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Mettl3 is required cell intrinsically in both muscle and neurons for development of functional flight muscle

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Zusammenfassung:

Die Entwicklung von Muskelgewebe ist ein hochkomplexer Prozess. Durch alternatives Spleißen und präzise Regulation der Genexprimierung zeigt dieses Gewebe sowohl zeitlich wie räumlich unterschiedliche Expressionsmuster von Isoformen seiner Gene. Über diese Mechanismen werden die physiologische Entwicklung der Sarkomere sowie ihre fein abgestimmten kontraktilen Eigenschaften realisiert. In Krankheiten wie Muskeldystrophie und Kardiomyopathien lässt sich eine Missregulation in der Exprimierung von Genisoformen beobachten. Daraus wird ersichtlich, warum es von Interesse ist diese Vorgänge zu erforschen und zu verstehen.

N6-Methyladenosin ist eine Modifikation der RNA von der man annimmt, dass sie viele physiologische Funktionen in der RNA Prozessierung übernimmt. Dazu zählen alternatives Spleißen, Stabilisierung der mRNA, Export und Translation. Dennoch bleiben viele Funktionen dieser Modifikationen bis heute unklar.

Kernelemente des zugehörigen m6A-Signalwegs wie Mettl3 und Mettl14 sind evolutionär hoch konserviert. Dieser Umstand erlaubt es potente genetische Werkzeuge, wie sie in Modellorganismen wie *Drosophila Melanogaster* verfügbar sind, einzusetzen. So können die Funktionen dieses evolutionär sehr alten Signalwegs in vivo näher beleuchtet werden.

In der vorliegenden Arbeit werde ich zeigen, dass Mettl3 eine wichtige Rolle bei der Entwicklung der indirekten Flugmuskulatur (IFM) und ihrer zugehörigen Motoneurone spielt.

Ein kompletter Verlust des essenziellen Enzyms Mettl3 in mutierten Fliegen führt zur Flugunfähigkeit und zu herabgesetzter Klettergeschwindigkeit. Konfokale Mikroskopie zeigte veränderte physikalische Dimensionen der IFM wie auch erhöhte Verzweigungsraten der Motoneurone. Mit Hilfe Muskel spezifischer RNAi-knockdowns konnten wir zeigen, dass ein Ausfall von Mettl3 im Muskelgewebe zu Flugunfähigkeit und zu veränderten Abmessungen der Sarkomere führt. Ein Ausfall von Mettl3 in neuronalem Gewebe hingegen offenbarte Defekte in der Verzweigung der Motoneurone.

Die veränderten Abmessungen im Muskel konnten durch die muskelspezifische Exprimierung von Mettl3 mittels Mef2-Gal4 in einem ansonsten globalen Mettl3 Knockdown geheilt werden. Analog dazu ließen sich die Verzweigungsdefekte durch Exprimierung von Mettl3 durch Elav-Gal4 nur in Neuronen beheben.

Aus diesen Erkenntnissen lässt sich eine muskel- und eine neuronenspezifische Aktivität von Mettl3 ableiten.

Mittels mRNA-Sequenzierung und qPCR zeigen wir, dass IFM, denen Mettl3 fehlt, veränderte Expressionsmuster von Genen zeigt, die in Verbindung zu Sarkomeren, Mitochondrien und Synapsen stehen. Im Zuge einer proteomweiten Massenspektrometrie und durch Experimente mit GFP-reportern fielen viele dieser Gene ebenfalls durch veränderte Expression auf.

Interessanterweise offenbarten unsere Experimente weiter signifikante Veränderungen im alternativen Spleißen muskelassoziierter Gene. Hier wurden unter anderem Isoformenwechsel der Titin ähnlichen Gene *bent* (bt, Projectin), *Myofilin* (Mf), *Zasp52* und *Unc-89* beobachtet.

Diese Daten bestätigen eine muskelspezifische Aktivität der m6A-Modifikation. Die Modifikation ist somit beteiligt in der Regulierung des alternativen Spleißens und der Exprimierung von muskelassozierten Genen, die für die physiologische Entwicklung der Muskelfibrillen erforderlich sind.

Abstract (English):

Muscle development is a highly organized and complex process. Through the precise regulation of gene expression and alternative splicing, muscles define temporal and spatial patterns of gene isoform expression that promote sarcomere assembly and fine-tune contractile properties. Misregulation of gene isoform expression is observed in diseases ranging from muscular dystrophies to cardiomyopathies, illustrating the importance of understanding this process. N6-Methyladenosine (m6A) is an RNA modification that is suggested to regulate multiple steps in RNA processing, including alternative splicing, mRNA stability, trafficking and translation efficiency, but many of its cellular functions still remain elusive. Core enzymes in the m6A-pathway such as Mettl3 and Mettl14 are highly conserved, providing the opportunity to utilize powerful genetic tools available in model organisms such as *Drosophila melanogaster* to understand *in vivo* functions of this ancient pathway. In this thesis, I show that Mettl3 plays an important role in the development of both the indirect flight muscles (IFMs) and their corresponding motor neurons.

Complete loss of the essential enzyme Mettl3 in mutant flies results in flightlessness and impaired climbing ability. Confocal imaging revealed altered sarcomere dimensions in IFM, as well as extensive over-branching of motor neurons. Using tissue-specific RNAi knockdown, we found that muscle-specific Mettl3 knockdown flies are flightless and have defects in sarcomere length, while neuronal-specific Mettl3 knockdown flies have defects in motor neuron branching. Sarcomere length defects could be rescued selectively by expression of Mettl3 with Mef2-Gal4 in muscle, while neuronal defects could be rescued selectively by expression of Mettl3 with Elav-Gal4 in neurons. This indicates muscle and neuron-intrinsic Mettl3 function. Using mRNA-Sequencing and qPCR, we show that IFM lacking Mettl3 has changes in gene expression of sarcomere, mitochondrial and synapse-associated genes, many of which are also misexpressed on the protein level as detected by whole-proteome mass spectrometry and GFP-tagged reporters.

Strikingly, our experiments revealed significant changes in muscle specific alternative splicing, including isoform switches in key sarcomere proteins such as the Titin-like gene *bent* (*bt*, Projectin), *Myofilin* (Mf), *Zasp52* and *Unc-89*. This data confirms a muscle-intrinsic function for m6A modification in regulating alternative splicing and protein expression levels during myofibrillogenesis that is necessary for proper growth in sarcomere length and muscle function.

1. Introduction

Muscle is a highly conserved tissue that is found in all animals. Muscle facilitates movement, and although different species develop multiple different types of muscle fibres, the smallest contractile unit of muscle, the sarcomere, can be found in all of them [1]. The main building blocks of sarcomeres are the proteins actin and myosin. Actin filaments, or thin filaments, are arranged in a cylindrical shape around piston shaped myosin filaments (or thick filaments), and densely packed in a hexagonal pattern to fill the available space as effective as possible [2]. Two actin filaments are anchored in opposite directions at the z-disc, while bipolar myosin filaments are anchored at the M-line. Interleaving of the thin and thick filaments at the A-band allows myosin to move along actin towards the z-disc, causing the structure to contract. Sarcomeres are organized end-to-end into long myofibrils, which span the length of the muscle.

The development of muscle tissue is a highly organized process. It starts with the presence of myoblasts, which are muscle precursor cells [1, 3]. During development, these myoblasts will migrate to sites of muscle formation and fuse to form a large syncytial myotube [4]. Myotubes grow and connect to tendon cells, providing the tension necessary for the assembly of myofibrils [5, 6]. Those fibrils can reach lengths up to several centimeters in humans and even around one millimeter in fruit flies [7]. The pre-myofibril structure initially built in growing myofibers is remodelled during subsequent maturation, undergoing addition of more sarcomeres and exchange or addition of components to enable mature contractile dynamics [1]. Myogenesis is therefore characterized by distinct stages with distinct morphological and physiological characteristics that enable the step-wise assembly of fully-functional adult muscle tissue.

Muscle fibers will not develop identically in every part of the organism but rather differentiate into different muscle types with specific morphologies and functional properties. Vertebrate skeletal muscles consist of a mixture of fast and slow twitch fibers.

Fast twitch fibers are easily fatigued, while slow twitch fibers are more fatigue resistant [2, 8]. On a molecular level, these fiber types express different isoforms of myosin, giving a molecular basis for those differences in function.

Fast twitch fibers express the type II version of myosin, together with a gene expression program that influences the muscle towards a more glycolytic and faster metabolism [9]. The slow twitching fibers, on the contrary, express the type I myosin and have a more oxidative and slower, yet more fatigue resistant, metabolism. Interestingly, myofibers exist on a continuous spectrum between “fast” and “slow”, and the muscle can adapt gene expression programs depending on physiologic need to fine-tune performance [10, 11]. Detailed reviews on muscle type identity and gene expression are available [9, 12], so I will not step into further detail here.

Like vertebrates, insects have different types of muscles and muscle fibers. In *Drosophila melanogaster* we can observe major architecture differences between tubular and fibrillar muscles, corresponding to synchronous and asynchronous muscles. Synchronous tubular muscles can be found all over the body of the animal, for example in its legs, abdomen or head. Here the fibers are organized around the nuclei in a peripheral, “tube-like” pattern [2, 13, 14]. Synchronous muscles are controlled by a corresponding nerve, and together they will form a unit [2, 13, 14]. Upon neuronal stimulation, calcium ions will be released. Those ions will then promote the cross-bridge cycle, causing the fibre to contract [2, 8, 9]. The ratio between neuronal input and muscle contraction here is 1:1.

On the other hand, the indirect flight muscles (IFM) that span across the thorax in regular bundles are fibrillar and asynchronous. Each fiber is a syncytial cell and the nuclei are arranged over the whole length of the fiber [6]. Asynchronous muscles, once initially activated by their nerve, can contract independently of neuronal input as they rely on a mechanical stretch-activation mechanism [14, 15].

This alternative mode of operation together with an increased stiffness due to a short titin isoform allows them to oscillate independently from neuronal input and at high frequencies with good power output [16, 17]. The transcription factor Spalt major (*salM*) is responsible for the fibrillar fate of the developing IFM, both through regulation of the fibrillar transcriptional program as well as by alternative splicing of genes and regulating the expression of those gene isoforms [12, 18, 19].

In vertebrates and in insects, distinct muscle fibers, for example heart or skeletal muscle, exhibit differences in mechanical properties like stiffness, in contractile properties including twitching speed and endurance, in general structural

architecture, and also in the neuronal control mechanism [9, 12, 17]. The mechanical differences are achieved by differential expression of muscle core components. Take for example Titin, a large protein with an extensible domain (PEVK-domain) that attaches the thick filaments to the thin filaments and z-disks and serves as the intermediate filament that determines sarcomere length, influences passive resting stiffness, and modulates contractile dynamics [20]. Long isoforms of this protein are found in skeletal muscles, dictating longer sarcomere lengths and lower resting stiffness, while shorter isoforms are found in mammalian cardiomyocytes concurrent with shorter sarcomere lengths and a higher mechanical stiffness compared to skeletal muscles [21]. The pattern of isoform expression therefore underlies the morphological and contractile properties of muscle fiber types.

There are multiple mechanisms cells use to regulate the expression of different gene isoforms. In addition to the regulation of transcription, translation or protein stability, a lot of regulation occurs on the level of RNA. RNA is modified in multiple ways, for example through splicing, capping or editing for particular nucleotides [22]. In particular, alternative splicing allows one gene to code for many different isoforms of associated proteins, dramatically increasing the proteome diversity [23]. In addition, RNA modifications can affect the trafficking, localization, stability and translation rate of mRNA transcripts [24, 25], giving cells the possibility to finely tune their functions and development.

One of these RNA-modification pathways is the m6A pathway. With its two main components Mettl3 and Mettl14, the methyl-transferase complex can attach a methyl group to an adenosine base of a target RNA strand. This particular N6-methyladenosine RNA-modification is one of the most abundant found in mRNA in eukaryotic cells [26, 27]. In particular, this mark is enriched in 5'-UTRs, around stop codons and within long exons, often at the consensus sequence RRACH, where R stands for adenosine or guanine and H for adenosine, cytosine or uracil [27-29]. The m6A mark has been shown to influence fundamental biological processes such as RNA splicing, polyadenylation, nuclear export, stabilisation, translation and decay [22]. It has been shown to impact neuronal tissue development, notably axonal growth and synaptic structure, in *Drosophila* larva [30]. There are further reported functions in the differentiation of C2C12 myoblasts and heart development in vertebrates [31].

The detailed functions of the pathway are still incompletely understood, as only recent methodologies such as methylation-specific immunoprecipitation and next generation sequencing have made it possible to further investigate its functions [24].

The methyl-transferase complex consists of multiple components, including Mettl3 and Mettl14, as well as for example WTAP [Fl(2)d], VIRMA (*Vir*), Zc3H13 (Flacc), RBM15, RBM15b (*Nito*) and HAKAI [29, 32, 33]. Mettl3 is the main enzyme responsible for methylation, or the “writer,” which can catalyse the methylation reaction. Mettl14 is a key scaffolding component that stabilizes the complex, and other components are thought to guide the complex to its target sites [22, 26]. Although protein constituents of the complex can be diverse across the species, Mettl3 and Mettl14 are the core members and are highly conserved [34, 35]. Other proteins in the cell such as FTO and ALKBH5 are capable of removing the methyl mark and thus acting as “erasers”. Comparable enzymes have not been identified yet outside of vertebrates, as FTO and ALKBH5 display poor sequence conservation across evolution [22, 36, 37].

The m6A mark can further be recognized by “reader” proteins, notably those containing the so called YTH-region, which are highly conserved. The presence of the mark increases the binding affinity of YTH-domain proteins 20- to 50-fold [22, 38, 39]. Reader proteins are reported to have distinct cytosolic or nuclear localisation patterns and to fulfil multiple tasks from mediating alternative splicing and RNA editing to directing mRNA transport, stability and translation dynamics [39, 40].

Since both “writer” and “reader” proteins are evolutionarily conserved, important insights into m6A pathway function as well as the discovery of novel pathway components have been reported in model organisms such as *Drosophila*. In this thesis, I explore the influence of the m6A pathway in muscle tissue *in vivo*, using *Drosophila melanogaster* as a model organism. In addition to conservation of the m6A pathway, *Drosophila melanogaster* is a well-established model organism in the field of genetics. Flies are easy to handle and they have a short generation time of 100 hours [41, 42]. They only have 4 chromosomes, which can be precisely manipulated with modern methods, and flies have a well-developed battery

of genetic tools such as the UAS/Gal4 system that permit precise control of spatial and temporal expression. *Drosophila* exhibits strong gene conservation with human genes, including 75% of all disease related genes, and has proven to be a useful model organism to understand conserved disease mechanisms [43]. Notably, muscle structure as well as the contractile mechanism of myosin-mediated contraction are well conserved, and *Drosophila* is also very suitable in the field of muscle studies [44]. *Drosophila melanogaster* is thus a suitable model organism for my project.

Development of the adult musculature, in particular the flight muscles, is well characterized in *Drosophila*. Two types of muscles are used for flight: the direct flight muscles (DFM) and the indirect flight muscles (IFM). The DFM are located in a fan like formation around the wing hinge and control the angle between the wings and the thorax. They are required for steering during flight and correct wing positioning [45]. DFM have a tubular muscle structure and are a synchronous muscle that respond to individual neuronal impulses.

The indirect flight muscles (IFM) consist of six large fibers that are spanning from anterior to posterior through the whole thorax (dorsolongitudinal muscles, DLM) and seven fibers, which span from the ventral to the dorsal side (dorsoventral muscles, DVM) [12]. IFM generate thrust by oscillating the thoracic cuticle, which causes the wings to move up and down. IFM have a distinct fibrillar structure and are asynchronous, meaning that their contractions are dissociated from neuronal impulses [12, 19]. Instead, a stretch-activation mechanism is employed to maintain the rapid, high-power contractions necessary to sustain flight [12, 19]. IFM has patterns of gene expression and alternative splicing that are distinct from those in tubular muscle [12, 19], and serve as a model system to test how regulation of gene expression and RNA processing contribute to muscle differentiation and contractility.

In this thesis project, I first demonstrate that *Mettl3* mutant flies display defects in behavioural properties like flight, climbing and wing positioning. I show that *Mettl3*^{-/-} IFM has a sarcomere phenotype, as well as underlying changes in gene and protein expression and alternative splicing, as assayed by whole-proteome mass spectrometry and mRNA-Seq, respectively. To define the tissue-specific requirement of *Mettl3*, I performed RNAi knockdown with the UAS/Gal4 system

specifically in muscle or neurons. My data show that Mettl3 is required in both tissues, as neuronal-specific RNAi leads to a severe motor neuron axon over-branching defect, while the sarcomere phenotype can be replicated by muscle-specific RNAi. Using RT-PCR, qPCR and GFP-tagged Fosmid reporters, our data demonstrates isoform-switches and misregulation of sarcomere gene expression, providing a possible mechanism to explain the observed structural and functional defects in IFM. I then perform tissue-specific rescue by expressing Mettl3 in a specific target tissue in the Mettl3 mutant background. My data thus demonstrate a novel, *in vivo* requirement for Mettl3 and m6A modification in muscle for the regulation of muscle-type specific sarcomere gene splicing and expression. Further, my results demonstrate that Mettl3 function in both muscle and neurons contribute to the observed behavioural defects.

2. Material and Methods

2.1 *Drosophila* husbandry

For this project I used *D. melanogaster* as a model organism. Fly work was performed with approval in Germany according to §15 GenTSV (license number 55.1-8791-14.1099). Fly stocks were maintained at room temperature using standard culture conditions. Experimental crosses were maintained at 18 °C, room temperature (~22 °C) or 27 °C, as noted. Fly food was prepared in a water-jacketed cooker by combining 16 L water, 150 g soy flour, 1.300 g corn flour, 300 g yeast, 130 g agar, 1.300 g molasses, and 650 g malt extract. After cooling, food was supplemented with 415 mL 10% Nipagin and 295 mL acid mix containing 3% phosphoric acid and 21% propionic acid. Food was aliquoted with a peristaltic pump, allowed to set at room temperature (RT) and stored at 4 °C until use.

The genotypes used in this study were: *w¹¹¹⁸*; as wildtype (Bloomington stock number: 3605), *y,w*; *Ime4^{Δ22-3}/TM3*, *Ser* (gift of M. Soller), ; *Df(3R)Exel6197(III) / Tm6b,Tb* (Bloomington stock number 7676), ; *Mef2-Gal4*; (gift of F. Schnorrer) [46]; *Elav-Gal4/Cyo*; (gift of L. Luo), *Elav-Gal4/Cyo; Mef2-Gal4/Tm6.Tb* (crossed by Shao-Yen Kao), *UAS-Dcr2;;Mef2-Gal4/(TM3,Sb)* (Gift of F. Schnorrer), ; *lf/Cyo; Sb/Tm3, Ser* (gift of F. Schnorrer), *UAS-Dcr2;; Ly/TM3, Sb* (gift of F. Schnorrer), ; *ElavGal4/Cyo ; Ime4^{Δ22-3}/TM3,Ser* (crossed by Maria Spletter), ; *UAS-Ime4/Cyo ; Ime4Df/TM3,Ser*

For the hairpins we used *Ime4 RNAis* (NIG stock number 5339-R, Bloomington stock numbers 80431, 80448, 80450, 41590 and VDRC stock number 20968). We also used *Mettl14 RNAis* (Bloomington stock number 64547 and VDRC stock numbers 48560, 105434, 38349) and *YT521-B RNAis* (Bloomington stock number 34627 and VDRC stock number 330558)

Furthermore we used GFP-tagged Fosmid lines inserted into attP^{VK33} available from VDRC including *Mef2-Gal4, 876 fln::GFP (VK33)*, *Mef2-Gal4, 501 Mf::GFP (VK33)*, *Mef2-Gal4, 519 Mhc::GFP (VK33)*, *Mef2-Gal4, 925 wupA::GFP (VK33)*, *Mef2-Gal4, 78 Act88F::GFP (VK33)*, *Mef2-Gal4, 500 Mhc::GFP (VK33)*, *Mef2-Gal4, 477 sls::GFP (VK33)*, *Mef2-Gal4, 478 Fhos::GFP (VK33)*, *w**; *P(UAS-GFP-FP4mito)119a* and *Mef2-Gal4, 1046 unc-89::GFP (VK33)* (all obtained from F. Schnorrer)

2.2 Behavioural tests

2.2.1 Flight test

Flight was tested with a tube made of acrylic glass. The tube itself was divided into 4 sections and had a little funnel at the very top of it. The flies to be tested were given into that funnel. Normal fliers would be found in the upper 2 sections, medium fliers in the lower 2 sections and finally flightless flies would fall straight to the bottom of the tube. The number of flies per test in each section was then counted [47]. At least 100 flies per genotype were assayed in groups of 20-30 flies, data were aggregated, and final results are reported as the percentage of total flies that were fliers, weak fliers, or flightless.

