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Extradenticle and Homothorax Control Adult Muscle Fiber Identity in *Drosophila*

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SUMMARY

Here we identify a key role for the homeodomain proteins Extradenticle (Exd) and Homothorax (Hth) in the specification of muscle fiber fate in *Drosophila*. *exd* and *hth* are expressed in the fibrillar indirect flight muscles but not in tubular jump muscles, and manipulating *exd* or *hth* expression converts one muscle type into the other. In the flight muscles, *exd* and *hth* are genetically upstream of another muscle identity gene, *salm*, and are direct transcriptional regulators of the signature flight muscle structural gene, *Actin88F*. Exd and Hth also impact muscle identity in other somatic muscles of the body by cooperating with Hox factors. Because mammalian orthologs of *exd* and *hth* also contribute to muscle gene regulation, our studies suggest that an evolutionarily conserved genetic pathway determines muscle fiber differentiation.

INTRODUCTION

The skeletal muscles of vertebrates are composed of heterogeneous individual fiber types that have distinct biochemical, mechanical, structural, and molecular characteristics (Punkt, 2002; Schiaffino et al., 1970; Toniolo et al., 2004). The differential expression of fiber-specific muscle genes distinguishes fibers at the molecular level and is used to classify fibers into distinct categories (Schiaffino and Reggiani, 2011). At the functional level, the abundance of particular fiber types in individual skeletal muscles is causatively related to muscle performance (reviewed in Zierath and Hawley, 2004), and a shift in fiber type ratio is characteristic of some congenital human myopathies (D'Amico and Bertini, 2008). Nevertheless, there is still much to learn concerning how muscle fiber identity is initially specified.

As in vertebrates, the somatic muscle system of the fruit fly, *Drosophila melanogaster*, comprises several types of fibers. Morphologically, there are two major muscle types in adult *Drosophila*: fibrillar muscles, which are exclusively present as indirect flight muscles and that provide the power for oscillatory flight (hereafter, we refer to these muscles as flight muscles); and tubular muscles, such as the jump muscles, leg muscles, and abdominal body wall muscles, which are neurogenic and are used for activities including walking and the initiation of flight (Bernstein et al., 1993). Molecular profiling validates the morphologically visible differences between fibrillar and tubular fiber types but also further segregates tubular muscles into distinct subtypes. For example, jump muscles and abdominal body wall muscles belong to the tubular type but express the muscle actin genes *Act79B* and *Act57B*, respectively (Fyrberg et al., 1983). The diversity of muscle fiber types, along with the genetic tractability of the organism, make *Drosophila* a useful model for studying genetic aspects of muscle fiber specification.

Some insight into how muscle fiber identity along the body axis is controlled in *Drosophila* has come from demonstration that homeotic selector (*Hox*) genes, such as *Ultrabithorax* (*Ubx*), can impact fiber-specific gene expression and muscle patterning (Roy and VijayRaghavan, 1997). However, no understanding of the mechanisms of possible *Hox*-dependent muscle identity specification has been gained to date. Hox proteins cooperate with the homeodomain proteins Hth and Exd to select and bind specific DNA sequences (Gebelein et al., 2002). Although no direct targets for these complexes have been identified in the muscle system, there is evidence that the vertebrate orthologs of Exd and Hth—termed Pbx and Meis, respectively—can contribute to the diversification of muscle types (Maves et al., 2007). However, simple Hox-dependent fiber identity regulation fails to explain how two substantially different fiber types in *Drosophila*, the flight and jump muscles, can both arise from myoblasts in the second thoracic segment that do not express homeotic selector genes (Roy et al., 1997; Roy and VijayRaghavan, 1997).

Here, we define a molecular mechanism that specifies fiber identity between the flight muscles and jump muscles, mediated by the homeodomain factors Hth and Exd. Since Exd and Hth orthologs are found in mammals as the muscle-expressed factors Pbx and Meis, respectively, our studies suggest a role for evolutionarily conserved factors in muscle fiber specification.

RESULTS

Identification of *exd* and *hth* as Critical Factors for Adult Muscle Gene Expression

To identify transcription factors that control fiber identity, we conducted RNA interference (RNAi)-based genetic screening using fiber-specific reporters as readouts. To achieve this, we first identified and cloned fiber-specific enhancers from the fibrillar flight muscle-specific *flightin* gene (Vigoreaux et al., 1993) and the tubular jump muscle-specific *Troponin C at 41C*

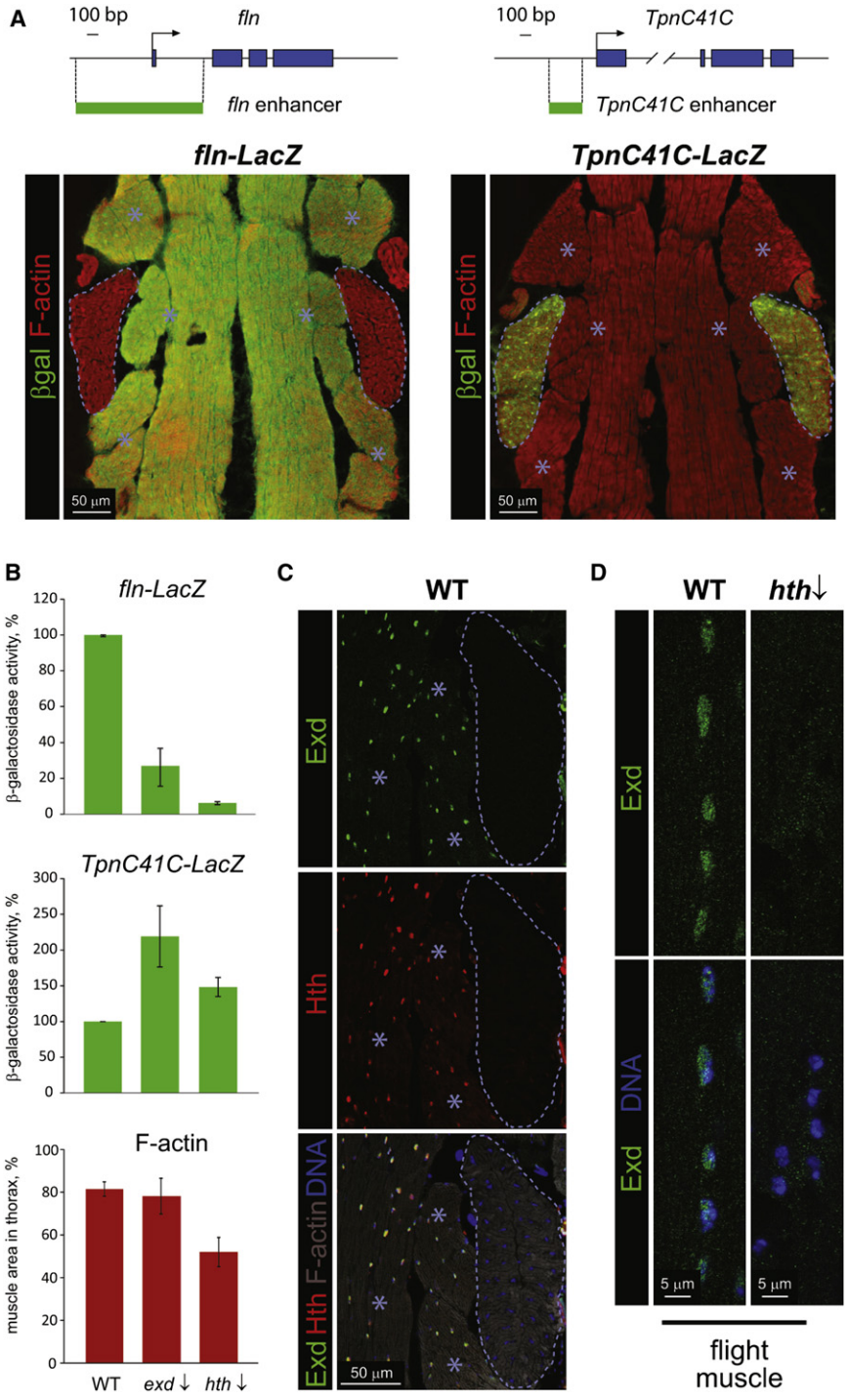

Figure 1. Identification of *exd* and *hth* as Important Determinants for Fiber-Specific Enhancer Activities

