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UNIVERSITY OF CALIFORNIA SAN DIEGO

SAN DIEGO STATE UNIVERSITY

The role of MEF2 and the invertebrate Ortholog of *Mkx*, CG11617, in myogenesis

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Elizabeth Munguia Trujillo

Committee in charge:

San Diego State University

Professor Richard M. Cripps, Chair
Professor Sanford I. Bernstein
Professor Ricardo M. Zayas

University of California San Diego

Professor Amy Kiger
Professor Karl Willert

2024

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Chair

University of California San Diego

San Diego State University

2024

DEDICATION

To my loving husband Ronnie,

My unwavering source of strength. Throughout the highs and lows of my Ph.D. journey, you stood by me with boundless love and unwavering support. You made sacrifices without hesitation, putting my dreams and aspirations above your own so that I could pursue my academic goals. Your sacrifices have not gone unnoticed, and I am forever grateful for your profound love and selflessness. This achievement is as much yours as it is mine.

To my sweet boy Ezra,

Your arrival into my life brought a newfound sense of purpose and determination, propelling me to achieve my dreams. Your sweet smile and gentle presence reminded me every day of the world I want to create for you—one filled with love, learning, and limitless possibilities.

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LIST OF ABBREVIATIONS

IFMs	Indirect flight muscles
KD	Knockdown
MEF2	Myocyte enhancer factor-2
Mkx	Mohawk

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Chapter 2 is currently being prepared for submission for publication. It is co-authored by Elizabeth M. Trujillo and Richard M. Cripps. The dissertation author was the primary investigator and author of this chapter.

Chapter 3, in full, contains unpublished material co-authored by Elizabeth M. Trujillo and Richard M. Cripps. The dissertation author was the primary investigator and author of this chapter.

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ABSTRACT OF THE DISSERTATION

The role of MEF2 and the invertebrate Ortholog of *Mkx*, CG11617, in myogenesis

by

Elizabeth Munguia Trujillo

Doctor of Philosophy in Biology

University of California San Diego, 2024
San Diego State University, 2024

Professor Richard M. Cripps, Chair

The study of muscle development and the transcription factors involved in this process is important for advancing our understanding of muscle biology, physiology, and pathology, with potential implications for human health, performance, and disease

treatment. The Myocyte enhancer factor-2 (MEF2) and the invertebrate ortholog to Mohawk (Mkx), CG11617, transcription factors play crucial roles in orchestrating muscle differentiation and fiber type specification. RNA-sequencing (RNA-seq) and immunofluorescence reveal that overexpression of MEF2 activates myogenesis in imaginal wing disc myoblasts by inducing sarcomere-like structures, significantly upregulating muscle gene expression, especially those important for myofibril formation and function, and primarily activating the embryonic and larval myogenic program. This broad activation of the embryonic and larval myogenic program suggests that additional regulatory factors are necessary to promote adult muscle-specific programs.

The *Drosophila* CG11617 may be essential in promoting the adult muscle-specific program based on previous research focused on the mammalian ortholog, *Mkx*, which promotes slow fiber type specification. Our study found that *CG11617* is essential for muscle development and proper myofibril formation, and it has nuclear localization in indirect flight muscles (IFMs) and nuclear and cytoplasmic localization in jump muscles. *CG11617* knockdown mutants also display lethality at the pharate adult stage, with disorganized IFM myofibrils and the absence of jump muscles, indicating its critical role in muscle structure integrity.

Furthermore, the RNA-sequencing analysis of *CG11617* knockdown mutants reveals significant upregulation of *upheld*, which encodes Troponin T, a gene crucial for muscle structure and function. Overexpression of Troponin T leads to muscle degeneration and dysfunction, potentially explaining the severe phenotypes observed in *CG11617* knockdown mutants. Despite these insights, the precise mechanistic role of *CG11617* in muscle fiber specification remains unclear.

Our findings highlight the multifaceted roles of MEF2 and CG11617 in muscle development and reveal the need for further research to elucidate the regulatory mechanisms governing skeletal muscle differentiation and function.

INTRODUCTION

Significance

The importance of understanding the intricacies of skeletal muscle fiber differentiation and specification, as well as the impact of muscle diseases on muscle function, cannot be overstated. Although treatments exist to alleviate specific symptoms associated with muscular dystrophies, including muscle weakness, movement impairments, and twitching, some interventions, such as immunosuppressant medications, pose heightened risks for immunocompromised patients. Therefore, gaining insights into how transcription factors, such as *Drosophila* CG11617 and *Mef2*, govern muscle fiber development not only presents an opportunity to advance our understanding of mammalian muscle biology but also holds the potential to address critical clinical challenges in the field of medicine.

Establishing proper myofiber development is essential for the functional integrity of the skeletal system

Origins of skeletal muscle

In vertebrates, the origins of skeletal muscles stem from the nascent paraxial mesoderm, which subsequently gives rise to the presomitic mesoderm, further subdividing into an immature posterior and a committed anterior region (Chal and Pourquié, 2017) (Figure 1A). The committed anterior region gives rise to somites, from which several different cell types emerge, including myotome cells (Chal and Pourquié, 2017) (Figure 1A).

The muscle progenitor cells (MPCs) are myotome cells that undergo differentiation to become myoblasts. These myoblasts populate the myotome and

proliferate to increase their numbers, undergo a series of differentiations, elongate to form myocytes, and subsequently merge to form multinucleated muscle cells known as myotubes (Deries and Thorsteinsdóttir, 2016) (Figure 1B). The myotube, considered an immature myofiber, contains multiple cell nuclei that are arranged in the middle of the myotube cell. Once the myofibers, also called muscle fibers, mature, the nuclei migrate to the periphery of the myofiber until they are anchored by an astral microtubular network below the plasma membrane (Roman and Gomes, 2018; Deries and Thorsteinsdóttir, 2016) (Figure 1B). The myoblasts that do not form myotubes undergo differentiation into satellite cells, also referred to as myosatellites, which can either remain in a quiescent state or re-enter the cell cycle to become myoblasts responsible for muscle repair (Deries and Thorsteinsdóttir, 2016). Muscle formation is a complex biological process that requires the precise and temporally coordinated orchestration of key regulatory events.

Muscle development stages

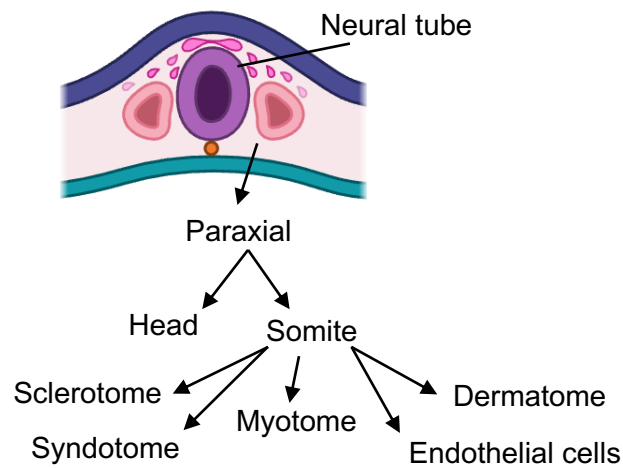
Conceptually, the main stages of muscle development are proliferation (MPCs to myoblast), differentiation (myoblast to myocyte), fusion (myocyte to myotube), and maturation (myotube to myofiber) (Knight and Kothary, 2011) (Figure 1B). Research has shown that the transcription factors Pax7 and Pax3 promote MPC proliferation and myogenic lineage commitment during muscle development (Olguín and Olwin, 2004; Buckingham & Rigby, 2014) while MyoD and Myf5 initiate myoblast differentiation (Weintraub and Tapscott, 1991), and myogenin, downstream of MyoD, regulates myoblast fusion into multinucleated myofibers (Cheng et al., 1993; Rawls et al., 1998) (Figure 1B). Downstream of these regulatory factors, Myomaker and Minion are crucial

transmembrane proteins necessary for myofiber formation (Millay et al., 2013; Leikina et al., 2018), and Mef2 also controls fusion-related gene expression to orchestrate myoblast fusion into multinucleated myofibers (Potthoff and Olson, 2007) (Figure 1B). MRF4 synergizes with MEF2 to induce myogenic differentiation (Molkentin et al., 1995), accumulating to levels sufficient to release the repression of ZEB, a vertebrate homolog of *Drosophila* Zfh-1, and allow for the activation of transcription (Postigo & Dean, 1997) (Figure 1B).

As we explore the intricate mechanisms governing muscle development, it is worth noting that Mohawk, the vertebrate ortholog of *Drosophila* CG11617, contributes to the determination of fiber types, enhancing our understanding of the functional diversity within the human skeletal muscle landscape (Anderson et al., 2012). Within the human skeletal muscle landscape, two primary fiber types are recognized: slow-twitch (Type 1) and fast-twitch (Type 2) fibers. Each of these fiber types play specific roles, allowing muscles to perform distinct functions (Scott et al., 2001). For example, Type 1 muscle fibers predominate in marathon runners while Type 2 muscle fibers predominate in sprinters (Schiaffino and Reggiani, 2012). Mohawk is known to promote Type 1 specification by repressing the transcription factor Sox6 during skeletal muscle differentiation (Anderson et al., 2012). A Mohawk null mutation in mice leads to the downregulation of myosin heavy chain 7 (Myh7) in differentiating myotubes validating its role in fiber type specificity (Anderson et al., 2012).

Despite having a strong understanding of the transcriptional control of general muscle differentiation, there is still much to learn about how individual muscle fibers are directed to assume fast or slow muscle fiber fates.

A



B

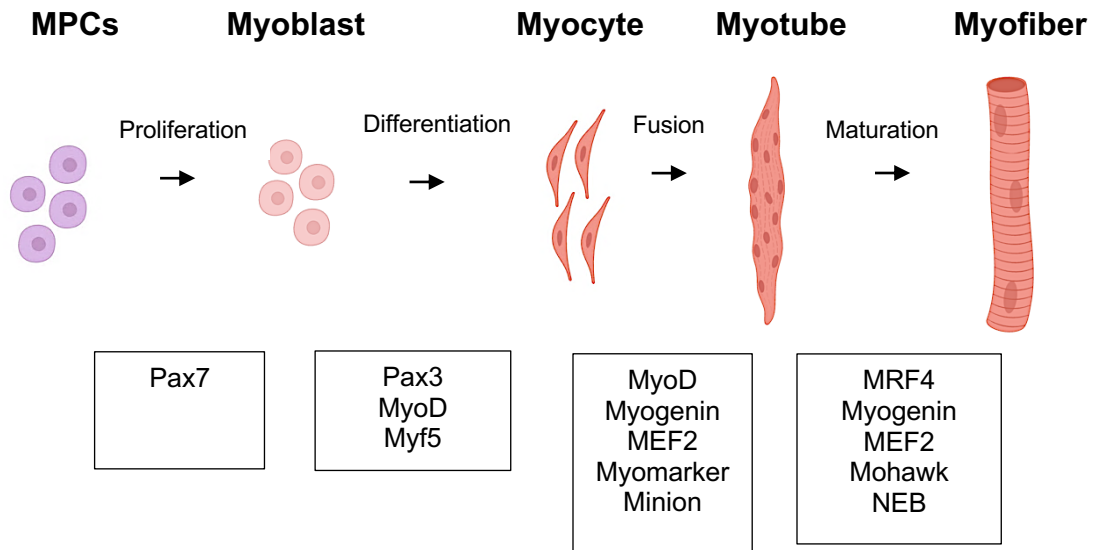


Figure 1. Origins of muscle development. (A) Schematic of the formation of the paraxial mesoderm. Adapted from Gilbert SF (2006). (B) Schematic of muscle cell differentiation. Adapted from Deries and Thorsteinsdóttir, (2016) & Knight and Kothary (2011). All images were created with BioRender.com

Myofiber structure

This complexity of muscle development extends to the structural level, where myofibers are composed of myofibrils, which, in turn, are structured from repeating contractile units known as sarcomeres that extend from z-disc to z-disc (Figure 2A). Sarcomeres consist of repeating arrays of thin actin and thick myosin filaments which gives skeletal muscles their characteristic striated appearance (Figure 2A). During muscle contraction, these thin actin and thick myosin filaments slide past each other in a complex physiological process known as the sliding filament theory of muscle contraction (Huxley and Niedergerke, 1954; Huxley HE, 1969). As the sarcomeres, within the myofibers shorten, myofibers contract. At the molecular level, muscle contraction depends upon the formation of cross-bridges, where myosin heads bind to the actin filaments. The binding event is initiated when calcium ions, released from the sarcoplasmic reticulum, bind to troponin situated on the actin filaments, resulting in the exposure of the actin-myosin binding sites (Sweeney and Stull, 1990; Ebashi and Endo, 1968). As the sarcomeres within the myofibers shorten, the myofibers contract, leading to movement and locomotion (Alberts et al., 2002; Huxley and Niedergerke, 1954; Hill AV, 1938). Despite having a strong understanding of the general mechanisms of muscle contraction, there is still much to learn about how individual myofibers develop their specific structural characteristics and functional roles.

Myofibril assembly and their sarcomeric proteins

Sanger *et al.* (2002) proposed a three-step model in which myofibrils assemble into skeletal muscle: premyofibrils, to nascent myofibrils, to myofibrils. First, actin filaments attach to alpha-actinin-rich z-bodies of mini sarcomeres while non-muscle

myosin II filaments, the vertebrate ortholog of the *Drosophila* zipper, localize between the z-bodies, creating mini-A bands (Figure 2B). Secondly, the premyofibrils begin to align with one another as titin, the vertebrate ortholog of *Drosophila* sallimus, and muscle-myosin II are recruited to form nascent myofibrils (Figure 2B). Thirdly, the z-bodies fuse to form the z-disc, creating mature myofibrils. At this stage, non-muscle myosin II is eliminated, and muscle-myosin filaments are aligned into A-bands (Figure 2B). The intricate process of myofibril assembly has been well studied; however, not all factors essential for the initiation of myogenesis have been fully identified.

Disruptions in myofiber development lead to fragile and damaged muscle fibers, associated with skeletal muscle diseases

Disruptions in myofiber development may lead to fragile and damaged muscle fibers, often associated with a spectrum of debilitating skeletal muscle diseases. Among these, Myotonic Dystrophy Type 1 (DM1) preferentially afflicts Type 1 muscle fibers over Type 2 (Krivickas et al., 2000). DM1 stems from the expansion of CTG trinucleotide repeats within the noncoding region of the DMPK gene and stands as the most common adult-onset form of muscular dystrophy, primarily affecting distal muscles within the human body (Bird TD, 2015). Krivickas *et al.* (2000) observed that in DM1, Type 1 muscle fibers exhibit many central nuclei, contrasting with the normal peripheral nuclei pattern. Moreover, Type 1 fibers experience profound atrophy and a pronounced reduction in force generation capabilities. As the disease advances, these compromised muscle fibers lead to debilitating weakness, followed by the loss of motor, vocal, and respiratory functions (Tews DS, 2002). Despite available treatments for ameliorating some symptoms associated with muscle diseases like DM1, therapeutic strategies aimed at stopping or reversing muscle atrophy remain nonexistent.

Vertebrate muscle development is a complex process that must be precisely orchestrated to ensure proper formation and the maintenance of muscle integrity throughout the individual's lifespan. There are several unanswered questions that can be addressed using a simple system such as *Drosophila melanogaster*. These questions include: What genetic pathways regulate muscle development? What are the cellular and molecular mechanisms underlying muscle differentiation and specification? How do mutations in muscle-related genes affect muscle formation?

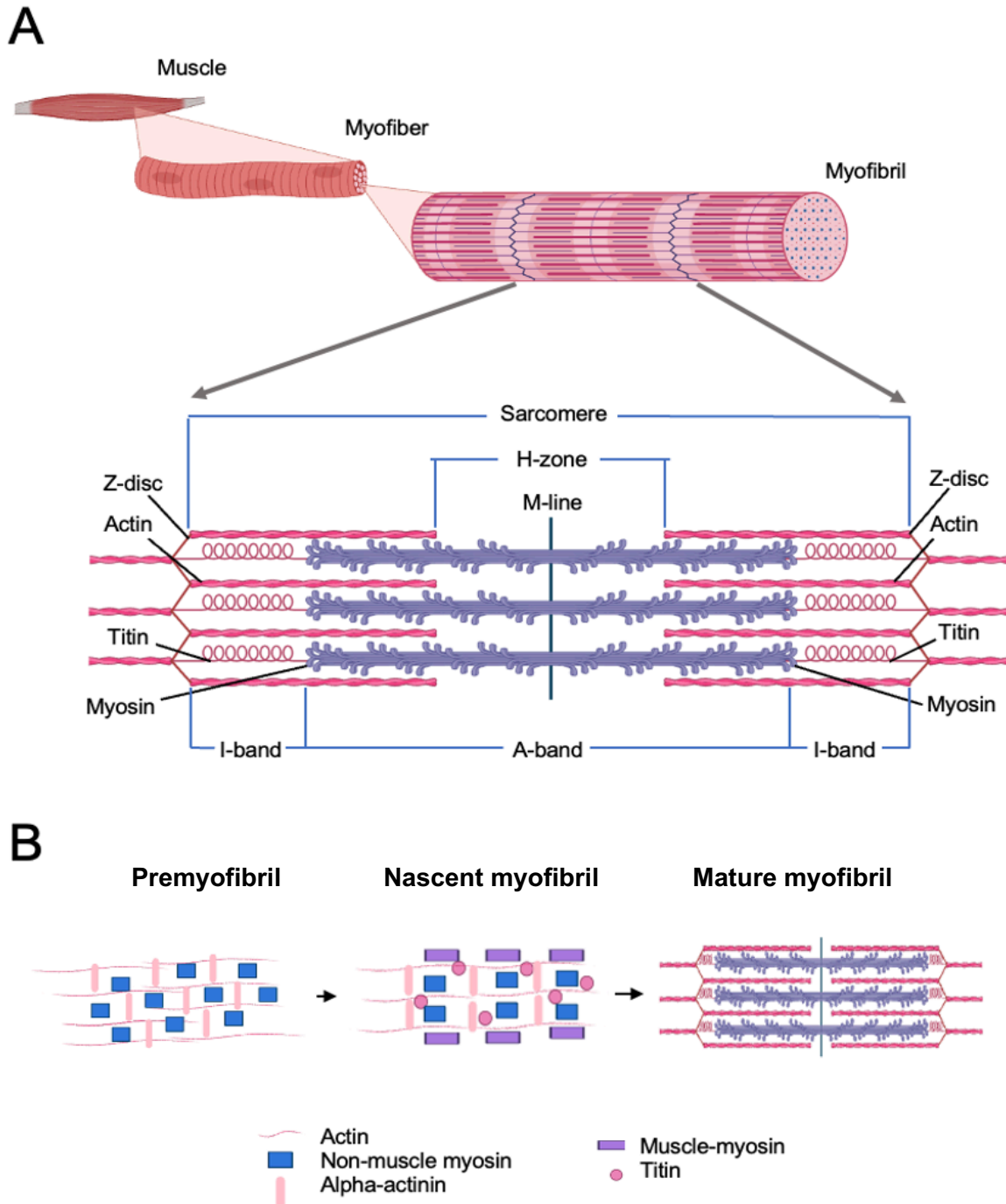


Figure 2. Myofibril composition and maturation. (A) Schematic of sarcomere structure. Adapted from Sanger et al. (2002) (B) Schematic of myofibrillogenesis three-step process. Adapted from White et al. (2007) & Sanger et al. (2002). All images were created with BioRender.com

***Drosophila melanogaster* as a model for muscle fiber differentiation and specification**

Drosophila melanogaster is a simple model organism that exhibits a single fiber type in flight muscles and two similar fiber types in jump muscles, unlike the mixture of fiber types characteristic of mammalian skeletal muscles (i.e. Type 1, Type 2a, Type 2b, Type 2x), where one type usually predominates (Brooke and Kaiser, 1970; Saltin and Gollnick, 1983; Pette and Staron, 2000). Additionally, *Drosophila* boasts shorter muscle development periods, a compact genome organized into four chromosomes, rapid sexual maturity within ten days, and prolific offspring production, facilitating large sample sizes for experimentation (Jennings BA, 2011).

Moreover, the substantial conservation of protein sequences between *Drosophila* and humans, encompassing approximately 40% identity among homologs and 80% to 90% identity within conserved functional domains, underscores the applicability of *Drosophila* in the study of muscle fiber specification and differentiation (Pandey and Nichols, 2011). This conservation extends not only to normal muscle development but also to the pathophysiology of skeletal muscle diseases (Jawkar and Nongthomba, 2020).

Reiter *et al.* (2001), who identified a remarkable 77% overlap between human disease genes and the *Drosophila* genome sequence through systematic BLAST analysis of 929 human disease genes, further accentuate the utility of *Drosophila* as a model organism for investigating muscle-related disorders. This suggests that *Drosophila* can serve as a valuable platform for the study of human disease genes and their functions.

Thoracic muscle development and differentiation in adult *Drosophila melanogaster* resemble that of vertebrates

The developmental and differentiation processes of thoracic muscles in adult *Drosophila melanogaster* closely parallel those seen in vertebrates. Unlike vertebrates, *Drosophila* undergoes two distinct phases of myogenesis: embryonic development which gives rise to larval muscles, and metamorphosis, which generates adult muscle fibers (Roy and VijayRaghavan, 1999). During early pupal stages, myoblasts migrate from imaginal discs, under the guidance of transcription factors, and this process culminates in the formation of physiologically diverse muscles that constitute the *Drosophila* thoracic muscular system (Lawrence, 1982; Bryantsev et al., 2012; Gunage et al., 2014; Vishal et al., 2020) (Figure 3). This complex system is comprised of fibrillar indirect flight muscles (IFMs), crucial in powering the wing stroke during flight (Bernstein et al., 1993; Spletter et al., 2018), and the tergal depressor of the trochanter muscle (TDT or jump muscle), responsible for the fly's jumping ability (Nachtigall and Wilson, 1967; Zumstein et al., 2004; Jaramillo et al., 2009). The IFMs, occupying a significant proportion of the adult thorax, are further categorized into dorsal-longitudinal muscles (DLMs; six pairs of medial fibers) and dorsoventral muscles (DVMs; three pairs of lateral muscles) (Bernstein et al., 1993; Fernandes and Keshishian, 1996) (Figure 3).

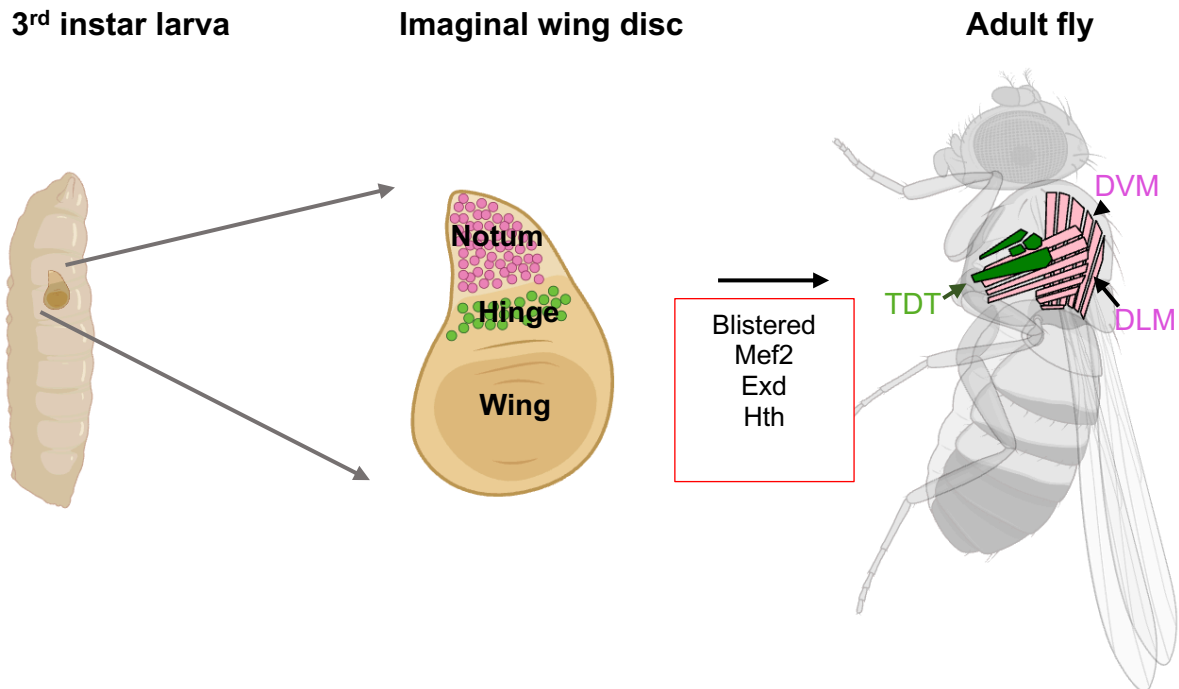


Figure 3. Schematic of the relationship between imaginal wing disc myoblasts and the adult thoracic muscle derivatives. Indirect flight muscles (IFMs) are composed of dorsal ventral muscles (DVM) and dorsal lateral muscles (DLM). Transcription factors are involved in the differentiation of myoblasts into thoracic muscles. All images were created with BioRender.com

Bryantsev *et al.* (2012) found that the transcription factors Extradenticle (Exd) and Homothorax (Hth) promote the specification of *Drosophila* flight muscles by regulating the expression of the flight muscle-specific actin gene Act88F, while the absence of Exd and Hth, or their co-expression with a Hox gene, may initiate tubular muscle fate. Interestingly, the mammalian orthologs of Exd and Hth, namely PBX and MEIS, respectively, have been shown to promote fast fiber fate in zebrafish and mice (Maves et al., 2007). In addition, DeAguero (2019) found that blistered (bs), the *Drosophila* homolog of Serum Response Factor (SRF), works with Exd and Hth to regulate muscle type differentiation of IFMs. When the gene *bs* is downregulated in all muscles, IFMs reveal immature and disorganized myofibrils, while other muscles are unaffected. These findings strongly suggest a conserved mechanism in muscle fiber specification, implying that *Drosophila* IFMs might employ the same mechanisms that dictate the development of fast-twitch muscle fibers in mammals.

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DISCLOSURES

The authors have no competing financial interests or other conflicts of interest.

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CHAPTER 1

Enhanced expression of the myogenic factor Myocyte enhancer factor-2 in imaginal disc myoblasts activates a partial, but incomplete, muscle development program

ABSTRACT

The Myocyte enhancer factor-2 (MEF2) transcription factor plays a vital role in orchestrating muscle differentiation. While MEF2 cannot effectively induce myogenesis in naïve cells, it can potently accelerate myogenesis in mesodermal cells. This includes in *Drosophila melanogaster* imaginal disc myoblasts, where triggering premature muscle gene expression in these adult muscle progenitors has become a paradigm for understanding the regulation of the myogenic program. Here, we investigated the global consequences of MEF2 overexpression in the imaginal wing disc myoblasts, by combining RNA-sequencing with RT-qPCR and immunofluorescence. We observed the formation of sarcomere-like structures that contained both muscle and cytoplasmic myosin, and significant upregulation of muscle gene expression, especially genes essential for myofibril formation and function. These transcripts were functional since numerous myofibrillar proteins were detected in discs using immunofluorescence. Interestingly, muscle genes whose expression is restricted to the adult stages were not activated in these adult myoblasts. These studies confirm a broad activation of the myogenic program in response to MEF2 expression and suggest that additional regulatory factors are required for promoting the adult muscle-specific program. Our findings contribute to understanding the regulatory mechanisms governing muscle development and highlight the multifaceted role of MEF2 in orchestrating this intricate process.

INTRODUCTION

Skeletal muscles are composed of myofibers organized into bundles, called fascicles. These myofibers are made up of myofibrils which, in turn, are structured from repeating contractile units known as sarcomeres that extend from Z-disc to Z-disc. Sarcomeres consist of overlapping arrays of thin actin and thick myosin filaments which gives skeletal muscles their characteristic striated appearance. As the sarcomeres within the myofibers shorten, the myofibers contract, leading to movement and locomotion (Alberts B et al., 2002; Huxley and Niedergerke, 1954; Hill AV, 1938). The proper functioning of skeletal muscles is vital to performing essential roles in respiration, stability, and movement. Many developmental deficiencies can disrupt normal physiological processes leading to an array of muscle dystrophies and/or myopathies (Thomas, 2013).