2.2.2 Climbing test

The climbing ability of the flies was tested using falcon tubes. Those tubes had a circular line in a 5 cm distance from the lid. Around 10 flies from a genotype to be tested were given into such a falcon tube. The tube was then sharply knocked on a hard surface, causing the flies to fall to the lid. Out of instinct, the flies inside start to crawl up the vial's walls immediately. This behaviour was recorded using the Open Camera application or a video recording application on a cell phone. The obtained video was then stopped at 3 seconds and at 5 seconds and the number of flies which made it above the 5 cm line in that time was recorded [46].

This assay was repeated 3 times for each sample group, with around 20 seconds of recovery time in between. Data are reported as the average of these three trials. At least 100 flies were tested in total for each genotype.

2.2.3 Wing angle assay

The wing angle of the flies was observed by observing groups of around 10 flies of a genotype. Normally those assays were made in parallel to the climbing assays. Flies which held out their wings were counted. At least 100 flies were observed, and data is reported as the percentage of total flies with held-out wings.

2.3 Imaging

2.3.1 Sample preparation

The myofibrillar structure and the motor neuron axonal branching pattern was analysed in adult thoraxes cut into halves longitudinally. The dissection was performed as described [5]. Neuronal tissue was analysed in whole adult brains. Tissues were dissected in 1x phosphate buffered saline (PBS), and then transferred to a buffer solution consisting of 1x PBS and 0.5% Triton-X100 (PBS-T) for permeabilization. All probes were fixed for ~25 mins in 4% paraformaldehyde at room temperature on a nutator. This step was followed by two 5-minute washing steps in PBS-T. Probes were stored at 4°C, or immediately stained as described below.

2.3.2 Immunohistochemistry

In this project, we used the following stains and antibodies: Rhodamine-Phalloidin (1:500, ThermoFisher) to stain actin, anti-horseradish peroxidase (HRP) 647 (1:100, ThermoFisher) to staining axon projections, rat anti-Kettin (1:100, MAC155/Klg16, Babraham Institute), guinea-pig anti-Ime4 (Hongay and Orr-Weaver, PNAS, 2011) (1:100, gift of A. Rockwell),

rabbit anti-GFP antibody (1:1000, ab290, Abcam). Secondary antibodies from Invitrogen were used 1:100 and included Alexa 488 goat anti-rat (A11006), Alexa 488 goat anti-rabbit (A11034), Alexa 647 goat anti-guinea pig (A21450), and Alexa 647 donkey anti-rat (A78947).

Samples were blocked with 5% normal goat serum in PBS-T for ~60 mins at room temperature on a nutator. Thoraxes were incubated overnight in antibody on a nutator at 4°C, and then washed 3 times in PBS-T. Thoraxes were incubated overnight at 4°C in secondary antibody and Rhodamine-phalloidin or HRP. Probes stained with HRP 647 were incubated for two nights to increase staining penetration and signal. Samples were washed in PBS-T 2x for 5 mins, 2 x for 10 mins, and 1x for 30 mins. HRP 647 stains were washed and additional 30 mins. Sample were mounted on slides with Vectashield containing DAPI (Biozol).

2.3.3 Confocal microscopy

Stained slides were analysed in the Core Facility Bioimaging at the LMU, Bio-medical Center (Martinsried, DE) using a Leica SP8X WLL upright confocal microscope, equipped with an HCPL FLUOTAR 10×/0.30 objective for 10-fold magnification and a HCPL APO 63×/1.4 OIL CS2 objective for 63-fold magnification. The microscope was running with the Leica LAS X software. The obtained images were viewed and quantified using the ImageJ-software (FiJI). For measurement of the sarcomeres, the Myofibril-J plugin developed by Drs. Giovanni Cardone and Maria Spletter was used. Imaris was used to trace axon projections. Data were tabulated and recorded in Excel, and plotted and analysed in GraphPad Prism.

2.4 Biochemical procedures

2.4.1 Western blot

In this study 8 brains or 12 IFM were dissected from adult flies of the genotype of interest. Samples were homogenised in 20 µl of freshly made SDS-buffer (2% SDS, 240 mM Tris, pH 6.8, 0.005% bromophenol blue, 40% glycerol, and 5% β-mercaptoethanol), incubated at 95°C for 3 min and stored at -20°C. Samples were run on 10% SDS-PAGE for separation and then transferred onto nitrocellulose membranes (Amersham Protran 0.2 µm NC) for 1 h at 120 V. Membranes were stained with Ponceau S (Sigma-Aldrich) to access the quality of the blotting. Membranes were de-stained and blocked with 5% non-fat milk solution in 0.5% Tween-TBS buffer (T-TBS) for 1 h and incubated for 1 h at room temperature with primary antibodies (guinea pig anti-lme4, 1:1000; rabbit anti-H2AZ, 1:2,000; mouse anti-GFP, 1:1000). Membranes were washed three times with T-TBS for 15 min and incubated with goat anti-guinea pig HRP-conjugated secondary antibodies (Bio-Rad, 1:10000) for 1 h at room temperature. After three rounds of washes, the membranes were developed using Immobilon Western chemiluminescent (Milipore) substrate and exposed to X-ray films (Fuji medical X-ray, Super RX-N) or imaged on a ChemiDoc MP (Bio-Rad).

2.4.2 Dot blot

For the dot blot experiments in this study brains or IFMs from 8 wildtype or mutant flies were dissected. The obtained samples were homogenized in 10x Tri-Star/Trizol. 20% of the total solution volume of chloroform was added. The solution was then shaken for 15 seconds and kept at room temperature for 10 minutes. This step was followed by centrifuging the solution for 5 min to separate the phases. The upper aqueous phase, containing the RNA, was transferred to a new tube and 1-2 µl glycogen carrier was added.

For precipitation, isopropanol was added at a ratio of 1:1, and the new solution was kept again for 10 mins at room temperature. After that, the solution was centrifuged at 4° C for 10 mins. Centrifugation was followed by washing the RNA precipitate with 70% ethanol 2x and centrifuging at 4° C for 10 minutes. Once finished, the supernatant was removed and the RNA pellet was air dried. The pellet itself was subsequently dissolved in DEPC treated water, including incubation at 50°C if necessary to help dissolve the pellet. To remove genomic DNA, ~1-25 µg of RNA samples were treated with 1 µl of DNase and 10 µl of NEB-DNase buffer, diluted with 100 µl of water. This solution was kept for 10 minutes at 37° C. For heat inactivation, 5 mM EDTA were added to the solution and samples were kept for an additional 10 minutes at 75° C. Samples were then stored until further use at -80° C.

For dot blotting samples of 500 ng, 750 ng and 1000 ng were given into 5-10 µl RNase free water. Those samples were then denatured for 10 minutes at 65° C and subsequently put on ice for 5 minutes. In the next step, an H-bond N+ Amer-sham membrane and a Whatman paper were wetted in 10 mM NaOH, 1 mM EDTA solution. The wetted Whatman paper was put on top of a dry piece of Whatman paper, and the wetted membrane was placed on top of the wetted paper. The prepared RNA samples were then spotted on the membrane and allowed to sink in. The membrane was crosslinked in a UV crosslinker at 120 mJ/cm² for ~ 1 min. The membrane was blocked in blocking solution for 1 hour at room temperature. The blocking solution consisted of 1x PBS, 0.02% Tween-20 and 5% non-fat-milk. The samples were then incubated with a rabbit anti-m6A antibody (Abcam) at 1:5000 dilution in the blocking solution at 4° C overnight. After incubation the samples were washed 3-6 times in a washing buffer consisting of 1xPBS and 0.02% Tween-20. The washing procedure was followed by a second incubation with an HRP conjugated goat anti rabbit antibody at 1:10000 dilution for 1 hour. After three rounds of washes as described above, the membranes were developed using Immobilon Western chemiluminescent (Milipore) substrate and exposed to X-ray films (Fuji medical X-ray, Super RX-N) or imaged on a ChemiDoc MP (Bio-Rad).

2.4.3 qPCR

For qPCR experiments, 200 IFM of adult flies were dissected as described [48]. Genotypes here were wildtype *w¹¹¹⁸* as control, mutant *Ime4^{Δ22-3}/Df(3R)Exel6197(III)* and *Mef2 Gal4 x NIG 5339-R* knockdown flies. RNA extraction was done as described in the dot blot method in 2.4.2. cDNA from those samples was prepared following the protocol of Luna Script RT Super Mix kit from New England Biolabs. Forward and reverse primers were designed to span introns and were ordered from MetaBion. Primers were diluted in water at 60°C to a concentration of 100 μmol.

For qPCR analysis, 1.5 μl primer solution, 6 μl cDNA and 7.5 μl SybrGreen were pipetted together. All wells to be analysed were then sealed with light-permissive plastic foil. The PCR plates were briefly centrifuged and remaining bubbles were removed by tapping. The prepared plates were finally placed in the QuantStudio3 qPCR machine, made by ThermoFisher. The machine was then set to its fast 7500 SybrGreen method and run on this programme. Obtained data was then transferred to Excel.

2.4.4 RT-PCR

For RT-PCR experiments, 200 IFM of adult flies were dissected as described [48]. Genotypes here were wildtype *w¹¹¹⁸* as control, mutant *Ime4^{Δ22-3}/Df(3R)Exel6197(III)* and *Mef2 Gal4 x NIG 5339-R* knockdown flies. RNA was extracted with Trizol as described in 2.4.2. cDNA was prepared with the Luna Script RT Super Mix kit (New England Biolabs). For the PCR reaction, 0.8 μl Taq-polymerase, 2.5 μl forward and 2.5 μl reverse primer, 1 μl cDNA and 1 μl dNTP were diluted in 40 μl water. 10x Taq buffer was added to a concentration of 5 μmol. Samples were amplified on a c1000 touch PCR-machine (BioRad). The machine was set for 34 cycles at 58°C and 1 minute annealing time. The amplified products were then loaded a 1.5% agarose gel containing 0.2 % ethidium bromide at a ratio of 1:750. A 1 kb+ marker (Invitrogen) was finally added to the gel.

The prepared gel was transferred to an electrophoresis basin and wired to 100V for 35 minutes. After this time, the gel was transferred to GelDoc quantum (Vilber) for documentation.

2.4.5 Proteomics (Mass Spectrometry)

200 IFMs from each control *w¹¹¹⁸*, mutant *Ime4^{Δ22-3}/Df(3R) Exel6197(III)*, and rescue *Mef2-Gal4/UAS-Ime4; Ime4^{Δ22-3}/Df(3R) Exel6197(III)* flies were dissected as described above in 2.4.4, and then snap frozen on dry ice after removing buffer. IFMs were harvested from groups of 10 flies, and then after batch thawing on ice, IFMs from 50 flies were pooled per biological replicate, with in total 4 replicates per genotype. Samples were processed using the PreOmics iST Sample Preparation Kit (Preomics, #0000.0061) following the manufacturer's instructions. Prepared peptides were submitted to the Protein Analysis Unit (ZfP) at the LMU Biomedical Center. Desalted peptides were injected in an Ultimate 3000 RSLC-nano system (Thermo) and separated in a 25-cm analytical column (75μm ID, 1.6μm C18, IonOpticks) with a 100-minute gradient from 5 to 60% acetonitrile in 0.1% formic acid. The effluent from the HPLC was directly electro sprayed into an LTQ Orbitrap XL (Thermo). Survey full scan MS spectra (from m/z 375–1600) were acquired with resolution R=60.000 at m/z 400 (AGC target of 3×10^6). The 10 most intense peptide ions with charge states between 2 and 5 were sequentially isolated to a target value of 1×10^5 , and fragmented at 27% normalized collision energy. Typical mass spectrometric conditions were: spray voltage, 1.5 kV; no sheath and auxiliary gas flow; heated capillary temperature, 250 °C; ion selection threshold, 33.000 counts. MaxQuant 1.6.14.0 (Cox et al., 2014)(159) was used to identify proteins and quantify by LFQ with the following parameters: Database, Uniprot_AUP000000803_Dmelanogaster_Isoforms_20210325.fasta; MS tol, 10 ppm; MS/MS tol, 20 ppm; Peptide FDR, 0.1; Protein FDR, 0.01 Min. peptide Length, 5; Variable modifications, Oxidation (M); Fixed modifications, Carbamidomethyl (C); Peptides for protein quantitation, razor and unique; Min. peptides, 1; Min. ratio count, 2. Additional analysis was performed in Perseus (160) (Tyanova and Cox, 2018).

To retain biologically relevant protein groups with “true negative” missing intensities between mutant and control samples (i.e., missing not at random, MNAR), we filtered the data by requiring at least three replicates in either group to contain a value, and we imputed missing values by replacement with a constant value (lowest observed intensity – 1). Differential expression was tested by t-test with FDR = 0.05. Results were exported and further analysis performed in R.

2.4.6 Transcriptomics (mRNA-Seq).

2 replicates of IFMs from 100 flies per genotype were dissected as previously described [48]. Genotypes included control *w¹¹¹⁸*, mutant *Ime4^{Δ22-3}/ Df(3R) Exel6197(III)*, and rescue *Mef2-Gal4/UAS-Ime4, Ime4^{Δ22-3}/ Df(3R) Exel6197(III)*. RNA was isolated with Trizol. Poly(A)+mRNA was purified using Dynabeads (#610.06, Invitrogen) and its integrity was subsequently verified on a Bioanalyzer (Agilent Technologies). The sequencing library was created with the library prep kit for Illumina (New England Biolabs) following the manufacturer protocol. Size selection was performed with SPRI select beads. For amplification of the obtained c-DNA samples, the oligonucleotides from Primerset 4 (NEB) of the kit were used. Amplification was then done by 7 PCR cycles. The quality of the library was verified once more with a Bioanalyzer (Agilent Technologies). Finally, sequencing was performed on an Illumina NextSeq at the LaFuga core facility of the LMU. Samples were sequenced as stranded, 150 bp paired-end to a depth of about 70 million reads.

Sequence data was mapped with STAR to ENSEMBL genome assembly BDGP6.22 (annotation dmel_r6.32 (FB2020_01)). Files were indexed with SAMtools and processed through featureCounts. Downstream analysis and visualization were performed in R using packages including DESeq2, DEXSeq, ggplot2, plyr, ComplexHeatmap, and viridis. Differential gene expression was analyzed with DESeq2, and differential exon use was determined with DEXSeq. Differentially expressed exons reflect both alternative splicing and alternative promoter use. Both packages were additionally used to generate normalized counts values. We employed previously annotated sets of genes, including sarcomere proteins (42) and all genes with the Flybase GO terms “muscle contraction,” “actin cytoskeleton,” “neuromuscular junction”, or “synapse”.

Plots were generated using ggplot2 or ComplexHeatmap. Unless otherwise stated, we used an adjusted p-value threshold of 0.05 for significance. If a $\log_2$ FoldChange threshold was used in a specific analysis, it is specified in the legend for the relevant figure panel.

2.5 Data processing

Obtained data was tabulated using Microsoft Excel. Sample numbers are stated, and were at least 10 individuals in all experiments. Statistical analysis and tests were performed with the GraphPad Prism software. For two-sample comparisons we used t-tests, and for experiments with three or more samples we used ANOVA with post-hoc Tukey tests of significance comparing all sample means. Images were initially processed in ImageJ (Fiji) as .tif files, and exported as .jpg for figures. Axonal tracing was done with Imaris. Figures and tables were created with Affinity Designer and Affinity Photo software.

3. Results

3.1 Behavioral assays

3.1.1 Flies lacking the *Mettl3* methylase are flightless and exhibit motor deficits

In a candidate screen, we observed that muscle-specific RNAi against *Mettl3* or *Mettl14* driven by *Mef2-Gal4* results in flightless flies [49], indicating that the m6A pathway may play an intrinsic role in muscle. With the aim of testing what role *Mettl3* plays in *Drosophila* muscle, we generated trans-heterozygous mutants of *Mettl3*^{Δ22-3} crossed to *Df(3R) Exel6197*, which spans the *Mettl3* locus. *Mettl3*^{Δ22-3} is a P-excision allele that deletes most of the coding region, including the catalytic domain [26]. With the obtained transheterozygous mutant flies, we then performed a panel of behavioural tests. We found that the mutant flies were almost completely flightless compared to wildtype (Fig.1 A). Also, we saw, that the mutant flies exhibited strong defects in their ability to climb. Only around 15% of mutant flies were able to climb 5 cm in 3 seconds, whereas around 80% of wildtype flies could climb this distance (Fig.1 B). Finally, we observed held out wings in around 40% of the assayed mutant flies, but we could not see any matching phenotype within the wildtype control group (Fig.1 C). These data are consistent with previously reported *Mettl3* phenotypes [22, 40], and demonstrate defects in the function of IFM, DFM, and leg muscles in flies lacking *Mettl3*.

3.1.2 *Mettl3* is expressed and functionally active in both brain and IFM

Previous studies have focused on the role of *Mettl3* in the fly nervous system and embryonic development [22, 26, 40, 50]. To demonstrate that *Mettl3* is also expressed in IFM, we performed immunostaining and Western blot. Using immunofluorescence and confocal imaging, we observed *Mettl3* expression in adult IFMs (Fig. 1 D-1 – D-3) that was absent in *Mettl3*^{Δ22-3} / *Df(3R) Exel6197* (Fig. 1 E-1 – E-3).

Interestingly, this Mettl3 signal was both nuclear and cytosolic. Mettl3 was also clearly detected by Western blot in both in dissected brain and IFM tissue from *w¹¹¹⁸* control flies, and this band was lost in *Mettl3^{Δ22-3} / Df(3R) Exel6197* tissues (Fig. 2 C). All together these experiments indicate that Mettl3 is expressed in both neurons and IFM, and also demonstrate that our mutant genotype is effective.

Mettl3 is the main methylase of the m6A pathway [40]. To test if it is functionally active in both muscle and neurons, we performed dot blots with an antibody that recognizes N6-methyladenosine. We could detect m6A marks on total RNA in both brain and IFM dissected from *w¹¹¹⁸* control flies (Fig. 2 A). *Mettl3^{Δ22-3} / Df(3R) Exel6197* flies are missing the main methylase Mettl3 and thus are expected to have a significant decrease of the m6A-mark in their RNA. Indeed, in neuronal as well as in muscle tissue we observed a significant decrease of m6A marks compared to the wildtype control flies (Fig. 2 A, B). This demonstrates that m6A methylation of RNA occurs in both brain, and presumably motor neurons, as well as in IFM.

3.1.3 Mutant sarcomeres are longer than in wildtype

To further understand why Mettl3 mutant flies are flightless, we analysed the myofiber and sarcomere structure of the mutant. IFM myofibers at 10x magnification looked normal spanning from anterior to posterior of the thorax in six bundles, and we saw no difference between the wildtype control and the mutant (Fig. 1 G-1 and G-2). However, at 63x magnification we observed mild defects and the mutant sarcomeres were different from the wildtype ones. Here we saw significantly longer sarcomeres of ~3.7 μm in *Mettl3^{Δ22-3} / Df(3R) Exel6197* flies compared to ~3.4 μm in wildtype *w¹¹¹⁸* (Fig. 1 I-2, I-3, J-2, J-3, F). However, Kettin was still correctly localized to the z-disc and z-disc structure was intact (Fig. 1 I-3, J-3). Myofibril width was also unaffected (Fig. 1 H). Sarcomere length in IFM is tightly regulated, and the observed increase in length would likely disrupt IFM contractility.

Figure 1. Mettl3 is expressed and regulates sarcomere length in *Drosophila* IFM.

(A-C) Plots quantifying behavioural defects in Mettl3 mutant flies in their ability to fly (A) (flightless, dark blue; weak fliers, light blue; normal flight ability, white), their ability to climb (B) (control, white; *Mettl3*^{Δ22-3}/*Df* mutant, blue) and their tendency to hold out their wings (C) (normal wing position, white; held-out wing position, blue). For those Experiments over 100 male flies were tested each on a time course of several days. Mutant flies were obtained from several independent crosses.

(D-E) Single-plane confocal images of wildtype (D1-3) and *Mettl3*^{Δ22-3}/*Df* mutant (E1-3) IFMs stained with antibodies against Mettl3. Mettl3 is detected both in the nucleus and cytoplasm and is not detected in the trans-heterozygous mutant. Scale bar = 5 μm. DAPI, cyan; Mettl3/lme4, magenta. For those experiments 7 thoraxes were imaged each.

