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Flight Muscle Progenitor Populations in Drosophila melanogaster

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DEFINING FLIGHT MUSCLE PROGENITOR POPULATIONS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

We use the *Drosophila* system to define how genes control muscle development and function. Many of the genes in the fruit fly, *Drosophila melanogaster*, are conserved with higher animals, and so our results can be important in understanding mechanisms of vertebrate muscle development and disease.

Study of the flight muscle progenitor populations in *Drosophila* gives clearer understanding of the genetic regulatory networks that lead to the development of flight muscle.

The direct flight muscles (DFMs) and indirect flight muscles (IFMs) arise from the myoblast population of the notal region of the *Drosophila* wing imaginal disc. Within the myoblast population, there are gene expression differences between the progenitor cells for the IFMs and DFMs (Sudarsan et al, 2001), but the different factors that specify each myoblast type are poorly understood.

Defining regulation of Vg expression in the imaginal disc myoblasts:
One difference between DFM and IFM populations is expression of Vestigial (Vg), a nuclear protein. Vg expression in the myoblasts is regulated through a novel 899 bp enhancer region specific to the wing disc myoblasts. To explore the regulation of the Vg in the myoblasts, DNA-protein binding assays were used to find functional transcription factor binding sites in the enhancer.

Defining gene expression in the myoblast populations in the wing disc:
The study of the genetics of the wing disc myoblast population, allows us to gain understanding of the genes important in the specification of flight muscles, as well as some insight into the differences between the IFM and DFM populations. To study this, successful isolation of the progenitor cells is achieved through fluorescently labeling the myoblasts and developing a protocol for generating a single cell suspension from the intact wing discs. Successful isolation of these cells will allow for genomics techniques such as RNA-seq for analyzing the gene expression differences in the two myoblast populations.

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INTRODUCTION TO MUSCLE DEVELOPMENT IN *DROSOPHILA*

Myogenesis

Myogenesis is the process by which muscles form. Morphologically, all mature muscles develop through the fusion of myoblasts into multinucleated fibers of muscle. Genetically, myogenesis occurs through switching expression of genes involved in myoblast determination to those encoding the proteins necessary for mature muscle function. Although the factors that control myoblast specification and differentiation have been defined over the past 20 years, relatively little is known about how adjacent myoblast populations contribute to different muscles and fiber types.

Drosophila as a model organism

Drosophila melanogaster has been widely used as a model organism in genetics and developmental biology due to conservation of important genetic networks with vertebrate development. *Drosophila* are genetically tractable, have a short generation time, and, most importantly, have been continuously studied as a model organism for over 100 years. As well as high conservation of the genetic networks, *Drosophila* can also be used a simplified system in some cases, with single proteins fulfilling a role in *Drosophila* where various semi-redundant proteins might in mammalian systems. *Drosophila* provide an ideal system for studying genetic networks.

Muscle pattern of *Drosophila*

The genesis of muscle during *Drosophila* development provides a paradigm for conserved processes, including founder cell maintenance, diversification of muscle types and final differentiation. The muscle pattern of *Drosophila* is composed of somatic muscle, muscle forming the vascular system and visceral muscle. All muscle types in *Drosophila* develop from the mesoderm. The mesoderm is then progressively subdivided and different regions of the mesoderm form the progenitors of the different muscle types (reviewed in Bate, 1993). This process is highly analogous to muscle development in vertebrates, where cardiac muscle, skeletal muscle and visceral muscle types of muscle all also arise from the mesoderm.

Drosophila are holometabolous insects, and therefore have both larval and adult phases of development. This requires two developmental stages specifying firstly the somatic muscles present and functional in the larval stage, and subsequently the muscles present and functional after pupal transformation. Additional to the functioning muscles, the larval stage also contains imaginal discs, internal areas of the larvae that will become parts of the appendages in the adult. In the pupal stage when many larval structures are broken down, the imaginal discs persist and go on to form adult structures.

Wing imaginal disc

The wing imaginal disc of *Drosophila* larvae is a key model system used to study cell differentiation or proliferation in response to patterning signals (Certel et al, 2000). The imaginal wing disc has key areas that are known to give rise to different areas of the adult wing (Figure 1). The notal region forms the thorax at the base of the wing, and has associated myoblasts that will differentiate into the muscles that in the adult are responsible for flight (Diaz-Benjumea and Cohen, 1993). Despite the extensive study of the development of the wing itself from the wing pouch, the early genetic hierarchy that leads to flight muscle differentiation has been largely under-investigated.

Wing muscle in the adult fly is made up of two distinct groups, the direct flight muscles (DFMs), responsible for changing wing direction, and indirect flight muscles (IFMs), which contribute to flight by deformation of the thorax (Bernard et al, 2006)(Figure 2).

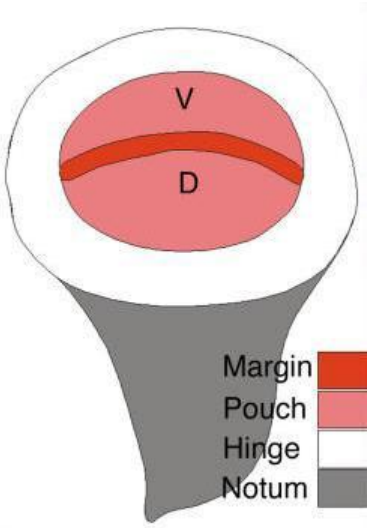

Figure 1: Schematic of the imaginal wing disc.
Myoblasts are found associated with the notum region of the wing disc. Image shows defined regions of the wing pouch, margin and hinge in relation to the notum. (Adapted from Le Borgne et al, 2005)

Myoblast diversification

Adult flight musculature provides an amenable system to address how signals influence muscle identity. Cell fate decisions across development may be the result of cell-autonomous or cell-non-autonomous factors, or an integration of the two.

Adult myoblast progenitors are siblings of embryonic muscle founder cells (Carmena et al, 1995), which are specified by combinations of regulatory factors (Baylies et al, 1998; Bate et al, 1991). Regulatory factors could specify adult myoblasts in the same way, but evidence suggests this may not be the case. Transplanted flight muscle myoblasts can contribute to diverse muscles in the adult fly and do not retain flight identity (Lawrence and Brower, 1982). This

experiment suggests that the adult myoblasts are a naive population, without a determined identity.

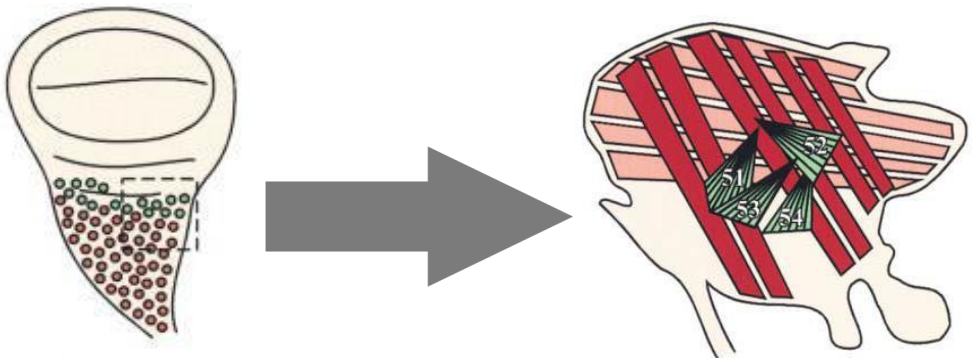

Figure 2: Myoblast region highlighted on wing disc in green, will populate the red and green flight muscles in the cross section *(adapted from Sudarsan et al, 2001) Wing disc schematic has myoblasts represented by red and green cells, for Vg+ and Vg- respectively. Myoblast cells populate adult flight muscles in cross section of the thorax, with Vg+ cells populating the red and pink indirect flight muscles, and Vg- cells populating the green direct flight muscles (51-54).*

However, reproduction of the transplantation experiments concluded that although the majority of the myoblasts of the disc are naïve, founder cells must exist in this population (Roy and VijayRaghavan, 1997). Founder cells in the embryo seed the fusion process of myoblasts to form a mature muscle fibre (Baylies et al, 1999). Myoblast fibers will take the identity of the founder cells (Rushton et al 1995), and so the bulk of myoblasts transplanted can contribute to other muscles. It was argued that the founder cells within the adult flight myoblast require information about their identity from regulatory factors, but also must be located within the correct position on the epidermis (Roy and VijayRaghavan,

1997). Therefore, flight myoblast specification must be the result of both autonomous and non-autonomous influences.