In mammals, the development of muscles involves a complex interplay of many regulatory genes, that control events from the fusion of myoblasts, through the formation of premyofibrils, to the maturation of myotubes into contractile fibers (reviewed in Lehka and Rędowicz, 2020; Sanger et al., 2002). The complex process of myofibrillogenesis is activated by transcription factors including Myocyte enhancer factor 2 (MEF2) and the myogenic basic helix-loop-helix proteins MyoD, myf-5, Myogenin, and MRF-4 (reviewed in Hernández-Hernández et al., 2017). Prominent among these genes is MEF2, whose central role in muscle development is to trigger myoblast fusion (reviewed by Haralalka and Abmayr, 2010; Bour et al., 1995; Brunetti et al., 2015; Bryantsev et al., 2012a) and muscle differentiation (Lilly et al., 1995; Bour et al., 1995; Lin et al., 1997). MEF2 additionally regulates cardiac development and

function (Desjardins and Naya, 2016), and neuronal development (reviewed in Dietrich, 2013), and has a direct involvement in human diseases such as cancers (reviewed in Chen et al., 2017). In *Drosophila melanogaster*, MEF2 regulates cardiac tube development (reviewed in Bilecky et al., 2023), pacemaker neurons (Blanchard et al., 2010), and immune responses (Clark et al., 2013). Moreover, MEF2 directly regulates the expression of numerous target genes that are essential for muscle development, including *Act57B*, *Mlc1*, *Mlc2*, and *Mhc* (Kelly et al., 2002; Elgar et al., 2008). However, despite an extensive list of known MEF2 target genes, MEF2 alone cannot convert naïve cells into muscles, either in mammals or *Drosophila* (Molkentin et al., 1995; Abmayr et al., 1995). By contrast, when expressed in a mesodermal environment, MEF2 can potentiate muscle gene expression in myoblasts. For example, in embryos, MEF2 plays a crucial role in initiating a genetic cascade that starts in the committed mesoderm, progresses to the somatic mesoderm, and ultimately results in the formation of skeletal muscle cells (Lilly et al., 1995; Bour et al., 1995).

Several previous studies have shown that differentiation can also be triggered in the wing imaginal disc myoblasts. These undifferentiated mesodermal cells are set aside in the embryo, proliferate during the larval stage, and differentiate during metamorphosis to ultimately give rise to the adult wing and thoracic muscles (Bate et al., 1991; Fernandes et al., 1991). The *Drosophila* thoracic muscular system is largely composed of fibrillar indirect flight muscles (IFMs) responsible for powering the wing stroke during flight (Bernstein et al., 1993; Spletter et al., 2018), and the tergal depressor of the trochanter muscle (TDT or jump muscle) responsible for jumping (Nachtigall and Wilson, 1967; Zumstein et al., 2004; Jaramillo et al., 2009). Premature

muscle differentiation can be triggered in wing disc myoblasts either by increasing expression of *Mef2* (Lovato et al., 2005; Soler and Taylor, 2009; Boukhatmi and Bray, 2018) or by knocking down expression of the *Zfh1* gene (Boukhatmi and Bray, 2018), that encodes a myogenic repressor (Postigo et al., 1999). However, to date, studies of muscle differentiation in the wing discs have relied predominantly upon assessing the accumulation of candidate myofibrillar proteins (Myosin heavy-chain and Tropomyosin) or F-actin; the extent to which a broader myogenic program is initiated has not been investigated.

Here, we carried out cell biological and transcriptomic analyses to identify the breadth of the myogenic program that MEF2 can trigger in wing disc adult myoblasts. We found that MEF2-induced myofibrillar proteins accumulated both into striated and non-striated structures. Some of these structures had characteristics of pre-myofibrils found in early differentiating muscles, but there was no extensive fusion of myoblasts into syncytia. At the transcriptomic level, there were over 147 up-regulated genes, mostly associated with muscle structure and function, and the increased expression of several of these was confirmed through immunofluorescence and quantitative RT-PCR (qPCR). Since the adult muscles express novel muscle proteins or unique isoforms of canonical muscle proteins, we also determined if the transcriptional profile from these adult myoblasts more closely resembled embryonic/larval or pupal/adult muscles. We found a strong bias towards the expression of embryonic/larval muscle protein genes, indicating that factors in addition to MEF2 are required for the proper formation of the more complex adult muscles.

MATERIALS AND METHODS

Drosophila stocks and crosses

All crosses were maintained in vials or bottles containing Jazz Mix medium (Genesee Scientific) at 25 °C, where they were allowed to lay eggs, and then transferred to 29 °C incubators after three days to maximize Gal4-induced expression. The following fly stocks were used: *1151-Gal4; fln-LacZ* (myoblast specific driver, Bryantsev et al., 2012b); *w UAS-Mef2 [26A]* (overexpression of *Mef2*, Lovato et al., 2005); and *w¹¹¹⁸* (control). To trigger premature *Mef2* expression in the imaginal discs, we crossed *1151-Gal4; fln-lacZ* to *w UAS-Mef2 [26A]*. For controls we crossed *1151-Gal4; fln-lacZ* to *w¹¹¹⁸*. Since both *1151-Gal4* and *UAS-Mef2* are on the X chromosome, we analyzed solely female offspring for these experiments.

RNA extraction

Wing imaginal discs were dissected from wandering third instar female larvae and then stored in Qiagen Buffer RLT, at -80 °C, until a total of 60 wing discs were collected, per replicate. Then, total RNA was extracted from each replicate using RNeasy Micro Kit (#74104; Qiagen, USA). Using a NanoDrop 2000 (Thermo Fisher Scientific, USA) the average yield extracted from control replicates was 1919.80 ng/μl (20 μl total extraction volume) and the average yield extracted from mutant replicates was 2387.97 ng/μl (20 μl total extraction volume). The quality of the RNA was determined by UC San Diego IGM Genomics Center using RNA ScreenTape® analysis (Agilent, USA). The average RIN^e for control and experimental samples was 10.0 (Appendix 1 - Figure S1).

Transcriptomic analysis

RNA-sequencing (RNA-seq) was conducted using Illumina NovaSeq 6000. Three single-end libraries were made from both control and experimental samples. No trimming was used. The modern aligner HISAT2 was used to map reads to Drosophila reference genome dm6 (*D. melanogaster* Aug. 2014 BDGP Release 6 + ISO1 MT/dm6) (Kim et al., 2019). Quality control was completed using the fastqcr R package. Next, to quantify the expression level of transcripts, the alignment files were used as inputs for featurecounts (Liao et al., 2014). Then, DESeq2 was used to identify significantly expressed genes between the control and experimental samples using these counts (Love et al., 2014). $P < 0.05$ was set as the threshold for significantly differentially expressed genes. Gene ontology (GO) analysis was completed with ClueGO, a CytoScape plug-in (Bindea et al., 2009). The raw and analyzed data were deposited in the Mendeley Data database under <https://doi.org/10.17632/777hyfy6h5.1>.

Quantitative RT-PCR

1 µg of total RNA from imaginal wing disc replicates was used to synthesize cDNA with iScript Adv cDNA kit for qPCR (#1725038; Bio-Rad) using C1000 Touch Thermal Cycler (Bio-Rad, USA). Then, SsoAdvanced Universal SYBR Green Supermix (#1725270; Bio-Rad, USA) was used for quantitative PCR (qPCR) using the CFX96 Real-Time System (Bio-Rad, USA). Each sample was run in triplicate and the transcripts were analyzed by $2^{-\Delta\Delta CT}$ method to calculate the relative gene expression. The threshold for significantly differentially expressed genes was set at a p-value below 0.05. The mean $\pm$ standard error of the mean was used for all graphs. The relative quantification was standardized to *Act5C* RNA levels. Primers used were as follows:

Act5C-Forward: 5'-GCAAGTACTCTGTCTGGATC-3'
 Act5C-Reverse: 5'-CCAGCAGAATCAAGACCATCC-3'
 Act57B Forward: 5'- TGCAGTTGCCTAGCACCA – 3'
 Act57B Reverse: 5'- GACCCCATCCGCTCTATC – 3'
 Act79B Forward: 5'- TGTCTCCAGCGTAAGACATCC – 3'
 Act79B Reverse: 5'- TTCCGGTCTTTTCTCGTCTC – 3'
 Act88F Forward: 5'- AGCTCTTCAAAGGCAGCAAC – 3'
 Act88F Reverse: 5'- ATTGTTGTGCGATGGGTTC – 3'
 TnI Forward: 5'- GAAGGTCTCCAAATACGAAA – 3'
 TnI Reverse: 5'- GCTGTTGTTGTTTATTGACTTC – 3'
 MHC Forward: 5'- AGAAGGCTGAGGAACTGC – 3'
 MHC Reverse: 5'- GTTCAAGTTGCGGATCTG – 3'

To assess patterns of alternative RNA splicing, diluted cDNA was used for PCR using OneTaq 2X Master Mix with Standard Buffer (New England Biolabs). The following primers were used.:

MHC Forward: 5'- ATGGTGAGCAGAGGAGGCA – 3'
 MHC Reverse: 5'- CGTTTTTCAGGAGCAAGGTCG – 3'
 Mlc1 Forward: 5'- GAGAGCTTGGATGACGAGCA – 3'
 Mlc1 Reverse: 5'- GGTTCAGCCAAAAGCAAAGC – 3'
 SIs Forward: 5'- CGCGCAGTATGTGCAAAAT – 3'
 SIs Reverse: 5'- AAACCGTTCCACGAAAAGTG – 3'

The PCRs were run for 20–35 cycles and the final products were resolved in a 2% agarose gel.

Immunofluorescent analysis

Wing imaginal discs from wandering third instar female larvae were dissected in 1X phosphate buffer saline (PBS) and fixed in 3.7% formaldehyde for 30 min at 4 °C, followed by immunolabeling. All primary antibodies were incubated with the samples overnight at 4 °C and secondary antibodies were incubated for 2 h at 22 °C. Samples were mounted in mounting media. Antibodies recognizing Myofilin (Mf), Myosin alkali light chain 1 (Mlc1), and Troponin T (TnT) did not show protein expression in the experimental discs (Table 2). However, we believe these antibodies are not functional in

our hands because cryosections of *w¹¹¹⁸* adult thoracic muscles did not show any specific staining (data not shown).

The following are the primary antibodies used:

1:500 Act88F (DSHB, Cat#BB8/20.11)
1:500 Actn (DSHB, Cat#BB8/384.1)
1:500 Bent (DSHB, Cat#P5-3B3)
1:500 CG43897 (Made in Dr. R. M.Cripps lab)
1:500 Fln (Dr. J. Vigoreaux)
1:500 Mf (DSHB, Cat#BB12/4.6)
1:500 Mhc (DSHB, Cat#3E8-3D3-s)
1:500 Mlc1 (DSHB, Cat#BB16-32.3)
1:500 Mlc2 (Vaziri et al., 2020)
1:500 Mlp84B (Clark et al., 2007)
1:500 Sals (Bai et al 2007)
1:500 Slis (DSHB, Cat#BB2/17.9)
1:500 Tm1 (DSHB, Cat#BB5/37.1)
1:500 TnT (DSHB, Cat#BB5-71.3)
1:500 TpnC (DSHB, Cat#BB14/7.1)
1:500 Unc-45 (Dr. S. I. Bernstein)
1:500 Unc-89 (DSHB, Cat#BB8/351.1)
1:500 Zasp52 (DSHB, Cat#1D3-3E4)
1:500 Zfh1 (Dr. R. Lehman)
1:500 Zip (Dr. J. Thomas)

Imaging and statistical analysis

Immunostained preparations were mounted in mounting media and imaged on an Olympus FluoView 3000 confocal microscope. Images were processed using Olympus Life Science software and assembled in Adobe Photoshop (2024) (version 25.0). All experimental and confocal images underwent equal changes in brightness and contrast.

To assess the extent of muscle protein accumulation relative to the total myoblast area, an area was drawn around MHC, F-actin, and MEF2 positive staining to calculate the fraction of the myoblast area occupied by MHC or F-actin. To calculate this

fraction, the area occupied by F-actin or MHC was divided by the area occupied by myoblasts (MEF2 staining).

To estimate the size of the myoblast pool, a border was also drawn around myoblasts, stained with anti-Cut, anti-MEF2 or anti-Zfh1 antibodies, and the area within that border was calculated using ImageJ. Immunopositive areas were defined as those that showed nuclear accumulation of the myoblast transcription factors. The error bars on all the graphs are standard error of the mean. Student's two-tailed *t*-test was used to calculate the p-value.

Data for qPCR was analyzed using parametric, two-tailed, unpaired Welch's *t*-test, using Microsoft^R Excel (version 16.77.1). GraphPad Prism software (Version 10.0.3) was used to generate all pie charts and bar graphs. The error bars are presented as the standard error of the mean. $P < 0.05$ was set as the threshold for significance and marked by asterisks.

RESULTS

Phenotypic analysis of MEF2-induced myogenesis in wing discs

We first studied the types of muscle-like structures that were formed in the wing imaginal discs of late third-instar larvae following *Mef2* over-expression. We used the *1151-Gal4* driver line, which is active in the adult myoblasts (Anant et al., 1998), and crossed females to either *w¹¹¹⁸* control males or to *UAS-Mef2* males (Lovato et al., 2005). Since both *1151-Gal4* and *UAS-Mef2* are X-linked, we analyzed only female offspring of control and experimental crosses. We assessed *Mef2* expression levels using RT-PCR (qPCR) and found that there was a 1.901 log fold change and a p-value

of 0.02 (p-value <0.05 being significant) among *1151>Mef2* discs compared to controls (Appendix - Figure S2), confirming the over-expression of *Mef2* in these samples.

While we never observed accumulation of Myosin heavy-chain (MHC) nor F-actin in control wing discs (Figure 4A and B; n = 20), 100% of experimental discs showed evidence of muscle differentiation (Figure 4C and D; n = 11). Within each disc, however, not all myoblasts showed evidence of differentiation: MHC and F-actin on average occupied 54.74% (n = 11) and 18.51% (n = 11), respectively, of the total area occupied by myoblasts as defined by MEF2 staining (Figure 4E–H). This was always most prominent at the proximal tip of the disc but frequently spread across the field of myoblasts. In addition, the regions that showed the most prominent differentiation by F-actin staining also showed the strongest MEF2 accumulation compared to adjacent myoblasts (Figure 4G), suggesting that once the myogenic program is initiated, *Mef2* expression can be further up-regulated.

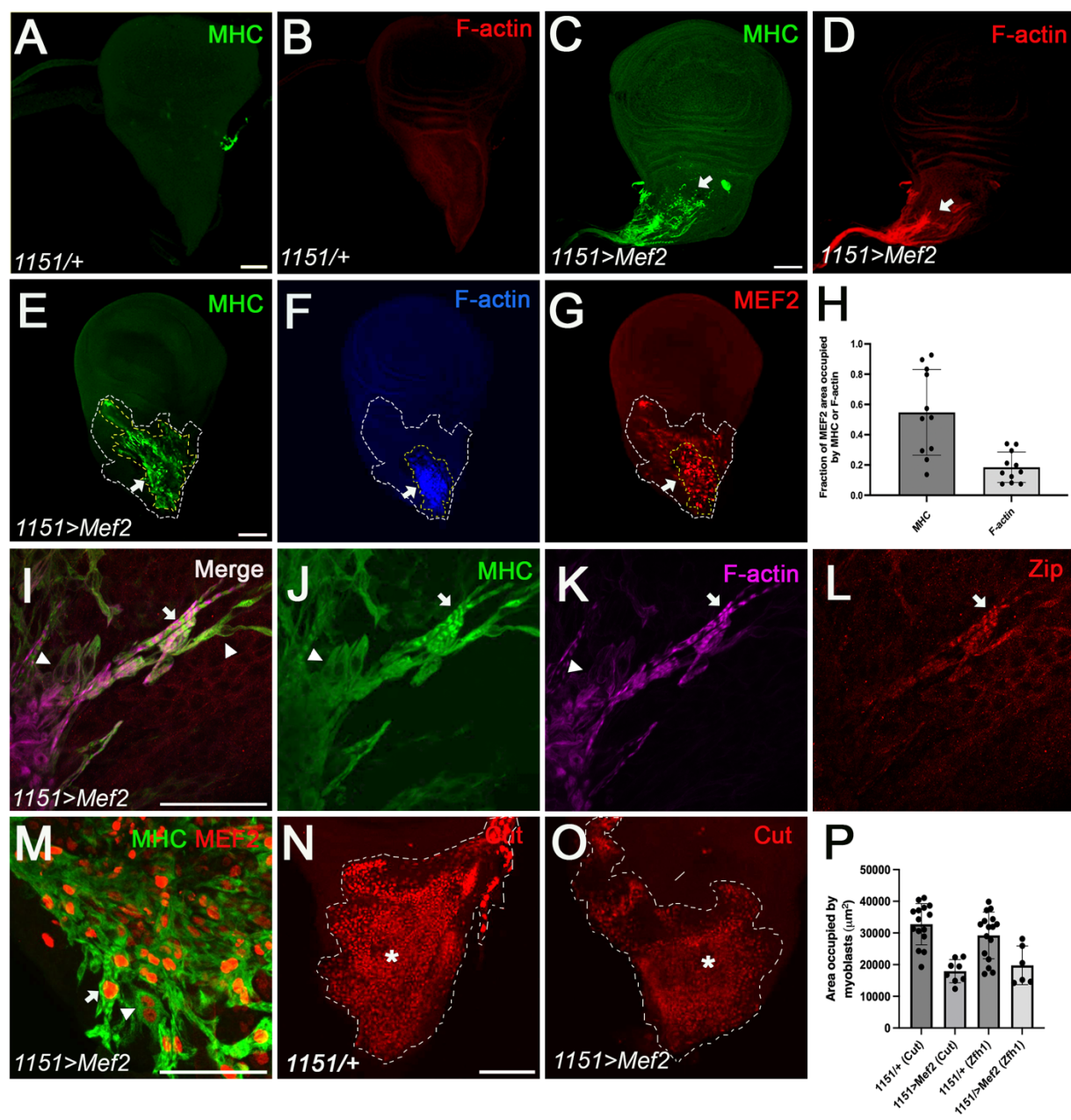
Next, we analyzed the myofibril-like structures that were formed in experimental discs. To achieve this, we performed immunofluorescent staining of wing discs using antibodies that recognized either muscle Myosin heavy-chain (MHC), or the non-muscle myosin heavy-chain, Zipper (Zip). We observed varying degrees of myofibril formation, where both striated myofibril-like structures and non-striated structures formed on the same disc (Figure 4I). MHC and F-actin accumulation were found in both striated and non-striated structures (Figure 4J and K), whereas Zip accumulation was the strongest in the striated structures (Figure 4L). Zip is involved in myofibril assembly, specifically myosin II filament assembly, localizing to the M-line in mature larval muscles (Bloor and Kiehart, 2001; Liu et al., 2008).

Since *Mef2* is required for myoblast fusion (Bour et al., 1995; Ranganayakulu et al., 1995) we also determined if there was evidence for muscle syncytia being formed. To assess this, we conducted immunofluorescent staining on experimental discs to detect the accumulation of MHC and MEF2. In regions where myogenic structures were not overlapping, we observed striated-like and non-striated-like structures (Figure 4M). However, it was challenging to definitively confirm the predominance of multinucleate or mononucleate cells because many of these structures overlap making it hard to see the individual cells, although in a few cases, we did observe what appeared to be binucleate cells (Figure 4M, arrowhead). In the next section, we assess the expression levels of myoblast fusion genes to gain a more comprehensive understanding of the potential formation of syncytia.

Finally, given that numerous myoblasts were beginning to differentiate, we hypothesized that this would impact the myoblast pool. To test this, we stained control and experimental discs with anti-Cut to measure the wing disc area occupied by myoblasts (Figure 4N–O). Cut encodes a homeoprotein that functions as a transcriptional factor in different cells, such as the myoblasts of imaginal wing discs. Quantification revealed that the Cut-positive myoblast area was reduced by 45.98% in the experimental discs ($n = 9$) compared to the controls ($n = 16$) ($p = 0.0019$) (Figure 4P). To add robustness to these data, an antibody against Zfh1 was also used to stain myoblasts (Appendix - Figure S3). We observed the myoblast area was reduced by 32.32% in the experimental discs ($n = 6$) compared to the controls ($n = 16$) ($p = 0.01143$) (Figure 1P). We conclude that myoblast number is consistently and

reproducibly reduced in the experimental discs, perhaps through the initiation of differentiation causing a reduction in myoblast proliferation.

Figure 4. Phenotypic analysis of MEF2-induced myogenesis in wing discs. (A–D) Control (*1151/+*) wing imaginal discs of late third-instar larvae upon *Mef2* over-expression show no expression of myosin heavy chain (anti-MHC, green) and F-actin (skeletal muscles; red) while experimental discs (*1151>Mef2*) show expression of both (arrow). Control $n = 20$. Experimental $n = 11$. Scale bar: 50 μm . (E–F) MHC and F-actin expression is more prominent at the proximal tip of *1151>Mef2* discs (arrow). Wing disc area occupied by MHC (yellow dashed line), F-actin (yellow dashed line), and myoblasts (white dashed line). Scale bar: 50 μm . (G) The strongest MEF2 accumulation is located where F-actin is expressed (yellow dashed line; arrow). Wing disc area occupied myoblasts (white dashed line). Scale bar: 50 μm . (H) MHC and F-actin occupied 54.74% ($n = 11$) and 18.51% ($n = 11$), respectively, of the area occupied by myoblasts. (I–L) *1151>Mef2* discs were stained with MHC, F-actin, and non-muscle myosin heavy-chain, Zipper (Zip). MHC and F-actin expression were found in both striated (arrow) and non-striated structures (arrowhead), while Zip expression was the strongest in the striated structures (arrow). Scale bar: 50 μm . (M) *1151>Mef2* discs stained for MHC and MEF2 showed both striated-like mononucleate (arrow) and non-striated bi-nucleate (arrowhead) cells. Scale bar: 50 μm . (N–O) Control (*1151/+*) and experimental (*1151>Mef2*) discs stained with Cut (myoblast, red, white dashed line) Scale bar: 50 μm . (P) Quantification of the area occupied by myoblasts revealed a significant decrease in Cut-positive myoblast pool by approximately 45.98% in the experimental discs ($n = 9$) compared to the controls ($n = 16$) ($p = 0.0019$); and a significant decrease in Zfh1-positive myoblasts by 32.32% in the experimental discs ($n=6$) compared to the controls ($n=16$) ($p = 0.01143$). The error bars on all the graphs were calculated by the standard error of the mean. All p -values <0.05 were deemed significant and calculated by two-tailed student's t-test.



Transcriptomic analysis of wing discs with elevated Mef2 expression

To assess the breadth of the myogenic program induced by MEF2, we conducted a transcriptomic analysis of whole wing discs isolated from control *1151/+* and experimental *1151>Mef2* female late third instar larvae. RNA-sequencing (RNA-seq) was conducted using Illumina NovaSeq 6000. Three single-end libraries were made from both control and experimental samples. Since paired-end data was not used, no trimming was required. Illumina reads used for differential gene expression in RNA-seq analysis are no longer required to undergo trimming when using modern aligners, such as HISAT2, which was used in this analysis (Williams et al., 2016). HISAT2 was used to map reads to the *Drosophila* reference genome dm6 (*D. melanogaster* Aug. 2014 BDGP Release 6 + ISO1 MT/dm6) (Kim et al., 2019). To ensure the Illumina data was acceptable, it was subjected to quality control using the fastqcr R package, and all categories received a passing score except for “per base sequence content” and “per sequence GC content” (Appendix - Figures S4–S12 and Table S1) (Wingett and Andrews, 2018; Andrews, 2023).

The “per base sequence content” always returns as a Fail score because the first 10–12 bases are a result of hexamer priming that occurs during RNA-seq library preparation so that reverse transcription can occur (Wang et al., 2009). Moreover, a Fail in “per sequence GC content” is usually due to overrepresented sequences, which was not observed here. GC content may affect differential expression results and downstream analysis such as Gene Ontology (Risso et al., 2011), so measuring %GC for all replicates was performed (Table 1). Our results showed that %GC content was between 47% and 50% in all samples, consistent with the GC-content range observed

in *Drosophila* genomes (Herold et al., 2008). In addition, the mapping rate was 95%.

Together, our results indicate high-quality sequencing data and successful alignment to the *Drosophila* genome.

Table 1. Summary of RNA-sequencing statistics and mapping results. Each replicate was mapped to the *D. melanogaster* Dm6 reference genome. All sequences were single-end reads. The average read-length was 101nt.

Library	% GC	Number of raw reads	Number of cleaned reads	Mapping rate
1151/+ replicate 1	47	30178968	28517245	95.26%
1151/+ replicate 2	48	31487326	29978815	95.65%
1151/+ replicate 3	48	32904070	30983815	95.50%
1151>Mef2 replicate 1	50	40241817	38715150	94.88%
1151>Mef2 replicate 2	48	32332760	30806108	95.24%
1151>Mef2 replicate 3	48	30027650	28541651	95.44%

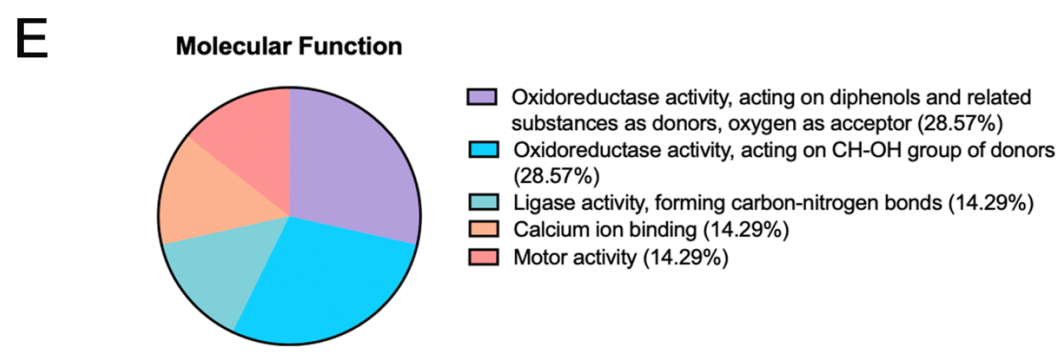
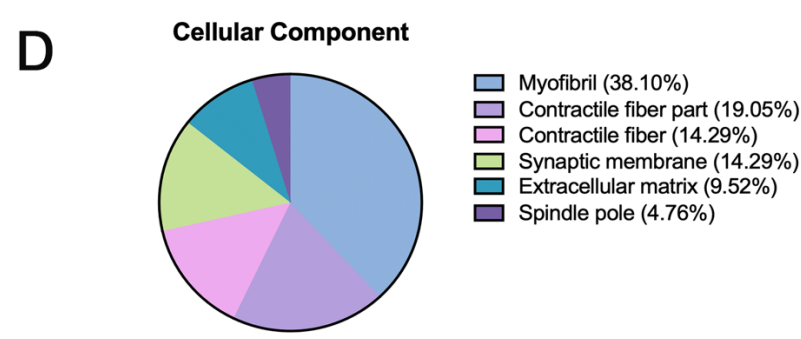
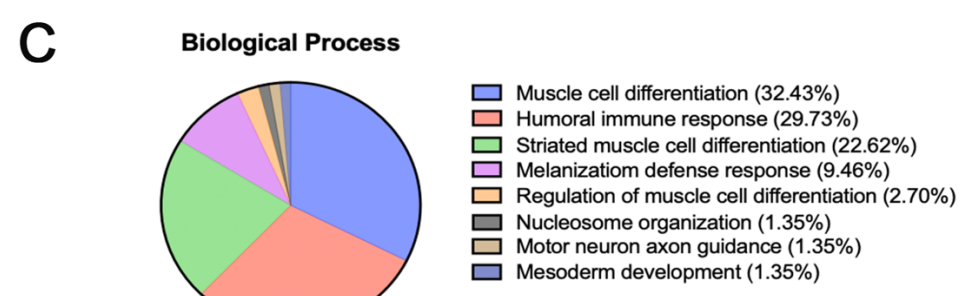
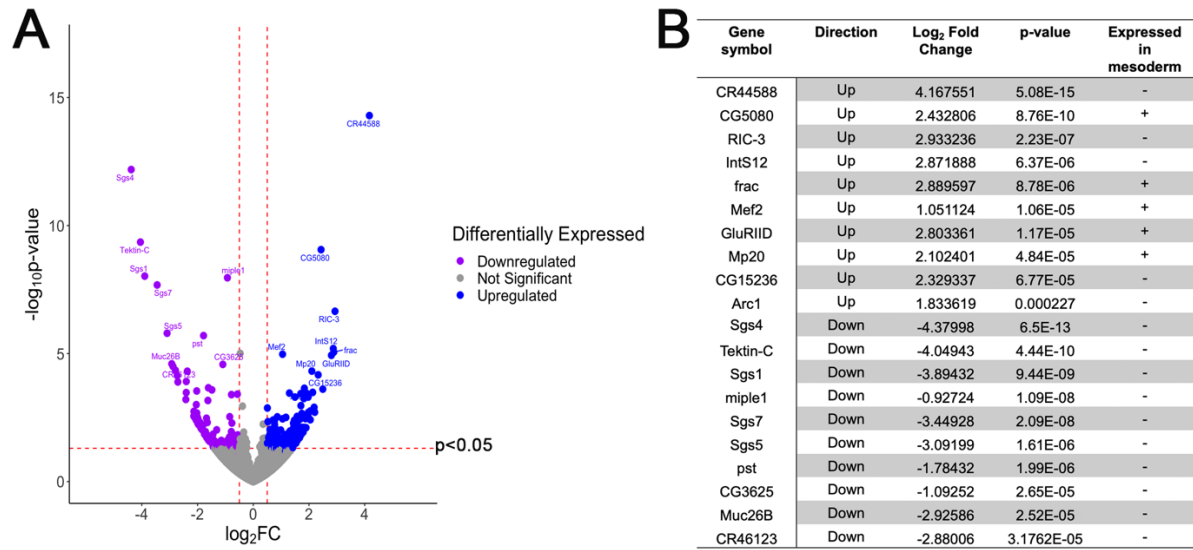
Next, to quantify the expression level of transcripts, the alignment files were used as input for featurecounts (Liao et al., 2014). Then, DESeq2 was used to identify significantly expressed genes between the control and experimental samples using these counts (Love et al., 2014). To visualize downregulated and up-regulated genes, a volcano plot was generated (Figure 5A). We established significance thresholds at $p\text{-value} < 0.05$ and a fold change cutoff of $\text{Log}_2\text{FC} > 0.5$ and $\text{Log}_2\text{FC} < -0.5$, up-regulated and downregulated, respectively, to enable the detection of smaller yet biologically relevant fold changes. This was necessary because the isolated wing discs contain significant amounts of other wing disc tissue in addition to the myoblasts, therefore we were concerned that changes in expression in the myoblasts might be masked by transcripts for the other tissues present. A table listing the top ten significantly up-regulated and downregulated genes were created, showing corresponding p-values,

Log₂ fold changes, and whether they are expressed in the mesoderm, based upon annotations at FlyBase.org (Gramates et al., 2022) (Figure 5B). From this list, we found that mesoderm-expressed genes are over-represented in the up-regulated fraction.