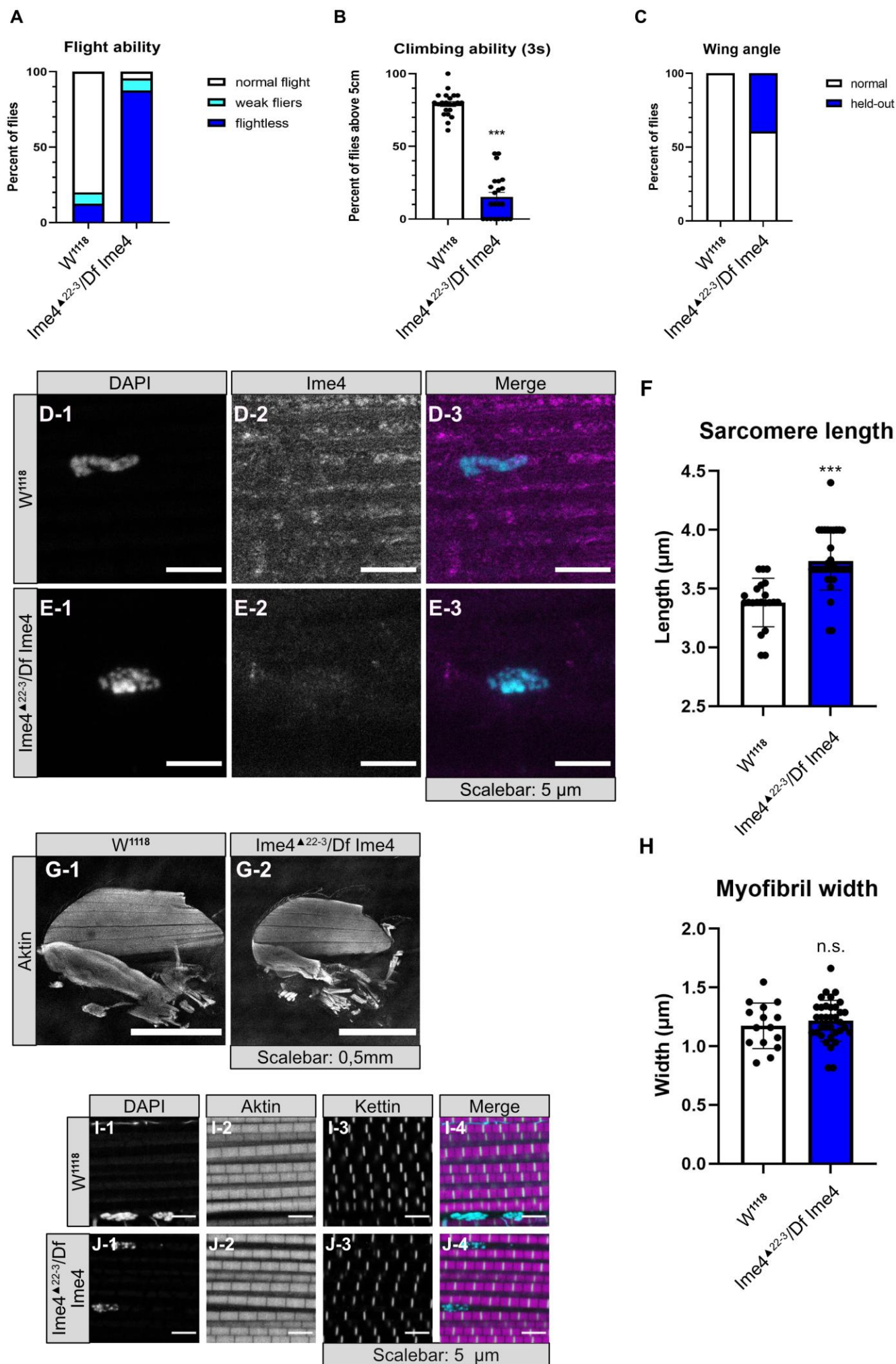
(F) Quantification of sarcomere length of the data shown in I-1 to J-4. Significance determined by t-test, n.s. = not significant; ***, p-value < 0.001.

(G) Confocal z-projections of wildtype and mutant IFM myofibers at 10x magnification. Myofibers are intact and grossly normal. Scalebar = 500 μm. phalloidin stained actin, grey. 5 thoraxes were imaged each.

(H) Quantification of myofibril width of the data shown in I-1 to J-4. Significance determined by t-test, n.s. = not significant; ***, p-value < 0.001.

(I-J) Single-plane confocal images of wildtype control *w*¹¹¹⁸ (H1-4) and *Mettl3*^{Δ22-3}/*Df* mutant (I1-4) IFM myofibrils. Although the sarcomere structure is intact, mutant sarcomeres are too long. Scalebar = 5 μm. DAPI stained nuclei, cyan; phalloidin labelled actin, magenta; Kettin-labelled z-discs, yellow. 5 thoraxes were imaged each.

Figure 1



3.1.4 Tissue-selective knockdown of *Mettl3* using RNAi in muscle or neurons

Previous studies have suggested that flight defects in *Mettl3* mutant flies can be attributed to neuronal deficits [50]. However, our data revealed a structural defect in IFM sarcomeres, and we hypothesized this may be due to muscle-intrinsic *Mettl3* function. To test if this is due to *Mettl3* function in muscle or neurons, we used tissue-specific RNA interference (RNAi) mediated by the UAS-Gal4 System. We selected *Mef2*-Gal4, which is expressed selectively in muscle tissue [51], and *Elav*-Gal4, which is expressed specifically in neurons, including motor neurons [52]. To find the most effective RNAi lines, we screened all available lines targeting *Mettl3* itself, its stabilising protein *Mettl14*, and the YTH-reader proteins. We selected the hairpin that generated the strongest and most consistent flightless phenotype for further analysis (Fig. 3). This hairpin was obtained from the NIG-fly stock center in Japan and had the stock number 5339-R, and will be referred to as *Mettl3*-IR^{NIG-5339}.

We performed a Western blot for *Mettl3* to check if the UAS-Gal4 system would in fact knock down tissue specifically. In the Western blot, we could detect *Mettl3* in wildtype *w¹¹¹⁸* brain and IFM, as well as in control samples when the drivers alone were crossed to the wildtype (Fig. 2 C). By contrast, we saw significant decreases in *Mettl3* protein expression as compared to the control when *Mettl3*-IR^{NIG-5339} was crossed to either *Elav*-Gal4 or *Mef2*-Gal4. We saw the same effect with *Mettl3*-IR^{BL80431} crossed *Mef2* Gal4. Importantly, with *Mef2*-Gal4 crosses we lost the *Mettl3* band in muscle tissue but not in brain, while with *Elav*-Gal4 crosses we lost the *Mettl3* band in brain (Fig. 2 C). This indicates that we can effectively knockdown *Mettl3* using RNAi in a tissue-specific manner.

Furthermore, we investigated the specificity of the NIG-5339 hairpin itself. To do this, we rescued the phenotype caused by this hairpin by overexpression of *Mettl3* using a UAS-*Mettl3* [53]. The experimental flies had the NIG-5339 hairpin driven in muscle by *Mef2*-Gal4, but we added back another UAS-cassette containing a functional copy of *Mettl3*. Approximately 20% of *Mettl3*-IR^{NIG-5339} die in the pupal case, and while lethality was significantly reduced to only 10% of *Mettl3*-IR^{NIG-5339}, *UAS-Mettl3* pupa (Fig. 4 I).

We saw a similar effect with jumping ability, where *Mettl3-IR^{NIG-5339}* flies jump significantly shorter distances than control *Mef2-Gal4* x *w¹¹¹⁸* flies. The jumping ability significantly increased once *Ime4* was overexpressed in *Mettl3-IR^{NIG-5339}*, *UAS-Mettl3* as compared to the muscle knockdown (Fig. 4 J). We were unable to rescue the flight phenotype, potentially indicating differences in *Mettl3* function in fibrillar IFM required for flight versus tubular leg and jump muscles required for jumping and eclosion. Taken together these experiments indicate that our *Mettl3* knockdown was specific.

Figure 2. Validation of mutant, RNAi, and overexpression tools reveals that *Mettl3* and the m6A mark are present in both brains and IFM.

(A) Dot-blot image of both brains and IFM dissected from control *w¹¹¹⁸* and *Mettl3^{Δ22-3}/Df* mutant flies. Anti-m6A antibodies were used to detect the mark in increasing amounts of total RNA ranging from 0.5 μg to 2 μg. The mutant rows show decreased signal intensities as compared to the control. For this experiment 8 IFMs of wildtype and mutant were analysed for 4 different concentrations. This experiment was carried out on 3 times on different days.

(B) Semi-quantitative analysis of the fold-change in dot blot intensities from (A). Data from three independent experiments were combined in the plot. *w¹¹¹⁸* (white) and *Mettl3^{Δ22-3}/Df* (blue), significance determined by t-test, n.s. = not significant; ***, p-value < 0.001.

(C) Western blots, stained with antibodies against *Ime4/Mettl3* with anti-H2AZ used as a loading control. *Ime4/Mettl3* protein is not detected in brain or IFM dissected from *Mettl3^{Δ22-3}/Df* mutant flies, but can be detected in both IFM and brain dissected from control *w¹¹¹⁸*. *Ime4* can be knocked down tissue specifically using RNAi hairpins targeting *Ime4* and a tissue specific Gal4 driver. *Mef2-Gal4* is expressed in muscle, while *Elav-Gal4* is expressed in neurons. NIG hairpin 5399-R and Bloomington hairpin BI8043 both target *Mettl3*. This data was obtained from 8 brains and 12 IFMs both of wildtype and mutant. This experiment was carried out on 4 times on different days.

(D) Western blot stained as in (C). *Mettl3* can be rescued tissue-specifically by expressing in in a target tissue (D). In *Mef2-Gal4* rescues, *Mettl3* is detected in IFMs but only weakly in brain. In *Elav-Gal4* rescues, *Mettl3* is detected in dissected brains but only weakly in IFMs. H2AZ was used as a loading control. This data was obtained from 8 brains and 12 IFMs both of wildtype and mutant. This experiment was carried out on 3 times on different days.

(E-F) Semi-quantitative analysis of the western blots for the tissue specific knockdown (C) and the rescue (D). Quantification shows strongly reduced expression in control tissues from *Mettl3^{Δ22-3}/Df* (blue), and tissue-specificity of knockdown (E) or rescue (F) with *Mef2-Gal4* (red) or *Elav-Gal4*

Figure 2

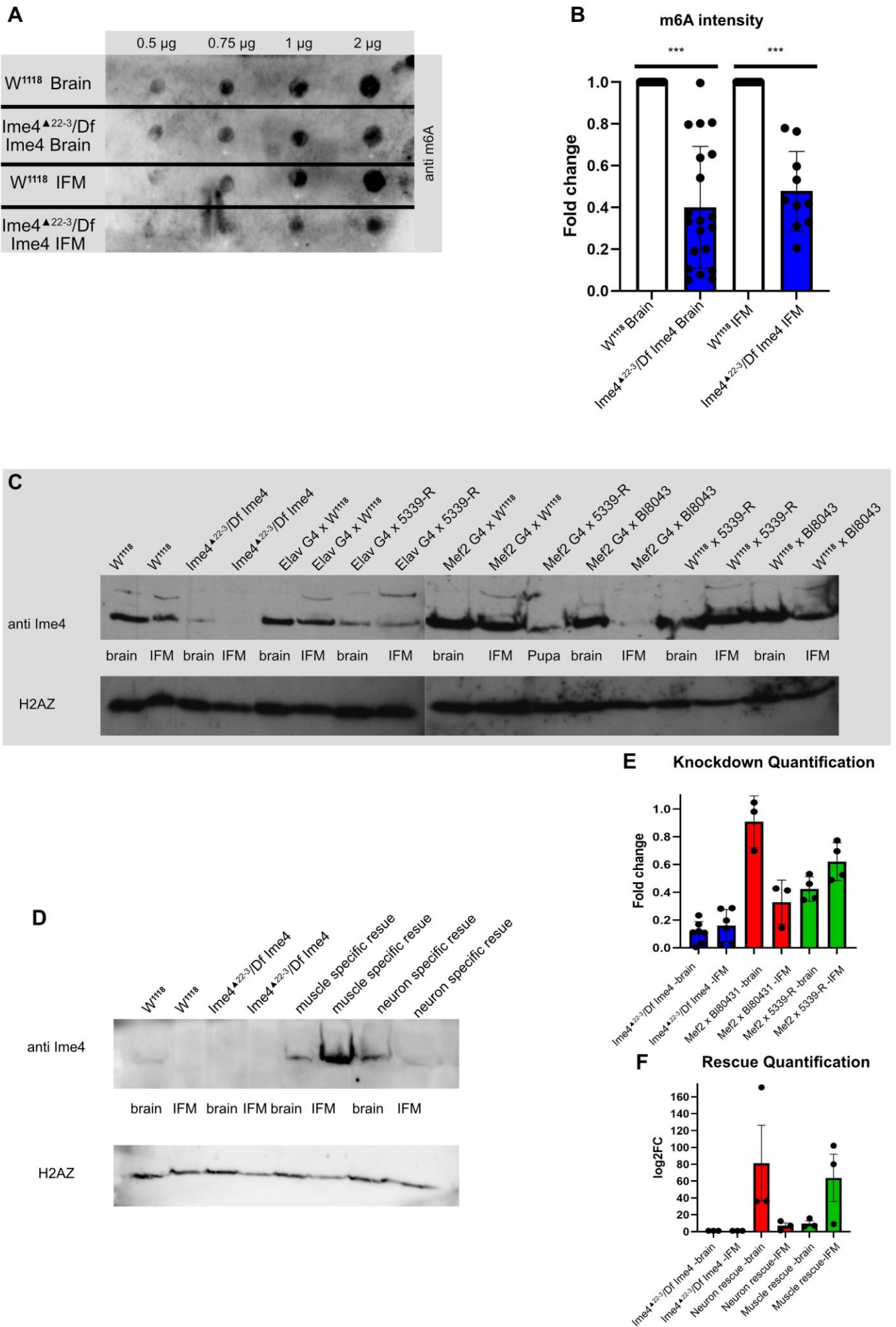


Figure 3. Screen identifies RNAi hairpins targeting Mettl3 and Mettl14 that both cause flightless phenotypes.

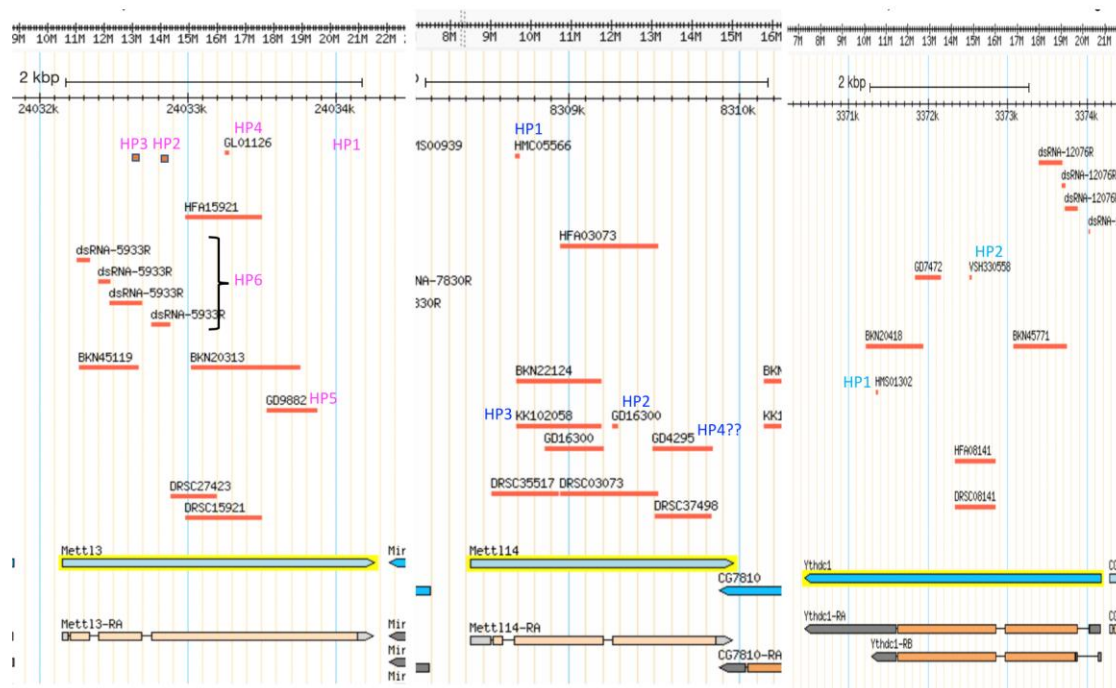
(A) Flybase screenshots showing the sequences targeted by all hairpins used to knockdown three genes of interest in the Mettl3 pathway used in this experiment (Mettl3, Mettl14 and Ythdc1). Mettl3 has m6A enzymatic activity, Mettl14 is an essential co-factor in the writer complex, and Ythdc1 is an m6A reader protein. The position of the hairpins relative to the target genes is marked.

(B) Plot showing the flight ability of RNAi knockdown flies with different RNAi hairpins. Hairpin 6 (HP6, NIG 9533-R) was identified as the strongest RNAi hairpin targeting *Mettl3*, and is used in subsequent experiments. For Mettl14, we identified V105434 from the Vienna stock center as the strongest hairpin resulting in lethality, while V48560 produced a strong flightless phenotype. Hairpins against Ythdc1 were either not effective, or Ythdc1 is not required in IFM, as we did not observe a strong flight phenotype. Together, independent hairpins targeting multiple components of the Mettl3 pathway identify a muscle-specific function of Mettl3 required for normal flight ability. N = total number of flies tested.

Figure 3

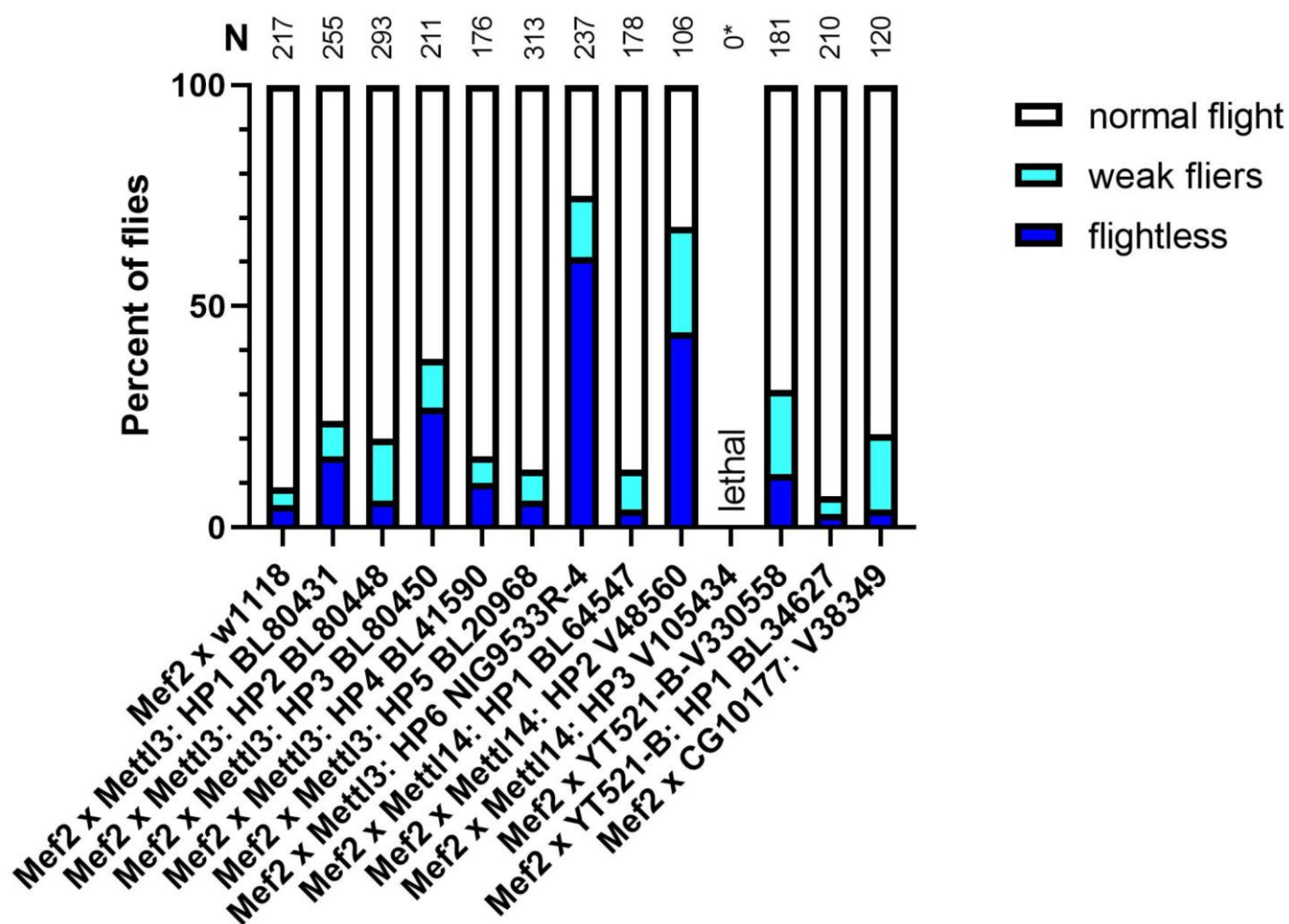
A

Hairpintargets



B

Mef2-Gal4 (all hairpins)



3.1.5 Muscle-specific *Mettl3* knockdown results in flightlessness

Having validated our RNAi reagents and tissue-specific drivers, we performed a panel of behavioural tests to determine which behaviours are dependent on *Mettl3* function in muscle versus neurons. We saw a strong flightless phenotype in the muscle specific knockdown when the NIG-5339 hairpin was crossed to the *Mef2-Gal4* driver. Around 60% of the tested flies were flightless and another ~15% were weak fliers (Fig. 4A). However, when we crossed the hairpin to the *Elav-Gal4* driver combined with *UAS-Dicer2* to enhance the neuronal specific *Mettl3* knockdown, we only saw 20% flightless flies and around 50% weak fliers (Fig. 4 A). This indicates that in IFM both neurons and muscle contribute to the flightless phenotype, with the *Mettl3* activity in the muscle itself playing a large role in the flight defect.