(A) Schematics of fiber-specific enhancers (green rectangles) from *fln* and *TpnC41C* genes and their location relative to the exons of the respective genes (blue boxes). Rightward arrows indicate transcription start sites. Lower panels for each gene show *lacZ* reporter activity in flight (asterisks) and jump (outlined) muscles in the thorax of adult flies. Reporter activity was assessed via immunofluorescent detection of β-gal on cryosections of pharate adults (green), counterstained with a fluorescent conjugate of phalloidin to identify actin filaments (red). Note that *fln-lacZ* expression is specific to the flight muscles, whereas *TpnC41C-lacZ* expression is strong in the jump muscles but absent in the flight muscles.

(B) Whole-fly reporter activities (green bars) and relative sizes of thoracic muscles (red bars) in control flies (WT) and flies with muscle-specific knockdown of *exd* (*exd*↓) or *hth* (*hth*↓). For β-gal assays, WT was normalized to 100%. Error bars represent standard deviation from four measured samples. Muscle size was calculated as a ratio of total muscle area to thorax area on frozen sections.

(C) Accumulation of Exd and Hth proteins in the thorax of pharate adults. Note the presence of Exd (green) and Hth (Red) staining in the flight muscles (asterisks) but not in the jump muscle (outlined). F-actin (visualized using fluorescent phalloidin, gray) is included to show muscles and nuclei are visualized using DAPI (blue).

(D) Localization of Exd in control (WT) and *hth* knockdown (*hth*↓) flight muscles. Nuclear enrichment of Exd (green) disappears in *hth* knockdown muscles.

See also Figure S1.

gene (Herranz et al., 2004). Transgenic flies harboring these enhancers fused to *LacZ* reporters selectively expressed β-galactosidase (β-gal) in flight and jump muscles, respectively (Figure 1A). The reporters were next crossed into a genetic background of the *1151-Gal4* transgenic line (Anant et al., 1998) that is used to specifically control binary gene expression (Brand and Perrimon, 1993) in the developing adult muscles. The resulting two lines were independently crossed to a library of *UAS-RNAi* lines (Dietzl et al., 2007) targeting transcription factors encoded by the *Drosophila* genome, and their offspring were quantitatively assayed for β-gal activity.

From 70 knockdowns assayed (for details, see Table S1 and Figure S6E available online), selective knockdown of *extradenticle* (*exd*) or *homothorax* (*hth*) caused the flight muscle-specific reporter to be detected at reduced levels, whereas in the same knockdowns the jump muscle-specific reporter profoundly increased in activity (Figure 1B). Of the genes assayed, *exd* and *hth* knockdowns were the only ones that had this effect upon reporter activities (Table S1). Similar effects were observed using independent *exd* or *hth* RNAi constructs targeted to different regions of each gene, several of which had no predicted off-targets (see Figure S6E and Experimental Procedures for details of knockdowns). Moreover, the changes in reporter activities could not be simply explained by changes in muscle size, as changes in muscle area measured

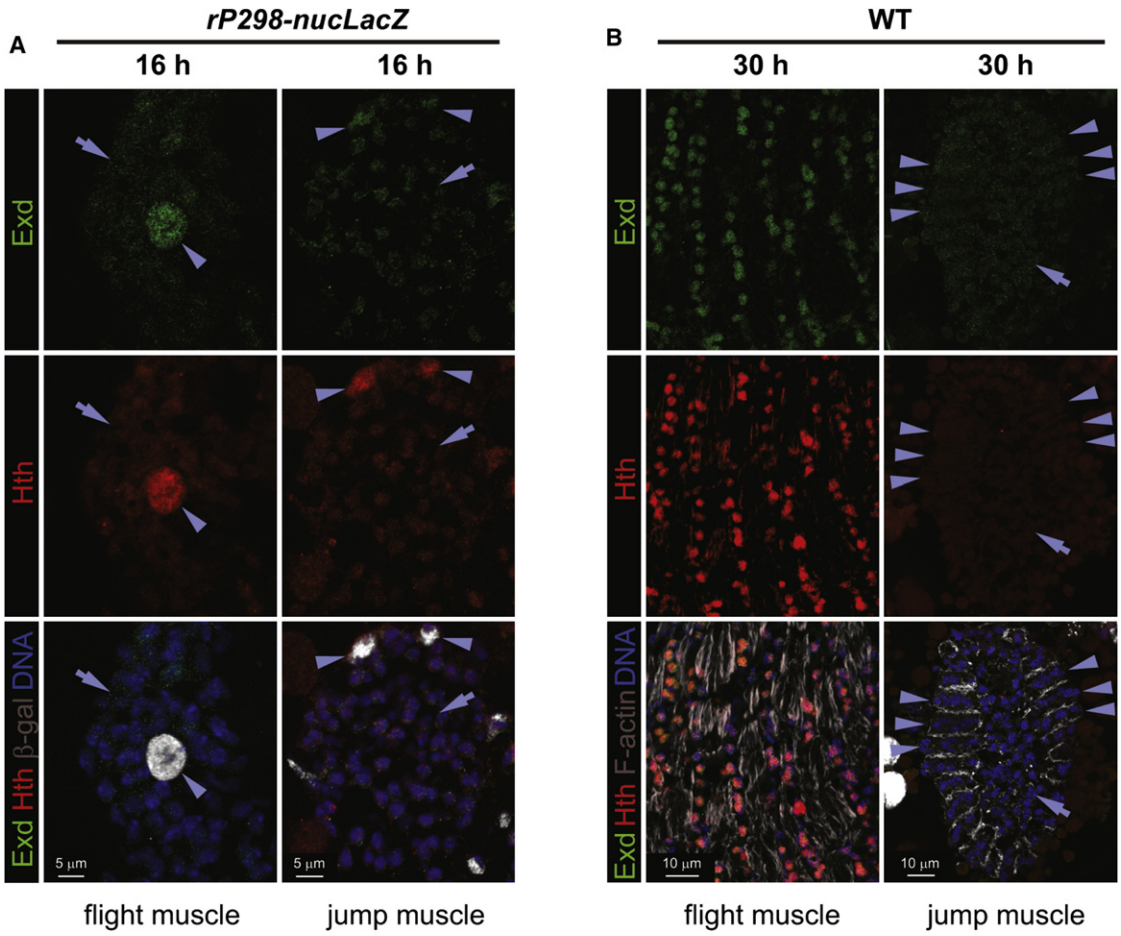

Figure 2. Dynamics of *exd* and *hth* Expression during Myogenesis of Flight and Jump Muscles

(A) Muscle templates in 16 hr APF pupa of the *rP298-nucLacZ* transgenic line, wherein *lacZ* expression (gray) is restricted to the muscle founder cells (Nose et al., 1998; Ruiz-Gómez et al., 2000). Note that Exd (green) and Hth (red) are present in nuclei of flight muscle founder cells (arrowheads in panel at left) but not in the surrounding fusion-competent myoblasts (arrows, also verified in Figure S2). Founder cells of the jump muscle (panels at right) are characteristically located at the periphery of the myoblast pool, and only weakly accumulate Exd and Hth.