Next, the differentially expressed genes were used for Gene Ontology (GO) enrichment analysis by means of ClueGO, a CytoScape plug-in, to analyze the biological processes, cellular components, and molecular functions of the 147 genes that were up-regulated in imaginal wing discs with *Mef2* over-expression (Bindea et al., 2009). Analyzing these data showed 32.43% of differentially expressed genes from the Biological Process category are involved in muscle cell differentiation, 38.10% from the Cellular Component category are myofibril genes, and 28.57% from the Molecular Function category have oxidoreductase activity (oxygen as acceptor) (Figure 5C–5E). These data suggest that as the myoblasts undergo differentiation, genes encoding structural proteins are expressed to ensure the proper development and function of myofibrils. In addition, oxygen, which serves as the last electron acceptor in the electron transport chain, enables the cells to generate adenosine triphosphate (ATP) for energy during muscle cell differentiation. Overall, muscle-related and energy-producing GO terms were associated with differentially expressed genes.

For down-regulated genes, although a total of 110 genes were significantly downregulated at Log₂FC < -0.5, ClueGO only returned the biological process category, showing 80% of the down-regulated genes as “response to ketone” and 20% as “germ cell migration”.

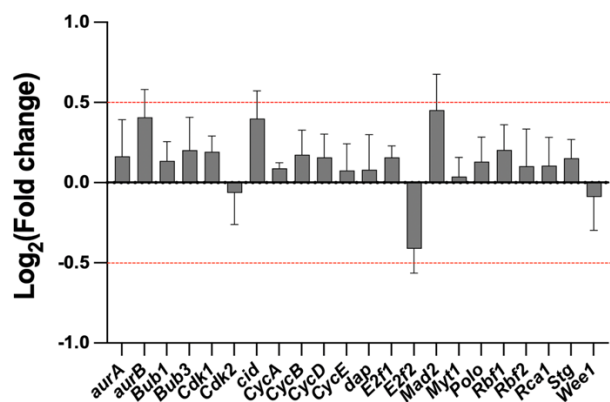
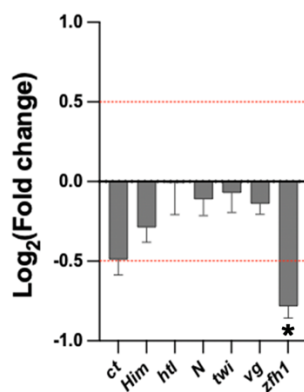
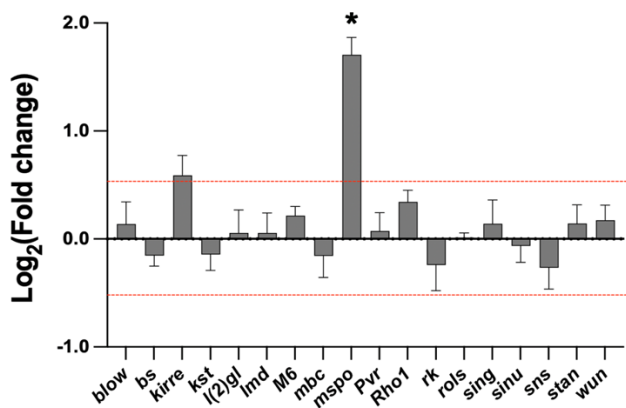
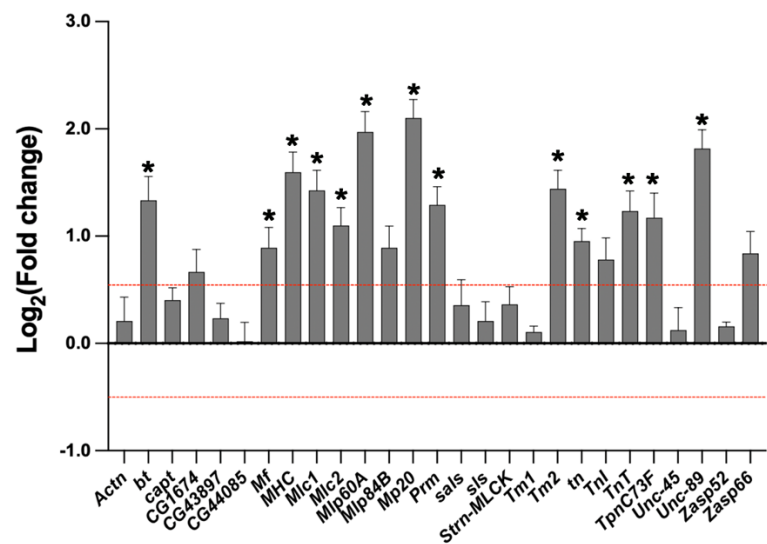
Figure 5. Analysis of differentially expressed genes upon Mef2 over-expression. Differentially expressed genes (DEGs) were determined using DESeq2 as described in Methods. (A) Volcano plot of DEGs. Log₂ fold change > or <0.5 plotted against p-value <0.05 was deemed significant. Significant downregulated genes are in purple, significant up-regulated genes are in blue, and non-significant genes are in gray. (B) Table of top 10 up-regulated and downregulated DEGs and their corresponding direction, log₂ fold change, and p-value. For each gene, a positive sign (+) indicates expression in the mesoderm while a negative sign (–) indicates no currently available data suggesting that the gene is expressed specifically in the mesoderm. (C–E) Pie chart of the Gene Ontology (GO) enrichment analysis of DEGs using ClueGO, a CytoScape plug-in, to analyze the biological processes, cellular components, and molecular functions that were up-regulated. Many differentially regulated genes in Biological Processes relate to muscle cell differentiation (32.43%), while in Cellular Component, the majority are myofibril genes (38.10%), and in Molecular Function, they are genes related to oxidoreductase activity (oxygen as acceptor; 28.57%).



To gain more specific insight into the differentially expressed genes, we mined the RNA-seq data for known genes associated with myoblast identity, cell cycle regulation, myoblast fusion, and myofibril assembly or function. We found that 0% of the cell cycle genes were differentially expressed (Figure 6A). Note that our data are from whole discs rather than purified myoblasts, and it is expected that cell cycle genes are broadly expressed in the growing wing discs. Therefore, changes in the expression of these genes solely in the myoblasts may be below the levels of detection.

When we mined the RNA-seq data for myoblast identity genes, we found that one was significantly down-regulated, *Zfh1* (Figure 6B). The down-regulation of *Zfh1* is consistent with our findings of a reduction of *Zfh1*-positive myoblasts (Figure 4P and Appendix - Figure S3) (Boukhatmi and Bray, 2018). We also observed a reduction in the expression level of *Cut* (Figure 6B) consistent with the reduction in the field of *Cut*-positive myoblasts (Figure 4P). However, this reduction was not significant, which may be due to the expression of *Cut* in other wing disc tissues, including the wing margin, that would not be affected by manipulation of *Mef2* expression in the myoblasts (Blochlinger et al., 1988, 1990; Ludlow et al., 1996).

Figure 6. Expression changes for genes associated with myoblast identity, cell cycle regulation, myoblast fusion, and myofibril assembly or function. (A–D) Significant differentially expressed genes (DEGs) in wing imaginal discs of late third-instar female larvae upon *Mef2* over-expression are indicated with an asterisk. Log₂ fold change greater than or less than 0.5 plotted p-value <0.05 was deemed significant (red dotted line). All graphs are Mean ± Standard Error (Student's two-tailed *t*-test). No significant DEGs were observed for the cell cycle genes category, while the myoblast identity genes category has one significantly down-regulated gene (*Zfh1*) and the myoblast fusion genes category has one significant upregulated gene (*mspo*). The myofibril gene category revealed 12 significant up-regulated genes, accounting for 48% of the genes in this category.

A**Cell cycle genes****B****Myoblast identity genes****C****Myoblast fusion genes****D****Myofibril genes**

We also determined if genes associated with myoblast fusion were up-regulated in the experimental samples. We observed that, of 18 genes analyzed, only *mspo* was significantly up-regulated (Figure 6C). These data are consistent with the minor proportion of syncytial muscle cells that we observed (see Figure 4). When we analyzed experimental discs in white pre-pupa, we observed striated-like structures and multinucleation (Fig. S13; white bracket). These data suggest that additional myoblast fusion genes are being activated during this later stage of development resulting in more evidence of myoblast fusion, although we have not tested this experimentally.

Finally, we assessed the expression levels of known sarcomeric protein genes. We observed that all genes studied showed up-regulation of expression in response to MEF2, and 48% of these genes showed significantly up-regulated expression (Figure 6D). Overall, our RNA-seq and GO analysis highlight the potential role of MEF2 in causing muscle differentiation and myofibril formation, although it appears that not all myogenic processes, such as myoblast fusion, are significantly impacted.

Immunofluorescence analysis of wing discs with elevated Mef2 expression

To validate the up-regulation of myofibrillar protein genes, we obtained antibodies recognizing the products of 16 of the up-regulated genes. We chose to detect the products of genes that were both significantly up-regulated and those that were not, to assess how representative was the RNA-seq analysis. Three antibodies failed specificity testing (see Materials and Methods), meaning that we tested 13 different myofibrillar proteins for accumulation in control and experimental discs (Table 2 and Figure 7). Figure 7 shows representative control and experimental discs stained with these 13 antibodies, as discussed in more detail below. Table 2 summarizes the

immunofluorescent analysis of the 13 polypeptides, where we quantify the percentage of discs that show protein accumulation in control and experimental samples, and the location of the protein product within normal myofibrils. In addition, we quantified the level of accumulation of each protein by calculating the fraction of the area covered by the stain, in relation to the myoblast pool. Appendix - Figure S14 shows the myoblast areas (as visualized using anti-MEF2) for each of the experimental discs shown here that demonstrates a roughly equivalent myoblast pool in all experimental discs. This observation emphasizes that differences in the extent of accumulation of different myofibrillar proteins, as shown below, does not arise from differences in the myoblast pool size.

Overall, we detected 12 of the 13 myofibrillar proteins in experimental wing imaginal discs but absent in control discs (Figure 7). We found that the sarcomeric protein Bent (Bt) (Tanentzapf and Perkins, 2014), the motor protein MHC (Wells et al., 1996; Standiford et al., 1997), the contractile protein myosin light chain 2 (MLC2) (Warmke et al., 1992) and the titin-like protein Obscurin (unc-89) (Katzemich et al., 2015) showed protein products accumulating in the experimental discs (Figure 7A–D'). For these four proteins, their transcripts were significantly up-regulated in the RNA-seq data compared to the control (Figure 6D). The fraction of myoblast area covered by each of these stains ranged from 0.8% for Unc-89 to 54% for MHC (Table 2).

Table 2. Polypeptide location in the myofibril and quantification of immunofluorescent stains of wing imaginal discs.

Polypeptide	Location in myofibril	Stain in 1151>w ¹¹¹⁸ (%)	Stain in 1151>Mef2 (%)	*Fraction of myoblast area covered by stain (% $\pm$ SEM)
Significantly up-regulated and stained				
Bent	Z-disc	0/8 (0%)	9/9 (100.00%)	37.24% $\pm$ 6.68
MHC	A band	0/20 (0%)	11/11 (100.00%)	54.74% $\pm$ 8.54
MLC2	Myosin filament	0/8 (0%)	6/8 (75.00%)	12.63% $\pm$ 4.64
Unc-89/Obscurin	M-line	0/10 (0%)	4/13 (30.77%)	0.80% $\pm$ 0.94
Significantly up-regulated, but did not stain (likely antibody not working)				
Mf	A band	0/15 (0%)	0/8 (0%)	0%
MLC1	Myosin filament	0/13 (0%)	0/12 (0%)	0%
TnT/up	Actin filament	0/12 (0%)	0/9 (0%)	0%
Not significantly up-regulated, but stained				
Actn	Z-disc	0/8 (0%)	7/7 (100%)	17.44% $\pm$ 5.68
CG43897	Z-disc	0/7 (0%)	5/8 (62.5%)	7.26% $\pm$ 2.92
Mlp84B	Z-disc	0/10 (0%)	8/15 (53.33%)	2.46% $\pm$ 0.82
Sals	Actin filament	0/10 (0%)	7/9 (77.78%)	8.88% $\pm$ 3.52
Slis	Z-disc	0/8 (0%)	7/9 (77.78%)	6.88% $\pm$ 2.47
Tm1	Actin filament	0/9 (0%)	8/10 (80%)	19.87% $\pm$ 11.62
TpnC	Actin filament	0/11 (0%)	12/14 (85.71%)	28.18% $\pm$ 7.75
Unc-45	Myosin filament	0/6 (0%)	3/9 (33.33%)	1.66% $\pm$ 1.49
Not significantly up-regulated and did not stain				
Zasp52	Z-disc	0/12 (0%)	0/13 (0%)	0%
Adult-specific genes not significantly upregulated				
Fln	Myosin filament	0/10 (0%)	0/8 (0%)	0%
Act88F	Actin filament	0/11 (0%)	0/11 (0%)	0%

*Proportion of the area containing protein accumulation compared to the total area of the myoblasts as revealed by MEF2 staining.

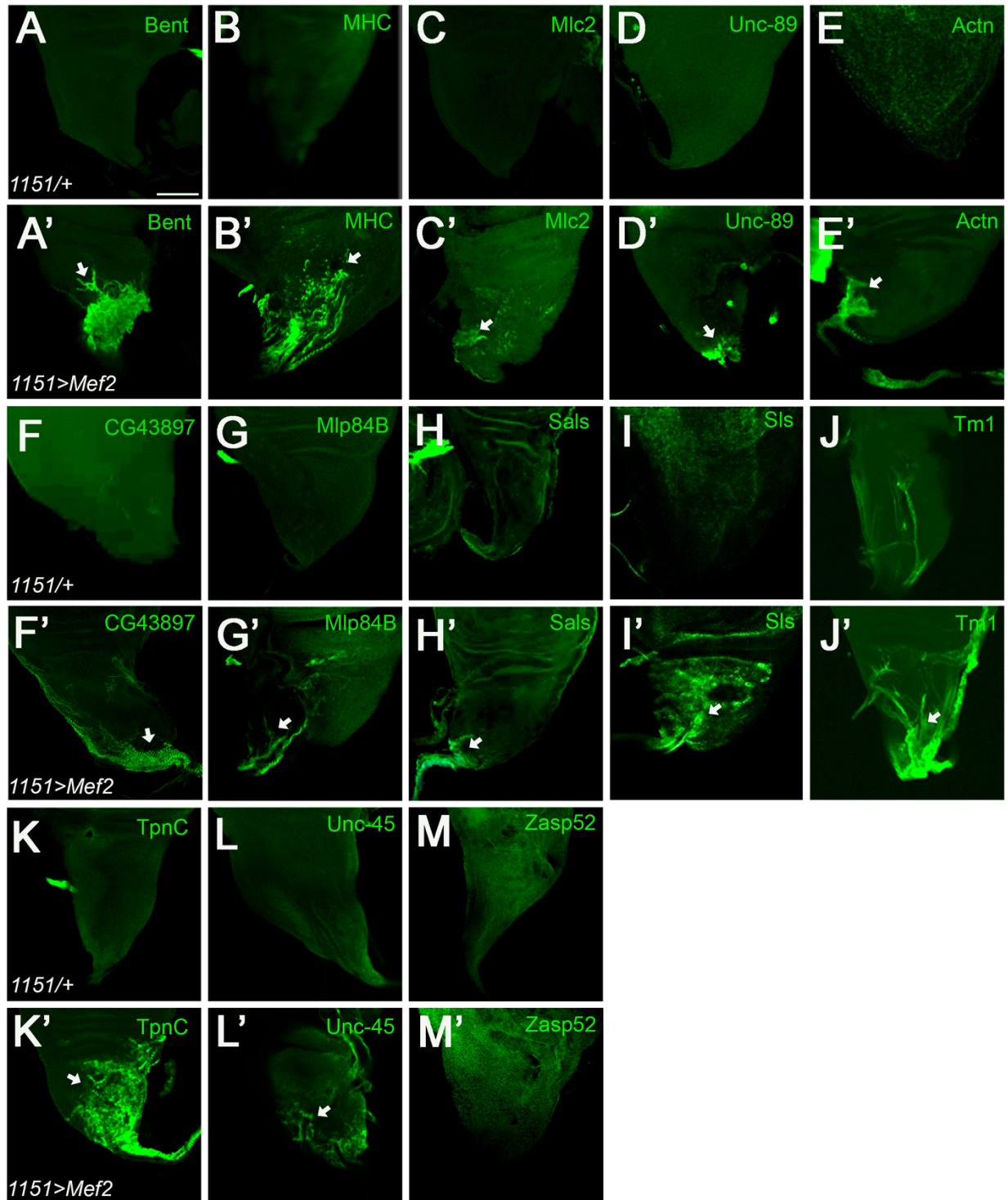
For eight proteins (α -actinin (Actn), CG43897, Muscle LIM protein at 84B (Mlp84B), Sarcomere length short (Sals), Sallimus (Slis), Tropomyosin (Tm1), Troponin C (TpnC) and Unc-45, we also observed significant accumulation in experimental but not control discs (Figure 7E–L'), even though their transcripts were not significantly up-regulated in the RNA-seq dataset. Actn aids in actin filament binding (Gaudet et al., 2011), CG43897 is predicted to be involved in mesoderm development (Furlong et al.,

2001), Mlp84B maintains muscle structural integrity (Clark et al., 2007), Sals promotes the elongation of actin filaments during muscle growth (Bai et al., 2007), SIs helps attach myosin filaments to the Z-disc (Burkart et al., 2007), Tm1 regulates muscle contraction (Gaudet et al., 2011), TpnC is required for myofibril assembly (Chechenova et al., 2017), and Unc-45 aides in myosin folding (Lee et al., 2011). The fraction of myoblast area covered by each stain ranged from 28.18% for TpnC to 1.66% for Unc-45 (Table 2), indicating that some genes were more responsive to activation by MEF2 than others.

Finally, Z band alternatively spliced PDZ-motif protein 52 (Zasp52), which accumulates in the Z-disc of developing myofibrils and attaches Actn to the Z-disc (Jani and Schock, 2007; Gaudet et al., 2011), was not significantly up-regulated in the RNA-seq data (Figure 6D) and did not show protein accumulation in control nor experimental discs (Figure 7M–M'). To test whether the lack of staining was attributed to a faulty Zasp52 antibody, we conducted cryosectioning and immunostaining of *w¹¹¹⁸* adult thoracic muscles. This analysis revealed staining for Zasp52, suggesting that this antibody is working (data not shown), however, this antibody likely recognizes an adult-specific isoform (Chechenova et al., 2013).

Taken together, these immunofluorescent studies broadly support the RNA-seq data in demonstrating that the products of up-regulated myofibrillar protein genes are detected in *Mef2*-over-expressing myoblasts. Nevertheless, the activation of the myogenic program is incomplete, at least at this stage of development.

Figure 7. Immunofluorescence analysis of late 3rd wing discs with elevated *Mef2* expression. All control *1151/+* did not show accumulation of the tested polypeptides, whereas many polypeptides were observed to accumulate in control discs. (A-A') Bent (Bt) is associated with the myosin thick filaments. (B-B') Myosin heavy chain (MHC) accumulates in myosin thick filaments and was used to visualize muscle differentiation. (C-C') Myosin light chain 2 (Mlc2) accumulates in the myosin II complex of the myofibril. (D-D') Obscurin (Unc-89) is a titin-like protein of the sarcomere. (E-E') α -actinin (Actn) is a Z-disc protein. (F-F') CG43897 accumulates in the Z-disc. (G-G') Mlp84B accumulates in the Z-disc. (H-H') Sarcomere length short (sals) is a sarcomeric protein expressed on the striated muscle thin filament. (I-I') Sallimus (Slis) links myosin filaments to the Z-disc and accumulates at the Z-disc. (J-J') Tropomyosin (Tm1) binds along the actin thin filament. (K-K') Troponin C (TpnC) is part of the troponin complex and is found along the actin thin filament. (L-L') Uncoordinated 45 (unc-45) binds to myosin and accumulates along the thick filaments. (M-M') Z band alternatively spliced PDZ-motif protein 52 (Zasp52) did not show accumulation on control and experimental wing discs. Scale bar: 50 μ m.



Up-regulated genes are characteristic of embryonic and larval muscle rather than pupal and adult muscle

The adult skeletal muscles of *Drosophila* are more complex than their larval counterparts (Miller, 1950), and this complexity is reflected in part by the existence of several muscle protein genes that are expressed in the pupal and adult muscles but not in the embryonic and larval stages, and several genes that are differentially spliced between larval and adult muscles (reviewed in Bernstein et al., 1993).

Since the imaginal myoblasts are precursors of the adult muscles, we investigated whether *Mef2* activates a larval or adult myogenic program, by mining the RNA-seq data for genes known to be expressed either at all stages of muscle development or just in the developing adult muscles.

For muscle actin, *Act57B* and *Act87E* are expressed throughout the life cycle, whereas *Act79B* and *Act88F* expression are specific to the adult muscles (Fyrberg et al., 1983; Sanchez et al., 1983). In the RNA-seq data, expression of both *Act57B* and *Act87E* were up-regulated, the latter significantly (Figure 8A); by contrast *Act79B* was only minimally up-regulated (and not significantly), and *Act88F* expression was down-regulated (not significantly) (Figure 8B).

For muscle Troponin C, *TpnC25D*, *TpnC47D*, and *TpnC73F* are expressed throughout the life cycle, whereas *TpnC4* and *TpnC41C* expression are specific to the adult muscles (Herranz et al., 2004; Chechenova et al., 2017). In the RNA-seq data, expression of *TpnC25D*, *TpnC47D*, and *TpnC73F* were all up-regulated, the latter significantly (Figure 8A); for the adult TpnC genes, *TpnC41C* expression was up-regulated (not significant) and *TpnC4* expression was slightly down-regulated (not significant) (Figure 8B).

There are also a small number of known adult-specific myofibrillar protein genes, including *flightin* (*fln*) (Vigoreaux et al., 1993) and *Zasp67* (Katzemich et al., 2013). Neither of these genes was significantly up-regulated in the experimental dataset (Figure 8B).

To provide validation for some of these findings, we carried out quantitative RT-PCR (qPCR) of experimental and control RNA samples, performed in triplicate for each genotype. First, we confirmed the robust and significant up-regulation of *Mhc* and *Tnl* in experimental RNA samples compared to controls (Figure 8C). Next, we assessed the expression of *Act57B*, *Act79B*, and *Act88F*. Only *Act57B*, which is expressed throughout the life cycle, showed significant upregulation in response to MEF2.

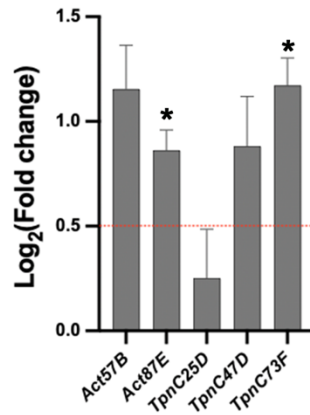
We also stained wing imaginal discs for the accumulation of Fln (*1151>w¹¹¹⁸* n = 10; *1151>Mef2* n = 8) and Actin88F (*1151>w¹¹¹⁸* n = 11; *1151>Mef2* n = 11) (Figure 8D–E'), and we validated Fln and Actin88F antibodies by immunostaining the thorax of 1-day old *w¹¹¹⁸* female flies (Figure 8F–8G). In support of the RNA-seq and qPCR assays, antibodies recognizing specifically Fln and Act88F did not stain experimental wing imaginal discs, although sections of adult muscles stained robustly with each antibody (Figure 8F–8G, Table 2).

To further analyze whether *Mef2* activates a larval or adult myogenic program, we assessed the patterns of alternative RNA splicing for genes that are differentially spliced between the flight muscles and larval muscles (Bernstein et al., 1993; Oas et al., 2014). In the RNA-seq dataset, only one gene encoding a splicing factor was differentially expressed. This gene, *smooth*, which was down-regulated, but it is not known to be expressed in the mesoderm. We used primers for RT-PCR that flanked

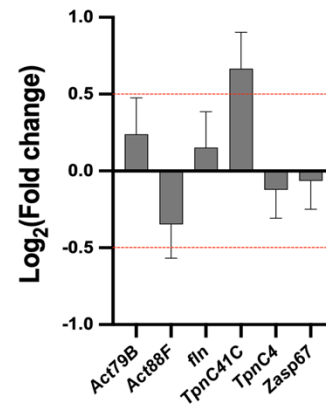
specific regions within *Mhc*, *Mlc1*, and *s/s* that show differential splicing between muscle types (Oas et al., 2014). We performed RT-PCR using RNA extracted from *w¹¹¹⁸* female adult thoracic muscles (T), *w¹¹¹⁸* whole larval muscles (L), control wing discs (C), and experimental wing discs (E) (Figure 8H). *Act5C* was used as a loading control. In the adult thoracic flight muscles, MHC exon 18 is included but excluded in other muscles. So, we designed a forward primer in exon 17, and a reverse primer in exon 19 to analyze this difference between control and experimental samples (Bernstein et al., 1986; Hastings and Emerson, 1991). As expected, we saw a band of 907 bp for the thoracic muscle sample (Figure 8H; lane 1) and no bands for the control experimental disc sample (Figure 8H; lane 3). We also observed that the experimental disc sample excludes exon 18 (Figure 8H; lane 4), similar to the larval muscle sample (Figure 8H; lane 2).

Figure 8. Up-regulated genes are characteristic of embryonic and larval muscle. (A–B) Bar graphs showing genes associated with embryonic and larval muscle, and pupal and adult muscles. Significant differentially expressed genes (DEGs) in wing imaginal discs of late third-instar female larvae upon *Mef2* over-expression are indicated with an asterisk. Log₂ fold change greater than or less than 0.5 plotted against p-value <0.05 was deemed significant (red dotted line). The embryonic and larval muscle genes, *Act87E* and *TpnC73F*, were significantly up-regulated. No significant DEGs were observed for the pupal and adult muscle genes category. (C) Quantitative RT-PCR (qPCR) of larval and adult genes revealed *Mhc*, *Tnl*, and *Act57B* to be significantly up-regulated. Log fold change greater than two was deemed significant (asterisk). (D–E) Immunofluorescence of late third instar female wing discs with elevated *Mef2* expression. *flightin* (*fln*) and *Actin88F* (*Act88F*) did not show expression in experimental wing discs. (D'–E') Cut and MEF2 stains show the myoblast pools for each disc. Scale bar: 50 µm. (F–G) Immunofluorescence of 1–2 day old female thoracic indirect flight muscles (IFMs). Fln and Act88F showed accumulation in the IFMs. Scale bar: 50 µm. (H) RT-PCR of *Mhc*, *Mlc1*, and *s/s* in *w¹¹¹⁸* female adult thoracic muscles (T), *w¹¹¹⁸* whole larval muscles (L), control wing discs (C), and experimental wing discs (E) revealed larval isoforms to be expressed in experimental wing discs. *Act5C* was used as a loading control. Lane numbers are depicted on the first row. *Mhc* exon 18 is included in T (907 bp; lane 1) but excluded in L and E (411 bp; lanes 2 and 4), and C revealed no bands (lane 3). *Mlc1* exon 5 is excluded in T (447 bp; lane 5) but included in L, C, and E (494 bp; lanes 6, 7, and 8). For *s/s*, exon 10 is excluded in T (106 bp; lane 9) and included in L and E (1075 bp; lanes 10 and 12), and C revealed no bands (lane 11). All graphs are Mean ± Standard Error (Student's two-tailed *t*-test).

