A large-scale systemic RNAi screen in the red flour beetle *Tribolium castaneum* identifies novel genes involved in arthropod muscle development

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Abstract

Although muscle development has been widely studied in *Drosophila melanogaster* there are still many gaps in our knowledge, and it is not known to which extent this knowledge can be transferred to other arthropods. To help closing these gaps we participated in a large-scale RNAi screen that used the red flour beetle, *Tribolium castaneum*, as a screening platform. The effects of systemic RNAi were screened upon double-stranded RNA injections into appropriate muscle-GFP tester strains. Injections into larvae were followed by the analysis of the adult thoracic muscle patterns, and injections into pupae by the analysis of the late embryonic/larval muscle patterns. Herein we focus on the results of our pupal injection screen for late embryonic/larval muscle phenotypes, which covered 8.500 of a total of ~16.500 genes of the *Tribolium* genome. Apart from many genes known from *Drosophila* as regulators of muscle development, a collection of genes previously unconnected to muscle development yielded larval body wall and leg muscle phenotypes. We present the main candidates that remained after being processed through a series of verification and selection steps. Further, we discuss why distinct though overlapping sets of genes are revealed by the *Drosophila* and *Tribolium* screening approaches.

Keywords:

muscle development; *Tribolium*; RNAi screen

Introduction

Muscle development in arthropods has been studied primarily in the dipteran *Drosophila melanogaster*, whereas much less is known about the regulatory mechanisms guiding muscle development in other arthropod orders. Thus, it is unknown whether more distantly-related arthropods, such as beetles, utilize largely the same processes and mechanisms to make muscles, or whether they differ in important aspects. Clearly, one shared process of holometabolous arthropods such as dipterans and coleopterans is that the musculature of an animal has to be developed twice; first the larval musculature during embryogenesis and second the adult musculature with completely different features during metamorphosis in the pupae.

In *Drosophila*, many of the genetic control mechanisms guiding the development of the multinucleated larval muscles of the body wall, also known as somatic muscles, have been uncovered during the past few decades. The earliest events in the hierarchy of zygotically active regulatory genes involve the activation of *twist* and *snail* in a ventral strip of cells in the blastoderm embryo, which are needed for the specification of the mesoderm and its invagination during gastrulation (Leptin 1991). *Tribolium castaneum* (*Tc*) *twist*, which is one of the few known regulators of mesoderm and muscle development in the red four beetle, fulfills similar early functions as *Drosophila twist* (Händel *et al.* 2005; Stappert *et al.* 2016). In *Drosophila* and likely in *Tribolium*, the subsequent spreading of the internalized mesoderm in tight contact with the overlying ectoderm is facilitated by FGF signals from the ectoderm (Wilson and Leptin 2000; Sharma *et al.* 2015). Upon formation of a mesodermal monolayer spanning the entire dorsoventral extent of the germ band, patterning events that are largely derived from the ectoderm subdivide the mesoderm along the anterior-posterior and dorsoventral axis within each parasegmental unit, which leads to the formation of the anlagen giving rise the somatic, cardiac, and visceral muscles. With regard to the somatic (and cardiac) mesoderm, a major inductive signal is provided by Wingless (Wg) (Baylies *et al.* 1995; Lee and Frasch 2000; Bodmer and Frasch 2010; Azpiazu *et al.* 1996; Riechmann *et al.* 1997; Lee and Frasch 2000). Subsequently, the somatic mesoderm is further subdivided by intersecting signals from the ectoderm, including Wg and Decapentaplegic (Dpp), into domains that are competent to respond to localized and temporally-regulated receptor tyrosine kinase (RTK) signals (Frasch 1999; Baylies and Michelson 2001).

These signals are mediated by the *Drosophila* epidermal growth factor receptor (EGFR) or, alternatively, the fibroblast growth factor receptor Heartless (Htl) and define groups of equivalent cells in terms of muscle formation (Carmena *et al.* 1998). The antagonistic actions of these RTK signaling activities and of Delta/Notch signaling activities within these equivalence groups ultimately results in the formation of two types of myoblasts within each group: A single muscle progenitor in which the RTK signaling cascade remains active and the Notch signaling cascade is inactive, and several adjacent cells with the opposite activity status of these signaling cascades. The muscle progenitor with high RTK and low Notch signaling activity divides asymmetrically and typically gives rise to two muscle founder cells that are programmed to form a single somatic muscle of a defined identity each. The specific identity of each muscle founder is defined by the expression and functions of defined combinations of so-called muscle identity genes, which are induced downstream of the respective RTKs and cooperating signals, and generally encode members of various families of transcription factors (de Joussineau *et al.* 2012; Dobi *et al.* 2015). Conversely, within the neighboring cells lacking RTK activities, high Notch signaling activities induce the expression of the transcription factor encoding *lameduck* (*lmd*) gene, which defines these cells as fusion-competent myoblasts that, *a priori*, are not committed to specific muscle fates (Duan *et al.* 2001; Ruiz-Gomez *et al.* 2002).

During the next important event, myoblast fusion, fusion-competent myoblasts fuse sequentially to each muscle founder cell and nascent myotube to generate a specific body wall muscle (Kim *et al.* 2015; Deng *et al.* 2017). The recognition and adhesion of the two types of myoblast occurs through the engagement of the immunoglobulin (Ig) domain proteins Sticks-and-stones (Sns) and Hibris (Hbs) on fusion-competent myoblasts with the related Ig domain proteins Kin of irre (Kirre) (aka, Dumbfounded, Duf) and Roughest (Rst, aka, IrreC) on the muscle founder cells. Downstream signaling cascades in both cell types lead to the differential assembly of polymerized actin structures at the prospective fusion site. Most prominently, within the fusion-competent myoblasts the extension of actin-propelled protrusions are thought to cause membrane breakdown and fusion pores upon invading the founder cells (Kim *et al.* 2015; Deng *et al.* 2017).

Towards the end and after myoblast fusion, the syncytial muscle precursors form extensions that migrate to the specific epidermal muscle attachment sites and make contacts with them. Several regulators involved in myotube guidance and the establishment of initial contacts have been identified (Schweitzer *et al.* 2010; Maartens and Brown 2015; Schulman *et al.* 2015). In many cases this involves the release of Slit proteins from tendon cells, their binding to Robo receptors on the myotubes for proper guidance, and subsequent arrest of migration upon the interaction of Robo with the LRT protein on the membranes of the tendon cells. In other cases, interactions between the trans-membrane protein Kon-tiki (Kon, aka Perdido,), its cytoplasmic partner, Grip (a PDZ domain-containing protein), and the cell-surface protein, Echinoid (Ed), are necessary for targeting muscles to their proper attachment sites and making initial connections. During muscle differentiation, myotendinous junctions and muscle-muscle connections at the attachment sites are stabilized by integrin-mediated adhesions to specific extracellular matrix structures between these cells, as well as by the intracellular linkage of integrin-associated proteins to the cytoskeleton in both muscle and tendon cells (Schnorrer and Dickson 2004; Schweitzer *et al.* 2010; Maartens and Brown 2015).

Muscle differentiation culminates in the assembly of the sarcomeric apparatus, proper positioning of the myonuclei, and the establishment of neuromuscular junctions (Nose 2012; Volk 2013; Schulman *et al.* 2015; Lemke and Schnorrer 2017). Two key regulators known to act in muscle differentiation in vertebrates, *MyoD* and *Mef2*, are present in *Drosophila* as single orthologs and regulate muscle differentiation (Michelson *et al.* 1990; Paterson *et al.* 1991; Bour *et al.* 1995; Lilly *et al.* 1995; Arredondo *et al.* 2001). *nautilus* (*nau*; *Drosophila MyoD*) is required for the formation and differentiation of a specific subset of larval muscles (Balagopalan *et al.* 2001). *Drosophila Mef2,* in addition to functioning in terminal muscle differentiation, has also an essential earlier role in myoblast fusion, likely via transcriptionally activating certain myoblast fusion genes (Sandmann *et al.* 2006; Brunetti *et al.* 2015).

Prior to adult muscle development, the vast majority of the larval body wall muscles are histolyzed in early pupae and the adult muscles are built (in most cases from scratch) from stem cell-like cells that have been set aside during embryogenesis and are called adult muscle precursors (AMPs) (Gunage *et al.* 2017). Many, but not all of the known

regulators of larval muscle development are being reutilized during adult muscle development (Gunage *et al.* 2017). Thoracic AMPs associated with wing and leg discs are being patterned by signals from the epidermal cell layer and thus assume differential muscle fates, such as direct versus indirect flight muscles in case of the wing disc (Sudarsan *et al.* 2001). The indirect flight muscles differ in their ultrastructure from all other fly muscles in that they exhibit fibrillar, stretch-activated myofibers instead of tubular myofiber organizations. The involvement of the transcription factor Spalt major (Salm) as a master regulator in fibrillar muscle development in the indirect flight muscles is currently one of the few documented examples of conserved regulatory processes during muscle development between *Drosophila* and *Tribolium* (Schönbauer *et al.* 2011).

While this brief synopsis shows that past research has yielded a considerable amount of information on the regulation of muscle development in *Drosophila*, there still remain many gaps in our knowledge. To fill these gaps, large-scale loss-of-function screens have been performed by RNA interference and classical mutagenesis (Schnorrer *et al.* 2010; Johnson *et al.* 2013; Hollfelder *et al.* 2014; Camuglia *et al.* 2018). Because each of these methods has its own limitations, such as the delayed action of inducible RNAi in *Drosophila* embryos and the lack of phenotypes with functionally-redundant genes, we chose to undertake an alternative approach. To identify new components required for normal development of the body wall (somatic) musculature in arthropods, and also to begin to understand the similarities and differences between dipterans and coleopterans in this process, we participated in a large-scale systemic RNAi screen in the red flour beetle *Tribolium castaneum*, termed „iBeetle“ (Schmitt-Engel *et al.* 2015). Our general strategy was to identify genes with interesting knock-down phenotypes in the somatic musculature in *Tribolium* and subsequently study the functions of their orthologs in *Drosophila* in more detail. Herein we describe an overview of this screen and provide a first description of the obtained muscle phenotypes. We include examples of genes from the screen that are orthologous to known regulators of muscle development in *Drosophila*. Of note, we identified and verified genes in the *Tribolium* screen that have not been implicated in muscle development in previous research.

Materials and Methods

Tribolium strains

All beetles were kept under standard conditions (Brown *et al.* 2009) on white wheat flour containing 5% dry yeast at 25 °C and shifted to 32 °C for the experiments. The following *Tribolium castaneum* stocks were used in this study: *San Bernardino (SB)*, *black* (Sokoloff *et al.* 1960), *piggyBac pig-19 (pBA19)* (Lorenzen *et al.* 2003), *D17Xred* (Schmitt-Engel *et al.* 2015).

Cloning of *Tc-Mef2* and *Tc-duf/Kirre*

To synthesize first strand cDNA the Omniscript RT kit (Qiagen) was used. 5 µg total RNA derived from early embryonic stages were reverse transcribed utilizing oligo(dT) primers and following the manufacturer's instructions. 1 µl of the cDNA synthesis reaction was subsequently used to amplify 1kb fragments of *Tc-Mef2* (*TC010850)* and *Tc-duf* (*TC002914)* by PCR using gene specific primers (*Tc-Mef2-F GTTTGATCGGTCCGTGCTAT*; *Tc-mef2-R GACCGCTCCAGGATATTGAA*; *Tc-duf-F ACGCGACCAGGAAATATCAC*; *Tc-duf-R GGAAGCTTGGTTCGGTGTAA*). The ~1kb *Tc-duf* fragment fully includes the iB_03469 sequences at its 3' portion. The amplified PCR fragments were gel purified and cloned into the pCR©II-TOPO© vector using the TOPO©TA Cloning© Dual Promoter kit (ThermoFisher Scientific) following the manufacturer's instructions.

Tc-mef2 and *Tc-Duf RNA* probe synthesis

To synthesize antisense DIG-labeled Riboprobes, 1 µg of linearized pCR©II-*Tc-mef2* or pCR©II-*Tc-duf/kirre* was *in vitro* transcribed utilizing the DIG RNA labelling kit (Roche) following the manufacturer's instructions. The DIG-labelled RNA was purified using the RNA cleanup protocol of the RNeasy Kit (Qiagen). *Tribolium* fixation and *in situ* hybridization were performed as described previously (Tautz and Pfeifle 1989; Patel *et al.* 1994).