(B) Nascent fibers of 30 hr APF pupa, showing continuing Exd and Hth presence only in flight muscles (panels at left). Newly formed myofibrils are detected with fluorescent phalloidin (gray) highlight fused myofibers. Developing fibers of the jump muscle (arrowheads in panels at right), as well as unfused myoblasts (arrow) do not accumulate Exd (green) and Hth (red).

See also Figure S2.

in thoraces of knockdown flies did not correlate with the changes in reporter activities (Figure 1B).

To understand if *exd* and *hth* have a direct impact upon muscle biology, we carried out antibody staining of cryosections from wild-type pharate adults to localize Exd and Hth accumulation. These stains revealed that *exd* and *hth* gene products were detected in the nuclei of almost all somatic muscles, including the flight muscles, but not in the jump muscle (Figure 1C). The levels of Exd and Hth accumulation varied noticeably among different tubular muscle types, being somewhat lower in leg muscles and more robust in abdominal body wall muscles (Figure S1). Nevertheless, the most striking difference was observed between the flight muscles and jump muscles (Figure 1C). Moreover, RNAi knockdown of *hth* also resulted in disappearance of the Exd protein from muscle nuclei (Figure 1D), consistent with the requirement of Hth protein for nuclear import of Exd (Rieckhof et al., 1997). Altogether, these data showed that Exd and Hth are unequally expressed in different muscles, and, while their presence in the flight muscles is required for flight muscle reporter activity, their loss coincided with a gain in jump muscle reporter activity.

To determine if *exd* and *hth* expression correlated with the earliest stages of adult muscle development, we assessed their expression in developing pupae at 16 hr after puparium formation (APF) and at 30 hr APF. We specifically concentrated upon the flight and jump muscles, given the focus of our study and the striking differences in Exd and Hth accumulation in the two muscle types. The earlier time point represents the onset of myoblast fusion, whereas the 30-hr APF time point represents the onset of fiber-specific gene expression (Bryantsev et al., 2012). The earliest expression of *exd* and *hth* in the flight muscle precursors mapped to single cells with large nuclei, that were surrounded by myoblasts containing smaller nuclei (Figure 2A, left panels). These single cells represented the founder cells for

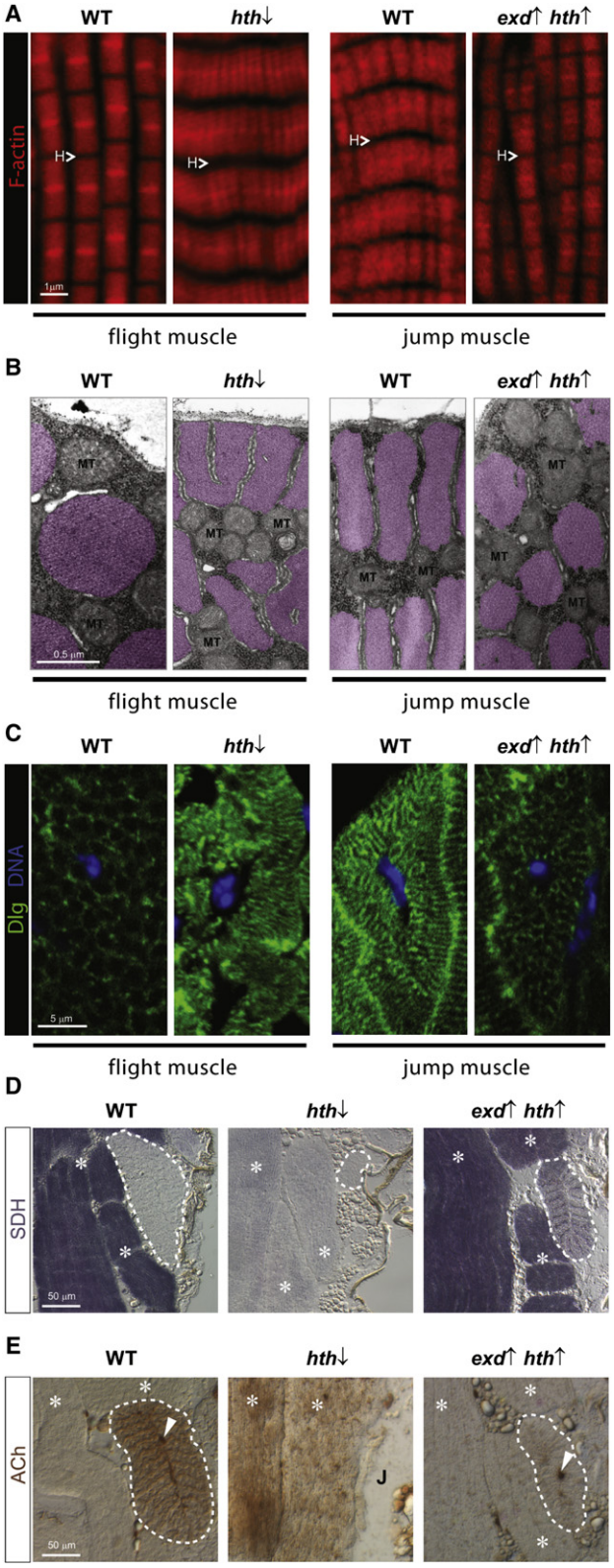

Figure 3. *hth* and *exd* Control the Muscle-Specific Properties of Flight and Jump Muscles

Fiber-specific features of flight and jump muscles in control (WT), *hth* knockdown (*hth*↓), and *exd* plus *hth* ectopic expression (*exd*↑ *hth*↑). Note the switching between flight and jump muscle properties upon manipulations of *hth* and *exd* expression. The following features are both distinct between flight and jump muscles, and altered upon manipulation of *exd/hth* expression.

(A) Patterns of myofibril striation visualized using phalloidin; H-zones are indicated.

(B) Shape and organization of myofibrils, visualized by electron microscopy of muscle transverse sections; myofibrils are highlighted in pink; MT, mitochondria.

(C) Nuclear positioning and T-tubule network on muscle transverse sections, revealed by DAPI (blue) and anti-Dlg immunostaining (green), respectively.

(D and E) Histochemical detection of SDH (D) and ACh (E) in the thorax. Jump muscles are outlined. Asterisks indicate flight muscles. Arrowheads indicate ACh-positive nerve. J, the position of the missing jump muscle.

