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Org-1, the *Drosophila* ortholog of Tbx1, is a direct activator of known identity genes during muscle specification

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SUMMARY

Members of the T-Box gene family of transcription factors are important players in regulatory circuits that generate myogenic and cardiogenic lineage diversities in vertebrates. We show that during somatic myogenesis in *Drosophila*, the single ortholog of vertebrate *Tbx1*, *optomotor-blind-related-gene-1* (*org-1*), is expressed in a small subset of muscle progenitors, founder cells and adult muscle precursors, where it overlaps with the products of the muscle identity genes *ladybird* (*lb*) and *slouch* (*slou*). In addition, *org-1* is expressed in the lineage of the heart-associated alary muscles. *org-1* null mutant embryos lack Lb and Slou expression within the muscle lineages that normally co-express *org-1*. As a consequence, the respective muscle fibers and adult muscle precursors are either severely malformed or missing, as are the alary muscles. To address the mechanisms that mediate these regulatory interactions between Org-1, Lb and Slou, we characterized distinct enhancers associated with somatic muscle expression of *lb* and *slou*. We demonstrate that these lineage- and stage-specific cis-regulatory modules (CRMs) bind Org-1 in vivo, respond to *org-1* genetically and require T-box domain binding sites for their activation. In summary, we propose that *org-1* is a common and direct upstream regulator of *slou* and *lb* in the developmental pathway of these two neighboring muscle lineages. Cross-repression between *slou* and *lb* and combinatorial activation of lineage-specific targets by Org-1–Slou and Org-1–Lb, respectively, then leads to the distinction between the two lineages. These findings provide new insights into the regulatory circuits that control the proper pattering of the larval somatic musculature in *Drosophila*.

KEY WORDS: Tbx1, Org-1, Muscle development, Transcriptional cascades, *Drosophila*

INTRODUCTION

The musculature and the central nervous system are two examples of tissues featuring a high diversity of cell types. In each hemisegment of the *Drosophila* embryo, about 30 distinct body wall muscles and six adult muscle precursors are formed and in the CNS the number of neuronal and glial cell types generated is an order of magnitude higher. Currently, we have only a limited picture of the regulatory processes that generate these great cellular diversities, but we know that several steps of muscular and neuronal development in *Drosophila* are accomplished by closely related processes. For example, the earliest progenitor cells, termed muscle progenitors and neuroblasts, respectively, seem to be defined by intersecting anterior-posterior and dorsoventral upstream regulators. In both tissues, these progenitors are preceded by larger clusters of promuscular or proneural cells, respectively, that are characterized by their expression of proneural genes from the Achaete-Scute complex. Lateral inhibition mediated by Delta/Notch signals singles out individual progenitor cells from these clusters (reviewed by Beckett and Baylies, 2006; Technau et al., 2006; Tixier et al., 2010). In the case of the muscle progenitors, receptor tyrosine kinase (RTK) signaling, particularly via epidermal growth factor (EGF)- and fibroblast growth factor (FGF)-receptors (Egfr and Htl, respectively), is required as a positive input to antagonize Notch (Carmena et al., 2002). An additional similarity is the occurrence of asymmetric cell divisions that involve the unequal segregation of the Notch-inhibitor Numb (reviewed by Karcavich, 2005; Beckett and Baylies, 2006; Tixier et al., 2010). In the case of muscle progenitors, these asymmetric divisions typically generate two distinct muscle founder cells that go on to form two different larval muscle fibers. In some instances, one sibling of a muscle founder cell gives rise to certain heart precursors or to a stem cell-like adult muscle progenitor that will contribute to the adult body wall musculature (reviewed by Figeac et al., 2007; Speicher et al., 2008). Subsequently, muscle founder myoblasts fuse with surrounding fusion-competent myoblasts to form distinct body wall muscle fibers. During both muscle and neuronal development, the increasing diversity of the newly generated cells is reflected in the distinct combinations of transcription factors expressed in them. Several of these factors are known to have key roles in defining the particular cell identities and have been called muscle identity factors (reviewed by Beckett and Baylies, 2006; Tixier et al., 2010).

Although the broad picture of these diversification events is known, there are major gaps in knowledge with regard to the specific design of the regulatory networks involved. In the case of *Drosophila* muscle development, a few lineages of muscle progenitors have been characterized in some detail. The first are those marked by the expression of the identity factor Even-skipped (Eve, a homeodomain protein) and have been best characterized with regard to the essential early-acting inputs. These particular muscle progenitors arise in the dorsal portion of the mesoderm and give rise to somatic muscle fibers as well as to specific cells of the dorsal vessel (Frasch et al., 1987). One of the two adjacent muscle progenitors marked by Eve generates the founder of muscle 1 (DA1), which requires *eve* function (Fujioka et al., 2005), whereas the other generates the founder of muscle 10 (DO2) (Speicher et

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al., 2008). The activation of *eve* in these muscle progenitors requires combinatorial signals from ectodermal Decapentaplegic (Dpp) and Wingless (Wg), the mesodermal competence factor Tinman, which itself is downstream of Dpp, as well as RTK signals via Egfr and Htl (Azpiazu and Frasch, 1993; Bodmer, 1993; Frasch, 1995; Wu et al., 1995; Carmena et al., 1998). All these combinatorial signaling and transcription factor inputs are directly integrated at the level of a mesoderm-specific enhancer element at the *eve* locus (Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002).

The step-wise specification of muscle identities has been addressed for muscle founders expressing the identity factor Collier (Col, a COE transcription factor; Knot – FlyBase). In abdominal segments, Col is expressed in the two adjacent dorsolateral muscle progenitors of muscles 3 (DA3) and 20 (DO5) as well as 18 (DT1) and 19 (DO4) and the corresponding promuscular clusters. The expression is only maintained in the founder and precursor of muscle 3/DA3, which requires Col for its development (Crozatier and Vincent, 1999). This dynamic expression is reflected in the activities of at least two cis-regulatory modules (CRMs) of *col*. An intronic CRM activates expression within both Col^+ promuscular clusters and the progenitors singled out from them, whereas an upstream CRM activates and maintains expression specifically in the muscle founder 3/DA3 and its muscle. The early-acting intronic CRM responds to positional and mesodermal inputs, which might involve binding by Twist and Tinman, whereas activation of the later-acting CRM in the M3/DA3 founder relies on the combinatorial binding of autoregulatory Col, Nautilus (Nau, also known as MyoD), and the Hox factors Antp and Ubx. In this manner, the early patterning and tissue-specific inputs are integrated with the axial inputs of the Hox genes that modulate the muscle pattern and fiber size in different body parts (Dubois et al., 2007; Enriquez et al., 2010).

The muscle lineages that are the focus of the present study are positioned laterally in the somatic mesoderm and are marked by the expression of the homeodomain factors Slouch (Slou) and Ladybird (Lb), which function as identity genes in these lineages [*lb* refers herein to the paralogous *ladybird early* (*lbe*) and *ladybird late* (*lbl*) genes]. Within this area of interest, Slou is expressed in the progenitor and sibling founders of muscles 5 (LO1) and 25 (VT1) and is crucial for the formation of these muscles (Dohrmann et al., 1990; Knirr et al., 1999). Lbe is expressed directly adjacent to Slou in a promuscular cluster and then two muscle progenitors. One of these progenitors forms muscle 8 (SBM) and a lateral adult muscle precursor (lAMP), which continue to express Lb, whereas the other forms a second Lb^+ lAMP and a sibling that probably undergoes apoptosis (Jagla et al., 1998; Knirr et al., 1999; Figeac et al., 2010). Genetic and genomic analyses indicate that Lb has important functions in the specification and/or differentiation of muscle 8 (Junion et al., 2007; Bataille et al., 2010).

The expression of *slou* and *lbe* appears to be controlled by an overlapping set of upstream regulators, because their mutually exclusive expression in the neighboring progenitors of muscles 5/25 and muscle 8/lAMP requires cross-repression by their respective gene products (Knirr et al., 1999; Junion et al., 2007). However, the nature of these shared regulatory inputs has been unknown. In the present study, we identify *org-1*, a *Drosophila Tbx1* ortholog that had not been characterized extensively prior to this work (Porsch et al., 1998; Lee et al., 2003), as a key activator of both *slou* and *lbe* in this pathway. *org-1* expression in the mesodermal areas and progenitors that will form muscles 5, 25, 8 and the Lb^+ lAMPs precedes that of *slou* and *lbe*, and the activation of these identity genes requires *org-1* activity. Consequently, the development of these muscles and the lAMPs is disrupted in *org-1* mutant embryos. We demonstrate that the activation of *slou* and *lbe* by *org-1* requires T-box binding motifs in their respective founder-specific enhancer elements and show in vivo occupancy of these elements by Org-1. Hence, *slou* and *lbe* appear to be direct target genes of *org-1* in the developmental pathway of these neighboring muscle lineages. In addition, *org-1* is expressed in the progenitor of the alary muscles and its function is needed for the development of these muscles, which form segmental anchors of the dorsal vessel.

