Muscle wasted: a novel component of the Drosophila histone locus body required for muscle integrity

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Summary
Skeletal muscles arise by cellular differentiation and regulated gene expression. Terminal differentiation programmes such as muscle growth, extension and attachment to the epidermis, lead to maturation of the muscles. These events require changes in chromatin organization as genes are differentially regulated. Here, we identify and characterise muscle wasted (mute), a novel component of the Drosophila histone locus body (HLB). We demonstrate that a mutation in mute leads to severe loss of muscle mass and an increase in levels of normal histone transcripts. Importantly, Drosophila Myocyte enhancer factor 2 (MeF2), a central myogenic differentiation factor, and how, an RNA binding protein required for muscle and tendon cell differentiation, are downregulated. MeF2 targets are, in turn, misregulated. Notably, the degenerating muscles in mute mutants show aberrant localisation of heterochromatin protein 1 (HP1). We further show a genetic interaction between mute and the Stem-loop binding protein (Slbp) and a loss of muscle striations in Lsm11 mutants. These data demonstrate a novel role of HLB components and histone processing factors in the maintenance of muscle integrity. We speculate that mute regulates terminal muscle differentiation possibly through heterochromatic reorganisation.

Key words: Muscle differentiation, Histone locus body, Heterochromatin

Introduction
Drosophila melanogaster provides a simple model system to study the development of the body wall muscles (analogous to vertebrate skeletal muscles). A stereotypic pattern of ~30 unique muscles in every embryonic hemisegment develop during mid-late embryogenesis, contract during late embryogenesis and in the developing larva where they are critical for motility (Bate, 1993; Broadie and Bate, 1993). Several muscle-specific genes are conserved with those of vertebrates and some principles of muscle development are similar (Abmayr et al., 2003; Baylies and Michelson, 2001; Taylor, 1998).

Early differentiation programmes, around embryonic stages 8-9, lead to the commitment of mesodermal progenitors that specify founder cells (FC), which fuse with several fusion-competent myoblasts (FCM) to form syncitial myotubes (Bate, 1990). The activation of subsequent differentiation programmes between stages 15-17, including muscle growth, extension and attachment to specialised epithelial cells lead to muscle maturation.

Drosophila Myocyte enhancer factor 2 (MeF2) appears to be central to the myogenic differentiation process and has diverse functions throughout myogenesis that include the temporal regulation of muscle genes (Elgar et al., 2008; Sandmann et al., 2006). Several studies have shown that chromatin states change in response to cellular signalling and gene activity (Baxter et al., 2004; Haff et al., 1990). Chromatin remodelling at muscle-specific loci is required to regulate muscle differentiation (Yahi et al., 2006). Histones and other chromatin remodelling complexes are components of intricate epigenetic mechanisms that organise genomes into discrete chromatin domains to regulate gene expression (Boyer et al., 2004; Quina et al., 2006). Histone levels are primarily controlled by regulating histone transcript abundance at the transcriptional and post-transcriptional levels (Berloco et al., 2001; Harris et al., 1991; Mitra et al., 2003; Stauber and Schumperli, 1988; Zhao et al., 2000). Unlike polyadenylated mRNAs, the 3’ end of metazoan histone mRNAs terminate in a stem loop (Marzluff, 1992). Several trans-acting factors (Wang et al., 2006; Martin et al., 1997; Galli et al., 1983; Mowry and Steitz, 1987) recruit a complex that triggers cleavage between the stem loop and the histone downstream element (HDE), forming the mature histone mRNA (Dominski et al., 2005; Kolev and Steitz, 2005; Wagner et al., 2007). Drosophila Stem-loop binding protein (SLBP) (Sullivan et al., 2001), U7snRNA- (Dominski et al., 2003) and the U7snRNP-specific proteins Lsm10 and Lsm11 (Azzouz and Schumperli, 2003) have been identified as factors required for histone pre-mRNA processing (Godfrey et al., 2006; Godfrey et al., 2009; Sullivan et al., 2001). U7snRNP particles have been shown to localise to the histone locus body (HLB), a distinct nuclear body that is often in close proximity to the Cajal body (CB) (Liu et al., 2006). The HLB is associated with the histone gene cluster and has been speculated to play multiple roles in assembly, modification, storage and delivery of the histone pre-mRNA processing machinery (Liu et al., 2006).

We present the isolation and characterisation of muscle wasted (mute) that appears to be a novel component of the HLB. Animals that lack mute function show the progressive loss of muscle mass and detached muscles during late embryonic development. Although early differentiation of myoblasts is not affected, terminal differentiation appears to be defective. We show that MeF2 and other terminal-muscle-differentiation genes are downregulated. In addition, levels of the predicted Drosophila homologue of atrogin-1 are increased, similar to that observed in vertebrate models of skeletal muscle atrophy (Glass, 2003; Gomes et al., 2001). Mute appears to regulate histone gene expression since levels of histone transcripts are significantly elevated and small amounts of...
misprocessed histone transcripts are also present in mute mutants. The aberrant localisation of Heterochromatin protein 1 (HP1) has led us to speculate that changes in heterochromatin may lead to genome instability and the misregulation of muscle genes. We also show genetic interactions between mute and Slhp, and the loss of striations in larval muscles of Lsm11 mutants. To our knowledge, this is the first report supporting the role of these factors in differentiated muscles.