See also Figures S3 and S4.

the flight muscles, since Exd and Hth colocalized with β-gal produced from the founder cell reporter, *rP298-nucLacZ* (Nose et al., 1998; Ruiz-Gómez et al., 2000). The surrounding cells were identified as myoblasts, since they expressed the muscle-specific marker MEF2 (Figure S2A). At this early stage, extremely low levels of Exd and Hth were sometimes observed in founder cell nuclei for the jump muscle (Figure 2A, jump muscle). However, whereas the Exd and Hth accumulation in the flight muscle nuclei was sustained through myoblast fusion and present at the onset of myofibrillogenesis (Figure 2B, "flight muscle"), these proteins were no longer detected in the jump muscles after the initiation of myoblast fusion (Figure 2B, "jump muscle"). These findings demonstrated that Exd and Hth are present in the flight muscles from their earliest time points in development. Moreover the expression of these two genes in flight muscle founder cells indicated that they might be critical determinants of flight muscle fate.

exd and *hth* Control a Cell Fate Decision between Flight and Jump Muscle Identities

Based upon the changes we observed in reporter gene expression in our knockdowns, and upon the sustained expression of *exd* and *hth* in the flight muscles but not the jump muscles, we hypothesized that the change in fiber-specific reporter expression in *exd* and *hth* knockdowns reflected a transition in flight muscle identity toward that of the jump muscle. The fibrillar fiber type of the flight muscles in wild-type adults shows several characteristic features (Figures 3A–3C, WT flight muscle), when compared to the tubular fiber type of the jump muscle (Bernstein et al., 1993) (Figures 3A–3C, WT jump muscle). The fibrillar muscles have: narrow H-zones in the cylindrical myofibrils, relatively weak coordination of Z discs in adjacent myofibrils, dispersed nuclei and T-tubule systems, and mitochondria intermingled with the myofibrils. By contrast, the jump muscles have cuboidal myofibrils with broad H-zones, myofibrils held in register relative to each other, nuclei located in a central lumen, an intricate T-tubule system, and mitochondria that segregate into a distinct zone between rows of myofibrils. In addition, the flight muscles show high levels of succinate dehydrogenase (SDH) activity and very low levels of acetyl cholinesterase (ACh) activity (Deak, 1977), whereas the converse is true of the jump muscles (Figures 3D and 3E, WT).

Flies with knockdowns of either *hth* or *exd* showed a striking transformation of the flight muscles into a jump muscle fate. We show here representative data for *hth* knockdowns;

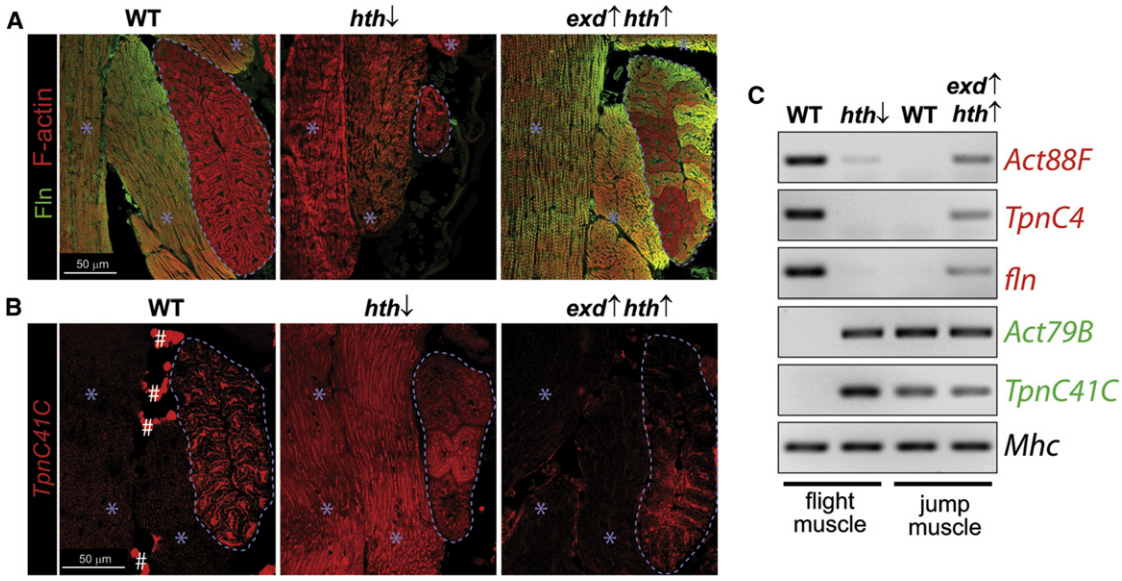

Figure 4. *hth* and *exd* Control the Molecular Identities of Flight and Jump Muscles

(A and B) Expression of characteristic structural genes in thoraces of the indicated genotypes. Asterisks mark flight muscles; jump muscles are outlined. (A) Accumulation of the Fln protein (green), revealed by immunostaining. Muscles are visualized using phalloidin (red). In WT, Fln accumulates in the flight muscles and not the jump muscle. Note the loss of this protein in the flight muscles of *hth* knockdowns, and the ectopic accumulation of Fln in the jump muscle when *exd and hth* are expressed there. (B) *TpnC41C* mRNA expression revealed by in situ hybridization (red); #, autofluorescent nonmuscle structures. In *hth* knockdown, *TpnC41C* transcripts are detected in the flight muscles. In *exd*↑ *hth*↑, several fibers of the jump muscle are negative for *TpnC41C* RNA.

(C) RT-PCR detection of fiber-specific genes from samples of indicated muscles from WT animals and those with *exd*/*hth* manipulations. Green and red fonts indicate flight and jump muscle markers, respectively; the pan-muscle-specific *Myosin Heavy Chain* (*Mhc*) serves as a reaction loading control. See also Figure S3 for similar effects of *exd* knockdown.

See also Figure S3 and S4.

nevertheless, similar effects were observed for each of several lines of *exd* or *hth* knockdown. Data for representative *exd* knockdowns are shown in Figure S3. The *hth* knockdown phenotype also most likely represents a double knockdown for *exd* plus *hth*, since Hth is required for nuclear localization of Exd (Figure 1D; Rieckhof et al., 1997); in addition, we generated a double-knockdown for both *exd* and *hth* in the adult muscles, and the phenotype was comparable to that observed for *exd* or *hth* knockdown alone (data not shown).

In the flight muscles of knockdown animals, myofibril and T-tubule system morphology, as well as mitochondria and nuclei localization, were more similar to those of the wild-type (WT) jump muscle (Figures 3A–3C, labeled *hth* ↓). Moreover, SDH levels in the knockdowns were markedly lower than wild-type, and ACh was upregulated in the flight muscles of knockdown animals (Figures 3D and 3E, *hth*↓). Clearly, loss of *exd* or *hth* function caused a major change in flight muscle fate toward that of the jump muscle. We note that, in the knockdowns, the sizes of the jump muscles were strongly reduced (outlined in Figure 3D). This might result from presumptive jump muscle myoblasts mistakenly fusing to nascent flight muscles that have transformed identity. Myoblast mixing between developing flight and jump muscles has been described before in a case of experimentally denervated muscles (Fernandes and Keshishian, 2005).

We next asked if ectopic expression of *exd* plus *hth* in the jump muscle would transform this muscle into a fibrillar fiber type. We used a jump muscle driver, *Act79B-Gal4* (Bryantsev et al., 2012), to control expression of *exd* plus *hth*, and we subjected the resulting flies to the same analyses described earlier. Here, we found that the jump muscle fibers were radically altered toward flight muscle fate (Figures 3A–3E, labeled *exd*↑ *hth*↑). These data indicated that Exd and Hth collaborate to induce flight muscle fate and that their absence leads to jump muscle identity being specified.

To determine if the observed morphological changes in muscle fiber identity were reflected at the molecular level, we studied fiber-specific gene expression in cryosections of control, knockdown, and gain-of-expression fibers. For Flightin, we observed a loss of this flight muscle-specific protein in *hth* knockdown animals, and there was an increase in the Fln protein in the transformed jump muscle fibers upon expression of *exd* plus *hth* using the *Act79B-Gal4* driver (Figure 4A). For *TpnC41C*, transcription was increased in the flight muscles in the *hth* knockdown and reduced in the jump muscles upon ectopic expression of *exd* plus *hth* (Figure 4B). In addition, we used microsampling followed by reverse transcriptase-PCR (RT-PCR) (Bryantsev et al., 2012), to collect samples from the wild-type and transformed flight and jump muscle fibers, and we analyzed these muscle samples for fiber-specific patterns of gene expression (Figure 4C). As observed for the physiological and molecular markers described earlier, there was a striking transition of the flight muscle to a jump muscle fate when *exd* or *hth* were knocked down, based upon loss of flight muscle markers. There was also a concomitant gain of the jump muscle-specific expression profile in the flight muscles of these knockdown individuals.

