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assembly and muscle growth in *Drosophila*

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Declaration of Authorship

I hereby declare that this thesis is my own work except when otherwise indicated.

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Christoph Langer

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1. Introduction

A complex muscle network enables higher organisms to perform deliberate movements, for example to catch prey and to escape from predators. For the correct execution of these behaviors, which decide over life and death, it is crucial that all muscles form at the right place and grow to the correct size.

In recent years we have learned much about how cells become committed to myoblast fate^{1 2} and thus to form muscles, nevertheless to date a systematic study of how muscles assemble and grow their contractile elements, the sarcomeres, so regularly is still lacking.

Drosophila is an optimal model system to study muscle growth and sarcomere assembly embryonic and larval muscles are built by single syncytical cells, which grow during the course of the larval development up to a thousand times in volume whilst staying transparent. This not only allows us to observe the muscle and sarcomeric structure with live markers in intact specimen but together with the powerful tool of the genome-wide inducible RNAi library³ it is possible to systematically dissect the molecular and cellular processes instructing muscle and sarcomere development.

1.1. Muscle development in *Drosophila*

Recent genome sequencing and annotation projects have proven vast similarities in the development of *Drosophila* and vertebrates at the molecular and genetic level. In particular it has been shown that 60% of *Drosophila* proteins share similarities with human proteins⁴ while this model organism has a far simpler genome composition, and the availability of a wide range of fast and low cost experimental techniques.^{5 6}

The following chapter will give a brief overview of muscle and sarcomere development in *Drosophila* and show how similar it is to ours.⁷

1.1.1. Muscle pattern of the *Drosophila* larva

Like in all higher organism, somatic musculature in a fly is very precisely arranged in a complex network, thus *Drosophila* larva has a stereotypic pattern of 30 muscles (or myofibers) per abdominal hemi segment. All of these larval muscles share common general properties for example contractibility, but each muscle has its own unique elements such as identifiable by size, shape, orientation, epidermal attachment and innervation.

To easily describe an individual muscle I will use the common lettering system reflecting topology used in Ref. 8, which labels according to muscle position (dorsal, lateral, ventral) followed by orientation (acute, longitudinal, oblique) followed by a number. Our focus was the large longitudinal muscles, which extend over approximately 30% of the circumference of the larva⁹. They consists, as typical in insects,¹⁰ of an outer layer (dorsal oblique (DO) stretching from antero-ventral to postero-dorsal) and an inner layer (dorsal acute (DA) muscles, which run from antero-ventral to postero-dorsal). (Figure 1.)

Every muscle is a single syncytical cell created by fusion, similar to the vertebrate primary fibers. Further each of these muscles is seeded by a single predecessor myoblast called the founder cell¹¹, to which, depending on the muscle, a distinct numbers of surrounding so-called “fusion competent cells” fuse.¹² Recent research^{13 14 15 16} support the notion that founder cells contain the “identity” information, which means, that: “each founder cell contains the unique information to direct the morphogenesis of a specific muscle”.²

1.2. Muscle formation and growth

Similar to vertebrates, *Drosophila* cardiac, somatic and visceral muscles all arise from the mesoderm.¹² I will strictly focus in this section on the somatic muscles since these have been the sole focus of my later research. (Figure 2.)

1.2.1. Specification segmentation of the mesoderm

Alike vertebrates, *Drosophila* starts out with a rather uniform mesoderm, which is then subdivide into segmental units and diversified by rather similar signals secreted from specific parts of the ectoderm: Dpp,^{17 18} which is related to the vertebrate TGF-beta family, wingless (Wg)^{19 20}, which is a member of the Wnt family, and hedgehog (Hh)²¹, which is related to Shh. All cells of the mesoderm layers share the common feature that they express twist (twi)²², and its targets heartless (htl), snail (sna), tinman (tin),^{23 24 25} and the myocyte enhancer factor 2 (mef2)^{26 27 28}.

The patterning along the dorsal ventral axis is prevalently determined by Dpp. It acts by maintaining dorsal expression of genes like tin and repressing ventrally expressed genes like pox meso (poxm).^{17 18} The anterior posterior division is mediated by the activity of the polarity segment genes even-skipped (eve) and sloppy-paired (slp) as well as the ectodermal signals Hh and Wg.²⁹ (Figure 3.)

The main goal of this subdivision process described above is to modulate the characteristic high uniform level of Twist, which is needed to specify the mesoderm. Slp with the help of the Wg pathway upregulates Twist expression^{19 20}, whereas Eve and Hh inhibit Twist expression mainly indirectly by interfering with Slp function.^{19 21} Notch is an additional crucial player in this subdivision.³⁰

The Eve/Hh domain gives rise mainly to the progenitors of the visceral mesoderm and fat body^{19 21 31}, while the Slp/Wg domain gives rise to the somatic muscles and the heart.^{19 20} (Figure 3.)

Although Twist, whose function is most similar to that of the vertebrate MRF family (containing MyoD, Myf5, myogenin and MRF4)³², sets the shift towards muscle development in motion, further factors are required. The Myocyte enhancer factor-2 (Mef2), which is member of the MADS family is one of them.²⁸ Originally identified and characterised in mammalian cell culture, its in vivo function especially its role during somatic muscle development, was most clearly addressed and established in *Drosophila*^{28 33 34 35}. Since it only has a single Mef2 gene in contrast to the four mef2 genes in mice with overlapping

patterns.³⁶ Mef2 binding sites^{37 38} are found in almost all known muscle specific genes, and it is known to synergistically act together with MRFs.³⁷

Mef2 is not only expressed in somatic muscles but also in heart and smooth muscles, in particular it has been identified as the first gene which controls the differentiation of multiple muscle cell types²⁸ at different levels are required for specific parts of muscle development.³⁹ In *Drosophila* Mef2 expression is activated by Twist^{40 41} and its expression is maintained by a direct positive feedback loop, in which Mef2 activates the Mef2 gene.⁴² A similar transcriptional loop with an additional intermediate player myogenin is evolutionary conserved in vertebrates.^{43 44 45 46}

1.2.2. Muscle specification & identity

In the Slp/Wg domain, at around 5 hours (after egg laying) clusters of equivalent cells appear that express the bHLH protein Lethal of Scute (L'sc). Inside these so called promuscular groups, the expression of L'sc is reduced to a single cell, and this is the so-called muscle progenitor cell.

Multiple signal pathways are needed during this specification step and the individual progenitor cell is progressively determined by a unique set of inter-cellular signals.¹⁴ This has been nicely summarised and intertwined integrative signalling networks are discussed in depth in ref. 2, As soon as progenitor cells are specified, they move closer to the ectoderm where they divide asymmetrically into usually two founder cells and thereby L'sc expression is lost.^{47 48} The same proteins that are involved during neural asymmetrical cell division have been shown to be involved also in the asymmetric division of muscle progenitors.^{47 48 49 50}

Inscuteable (Insc) delivers the positional information required for Numb segregation, which then represses Notch in one daughter cell.⁵¹ Currently there are two models how Numb inhibits Notch function, which are not necessarily exclusive. The two proteins linked to them are: 1) alpha-Adaptin⁵² and/or 2) Sanpodo (Spdo).^{53 54} Further players are: Partner of Numb (Pon) described in ref. 50 and Abstract (Abs) depicted in ref. 55.

Thus both intrinsic and extrinsic cues are necessary to specify fate of muscle founders, and the asymmetrical nature of progenitor division give rise to unique fates.^{13 16 21 49} Involved in this process are also 11 so called muscle identity genes (ref. 2). Most are transcriptional regulators which are expressed in different but sometimes overlapping founder cell subsets and are presumed to specify the fate of individual muscles and lead to partial or full muscle identity determination.

1.2.3. Myoblast fusion

Following specification myoblasts start the fusion program, which results in a common trait that the *Drosophila* muscles share with almost all vertebrates, namely that they are multinucleate syncytia. Many of the key players of this process have been identified in *Drosophila*^{56 57 58 59 60} due to powerful molecular genetic tools available.

The first step in this series of events, is that the “naïve” fusion competent myoblasts must identify and move to the founder cells, to initiate first contact and adhere. It is important to note that fusion is asymmetric neither fusion-competent cells nor founder cells fuse with themselves.²⁹

This first step is mediated by the members of the Ig-superfamily, sticks and stone (sns),⁶¹ dumbfounded (also called kine of irreC),⁶² and irreC (irregular-chiasma-C also called roughest (rst))⁶³ and regulated by a fourth fibronectin type III domain (fth).^{59 64 65} Their interactions lead to changes in both function and arrangement of the cytoskeleton. So far the founder cells are better understood and it's believed that Duf is linked to the Rac small GTPases⁶⁶ both via adapter proteins Rolling pebbles/Antisocial (Rols /Ants)^{67 68 69} and Myoblast city (Mbc)⁷⁰ as well as via the guanine-exchange factor named Loner and Arf6 GTPase.⁷¹ The asymmetry in fusion, is linked to molecular asymmetry: Sns and Hbs are selectively expressed only on the fusion competent myoblasts, Duf and its downstream partners Rols/Ants and Loner are only expressed in founder cells. All of the proteins mentioned above have relatives in vertebrates.⁶⁰

1.2.4. Muscle migration and attachment

Muscle migration is a three-step process ⁷², starting with migration of founder cells relative to each other, followed by the fusing of FCMs with the founder cells and the formation of polarized myotubes with a highly dynamic leading edge at each end. Similar to the growth cones of neurons, these tips seem to directly migrate towards a specific target cell (stripe expressing tendon cells ⁷³) and ends with the recognition of a specific attachment site and the establishment of a stable connection.

Little is however known, how myotubes actually locate their specific tendon cell targets. Current models suggest that distinct sets of surface or secreted proteins act as a molecular recognition system. Three molecular system have been so far identified. The Slit and Robo family receptors repel axons and myotubes away from the midline ^{74 75} and later attract some myotubes to their intersegmental attachment site ⁷⁶, derailed (drl) controls axon-routing across the midline ⁷⁷ and helps some myotubes selecting the right intersegmental attachment site ⁷⁸ and finally Kon-tiki (kon), which promotes directed migration as well as target recognition in a subset of ventral myotubes ⁷⁹.

Slit and Robo as well as drl have been originally defined by their role in axon guidance but not all guidance and recognition molecules are shared between neurons and muscles. Kon, which is a large transmembrane protein enriched at the myotube tips during targeting, has no effect on the nervous system. Kon interacts via a PDZ-binding domain with the cytoplasmatic adaptor Dgrip ⁸⁰ and seems to signal into an evolutionary conserved molecular pathway for muscle targeting ⁷⁹.

Integrin and its associated proteins are the crucial for stable attachment between the muscles and the tendon matrix and between the tendon matrix and the tendon cells ^{81 82} by forming so-called hemiadherens junctions. (=a junctions between the ECM and the actin cytoskeleton). Talin is involved in linking integrins to the actin cytoskeleton ⁸², just like ILK ⁸³, which mediates a subset of the interactions between integrins and the cytoskeleton in particular the attachment sarcomeric actin to the ends of the muscles.

1.3. The sarcomere

Up to now I have given a brief overview of all the delicate steps needed to form a fully intact muscle network but one major component is missing namely the sophisticated and highly ordered molecular machinery, that generates the power and function of muscles.

Characterisation of these muscle proteins, deploying diverse experimental approaches has been mainly focussed onto their functional properties. Little insight into the regulation, stability assembly and growth of this machinery is available. As stated by J. Vigoroux : “Despite much progress, the broad characterization of many of the mutants' effects has yet to uncover the mechanisms that regulate and dictate the assembly of the myofibril. “ in ref. 84.

1.3.1. Sarcomere structure and function

Sarcomeres are the smallest functional subunit of a muscle, these incredibly regularly assembled repetitive units of contractile proteins are the molecular machinery of the muscle and produce its force. Comprehensive and detailed reviews on this topic are ref. 84 and ref. 85 as well as ref. 86 and ref. 87 which point out the long tradition of genetic experiments on this topic carried out in *Drosophila*.

The key players have been very well conserved in evolution, from animals as simple as jellyfish up to vertebrates ⁸⁸ and these are those I would like to briefly describe in this section (Figure 4).

The main mechanism of force generation is rather simple and is done by two main components: the thin actin-filaments and the thick myosin-filaments, by sliding over each other and thereby shortening the sarcomere. Myosin is the molecular motor protein and produces the force in the so called Lymn-Taylor cycle.⁸⁹

Myosin starts in the rigor configuration,⁹⁰ binding of ATP weakens the binding of myosin to the filamentous-actin (f-actin) releasing it. The following hydrolysis of ATP causes (extension and displacement of the head along the actin fibre) the cocked conformation, which is the conformational state that is re-

quired for the following power stroke. Myosin-ADP-Pi then binds to actin and the dissociation of the inorganic phosphate (Pi) tightens the grip. This is immediately followed by the release of the ADP and the so-called power stroke, which changes the myosin back into its rigor shape and thereby pulls the molecule along the thin filament. This classical textbook knowledge has been updated ⁹¹ and suggests that rather by rolling of the myosin head during the power stroke, only the distal part of the myosin cross-bridge moves, acting like a swinging lever-arm. (=swinging lever-arm hypothesis ⁹²).