MATERIALS AND METHODS

Drosophila strains

We used *lbl-SBM-lacZ* (Philippakis et al., 2006) (from A. M. Michelson, NIH/NHLBI, Bethesda, MD, USA), *RRHS59-lacZ* (Knirr et al., 1999), $Dp(1;3)sn^{13a1}$, w^{1118},$Mi\{ET1\}org\text{-}1^{MB01466}$ and w^{1118};noc^{Sco}/*SM6a,P{hsILMiT}2.4* (Bloomington Stock Center, Indiana University). Forced pan-mesodermal expression of *UAS-org-1* with *2xPE-GAL4*;how^{24B}*-GAL4* (Wang et al., 2005) was carried out at 28°C.

Generation of *org-1* mutations

The viable insertion $Mi\{ET1\}org\text{-}1^{MB01466}$ was used in a imprecise excision screen with Minos transposase as described by Metaxakis et al. (Metaxakis et al., 2005). Balanced *Pax6-EGFP*-negative females in F2 were crossed individually with *FM7c* males. Of the 591 excision lines screened for X-chromosomal lethality, the molecular lesions in two lines, $org\text{-}1^{OJ423}$ and $org\text{-}1^{OJ487}$ were defined by genomic PCR and sequencing of the shortened bands obtained.

Generation of an *org-1* cDNA, *UAS-org-1* and Org-1-specific antibodies

The 5′ portion (222 bp upstream of initiator ATG to *Nsi*I site) of the cDNA was generated by RACE (SMART RACE Kit, Clontech) from cDNA of 8- to 12-hour-old embryos and was combined with a PCR-derived fragment of the 3′ portion (*Nsi*I to 24 nucleotides downstream of stop codon) using an cDNA library (from 8- to 12-hour-old embryos) as a template. The combined and sequenced *org-1* cDNA#1 (2385 bp) was cloned as an *Eco*RI (5′)/*Hin*dIII (3′) fragment into *pBluescript SK+* and as an *Eco*RI/*Sal*I fragment into *Eco*RI/*Xho*I of *pUAST* (Brand and Perrimon, 1993). The *pUAST* construct was injected into *yw* (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

A fragment from a PCR product of *org-1* cDNA#1 (using primers introducing *Bam*HI and *Eco*RI sites directly upstream of the first ATG and at position 861 of the ORF, respectively) was cloned into *Bam*HI/*Eco*RI cut *pET30a* (Novagen). Bacterially expressed recombinant protein corresponding to the N-terminal portion of Org-1 including the first six residues of the T-box was purified by His affinity chromatography and injected into rats (Covance, Denver, PA, USA) for antibody generation.

Construction of reporter and rescue constructs

Supplementary material Table S1 shows the genomic regions that were PCR-isolated from *yw* genomic DNA and used for reporter constructs. *HN39*, *SK10* and *SK16* were cloned into *Eco*RI/*Bam*HI of *pCaSpeR-hs-43-βgal* (Thummel and Pirrotta, 1992). For *SK16-GFP*, the genomic fragment was cloned into *Eco*RI/*Bam*HI of *pGreen H-Pelican* (Barolo et al., 2000). The *slou-SK19* and *lbl-SBMs* fragments were cloned into *Eco*RI/*Kpn*I of a modified *pH-Stinger* vector, which has attB sequences inserted into its *Avr*II site (*pH-Stinger-attB*; H.J. and M.F., unpublished). For the analogous creation of *SK16 OrgI-VIImut-GFP*, *SK19 OrgI-IIImut-GFP* and *lbl-SBMs OrgI-VImut-GFP* reporters the Org-1 binding sites within the *SK19*, *SK16* and *lbl-SBMs* sequences were predicted using a positional weight matrix generated by SELEX with Org-1-GST [H.J. and M.F., unpublished; Target Explorer (Sosinsky et al., 2003)] and mutated via site-directed mutagenesis or de novo DNA synthesis (MrGene, Regensburg, Germany) (supplementary material Table S1). For *org-1-HN39-GFP*, the *HN39* fragment was cloned into *Eco*RI/*Bam*HI of *pH-Stinger-attB*. For *org-1-HN39-org-1*, *org-1-HN39-slou* and *org-1-HN39-lbe, org1 cDNA* (this

study), *slou cDNA* (Dohrmann et al., 1990) and *lbe cDNA* (Jagla et al., 1998) with added C-terminal Myc-tags were cloned into *Age*I/*Hpa*I of *org-1-HN39-pH-Stinger-attB*, substituting the EGFP-cassette. *pH-Stinger* constructs were transformed into *yw* and the *pH-Stinger-AttB* constructs into strains with the landing sites *ZH-35B* (Bischof et al., 2007) or *AttP2* (Groth et al., 2004). At least four independent transformed lines were tested for transgene activity.

Chromatin immunoprecipitation (ChIP) assays

Chromatin preparation using ChIP was performed using staged *2xPE-GAL4; how^{24B}-GAL4xUAS-org1;UAS-org1;UAS-org1* embryo collections and two different Org-1 antibodies as described (Sandmann et al., 2006). The precipitated DNA was purified with MinElute PCR purification kit (Qiagen, Hilden) and quantified by qPCR using the Brilliant II SYBR Green QPCR Master Mix (Agilent) on an Mx3000P system (Stratagene). Three independent precipitations per amplicon were analyzed (supplementary material Table S1).

Immunohistochemistry

Embryo staining and in situ hybridization were performed as described previously (Azpiazu and Frasch, 1993; Knirr et al., 1999) with the following antibodies and probes: rat anti-Org-1 (1:100, this study), mouse anti-Lb monoclonal (1:10) (Jagla et al., 1998), rabbit anti-S59 (1:100) (Dohrmann et al., 1990), rabbit anti-β3-Tubulin (1:3000; gift from R. Renkawitz-Pohl, University of Marburg, Germany), mouse anti-Ubx monoclonal (1:50; gift from R. White, University of Cambridge, UK), chick anti-Twist (1:200, affinity-purified; Dominik Müller and M.F., unpublished), rat anti-Tropomyosin (1:200; Babraham, Cambridge, UK), rabbit anti-GFP (1:2000; Molecular Probes), mouse anti-GFP (1:2000; Molecular Probes), rabbit anti-βGal (1:1500; Promega), mouse anti-βGal (40-1a, 1:200; Developmental Studies Hybridoma Bank, University of Iowa, USA) and digoxygenin-labeled antisense transcripts from *caup* cDNA (Gomez-Skarmeta et al., 1996). All mutant lines were balanced with *lacZ* or GFP balancers to identify mutant embryos. Pictures were taken with a Zeiss Axio Imager equipped with a Zeiss ApoTome (20×/0.8 Plan-Apochromat; 40×/1.3 Plan-Apochromat Oil) or a Leica SP5 II (63×/1.3 PL APO Glycerol). Projections were created using Axiovision 4.8 or Leica LAS AF.

RESULTS

org-1 is expressed in two characterized somatic muscle lineages and in alary muscles

org-1, the *Drosophila* ortholog of vertebrate *Tbx1* (see Fig. 1 for details on the gene locus), showed a striking and dynamic pattern of expression in the developing mesoderm. During early embryonic stage 9, Org-1 protein started to be expressed in narrow segmental stripes within the somatic mesoderm and by stage 10, Org-1 expression also appeared in the 11 segmental clusters of trunk visceral mesoderm precursors (Fig. 2A,B). As previously reported for *org-1* mRNA (Lee et al., 2003), during stages 10-11 Org-1 protein expression in the visceral mesoderm became restricted to the visceral muscle progenitor and founder cells (Fig. 2B-D). Importantly, somatic mesodermal expression also refined during stage 11 and remained active in a few individual cells per hemisegment, which we identified as somatic muscle progenitors (Fig. 2D). During stage 12, these muscle progenitors divided into Org-1$^+$ somatic muscle founder cells (Fig. 2E). After myoblast fusion, the abdominal expression of Org-1 could be assigned to somatic muscles 5 (M5, also known as LO1) and 25 (M25/VT1), which are derived from sibling founder cells, muscle 8 (M8/SBM) and two lateral adult muscle precursors (lAMPs), one of which is a sibling of the M8 founder. In addition, the alary muscle expressed Org-1 and was formed from one of the Org-1$^+$ founder cells located in dorsolateral mesodermal areas (Fig. 2F,G). These assignments were supported further by the expression analysis of an *org-1* reporter construct driven by a downstream enhancer, *HN39*, which faithfully recapitulated *org-1* expression in the progenitors, founders, lAMPs, and fibers of the somatic muscles (Fig. 1, Fig. 2H-J). During stage 14, *org-1* mRNA and protein expression became extinct in M5 and M8, but owing to the longer half-life of β-galactosidase (βGal), reporter signals perdured in these muscles until the end of embryogenesis (Fig. 2G-I′; C.S. and M.F., unpublished).