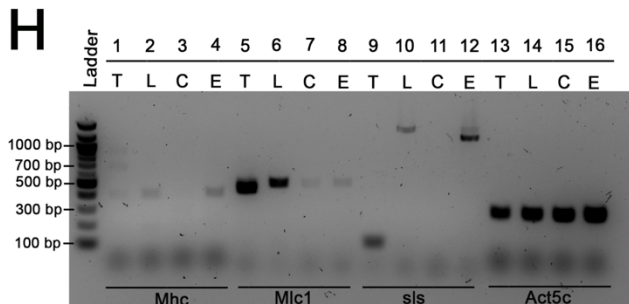
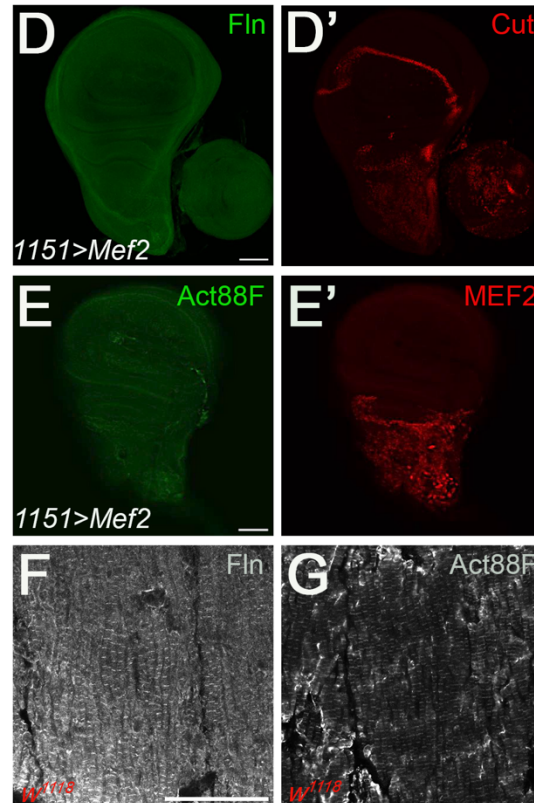
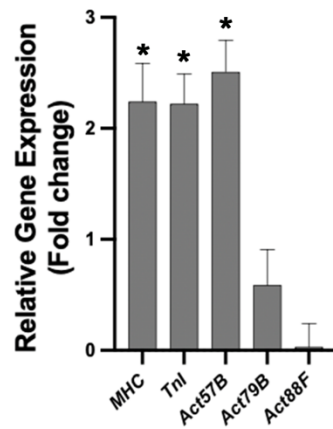
A Embryonic and larval muscle genes



B Pupal and adult muscle genes



C qPCR of larval and adult genes



T: adult thoracic muscles
L: larval muscles
C:1151/+
E:1151>Mef2

Next, we assessed *Mlc1* transcripts, which differ between larval and adult transcripts by 47 nucleotides, because *Mlc1* exon 5 is excluded in thoracic flight muscles but included in larval muscles (Falkenthal et al., 1987). For the thoracic muscle sample, we saw a band at 447 bp (Figure 8H; lane 5) and a band at 494 bp for the larval muscle sample (Figure 8H; lane 6). For imaginal discs, we observed bands at 494 bp in both control and experimental disc samples (Figure 8H; lanes 7 and 8), similar to what was observed for the larval muscle sample.

Lastly, we analyzed *s/s*, which encodes a Titin isoform. Transcripts of the *s/s* gene exclude exon 10 in flight muscles, whereas it includes this exon in jump muscles (Oas et al., 2014). We observed a band size of 106 bp for the thoracic muscle sample (Figure 8H; lane 9) and a band size of 1075 bp for the larval muscle sample (Figure 8H; lane 10). The control wing disc sample did not reveal a band (Figure 8H; lane 11) but the experimental wing disc sample revealed two bands, one of which was at 1075 bp, similar to the larval muscle sample, and the other band was slightly smaller (Figure 8H; lane 12).

Overall, we conclude that the predominant myogenic splicing program that is up-regulated in wing imaginal discs more closely reflected the embryonic/larval program, rather than the formation of the more complex pupal/adult muscles. Interestingly, whereas two out of three myofibrillar protein genes assayed did not show transcripts in the control discs, we observed low levels of *Mlc1* transcripts in control discs. This suggests that some aspects of the myogenic program may be poised to be activated even in control discs, and is consistent with the detection of *Act57B* transcripts in wild-type imaginal discs (Butler et al., 2003).

DISCUSSION

In this paper, we found that enhanced expression of *Drosophila Mef2* in adult myoblasts of the larval wing imaginal discs induced an incomplete myogenic response. Transcriptomic analysis, coupled with immunofluorescence and qPCR, of whole wing discs from control and experimental female larvae revealed a preference for the expression of muscle structural genes. Genes most strongly activated tended to be those expressed throughout normal development, rather than those genes specific for the pupal or adult stages.

Heterogeneity in muscle differentiation stages

Immunofluorescent analysis of discs displaying muscle differentiation revealed striations in certain myofibril-like structures, whereas others lacked this characteristic. One interpretation of this observation is that the two different phenotypes represent successive stages of myofibril assembly occurring, beginning with the accumulation of myofibrillar proteins, and subsequent assembly into ordered structures. Myofibril assembly is thought to occur first through the accumulation of alpha-actinin and titin into Z-bodies, and subsequent assembly into pre-myofibrils containing mostly myofibrillar components plus non-muscle myosin (encoded by *zipper* in *Drosophila*). Muscle myosin subsequently displaces the non-muscle isoform as the myofibrils mature (Sanger et al., 2002; reviewed in Sparrow and Schöck, 2009). In this scenario, the un-ordered deposits of muscle proteins may be awaiting assembly. However, during normal pre-myofibril formation, these proteins are generally present at much lower levels. Furthermore, given the presence of muscle MHC both in deposits and in striated structures, it seems more likely that the deposits represent proteins that have failed to organize normally,

perhaps because the normal sequential expression of myofibrillar protein genes is not occurring in this system.

Why, then, do some regions of the disc show organized structures whereas others do not? We imagine that the timing and level of accumulation of different myofibrillar proteins is somewhat stochastic between different cells, and it is possible that some proteins need to be present at significant levels to trigger ordered assembly. In this case, such “organizer” proteins should be more frequently present in striated structures and never in un-ordered deposits. Along these lines, we note that CG43897 and Zip almost exclusively accumulated in striated structures, suggesting either that they have organizer properties in the nascent myotubes, or are closely associated with proteins that may organize myofibril assembly.

MEF2 accumulation and differentiation varied between myoblasts

Both MEF2 accumulation and the region of differentiation varied across the myoblast field: muscle differentiation more prominently occurred at the proximal region of the imaginal wing disc, where MEF2 accumulation was also more pronounced. The explanation for this regional localization is not clear, although Cut protein is known to repress differentiation and shows reduced expression in proximal myoblasts as a result of Wingless signaling repressing Cut accumulation (Sudarsan et al., 2001). Therefore, it is possible that differentiation occurs as a product of distance from higher *cut* expression, and differentiation might be potentiated by simultaneously knocking down *cut* expression. Variation in MEF2 accumulation might arise in myoblasts that are activated earlier for differentiation, and along these lines, MEF2 is known to activate its

own expression in maturing muscles (Cripps et al., 2004), therefore those cells showing higher MEF2 accumulation might have activated this positive autoregulatory loop.

We also note the fundamental differences in detection that we are using when assessing transcript levels and protein accumulation. Are transcripts for a particular gene up-regulated in all myoblasts, or in just a subset of myoblasts? Since *Mef2* expression is controlled directly by the *1151-Gal4* driver, it must be up-regulated in all myoblasts, even though its expression appears to be further potentiated in a subset of myoblasts. For myofibrillar protein genes, it is as yet unclear if their expression is increased in all myoblasts or in just a subset of them. If the former, this would raise the interesting possibility that translational efficiency may differ between myoblasts. This question could partially be addressed by comparing protein accumulation with transcript levels using *in situ* hybridization.

Failure of MEF2 to induce myoblast fusion

Our results pose another question: why is myoblast fusion rarely observed, despite the knowledge that fusion genes are MEF2 targets? Our RNA-seq data analysis of fusion gene expression showed that only *m-spondin* (*mspo*) is significantly up-regulated, however, expression of other fusion genes that are known MEF2 targets such as *sns*, *rols*, *mbc*, and *kirre* were not significantly altered. It is possible that MEF2 requires the function of additional co-factors to trigger broad expression of myoblast fusion genes, and these co-factors are not activated by enhanced levels of MEF2. One likely candidate is the C2H2 zinc finger transcription factor Lameduck, which is both required for myoblast fusion and upstream of *Mef2* (Duan et al., 2001; Ruiz-Gomez et al., 2002; Cunha et al., 2010). It is also possible that other factors expressed in the

myoblasts may function to suppress myogenesis (including myoblast fusion). These include the known myogenic repressor, Zfh1 (Postigo et al., 1999).

Confirmation of RNA-sequencing data

One source of variability in our data was the significance of gene expression changes when comparing RNA-seq and qPCR datasets: some genes whose expression was not significantly altered in the RNA-seq showed significant changes in gene expression by qPCR, and vice versa. In addition, we also observed several gene products accumulating in the imaginal discs despite transcripts of the parent gene not being significantly up-regulated. The first obvious explanation for this difference could be the low number of replicates. For both datasets, we used three biological replicates, which have a poorer statistical power than a larger set. Statistically speaking, obtaining more replicates will decrease random variability and improve reproducibility. A paper published by Schurch et al. (2016) used a full set of 42 biological replicates, in each of two conditions, to evaluate how many replicates are needed to identify significantly differentially expressed (SDE) genes. They found that when using DESeq2, at least 12 replicates should be used to identify SDE genes for 0.5- and 2.0-fold changes. In this study, we used DESeq2 and three replicates to identify SDE genes for 0.5-fold changes, therefore some level of false positives or false negatives could have arisen. On the other hand, RNA-seq was a valuable guide to identifying many genes whose changes in expression were validated either by qPCR or by immunofluorescence.

We also postulate that differences in normalization methods and data analysis pipelines used for RNA-seq and qPCR may contribute to this variability. The normalization process, using DESeq2 for RNA-seq analysis, adjusts variations by

dividing the counts for each gene by the size factor for that sample. This method of normalization ensures that the counts are adjusted for differences in library size before performing a log transformation of the counts. On the other hand, qPCR relies on reference genes for normalization. The choice of reference genes is crucial and is typically validated during the experimental design, as was done in this experiment. Nevertheless, a consistent trend emerges from our data set, that being the ability of MEF2 to preferentially activate the larval myogenic program.

Activation of embryonic and larval vs pupal and adult genes

The adult muscles of *Drosophila* are more complex than those of the larva, and in particular, the thoracic muscles show at least two distinct fiber types that are not present in larval muscles (reviewed in Bernstein et al., 1993). Since the wing disc myoblasts are the precursors of these complex adult muscles (Bate et al., 1991; Fernandes et al., 1991; Sudarsan et al., 2001) we tested whether the muscle differentiation that occurred was reflective of adult muscles or larval muscles. Based upon the RNA-sequencing, qPCR, PCR, and immunofluorescence analysis, there was no strong evidence of up-regulation of adult muscle genes or splice variants. This suggests that MEF2 appears to be more active in driving the expression of genes required for muscle development during embryonic and larval stages rather than the pupal and adult stages. As the organism transitions to the pupal and adult stages, the role of MEF2 may evolve, requiring other regulatory factors to take the lead in driving the expression of genes essential for mature muscle tissue maintenance and function. Along these lines, of the 147 genes that were up-regulated, 27% had nearby MEF2 binding sites, as determined using embryonic ChIP-seq. By contrast, none of the adult

muscle genes had adjacent MEF2 binding sites in the same data set, even though *TpnC41C* is a known MEF2 target (Chechenova et al., 2015). While these data are derived from MEF2 binding in embryos, they provide some guidance as to why MEF2 enrichment did not activate the adult myofibrillar protein genes that we analyzed.

Adult flight muscle identity is dependent upon a regulatory suite of factors including Spalt-major (Schönbauer et al., 2011), Extradenticle plus Homothorax (Bryantsev et al., 2012b), and H15 (Katti et al., 2022), most of which commence expression early during pupal development. Therefore, it is likely that the myoblasts of the wing disc have not yet assumed an adult-like identity, and myotubes arising from these myoblasts follow a default larval pattern of differentiation. It remains to be determined if the expression of these adult myogenesis factors can trigger adult patterns of gene expression in the naive wing disc myoblasts.

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DISCLOSURES

The authors have no competing financial interests or other conflicts of interest.

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CHAPTER 2

The localization of *Drosophila melanogaster* CG11617 and its physiological relevance upon knockdown in muscle development

ABSTRACT

Drosophila melanogaster is a great model organism for studying the role of the invertebrate ortholog to *Mohawk* (*Mkx*), termed CG11617, a transcription factor that promotes the expression of slow fiber type specification. Formation of the *Drosophila* thoracic indirect flight muscles (IFMs) closely resemble that of fast-twitch (Type 2) muscle fibers in humans, and development of the tergal depressor of the trochanter muscles (TDT or jump muscles) may more closely resemble slow-twitch (Type 1) muscle fibers. In the mammalian counterpart, skeletal muscles are composed of a mixture of fiber types, where one fiber type predominates the other. In this study, we examined the localization of CG11617 and the structural integrity of *Drosophila* CG11617 knockdown mutant IFMs and jump muscles. Our analysis reveals nuclear localization of CG11617 in IFMs, whereas the jump muscles reveal nuclear and cytoplasmic localization between the myofibrils. CG11617 knockdown mutants also showed lethality in the pharate adult stage. Upon closer examination, these mutant flies presented fibrillar disorganization of IFMs and the absence of jump muscles. In addition, CG11617 may be needed to repress jump muscle genes in IFMs, therefore repressing IFMs from developing a jump muscle-like phenotype. Together, these findings may account for the overall skeletal muscle impairment and pupal lethality observed in the knockdowns.

INTRODUCTION

Muscle fibers in vertebrates and developing muscles in the *Drosophila* pupa share key similarities in their formation and function, reflecting a conserved evolutionary process (Taylor MV et al., 2006). In vertebrates, muscle fibers are categorized into two main types, such as slow-twitch (Type 1) and fast-twitch (Type 2) fibers, which are specialized for endurance and rapid contraction, respectively (reviewed in Mukund and Subramaniam, 2020). These muscle fibers develop through a complex process involving the differentiation of myoblasts into mature muscle cells, guided by specific transcription factors and signaling pathways. Key transcription factors, such as MyoD, Myf5, and members of the MEF2 family, help orchestrate the intricate developmental events, ensuring the proper formation and specialization of skeletal muscle fibers (reviewed in Hernández-Hernández et al., 2017).

Moreover, many transcription regulators, such as the vertebrate *Mkx*, and signaling pathways, such as Notch, Wnt, and IGF1, involved in muscle development, are conserved between *Drosophila* and mammals. Notch signaling has been shown to maintain the balance between the proliferation and differentiation of muscle precursor cells (Shang et al., 2016); Wnt signaling influences the fate of satellite cells (Grainger et al., 2018; Otto et al., 2008); and IGF1 is known to promote muscle growth and regeneration (Ahmad et al., 2020). Additionally, earlier studies show that the *Mus musculus* *Mkx* transcription factor is the most related murine protein to *Drosophila* CG11617. The homeodomain protein sequence identity between *Mkx* and CG11617 is 46% (Anderson et al., 2006), suggesting a conserved role between these orthologs.

Like vertebrates, the *Drosophila* skeletal muscle development and differentiation are complex processes that are regulated by many genetic and molecular mechanisms. *Drosophila* provides a powerful model for studying muscle development, particularly during the pupal stage when indirect flight muscles (IFMs) and jump muscles undergo significant growth and differentiation (as reviewed in Laurichesse and Soler, 2020). In early muscle development, mesodermal cells give rise to founder cells and adult muscle precursors, which fuse to templates and form myotubes in the pupal stage (as reviewed in Morriss et al., 2012). These myotubes are then further specified to become either flight muscles or jump muscles, which resemble vertebral fast-twitch or slow-twitch muscle fibers, respectively. Understanding these processes will contribute to our broader knowledge of muscle development and muscle fiber specification.

In this study, we examined the localization of CG11617 as well as the structural integrity of *Drosophila* CG11617 knockdown mutant indirect flight muscles (IFMs) and jump muscles. We found that CG11617 expression begins 24 hours after pupal formation (APF), and in adults, CG11617 is localized to the IFM nucleus; however, in the jump muscles, it is localized to the nucleus and cytoplasm between the myofibrils. Moreover, the knockdown of CG11617 in the developing adult thoracic muscles resulted in fibrillar disorganization of the IFMs, the complete absence of jump muscles, and lethality at the pharate adult stage. Interestingly, CG11617 knockdown mutant thoracic muscles do not form properly, which may result from the reduced myoblast pool we observed in the imaginal wing discs. Together, these findings may account for the overall skeletal muscle impairment and pupal lethality observed in the knockdowns.

MATERIALS AND METHODS

Drosophila stocks and crosses

All crosses were maintained in vials or bottles containing Jazz Mix medium (Genesee Scientific) at 25°C, allowed to lay eggs, and then transferred to 29°C incubators after three days to maximize Gal4-induced expression. The following fly stocks were used for *CG11617* knockdown analysis: *1151-Gal4*; *fln-LacZ* (myoblast specific driver, Bryantsev et al., 2012b); *UAS-RNAi [CG11617-30533]* (Vienna Stock Center, VDRC ID #10928); *UAS-RNAi [CG11617-30534]* (Vienna Stock Center, VDRC ID #10929); *UAS-RNAi [CG11617-RNAi(II)]* (Bloomington Drosophila Stock Center, BDSC ID #98603) and *w¹¹¹⁸*. To trigger *CG11617* knockdown in the thoracic muscles, we crossed female *1151-Gal4* driver to *UAS-RNAi* male adult flies. Three *CG11617* knockdown lines were generated using the three *UAS-RNAi* lines; *1151>UAS-RNAi [CG11617-30533]*, *1151>UAS-RNAi [CG11617-30534]* and *1151>UAS-RNAi [CG11617-RNAi(II)]*. We also crossed female *1151-Gal4*; *fln-lacZ* to male adult *w¹¹¹⁸* flies to generate the *1151/+* Control line. Since both *1151-Gal4* and *UAS-RNAi* are on the X chromosome, we analyzed female offspring.

We utilized a *UAS-Dicer* line from the Bloomington Drosophila Stock Center (BDSC, ID #59022) to ensure that the *CG11617* KD phenotype was not due to an off-target effect. A stable stock was generated by crossing females from the *1151-Gal4* driver line with males carrying *UAS-Dicer2*, marked with the balancer chromosome TM6 (*1151*; *UAS-Dicer2/TM6* stable stock). This balancer chromosome, with a tubby phenotype, was crossed to males from *UAS-RNAi [CG11617-30533]* to generate *1151 > RNAi[30533] + Dicer-2*, selecting female offspring. Females from this stable stock line were also crossed to males from *UAS-RNAi [CG11617-30534]* to generate *1151 >*

RNAi[30534] + *Dicer-2*, selecting non-tubby female offspring. Lastly, females from the stable stock line were crossed to males from *UAS-RNAi* [*CG11617-RNAi(II)*] to generate *1151 > RNAi*[*RNAi(II)*] + *Dicer-2*, selecting straight-wing female offspring.

The following fly stocks used for ectopic expression of CG11617 in fat cells: *Cg-Gal4* (fat cell-specific driver, Bloomington Drosophila Stock Center ID# 7011); *UAS-CG11617-HA* (Zurich ORFeome Project, ORF ID #F000068); and *w¹¹¹⁸*. To trigger ectopic expression of CG11617 in fat cells, we crossed female *Cg-Gal4* driver to male *UAS-CG11617-HA* to generate the Experimental fly line *Cg-Gal4>CG11617-HA*. For the Control line, we crossed female *Cg-Gal4* driver to male adult *w¹¹¹⁸* to generate *Cg-Gal4/+* flies. We analyzed female offspring.

The following fly stocks were used to generate a CG11617 Rescue line: A10 (*w*; *CyO/Bl*; *TM2,Ubx/TM6*) (Bloomington Drosophila Stock Center), *UAS-CG11617-HA* (Zurich ORFeome Project, ORF ID #F000068), and *UAS-RNAi* [*CG11617-30533*] (Vienna Stock Center, VDRC ID #30533). Two separate crosses were made. The first, A10 female flies were crossed to *UAS-CG11617-HA* males. From this line, females with curly wings (*CyO*) were collected. For the second cross, A10 female flies were crossed to *UAS-RNAi* males. From this second line, males with bristles on their halteres were collected (*TM2*). Then, curly-wing (*CyO*) female flies from the first cross were crossed to males with bristles on the haltere (*TM2*) to generate a *CyO/UAS-CG11617 RNAi*; *TM2/UAS-CG11617-HA* line. Males and female offspring were collected and crossed to each other to generate *UAS-CG11617-RNAi/UAS-CG11617-RNAi*; *UAS-CG11617-HA/UAS-CG11617-HA* stable stock fly line. Finally, stable stock male flies with straight wings but no bristles on their halteres (non-*TM2*) were crossed to *1151-Fln-Gal4* driver

females. This final cross generated the *1151-Gal4 > UAS-CG11617-RNAi + UAS-CG11617-HA* (Rescue) line, from which female offspring were collected for analysis.

The following fly stocks were used for *CG11617* knockdown analysis of thoracic muscles. A *Fln-Gal4* driver, which is activated only after the onset of muscle differentiation was used to assess the thoracic muscles up to the adult stage (Bryantsev et al., 2012b). Female *Fln-Gal4* driver flies were crossed to either male *w¹¹¹⁸* to generate *Fln-Gal4/+* (Control) offspring or to male *UAS-RNAi [CG11617-30533]* (Vienna Stock Center, VDRC ID #30533) to generate *Fln-Gal4>CG11617 KD* (Experimental) offspring. We analyzed female offspring for these experiments.

CG11617 protein expression and validation

The *CG11617* LD22691 cDNA clone was obtained from the Drosophila Genomics Resource Center (RRID: DGRC_5950). This clone contains the entire coding sequence for the *CG11617* isoform (RE08174). *CG11617* was subjected to PCR using the following oligonucleotides:

Forward: 5' – GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG
AGA TAG AAC CAT GGA CAA GTC AAC GCG GCC – 3'

Reverse: 5' – GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GAT ATG
GTG CCA GCG CTG CC – 3'

This resulted in a PCR product that was recombined into the Gateway vector pDONR221, using BP Clonase Gateway cloning (Thermo Fisher). Next, LR Clonase (Invitrogen; REF:56484; LOT# 2417590) was used to transfer the *CG11617* coding sequence into the pEXP1 destination vector. The pEXP1 adds an N-terminal 6x-His-Tag to *CG11617* to detect *CG11617* indirectly with an antibody against 6x-His and to enable

affinity purification of the expressed protein. This pEXP1/CG11617 plasmid was then transformed into XL1-Blue competent *Escherichia coli* (LOT# 25628898; Invitrogen). The resulting colonies were cultured under ampicillin selection (100 µg/ml) in LB broth at 37 °C on a shaker. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN; REF: 27106; LOT#163041120), and the plasmids were sequenced to ensure successful cloning. Two 500 mL LB broths were then inoculated with the starter culture of the correct plasmid inserted into BL21-derived *E. coli* cells and allowed to grow to an OD₆₀₀; however, efficient growth was not detected. We used BL21, BL21 DE3, and BL21 DE3 pLysS; however, efficient growth was also not detected, which we hypothesized was due to the leaky expression of CG11617 being toxic to the cells. Therefore, induction of CG11617 protein expression by lambda CE6 bacteriophage was utilized.

Before induction of the CG11617 protein, we first amplified the lambda CE6 bacteriophage using the Plate Lysate Amplification Method and titrating the Lambda CE6 Bacteriophage protocol according to the Lambda CE6 Induction Kit (Agilent Technologies). We determined that 0.66 ml of the bacteriophage was needed for a total concentration of 5.8×10^{11} pfu/ml, in a 50 ml culture. The bacteriophage provides precise control of protein expression, which is crucial for expressing toxic proteins such as CG11617. This approach ensured that T7 RNA polymerase was absent until the time of induction.

pEXP1/CG11617 plasmid was propagated into the LE392 host strain according to the Transformation Protocol. The transformation reaction was spread directly onto LB-ampicillin agar plates and incubated at 37°C. A single colony of these cells was

inoculated into 5 ml of NZCYM broth with 20% maltose containing ampicillin overnight in a 37°C shaker. In the morning, 1.0 ml of the overnight culture was centrifuged and resuspended in 1.0 ml of fresh NZCYM broth with 20% maltose, and subsequently pipetted into 50 ml of fresh NZCYM broth with 20% maltose. The culture was grown to an OD₆₀₀ of 0.3, and glucose was added for a final concentration of 4 mg/ml. Next, the culture was grown to OD₆₀₀ = 0.82, and 500 µl of 1M MgSO₄ was added for a final concentration of 10 mM. A 500 µl portion of the culture was removed to serve as the uninduced control.

Induction was performed by adding 0.66 ml of the bacteriophage, and samples were taken at 1-hour, 2-hour, and 3-hour time points after infection. At each time point, a 500 µl portion of the culture was removed, centrifuged, and the pellet resuspended in 80 µl of 1X SDS sample buffer for SDS-PAGE.

Protein purification was then conducted using a Protein Purification Kit (Invitrogen) against 6x-His-Tag on CG11617. Then, to assess the efficiency of protein expression and purification, a Western Blot was conducted following a standard protocol. Mouse anti-6x-His-Tag (REF #MA1-21315-HRP, LOT #YD371684, Invitrogen) primary antibody at 1:1000 dilution, and horseradish peroxidase (HRP)-linked goat anti-rabbit IgG at 1:5,000 dilution (Invitrogen) were used to detect the primary antibodies on the nitrocellulose membrane.

CG11617 antibody production and validation

The purified CG11617 protein, at a concentration of 6.556 mg/ml in 250 mM imidazole, was sent to ProSci, Inc. (Poway, CA). They developed the CG11617 rabbit antiserum by immunizing two New Zealand white female rabbits (#22533 and #22534).

Western blot using w¹¹¹⁸ whole female fly control line was used to validate the antibody. A total of four test bleeds and one pre-immune serum were tested on SDS-PAGE per animal. Bleed #4 from animal #22534 worked the best. An antibody against pan-actin (LOT# 4868S, Cell Signaling Technology) was used as the loading control.

The specificity of the antibody was validated using *Cg-Gal4>CG11617-HA* and *Cg-Gal4>w¹¹¹⁸* fly crosses. A monoclonal antibody against HA (REF #26183-HRP, LOT# WI330737; Invitrogen) was used to validate the specificity of the CG11617 antibody further.

Horseradish peroxidase (HRP)-linked goat anti-mouse IgG (HRP; 1:5,000; Invitrogen) and anti-rabbit IgG (HRP; 1:5,000; Invitrogen) were used to detect the primary antibodies on the nitrocellulose membrane.

Immunostaining thoracic muscles, abdominal fat cells, and imaginal wing discs

Drosophila pharate adults from Control and CG11617 KD lines were submerged in a Tissue-Tek medium, frozen in liquid nitrogen, and sectioned using an HM525 NX Thermo Scientific cryostat machine (Jaramillo et al., 2009). 10 - 12µm sections were dried on slides, fixed in 3.7% formaldehyde in 1X PBS for 8 minutes, and washed three times with phosphate buffer saline (PBS) containing 0.1% Triton-X 100 (PBTx). All primary antibodies were diluted in 1% (bovine serum albumin) BSA in PBTx for immunostaining and incubated with the samples overnight, in a humidity chamber. Then, the sections were washed with PBTx, and secondary antibodies were added and incubated for 2 hours, in a humidity chamber.

Adult experimental and control female fly abdomens were dissected in 1X PBS for the abdomen fat cell immunofluorescent analysis. Then, they were fixed in 3.7%

formaldehyde for 30 minutes at 4°C, followed by immunolabeling. All primary antibodies were incubated with the samples overnight at 4°C and secondary antibodies were incubated for 2 hours at 22°C. Samples were mounted in mounting media. All antibodies were diluted in 1% BSA (Bovine serum albumin 1% w/v). For immunofluorescent detection, the following primary antibodies and their concentrations were used:

- 1:500 HA (Invitrogen; LOT# XA337820)
- 1:500 CG11617 (this study)
- 1:500 MEF2 (Vishal et al., 2020)
- 1:500 β -integrin (DSHB CG.6G11)
- 1:500 22c10 (DSHB; Antibody Registry ID: AB_528403)

Wing discs collected from wandering 3rd instar female larvae were dissected in 1X PBS, fixed in 4% formaldehyde, and labeled using an antibody against Cut (2B10) (DSHB #AB_528186; 1:500) to visualize the myoblasts.

Alexa-conjugated secondary antibodies, Alexa 488 (Roche) and Alexa 568 (Roche), were diluted to 1:500 in 1% BSA. Phalloidin 647 (Roche) was diluted to 1:200 in 1% BSA to visualize skeletal muscles (F-actin). DAPI (Sigma) was diluted to 1:10000 in 1% BSA to visualize the nucleus.