We next examined climbing ability. In the muscle-specific *Mettl3* knockdown with *Mef2-Gal4*, we did not see a distinguishable phenotype: their climbing ability was not significantly different from the control (Fig. 4B). By comparison, in the neuronal specific knockdown we saw a strong climbing phenotype that was comparable in severity to that of *Mettl3^{Δ22-3} / Df(3R) Exel6197* (Fig. 4 B). We interpret this to mean that neuronal deficits, rather than muscle deficits, are responsible for the climbing phenotype. Held-out wings were observed with both muscle and neuronal-specific knockdown, but *Mettl3^{Δ22-3} / Df(3R) Exel6197* animals had a much strong held-out wing phenotype (Fig. 4 C).

3.1.6 Sarcomeres in muscle-specific knockdown IFM show defects

We next examined sarcomere structure in *Mef2-Gal4* driven *Mettl3-IR^{NIG-5339}* flies. We expected that if *Mettl3* plays an intrinsic role in IFM, we should observe a sarcomere defect like we observed in *Mettl3^{Δ22-3} / Df(3R) Exel6197* flies (Fig. 1 I-1 – J-4). In the muscle knockdown, we observed changes in sarcomere structure. Although myofibrils with regular sarcomeres formed, the length of the sarcomeres was variable as compared to the control (Fig. 4 D-1 – F-4, H). Here the sarcomeres were not uniformly longer, but instead we also saw sarcomeres which were even shorter or longer than wildtype.

Short sarcomeres may indicate hypercontraction [46], and the efficiency of knock-down in different flies could result in the observed variability in sarcomere length. We also saw overgrown Z-disk areas, known as zebra bodies, distributed irregularly in the IFMs in the muscle-specific knockdown (Fig. 4 G-1 – G4), which is indicative of hypercontraction or z-disc instability. We conclude that sarcomere defects observed in IFMs of *Mettl3*^{Δ22-3} / *Df(3R) Exel6197* and *Mef2-Gal4* > *Mettl3-IR*^{NIG-5339} are the result of muscle-intrinsic function of *Mettl3*.

Figure 4. Tissue specific RNAi knockdown supports a requirement for *Mettl3* in both muscles and neurons.

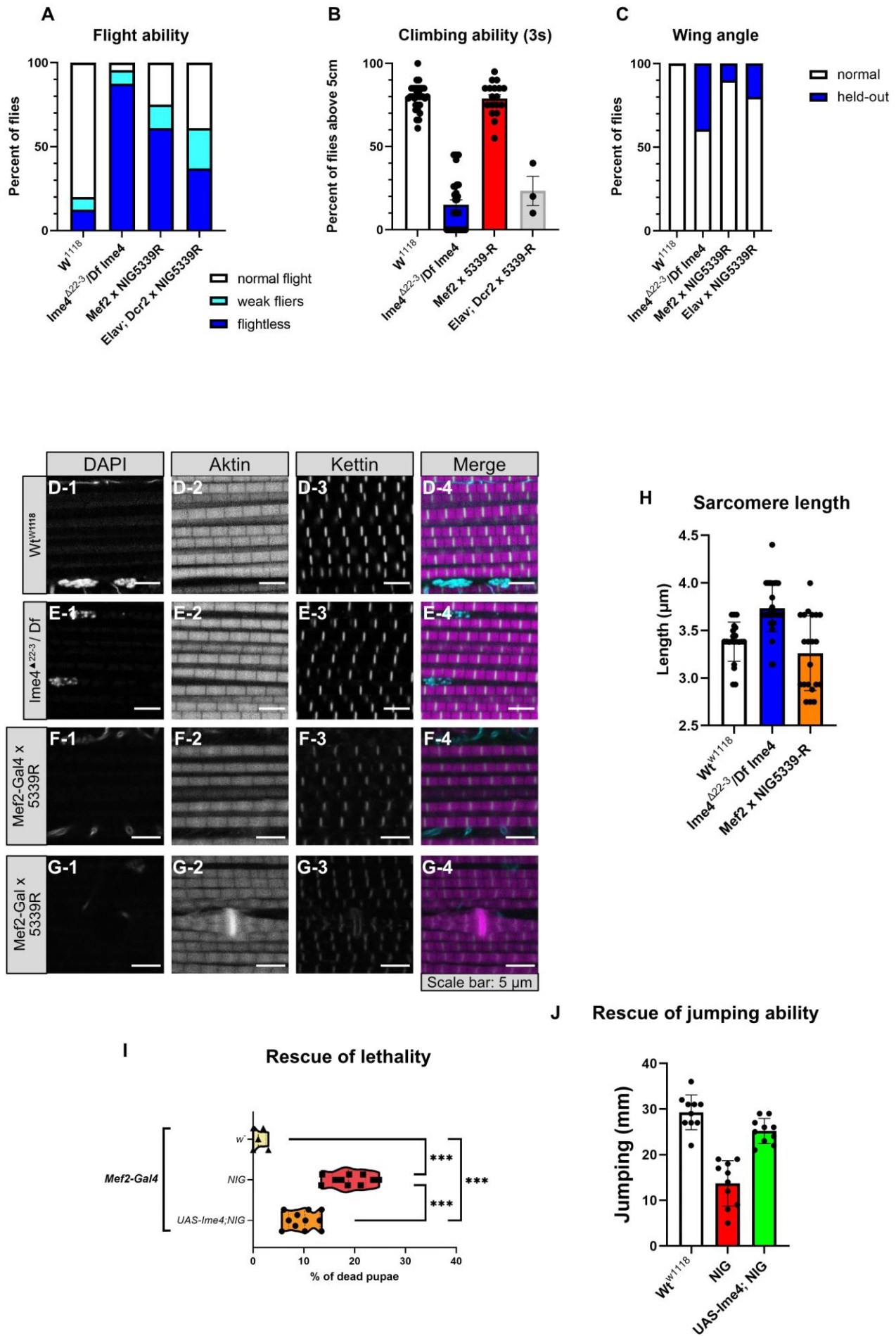
(A-C) Plots quantifying behavioural defects in *Mettl3* mutant flies and *Mettl3* tissue-specific RNAi knockdown flies in their ability to fly (A), their ability to climb (B) and their tendency to hold out their wings (C). *Mef2-Gal4* driving in muscle results in impaired ability to fly, while *Elav-Gal4* driving in neurons impairs both flight and climbing. *Dcr2* enhances knockdown efficiency. Flight ability was assayed from over 100 male flies each from several crosses. Climbing ability and wing angle were assayed from 60 male flies each, also from several crosses.

(D-G) Single-plane confocal images of wildtype control *w¹¹¹⁸* (D1-4), *Mettl3*^{Δ22-3}/*Df* mutant (E1-4) and *Mef2-Gal4* x 5339R knockdown (F1-4, G1-4) IFM myofibrils. The sarcomere structure is intact but mutant and knockdown sarcomeres show defects in sarcomere length. The formation of zebra bodies (actin inclusions at the z-disc) (G1-4) is also prevalent in muscle specific knockdowns. Scalebar = 5 μm. DAPI stained nuclei, cyan; phalloidin labelled actin, magenta; Kettin-labelled z-discs, yellow. 5 thoraxes were imaged each.

(H) Quantification of sarcomere length of the data shown in D-1 to G-4.

(I-J) Plots quantifying the percentage of dead pupae (I) and the jumping ability (J) in *w¹¹¹⁸*, *Mef2-Gal4* x 5339R knockdown and flies with an *UAS-Ime4* rescue construct. Lethality is significantly reduced and jumping ability increased as soon as *Ime4* is overexpressed in the knockdown, indicating the specificity of the hairpin. Significance determined by ANOVA with posthoc Tukey, p-value < 0.001, ***). Lethality data was assayed from 5 wildtype replicates, 10 *Mef2-Gal4* x 5339R replicates, and 11 *UAS-Ime4* rescue constructs. Jumping data was obtained from 10 male flies per genotype. This experiment was conducted twice.

Figure 4



3.1.7 Tissue-specific expression of *Mettl3* is unable to rescue mutant behavioural phenotypes

To test if *Mettl3* indeed has a tissue-intrinsic function in muscle, we performed muscle-specific rescue of *Mettl3* mutants. We made use of the Gal4-UAS system to express specifically in muscles with *Mef2*-Gal4 or neurons with *Elav*-Gal4 in a mutant background. One fly line we built had the tissue specific driver on the second chromosome and *Mettl3*^{Δ22-3} on the third: *Mef2-Gal4* / *Cyo*; *Mettl3*^{Δ22-3} / *TM3*, *Ser*. The other fly line had a UAS-*Mettl3* cassette on the second and *Df(3R) Exel6197* on the third chromosome: *UAS-Ime4* / *Cyo*; *Df(3R) Exel6197* / *TM3*, *Ser*. When those two lines were crossed together, we obtained mutant progeny which still could express working *Mettl3* in muscles or neurons. (Fig. 5) We validated that our UAS constructs were expressed in a tissue-specific manner using Western blot. While the *Mettl3* band is absent in IFM and brain dissected from mutant flies, *Mef*-Gal4 rescue flies express *Mettl3* in IFMs and *Elav*-Gal4 rescue flies expression *Mettl3* in neurons (Fig. 2 D).

This experiment therefore indicates that our rescue construct is working and *Mettl3* is expressed tissue-specifically.

We next performed the same behavioural assays with the rescue flies that we had tested with the *Mettl3*^{Δ22-3} / *Df(3R) Exel6197* mutant and the *Mettl3-IR*^{NIG-5339} knockdown flies. At room temperature, control *w*¹¹¹⁸ flies were able to fly, and ~90% of mutant and control flies were flightless (Fig. 6 A). We thus do not see background noise or leaky expression of the Gal4-UAS system under our conditions. Neither the neuronal nor the muscle rescue could rescue flight ability (Fig. 6 A). The neuronal rescue performed best with ~70% flightless or weak fliers. These data suggest that *Mettl3* may be required in both muscle and neurons to enable flight.

We were additionally unable to rescue climbing or held-out wing phenotypes by expression of *Mettl3* in selectively muscle or neurons. In our climbing assays, only ~15% of mutant flies made it over the 5 cm line in 3 seconds. In the control groups (just the cassette or just a driver) as well as the *Mef2*-Gal4 and *Elav*-Gal4 rescue flies, only ~20-35% of flies were able to climb 5 cm in 3 seconds, and thus the rescue did not perform significantly better than the control groups (Fig. 6 B).

The rescue flies also did not differ significantly from controls and the mutant in the percent of flies with held-out wings (Fig. 6 C). In summary, at room temperature the *Mettl3* phenotypes in flight, climbing, and wing positioning could not be rescued tissue specifically.

To increase the level of *Mettl3* expression, we repeated the behaviour experiments after raising the rescue flies at 27° C. At this temperature, we saw a much better rescue with approximately 50% of flies able to fly with both *Mef2-Gal4* and *Elav-Gal4* rescue (supplementary Fig. 13). However, we also observed that the control group with UAS-*Mettl3* and lacking a specific driver was also performing better with around 40 % able to fly, but the control group containing only the *Gal4* driver performed comparable to the mutant genotype. This suggests that at 27° C the UAS-cassette gets leaky and hence its expression is not strictly limited to one tissue any more. Nevertheless, these results suggest that the flight phenotype can be rescued when *Mettl3* expression is not limited to only muscular or neuronal tissue, but will be expressed in both instead.

This observation supports our hypothesis that the mutant phenotype is in fact coming from a neuronal and a muscular side.

3.1.8 Muscle-specific *Mettl3* expression can rescue the sarcomere phenotype

Our RNAi data showed that *Mettl3* has an intrinsic function in muscle to regulate sarcomere length (Fig. 4 F-1 – F-4, G-1 – G-4, H). We reasoned that even if flight ability was not restored, our muscle-specific rescue might restore normal sarcomere structure. To test this, we imaged the sarcomere structure of our *Mef2-Gal4* rescue flies and all the matching controls. We found that *Mettl3*^{Δ22-3} / *Df(3R) Exel6197* mutants as well as controls including just the UAS-cassette (*UAS-Mettl3* / +; *Mettl3*^{Δ22-3} / *Df(3R) Exel6197*) or just one of the two *Gal4* drivers (*Mef2-Gal4* or *Elav-Gal4* / +; *Mettl3*^{Δ22-3} / *Df(3R) Exel6197*) had a sarcomere length of about 3.7 μm (Fig. 6 G). Sarcomeres in IFM from the neuronal specific rescue (*Elav-Gal4/UAS-Mettl3*; *Mettl3*^{Δ22-3} / *Df(3R) Exel6197*) were also about 3.7 μm long and not significantly different from the mutant (Fig. 6 G).

Remarkably, the muscle-specific rescue (*Mef2-Gal4 / UAS-Mettl3; Mettl3^{Δ22-3} / Df(3R) Exel6197*) had an average sarcomere length of ~3.4 μm, and was therefore much closer to the wildtype control in length (Fig. 6 D-1 - F-4, G). This data suggests that muscle-specific expression of Mettl3 is sufficient to rescue the defects in sarcomere length in *Mettl3* mutants, demonstrating a muscle-intrinsic function for Mettl3.

Fig. 5 Crossing scheme used to obtain tissue-specific Mettl3 rescue genotypes.

Crossing scheme illustrating how all four genetic elements were combined into a single fly to obtain the rescue genotypes. In the first and second stage of the scheme (top two boxes), the Mettl3 mutant chromosome (*Mettl3^{Δ22-3}*), the Mettl3 deficiency (Df) (*Df(3R) Exel6197(III)*), the UAS-Mettl3 construct (*UAS-Mettl3-HA^{attP40}/CyO*) and a Gal4 driver (*Mef2-Gal4/CyO*) are double balanced. This has to be done in two steps to generate stably balanced fly lines. The second chromosome was balanced over CyO, while the third was balanced over TM3, Ser (Ser). In the third stage, one mutant copy of Mettl3 and one element of the UAS/Gal4 system are combined in the same fly. Since the full mutant genotype is sterile, the flies must be heterozygous for the Mettl3 mutation to be maintained as a stock. In the last stage (bottom) which generates the rescue fly (genotype in bold at the bottom, *Mef2-Gal4 / UAS-Mettl3-HA^{attP40}; Mettl3^{Δ22-3} / Df(3R) Exel6197(III)*), all genetic components are brought together generating an experimental fly that is homozygous mutant for Mettl3 with a UAS-Mettl3 expressed in a tissue determined by the tissue specific Gal4 driver. This scheme is an example, as instead of Mef2-Gal4 (Mef2), other drivers could also be used as long as they are on the same chromosome (Chromosome II). Df = *Df(3R) Exel6197(III)*; lme4- = *Mettl3^{Δ22-3}*; UAS-Mettl3 = *UAS-Mettl3-HA^{attP40}/CyO*; Mef2-G4/Mef2 = *Mef2-Gal4/CyO*.