The expression of Org-1 during somatic muscle development was examined in more detail by double staining with *org-1-HN39-lacZ* and probes reflecting the expression of two known muscle identity genes, *slouch* (*slou*) and *ladybird* (*lb*). As shown in Fig. 3A,A′, by stage 11 *org-1* expression became restricted to two neighboring somatic muscle progenitors per hemisegment, which were also positive for *org-1-βGal*. Shortly thereafter, the appearance of a third Org-1$^+$ progenitor gave rise to a three-cell cluster at this position (Fig. 3A-B′). Double staining for βGal and Lb demonstrated that the two dorsal cells within this cluster correspond to the Lb$^+$ muscle progenitors (Fig. 3F,F′). They divide into four Org-1$^+$ (and Lb$^+$) cells (Fig. 3C,C′,F,F′), although one of these rapidly became undetectable. The observation of about one extra Lb$^+$ cell in this area upon blocking cell death (Figeac et al., 2010) suggests to us that this fourth cell normally undergoes apoptosis. Consistent with previous findings with Lb as a marker, one of the three Org-1$^+$ and Lb$^+$ cells was seen to form M8, whereas the other two formed the two lAMPs (Fig. 3G,G′). M8 and the lAMPs maintained βGal signals and Lb expression but M8 rapidly lost Org-1 expression after myoblast fusion (Fig. 3D-E′,G,G′).

The ventral muscle progenitor shown in Fig. 3A in each hemisegment became positive for Slouch (Slou; data not shown) and for βGal driven by the *RRHS59* enhancer upstream of *slou*

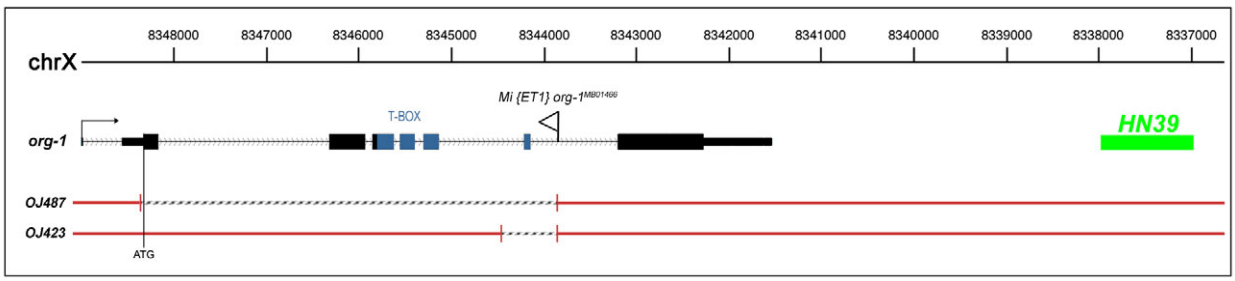

Fig. 1. *org-1* gene locus and imprecise excision-induced deletion alleles. *org-1* gene map (genome annotation R5.19) showing transcribed regions in black, regions coding for the T-Box DNA binding motif in blue and deleted regions in the imprecise excision alleles *org-1^{OJ423}* and *org-1^{OJ487}* underneath. The *HN39* genomic region used for *org-1-lacZ*, *org-1-GFP* and the rescue constructs is shown in green.

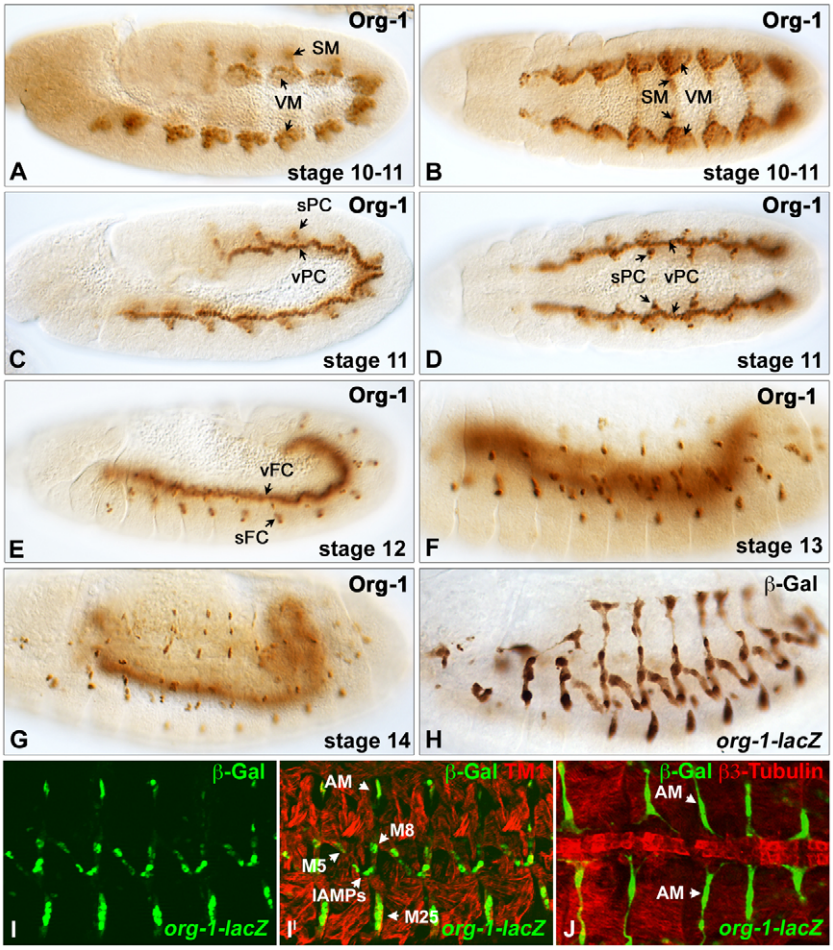

Fig. 2. Org-1 protein is expressed in visceral and a subset of somatic muscle progenitor cells. (**A**,**B**) Org-1 protein is found during stage 10 (A, lateral view; B, ventral view) in all cells of the trunk visceral mesoderm (VM) and in narrow stripes of cells in the somatic mesoderm (SM). (**C**,**D**) At stage 11 (C, lateral view; D, ventral view), Org-1 expression domains in the segmented mesoderm become confined to progenitor cells of the visceral musculature (vPC) as well as to a specific subset of somatic muscle progenitor cells (sPC). (**E**,**F**) During germ band retraction (E), Org-1 is present in visceral (vFC) and somatic (sFC) founder cells, derived from the progenitors, and during stage 13 (F) neighboring nuclei become positive after being incorporated into Org-1$^+$ muscle precursors upon myoblast fusion. (**G-J**) At stage 14, nuclear Org-1 (G) and cytoplasmic β-Gal (H-J) expression driven by *org-1-HN39-lacZ* are present in muscles 5 (LO1), 25 (VT1) and 8 (SBM), alary muscles (AM) and lateral adult muscle precursors (lAMPs). The muscle pattern is visualized by Tropomyosin (TM1) or β3-tubulin antibody staining.

(Fig. 3H,H′) (Knirr et al., 1999). This progenitor divides into the founder cells of M5 and M25 (Fig. 3C-D′) (Dohrmann et al., 1990). Whereas M25 continued expressing Org-1 until the end of embryogenesis, Org-1 expression in M5 was gradually lost (Fig. 3E,E′,I,I′).

During early stage 12, a new muscle progenitor located dorsally to the Org-1$^+$ and Lb$^+$ muscle 8 founder started expressing Org-1 and divided into two Org-1$^+$ and *org-1*-βGal$^+$ muscle founders (Fig. 3C,C′). The dorsal sibling corresponds to the founder of the alary muscle. It fuses with dorsally adjacent fusion-competent myoblasts, which showed gradually increasing Org-1 signals (Fig. 3D,D′, see also Fig. 2F,G), and elongated towards the dorsal vessel. Owing to its rapid loss of Org-1 expression, the fate of the sibling of the alary muscle founder remains unknown.