Imaging and statistical analysis

Immunostained preparations were imaged on an Olympus FluoView 3000 confocal microscope after the mounted samples were left to dry for three hours. All images were processed using the Olympus Life Science software and assembled in Adobe Photoshop 2024 (version 25.0). All experimental and confocal images underwent equal changes in brightness and contrast.

ImageJ was used to calculate the fraction of *Drosophila* thorax occupied by the area of the indirect flight muscles (IFMs) to determine the fraction of the wing disc area occupied by the myoblast.

GraphPad Prism software (Version 10.0.3) was used to generate bar graphs. The error bars on all the graphs are presented as the standard error of the mean. A p-value of $p < 0.05$ was set as the threshold for significance and marked by asterisks. Student's two-tailed t-test was used to calculate the p-value.

Flight Tests

Flies were released from a vial situated in the center of a graded flying chamber. To classify each fly's flying ability, the landing location was recorded as upward (U), horizontally (H), downward (D), or not flying at all (N) (Miller et al., 2008).

RESULTS

Validation of CG11617 protein and antibody

To induce the expression of the CG11617-6x-His tagged protein, Lambda CE6 phage was utilized to deliver T7 RNA polymerase into the LE392 *Escherichia coli* host strain. Lambda CE6 bacteriophage is ideal for protein expression control by allowing a pause for the delivery of T7 RNA polymerase into the cells until the desired time of induction. We waited for OD₆₀₀ to reach 0.82 before beginning induction. This allowed for the successful expression of CG11617-6x-His tagged protein and determined the optimal time for its maximum expression. The Western blot showed no band at 0 hours (before induction), a faint band intensity at 38 kDa one hour after induction, a moderate band intensity two hours after induction, and the strongest band intensity three hours

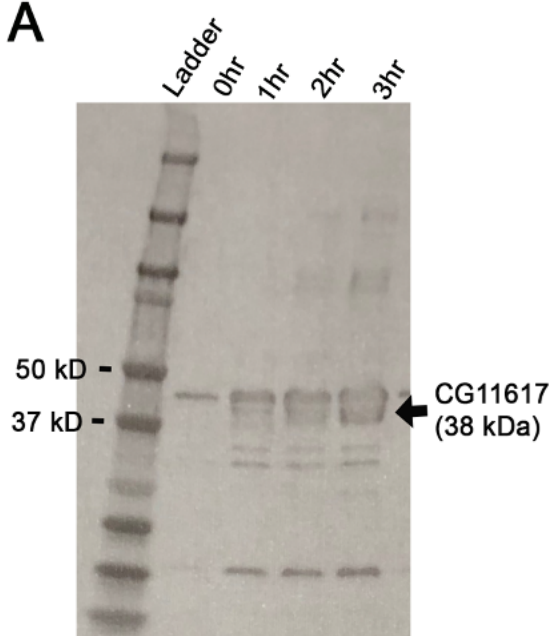
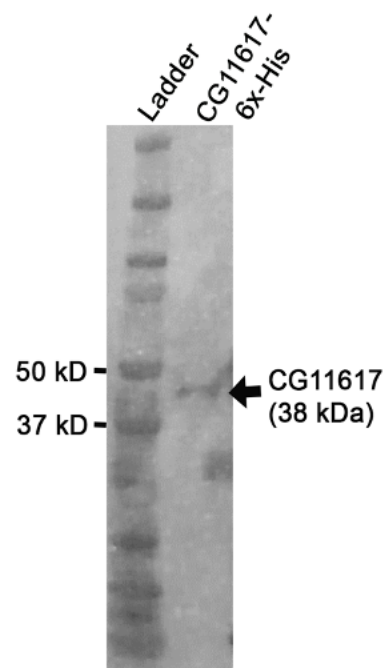
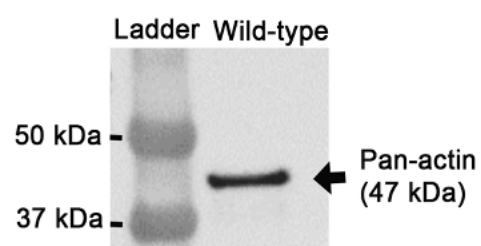
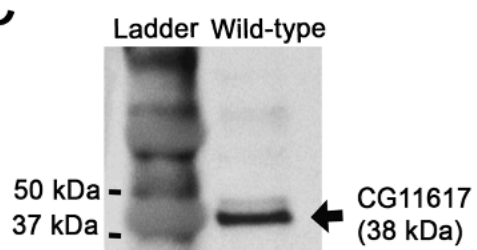
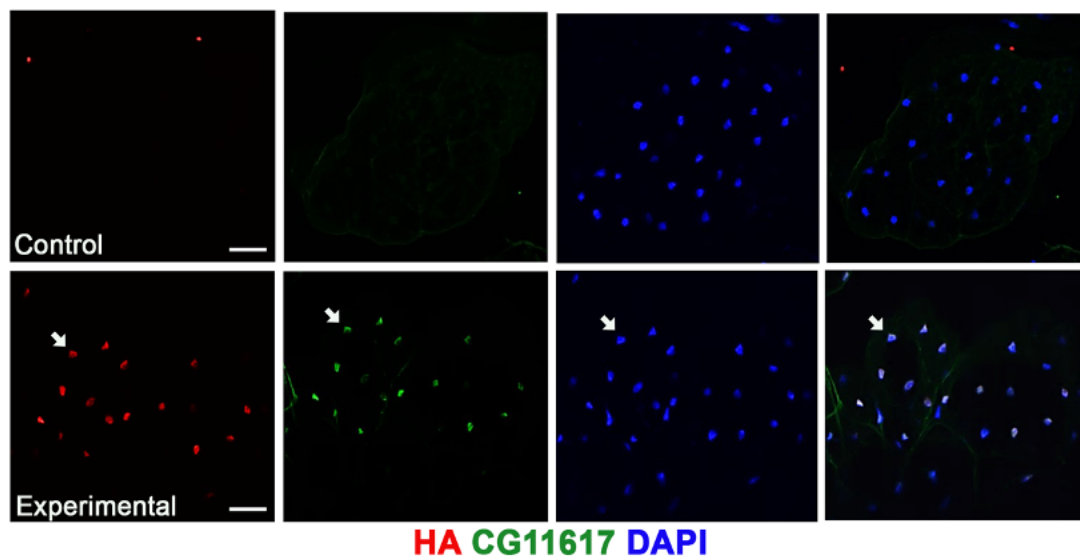
after induction (Figure 9A). This Western blot suggests that maximum CG11617 protein expression occurs 3 hours after induction with lambda CE6 bacteriophage.

After using standard nickel metal ion affinity binding to CG11617-6x-His tagged protein, we validated the purity of the CG11617-6x-His tagged protein using an antibody against 6x-His tag. The Western blot revealed a band of the correct size, 38 kDa (Figure 9B). We then sent 6.556 mg/ml in 250 mM imidazole of the protein to ProSci, Inc. (Poway, CA) for antibody production, where two New Zealand white female rabbits were immunized. We utilized Western blot to test each bleed (a total of four) using control (w^{1118}) whole adult female flies. We found that rabbit #22534, test bleed #4, worked the best recognizing the 38 kD protein that we had purified, with minimal background suggesting good specificity with the antibody (Figure 9C).

To further test the specificity of the CG11617 antibody we utilized the Gal4-UAS system (Brand & Perrimon, 1993) to ectopically express CG11617 mRNA in the fat body of adult female flies. Female adult *Cg-Gal4* driver was crossed to *UAS-CG11617-HA* male adult flies to generate *Cg-Gal4>CG11617-HA* (Experimental) offspring, which should express an HA-tagged isoform of CG11617 in the adult fat body. The Control line was established by crossing the female adult *Cg-Gal4* driver to w^{1118} generating *Cg-Gal4>w¹¹¹⁸* (Control). We immunofluorescently stained the abdomen of the female flies with antibodies that recognized human influenza hemagglutinin (HA; red) tagged to CG11617, CG11617 (green), and the nucleus (DAPI; blue). We observed the expression of HA and CG11617 in the *Cg-Gal4>CG11617-HA* (Experimental) abdominal fat body samples, but not in the *Cg-Gal4>w¹¹¹⁸* (Control) samples (Figure

9D). This suggests that our antibody is specific to CG11617 and can be utilized for further analysis.

Figure 9. CG11617 protein and antibody validation. (A) The western blot showed no band at 0 hour (before induction), a faint band intensity at 38 kD one hour after induction, a moderate band intensity 2 hours after induction, and the strongest band intensity 3 hours after induction. (B) Western blot of purified CG11617-6x-His tagged protein shows a band at 38 kD. (C) We used a wild-type (w^{1118}) whole fly and observed a band size of the correct size, at 38 kD (left gel). Pan-actin was used as a loading control (right gel). (D) We observed the expression of HA and CG11617 in the Cg-Gal4>CG11617-HA (Experimental; arrow) abdominal fat body samples, but not the Cg-Gal4> w^{1118} (Control) samples. Scale bar: 50 μ m.

A**B****C****D**

Expression pattern of CG11617 in *Drosophila* thoracic muscles

To determine the expression pattern of CG11617, antibodies against Myocyte enhancer factor-2 (MEF2) to visualize the myoblasts (Figure 10A; top row; arrowhead), and CG11617 to visualize CG11617 expression were used (Figure 10A; bottom row; arrowhead). Myoblasts are precursor muscle cells that give rise to the adult thoracic muscles (Bate et al., 1991; Dobi et al., 2015). We selected Control (w^{1118}) samples at 16 hours after pupal formation (APF), 24 hours APF, 36 hours APF, and 48 hours APF for immunolabeling because these developmental time points cover the events of myoblast fusion, myotube formation, myofibril assembly, and myofibril growth, respectively, and whose proper function is critical in muscle development (Spletter et al., 2018). Control flies did not show accumulation of CG11617 at 16 hours APF, while MEF2 was detected, indicating the presence of myoblasts. Moreover, CG11617 expression was visible 24 hours APF, 36 hours APF, and 48 hours APF (Figure 10A; arrowhead). This suggests that CG11617 expression begins between 16 and 24 hours APF, during the pupal stage, and may function later in the muscle differentiation process since it is not present during the myoblast fusion period (16 hours APF) but during myotube formation.

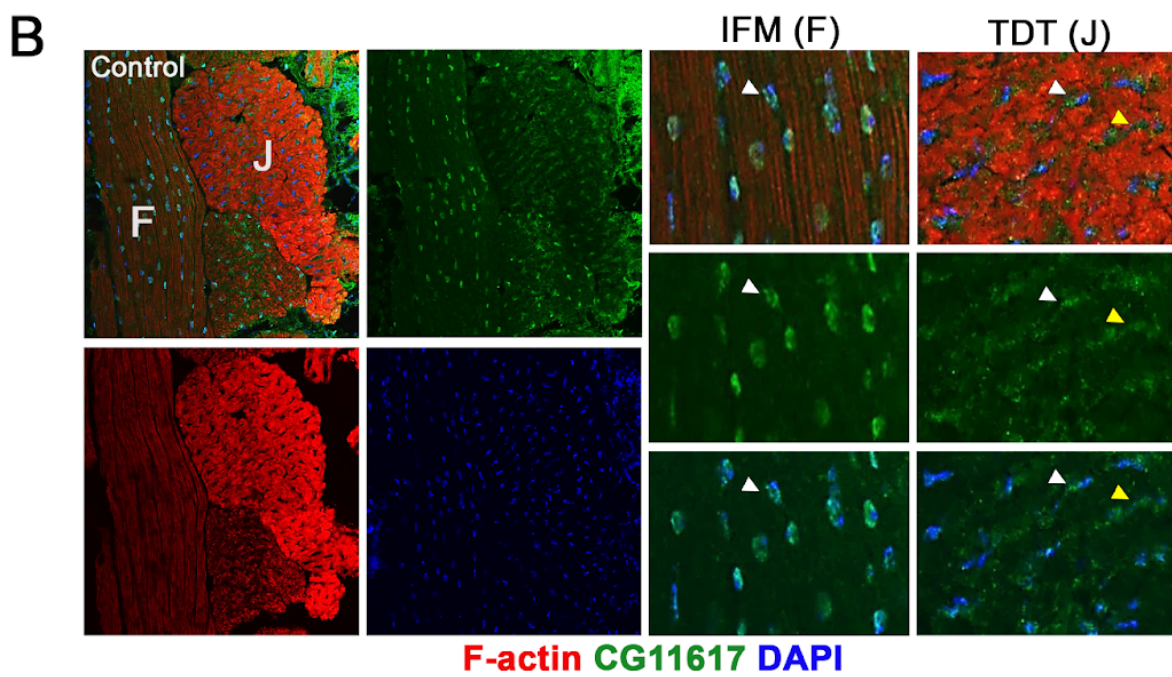
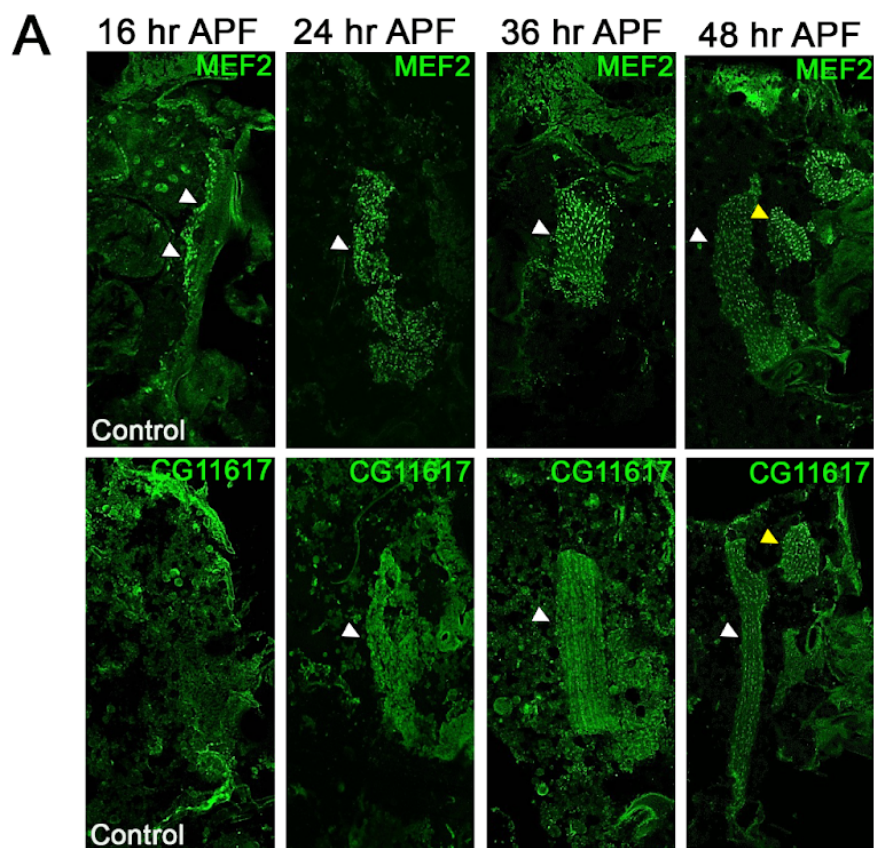
CG11617 is localized to the adult muscle nucleus

To uncover where CG11617 is localized in Control (w^{1118}) *Drosophila* adult thoracic muscles, we utilized our antibody against CG11617 (green), F-actin to visualize skeletal muscles (red), and DAPI to visualize the nucleus (blue). We cryosectioned through the thorax of the flies, and the thoracic muscles showed a punctate stain in the indirect flight muscles (F), colocalized with nuclear staining, indicating that the epitope is localized to the nucleus (Figure 10B). The jump muscles (J) showed nuclear and

cytoplasmic staining between the myofibrils (Figure 10B). These findings show a difference in CG11617 sub-cellular localization between the indirect flight muscles and jump muscles. This suggests that an unknown CG11617 co-factor or post-translational modification may impact CG11617 localization, resulting in the distinct differentiation between these two muscle fiber types.

Figure 10. Expression pattern and localization of CG11617 in adult thoracic muscles.

Expression pattern and localization of CG11617 in adult thoracic muscles. (A) Expression of MEF2 (top panels) and CG11617 (bottom panels) at 16 hr APF, 24 hr APF, 36 hr APF, and 48 hr APF. White arrowheads in the top panels point to MEF2-positive cells that are localized near the body wall of the thorax at 16 hr APF and migrate inwards to fuse and develop into the flight and jump muscles of the fly. The arrowheads in the bottom panel point to CG11617-positive cells where expression of CG11617 begins at 24 hr APF. All yellow arrowheads point to the jump muscles. (B) CG11617 punctate stain in the nucleus of indirect flight muscles (F), and nuclear and cytoplasmic staining between the myofibrils in jump muscles (J). White arrowhead points to the nucleus. Yellow arrowhead points to the cytoplasm. CG11617 (green), F-actin to visualize skeletal muscles (red), and DAPI to visualize the nucleus (blue).



Analyzing CG11617 in RNAi knockdown lines

To assess the role of CG11617 in muscle development, we generated CG11617 knockdown flies using the Gal4-UAS system (Brand & Perrimon, 1993) to express RNAi targeting CG11617 mRNA in developing muscles. Female adult *1151-Gal4* driver flies were crossed to *UAS-RNAi [CG11617-30533]* male adult flies to generate *1151 > UAS-RNAi [CG11617-30533]* or to male *w¹¹¹⁸* to generate *1151/+* (Control) offspring. We also generated two additional CG11617 knockdown lines using two UAS-RNAi lines: *UAS-RNAi [CG11617-30534]* and *UAS-RNAi [CG11617-RNAi(II)]*. Control and *1151 > CG11617 RNAi* pharate adult flies were cryosectioned through the thorax and immunostained to visualize the thoracic muscles. We used phalloidin, which recognizes F-actin, to visualize the skeletal muscles (red) and DAPI to visualize the nucleus (blue). Control flies show normal thoracic muscle structures of indirect flight muscles (IFMs; F) and jump muscles (J) (n=5) (Figure 11A). *1151 > UAS-RNAi [CG11617-30534]* and *1151 > UAS-RNAi [CG11617-RNAi(II)]* did not generate a knockdown phenotype (data not shown). However, *1151 > UAS-RNAi [CG11617-30533]* (CG11617 KD) flies show fibrillar disorganization of the IFMs (F) and the absence of jump muscles (J) (n=5) (Figure 11A). This fly line also exhibited 100% lethality at the adult pharate stage.

Validation of CG11617 knockdown line with Dicer-2

To determine whether the phenotype that arises from knocking down CG11617 is from an off-target effect, we generated Dicer lines with three available CG11617 RNAi lines. Dicer can achieve a more efficient and specific knockdown of CG11617, enhancing the RNAi effect because the resulting small interfering RNAs (siRNAs) are

more specific to *CG11617* mRNA, increasing the precision of knocking down this gene (reviewed in Wilson and Doudna, 2013). Dicer works by recognizing and binding to double-stranded RNA (dsRNA), cleaving dsRNA into smaller fragments with the aid of its RNase III domains. The siRNA duplex is then transferred to the RNA-induced silencing (RISC) complex, where the siRNA duplex is unwound and the complementary strand is degraded (reviewed in Wilson and Doudna, 2013). The retained guide strand directs the Argonaute protein in the RISC complex to cleave complementary mRNA molecules for sequence-specific silencing upon binding of mRNAs (reviewed in Wilson and Doudna, 2013).

To establish Dicer crosses, we generated a stable stock by crossing females from the *1151-Gal4* driver line with males carrying *UAS-Dicer2* marked with the balancer chromosome TM6, which is the tubby phenotype. The resulting stable stock line is *1151; UAS-Dicer2/TM6*. The TM6 balancer chromosome aids in maintaining the stability of the genetic line during subsequent crosses. Females from this stable stock line were crossed to males from *UAS-RNAi [CG11617-30533]* to generate *1151 > RNAi[30533] + Dicer-2*, selecting female offspring. Females from this stable stock line were also crossed to males from *UAS-RNAi [CG11617-30534]* to generate *1151 > RNAi[30534] + Dicer-2*, selecting non-stubble female offspring. Lastly, females from the stable stock line were crossed to males from *UAS-RNAi [CG11617-RNAi(II)]* to generate *1151 > RNAi[RNAi(II)] + Dicer-2*, selecting straight-wing female offspring. The *1151 > RNAi[30533] + Dicer-2* line revealed 100% of the adult pharate flies to be non-viable (Table 3). While *1151 > RNAi[30534] + Dicer-2* and *1151 > RNAi[RNAi(II)] +*

Dicer-2 adult pharate flies showed 22.72% and 17.65%, respectively, to be non-viable (Table 3).

The 100% lethality observed at the adult pharate stage of *1151 > RNAi[30533] + Dicer-2* female flies indicates that knocking down *CG11617* may be critical for survival during the late stages of pupal development.

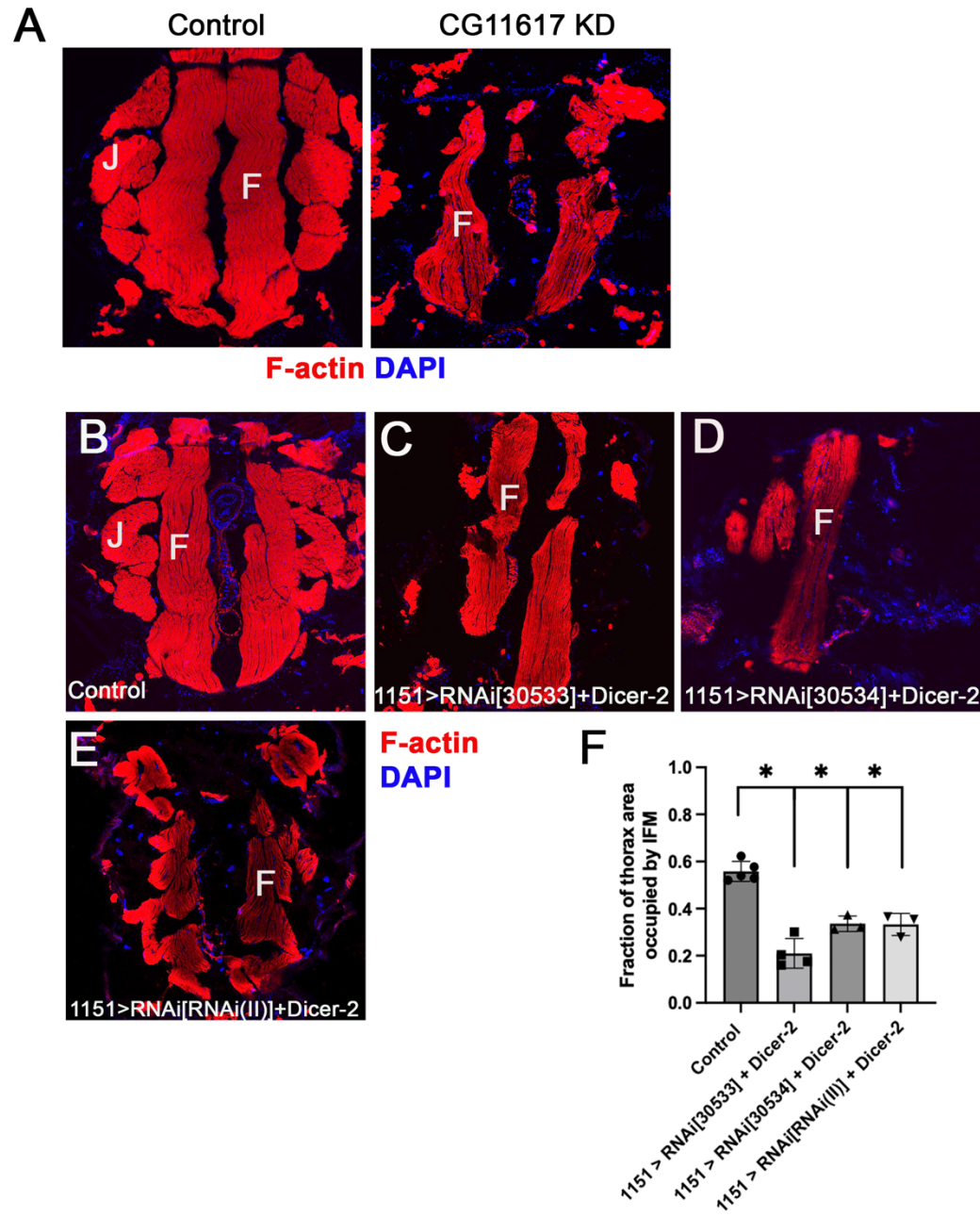
Table 3. Lethality of *Dicer-2* crosses.

Fly line	Viable	Non-viable	Total flies assessed	Percent non-viable
<i>1151 > RNAi[30533] + Dicer-2</i>	0	26	26	100%
<i>1151 > RNAi[30534] + Dicer-2</i>	34	10	44	22.72%
<i>1151 > RNAi[RNAi(II)] + Dicer-2</i>	42	9	51	17.65%

Next, we analyzed the development of the *Dicer-2* lines' thoracic muscles. We cryosectioned through the thorax of the *Dicer* lines and stained with β -integrin (muscle fiber periphery; cyan), phalloidin, which recognizes F-actin (skeletal muscles; red), and DAPI (nucleus; blue). Immunofluorescent images showed normal development of Control indirect flight muscles (IFMs; F) and jump muscles (J) ($n = 3$) (Figure 11B), severe disorganization of IFMs (F) and absence of jump muscles (J) in *1151 > RNAi[30533] + Dicer-2* flies ($n = 4$) (Figure 11C), and fibrillar disorganization of IFMs (F) and absence of jump muscles in both *1151 > RNAi[30534] + Dicer-2* flies ($n = 2$) (Figure 11D) and *1151 > RNAi[RNAi(II)] + Dicer-2* flies ($n = 3$) (Figure 11E). Lastly, we quantified the fraction of thorax occupied by IFMs and observed a significant loss of muscle tissue in all *CG11617* RNAi knockdowns compared to the Control (Figure 11F).

All three knockdowns produced the same phenotype, indicating a genuine requirement for *CG11617* in muscle development.

Figure 11. Muscle loss upon *CG11617* knockdown in indirect flight muscles. (A) Immunostaining of 1151 > *w¹¹¹⁸* (Control) flies show normal development of indirect flight muscles (IFMs; F) and jump muscles (J) (n = 5). Immunostaining of *CG11617>KD* flies show fibrillar disorganization of the IFMs (F) and the absence of jump muscles (n = 5). F-actin (skeletal muscle; red) and DAPI (nucleus; blue). (B) 1151>*w¹¹¹⁸* + Dicer (Control) IFMs (F) show and jump muscles (J) (n = 3) show normal development. (C) Severe disorganization of IFMs (F) and the absence of jump muscles (J) were observed in 1151 > RNAi[30533] flies (n = 4). (D) Fibrillar disorganization of IFMs (F) and jump muscles (J) was seen in 1151 > RNAi[30534] + Dicer-2 flies (n = 2) and in (E) 1151 > RNAi[RNAi(II)] + Dicer-2 flies (n = 3). (F) Quantification of IFM muscle loss shows significant muscle loss in 1151 > RNAi[30533] flies (n = 4; p-value = 2.2×10^{-5}), 1151 > RNAi[30534] flies (n = 3; p-value = 0.00025), and 1151 > RNAi[RNAi(II)] flies (n = 3, 0.00042). The error bars were calculated by the standard error of the mean. All p-values < 0.05 were deemed significant and calculated by student's two-tailed t-test. All images were taken at 20X magnification.



Validation of CG11617 KD line with Rescue line

To further validate that the most pronounced phenotype is not due to an off-target effect, we conducted a rescue experiment. We expressed *CG11617-HA* in the CG11617 KD flies by adding the previously validated *UAS-CG11617-HA* line. We cryosectioned through the thorax of Rescue female pharates and stained the muscles with phalloidin that recognized F-actin (red), the nucleus with DAPI (blue), and the periphery of the myofibers with β -integrin (cyan). We saw restored IFMs (F) and jump muscles (J) in the Rescue line (n = 4) (Figure 12A) suggesting that the observed phenotypic changes are indeed due to *CG11617* knockdown and not off-target effects.

The *UAS-RNAi [CG11617-30533]* line that generated the more severe phenotype was then utilized to ensure a more pronounced and observable effect on muscle fiber type specification, facilitating a clearer and more definitive insight into the gene's function.

CG11617 knockdown disrupts normal muscle development, and the Rescue line restores it

To address the impact of loss of tissue, we calculated the fraction of the thorax area occupied by the indirect flight muscles (IFMs). These results showed a significant reduction of IFM tissue in the CG11617 KD flies (n = 5) compared to the Control (n = 5) (p-value = 0.00018) (Figure 12B). We also found that the Rescue IFM tissue (n = 4) was restored, comparable to the Control (n = 5) (Figure 12B).