Figure 5

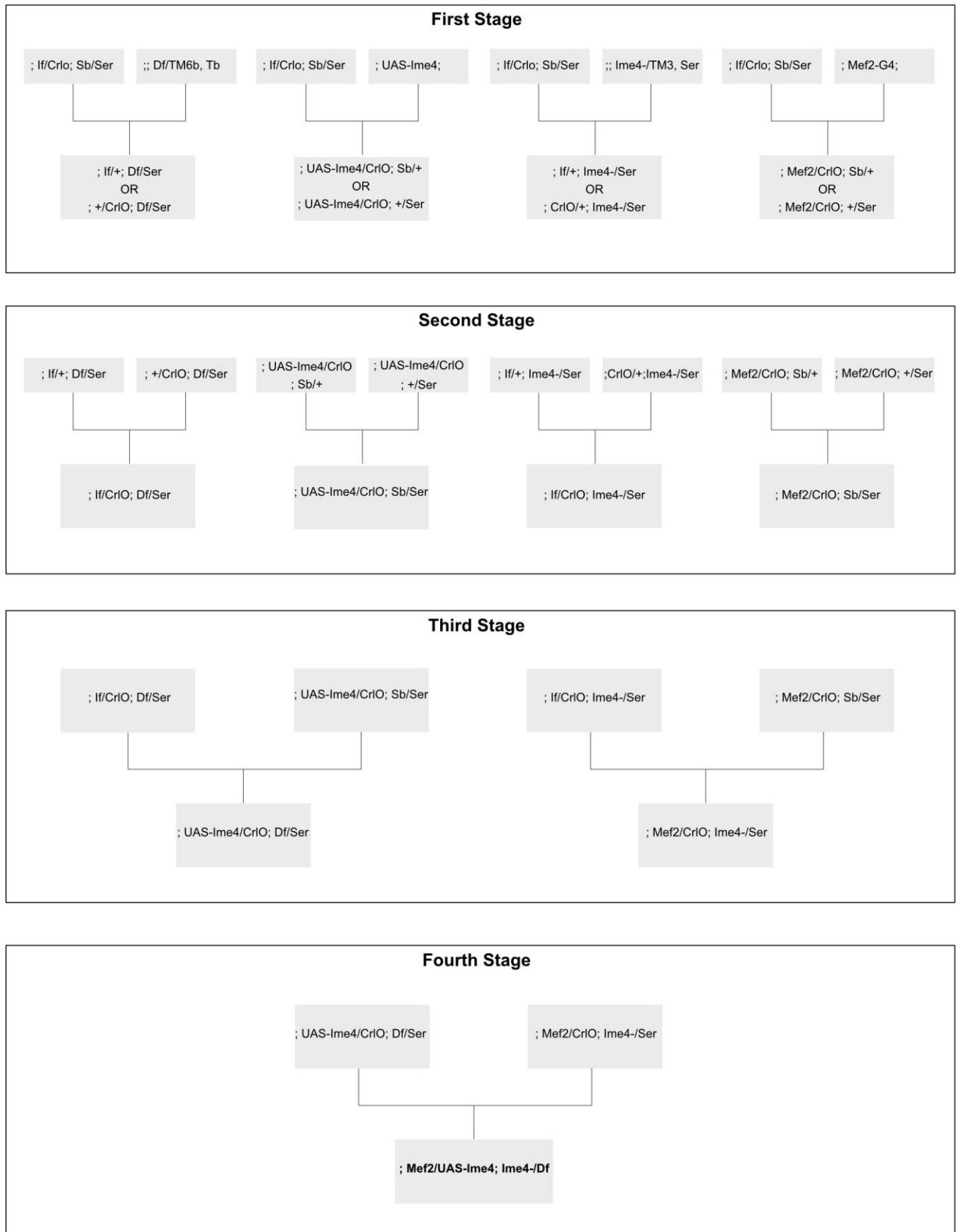


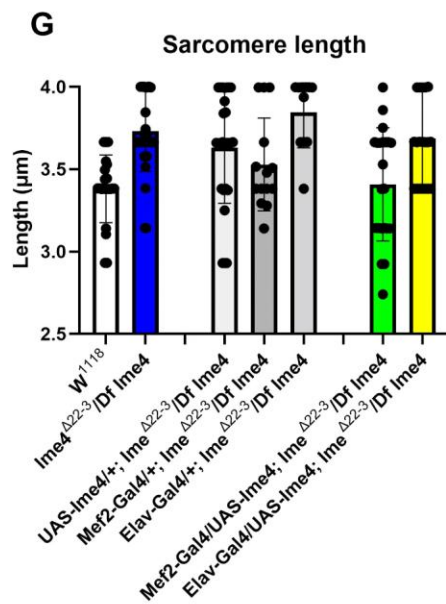
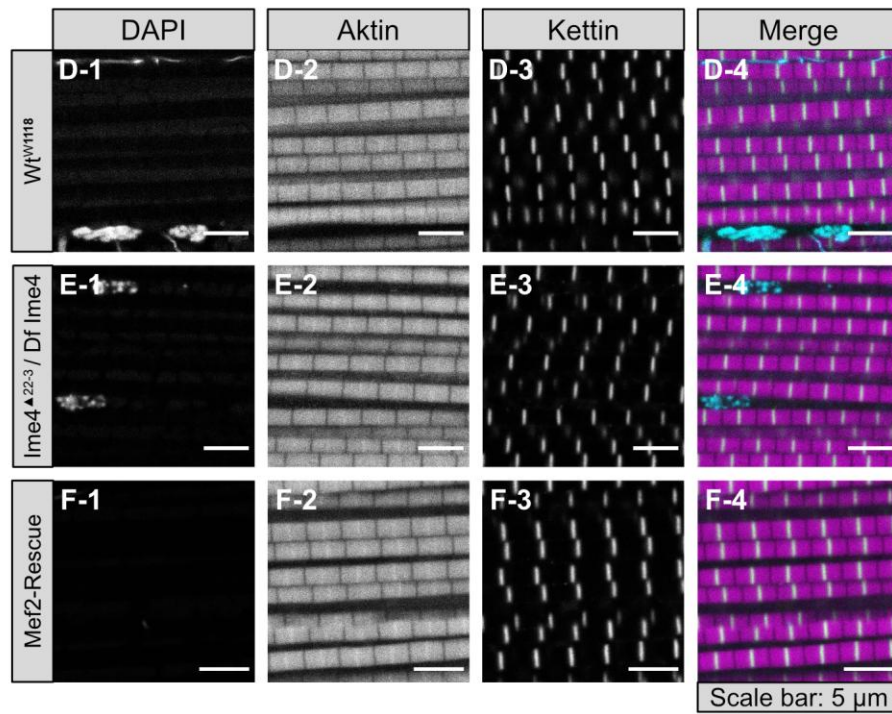
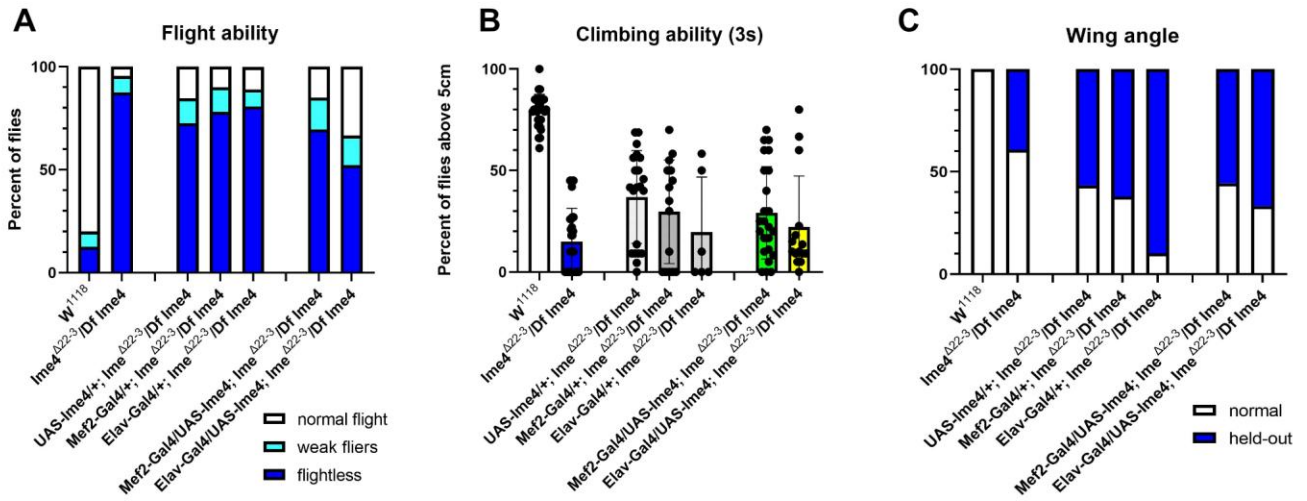
Figure 6. Tissue specific rescue can restore the sarcomere structure but not higher functions like flight, climbing, and wing posture.

(A-C) Plots quantifying behavioural defects in *Mettl3* mutant, control, and tissue-specific rescue flies in their ability to fly (A), their ability to climb (B), and their tendency to hold out their wings (C). *Mef2-Gal4* drives in muscle, while *Elav-Gal4* drives in neurons. Flight ability was assayed from over 100 male flies each from several crosses. Climbing ability and wing angle were assayed from over 60 male flies each, also from several crosses.

(D-F) Single-plane confocal images of wildtype control *w¹¹¹⁸* (D1-4), *Mettl3^{Δ22-3}/Df* mutant (E1-4), and muscle specific rescue (F1-4) IFM myofibrils. Mutant sarcomeres are too long, whereas wildtype and muscle specific rescue sarcomeres are indistinguishable. Scalebar = 5 μm. DAPI stained nuclei, cyan; phalloidin labelled actin, magenta; Kettin-labelled z-discs, yellow. 5 thoraxes were imaged each.

(G) Quantification of sarcomere length of the data shown in D-1 to F-4. *Mef2-Gal4* (green) but not *Elav-Gal4* (yellow) is able to rescue the sarcomere length defect.

Figure 6



3.1.9 Sarcomere proteins are misregulated in *Mettl3* RNAi IFM

Having demonstrated that *Mettl3* plays a role in *Drosophila* IFM in sarcomere development, we next investigated how loss of *Mettl3* affects the expression of sarcomere proteins. To test the expression levels of certain muscle related proteins, we made use of GFP-tagged Fosmid reporter lines. Fosmid reporter lines contain a genomic fragment with a third copy of a C-terminally GFP tagged gene of interest inserted into attP-VK33 [54]. The inserted GFP-tagged genes are under the control of native regulatory elements, similar to genomic rescue constructs. The reporter lines were recombined with *Mef2-Gal4*, and then crossed to *w¹¹¹⁸* as a control and *Mettl3-IR^{NIG-5339}* to knock-down *Mettl3* expression. The obtained progeny were then imaged with the same confocal settings to quantitatively compare GFP fluorescence intensities.

We examined several proteins including Flightin (Fln), Myofilin (Mf), Obscurin (Unc-89), and Actin88F which are localized to different parts of the sarcomere [1]. Fln and Mf both localize to the thick filaments, and were significantly decreased in the knockdown background (Fig. 7 C-1 – F-4, M, N). The mean GFP intensity level of Fln-GFP in control was ~55 (mean brightness value) (Fig. 7 M), while in the knockdown background was only ~20 (Fig. 7 M), a fold change of 2.75. Mf-GFP intensity averaged ~60 in control, but was significantly decreased to ~15 in the RNAi (Fig. 7 E-1 - F-4, N), a 4-fold change. Unc-89 sits at the sarcomere M-line. In control, Unc-89-GFP had a mean GFP intensity around ~25, while in the knockdown this dropped significantly to ~9 (Fig. 7 G-1 - H-4, O), a fold change of 2.8. This decrease in expression appears to affect not only specific sarcomere proteins, as Act88F, which forms the thin filaments, displayed also a significant decrease between the wildtype control and the RNAi knockdown (Fig. 7 A-1 - B-4, K). This suggests that *Mettl3* normally regulates the expression of select sarcomere proteins.

3.1.10 Expression of UAS-mito-GFP shows a strong decrease in muscle specific *Mettl3* knockdown

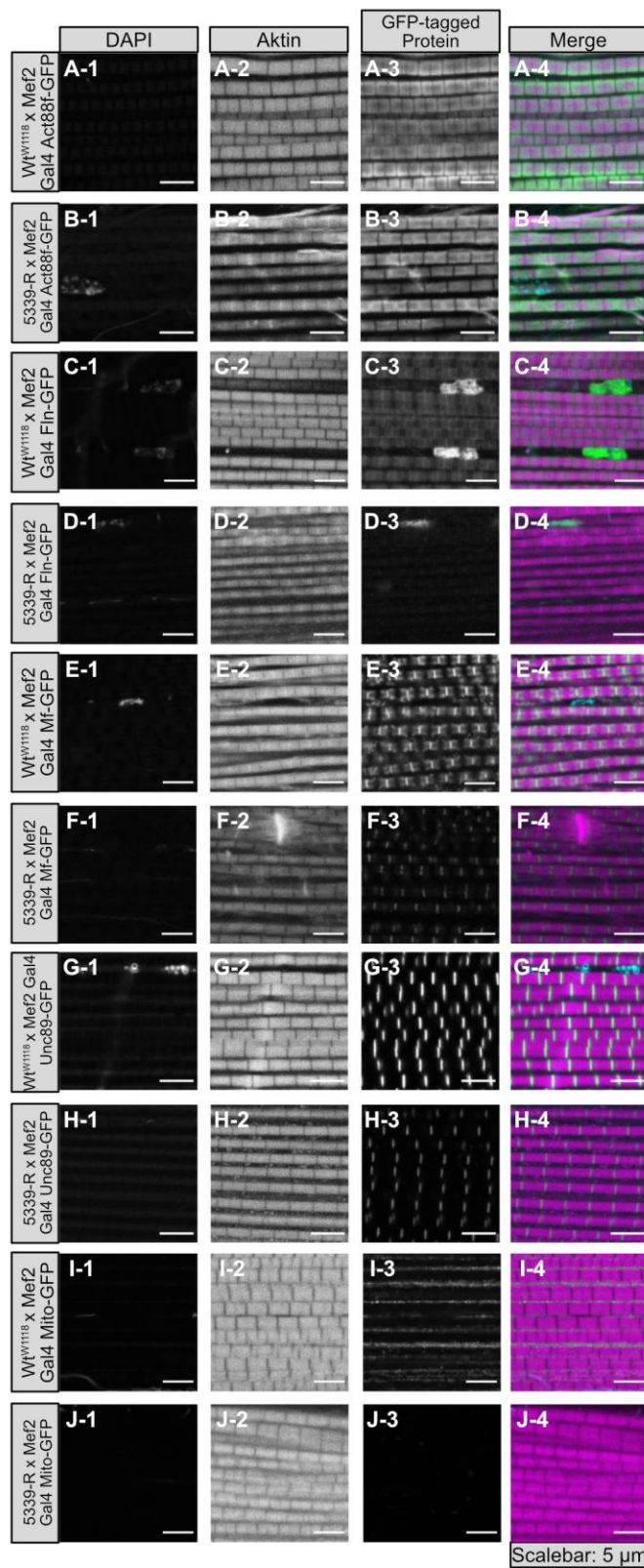
In addition to sarcomere components, we were interested in potential differences in mitochondria that might contribute to sarcomere or functional defects in IFM. Normally the mitochondria within the IFM in *Drosophila* will fuse during development [55]. It is proposed that mitochondria form long, string-shaped patterns between the myofibrils and spanning alongside them to cope with the strong energy demand of IFM tissue. To explore the mitochondria, we made use of a UAS-mito-GFP reporter line, which is a GFP-tagged version of the enzyme Cox8A, which is located in complex IV within the mitochondria [56]. In the control, the mitochondrial signal was located in strings or an elongated network structure alongside the myofibrils (Fig. 7 I-3). In the *Mettl3-IR^{NIG-5339}* knockdown however, the signal was significantly reduced (Fig. 7 J-3, P) and the mitochondrial network was much less elaborate. The mean brightness intensity of the control was ~55, whereas in the knockdown was around ~7. Thus, we see a 7.8-fold decrease in mitochondrial signal upon *Mettl3* knockdown, suggesting that mitochondrial associated genes are also affected.

Figure 7. Expression of multiple sarcomere proteins is decreased in *Mettl3* knockdown flies.

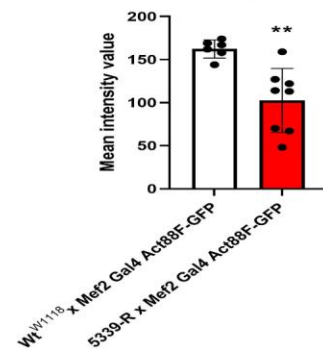
(A-J) Single-plane confocal images of GFP-tagged reporter expression in IFM for GFP-tagged Fosmid including Act88F (B1-4), Fln (D1-4), Mf (F1-4), and Unc-89 (H1-4), as well as Mito-GFP (J1-4). Reporters were crossed to wildtype control *w¹¹¹⁸* (A, C, E, G and I 1-4) to establish baseline expression, and then *Mettl3* RNAi 5593R to determine the change in expression after loss of *Mettl3*. Scale-bar = 5 μ m. DAPI stained nuclei, cyan; phalloidin labelled actin, magenta; GFP-tagged proteins, yellow. 5 thoraxes were imaged each.

(K-P) Quantification of signal intensities of the data shown in A-1 to J-4. Significance determined by t-test, n.s. = not significant; *, p-value < 0.1; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

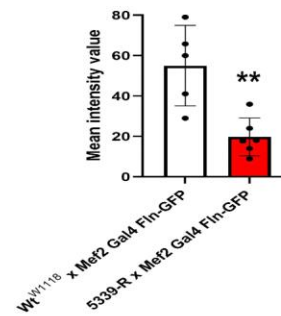
Figure 7



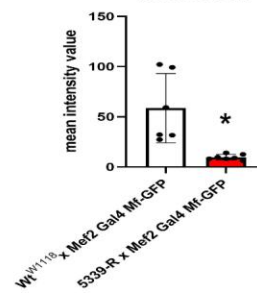
K Act88F intensity



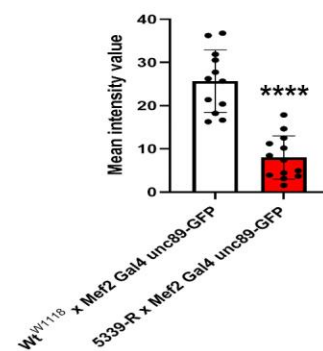
M Fin intensity



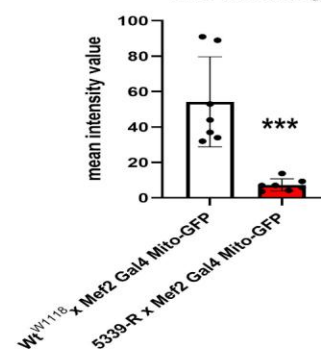
N Mf intensity



O Unc-89 Intensity



P Mito intensity



3.1.11 Proteomic analysis of Mettl3 mutant IFMs

We extend our analysis of proteins misregulated after loss of Mettl3 to the entire proteome, we performed a label free mass spectrometry analysis of control *w¹¹¹⁸* and mutant *Mettl3^{Δ22-3} / Df(3R) Exel6197* IFMs. To validate the data, we first plotted the distribution of pre-imputation intensities for each replicate and sample. All 8 curves had peak intensities around ~27, and were roughly the same height with similar smooth distributions (Fig. 8 A). This shows our data are consistent, and the samples are comparable and thus meaningful.

A principal component analysis (PCA) was also done to visualize the variation between the samples and individual replicates of our data in multi-dimensional space (Fig.8 B). A PCA analysis is a dimensionality-reduction approach that standardizes the range of continuous variables, computes a covariance matrix to identify correlations between those variables, generates eigenvectors and eigenvalues to identify the principal components, creates feature vectors of the top principal components that capture the greatest variance, and then recasts the data along the principal component axes. The PCA analysis revealed that 56% of the variance in the proteomics data was captured by the first two principal components, and the wildtype control replicates did cluster tightly and were clearly separated from the mutant replicates in the first dimension. This piece of data thus indicated that the control and the mutant samples were clearly distinct from each other, and can be reliably used for differential protein expression analysis.

We then performed a differential protein expression analysis based on the LFQ intensity values. We visualized our data with a volcano plot, where we plotted the Difference between the mutant genotype versus the wildtype control on the x-axis, and the significance (-log p-value) on the y-axis (Fig.8 C). The data were distributed in a funnel shape, allowing us to clearly see that several hundred proteins are significantly up- or downregulated in the mutant genotype. Mettl3 was downregulated in the mutant, as expected. We also confirmed multiple sarcomere proteins as downregulated in the proteomics data, and found that genes previously identified as having a muscle-specific RNAi phenotype were significantly downregulated in our data. (Appendix Table)

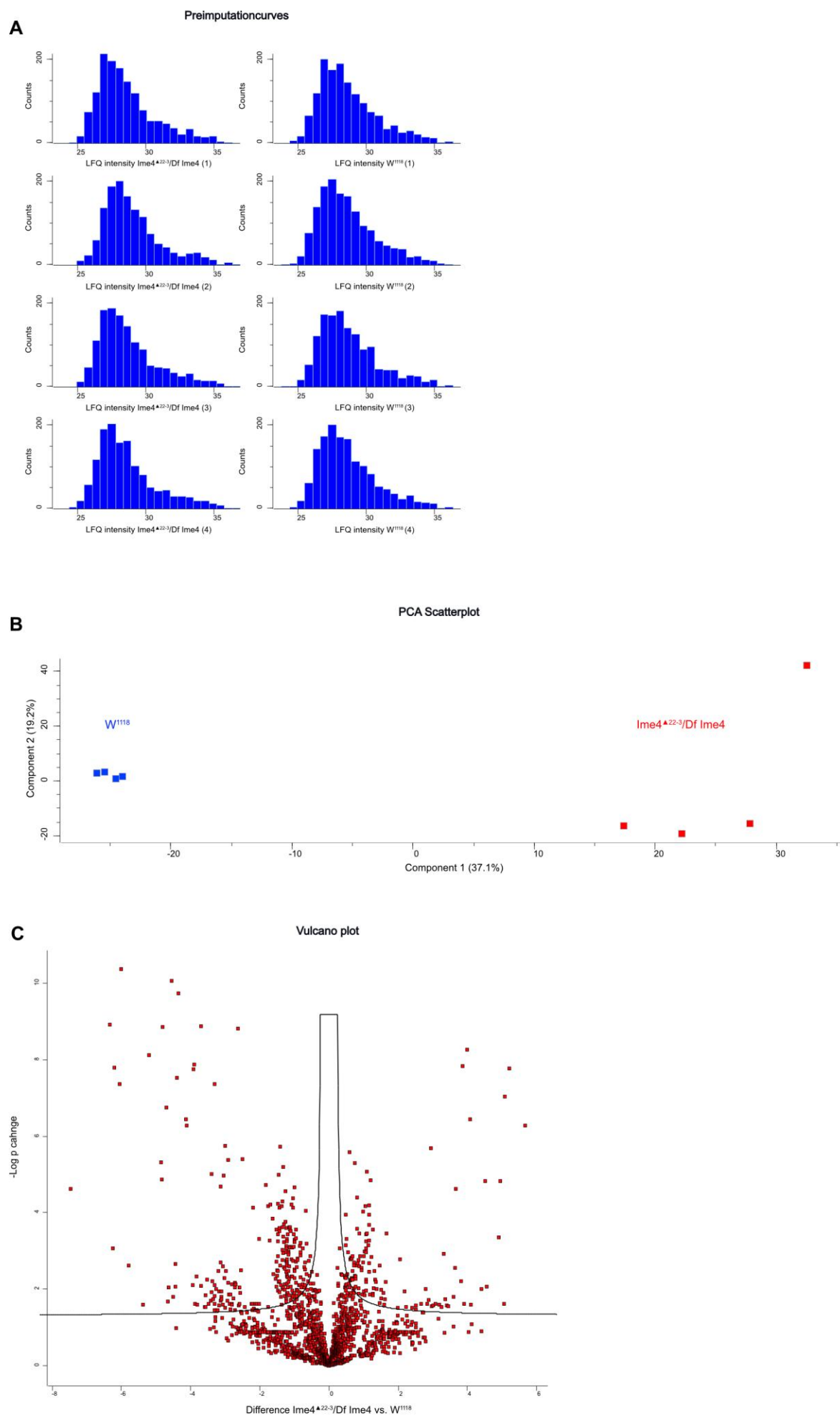
At the time this thesis was written, the data from this experiment still needed to be fully analysed and interpreted. However, we could already draw some conclusions here. We can clearly see significant changes in protein expression in IFMs from *Mettl3* mutant animals. This suggests *Mettl3* has a function in muscle, and that there are distinct changes in expression of *Mettl3* target genes that may underly the observed flightless phenotype. This nicely supports our previous findings regarding altered protein levels in the mutant fly.

Figure 8. *Mettl3* mutant flies show significant changes in protein expression.

(A) Intensity distribution plots for quality control of proteomics samples including the four *w¹¹¹⁸* and four *Mettl3^{Δ22-3}/Df* mutant samples tested in this experiment. The preimputation values show a uniform distribution with their peaks at around ~27 and are roughly the same height and width. For both genotypes in this figure, 50 IFMs went into one replicate. There were 4 replicates, which makes 200 IFMs in total for every genotype.

(B) Principal component analysis shows that the largest component of variance is between control and the mutant samples, which are clearly distinct from each other. The individual biological replicates for each genotype cluster together.

(C) Volcano plot showing that many proteins are differentially expressed between control and *Mettl3* mutant (*Mettl3^{Δ22-3}/Df*) samples. Difference between the control and mutant samples is plotted on x-axis. The significance is plotted on the y-axis as -log p-value. Smooth lines represent an FDR < 0.05. Zero values (peptides missing in one genotype) were replaced with an intensity value two points lower than the lowest recorded intensity, and thus form the “wings” on the plot. Loss of *Mettl3* results in large changes in protein expression in dissected IFM samples.