In summary, *org-1* shows a highly restricted pattern of expression in the somatic mesoderm, which precedes and overlaps with the expression of the previously characterized muscle identity genes *slou* and *lb*. In addition, *org-1* represents the first clear marker for the founders and fibers of the alary muscles, which attach segmentally to the dorsal vessel.

org-1 functions as a muscle identity gene in all *org-1*$^+$ lineages

The function of *org-1* during somatic muscle development was analyzed with two lethal alleles made by imprecise Minos-element excisions (Fig. 1), which had identical phenotypes. Both alleles showed complementation of hemizygous lethality by *Dp(1;3)sn*^{13a1}, a duplication covering *org-1*, and appeared to be null mutants. *org-1*^{OJ487} had the translation initiation codon as well as the first six exons deleted and did not show any detectable Org-1 protein, whereas *org-1*^{OJ423} caused a C-terminal truncation of the protein including a portion of the T-Box DNA-binding domain (Fig. 1). In hemizygous *org-1* mutant embryos, M5 and M8 were not identifiable. Instead, there were small syncytia with variable shapes located near the normal ventral attachment sites of these two muscles, which did express differentiation markers such as β3-Tubulin. This phenotype exhibited 100% penetrance. Fibers resembling M25 appeared to be present, although they were highly variable in shape and often connected to incorrect attachment sites (Fig. 4B-B‴, compare with 4A-A‴). The formation of the alary muscles was also disrupted in *org-1* mutant embryos, in which these muscles were completely missing (Fig. 4D, compare with 4C).

Although *org-1-HN39-lacZ* expression was activated in the progenitor and founder cells of M5, M8, M25 and the alary muscles, this expression rapidly disappeared upon myoblast fusion in the *org-1* mutant background, as did the truncated protein detected in *org-1*^{OJ423} mutant embryos (Fig. 4F, compare with 4E; data not shown). Hence, normally there is direct or indirect autoregulation.

Because *org-1* expression overlaps with that of *slou* and *lb* in the somatic mesoderm and *org-1* mutations affect *slou*$^+$ and *lb*$^+$ muscles, we tested whether *org-1* is required for the expression of these two muscle identity genes. Indeed, we found that *lb* expression was never activated in the somatic mesoderm of *org-1* mutants and the Lb protein could be detected neither in the

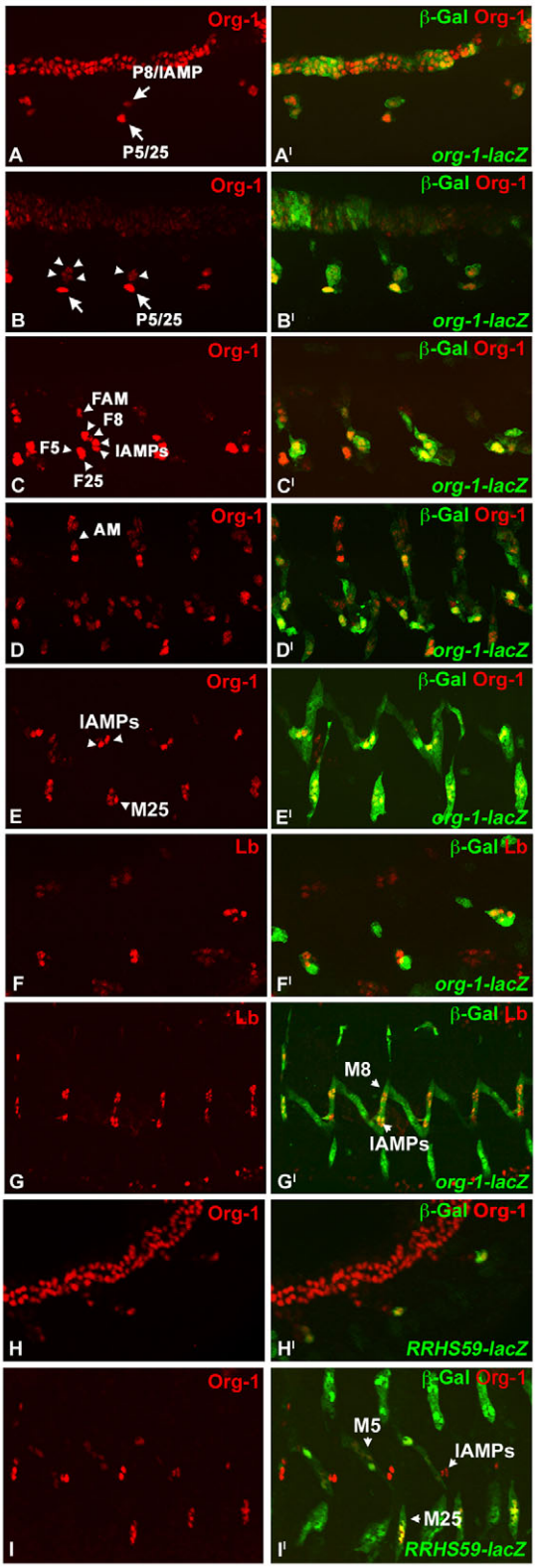

Fig. 3. The expression patterns of *org-1* and *org-1-lacZ* overlap with those of the muscle identity genes *ladybird* (*lb*) and *slouch* (*slou*). (**A**,**A′**) During stage 11, Org-1 protein as well as *org-1-lacZ* reporter (β-Gal) activity are present in the progenitor cells (arrows) of muscles 5/25 (L01/VT1) and M8 (SBM)/lAMP in each abdominal segment. (**B**,**B′**) During stage 12, the Org-1$^+$ *org-1-lacZ*$^+$ progenitors cells divide to generate muscle founder cells and lAMPs (arrowheads). Arrows indicate progenitor cells. (**C**,**C′**) At late stage 12, Org-1 and *org-1-lacZ* are present in the founder cells of muscles 5, 25 and 8 and the lAMPs, and are newly expressed in alary muscle founders (FAM). (**D**,**D′**) Org-1 and *org-1-lacZ* in muscle precursors during myoblast fusion and in lAMPs at stage 13. AM, alary muscle. (**E**,**E′**) At stage 14, Org-1 expression remains in M25 and lAMPs (and alary muscles; not depicted) but recedes in M5 and M8. (**F**,**F′**) In early stage 11 embryos, Ladybird protein (Lb) can be detected in two (left and middle) and in more advanced (right) segments in four of the *org-1-lacZ*$^+$ muscle founders. (**G**,**G′**) At stage 15, these founders have given rise to M8 and the lateral adult muscle precursors (lAMPs). (**H-I′**) At stage 11, Org-1 protein expression is also observed in one *slou-RRHS59-lacZ*$^+$ muscle progenitor (H,H′) and at stage 15 Org-1 overlaps in muscle 5 and 25 with the *slou-lacZ* signals (I,I′).

progenitors, founders and fibers of M8 nor in the lAMPs (Fig. 5B,D, compare with 5A,C). Staining for Twist, which marks all adult muscle precursors, showed that, unlike adult muscle precursors at other positions, the number of lAMPs was strongly reduced. In ~50% of the hemisegments no lAMPs were detectable and in the remaining ones only one instead of two lAMPs was seen, which failed to express Lb (Fig. 5C,D). Thus, *org-1* is required for proper formation of lAMPs.

As observed for Lb, the expression of the Iroquois homeobox genes *caupolican* and *araucan* is specifically missing at the normal positions of M8 in *org-1* mutants, showing that the Iroquois genes are also expressed downstream of *org-1* in M8 and confirming that M8 is not properly specified in the absence of Org-1 (Fig. 5E,F; data not shown). *caup* expression was unaffected (i.e. neither reduced nor expanded) in the *org-1*-independent lateral transverse muscles, in which the Iroquois genes were recently shown to act as muscle identity genes (Carrasco-Rando et al., 2011).

In the lineages of M5 and M25, the expression of Slou and *slou-RRHS59-lacZ* was absent during all phases of their (aberrant) development (Fig. 5H,J, compare with 5G,I; data not shown). As expected, the expression in M18 and M27, which lack Org-1 also during normal development, was unaffected. Together, these observations define *org-1* as a crucial upstream regulator of the identity genes *lb*, *slou* and *caup* in the developmental pathway giving rise to M5, M8, M25 and the lAMPs.

Additional experiments addressed the question of whether *org-1* is simply required to activate *lb* and *slou* in the precursor cells or whether it plays additional essential roles in the development of the respective muscles. Specifically, we tested whether restoring the expression of *lbe* or *slou* in *org-1* mutant backgrounds would rescue the observed defects in the development of M8 and M5/M25, respectively. Whereas the expression of *org-1* in *org-1* mutant backgrounds under the control of the *HN39-org-1* enhancer was able to rescue these muscles to their normal morphology, albeit at a low frequency (supplementary material Fig. S1D, compare with S1B,C), neither expression of *lb* (supplementary material Fig. S1F, compare with S1B,D,E) nor of *slou* (supplementary material Fig. S1H, compare with S1B,D,G) under the same conditions was ever seen to provide any rescue. This strongly suggests a role for *org-1* in activating other essential target(s) in parallel to *lb* and *slou* during the development of these muscles.