We then examined CG11617 protein levels via immunofluorescence and *CG11617* mRNA levels via qPCR in CG11617 KD and Rescue line IFMs. We cryosectioned through the thorax of female adult pharate CG11617 RNAi knockdown flies and used antibodies against CG11617 (green), β -integrin (muscle fiber periphery;

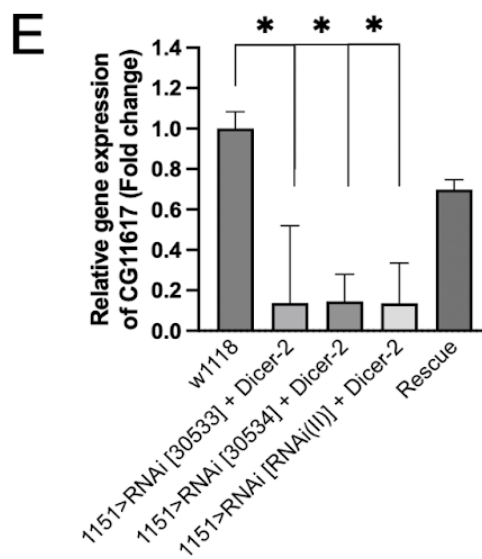
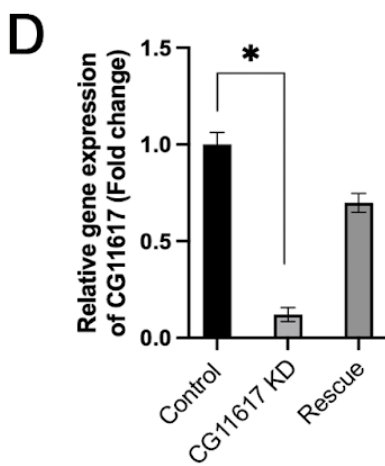
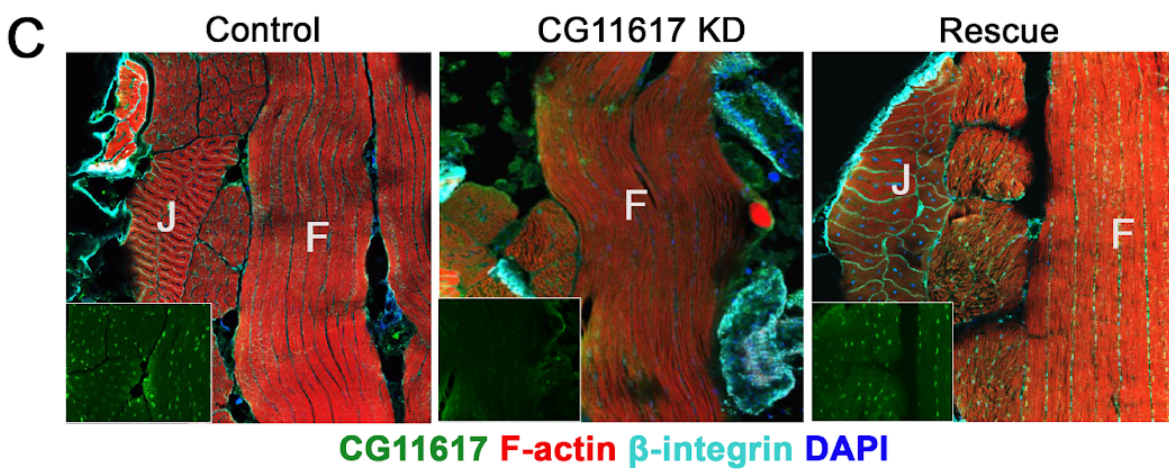
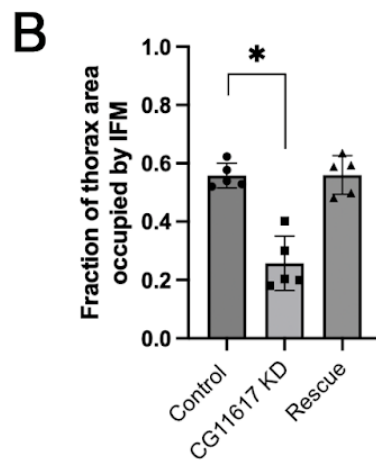
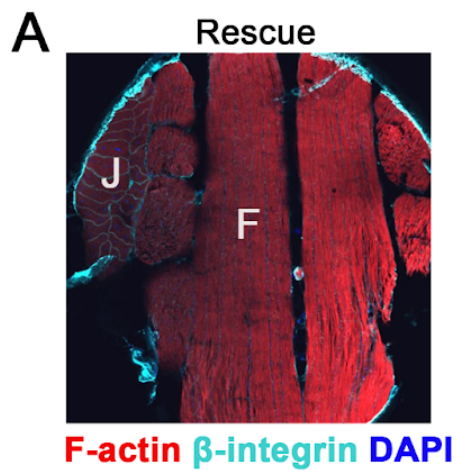
cyan), phalloidin, which recognizes F-actin (skeletal muscles; red), and DAPI (nucleus; blue). The fluorescent images revealed decreased levels of *CG11617* in the *CG11617* KD flies (n = 3) and restored expression in the Rescue line (n = 3) (Figure 12C).

Quantifying *CG11617* mRNA expression levels revealed significantly reduced expression in *CG11617* KD flies (n = 6) (p-value = 0.01804) and restored expression levels in the Rescue flies (n = 6) (Figure 12D).

Lastly, we quantified the mRNA levels of *CG11617* with qPCR to assess *CG11617* knockdown efficiency in our Dicer lines. We used six thorax samples per line. We found that the expression levels were significantly reduced in all three *CG11617 RNAi + Dicer-2* lines (1151> UAS[*CG11617-30533*]; n = 6; p-value = 0.0019) (1151> UAS[*CG11617-30534*]; n = 6; p-value = 0.00014) (1151> UAS[*CG11617-RNAi(II)*]; n = 6; p-value = 0.00346) and restored in the Rescue line (n = 6) compared to the Control line (Figure 12E). These data further validate that our *RNAi[30533]* and Rescue lines are suitable for further experimental use.

Overall, these findings may account for the overall skeletal muscle impairment and pupal lethality observed in the knockdowns and demonstrate the necessity for *CG11617* in adult muscle development. However, it remains unclear whether IFMs and jump muscles degenerate over time or do not form properly during the first or second stage of myogenesis.

Figure 12. Restored thoracic muscles and CG11617 expression in the Rescue line. (A) Immunofluorescent images of Rescue female pharates revealed restored IFMs (F) and jump muscles (J) (n = 4). Phalloidin (F-actin; red; muscles), β -integrin (muscle periphery; cyan), and DAPI (nucleus; blue). 20X magnification. The left lower frame is a zoomed-in portion of the IFMs. (B) The fraction of the thorax area occupied by CG11617 KD IFMs shows a significant loss of muscle and the Rescue line IFMs show restored IFM tissue comparable to the control. (C) Immunofluorescent images show reduced levels of CG11617 in the IFM of CG11617 KD flies. Phalloidin (F-actin; red; skeletal muscles), β -integrin (muscle periphery; cyan), and DAPI (nucleus; blue). 40X magnification. (D) CG11617 expression levels were quantified revealing a significant reduction in CG11617 KD flies and restored expression in the Rescue flies. (E) Quantification of CG11617 expression levels shows a significant reduction in all three CG11617 RNAi + Dicer-2 lines and restored expression levels in the Rescue line. The error bars, on all the graphs, were calculated by the standard error of the mean. All p-values < 0.05 were deemed significant and calculated by two-tailed student's t-test.



Reduced myoblast pool in CG11617 knockdown imaginal wing discs

To determine if *CG11617* loss-of-function affects myoblast pool or pupal myogenesis, as an indication of skeletal muscle degenerating or improper development, an antibody against Cut (Ct) was used to visualize the myoblast pool in the notum of Control and CG11617 KD wandering 3rd instar female larva imaginal wing discs (Figure 13A). We observed an 18.63% reduction in the experimental discs' (n=5, SEM= 0.00636) myoblast pool compared to the control (n=5, SEM = 0.003) (p-value = 0.02) (Figure 13B). Roy & VijayRaghavan's (1998) observation suggests that a low myoblast pool can lead to muscle degeneration. Therefore, a low myoblast pool may account for the fibrillar disorganization, and the absence of jump muscle development observed in CG11617 KD flies.

We then aimed to investigate whether a decrease in the myoblast pool arises from knocking down *CG11617*. We immunostained *1151/+* and CG11617 KD imaginal wing discs of 2nd instar (L2) and wandering 3rd instar (L3) female larva with our antibody against CG11617. We examined these stages because RNA-seq CG11617 expression data using whole organism samples shows moderate CG11617 expression between L2 and L3 stages (Flybase Consortium, 2024). We expected a reduction of CG11617 expression in the imaginal wing discs, however, there was no expression in either *1151/+* or CG11617 KD wing disc samples (data not shown). To ensure that CG11617 can be detected in the imaginal wing discs we overexpressed CG11617 using an *1151-Fln* drive line and our *UAS-CG11617-HA* line to produce an *1151>CG11617-HA* fly line. We saw the expression of CG11617 in the L3 wing discs, ruling out that our CG11617 antibody cannot detect this protein in the wing discs (Figure 13C).

Overall, these data indicate that CG11617 is not detectably expressed in L2 and L3 wing discs. We are unclear as to why the knockdown results in a reduced myoblast pool; it may be that the CG11617 protein is present and necessary for myoblast number but below the level of detection. Alternatively, it may be that earlier expression of the RNAi affects the initial size of the myoblast pool.

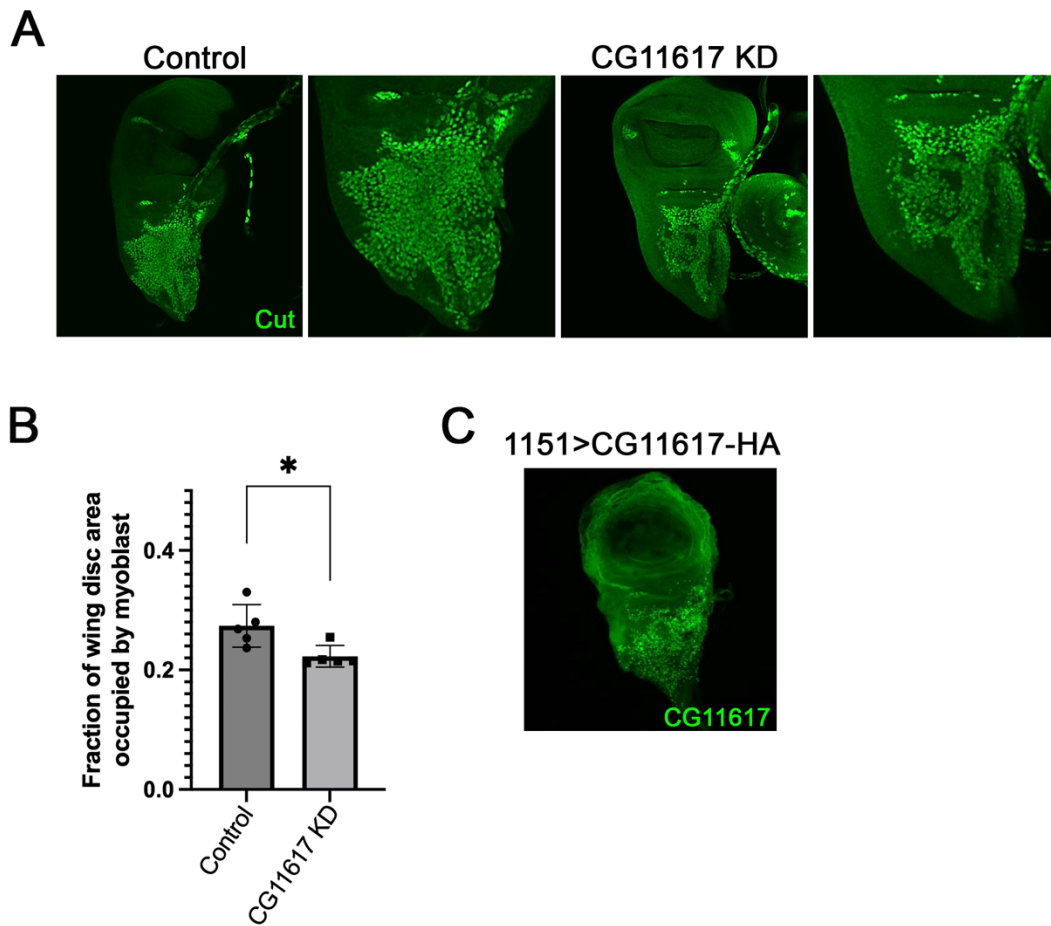


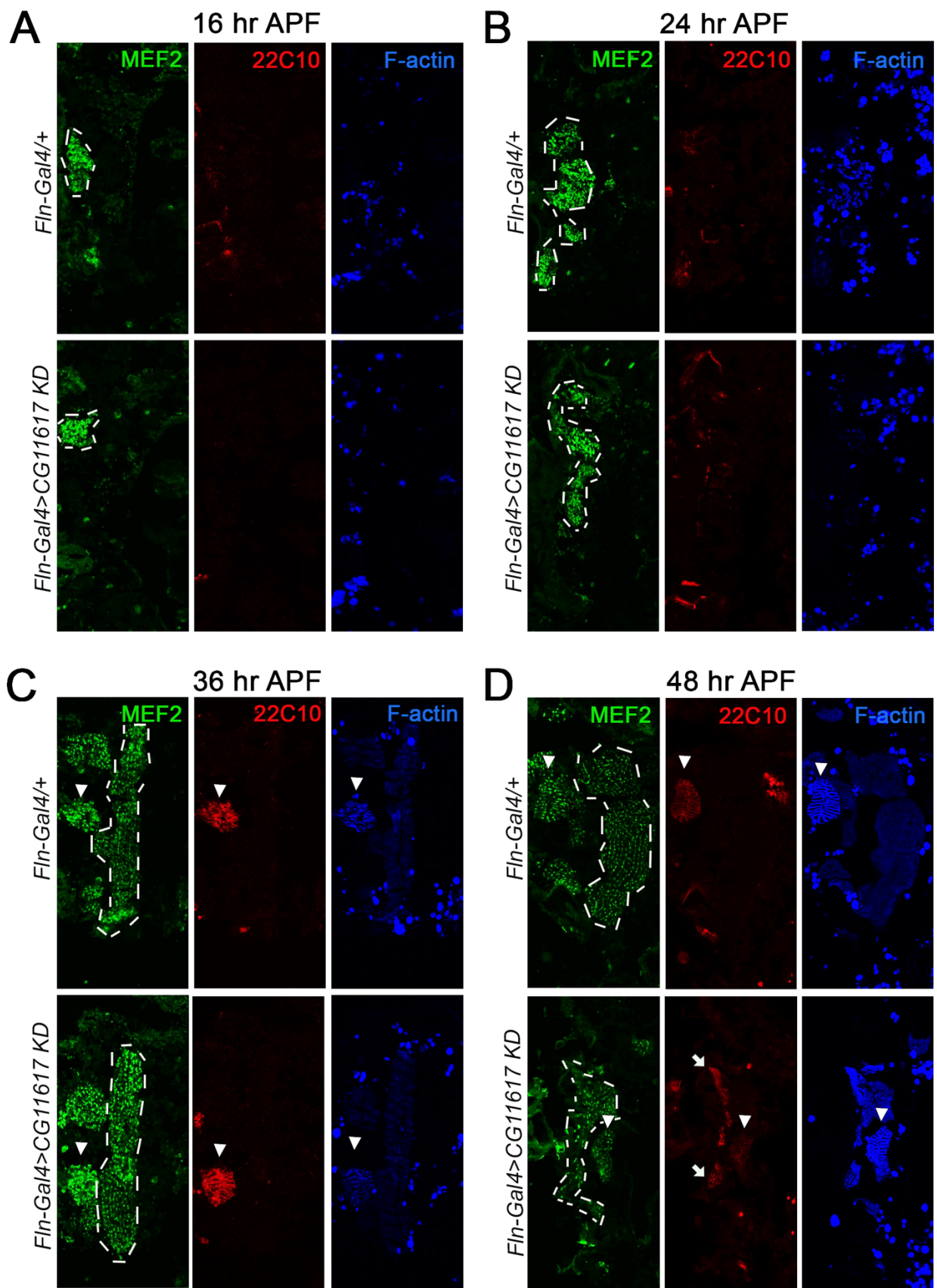
Figure 13. Myoblast pool is reduced in CG11617 KD 3rd instar imaginal wing discs. (A) Stained imaginal wing discs of Control and CG11617 KD 3rd instar larva. Cut (myoblast, green). 20X magnification. The panel to the right is a zoomed-in image. (B) Wing disc area occupied by myoblast divided by the total area of the wing disc (n=5). Error bars were reported as standard error of the mean. The p-value is 0.02. (C) Overexpression of CG11617 in imaginal wing disc myoblasts shows the expression of CG11617 in 3rd instar (L3) female larva wing discs. 20X magnification.

Developmental time points

Muscle degeneration occurs over time in IFMs and jump muscles, as evidenced by *CG11617* knockdown mutants generated with a *Fln-Gal4* driver, which is activated only after the onset of muscle differentiation (Bryantsev et al., 2012b). We utilized a *Fln-Gal4* driver to specifically knock down *CG11617* expression in the flight muscles, aiming to obtain live adults for subsequent analysis. This approach also mitigated potential complications from a reduced myoblast pool, as the *Fln-Gal4* driver becomes active only 24 to 30 hours after pupal formation. Female *Fln-Gal4* driver flies were crossed to either male *w¹¹¹⁸* to generate control (*Fln-Gal4/+*) offspring or to male *CG11617-RNAi[30533]*, to generate experimental (*Fln-Gal4>CG11617 KD*) offspring.

Female offspring were cryosectioned through the thorax and immunostained to visualize the thoracic muscles. We selected 16 hours after pupal formation (APF), 24 hours APF, 36 hours APF, and 48 hours APF for immunolabeling because these developmental time points cover the events of myoblast fusion, myotube formation, myofibril assembly, and myofibril growth, respectively, and whose proper function is critical in muscle development (Spletter et al., 2018). We used antibodies against MEF2 to visualize the myoblasts (green), 22c10 to visualize the tubular muscle cells (red), and F-actin to visualize the skeletal muscles (blue). Control flies show normal development of both IFMs (white dashed border) and jump muscles (white arrowheads), however, experimental flies' IFMs show tubular cell formation (white arrows), similar to jump muscle morphology (Figure 14A-14D).

Figure 14. *CG11617* is essential for normal indirect flight muscle development. Horizontal sections through the thorax of *Fln-Gal4/+* and *Fln-Gal4>CG11617* KD females stained for the accumulation of MEF2 (myoblasts, green), 22C10 (jump muscle cells, red), and F-actin (skeletal muscles, blue) in flight (white dashed border) and jump muscles (white arrowheads) (A) 16 hours after pupal formation (APF) (B) 24 hr APF (C) 36 hr APF and (D) 48 hr APF. White arrows point to tubular cell formation within indirect flight muscles (IFMs). *Fln-Gal4>CG11617* KD IFMs show abnormal muscle morphology. 20X magnification.



Pharate adults and adults show tubular formations in the indirect flight muscles

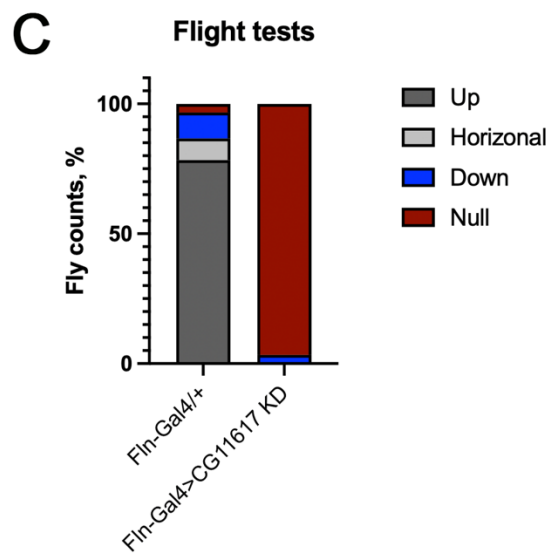
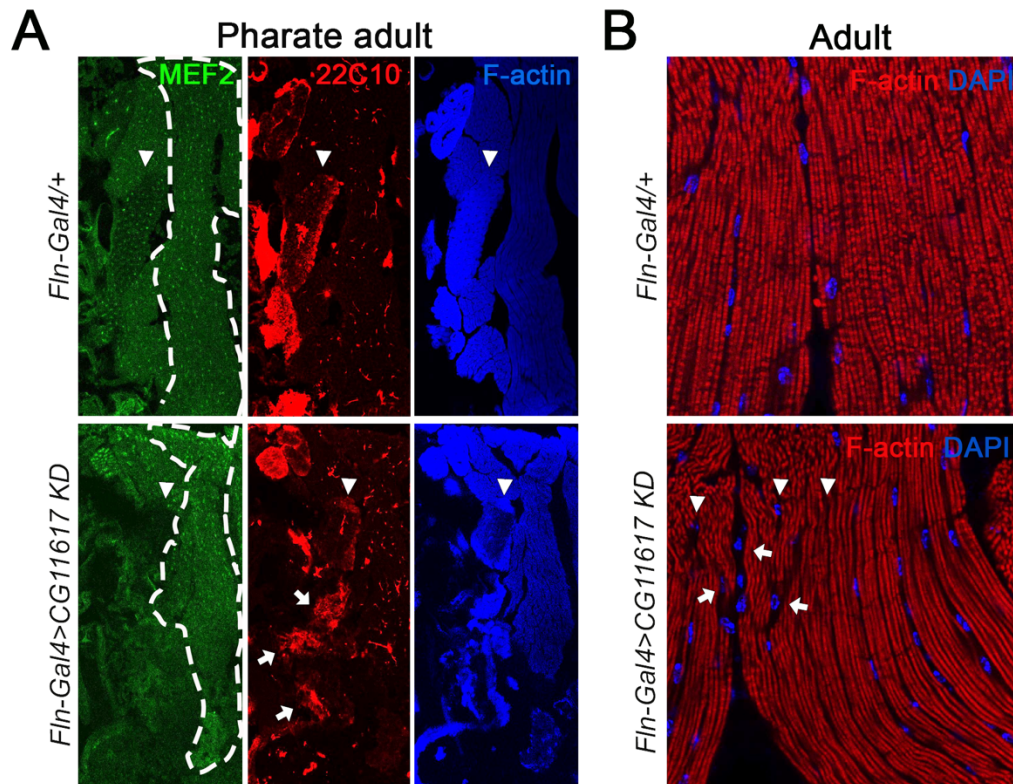
Next, we cryosectioned through the thorax of female pharate adults using antibodies against 22c10 (tubular muscles; red), MEF2 (myoblasts; green), and F-actin (skeletal muscles; blue) to visualize the muscle morphology. We found that pharate adult *Fln-Gal4>CG11617 KD* indirect flight muscles (IFMs) show an increase in tubular-like formations, which is characteristic of the tubular-shaped myofibrils found in jump muscles (Figure 15A).

We then assessed the IFMs of adult female flies to determine if there were any morphology changes as a result of *CG11617* knockdown. We used phalloidin to stain the skeletal muscles (F-actin; red) and DAPI to stain the nucleus (blue). Our immunofluorescent images of *Fln-Gal4/+* IFMs showed normal morphology: myofibrils are densely packed and parallel, and actin (thin) and myosin (thick) filaments show regular arrangement within the sarcomeres. Also, these flies' nuclei are localized to the periphery of the muscle fibers (Figure 15B). On the other hand, *Fln-Gal4>CG11617 KD* IFMs showed loosely packed myofibrils and instances of central nuclei (Figure 15B; arrowheads and arrows).

Since the morphology of IFMs *Fln-Gal4>CG11617 KD* flies is altered, we conducted flight tests to determine if their flight ability is affected. Our analysis revealed that 59/60 (98.33%) of the adult female *Fln-Gal4>CG11617 KD* flies were flightless while 1/60 (1.66%) flew down (Figure 15C). Overall, this data suggests that the combined effects of abnormal tubular cell formations, loosely packed myofibrils, and centrally localized nuclei lead to a significant reduction in the structural integrity and functional efficiency of the IFMs, ultimately impairing the flies' ability to fly.

Figure 15. *CG11617* knockdown leads to tubular-like formations in the IFMs.

(A) Horizontal sections through the thorax of *Fln-Gal4/+* (n = 4) and *Fln-Gal4>CG11617* KD (n = 4) females stained for the accumulation of MEF2 (myoblasts, green), 22C10 (jump muscle cells, red), and F-actin (skeletal muscles, blue) in flight (white dashed border) and jump muscles (white arrowheads). 20X magnification. (B) Horizontal sections through the thorax of *Fln-Gal4/+* (n = 6) and *Fln-Gal4>CG11617* KD (n = 6) females stained for skeletal muscles (F-actin; red) and nucleus (DAPI; blue). 60X magnification. Central nuclei are represented by arrow (horizontal) and arrowheads (perpendicular). (C) The flight ability of *Fln-Gal4>CG11617* KD flies (n = 60) is altered compared to *Fln-Gal4/+* flies (n = 60). The fly's landing location was recorded as upward (U), horizontally (H), downward (D), or not flying at all (N).



DISCUSSION

In this paper, we analyzed the localization of CG11617 and found that it may have muscle-type-specific functions. In indirect flight muscles (IFMs), the nuclear localization of CG11617 implies a role in regulating nuclear processes, possibly involved in gene expression or other nuclear functions specific to the flight muscles. The nuclear localization of CG11617 could be critical for maintaining the unique structural and functional properties of IFMs, which are known for their high-frequency contractions necessary for flight (Bernstein et al., 1993; Spletter et al., 2018). In jump muscles, the localization of CG11617 in both the nucleus and the cytoplasm suggests that CG11617 could be exported from the jump muscle nuclei in order to moderate its function. This dual localization could be essential for the unique functions of jump muscles, which are necessary for *Drosophila*'s jumping ability (Nachtigall and Wilson, 1967; Zumstein et al., 2004; Jaramillo et al., 2009).

The difference in CG11617 sub-cellular localization between IFMs and jump muscles raises questions about the regulatory mechanisms that govern its localization. It suggests that CG11617 localization is likely influenced by muscle type-specific factors such as co-factors or post-translational modifications. One hypothesis is that an unknown co-factor or post-translational modification specific to jump muscles influences CG11617 localization, enabling its cytoplasmic presence. Investigating these factors could provide insights into the regulatory mechanisms that control CG11617 localization and function. Interactions with other proteins could dictate the distribution of CG11617 within these two muscle fiber types. For example, *Vestigial* (Vg) and *scalloped* (Sd) are two proteins that need to be co-expressed for nuclear localization to regulate gene

expression patterns specific to the *Drosophila* wing (Halder et al., 1998). In addition, the cytoplasmic presence of CG11617 between the jump muscle myofibrils implies that CG11617 is likely exported from the jump muscle nuclei because its transcriptional function is not desired in this tissue, or that less of it is imported into the nucleus. Thus, CG11617 may function as a transcriptional repressor of jump muscle gene expression, leading to its exclusion from the jump muscle nuclei.

Does the RNAi phenotype reflect the true effect of *CG11617*'s loss of function? We incorporated Dicer to ensure that our phenotype was not due to an off-target effect by generating three *1151 > RNAi + Dicer-2* crosses. These crosses resulted in the improper development of IFMs and jump muscles (Figure 11C-E), as observed in *CG11617* KD flies (Figure 11A), and significantly lower levels of *CG11617* expression (Figure 12E), as found in *CG11617* KD flies (Figures 12C and 12D). To further validate that the RNAi phenotype was true, we produced a Rescue line that restored the levels of *CG11617* (Figures 12C, 12D, and 12E) and the development of normal thoracic muscles (Figure 12A). Together, these data support that we specifically targeted *CG11617* and observed the true effect of *CG11617*'s loss of function.

The expression pattern of *CG11617* suggests that it may play a significant role in the later stages of muscle differentiation, particularly in myotube formation, myofibril assembly, and/or muscle growth. The absence of *CG11617* expression during early myoblast fusion implies that it is not essential in the initial stages of muscle cell fusion but may be important in subsequent muscle development processes. This was further analyzed by knocking down *CG11617*, which resulted in the improper development of IFMs and the absence of jump muscles (Figures 11A and 11F). These data suggest that

CG11617 is crucial for the normal development and growth of both IFMs and jump muscles and may not be necessary for the initial fusion of myoblasts in the early stages of the pupa.

Is there a muscle fiber identity switch in the IFMs upon *CG11617* knockdown? The *Fln-Gal4 > CG11617 KD* flies, which utilize a *Fln-Gal4* driver activated exclusively after the onset of muscle differentiation, were used to assess thoracic muscle development into adulthood. These flies exhibited tubular formations similar to those observed in jump muscles. Interestingly, the experimental adult female flies were unable to fly, indicating that the tubular morphology interferes with flight function. This correlation between structure and function highlights the importance of proper IFM development for the flies' flight capability. Future studies could utilize RNA-sequencing to assess the downregulation of muscle-specific genes such as homothorax (*hth*) and extradenticle (*exd*) in *CG11617 KD* IFMs. Research by Bryantsev et al. (2012b) revealed that *exd* and *hth* are expressed in IFMs but absent in jump muscles, so a downregulation of these genes could indicate a flight muscle to jump muscle identity switch in the IFMs.