We also documented at the molecular level jump muscles taking on flight muscle fate when *exd* and *hth* were ectopically coexpressed. We note that, in this case, not all of the individual fibers comprising the jump muscle were completely transformed. This might result either from a requirement for factors in addition to *exd* and *hth* to completely alter jump muscle fate or from incorrect timing of expression initiation, given that the *Act79B-Gal4* driver has a relatively late onset in adult myogenesis.

Overall, our data strongly confirmed the radical changes in flight and jump muscle phenotypes that resulted from manipulation of *exd* and *hth* expression. While we cannot assess every single fiber-specific property of the muscles, the transformations in ultrastructure, T-tubule morphology, patterns of gene expression and muscle cell biochemistry lead strongly to the conclusion that Exd/Hth specify flight muscle versus jump muscle fate.

Experimentally induced changes in fiber-specific expression were not limited to the flight and jump muscles. We detected abnormal expression of the *TpnC41C-LacZ* reporter in abdominal wall muscles in response to *hth* knockdown (Figure S4). Notably, these muscles do not express *TpnC41C* gene or its reporter under normal conditions (Figure S4; Herranz et al., 2004). This observation supports the conclusion that Exd/Hth contribute to muscle identity in other muscles of the body.

Exd and Hth Control a Regulatory Cascade Specifying Fibrillar Muscle Fate

The zinc finger transcription factor *spalt-major* (*salm*) was recently shown to promote flight muscle fate in *Drosophila* (Schönbauer et al., 2011). To understand the role of *salm* in the context of *exd*- and *hth*-mediated identity specification, we analyzed *salm* expression by quantitative RT-PCR (RT-qPCR) and by immunofluorescence in normal and transformed muscles. In WT samples, significant *salm* expression was detected in the flight muscles, with lower levels consistently observed in the jump muscles. Jump muscle transcript levels were approximately 40% of that in the flight muscles (Figures 5A and 5B). This difference was also observed in microsamples of muscle tissues, using endpoint RT-PCR (Figure 5C). Apart from the flight and jump muscles, Salm protein was not detected in any other muscle (Schönbauer et al., 2011; data not shown). These observations suggest that the presence of Salm alone is not sufficient to induce flight muscle fate. In *hth* knockdowns, there was a reduction in *salm* expression in the transformed flight muscles, as detected both by endpoint RT-PCR and by immunofluorescence (Figures 5C and 5D, *hth*↓). When *exd* plus *hth* were expressed in the jump muscle, those fibers that were transformed toward fibrillar muscle fate showed an increase in *salm* expression compared to control jump muscles (Figures 5C and 5D, *exd*↑ *hth*↑). We also carried out the converse experiment, where *salm* expression was manipulated in the adult muscles: under knockdown conditions, Salm levels were undetectable by immunofluorescence (Figure S5, *salm*↓), yet Exd and Hth levels in the flight muscles were unchanged (Figure 5E, flight muscle, *salm*↓). When *salm* was overexpressed in the jump muscle (Figure S5, *salm*↑), there was no induction of *exd* nor *hth* in the jump muscle, and there was no evidence of a transition of the jump muscle to fibrillar muscle fate (Figure 5E, jump muscle, *salm*↑). While these epistatic interactions can be difficult to interpret since the cells are also changing fate, the data strongly suggest that expression of *exd* and *hth* are required for the elevated expression of *salm* in the flight muscles and provisionally place *exd/hth* genetically upstream of *salm* in a pathway for fiber type specification. Since high levels of *salm* expression only correlated with the transformed jump muscle fibers upon *exd/hth* expression, there are perhaps further interactions between these factors to ensure mutual enrichment in flight muscles. In addition, since there is still transformation of the flight muscles to a tubular muscle fate in *salm* knockdowns, despite sustained accumulation of Exd and Hth in these mutants (Schönbauer et al., 2011; Figure 5E, *salm*↓), at least some of the function of Exd and Hth to promote fibrillar fiber fate must occur through upregulation of *salm*.

Exd/Hth Act to Regulate Expression of Structural Genes across Different Fiber Types

To date, no direct targets of Exd/Hth or Salm have been identified in muscles. Within the full-length *Act88F* enhancer (Bryantsev et al., 2012) that shows flight muscle activity (Figure 6A), we identified an evolutionarily conserved 52 base pair (bp) core element that we termed Act88F(Reg1), or Reg1 (Figure 6B). When Reg1 was tested on its own for in vivo enhancer activity, this small enhancer retained flight muscle specificity (Figure S6A), and had activity similar to that of the full-length *Act88F* enhancer (Figure S6B). This short element contained consensus binding sites for Exd/Hth, and an adjacent site for homeotic factors, that were conserved across multiple *Drosophila* species (Figure 6B). Electrophoretic mobility shift assays (EMSA) revealed that Exd/Hth could bind specifically to this sequence, and that DNA binding required functional homeodomains for both Exd and Hth, indicating that these factors can form a complex on the *Act88F* enhancer sequence. Moreover, binding of Exd and Hth to Reg1 required the integrity of their identified sites (Figure 6C). Conversely, no binding sites for Salm were observed in this sequence, and Salm protein did not interact with the Reg1 element, either in isolation or in collaboration with Exd/Hth. As a control, the purified Salm protein was able to bind to a control sequence from the *rhomboid* gene (Li-Kroeger et al., 2008) (Figure S6C).

To determine if Exd/Hth are required in vivo for *Act88F* enhancer activity, we assessed the activity of Reg1 under conditions of *hth* knockdown. This resulted in a loss of Reg1 enhancer activity in the flight muscles (Figure 6D, *hth*↓). One could interpret this result as a direct requirement for Hth in *Act88F* expression; however, since *hth* knockdown also causes a fiber type transformation, the loss of enhancer activity might simply have reflected a change in muscle fate. Therefore, we tested the activity of the Reg1 enhancer under normal genetic conditions, but with its Hth or Exd sites mutated. Here, there was also a loss of enhancer activity (Figure 6D; Figure S6D). We conclude that Exd/Hth are direct and positive regulators of *Act88F* through these sites, providing a molecular link between the regulators of fiber identity and a structural gene that is characteristic of that fiber. This result is of further significance, since manipulation of *salm* levels did not affect *Act88F* expression (Schönbauer et al., 2011), indicating that the impact of *exd/hth* upon at least some flight muscle genes does not occur through *salm*.

