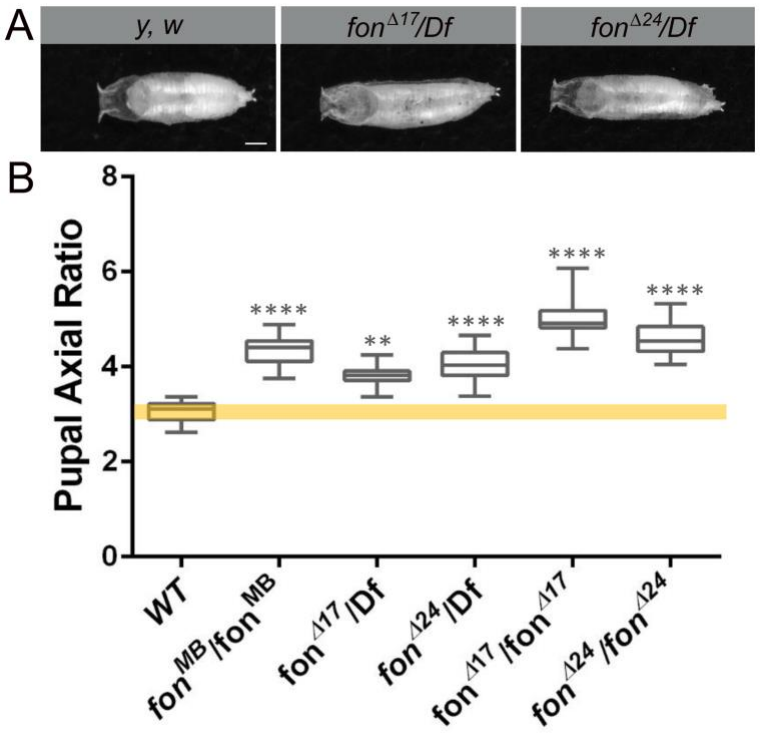

Figure 2.A Mutations in *fon* result in elongated pupal phenotypes.

(A) Pupal cases of control (*WT*) or *fon* mutations ($fon^{\Delta 17}$ and $fon^{\Delta 24}$) analyzed over a deficiency chromosome (Df) that removes the *fon* locus. (B) Measurement and quantitation of axial ratios (length/width) of the indicated pupal genotypes demonstrate that *fon* mutants are defective in the ability to shorten their pupal case ($17 \leq n \leq 35$ for each genotype). Mean ± SD; *P*-values: ** $P < 0.01$, **** $P < 0.001$. Bars, 0.75 µm.

The extracellular Fon protein is essential for stable muscle attachment

To examine whether defects in muscle structure and/or function could be responsible for the elongated *fon* pupal phenotype, we dissected and immunostained muscles in late wandering L3 larvae, just prior to puparium formation. Fillets of *WT* L3 individuals revealed a precise pattern of segmentally repeated myofibers that were rectangular in shape and firmly attached to other muscles or directly to the larval exoskeleton (Figure 2.2A). As expected in *WT* animals, the dorsal oblique muscles 9 and 10 (Figure 2.2A, DO1 and DO2, respectively) were in close proximity at the segment borders (Figure 2.2A, asterisks), with no obvious gaps between adjacent muscles in higher magnification confocal images viewed in an XY (Figure 2.2D-D′) or XZ planes (Figure 2.2, G-G′).

Dissection of L3 *fon* mutants ($fon^{\Delta 24}/Df$ or fon^{MB}/fon^{MB}) revealed two obvious muscle phenotypes. First, myofibers were detached and rounded due to their inability to

remain attached during muscle contraction (Figure 2.2B-C, carets). The penetrance of detached muscles varied among *fon* mutant genotypes, ranging from 28.8 to 54.2% of all muscles examined (Figure 2.2J and Table 2). Loss of Fon affected all muscle subsets and both direct and indirect linkages. The second morphological phenotype we observed in *fon* mutants was large gaps between muscles 9 and 10 (Figure 2.2B-C, asterisks; E-F, K; Table 3). Since Fon is a secreted protein [24, 39], we hypothesized that the apparent gaps and detached muscles were a consequence of changes in the extracellular environment rather than intracellular defects. Consistent with this, we did not observe detachment of the actin cytoskeleton from the sarcolemma in *fon* mutants (Figure 2.2H-I, arrowheads).

Table 2. Muscle detachment in L3 larvae of the indicated genotypes.

	Detached Muscles (% of total muscles)	Number of muscles analyzed (n)	Duplicated Muscles (% of hemisegments)	Number of hemisegments (n)
yw	2.0 %	2,667	2.3 %[a]	127
$fon^{\Delta 17}/Df(2L)Exel6043$	29.9 %	3,801	0.0 %	181
$fon^{\Delta 24}/Df(2L)Exel6043$	54.2 %	2,457	0.0 %	117
$fon^{MB}/Df(2L)Exel6043$	28.8 %	3,969	0.0 %	189
$fon^{\Delta 24}/+$	6.3 %	2,772	4.5 %[a]	132
$Tig^{A1}/+$	9.9 %	2,898	0.0 %	140
$+; fon^{\Delta 24}/Tig^{A1}, +$	20.9 %	1,995	21.0 %[a-d]	95

[a] Lateral Transverse (muscles 21-24)
[b] Lateral Longitudinal (muscle 4)
[c] Dorsal Oblique (muscles 9,10,11,19)
[d] Dorsal Acute Muscles (muscles 1,2,3)

Table 3. Gap distance between muscles 9 and 10.

Genotype	Average gap distance (µm)	Gap distance range (µm)	(n)

yw	6.44	3.0-11.2	25
$fon^{\Delta 17}/Df(2L)Exel6043$	12.3	7.3-20.0[a]	35
$fon^{\Delta 24}/Df(2L)Exel6043$	24.1	12.8-37.4[b]	35
$fon^{\Delta 24}/+$	8.5	3.8-16.1	35
$Tig^{A1}/+$	7.2	3.1-15.1	35
$+; fon^{\Delta 24}/Tig^{A1}, +$	11.8	5.5-22.5	31

[a]Outlier = 64.5 μm
[b]Outlier = 81.2 μm

Structurally, both vertebrate and invertebrate MASs are composed of integrin heterodimer complexes located within the plasma membranes of muscle cells that link the ECM to the internal muscle contractile apparatus. Mammals display 18 α- and 8 β-subunits, so far known to comprise 24 distinct integrin heterodimers [40]. *Drosophila* has only 5 α- and 2 β-position specific (PS) integrin chains, (called αPS1-5, βPS, and βv) that assemble into cell-type-specific heterodimer complexes [41]. The αPS2βPS integrin subunits accumulate at the ends of migrating myofibers, while the αPS1βPS heterodimer is found solely on the surface of target tendon cells [3, 4, 7]. Thus, we next examined if the detached muscles in *fon* mutants could be due to a loss or mislocalization of integrin protein. We found that the βPS subunit accumulated normally at the muscle cell surface in both *WT* (Figure 2.2D-D′, arrowhead) or *fon* mutant (Figure 2E and F, arrowhead) larvae. Examination of the localization and relative protein levels of αPS2 and Talin, an indicator of intact integrin signaling, were also unaffected, as was the accumulation of Tig (Figure B1). Consistent with previous results that basement membrane components are absent from the MTJ [6], we found that loss of Fon did not alter the localization of Perlecan or Collagen IV, the latter of which is encoded by the *viking* (*vkg*) gene (Figure B1).

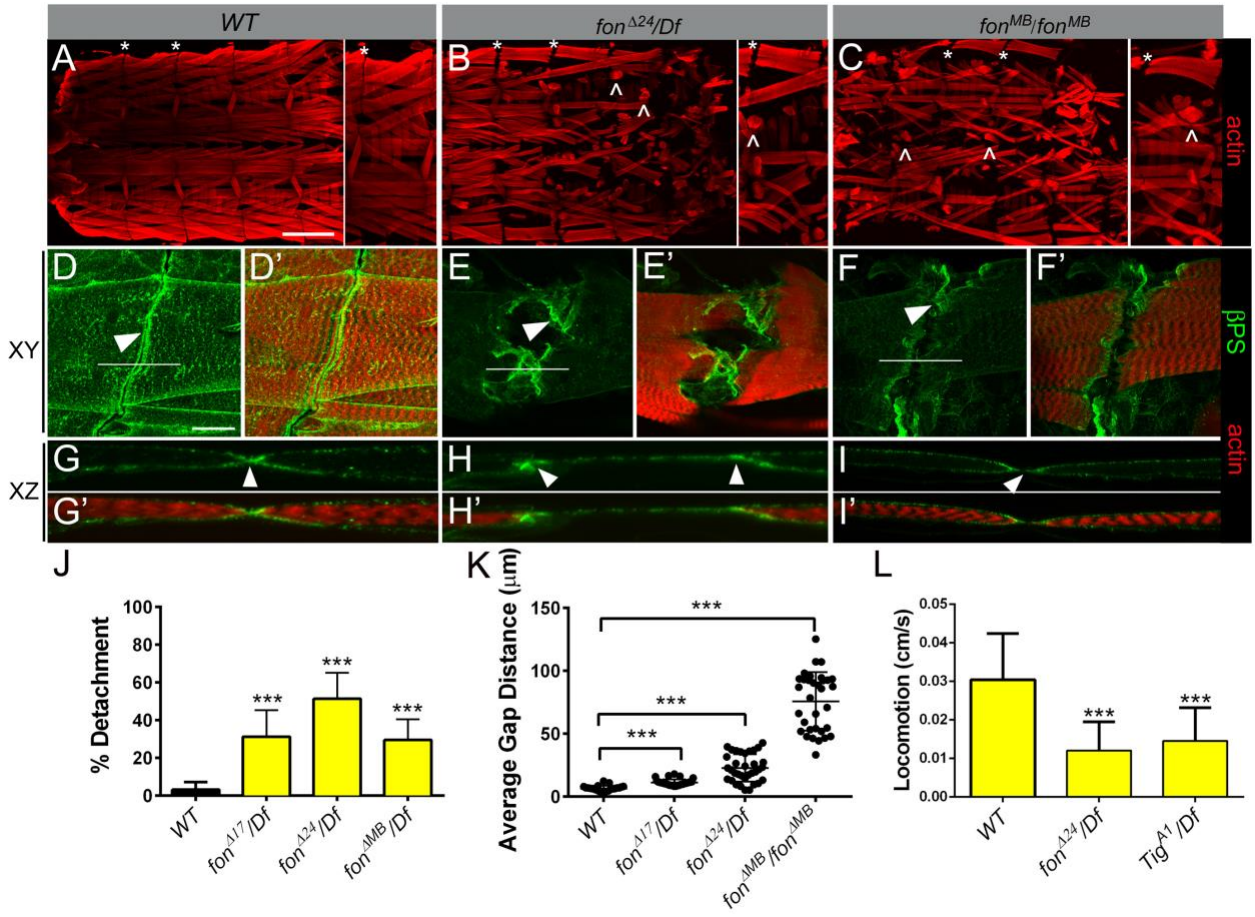

Figure 2.B Fon is required for muscle attachment stability and larval locomotion.

(A–I′) Internal views of larval fillets at low (A–C) or high magnification (D–I′) in *WT* or *fon* mutant larvae in XY (A–F′) or XZ (G–I′) focal planes. (A) *WT* larval muscles are rectangular when firmly attached to other muscles or tendon cells. (B and C) Deletion of the *fon* locus (*fon*$^{\Delta 24}$*/Df*; B) or an insertion that disrupts *fon* (*fon*^{MB}*/fon*^{MB}; C) results in many detached muscles (carets). (D–I′) Phalloidin (red) and βPS integrin (green) staining in the indicated genotypes. (D and G) *WT* dorsal oblique muscle 10 (DO2) shows accumulation of βPS integrin at attachment sites between adjacent muscles (white arrowhead). (E, F, H, and I) *fon* mutant DO2 muscles reveal large gaps between adjacent hemisegments, yet retain βPS integrin accumulation at muscle edges (white arrowheads). (J) Analysis of different *fon* mutant alleles show an increased percentage of detached myofibers. (K) The dorsal oblique muscles 9 and 10 (DO1 and 2) exhibit a variable, but significant increase in gap distance between adjacent hemisegments in *fon* mutants. (L) L3 wandering larvae ($29 \leq n \leq 46$ for each genotype) with mutations in the *fon* locus traverse across agar plates at a velocity lower than their *WT* counterparts, but similar to *Tig* mutants. Mean ± SD; *P*-values: *** $P < 0.005$. Bars, 200 μm for A–C; 50 μm for D–I′.

The severe muscle detachment observed in *fon* mutants would be predicted to affect organismal movement. Indeed, *fon* mutant larvae moved across agar plates at a significantly decreased rate compared to *WT* individuals (Figure 2.2L). Loss of Fon also did not affect the bouton number of type I synapses (Figure B.2), suggesting that the locomotion defects are not an indirect effect of defective neuronal connections. Taken together, these data demonstrate that Fon is essential for the attachment of muscles and does not affect the linkage of actin filaments or the relative membrane localization of integrins and known ECM proteins.

Fon protein accumulates at MASs

Photomicrographs of larvae expressing a Fon–GFP fusion construct driven by *da*-Gal4 were described as having a banded pattern of cuticular fluorescence [27, 28]. We confirmed that this striped pattern corresponded to MASs between adjacent hemisegments. Dissection of L3 larval pelts from animals expressing Fon–GFP driven by the ubiquitous *tubulin* (*tub*)-Gal4 [or fat body and salivary gland *pumpless* (*ppl)*-Gal4] driver revealed an accumulation of Fon–GFP at the distal ends of muscles (Figure 2.3A–F), although the amount of Fon varied depending on whether the muscle subsets were directly or indirectly linked to the cuticle. In general, Fon weakly localized to the ends of all muscles that were directly attached through tendon cells to the cuticle. Figure 3B shows an example of Fon accumulation at the distal ends of one such set of directly attached muscles (lateral muscles 21–23). Strong accumulation of Fon–GFP was evident between adjacent muscles in each hemisegment at indirect muscle attachment sites (Figure 3, C–F). Views of Fon–GFP accumulation in XY (Figure 2.3D) or XZ (Figure 2.3E-

F) planes revealed a heavy localization of Fon–GFP between adjacent muscles and an accumulation under the muscles, likely responsible for the cuticle attachment. Notably, Fon also colocalized with the extracellular integrin ligand Tig at indirect attachment sites (Figure 2.3E, arrowheads), further supporting Fon as an ECM protein. Interestingly, larvae in which one copy of both *fon* and *Tig* were removed showed an increase in the gap distance between adjacent muscles, suggesting that *fon* and *Tig* exhibit dominant interactions and may function together in the ECM (Figure B.3). Note that the accumulation of Fon–GFP protein was not altered in *Tig* mutant muscles (Figure B.1).

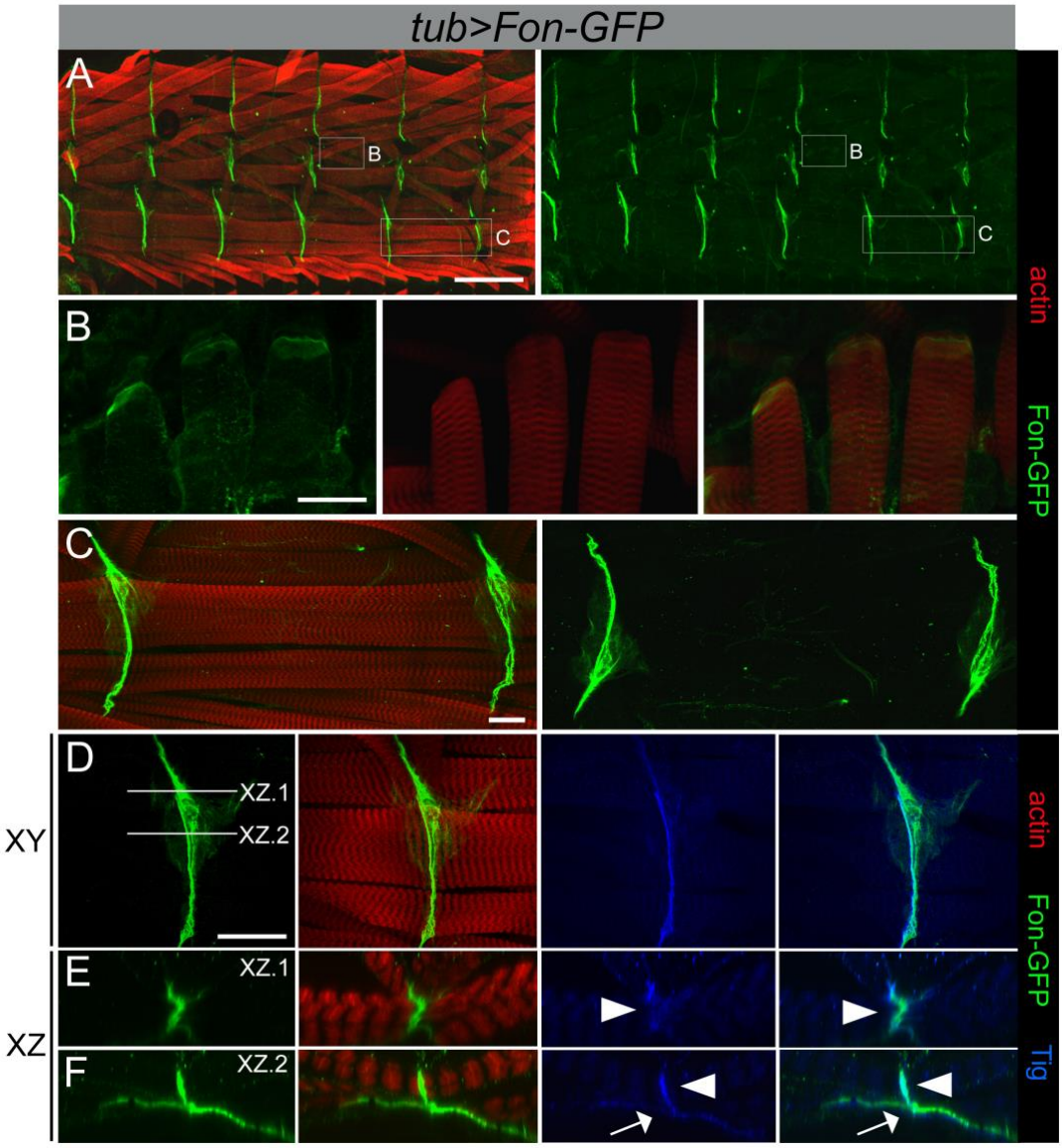

Figure 2.C Fon accumulates at muscle attachment sites.

(A–F) Ubiquitous expression of a Fon–GFP fusion protein (green) using the *tubulin*(*tub*)-Gal4 driver. Muscles are labeled with phalloidin (F-actin; red). Fon–GFP is enriched at the ends of attached myofibers at direct (muscle–cuticle; white arrows) and indirect (muscle–muscle; white arrowheads) attachments shown in a low magnification view of half a larval fillet (A) or in high magnification views (B–F). Fon–GFP weakly accumulates at the ends of lateral muscles at direct attachments (B) and is found at high levels between muscles at indirect attachment sites, such as the ventral muscles 6 and 7 (C–F). (D–F) The photographs in E and F represent the XZ plane of the lines indicated in D. Fon–GFP colocalizes with anti-Tig immunostaining (blue) at indirect attachments in regions of muscle-to-muscle contacts (E, arrowheads), but weakly within sites where muscles associate with the tendon cell (F, arrows), where Fon–GFP is more prominent. Bars, 200 µm for A; 50 µm for B and D; and 25 µm for C.

To determine whether Fon accumulation at MASs is produced locally or secreted from the fat body to circulate in the hemolymph as in coagulation [24], we utilized an RNAi strategy using tissue-specific Gal4 drivers. A ubiquitous decrease in Fon levels (*da*-Gal4) led to drastic muscle detachment (Figure 2.4A, E; carets), demonstrating the efficacy of our *fon* RNAi approach. The myofibers in muscle-specific *24B > fon* RNAi larvae remained attached, but occasionally were stripped away during dissection or missing, possibly resulting from specification or patterning defects in embryonic myogenesis (Figure 2.4B, bracket, and E). Expression of *fon RNAi* by the tendon cell promoter *stripe* (*sr*) did not produce detached muscles (Figure 2.4C and E), while use of the fat body driver *ppl*-Gal4 resulted in severe myofiber detachment (Figure 2.4D, E; carets). Effective knockdown of *fon* by RNAi was confirmed using quantitative PCR (qPCR) (Figure 2.4F).

To further test the idea that Fon is produced in fat body, we examined the consequences of blocking Fon protein secretion in this tissue. Larvae expressing Fon–GFP in fat body cells under control of the *ppl* driver were dissected live and incubated in a solution of either DMSO or the ER → Golgi inhibitor BFA dissolved in DMSO. Dissection and imaging of control DMSO-treated fat body tissue revealed a low level of Fon–GFP inside cells (Figure 2.4G-G′) with increased internal Fon–GFP accumulation upon treatment with BFA (Figure 2.4H-H′). This block in Fon secretion after BFA treatment was confirmed by quantitation of the relative fluorescence intensity of Fon–GFP (Figure 2.4I). These data demonstrate that Fon is secreted from the fat body and is incorporated into MASs.