Knockdown of *CG11617* expression in thoracic muscles revealed profound changes in myofibril structure and function, resulting in the improper development of flight and jump muscles and pupal lethality. Understanding the factors that control *CG11617* localization could provide deeper insights into its role in muscle development and function, highlighting the complexity of protein localization and its impact on cellular differentiation and specialization. In addition, understanding the molecular basis for the muscle fiber fate transformation observed in *CG11617 KD* IFMs will provide valuable

insights into the potential pathways for addressing flightlessness caused by similar morphological abnormalities.

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DISCLOSURES

The authors have no competing financial interests or other conflicts of interest.

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CHAPTER 3

Unraveling the mechanistic role of the *Drosophila melanogaster* transcription factor CG11617 in muscle fiber fate

ABSTRACT

The mammalian *Mohawk* (*Mkx*) transcription factor plays a crucial role in skeletal muscle development by promoting the expression of slow fiber fate. However, the mechanistic details of its function remain unclear. Utilizing the *Drosophila* ortholog, *CG11617*, we utilized *CG11617* knockdown mutant flies to examine the myofibril structure of thoracic muscles and investigated the mechanistic role of *CG11617* in muscle fiber specification and differentiation by RNA-sequencing. We found that the muscle structure of *CG11617* knockdown mutant IFM myofibrils show similar cross-striations to wild-type jump muscles and both fibrillar muscle and tubular muscle fiber identity. This data suggests that IFMs may be switching to tubular muscle fiber identity upon *CG11617* knockdown and this may influence skeletal muscle precursor differentiation towards slow fiber types. In addition, the RNA-sequencing analysis of *CG11617* mutant flies revealed that *upheld*, which encodes for Troponin T, a gene important for the proper development of muscle structure and flight ability, was upregulated and significant. The overexpression of *upheld* resulted in severe degeneration of thoracic muscles but no evidence for muscle fiber fate switch in *CG11617* mutant flies. Although RNA-seq is a powerful tool for investigating developmental mechanisms, it did not reveal a clear path for the mechanistic role of *CG11617*.

INTRODUCTION

In vertebrates, the two predominant muscle fiber types that characterize muscle tissue are slow-twitch (Type 1) and fast-twitch (Type 2) muscle fibers. They are categorized into distinct types based on their contractile and metabolic properties (reviewed in Mukund and Subramaniam, 2019). Despite extensive research to identify transcription factors involved in fiber-type fate pathways, the precise molecular mechanisms and regulatory interactions remain incompletely understood.

Previous studies have begun to unravel the role of specific transcription factors in fiber-type fate pathways. One such transcription factor is Mohawk (*Mkx*), which has been implicated in the regulation of muscle precursor differentiation and fiber type specification. *Mkx* is expressed in embryonic precursors of skeletal muscle and functions as a transcriptional repressor by inhibiting the differentiation of MyoD-induced myoblasts (Anderson et al., 2009). In addition, Sox6, a transcription factor involved in the terminal differentiation of skeletal muscle, has been shown to participate in fiber type specification in skeletal muscle by repressing the transcription of the slow-twitch myosin heavy chain (MHC) isoform (*Myh7*) (Hagiwara et al., 2007). When *Mkx* was knocked out in isolated skeletal muscle satellite cells from 5-month-old *Mkx*^{-/-}, Sox6 was upregulated and the slow-twitch *Myh7* isoform was downregulated compared to wild-type littermates (Anderson et al., 2009). These findings suggest that *Mkx* promotes slow fiber fate during muscle development through inhibition of Sox6 activity.

Studying the *Drosophila* ortholog, *CG11617*, will enhance our understanding of transcription factors involved in muscle fiber-type fate pathways. Previous research shows that the transcription factors Extradenticle (*Exd*) and Homothorax (*Hth*) are

crucial for the specification of *Drosophila* flight muscles by controlling the expression of the flight muscle-specific actin gene Act88F (Bryantsev et al., 2012b). In contrast, when Exd and Hth, or their co-expression with a Hox gene, are absent, the development of tubular muscles is triggered instead. In addition, the mammalian equivalents of Exd and Hth, PBX and MEIS, respectively, have been found to promote fast fiber formation in zebrafish and mice (Maves et al., 2007). Furthermore, it was discovered that blistered (*bs*), the *Drosophila* homolog of Serum Response Factor (SRF), interacts with Exd and Hth to regulate muscle type differentiation in indirect flight muscles (IFMs) (DeAguero et al., 2019). Downregulating *bs* across all muscles leads to immature and disorganized myofibrils in IFMs, while other muscle types remain unaffected. These results suggest a conserved mechanism underlying muscle fiber specification, indicating that *Drosophila* IFMs might utilize the same processes that guide fast-twitch muscle fiber development in mammals.

Furthermore, the flight muscle specification mechanisms in *Drosophila* closely resemble those governing fast-twitch (Type 2) muscle fibers in mammals, while the formation of thoracic jump muscles is analogous to the specification of slow-twitch (Type 1) muscle fibers (Maves et al., 2007; Bryantsev et al., 2012b; Jawkar and Nongthomba, 2020). Also, *Drosophila* is a simple model organism that can be used to study muscle fiber specification. It displays a single fiber type in its flight muscles and two similar fiber types in its jump muscles, in contrast to the mixture of fiber types characteristic of the mammalian skeletal muscles (i.e., Type 1, Type 2a, Type 2b, and Type 2x), where one type usually predominates (Brooke and Kaiser, 1970; Saltin and Gollnick, 2011; Pette and Staron, 2000).

In this study, we utilized RNA-sequencing (RNA-seq) and immunofluorescence to examine the mechanistic role of *CG11617* in muscle fiber type specification. RNA-seq was used to identify the genes that are up-regulated by *CG11617* knockdown in indirect flight muscles (IFMs). We also examined the muscle structure of IFMs by utilizing beta-galactosidase (β -gal) as a reporter for the jump muscle-specific TpnC41C gene (Chechenova et al., 2017) and antibodies against the flight muscle-specific genes *homothorax* (*hth*) and *extradenticle* (*exd*), which are evolutionary conserved in muscle fiber specification (Maves et al., 2007; Bryantsev et al., 2012b). We found TpnC41C expression in IFMs (normally expressed in the jump muscles) and IFM structure that resembles the jump muscles. Together, these findings suggest that there is a muscle fiber fate switch in the IFMs of *CG11617* knockdown mutants.

MATERIALS AND METHODS

Drosophila stocks and crosses

All fly crosses were maintained in vials or bottles containing Jazz Mix medium (Genesee Scientific) at 25°C, allowed to lay eggs, and then transferred to 29°C incubators after three days to maximize Gal4-induced expression. The following fly stocks were used for *CG11617* knockdown analysis: *Fln-Gal4* (activated only after the onset of muscle differentiation, Bryantsev et al., 2012b), *UAS-RNAi [CG11617-30533]* (Vienna Stock Center, VDRC ID #30533) and *w¹¹¹⁸*. We crossed female *Fln-Gal4* drivers with *UAS-CG11617 RNAi[30533]* male adult flies to trigger *CG11617* knockdown in the flight muscles to generate *Fln-Gal4 > CG11617 KD* (*CG11617 KD*) offspring. For controls, we crossed female *Fln-Gal4* with male adult *w¹¹¹⁸* flies to generate *Fln-Gal4/+*

(Control). We analyzed female offspring for these experiments because both *Fln-Gal4* and *UAS-CG11617* are on the X chromosome.

The following fly stocks were used to determine muscle fiber identity switch: *1151-Gal4; TpnC41C-lacZ* female virgin flies were crossed to either *UAS-RNAi [CG11617-30533]* (Vienna Stock Center, VDRC ID #30533) male flies to generate *1151 > CG11617 KD + TpnC-41C-lacZ* mutant offspring or to *w¹¹¹⁸* male flies to generate *1151-Gal4; TpnC41C-lacZ* control offspring. The enhancer-lacZ constructs bound to the tubular muscle-specific *Troponin C at 41C* (*TpnC41C*) gene (Herranz et al., 2004) will selectively express β -galactosidase (β -gal), enabling visualization of TpnC41C accumulation.

Immunostaining of indirect flight muscle myofibrils and imaginal wing discs

Control and CG11617 KD pharate adults were submerged in Tissue-Tek medium, frozen in liquid nitrogen, and sectioned using an HM525 NX Thermo Scientific cryostat machine (Jaramillo et al., 2009). These 10 μ m-12 μ m sections were dried on slides and fixed in 3.7% formaldehyde in 1X phosphate buffer saline (PBS) for 30 minutes at 4°C. Then, they were washed with 0.3% PBTx two times, for 5 minutes each, and washed one final time, for 30 minutes, on a rotator.

The primary antibodies were diluted in 1% bovine serum albumin (BSA) and incubated with the samples overnight in a humidity chamber. Samples were then washed with 0.3% PBTx for 5 minutes on a rotator. The secondary antibodies were added, incubated for 2 hours, and kept in a humidity chamber. Lastly, these samples were mounted in mounting media.

For immunofluorescent detection, the following primary antibodies were used:

1:500 CG11617 (made in Dr. R.M. Cripps lab)
1:500 β -galactosidase (Promega; REF:Z378A; LOT# 0000557908)
1:500 Homothorax (Dr. H. Sun)
1:500 Extradenticle (DSHB; AB_10683835)

Alexa conjugated secondary antibodies, Alexa 488 (Invitrogen) and Alexa 568 (Invitrogen), were diluted to 1:500 in 1% BSA. Phalloidin 647 (Invitrogen) was diluted to 1:200 in 1% BSA to visualize skeletal muscles (F-actin). To visualize the nucleus, DAPI (4',6-diamidino-2-phenylidole-dihydrochloride) was diluted to 1:10,000 in 1% BSA.

Indirect flight muscle dissection and RNA extraction

Indirect flight muscles (IFMs) were dissected from pharate adult female *Drosophila* thoraces in a cooled, 1% sucrose solution. IFMs were then transferred to 350 μ l of RLT lysis buffer supplied by the Qiagen RNeasy Mini kit (REF#74104; Qiagen, USA) and frozen at -80 °C until a total of 60 IFMs were collected per replicate. Whole RNA was extracted from IFMs according to the Qiagen RNeasy Mini protocol. A NanoDrop 2000 (Thermo Fisher Scientific, USA) was used to determine the average yield extracted from control replicates: 852.57 ng/ μ l (30 μ l total extraction volume) and the average yield extracted from mutant replicates: 389.47 ng/ μ l (30 μ l total extraction volume). The quality of the RNA was determined by the UC San Diego IGM Genomics Center using RNA ScreenTape® analysis (Agilent, USA). The average RIN^e for Control and CG11617 KD samples was 9.2 (Appendix 2 - Figure S1).

Transcriptomic analysis

Illumina NovaSeq 6000 was used for RNA-sequencing (RNA-seq). UC San Diego IGM Genomics Center created three single-end libraries from both Control and

CG11617 KD samples. Trimming with the R package Trimmomatic was utilized to remove the Illumina adapters (Bolger et al., 2014):

```
>AGATCGGAAGAGCACACGTCTGAACTCCAGTCA  
>AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
```

In addition, we used Trimmomatic LEADING:3 to remove leading low-quality reads below quality 3, TRAILING:3 to remove trailing low-quality reads below quality 3, and SLIDINGWINDOW:4:20 to scan the read with a 4-base wide sliding window and cut when the average quality per base drops below 20. Next, the reads were mapped to the *Drosophila* reference genome dm6 (*D. melanogaster* Aug. 2014 BDGP Release 6 + ISO1 MT/dm6) using the modern aligner HISAT2 (Kim et al., 2019). Then, the fastqcr R package was utilized to analyze the quality of the aligned sequences (Andrews S, 2010; Kassambara A, 2023) (Appendix 2 - Figure S2-S9).

Next, the transcript expression level was quantified using the alignment files as inputs for featurecounts (Liao et al., 2014). These counts were then used to identify significantly expressed genes in the data set using DESeq2 (Love et al., 2014). $\text{Log}_2\text{Fold Change} > 0.5$ or < -0.5 , and $p < 0.05$ was set as the threshold for significantly up-regulated or downregulated expressed genes, respectively.

Lastly, Gene ontology (GO) analysis was completed with ClueGO, a CytoScape plug-in, to analyze the biological processes, cellular components, and molecular functions that were differentially expressed in IFMs upon CG11617 knockdown (Bindea et al., 2009).

Immunofluorescent analysis

Cryosections were imaged on the Olympus FluoView 3000 confocal microscope. The Olympus Life Science software was used to process all images. Then, the images

were assembled in Adobe Photoshop 2024 (version 25.0). All control and experimental confocal images underwent equal changes in brightness and contrast.

Statistical analysis

GraphPad Prism software (Version 10.0.3) was used to generate all pie charts and bar graphs. The error bars are presented as the standard error of the mean. A p-value of less than 0.05 (p-value < 0.05) was set as the threshold for significance and marked by asterisks. Student's two-tailed t-test was used to calculate the p-value.

Quantification of *exd* and *hth* expressions was conducted by analyzing hyperstack images of 5 slices (z-stack). The ImageJ 1.54g software was utilized to measure the expression of *exd* and *hth* in each nucleus within a 1035x1035 pixel frame (Shihan *et al.* 2021). The background fluorescent intensity (Background) for Control and CG11617 KD pharate adult flies was measured. Then, the mean fluorescent intensity (MFI) in a region of interest (ROI), which is each nucleus present in the frame, for this experiment was measured. We then calculated the Final MFI according to the following equation:

$$\text{Final MFI} = \text{MFI of an ROI} - \text{MFI of Background}$$

We subtracted the corresponding Background from each nucleus MFI and calculated the standard error of the mean (SEM). The Final MFI, SEM, and p-value for Control and CG11617 KD samples were calculated using Excel (Version 16.87).

The number of nuclei in indirect flight muscles of 1035 x 1035 frames was manually calculated.

RESULTS

Transcriptomic analysis of indirect flight muscles upon *CG11617* knockdown

To examine the mechanistic role of *CG11617* in muscle fiber type specification, we conducted a transcriptomic analysis of indirect flight muscles (IFMs) from *Fln-Gal4/+* (Control) and *Fln-Gal4>CG11617 KD* (*CG11617* KD) samples. Three single-end libraries were made from Control and *CG11617* KD samples. Then, RNA-sequencing (RNA-seq) was conducted using Illumina NovaSeq 6000. Trimming was done with the R package, Trimmomatic, and then HISAT2 was used to map the reads to the *Drosophila* reference genome dm6 (*D. melanogaster* Aug. 2014 BDGP Release 6 + ISO1 MT/dm6) (Kim et al., 2019). The fastqcr R package was utilized for quality control of the Illumina data (Kassambara A, 2023). For Control and *CG11617* KD replicates all categories received a passing score except for “per sequence GC content” (Appendix - Figure S6) (Wingett and Andrews, 2018; Andrews S, 2010).

A Fail in “per sequence GC content” arises when there are overrepresented sequences in the data set. Control and *CG11617* KD replicates triggered a warning for overrepresented sequences, but none of these sequences matched common contaminants (Appendix 2 - Table S1). A sequence is considered overrepresented when it is found to represent more than 0.1% of the total (Andrews S, 2010). We measured the % GC for all replicates to ensure that each % GC fell within the acceptable range for *Drosophila*, 40%–60%, and that differential expression results and subsequent downstream analysis such as Gene Ontology would not be affected (Risso et al., 2011; Herold et al., 2008). Our results showed the % GC content range was between 41%-46% (Table 4), consistent with the acceptable range for *Drosophila*. In

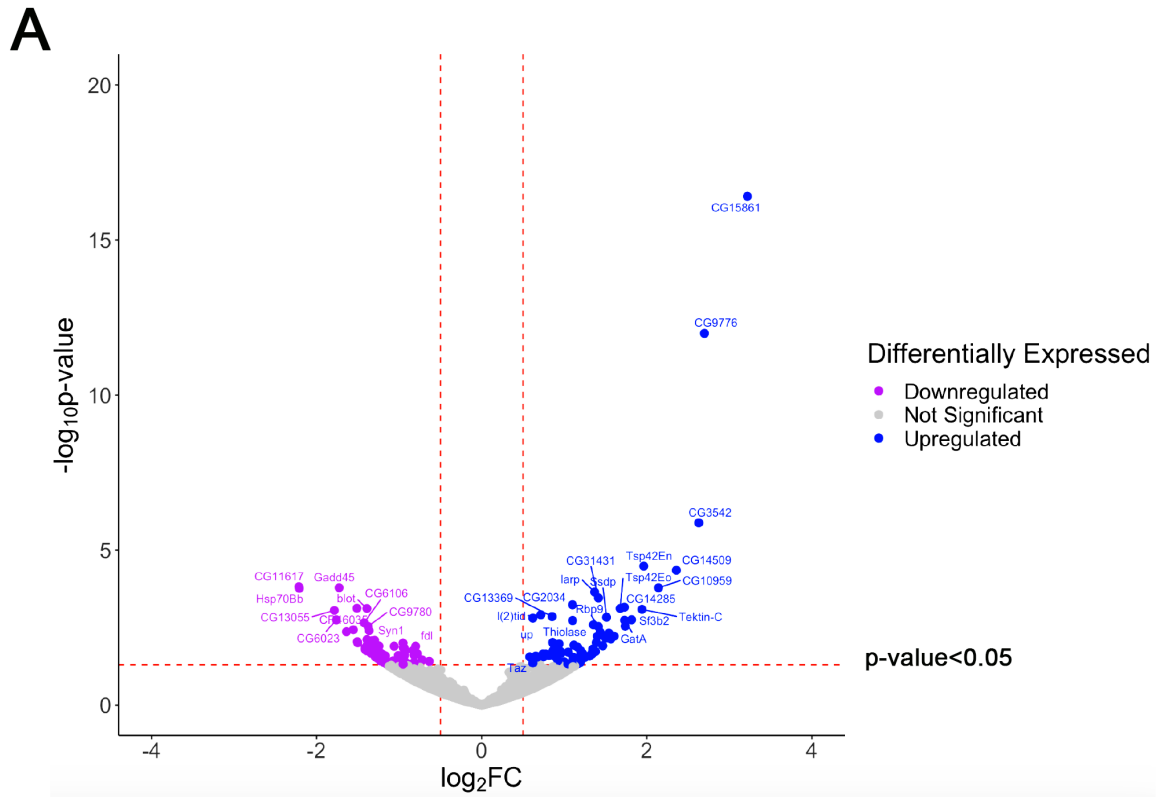
addition, the mapping rate was between 89.99% and 97.10%, suggesting good-quality sequence fragments. Moreover, the average read length across all samples was 86 base pairs, suggesting uniformity. Together, our results indicate good-quality sequencing data and successful alignment with the well-annotated *Drosophila* genome.

Next, the alignment files were used to quantify the expression level of the transcripts, and DESeq2 was utilized to identify significantly expressed genes (Liao et al., 2014; Love et al., 2014). A volcano plot was generated to visualize downregulated and up-regulated genes, establishing significance thresholds at $p\text{-value} < 0.05$ and a fold change cutoff of $\text{Log}_2\text{FC} > 0.5$ and $\text{Log}_2\text{FC} < -0.5$, up-regulated and downregulated, respectively (Figure 16A). This fold change cutoff was established to ensure that smaller, yet biologically relevant, fold changes were detected. We analyzed the top 10 up-regulated and downregulated genes to determine whether there was a relationship between the localization of expression or the function of these genes. We found that the top 10 significantly up-regulated genes are mostly expressed during the embryonic stage and regulate gene expression (Figure 16B). One of these genes, CG14285 is currently uncharacterized, and its function is unknown. The top 10 downregulated genes are mostly expressed during the embryonic stage and have a wide range of functions (Figure 15C). One of these genes, the long non-coding RNA, CR46035, is uncharacterized, and its function is unknown. Also, CG13055 was significantly downregulated and is expressed during the late pupal stage, but its function is also unknown (Flybase Consortium, 2024).

Table 4. Summary of RNA-sequencing statistics and mapping results. Each replicate was mapped to the *D. melanogaster* Dm6 reference genome. All sequences were single-end reads. The average read-length was 86nt.

Library	% GC	Number of raw reads	Number of cleaned reads	Mapping rate
<i>Fln-Gal4/+</i> replicate 1	47	32248179	30626827	97.10%
<i>Fln-Gal4/+</i> replicate 2	46	482239	439581	93.49%
<i>Fln-Gal4/+</i> replicate 3	47	537481	490364	94.42%
<i>Fln-Gal4>CG11617 KD</i> replicate 1	41	480534	436996	91.66%
<i>Fln-Gal4> CG11617 KD</i> replicate 2	41	467264	430072	92.76%
<i>Fln-Gal4> CG11617 KD</i> replicate 3	42	553038	493262	89.99%

Figure 16. Analysis of differentially expressed genes upon *CG11617* knockdown. DESeq2 was utilized to determine Differentially expressed genes (DEGs) using as described in Materials and Methods. (A) Volcano plot of DEGs. Log₂ fold change > or < 0.5 plotted against p-value < 0.05 was deemed significant. Significant downregulated genes are in purple, significant up-regulated genes are in blue, and non-significant genes are in gray. (B) Table of top 10 up-regulated and (C) downregulated DEGs and their corresponding log fold change, p-value, expression location, and function. Some genes have no known data on expression location and/or function, based on flybase.org.



B

Gene	Log2FC	p-value	Expression location - Function
CG15861	3.21864	3.9E-17	Embryo hindgut and large intestine
CG9776	2.698184	1.02E-12	Embryonic stage nucleoplasm
CG3542	2.629874	1.33E-06	Embryonic/larval muscle – mRNA splicing
CG14509	2.358682	4.51E-05	Early larva and late pupal stage axons – regulates membrane potential
CG10959	2.14178	0.000164	Early embryonic stage – regulate gene expression by DNA binding activity
Tsp42En	1.963023	3.3E-05	Embryonic and late pupal stage – brings multiple proteins into the membrane region
CG14285	1.728741	0.000698	No data
CG31431	1.414478	0.000353	Early larva and late pupal stage plasma membrane
larp	1.368523	0.000227	Embryo development - encodes a protein that associates and interacts with poly(A)-binding protein
CG2034	1.099835	0.000584	Embryonic and adult stages – establish mitotic spindle asymmetry

C

Gene	Log2FC	p-value	Expression location - Function
CG11617	-2.2149	0.000153	Embryonic/larval muscle nucleus – DNA binding
Hsp70Bb	-2.20937	0.000171	Pupal stage - ATP binding; protein folding chaperone; heat shock protein binding
Gadd45	-1.72731	0.000164	Late pupal stage - JNK cascade; regulation of egg-laying behavior; regulation of cell cycle
CR46035	-1.51229	0.000754	No data
CG13055	-1.7857	0.000886	Late pupal stage – no known function
CG6023	-1.76157	0.001785	Early embryonic and late female adult stage – no known function
CG6106	-1.42591	0.002222	Embryonic/larval muscle system - nucleotide synthesis
mas	-1.41496	0.01535	Embryonic stage - somatic muscle attachment and development of axonal pathways
blot	-1.39056	0.000768	Embryonic stage - neurotransmitter transport; amino acid transport; sodium ion transmembrane transport
CG9780	-1.37774	0.002913	Embryonic and late pupal stages - proteolysis

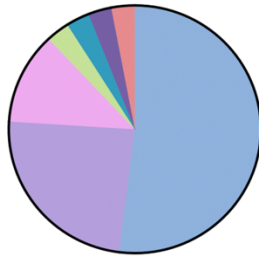
Afterward, Gene Ontology (GO) enrichment analysis using ClueGO, a CytoScape plug-in, was utilized to analyze the differentially expressed genes and their biological processes, cellular components, and molecular functions when *CG11617* was knocked down in order to determine the molecular mechanism for *CG11617* in IFMs (Bindea et al., 2009). Our analysis of significantly up-regulated genes showed a large fraction, 51.52% of differentially expressed genes from the Biological Process category are involved in embryonic development, 80.0% from the Cellular Component category are microtubule cytoskeleton, and 50.0% from the Molecular Function category have adenyl nucleotide binding (Figure 17A). Genes typically associated with early embryonic development may be reactivated or expressed at higher levels in adult *CG11617* KD IFMs because these IFMs might be undergoing remodeling or repair. In addition, IFM tissues under stress may cause up-regulation of the microtubule cytoskeleton genes in order to help reinforce the structural integrity of the IFMs and prevent further damage. Furthermore, the up-regulation of adenyl nucleotide binding genes may be necessary for an increase in mitochondrial activity to provide the necessary energy (in the form of ATP) for muscle repair. RNA binding genes may also be activated to regulate the expression of genes involved in muscle development, maintenance, and function.

We also analyzed significantly downregulated genes, which revealed that a large fraction, 40.4% of differentially expressed genes from the Biological Process category, are involved in the regulation of growth, and 66.67% from the Cellular Component category are intrinsic components of the plasma membrane (Figure 17B). These findings suggest that genes important for the regulation of IFM growth and maintaining the integrity of the plasma membrane are impacted by *CG11617* knockdown. However,

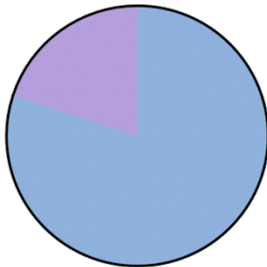
there was no output for Molecular Function, maybe because of the wide range of functions within this group.

Overall, GO terms did not reveal any useful information as to what the mechanism for *CG11617* in IFMs might be. However, it did provide insights into how the IFMs' growth regulation may be impacted, and the flies' attempt to restore normal IFM tissue when faced with skeletal muscle damage.

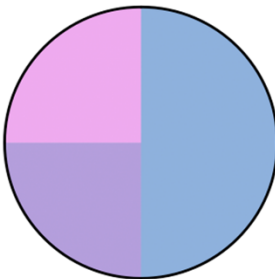
Figure 17. GO analysis of differentially expressed genes upon *CG11617* knockdown. Pie chart of the Gene Ontology (GO) enrichment analysis of DEGs using ClueGO, a CytoScape plug-in, to analyze the biological processes, cellular components, and molecular functions that were up-regulated or downregulated. (A) Many differentially up-regulated genes in Biological Processes relate to embryonic development (51.52%), while in Cellular Component, the majority are microtubule cytoskeleton (80.0%), and in Molecular Function, they are genes related to adenyl nucleotide binding (50.0%). (B) Many differentially downregulated genes in Biological Processes relate to regulation of growth (40.0%), while in Cellular Component, the majority are intrinsic components of the plasma membrane (66.67%).

A**Biological Process**

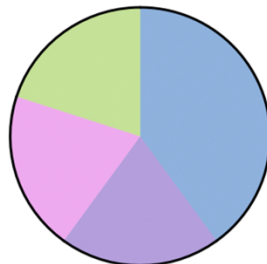
- Embryo development (51.52%)
- Germ cell development (24.24%)
- Cellular lipid metabolic process (12.12%)
- Cell surface receptor signaling pathway (3.03%)
- Mitochondrion organization (3.03%)
- Phosphorylation (3.03%)
- mRNA metabolic process (3.03%)

Cellular Component

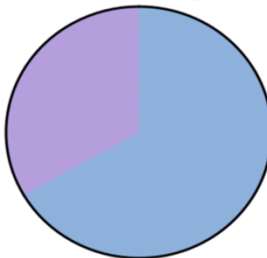
- Microtubule cytoskeleton (80.0%)
- Mitochondrion (20.0%)

Molecular Function

- Adenylyl nucleotide binding (50.0%)
- Cytoskeletal protein binding (25.0%)
- RNA binding (25.0%)

B**Biological Process**

- Regulation of growth (40.0%)
- Cellular response to stress (20.0%)
- Microtubule-based process (20.0%)
- Movement of cell or subcellular component (20.0%)

Cellular Component

- Intrinsic component of plasma membrane (66.67%)
- Endoplasmic reticulum (33.33%)

To determine a potential mechanism for how *CG11617* promotes muscle fiber differentiation and specification, we analyzed the RNA-seq data for muscle genes that encode myofibrillar proteins or are muscle-specific genes. In Chapter 2, we found that the thoracic muscles are severely affected in *CG11617* KD mutants. Here, we hypothesize that this is due to a downregulation of myofibrillar proteins required for myofibril assembly or maintenance. The decreased levels of these proteins may impair myofibril integrity, ultimately affecting their normal function of contraction. Furthermore, we speculate a downregulation of flight muscle-specific genes and an up-regulation of jump muscle-specific genes in the *CG11617* KD IFMs as indicators of muscle fiber fate switching.

Genes that code for myofibrillar proteins are *fln* that is required for myosin thick filament assembly (Vigoreaux et al., 1993), the motor protein *myosin heavy chain (mhc)* (Wells et al., 1996; Standiford et al., 1997), *myosin light chain 1 (mlc1)* (Falkenthal et al., 1987) and *myosin light chain 2 (mlc2)* are essential components of the muscle contraction machinery that regulate ATPase activity (Warmke et al., 1992), *Mlp84B* maintains muscle structural integrity (Clark et al., 2007), *Tm1* (Gaudet et al., 2011) and *Tm2* (Karlik CC et al., 1984) stabilizes actin filaments to facilitate efficient muscle contraction, *TnI (wupA)* is part of the troponin complex that inhibits muscle contraction in the absence of calcium (Nongthomba et al., 2004), and *upheld (up)* helps regulate muscle contraction by enabling calcium binding to the thin filaments and regulating muscle contraction (Benoist et al., 1998).