However further proteins needed to build this highly organised almost crystalline like arrays of sarcomeres. The beginning and the end of the sarcomere is by definition the so-called Z-disk, ⁹³ that is shared by neighbouring sarcomeres. ZASP ^{94 95 96} which organises the Z disk is activated by integrins and recruits alpha-actinin, ⁹⁵ which cross-links and anchors thin filaments to them by alpha-actinin. ^{85 93} On this end (plus end) these actin filaments are capped by CAPZ, on the other (minus) end this is done by Tropomodulin. Further Tropomyosin and Troponin are needed for the proper interaction of actin with myosin and controlled contraction. ⁹⁷ In the centre of the sarcomere is the so called M-line here the thick filaments (= essentially myosin bundles) are crosslinked and anchored and the long elastic protein Titin is a that connects the z disk with the thick filament. ^{98 99 100}

1.3.2. Sarcomere assembly

At the moment there are two rather recent reviews ^{101 102} that deal with the assembly and maintenance of sarcomere. Both emphasize the complexity of this process that leads to this remarkably regular machinery. So far most experiments have been done in cardiomyocyte cell cultures or if in tissue, were descriptive histological studies on an electron microscopic level. (As mentioned by Boateng et al. ¹⁰¹ in 2007 there is a great need for new in vivo models to study this dynamic process during development.

The latest ¹⁰² focuses on the role of integrin creating so called protocostameres, these early integrin adhesion sites which in turn nucleate alpha-actinin accumulation leading to the so called Z-body precursor to which the

early cortical premyofibrils are attached. Z-body¹⁰³ are membrane associated aggregates (which contain alpha-actin, titin and ZASP) and are believed to be the precursors of the Z-discs. This coincides with observations made, that first myofibrils are always observed close to the membrane^{104 105} and thus it is believed that sarcomere assembly begins at the periphery of the cell. These early precursors of myofibrils called premyofibrils¹⁰⁶ strongly resemble actin stress fibres, containing clusters of alternating patches of alpha-actinin and non-muscular myosin.^{107 108 109 110}

The model suggests that further maturation of these premyofibrils to the regular myofibrils, which occurs over the entire length of the muscle simultaneously,^{105 111 112} is activated by early contractile forces: probably due to the signalling of elastic proteins like titin together with thick and thin filament regulatory mechanisms.¹⁰² This model is briefly summarised in figure 5.

Addition of new sarcomeres during muscle growth is still a big black box.

1.3.3. Sarcomeric size control

Although larval muscles grow over ten fold in length during development, sarcomeres remain roughly the same size during development (data not shown). Since the length of thick filaments is fairly constant¹¹³ thin filaments seem to play the important role in determining the size of the sarcomere.^{113 114} Consequently their length is precisely specified to achieve optimal overlap of thick and thin filaments and thus contractility¹¹⁵.

As mentioned previously actin filaments are capped in muscles on both sides, it is generally accepted in this field that thin filament length is governed by the inhibitory function of Tmod on the pointed end of the actin filament.^{116 117 118}

In vertebrates the favored mechanism controlling this process was nebulin acting as molecular ruler and based mainly on the structural evidence and certain properties of this molecule.^{119 120 121 122} In the classic textbook “Molecular Biology of the Cell” by Bruce Alberts et al. nebulin is mentioned as one of the prime examples of this molecular ruler mechanism.

Only very recently, this has been proven wrong in ref. 123 and as thoroughly discussed in ref. 124: A nebulin-independent mechanism, based on pointed

end dynamics seems to primarily specify thin filament length. This fits well to the previous discovery of SALS (sarcomere length short), which seems to antagonize Tmod.¹²⁵

With the dismantling of this dogma, pointed end dynamics steps into a new lime light, how this process is controlled is still a big mystery. It seems as if this problem is more complex and evolutionary older than previously thought, which makes it a perfect candidate for a systematic approach in *D. melanogaster*.

1.4. Systematic genetic analysis of muscle morphogenesis and function in *Drosophila*

With the discovery of the RNA interference effect (an endogenous cellular effect that leads to degeneration of double stranded RNA and consequently selective destruction of messenger RNA with identical sequence patches [=the interference] and thus leading to gene silencing) a new powerful tool was added to the geneticians repertoire.^{126 127} I will not go into details here how the RNAi effect work, as this is nicely done by ref. 128 and ref. 129.

This allows us in a relatively easy fashion to target a specific gene to produce a loss of function mutant. This has been successfully used for genome wide screens in cell cultures.^{130 131 132} However the real advantage arises with the generation of in vivo inducible RNAi libraries³, it is now possible to use this reverse genetic method in a forward genetic manner to screen whole genomes, which allows us to immediately link a newly discovered phenotype to its correspondent gene.

Another great advantage is that this can be done in tissue specific manner through the usage of the GAL4-UAS driver system.¹³³ Especially since as previously described muscles consists of multinuclear cells which renders the classical clonal mutation unsuitable, since the efficiency is not high enough to eliminate the gene in gene from all nuclei of the muscle.

2. Results

The work during my diploma thesis contributed to following papers:

C. C. H. Langer*, R. K. Ejmont*, C. Schönbauer*, F. Schnorrer, P. Tomancak

In vivo RNAi Rescue in *Drosophila melanogaster* with Genomic Transgenes from *Drosophila pseudoobscura*. PLoS ONE (2010)

F. Schnorrer, C. Schönbauer, **C. C. H. Langer**, G. Dietzl, M. Novatchkova, K. Schernhuber, M. Fellner, A. Azaryan, M. Radolf, A. Stark, K. Keleman, B. J. Dickson

Systematic genetic analysis of muscle morphogenesis and function in *Drosophila*. Nature (2010)

In the following I will summarise my contributions to both publications. Figures or segments previously published will be referenced with ‡ for Langer et al. and ‡‡ for Schnorrer et. al..

2.1. Larval muscle screen

As previously discussed the VDRC library³ enabled us to perform a systematic muscle specific RNAi screen. Schnorrer et al. screened 17702 lines corresponding to 10462 genes, which represent 75% of the annotated *Drosophila* genome as described in ‡‡.

2.1.1. Larval screen design

To distil the relevant information out of this large number of genes, it is important to have an efficient primary screen. It consisted of two steps: a lethality test and for those surviving a flight performance assay, which is a fast and sensitive read out for muscle function.

As one would predict a large number of UAS-IR transgenes, when crossed with the pan muscle driver Mef2Gal4, resulted in lethality. In total 1963 genes were lethal (18.8%). From these lethals I focused on the embryonic and larval lethal genes (587) as this class should contain the genes involved in larval sarcomere function and assembly as well as muscle growth. Since one would expect disrupting these processes would have a severe impact on survival rate at a very early stage. (Figure 6)

Lethality is a rather ill defined phenotype and one would predict any of the so-called “house keeping” genes, to result in such a phenotype. One needs to identify the interesting genes amongst the many house-keeping genes by an intelligent muscle specific secondary assay. Our approach was to visualizing the sarcomeric Z-bands in vivo and subsequently the sarcomere structure & organization. All this becomes eminent if we look at the bioinformatic analysis performed by Novatchkova and Stark ^{##}. The GO-term heat map in Figure 7 indicates a strong enrichment for muscle specific genes in the lethal group but also the enrichment for genes involved in more general affairs like transcription & splicing, metabolism & mitochondria and genes involved in the protein synthesis machinery.

The larval screen needed to be relatively fast since the set of 587 candidate genes was large. I decided to focus on all genes for which a homozygous viable RNAi lines was available and crossed these 563 lines, corresponding to 436 genes to Mef2GAL4 and ZCL0663, a GFP trap in ZASP66 labeling the Z-line (ref) in a total I performed 934 assays. Consequently the screen consisted of the four steps depicted in the following schematic. Ultimately resulting in a snapshot of the muscle and sarcomere organization of intact animals. (Figure 8)

2.1.2. Muscle and sarcomere morphology classes

This secondary assay focused on the body wall muscles of the larva, in particular the large DO/DA muscles, reasoning that defects in these muscles would most likely result in embryonic or larval lethality. The DO/DA were chosen because they are large, thus easy to locate and observe, and also they

are big strong muscles, which are crucial and heavily used for locomotion. Our observation was that muscle phenotypes started to emerge or were strongest in these sets of muscles.

As depicted in previous Figure 6, I focused on a set of 436 genes and analyzed defects either in the overall muscle morphology or sarcomeric organization. For a detailed list of genes please refer to the original research paper. ^{††} All 934 assays performed were entered into a Filemaker database, specially designed not only to manage the large number of pictures but also to annotate and link to important information from Flybase. Further barcodes were used to easily handle the stocks. Thus we could use the transformant IDs instead of gene names to identify a line and achieve an essentially blind and unbiased assay. Further this database was crucial during the development of an ideally **mutually exclusive** and **collectively exhaustive** (MECE) classification system.

“Eventually we distinguished three classes of defects in muscle morphology (Fig. 9 c–k): ‘split myofibril’ (53 genes), ‘missing muscles’ (8 genes) and ‘rounded muscles’ (13 genes), in which muscles are either split into thinner myofibrils, are missing or have the rounded appearance characteristic of muscles that undergo normal morphogenesis but fail to form stable attachments. The ‘split myofibril’ class includes several signalling molecules, such as the FGF receptor *heartless*, *Anxb11*, the actin regulator *α-actinin* and several RNA-binding proteins (for example, CG5800; Fig. 9g). In these mutants, the muscles still generally attach to the appropriate tendon cells.” ^{††} This class also enriches for ribosomal proteins and other genes involved in protein synthesis as can be seen in Figure 16. However most of these genes share a common combination of phenotypes. Not only do they show the “splitting myofibril” and the later described sarcomeric phenotype called “fading Z” but also a dramatic increase in Z distance (data not shown).

“The ‘missing muscles’ class includes splicing factors and *His2A* (Fig. 9f). The ‘rounded muscles’ class includes known muscle attachment factors such as integrins, *ILK* and *rhea*, the fly *Talin* orthologue (Fig. 9d), as well as the fly *Parvin* orthologue.” ^{††} Muscle myofibrils in this set start tearing at one of the muscle attachment sites. The detached muscle and/or myofibrillar structure

then contracts to the characteristic roundish shape. This phenotype is characteristic for its severer and early onset. Specimens showing this phenotype generally do not survive past the L1 stage and most of them die as late stage embryos.

“We also defined three classes of defect in sarcomeric organization (Fig. 10b–l): ‘fading Z’ (94 genes), ‘spotty Z’ (50 genes) and ‘clumpy Z’ (18 genes)”

‡ The fading Z phenotype is characteristic for weakening of the marker signal further the z-bands themselves are broader, typical is also the late phenotype onset, 106 of the 140 (76%) transformants (TF) belonging to this class die at L2 or L3 stage, further the phenotype is most prominent respectively starts at the central DOs/DAs thus in the most used segments, hinting towards a degenerative phenotype respectively contain genes that are involved in the stability of the sarcomere. The spotty Z-phenotype is characteristic for the discontinuous or disrupted Z-line appearance, it starts as slight discontinuous Z-patterning up to fully disrupted sarcomeres where the Z-bands are erratically distributed in spotty fashion throughout the muscle. In contrast to the “fading Z” phenotype the onset is far earlier. 25 of 58 TF (43%) die already as embryos or L1 larva, together with the later mentioned candidates this hints that genes class are involved in the building up and/or are crucial structural components of the sarcomere. The final class “clumpy Z” although it can have similar severe penetrance (up to a nonexistent Z-patterning), it is clearly distinguishable from the “spotty Z” phenotype. Rather than being discontinuous and disrupted, Z-bands of this set seem to clump together forming large irregular aggregates.

“The ‘fading Z’ class is exemplified by a-actinin (Actn; Fig. 10f), and the ‘spotty Z’ class by how (Fig. 10e), which encodes an RNA-binding protein required for muscle development¹⁷⁷, and bent (Fig. 10c), which encodes a titin- or projectin-like protein¹⁷⁸. Most of the known sarcomeric components (bt, sls, Mhc, actin, Tm2, TpnC47D, and TpnC73F) fell into the spotty Z class. The ‘clumpy Z’ class includes flare (CG10724), the recently identified fly orthologue of actin interacting protein 1 (AIP1), a regulator of F-actin disassembly¹⁷⁹ and Basigin (Bsg), a muscle trans- membrane protein required for formation of the neuromuscular junction¹⁷⁹ (Fig. 10g, h).” ‡

Since the spotty set is particularly promising (see Figure 16), we retested all of the candidates with an independent sarcomere marker (Mhc-GFP [Wee-P26]¹⁸⁰). 43 out of 50 genes come up with the same or similar phenotype (Figure 11), due to the structure of sarcomeres huge distortion in the z bands will subsequently lead to a phenotype in the m-line. For 5 genes (cag, vrille, Spt5, CG16812, ciboulot) the original phenotype could not be reproduced. Shaking B and Proteasome 25kD subunit did show a stronger although slightly different phenotype (rather fading then dotted) with Mhc-GFP as a marker (Figure 11 d,h). CG17927 or Mhc was as a first proof for the efficiency of our knockdown, no signal other than auto fluorescence could be detected (Figure 11 b).

2.1.3. Sarcomere measurements

Further we were interested in genes that show a defect in sarcomere addition and growth. To determine these we decided to engage in Z-distance (i.e. distance between Z-lines) measurements (see 5.1.4), with the rationale that genes involved in sarcomere addition during growth or sarcoma length regulation should show abnormalities. As one can see in Figure 17, sarcomere size is rather constant during larval growth and only a slight decrease in size during the late L3 phase is visible.

Since we had no automated process for measuring Z-distances and due to its time consuming nature, a priority set was first determined. We aggregated additional information about our genes from Flybase¹³⁵, BLAST¹³⁷ and SMART¹³⁸ in our database. We ranked them according to predicted molecular function, domains and conservation and excluded so called housekeeping genes, in particular mitochondrial and ribosomal proteins, transcription and splicing factors and general cell signal molecules. Our final list included a set of 108 TFs (transformants) of 81 genes.