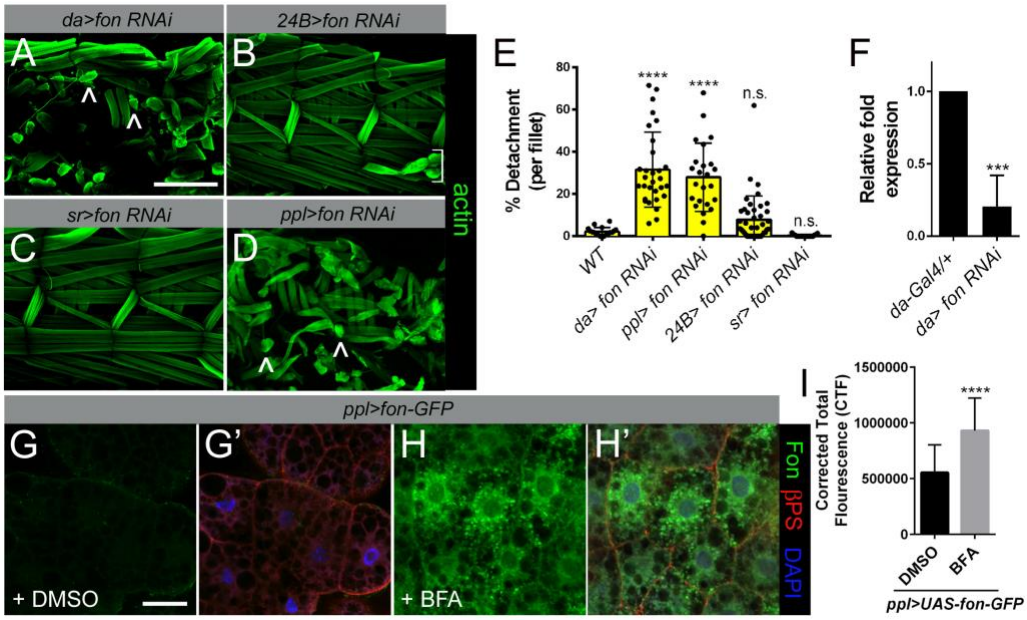

Figure 2.D Fat body-produced Fon is required for stable muscle attachments.

(A–D) Visualization of larval muscles (F-actin; green) after *fon RNAi* knockdown using the indicated Gal4 drivers. Driving *fon RNAi* under expression of the ubiquitous *da* (A) or the fat body driver *ppl* (D) regulatory sequences results in muscle attachment defects (carets). Knockdown of *fon RNAi* in the muscle (*24B*-Gal4) occasionally results in missing muscles (B, bracket), while knockdown in tendon cells [C; *stripe (sr)*-Gal4] does not alter muscle attachment stability. (E) Percent muscle detachment in the indicated genotypes. Muscles within each fillet were scored as being detached or intact ($n \geq 19$ for each genotype). (F) qPCR reveals that *fon mRNA* levels are decreased upon *fon RNAi* knockdown (*da* > *fon RNAi*) compared to control (*da*-Gal4) L3 larvae. (G–H′) βPS integrin (red) and DAPI (blue) label fat body tissue from *ppl* > *fon-GFP* L3 larvae fed control DMSO or BFA. BFA treatment blocks efficient Fon–GFP (green) transport out of fat body cells. (I) Quantitation of mean fluorescence intensity within individual fat body cells ($n = 12$ for each untreated and BFA-treated samples). Mean ± SD; *P*-values: **** $P < 0.001$, *** $P < 0.005$. Bars, 200 μm for A–D; 50 μm for F and G.

Fon is a critical regulator of MAS architecture

We next utilized TEM to analyze the muscle, tendon, and cuticular structures present in sagittal sections of *WT* or *fon* mutant L3 larvae to explore how loss of Fon alters the ultrastructure of indirect attachment sites. Consistent with previous reports [5, 6], TEM images revealed a regular arrangement of horizontally oriented cuticular (c)

laminae underneath epidermal and tendon (t) cells (Figure 2.5A-A′). Extracellular electron-dense material accumulated between the interdigitating sarcolemma of the incoming myofibers (m) and the tendon cell membranes (Figure 2.5B, large black arrow). Apical junctions at the base of the tendon cell (Figure 5C, small black arrow) connect muscle attachment fibers (MAFs) [42], also called tonofilaments [5], located within cuticle pore canals to the muscle–tendon interface at basal cell junctions. In stark contrast, loss of Fon revealed an unorganized and highly convoluted cuticular structure (Figure 2.5E-E′) and a loss of apical junctional complexes in tendon cells (Figure 2.5G, small black arrow). Most significantly, while the muscle and tendon cell membranes were adjacent to one another, there was a complete loss of electron-dense ECM components and cell interdigitation (Figure B.4 and Figure 2.5F, asterisk). These data show that Fon is essential in the organization of ECM components at the MTJ.

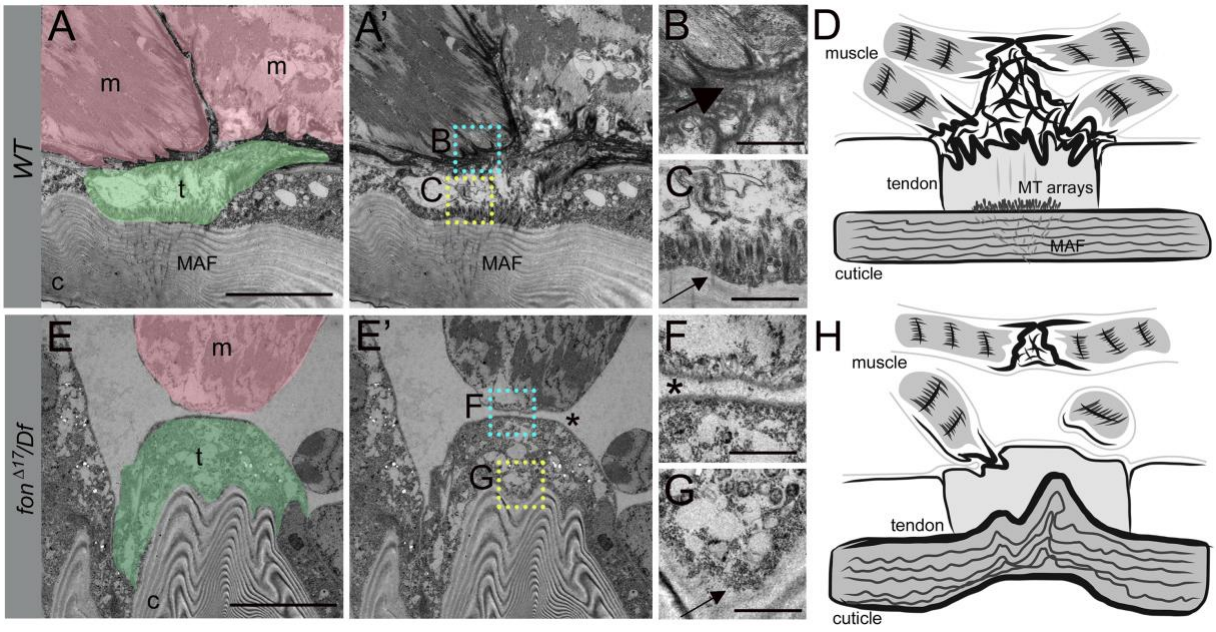

Figure 2.E Loss of Fon alters cuticle integrity, tendon cell cytoarchitecture, and ECM accumulation.

(A–C and E–G) TEMs of an indirect muscle–tendon attachment site in *WT* (A–C) or *fon*$^{\Delta 17}$*/Df* mutants (E–G). (A and A′) Muscles (m; pink) are interlaced with tendon cells (t; green) at control MTJs. The insets correspond to high magnification images in B (cyan) and C (yellow). (B) An electron-dense ECM matrix is observed between the sarcolemma and tendon cell membranes (large arrow). (C) Apical junctions present at the base of tendon cells (small arrow) are associated with MAFs (A and A′) that extend into the cuticle. (D) Generalized schematic representation of *WT* muscle attachment. (E and E′) The detached muscle (m; pink) in this *fon* mutant remains close to the tendon cell (t; green), which is attached to a highly convoluted cuticle (c). The insets correspond to close-up images in F (cyan) and G (yellow). (F) There is a loss of electron-dense ECM material and membrane interdigitation between the muscle and tendon cell (asterisk). (G) Note the absence of tendon cell junctional complexes at the base of the tendon cell (G, small arrow). (H) Illustration reveals the dramatic loss of muscle attachment, ECM accumulation, and morphological abnormalities associated with mutations in *fon*. Bars, 10 μm for A and E; 1 μm for B, C, F, and G.

A set of conserved clotting proteins are required at MASs

Fon and Tig are present in the hemolymph clot [25, 26]. Thus, we wondered whether other ECM and/or clotting factor proteins are necessary for *Drosophila* muscle attachment. We chose a *fon*-sensitized RNAi approach for two reasons: (1) to target

genes that may interact with *fon* in the ECM and (2) because characterized mutations were not available for many of the candidate clotting genes.

To examine the contribution of RGD- and KGD-containing integrin ligands in larval myofiber attachment stability, we performed candidate RNAi knockdown of *Tig* and *Tsp* in a genetically sensitized *fon* mutant ($fon^{\Delta 24}$/+; *da*-Gal4) background. Ubiquitous knockdown of *Tig RNAi* (Figure 2.6B), *Tsp RNAi* (Figure 2.6C), or $fon^{\Delta 24}$/+; *da*-Gal4 (Figure 2.6E) alone resulted in a low level of detached muscles (Figure 6I). In contrast, a significant increase in the number of detached muscles (Figure 2.6I) was observed upon a 50% reduction in *fon* copy number in a *Tig* (Figure 2.6F) or *Tsp* (Figure 2.6G) RNAi genetic background. Since both Tig and Tsp are extracellular proteins, we utilized secreted Gelsolin (Gel), a protein that also circulates in the hemolymph [25, 43] as a negative control. RNAi knockdown in two independent lines of *Gel* alone (Figure 2.6D), or in combination with a reduction in *fon* (Figure 2.6H), did not affect the ability of muscles to remain attached (Figure 2.6I). qPCR results demonstrating RNAi knockdown are shown in Figure 2.6J.

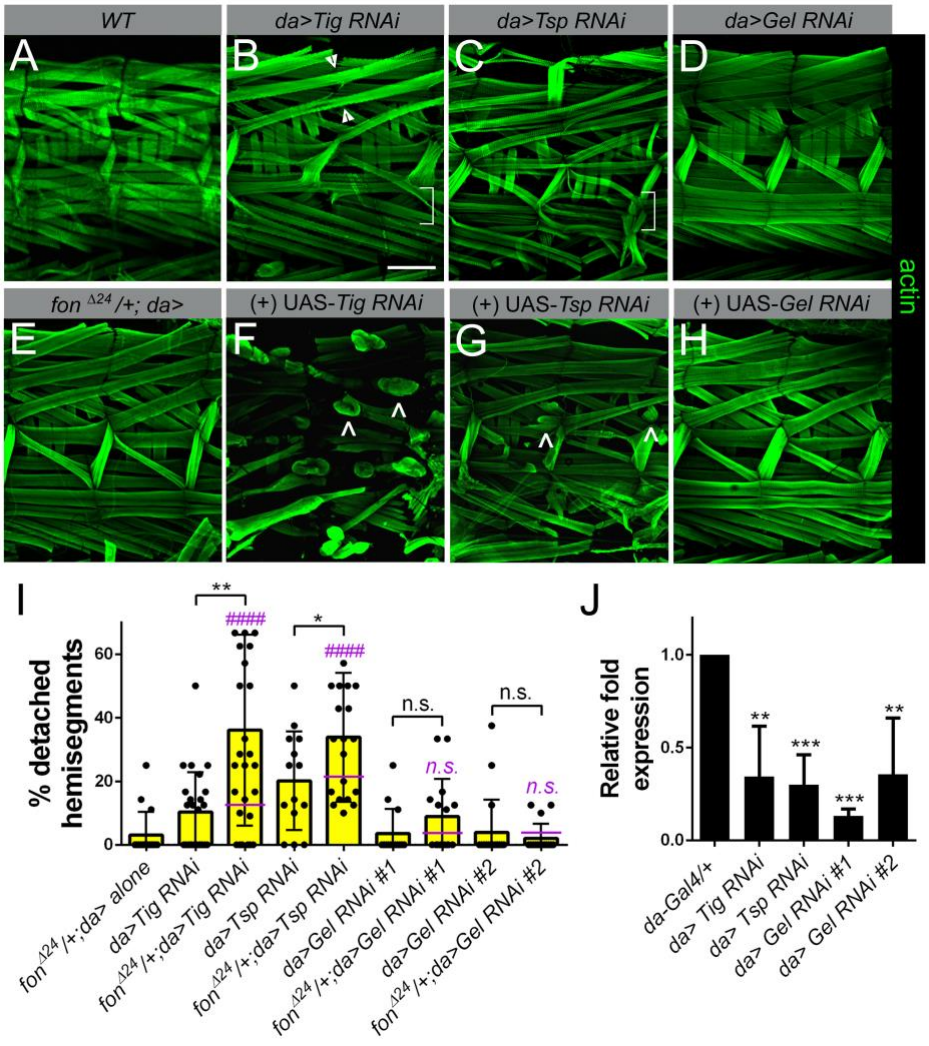

Figure 2.F RNAi knockdown of Tig or Tsp enhances *fon*-mediated muscle detachment.

(A–H) Two hemisegments of the larval musculature stained for F-actin in a *WT* control, the indicated RNAi lines alone (B–D) or in a sensitized *fon* ($fon^{\Delta 24}$/+) genetic background (E–H). (B) RNAi knockdown of *Tig* results in missing muscles 6 and 7 (bracket) or muscles that lift off of the cuticle (indented arrowheads). (C and D) Loss of *Tsp* (C) mildly affects muscle attachment, while G*el* (D) alone has no effect. Compared to the *WT*appearance of $fon^{\Delta 24}$/+, *da*-Gal4 alone (E), an enhancement of detached muscles (arrows) is observed upon concurrent expression of *Tig RNAi* (F) or *Tsp RNAi* (G), but not *Gel RNAi* (H). (I) Quantitation of muscle detachment in the indicated genotypes in a *fon*-sensitized background ($14 \leq n \leq 29$ for each genotype). The purple lines illustrate the predicted additive effects of each individual contribution. (J) qPCR results showing that the indicated RNAi lines effectively knockdown *Tig*, *Tsp*, and *Gel* transcripts. Mean ± SD; *P*-values: *** $P < 0.005$, ** $P < 0.01$, * $P < 0.05$; n.s. = not significant. Notations in purple indicate comparisons to $fon^{\Delta 24}$/+; *da*-Gal4 alone, *P*-values: #### $P < 0.001$, n.s. = not significant. Bars, 100 µm for A–G.

We utilized our genetic interaction assay to test the requirement for multiple candidate proteins found in the hemolymph clot, including Imaginal disc growth factor 4 (Idgf4), Retinoid- and fatty acid-binding glycoprotein (RfaBp/ApoL1), Larval serum protein 1γ (Lsp1γ), and Larval serum protein 2 (Lsp2) (data not shown). Only RNAi knockdown of Lsp1γ (Figure 2.7D) resulted in detached muscles (Figure 2.7B-C, carets) although penetrance of the phenotype was not increased in a heterozygous *fon* (*fon*$^{\Delta 24}$/+) background (Figure 2.7C). To further assess how Lsp1γ may contribute to larval muscle attachment, we expressed Lsp1γ–GFP transgenic flies under UAS control. As Lsp1γ is secreted from fat body tissue [44], we expressed this fluorescently tagged fusion protein with *ppl*-Gal4 (Figure 2.7E-E′). Lsp1γ–GFP accumulated at junctions where muscles were directly attached to the cuticle, such as the lateral transverse muscles 21–23 (Figure 2.7E-F, small arrows). Lsp1γ–GFP was also observed at indirect muscle sites that meet at the hemisegmental border (Figure 2.7E-E′, white arrowheads). Closer examination revealed a block-like appearance of Lsp1γ–GFP accumulation (Figure 2.7G-G′), consistent with the location of tendon cells along the cuticle as visualized by the tendon cell marker Sr (Figure 2.7I-I′). Scanning from the top of the muscle toward the cuticle revealed an accumulation of Lsp1γ–GFP underneath (Figure 2.7H-H ′, white arrow), but not between muscles in adjoining hemisegments (white arrowhead), further demonstrating that Lsp1γ is located near tendon cells. This is in contrast to Tig, which primarily localizes to the junctions between muscles (Figure 2.3D–F).

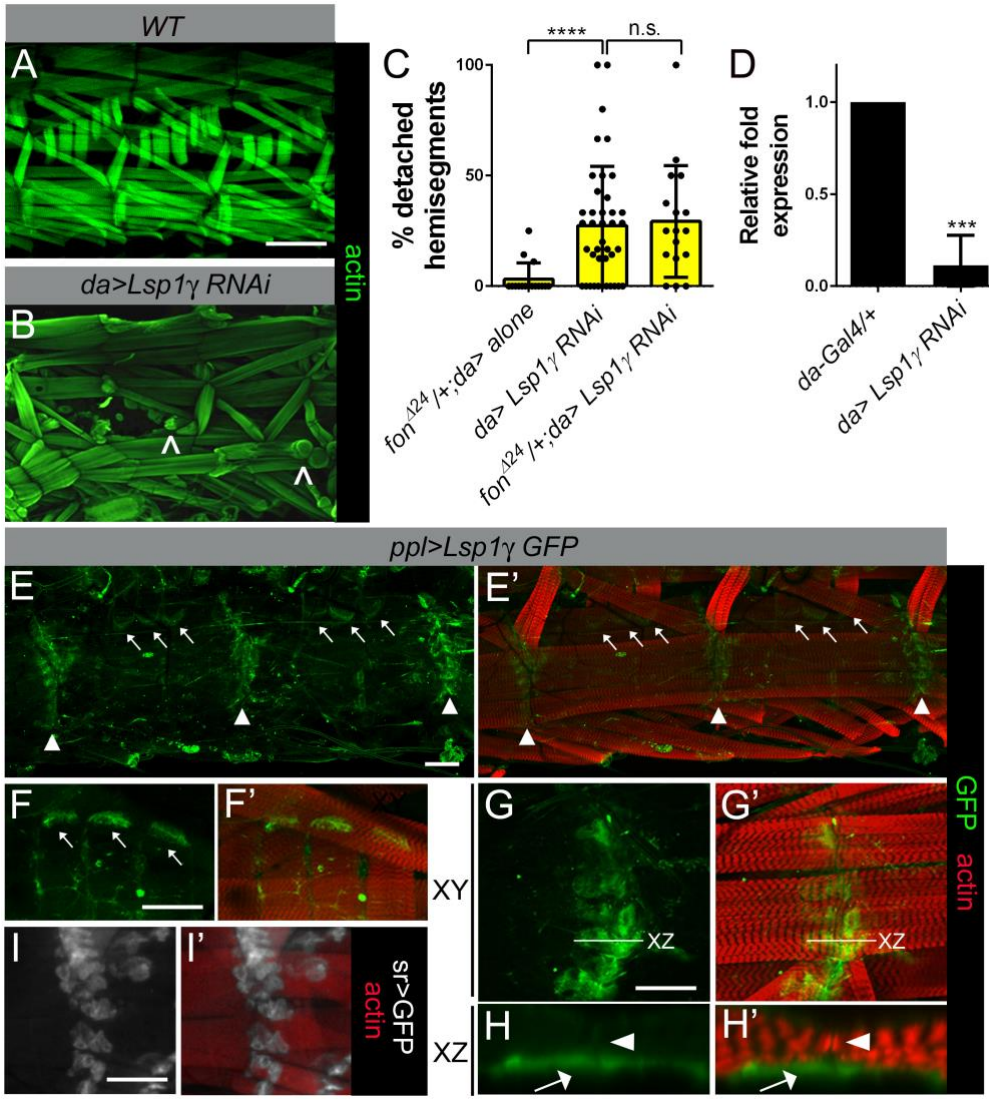

Figure 2.G The clotting factor Lsp1γ accumulates at MASs.

(A and B) Three hemisegments of the larval musculature stained for F-actin. (A) The normal pattern in *WT* larvae. (B) Ubiquitous induction of *Lsp1γ RNAi* reveal detached muscles (carets). (C) Quantitation of muscle attachment defects present in *Lsp1γ* knockdown larvae ($16 \leq n \leq 40$ for each genotype). Note that the penetrance is not increased in a *fon*-sensitized background. (D) *Lsp1γ* RNA is decreased upon RNAi knockdown as determined by qPCR. (E–H′) *Lsp1γ*–GFP fusion protein accumulates at sites consistent with tendon cell localization. (E–F′) The fusion protein is found at the ends of lateral muscles (small arrows in E, E′, and F) and on tendon cells at the segmental borders (arrowheads in E and E′). (G–H′) An XY (G and G′) or XZ (H and H′) scan through the plane shown in G reveals Lsp1γ accumulation on tendon cells (arrows), but not between adjacent muscles (open arrowhead). (I) Confirmation of tendon cell localization at segmental borders as visualized by GFP under control of the *sr* tendon cell promoter. Mean ± SD; *P*-values: **** $P < 0.001$, *** $P < 0.005$, n.s. = not significant. Bars, 150 μm for A and B; 50 μm for D–F and H.