Myoblasts of the wing disc express Twist and Mef2 uniformly

In the wing imaginal disc, Twist (TWI) is expressed in the adult muscle progenitors before differentiation into adult muscles (Cripps et al, 1998). TWI and Notch are responsible for preventing differentiation into adult flight muscles, and maintaining the myoblast population (Anant et al, 1998). TWI function is required during late larval stages for DLM patterning (Cripps and Olson, 1998). Adult flies have six pairs of dorsal longitudinal indirect flight muscles (DLMs), but have only three pairs of DLMs when TWI function is reduced (Cripps and Olson, 1998). Expression of TWI declines during pupation where myoblasts fuse to form adult muscles (Bate et al, 1991). Sustained TWI expression represses differentiation into mature muscles, but is essential in formation of progenitor myoblasts (Baylies and Bate, 1996).

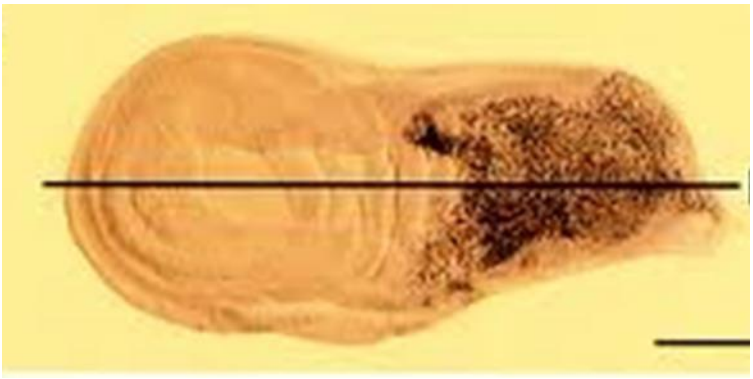

Figure 3: Mef2 expression in the myoblast region of the wing disc: *Third instar larvae wing discs were reacted with anti-Mef2 polyclonal antibody shows uniform expression of Mef2 in the myoblast wing discs. Bar, 50 µm. (Adapted from Cripps et al, 1998)*

Mef2 is also expressed uniformly in the flight muscle myoblasts (Figure 3) (Cripps et al, 1998; Sudarsan et al, 2001) and has been found to promote differentiation (Lovato et al, 2005 and Bernard et al, 2006). *Mef2* is expressed in all muscle cells from gastrulation and throughout embryogenesis, is regularly used as a marker for muscle during embryonic development (Cripps et al, 1998), and is expressed in the 3rd instar wing imaginal disc population (Figure 3). Embryos lacking *Mef2* exhibit severe defects in myogenesis, with a lack of muscle fibres (Bour et al, 1995). *Mef2* function is also important in adult myogenesis in determining number of DLM fibers, since hypomorphic Mef2 mutants that survive to adulthood lose DLMs (Ranganayakulu et al, 1995), and *Mef2* knockdown during the pupal stage results in loss of adult muscles (Bryantsev et al, 2012).

The population of myoblasts on the imaginal disc were initially thought to be an undifferentiated population able to give rise to either type of flight muscle, since expression of both *Mef2* and *twi* is uniform (Lawrence and Bower, 1982; Sudarsan et al, 2001). However, mature IFMs and DFMs differ in their size, location, contractile properties and innervations. More recently, it has been shown that the myoblasts belong to distinct groups that populate either IFMs or DFMs (Sudarsan et al, 2001; Bernard et al, 2009).

Vestigial and CUT expression in the myoblasts are stabilized by a mutually repressive feedback loop

By the third larval instar, the wing disc myoblasts can be divided into two populations with distinctive properties. In third instar larvae, Vestigial (Vg) and low levels of Cut are expressed in cells that will contribute to the indirect flight muscles (IFMs) (Sudarsan et al, 2001). High levels of Cut (but not Vg) are required for the development of the direct flight muscles (DFMs) (Sudarsan et al, 2001). Wingless signaling from the ectodermal cells were shown to be required for the maintenance of these populations. Distinction between the two groups is also maintained by a mutually repressive feedback loop between Vg and Cut (Sudarsan et al, 2001).

Vg is a nuclear protein and transcription factor expressed in the embryonic wing discs. Vg is a co-factor for Scalloped, a *Drosophila* ortholog of mammalian Transcription Enhancer Factor -1. In wing development, Vg is necessary in the

patterning of the wing: loss of Vg leads to aberrant wing development (Lindsley and Zimm, 1992), and ectopic expression of Vg leads to ectopic wing development (Kim et al, 1996; Klein and Martinez- Arias, 1999).

Despite the well characterized role of Vg in wing proper development, the role of Vg in the myoblast cells has been under-studied. In flight muscles, homozygous Vg^{null} mutants were found to have DLM degeneration through apoptosis and a total absence of DVMs in the adult (Bernard et al, 2003), but the regulation of Vg expression in the myoblasts is poorly understood.

Control of Vg expression

Vg is expressed in the imaginal disc by the action of various signaling pathways that control expression through different enhancers. Three published enhancers control the expression of Vg in the wing disc. The Boundary and Quandrant enhancers (Vg^{BE} and Vg^{QE}) control the expression of Vg in the wing pouch of the wing disc, and are regulated through the activities of major signaling pathways, Notch, Wingless and decapentaplegic (Klein and Martinez-Arias, 1999). However, neither the Boundary nor Quadrant Enhancers are active in the myoblasts of the notal region (Klein and Martinez-Arias, 1999), (Figure 4).

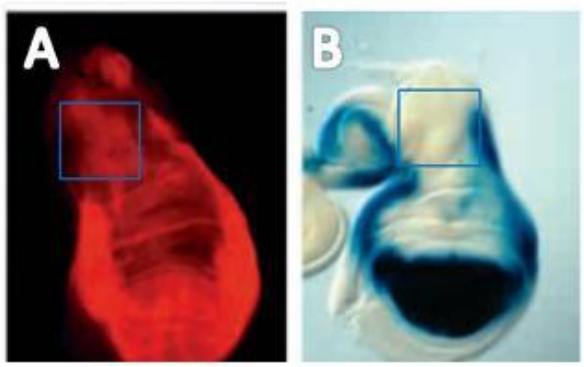

Figure 4: Known Vg enhancers do not account for myoblast expression *A) Vg Ab stain shows total Vg expression in wing disc. B) Boundary and Quadrant enhancers driving lacZ, there is no lacZ expression in the myoblast region indicated by blue outline.(Adapted from Klein and Martinez, 1999)*

Additionally, a third enhancer of 822bp known as the Adult Muscle Enhancer controls expression of Vg in the differentiated IFM muscles of the adult during the pupal stage (Bernard et al, 2009). The three characterized enhancers do not control the expression of Vg in the myoblasts of the wing disc.

A novel Vg enhancer of 899 bp has putative consensus binding sites for Twi, Su(H) and MEF2, and is upstream of both Vg^{AME} and Vg^{QE} the in the fourth intron (Figure 5). This enhancer was found to control expression of Vg in the myoblasts, and so further study of the enhancer is required to better understand Vg regulation.