Due to the setup of the general screen (per TF pictures of only 2-4 individual larva were taken) a cutoff for candidates which is well above the average of the wt $7.2 \pm 0.4 \mu\text{m}$ ($n = 21$) but well below the range of our positive control Tmod^{116 117 118} which averaged $11.5 \mu\text{m}$ (TF32601) and $13.8 \mu\text{m}$ (TF32602)

depending on the TF's knockdown efficiency was chosen. This cutoff leads to the list shown in Figure 18.

Candidates with a decrease in Z-distance (CG6842, CG4198, CG31635, CG6340, CG31605) weren't only fewer but also the effects were less pronounced. Thus a high quantity retest to proof significance was performed. For this test wild type and knockdown larva were timed (i.e. parents were flipped out of the vial after 6 hours of egg laying).

CG31635 and CG6340 could not be retest due to unavailability of the stock at the time. Bsg (CG31605) believed to be a small-Z candidate turned out to have a rather unique irregular Z-spacing instead (data not shown). CG6842 and CG4198 both showed a significant decrease in Z-distance, in particular the Z-distance CG6842 further decreased over time as can be seen in Figure 19.

2.2. RNAi phenotype validation

As with any screen, quality assessment is crucial. This is discussed in details in §§ and section 3.1. However these methods only tell us about the success of a screen as a whole. Consequently only the validation a specific phenotype per se, transforms a screen hit into a candidate gene for further scientific inquiries.

We confirmed efficient knockdown of several of these proteins by antibody staining in the supplementary figures of the original research paper. §§ While this gives us some evidence how efficient the knockdown works, it not exclude that the phenotype is caused by an unspecific effect leading to a false positive result.

In general there are two sources of false positives in our screen: (a) by the so-called off-target effect (=unspecific knockdown of genes other than the anticipated target) and (b) due to the randomly inserted hairpin-constructs, by miss-expression of neighboring genes.

While the later can be accounted for, by using second-generation libraries (VDRC's phiC31 RNAi library) which use site specific integration of the hairpin

construct at so called landing sites,^{139 140} the earlier can only be excluded by further independent approaches to validate a target.

Prime choice would be to recapitulate the RNAi phenotype via classical mutants. However if these are not available, we strongly rely on the location of a suitable P-element next to our gene¹⁴¹, to be able to create a gene deletion. This can be done with classical methods like imprecise excision^{142 143} or FRT mediated deletion,¹⁴⁴ which can even be used in tissue specific manner.¹⁴⁵ Due to the bias of the P-element integration^{146 147} and so-called “cold spots”, in some cases only direct gene targeting methods (similar to the ones in mice) are possible. However these are similar laborious^{148 149} and thus unsuitable for the validation of multiple candidates of a screen. Classical non-conditional mutants however share one major disadvantage; they may display an uninterpretable pleiotopic phenotype, due important functions of the gene in various different tissues.

Creating a RNAi hairpin that targets a completely different region of that protein would be a further option. This is done with the notion in mind that a valid phenotype should be reproduced; however mimicking an off-target effect should be highly unlikely. Unfortunately the creation of multiple efficient non-overlapping hairpins is not possible for all proteins.

Rescuing the phenotype by a mRNA resistant to the RNAi effect would be a rigorous specificity proof. This is possible due to codon redundancy, which can be used to create mRNA that is dissimilar on a nucleotide level (thus RNAi resistant), however codes for a protein of identical amino acid sequence. Synthesizing such mRNA is expensive and laborious, thus we came up with a new method that uses evolutionary conservation of gene function.

2.2.1. Pseudoobscura fosmid rescue

As a proof of principal we focused on the three following genes:

Cg25C, a collagen IV homologue, which has so far shown to be strongly expressed in embryonic hemocytes and supposed to have an important function in the basement membrane¹⁵⁰. This gene was part of the larval lethal class in our previous screen.

CG32528, the fly parvin homolog which is predicted by SMART ^{i 151} to have a similar structure (sharing the tandem calponin homology (CH) domains) as the mouse alpha-parvin, which was identified as an actin-binding protein ^{152 153}. In our previous screen it turned out to be early larval lethal and showed a severe myospheroid phenotype (previously described as “rounded muscles”). Sar1, a small GTPase, which has so far been implicated to have a role in vesicle transport ¹⁵⁴ and heart formation ¹⁵⁵ in the embryo. In our previous screen we showed the knockdown to be larval lethal and to have a strong sarcomere defect (previously described as “fading-Z”) both in the myosin thick filaments and the Z-line. In severe cases a partial loss of the sarcomere was visible.

Since *Drosophila* has a second collagen IV homolog, called Vkg present in the basement membrane around the larval muscles ¹⁵⁰, we first tested if the collagen IV antibody specifically recognizes Cg25C. First attempts have been made in the Supplementary Figure 3 of the original research paper by looking at classical mutants. [‡] This was further corroborated by the fact, that collagen IV levels detected by the antibody in the wild type (Figure 12a) were identical to the RNAi knockdown of Vkg (Figure 12d). However levels were severely reduced in the Cg25C knockdown (Figure 12b) indicating that the anti collagen IV antibody indeed recognizes Cg25C.

The specificity of the RNAi-knockdown can be shown by the *D. pseudoobscura* fosmid rescue in Figure 12e. It depicts a significant but not complete rescue in larval growth. This can either be caused by the *D. pseudoobscura* version not being fully functional or the construct being not fully immune the IR-effect induced by the hairpin. Staining of the antibody argues for the latter, since Cg25c is localized around the muscle in a similar fashion (=evidence for function) but it's expression is markedly reduced. (Figure 12c). In conclusion this shows, that the muscle specific knockdown of Cg25C can be rescued by the *pseudoobscura* fosmid. (C. Schönbauer completed this experiment)

It is known that the integrin complex is needed for stable attachment to the tendons ¹⁵⁶. Parvin knockdown resulted in an early larva lethal phenotype dis-

ⁱ Tool available at: <http://smart.embl-heidelberg.de/>

playing a myospherid phenotype, (Figure 13a) which could be entirely rescued by the *D. pseudoobscura* fosmid (Figure 13 b-d). Similar the growth phenotype could be rescued; interestingly this could be improved by doubling the copy number of the fosmid (Figure 13e and Figure 14). These flies survive up to the early pupae stage.

We therefore conclude that *Drosophila* parvin plays a role in muscle attachment most likely via an integrin dependent mechanism as mouse parvin has been shown to be a part of the integrin complex¹⁵⁷ and integrin mutants display a similar myospherid phenotypes¹⁵⁶.

Finally, we analysed *sar1*, which displayed a sarcomere phenotype as mentioned before. Our fosmid rescue could completely rescue the phenotype (Figure 15a-d) as well as larval growth (Figure 15e) thus demonstrating a specific role of *sar1* in sarcomere formation and muscle growth.

3. Discussion

3.1. Quality assessment of the RNAi screen

Two factors mainly determine the quality of a screen; it's accuracy and the amount of novel information that can be gained through it.

The accuracy can be estimated using a set of positive and negative control genes. These are genes, which have been shown to play (or not play) a role in muscle function in classical genetic studies. (A detailed list can be found in the original research paper ^{††}). From the positive control set of 77 genes we could pick up 73 with our screen giving us a false-negative rate of just 5%. From the 121 lines of the 79 genes we used as negative control set, only 1 tested positive, thus resulting in a false positive rate of just 1.3%. This concludes that our screen has a high sensitivity (true positive rate), an even higher specificity (true negative rate) and an accuracy of nearly 97% in the primary screen.

Ultimately however the aim of every screen is to indentify new molecules involved in the specific biological process investigated. More than half of the genes in the positive set of our primary screen were either not annotated or their role in muscle development was unknown. This shows the great potential of this screen. If we look at the positive control set, in particular genes which code for proteins that are integral parts of the sarcomere, the candidates die at an early stage after knockdown, e.g. sls (titin), actn, troponin, mhc, if (α -integrin), bt (projectin), tropomyosin.

The primary screen for lethality (see Figure 7) shows a slight enrichment for muscle specific genes but also a strong enrichment for genes involved in more general processes like protein synthesis, transcription (splicing) and metabolism (mitochondria). Hence, we needed a more muscle specific assay (i.e. the larval muscle screen) to distinguish from these genes, that I would like to summarize as housekeeping genes.

3.2. Larval muscle screen

As discussed previously the early lethal set will most likely contain many genes that have a cellular housekeeping function.

If we take a look at the GO-heat map in Figure 16, we will see that one lethality stages seems particular interesting. This is the embryonic lethal group, it is a rather small group and contains many well-known sarcomeric proteins (for details see ^{††}). Interestingly this group is nearly absent of crucial housekeeping genes, we reason that either high maternal contribution or high zygotic expression of these genes, leads to insufficient knockdown at this very early stage.

An particular interesting muscle phenotype that coincides with this early lethality (i.e. 85% die either in the embryonic (7 out of 13) or early larval (4 out of 13) state) is the “round muscle” phenotype. Knockdown of these genes lead to muscle detachment during muscle growth and maturation. It includes many known muscle attachment factors such as integrins, ILK and rhea, the fly Talin orthologue, as well as the fly Parvin orthologue.^{††} A particular promising new gene belonging to this lethality group which also shows the round phenotype is CG5186 (slim - scruin like at the midline). Scruin was originally found in *Limulus* sperm ¹⁵⁸ where it stabilizes unusually long actin filaments, presumably by cross-linking of adjacent filaments. A homolog the kelch-encoded protein serves a similar function during the formation of actin filament bundles in the ring canals of developing *Drosophila* follicles ¹⁵⁹ Lack of kelch does not impair the formation of individual actin filaments but loosens their bundling. Little is known about slim itself in *Drosophila* other than it's presumed expression in the midline, postulated by Butland et al. in 1999 in a conference abstract ¹⁶⁰. During our observation sarcomeres were still visible, however the structures was distorted and muscles or the internal sarcomeric structure seemed to detach. Thus slim seems to have a similar stabilizing function on the structural integrity of the muscle cytoskeleton and in particular the sarcomeres. Slim was chosen as a candidate for a future site specific FRT deletion.

The bioinformatic data (Figure 16) shows for the next lethality group (i.e. the larval lethal set), that the muscle relevant (=specific) genes are masked by a high number of housekeeping genes. Nevertheless one can also see that our originally postulated idea worked i.e. by looking at the sarcomere and muscle morphology we could find certain phenotypes enriching for genes that are involved in muscle and sarcomere development.

The previous mentioned muscle phenotype “round muscles” shows this quite dramatically. It strongly enriches for muscle specific genes (in particular muscle attachment and development genes) and shows no particular enrichment in the housekeeping genes (shown in Figure 16 as protein synthesis, transcription, splicing, metabolism and mitochondria genes.)

If we look at the sarcomeric classes, wild type and fading-Z seem to capture the housekeeping specifically both are in fact slightly deriched in muscle specific genes.

The most promising sarcomere class is the spotty-Z phenotype strongly enriching in genes involved in sarcomere and general muscle structure proteins as well as genes involved in muscle contraction. “Spotty-Z” harbors genes that show severe sarcomeric abnormalities, which are either due to sarcomeric instability or incorrect assembly during growth. Many of these genes are highly conserved and thus will be the beginning of further studies, two noteworthy are:

Chd64, a new gene with a single CH domain and good embryonic muscle expression ¹⁶¹, shows a very strong spotty-Z phenotype. Recently it has been shown to be part of the heart proteom. ¹⁶² Another heart player htl has the same phenotype.

Ciboulot, a actin binding protein with a thymosin beta actin-binding motif and muscle primordium expression, has been shown to regulate actin assembly during *Drosophila* brain metamorphosis ¹⁶³ and might have similar functions during sarcomere assembly.

3.3. Sarcomere measurements

This group might hold the most promising genes in particular concerning sarcomere assembly and muscle growth regulation. However a further retest using over expressions constructs is highly recommended. This is rather easy using the fosmid libraries developed in the Tomancak lab^{164 ‡} which are optimized for liquid culture recombineering.^{164 165} It could be done for all candidates in single experiment.

The decreased Z-distance candidates are few and promising. Unfortunately the more common increased Z-distances phenotype could also be a more degenerative effect. As mentioned previously this increase in Z-distance correlates strongly with the fading-Z sarcomere phenotype and this phenotype strongly enriches for genes involved in protein synthesis (see Figure 16). One can reason that general shortage in sarcomere proteins leads to a decrease in newly incorporated z-Bands during larval growth.

Since these genes have been excluded from the sarcomere measurement screen based on their bioinformatic data and known muscle players like *actn* can show the fading-Z phenotype, this set is still likely to include interesting genes for further studies.

3.4. Rescue of RNAi phenotype with *D.pseudoobscura* fosmids

We could show a significant rescue of all the three genes analyzed *Cg25C*, *parvin* and *sar1*.

However we could not completely rescue the lethality. We believe this is most likely due to the insufficient dissimilarity between *D. melanogaster* and *D. pseudoobscura* and thus the RNA is still knocked down to a certain extend. We base this conclusion on the evidence, that *D. pseudoobscura* protein can fully rescue classical mutants^{‡ 172}. Further we could show that increasing the copy number, leads to a better rescue, indicating that the *D. pseudoobscura* gene is limiting.

However if a phenotype can be rescued, although incomplete, the specificity of the hairpin construct doesn't need to be questioned any longer and consequently this method will become an integral part of a genetics toolbox, when working on a system wide level.

4. Conclusion & Outlooks

We successfully performed an in-depth secondary assay for a muscle specific in-vivo genome wide screens. We looked at a set of 436 early lethal genes and assigned them a morphological muscular and sarcomeric phenotype class. We thereby indentified genes that play a role in sarcomere stability and growth in *Drosophila*.

Further the sarcomere distance measurement screen, focusing on the bioinformatically most promising genes, has revealed a number genes potentially involved in sarcomere length and growth control. Using the new fosmid library presented future over expression studies of this genes can be systematically performed with ease.