Figure 8

3.2 Neuronal structure

Previous studies [57-59] as well as our behavioural data from mutant and Elav-Gal4 knockdown experiments suggest that *Mettl3* and the m6A pathway play an important role in neuronal development. To determine if there are also neuronal defects in the motor neurons that innervate the IFMs, we analysed the motor neuron axons that innervate IFM from wildtype control, mutant, muscle- and neuronal specific knockdown, muscle- and neuronal specific rescue and all the control lines. Thoraxes were stained for HRP to make the axons, deriving from the motoneurons, visible [30].

Normally, the motor neuron axons fasciculate after exiting the ventral nerve cord and extend centrally in the thorax to the IFMs [60]. After reaching the IFMs, the primary branch forms secondary and higher order branches. Under physiological conditions, those axon branches do not cross or overlap and the overall silhouette looks like a tree. This pattern we also could observe in our wildtype control (Fig. 9 A-1). Strikingly, when we examined the mutant *Mettl3^{Δ22-3} / Df(3R) Exel6197* flies, we saw a dramatic overbranching phenotype. The axons crossed each other frequently and the sparse, tree like silhouette was lost (Fig. 9 B-1). The axon branches were not only more numerous, but also made sharper bends. We conclude that loss of *Mettl3* results in an axon overbranching phenotype in the adult IFM motor neurons, consistent with the phenotype reported for larval body wall motor neurons [50].

We next tested if this axon overbranching phenotype is tissue-specific using RNAi. Using Elav-Gal4 knockdown, we saw a comparable pattern with neuronal specific knockdown of *Mettl3* (Fig. 9 D-3) to that observed in the mutant (Fig. 9 B-3). The axonal branches grew in a more chaotic manner than in the control and developed more branches. The Mef2-Gal4 muscle knockdown did not display an axon phenotype, and was comparable to the control (Fig. 9 C-3). These data confirm that axon overbranching results from loss of *Mettl3* selectively in the neurons.

If *Mettl3* function in neurons leads to overbranching, we hypothesized that neuron but not muscle-specific rescue would revert the overbranching phenotype. We therefore checked the motor neuron innervation in the two tissue-specific rescue experiments (Fig. 9 E-1 to F-3).

The neuronal specific rescue was indeed more effective and resulted in a near-wildtype branching pattern. Despite the high level of natural variation in motor neuron axon innervation patterns, our data indicate that overbranching contributes to the disrupted IFM function upon loss of *Mettl3*.

To further analyse the data, we could obtain from those images and calculate the average number of branches one filament has, we performed axonal tracing with the help of Imaris. This task was more difficult than expected, as Imaris had trouble accurately reconstructing the axon structures, especially the primary branches where the axons were thick. Imaris often treated those regions as many single fibres although it was one single, thick fibre. This was most pronounced in physiological control conditions, leading to inflated total axonal length and branch point counts. In addition, backfilling of trachea with DAPI-containing mounting medium could not be distinguished by Imaris from actual axonal staining. Future analysis will be required to generate a more accurate quantitative analysis.

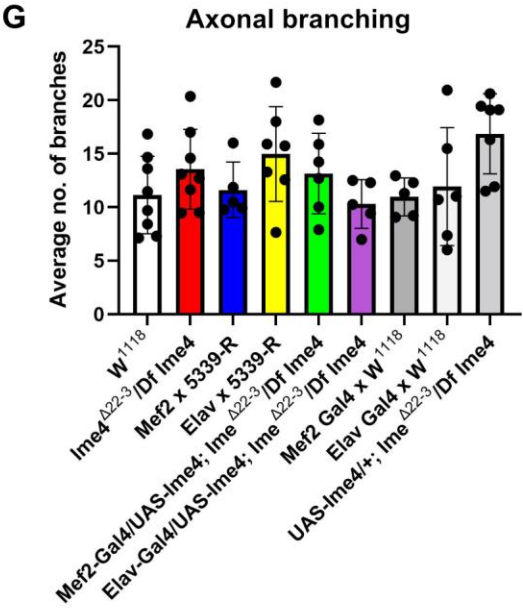
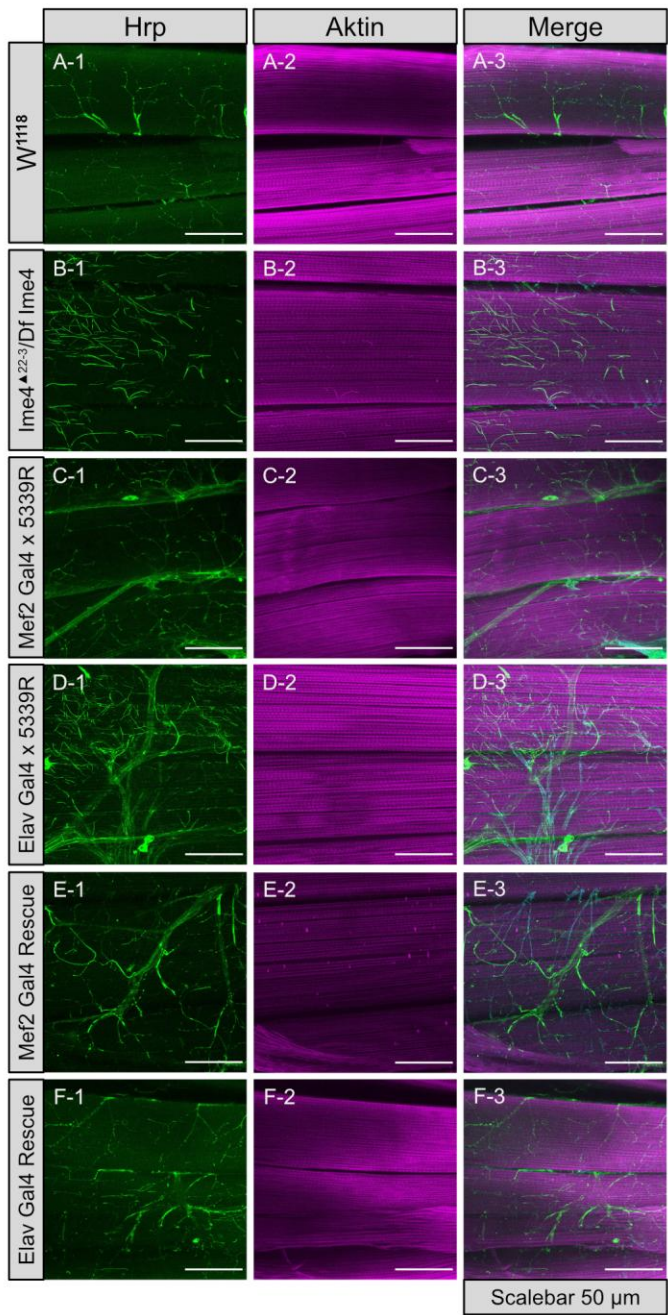
Using the dataset I generate in Imaris, the data were then further analysed and the mean number of branches was quantified and compared between the genotypes (Fig. 9 G). The variance in this data was quite big, and an ANOVA did not reveal a significant difference between samples, as the p-value was 0.0548. We could observe tendencies which supported our visual observations. The mean number of branches was increased in both mutant and the neuronal specific knockdown compared to wildtype. The muscle specific rescue and just the UAS-cassette containing *Mettl3* over the mutant background showed elevated results. Matching to our previous findings and assumptions, we saw lower levels in the muscle specific knockdown, the neuronal specific rescue and the drivers crossed to wildtype. In summary, when our data are taken together, we conclude that the loss or depletion of *Mettl3* leads to an overgrowth phenotype of motoneuron axons that innervate the IFM of *Drosophila*.

Figure 9. *Mettl3* mutants and RNAi knockdown flies exhibit motor neuron axon overbranching.

(A-F) Maximum projection confocal images of wildtype control *w¹¹¹⁸* (A1-4), *Mettl3^{Δ22-3}/Df* mutant (B1-4), muscle specific *Mettl3* knockdown (C1-4), neuronal specific knockdown (D1-4), muscle specific rescue (E1-4) and neuronal specific rescue (F1-4) IFM. Mutant IFMs show overbranched axons. Scalebar = 50 μm. HRP stained axons, cyan; phalloidin labelled actin, magenta. 5 thoraxes were imaged for each genotype.

(G) Quantification of axonal branching of the data shown in A-1 to F-4. Due to technical issues with the tracing software used, this data may not be consistent.

Figure 9



3.3 mRNA-Seq analysis

In this part of the project, we wanted to understand how loss of *Mettl3* affects the muscle on the molecular level. To do this, we sequenced and analysed RNA in wildtype and mutant flies to better understand what is happening on the transcriptome level.

3.3.1 mRNA sequencing revealed altered transcriptional patterns of muscle related genes in *Mettl3* mutant flies

To further explore the differences between wildtype and mutant flies, we dissected IFM from both control *w¹¹¹⁸* and mutant *Mettl3^{Δ22-3} / Df(3R) Exel6197* and performed mRNA-Seq. We then performed a differential expression analysis on the gene level using DESeq2 and on the exon level using DEXSeq. We generated Volcano plots and saw hundreds of genes and exons that were significantly up or down regulated in mutant IFMs (Fig. 10), suggesting that loss of *Mettl3* affects a broad set of targets.

To better understand what types of genes were misregulated, we looked for overall trends in the expression of muscle-associated, neuronal, synaptic, and mitochondrial genes. The sets of genes included in the analysis include genes with an RNAi phenotype in muscle (Spletter, eLife, 2018), sarcomere genes (Spletter, eLife, 2018), mitochondrial associated proteins (Yim, NAR, 2020), and genes annotated in Flybase with the GO term “synapse” or “neuromuscular junction”. We saw a trend that genes that were reported to have a muscle-specific RNAi phenotype [47], and thus are important for muscle development or function, were downregulated in *Mettl3* mutant IFM (Fig. 10 A). *Mettl3* itself was significantly downregulated in the mutant samples, as expected.

By contrast, strikingly fewer synapse or neuronal associated genes were significantly altered, and those that did change tended to be upregulated (Fig. 10 B). This was also reflected in violin plots, where synapse and neuromuscular junction (NMJ) genes were as a group significantly upregulated (Fig. 10 E). This data supports our earlier conclusions. There are distinct gene expression changes in muscle-related genes, demonstrating a function for *Mettl3* in muscle.

Increased expression of NMJ components is consistent with an axon overbranching phenotype.

We performed a similar analysis to understand which genes have exons that are differentially expressed in *Mettl3* mutant muscle. Exons detected by DEXSeq can reflect either alternative splicing, alternative 5' exon use, or alternative 3' exon use. We found that exons from genes with a muscle-specific RNAi phenotype were both up and down regulated (Fig. 10 C), with certain exons of sarcomere genes notably used less frequently than in control IFM (Fig. 10 F). Exons of most synapse genes were not significantly altered, and notably the majority of significantly changed synapse exons were upregulated (Fig. 10 D). Taken together, this data highlights that the loss of *Mettl3* causes many changes in exon use and alternative splicing on the transcript level. Those changes affect both muscle and neuronal associated genes, and further support the hypothesis that the m6A-pathway is involved in regulating splicing in IFM.

Figure 10. *Mettl3* mutants exhibit altered patterns in gene expression and exon use.

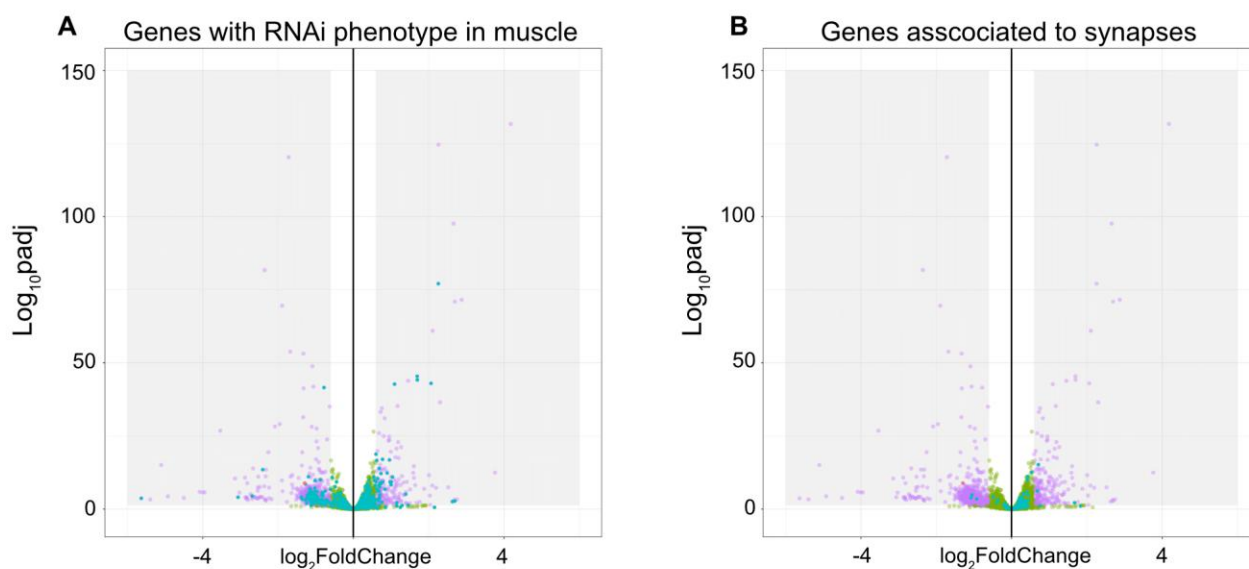
(A-B) Volcano plots from mRNA-Seq data showing changes in gene expression between control and mutant *Mettl3*^{Δ22-3}/*Df* IFM. Data was analysed at the level of gene expression with DESeq2. Genes that are significantly misregulated were filtered by a log₂(fold change) > 0.5 and a log₁₀(adjusted p-value) > 0.05 (purple dots, grey boxes). Data are overlayed with genes associated with an RNAi phenotype in muscle (A, cyan dots) or genes annotated with the GO term “synapse” (B, cyan dots). Genes with an RNAi phenotype in muscle (from Schönbauer *et al.*, Nature, 2010) are significantly downregulated in *Mettl3* knockdown, while a subset of synaptic genes show increased expression. For those experiments IFMs of 100 flies per genotype were assayed in two replicates.

(C-D) Volcano plots from mRNA-Seq data analysed for changes in exon use using DEXSeq. Significant exons were filtered by a log₂(fold change) > 1.5 and a log₁₀(adjusted p-value) > 0.05 (purple dots, grey boxes). Plots were overlayed with genes associated with RNAi phenotype in muscle (C, cyan dots) or genes annotated with the GO term “synapse” (D, cyan dots) in *Mettl3* mutant *Mettl3*^{Δ22-3}/*Df* versus wildtype control IFM. The *Mettl3* gene or exons are coloured in red. The fold change between *Mettl3* mutant: wildtype control and is plotted on the x-axis as log₂(Fold Change). The adjusted P-value is plotted on the y-axis (log₁₀).

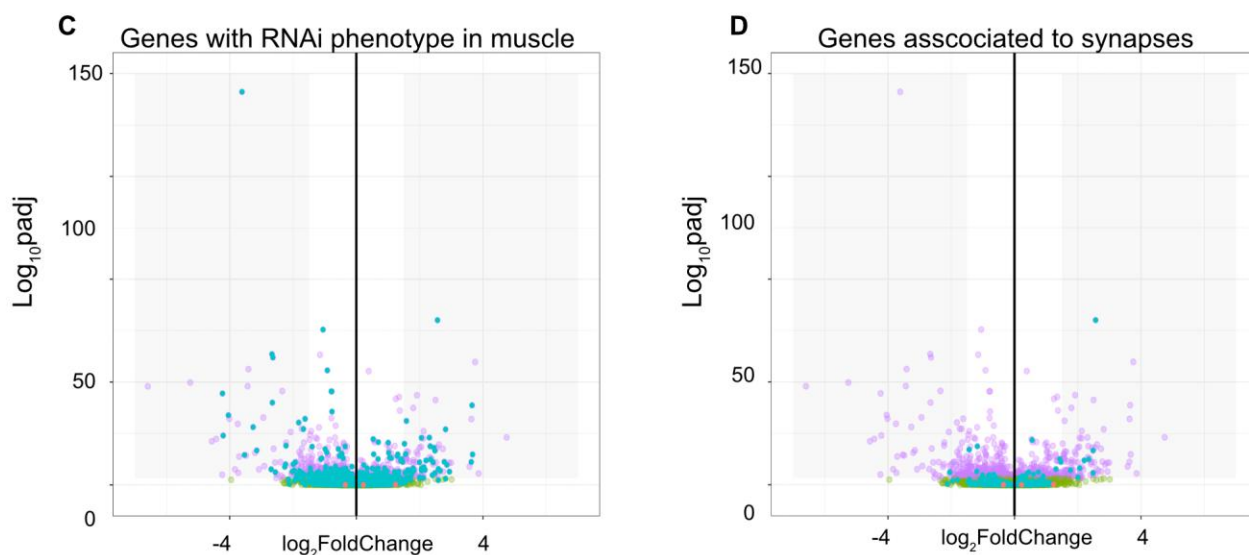
(E-F) Violin plots showing the changes between wildtype control and *Mettl3* mutant on a gene (E) and exon (F) level. On the x-axis the gene sets examine (including genes with an RNAi phenotype in muscle, sarcomere genes, mitochondrial genes, and genes included in GO terms “synapse” and “neuromuscular junction”) are labelled. The log₂ fold change in expression is plotted on the y-axis. Each plot is overlayed with the associated box plot in white.

Figure 10

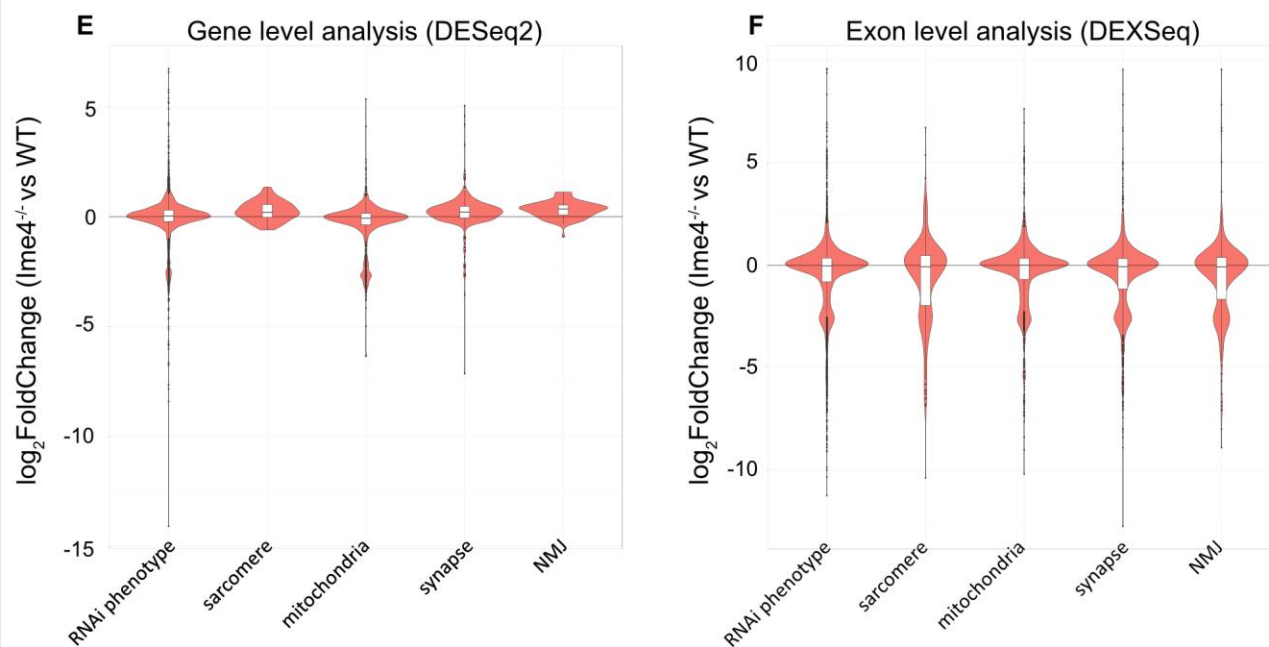
Gene level analysis (DESeq2), *Ime4* mutant vs wildtype



Exon level analysis (DEXSeq), *Ime4* mutant vs wildtype



Changes in muscle, mitochondrial and neuronal genes



3.3.2 Decreased expression of *Mettl3* causes changes in mRNA levels of muscle related proteins

To further extend our understanding of transcriptional changes caused by the loss of *Mettl3* and to validate our mRNA-Seq results, we performed qPCR experiments to quantify and compare the amounts of mRNA coding for selected proteins. We dissected IFM from control *w¹¹¹⁸*, mutant *Mettl3^{Δ22-3} / Df(3R) Exel6197* and muscle-specific RNAi animals (*Mettl3-IR^{NIG-5339}*). Our samples are therefore highly enriched for IFM tissue, although any mRNA transcripts present in the motor neuron axons will also be detected. In both the mutant and the muscle specific knockdown, we saw a decrease of *Mettl3* mRNA of greater than 50% (Fig. 11 A). This validates our mutant as well as the knockdown efficiency of our RNAi line.