Double-stranded RNA preparation and injections for RNAi

To synthesize dsRNA of *Tc-mef2* and *Tc-Duf*, 1 ng of pCR©II-Tc-mef2 or pCR©II-Tc-Duf/Kirre were used in a PCR reaction using T7 and T7-SP6 primers. The amplified

fragments were purified using the QIAquick Gel Purification Kit (Qiagen) and dsRNA was produced as described in (Bucher *et al.* 2002). To induce parental RNAi 1 µg/µl of Tc-mef2 or Tc-Duf dsRNA were injected into adult females as described in van der Zee *et al.* (2006).

The procedure of the iBeetle larval and pupal RNAi injection screen and the procedure for the analysis of late embryonic/early larval muscles are described in detail in Schmitt-Engel *et al.* (2015). The dsRNAs used in the iBeetle Screen were obtained from Eupheria Biotec GmbH (Dresden). To generate the dsRNAs for the rescreen cDNA was generated using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The cDNA was then used to amplify fragments by PCR with the same primers as used by Eupheria Biotec GmbH (Dresden) for the original iBeetle Screen. The sequences of the dsRNA fragments in the primary screen (iB dsRNAs; annotated in the format iB_nnnnn) and of the dsRNA fragments non-overlapping with the iB fragments (annotated as iB_nnnnn_2) are accessible in http://ibeetle-base.uni-goettingen.de/gb2/gbrowse/tribolium/ (select track "iB dsRNA"). These dsRNAs were synthesized from PCR products using the MEGAscript™ T7 Transcription Kit (Ambion).

Research materials and data availability

Materials produced in this study are available upon request. The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures with the exception of sequence information (e.g., for amplification primers) that is available at http://ibeetle-base.uni-goettingen.de/gb2/gbrowse/tribolium/.

Results

Comparison of somatic muscle patterns between *Tribolium* and *Drosophila*

The RNAi screen took advantage of the systemic nature of parental RNA interference in *Tribolium castaneum* (Bucher *et al.* 2002). For screening we employed the *pig-19* line, which carries a piggyBac insertion in the 3' UTR of *TC003326* (*Actin-87E-like*) that expresses EGFP in all larval somatic and visceral muscles (Lorenzen *et al.* 2003). Before screening we used this expression pattern to characterize the wild type pattern of the larval body wall musculature in more detail. As shown in Fig. 1A - E, each abdominal segment displays a stereotypical arrangement of ca. 28 syncytial muscle fibers underneath the body wall, excluding ventral areas where the CNS is located and the dorsal midline where the unlabeled dorsal vessel is positioned. In the thoracic segments, which unlike *Drosophila* embryos carry appendices, a modified muscle pattern is observed (Fig. 1A - D) and a stereotypic muscle pattern is also seen in the legs (Fig. 1F). The muscle arrangement in each abdominal segment into dorsal, lateral, and ventral groups, as well as their orientations as longitudinal, oblique, transverse, and acute muscles within these groups, are strongly reminiscent of the well-characterized pattern of abdominal body wall muscles in *Drosophila* (Fig. 1G - I) (Bate 1993). However, the details differ and due to the current lack of conserved markers for individual muscles or sets of muscles it is presently unclear whether any of these muscles are homologous between the two arthropod species.

Additional evidence for similarities in muscle development between the two arthropod species came from the expression and function of several *Tribolium castaneum* (*Tc*) orthologs of known key regulators of muscle development in *Drosophila.* The expression of the early mesodermal regulator Twist in the embryonic mesoderm of *Tribolium* has been documented extensively (Händel *et al.* 2005; Stappert *et al.* 2016), and as expected, RNAi against *Tc-twist* in *pig-19* led to a complete absence of muscles (data not shown, see http://ibeetle-base.uni-goettingen.de). *TC002914*, the single ortholog of *Drosophila kirre* (aka *duf*) and *roughest* (*rst*), which in *Drosophila* are essential for myoblast fusion in a functionally redundant manner, is expressed in somatic mesodermal cells of the body wall and limbs in *Tribolium* embryos (Fig. 1J). Whereas *Drosophila kirre* is expressed only in muscle founder cells its paralog *rst* is expressed in both founder and

fusion-competent myoblasts (Ruiz-Gomez *et al.* 2000; Strunkelnberg *et al.* 2001). The seemingly broader expression of *TC002914* as compared to *Drosophila kirre* may suggest that the expression of *TC002914* is more akin to that of *rst* in *Drosophila*. This interpretation is supported by the expression of *Tc-sticks-and-stones* (Tc-*sns*) (*TC032336*), which is similar albeit slightly narrower in the somatic mesoderm as compared to *Tc-kirre*/*rst* (data not shown). Potentially, *Tc-sns mRNA* is restricted to fusion-competent myoblasts like *Drosophila* Sns, which regulates myoblast fusion upon interaction with Kirre. Importantly, RNAi knock-down upon injections of *Tc-kirre*/*rst* dsRNA into in *pig-19* adult females led to strong reductions in both numbers and sizes of GFP-stained muscles (Fig. 1K, K'), which corresponds to analogous phenotypes in *kirre rst* double mutants in *Drosophila* (Ruiz-Gomez *et al.* 2000; Strunkelnberg *et al.* 2001). In the most severe examples, only few small muscle fibers were present (Fig. 1K), whereas in milder cases presumably resembling partial knock-downs a fraction of the muscles were present but many others were very thin or missing (Fig. 1K'). Incidentally, this example also illustrates that RNAi screens in *Tribolium* have the potential to identify myogenic regulators that may have been missed in *Drosophila* screens due to the presence of functionally-redundant paralogs, if these have only a single ortholog in the beetle. Another example of similarities in myogenic regulation between the two arthropod species is provided by *Mef2*, which in *Drosophila* is expressed in all muscle progenitors (and muscles) and encodes a crucial muscle differentiation factor (Lilly *et al.* 1994; Nguyen *et al.* 1994). In *Tribolium* embryos, the *Mef2* ortholog *TC010850* is also expressed broadly in the somatic mesoderm of the body wall and the limb (Fig. 1L). Like with *Drosophila Mef2* mutant embryos, knock-down of *TC010850* upon dsRNA injections into adult females caused almost complete loss of muscles as detected by GFP in *pig-19* embryos (Fig 1M, M') (Bour *et al.* 1995; Lilly *et al.* 1995). Additional examples of phenotypes of genes orthologous to known regulators of *Drosophila* myoblast fusion are shown in the accompanying paper (Schultheis et al., 2018b).

Regimen of screening and verification of candidate genes

The main screen ('pupal injection screen'), which we focus here upon, involved injections of dsRNAs (named iB RNAs) for a total of ~ 8,500 genes into the body walls of female *pig-19* pupae. After crossing the eclosed females (if viable and fertile) with *black* males the GFP-marked muscle patterns of their offspring were analyzed live under a

fluorescence microscope in late embryonic stages and newly hatched first instar larvae, as shown for controls in Fig. 1. The screen was performed in two consecutive rounds and included screenings for various additional phenotypes by the screening consortium, as was described in an overview of the results of the first screening round of ~5,300 genes (Schmitt-Engel *et al.* 2015). A parallel screen ('larval injection screen') involved injections of a total of ~5,000 iB dsRNAs into L6 stage larvae of the *D17Xred* strain and the analysis of the GFP-marked late pupal thoracic muscle patterns (as well as additional phenotypes), as described in Schmitt-Engel *et al.* (2015).

In the first-pass screen with pupal dsRNA injections, 229 of the ~8,500 tested genes were annotated with specific embryonic muscle phenotypes upon iB dsRNA injections that were not deemed to be secondary to broader disruptions such as segmentation defects, severe embryonic malformations, early developmental arrest, etc. (Fig. 2). In the first-pass larval injection screen, 96 of the ~5000 tested genes were annotated with specific pupal muscle phenotypes, only six of which were also found in the pupal screen. In the following, we focus particularly on the genes with embryonic muscle phenotypes detected in the pupal injection screen. As shown in Table 1, 24 of these corresponded to genes with *Drosophila* orthologs that have been implicated in various aspects of *Drosophila* muscle development. Although in this first-pass screen most of the muscle phenotypes were not characterized and annotated in detail, the phenotypes for the knock-downs of several *Tribolium* genes were reminiscent of the muscle phenotypes of mutations in their orthologs in *Drosophila*. For example, knock-down of the *Tribolium* ortholog of the *Drosophila* muscle identity gene *org-1* led to the absence of specific muscles, including the segment border muscles like in *Drosophila* (Fig. 3B, cf. Fig. 3A) (Schaub *et al.* 2012). Likewise, knock-down of the ortholog of *nautilus* (*nau*; *Drosophila MyoD*) led to a loss of muscles and reduction of the GFP differentiation marker, although the observed phenotype appears more severe as compared to *Drosophila nau* mutants (Balagopalan *et al.* 2001) (Fig. 3C). Also knock-down of the *kon-tiki* (*kon*) ortholog caused the absence of subsets of muscle fibers, which in *Drosophila kon* mutants is attributed to defects in myotube migration and attachments in subsets of muscles (Schnorrer *et al.* 2007) (Fig. 3D). Knock-downs of the *Tribolium* orthologs of *inflated* (*if*) (Fig. 3E) and *stripe* (*sr*) (Fig. 3F) led to the appearance of spherical myotubes. This is likely due to disrupted muscle attachments because of weakened integrin-mediated adhesions with tendon cells (in the case of *if*) or the absence of differentiated tendon

cells (in case of *sr*), as shown previously in *Drosophila* mutants of their respective orthologs (Brown 1994; Volk and VijayRaghavan 1994). A smaller number of muscle regulatory genes known from *Drosophila* showed phenotypes in *Tribolium* late pupal muscles upon knock-downs in the larval injections screen (Table 1 and data not shown). In sum, among the *Tribolium* orthologs of 76 *Drosophila* genes connected with various roles in muscle development, 28 showed a knock-down phenotype in late embryonic or (more rarely) in late pupal muscles. For 26 genes no such phenotype was annotated in the first pass screen, 8 were not screenable for muscle phenotypes due to lethality, sterility, or broad embryo disruptions prior to muscle formation, and 14 have not been screened yet by dsRNA injections (Table S1). These data, as well as the data for muscle phenotypes upon knock-downs of genes previously not implicated in muscle development can be accessed in the searchable iBeetle database (http://ibeetle-base.uni-goettingen.de), along with other morphological defects that were screened for in these large-scale RNAi screens (Dönitz *et al.* 2015; Dönitz *et al.* 2018).

In the next step, we performed a rescreen with 102 of the 229 iB dsRNAs that had annotated muscle phenotypes in the first-pass screen in order to confirm these phenotypes. Because of our aim to identify new *Drosophila* genes with functions in myogenesis we omitted the genes that have *Drosophila* orthologs with known roles in myogenesis and those that lack *Drosophila* orthologs (except for a few with striking and highly penetrant phenotypes). In addition, iB dsRNAs that produced phenotypes with very low penetrance were omitted, as were those that upon closer inspection of the database were likely to yield indirect effects on muscle development. In this rescreen, again performed in *pig-19*, the muscle phenotypes were confirmed for 54 of the original iB dsRNAs (Figure 2). Particularly in the first round of first pass screening, it turned out that many of the false-positives were due to GFP leakage from muscles upon injury of the late stage embryos or larvae during preparation and mounting. In the second round of first pass screening this effect was taken into account.

In a second rescreen, 40 of the corresponding genes were tested again with the aim to exclude off-target effects and possible strain specific effects. To reduce the work load, 14 genes were omitted, including some that showed only maternal expression in *Drosophila* or others that encoded enzymes with potentially broader or "house-keeping" functions. These included for example *TC010977* and *TC002552* that encode an elongase

of very long fatty acids and a cytochrome P450, respectively. However we note that genes of this type may still have interesting functions in muscle tissues (see Wang *et al.* 2016; Xu *et al.* 2018). For the 40 selected genes, new dsRNAs with sequences that did not overlap with the original iB dsRNA sequences were injected into female pupae from the San Bernardino (SB) strain, and the muscle patterns were analyzed in embryos from a cross of these females with *pig-19* males. In addition, the original iB dsRNA fragments were tested by analogous SB pupa injections. These tests served to rule out off-target effects and to confirm an essential role in muscle formation in different genetic backgrounds. As a result, the knock-down phenotypes in the embryonic musculature were confirmed for 28 of the 40 genes with the non-overlapping dsRNAs (Fig. 2). Their phenotypes and additional information on the affected genes are shown in Table 2. These genes show a broad spectrum of distinct muscle phenotypes and the encoded proteins belong to a variety of different protein classes, most of which have not been implicated in muscle development in *Drosophila*.