Further experiments in particular using adult muscle specific drivers like the previously mentioned 1151-Gal4 will allow us to monitor the sarcomeric assembly in adult muscles. With new imaging techniques developed in the Schnorrer lab, it is possible to follow the muscle development and sarcomere assembly in real-time during the pupal stage. This is much easier then in larva, due to the intrinsic immobility and will be very helpful to finally address many of the open questions concerning sarcomeric assembly during muscle growth.

We have shown the power of inducible RNAi transgenes to systematically dissect a complex developmental process. Together with the rapid fosmid rescue system we have proven once more the strength of *Drosophila* as an experimental system to analyze muscle development and growth.

5. Materials and Methods

5.1. RNAi screen

For further details please refer to the original research paper, segments previously published are again marked with ‡‡

5.1.1. *Drosophila* stocks

Stock maintenance and crosses were performed according to standard procedures.⁵

5.1.2. Larval assay ‡‡

RNAi hairpins from the VDRC collection³ were crossed to *Mef2-GAL4*, *ZCL0663*, a GFP trap in *CG6416* (*ZASP66*) labelling the Z-line¹⁶⁶, and progeny assayed after 2–5 days at 27°C, depending on the lethality stage observed in the initial screen. Larvae were immobilised by placing them into 65°C water for about 1 sec, and then mounted in 50% glycerol. Images were acquired on a Zeiss Axiophot or Zeiss AxiomagerZ1 at 10x and 20x and analysed with Metamorph software.

5.1.3. Embryo and larval staining ‡‡

Embryos were stained as described⁷⁹ with mouse anti-Mhc 3e8 (1:100; ref. 167) and rat anti-Projectin MAC150 (1:100) (Babraham Institute). For larval stainings, larva-filets were prepared as described¹⁶⁸ with the modification that dissections were done in relaxing solution (20mM phosphate buffer, pH 7.0; 5 mM MgCl₂; 5 mM EGTA, 5 mM ATP). Samples stained with rat anti-How (1:100; ref. 169) were fixed with 4% paraformaldehyde (PFA), samples stained with rat anti- α -Actinin MAK276 (1:3; ref. 170) (Babraham Institute) were fixed with ice cold MeOH. All incubations were performed in PBST (PBS

+ 0,2% Tx100) instead of PBSTween. Mouse anti-Mhc 3e8 was used (1:30; ref. 167). Images were taken with a Leica SP2 with a 40x objective and processed with ImageJ and Photoshop.

5.1.4. Sarcomere measurements

The distance between the Z-disks was measured using the images of the larval screen. Calibration data was acquired for all the microscopes used. MetaMorph was used to manually place line scans orthogonal to the muscle striation pattern onto 2-4 DO muscles of the segment A2 or A3 and an arithmetic average was calculated. A minimum of 2 independent larva were measured per test. Initial candidates were retested in higher numbers.

5.2. Fosmid rescue

For further details please refer to the original research paper, segments previously published are again marked with ‡

5.2.1. Fly strains and genetics ‡

All crosses were done at 27 °C to increase GAL4 activity. All hairpins were obtained from the VDRC stock centre. All fosmids were inserted at the same site on the third chromosome (attP2¹³⁹) using site specific phiC31 integrase¹⁴⁰ and were recombined with Mef2-GAL4 also located on the third chromosome¹⁷¹. Recombinants were easily identified by dsRed expression in the ocelli (expression in the eye is quenched by [white +]). If the hairpin was located on the third chromosome it was also recombined with the fosmid enabling to test for rescue in the presence of two copies of the fosmid. Cg25C, sar1, and vkg mutants as well as Df(2L)Exel7022 deleting both vkg and Cg25C were obtained from Bloomington. A GFP trap in CG6416 was used to label the Z-line in larvae¹⁶⁶. w[1119] was used as wild type and is indicated by “+”. Recombinant chromosomes are indicated by “,”; homologous chro-

mosomes by “/” .

5.2.2. Phenotypic analysis of larval muscles ‡

The larva-filets for immuno-stainings of larval muscles were prepared as described ¹⁶⁸. All dissections were done in relaxing solution (20 mM phosphate buffer, pH 7.0; 5 mM MgCl₂, 5 mM EGTA, 5 mM ATP). Samples were fixed with 4% paraformaldehyde (PFA) in relaxing solution. Antibody incubations and subsequent washing steps were performed in PBS with 0,2% Triton X-100 instead of PBS-Tween. Samples were stained with rabbit anti-Kettin Ig 1/3 (1:100) ¹⁷³, mouse anti- Mhc 3e8 (1:100) ¹⁶⁷, mouse anti-Collagen IV (1:100) ¹⁷⁴, and rhodamine phalloidin or Alexa dye labelled secondary antibodies (Molecular Probes).

To analyse muscles of intact larvae the larvae carrying the CG6416 GFP trap were immobilised by dipping in 65 °C water for about 1 sec, and then mounted in 50% glycerol. Images were acquired on a Zeiss AxiolmagerZ1 at 20x and analysed with ImageJ software.

To score for larval growth well fed, mated males and females were incubated in a vial for about 24 h, adults were removed and the vial was incubated for another 48 h or 72 h depending on the strength of the RNAi phenotype. All relevant crosses were done in parallel at the same time blind to the genotype. Larvae were immobilised by placing into 65 °C water for about 1 sec, and then mounted in 50% glycerol. Images were acquired on a Leica M2FLIII with a ProgRes C14 at 1.25x magnification and larval length from head to tail was measured with Photoshop.

6. Figures

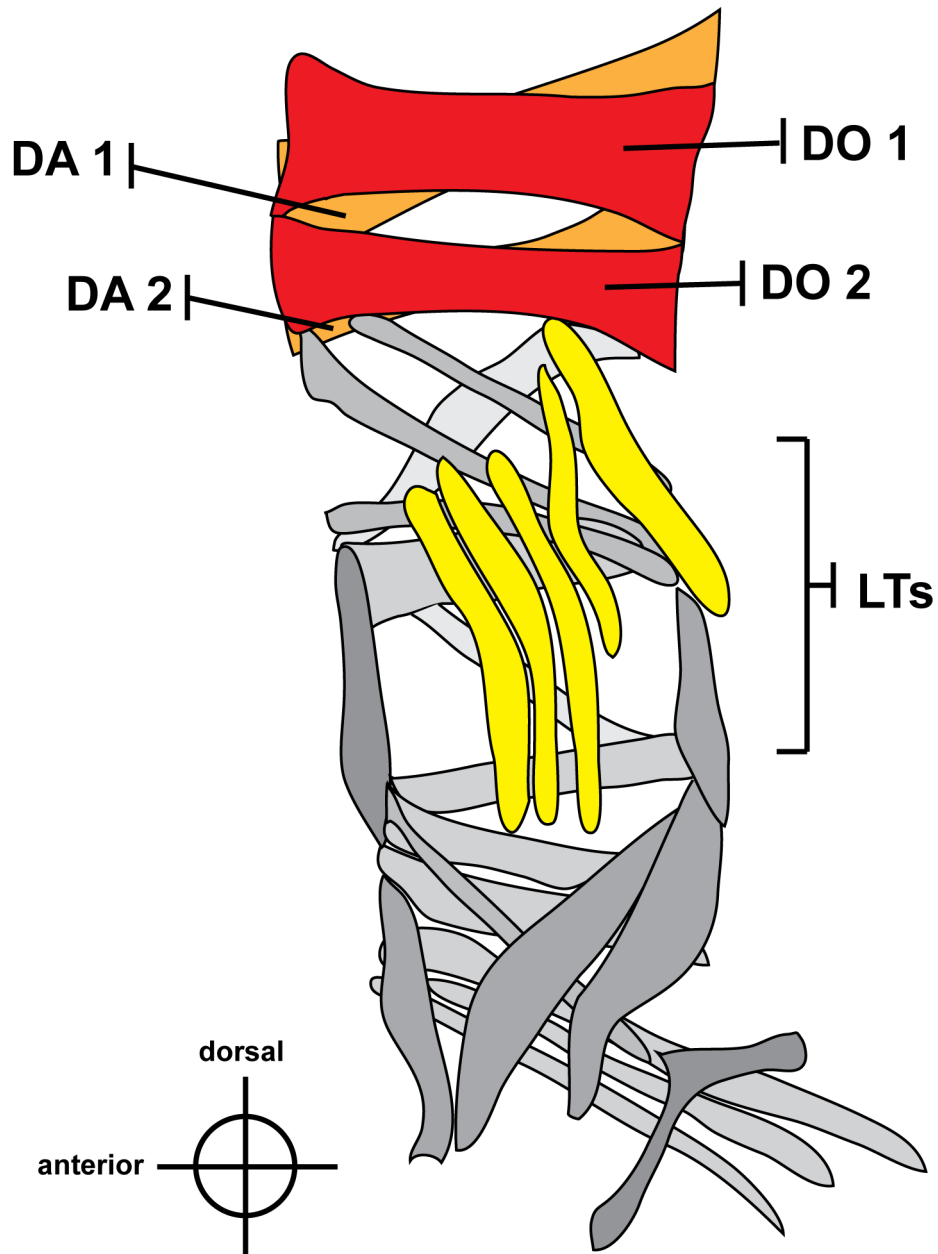


Figure 1| Somatic muscle pattern of the *Drosophila* larva.

This cartoon shows the external view of the abdominal segments A2-A7 muscle pattern. (Adapted from ref. 2) Highlighted are in red the muscles examined in our study (= dorsal oblique muscles) and in yellow the lateral transverse muscles, which were used for easy anterior-posterior orientation of the pictures.

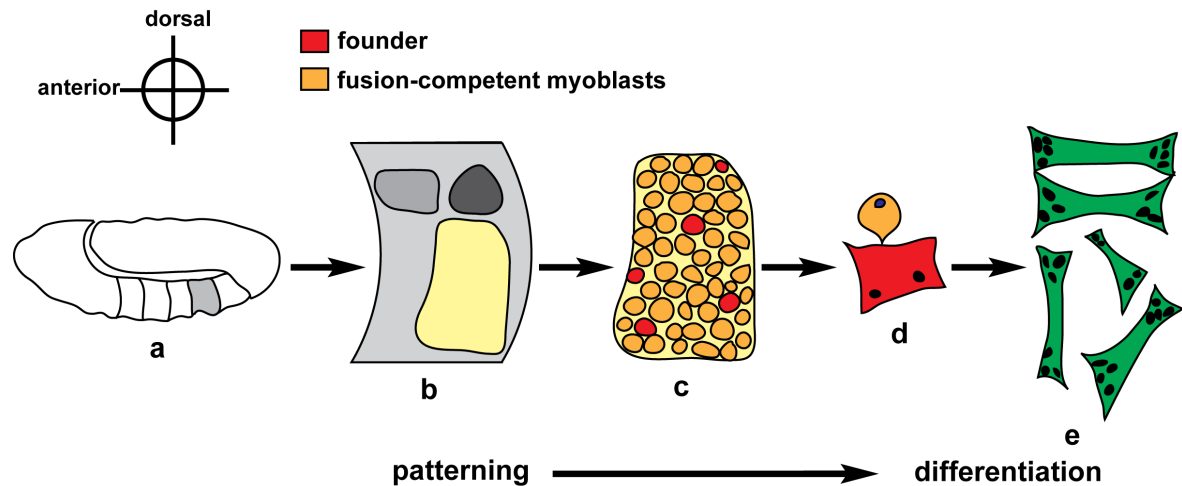


Figure 2| Overview somatic muscle development in *Drosophila*.

(a) This cartoon shows the main steps from an extended germ band embryo (a) up to the fully differentiated array of 30 distinct muscles fibers per hemi-segment at the end of embryogenesis. (b) Patterning and segmentation of the mesodermal hemi-segment into the progenitors of the different muscle groups is achieved by a multitude of intrinsic transcription regulators and extrinsic signals. (c) The somatic mesoderm gives rise to founder and fusion competent myoblasts. (d) Founders fuse with fusion competent myoblasts and form syncytical muscle precursors. (e) Further differentiation leads to a complex muscle system. (Adopted from ref. 175)

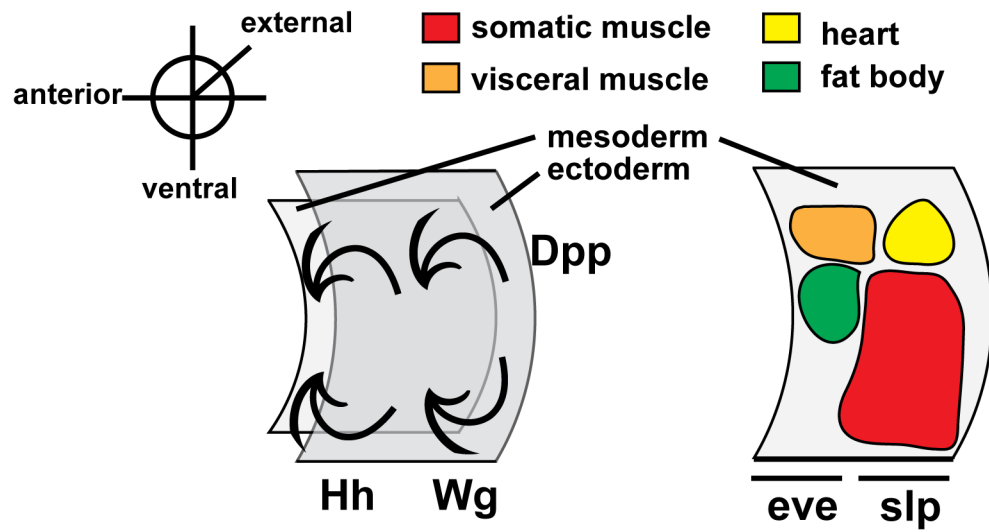


Figure 3| Mesoderm subdivision.