Discussion

The unexpected lethality of fon mutant alleles led us to the surprising finding that a subset of clotting factors also acts in the independent process of muscle attachment. Further identification of a common suite of secreted proteins (Tig and Lsp1γ) that function in both coagulation and muscle attachment suggests that these ECM proteins share a unique structural role in providing rigidity and strength during wound healing and MTJ attachment, yet remain elastic to withstand forces generated by muscle contraction and organismal movement.

Seminal studies in *Drosophila* uncovered the role of integrins in muscle attachment over 20 years ago. Since this discovery, the requirement for integrins and integrin-associated proteins in vertebrate muscle attachment has reinforced evolutionarily conserved mechanisms that underlie MTJ structure and function [2, 3, 45]. The prevailing notion in muscle biology is that the stable attachment of muscles depends upon integrin-mediated noncovalent interactions that link the internal muscle cell cytoskeleton to the external environment during active muscle contraction. Moreover, the majority of ECM proteins (*e.g.*, Tig, Tsp, and Laminin) identified that function in muscle–tendon attachment directly bind integrin heterodimers through RGD or KGD binding motifs [46]. Neither the Fon nor Lsp1γ protein sequences contain these tripeptide sequences, suggesting they are not integrin ligands. Interestingly, the lack of integrin binding motifs implies that these, and possibly other, secreted proteins accumulate in the extracellular space and associate with integrin ligands to maintain MTJ integrity. Whether these proteins physically associate in the extracellular environment remains to be determined.

Based upon the data presented here, our current model suggests that Fon is a key ECM component required for MAS stability. First, the muscle detachment phenotypes in *fon* mutants appear consistently stronger than loss of Tig, Tsp, or the α-Laminin chain encoded by the *wb* gene [17]. More importantly, loss of Fon in TEM studies reveals not just a loss of muscle attachment, but a complete absence of ECM components and a loss of membrane interdigitation between the sarcolemma and tendon cell (Figure 2.5D, H). We postulate that the lack of mechanical tension at the MTJ compromises the stability of cytoskeletal arrays attached to apical junctions in tendon cells, leading to a loss of muscle attachment fibers in lamellae-associated pore canals and a loss of cuticle organization in *fon* mutants.

Surprisingly, we find that Lsp1γ accumulates on tendon cells to mediate the attachment of muscles to the underlying cuticle, adding a novel role to the repertoire of potential Lsp functions. This secreted protein is a member of the insect hexamerin family and is widely regarded as a nutrient storage protein [47]. Since hexamerins accumulate during late larval stages and are not detected in pupae or adults, it was proposed that these proteins store amino acids and thus energy reserves during nonfeeding stages. This idea was supported by experimental evidence in multiple organisms whereby hexamerin storage and degradation are correlated with stage- and sex-specific usage. Other possible roles for hexamerins include ecdysteroid binding and transport, cuticle formation, and humoral immune defense, although it is worth noting that these diverse roles have been demonstrated in insects other than *Drosophila*. Genetic studies in the fly model reported that null mutations in all three *Lsp1* genes (α, β, and γ) are viable [48].

However, another study observed Lsp1 proteins in these null animals by electron microscopy, questioning the validity of the null alleles [49].

In addition to its role in the immune response and energy storage, the fat body serves as a source of secreted protein to regulate the development of multiple organs. Fon, Tig, and Lsp1γ are secreted from fat body tissue and circulate in the hemolymph to reach their final destination. We provide evidence that Fon is secreted via the canonical secretory pathway as treatment with BFA blocks Fon–GFP transport out of fat body cells. Furthermore, knockdown of Fon in the fat body using targeted RNAi causes muscle detachment. There is ample evidence for fat body secretion of proteins into the hemolymph and subsequent localization to target tissues, including Collagen IV (Col IV) into the basement membrane (BM) and the chitinase Serpentine (Serp) into the trachea [32, 50]. While it is not clear how Col IV is targeted to the BM, synthesized Serp is secreted into the hemolymph and is transcytosed across epithelial cells to reach the tracheal inner lumen. This type of mechanism seems unlikely for Fon, Tig, or Lsp1γ transport as reaching MASs does not require crossing cell layers. While it is not yet clear how circulating hemolymph proteins become targeted to other tissues, possibilities include diffusion or lipid/protein-based transport systems.

Over 85% of the Fon residues are polar or hydrophobic, including an abundance of Ser, Ala, and Gly residues not uncommon in extracellular proteins. Fon protein appears to be composed of unstructured regions with no discernible predicted secondary structure or predicted conserved domains. Yet, clearly Fon is crucial for organization of the ECM at MASs. Thus, further insight into Fon structural and/or biochemical properties may shed light on this exciting new role for Fon.

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Chapter 3 - “A Tissue Communication Network Coordinating Innate Immune Response During Muscle Stress”

This chapter has been submitted for consideration as a journal article:

Green, N., Walker, J., Bontrager, A., Zych, M., Geisbrecht, E. A tissue communication network coordinating innate immune response during muscle stress.

Abstract

Complex tissue communication networks function throughout an organism’s lifespan to maintain tissue homeostasis. Using the genetic model *Drosophila melanogaster*, we have defined the activation of immune responses during muscle stress. In *fon* mutants, we observe recruitment of hemocytes and the accumulation of melanin at muscle attachment sites (MASs) with detached muscles. Loss of *fon* also initiates Toll signaling in the fat body and the increased expression of the Toll-dependent antimicrobial peptide (AMP), *drosomycin*. Interestingly, genetic interactions between *fon* and various Toll pathway components enhance muscle detachment. Similarly, overexpression of AMPs in a *fon*-sensitized background increases muscle detachment and drives a muscle hypercontraction phenotype. In addition to Toll signaling, the JAK/STAT pathway is activated in muscle following the induction of specific muscle stresses such as

hypercontraction, detachment, and oxidative stress. This work identifies a reciprocal signaling network linking JAK/STAT and Toll pathways in a multi-organ immune response to altered muscle physiology.

Introduction

In insects, detection of foreign molecules activates a robust immune signaling cascade coupled with biological outputs to minimize damage to the host. Innate immunity can be broken down into the humoral arm, which uses signaling pathways for antimicrobial peptide (AMP) and target gene expression, and the cellular arm, which regulates the mobilization of hemocytes and encapsulation [1]. Signaling during immune challenge proceeds through two canonical pathways, Toll (Tl) and Immune Deficiency (Imd), depending on the type of infection [2]. Both the Toll and Imd pathways require binding of an extracellular ligand to transmembrane receptors to activate signal transduction and a series of intracellular events which lead to the nuclear translocation of an NF-κB transcription factor, Dorsal/Dif or Relish, respectively [3-7]. Once in the nucleus, these transcription factors induce the expression of antimicrobial peptides (AMPs) and other immune-responsive genes to generate the humoral immune response. In addition to the primary Toll and Imd cascades, the activation of additional signaling pathways such as JAK/STAT, JNK, and Hippo allow for immune-based gene expression to be tailored for individual wounds or infections [8-17]. More recently, these secondary immune pathways have gained recognition for roles in influencing both cellular activities and the molecular mechanisms linking localized and systemic immune responses [12, 18-20].

Because an efficient immune response often requires the efforts of multiple signaling cascades, complex crosstalk and feedback loops link both humoral and cellular actions. The cellular arm of the innate immune response in insects focuses on activities performed by circulating blood cells called hemocytes [1, 21]. *Drosophila* possesses three types of hemocytes: migratory plasmatocytes similar to macrophages, lamellocytes for encapsulation of pathogens, and crystal cells which are crucial for releasing molecules essential for melanization [22, 23]. Recruitment of hemocytes is followed by phagocytosis and encapsulation of microbes through melanization. Melanization is an arthropod-specific response used to neutralize pathogens and strengthen the hemolymph clot at wound sites. Accumulation of melanin occurs following the enzymatic conversion of tyrosine derivatives such as L-DOPA by phenoloxidase (PO) which is secreted from a subset of hemocytes known as crystal cells [2, 24, 25]. Mutations in genes important for hemocyte function and regulation of PO activity result in the formation of melanotic tumors [26]. Interestingly, constitutively active mutants of both *Tl* and *hopscotch* (*hop*) have melanotic tumors which accumulate in *Drosophila* larvae and adults, exposing relationships between the melanotic cascade and two major immune signaling pathways, Toll and JAK/STAT [27, 28]. Furthermore, Toll signaling is required for both hemocyte recruitment and melanization [29]. These observations suggest an intricate orchestration of the cellular and humoral systems during the immune response, the details of which remain to be elucidated.

Recently, several examples in invertebrate and vertebrate models have highlighted the importance of muscle tissue in the innate immune response. During parasitoid wasp infections, *Drosophila* larval muscles show activation of JAK/STAT

signaling, which is coordinated with Toll signaling in the fat body [30]. Muscle-specific knockdown of JAK/STAT signaling led to a decreased capacity to encapsulate wasp eggs and reduced the mobilization of lamellocytes, indicating that muscle tissue plays a significant role in managing this type of infection [20]. Gene profiling during *P. aeuroginosa* infection revealed an increase in muscle structural genes [31]. Further exploration of these observations emphasized that *Drosophila* indirect flight muscles (IFMs) are an immune-responsive tissue essential for surviving bacterial challenges during adulthood [32]. During an infection, AMP production occurs in the adult IFMs and importantly, reduction of AMPs by knocking down Toll or Imd pathway components, or compromising IFM structural integrity limits an individual's survival [32]. Similar trends in fish species showed that the immune-responsiveness of muscles is a conserved feature of immune activation [32, 33].

Gene expression profiles obtained from *Drosophila* mutants with hypercontraction-induced myopathy [34] and human muscular dystrophy patients [35-37] revealed upregulation of genes involved in actin-dependent remodeling and chaperone transcripts as well as a downregulation of metabolic and mitochondrial genes characteristic of metabolic stress in dystrophic muscle. Surprisingly, innate immune transcripts were also upregulated. The invasion of immune cells and inflammation following muscle injury as a repair mechanism is a common occurrence in vertebrates [38]. However, individuals with myopathies and muscular dystrophies often experience persistent immune responses in damaged muscle which may contribute to disease progression [39-42]. In *Drosophila*, prolonged immune activation is capable of driving tissue damage in the neural system [43]. Specifically, overexpression of AMPs in nervous tissues is sufficient to drive

neurodegenerative phenotypes [43]. The capacity for immune responses to act not only in response, but to potentially drive tissue damage emphasizes the necessity for understanding fundamental mechanisms regulating immune and tissue physiology.

We have uncovered a tissue communication network linking muscle maintenance and innate immune signaling. While characterizing the muscle phenotypes of *fondue* (*fon*) mutants [44], we noted several immune responses related to muscle tissue, including the deposition of melanin at MASs and hemocyte recruitment to detached and damaged muscle. A closer examination of immune signaling revealed Toll activation in the fat bodies of *fon* mutants. Overactivation of key regulatory points in the Toll pathway as well as AMPs in a *fon*-sensitized background enhanced muscle detachment and hypercontraction, respectively. To understand how systemic Toll activation could be achieved, we looked for local signal activation of immune and stress responses in muscle tissue to link the tissue network. Within muscle issue, JAK/STAT signaling is initiated following specific classes of muscle stresses. Furthermore, JAK/STAT signaling induced through the constitutively active JAK allele, $hop^{Tum\text{-}l}$, is capable of activating Toll signaling, and weakly in the reverse. Therefore, muscle tissue maintenance is coordinated with innate immunity in a multi-organ response mediated through local JAK/STAT signaling and systemic Toll activation.

Materials and Methods

Fly Genetics

Flies were raised on standard cornmeal medium at 25° C unless otherwise specified. The control stock used in all experiments was w^{1118} . Two *fon* null alleles, $fon^{\Delta 17}$ and $fon^{\Delta 24}$

[45] were used to remove *fon* and paired with the deficiency stock, *Df(2L)Exel6043*. Other mutant alleles used in experiments were *Brkd*^{J29} (a gift from T. Littleton), *hop*$^{Tum\text{-}l}$ (BL-8492), *Mhc*^{S1} (a gift from T. Littleton), *Tig*$^{\Delta x}$ [46], and *tn*$^{\Delta A}$ [47]. The following GAL4 lines were used to direct tissue-specific expression: *da*-GAL4 (originally BL-37291 outcrossed ten times to w^{1118} to remove background lethals), *Cg*-GAL4 (BL-7011), and *ppl*-GAL4 (a gift from L. Dobens). Stocks analyzed in screens include: UAS-*dl* (BL-9319), UAS-*Dif* (BL-22201), UAS-*SPE* (a gift from Won-Jae Lee [48]), UAS-*Drs* (a gift from D. Wassarman), UAS-*Metc*h (a gift from D. Wassarman), UAS-*Dro* (a gift from D. Wassarman), UAS-*Toll*^{10B} (BL-58987), UAS-*cact RNAi* (BL-31713), UAS-*Tig RNAi* (BL-31570; RNAi validation in [44]), UAS-*tn RNAi* (BL-31588; RNAi validation in [49]), UAS-*park RNAi* (BL-38333; RNAi validation in [49]), and UAS-*SOD* (BL-24754). Reporter stocks used in experiments were 10xStat92E-GFP (BL-26197).

Mutant alleles and genetic constructs were maintained over the appropriate balancer chromosome: *FM7C* (I), *Cyo-Act-GFP* or *Cyo*, *Tb* (II), or *TM6, Tb* (III). Individuals were chosen by selection against the *Tb* or *GFP* marker for 2nd and 3rd chromosome crosses or gender in 1st chromosome balanced alleles. All temperature-dependent crosses were performed at 29°C with the following exceptions: 1) crosses involving temperature-sensitive alleles *Brkd*^{J29} and *Mhc*^{S1} were raised at 29° C and heat shocked for 1 hour at 37° C immediately before dissection; 2) to bypass embryonic development, *hop*$^{Tum\text{-}l}$ crosses were shifted from 18° C to the permissive temperature at 29° C following embryogenesis; 3) experiments using *cact RNAi* were performed at 25° C to avoid early lethality in combination with the *fon*-sensitized background.

Immunostaining & Microscopy

Larvae were dissected to either retain fat body tissue or isolate muscle fillets and fixed in 4% formaldehyde. Tissues were stained with the following primary antibodies: mouse anti-Dl (1:200, DSHB) and rabbit anti-Hemese (1:1000, a gift from D. Hultmark). Fluorescence was detected using the following secondary antibodies: Alexa Flour anti-mouse 488 and Alexa Flour anti-rabbit 488 (1:400, Molecular Probes). F-actin was labeled with Phalloidin 488, 594, or 647 (1:400, Molecular Probes). Images were captured using a Zeiss 700 confocal microscope. Image processing and analysis was performed using a combination of Zen Black (Zeiss), ImageJ (NIH), and Adobe Photoshop.

qPCR Analysis

Transcript levels were assessed using quantitative PCR (qPCR) to verify RNAi knockdown and to compare gene expression amongst genotypes. Total RNA was collected from individual wandering L3 larvae in triplicate using the RNeasy Mini Kit (Qiagen). Fat body-specific RNA was obtained by isolating the fat bodies of five L3 larvae and homogenizing isolated tissues in ice cold RLT buffer (Qiagen). Synthesis of cDNA from 125 ng RNA was performed using the qScript XLT cDNA Supermix kit (QuantaBio). Dilutions of cDNA were optimized according to each primer set and combined with PowerUp SYBR Green Master Mix. The following primers and cDNA dilutions were used: *rp49* forward 5'-GCCCAAGGGTATCGACAACA-3', reverse 3'-GCGCTTGTTCGATCCGTAAC-5' (1:50) (generated via FlyPrimer Bank; Hu et al., 2013) *Drs* forward 5'-CCCTCTTCGCTGTCCTGA-3', reverse 3'-GCGTCCCTCCTCCTTGC-5' (1:50) [50]; *cact* forward 5'-CTCACTAGCCACTAGCGGTAA--3', reverse 3'-

CCCGAATCACTGGTTTCGTTT-5' (1:50) [30]. Quantitative transcript levels were obtained using the $2^{-\Delta\Delta Ct}$ method and graphed as Mean ± SEM using GraphPad 6.0.

Phenotypic Quantification & Statistical Analysis

Detachment. Images were quantified as described in [44]. Muscles were considered detached if muscles had rounded up following detachment or if muscles were beginning to strip away from the attachment site. Percent detachment was calculated by dividing the number of hemisegments containing one or more detached muscles by the total number of hemisegments within the fillet. These percentages were compiled in GraphPad 6.0 and graphically represented as a dot plot. ***Hypercontraction***. Muscles containing differentially compressed regions of sarcomeres were scored as 'hypercontracted' as previously defined [34, 51]. Percent hypercontraction was calculated in the same manner as percent detachment, input into GraphPad 6.0 and graphed as mean ± SD dot plots. ***Statistical analysis***. Statistical analyses were performed in GraphPad 6.0 using the Kruskal-Wallis test to analyze non-Gaussian distributions of three or more unmatched genotypes. Significance values are listed for each quantification within the figure legend.

DOPA incubation

Larvae were live dissected and washed in cold PBS. Muscle fillets were then incubated in L-DOPA solution (60 mM dissolved in PBS) for 1 hour at 25°C in the dark to allow melanization to proceed. Fillets were then washed and fixed in 4% formaldehyde. Images were taken on a Nikon 80i and processed using ImageJ (NIH) and Adobe Photoshop.

Axenic Conditions

Axenic larvae were generated using the protocol described in Sabat et al., 2015. In a sterilized hood, embryos were dechorionated and sterilized using bleach and 70% ethanol solutions. Sterilized embryos were transferred to autoclaved food vials until individuals matured to the larval stage. Larvae were then collected and dissected as described above. The microbe status of axenic lines was analyzed by growing overnight cultures on Luria Broth (LB) containing larval lysates from either normal or axenic conditions. No bacterial growth was observed in axenic lines compared to an LB only control, whereas larval lysates not sterile and grown on normal food showed obvious bacterial growth.

Results

Loss of Fon activates innate immune processes.

The role of *fon* was previously characterized in coagulation and muscle attachment [44, 45, 52, 53]. During our analysis of Fon at the MASs, we noted several phenotypes in *fon* mutants corresponding to activation of the innate immune system. It was known that mutations in *fon* result in diffuse melanization at wound sites [53]. In *WT* larvae, unchallenged individuals are free of melanization with the exception of an external injury with very localized melanization or an encapsulated melanin deposit following the survival of an infection [2, 25, 54]. A small percentage of *fon* mutants present a unique melanization phenotype where melanin spontaneously accumulates at sites of muscle attachment (Figure 3.1D). To induce melanization, fillets were dissected and incubated in the presence of the PO substrate L-DOPA. In *WT* fillets, melanin does not accumulate at MASs but may be found along sites of dissection and within the cuticle (Figure 3.1B-C).

However, *fon* mutant fillets in the presence of L-DOPA accumulate melanin at MASs similar to endogenous MAS melanization (Figure 3.1D-F). During a wounding event, hemocytes are recruited to the sites of tissue damage as part of the cellular arm of the immune response. In third instar larvae (L3), hemocytes can be circulating throughout the hemolymph or reside along the dorsal vessel or in haematopoietic pockets between body wall muscles and epithelia [55-59]. Hemocytes are randomly distributed along larval muscles, but do not specifically localize to the MAS. (Figure 3.1G). In *WT* fillets with mechanical damage to muscles, hemocytes are recruited to sites of tissue damage (Figure 3.1H). Similarly, hemocytes are targeted to detached muscles and attachment sites where muscles have begun to pull away from one another upon genetic loss of *fon* (Figure 3.1I). Together these data indicate that loss of Fon and the subsequent muscle detachment are linked with the initiation of both humoral and cellular arms of the innate immune response.