Discussion

The iBeetle RNAi screen for muscle defects in *Tribolium* complements analogous screens in *Drosophila*

Using *Tribolium* as high-throughput RNAi screening platform, we uncovered a number of genes that had not been implicated in muscle development in previous work in *Drosophila*. Conversely, not all orthologs of known *Drosophila* muscle genes were recovered (27 out of 62; these *Drosophila* genes were identified via both forward and reverse genetics). This suggests that the different properties of these alternative screening platforms sometimes reveal different facets of a given biological process.

In *Drosophila*, several forward genetic screens using fluorescent reporter lines for somatic muscles have been performed. In two screens *MHC-tauGFP*, which marks all somatic muscles similar to the EGFP enhancer trap line used herein in *Tribolium,* was employed to screen for late embryonic muscle phenotypes upon ethyl methanesulfonate (EMS) mutagenesis (Chen and Olson 2001; Chen *et al.* 2008). Although the full screens have not been published, several mutants in genes regulating myoblast fusion, myotube targeting and attachment, and muscle maturation have been recovered from these (Chen and Olson 2001; Chen *et al.* 2003; Schnorrer *et al.* 2007; Johnson *et al.* 2013). Other EMS mutagenesis screens employed a cytoplasmic RFP reporter or a nuclear dsRed reporter driven by the founder cell enhancers of the muscle identity gene *org-1* and *apterous* (*ap*), respectively, in small subsets of muscles. From the *org-1*-RFP screen, mutants in the genes for the extracellular matrix proteins laminin β and collagen IV α1 have been reported to date, which revealed important roles of these proteins in muscle attachments, e.g., to the cardiac ECM (Hollfelder *et al.* 2014). From the ap::NLSdsRed screen, genes such as *esconsin* (*ens*) were identified that regulate nuclear positioning within myotubes (Metzger *et al.* 2012). For a number of reasons, including incomplete coverage of the genome and the possible failure to detect more subtle muscle phenotypes, none of these screens reached saturation. RNAi screens for muscle phenotypes upon gene knock-downs have been performed in *Drosophila* as well, which used lethality and locomotion or flight behavior as initial screening criteria. Instead of injections with dsRNAs, these screens employed inducible expression of dsRNAs with the UAS/GAL4 system and muscle-specific drivers. Due to the generally

low knock-down efficiency of this method in embryonic stages, screening for phenotypes was largely confined to larval, pupal, and adult stages. In a large-scale RNAi screen with *Mef2*-GAL4 driving transgenic UAS-IR RNAi insertions, initial screening was for lethality and flightlessness, and follow-up analyses involved the examination of sarcomeric GFP markers (Schnorrer *et al.* 2010). This screen identified a large number of known and yet uncharacterized muscle-intrinsic players acting in muscle morphogenesis and function, including *spalt*, which turned out to be an evolutionarily-conserved master regulator of fibrillar flight muscle development during metamorphosis in arthropods (Schönbauer *et al.* 2011). Among the 23 genes with verified *Tribolium* muscle phenotypes (Table 2) that have *Drosophila* orthologs covered in the Schnorrer screen, only six were annotated for phenotypes in the primary screen in *Drosophila* (*CG11526*: early pupal lethality; *ths*: weak fliers; *sau*: semilethality; *babo*: late pupal lethality; *croc*: weak fliers; *MTA1-like*: flightlessness) and none of these were followed up with analyses of muscle phenotypes. Reasons for the viability and normal flight capabilities of the other 17 could include, 1) redundant gene functions in *Drosophila*, as exemplified by *Nostrin* in the accompanying paper (Schultheis et al, 2018b), or mild defects not leading to lethality or overt flight defects; 2) divergent functions of the fly orthologs; 3) delayed functional knock-down in *Drosophila* embryos and absence of post-embryonic function in muscle development; 4) ineffective inverted repeat RNAs in *Drosophila* or false-positives in *Tribolium* in spite of the verification steps taken. In a recent small scale screen of 82 genes for larval locomotion defects, four genes with orthologs positive in the iBeetle screen were included: *twi* and *Vrp1*, which caused lethality, *if* which caused increased larval locomotion upon knock-down, and *nau* which lacked any locomotion phenotype. *singed* (*sn*, *Drosophila fascin*), which decreased locomotion, was shown to affect myoblast fusion to a similar degree as our *Nost cip4* double mutants (Camuglia *et al.* 2018; Schultheis et al, 2018b). *Tc-sn* (*TC006673*) was not annotated with a muscle phenotype in the iBeetle screen.

The iBeetle screen for genes with knock-down phenotypes in the somatic muscles had the technical advantage provided by the systemic agency of RNAi in *Tribolium* (Bucher *et al.* 2002), which made the injection work more economical due to the ease of injecting larvae and pupae and the recovery of large numbers of offspring from each injected female animal. In addition, this procedure is expected to knock down both the maternal and zygotic contributions of genes, and thus has the potential to uncover

genes that may have been missed in the *Drosophila* screens due to maternal rescue. In contrast to the mesoderm-specific RNAi screens in *Drosophila* but similar to the EMS screens, the iBeetle RNAi screen involved global knock-downs of gene functions. Hence, in addition to genes with muscle-intrinsic functions, genes with non-autonomous functions in muscle developments could be recovered as exemplified by *stripe* (Fig. 2), which in *Drosophila* is essential for muscle attachments through its function in determining epidermal tendon cell fates (Becker *et al.* 1997; Vorbrüggen and Jäckle 1997). On the other hand, global knock-downs increase the risk of secondary effects on muscle development, e.g., due to early effects of genes on developmental events prior to muscle development such as embryonic patterning, cell proliferation, etc.. These effects were minimized because the iBeetle screen included careful analyses of the larval cuticles with the aim to recover patterning genes as well (Schmitt-Engel *et al.* 2015; Ansari *et al.* 2018). Thus, even if some of the genes on our shortlist (Table 2) have additional roles in other tissues, their most prominent functions are expected to be in the development of the somatic musculature, either via mesoderm-intrinsic or via non-autonomous mechanisms.

As for any other screen, it is clear that we missed many genes affecting *Tribolium* muscle development. Among the *Tribolium* orthologs of 62 genes known to affect *Drosophila* muscle development that were included in the iBeetle screen, 27 were recovered through their knock-down phenotypes in the musculature. This is a surprisingly low portion given that the positive controls of genes with a variety of developmental functions included in the screen indicated a detection rate of about 80-90% (Schmitt-Engel *et al.* 2015). Further analyses are required to test whether this is due to false negative annotations in the screen or to biological differences in the muscle developmental program between the two arthropod species. One potential reason for missing larval muscle phenotypes in false-negatives could be the relatively long time delay between the pupal injections and the onset of embryonic muscle development, which in some cases may reduce the dsRNA concentrations below a critical threshold. As indicated by the rather severe phenotypes obtained with adult injections of *Tc-duf* dsRNAs as compared to those with pupal injections for several other myoblast fusion genes, adult injections may sometimes provide stronger effects. We did not systematically explore this possibility because pupal injections were necessary for other participants in the consortium, who screened for ovary and oogenesis defects (Schmitt-

Engel *et al.* 2015). As in other screens with pan-muscle markers, it is also likely that some genes with more subtle knock-down phenotypes, e.g., affecting individual or small subsets of muscles, were missed and indeed, the majority of recovered phenotypes affected muscles globally (for an exception, see *TC009963*, Table 2).

Novel genes identified in the iBeetle screen

Of note, many *Tribolium* genes were recovered for which their orthologs were not known to affect muscle development in *Drosophila*. A few genes lack any orthologs in *Drosophila* although some of them do have orthologs in vertebrates. A notable example for the latter is *Tc-Rbm24* (*TC001720*), for which knock-downs exhibited severe muscle phenotypes (Table 2; Schmitt-Engel *et al.* 2015). *Tc-Rbm24* mRNA is specifically expressed in the developing and mature embryonic somatic musculature and, more weakly, in the dorsal vessel (as well as in the CNS; DS and MF, data not shown). During our screen, mouse *RNA-binding motif protein 24* (*Rbm24*) (and presumably its paralog *Rbm38* (Miyamoto *et al.* 2009)) was reported to play a major role in embryonic skeletal muscle development by regulating alternative splicing of a large number of muscle-specific primary transcripts (Yang *et al.* 2014). It is conceivable that in *Drosophila* the role of *Tc-Rbm24* in muscle development is exerted by other members of the RRM superfamily of RNA binding proteins. Starting to explore this possibility, we found that some *Drosophila* RRM superfamily members are expressed in the embryonic somatic mesoderm (*boule*, *CG33714*, *Hrb87F*; in the case of *boule* exclusively so; DS and MF, data not shown). In addition to *Tc-Rbm24*, three other genes encoding putative *Tribolium* RNA binding proteins were recovered (*TC010637*, *TC006055*, *TC010693*; Table 2), reinforcing the important contribution of RNA metabolism in regulating normal muscle development (see also examples from *Drosophila*; Volk *et al.* 2008; Johnson *et al.* 2013; Oas *et al.* 2014; Spletter *et al.* 2015).

Several other identified genes shown in Table 2 encode predicted chromatin regulators, which are likely to influence gene regulatory programs during muscle development (*TC005276, TC005276, TC006419, TC009963)* and possibly have additional, perhaps less prominent roles in other tissues. *Tc-croc* (*TC002813*, Table 2), which encodes a forkhead domain transcription factor, exclusively affects the ventral muscles, suggesting that it may act as a muscle identity gene in *Tribolium*. Interestingly, *Drosophila croc* appears to be expressed in subsets of ventral mesodermal cells during early muscle development,

but we have been unable to detect any ventral muscle defects in *Drosophila croc* mutant embryos (Häcker *et al.* 1995; MW and MF, unpublished data).

The *Drosophila* and mammalian counterparts of TC032839 protein, Unc-76 and FEZ1, respectively, bind to the Kinesin-1 Heavy Chain (KHC). FEZ1 binding in combination with JNK interacting proteins (JIP1, and perhaps similarly JIP3) was shown to release Kinesin-1 autoinhibition and thus activate the motor protein for microtubule binding and motility (Blasius *et al.* 2007; Koushika 2008). In accordance with this molecular interaction, *Drosophila* and *C. elegans unc-76* were shown to be required for axonal outgrowth and transport (Bloom and Horvitz 1997; Gindhart *et al.* 2003). In *Drosophila* kinesin and kinesin-associated proteins, including JIP1/Aplip1 and JIP3/Synd, were shown to regulate nuclear positioning within muscle syncytia (Metzger *et al.* 2012; Schulman *et al.* 2014; Auld *et al.* 2018). Therefore we presume that Unc-76 likewise is involved in this regulatory pathway. *Tc-Unc-76* and *Dm-Unc-76* are both expressed in the somatic mesoderm (and more prominently in the CNS, as well as maternally), but unlike with RNAi in *Tribolium*, CRISPR/Cas9-generated zygotic null mutants did not show any overt muscle morphology phenotype in *Drosophila* embryos (DS, MW, and MF, unpublished data). Therefore, future analyses should investigate myonuclear positioning in *Drosophila* mutants that lack both the maternal and the zygotic contributions of *Unc-76*.

In addition, three signaling components were identified, namely Tc-fgf8, Tc-Babo, and Tc-Pvf3. *TC-fgf8* (*TC000278)* encodes the single *Tribolium* FGF8 member and is a putative ligand for the single, mesodermally-expressed FGF receptor Tc-fgfr, both of which were recently shown to be required for maintaining the expression of Tc-Twist in the somatic mesoderm of late stage embryos (Sharma *et al.* 2015). This requirement, along with a possible requirement for the activation of yet undefined differentiation genes, could explain the complete absence of *pig-19* muscle GFP in *Tc-fgf8* RNAi embryos. It will be interesting to investigate in more detail how far muscles can develop in the absence of FGF8 signals. *Tc-babo* (*TC003240*) encodes a *Tribolium* TGF-beta type 1 receptor. The thin-muscle phenotype upon knock-down is reminiscent of phenotypes obtained upon knock-downs of myoblast fusion genes (Schultheis et al., 2018b) and in this case could perhaps be due to under-proliferation of the fusion-competent myoblasts. *Tc-Pvf3* (*TC008417*) encodes a putative ligand for the *Tribolium* PDGF/VEGF

related receptor. In *Drosophila* this ligand/receptor interaction is required for normal proliferation, migration, and maintenance of hemocytes (Parsons and Foley 2013; Sopko and Perrimon 2013). Hemocytes, in turn, are required for the deposition of extracellular matrix components in the *Drosophila* body wall (Matsubayashi *et al.* 2017). Thus it is conceivable that the phenotype of rounded muscles seen upon *Tc-Pvf3* knock-down is due to muscle detachments as a result of deficient extracellular matrix at their attachment sites (Maartens and Brown 2015). Following up on these avenues, and likewise on the exact involvement of the other genes identified in the screen, could yield unexpected insights into new aspects in the regulation of arthropod muscle development (for additional preliminary and unpublished data see Schultheis, 2016 (in German)).