This cartoon shows how the intrinsic transcription regulators (*eve*, *slp*) and extrinsic signals coming from the overlaying ectoderm (DPP, Hh, Wg) contribute to the patterning of the mesoderm. (Adopted from ref. 29)

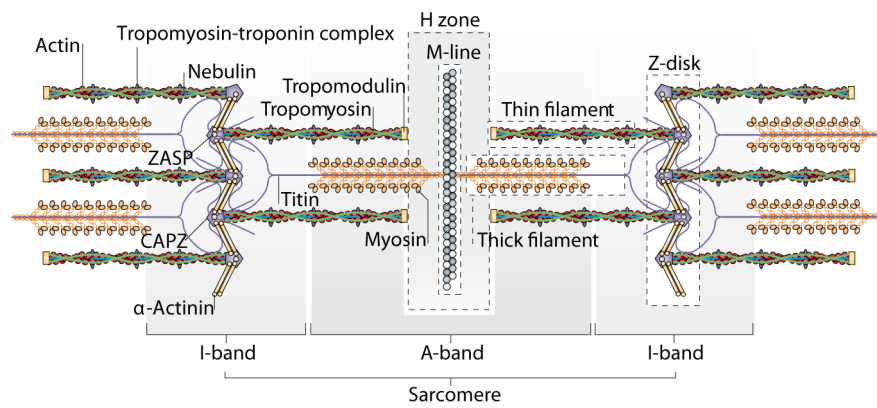


Figure 4 | Sarcomere overview.

Schematic overview of a sarcomere, depicting important proteins and regions discussed in this work. (Adopted from ref. 176.)

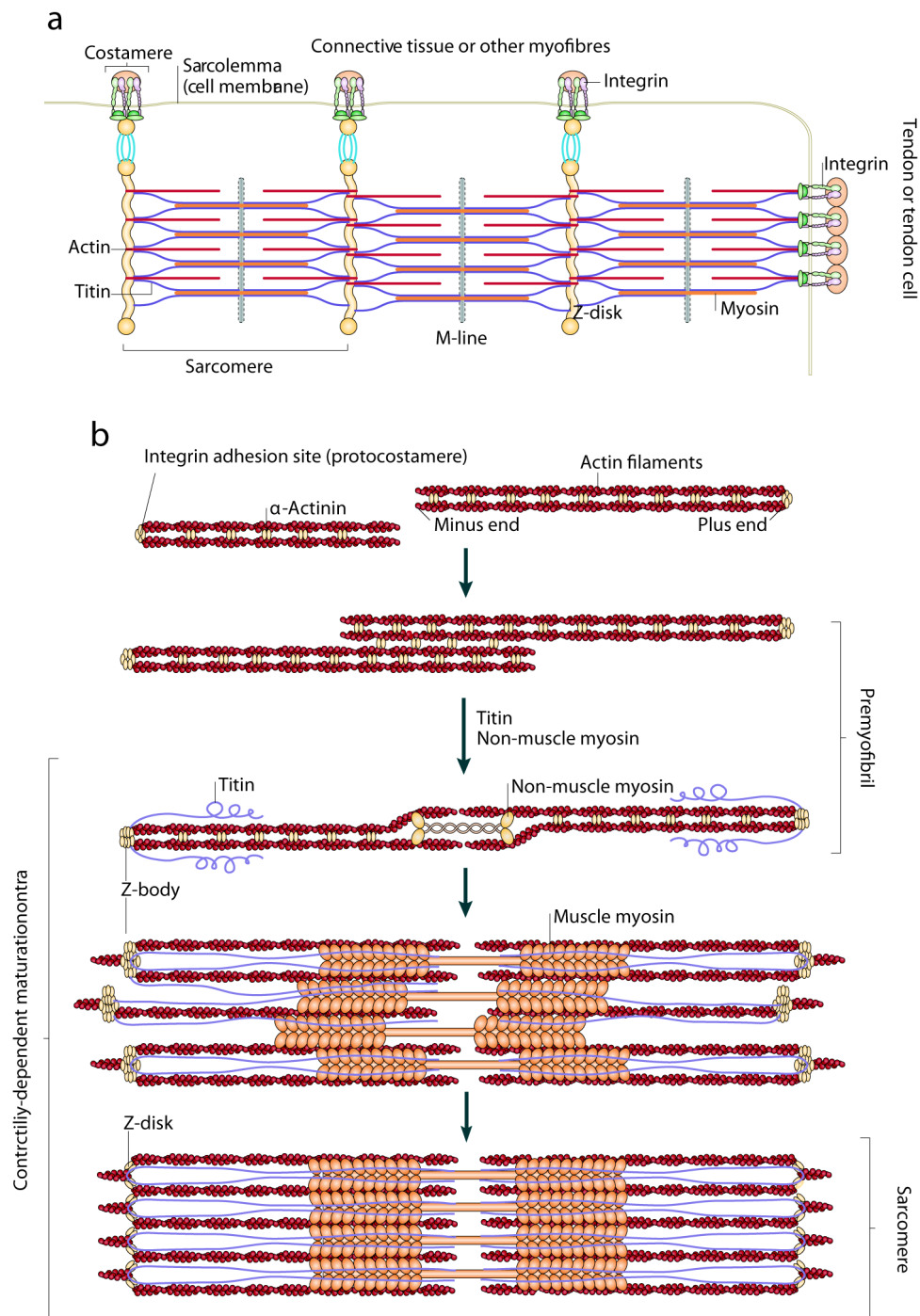


Figure 5| Model of myofibril assembly.

(a) Depicting costameres along the membrane that give rise to z-bodies and later z-discs. (b) Illustrates the steps of myofibril assembly: starting with the accumulation of alpha-actinin at still irregular spaced integrin adhesion sites, the growing of the actin filaments towards each other and finally the cross

linking by alpha actinin and then non-muscular myosin leading to stress fiber like premyofibrils. Replacement of the non muscular myosin by muscular myosin and incorporation of further muscle proteins (especially titin) together with first contractile motions lead to the maturation and the precise spacing of mature sarcomeres. (Adopted from ref. 176.)

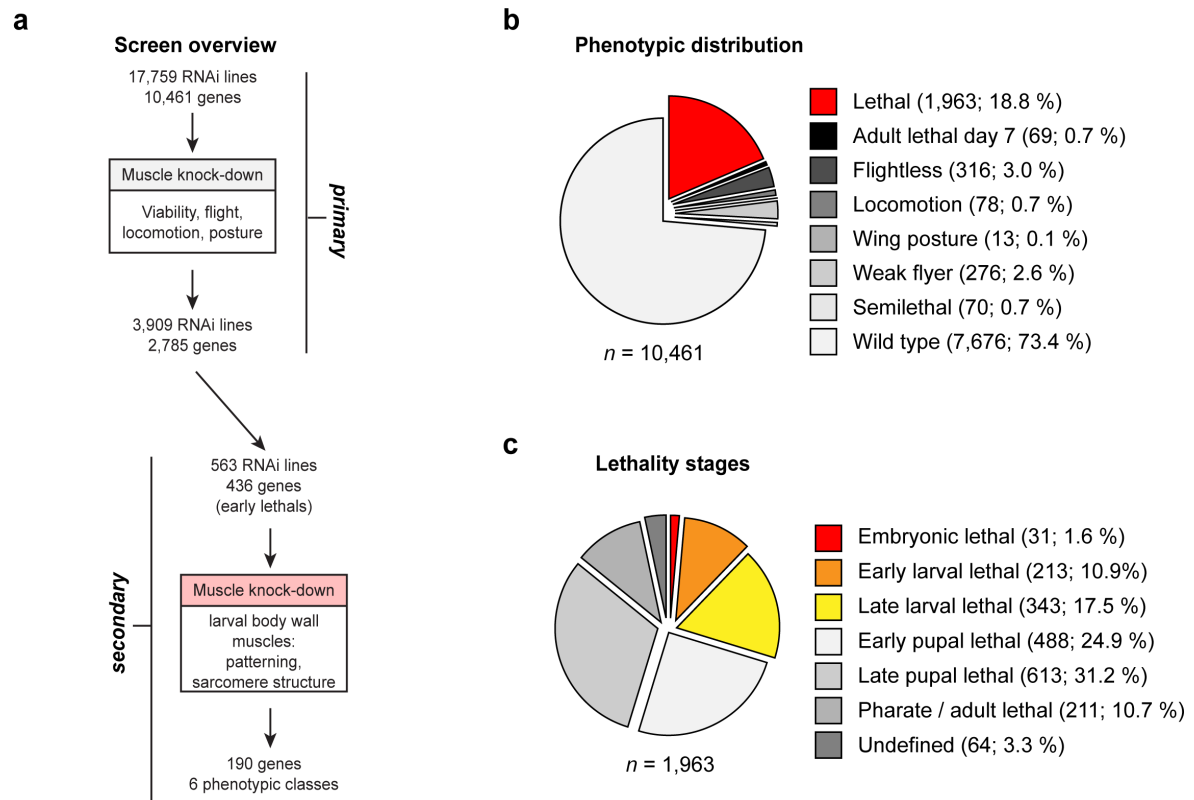


Figure 6| Screen overview and phenotypic classification. ^{##}

(a) Overview of primary and secondary *Mef2-GAL4* screens. Genes identified in the primary screen were assigned to specific classes according to viability, flight, locomotion and wing posture, and selected genes assayed in secondary screens according to their phenotype. (b) Distribution of genes into phenotypic classes in the primary muscle screen. (c) Distribution of the lethality stages for all essential genes.

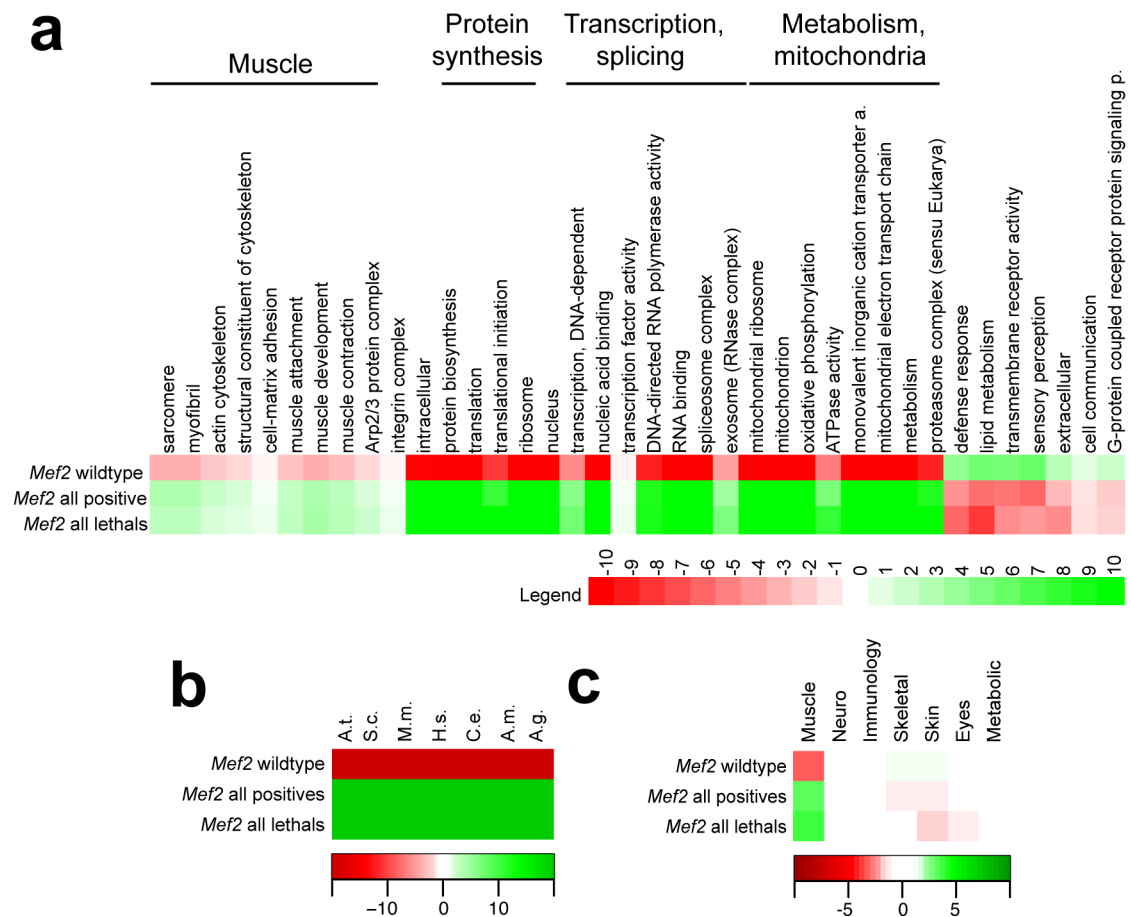


Figure 7 | Bioinformatic analysis. ##

a, GO-term enrichment in *Mef2* lethals of the primary screen. Conservation across species (**b**) and link to clinical diseases (**c**).