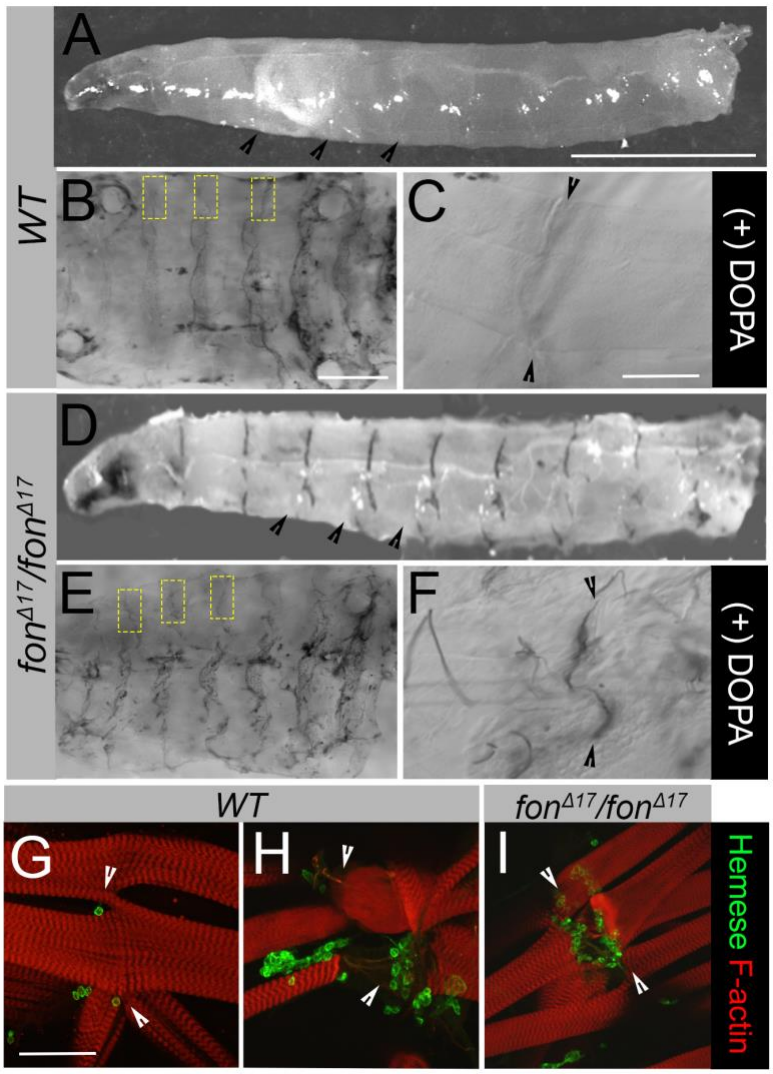


Figure 3.A Loss of Fon activates immune processes.
(A-F) Presence of melanin in *WT* versus *fon* mutants visualized in exterior views and dissected muscle fillets. (G-I) Distribution of hemocytes at MASs in filleted larvae. (A) *WT* larvae lack a visible melanization response throughout the body cavity or MASs (black arrows). (B) Addition of the phenoloxidase substrate, DOPA, allows for conversion into melanin which collects non-specifically throughout the cuticle of *WT* larvae. (C) *WT* muscle attachments (black arrows) taken at higher magnifications are free of melanin. (D) Melanin is spontaneously deposited at MASs (black arrows) in low percentages (~5%) of *fon* mutant larvae. (E) Melanization at MASs (yellow boxes) can be induced by providing excess DOPA substrate to dissected fillets. (F) High magnification of melanin deposits at muscle attachments of *fon* larvae observed upon addition of DOPA (black arrows). (G) Hemocytes stained with Hemese are found at low levels near intact MASs. (H, I) In *WT* muscles that have been mechanically damaged during dissection or upon *fon*-mediated muscle detachment, hemocytes are recruited to sites of muscle attachment and/or damaged muscles. Scale bars 1 mm A,D; 1 mm B,E; 100 μm C,F; 100 μm G-I.

Fon was identified in a genetic screen using elongated pupal morphology as a phenotype for novel muscle mutants [44]. Compared to *WT* pupae, loss of *fon* results in pupae with greater axial ratios and/or curved pupal cases due to defective muscle contraction [44, 53]. The muscle morphology of dissected *WT* larval fillets features repeating hemisegments of broad, rectangular muscles stably anchored at MASs (Figure 3.2B). In *fon* mutants, unstable muscle attachments lead to muscle detachment which generates extensive tissue damage (Figure 3.2D, arrows). In the absence of pathogens, muscle morphology is maintained in *WT* fillets and disrupted in *fon* mutants (Figure 3.2C,E).

In previous studies that examined the role of Fon in immunity, *fon* mutants constitutively expressed the Toll-specific AMP, Drosomycin (Drs), and *fon* itself was identified as a Toll-responsive gene [53]. We utilized the subcellular localization of Dorsal (Dl) to show activation of the Toll pathway upon loss of *fon*. In *WT* larvae without infection or damage, Toll signaling is inactive and the NF-κB transcription factor, Dl, is localized throughout the cytoplasm of the cell (Figure 3.2A, B'). Upon Toll activation, Dl translocates into the fat body nucleus to initiate transcription of Toll-responsive genes (Figure 3.2A,D'). Dl staining is concentrated in the nuclei of *fon* null mutants which implies that a loss of Fon activates Toll signaling (Figure 3.2D'). The nuclear localization of Dl is absent in the muscle tissue of both *WT* and *fon* mutants, narrowing Toll activation to a systemic rather than local response (Figure B.1). To determine whether microbes are important for Toll activation in *fon* mutants, the same experiments were performed under axenic, or germ-free, conditions. The appearance of muscle detachment and the translocation of Dl into the nucleus indicate that the consequences from loss of *fon* are pathogen-independent

(Figure 3.2D-E'). Previously, Drs expression in unchallenged *fon* mutants was detected using a *Drs*-GFP reporter [53]. Complimentary to these studies and our observations that *fon* mutants activate Toll in the fat body, *Drs* transcripts are dramatically increased in isolated fat bodies lacking *fon* (Figure 3.2F).

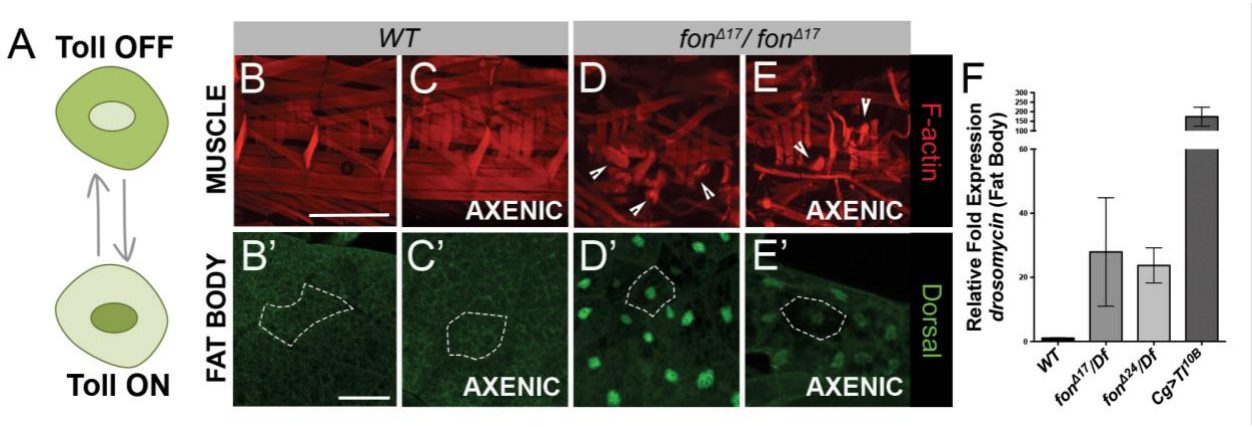


Figure 3.B Toll signaling is activated in *fon* mutants.

(A) Schematic showing Dl localization during Toll signaling. Dorsal is primarily cytoplasmic when Toll signaling is turned off in L3 fat bodies. Following Toll activation, Dl moves into the nucleus to induce gene expression. (B-E) Muscle fillets of dissected L3 *WT* and *fon* mutants raised in either normal or axenic conditions and visualized with Phalloidin (F-actin; red). (B'-E') Dl localization (green) in larval fat body tissue. Individual cells outlined in white. (B-C) *WT* muscles are rectangular and firmly anchored to adjacent muscles and tendon cells in both the presence and absence of pathogens (axenic). (D-E) Muscles round up and detach upon loss of *fon* independent of the presence of pathogens. (B'-C') Dl is localized to the cytoplasm of fat body cells regardless of the presence or absence of pathogens. (D'-E') Dl is enriched in the nucleus of *fon* mutant fat body cells in both normal and axenic conditions. (F) Relative transcript levels of *Drs* RNA collected from the pooled fat bodies of *WT, fon* alleles (*Δ17* and *Δ24*), and *Tl* overexpression, *Cg>Tl^{10b}.* Mean ± SEM. Scale bars 500 μm B-E; 50 μm B'-E'.

fon genetically interacts with genes that activate Toll signaling.

Because loss of Fon induces systemic Toll activation, we wanted to determine if canonical components of Toll signaling are required. We implemented a *fon*-sensitized background screen using the GAL4/UAS system to target genes at regulatory points in Toll activation

[60]. In both *WT* and *fon* heterozygotes, little muscle detachment is observed (Figure 3.3A, E). When crossed to candidate genes that interact with *fon*, genetic interactions would lead to an enhancement of muscle detachment greater than that of the knockdown or overexpression of the candidate alone. Candidates were chosen to simulate the genetic activation of Toll signaling, i.e.,either the overexpression of genes (UAS-*SPE*, UAS-*dl*, US-*Dif*, UAS-*Toll*^{10B}) at key activation steps or the knockdown of inhibitors (UAS-*cact RNAi*) present in the pathway.

We first tested the effects of knocking down *cact* due to elongated pupal phenotype observed in both mutants [61]. The NF-κB inhibitor, *cact*, is responsible for sequestering the transcription factors, Dif and Dl, in the nucleus until signal transduction has occurred. When *cact* is ubiquitously knocked down using RNAi, muscles remain intact (Figure 3.3B,I). Knocking down *cact* in the *fon*-sensitized background causes dramatic, widespread muscle detachment denoting a genetic interaction between *fon* and *cact* (Figure 3.3F,I). We note that only small decreases to *cact* transcripts are necessary to induce muscle detachment as knockdown of *cact* at 29°C in the *fon*-sensitized background results in lethality (Figure 3.3J). Overexpression of Dl in the *fon*-sensitized background results in low levels of muscle detachment, although this trend is not statistically significant (Figure 3.3G,I). Because Fon is present in the extracellular space, we tested extracellular proteins involved in the proteolytic activation of Spatzle (Spz). Spatzle-processing enzyme (SPE) is a protease required for cleavage of the proenzyme, pro-Spz, into its active form (Spz) which may bind Toll to initiate signal transduction [62]. Overexpression of SPE alone is not sufficient to cause muscle detachment (Figure 3.3D,I). When a single copy of *fon* is removed and SPE is overexpressed, muscle

attachment is disrupted (Figure 3.3H,I). Genetic interactions between *fon* and two important pathway members, *Tl* and *Dif*, could not be determined due to lethality of these crosses at temperatures as low as 18°C (Figure 3.3I). This *fon*-sensitized assay acts as an important tool for uncovering the implications of systemic Toll activation in maintaining muscle tissue homeostasis.

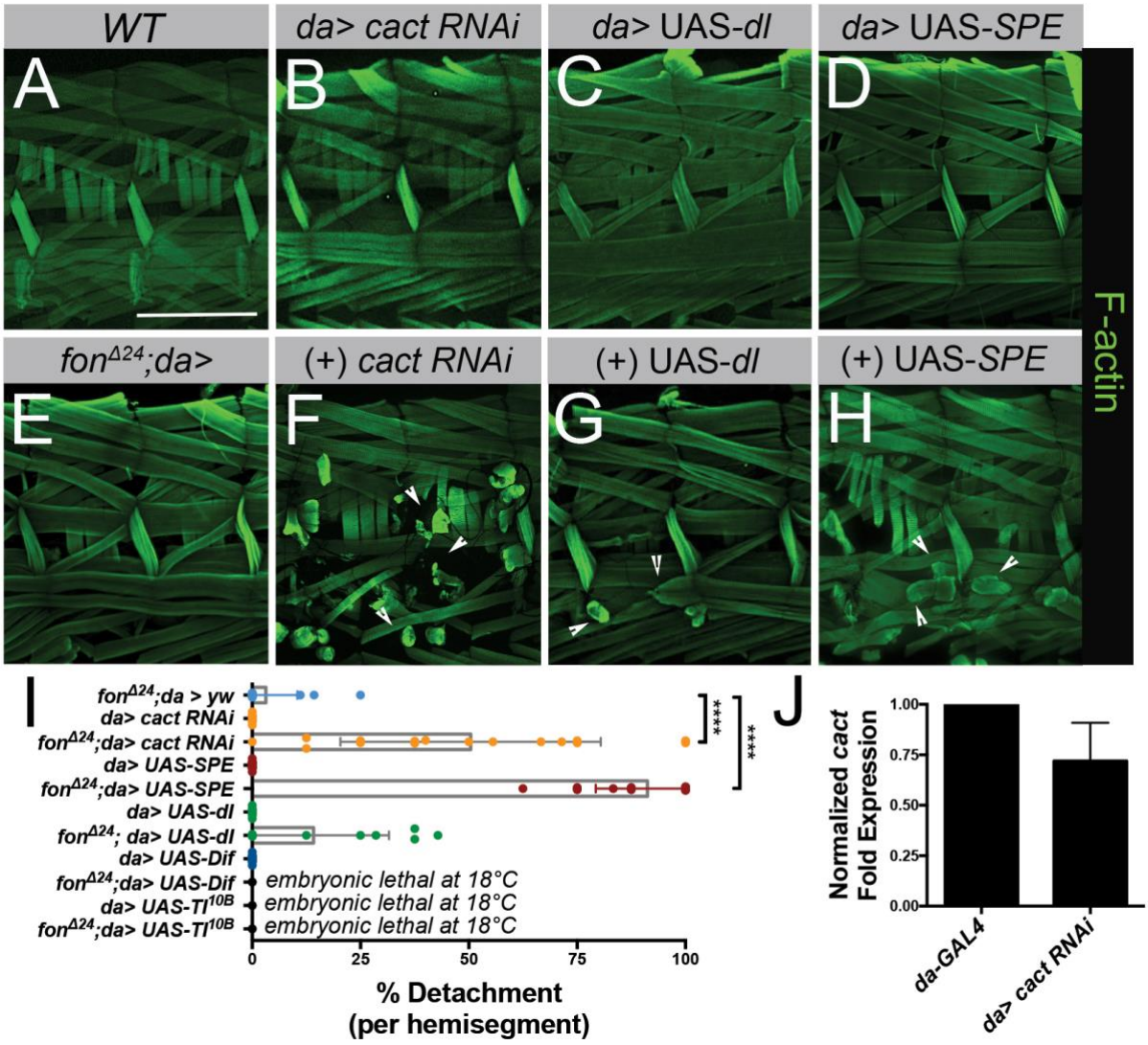


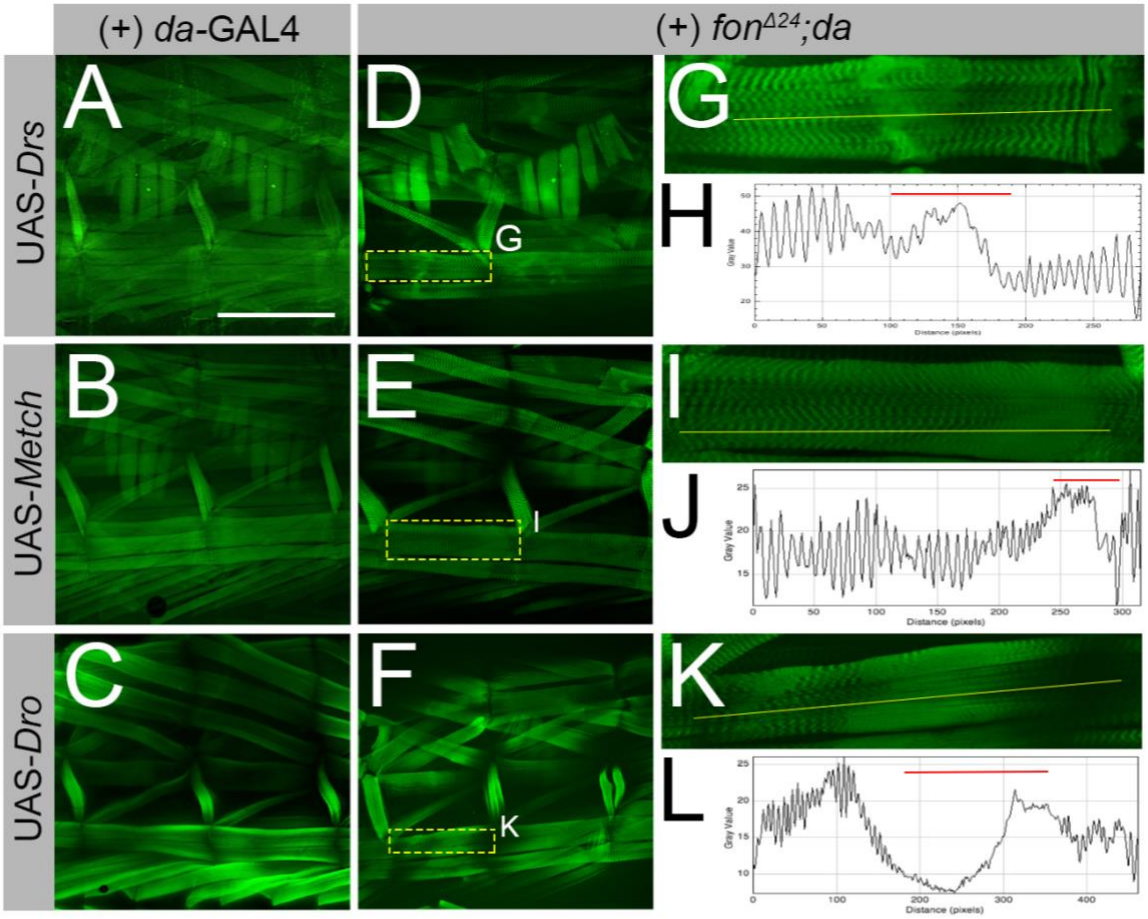
Figure 3.C Genetic interactions between *fon* and Toll pathway components enhance muscle detachment.

(A-H) Two hemisegments from muscle fillets stained with Phalloidin (F-actin; green) in a *WT* control, selected RNAi lines alone (B-D) or in a *fon*-sensitized genetic background, *fonΔ24/+; da*-GAL4 (E-H). (A,E) Muscle fillets of *WT* and *fon*$^{\Delta 24}$*/+;da* sensitized

background have muscles that are absent of morphological defects. (B) RNAi knockdown of the NFκB inhibitor, *cact,* does not disrupt muscle attachment. (C-D) Overexpression of Dorsal or SPE has no obvious consequences to the MAS. (E-H) In comparison to heterozygous $fon^{\Delta 24}$/+, loss of *cact* (F) and the overexpression of *dl* (G) or *SPE* (H) in a *fon*-sensitized background significantly enhances muscle detachment (arrows). (I) Quantification of muscle detachment of select genotypes ($10 \leq n \geq 21$). Lethality of UAS-*Dif* and UAS-$Toll^{10B}$ combinations at and above 18°C prevented a similar larval analysis. (J) Effectiveness of RNAi knockdown of *cact* transcripts determined by qPCR. Mean ± SEM; *P*-values determined via Kruskal-Wallis statistical test: **** $P < .0001$. Scale bars 500 μm A-H.

The major output of Toll signaling is the expression of AMPs which eliminate pathogens through mechanisms which require more extensive definition [63]. Data indicate that excessive or persistent AMP expression can have detrimental effects on host tissues, especially in the absence of pathogens [43, 64]. Overexpression of AMPs have recently been shown to be a driving force in *Drosophila* neurodegeneration [43]. We reasoned that a potential mechanism for disrupting muscle tissue could come from the constitutive expression of AMPs as a result of systemic Toll activation. Using the same sensitized background approach, we examined the effects of overexpressing specific AMPs on muscle architecture. Drosomycin is an AMP expressed as a result of Toll, but not Imd signaling [65]. While ubiquitous overexpression of *Drs* does not disrupt larval muscle morphology (Figure 3.4A,M), increased levels of Drs in a *fon*-sensitized background causes low levels of detachment (Figure 3.4M). Notably, hypercontracted regions of muscles are observed at significant levels in this combination (Figure 3.4D,G,M). In both *C. elegans* and *Drosophila,* hypercontraction can act as a precursor to muscle detachment [66-71]. Similar increased trends in detachment and hypercontraction are observed for overexpression of the AMPs, Metchnikowin (Metch) and Drosocin (Dro) (Figure 3.4 E-F, I-N). Metchnikowin expression results from a

combination of Toll and Imd activation, whereas Drosocin is regarded as an Imd-specific AMP [65]. Despite the fact that each of these AMPs can be activated by either Toll or Imd pathways and act through different mechanisms, the presence of large quantities of AMPs in larvae sensitized to muscle detachment leads to a breakdown of muscle maintenance processes.


(+) da-GAL4
(+) fon^Δ24;da
UAS-Drs
UAS-Metch
UAS-Dro
A
B
C
D
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K
L
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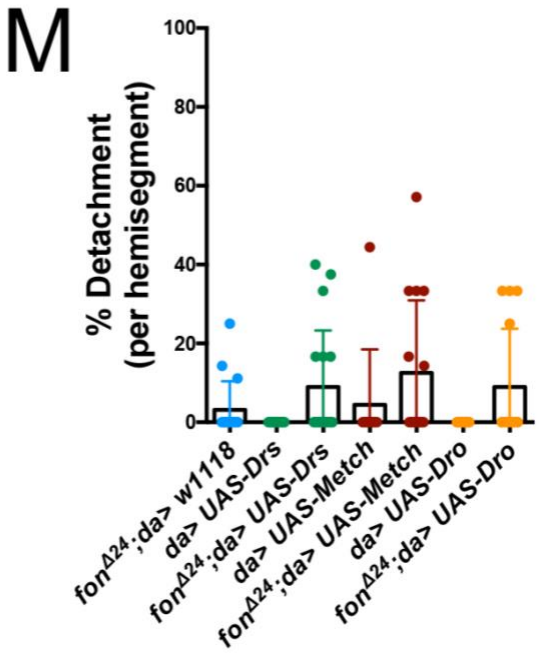
Gray Value
Distance (pixels)


M
% Detachment
(per hemisegment)
fonΔ24;da> w1118
da> UAS-Drs
fonΔ24;da> UAS-Drs
da> UAS-Metch
fonΔ24;da> UAS-Metch
da> UAS-Dro
fonΔ24;da> UAS-Dro

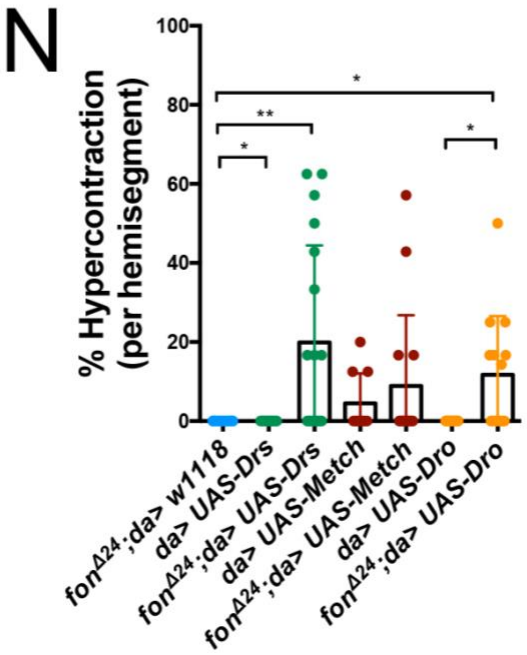

N
% Hypercontraction
(per hemisegment)
fonΔ24;da> w1118
da> UAS-Drs
fonΔ24;da> UAS-Drs
da> UAS-Metch
fonΔ24;da> UAS-Metch
da> UAS-Dro
fonΔ24;da> UAS-Dro

Figure 3.D Overexpression of AMPs disrupts muscle maintenance.