Conclusion

The iBeetle RNAi screen has identified numerous genes that are candidates for regulators of arthropod muscle development. In many cases, their orthologs were already known to play roles in muscle development in *Drosophila*, and in some cases in vertebrates. A significant number of the genes identified in *Tribolium* had not been recovered in previous *Drosophila* work before. It will be interesting to examine the roles of identified genes previously not implicated in muscle development in detail in both *Tribolium* and in *Drosophila*. In *Tribolium*, the functional studies by RNAi can now be complemented by CRISPR/Cas9 induced mutations and engineered gene loci (Gilles *et al.* 2015). In parallel, more detailed analyses of the process of *Tribolium* muscle development will provide interesting insight into the similarities and differences of muscle development in beetle as compared to fly embryos. In addition, the *Drosophila* orthologs of newly identified genes with muscle phenotypes in *Tribolium* can be studied for potential functions in fly muscle development and added to the well-developed framework of regulatory networks in this system. The accompanying paper (Schultheis et al., 2018b) presents an example of this approach by showing that the *Drosophila* ortholog of the F-Bar domain encoding gene *Tc-Nostrin*, identified in the iBeetle screen through its muscle phenotype, together with related F-Bar proteins plays a role in *Drosophila* myoblast fusion and the morphogenesis of adult midgut muscles. Functional redundancy in the fly previously had impeded the identification of this role in *Drosophila*.

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References

Ansari, S., N. Troelenberg, V. A. Dao, T. Richter, G. Bucher *et al.*, 2018 Double abdomen in a short-germ insect: Zygotic control of axis formation revealed in the beetle *Tribolium castaneum*. Proc Natl Acad Sci U S A 115**:** 1819-1824.

Arredondo, J. J., R. M. Ferreres, M. Maroto, R. M. Cripps, R. Marco *et al.*, 2001 Control of *Drosophila paramyosin/miniparamyosin* gene expression. Differential regulatory mechanisms for muscle-specific transcription. J Biol Chem 276**:** 8278-8287.

Auld, A. L., S. A. Roberts, C. B. Murphy, J. M. Camuglia and E. S. Folker, 2018 Aplip1, the *Drosophila* homolog of JIP1, regulates myonuclear positioning and muscle stability. J Cell Sci 131.

Azpiazu, N., P. Lawrence, J.-P. Vincent and M. Frasch, 1996 Segmentation and specification of the *Drosophila* mesoderm. Genes Dev. 10**:** 3183-3194.

Balagopalan, L., C. A. Keller and S. M. Abmayr, 2001 Loss-of-function mutations reveal that the *Drosophila nautilus* gene is not essential for embryonic myogenesis or viability. Dev Biol 231**:** 374-382.

Bate, M., 1993 The mesoderm and its derivatives in *The development of Drosophila melanogaster*, edited by M. Bate and A. Martinez-Arias. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Baylies, M., A. Martinez Arias and M. Bate, 1995 *wingless* is required for the formation of a subset of muscle founder cells during *Drosophila* embryogenesis. Development 121**:** 3829-3837.

Baylies, M., and A. Michelson, 2001 Invertebrate myogenesis: looking back to the future of muscle development. Curr Opin Genet Dev 11**:** 431-439.

Becker, S., G. Pasca, D. Strumpf, L. Min and T. Volk, 1997 Reciprocal signaling between *Drosophila* epidermal muscle attachment cells and their corresponding muscles. Development 124**:** 2615-2622.

Blasius, T. L., D. Cai, G. T. Jih, C. P. Toret and K. J. Verhey, 2007 Two binding partners cooperate to activate the molecular motor Kinesin-1. J Cell Biol 176**:** 11-17.

Bloom, L., and H. R. Horvitz, 1997 The *Caenorhabditis elegans* gene *unc-76* and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. Proc Natl Acad Sci U S A 94**:** 3414-3419.

Bodmer, R., and M. Frasch, 2010 Development and aging of the *Drosophila* heart, pp. 47-86 in *Heart Development and Regeneration*, edited by N. Rosenthal and R. P. Harvey. Academic Press, Oxford.

Bour, B., M. O'Brien, W. Lockwood, E. Goldstein, R. Bodmer *et al.*, 1995 *Drosophila* MEF2, a transcription factor that is essential for myogenesis. Genes Dev. 9**:** 730-741.

Brown, N., 1994 Null mutations in the alpha PS2 and beta PS integrin subunit genes have distinct phenotypes. Development 120**:** 1221-1231.

Brown, S. J., T. D. Shippy, S. Miller, R. Bolognesi, R. W. Beeman *et al.*, 2009 The red flour beetle, *Tribolium castaneum* (*Coleoptera*): a model for studies of development and pest biology. Cold Spring Harb Protoc 2009**:** pdb emo126.

Brunetti, T. M., B. J. Fremin and R. M. Cripps, 2015 Identification of singles bar as a direct transcriptional target of *Drosophila* Myocyte enhancer factor-2 and a regulator of adult myoblast fusion. Dev Biol 401**:** 299-309.

Bucher, G., J. Scholten and M. Klingler, 2002 Parental RNAi in *Tribolium* (*Coleoptera*). Curr Biol 12**:** R85-86.

Camuglia, J. M., T. R. Mandigo, R. Moschella, J. Mark, C. H. Hudson *et al.*, 2018 An RNAi based screen in *Drosophila* larvae identifies fascin as a regulator of myoblast fusion and myotendinous junction structure. Skelet Muscle 8**:** 12.

Carmena, A., S. Gisselbrecht, J. Harrison, F. Jimenez and A. Michelson, 1998 Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. Genes Dev. 15**:** 3910-3922.

Chen, D., A. Ahlford, F. Schnorrer, I. Kalchhauser, M. Fellner *et al.*, 2008 High-resolution, high-throughput SNP mapping in *Drosophila melanogaster*. Nat Methods 5**:** 323-329.

Chen, E. H., and E. N. Olson, 2001 Antisocial, an intracellular adaptor protein, is required for myoblast fusion in *Drosophila*. Dev Cell 1**:** 705-715.

Chen, E. H., B. A. Pryce, J. A. Tzeng, G. A. Gonzalez and E. N. Olson, 2003 Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector ARF6. Cell 114**:** 751-762.

de Joussineau, C., L. Bataille, T. Jagla and K. Jagla, 2012 Diversification of muscle types in *Drosophila*: upstream and downstream of identity genes. Curr Top Dev Biol 98**:** 277-301.

Deng, S., M. Azevedo and M. Baylies, 2017 Acting on identity: Myoblast fusion and the formation of the syncytial muscle fiber. Semin Cell Dev Biol 72**:** 45-55.

Dobi, K. C., V. K. Schulman and M. K. Baylies, 2015 Specification of the somatic musculature in *Drosophila*. Wiley Interdiscip Rev Dev Biol 4**:** 357-375.

Dönitz, J., L. Gerischer, S. Hahnke, S. Pfeiffer and G. Bucher, 2018 Expanded and updated data and a query pipeline for iBeetle-Base. Nucleic Acids Res 46**:** D831-D835.

Dönitz, J., C. Schmitt-Engel, D. Grossmann, L. Gerischer, M. Tech *et al.*, 2015 iBeetle-Base: a database for RNAi phenotypes in the red flour beetle *Tribolium castaneum*. Nucleic Acids Res 43**:** D720-725.

Duan, H., J. B. Skeath and H. T. Nguyen, 2001 *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. Development 128**:** 4489-4500.

Frasch, M., 1999 Controls in patterning and diversification of somatic muscles during *Drosophila* embryogenesis. Curr. Opin. Genet. Devel. 9**:** 522-529.

Gilles, A. F., J. B. Schinko and M. Averof, 2015 Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. Development 142**:** 2832-2839.

Gindhart, J. G., J. Chen, M. Faulkner, R. Gandhi, K. Doerner *et al.*, 2003 The kinesin-associated protein UNC-76 is required for axonal transport in the *Drosophila* nervous system. Mol Biol Cell 14**:** 3356-3365.

Gunage, R. D., N. Dhanyasi, H. Reichert and K. VijayRaghavan, 2017 *Drosophila* adult muscle development and regeneration. Semin Cell Dev Biol 72**:** 56-66.

Häcker, U., E. Kaufmann, C. Hartmann, G. Jürgens, W. Knöchel *et al.*, 1995 The *Drosophila* fork head domain protein Crocodile is required for the establishment of head structures. EMBO J 14**:** 5306-5317.

Händel, K., A. Basal, X. Fan and S. Roth, 2005 *Tribolium castaneum twist*: gastrulation and mesoderm formation in a short-germ beetle. Dev Genes Evol 215**:** 13-31.

Hollfelder, D., M. Frasch and I. Reim, 2014 Distinct functions of the laminin beta LN domain and collagen IV during cardiac extracellular matrix formation and stabilization of alary muscle attachments revealed by EMS mutagenesis in *Drosophila*. BMC Dev Biol 14**:** 26.

Johnson, A. N., M. H. Mokalled, J. M. Valera, K. D. Poss and E. N. Olson, 2013 Post-transcriptional regulation of myotube elongation and myogenesis by Hoi Polloi. Development 140**:** 3645-3656.

Kim, J. H., P. Jin, R. Duan and E. H. Chen, 2015 Mechanisms of myoblast fusion during muscle development. Curr Opin Genet Dev 32**:** 162-170.

Koushika, S. P., 2008 "JIP"ing along the axon: the complex roles of JIPs in axonal transport. Bioessays 30**:** 10-14.

Lee, H., and M. Frasch, 2000 Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target *sloppy paired*. Development 127**:** 5497-5508.

Lemke, S. B., and F. Schnorrer, 2017 Mechanical forces during muscle development. Mech Dev 144**:** 92-101.

Leptin, M., 1991 *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm formation. Genes and Development 5**:** 1568-1576.

Lilly, B., S. Galewsky, A. Firulli, R. Schulz and E. Olson, 1994 D-MEF2: a MADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during *Drosophila* embryogenesis. Proc Natl Acad Sci U S A 91**:** 5662-5666.

Lilly, B., B. Zhao, G. Ranganayakulu, B. Paterson, R. Schulz *et al.*, 1995 Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. Science 267**:** 688-693.

Lorenzen, M. D., A. J. Berghammer, S. J. Brown, R. E. Denell, M. Klingler *et al.*, 2003 piggyBac-mediated germline transformation in the beetle *Tribolium castaneum*. Insect Mol Biol 12**:** 433-440.

Maartens, A. P., and N. H. Brown, 2015 The many faces of cell adhesion during *Drosophila* muscle development. Dev Biol 401**:** 62-74.

Matsubayashi, Y., A. Louani, A. Dragu, B. J. Sanchez-Sanchez, E. Serna-Morales *et al.*, 2017 A Moving Source of Matrix Components Is Essential for De Novo Basement Membrane Formation. Curr Biol 27**:** 3526-3534 e3524.

Metzger, T., V. Gache, M. Xu, B. Cadot, E. S. Folker *et al.*, 2012 MAP and kinesin-dependent nuclear positioning is required for skeletal muscle function. Nature 484**:** 120-124.

Michelson, A. M., S. M. Abmayr, M. Bate, A. Martínez-Arias and T. Maniatis, 1990 Expression of a *MyoD* family member prefigures muscle pattern in *Drosophila* embryos. Genes and Development 4**:** 2086-2097.

Miyamoto, S., K. Hidaka, D. Jin and T. Morisaki, 2009 RNA-binding proteins Rbm38 and Rbm24 regulate myogenic differentiation via p21-dependent and -independent regulatory pathways. Genes Cells 14**:** 1241-1252.

Nguyen, H., R. Bodmer, S. Abmayr, J. McDermott and N. Spoerel, 1994 *D-mef2*: a *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. Proc Natl Acad Sci U S A 91**:** 7520-7524.