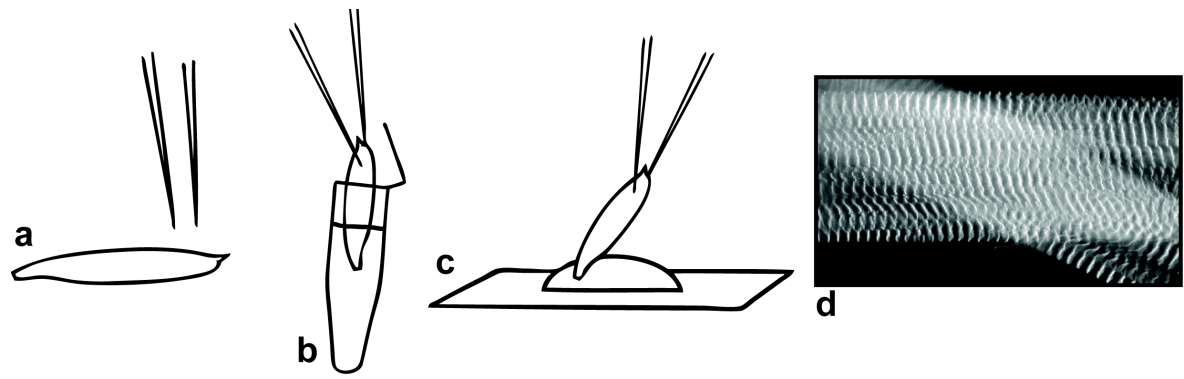


Figure 8| Secondary screen working steps.

(a) After 2-5 days at 27°C larva were picked with forceps, **(b)** placed for 2'' into 60°C H₂O to immobilise, **(c)** mounted in 50% glycerol and **(d)** imaged at 10x and 20x.

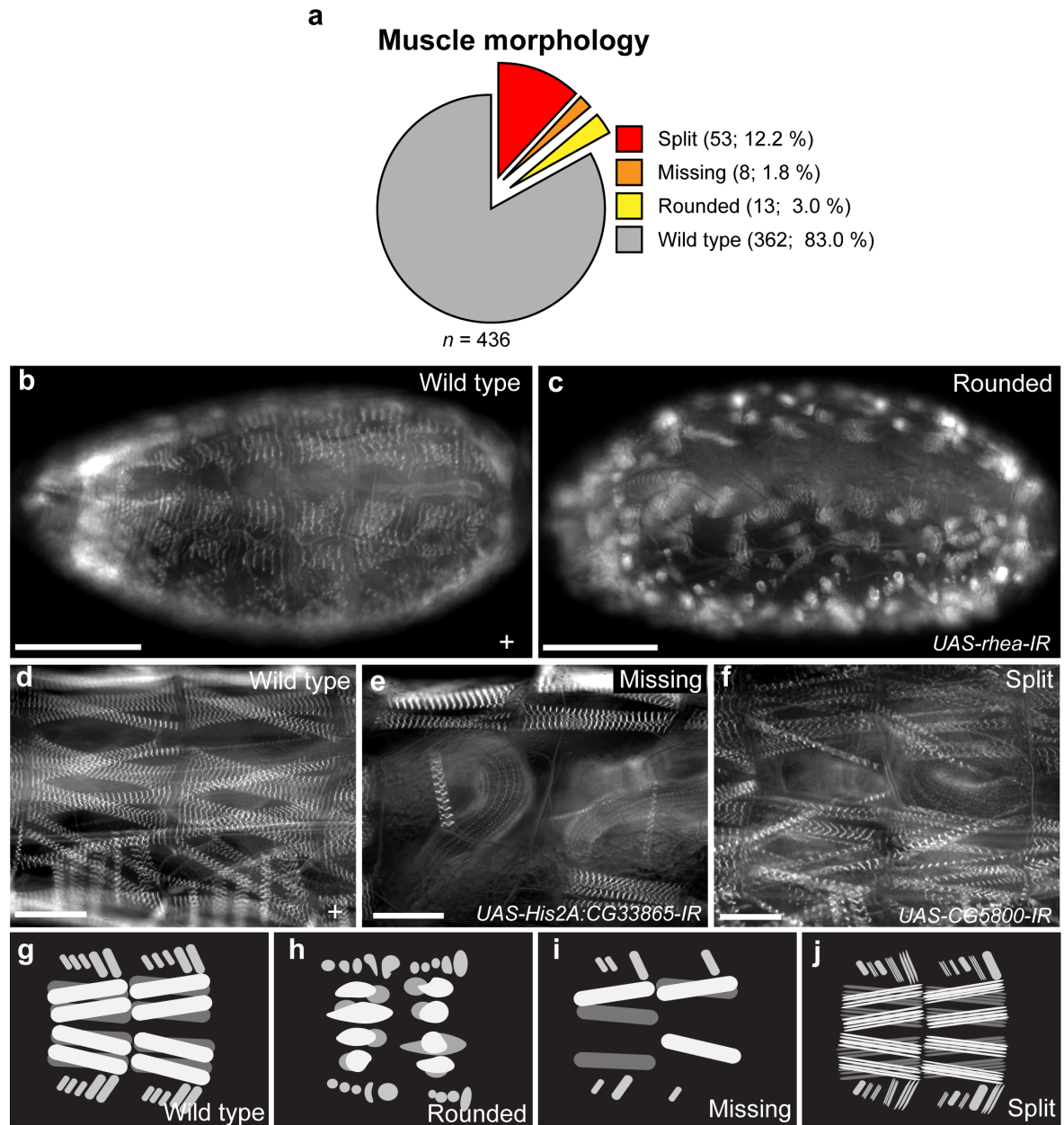


Figure 9 | Muscle morphology. ^{##}

(a) Muscle morphology classes of the larval body muscles. **b-j** wild type and mutant phenotypes, normal muscles in wild-type embryos **(b)** compared to rounded muscles in *UAS-rhea-IR* (TF40400) **(c)**, wild-type L3 larval muscles **(d)**, missing muscles in *UAS-CG33865-IR* (TF39116) **(e)** and split muscles in *UAS-CG5800* (TF27519) **(f)**. Scale bars: 100 μm **(b,c)** and 50 μm **(d - f)**.

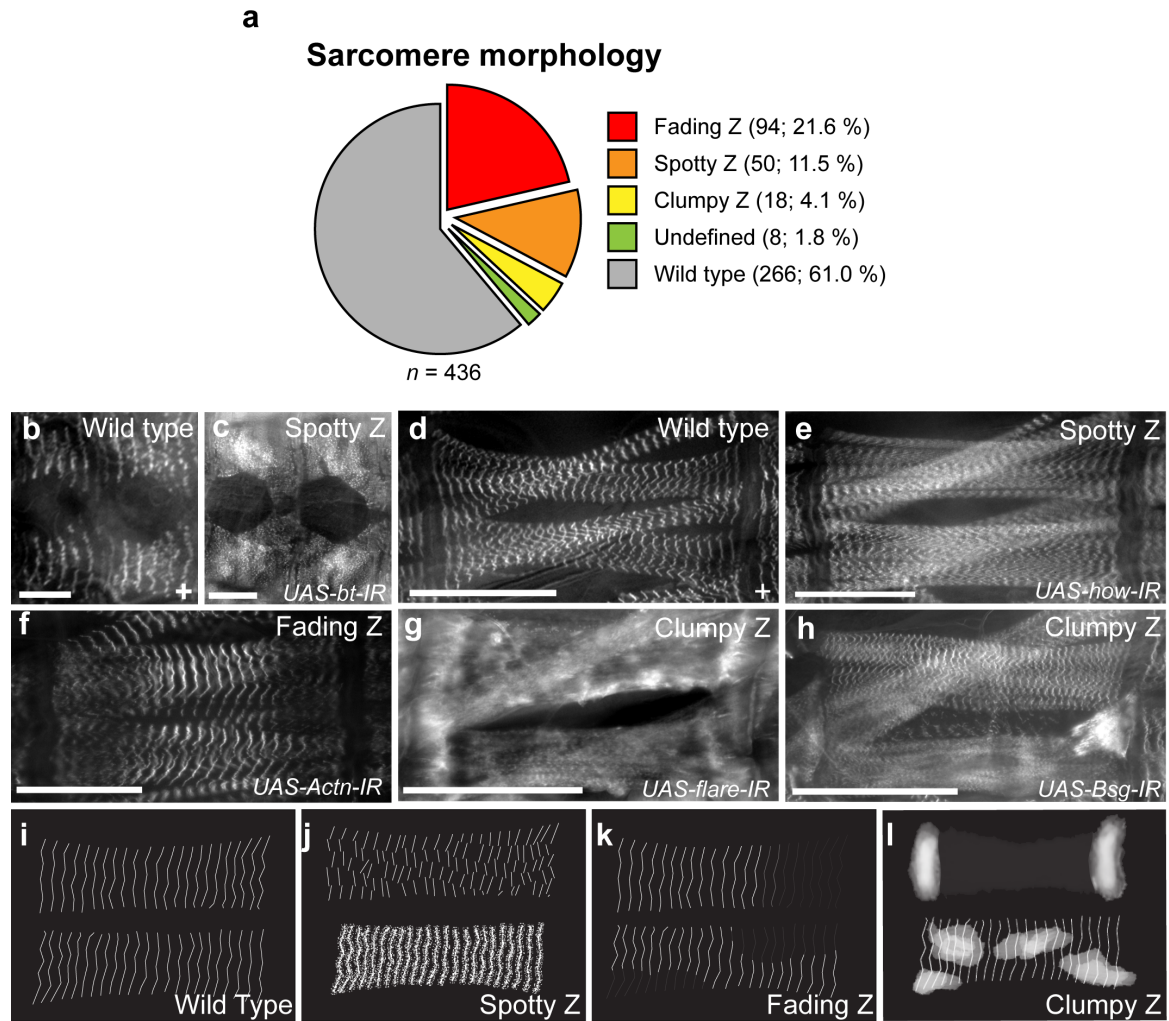


Figure 10 | Sarcomere morphology.**

(a) Sarcomere morphology classes of the larval body muscles. **b-l**, Sarcomere morphology phenotypes, wild-type embryonic sarcomeres (**b**) compared to *UAS-bt-IR* (TF46253) displaying spotty Z sarcomeres (**c**), and wild type L3 sarcomeres (**d**) in comparison to spotty Z in *UAS-how-IR* (TF13756) (**e**), fading Z in *UAS-Actn-IR* (TF7760) (**f**), and clumpy Z in *UAS-flare* (TF22851) (**g**) and *UAS-Bsg-IR* (TF43307) (**h**). Scale bars: 50 μ m (**b** - **h**).

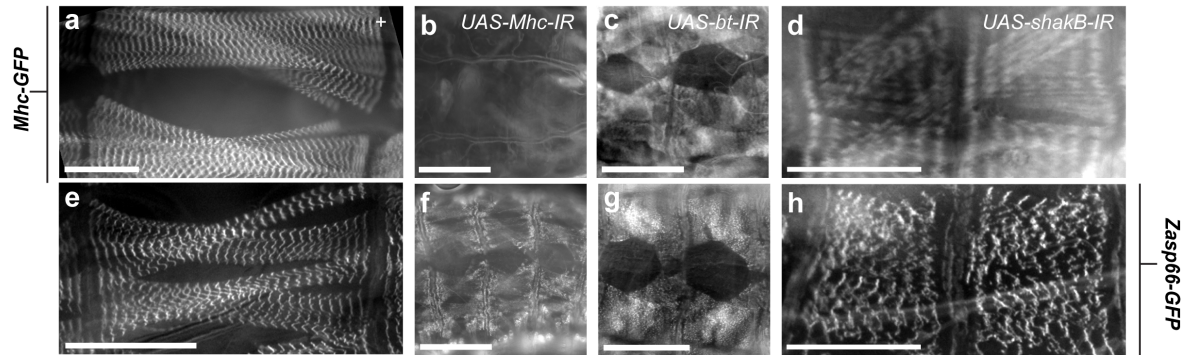


Figure 11 | Spotty Z class retest.

Looking at the spotty Z sarcomere morphology phenotype with Mhc-GFP and comparing it to the previous Zasp66-GFP. Showing wildtype (**a,e**), UAS-Mhc-IR (TF 7164) (**b,f**), UAS-bt-IR (TF46253) (**c,g**) and UAS-shakB (TF26801) (**d,h**). Scale bars: 50 μ m (**a - h**).

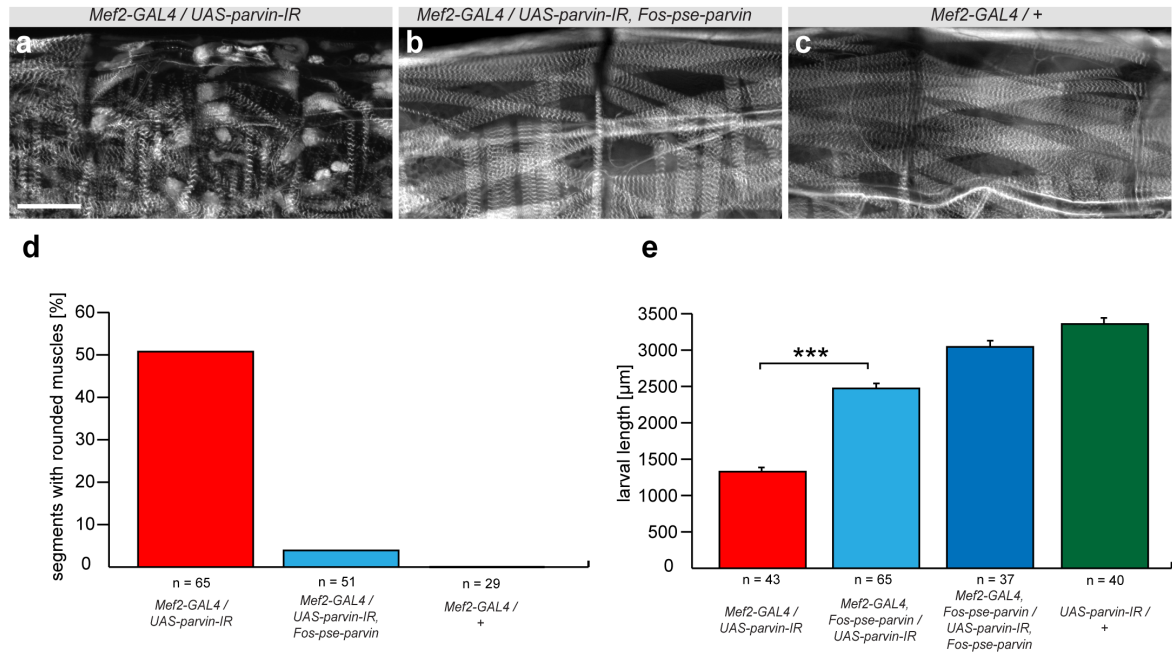


Figure 12 | Phenotypic rescue of Cg25C by *D.pseudoobscura* fosmid.[‡]

(a–d) Collagen IV (green) wraps the larval muscles in wild type (a) and is strongly reduced in Mef2-GAL4/UAS-Cg25C-IR (TF104536) (b) but rescued by FlyFos-pse-Cg25C (c); Collagen IV levels are not altered in Mef2-GAL4/UAS-vkg-IR (TF106812) (d); actin is visualised with phalloidin; size bar corresponds to 25 μm. (e) Quantification of larval size in Mef2-GAL4/UAS-Cg25C-IR (TF104536) larvae (red) rescued by FlyFos-pse-Cg25C (blue) and wild type (green). ***p<0.0001 (unpaired two-tailed t-test). 72–96 h after egg laying were assayed. Error bars indicate standard error of the mean (SEM).