(A-C) Muscle morphology in two hemisgements of L3 muscle fillets (green; F-actin) following AMP overexpression. Overexpression of AMPs in a *fon*-sensitized genetic background at low magnification (D-F) and high magnification with line plot analysis (G-L). (A-C) Muscle tissue exposed to overexpression of Drs, Metch, and Dro display no visible morphological defects. (D-F) Low magnification images of UAS-*AMP* overexpression in the *fon*-sensitized background. (G, I, K) High magnification images of insets (yellow box) following AMP overexpression of *Drs*, *metch*, and *Dro* in a *fon*-sensitized background. Compared to AMP overexpression alone, the combination of one copy of *fon* and heightened levels of *Drs, metch,* and, *Dro* cause an increasing trend of muscle detachment (M) and significant levels of hypercontraction (H, J, L, N). (G-L) Representative line plot analysis of F-actin sarcomeric staining across a single ventral lateral L3 muscle (yellow line). Regions of compressed sarcomeres are indicated by red lines on individual line plots. (M, N) Quantification of muscle detachment ($13 \leq n \geq 18$) (M) and hypercontraction ($13 \leq n \geq 18$). (N) in indicated genotypes. Mean $\pm$ SD; *P*-values determined via Kruskal-Wallis statistical test: * $P < .05$, ** $P < .005$. Scale bars 500 μm A-F; 100 μm G, I, K.

Coordination of JAK-STAT and Toll signaling during muscle stress.

The role of JAK-STAT signaling in tissue stress has been well documented [72]. More recently, a novel role for JAK-STAT signaling within muscle tissue has been described in the *Drosophila* response to wasp parasitic nematode infections [20, 73]. We wanted to determine whether JAK-STAT signaling could act as a local response to pathogen-independent muscle tissue stress using the JAK-STAT reporter, 10xSTAT92E-GFP. When JAK-STAT signaling is active, the transcription factor STAT92E undergoes increased expression and translocates into the nucleus. In healthy muscle, JAK/STAT signaling is held at very low levels throughout muscle tissue (Figure 3.5, A-A'). In the constitutively active JAK mutant, $hop^{Tum\text{-}l}$, STAT expression is enhanced in the cytoplasm and the nucleus (Figure 3.5B-B'). We anticipated muscle detachment which occurs upon loss of *fon* would cause muscle tissue stress and activate JAK-STAT signaling. Indeed, *fon* mutants have muscles with dramatically increased STAT reporter expression in both the cytoplasm and nucleus (Figure 3.5C-C'). In addition to muscle detachment, we tested

two temperature-sensitive hypercontractile mutants, *Mhc*^{S1} and *Brkd*^{J29}, to determine the impact of other muscle stresses on JAK-STAT signaling. Both hypercontractile alleles have been reported to cause hypercontraction which leads to degeneration of the IFMs [34, 51]. At permissive temperatures, both *Mhc*^{S1} and *Brkd*^{J29} exhibit hypercontraction and active JAK-STAT signaling, suggesting that JAK-STAT acts as the local mediator of muscle damage (Figure 3.5D-E').

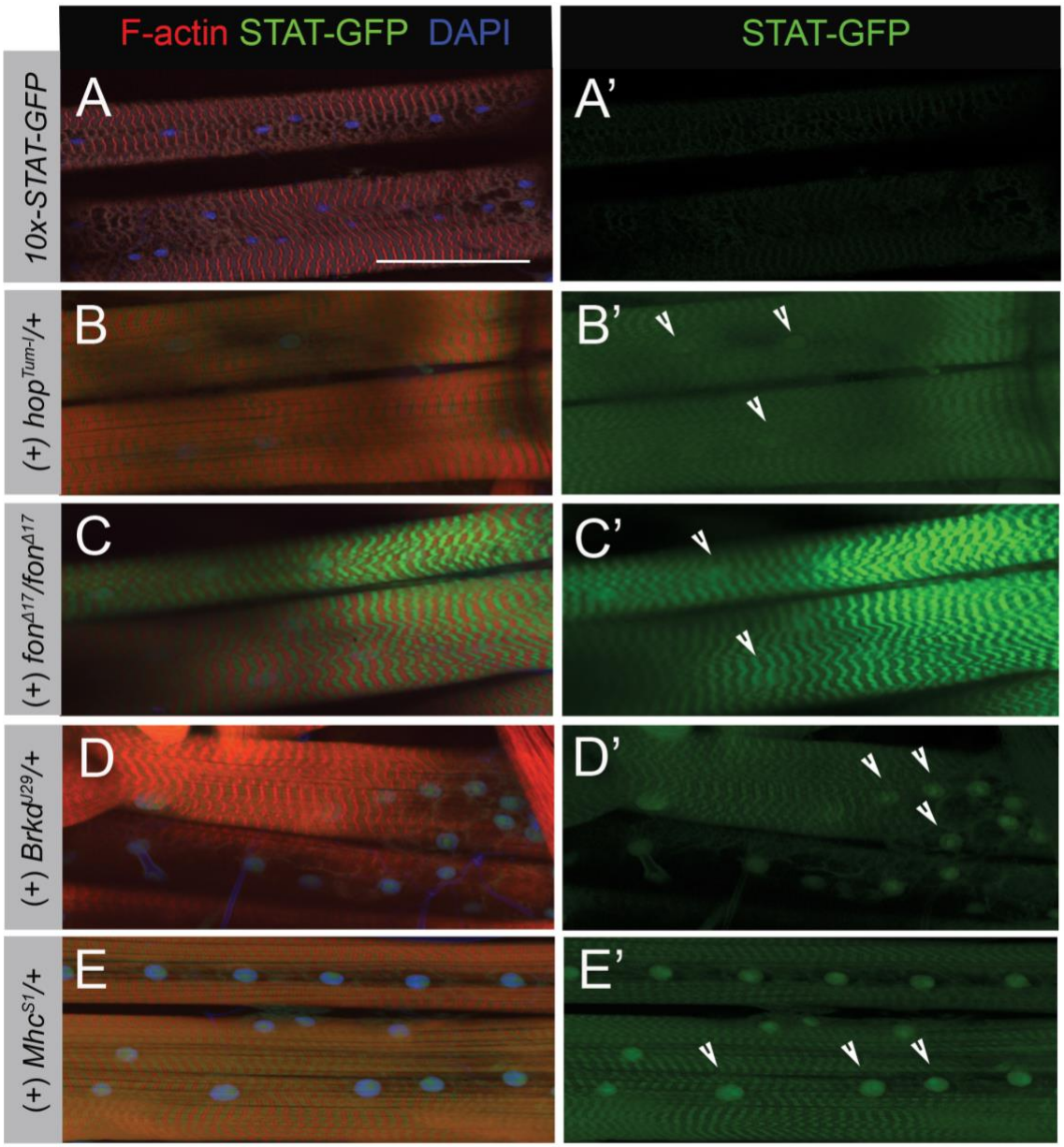


Figure 3.E JAK/STAT signaling is a local response to muscle stress.
(A-E') Expression of 10x-STAT92E-GFP (green) in L3 larval muscle stained with Phalloidin (F-actin; red) and DAPI (blue). (A-A') In normal muscle, STAT-GFP expression is at low levels. (B-B') Activation of JAK/STAT signaling using the constitutively active JAK allele, $hop^{Tum\text{-}l}$, increases STAT-GFP levels in both the cytoplasm and nucleus (arrows) as anticipated. (C-C') Loss of *fon* causes dramatic increases in STAT-GFP both cytoplasmically and within the nucleus. For clarity and consistency, a muscle which remained attached was imaged. (D-E') In hypercontractile mutants, Mhc^{S1} and $Brkd^{J29}$, STAT-GFP expression is increased throughout muscle tissue and concentrates in the nucleus (arrows). Scale bars 100 μm A-E'.

We next wanted to determine to what extent various muscle stresses could activate JAK/STAT in muscle tissue. We screened genes that have been characterized in tissue degeneration, detachment, oxidative stress, or mitochondrial stress. Similar to a loss of *fon*, RNAi knockdown of *Tig* results in muscle detachment, although at lower percentages and at only indirect attachments [46]. Loss of tissue integrity via *Tig* knockdown, but not the myofibrillar unbundling phenotype present in *tn RNAi* fillets, activates JAK/STAT suggesting that activation may require the loss of ECM or membrane integrity (Figure 3.6B-B', E-E'). Tissue balance of reactive oxygen species (ROS) is maintained by a series of enzymes including superoxide dismutase (SOD). Both overexpession and knockdown of SOD have been reported to increase levels of ROS which lead to tissue responses [74, 75]. Disruption of ROS levels results in active JAK/STAT signaling at comparable levels to *Tig* knockdown (Figure 3.6C-C'). Mitochondrial stress weakly activates JAK/STAT signaling upon knockdown of *parkin* (*park*), although no obvious morphological defects in muscle are observed (Figure 3.6D-D'). Because *cact* has reported roles in *Drosophila* larval muscle maintenance [76, 77], we examined *cact RNAi* fillets for the initiation of JAK/STAT signaling, but loss of *cact* was not capable of signal activation (Figure 3.6F-F'). Therefore, JAK/STAT activation in

muscle tissue is restricted to a subset of muscle stresses and not a general response to all tissue stresses.

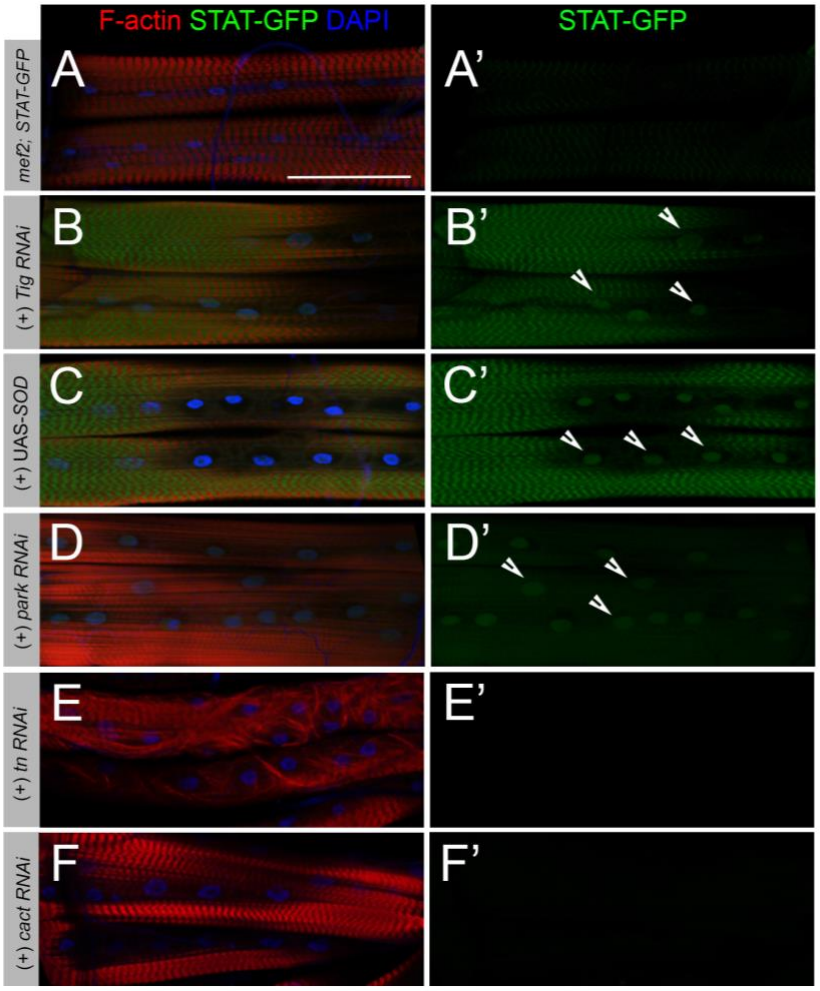


Figure 3.F JAK/STAT is activated in specific, but not all types of muscle stress.

(A-H) Muscle phenotypes (F-actin; red) of select genotypes inducing muscle stress via muscle-specific GAL4/UAS. (A'-H') STAT expression in L3 larval muscles as measured with the 10xSTAT92E-GFP reporter (GFP; green). (A-A') STAT levels are low or nearly undetectable in the cytoplasm and nucleus of unstressed larval muscles. (B-B') RNAi knockdown of *Tig* in muscles leads to weakened tendon cell anchoring, but muscle attachments maintained across hemisegments. Partial detachment is associated with cytoplasmic and nuclear increases in STAT expression (arrows). (C-C') Perturbations to oxidative stress induced by muscle-specific overexpression of *SOD* causes STAT levels to increase throughout muscle and STAT becomes detectable in the nucleus (arrows). (D-D') Reducing *park* transcripts in muscle disrupts mitochondrial dynamics and a weak, but visible presence of STAT in the nucleus (arrows). (E-F') Myofibrillar unbundling and muscle degeneration driven by knockdown of *tn* or the knockdown of *cact* in muscle is not capable of activating JAK/STAT signaling. Scale bars 100 μm A-F'.

Previous experiments identified overlap between JAK/STAT and Toll signaling during infection [20, 78-80]. We wanted to understand how these two tissues could coordinate to initiate the innate immune system in response to muscle stress. To determine potential reciprocity between fat body and muscle, we chose to artificially overactivate JAK-STAT signaling via the temperature sensitive allele, $hop^{tum\text{-}l}$, or Toll signaling driven by the constitutively active transgene, UAS-Tl^{10B}, expressed in a tissue-specific manner. In $hop^{Tum\text{-}l}$ larvae, muscles appear relatively healthy similar to *WT* muscles, with occasional disruptions in muscle morphology (Figure 3.7A,C). When JAK-STAT signaling is constitutively active, we observe Dl translocating into the nucleus due to active Toll signaling (Figure 3.7D). STAT-GFP expression is largely absent from stable muscle tissue (Figure 3.7E-E'), but is enhanced at permissive temperatures for the activation of the constitutively active JAK allele, $hop^{Tum\text{-}l}$ (Figure 3.7F-F'). Conversely, when Toll signaling is constitutively activated, JAK-STAT signaling is turned on at low levels and is a largely cytoplasmic effect (Figure 3.7G-H'). These data suggest that the crosstalk between these two pathways exists in a reciprocal manner, although JAK/STAT signaling has a greater ability to activate Toll signaling than in the opposite direction.

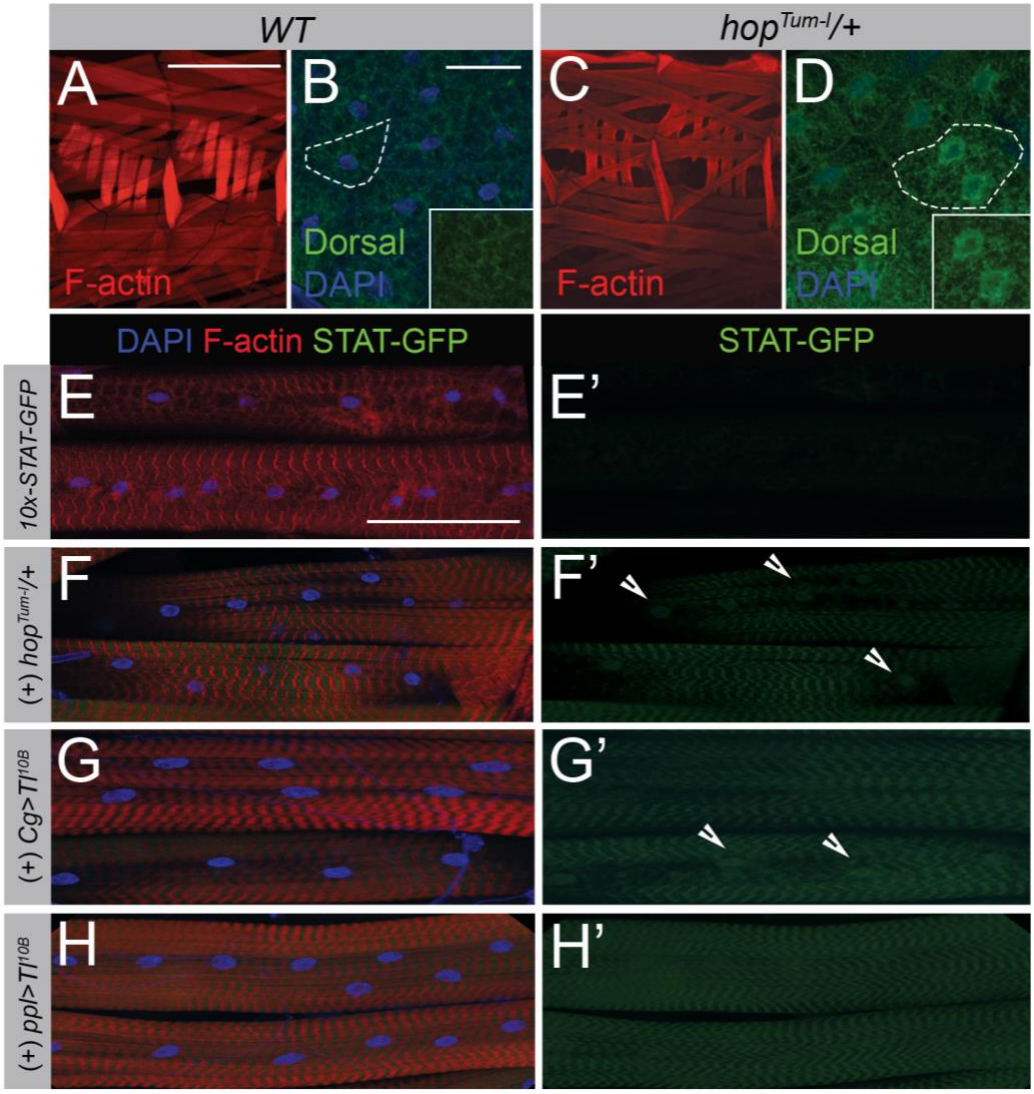


Figure 3.G Active JAK/STAT signaling stimulates systemic Toll signaling.

(A-D) Impact of JAK/STAT signaling in L3 muscle on systemic Toll signaling. (E-H') Analysis of JAK/STAT signaling following Toll activation. (A) Two hemisegments of *WT* muscle (F-actin; red) with stable attachment sites. (B) NFκB transcription factor, Dorsal (green) localizes to the cytoplasm of fat body cells (outline, inset). (C) Constitutively active *hop*$^{Tum-l}$ mutants have muscles with no visible defects. (D) Dorsal translocates into the nucleus of fat body cells following JAK/STAT activation via the *hop*$^{Tum-l}$ mutation (outline, inset). (E-E') In *WT* muscles, expression levels of the STAT reporter *10x-STAT92E-GFP* (green) are low. (F-F') Activation of the *Drosophila* JAK allele, *hop*$^{Tum-l}$, causes 10X-STAT-GFP levels to increase in muscle tissue and move into the nucleus (arrows). (G-H') Initiation of Toll signaling using a constitutively active Toll construct *UAS-Tl*^{10b} expressed in the fat body/hemocytes (G-G') or fat body/salivary glands (H-H') increases STAT levels in larval muscle. Scale bars 50 μm A,C; 50 μm B,D; 100 μm E-H'.

Discussion

We have made the unanticipated discovery that innate immune activation occurs upon muscle stress at both local and systemic levels. In our initial characterization of *fon* mutants, we identified a suite of proteins that are secreted from the fat body and then recruited to the clot during wounding or to the MAS during normal development [44]. The dual use of secreted hemolymph proteins in forming the clot and MAS could be explained by analogous structural requirements. However, immune phenotypes observed in *fon* mutants (Figure 3.1) indicated that a broader set of immune responses were activated upon loss of proteins crucial to maintaining muscle tissue integrity. Using *fon* mutants as a tool, we identified two signaling pathways, JAK/STAT and Toll, which are activated upon muscle stress (Figures 3.2, 3.5). During infection, *fon* expression is increased in response to Toll signaling [53] and *fon* transcripts are upregulated in expression profiles of Mhc^{S1} mutants [34]. Precedence for mechanical damage to muscle tissue activating NF-κB pathways was previously reported in the *mdx* mouse model of Duchenne muscular dystrophy (DMD) and patients with a variety of muscle diseases including DMD, skeletal muscle atrophy, and cachexia-induced muscle wasting [81-89]. In addition to Toll acting as the main signaling pathway in many types of infections, mitigating vertebrate TLR signaling implicated in the pathology of myositis and inflammatory myopathies is being explored for therapeutic intervention [90, 91]. *Drosophila* has yielded many insights into vertebrate Toll-like receptor (TLR) immune response which garnered a Nobel Prize in 2011 [65]. Because of the largely conserved nature of Toll signaling between *Drosophila* and humans [92, 93], determining the relationship between tissue stress and Toll

signaling could have benefits in defining new methods of immune activation and in providing novel perspectives on pathological conditions.