Nose, A., 2012 Generation of neuromuscular specificity in *Drosophila*: novel mechanisms revealed by new technologies. Front Mol Neurosci 5**:** 62.

Oas, S. T., A. L. Bryantsev and R. M. Cripps, 2014 Arrest is a regulator of fiber-specific alternative splicing in the indirect flight muscles of *Drosophila*. J Cell Biol 206**:** 895-908.

Parsons, B., and E. Foley, 2013 The *Drosophila* platelet-derived growth factor and vascular endothelial growth factor-receptor related (Pvr) protein ligands Pvf2 and Pvf3 control hemocyte viability and invasive migration. J Biol Chem 288**:** 20173-20183.

Patel, N. H., B. G. Condron and K. Zinn, 1994 Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles. Nature 367**:** 429-434.

Paterson, B. M., V. Walldorf, J. Eldrigde, A. Dübendorfer, M. Frasch *et al.*, 1991 The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. Proc Natl Acad Sci USA 88**:** 3782-3786.

Riechmann, V., U. Irion, R. Wilson, A. Grosskortenhaus and M. Leptin, 1997 Control of cell fates and segmentation in the *Drosophila* mesoderm. Development 124**:** 2915-2922.

Ruiz-Gomez, M., N. Coutts, A. Price, M. Taylor and M. Bate, 2000 *Drosophila* Dumbfounded: a myoblast attractant essential for fusion. Cell 102**:** 189-198.

Ruiz-Gomez, M., N. Coutts, M. Suster, M. Landgraf and M. Bate, 2002 *myoblasts incompeten*t encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*. Development 129**:** 133-141.

Sandmann, T., L. J. Jensen, J. S. Jakobsen, M. M. Karzynski, M. P. Eichenlaub *et al.*, 2006 A temporal map of transcription factor activity: Mef2 directly regulates target genes at all stages of muscle development. Dev Cell 10**:** 797-807.

Schaub, C., H. Nagaso, H. Jin and M. Frasch, 2012 Org-1, the *Drosophila* ortholog of Tbx1, is a direct activator of known identity genes during muscle specification. Development 139**:** 1001-1012.

Schmitt-Engel, C., D. Schultheis, J. Schwirz, N. Ströhlein, N. Troelenberg *et al.*, 2015 The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology. Nat Commun 6**:** 7822.

Schnorrer, F., and B. J. Dickson, 2004 Muscle building; mechanisms of myotube guidance and attachment site selection. Dev Cell 7**:** 9-20.

Schnorrer, F., I. Kalchhauser and B. J. Dickson, 2007 The transmembrane protein Kon-tiki couples to Dgrip to mediate myotube targeting in *Drosophila*. Dev Cell 12**:** 751-766.

Schnorrer, F., C. Schönbauer, C. C. Langer, G. Dietzl, M. Novatchkova *et al.*, 2010 Systematic genetic analysis of muscle morphogenesis and function in *Drosophila*. Nature 464**:** 287-291.

Schönbauer, C., J. Distler, N. Jährling, M. Radolf, H. U. Dodt *et al.*, 2011 Spalt mediates an evolutionarily conserved switch to fibrillar muscle fate in insects. Nature 479**:** 406-409.

Schulman, V. K., K. C. Dobi and M. K. Baylies, 2015 Morphogenesis of the somatic musculature in *Drosophila melanogaster*. Wiley Interdiscip Rev Dev Biol 4**:** 313-334.

Schulman, V. K., E. S. Folker, J. N. Rosen and M. K. Baylies, 2014 Syd/JIP3 and JNK signaling are required for myonuclear positioning and muscle function. PLoS Genet 10**:** e1004880.

Schultheis, D., 2016 Identification and characterization of new genes regulating myogenesis via a genome-wide RNAi screen in *Tribolium castaneum*, pp. 394. Thesis, *Naturwissenschaftliche Fakultät / Department Biologie*. Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU).

Schultheis, D., J. Schwirz and M. Frasch, 2018b RNAi screen in *Tribolium* reveals involvement of F-BAR proteins in myoblast fusion and visceral muscle morphogenesis in arthropods. BioRxiv submitted.

Schweitzer, R., E. Zelzer and T. Volk, 2010 Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. Development 137**:** 2807-2817.

Sharma, R., K. Beer, K. Iwanov, F. Schmohl, P. I. Beckmann *et al.*, 2015 The single *fgf* receptor gene in the beetle *Tribolium castaneum* codes for two isoforms that integrate FGF8- and Branchless-dependent signals. Dev Biol 402**:** 264-275.

Sokoloff, A., H. M. Slatis and J. Stanley, 1960 The black mutation in *Tribolium castaneum*. J Heredity 51**:** 131-135.

Sopko, R., and N. Perrimon, 2013 Receptor tyrosine kinases in *Drosophila* development. Cold Spring Harb Perspect Biol 5.

Spletter, M. L., C. Barz, A. Yeroslaviz, C. Schonbauer, I. R. Ferreira *et al.*, 2015 The RNA-binding protein Arrest (Bruno) regulates alternative splicing to enable myofibril maturation in *Drosophila* flight muscle. EMBO Rep 16**:** 178-191.

Stappert, D., N. Frey, C. von Levetzow and S. Roth, 2016 Genome-wide identification of *Tribolium* dorsoventral patterning genes. Development 143**:** 2443-2454.

Strunkelnberg, M., B. Bonengel, L. M. Moda, A. Hertenstein, H. G. de Couet *et al.*, 2001 rst and its paralogue *kirre* act redundantly during embryonic muscle development in *Drosophila*. Development 128**:** 4229-4239.

Sudarsan, V., S. Anant, P. Guptan, K. VijayRaghavan and H. Skaer, 2001 Myoblast diversification and ectodermal signaling in *Drosophila*. Dev Cell 1**:** 829-839.

Tautz, D., and C. Pfeifle, 1989 A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma 98**:** 81-85.

van der Zee, M., O. Stockhammer, C. von Levetzow, R. Nunes da Fonseca and S. Roth, 2006 Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. Proc Natl Acad Sci U S A 103**:** 16307-16312.

Volk, T., 2013 Positioning nuclei within the cytoplasm of striated muscle fiber: cooperation between microtubules and KASH proteins. Nucleus 4**:** 18-22.

Volk, T., D. Israeli, R. Nir and H. Toledano-Katchalski, 2008 Tissue development and RNA control: "HOW" is it coordinated? Trends Genet 24**:** 94-101.

Volk, T., and K. VijayRaghavan, 1994 A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo. Development 120**:** 59-70.

Vorbrüggen, G., and H. Jäckle, 1997 Epidermal muscle attachment site-specific target gene expression and interference with myotube guidance in response to ectopic stripe expression in the developing *Drosophila* epidermis. Proc Natl Acad Sci U S A 94**:** 8606-8611.

Wang, B., H. Zeng, Z. Wen, C. Chen and D. W. Wang, 2016 CYP2J2 and its metabolites (epoxyeicosatrienoic acids) attenuate cardiac hypertrophy by activating AMPKalpha2 and enhancing nuclear translocation of Akt1. Aging Cell 15**:** 940-952.

Wilson, R., and M. Leptin, 2000 Fibroblast growth factor receptor-dependent morphogenesis of the *Drosophila* mesoderm. Philos Trans R Soc Lond B Biol Sci 355**:** 891-895.

Xu, J., D. Liu, H. Yin, H. Tong, S. Li *et al.*, 2018 Fatty acids promote bovine skeletal muscle satellite cell differentiation by regulating *ELOVL3* expression. Cell Tissue Res 373**:** 499-508.

Yang, J., L. H. Hung, T. Licht, S. Kostin, M. Looso *et al.*, 2014 RBM24 is a major regulator of muscle-specific alternative splicing. Dev Cell 31**:** 87-99.

Figure Legends

Figure 1 Somatic muscle pattern in *Tribolium castaneum* and *Tribolium* orthologs of known *Drosophila* myogenesis regulators

(A) to **(G)** show *Tribolium castaneum pig-19* enhancer trap 1st instar larvae and embryos imaged live for EGFP expression in the somatic musculature. **(A)** Newly hatched 1st instar larva, view of dorsal muscle pattern. **(B)** Newly hatched 1st instar larva, view of dorsal-lateral muscle pattern. **(C)** Newly hatched 1st instar larva, view of ventral-lateral muscle pattern. **(D)** Newly hatched 1st instar larva, view of ventral muscle pattern. **(E)** Late stage embryo prior to hatching, view of lateral muscle pattern. **(F)** View of 1st instar larval leg muscle pattern. **(G)** Newly hatched 1st instar larva, high magnification view of muscle pattern in two abdominal segments (composite of a dorsal-lateral and a ventral-lateral view from two different animals). **(H)** Schematic representation of late embryonic muscle pattern in an abdominal segment from *Tribolium castaneum*. Note that the external-to-internal orders of the muscles and the exact numbers of DL and VL muscles are tentative. **(I)** Schematic representation of late embryonic muscle pattern in an abdominal segment from *Drosophila* (abbreviations: DA: dorsal acute; DL: dorsal longitudinal; DO: dorsal oblique; LT: longitudinal transverse; SBM: segment border muscle; VA: ventral acute; VL: ventral longitudinal; VO: ventral oblique muscles; for nomenclature see (Bate 1993)). **(J)** *In situ* hybridization of *Tribolium* embryo (early germ band retraction stage) for mRNA of *TC002914* (ortholog of *Drosophila duf/rst*). Arrow head: somatic mesoderm in abdominal segment. Arrow: Somatic mesoderm in leg. **(K)** Late stage *pig-19* embryo from adult female injected with dsRNA for *TC002914*, imaged live for EGFP. Only few and very thin muscle fibers are present (arrows). **(K')** Example of milder phenotype in *TC002914* knock-down embryo as in (K), showing residual large muscles, very thin muscles (arrows), and gaps where muscles are missing. **(L)** *In situ* hybridization of *Tribolium* embryo (retracted germ band stage) for mRNA of *TC010850* (ortholog of *Drosophila Mef2*). Arrow head: somatic mesoderm in abdominal segment. Arrow: Somatic mesoderm in leg. **(M)** Late stage *pig-19* embryo from adult female injected with dsRNA for *TC010850*, imaged live for EGFP. Few muscles are present that are very thin

(arrows). **(M')** Example of milder phenotype in *TC010850* knock-down embryo as in (M).

Figure 2 Progression of iBeetle RNAi screen for genes with knock-down phenotypes in muscles upon dsRNA injections into *Tribolium pig-19* pupae.

Green bars show numbers of screened and selected genes in the first round and blue bars the corresponding numbers in the second round of screening (blue-green bars in (B) to (F) include "stragglers" from the first-round) (logarithmic scale). **(A)** A total of 8.500 genes were screened by dsRNA ("iB fragment") injections in the two rounds. **(B)** 126 out of 3.500 injected dsRNAs from the first round and 103 out of another 1.800 injected dsRNAs from the first round and from 3.200 of the second round were annotated with muscle phenotypes in the primary screens. **(C)** 102 from the 229 genes in (B) were selected for rescreens. **(D)** 54 of the 102 genes from (C) were confirmed for embryonic muscle phenotypes upon re-injection of the original iB dsRNA fragments into *pig-19* pupae. **(E)** 40 of the 54 genes from (D) were selected for independent verification of the observed muscle phenotypes. **(F)** 28 of the 40 genes from (E) showed confirmed muscle knock-down phenotypes upon pupal injections of dsRNAs non-overlapping with the original iB dsRNA fragments ("NOFs") into a different *T. castaneum* strain, *San Bernardino* (*SB*).

Figure 3 Examples of muscle phenotypes of *Tribolium* orthologs of known *Drosophila* myogenesis regulators from primary screen

Shown are lateral or dorsal-lateral views of late stage *pig-19* embryos prior to hatching, live imaged for EGFP. **(A)** Control embryo from uninjected female pupa. DO: Dorsal oblique muscles; SBM: segment border muscles. **(B)** Embryo with RNAi knock-down of *TC015327* (*Tc-org-1*). Arrows indicate areas of missing or strongly reduced SBM muscles. **(C)** Embryo with RNAi knock-down of *TC015855* (*Tc-nau/MyoD*). **(D)** Embryo with RNAi knock-down of *TC004764* (*Tc-kon*). Arrows indicate areas of missing DO muscles. **(E)** Embryo with RNAi knock-down of *TC001667* (*Tc-if*). **(F)** Embryo with RNAi knock-down of *TC004846* (*Tc-sr*).