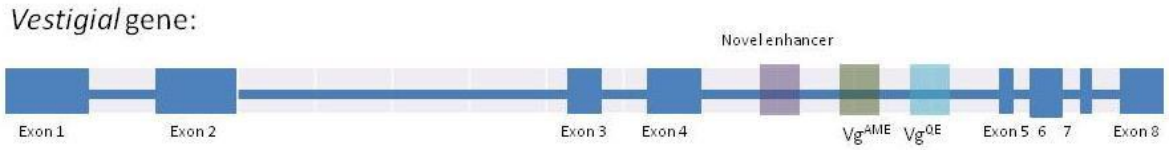

Figure 5: Schematic of Vestigial gene with enhancer region. *Novel 899bp enhancer shown in purple is in intron 4. Vg^{AME} (green) and Vg^{QE} (light blue) are downstream of this enhancer in intron 4.*

SIGNIFICANCE: VG-LIKE IN MAMMALIAN MUSCLES

The conserved nature of Vg and the importance of Vg homologs in vertebrate myogenesis justifies investigation into Vg regulation and function in *Drosophila*. Vg functions as a heterodimer with Scalloped (Sd) in order to control gene expression in myogenesis (Paumard-Rigal et al, 1998). Whereas Vg expression is restricted to a subset of cell types, Sd expression is dynamic and found in a much wider range of cell types, suggesting that Vg expression regulates the function of the heterodimer (Sudarsan et al, 2001; Delanoue et al, 2004).

The mammalian ortholog of Sd is Transcription Enhancer Factor-1 (TEF-1), or TEAD1, and has the critical function of controlling muscle specific gene expression. There are four Vestigial–like (VgII) genes in mammals (Vaudin et al 1999; Maeda et al 2002).These mammalian orthologs have highly restricted expression during development. *Vgll2* is expressed almost exclusively at sites of myogenesis, physically interacts with TEAD1 and potentiates MEF2 dependent gene activation (Maeda et al 2002). Additionally, *Vgll2* morphants in chick show attenuation of muscle gene expression in embryos (Chen et al 2004).

It is clear that Vgl expression in skeletal muscle during development is important in higher animals, and yet regulation and targets of Vgl remain poorly defined. This study will provide a framework through which higher regulation and roles of Vgl can be explored. This could be particularly important in understanding the etiology of muscular diseases, given the pervasive expression of Vgl in skeletal muscle.

1) REGULATION OF VG EXPRESSION IN IMAGINAL DISC MYOBLASTS

Overview

The Vg 899bp enhancer region was identified due to its involvement in the mutant Vg^{f02736}. Vg^{f02736} has a transposable element insertion at 242bp in the 899bp enhancer and has ablation of the Vg protein expression in the myoblast region. Our hypothesis is that the loss of Vg expression is caused by the transposable element insertion disrupting interactions between active transcription factor binding sites within the enhancer. A number of consensus sequences that could potentially bind transcription factors have been identified in the enhancer, significantly Suppressor of Hairless (Su(H)), an integral part of the Notch pathway (Jarriault et al, 1995) and Twi binding sites. As this is a novel enhancer, the transcription factor binding and regulation of this region is uncharacterized. Through EMSA analysis, consensus sites that were able to bind transcription factors were identified, and inform the understanding of the regulation of Vg protein in the myoblasts.

MATERIALS AND METHODS

Fly stocks and crosses

Fly stock *Vg*^{f02736} was obtained from Bloomington *Drosophila* Stock Center (Bloomington, Indiana), *Vg899bp-LacZ* was generated by Dr. Tyanna Lovato. All stocks were maintained on Fisher-Scientific Jazz Mix medium.

Immunohistochemistry

Late third instar larvae were collected and washed in PBS. They were placed in PBS and wing discs were dissected carefully using forceps. Wing discs were fixed in 4% paraformaldehyde for 10 minutes and then washed with PBS containing 0.1% (vol/vol) Triton X-100 before incubation with primary antibodies. The following primary antibodies were used: rabbit anti-Vg were used at 1:50 dilution; anti-β-galactosidase monoclonal (Promega) at 1:250 and monoclonal antibody against Cut (Developmental Studies Hybridoma Bank) used at 1:100 dilution. Discs were mounted on 1mm VWR micro slides in 80% glycerol for imaging. Images were obtained using LSM 780; Carl Zeiss confocal microscope at room temperature. All images were acquired through Zen 2011 (black edition; Carl Zeiss) and edited using Photoshop (Adobe).

Electrophoretic mobility shift assays (EMSA)

Twi binding assays were performed as described in Cripps et al, (1998) with slight modifications. Briefly, in addition to the probe, 1 µg of poly[d(I–C)]; 3 µl of TnT lysate and 1 µl of a 10× buffer consisting of 400 mM KCl, 150 mM HEPES at pH 7.9, 10 mM EDTA, 5 mM DTT, 50% glycerol as described; 0.5 µg of Herring

sperm DNA was also used to reduce non-specific binding.

For competition experiments, the E1 Mef2 enhancer fragment oligonucleotide was used as a known binding control, and competed with x100 concentration oligonucleotide probes for Twi site 1, 2, 3, 4, 5, 6 and 7 as well as mutant Twi binding sites 2, 3 and 5. Mef2E1 enhancer probe as in Cripps et al, 1998, the experimental oligonucleotides had the sequences in the Table 1:

Table 1: EMSA probe sequences

Target site in 899bp enhancer:	Forward primer (5'✳)	Reverse primer (5'✳)
Twi consensus site 1	GGATCAAGTTGCCACTTGCCACTCGCTT	GGAAGCGAGTGGCAAGTGGCAACTTGAT
Twi consensus site 2	GGTACATACATACATATGTACATATATA	GGTATATATGTACATATGTATGTATGTA
Twi consensus site 3	GGAATACCCCAACATGTGCGTTGTTTAT	GGATAAACAACGCACATGTTGGGGTATT
Twi consensus site 4	GGGAAGCCTTGTCAAGTGAAGATTTTCA	GGTGAAAATCTTCACTTGACAAGGCTTC
Twi consensus site 5	GGATTATCCTCGCATTTGAATGCCGAGC	GGGCTCGGCATTCAAATGCGAGGATAAT
Twi consensus site 6	GGGAATGCCGAGCAGGTGTTGGCTGGCG	GGCGCCAGCCAACACCTGCTCGGCATTC
Twi consensus site 7	GGGCCACACCGCCAATTGCAGTTAAACC	GGGGTTTAACTGCAATTGGCGGTGTGGC
Mutant Twi site 2	GGTACATACATA**GAATTC**TACATATATA	GGTATATATGTA**GAATTC**TATGTATGTA
Mutant Twi site 3	GGAATACCCCAA**GAATTC**CGTTGTTTAT	GGATAAACAACG**GAATTC**TTGGGGTAT
Mutant Twi site 6	GGGAATGCCGAG**GAATTC**TTGGCTGGCG	GGCGCCAGCCAA**GAATTC**CTCGGCATTC

Twi consensus sequence underlined; Mutant Twi consensus✳EcoRI site underlined and bold.

RESULTS

Vg^{f02736} 899bp enhancer drives expression in the myoblast region

LacZ expression driven by Vg 899bp enhancer overlaps with the expression of Vg and Cut in the myoblasts of the wing disc (Figure 6). Vg expression in the distal cells is absent, and only Cut is expressed, whereas in the proximal cells Vg is present and Cut expression is lower. Disruption of this enhancer region in the vg^{f02736} mutants, where there is an insertion of a transposable element into the fourth intron of *vg,* after a Su(H) binding site and the between putative Twi binding sites.