We also observed increases in muscle-based activation of JAK/STAT signaling, which has been described in many tissue stresses to act as a local mediator of immune induction and gene expression [72, 94]. We show that specific muscle stresses activate JAK/STAT signaling (Fig. 5, 6), further emphasizing that immune pathways are responsive to the physiological states of tissues. Our experiments indicate that JAK/STAT signaling is capable of activating Toll in the fat body to drive the systemic immune response (Figure 3.7; Figure 3.8, large arrow). The reverse condition where Toll signaling is constitutively activated in primary immune tissues shows slight increases in muscle-based STAT expression (Figure 3.7; Figure 3.8, small arrow). These data suggest an imbalance in the reciprocal signaling between JAK/STAT and Toll signaling, where JAK/STAT is sufficient to activate Toll at full levels but that Toll signaling requires additional factors to strongly induce the JAK/STAT pathway in muscle. It should be noted that the overall robustness of immune responses following muscle stress are attenuated in comparison to those observed during infection. One explanation for this difference could be the time scale and urgency required to eradicate invading pathogens which is not as imperative in sterile tissue damage.

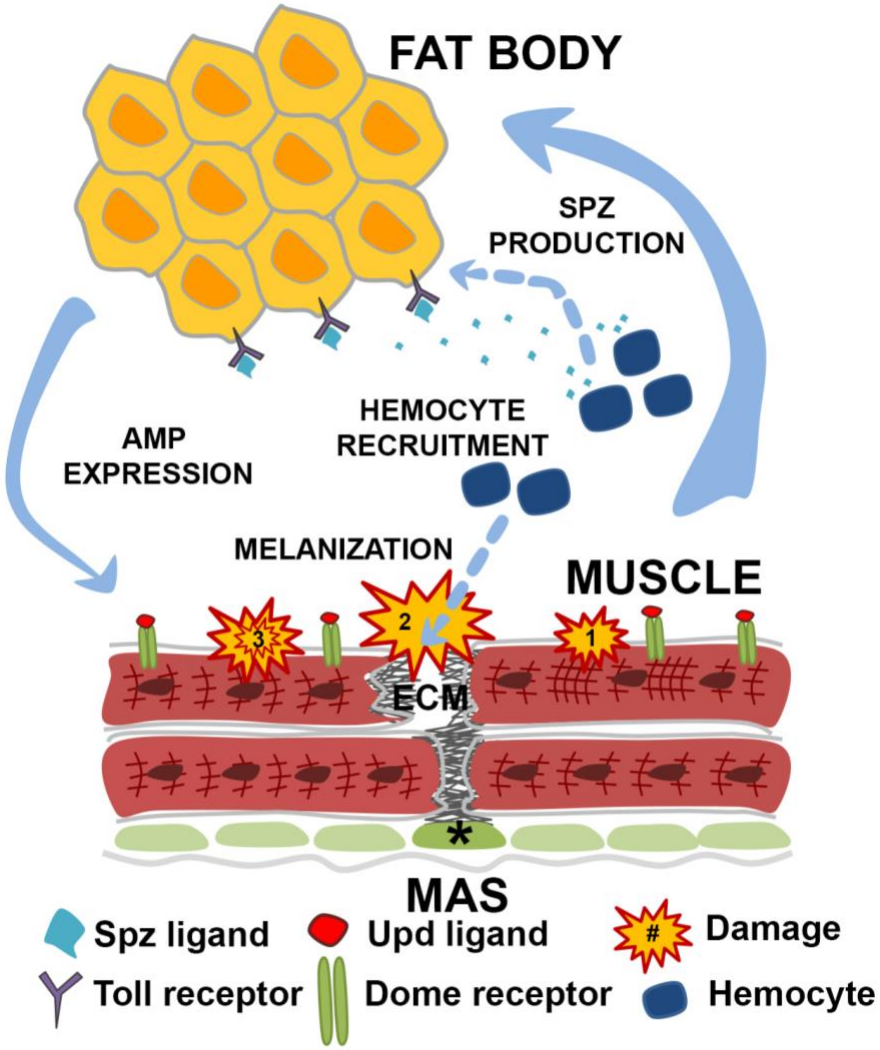


Figure 3.H Model of damage-based tissue communication.

Schematic representation of the tissue communication network activated following disruptions to muscle homeostasis. Muscle health depends on muscle integrity and the strong attachment of muscle (red) to tendon (green, asterisk) via ECM interactions. Muscle hypercontraction (Damage 1) and weakened MASs that progress to detachment (Damage 2) generate stress responses that activate local and systemic immune responses. Muscle damage prompts a series of cellular immune responses including hemocyte recruitment and melanization. Locally, JAK/STAT signaling is activated in muscle tissue to induce expression of immune-responsive genes. The systemic immune response is activated following JAK/STAT signaling to induce hemocytes to produce the Toll ligand, Spz, which binds to Toll receptors. Following Spz-Toll binding, Dorsal moves into the nuclei of fat body cells to activate Toll-responsive genes such as the AMP, *drosomycin*. The increase in AMP levels without a pathogenic target causes AMPs to act on muscle and other tissues resulting in a potentially pathological cycle (Damage 3). Directionality of signaling network represented by size of solid arrows. Dotted line arrows indicate the movement within the model system.

Upon muscle stress, many immune responses are induced, including the recruitment of hemocytes which are known to secrete a variety of bioactive molecules at wound sites [95]. A subset of hemocytes called crystal cells are responsible for secreting PO, which drives the reaction to produce melanin. Melanization is a critical step in hardening and stabilizing the clot when melanin polymerizes around the soft clot structure. Hemocyte localization to damaged MASs and the strengthening role of melanization provides solid rationale for the involvement of hemocytes in a stabilizing response at *fon* mutant MASs. In particular, the *Drosophila* immune response against wasp parasitoid infections utilizes a muscle-hemocyte-fat body communication network via secreted ligands to mount an efficient and successful immune response [20]. In Toll signaling, the ligand Spz is produced and secreted primarily from hemocytes [96]. Upd ligands necessary for JAK/STAT activation are also secreted by hemocytes [20, 73] making these mobile cells a prime candidate for sensing and responding to both muscle and fat body through ligand expression (Figure 3.8). Skeletal muscle has also been shown to produce Upd ligands during homeostatic communication and may act as a source of signaling ligands in a long-range secretion mechanism [97]. At present, our efforts are concentrated on identifying the muscle stresses that activate this immune network with future work concentrated on dissecting the molecular details of how these three tissues are coordinated during immune activation.

Once activated, Spz binds to Toll and induces signal transduction to allow Dl to move into the nucleus to initiate transcription of molecules such as *Drs*. In a normal infection the dramatic expression of AMPs target pathogens, eliminating and deactivating foreign molecules through a variety of destructive mechanisms. When AMPs lack explicit

pathogenic targets, AMPs inappropriately turn on healthy tissue creating a paradox for innate immune activation during sterile tissue damage [43]. In the context of muscle damage, AMPs may act on stressed muscle tissue to exacerbate damage, which we see in the enhanced levels of muscle detachment following overexpression of AMPs (Figure 3.4). In *Drosophila*, excessive levels of AMPs have already been shown to induce neurodegeneration when activated through neural bacterial infections or artificial tissue-specific expression using the GAL4/UAS system [43]. Our result that overexpression of AMPs in a *fon*-sensitized background induces muscle detachment suggests that in the absence of pathogens, AMPs produced during innate immune responses can contribute to tissue damage that initially stimulated immune responses (Figure 3.8).

Models for initiating innate immune responses can be mediated through either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Examples of 'damage-based' innate immune activation can be found throughout the invertebrate and vertebrate literature (for reviews see [98-100]). We show that both muscle detachment and systemic immune activation are pathogen-independent which could implicate the release of a DAMP as part of this tissue network (Figure 3.2). More recently, efforts to understand DAMP-based immune activation have focused on the identification of molecules capable of initiating immune signaling upon tissue damage. Currently, DAMPs that have identified are intracellular components such as chromatin, nucleotides (ATP), ROS, cytoskeletal components (Mhc, actin), or fragments of ECM or basement membrane released by matrix metalloproteases that are recognized as foreign and elicit immune responses, often through TLR signaling [99]. Complimentary studies in *Drosophila* and vertebrates showed that F-actin which can be released during tissue

damage was sufficient to elicit an immune response [94, 101]. Torn and leaky membranes characteristic of muscle damage and long-term diseases present prime opportunities for integrating DAMP release and the immune phenotypes noted in clinical descriptions. However, very few DAMPs have been identified in *Drosophila* despite the genetic and molecular tools that could be used to expose novel conserved DAMPs.

While muscle is recognized as an energy sensor and endocrine source, the complex physiology between muscle and immune tissue is only beginning to be explored. Muscle tissue has previously been shown to be an important immune responsive tissue and producer of myokines which alter the activities of local and distant tissues [102, 103]. Muscle is particularly sensitive to altered physiology, yet must withstand substantial stresses during movement which presents significant challenges in maintaining homeostasis. A major gap in our understanding lies in how disruptions to muscle contractility can translate to altered mechanical stresses, and how the immune response is impacted by alterations to muscle integrity. More importantly, minimized functional redundancy in signaling pathways and a lack of an adaptive immune system to complicate interpretations of signaling pathways make *Drosophila* musculature an ideal tissue for unraveling the biological phenomena regulating a balance between tissue homeostasis and immune activation.

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Chapter 4 - Conclusions & Future Directions

Conclusions

Our work demonstrates the power of unbiased forward genetic screens and *Drosophila* genetics in the dissection of complex biological phenomena. An initial genetic screen based on a novel pupal marker led us to identify a gene which had only been studied in the context of innate immunity. Similarities in the matrix structures of the hemolymph clot and the MAS make the usage of ECM proteins in both processes a logical strategy during development. However, we did not anticipate that our characterization of a novel muscle gene, *fon*, would lead us to define a tissue communication network encompassing multiple tissues upon muscle stress. In conjunction with patient data and related examples emerging from other model organisms, this project identifies an intrinsic link between muscle and immune tissues. Due to the versatility of *Drosophila* genetics and the breadth of tools developed for both muscle biology and innate immunity, the *Drosophila* MAS presents a unique opportunity to understand the integration of multiple signaling pathways during tissue maintenance.

Secreted hemolymph proteins serve dual functions during larval development.

Tissue integrity relies heavily on extracellular proteins forming cellular attachments. Muscle tissue poses a unique challenge on extracellular structure in that ECM must be resilient enough to endure repeated contractions over the lifetime of an individual, but also possess enough elasticity to allow for tissue flexibility and force transmission. The removal of ECM proteins from the MAS can have a broad range of effects, ranging from undetectable consequences on muscle attachment, such as those observed in the loss

of Masquerade [1] or M-spondin [2], or the catastrophic failure of MASs caused by mutations to integrin subunit genes, *myospheroid* and *inflated* [3, 4]. These differential consequences upon loss of MAS components points to a hierarchical order of proteins involved in organizing and maintaining the ECM during development. Three major questions emerge when thinking about the structure and composition of the *Drosophila* ECM: 1) What role are secreted proteins playing in ECM stabilization?; 2) Do organisms use conserved strategies to maintain the ECM?; and 3) What is the origin and mechanism for the incorporation of secreted proteins into ECMs during development?

Our characterization of *fon* led to the discovery that a specific set of secreted hemolymph proteins previously characterized for roles in coagulation were also essential for maintaining muscle attachment [5]. However, the molecular mechanisms for maintaining attachment by Fon, Tig, and Lsp1γ remain undetermined at this time. Based on mutant phenotypes and localization methods, we can predict regional areas of these proteins within the MAS ECM. Fon-GFP and Tig antibody staining localizes throughout the tendon belt of indirect muscle attachments with only Fon-GFP found at direct MASs [5, 6]. Contrary to Tig and Fon, Lsp1γ-GFP localizes in a block-like pattern on the tendon face rather than throughout the MAS. Knockdown of both either Tig or Lsp1γ using RNAi can cause muscle detachment at low to moderate levels. However, muscles lacking *fon* have a strong and universal effect on all muscle attachments (direct and indirect) identifying Fon as a crucial ECM organizer. In fact, the severity of muscle detachment in *fon* mutants ranks above many well-studied proteins such as Tsp, Laminin W (LanW), or Tig, and is lesser only in comparison to integrin subunits βPS and alphaPS2. The necessity of integrin complexes have earned them the status of crucial mediators in

muscle attachment. However, it should be noted that integrins localize properly in *fon* mutants suggesting that the loss of Fon is an ECM protein that stabilizes MASs in an integrin-independent manner.

Many of the proteins identified as a part of the *Drosophila* MAS have no obvious or very subtle effects when mutated. It has been hypothesized that the absence of mutant phenotypes stems from functional redundancy with other MAS proteins. The presence of muscle detachment in *fon*, *Tig*, and *Lsp1γ* mutants would indicate that these proteins possess a necessary and unique function within the MAS which is not compensated for by functional redundancy. Extracellular proteins possess general amino acid characteristics such as repetitive motifs that provide structural flexibility though individual proteins may greatly differ in amino acid sequence. Fon protein structure does not contain any defined domains and is predicted to be intrinsically disordered according to secondary structure prediction software. However, these predictions are based of our knowledge of existing protein structures and cannot account for novel protein domains or intrinsically disordered regions which take shape only within the appropriate biological context. Because Fon, Tig, and Lsp1γ are restricted to Dipterans (with the exception of Lsp1γ which is found more broadly in insect species), it is possible that vertebrates possess proteins containing similar structural features performing analogous roles at the MAS. The topic of ECM composition and the maintenance of tissues through cellular attachments is a fundamental question that applies to all organisms. By not limiting our efforts to conserved proteins, we have identified a group of imported secreted proteins that had not previously been considered for functions in muscle attachment.

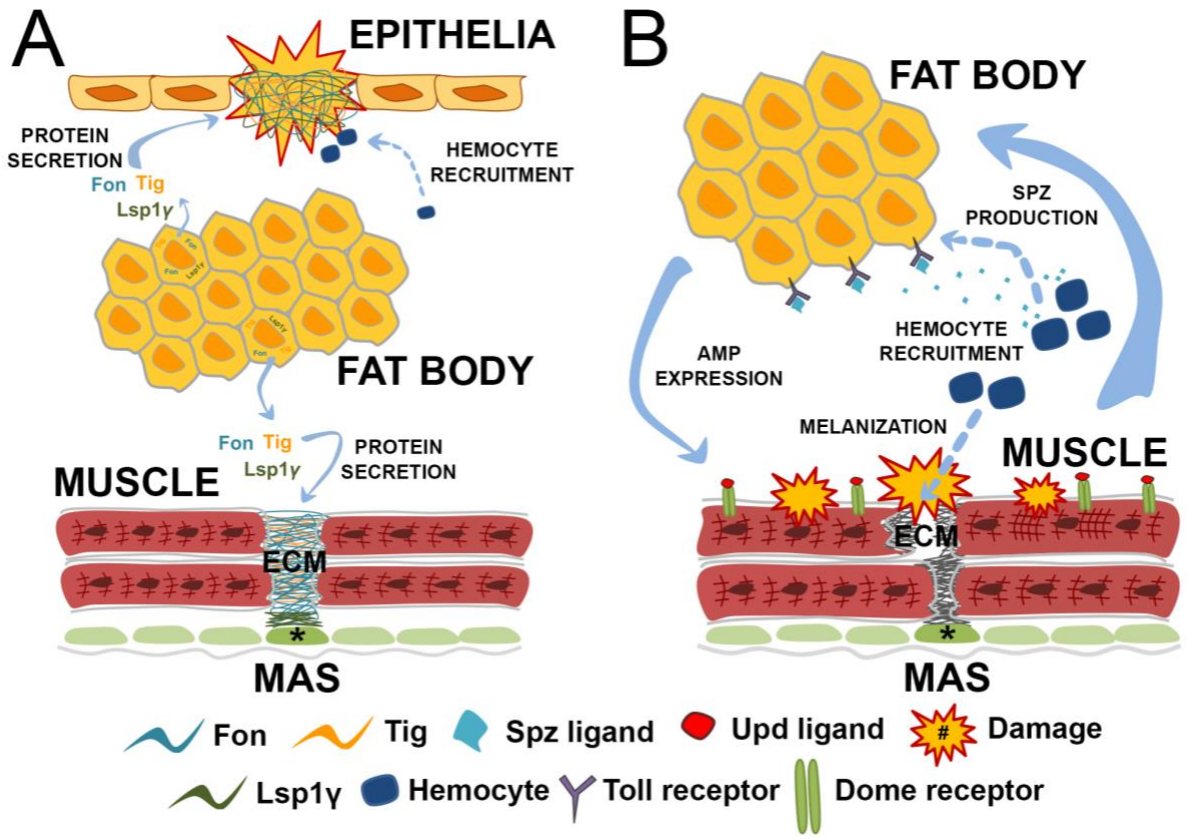


Figure 4.A Biological intersections in *Drosophila* innate immunity and muscle development.

(A) A common suite of coagulation proteins functions in coagulation and muscle attachment. Secreted hemolymph proteins Fon, Tig, and Lsp1γ are synthesized in the fat body and secreted into the hemolymph throughout the larval stage. In a wounding event, specific hemolymph proteins are recruited to the site of damage to from the clot matrix. Immune cells (hemocytes) are recruited through signaling to the wound site to secrete additional proteins to crosslink and melanize the clot for stability. Apart from the conditional use of these proteins during coagulation, Fon, Tig, and Lsp1γ are incorporated into the larval MAS as required components for maintaining ECM integrity and muscle attachment. Both Tig and Lsp1γ have a more restricted localization pattern than Fon which is indicated in the model. (B) Our proposed model for tissue communication which activates the immune system locally and systemically upon muscle various muscle stresses. See Figure 3.8 for detailed description. Muscle, hemocytes, and fat body coordinate through activation of signal transduction, ligand release, and gene expression to alter organismal physiology in response to tissue damage.

Tissue development is regulated by complex physiological networks.

A universal theme in our work has been the coordination of multiple tissues in tissue maintenance. In Chapter 2, we identified a subset of hemolymph proteins which is manufactured primarily in the fat body (Figure 2. 4) and then secreted into the hemolymph (original identification in [7-10]). We showed that Fon and Lsp1γ are incorporated into MASs to maintain ECM integrity during larval development (Figure 4.1A). In our characterization of Fon, we isolated fat body as the tissue of origin using two methods: 1) by universally blocking protein secretion using BFA, we observed the accumulation of Fon-GFP within fat body cells (Figure 2.4G-I); and 2) using tissue-specific RNAi of *fon* and assaying for the presence of muscle detachment (Figure 2.4A-E). The dual use of a subset of secreted proteins in the hemolymph is predicated on the continued production of these proteins via fat body making it an essential tissue for muscle attachment.

In chapter 3 we pursued observations from the characterization of *fon* which led to the assembly of a multi-tissue model of signaling upon loss of muscle homeostasis. We propose the coordination of muscle, fat body, and hemocytes following the release of cellular signals for activation of innate immune signaling. We are currently working to solidify whether innate immune activation is simply a consequence or a driving factor for pathogenesis in our model of muscle maintenance. As our knowledge about innate immune activation grows, scientists are finding novel mechanisms for immune induction including the involvement of new immune-responsive tissues and complex tissue relationships required for mounting an effective immune response. Research into autoimmune diseases and the effects of chronic inflammation highlight how immune responses may act as a double-edged sword. A robust, short-term immune response is

required for an organism's survival from pathogenic microbes, yet long-term or inappropriate activations of innate immune components damages the very tissues these responses are designed to protect. Similar to many tissue maintenance processes, tight regulation of the innate immune system diminishes as we age. Understanding the intersection of tissue homeostatic processes and how regulation becomes less sensitive as we age is an important developmental question and may provide insight into why we see dramatic changes to cells at the end of their lifetime.