In addition to Slou or Lb expression, the Org-1$^+$ founder cells were also positive for Ultrabithorax (Ubx) protein during stage 12 in abdominal segments (Fig. 5K). In *org-1* mutant embryos, not

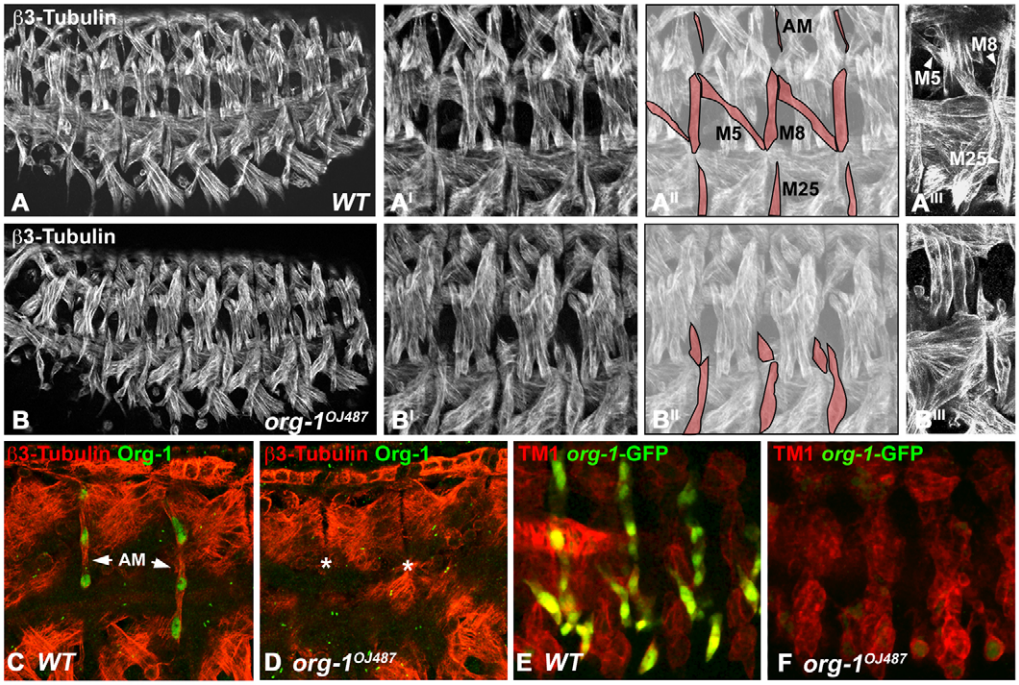

Fig. 4. Loss of *org-1* function leads to muscle specification defects. (**A-B‴**) Comparison of the segmental muscle patterns of stage 15 *WT* and *org-1*^{OJ487} mutant embryos (visualized with anti-β3-tubulin) reveals lost or mis-shapen M8, M5, M25 and alary muscles in mutants. In A″ and B″ the phenotypic differences between the *WT* (A″) and the *org-1*^{OJ487} null mutant (B″) situations are highlighted. Projections of confocal slices containing wild-type M8, M5, M25 (A‴) and their mutant counterparts (B‴) are shown. (**C**) In the wild type (WT), the alary muscles (AM), marked by Org-1 and β3-Tubulin, extend from the tip of M8 towards the dorsal vessel. (**D**) In *org-1*^{OJ487} mutant, alary muscles are absent (asterisks). (**E**) *org-1*-GFP expression marking all Org-1$^+$ muscle precursors and lAMPs in stage 14 wild-type background (TM1, Tropomyosin). (**F**) Severely reduced *org-1*-GFP expression in stage 14 *org-1*^{OJ487} mutant background.

only Slou and Lb but also Ubx expression was abolished in these founder cells (Fig. 5L). Hence, *Ubx* is another example of an *org-1* downstream gene within this subset of somatic founder cells and probably acts in the regional modulation of the respective muscle identities.

The potency of *org-1* in muscle specification was also evident from experiments in which we forced its ectopic expression. Pan-mesodermal expression of Org-1 caused severe disruptions of the somatic muscle pattern in 100% of scored segments (Fig. 6A-B′). This effect was accompanied by a dramatic expansion of *lb* expression (Fig. 6D,D′, compare with 6C,C′). Slou expression was expanded to a lesser degree with pan-mesodermal Org-1 and the expression domains of Lb and Slou remained mutually exclusive (data not shown), indicating that cross-repression between Slou and Lb (Knirr et al., 1999; Junion et al., 2007) remains intact in this situation. Together with the observed specific increase of Twist-marked lAMPs upon ectopic expression of Org-1 (Fig. 6F, compare with 6E), these data suggest that ectopic Org-1 can shift the identities of other muscle founders and muscles towards those of M8, lAMPs and (to a lesser degree) M5 and M25.

Org-1 is a direct upstream regulator of *slou* and *lb* in the somatic mesoderm

To determine whether Org-1 is a direct activator of *slou* and *lb* expression, we examined relevant cis-regulatory modules of these two genes. The previously defined *lbl-SBM* enhancer element (Philippakis et al., 2006) and a 925 bp subfragment, *lbl-SBMs*, defined by us (Fig. 7A) showed reporter activity in the Lb$^+$ muscle founder cells as well as in M8 and the lAMPs (Fig. 7B,D,F), thus recapitulating the somatic mesodermal Lb expression pattern. As shown for the Lb protein, *lbl-SBM* enhancer activity depends on *org-1* (Fig. 7C). Conversely, it is dramatically expanded upon ectopic pan-mesodermal *org-1* expression (data not shown).

Our dissection of the *slou-RRHS59* element revealed two separate genomic regions that were active in the *slou*$^+$ and *org-1*$^+$ muscle lineage. A 622 bp element, *slou-SK19*, and a slightly shorter version of it, *slou-SK10* (Fig. 7H), were already active in muscle progenitors and muscle founders of all Slou$^+$ muscles (Fig. 7I,K). In addition, a non-overlapping 745 bp element located proximally to *slou-SK19*, termed *slou-SK16* (Fig. 7H,M), initiated expression only in founder cells and remained active in muscles. Apart from the *slou*$^+$ M5 and M25, *SK16* also drove ectopic expression in M8, apparently because it lacks sequences that are subject to Lb-dependent repression (Fig. 7N,P; there is also weak ectopic activity in M21 and M23). In *org-1* mutant backgrounds, the activity of the two distinct *slou* enhancers *SK10* and *SK16* was specifically absent in the lineages that normally express *org-1*, namely the progenitors and founders of M5 and M25 (*SK10*; Fig. 7J) and the founders and fibers of M5/M25 and M8/LaP (*SK16*; Fig. 7O). As expected, native and ectopic enhancer activity in other muscles and their precursors that normally are independent of *org-1* was unaffected by *org-1* mutation (M18, M27, M21, M23; Fig. 7J,O). The observed dependency of *lb* and *slou* enhancer activities on *org-1* suggested that these enhancers could be transcriptional targets of Org-1.

Sequence analysis of these *lb* and *slou* enhancer elements in combination with data from SELEX experiments with Org-1 fusion proteins identified six putative Org-1 binding motifs within *lbl-*