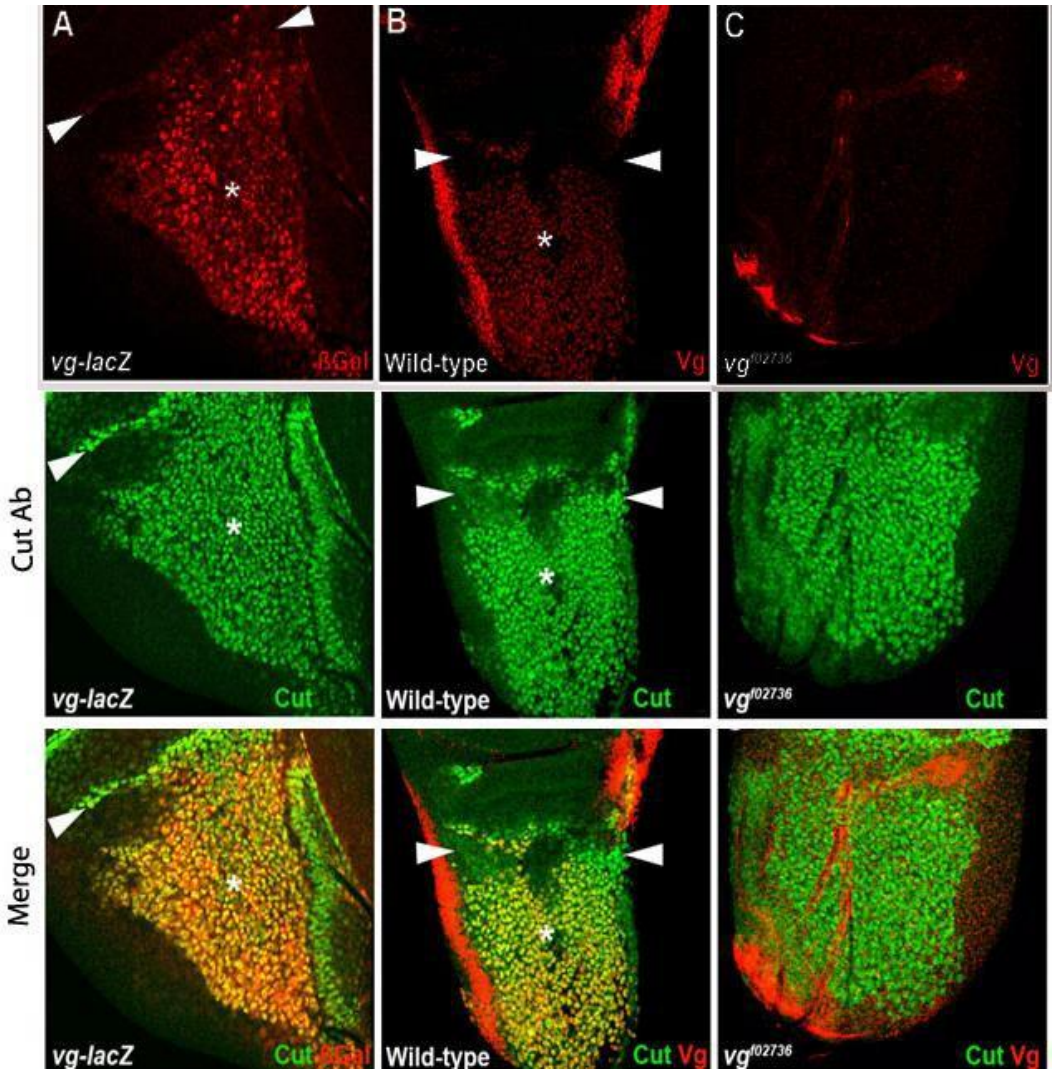

Figure 6: Vg expression in myoblasts arises from an enhancer in the fourth intron

A: When fused to lacZ, the 899bp enhancer directs strong ßGal (Red) accumulation in the adult myoblasts (asterisk). Note area of reduced ßGal accumulation in the portion of the wing disc between the arrowheads. B: In wild-type, Vg accumulation (Red) is detected in the myoblasts (asterisk) and more strongly in myoblasts close to the notum. Note that Vg levels are reduced in the myoblasts between the arrowheads. C: In vg^{f02736} mutants, Vg does not accumulate in the myoblasts. In all panels, adult myoblasts are visualized using anti-Cut (Green).

Despite early evidence that TA E-box sites (ie CA**TA**TG) were preferred by TWI, TWI sites were found by ChIP-seq near ChIP summits to be CA E- box (CACATG) sites, with a high CABVTG box frequency (where B can be a C, G or T and V can be an A, C or G nucleotide)(Ozdemir et al, 2011). However, all CANNTG E box sites can show some binding to TWI and so each of the CANNTG E-boxes in the enhancer region were studied (Figure 7).

Figure 7: Vg 899 bp enhancer: *Enhancer bounded by primers (italic underlined) with Twi consensus sequences highlighted in red. Consensus sequences are numbered 1-7 from the 5' end. EMSA identified sites in blue outline. Su(H) consensus sequence identified with green outline.*

EMSA analysis of Twist binding to 899bp Vg enhancer confirms three Twist binding sites are functional in vitro

The Twist E-box CANNTG consensus sequence features seven times in the 899bp enhancer region, Twi sites 1-7. Twi sites 1, 2, 3 and 6 are all CABVTG sites, which are found to have occupied Twi sites at a higher rate than other, CANNTG Twi consensus sequences by ChIP-on-chip (Ostemir et al, 2010). Probes of 28bp were designed for each Twi site 1-7.

A known Twi binding site, the E1 enhancer from *Mef2* (Cripps et al, 1998), was radioactively labeled and used in a 1 in 100 competition EMSA (Figure 7). From this EMSA, Twi sites 2, 3 and 6 were shown to compete with the known E1 enhancer Twi site.

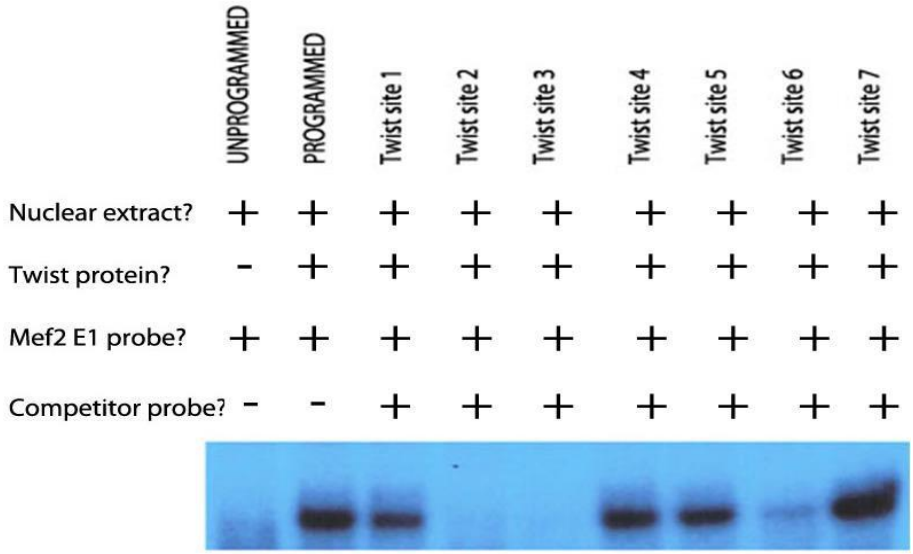

Figure 8: Competition EMSA for Twist binding: *Unprogrammed sample of labeled probe with no TWI protein; Programmed TWI+ Mef2 E1 enhancer Twi site; TWI sites 1-7. Nuclear extract in all lanes, Twist protein expressed by nuclear extract in programmed through Twist site 7. Competitor Mef2 E1 probe in all lanes.*

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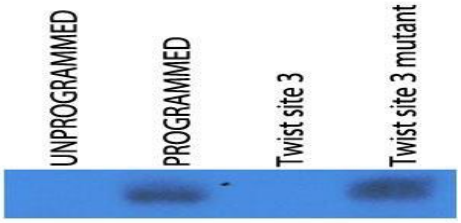

Figure 9: Twi site 3 competition EMSA: *Unprogrammed lane: no Twi protein added; Programmed: Twi protein and E1 enhancer control; TWI site 3: competition with Twist site 3 probe x100 concentration and Mef2 E1 enhancer probe; Mutant TWI site 3:competition with Twist site 3 mutant probe and Mef2 E1 enhancer probe.*

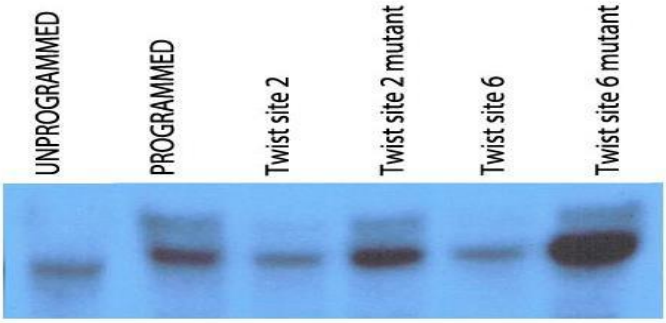

Figure 10: Twi site 2 and 6 competition EMSA. *Unprogrammed lane: no Twi protein added; Programmed: Twi protein and E1 enhancer control; TWI site 2: competition with Twist site 2 probe x100 concentration and Mef2 E1 enhancer probe; Mutant TWI site 2:competition with Twist site 2 mutant probe and Mef2 E1 enhancer probe. TWI site 6: competition with Twist site 6 probe x100 concentration and Mef2 E1 enhancer probe; Mutant TWI site 6: competition with Twist site 6 mutant probe and Mef2 E1 enhancer probe.*