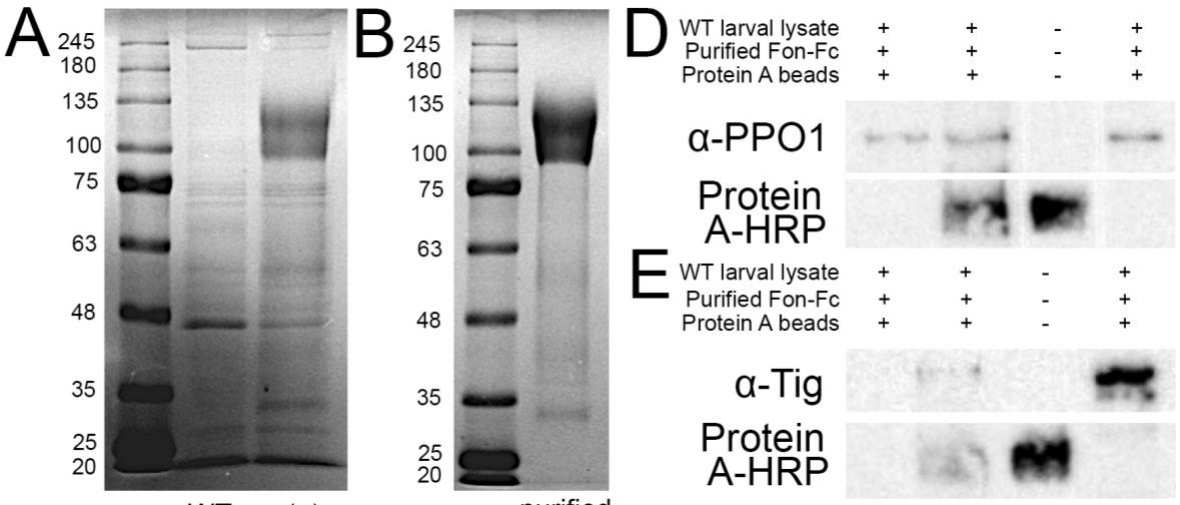
Future Directions

How are proteins targeted to the MAS during development?

All extracellular proteins must be made intracellularly, processed, and secreted for transport to their required location. Many proteins found at the MAS are made within muscle or tendon cells and undergo a localized secretion to their functional site, including integrins, Tsp, and a small muscle contribution of Tig (for review see [11]). However, many MAS and basement membrane components are made in the fat body, undergo secretion into the extracellular environment and are organized into matrices distantly. More recently, an example of an enzyme originating from the fat body was shown to localize and act as an essential player in tracheal development [12].

Fat body and salivary gland tissues have been viewed as protein secretion factories, producing proteins required throughout development. Therefore, it is not surprising that large quantities of proteins utilize this synthesis machinery. What remains mysterious is how these proteins are both delivered and targeted to developing tissues. Key to understanding the targeting and endogenous function of Fon will be identifying

genetic interactors and *in vivo* binding interactions which may act as co-transporters or on-site binding partners to incorporate Fon into MASs. This can be accomplished through biochemical approaches such as immunoprecipitation-mass spectrometry (IP-MS/MS) and validation through co-immunoprecipitaiton (Co-IP) additionally paired with genetic analyses such as transheterozygote analysis and candidate enhancer screens. We have performed exploratory IP-MS/MS experiments using purified Fon (in conjunction with collaborators for protein expression (Samuel Bouyain, University of Missouri-Kansas City; UMKC) and mass spectrometry (Steven Hartson and Janet Rogers, Oklahoma State University; OSU) to simultaneously identify previously unknown MAS proteins or proteins which may bind Fon in larval hemolymph (Figure 4.2). Our results confirm the genetic interaction of Fon with Tig described in Chapter 2 (Figure 2.5) with Western blotting (Figure 4.2C, E). Using these studies as a proof-of-concept, future work will incorporate other genetic backgrounds and tissue-specificity to more selectively isolate protein composition present in normal and weakened muscle tissues. Ideally, the identification of new candidate binding partners and improvement of *in vivo* biochemical and microscopy-based protein detection will lead to a mechanistic explanation of Fon accumulation at MASs in the future.



C

	Experiment #1 (1432)		Experiment #2 (1470)		Experiment #3 (1476)	
	Control	Fon-Fc	Control	Fon-Fc	Control	Fon-Fc
Fondue, CG15825-PB, isoform B	0.0%	20.8%	0.0%	16.30%	0.0%	8.3%
Dipeptidase B, isoform A, GN=Dip-B	0.0%	31.1%	9.9%	34.50%	8.5%	45.8%
Phenoloxidase 2, GN=PPO2	0.0%	34.0%	15.6%	28.60%	12.8%	22.5%
Phenolodxidase 1, GN=PPO1	0.0%	13.5%	0.0%	0.00%	0.0%	0.0%
Tiggrin, GN=Tig	0.0%	2.4%	3.6%	9.40%	5.0%	10.5%
CG11208; Thiamine pyrophosphate enzyme	0.0%	17.9%	0.0%	22.00%	0.0%	27.5%
CG2233, GN=CG2233, unknown	0.0%	5.3%	0.0%	18.70%	0.0%	0.0%
CG30069, GN=CG30069	0.0%	0.0%	0.0%	3.90%	0.0%	5.0%
DNA-binding protein modulo, GN=mod	0.0%	0.0%	0.0%	9.50%	0.0%	8.5%
Serine/threonine-protein phosphatase PP2A, GN=Pp2A-29B	0.0%	0.0%	0.0%	5.40%	0.0%	5.5%
CG1970, isoform B, GN=ND-49	0.0%	0.0%	0.0%	7.20%	0.0%	0.0%
CIN85 and CD2AP orthologue, GN=cindr	0.0%	18.1%	0.0%	0.00%	0.0%	0.0%
Paramyosin, GN=Prm	0.0%	9.6%	0.0%	0.0%	0.0%	8.6%
Clathrin heavy chain, GN=Chc	0.0%	1.7%	0.0%	0.0%	3.0%	14.1%
Cheerio, isoform M, GN=cher	0.0%	3.2%	0.0%	0.00%	0.0%	8.9%
AP-2 complex subunit alpha, GN=AP-2alpha	0.0%	3.2%	0.0%	0.00%	0.0%	8.4%
Adaptor protein complex 1/2, beta subunit	0.0%	3.3%	0.0%	0.00%	0.0%	4.6%

Figure 4.B Biochemical identification of Fon-interacting proteins from larval lysates.

(A-B) Coomassie-stained SDS-PAGE gel of purified Fon-Fc protein bait pulldown with analyzed conditions. (A) Pulldowns of either Protein A bead incubation with WT larval lysates alone (control) or co-incubation of beads bound to tagged, purified Fon-Fc with WT larval lysates (Fon-Fc experiment). (B) Purified Fon-Fc is heavily glycosylated and runs around ~110-120 kDa with faint lower bands showing weak degradation species. (C) Table of putative Fon binding proteins following mass spectrometry analysis. Three biological replicates of control and experimental pulldowns were analyzed and compared to isolate proteins significantly enriched in experimental lanes. Values listed for each condition are percent coverage. (D) Western blot of Fon-Fc binding to mass spectrometry candidate, PPO1. (E) Western blot of Fon-Fc binding to mass spectrometry candidate, Tig.

Mass spectrometry experiments also identified phenoloxidases PPO1 and PPO2 as putative Fon interactors (Figure 4.2C,D). It remains unknown why loss of *fon* results in the MAS-patterned melanization, but the intersection between this immune response and the maintenance of muscle attachment was a vital piece of evidence in pursuing the relationship between immune signaling and muscle damage. In addition to the MAS, we have also observed melanin accumulation along the dorsal vessel (data not shown; see Figure 3.1 for MAS phenotypes). Therefore, we observe the accumulation of melanin at sites where Fon-GFP normally localizes (also see Future Directions, Figure 4.5). We know that hemocytes are recruited to the sites of damaged tissues in both wounding and a loss of tissue integrity such as muscle detachment (Figure 3.1G-I). Our analysis used a universal hemocyte marker, Hemese, so we cannot identify specific hemoycte subtypes being recruited to MASs. However, it stands to reason that the presence of melanization at MASs implicates the recruitment of crystal cells to sites of damage similar to wounding events.

Reports of patterned melanization are relatively rare in comparison to the breadth of mutant melanin phenotypes described in *Drosophila* literature [13]. Many of these examples are mutations to inhibitors of the melanization pathway that localize in a tissue-specific manner in comparison to those exhibiting melanotic tumors within the hemocoel [14, 15]. One reasonable explanation for melanization at mutant *fon* MASs is for strengthening of weakened ECM. Formation of the hemolymph clot includes two phases: 1) formation of the soft clot through the binding and crosslinking of ECM proteins and 2) hardening of the clot through the deposition of melanin. Presumably, recruitment of hemocytes occurs in the response to a signal released by damage muscle. Whether this

is the ‘damage signal’ sufficient to induce the full complement of immune responses or restricts activation to the mobilization of hemocytes remains to be determined. Further investigation into specific regions of tissues could provide an explanation to both the utility and novel signals used in the initiation of the immune response.

Do the mechanisms coordinating muscle homeostasis and innate immunity change throughout development?

Drosophila larval muscles must undergo extensive and rapid growth of muscle tissue making this a useful system for probing the molecular details of tissue maintenance. We decided to look earlier in development during embryonic muscle development to distinguish whether the function of Fon was restricted to larval maintenance. Embryonic muscles are fully formed in stage 16 and persist throughout the larval stage until undergoing remodeling during pupal morphogenesis to fit the adult body plan.

We see the incorporation of Fon-GFP into embryonic MASs prior to the end of muscle development (Figure 4.5C). Similar to larval muscle fillets, loss of *fon* in embryos results in weakened muscle attachments which present as spindle-shaped MASs or detached muscles (Figure 4.3G, Figure 4.5B). Alternatively, overexpression of Fon-GFP during embryogenesis also caused muscle defects, although the severity of these phenotypes fell in the mild-moderate categories unlike loss-of-function embryos which demonstrate more severe levels of detachment (Figure 4.3G). Overexpression and loss of Fon also causes disruptions to muscle patterning including muscles which are missing or target to the incorrect tendons resulting in aberrant muscle attachments (Figure 4.3H). Therefore, alterations to Fon levels during embryogenesis have deleterious effects which compromise embryonic muscle structure.

Assuming that Fon is expressed in a fat body-specific manner as seen in larval stages, we used *ppl*-GAL4 to drive overexpression in the above studies. We also attempted to ubiquitously overexpress Fon-GFP using the weak driver, *da*-GAL4, but this manipulation proved to be highly lethal during embryogenesis. One explanation for this lethality could be the effect of overexpression on epithelial integrity leading to the epithelial blebbing phenotype described in Figure 4.3I-K). In an effort to understand the time point at which embryonic lethality was occurring in *da> Fon-GFP* embryos, we analyzed embryos at stages proceeding the completion of muscle development. Visualizing Fon-GFP staining, we noted that the epithelial surface of embryos resembled *armadillo* (*arm*; β-catenin) mutants [16]. In embryos overexpressing Fon-GFP ubiquitously, we see that regions with intact epithelia have Arm outlining cells (Figure 4.3J), whereas regions where Arm localization has been lost corresponds to a loss of epithelial integrity and blebbing from the epithelium (Figure 4.3K). This is most likely due to the ‘soaking up’ or premature binding of Fon to a crucial protein involved in epithelial adhesion. The misexpression of Fon at early stages disrupts the developmental program, although the identification of the specific protein or protein complexes leading to the re-allocation of Arm may provide new insight into Fon binding partners during embryogenesis or larval stages.

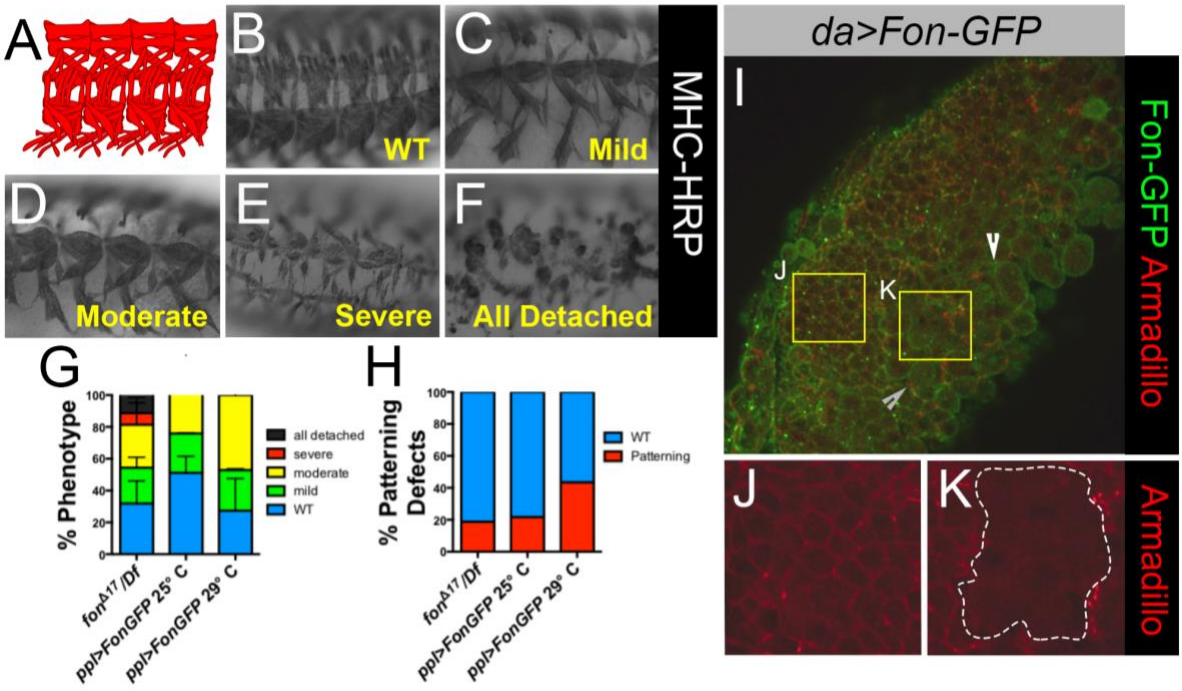


Figure 4.C Fon is essential during embryonic muscle development

Characterization of muscle phenotypes induced by removal or increased levels of Fon throughout embryogenesis. (A) Schematic of embryonic muscle hemisegments. (B-F) Phenotypic groupings used in the quantification of embryonic loss-of-function and overexpression genotypes. (C) Mild muscle defects are typified by slightly narrowed MASs, but no muscle detachment. (D) In the moderate class, MASs are narrowed to a spindle shape and low levels of muscle detachment occur. (E-F) Severe muscle defects include missing muscles and the detachment of the majority of visible muscle. If all present muscles are detached, these embryos were classified in a more extensive category, 'all detached'. (G-H) Quantification of detachment-based phenotypic classes and patterning defects seen in *fon* loss-of-function and overexpression via the primarily fat-body driver, *ppl*-GAL4. (I-K) Ubiquitous overexpression of Fon-GFP (green) in early embryogenesis causes lethality and an epithelial blebbing phenotype (arrows, Armadillo; red). (J-K) Region of epithelia with intact epithelial cells (J) compared to a region of compromised epithelium (K). The dotted white outline differentiates normal Armadillo staining from loss of integrity in the epithelial layer.

In addition to the role of Fon in somatic muscle development, we also discovered that Fon is also involved in larval cardiac development. In both external visualization and ventral dissection of *Drosophila* larvae, Fon-GFP is localized along the dorsal vessel (Figure 4.4A). Upon closer examination, Fon-GFP (green) can be found on pericardial

cells (pc) and at several points of cellular attachment such as the insertions of cardiomyocytes (solid arrow) and the attachment of alary muscles (lined arrow) to the dorsal vessel (Figure 4.4B). Because loss of *fon* altered ECM integrity in somatic muscle attachments, we wanted to look at cardiac ECM structure. As expected, cardiac ECM (pericardin, green) is reduced and disorganized around a thinned dorsal vessel compared to *WT* dorsal vessel morphology (Figure 4.4C-D'). Not surprisingly, destabilization of ECM leads to altered cardiac outputs. Normal cardiac BPM is increased in either loss or overexpression of *fon* (Figure 4.4E). This increase in pumping rate could be explained as a compensatory mechanism for decreased efficiency in larvae with altered Fon levels.

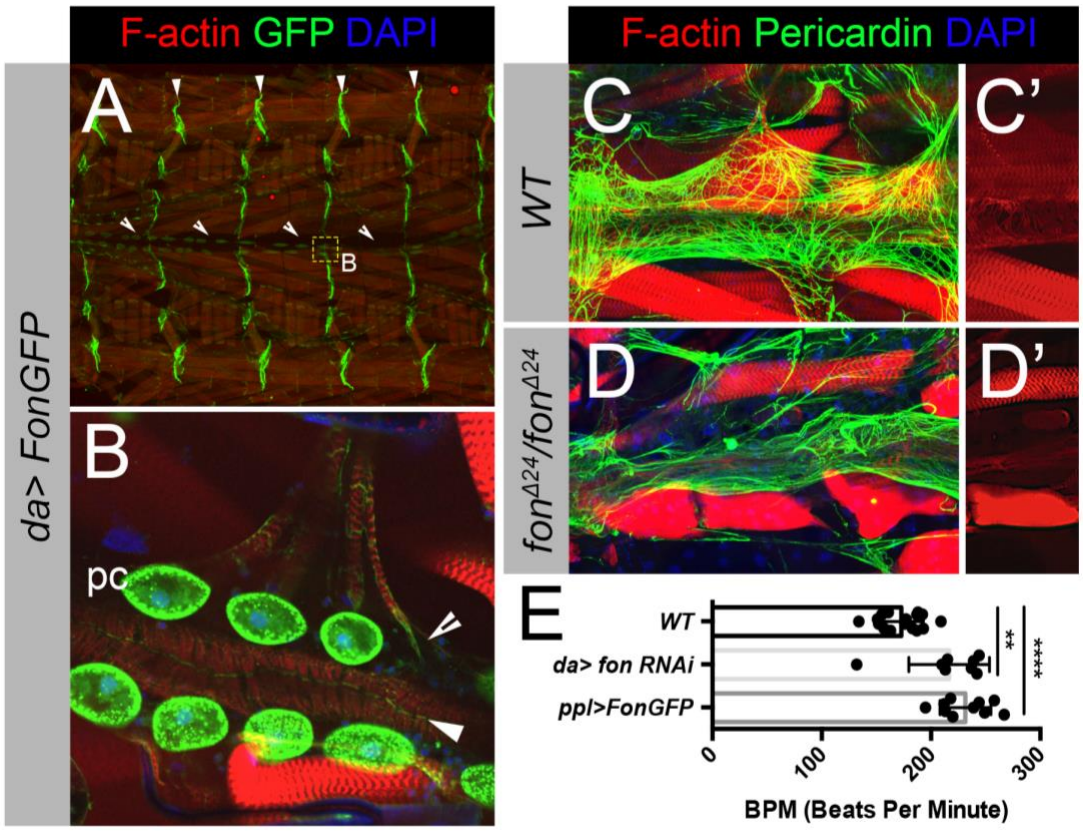


Figure 4.D Fon has a role in cardiac muscle development.

(A-B) Localization of Fon-GFP (green) within larval cardiac muscle (F-actin; red). (C-D') Larval dorsal vessel and cardiac ECM in of *WT* in comparison to *fon* mutants. (A)

Ubiquitously overexpressed Fon-GFP localizes to MASs and the dorsal vessel (solid arrows) in ventrally dissected L3 larvae. (B) High magnification image of the dorsal vessel and associated cardiac structures within the boxed region of panel A. Fon-GFP strongly collects on the surface of pericardial cells (pc). Like the trend seen in somatic musculature, Fon-GFP is localized to cardiac attachments such as contacts between cardiomyoctyes (solid arrow) and the junction of dorsal vessel and alary muscles (lined arrow). (C-C') *WT* dorsal vessel is surrounded by a meshwork of proteins forming the cardiac ECM (pericardin; green). Normal dorsal vessels feature a consistent diameter and actin patterning (F-actin; red). (D-D') Loss of *fon* leads to a reduction in ECM and a loss of structural integrity in the cardiac ECM. Morphological changes to the dorsal vessel including thinning and disruptions in cardiomyocytes are also observed.(E) Quantification of heart rate as a functional measurement in selected genotypes. Mean ± SD; *P*-values determined via Kruskal-Wallis statistical test: ** *P* <.005; **** *P* <.0001.

The above data show that Fon is a necessary factor throughout early muscle development and have been compiled in Figure 4.5. Early muscle development is focused on the formation and maintenance of musculature that persists through larval development. Null *fon* alleles result in pupal lethality [17, 18]. We wanted to determine whether Fon had a role in adult development if we bypassed pupal lethality using genetic tools. We turned to the temperature-dependent GAL-UAS system to knockdown *fon* using RNAi after pupal development had finished. Knocking down *fon* during adult muscle development produces no obvious defects in tissue morphology (Figure 4.5G, H). Overexpression of Fon-GFP does not seem to affect adult muscle integrity either (Figure 4.5I). However, similar to the localization of Fon-GFP in embryonic and larval muscle, Fon-GFP localizes to points of attachment in the adult thorax collecting along the cuticle and in between the flight muscles (Figure 4.5I). This suggests that although Fon is essential in embryogenesis and larval muscle maintenance, Fon is dispensible in the maintenance of adult muscle morphology.