Using immunostaining, we had identified the jump muscle as one of the only somatic muscles in adult thoraces that did not

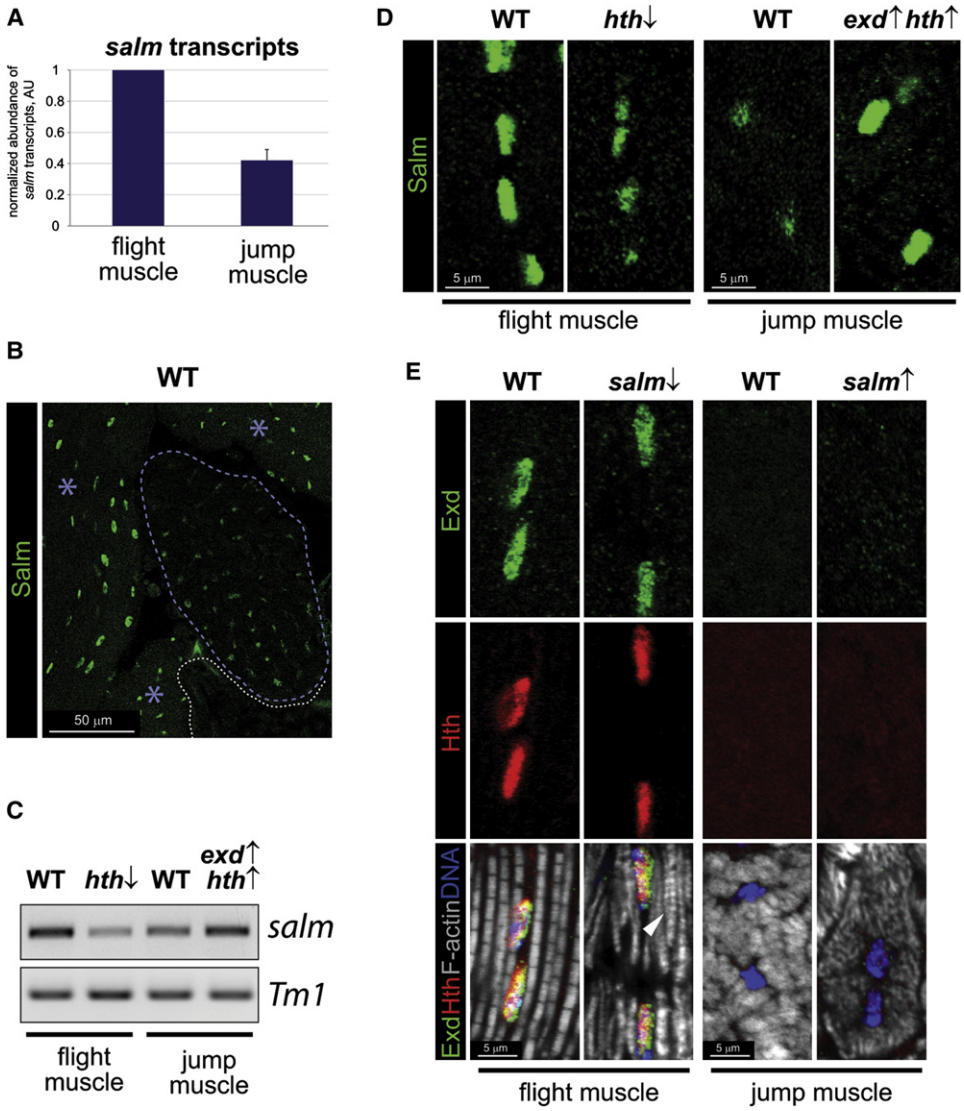

Figure 5. Regulation of *salm* Expression in Flight and Jump Muscles

(A) Results of real-time qPCR analysis of *salm* expression in isolated flight and jump muscles. *salm* levels are normalized to pan-muscle-specific *Mhc* transcripts and arbitrarily set to 1.0 in flight muscles. Error bars represent standard deviation.

(B) Salm protein accumulation in the thorax detected by immunofluorescence. Note reduced Salm (green) in the jump muscle (dashed line) compared to the flight muscles (asterisks), and the absence of Salm in other tubular muscles (white dotted line).

(C) RT-PCR detection of *salm* transcripts from microsamples of flight and jump muscles of WT animals, and animals with *exd/hth* manipulations. Note the reduction in *salm* transcripts in flight muscles of *hth* knockdown animals, and the increase in jump muscles of overexpression animals. *Tropomyosin1* (*Tm1*) levels were used as a loading control for muscle transcripts.

(D) Nuclear Salm (green) accumulation is reduced in flight muscles when *hth* is knocked down and increased in the jump muscles in response to ectopic *hth* plus *exd* expression.

(E) Exd (green) and Hth (red) expression in nuclei of muscles with experimentally manipulated expression of *salm* (↓ and ↑ indicate knockdown and overexpression, respectively). Exd/Hth levels are not reduced when Salm levels are reduced, and they are not ectopically induced in the jump muscles when *salm* expression is elevated there. Arrowhead points to a myofibril with altered striation pattern, indicative of transformation of the flight muscle toward a tubular muscle phenotype, caused by *salm* knockdown.

See also Figure S5.

express *exd* and *hth* (Figure S1). Interestingly, many *exd/hth*-expressing somatic muscles (such as abdominal muscles and leg muscles) belong to the tubular fiber type, demonstrating features more similar to those of the jump muscle than the flight muscle. How do we reconcile the presence of Exd/Hth proteins in many tubular muscle types with the ability of these factors to promote flight muscle fate? The flight and the jump muscles arise from the T2 thoracic segment, and the T2 musculature is unique in not expressing homeotic (Hox) genes encoded by the Antennapedia or Bithorax complexes (Roy and VijayRaghavan, 1997) that are critically involved in body segment specification (Foronda et al., 2009). We therefore reasoned that the activities

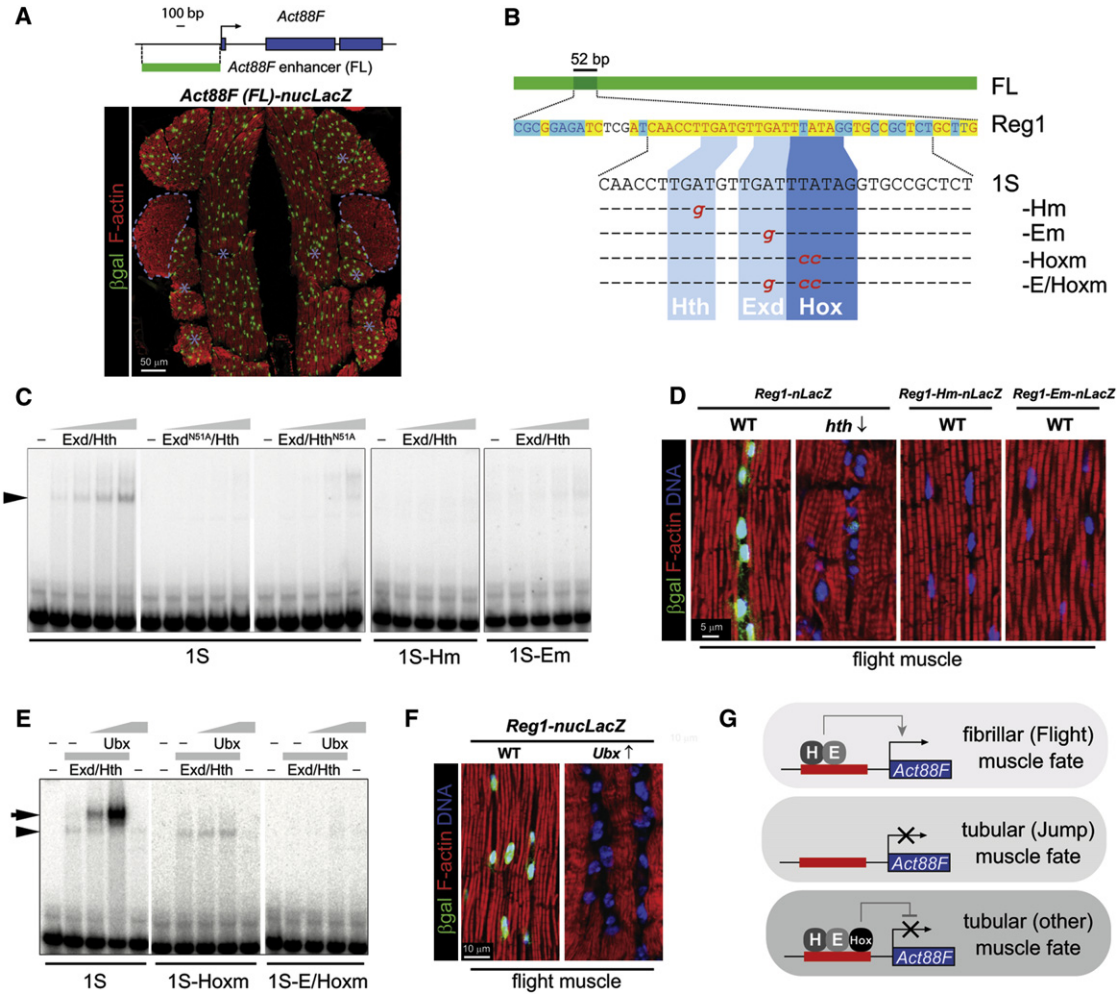

Figure 6. Direct Transcriptional Regulation of Muscle Fiber Identity by Hth and Exd