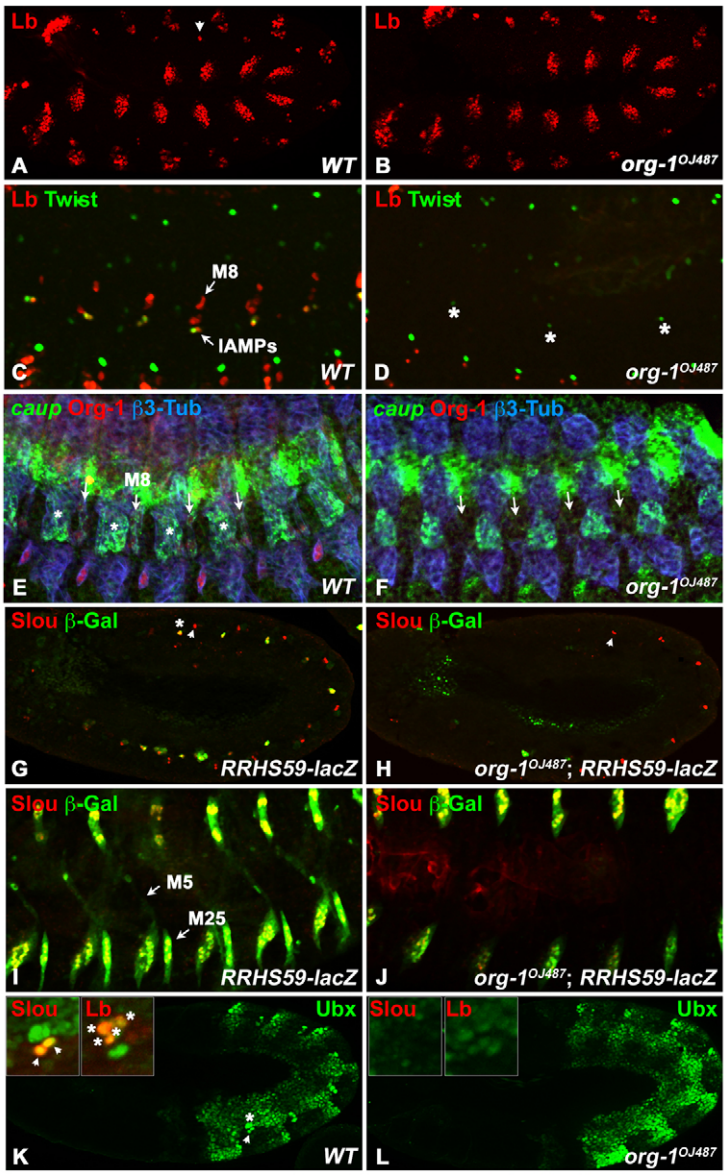

Fig. 5. The expression of the muscle identity genes *ladybird*, *caupolican* and *slouch* is regulated by *org-1*. (**A**) In stage 11 wild-type embryos, Lb is detected in two muscle progenitor cells in each abdominal segment (arrowhead). (**B**) In the *org-1* mutant background, Lb expression in the progenitors is absent. (**C**) Stage 14 wild-type embryo showing Lb expression in M8 and the lAMPs (identified by Twist). (**D**) In an *org-1* mutant embryo, Lb expression in M8 and the lAMPs is abolished. In ~50% of the segments one weakly Twist$^+$ lateral cell remains (asterisks). (**E**) Stage 14 wild-type embryo showing mesodermal *caupolican* (*caup*) mRNA expression (green) in M8 (arrows) and in M21-24 (asterisks) (blue, β3-Tubulin; red, Org-1). (**F**) In *org-1* loss-of-function embryos, *caup* expression at the normal positions of M8 is absent (arrows). (**G**) In a stage 12 wild-type embryo carrying *slou-lacZ*, β-Gal protein is observed in one Slou$^+$ muscle progenitor (P5/25; asterisk) but not yet in a second progenitor (P26/27; arrowhead) per hemisegment. (**H**) In the *org-1^{OJ487}* background, both Slou protein and *slou-lacZ* activity in P5/25 and the two corresponding muscle founders are absent. Arrowhead indicates progenitor of M26/27. (**I**) Stage 15 embryo with Slou and *slou-lacZ* signals in four distinct muscles per hemisegment. (**J**) In the *org-1^{OJ487}* background at stage 15, Slou expression and *slou-lacZ* signals in M5 and M25 are missing. (**K**) In a stage 12 wild-type embryo, high levels of Ultrabithorax protein (Ubx) are found in the Org-1$^+$ muscle founders, where it shows co-expression (insets) with either Slou (arrowheads) or Lb (asterisks). (**L**) Loss of Org-1 function causes loss of Ubx as well as Slou and Lb expression in these founder cells.

SBMs, three within *slou-SK19* and seven within *slou-SK16* (Fig. 7A,H,M,R). Anti-Org-1 ChIP analysis with chromatin from stage 11-12 embryos (*2xPE; how24B>>UAS-org-1*) clearly confirmed in vivo binding of Org-1 to the binding sites in the *SK19* and *lbl-SBMs* enhancer fragments, whereas chromatin including the binding sites within the *SK16* fragment was enriched less strongly (Fig. 7R).

When we introduced two to three base pair changes into each of these motifs at positions known to be important for T-box protein binding (Müller and Herrmann, 1997), we observed dramatic changes in all reporter expression patterns. The *lbl-SBMs OrgI-VImut* reporter showed a total loss of activity in the Lb$^+$ muscle progenitor and founder cells (Fig. 7E) as well as strong downregulation in M8 and the lAMPs (Fig. 7G). Reporter activity of *slou-SK19 OrgI-IIImut* in the Org-1$^+$ precursor cells was completely abolished (Fig. 7L), and also *slou-SK16 OrgI-VIImut* was specifically inactivated in all Org-1$^+$ muscles, namely M5, M8 and M25 (Fig. 7Q). By contrast, the activity in the Slou$^+$/Org-1$^-$ muscle M18 and the ectopic activity in the Slou$^-$/Org-1$^-$ muscles M21 and M23 was unaffected, thus providing an internal control for the specificity of the effects (Fig. 7Q). Altogether, these data strongly suggest that the normal activation of *lb* and *slou* in the founder cells and muscles of these *org-1*$^+$ muscle lineages requires binding of Org-1 to some or all of the Org-1 binding motifs within the *lbl-SBMs*, *slou-SK19* and *slou-SK16* enhancer sequences.

DISCUSSION

Our analysis of the expression and function of *org-1* in somatic muscle development has established this gene as a new and crucial representative of muscle identity genes in *Drosophila*. The data have provided new insights into developmental controls in two well-defined muscle lineages and somatic muscle development in general. These lineages include one dependent on the homeobox gene *slou*, which gives rise to muscles M5 and M25 (also known as 'cluster I') (Dohrmann et al., 1990), and another dependent on the *lb* homeobox genes, which gives rise to the segment border muscle M8 and lateral adult muscle precursors (lAMPs) (Fig. 8). They arise from promuscular clusters and muscle progenitors abutting each other in the ventrolateral somatic mesoderm. Until now, it was assumed that *slou* and *lb* are positioned at the top of

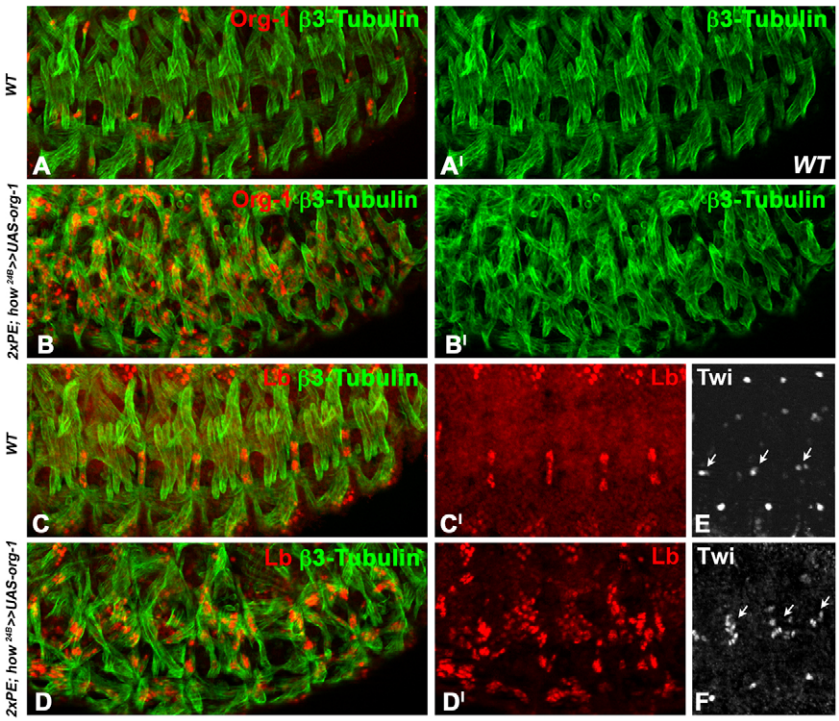

Fig. 6. Ectopic Org-1 expression induces founder cell fate transformations. (**A-B′**) Severe disruption of the normal somatic muscle pattern (A,A′) marked by b3-Tubulin (green) as a result of pan-mesodermal overexpression (B,B′) of Org-1 (red in A,B). (**C-D′**) Lb expression (red), normally restricted to one M8 and two lAMPs per hemisegment (C,C′), strongly expands upon pan-mesodermal Org-1 expression (D,D′). (**E**,**F**) Ectopic Org-1 expression leads to increased formation of Twi$^+$ lAMPs (F, arrows) in comparison with the wild type (E, arrows).

the regulatory hierarchy set up within each of these lineages, as their expression and function is already detected at the progenitor cell stage prior to asymmetric divisions into different founder cells (Dohrmann et al., 1990; Jagla et al., 1998; Knirr et al., 1999). As in the model proposed for the *even-skipped*-expressing lineage in the dorsal somatic mesoderm, the progenitor-specific expression of *slou* and *lb* was assumed to be determined directly by the antagonistic actions of various more broadly active activators and negative influences through lateral inhibition via Notch (Bate et al., 1993; Jagla et al., 1998). Based on genetic assays using the expression of *slou* and *lb*, and the formation of the respective muscles as outputs, good candidates for such activators included localized receptor tyrosine kinase (RTK)/Ras/MAPK signals (Baylies and Michelson, 2001; Bidet et al., 2003) (H.N. and M.F., unpublished) in conjunction with the relatively broadly expressed mesodermal transcription factors Tinman (Azpiazu and Frasch, 1993; Jagla et al., 1998), Six4 (in the case of *lb*) (Clark et al., 2006), Twist (Cox and Baylies, 2005) and Sloppy paired (Lee and Frasch, 2000). Ectodermal Wg signals were also found to regulate these Slou$^+$ and Lb$^+$ lineages (Baylies et al., 1995; Jagla et al., 1998), although at least in the case of *slou* these apparently act indirectly by upregulating *twi* and *slp* expression (Baylies et al., 1995; Lee and Frasch, 2000; Cox and Baylies, 2005).