We then analysed a number of sarcomere proteins, to determine how *Mettl3* affects the expression of key structural components of muscle.

The *bent* (*bt*) locus encodes Projectin, a large Titin-like protein which is associated with the intermediate filament complex in IFMs and contributes to the stiffness of the muscle. [61]. In agreement with our mRNA-Seq data, we observed a 7-fold increase in *bt* transcripts in mutant and a 10-fold increase in the muscle specific knockdown (Fig. 11 B). Act88F is the Actin isoform expressed specifically in IFM which is the major component of the thin filament [62]. Act88F (Fig. 7 A1-B4), qPCR showed a significant decrease of ~90% in *Act88F* expression in both the mutant and the muscle specific knockdown (Figure 9 D). These findings were supported by the results obtained from the Fosmid reporter experiments; here we also saw a significant decrease in intensity in mutant IFM. (Fig. 7 B-3 and K) Flightin (Fln) is a thick-filament associated protein expressed specifically in differentiated IFM that is necessary for flight and sarcomere structure [63]. Consistent with the findings from the Fln Fosmid reporter (Fig. 7), *fln* mRNA levels were around 80% reduced in mutant and about 60% less in the RNAi knockdown (Fig. 11 C). These results are consistent with both our mRNA-Seq and our Fosmid-reporter data, and show that a decrease or loss of *Mettl3* expression alters the expression of key muscle-related mRNAs and proteins.

3.3.3 Decreased expression of Mettl3 in an increase in the mRNAs of neuronal- and synaptic related proteins

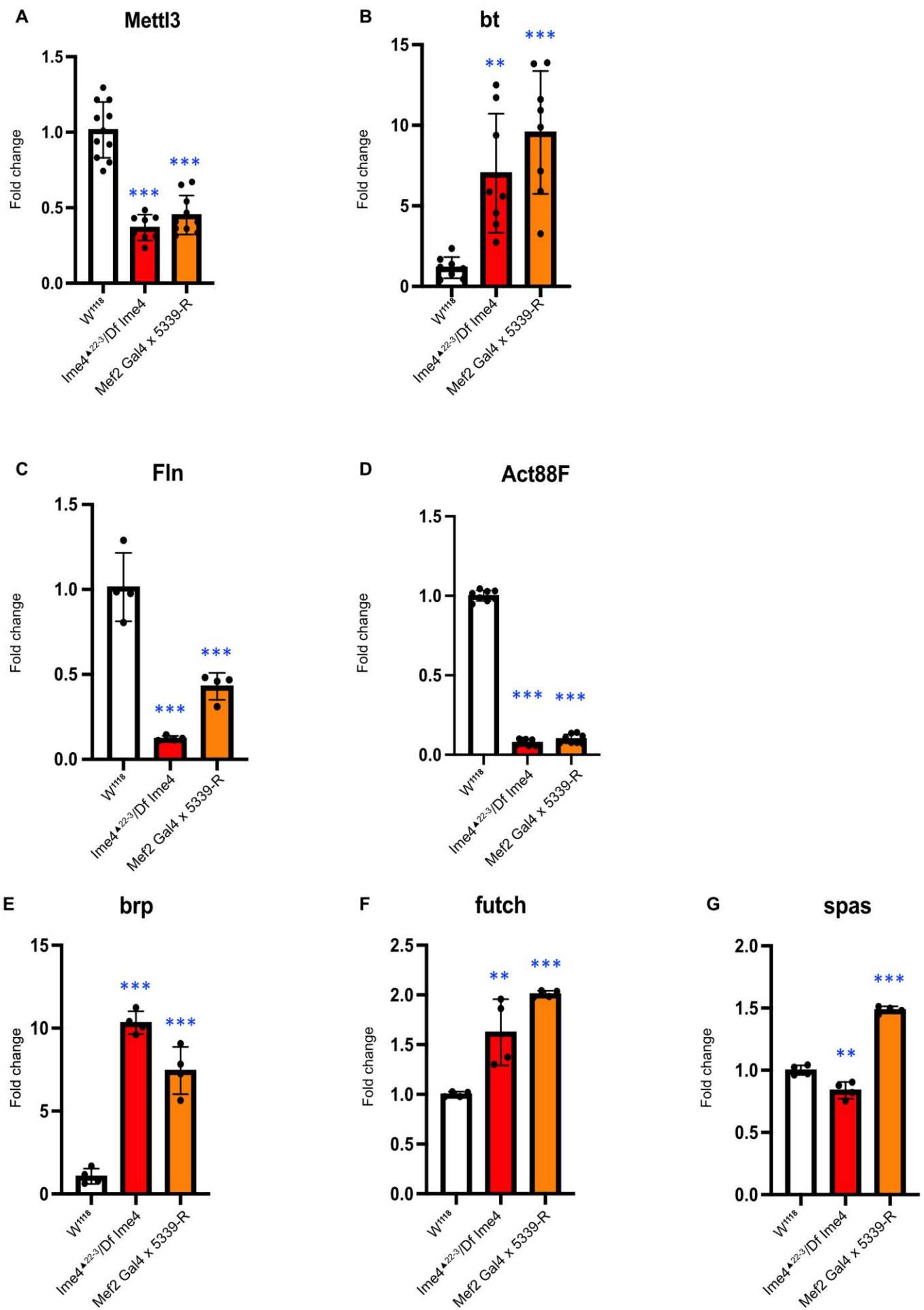
Our results above demonstrated that IFM motor neuron axons are over branched (Fig. 9) and our mRNA-Seq data showed that mRNA levels of synaptic components are increased after loss of Mettl3 (Fig. 10). To validate this finding, we performed RT-qPCR for several neuronal or synaptic targets. Bruchpilot (Brp) is a cytoskeletal protein which contributes to size regulation of synaptic vesicles, calcium channel clustering, and is critical for the structural integrity of the T-bar region within synapses. [64] In Mettl3 mutant flies, where mRNA levels of Mettl3 were strongly decreased, we measured an approximately 10-fold increase of mRNA coding for *brp*, while in the muscle-specific RNAi knockdown we observed about a 7-fold increase (Fig. 11 E). Futsch is a microtubule binding protein involved in the formation of synaptic buttons at the neuromuscular junction [65]. *futsch* expression saw a 1.6-fold increase in mutant and a 2-fold increase in the RNAi knockdown (Fig. 11 F).

Spastin (Spas) is a protein which assembles into hexamers and severs microtubules along their lengths, contributing to neuronal axon transport, synapse formation and dendrite arborization [66]. Although *Spas* expression was not significantly affected in the mutant IFM, we detected a 1.5-fold increase in the muscle specific RNAi knockdown (Fig. 11 G). Our experiments therefore confirm our mRNA-Seq results and show that depletion of Mettl3 results in increased expression of synaptic components including *brp*, *Spas*, and *futsch*. These results are consistent with the over branching phenotype we describe in Mettl3 mutants.

Figure 11. Mettl3 Mutants and RNAi knockdown flies show significant changes in the amount of mRNA for selected proteins.

(A-G) Semi-quantitative RT-qPCR analysis of gene expression. Plots quantifying the fold change in mRNA expression. Control *w¹¹¹⁸* was normalized to 1. Significance determined by ANOVA and posthoc Tukey, n.s. = not significant; **, p-value < 0.01; ***, p-value < 0.001. For those experiments 200 IFMs of each genotype were assayed in two biological replicates of 100 IFMs each. Three to five technical replicates were performed from each biological replicate.

Figure 11



3.3.4 Decreased expression of *Mettl3* causes defects in alternative splicing and isoform expression of Titin-like protein Projectin

To validate the defects in exon usage we detected in our mRNA-Seq data, we performed semi-quantitative RT-PCR experiments. Here we were especially interested in possible isoform switches. Although this part of the study was not yet completed when this thesis was written, there was already one particular result complete enough to be presented in this work. Projectin, encoded by the *bent* (*bt*) gene, is a large, Titin-like protein which is associated with the intermediate filaments and contributes to the stiffness of IFM [61]. In *Drosophila* there are five annotated splice isoforms of this protein, including a short version with a short PEVK domain found exclusively in IFM and long versions that include additional PEVK domain exons which are associated with tubular muscles [67]. It has been shown that expression of long Projectin isoforms in IFM results in reduced stretch activation and altered IFM kinetics [68]. Newer publications show, that a knockdowns for *bent* are lethal [47]

We designed forward and reverse primers that span the alternatively-spliced PEVK region of *bt*, from exon 14 to exon 25 of that gene. The short isoform of *bt* contains exons 14-16-25 and has an RT-PCR product with a length of around 181 base pairs. The long isoforms of *bt* expressed in adult flies, on the other hand, contain additional exons including 14-15-16-17-18-20-21-25 and produce a RT-PCR products that are 800 and 900 base pairs long (Fig.12 A). In IFM from control flies, we could only detect the band for the short isoform at around 100 base pairs (Fig. 12 B).

This band was also visible in the *Mettl3* mutant and the knockdown samples, but in addition we saw a band at 800 base pairs and a prominent band at 900 base pairs (Fig. 12 B). Quantification of the data revealed a significant decrease in the use of the short isoform in both mutant and knockdown IFM, and a corresponding increase in the use of the longer, tubular isoforms (Fig.12 C). These results show that decreased expression or loss of *Mettl3* results in the expression of tubular muscle specific isoforms of *bt* in IFM, which is not physiological.

This shows that *Mettl3* and hence the m6A-pathway is involved in the correct splicing of *bt* in IFM, and further identifies a mechanism that underlies the flight defect observed in *Mettl3* mutant and RNAi knockdown flies.

Figure 12. Mettl3 controls an isoform switch in Titin-like Projectin.

(A) Plot showing the exons in the PEVK region of Projectin (*bent*, *bt*). Red arrows stand for the primers used in this experiment. The short IFM isoform of *bt* contains exons 14, 16 and 25. The long, tubular isoforms contain exons 14-15-16-17-18-20-21-25.

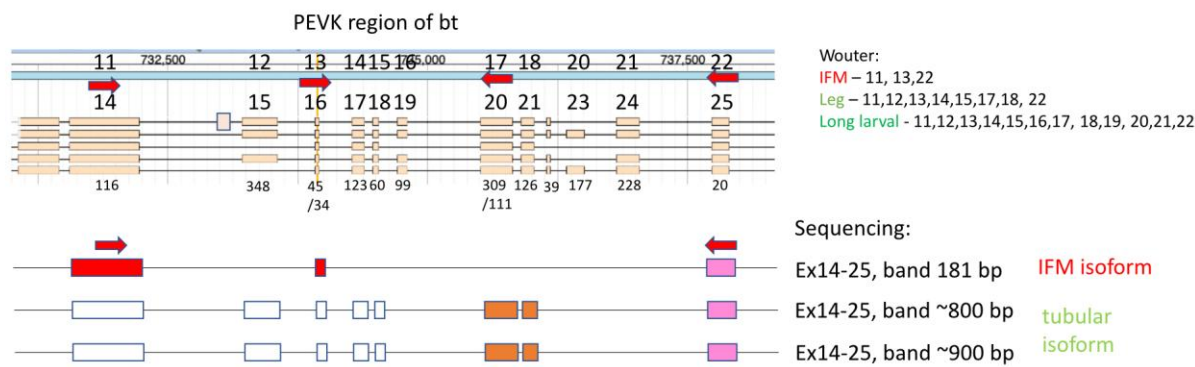
(B) Gel image of the PCR result with primers for exon 14-25 for dissected IFMs from wildtype control, Mettl3 mutant and Mettl3 knockdown. Wildtype control only shows one band at around 100 base-pairs, whereas the mutant and the knockdown probes show an additional band at around 900 base-pairs. For those experiments 200 IFMs from flies of each genotype were assayed in one replicate.

(C) Plot quantifying the isoform use from the data obtained in B. Significance determined by ANOVA with posthoc Tukey, n.s. = not significant; *, p-value < 0.1; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

Figure 12

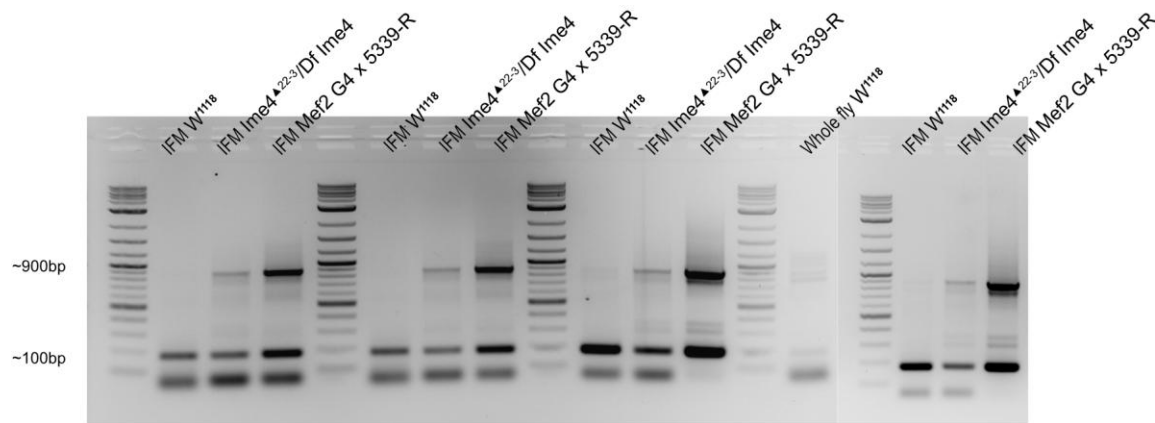
A

Analysed Isoforms



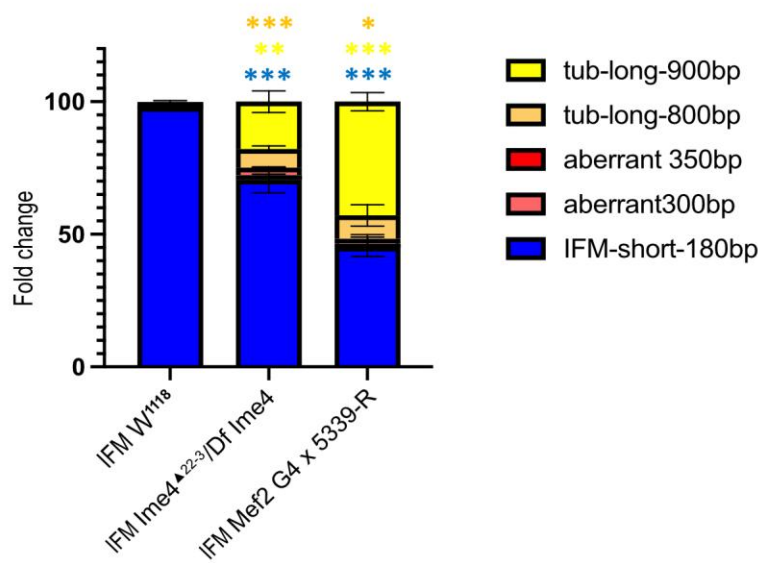
B

RT-PCR gel for exon 14-25 primers



C

bt Ex14-25 isoform use



4. Discussion

In this study, we identified a novel function for the m6A pathway in *Drosophila* indirect flight muscle. We show by tissue-specific RNAi and tissue-specific rescue that Mettl3 has an intrinsic function in muscle, as well as a function in the IFM motor neurons. Our proteomics and mRNA-Seq data reveal changes in expression of many muscle related genes. We confirm changes in expression of Act88F, Fln, Unc-89 (Obscurin), and Projectin (*bent*) using GFP-tagged Fosmid reporters or RT-qPCR. We moreover confirm an isoform switch from short to long Projectin isoforms that causes flightlessness. The data presented here thus extend our understanding of the role of the m6A pathway and Mettl3 in muscle and demonstrate the conservation of Mettl3 pathway from flies to mammals.

4.1 Flightlessness of Mettl3 mutants is not a purely neuronal phenotype

Previous studies have suggested that the flightless phenotype, impaired climbing ability and held-out wings observed in Mettl3 mutants are of a neuronal nature [26, 57]. In this study we found that the loss of Mettl3 in mutant flies indeed leads to over branching and axon overgrowth of IFM motor neurons (Fig. 9). We further show increased mRNA levels coding for the proteins Bruchpilot, Futsch and Spas, which are key synaptic components [64-66] (Fig. 11). These data are consistent with previous studies in *Drosophila* larvae that showed over branching and increased bouton formation in Mettl3-mutant sensory neurons [50, 69]. Further, mis-splicing of *trio* leading to an imbalance in Rac and RhoGTPase activity has been implicated in axon branching defects in class IV sensory neurons in Nab2 mutants with aberrant m6A methylation [70]. Future experiments will be necessary to determine if Mettl3 activity helps promote axon pruning or alternatively limits axon branch formation in both the larval and adult peripheral nervous system. This data indicates that the m6A pathway is involved in the normal development of the adult nervous system in *Drosophila*.

Our data demonstrate that Mettl3 activity in both the muscle and neurons contribute to observed behavioural defects in Mettl3 mutants. Using tissue specific RNAi, we showed that flies performed motor tasks like climbing worse when

Ime4 was knocked down specifically in neurons, but climbing was not as strongly affected when Mettl3 was knocked down in muscle (Fig. 4 B). The same tendency was also observed for the held-out wings phenotype (Fig. 4 C). By contrast, loss of flight ability was more pronounced with muscle-specific knockdown of Mettl3 than with neuronal-specific knockdown (Fig. 4 A). One possible explanation is that fibrillar IFM, unlike other muscle-fibre types like tubular muscles, are stretch-activated, asynchronous, and can oscillate at high frequencies autonomously once activated [71]. While fibrillar IFM may require neuronal activity to initiate flight, it is able to contract independent without sustained neuronal input. Tubular muscles, like leg or jump muscle, are synchronous and their contraction is dependent on continuous and sustained neuronal input. This type of relationship between muscles and motor neuron function may be reflected in different human disorders, for example facioscapulohumeral dystrophy (FSHD), oculopharyngeal muscular dystrophy, or limb-girdle muscular dystrophy, where molecular characteristics of different sets of muscles make them more susceptible and show differential levels of involvement in the disease [72-74]. Taken together our data show that behavioural defects such as flight involve Mettl3 function in both neurons and muscle.

4.2 Mettl3 is required in muscle to regulate sarcomere dimensions in IFM

Recent studies in mammals have identified a function for Mettl3 in regulation muscle hypertrophy [75], in cardiac development [76], and in myoblast division and differentiation [77]. Our data demonstrate that Mettl3 also plays an important role in *Drosophila* indirect flight muscle, illustrating a conserved function for the m6A pathway in muscle development. Our confocal data revealed altered sarcomere structure in Mettl3 mutant IFM, where the sarcomeres were significantly longer in the mutant than in the wildtype control (Fig. 1, I1-J4). Sarcomere dimensions were also altered in the muscle specific knockdown and appeared either too be too long or too short (Fig. 4 D1-G4).

These data suggest that misregulation of Mettl3 affects sarcomere length, and may lead to an imbalance in isoform-specific splicing leading to hypercontraction of the muscle fibres. The appearance of zebra-bodies within the sarcomeres indicates abnormal thin-filament assembly or stability. Notably those changes in the sarcomere dimensions could not be found in the neuronal specific

knockdown, and thus must derive from loss or depletion of Mettl3 within the muscle itself.

Also, the circumstance, that this cellular defect can be rescued tissue specifically as we have shown again highlights the muscular nature of this cellular phenotype.

Taken together also this part of our experiments showed once again, that the m6A-pathway is involved in the natural development of working muscle fibres.

Recent studies described, that in mice, Mettl3 is involved in the myostatin pathway on a posttranscriptional level. This pathway is critical for the control of muscle size and growth [75]. Also in mice, Mettl3 is furthermore involved in proliferation and differentiation of muscle stem cells. Hence it controls myogenesis and regeneration of mammalian muscle tissue [78, 79]. In other studies, it is stated, that Mettl3 suppresses muscle specific miRNAs in C2C12 myoblasts and plays an important role in their differentiation [31].