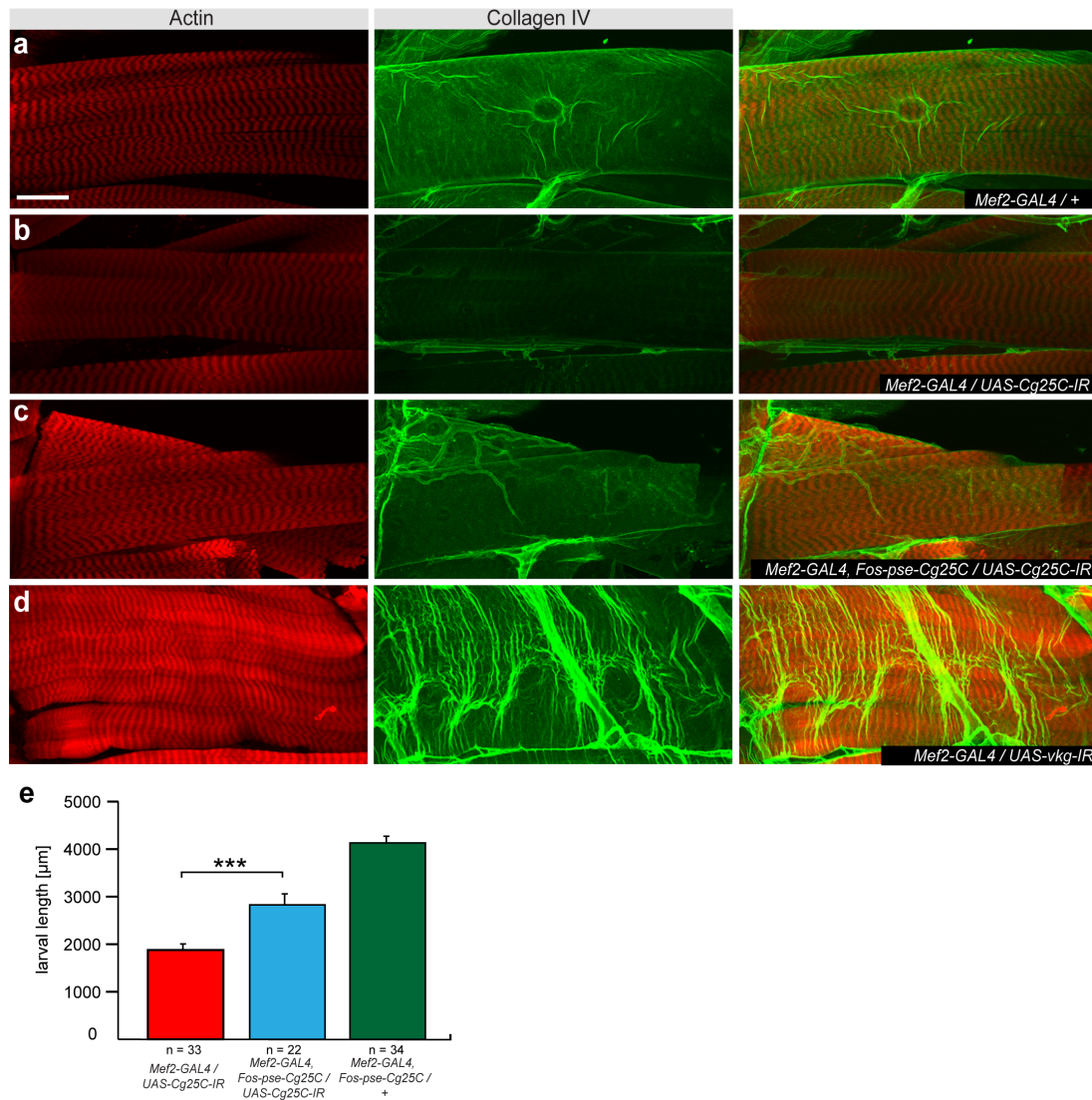


Figure 13 | Phenotypic rescue of parvin by *D.pseudoobscura* fosmid.[‡]

(a–c) Rounded/myospheroid muscle phenotype in Mef2-GAL4/UAS-parvinIR (TF11670) **(a)** is rescued by FlyFos-pse-parvin **(b)** to wild type **(c)**; size bar corresponds to 100 μm. **(d)** Quantification of myospheroid phenotype rescue, percentage of segments containing rounded muscles are shown, below the total numbers of segments scored. **(e)** Quantification of larval size in Mef2-GAL4/UAS-parvinIR larva (red), rescued by one (light blue) or two copies of FlyFos-pse-parvin (dark blue), compared to wild type (green). Larvae 48–72 h after egg laying were assayed. Error bars indicate standard error of the mean (SEM), *** $p < 0.0001$ (unpaired two-tailed t-test) compared to rescued larvae.

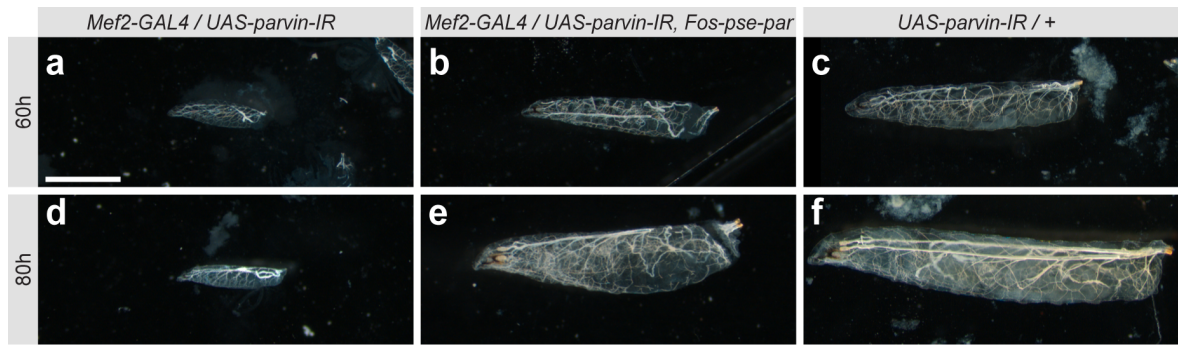


Figure 14 | Rescue of parvin knock-down. ‡

Larva of 48–72 h (**a–c**) or 72–96 h (**d–f**) were imaged at the same magnification. *Mef2-GAL4/UAS-parvinIR* (TF11670) (**a, d**) stay tiny compared to *Mef2-GAL4/UAS-parvinIR, FlyFos-pse-parvin* (**b, e**) and *UAS-parvin-IR/ +* control larvae (**c, f**). Size bar corresponds to 1 mm.

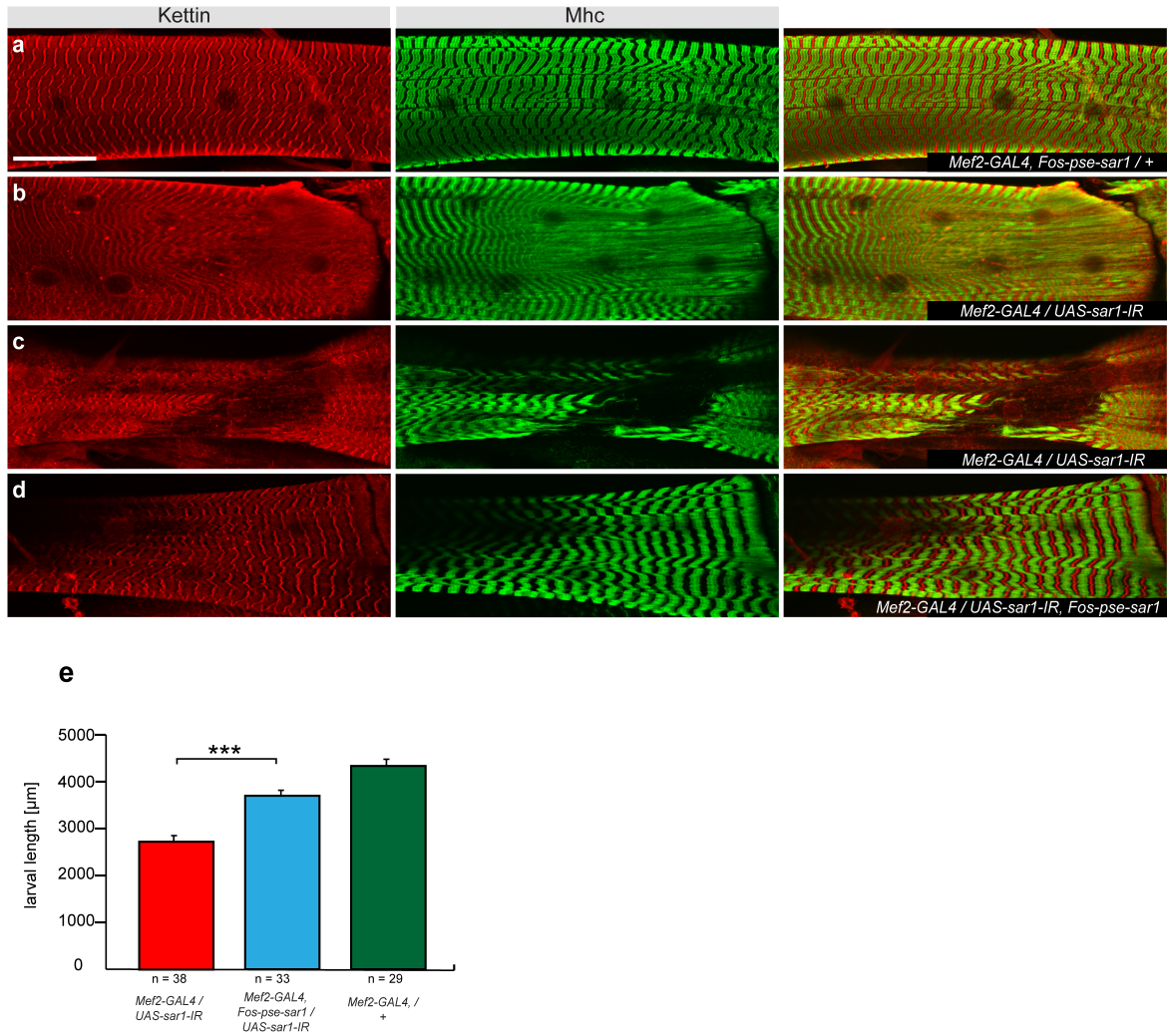


Figure 15 | Phenotypic rescue of sar1 by *D.pseudoobscura* fosmid.[‡]

(a–d) Fading Z- and M-line or loss of sarcomeres in *Mef2-GAL4/UAS-sar1-IR* (TF34191) (b, c) is rescued by *FlyFos-pse-sar1* (d) to wild type (a). Z-lines are visualised with anti-Kettin (red), M-lines with anti-Mhc antibody (green); size bar corresponds to 50 μm . (e) Quantification of larval length in *Mef2-GAL4/UAS-sar1-IR* larvae (red), compared to *FlyFos-pse-sar1* rescued (blue) and wild type (green). Larvae 72–96 h after egg laying were assayed. Error bars indicate standard error of the mean (SEM), *** $p < 0.0001$ (unpaired two-tailed t-test) compared to rescued larvae.