(A) Schematic of the full-length *Act88F* enhancer and immunofluorescent image depicting the activity of the enhancer in flight muscles (asterisks) but not in jump muscles (outlined). Enhancer activity is detected by nuclear accumulation of β-gal (green) in nuclei of muscles (red).

(B) Schematic of the *Act88F* enhancer showing sequence and conservation of the 52-bp minimal enhancer, termed Reg1. Yellow and blue colors indicate absolute and significant nucleotide conservation, respectively, in 12 *Drosophila* species. Sequences of probes used for EMSA are shown, and putative binding sites for Exd, Hth, and Hox proteins are shaded. Mutations of the binding sites used for EMSA are shown in lowercase letters, with the rest of the sequences remaining intact (dashes).

(C–F) EMSA results are shown in (C) and (E); arrowheads and arrows mark Hth/Exd and Hth/Exd/Ubx bound probes, respectively. Grey marks indicate relative protein concentration added to reactions. N51A is a homeodomain mutation that prevents DNA binding of the mutated polypeptide. Note that Exd/Hth must both be present for DNA binding to occur and that Ubx can bind to these factors to make a higher-order complex with DNA. In (D) and (F), changes in in vivo expression of the minimal *Act88F* enhancer, Reg1, are shown in response to indicated genetic manipulations and point mutations. Mutation abbreviations are synonymous to those indicated in (B). Note that Reg1 activity is lost in the absence of Hth or when Hth or Exd binding sites are mutated. Reg1 activity is also lost when *Ubx* is expressed in the flight muscles.

(G) Model for the regulation of flight muscle structural genes (modeled on *Act88F*) by Exd (E), Hth (H), and Hox proteins. Depending upon the factors present, these *Drosophila* somatic muscles acquire the fate of flight, jump or other tubular fibers.

See also Figure S6.

of *exd* and *hth* to promote flight muscle fate could be blunted by the coexpression of a *Hox* gene. We tested this idea first by determining if Hox proteins could interact with the DNA/Exd/Hth complex formed on the *Act88F* enhancer sequence. We found that several Hox proteins, including Ultrabithorax (Ubx), could form a complex upon the *Act88F* enhancer with Exd and Hth. Moreover, the formation of this complex was dependent upon the binding sites for Exd or Hox (or both) (Figure 6E). Next, we analyzed the effects upon Reg1 enhancer activity of ectopically expressing *Ubx* in the flight muscles. Here, there was a repression of Reg1 enhancer activity, and a reprogramming of the large thoracic muscles to a nonflight muscle fate, as judged by changes in their myofibril morphology and clustering of their nuclei in a lumen (Figure 6F). These data supported the hypothesis that Exd and Hth promote flight muscle fate, whereas tubular muscle fate is initiated either in the absence of *exd/hth* expression, or when these two genes are coexpressed with a *Hox* gene (summarized in Figure 6G).

DISCUSSION

Our results demonstrate a dramatic effect upon muscle fiber identity of the two factors Exd and Hth. The fact that muscle fiber type can be profoundly influenced by the activity of the two genes defines a central mechanism for the control of fiber identity and begins to expose the entire fiber specification pathway.

In a recent study by Schönbauer et al. (2011), a genetic factor that controls transition from tubular leg muscle to the fibrillar fiber type was identified as *salm*. The authors demonstrated that tubular leg muscles could be transformed into the fibrillar type by ectopic expression of *salm*. In our study, we expand these observations to show that the mechanism of Salm action is less straightforward: *salm* is expressed in the tubular jump muscle, suggesting that its pro-fibrillar action may require cooperation with additional factors. Our data suggest that Salm cofactors could be Exd and Hth: their absence in the jump muscle prevents this muscle from acquiring a fibrillar fiber phenotype despite its expression of *salm*; also, ectopic expression of *salm* in leg muscles promotes fibrillar fate, perhaps because the leg muscles also express *exd* and *hth* (Figure S1; Schönbauer et al., 2011).

We also note that, in the flight muscles, Exd and Hth maintain their localization in the absence of Salm. Moreover, despite the sustained accumulation of Exd and Hth, loss of Salm nevertheless results in transformation of the flight muscles toward a tubular fate (Schönbauer et al., 2011). This indicates that Exd and Hth have at least some requirement for Salm to promote fibrillar muscle fate, and it will be interesting in the future to identify the respective roles of these factors directly interacting with other fiber-specific enhancers.

We also provide a direct mechanistic link between the determinants of fibrillar fate, *exd*/*hth*, and the actin gene characteristic of the flight muscles, *Act88F*. Whether fibrillar muscle genes are direct targets of Exd/Hth or Salm, or both, is yet to be determined; nevertheless, the identification of fiber-specific enhancers described here and elsewhere (Bryantsev et al., 2012) will provide new mechanistic insight into this process.

Since diverse fiber types are characteristic of many vertebrate muscles, our findings may relate directly to vertebrate myogenesis. In zebrafish, slow muscle fate is promoted by the activities of PBX and MEIS, which are the vertebrate orthologs of Exd and Hth, respectively (Maves et al., 2007). In mice, PBX and MEIS are cofactors for myogenic determination genes, where they facilitate transcription factor binding to nonconsensus target sites, and this effect might function to fine tune muscle fiber fate (Heidt et al., 2007). Thus, diverse lines of evidence suggest a robust and conserved mechanism for fiber type specification, acting through PBX1/Exd and MEIS/Hth.