4.3 Mettl3 and the m6A pathway regulate alternative splicing in IFM

When we performed antibody staining for Mettl3, we could see a clear nuclear signal in IFM, suggesting Mettl3 may play a role in the nucleus. Our RNA sequencing from dissected IFMs revealed many genes coding for muscle related proteins that are changed in the Mettl3 mutants. Some of these are changed at the level of gene expression, while others displayed differential exon use in muscle related genes. This data indicates that Mettl3 may play a role in the nucleus in muscle tissue to regulate alternative splicing. Mettl3 has also been reported to regulate splicing during germline development, notably controlling sex differentiation through regulation of sex-specific splicing of *Sxl* [26]. Mettl3 in mammals is also reported to regulate alternative splicing. For instance, it has been published, that Mettl3 and its m6A mark are involved in alternative splicing events in pig skeletal muscles. Here they could contribute to the differentiation between oxidative and glycolytic muscles [80]. Apart from alternative splicing in muscle tissue it has also been suggested, that Mettl3 also regulates breast cancer-associated alternative splicing switches [81]. Furthermore, Mettl3 seems also to be involved in mammalian fertility due to alternative splicing. It has been found out, that Mettl3 regulates spermatogonial differentiation and meiosis in mice [82].

Our RT-PCR experiments moreover showed an isoform switch of *bent*, which encodes the Titin-like protein Projectin and is required for physiological function of IFM in *Drosophila* [61]. There are multiple splice isoforms of *bent*, but the distinction between long and short isoforms is of physiological relevance. Tubular muscles express long isoforms of Projectin, while IFMs express short isoforms which are thought to contribute to the high passive stiffness of IFM tissue. Short Projectin isoform expression is also essential for stretch-activation and cannot be compensated by longer isoforms [67, 68]. It is likely that this isoform switch alone strongly contributes to the flightless phenotype in mutant as well as in muscle specific knockdown flies. Muscles are a highly complex and organized tissue; thus, they are sensitive to wrong isoforms.

4.4 mRNA transcript-level changes in Mettl3 mutant IFM are reflected in the proteome

Our data showed corresponding changes on the mRNA and proteomic level, likely capturing changes both on transcriptional level as well as any effect on mRNA stability or translation. Proteomics analysis revealed that many peptides and hence proteins are changed in the mutant background. Immunofluorescence microscopy of GFP-tagged Fosmid reporter lines confirmed a significant decrease in expression of muscle associated proteins like Myofilin and Fln, which were also downregulated in mRNA-Seq and proteomics data from muscle specific Mettl3 knockdown IFM. Furthermore, we could show shifts in expression of specific categories of proteins, such as mitochondrial or sarcomere related proteins. For example, expression of sarcomere related proteins as well as synaptic proteins increased more strongly on the proteomics level than on the gene level. This effect could be explained by the function of the m6A-pathway in translation [22]. In fact, many m6A target sites have been identified within 5'-UTRs of genes in both vertebrates and *Drosophila* [22, 27]. Methylation in this region could be read by YTH reader proteins associated with translation complexes, NMD machinery, or mRNA trafficking, and either inhibit or promote the eventual translation of a particular protein [83].

All the proteins analysed here by Fosmid reporter lines were in fact previously identified as direct-targets of the m6A-pathway by meRIP [29]. In future experiments that are beyond the scope of this work, it would be interesting to take a

closer look into the translation process itself, for example, to pull down ribosomes in mutant and wildtype flies and analyse which mRNAs get in fact translated. This would increase our insight into potential cytoplasmic roles of the m6A pathway in IFM.

One of the genes misregulated in our data illustrates the potentially complex relationships between alternative splicing, proteomics, and Mettl3 function. The GFP-tagged Fosmid reporter line for Myofilin showed a decrease in expression, but both RT-PCR and proteomics showed quite the opposite, an increase of Myofilin expression. A check of the position of the GFP tag itself showed that it is located just before the 5'-UTR of *Mf* exon 1 and is isoform specific. *Mf* encodes 17 transcripts, only 5 of which are annotated to use this particular termination (Flybase). RT-PCR also showed that in the mutant background, there seems to be yet another isoform switch in *Mf* expression from the exon1-exon3 event (which would be the GFP-tagged isoform) to the exon1-exon2-exon3 event (which would not include the GFP-tag because exon2 encodes a stop codon upstream of the GFP). When we looked for individual peptides within our proteomics data, we found one which covered the exactly the splice junction area between exon2 and exon3 which was significantly upregulated in Mettl3 RNAi as compared to control IFM in the proteomic analysis. Thus, we saw a decrease with our Fosmid reporter approach but an increase in expression with our other two assay methods. This highlights how apparent inconsistencies between different assays for gene and protein expression can reveal biological regulatory mechanisms impacted the Mettl3 activity and m6A pathway function.

4.5 Conclusion

The results discussed in this thesis lead us to the conclusion that the m6A-pathway is indeed involved in muscle as well as neuronal tissue in *Drosophila*. The flightless phenotype of *Mettl3* mutant flies, based on our data, results from IFM defects including isoform switches of key sarcomere components, altered sarcomere morphology, and possible mitochondrial alterations. The flightless phenotype is further exacerbated by over branching of IFM motor neurons. Our data

therefore highlight a role for Mettl3 and m6A modification in both tissues, and provide an example how tissue-specific phenotypes synergize to produce organism-level behavioural defects.

There are several limitations of our study, and a lot of future experiments are conceivable. While some analysis of the sequencing and massSpec data was completed, the data still needs to be fully analysed and to be examined in the context of our existing work. This may reveal additional signatures or trends in the data that are not discussed in this work. We have tested a number of potential targets by RT-PCR, but a more extensive list of targets could be tested by RT-PCR. The differences in mRNA versus protein level expression for the selected targets could also be assayed by Western Blot, to better define the connection between RNA and protein expression levels. This would also likely shed more light on which targets are regulated at the level of translation or mRNA stability in the cytoplasm, versus those targets whose alternative splicing is regulated in the nucleus. There are also a lot more genes with potential isoform switches to be confirmed and further investigated. Here it would be particularly interesting to analyse also mitochondrial genes. Ongoing work in the Spletter lab will seek to address some of these open questions regarding Mettl3 and m6A pathway function in IFMs.

The data presented here extends our knowledge about the m6A pathway in muscle tissue. Our work identifies important roles for Mettl3 in regulating alternative splicing of structural genes as well as potentially regulating development shifts in expression through regulating the stabilization of select transcripts during myogenesis.

Although our findings were made in a model organism, *Drosophila* is an excellent model organism to understand disease also in humans due to genetic conservation [43]. Existing reports of Mettl3 function during hypertrophy and cardiomyogenesis also indicate an important function for Mettl3 during growth and remodelling of human muscle [84]. Further, the m6A pathway has been reported to play an important role in heart diseases and muscle dystrophy in humans [43], indicating that our findings are disease relevant. Our work contributes to a better understanding of Mettl3 function in muscle, and possibly may one day contribute to development of new therapy approaches for diseases affected by abnormal m6A pathway activity.

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Appendix

Mass Spec results for sarcomere specific proteins

Gene name	P value (log)	Difference Mutant vs. Wildtype	LFQ intensity Mutant 1	LFQ intensity Mutant 2	LFQ intensity Mutant 3	LFQ intensity Mutant 4	LFQ intensity Wild-type 1	LFQ intensity Wild-type 2	LFQ intensity Wild-type 3	LFQ intensity Wild-type 4
wupA	1.51298761	2.68943548	27.3600121	26.9054089	24	28.492321	24	24	24	24
parvin	1.77046056	2.61506367	27.5074863	27.5694981	27.3476486	27.2152119	24	24	24	27.1795902
TpnC41C	0.7578083	2.4800601	31.2266483	29.4435387	32.0211525	31.3304195	29.9411182	30.4016171	24	29.7587833
TpnC25D	0.74669054	2.27372122	28.3733501	28.0682011	24	28.6954117	24	28.042078	24	24
Mhc	1.09561062	2.19587088	30.3305264	34.0319862	30.5992127	32.3605042	29.0900269	30.3146248	28.2570095	30.8770847
Zasp52	4.62926254	1.94394112	25.9016399	25.5867481	26.3906231	25.8967533	24	24	24	24
Ilk	1.25729165	1.76131821	27.3548946	26.8325558	26.5403042	27.241909	26.7124043	24	24	26.2119865
TpnC73F	0.72322748	1.6871686	28.9566803	29.7473202	28.1580296	28.7267647	27.9415093	28.8753433	24	28.0232678
Lmpt	0.44805845	1.65525389	29.1234913	30.6242714	31.286314	29.2221413	31.414175	29.327488	24	28.8935394
Mlp84B	1.11417938	1.49654436	27.6360951	27.689991	27.4708824	27.3143215	26.3055134	27.1033955	26.7162037	24
Tm1	0.33180333	1.46251917	29.7269421	28.6643124	24	28.891571	28.6656342	24	28.7671146	24
Zasp66	2.84068827	1.22836208	31.758276	31.8956928	32.065033	32.3142204	30.6667023	30.3394737	31.2229691	30.8906288
rhea	4.83667004	1.21154213	28.5343781	28.8431702	28.6778812	28.7918491	27.4402351	27.5694256	27.3468037	27.6446457
Pax	4.18310106	1.15101767	27.3530445	27.5816155	27.2571888	27.4438744	26.0152721	26.4555702	26.2099056	26.3509045
tmod	3.46005639	1.10278082	29.5449524	29.3693619	29.4098129	29.3129177	28.2008362	27.9823418	28.6607475	28.3819962
Act57B	0.71783921	0.99925709	32.7331963	30.2255001	32.4118271	32.929718	31.5007114	31.5442066	30.497242	30.7610531
Mf	1.8613884	0.95730019	34.5749741	33.5117111	34.4034348	34.1855621	33.3048401	32.8358078	33.5603561	33.1454773
Prm	2.7592242	0.85669041	33.095974	33.4270477	33.3406944	33.5475426	32.6708832	32.165638	32.7256584	32.4223175
Msp300	4.40567066	0.80465365	31.6042595	31.8246422	31.5797005	31.5122032	30.8440094	30.783083	30.9098492	30.7652493
Act79B	2.35551174	0.73891258	33.4572105	33.5459328	33.8683891	33.476532	32.7450295	32.9279366	33.1808739	32.5385742
up	0.80416316	0.57448864	32.4143753	31.5676498	32.9991531	32.9950981	31.678503	32.2054825	31.9348412	31.8594952
Mlc1	0.87400868	0.55380726	34.9654656	36.0806198	35.4628601	35.2354927	34.7382202	34.4151955	34.9421387	35.4336548
if	0.98049614	0.54572678	27.2111321	27.8709717	27.5187092	26.817997	26.3359604	26.9244499	27.1823406	26.7931519
beta-Spec	2.38985472	0.52810574	33.3665886	33.5858688	33.2928276	33.1237831	32.7315598	32.8815613	32.6748047	32.9687195
Mhc	1.34324028	0.4956274	35.5319176	36.1796455	35.4872971	35.2856445	35.1501312	35.0441704	35.1014557	35.2062378
Tm1	0.52802144	0.49130011	31.0297985	29.3812332	30.2831707	30.9942226	30.0758438	30.108738	29.3745327	30.1641102
alpha-Spec	1.77057409	0.4322772	31.0447254	31.014183	30.5817223	30.8769398	30.2826176	30.6559963	30.3832951	30.4665527
Tm1	0.15721458	0.41906881	26.7662296	24	27.0068874	24	24	26.0310326	24	26.0658093
Mlp60A	0.0961933	0.40029097	24	29.0297852	24	24	24	24	24	27.4286213
up	0.92928163	0.3952961	33.0500565	33.5500679	33.4509583	33.866272	33.0392761	32.8220596	33.0082321	33.4666023
stv	0.33472192	0.37746954	26.08671	27.4780006	25.2736969	26.0848885	26.0486202	25.7718296	25.4689884	26.1239796
Prm	2.39533037	0.34732246	33.3202591	33.4935722	33.1691208	33.3826523	32.9861908	32.9318161	32.9601669	33.0981407
chic	0.11272922	0.32309198	26.7569733	27.0265427	24	24	24	26.4823151	26.0088329	24
zormin	0.89645061	0.32234573	28.9886665	28.6743164	28.8960533	28.3875122	28.4361095	28.1462765	28.3371773	28.7376022
Act88F	1.81844623	0.3030138	36.428875	36.8015976	36.5692139	36.4900208	36.2485924	36.1701965	36.3238182	36.3350449
Mhc	0.06931339	0.30111361	28.2072735	28.7690697	24	28.7103348	28.0140362	28.4003964	24	28.067791
fln	0.97460797	0.29766369	34.6609764	35.1147766	34.9319191	34.6407585	34.7557831	34.2423248	34.5697212	34.5899468
sls	2.07208876	0.27155018	32.3001556	32.1935463	32.3672905	32.3609505	32.0516777	31.8755493	32.1541824	32.0543327
wupA	1.0094717	0.26801872	34.5442658	34.543747	34.9414406	34.6210213	34.2237473	34.3665237	34.6774521	34.3106766
Strn-Mlck	2.55329331	0.25101185	34.3533058	34.2825089	34.3728905	34.4460373	34.1325455	33.9971542	34.1578293	34.1631661
Mhc	1.28925616	0.21814346	36.1748161	36.1829033	36.3443031	36.1956825	36.0032196	35.7832451	36.152874	36.0857925
TpnC4	0.62040315	0.16947651	33.9413109	34.3095245	34.2397652	34.4022293	33.8347931	34.2322083	34.1092987	34.0386238

Gene name	P value (log)	Difference Mutant vs. Wildtype	LFQ intensity Mutant 1	LFQ intensity Mutant 2	LFQ intensity Mutant 3	LFQ intensity Mutant 4	LFQ intensity Wild-type 1	LFQ intensity Wild-type 2	LFQ intensity Wild-type 3	LFQ intensity Wild-type 4
SERCA	0.52346419	0.16900349	33.5782394	33.1299667	33.6185074	33.5922318	33.1139946	33.2047081	33.5179405	33.4062882
Zasp52	0.04269839	0.14631558	27.7378273	27.3778248	27.2200203	24	27.2879772	27.0078507	24	27.4545822
Actn	1.29014151	0.13796711	33.5685349	33.5653572	33.5198135	33.5944595	33.5074616	33.2636032	33.471817	33.4534149
hts	0.3256251	0.11687899	31.3484726	30.8405609	30.8203888	30.8715153	30.8798637	30.9980621	30.6085835	30.9269123
Mlc2	0.23404638	0.10820293	35.0122604	34.4397965	35.2792931	35.1119041	34.852478	34.8746338	34.7432442	34.9400864
Mp20	0.11518868	0.08267546	28.7853451	28.5383816	28.2122459	28.8106308	28.7354412	28.6277371	27.8317719	28.8209515
TpnC47D	0.0197847	0.06585693	24	24	24	27.3286018	24	27.0651741	24	24
bt	0.20543382	0.0610199	32.9348564	32.5974159	33.0284996	32.9262581	32.86129	32.6461372	32.9766121	32.7589111
Zasp52	0.12783821	0.03174591	33.0956574	33.1485863	33.1708832	33.1031189	33.1427269	32.8666229	33.3082352	33.0736771
wupA	0.02508881	0.02729464	28.0809593	27.8919182	27.8490009	27.2088089	27.0263329	27.4947262	28.542408	27.8580418
Tm1	0.02514611	0.01014137	34.834507	34.7028008	35.1524773	35.0973015	34.8493118	34.7361527	35.0514832	35.1095734
up	0.00476567	0.0014286	34.7720032	34.5107651	34.6826935	34.9037399	34.7827339	34.5462799	34.7537918	34.7806816
Tm1	0	0	24	24	24	24	24	24	24	24
tmod	0	0	24	24	24	24	24	24	24	24
bt	0	0	24	24	24	24	24	24	24	24
Msp300	0	0	24	24	24	24	24	24	24	24
Mhc	0	0	24	24	24	24	24	24	24	24
SERCA	0	0	24	24	24	24	24	24	24	24
Lasp	0	0	24	24	24	24	24	24	24	24
Tm1	0	0	24	24	24	24	24	24	24	24
Zasp66	0	0	24	24	24	24	24	24	24	24
Zasp67	0	0	24	24	24	24	24	24	24	24
RyR	0.00191539	0.00952959	28.669323	24	24	24	24	24	28.7074413	24
Arp3	0.01168319	-0.022892	24	24	24	25.8204823	24	24	24	25.9120503
Mlp60A	0.17775536	0.06187534	30.3823681	30.3303127	30.6743832	30.310297	30.4260235	30.2266483	30.7243595	30.567831
Unc-89	0.34623561	0.07378006	31.6434498	31.9489346	31.7465115	31.6194687	31.9258862	31.6734982	31.8168068	31.8372936
RyR	0.39761557	-0.0779953	30.7166939	31.0442696	30.7505493	30.7290688	30.8904114	30.7914925	30.9639587	30.9067001
Tm2	0.22894723	-0.1106863	34.8245392	34.1449776	34.8105774	34.9570122	34.6150017	34.7721977	34.9114838	34.8811684
Zasp67	0.72191525	0.19101906	31.6340008	31.7323875	31.779604	32.1798821	32.0719528	31.9608555	32.1311493	31.925993
CanB2	0.37242398	0.19749403	27.5687008	26.7242947	27.2701721	27.4574814	27.6620731	27.6927204	27.1470528	27.3087788
Strn-Mlck	0.95497691	0.22316313	28.6012325	28.0923481	28.487154	28.3068256	28.5066547	28.6306896	28.7003479	28.5425205
Bsg	0.37537575	0.27148151	30.1554489	28.9677143	30.0240612	30.0220776	29.8059502	29.8769531	30.4720841	30.1002407
Chd64	0.87177602	0.42488623	29.145134	28.5812206	28.2391796	28.7222099	29.3037586	29.4153042	28.9329777	28.7352486
Prm	0.13128421	0.42523146	27.6539669	29.0485878	29.2169476	24	27.7517471	27.6787548	28.2762203	27.9137058
Mf	0.15222329	0.47282744	24	27.5057449	24	26.9582577	24	26.7423973	26.3008423	27.3120728
Lmpt	2.44925156	0.48757839	29.0625515	29.2636719	29.2103424	29.0858288	29.6166153	29.4501629	29.8986511	29.6072788
cpb	0.21558928	0.50790167	26.7480412	24	26.1985798	24	25.942728	26.709383	24	26.3261166
Tm2	0.61502248	0.55331516	33.6229858	32.0189972	33.9242401	33.4950028	33.7064591	33.874176	33.8821259	33.8117256
Hsp83	2.11661594	0.75894737	29.7003155	30.5506878	30.1749802	29.8349228	30.8393612	30.8845291	30.7356987	30.8371067

Gene name	P value (log)	Difference Mutant vs. Wildtype	LFQ intensity Mutant 1	LFQ intensity Mutant 2	LFQ intensity Mutant 3	LFQ intensity Mutant 4	LFQ intensity Wild-type 1	LFQ intensity Wild-type 2	LFQ intensity Wild-type 3	LFQ intensity Wild-type 4
flr	0.55255618	-0.9004159	26.8129883	24	27.3387508	26.7645855	27.1547985	27.043747	27.347311	26.9721317
tn	3.56471927	0.95894671	26.3828945	26.8389397	26.5098591	26.8574486	27.7176418	27.5958386	27.5092239	27.6022244
unc-45	0.81129848	-0.9700861	25.0115433	27.7730389	26.5087414	26.7423973	27.8578053	27.6557407	27.075716	27.3268032
mys	0.29846376	1.01912737	27.786562	24	24	27.5277576	28.0493164	27.3235416	24	28.017971
Msp300	0.86266266	-1.2848444	24	24	24	24	24	26.311779	26.8275986	24
Dg	1.59439797	1.46490765	24	24	24	24	26.1757145	24	25.9238167	25.7600994
sls	0.70251925	1.63236046	28.3973389	24	28.4722385	28.6244984	28.7570038	29.0233746	29.0625515	29.1805878
sals	2.18082398	2.52025461	26.506321	25.8986454	26.4153194	24	28.4113483	28.4822006	27.60569	28.4020653

Significant probes are written in bold letters

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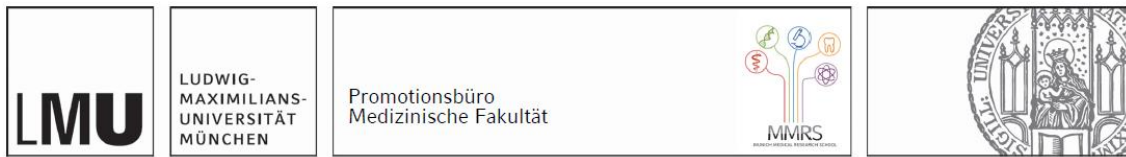
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I had a really good time and will miss you all!

Confirmation of congruency**Eidesstattliche Versicherung**

Heemken, Jakob

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Mettl3 is required cell intrinsically in both muscle and neurons for development of functional flight muscle

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Publications

A candidate RNAi screen reveals diverse RNA-binding protein phenotypes in *Drosophila* flight muscle.

(Cells, 2021 by Kao, Nikonova, Chaabane, Sabani, Martitz, Wittner, Heemken, Straub, Spletter; <https://pubmed.ncbi.nlm.nih.gov/34685485/>)