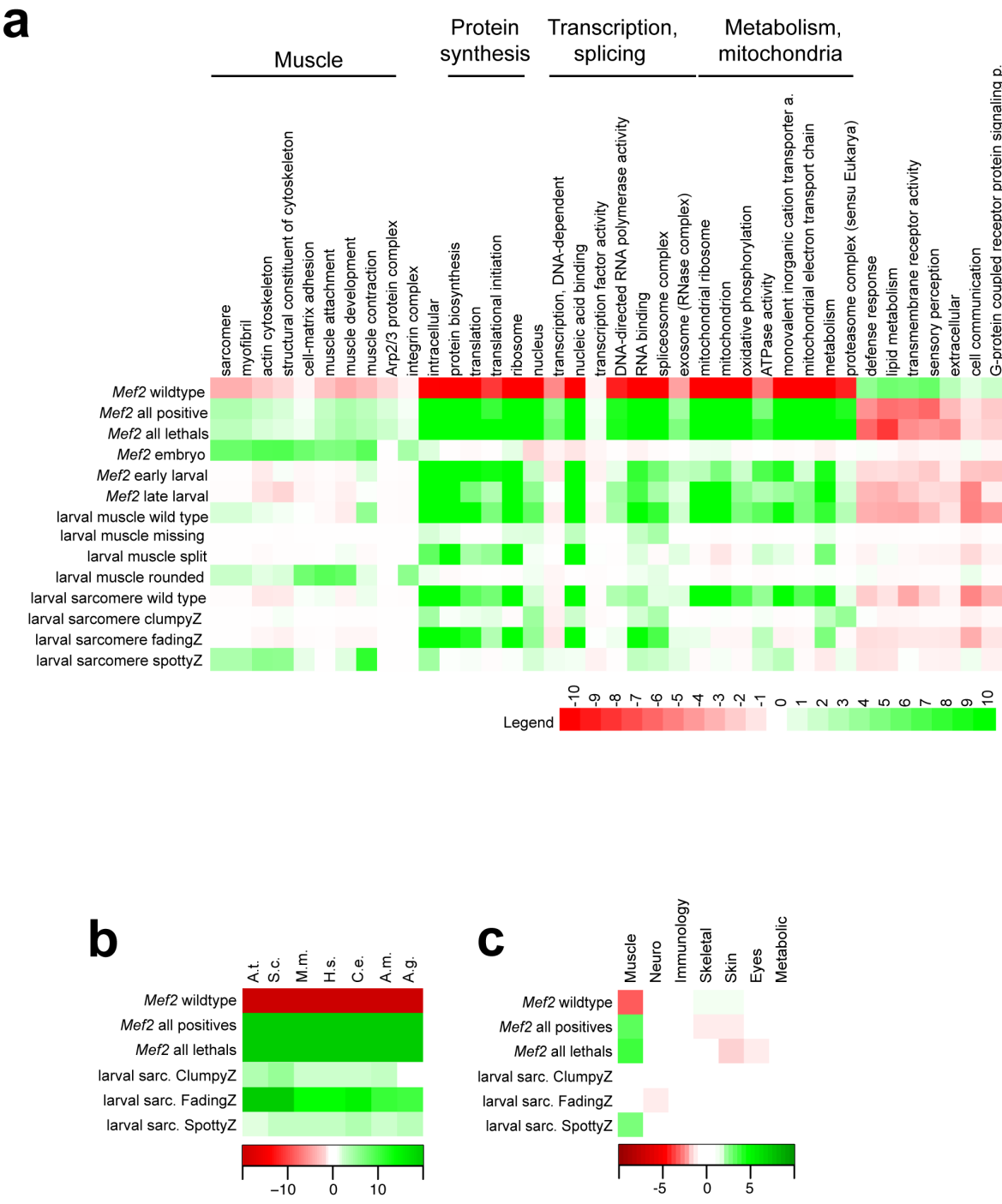


Figure 16 | Bioinformatic analysis (extended). ##

a, GO-term enrichment in *Mef2* lethals of the primary screen. Conservation across species (**b**) and link to clinical diseases (**c**).

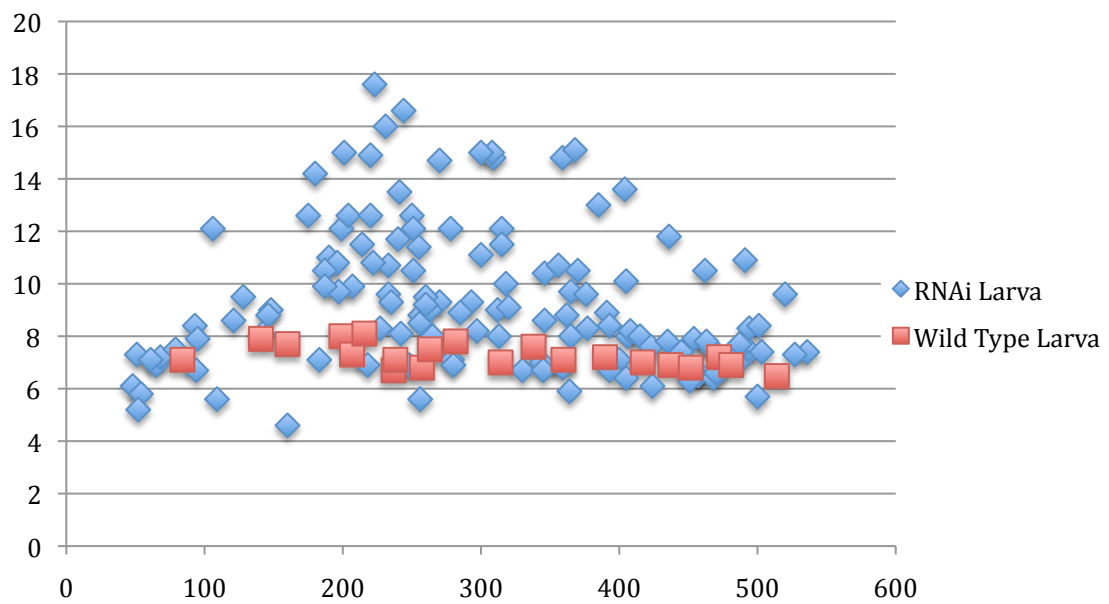


Figure 17 | Z-distance measurements.

Graph depicting the Z-distance measurements of w- drosophila larva in red and a random selection of knockdown larva in blue. X-axis corresponds to the length of the DO muscle in segment A2 or A3 in μm and y-axis corresponds to the Z-distance in μm .

TFs	CG	
7760/-1/-2	CG4376	Actn
2902	CG4006	Akt1
22445	CG6815	bor
17473	CG1430	bys
32325/-7	CG14805	CG14805
37842/-3	CG15630	CG15630
28900	CG8435	CG8435
21549	CG4944	cib
44829	CG8711	cul-4
889/900/33295	CG5820	Gp150
992	CG18402	InR
41403	CG13746	MrgBP
19692/-3	CG11518	pygo

Figure 18 | Z-distance candidates >10 μ m

The table shows CG number, TF number and gene symbol of candidates that scored reproducibly > 10 μ m during the Z-distance measurements.

	TF 35126 (96h)	TF 35126 (84h)	TF 26421 (84h)
	vs	vs	vs
	WT (96h)	WT (84h)	WT (84h)
Unpaired t test			
P value	0.0008	0.0497	0.0264
P value summary	***	*	*
Are means signif. different? (P < 0.05)	Yes	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
t, df	t=4.59 df=11	t=2.15 df=14	t=2.50 df=13
Mean ± SEM of column A (RNAi)	6.6396 ± 0.1205 N=7	6.9018 ± 0.1412 N=8	6.6747 ± 0.2301 N=7
Mean ± SEM of column B (WT)	7.3825 ± 0.1033 N=6	7.2856 ± 0.1089 N=8	7.2856 ± 0.1089 N=8

Figure 19 | Small Z-distance candidates

The table shows the results of the timed retest of the knockdown vs. wild type larva.

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8. Appendix

8.1. Abstract (English)

Muscles enable a higher organism to perform fast moves, e.g. to catch prey, or slow ones, e.g. to read this abstract. For the correct execution of these behaviors all muscles have to form at the right place and grow to the correct size.

In recent years we have learned much about how cells become committed to myoblast fate and thus to form muscles however a systematic study of how muscles grow and assemble their contractile units, the sarcomeres, so regularly is lacking. *Drosophila melanogaster* is an ideal model system to study muscle growth and sarcomere assembly as *Drosophila* larval muscles are built from single cells which grow up to 1000 times in volume from the embryo until the late larval stage. At the same time the larvae stay transparent and the sarcomere organization can be observed using live markers in intact animals.

We applied genome-wide transgenic RNAi to systematically test 75% of the fly genes for a function in muscle growth and sarcomere assembly. We determined size and shape of a defined class of *Drosophila* muscles to classify each gene into a muscle morphology category. Similarly, we measured distance and regularity as well as the shape of the muscle Z-bands to assign each gene into a sarcomere morphology category. Bioinformatic analysis of these data found a strong enrichment of muscle key players in the identified genes. Thus, these data will be an invaluable resource for future detailed studies to unravel the molecular regulation of muscle growth and sarcomere assembly during life of an organism. Further we developed a method to demonstrate specificity of RNAi phenotypes in a systematic manner using cross-species transgenesis.

8.2. Abstract (German)

Muskeln erlauben es höheren Organismen eine Vielzahl unterschiedlichster Bewegungen auszuführen vom Fangen einer Beute bis hin zum Lesen dieser Zusammenfassung. Dass alle Muskeln an der richtigen Stelle zur richtigen Größe herangewachsen sind, ist Voraussetzung für die korrekte Ausführung dieser Tätigkeiten.

In den letzten Jahren konnten wir viel über den Entwicklungspfad von Zellen zu Myoblasten und weiter zu Muskeln in Erfahrung bringen. Jedoch fehlt eine systematische Studie wie Muskeln wachsen und ihre höchst regelmäßigen kontraktile Einheiten, die sogenannten Sarkomere, zusammenfügen. *Drosophila melanogaster* ist ein idealer Modelorganismus um Muskelwachstum und Sarkomeraufbau zu beobachten, da ihre Muskeln aus einer einzigen Zelle bestehen und in der Entwicklung vom Embryo bis zur späten Larve um das 1000-fache im Volumen wachsen. Gleichzeitig bleiben Larven transparent und die muskelinterne Organisation kann direkt anhand visueller Marker in intakten Individuen beobachtet werden.

Wir testeten systematisch, mithilfe von genomweiter transgener RNAi, 75% des Fliegen-genoms auf eine Funktion im Muskelwachstum und Sarkomeraufbau hin. Wir bestimmten die Größe und Form einer spezifischen Gruppe von Drosophila Muskeln und ordneten jedem Gen eine Muskelmorphologiekategorie zu. Des Weiteren bestimmten wir die Abstände, Regelmäßigkeit sowie die Form der Z-Bänder in den Muskeln und ordneten jedem Gen eine Sarkomermorphologiekategorie zu. Eine bioinformatische Analyse dieser Daten fand die starke Anreicherung der bekannten muskelspezifischen Hauptakteure unter den identifizierten Genen. Somit werden diese Daten eine unschätzbare Ressource für zukünftige detaillierte Studien sein, deren Ziel es ist die molekulare Regulation von Muskelwachstum und Sarkomeraufbau während des Lebens eines Organismus zu entschlüsseln. Zusätzlich haben wir eine Methode entwickelt um die Spezifität von RNAi-Phänotypen systematisch mithilfe von Cross-Species-Transgenese zu demonstrieren.

8.3. Curriculum Vitae

Christoph C. H. Langer

Language skills:

German (mother tongue)
English (fluent)
French (basics)

Education:

1997 – 2005

Gymnasium St. Martin
English as a medium of instruction „bilingual“

Oct. 2005 – present

University of Vienna
Diploma (MA) program in Molecular Biology
Specialized in: Genetics, Developmental Biology and Neurosciences

Oct. 2009 – present

University of Vienna
Bachelor/Master program Scientific Computing

Research experience:

Feb. – Mar. 2007

Internship - **University of Vienna** - *Department of Biochemistry*
Gustav Ammerer Group
Project: Assistance at the revision of the paper: “Activation of the G2/M-specific gene CLB2 requires multiple cell cycle signals.” *Mol Cell Biol.* 2007 Dec

Jul. – Nov. 2007

Internship - **IMP** – *Vienna*
Barry Dickson Group
Project: Muscle Dynamics & Development – Design, Establishing & Realization of a secondary *D. melanogaster* larva muscle screen.

Feb.- Mar. 2008

Internship - **IMBA** – *Vienna*
Josef Penninger Group
Project: Construction of a transgenic mouse (Red/ET recombineering) and working with several in vivo & in vitro mouse cancer models

Apr. 2008 – Oct. 2009

Diploma (Master) Thesis - **MPI of Biochemistry** – *Munich*
Frank Schnorrer Group
Thesis Title: Identification and characterization of genes required for sarcomere assembly and muscle growth in *Drosophila*.

Teaching experience:

Nov. 2008 – Jan 2009

Project supervision of an intern at the MPI
Project: Generation of classical fly mutants by imprecise excision

Mar. 2010 – Jun. 2010

Tutoring a post-graduate course at the University of Vienna
Course: Computational Drug Design

Mar. 2011 – Jun. 2011	<i>Tutoring a post-graduate course at the University of Vienna</i> <u>Course:</u> Computational Drug Design <i>Tutoring a undergraduate course at the University of Vienna</i> <u>Course:</u> Software Architecture
Mar. 2012 – Jun. 2012	<i>Tutoring a post-graduate course at the University of Vienna</i> <u>Course:</u> Computational Drug Design
	Scientific activities:
Sep. 2007	20th European Drosophila Research Conference - Vienna, Austria
May 2008	3rd MicroSymposium on Small RNAs - Vienna, Austria
Jul. 2008	XX International Congress of Genetics - Berlin, Germany
	Social activities:
Mar. 2011 – Jun. 2011	Co-initiator and manager of an interdisciplinary event series in Vienna (more info on vierfarben.org)
	Publications:
Dec. 2009	Systematic genetic analysis of muscle morphogenesis and function in <i>Drosophila</i> <i>F. Schnorrer, C. Schönbauer, C.C.H. Langer, G. Dietzl, K. Schernhuber, M. Fellner, A. Azaryan, M. Novatchkova, A. Stark, K. Keleman and B.J. Dickson - Nature</i>
Jan. 2010	In vivo RNAi Rescue in <i>Drosophila melanogaster</i> with Genomic Transgenes from <i>Drosophila pseudoobscura</i> <i>C.C.H. Langer, R.K. Ejmont, C. Schönbauer, F. Schnorrer, P. Tomancak - PLoS ONE</i>

Vienna: July 22th 2013

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