Table 1 Compilation of screening data for *Tribolium* orthologs of *Drosophila* genes known to regulate various aspects of muscle development with clear *Tribolium* muscle phenotypes (from primary screen; see also http://ibeetle-base.uni-goettingen.de)

Table 2 Features and muscle phenotypes of selected genes and confirmed in rescreens with original (iB) and non-overlapping (NOF) dsRNA fragments

Supplementary file

Table S1 Compilation of screening data for *Tribolium* orthologs of *Drosophila* genes known to regulate various aspects of muscle development with no annotated *Tribolium* muscle phenotypes, uninterpretable muscle phenotypes, or no injected dsRNAs.

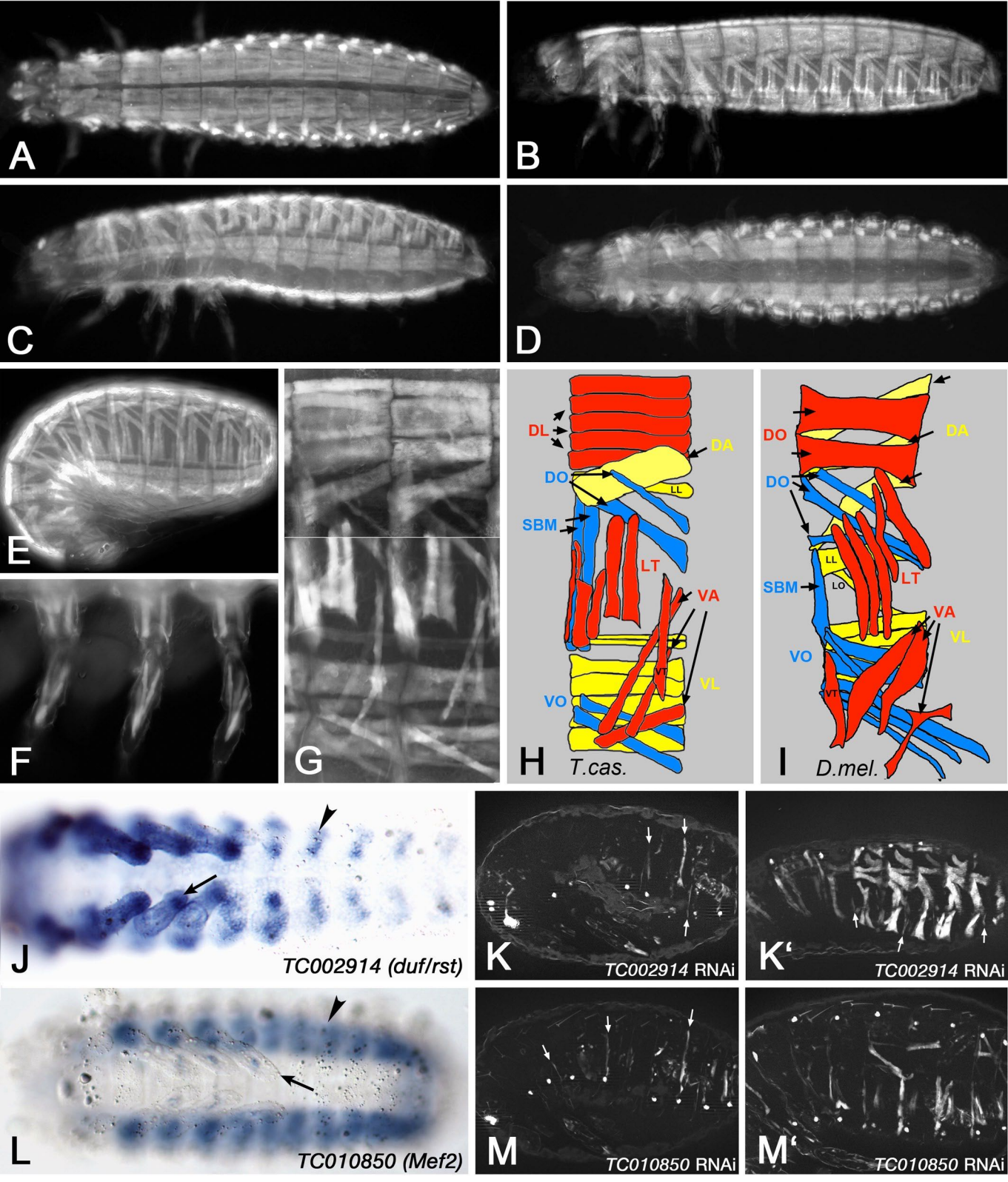

A
B
C
D
E
F
G
DL
DA
DO
LL
SBM
LT
VA
VT
VL
VO
H
T.cas.
DO
DA
DO
LL
LO
SBM
LT
VA
VL
VO
VT
I
D.mel.
J
TC002914 (duf/rst)
K
TC002914 RNAi
K'
TC002914 RNAi
L
TC010850 (Mef2)
M
TC010850 RNAi
M'
TC010850 RNAi

Fig. 1

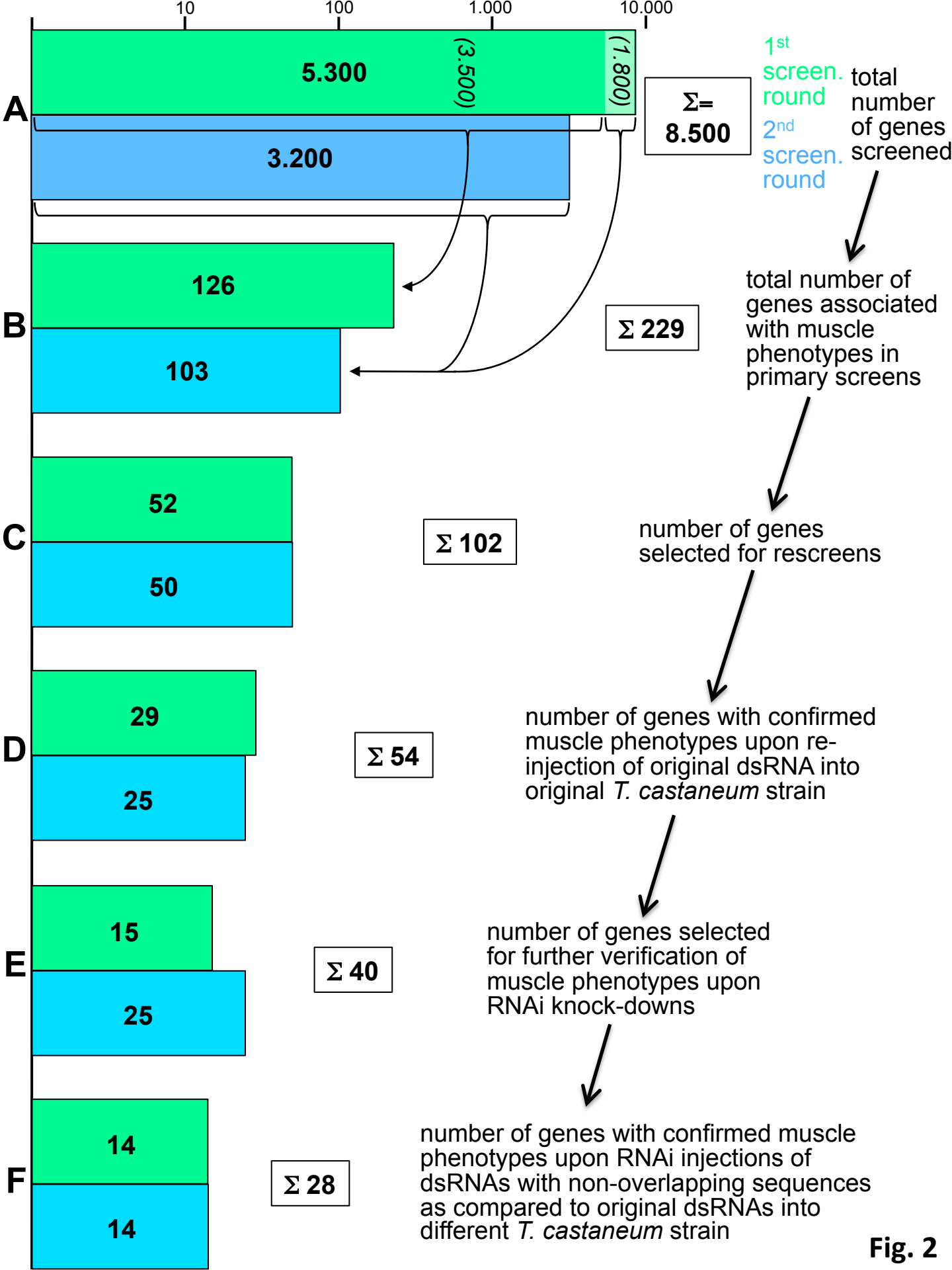

10
100
1.000
10.000
A
5.300
(3.500)
(1.800)
3.200
Σ= 8.500
1st screen. round
2nd screen. round
total number of genes screened
B
126
103
Σ 229
total number of genes associated with muscle phenotypes in primary screens
C
52
50
Σ 102
number of genes selected for rescreens
D
29
25
Σ 54
number of genes with confirmed muscle phenotypes upon re-injection of original dsRNA into original T. castaneum strain
E
15
25
Σ 40
number of genes selected for further verification of muscle phenotypes upon RNAi knock-downs
F
14
14
Σ 28
number of genes with confirmed muscle phenotypes upon RNAi injections of dsRNAs with non-overlapping sequences as compared to original dsRNAs into different T. castaneum strain

Fig. 2

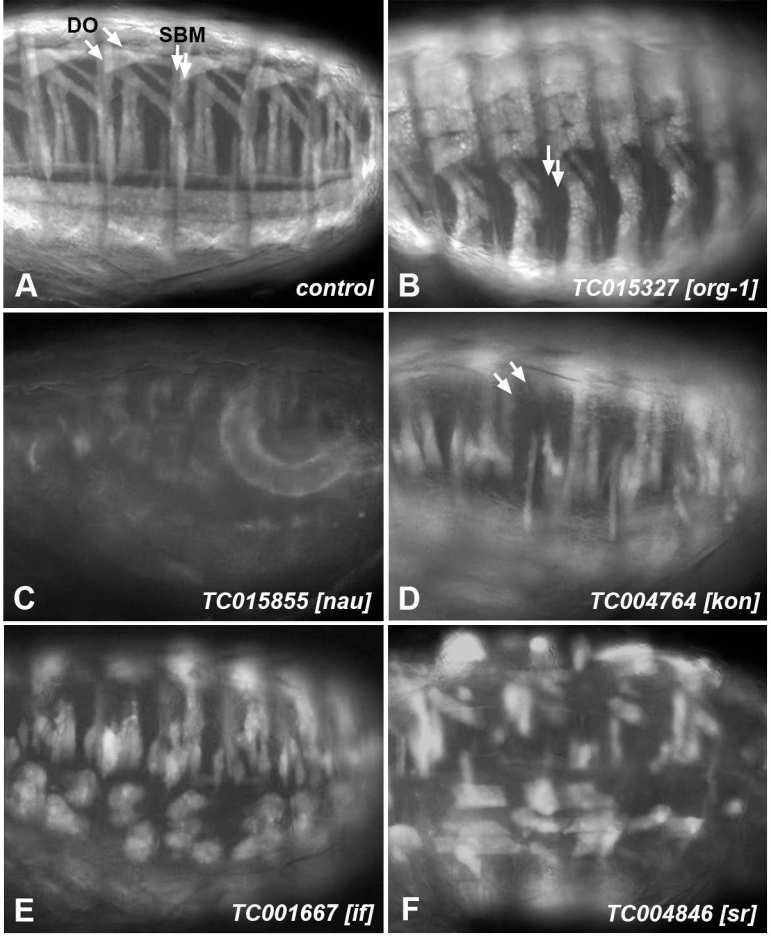

DO
SBM
A
control
B
TC015327 [org-1]
C
TC015855 [nau]
D
TC004764 [kon]
E
TC001667 [if]
F
TC004846 [sr]