When assessing the RNA-seq data, we found that most of these genes varied in expression, and only one gene, *upheld (up)*, which encodes for Troponin T (TnT), was

significantly up-regulated (Figure 18). Troponin T, a gene important for the proper development of muscle structure and flight ability (Nongthomba et al., 2004), was up-regulated and significant. The overexpression of *TnT* has been shown to cause severe degeneration and muscle dysfunction through the loss of thin filaments and ultrastructural perturbations to the sarcomere (Marco-Ferreres et al., 2005). Up-regulation of the *up* gene in our dataset may account for the phenotype observed in *Fln-Gal4 > CG11617 KD* flies when the thoracic muscle development into adulthood was analyzed (Chapter 2).

We then determined if the expression of muscle-specific genes was altered upon *CG11617* knockdown, indicating a muscle fiber identity switch in the IFMs. This is based on Chapter 2 findings, where we observed tubular-like formations in the IFMs. *Drosophila Act79B* is predominantly expressed in the adult jump muscle, while *Act88F* is predominantly expressed in the indirect flight muscles (IFMs) (Fyrberg et al., 1983). In addition, extradenticle (*exd*) and homothorax (*hth*) are expressed in the IFMs but absent in the jump muscles (Bryantsev et al., 2012b). We also analyzed the levels of *TpnC4* which is predominantly expressed in IFMs (Chechenova et al., 2017). Our RNA-seq data revealed an unexpected up-regulation of flight muscle-specific genes *Act88F*, *exd*, *hth*, and *TpnC4* (Figure 18). This finding suggests a compensatory mechanism that enhances the expression of flight muscle-specific genes, especially *Act88F* whose expression levels are the highest, in *CG11617 KD* mutants. Further investigation is needed to understand the underlying reasons for this up-regulation and its implications for muscle function and development in *Drosophila*.

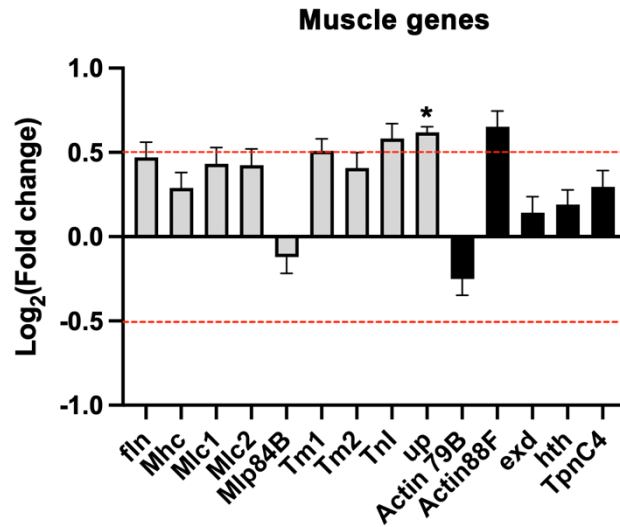


Figure 18. *Upheld* is revealed to be significantly up-regulated upon *CG11617* knockdown. Bar graphs show muscle genes that encode myofibrillar proteins (dark gray) or are muscle-specific genes (black). Significant differentially expressed genes (DEGs) of female adult pharate indirect flight muscles (IFMs) upon *CG11617* loss-of-function are indicated with an asterisk. Log₂ fold change greater than 0.5 or less than -0.5 plotted against p-value < 0.05 was deemed significant (red dashed line). *Upheld* (*up*) was significantly up-regulated in female adult pharate IFMs. The error bars were calculated by the standard error of the mean.

Indirect flight muscles of *CG11617* knockdown mutants show similar cross-striations to jump muscles

We complemented the transcriptomic analysis with ultrastructural and immunofluorescent studies of Control and *CG11617* knockdown thoracic muscle samples. To determine whether knocking down *CG11617* results in morphological appearance changes of the IFM myofibrils, as an indicator of fiber fate switch, we utilized *1151-Fln* driving the *CG11617* RNAi[30533] to generate *1151 > CG11617-RNAi[30533]* (*CG11617* KD).

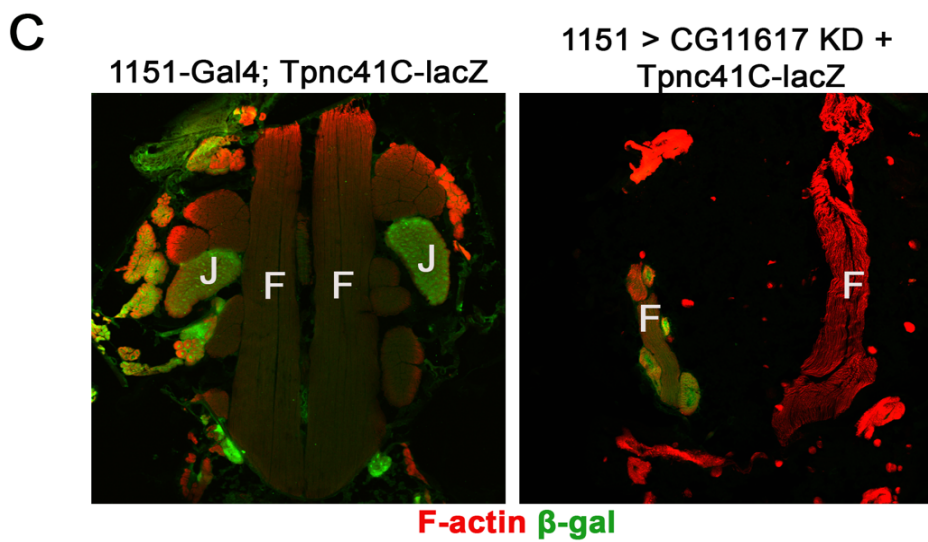
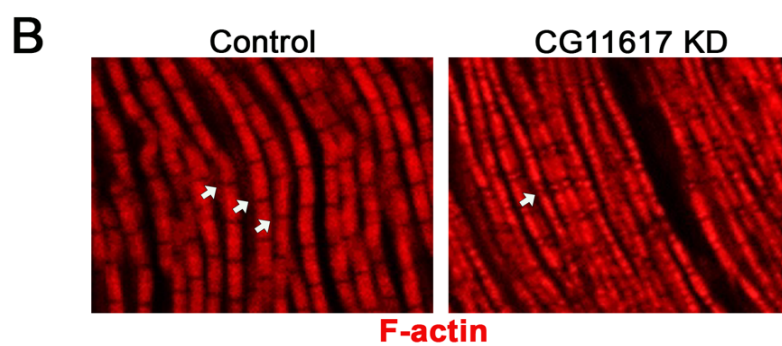
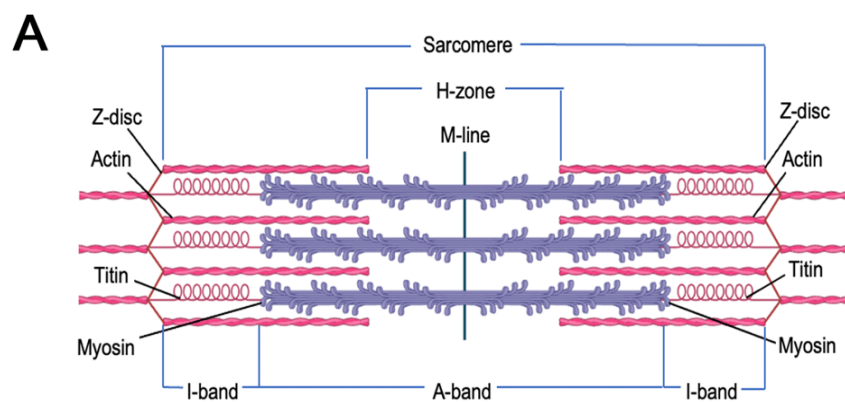
Normally developed myofibrils of the IFMs exhibit narrow H-zones, with cross striations that are staggered relative to adjacent myofibrils (Bryantsev et al., 2012b). On the other hand, jump muscles have cuboidal myofibrils with broad H-zones where cross striations are aligned to each other (Bryantsev et al., 2012b). The H-zone is the central region within the sarcomere that does not contain thin (actin) filaments (Figure 19A). We examined the cross striations in the Control IFMs and found H-zones were staggered (n = 8) (Figure 19B). However, the muscle structure of *CG11617* KD IFM myofibrils revealed similar cross-striations to Control jump muscles, where the H-zones are aligned to each other (n = 6) (Figure 19B). These results suggest that there is a muscle fiber fate switch in *CG11617* KD mutants.

***CG11617* may control fiber identity**

To further validate whether there is a muscle fiber identity switch in the IFMs, we used a *151-Gal4; TpnC4-LacZ* driver line because this line contains enhancer-lacZ constructs bound to the tubular muscle-specific *Troponin C at 41C* (*Tpnc41C*) gene (Herranz et al., 2004). These lacZ reporters bound to TpnC41C will selectively express β -galactosidase (β -gal), enabling us to visualize TpnC41C accumulation. Female flies

from *1151-Gal4; Tpnc41C-lacZ* were crossed with CG11617 RNAi[30533] male flies to generate *1151 > CG11617 KD + Tpnc41C-lacZ* mutant offspring or with male flies from *w¹¹¹⁸* to generate *1151-Gal4; Tpnc-41C-lacZ* control offspring. Adult pharate control flies showed Tpnc41C accumulation, as revealed by green β -gal expression, in the jump muscles but was absent in the IFMs (n = 5) (Figure 19C). Interestingly, the adult pharate mutant flies revealed Tpnc41C accumulation in regions within the IFMs, opposite of what was observed in the control fly line (n = 3) (Figure 19C). These mutant flies show both fibrillar and tubular muscle fiber identity in the IFMs, which is a significant finding. Overall, this data suggests that IFMs may be switching to a tubular muscle fiber identity upon *CG11617* knockdown.

Figure 19. Knockdown of *CG11617* gives rise to muscle fiber fate switch in sections of IFMs. (A) Schematic of sarcomere structure. Adapted from Sanger et al. (2002). Generated with Biorender. (B) Cross striation of *CG11617* knockdown mutants resemble jump muscle structure. Control n = 8. *CG11617* n = 6. Magnification 120x. (C) *CG11617* knockdown reveals muscle fiber identity switch. β -galactosidase (β -gal; green) expression is abnormally expressed in IFMs of mutant flies. F-actin (muscles; red). Control (n = 3). *CG11617* (n = 5). Magnification 20x.



Immunostaining analysis reveals muscle fiber identity switch in sections of the indirect flight muscles

Since our previous images revealed tubular-like formations in the IFMs of CG11617 KD flies (Chapter 2 and Figure 19C), we speculated that our RNA-seq analysis would not reveal significant changes in muscle gene expression because the expression of muscle-specific genes might be too small to detect, given the lower IFM tissue in the mutants. Therefore, we conducted immunostaining for the flight muscle-specific genes *homothorax* (*hth*) and *extradenticle* (*exd*) to determine any gene expression alterations in the IFMs. We crossed *Fln-Gal4* driver females to UAS-CG11617-RNAi[30533] male flies (CG11617 KD) or to *w¹¹¹⁸* males (Control). The *Fln-Gal4* driver causes knockdown of *CG11617* in the thoracic muscles and because it is activated only after the onset of muscle differentiation, it allows the mutant offspring to reach adulthood, therefore allowing us to examine mature myofibrils (Bryantsev et al., 2012b). Research by Bryantsev et al. (2012b) revealed that *exd* and *hth* are expressed in IFMs but absent in jump muscles. The immunostains of CG11617 KD IFMs revealed similar levels of *exd* protein (n = 3) (Figure 20B'') and *hth* protein (n = 3) (Figure 20D'') when compared to the Control IFMs (n = 3) (Figures 20A'' and 20C'').

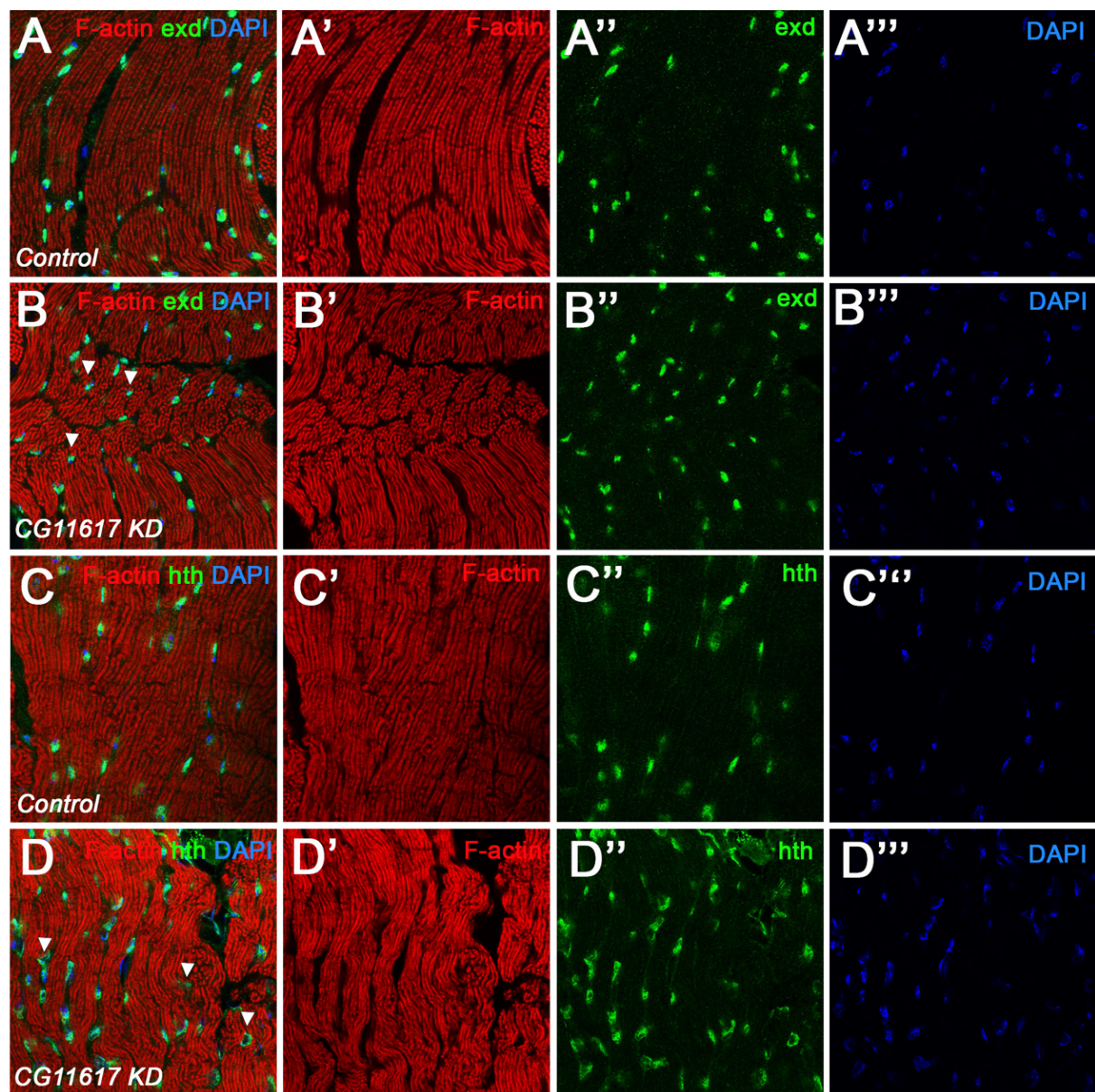


Figure 20. *CG11617* knockdown alters the expression of flight muscle-specific genes extradenticle and homothorax. (A - A''') Extradenticle (*exd*; green) is unchanged in *CG11617* KD IFMs ($n=3$) compared to Control flies ($n = 3$). (B - B''') Homothorax (*hth*; green) expression is unchanged in *CG11617* KD IFMs ($n = 3$) compared to Control flies ($n = 3$). F-actin (muscles; red). DAPI (nucleus; blue). Central nuclei (arrowheads).

We then quantified the expression of *exd* and *hth* in each nucleus within a 1035x1035 pixel framed image, according to the protocol in Shihan *et al.* (2021). We measured the background fluorescent intensity (Background) for Control and CG11617 KD pharate adult fly IFMs. Then, we measured the mean fluorescent intensity (MFI) in a region of interest (ROI), which is each nucleus present in the frame. We then calculated the Final MFI according to the following equation:

$$\text{Final MFI} = \text{MFI of an ROI} - \text{MFI of Background}$$

We subtracted the corresponding Background from each nucleus MFI and calculated the standard error of the mean (SEM). We found that the Final MFI was not significantly different for *exd* (p-value = 0.144) between Control (n = 38) and CG11617 KD (n = 48) (Figure 21A). We also found that the Final MFI for *hth* was higher, but not significantly different (p-value = 0.189) between Control (n = 27) and CG11617 KD (n = 38) (Figure 21B). The average CG11617 KD *exd* fluorescent intensity was also higher, 37.779, compared to the Control, 33.348. The average CG11617 KD *hth* fluorescent intensity was 31.817, and Control showed 27.910 fluorescent intensity. This data suggests that a compensatory alternative pathway may be activated, driving an increase of *exd* and *hth* levels in an attempt to establish homeostasis and possibly regenerate IFMs.

Next, we determined whether CG11617 KD IFMs are undergoing regeneration and repair processes by calculating the number of nuclei in 1035 x 1035 pixel frames. Our analysis revealed a significant increase in nuclei in CG11617 KD flies (n = 4) compared to the Control (n = 3) (p-value = 0.046) (Figure 21C). Interestingly, we also found instances of centrally located nuclei (Figure 21C; white arrowheads), which is

characteristic of skeletal muscle diseases such as Duchenne Muscular Dystrophy (DMD) (Wang et al., 2000) and Emery-Dreifuss Muscular Dystrophy (Gueneau et al., 2009) whose myofibers are undergoing repair. The increase in nuclei may be attributed to the newly incorporated nuclei that first localize at the center of the myofiber before moving to the periphery (as reviewed in Folker and Baylies, 2013). This nuclear positioning is characteristic not only of muscle repair but also occurs during muscle development (as reviewed in Folker and Baylies, 2013). Overall, our data suggests that *Drosophila* IFMs may be undergoing a similar process of damage and repair cycling, or an attempt at muscle development, triggering a compensatory pathway to address muscle damage upon *CG11617* knockdown.

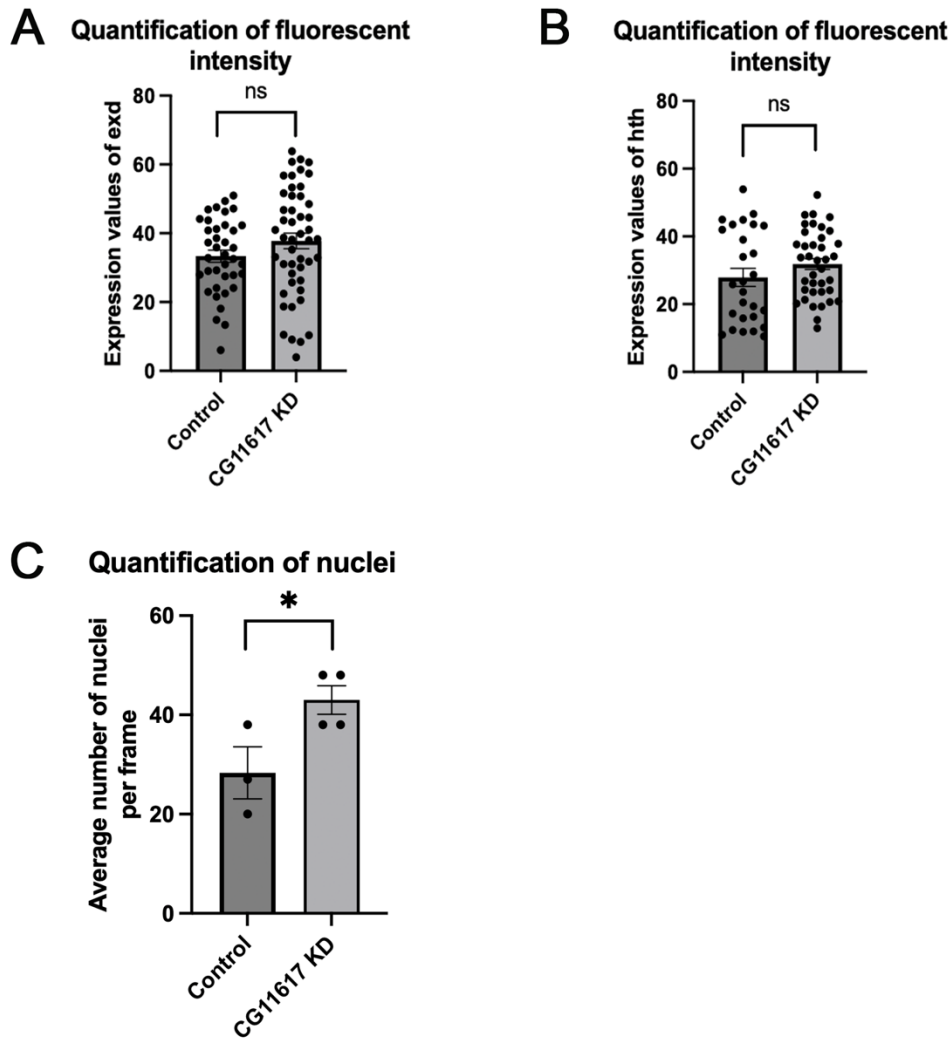


Figure 21. Quantification of extradenticle and homothorax fluorescent intensity in IFMs. (A) Final MFI was not significantly different for exd (p-value = 0.144) between Control (n = 38) and CG11617 KD (n = 48). The average CG11617 KD exd fluorescent intensity was 37.779, and Controls showed 33.348 fluorescent intensity. (B) We also found that the Final MFI for hth was not significantly different (p-value = 0.189) between Control (n = 27) and CG11617 KD (n = 38). The average CG11617 KD hth fluorescent intensity was 31.817, and Control showed 27.910 fluorescent intensity. (C) Quantification of nuclei in a 1035 x 1035 frame for Control (n = 3) and CG11617 KD (n = 4) revealed a significant increase (p-value = 0.046) in CG11617 KD IFMs.

DISCUSSION

Overall, the RNA-sequencing (RNA-seq) analysis revealed the broad effects of knocking down *CG11617* at the transcriptional level. It identified genes whose expression is significantly downregulated or up-regulated, suggesting that these genes are directly or indirectly regulated by *CG11617*. In addition, the Gene Ontology (GO) terms categorized the functional roles of the differentially expressed genes and revealed those that remain uncharacterized and might play regulatory roles in response to *CG11617* knockdown in the indirect flight muscles (IFMs). Lastly, immunostains revealed that other factors, in addition to *CG11617*, might be involved in muscle fiber type specification.

In this study, RNA-seq analysis suggests that *CG11617* knockdown impacts multiple biological pathways and processes. The top ten downregulated genes, primarily expressed during the embryonic stage, display a wide range of functions. Among these downregulated genes is a long non-coding RNA (lncRNA), CR46035. The role of this lncRNA remains largely unknown, highlighting a gap in our understanding of its potential contribution to muscle development and function. LncRNAs are RNAs that are longer than 200 nucleotides and are not translated into functional proteins; however, depending on their subcellular localization, cytoplasm, or nucleus, lncRNAs can have unique and important cellular functions (Mas-Ponte, D. et al., 2017; reviewed in Statello et al., 2021; reviewed in Ferrer and Dimitrova, 2024). Those present in the cytoplasm have functions that range from protein trafficking (Willingham et al., 2005; Kino et al., 2010) to translation regulation (Yoon et al., 2012; Schein et al., 2016) and miRNA decoys (Cesana et al., 2011). lncRNAs were also discovered to function within the

nucleus as antisense transcripts or decoys for splicing factors, resulting in aberrant splicing (Beltran et al., 2008; Tripathi et al., 2010).

Previous studies from our lab have shown that CG11617 is localized to the nucleus in indirect flight muscles (IFMs) and to both the nucleus and cytoplasm in jump muscles (see Chapter 2). The downregulation of CR46035 in our data set raises the question of whether CR46035 is primarily present in the nucleus or cytoplasm and if it moderates CG11617's function in IFMs and/or jump muscles. To further investigate the role of CR46025, methods to map RNA molecules, such as fluorescence *in-situ* hybridization, can be used.

The RNA-seq analysis also revealed unexpected results: *Act88F* was not significantly downregulated and was, in fact, up-regulated (Figure 18). We anticipated a downregulation of *Act88F* in these knockdown mutants because it is predominantly expressed in flight muscles (Fyrberg et al., 1983). One possible explanation for this finding is that the loss of IFM tissue in CG11617 KD mutants triggers a compensatory mechanism, leading to the upregulation of *Act88F* in an attempt to promote IFM development or maintenance. This compensatory response could be an effort by the fly to counteract the loss of IFM tissue and restore normal function. Similar compensatory mechanisms have been observed in *Drosophila* jump muscles. Upon knockdown of the predominant actin in jump muscles, *Act79B*, *Act88F* was found to functionally substitute for it (Dohn and Cripps, 2018). The functional compensation of *Act88F* in *Drosophila* IFMs has not been tested and can be a subject of future analysis.

Furthermore, examining *Drosophila* IFMs upon CG11617 did not reveal a clear pathway for the mechanistic role of CG11617 in muscle fiber type specification. We

analyzed adult pharate female flies, and it is possible that the main role of *CG11617* is earlier in pupal development. Chromatin immunoprecipitation (ChIP-seq) may be conducted using *Drosophila* at the mid-pupal stages, 24 hours, and 48 hours after puparium formation (APF). ChIP-seq will help identify where *CG11617* binds on the genome, elucidating its role in regulating gene expression and potentially helping us map out a pathway for how muscle fibers develop and become specialized.

In summary, we found that knocking down *CG11617* resulted in the switching of a number of IFM fibers to jump muscle fiber identity and that other factors, in addition to *CG11617*, might be involved in muscle fiber type specification. Additionally, as more genes are discovered and their functions elucidated, we can gain a clearer understanding of the mechanistic role of *CG11617* in muscle fiber type specification.

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DISCLOSURES

The authors have no competing financial interests or other conflicts of interest.

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CONCLUSION

Understanding skeletal muscle fiber differentiation and specification through the investigation of transcription factors such as *Drosophila CG11617* and *Mef2* is crucial for advancing our knowledge of muscle biology and for providing essential insights into addressing muscle-related diseases. Although significant strides have been made to uncover the mechanisms of muscle development, the specification of muscle fiber types and the precise molecular events underlying myofibril assembly and function remain largely unclear.

In Chapter 1, we found that enhanced expression of *Drosophila Mef2* in adult myoblasts of the larval imaginal wing discs induces an incomplete myogenic response, primarily activating genes associated with early muscle development rather than those specific to pupal or adult stages. Our data also revealed heterogeneity in muscle differentiation stages, failure of *Mef2* to induce the broad expression of myoblast fusion genes, and incomplete myofibril assembly. These data suggest that additional co-factors or regulatory elements are required to fully activate myoblast fusion and adult muscle differentiation. Future research should focus on identifying these co-factors, investigating the role of specific organizer proteins in myofibril assembly, and exploring the regulatory mechanisms that govern the transition from larval to adult muscle identity.

Our findings in Chapter 2 revealed that *CG11617* exhibits muscle-type-specific localization, with nuclear presence in indirect flight muscles (FMs), and localization to both the nucleus and the cytoplasm in jump muscles, suggesting distinct regulatory roles in each muscle type. Our findings also indicate that *CG11617* is crucial for the

proper development of IFMs and jump muscles, with its knockdown leading to significant morphological changes and impaired muscle function. Future research should investigate the regulatory mechanisms controlling *CG11617* localization, its interaction with potential co-factors or post-translational modifications, and its role in muscle fiber identity and differentiation. Understanding these aspects will provide valuable insights into the molecular pathways involved in muscle development and function.

In Chapter 3, our RNA-sequencing analysis showed that knocking down *CG11617* has significant effects on gene expression, with several genes being either upregulated or downregulated, suggesting a direct regulatory role. Despite identifying some differentially expressed genes, the precise mechanistic pathway for *CG11617* in muscle fiber specification remains unclear, in both IFMs and jump muscles. Future studies using chromatin immunoprecipitation sequencing (ChIP-seq) to identify *CG11617*'s genomic binding sites during the pupal developmental stage will provide deeper insights into the role of *CG11617* and its associated factors in muscle development and specification.

Exploring the utility of *Drosophila melanogaster* as a model organism can provide valuable insights due to conserved transcription factors with humans and simpler muscle structure. Future research to identify the factors involved in myofibril assembly and how different transcription factors and proteins influence muscle development and fiber fate will help understand the mechanisms that can lead to better therapeutic strategies for muscle diseases and improve interventions aimed at reversing muscle atrophy.