FUTURE DIRECTIONS

Having confirmed the *in vitro* binding of Twi to the Twi sites 2, 3 and 6, in the future we intend to confirm the identified binding sites are active *in vivo.* Introducing mutations into the consensus sequence of the identified Twi binding sites on a plasmid system where the 899bp Vg enhancer drives LacZ expression should result in loss of LacZ expression in myoblasts *in vivo*. This ablation of enhancer activity would show not only that these sites are active *in vivo*, but also that Twi is necessary for the function of this enhancer in the myoblasts. In addition to Twi, preliminary data from this lab suggests that the Su(H) consensus site in the enhancer is functional under EMSA conditions (unpublished data) and work is continuing using the same Vg 899bp enhancer-*LacZ* system to mutate the Su(H) site to see resulting enhancer functionality. It is possible due to the location of the insertion in the 899bp region that the Su(H) site binding is disrupted separate from the effect of Twi site disruption and these experiments would therefore clarify the roles of the these two transcription factors in the regulation of Vg in the wing disc myoblasts.

2) DEFINING GENE EXPRESSION IN THE MYOBLAST POPULATIONS IN THE WING DISC

Overview

The identification of two populations of cells in the myoblast region of the wing disc (Sudarsan et al, 2001) has led to questions concerning which genes may be expressed differentially, and how those genes may be involved in specifying different muscle cell fates. To investigate this, we must first develop a method for separating the two cell populations for individual analysis. Published methods for homogenizing Drosophila wing discs do not maintain cell viability (Neufeld et al, 1998), and therefore cannot be used to gain insight into the transcriptional profile of the cell populations. The methods described allow for this isolation of viable labeled cells, permitting analysis of the gene expression of each population in the future.

MATERIALS AND METHODS

Construct creation

Vg 899bp enhancer amplified from genomic DNA extracted from W118 flies using forward primer 5' TTCGCCTCCACTCATTG 3' and reverse primer 5' GTTGTTAAGCAGCCTGTG 3' and inserted into a pUC8 backbone driving expression of fluorescent protein DsRed (PRed H-Stinger, #1204, *Drosophila* Genomics Resource Center). Sequencing was performed using BigDye Terminator v3.1 cycle sequencing kit (Life technologies, cat. no. #4337455).

Germline transformation

Transgenic flies for this study were obtained by P-element-mediated transformation (Rubin and Spradling, 1982). Yellow-white (yw) embryos were injected with the cloned transforming plasmid and Δ2-3 transposase source. Several independent transgenic lines were identified by eye color change and tested for expression of transgene.

Fly stocks and crosses

All experimental crosses were carried out at 25˚C, except UAS-Gal4 systems, which were crossed at 29˚C. Line *Mef2-Gal* and Line *UAS-GFPx2* were obtained from Bloomington *Drosophila* Stock Center, (Bloomington, IN, USA).

Imaging

Late third instar larvae were collected and washed in PBS. They were placed in PBS and wing discs were dissected carefully using forceps. Dissected wing discs

were imaged for expected expression of fluorescent proteins. Wing discs were fixed in PFA for 5 minutes and stained with DAPI for nuclear staining. Discs were mounted on 1mm VWR micro slides in 80% glycerol for imaging. Images were obtained using LSM 780; Carl Zeiss confocal microscope at room temperature. All images were acquired through Zen 2011 (black edition; Carl Zeiss) and edited using Photoshop (Adobe).

Homogenization of wing discs

100-200 wing discs were homogenized manually using the 1 mL loose tissue grinder dounce homogenizer (Wheaton, cat. #432-1270) and disrupted using 4-5 strokes of the pestle. Wing discs were then incubated in 0.05% trypsin-EDTA (GIBCO, Grand Island, NY) for 10 minutes shaking Resulting single cells were spun at 1000 g for 5 minute, trypsin-EDTA supernatant removed and washed with PBS, before being resuspended in PBS and transported on ice for FACS.

Cell viability

Cell viability was assayed using 10 µg/ml Propidium Iodide (PI) (Invitrogen cat #P3566) for 5 minutes, and Hoescht 33258 (Invitrogen, cat #H3569). .

Fluorescently activated cell sorting

The cells were sorted using a high-speed Legacy MoFlo cell sorter (Beckman Coulter), equipped with UV and argon lasers (Biomedical Sciences FACS facility, University of New Mexico, NM). Cells were sorted by gating for either GFP-positive or DsRed-positive events. Cells were sorted directly into RLT buffer (Qiagen cat #74104) for lysis and RNA extraction or into PBS for imaging cell viability after sorting.

RNA extraction

RNA extractions were performed using Qiagen RNeasy kit (Qiagen cat #74104) according to manufacturer's protocol (including DNAase treatment steps) and resuspended in 30µl of RNAse-free water. Concentration of extracted RNA was assayed using fluorimetric analysis using Quant-iT RiboGreen (Life technologies), and Qubit (Life technologies).

Expression analysis

Total collected RNA was used to synthesize cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers (Roche). Diluted cDNA was used as template for subsequent PCR analysis with OneTaq (Invitrogen) and the following pairs of gene-specific primers: Forward 5'-GCCAGCAGTCGTCTAATCCA-3' and reverse 5'-CCGTGCTCAATGGGGTACTT-'3.

RESULTS

Molecular cloning generates enhancer-fluorescent protein constructs

To label the live cells, genes expressed in each sub-population were used to drive expression of fluorescent proteins. MEF2 is a marker for muscle in both the larval and adult fly and is present in the whole population of wing disc myoblasts. As discussed, Vg is expressed in a subsection of the myoblasts, the putative IFM progenitors, driven by the 899bp enhancer. The novel Vg 899bp enhancer was inserted into the pRed H-Stinger plasmid to drive nuclear localized DsRed.T4 expression (Figure 11).

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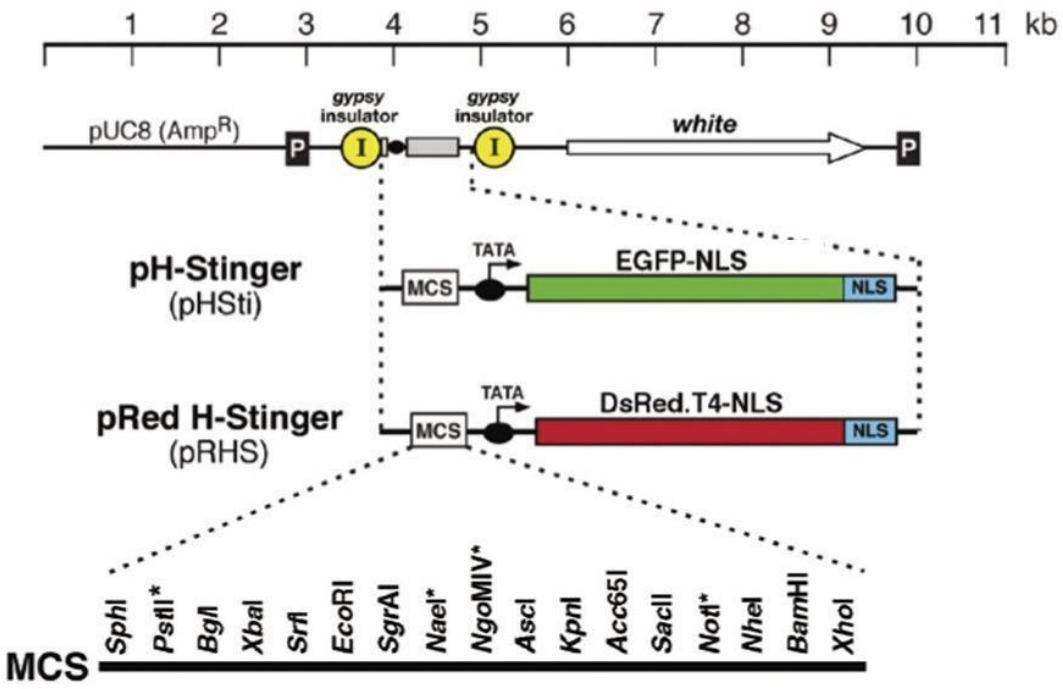