Fig. 3

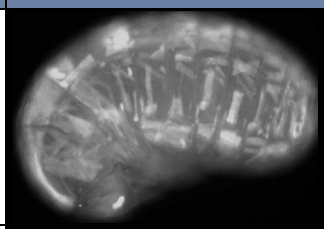
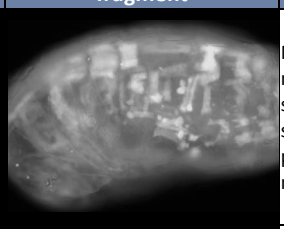
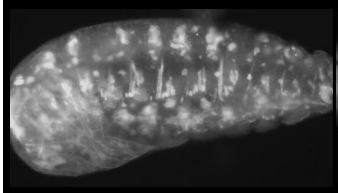
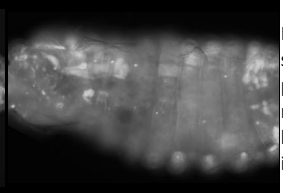
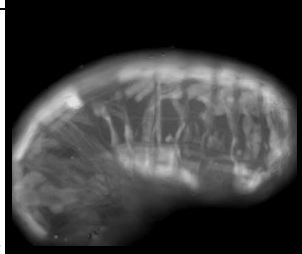
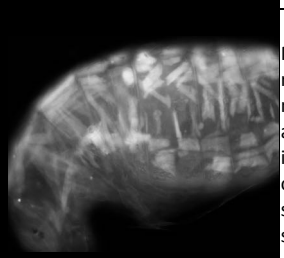
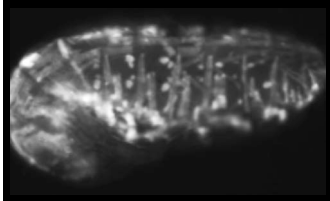
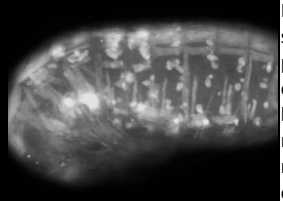
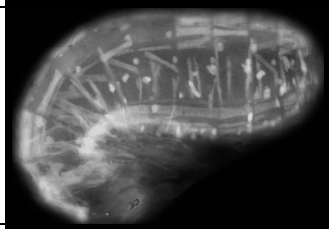
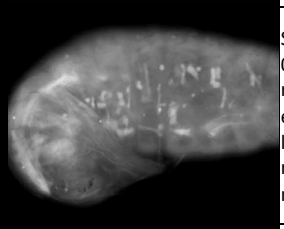
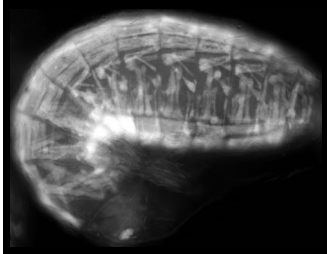
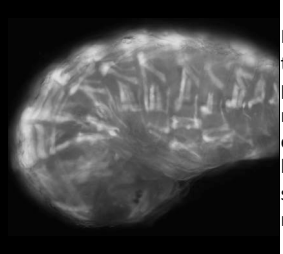
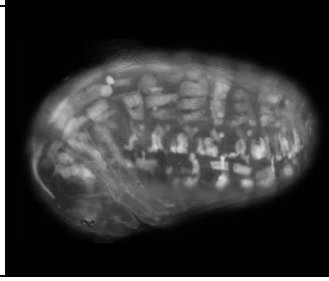
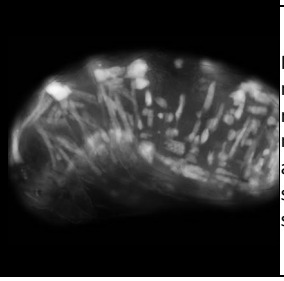
Table 1 Compilation of screening data for *Tribolium* orthologs of *Drosophila* genes known to regulate various aspects of muscle development (primary screen, see http://ibeetle-base.uni-goettingen.de)

			Pupal Screen	Larval Screen
***Drosophila* Gene**	**Tc#**	**iB dsRNA#**	**Muscle phenotype**	**Muscle phenotype**
Ced-12 (Elmo)	*TC002742*	08003	✔	---
Crk	*TC004767*	07501	✔	---
dock	*TC013803*	02209	X	✔
duf (kirre), rst	*TC002914*	03469	X [2)]	✔
HLH54F	*TC002349*	06703	✔	---
htl	*TC004713*	03821	✔ (no muscle-GFP)	X [1)]
if	*TC001667*	03227	✔	---
kon	*TC004764*	07523	✔	---
LanA	*TC003460*	03560	✔	✔
LanB1	*TC005184*	08660	✔	---
LanB2	*TC010540*	01705	✔	X
lmd	*TC030749*	06061	✔	---
nau (MyoD)	*TC015855*	05915	✔	---
ndg	*TC008043*	01306	✔	X
numb	*TC012074*	05163	✔	X
org-1	*TC015327*	05796	✔	---
ptc	*TC004745*	03831	✔	X [1)]
simj	*TC009782*	04697	✔	X
siz	*TC005167*	03913	✔	X
salm	*TC013501*	10041	✔	--- [3)]
sr	*TC004846*	03857	✔	X [1)]
stumps (dof)	*TC011323*	02631	✔	X
Syd	*TC003186*	02682	✔	X
twi	*TC014598*	09112	✔	---
vg	*TC010897*	04931	X	✔
Vrp1	*TC012341*	05218	✔	---
wb	*TC014773*	05688	X [1)]	✔

✔ muscle phenotype annotated
X no muscle phenotype annotated
--- no dsRNA injected
[1)] not screenable as RNAi caused lethality prior to muscle development (pupal, adult, or early embryonic)
[2)] but strongly depleted musculature in experiments with injections into adult beetles (see Fig. 1)
[3)] see reported *Tribolium* muscle phenotype in Schönbauer et al., Nature 479, 406-9 (2011)

Table 2

Table 2A Features and muscle phenotypes of selected genes from **1st screening round** and confirmed in rescreens with original (iB) and non-overlapping (NOF) dsRNA fragments

iB# TC gene	Ortholog *D.mel.*	Gene product/ function	Muscle phenotype		Muscle phenotype	Other phenotype
			iB fragment	Non-overlapping fragment		
00174 *TC 000807*	*ALiX*	- BRO1 domain - multivesicular body sorting (ESCRT) for receptor degradation - actin remodeling in muscles			Many muscles missing, round syncytia and small GFP+ cells present, other muscles thinner	Cuticle head bristle pattern irregular leg: tibiotarsus rounded and shortened
00289 *TC 001720*	*Rbm24* (vertebr. no *D.mel.*)	- RNA binding - target of MyoD (Xenopus) - required for sarcomere assembly & heart contractility in zebrafish - muscle diff. mouse			Many round syncytia present, few muscle fibers left except some in lateral areas	Larval screen wings not closed
01111 *TC 007027*	*GlcAT-I*	- glycosyl transferase - proteoglycan biosynthesis - humans: assoc. with cardiac & joint defects - *D.mel*: Expressed maternally & in gut			Muscles missing, remaining muscles thicker and often with incorrect orientations, some round syncytia	Cuticle: bristle phenotype (dorsal ridge row /abdomen) Larval screen molt delay Shortened antennae, wings
01159 *TC 032839*	*Unc-76*	- kinesin binding - required for axonal transport & outgrowth (*D.mel.* & *C. elegans*) - *D.mel*: expressed in CNS & somatic mesoderm			Many round syncytia present, especially in lateral areas, remaining muscle fibers often thinner	Larval screen Elytra blistered, wings not closed
01319 *TC 008216*	*Hsl*	- predicted triglyceride lipase - vertebrate homolog: Lipe (hormone-sensitive lipase) - cholesterol metabolism			Similar to 01159: Many round syncytia especially laterally, remaining muscles thinner	none recorded
01718 *TC 010637*	*CG6961 CG18259*	- mRNA binding protein - vertebr. homolog: Poldip3/SKAR/ PDIP46 - links mTOR/S6K1-mediated translational stimulation of spliced mRNAs			Muscles very thin in middle portions but normal-sized at either end (bar-bell shape), some muscles missing	Some larvae with segmentation defect Larval screen Wing blisters Antennae bent, short
01726 *TC 010759*	*Strip*	- vertebrate homolog: Fam40A - STRIPAK complex component - actin elongation via Hippo/Ena (at NMJ)			Many muscles missing, remaining muscles shorter and thickened, some round syncytia present	Cuticle head capsule decreased, leg segments shortened, squat shape of abdomen and thorax Larval screen Wing blisters