Our current findings have uncovered an additional layer of regulation upstream of *slou* and *lb* within the M5/M25 and M8/lAMP lineages that involves *org-1* (Fig. 8). The initial expression of *org-1* occurs in segmented areas of the lateral somatic mesoderm, from which the *org-1*$^+$ M5/M25 and M8/lAMP progenitors emerge. These areas probably delineate the two abutting promuscular clusters from which these progenitors are singled out (as well as a third one dorsolaterally, from which the progenitor and founder of the alary muscle is formed). Although this early expression of *org-1* is not required for progenitor formation per se (based upon the normal pattern of *org-1:HN39-lacZ* in progenitors of *org-1* mutants; C.S. and M.F., unpublished), *org-1* is crucial either during this phase or during early phases of progenitor formation for activating *slou* and *lb* in the respective progenitors. Hence, we propose that the previously identified regulators of *slou* and *lb*, including *Notch*, RTKs, *tin*, *wg*, *twi* and *slp*, act predominantly in establishing progenitor-specific expression of *org-1*, which in turn activates *slou* and *lb*. However, a plausible alternative to this linear model of regulation would be a feed-forward model, in which some of the above upstream regulators are re-employed during the second step to activate *slou* and *lb* together with mandatory Org-1. Whether *org-1* activates *slou* or *lb* in any given progenitor would depend on the outcome of the previously reported mutual inhibition between *slou* and *lb*. We think it is unlikely that the outcome of this process is completely random; instead, we favor a mechanism involving an initial bias towards one or the other. Such biases could, for example, arise through slight differences in the spatial activities of the EGF and FGF receptors (Egfr and Htl), or of transcription factors such as Six4, coupled with differential responses of *slou* and *lb* to these regulators. Regardless of the specific mechanism, this principle of joint activation followed by mutual repression allows for the differential specification of directly neighboring cells, in this case of P5/25 and P8/lAMP, that are under the influence of common upstream regulators.

The combination of genetic data and functional enhancer analysis provides convincing evidence that both *slou* and *lb* are direct transcriptional targets of Org-1. This conclusion also fits nicely with the observation that a *slou* enhancer fragment used herein, *SK16*, is active ectopically in M8 and that Org-1 and the putative Org-1 binding sites are required for its activity not only in M5 and M25, but also in M8. By contrast, an adjacent enhancer fragment, *SK10*, is not active in M8 and thus reflects the endogenous Org-1-dependent pattern of *slou*. Presumably unlike *SK16*, this element still includes *lb*-dependent repression elements that block its activation by Org-1 in M8.

The *lbl-SBM* enhancer (1.36 kb) was identified initially in a bioinformatics approach for sequences near founder cell-expressed genes that are enriched with binding motifs for Twi, Tin, dTCF

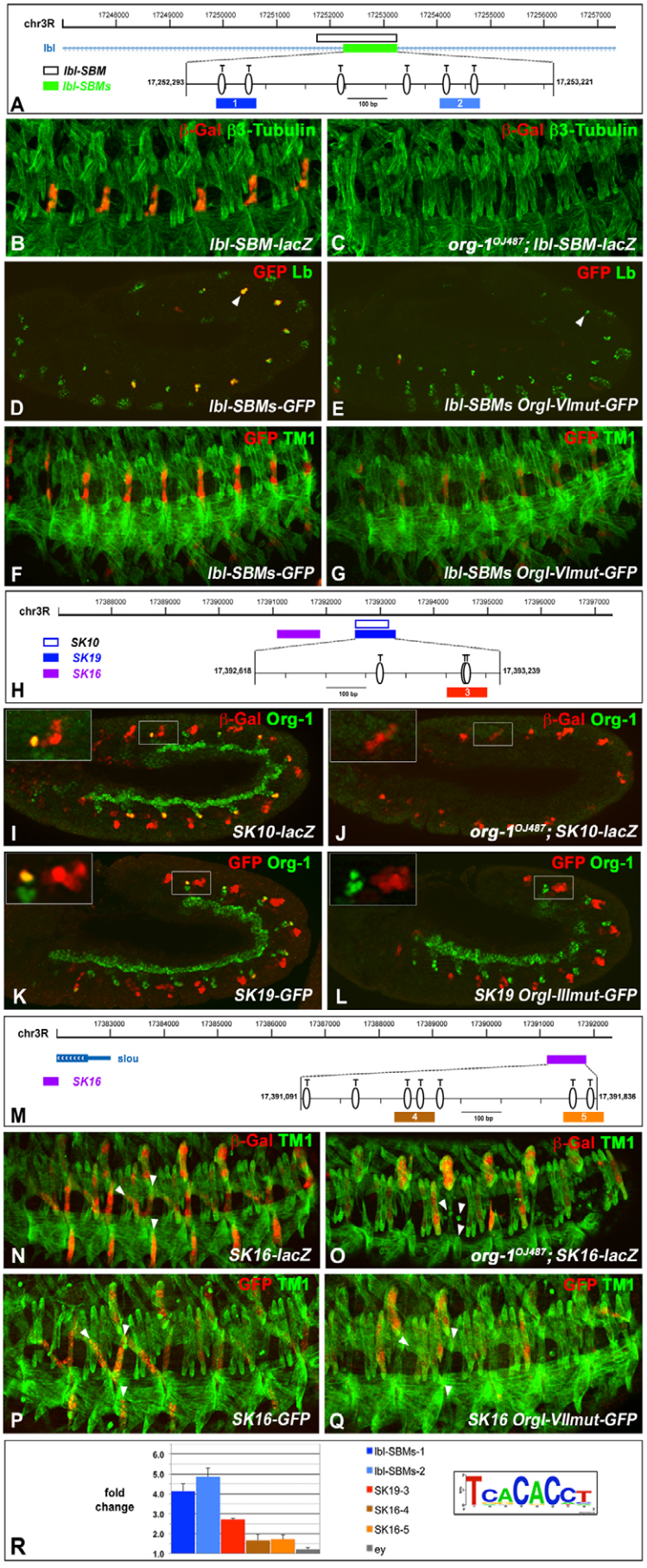

Fig. 7. The Org-1 binding sites in somatic mesodermal *ladybird* and *slouch* CRMs are essential for lineage-specific enhancer activity. (**A**) Schematic of the genomic region used for the generation of the *lbl-SBM* (boxed, black) and *lbl-SBMs* (green) reporters. Predicted T-Box binding sites are indicated in detailed view below. (**B**) *lbl-SBM*-driven *lacZ* expression in M8 and lAMPs in stage 15 wild-type embryo. (**C**) Stage 15 *org-1* mutant embryo carrying *lbl-SBM-lacZ* showing complete absence of β-Gal expression. (**D**) *lbl-SBMs-GFP* reporter recapitulates expression of Lb protein in somatic muscle founders (arrowhead). (**E**) The *lbl-SBMs Orgl-VImut-GFP* enhancer derivative with all predicted Org-1 binding sites mutated shows loss of reporter activity in somatic muscle founders (arrowhead) (stage 12, anti-GFP, anti-Lb). (**F**) *lbl-SBMs* reporter expression in M8 and lAMPs at stage 15. (**G**) The *lbl-SBMs Orgl-VImut-GFP* enhancer construct displays reduced reporter activity in M8 and the lAMPs (stage 15, anti-GFP, anti-TM1). (**H**) Schematic of the genomic region used for the *SK10* (boxed) and *SK19* (solid blue) reporter constructs with predicted T-Box binding sites. (**I**) In a stage 10 embryo, the *slou-SK10-lacZ* reporter includes activity in the Org-1$^+$ P5/25 progenitors. (**J**) In *org-1* mutant backgrounds, the *slou-SK10-lacZ* signals are absent in P5/25 progenitors. (**K**) The *slou-SK19-GFP* enhancer element is active in Slou$^+$ progenitor cells including the Org-1$^+$ P5/25. (**L**) The *slou-SK19 Orgl-IIImut-GFP* reporter with mutated Org-1 binding sites in *SK19* shows loss of activity in the Org-1$^+$ progenitors (stage 12, anti-GFP, anti-Org-1). (**M**) Schematic of the genomic region used for the *SK16* reporter construct (purple). Predicted T-Box binding sites are indicated. (**N**) *slou-SK16-lacZ* reporter activity in the Org-1$^+$ muscles 5, 25 and 8 (arrowheads) as well as in M18 (plus weak ectopic activity in M21 and M23). (**O**) In *org-1* mutant background, the *slou-SK16-lacZ* reporter fails to be activated in remnants of M5, M25 and M8 (arrowheads), but remains active in M18, M21 and M23. (**P**) *slou-SK16-GFP* reporter activity in Slou$^+$ muscles 5, 25 (arrowheads) and 18 as well as in M8 (arrowhead), M22 and M24. (**Q**) The *slou-SK16 Orgl-VIImut* enhancer derivative with mutated Org-1 binding sites fails to activate GFP in muscles 5, 25 and M8 (arrowheads) (stage 15, anti-GFP, anti-TM1) whereas expression in M18, M21 and M23 remains. (**R**) Anti-Org-1 ChIP was assayed by qPCR using amplicons covering T-Box binding sites (color-coded in A,H,M) and an *ey* exonic amplicon as negative control. Each bar represents the average of three independent biological replicates normalized to negative controls from the *C15* gene (see supplementary material Table S1). The sequence logo shows the Org-1 consensus motif identified by SELEX (H.J. and M.F., unpublished).