Figure 11: Schematic of plasmids. *Vg 899bp and Mef2 175bp enhancer insertion into MCS region of pRed H-Stinger and pH-Stinger in order to drive expression of DsRed.T4-NLS and EGFP-NLS respectively (Mef2 175bp construct was non-functional). White gene allows for selection based on eye colour.*

Transgenic lines generated by injection

The completed construct was then used to create transgenic lines. 100 flies injected with the construct were crossed with YW flies. Transgenic animals were then identified in the G1 generation by eye color. Homozygous lines were generated by standard genetic crosses. Two lines of Vg.DsRed.T4.NLS were generated. The inserts were mapped and shown to be on chromosome 2 and 3.

Confirmation of fluorescent protein expression in myoblasts

Homozygous flies were dissected and wing discs removed at the 3rd instar for imaging. Imaging with fluorescent microscope confirmed expected expression patterns of DsRed in the wing disc for Vg.DsRed.T4.NLS. Vg.DsRed.T4.NLS flies did not have expression outside the notal region of the wing imaginal disc, where Vg expression is driven by different enhancers (Figure 12).

Mef2>GFP labeled myoblasts generated with UAS-Gal4 system

The Mef2 175 bp enhancer was inserted in the pH Stinger plasmid (Figure 11) to drive expression of nuclear localized eGFP intended to label the Mef2 population. The construct was sequenced to confirm correct insertion, and injected into flies as above. Resulting transgenic flies had strong GFP expression in the salivary glands, however, the 175bp Mef2 enhancer was unable to drive strong GFP expression in the myoblasts and downstream FACS analysis of these discs could not separate the GFP expression from auto-fluorescence at FITC emission (Supplementary Figure 1).

In order to label the Mef2-positive population, the UAS-GAL4 system was used. *Mef2-Gal4* and *UAS-GFPx2* lines were crossed and successfully labeled the region (Figure 12).

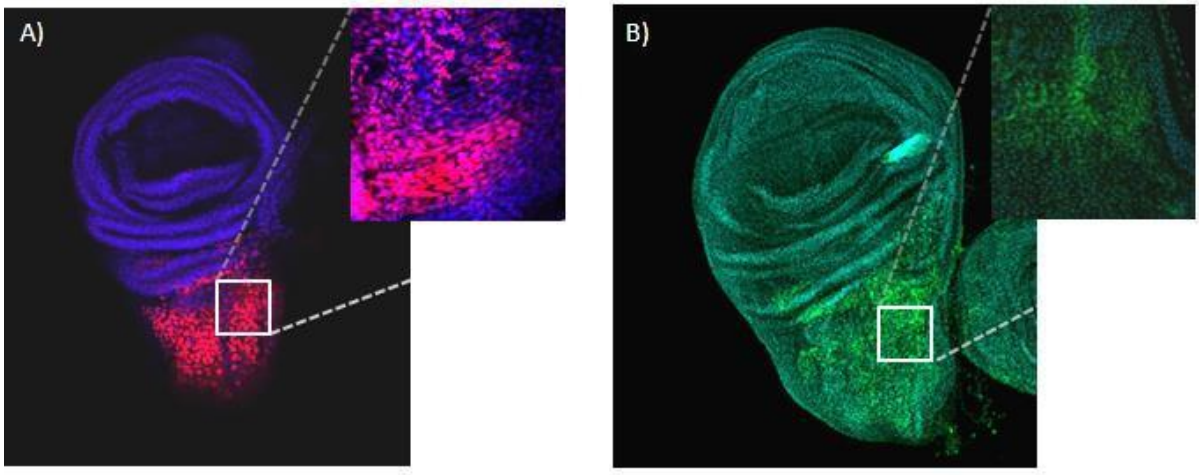

Figure 12: Confirmation of fluorescent labels *A) Vg.DsRed.NLS whole wing disc. DsRed is shown in red, DAPI in blue. B) Mef2>GFP whole wing disc. GFP is shown in green, DAPI in blue.*

Homogenization of wing discs generates an intact single cell suspension

It is not possible to isolate a large number of labeled cells from whole larvae using FACS (Supplementary Figure 2). Wing disc cells need to be single cell suspensions for FACS. Previously published wing disc cell homogenization methods were solely enzymatic (Neufeld et al, 1998) and resulted in high levels of cell death assayed by PI and Hoescht stain. Cell viability is essential for transcriptomic analysis of the FACS analyzed cells due to rapid degradation of RNA in dead cells (Gallege Romero et al, 2014) and so the single cell suspension must consist of live cells.

Preparation of single cell suspensions from various tissue types commonly involves initial mechanical disruption of the tissues, and various sources have

used tissue grinders to achieve the disruption (Chan et al, 1971; Shigenobu et al, 2006; Bryantsev et al, 2012).

Therefore, in order to gain better enzyme accessibility, the dissected wing discs in Trypsin-EDTA were transferred into a Dounce homogenizer and the tissue disrupted using 4-5 gentle stokes and then incubated in trypsin-EDTA for 10 minutes shaking. The action of the Dounce tissue grinder needs to be limited or will result in membrane rupture and cell death. ~85% of the cells remain intact and alive after both cell homogenization and FACS (Figure 13).

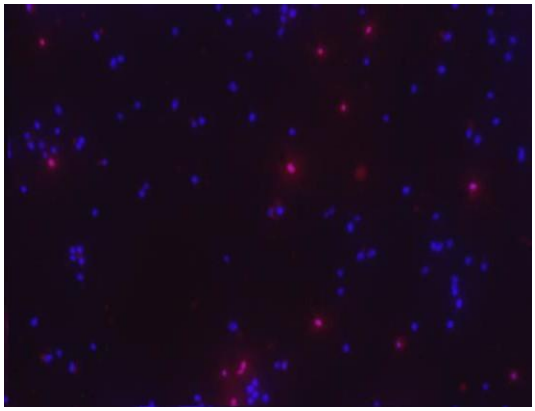

Figure 13: Cell viability after homogenization and cell sorting. *Propidium iodide stain (magenta) indicates dead cells, Hoescht 33322 (blue) indicates total cells. Homogenized cell suspensions (n=3) were imaged to show 14.9% of cells are dead after homogenization and sorting*

FACS gives isolated labeled populations

Single imaginal discs contain 10,000 cells (Klebes et al, 2002), but have <2,000 myoblasts (Ueyema et al, 2010). Around 80-90% of the myoblasts in the wing disc label with Vg antibody (Figure 6B) (Sudarsan et al, 2001). The labeled

myoblasts were sorted by gating based on absorption profile of YW wing discs. Both GFP+ and DsRed+ populations made up between 6-18% of the total wing disc cells (Figure 14). The cell sorts gave between 10,000-25,000 labeled cells (either Mef2+ or Vg+) and between 100,000 and 250,000 unlabeled non-myoblast wing disc cells.