EXPERIMENTAL PROCEDURES

Genomic fragments of *fln* and *TpnC41C* for enhancer analysis were PCR amplified, cloned into pCHAB and used for P-element transformation of flies, similarly to what has been described (Bryantsev et al., 2012). For *Act88F* enhancers, the PCR-amplified fragments carrying flanking attB sites were recombined into pDONR-nLacZ-attb, a Gateway-compatible derivate of the plasmid pnLacZattB (provided by Dr. Basler, University of Zurich, Zurich, Switzerland) and incorporated into the genome via phiC31 integrase at the identical landing site at 86Fb (Bischof et al., 2007). Expression constructs for *exd* and *hth* were created by RT-PCR of pupal RNA samples, generating the RA isoform of *exd* and the RE isoform of *hth*, and cloned into pUASTattB for making transgenic lines. *Drosophila* methods were carried out using standard approaches, and nomenclature is as described at Flybase.org. Fly stocks were obtained from the Bloomington *Drosophila* Stock Center or from the Vienna *Drosophila* RNAi Center (VDRC). Among several lines tested, the lines 100687 (Vienna) and 34637 (Bloomington) were the most effective in *exd* and *hth* knockdown, respectively. Salm levels in muscles were downregulated with the RNAi transgenic line 101052 (VDRC) using the published protocol (Schönbauer et al., 2011), and upregulated with *Act88F-Gal4* (Bryantsev et al., 2012) and *UAS-Salm* (Dr. Schnorrer, Max Planck Institute of Biochemistry, Martinsried, Germany). Crosses were carried out at 29°C unless indicated. β-gal assays were carried out as described in the Supplemental Experimental Procedures. Cryosections were analyzed as described before (Jaramillo et al., 2009), and antibodies were obtained from The University of Iowa Developmental Studies Hybridoma Bank unless otherwise indicated. The anti-Salm antibody was first described elsewhere (Xie et al., 2007). Histochemical stains were as described by Deak (Deak, 1977). Electron microscopy used an established protocol (O'Donnell et al., 1989). For in situ hybridization, Stellaris probes for *TpnC41C* were generated by Biosearch Technologies. The in situ hybridizations were carried out according to the probe manufacturer's protocol for frozen tissue sections. Muscle-specific samples for RT-PCR were collected as described in Supplemental Experimental Procedures. Electrophoretic mobility shift assays (EMSA) were performed as previously described (Gebelein et al., 2002), using 2.5 ng, 5 ng, 10 ng, and 20 ng of Exd/Hth in Figure 6C; and a constant 10 ng of Exd/Hth and either 5 ng or 50 ng of Ubx as indicated in Figure 6E.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.08.004.

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REFERENCES

Anant, S., Roy, S., and VijayRaghavan, K. (1998). Twist and Notch negatively regulate adult muscle differentiation in Drosophila. Development *125*, 1361–1369.

Bernstein, S.I., O'Donnell, P.T., and Cripps, R.M. (1993). Molecular genetic analysis of muscle development, structure, and function in Drosophila. Int. Rev. Cytol. *143*, 63–152.

Bischof, J., Maeda, R.K., Hediger, M., Karch, F., and Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA *104*, 3312–3317.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Bryantsev, A.L., Baker, P.W., Lovato, T.L., Jaramillo, M.S., and Cripps, R.M. (2012). Differential requirements for Myocyte Enhancer Factor-2 during adult myogenesis in Drosophila. Dev. Biol. *361*, 191–207.

D'Amico, A., and Bertini, E. (2008). Congenital myopathies. Curr. Neurol. Neurosci. Rep. *8*, 73–79.

Deak, I.I. (1977). A histochemical study of the muscles of Drosophila melanogaster. J. Morphol. *153*, 307–316.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151–156.

Fernandes, J.J., and Keshishian, H. (2005). Motoneurons regulate myoblast proliferation and patterning in Drosophila. Developmental Biology *277*, 493–505.

Foronda, D., de Navas, L.F., Garaulet, D.L., and Sánchez-Herrero, E. (2009). Function and specificity of Hox genes. Int. J. Dev. Biol. *53*, 1404–1419.

Fyrberg, E.A., Mahaffey, J.W., Bond, B.J., and Davidson, N. (1983). Transcripts of the six Drosophila actin genes accumulate in a stage- and tissue-specific manner. Cell *33*, 115–123.

Gebelein, B., Culi, J., Ryoo, H.D., Zhang, W., and Mann, R.S. (2002). Specificity of Distalless repression and limb primordia development by abdominal Hox proteins. Dev. Cell *3*, 487–498.

Heidt, A.B., Rojas, A., Harris, I.S., and Black, B.L. (2007). Determinants of myogenic specificity within MyoD are required for noncanonical E box binding. Mol. Cell. Biol. *27*, 5910–5920.

Herranz, R., Díaz-Castillo, C., Nguyen, T.P., Lovato, T.L., Cripps, R.M., and Marco, R. (2004). Expression patterns of the whole troponin C gene repertoire during Drosophila development. Gene Expr. Patterns *4*, 183–190.

Jaramillo, M.S., Lovato, C.V., Baca, E.M., and Cripps, R.M. (2009). Crossveinless and the TGFbeta pathway regulate fiber number in the Drosophila adult jump muscle. Development *136*, 1105–1113.

Li-Kroeger, D., Witt, L.M., Grimes, H.L., Cook, T.A., and Gebelein, B. (2008). Hox and senseless antagonism functions as a molecular switch to regulate EGF secretion in the Drosophila PNS. Dev. Cell *15*, 298–308.

Maves, L., Waskiewicz, A.J., Paul, B., Cao, Y., Tyler, A., Moens, C.B., and Tapscott, S.J. (2007). Pbx homeodomain proteins direct Myod activity to promote fast-muscle differentiation. Development *134*, 3371–3382.

Nose, A., Isshiki, T., and Takeichi, M. (1998). Regional specification of muscle progenitors in Drosophila: the role of the msh homeobox gene. Development *125*, 215–223.

O'Donnell, P.T., Collier, V.L., Mogami, K., and Bernstein, S.I. (1989). Ultrastructural and molecular analyses of homozygous-viable Drosophila melanogaster muscle mutants indicate there is a complex pattern of myosin heavy-chain isoform distribution. Genes Dev. *3*, 1233–1246.

Punkt, K. (2002). Fibre types in skeletal muscles. Adv. Anat. Embryol. Cell Biol. *162*, III–XV, 1–109.

Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M., and Mann, R.S. (1997). Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. Cell *91*, 171–183.

Roy, S., and VijayRaghavan, K. (1997). Homeotic genes and the regulation of myoblast migration, fusion, and fibre-specific gene expression during adult myogenesis in Drosophila. Development *124*, 3333–3341.

Roy, S., Shashidhara, L.S., and VijayRaghavan, K. (1997). Muscles in the Drosophila second thoracic segment are patterned independently of autonomous homeotic gene function. Curr. Biol. *7*, 222–227.

Ruiz-Gómez, M., Coutts, N., Price, A., Taylor, M.V., and Bate, M. (2000). Drosophila dumbfounded: a myoblast attractant essential for fusion. Cell *102*, 189–198.

Schiaffino, S., and Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. Physiol. Rev. *91*, 1447–1531.

Schiaffino, S., Hanzlíková, V., and Pierobon, S. (1970). Relations between structure and function in rat skeletal muscle fibers. J. Cell Biol. *47*, 107–119.

Schönbauer, C., Distler, J., Jährling, N., Radolf, M., Dodt, H.U., Frasch, M., and Schnorrer, F. (2011). Spalt mediates an evolutionarily conserved switch to fibrillar muscle fate in insects. Nature *479*, 406–409.

Toniolo, L., Patruno, M., Maccatrozzo, L., Pellegrino, M.A., Canepari, M., Rossi, R., D'Antona, G., Bottinelli, R., Reggiani, C., and Mascarello, F. (2004). Fast fibres in a large animal: fibre types, contractile properties and myosin expression in pig skeletal muscles. J. Exp. Biol. *207*, 1875–1886.

Vigoreaux, J.O., Saide, J.D., Valgeirsdottir, K., and Pardue, M.L. (1993). Flightin, a novel myofibrillar protein of Drosophila stretch-activated muscles. J. Cell Biol. *121*, 587–598.

Xie, B., Charlton-Perkins, M., McDonald, E., Gebelein, B., and Cook, T. (2007). Senseless functions as a molecular switch for color photoreceptor differentiation in Drosophila. Development *134*, 4243–4253.

Zierath, J.R., and Hawley, J.A. (2004). Skeletal muscle fiber type: influence on contractile and metabolic properties. PLoS Biol. *2*, e348.