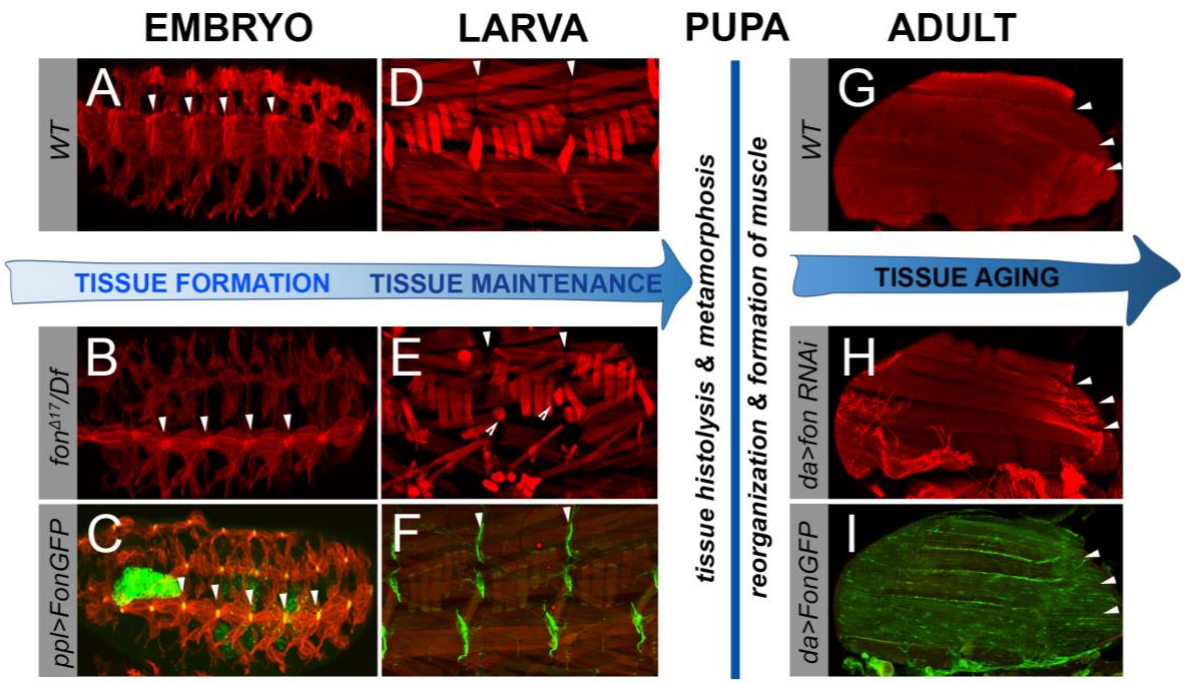


Figure 4.E Profile of Fon throughout Drosophila muscle development.

(A-C) *WT* and *fon* loss-of-function or overexpression (UAS-Fon-GFP; green) phenotypes during embryonic muscle development following the completion of muscle formation in st. 16 embryos (F-actin; red). (D-F) L3 larval muscle fillets in the presence of normal *fon* and the effects of altered expression levels. (G-I) Adult muscle development is not affected by perturbation to Fon. At all stages of *Drosophila* development, *WT* muscles appear rectangular and are strongly anchored at MASs (A, D, G; solid arrows). (B-C) Both removal and overexpression of *fon* leads to weakened or spindle-shaped MASs (solid arrows) and a ranging levels of detachment during embryogenesis (see Figure 4.1). During embryonic development, Fon-GFP clearly localizes to the fat body and MASs. (D) Embryonic muscles persist through third instar (L3) larval development (red). (E) Loss of *fon* destabilizes MASs resulting in detached muscles (lined arrow?). (F) Fon-GFP expressed in the fat body accumulates at indirect and direct attachments sites. Overexpression of *fon* does not produce notable effects on muscle morphology in the larval stage. (G) Following histolysis and remodeling during pupariation, six flight muscles (red) occupying the thorax form attachment sites to the cuticle at either end and along muscle contacts (solid arrows). (H-I) Adult thorax bissections show that neither knockdown of overexpression of *fon* has visible effects on adult musculature. Similar to previous stages, Fon-GFP is localized to sites of attachment when driven by the ubiquitous driver, *da*-GAL4. Solid arrows indicate attachment sites; lined arrows denote detached muscles.

In Figure 4.1B, we outline a model for immune activation in cases of larval muscle stress. In the future, we will be exploring whether this model applies to cases of adult

muscle stress. First, do we see immune activation following muscle stress? Other groups have found that *Drosophila* muscle is an essential tissue for the activation of immune signaling, the production of AMPs, and survival of infection [19]. Adult muscle is an ideal system for looking at issues of tissue aging and disease due to the length of this stage which lasts well beyond the hours and days of embryogenesis and larval development.

Secondly, if adult muscle stress elicits an immune response, does the mechanism for tissue communication change during immune activation? The adult tissues corresponding to our model have be reorganized and may or may not utilize the same signaling pathways, which could change how immune-responsive tissues communicate at this stage. Furthermore, the ligands and immune regulation of these tissues could be altered resulting in differential immune responses compared to those observed in a loss of larval muscle homeostasis. Last, can the immune response be modulated or suppressed in response to muscle stresses. We show that an overexpression of AMPs in muscles sensitized through a *fon* mutation enhance muscle detachment (Figure 3.4). This observation in conjunction with data from the *Drosophila* neuroscience field as well as clinical descriptions of chronic inflammation in human patients with muscle disease supports the idea that the protective effects of an immune response can transition to damage-inducing over time. Using *Drosophila* adult muscle and the variety of genetic tools already utilized in our larval studies we can more accurately dissect long-term effects of our tissue communication network. Ideally, a more comprehensive understanding of tissue communication over development will yield information about how to balance the beneficial short-term effects of immune responses while minimizing the harmful effects of chronic inflammation.

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Manhattan
KS 66506

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Appendix B - Chapter 2 Supplemental Materials

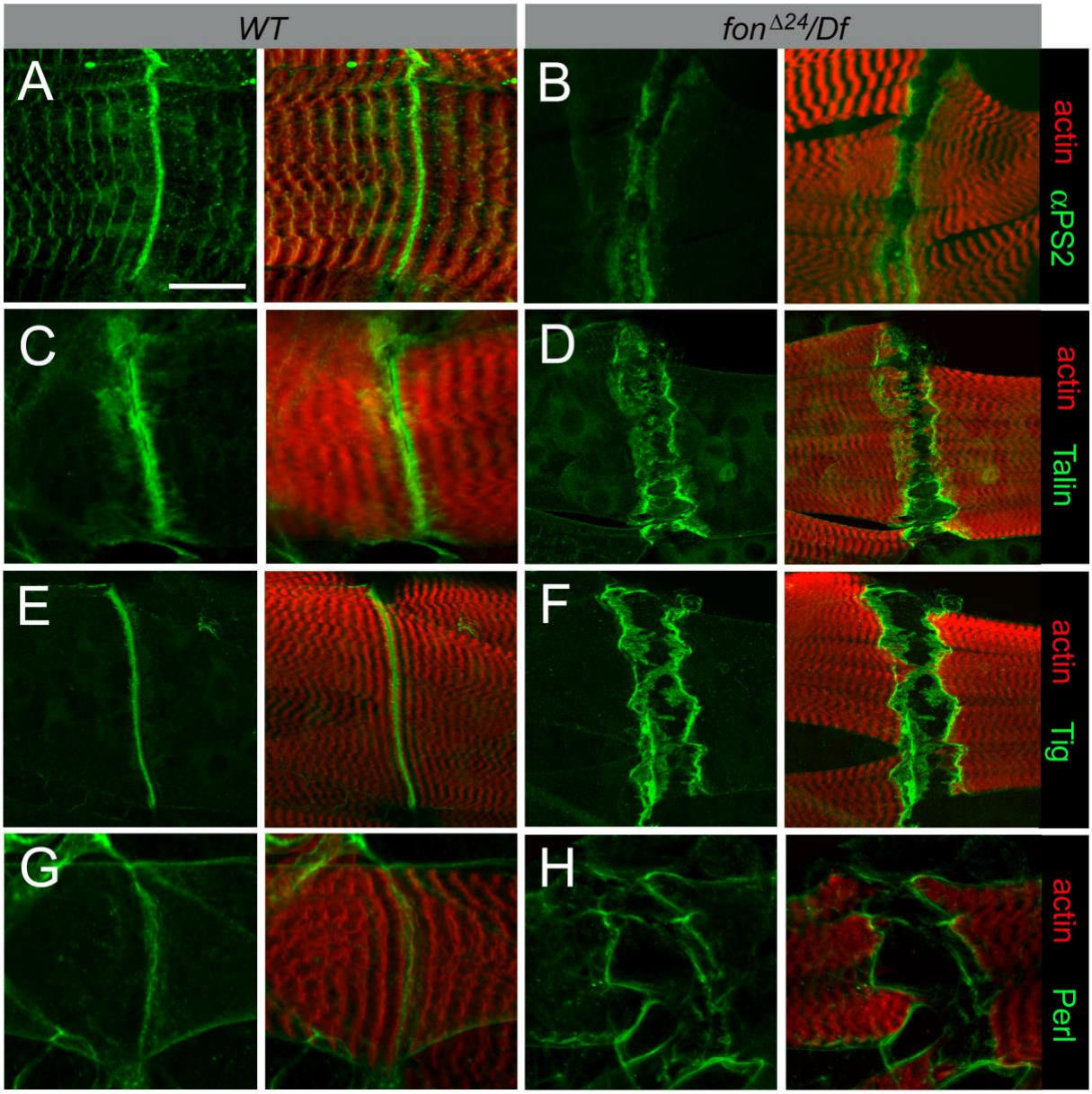

Figure B.1 Localization of known MAS proteins.

(A-J') Confocal micrographs of *WT* (A,C,E,G, I,) or *fonΔ17/Df* mutants (B,D,F,H,J) immunostained with antibodies against the indicated proteins. αPS2 integrin (A,B), Talin (C,D), Tig (E,F), Perl (G,H) and Vkg-GFP (I,J) all accumulate normally in WT or *fon* mutants. (K-L'). *WT* (K) or *tigΔX/tigA1* mutants (L) both contain Fon-GFP at muscle attachment sites. Scale bars, 50 μm for A-H.

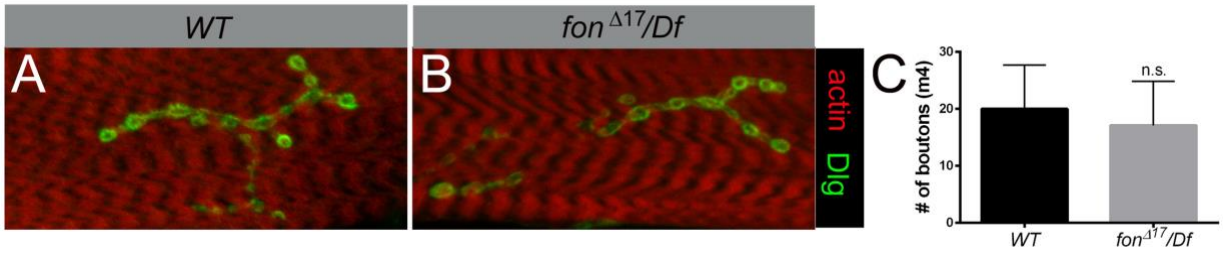

Figure B.2 Bouton number is not changed in *fon* mutants.

(A-C) The relative number of boutons (Dlg; green) that innervate muscle 4 (F-actin; red) is similar in *WT* (A) or *fon*$^{\Delta 17}$*/Df* (B) mutants as quantitated (C) (28 ≤ n ≤ 40 for each genotype).

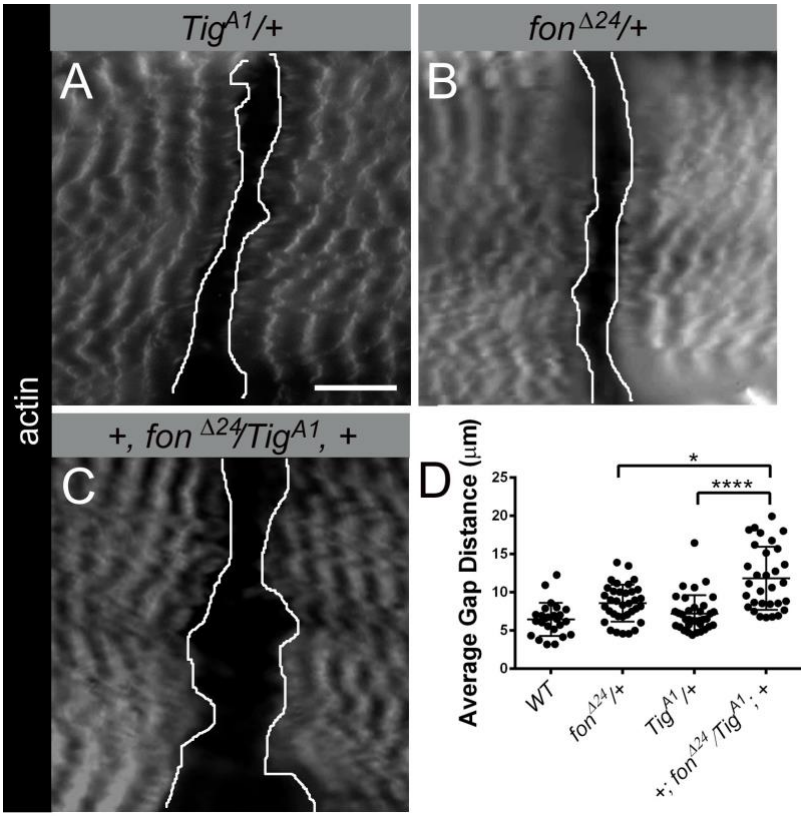

Figure B.3 Decrease in muscle attachment integrity in +/*fon*, *Tig*/+ trans-heterozygotes.

(A-C) Confocal micrographs of the junction between dorsal oblique muscles 9 (m9). White lines demarcate the edge of muscle ends used to determine gap distance. (D) Quantification of the gap distances. P-values: ****p<0.001; *p<0.05. Mean = +/- SD Scale bars, 20 μm for A-C.

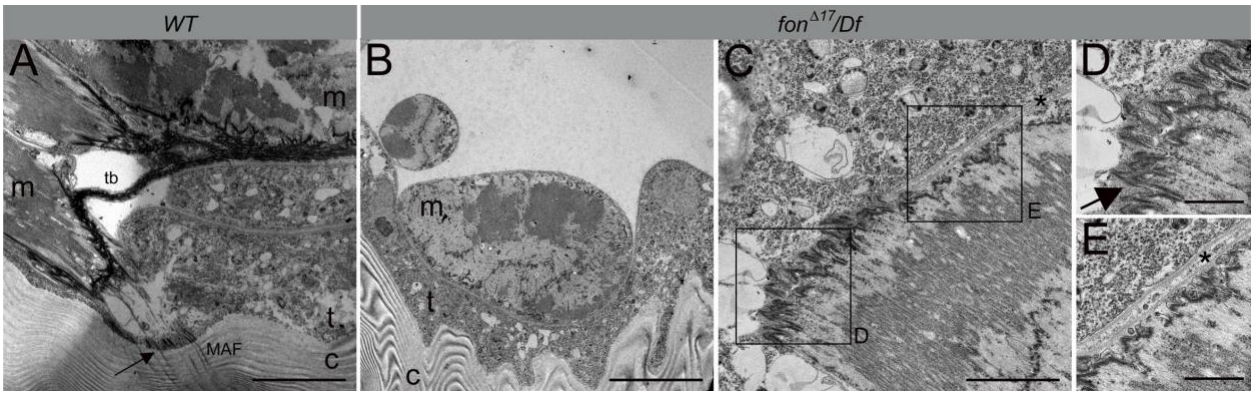

Figure B.4 TEM images of the MAS in control or *fon* mutants.

(A) In a *WT* example, muscles (m) are attached to a tendon cell (t) via a tendon belt (tb) of ECM-rich material. Muscle attachment fibers (MAF) traverse the cuticle (c) and are associated with hemiadherens junctions located at the apical region of the tendon cell. (B) A completely detached muscle (m) lies adjacent to its tendon (t) cell. Note the lack of membrane interdigitation and matrix material accumulation. (C-E) A partially detached muscle exhibits normal, finger-like processes at the muscle-tendon interface in one region of the MAS (C,D), while an adjacent area lacks extensive surface contacts and a loss of ECM material (D,E). Scale bars, 10 μm for A,B; 5 μm for C; 1 μm for D,E.

Table 4 Candidates from genetic screen with abnormal pupal morphology.

BDSC number[a]	Abbreviated Genotype	Putative gene disrupted	Pupal morphology	Muscle morphology phenotype in L3 larvae
BL2162[b]	*pb[6] pb[12] Antp[72j]/TM3, Sb[1]*	*Antp*	long, thin	None observed
BL3767	*N[spl-1] sn[1] l(1)EN6[1] bb[*]/Binsc, Sxl[f1] oc[1] ptg[1]*	*l(1)EN6*	long, thin	None observed
BL4640	*l(1)1Fc^{6}/FM7a*	*l(1)1Fc*	thin	None observed
BL4888	*wg[Gla-1] Bc[1]/CyO, P{w[+mW.hs]=Ubi-GFP.S65TPAD1*	unknown	curved	None observed
BL10134	*P{w[+mC]=lacW}Trxr-1[G0481]/FM7c*	*Trxr*	long	None observed
BL10773	*P{w[+mC]=lacW}E7-3-63/TM3, Sb[1]*	unknown	long, thin	None observed
BL12185	*P{lacW}Rho1[k02107b]/CyO*	*Rho1*	thin	None observed
BL17972	*PBac{w[+mC]=RB}Eno[e01615]/CyO*	*Eno*	thin	None observed
BL26635	*P{w[+mC]=EP}rtv[G17427]/FM6*	*Rtv*	thin	Muscle detachment
BL29262	*Mi{ET1}fon[MB11923]/SM6a*	*Fon*	long; curved	None observed
BL29696	*P{w[+mC]=EP}Cchl[G6848]/TM3, Sb[1] Ser[1]*	*Cchl*	thin	None observed
BL30708	*Mi{y[+mDint2]=MIC}Pka-R1[MI00323]/TM6B, Tb[1]*	*Pka-R1*	long, thin	None observed
BL35861	*Mi{y[+mDint2]=MIC}plx[MI02460]/TM3, Sb[1]*	*Plx*	curved	None observed
BL35888	*Mi{y[+mDint2]=MIC}Ten-m[MI02844]/TM3, Sb[1] Ser[1]*	*Ten-m*	long	None observed
BL38104	*coro[ex8]/CyO*	*coro*	curved	None observed

[a] BDSC = Bloomington *Drosophila* Stock Center
[b] line discarded from BDSC

Table 5 Primers used for qPCR.

Gene	Forward primer	Reverse primer	cDNA dilution
rp49	5'-GCCCAAGGGTATCGACAACA-3'	5'-GCGCTTGTTCGATCCGTAAC-3'	1:100
fon	5'-GTCCCCACTTCCGAGTATTACA-3'	5'-CCTCGTGATGAACGTAACGCT-3'	1:25
Tig	5'-TGGAACACTTCAAGGTGTGG-3'	5'-TGATACCCGGATTGTTCACG-3'	1:25
Tsp	5'-ACCTCGTCGAAAGTTGCACA-3'	5'-GGATCGGATGGTGCTTGTCT-3'	1:100
Gel	5'-GAAACACGACTATTCCAGGTCAA-3'	5'-TGATAGCCTTTAGCTTCTCAACG-3'	1:100
Lsp1γ	5'-TGAGCCTCTAATGTTCGAGGA-3'	5'-AAAAACTCTCCCTTGGGAAGC-3'	1:100

Appendix C - Chapter 3 Supplemental Materials

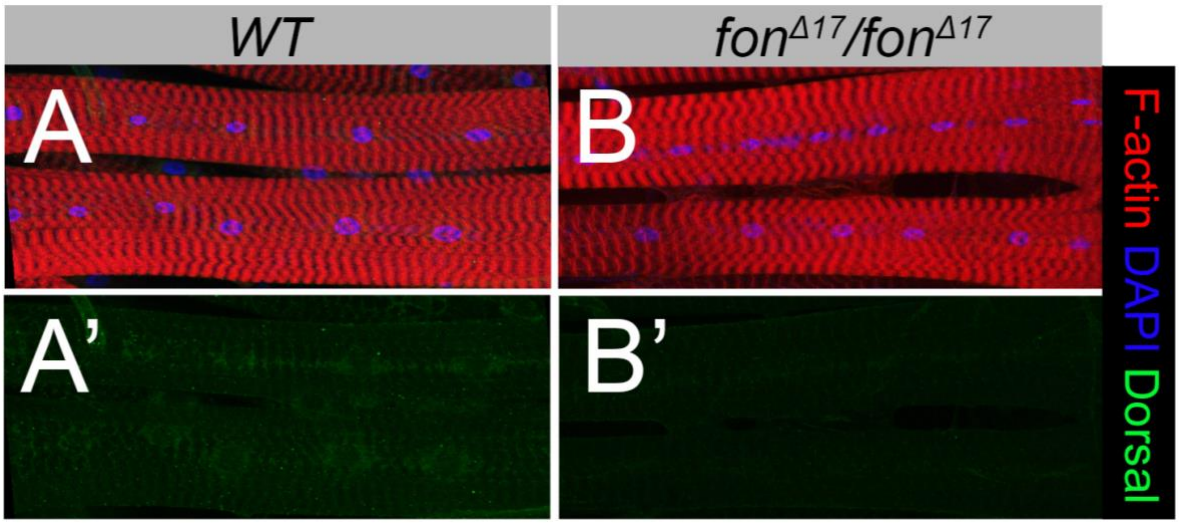

Figure C.1 Toll signaling is not activated in the muscles of *fon* mutants.
(A-B') Dl expression (green) in L3 ventral longitudinal muscles (F-actin; red) of *WT* and *fon* mutants. (A-A') Dorsal is faintly detected in *WT* larval muscles with stable MASs. (B-B') Localization of Dl within detached muscles is difficult to detect. However, in muscles that remain attached loss of *fon* does not induce increasing levels of Dl in either the cytoplasm or nucleus of muscle tissue.