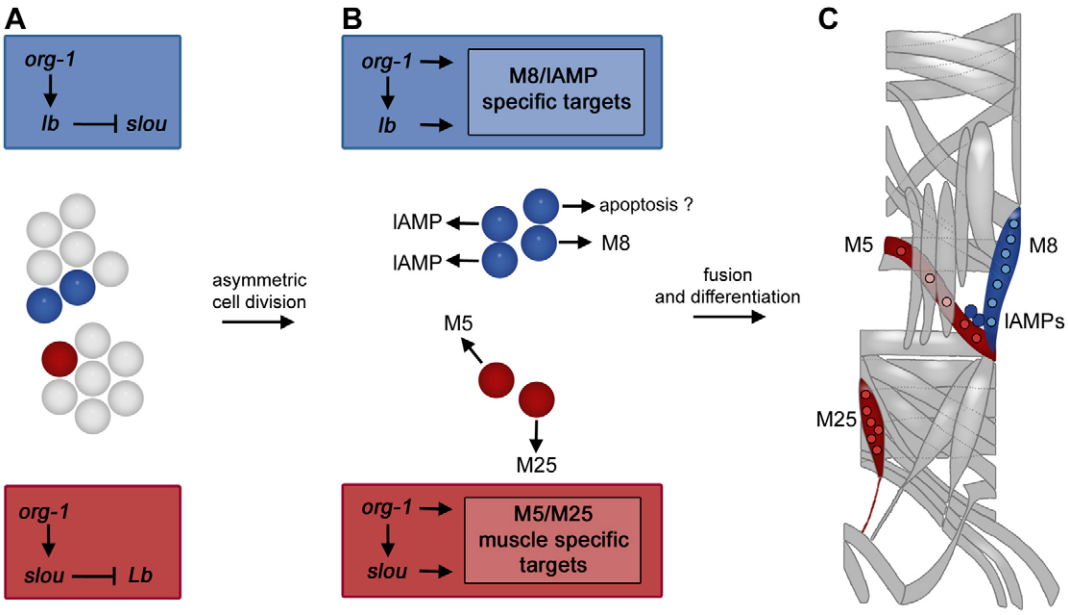

Fig. 8. *Org-1* is a direct regulator of *ladybird* and *slouch* and is required for cell fate specification and differentiation in distinct lateral muscle cell lineages. (**A**) During early development, cell-specific *org-1* expression leads to the direct activation of either *slouch* or *ladybird* in the corresponding progenitor cells in combination with mutual repression between *slouch* and *ladybird*. (**B**) After asymmetric cell division, *org-1* activity together with *slou* or *lb* expression is required for cell fate specification of muscles 5 and 25 (*slou*) as well as the Lb$^+$ cell lineages. This leads to identity-determining target activation in each founder cell and lAMP specification. (**C**) Following myoblast fusion, activation of lineage-specific expression profiles in all nuclei of the syncytia induces muscle-specific differentiation events and formation of the mature muscles.

(Pan – FlyBase; Wg signaling effector), Pointed (RTK signal effector) and Mad (Dpp signaling effector), and also showed increased evolutionary conservation (Philippakis et al., 2006). However, this enhancer had a relatively low score and, furthermore, the Tin and Twi motifs with decent matches to bona fide binding sites for these factors lie outside of our shortened version, *lbl-SBMs*. Additionally, Smad proteins are not expected to be active in this ventrolateral region. It is possible that this enhancer was picked up with this algorithm largely because of the adjacent high-scoring cardiac enhancer. Nevertheless, some of the remaining binding motifs within *lbl-SBMs*, e.g. those for dTCF and Pnt, might contribute to the enhancer activity in progenitors and founders together with the essential Org-1 binding sites. The residual activation of the *lbl-SBMs* enhancer with mutated Org-1 binding motifs in the differentiated M8 shows that the maintenance of enhancer activity at later stages depends on regulators other than Org-1, which agrees with the observation that Org-1 is no longer expressed in M8 during these stages. This maintenance might be regulated by inputs shared with two separate later-acting enhancer elements upstream of *lbe* (termed LAMPE) and *lbl* (termed LME). Both of these elements include homeodomain binding motifs that probably mediate autoregulation by Lb, in addition to ETS domain and Mef2-binding motifs, respectively, that also contribute to full activity in M8 (Figeac et al., 2010). In *org-1* mutants, the *lbl-SBM* enhancer is inactive not only early but also late, possibly because Lb protein is not available for autoregulation.

Unlike in *slou* mutants or embryos with ectopic *lb* expression, in *org-1* mutants the muscles derived from the progenitors of M5/25 and M8/lAMP are not transformed into other muscles with recognizable identities. Instead, they form syncytia with variable shapes that do not appear to be properly attached to tendon cells. This phenotype is explained by the requirement of *org-1* for the activation of both *slou* and *lb*. Whereas in *slou* mutants the progenitor of M5/M25 expresses ectopic *lb*, which transforms it into a second progenitor of M8/lAMP (Knirr et al., 1999), in *org-1* mutants de-repression of *lb* is not possible as *org-1* is also needed for the activation of *lb*. Hence, neither of the two progenitors is able to express any of these identity genes that would allow them to form identifiable muscle fibers, although they still divide into founder cells that form syncytia. Only the founder of M25 forms a syncytium that resembles M25 to some extent, perhaps because it still expresses an unknown, *org-1*-independent identity gene that can establish some of the characteristics of M25 in the absence of *slou* and *org-1* function.

Vertebrate *Tbx1* is also expressed prominently in mesodermal areas that form muscles, although in this case not in the trunk region but rather in the core mesoderm of the pharyngeal arches that form branchiomeric muscles of the head (Kelly et al., 2004; Sambasivan et al., 2009). Notably, *Tbx1* is also expressed in pharyngeal arch core mesoderm derived from anterior portions of the second heart field, which makes prominent contributions to the outflow tract and right ventricle of the heart (Huynh et al., 2007; reviewed by Vincent and Buckingham, 2010). All of these muscle and heart tissues are disrupted upon mutation of *Tbx1* in the mouse and are also affected in patients suffering from the DiGeorge (or velo-cardio-facial) syndrome, in which mutations and deletions cause haploinsufficiency of *TBX1* (reviewed by Baldini, 2004). We do not see any obvious connections between *Drosophila org-1* function and the cardiogenic roles of vertebrate *Tbx1*, as *org-1*-expressing cells do not contribute to the dorsal vessel proper and the *org-1*$^+$ alary muscles are specialized skeletal muscle fibers rather than genuine cardiac muscles. However, conceivably some of the regulatory interactions upstream or downstream of *Tbx1*/*org-1* during the development of vertebrate head muscles and

Drosophila trunk muscles have been conserved. This could, for example, be the case during tongue muscle development, in which the *lb* ortholog *Lbx1* is co-expressed with *Tbx1* (Brohmann et al., 2000; Zoupa et al., 2006; Shih et al., 2008). The identification of larger numbers of upstream regulators and targets of both *org-1* and *Tbx1* will be required in order to obtain a clearer picture of the evolutionary conservation and divergence of the developmental functions of these genes.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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