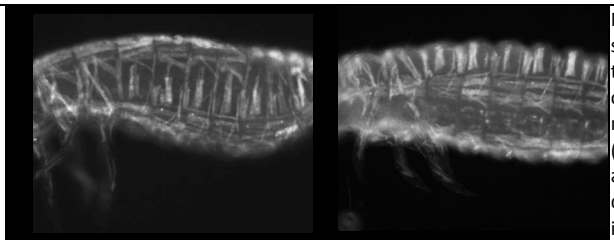
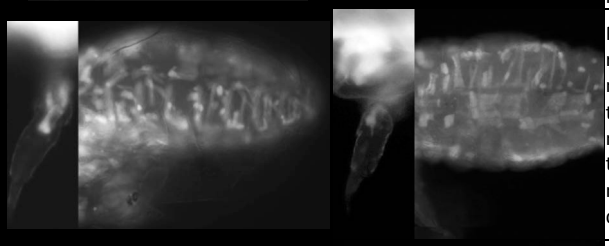
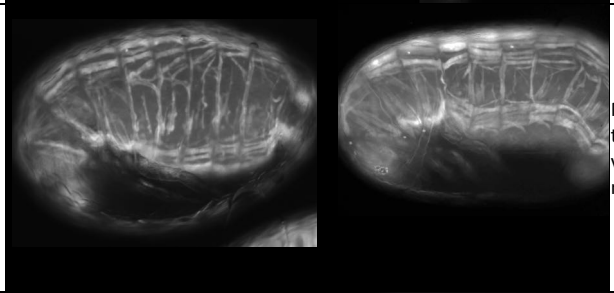
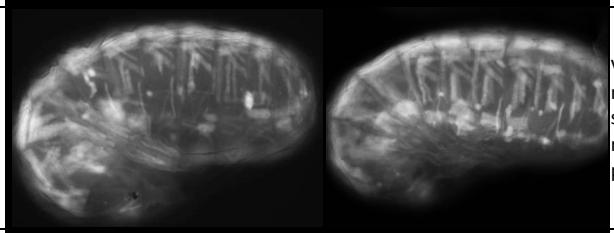
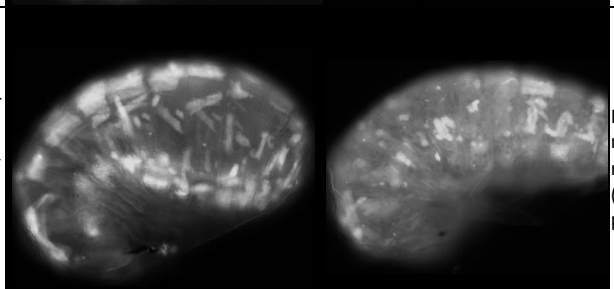
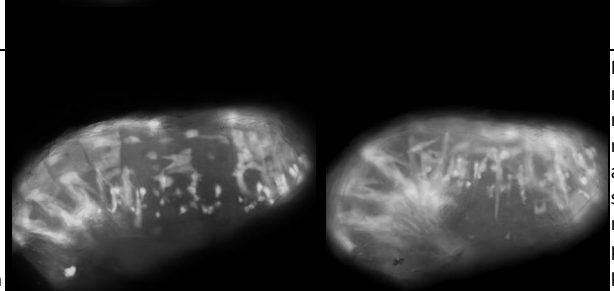
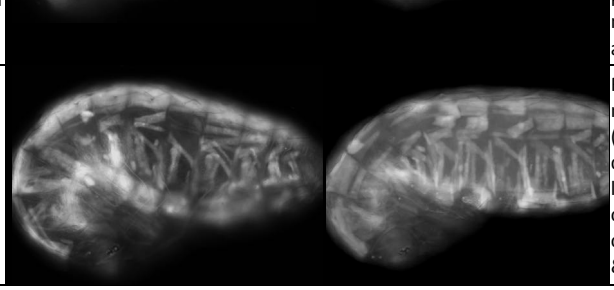
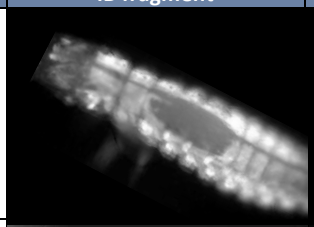
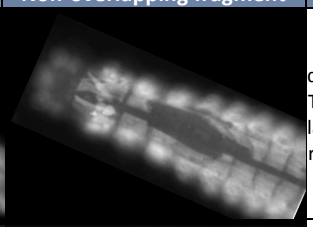
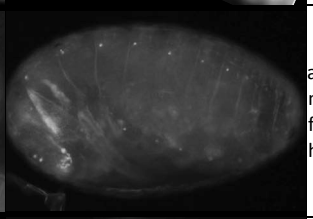
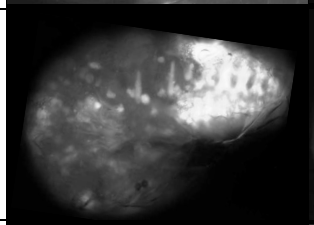
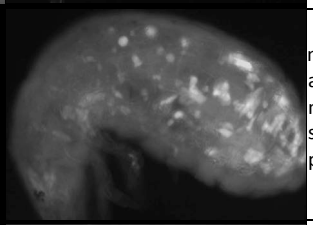
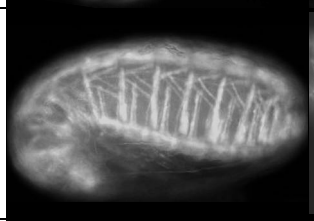
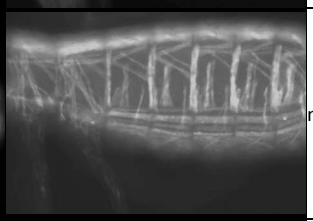
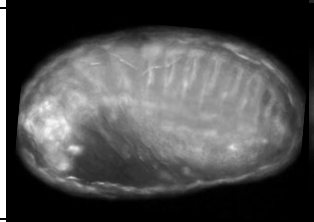
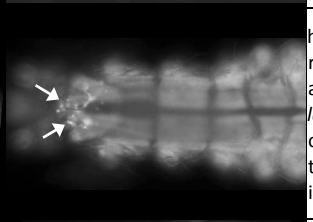
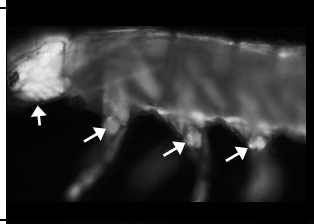
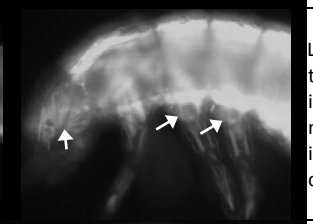
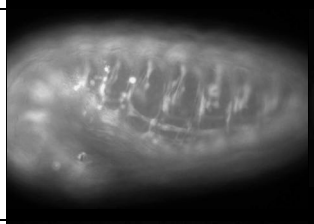
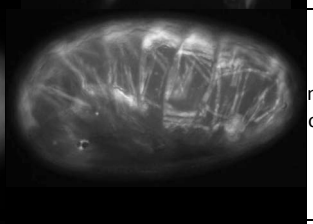
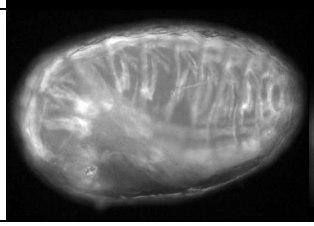
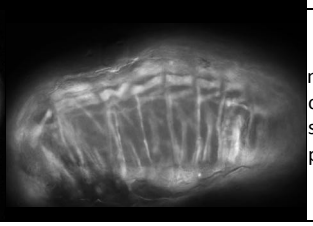
02205 *TC 013784*	*Nost*	- F-Bar protein - potentially involved in cell membrane bending - see main body of publication		Most muscles significantly thinner, occasionally muscles absent (both examples are from iB-dsRNA injections)	Larval screen: Oblique dorsal thoracic muscles affected in pupae
02248 *TC 014101*	*verm*	- chitin binding protein - LDL receptor class I - *D.mel:* tracheal development, expr. in tracheal pits & trachea		Many muscles rounded, others missing or thinner, leg muscles thickened and not extended distally (see left)	Larval screen: Larval lethality after injection
02588 *TC 034740*	*apolpp*	- retinoid- and fatty acid-binding glycoprotein - proapolipophorin family - van Willebrand factor type D domain - *D.mel:* expressed in amnioserosa & fat body		Muscles very thin and variously missing	Cuticle most bristles missing, cuticle thin Larval screen: all animals died during metamorphosis
03487 *TC 003028*	*axed*	- BTB/POZ & BACK domain - no vertebrate ortholog - *D.mel.*: injury-induced axon degeneration		Various muscles missing, some shortened or round syncytia present	Larval screen: all animals died during metamorphosis
03581 *TC 003570*	*sv* (*Pax2*)	- Pax domain transcription factor - in D.mel. only expressed in CNS & PNS - functions in neuronal development		Many muscles missing, some round syncytia (large & small) present	Cuticle tibiotarsus & head appendages rounded and shortened, bristles absent (as also seen in *Drosophila*) Larval screen: all animals died during metamorphosis
04483 *TC 008417*	*Pvf3*	- PDGF/VEGF-related factor 3 - VEGF receptor binding - *Drosophila*: acts in hemocyte migration, which is required for ECM deposition (expr. in head, ventral midline, ectoderm)		Many muscles missing, remaining muscles with abnormal shapes, small round syncytia present, middle portion of larva more strongly affected	Larval screen elytra not closed, surface & color irregular
05264 *TC 012539*	*Dscam*	- Immunoglobulin superfamily - *Drosophila*: axon guidance receptor		Random muscles missing (particularly dorsal & ventral longitudinal ones), others often shortened & disarranged	Cuticle: Head bristle pattern irregular Larval screen Stink gland phenotype

Table 2B Features and muscle phenotypes of genes selected genes from **2nd screening round** and confirmed in rescreens with original (iB) and non-overlapping (NOF) dsRNA fragments

iB# TC gene	Ortholog *D.mel.*	Gene product/ *D.mel.* function	Muscle phenotype		Muscle phenotype	Other phenotype
			iB fragment	Non-overlapping fragment		
00988 *TC 006055*	*TBPH (TAR DNA-binding protein-43 homolog)*	- RNA-binding protein of the hnRNP family - roles include synaptic growth of motor neurons and glial wrapping			dorsal muscles in T3 to A3 missing, lateral muscles rounded	urogomphi smaller
03004 *TC 000278*	*pyr, ths* (low similarity)	- FGF8			all muscles missing except for remnants in head	none recorded
03251 *TC 001762*	*sau (sauron, aka rotini, GOLPH3)*	- phosphatidyl-inositol-5-phosphate binding; Rab GTPase binding - cytokinesis, trans-Golgi trafficking			muscles spherical and many missing, most severe in anterior portion	none recorded
03525 *TC 003240*	*babo (baboon)*	- TGF-beta receptor (type I) - neurogenesis (pupal neuroblast & disc cell proliferation, axon & dendrite migr.)			muscles thinner	gena triplet seta number increased Larval Screen: Pupal molt delayed
04897 *TC 010693*	*CG4266*	- RNA processing RBM16/Scaf4/ Scaf8			head muscles rounded/degenerated *larval injections:* oblique dorsal thoracic muscles in pupae thinner	larval injections: pupal wings not closed Oblique dorsal thoracic muscles thinner in pupae
06557 *TC 001618*	no direct ortholog, several homologs	- crotonase-like superfamily (ethylmalonyl-CoA decarboxylase, Enoyl-CoA hydratase, etc.)			Leg musculature thicker, especially in coxa, head muscles increased (not as clear with NOF)	none recorded
06574 *TC 030731*	*aralar1*	- EF-hand domain - mitochondrial malate-aspartate NADH shuttle protein			muscles thinner, others missing	none recorded
06673 *TC 005276*	no direct ortholog, closest to *Psc, Su(z)2, l(3)73Ah*	- RING and RAWUL domain-containing protein - chromatin regulators			muscles missing, occasionally spherical muscles present	cuticle defects in prothorax and head

07983 *TC 002813*	*croc (crocodile)*	- forkhead domain transcription factor - embryonic head development			specifically ventral muscles missing or abnormal	head defects
08029 *TC 010342*	*CG8009*	- Nuclear envelope phosphatase-regulatory subunit 1 related - may activate lipin-like phosphatases			spherical muscles present, muscles missing	larvae with dorsal bent, tibiotarsae of legs shortened
08779 *TC 008832*	*MTA1-like*	- chromatin remodeling - NURD complex			dorsal-lateral muscle pattern irregular (oblique muscles largely missing)	head, thoracic, and abdominal cuticle defects with lower penetrance than muscle defects
08916 *TC 006419*	*CG10348*	- Evi1/PRDM16 zinc finger homology - implicated in chromatin remodeling/ transcriptional activation			muscles shortened, rounded, and missing	head cuticle defects
09244 *TC 009963*	*calypso (aka BAP1)*	- Polycomb repressive deubiquitinase - chromatin silencing (incl. Hox genes)			prothorax musculature specific subset not present (see arrows)	abdominal segment number increased, posterior terminus may be transformed

Table 2C Control GFP patterns

Controls			Left: late stage embryo prior to hatching Right: newly-hatched larva (lower magnification)

Table S1 Compilation of screening data for *Tribolium* orthologs of *Drosophila* genes known to regulate various aspects of muscle development with no annotated muscle phenotypes, uninterpretable muscle phenotypes, or no injected dsRNAs.

No muscle phenotypes with pupal injections and larval injections (if performed) were annotated for iB dsRNA injections for the following muscle development-related genes (*Drosophila* gene name (alphabetical)/*Tribolium* gene name/iB dsRNA fragment tested):

aret/*TC012080*/iB_05164 (not done with larval injection)
CLIP190/*TC031243*/iB_01909
Fit1/Fit2/*TC010123*/iB_01652 (all animals died in both screens after injection)
H15/*TC013513*/iB_05442
Hand/*TC004726*/iB_03827
how/*TC000827*/iB_03083 (all animals died after pupal injection) (not done with larval injection)
Ilk/*TC002476*/ iB_00385 (but wing blisters & possible muscle phenotype w. larval injections) (lethal after pupal injection)
kette (hem)/*TC001541*/iB_00258 (animals sterile after pupal injection)
Klc/*TC007992*/iB_09878 (animals sterile after pupal injection)
lbe/*TC011748*/iB_05096
mid/*TC014296*/iB_05605 (not done with larval injection)
mp20/*TC007135*/iB_01129
msh/*TC011744*/05095
msh (Dr)/*TC012748*/iB_05308 (embryonic muscle defects potentially obscured by early defects)
parvin/*TC000078*/iB_00014 (but wing blisters w. larval injections; animals sterile after pupal injection)
poxm/*TC008838*/iB_04548
Ptx1/*TC032174*/iB_07879 (not done with larval injection)
rhea/*TC033529*/iB_01537 (for pupal injections; no adults obtained with larval injections)
rols/*TC002665*/iB_07027 (not done with larval injection)
Scar/*TC006152*/iB_01003 (strongly reduced egg lays after pupal injection, no embryonic cuticle)
sd/ *TC032219*/09689 (disrupted embryo morphology, > 80% inside-out)
sing/*TC012091*/iB_05167
six4/*TC003852*/iB_03634
slou/*TC012332*/iB_05216
slow/*TC005670*/iB_07509 (not done with larval injection)
sns, hbs/ *TC032336*/iB_03544
Tsp/*TC033883*/iB_01372 (> 80% not fertilized/not developed and no cuticle)

Not injected (---), or not screenable for muscle phenotypes due to lethality prior to muscle development (pupal, adult, or early embryonic) upon larval and pupal injections, were dsRNAs for the following muscle development-related genes:

abba/*TC031245*/iB_01986 (--- with larval injections)
ara/caup/TC031040/iB_03594 (not screenable)
Arp2/*TC000144*/iB_00026 (not screenable)
Arp3/*TC002963*/iB_00483 (not screenable)
blow/*TC014242*/---
col (***kn***)/*TC001270*/---
dia/*TC006029*/iB_00980 (no embryonic cuticle)
Dhc64C/*TC008801*/---
Dlc90F/*TC010513*/---
Dlc90F/*TC009745*/---
ens/*TC010873*/---
Grip/*TC034397*/---
hoip/*TC002918*/---
lms/*TC033532*/---
mbc/*TC012454*/iB_10628 (early embryonic lethality + no cuticle)
Mef2/*TC010850*/iB_04920 (not screenable)
mew/*TC034177*/---
mys/*TC011707*/---
mys/*TC013706*/---
stk/*TC011050*/---
Wasp/*TC013303*/---