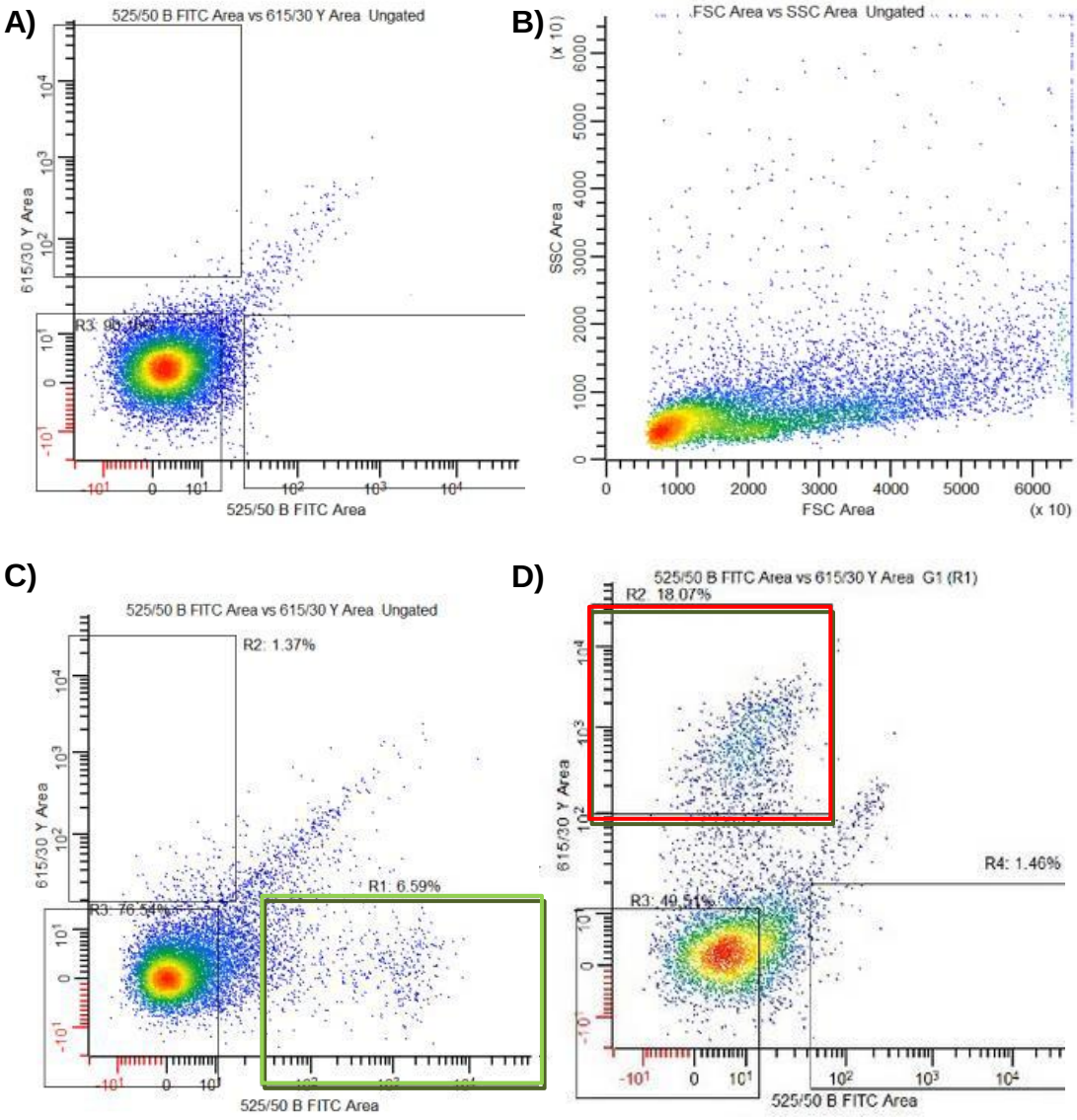

Figure 14 FACS plots give isolated populations *. A) Control YW 615/30 Y axis indicates red fluorescence absorbance. 525/50 X axis indicates green fluorescence absorbance B) Forward-scattered light (FSC) is proportional to cell-surface area (FSC Area) vs Side scattered light (SSC) area proportional to cell complexity. Gating for cells that are above 10,000 FSC area eliminates cell debris. C) Mef2>GFP population shown in R1 (outlined in green) as 6.59% of cells. D) Vg.DsRed.NLS population shown in R2 (outlined in red) as 18.07% of total cells.*

RNA extractions from FACS products were unsuccessful

The single cell populations were initially sorted into RLT lysis buffer (Qiagen RNeasy kit) and immediately used for RNA extraction. The RNA extractions gave an RNA concentration out of the range that can be measured accurately using Nanodrop 2000 (Thermo scientific) and too low to measured by Qubit (Life technologies) (Table 2). After variations in RNA extraction were unsuccessful, cell pellets from FACS products were sent to a genomic service company (Genewiz Inc.) for RNA extraction. Results from these extractions are shown in Table 3. No labeled cell RNA extractions gave a quantifiable RNA concentration.

Table 2: RNA extractions quantified

Sample/Name	Sample Vol. (ul)	Nanodrop 2000				Qubit	
		Nucleic Acid Conc. (ng/ul)	Total Amount (ng)	A260/ A280	A260/ A230	Nucleic Acid Conc. (ng/ul)	Total Amount (ng)
1A: No Label	41	2.9	118.9	2.26	0.23	Too low	N/A
1B: DsRed+	41	3.5	143.5	2.48	0.13	Too low	N/A
2A: No label	38	5	190	1.9	0.21	Too low	N/A
2B: DsRed+	38	3.7	140.6	2.07	0.46	Too low	N/A
3A: No label	41	3.9	159.9	1.9	0.09	Too low	N/A
3B: DsRed+	41	3.1	127.1	1.72	0.07	Too low	N/A
4A: No label	41	5.4	221.4	1.62	0.31	Too low	N/A
4B:DsRed+	41	3.6	147.6	2.07	0.26	Too low	N/A

Samples 1-4 are RNA extractions from 4 separate cell sorts Quantification of RNA extraction by Genewiz. All populations gave RNA concentrations that were too low for library construction for RNA-seq (Qubit measurements). Nanodrop 2000 readings are inaccurate below 20ng/µl RNA.

Table 3: Cell pellets RNA extraction

Sample	Qubit		
	Vol. µl	Nucleic acid concentration (ng/ µl)	Total Amount (ng)
1A-No label	10	5.64	56.4
1B-DsRed+	10	too low	too low
2A-No label	10	too low	too low
2B-DsRed+	10	too low	too low
3A-No label	10	5.66	56.6
3B-DsRed+	10	too low	too low
4a-No label	10	5.72	57.2
4b-DsRed+	10	too low	too low

1-4 are 4 separate cell sorts of 15,000- 20,000 DsRed+ cells and 150, 000-250,000 unlabeled cells. All DsRed labeled samples were unable to give a quantifiable concentration of RNA. 3 unlabelled samples had low but quantifiable RNA concentrations.

To confirm the RNA extraction process was not at fault, RNA was extracted using the same protocol from 5 homogenized larvae to give 210ng/µl of RNA. This, and the successful RNA extraction and quantification from the unlabeled samples with an order of magnitude more cells, suggests there may be insufficient RNA in the labeled cell samples for extraction by these methods.

To test whether RNA has been successfully extracted, but at a concentration too low for accurate quantification, an RT-PCR was performed after RNA extraction using intron-spanning primers for the constitutively expressed gene *Actin 5C*. Genomic DNA contamination will give a different length product than cDNA due to intron inclusion. Whole larvae cDNA was compared to cDNA constructed from

RNA extraction products from either DsRed+ or unlabelled cells. Both DsRed+ and unlabeled (DsRed-) cells had sufficient RNA to allow for *Actin5C* cDNA production suggesting that low concentrations of RNA are successfully extracted, but that these concentrations are insufficient for accurate quantification or RNA-seq purposes.

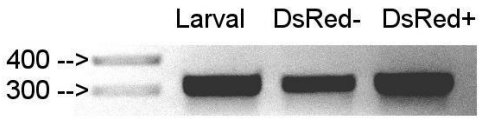

Figure 15: Actin 5C 316bp product. *Larval lane represents larval RNA extracted from 5 3rd instar larvae. DsRed+ and DsRed- populations from FACS both had sufficient Actin5C RNA to produce cDNA.*

FUTURE DIRECTIONS AND DISCUSSION

The goal of these experiments is to use RNA from sorted cells for transcriptomic analysis, specifically RNA-seq. RNAseq is a next generation-sequencing based transcriptome survey that allows analysis of expression of the cell populations to occur without prior knowledge of expected genes.

Since RNA extractions of labeled cells have been so far unsuccessful at producing a quantifiable amount of RNA, but some unlabelled cell RNA extractions have yielded low concentrations of RNA, extracting the concentration of RNA needed for RNA-seq from the labeled populations would require scaling up the dissection of wing discs. Mass isolation of discs can give 1000s of isolated wing discs (Fristom et al, 1965) without laborious disc dissections. Additionally, recent automations of mass isolation protocols use density gradient centrifugation, which gives a relatively impure preparation of wing discs with contamination from other discs. This preparation is then followed by large particle flow cytometry to gain only wing discs (Marty et al, 2014).

This would likely require additional fluorescent labeling for wing disc detection during sorting. The Mef2>GFP flies do not label the wing disc myoblasts exclusively, as other myoblast populations in the larva are also labeled. A wing disc specific label such as the Spalt major enhancer driving fluorescence (Salm>eGFP) would allow for isolation of wing discs (Marty et al, 2014) (Schonbauer et al, 2011).

Alternatively, recent advances in RNA-seq mean it is possible to obtain transcriptome measurements from single cells (Wu et al, 2013), and various methods have been reported for single cell RNA-seq experiments (Tang et al, 2010) (Ramskold et al, 2012). Multiple single cell RNA-seq experiments on cells from a population can recapitulate the complexity of the transcriptome of that population, while also defining the heterogeneity of the sequenced tissue. Isolation of single wing disc myoblast cells would be a possible, although costly, solution.

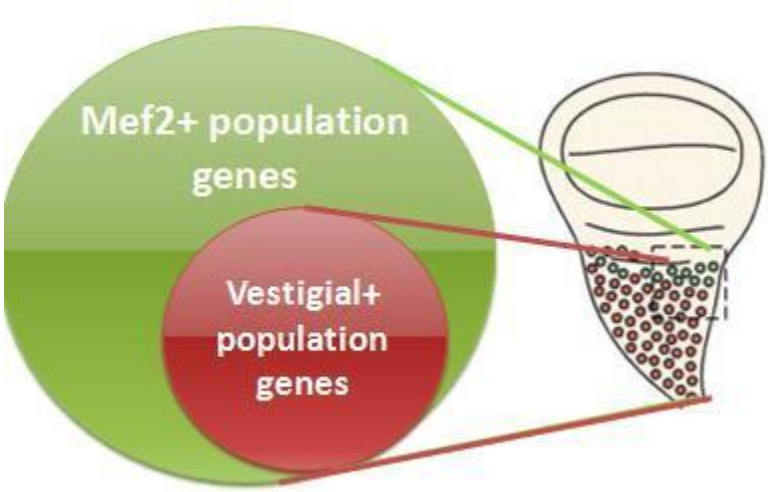

Figure 16 Schematic of expected results from RNA-seq analysis of myoblast populations.

If future RNA extraction and RNA-seq are successful, this will provide the gene expression profiles of the GFP+ Mef2 population; DsRed+ Vg population and both eGFP- and DsRed- populations of non-myoblast cells (Figure 16). Comparisons between these transcriptional profiles would allow for candidate genes with roles in specifying muscle generally or specifically IFMs or DFMs to be investigated. Initial identification of candidate genes is aided by to the large database of annotated *Drosophila* genes, Flybase (Flybase.org, 2015).

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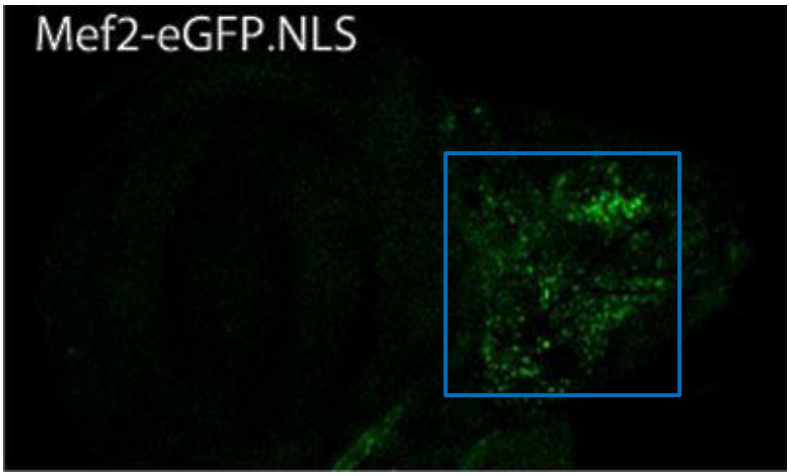
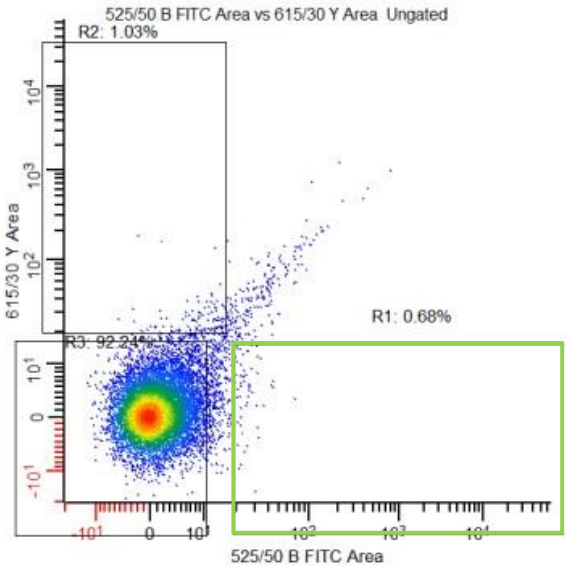
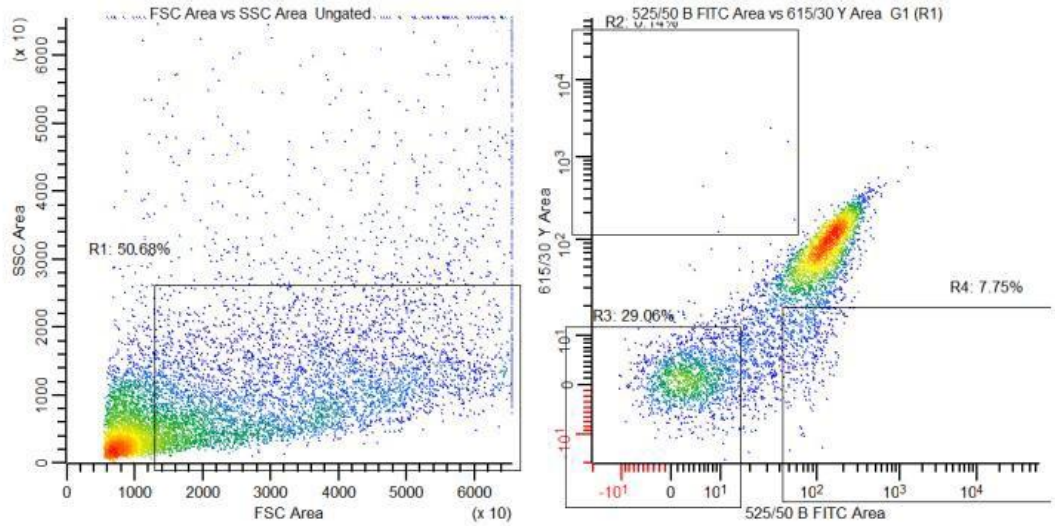
SUPPLEMENTARY FIGURES





Supplementary 1: A) Wing disc from transgenic Mef2 175bp enhancer-eGFP.NLS fly. GFP expression is increased in the myoblast region (outlined in blue), but expression is weak. B) FACS analysis of cells from these discs were unable to distinguish the GFP population from auto-fluorescence (Region R1 at 0.68% of total cells)



Supplementary 2: Whole Larva Vg 899bp- DsRed FACS after homogenization. A) Greater variation in cell size and complexity than in disc only FACS B) Whole larvae have strong 525/50 (green) auto-fluorescence compared to discs, and DsRed+ cells make up less than 0.2% of the total cells.