Results

Identification and molecular cloning of mute

To identify novel genes required for myogenesis, we performed an EMS-mutagenesis screen of the second chromosome. Embryos homozygous for an allele 1281, showed a severe loss of muscle mass during late embryogenesis (Fig. 1A,B). Using deficiency mapping, we found Df(2R)Exel 6065 (breakpoints 53D14-53F8) produced the degenerative muscle phenotype when in trans with 1281. We named this locus muscle wasted (mute) and the EMS allele as mute1281. Upon sequencing, we identified a mutation in CG34415 that lies at the 53E2 locus, on the right arm of chromosome 2 (Fig. 1C). A base change from C to T was detected at position 559 of the coding sequence. CG34415 spans ~6.8 kb of the genome and is predicted to encode two isoforms that we verified by northern analysis using total RNA from staged wild-type (WT) embryos. Both isoforms, mute-L (long) and mute-S (short), are expressed from 0-5 hours after egg laying (AEL) and are not detectable at later stages of embryogenesis. mute-L migrates at ~5.5 kb and mute-S at ~3.3 kb (Fig. 1E).

The mutation in mute1281 changed amino acid Q187, producing a premature stop codon, TAA, which is predicted to produce a truncated Mute-L protein (Fig. 1C, red triangle). A P-element insertion P{GawB}NP1160, 90 bases upstream of the ATG of Mute-L also resulted in a similar degenerative muscle phenotype when present in trans with mute1281 (Fig. 1C, green triangle, data not shown). mute-L and mute-S encode predicted proteins of 1739 and 665 amino acids in length, respectively (Fig. 1D). Simple modular architecture research tool (SMART) homology searches revealed that the N-terminal region of Mute-L [amino acid (aa) 216-1088] is weakly homologous to the Atrophin-1 protein family. The C terminal region (aa 1658-1732) is weakly homologous to the SANT (Swi3, Ada2, N-CoR and TFIIB) DNA-binding domain. InterPro predicts the presence of a homeodomain-like region (aa 1657-1735) and two paired amphipathic helix (PAH) repeats (aa 1121-1198 and 1204-1279) in Mute-L, regions also common to Mute-S (Fig. 1D). Some PAH-repeat-containing proteins are known to function as components of co-repressor complexes in transcriptional silencing (Wang et al., 1990). SANT and homeodomains function in the regulation of transcription (Boyer et al., 2004; Hueber and Lohmann, 2008). Interestingly, BLAST searches revealed that the PAH repeats of Mute-L are 28% identical to that of zebrafish Ugly duckling (Udu), a nuclear protein required for somite development (Lim et al., 2009; Liu et al., 2007) and 27% identical to that of human GON4L or YARP (YY1AP-related protein 1), a nuclear protein with suggested transcriptional-regulator-like functions (supplementary material Fig. S1).

mute is required for maintenance of muscle mass and integrity

Somatic muscles were analysed from stage 14, during the peak of myogenesis, up to the end of embryogenesis at stage 17, when the muscles are fully developed. The phenotype in mute1281 homozygotes was less severe compared with mute1281/Df(2R)Exel 6065 embryos in ~10% of mutant embryos analysed (n=4/40). Hence, mute1281/Df (2R)Exel 6065 embryos were analysed in all subsequent experiments, to ensure the removal of one copy of the entire mute locus. The size and shape of the dorsal muscles DO (outlined in dashed lines in Fig. 2) and DA (outlined in solid lines), were compared. At stage 14, no significant difference in muscle morphology or mass was observed between mute1281/Df and WT embryos (Fig. 2A,B). By stage 15, some muscles appeared thinner than their WT counterparts (Fig. 2C,D). Also, some muscles were no longer detectable in mute1281/Df embryos (Fig. 2D, asterisks). Although only dorsal muscles are shown at these stages, it should be noted that most somatic muscles showed similar defects. At stage 16 many muscles appeared significantly thinner in mute1281/Df embryos compared with their WT counterparts (Fig. 2E-L, outlined). There was no bias towards any specific muscle type. Some appeared to be detached at least at one end (Fig. 2F,H,J, arrows). Owing to the reduced muscle mass, gaps between muscle subsets were larger, making the underlying visceral mesoderm more visible (Fig. 2F, v). At stage 16 some muscles could not be detected (Fig. 2F,J,
were expressed under the control of the mesodermal drivers

mute in the somatic muscles labelled with anti-MHC (red). DO and DA muscles are outlined with dashes and solid lines, respectively. (A,B) At stage 14 muscle mass in mute1281/Df is comparable to that in WT. (C,D) By stage 15 some muscles appear thinner. Gaps between muscles begin to appear (D, asterisk). (E-J) At stage 16 muscles are significantly thinner. Dorsal, lateral and ventral groups are equally affected. Some muscles appear to be missing (F,J, asterisk). The underlying visceral mesoderm is more visible because of wide gaps (F,'v*'). (K,L) Stage 17 WT and mute1281/Df somatic muscles labelled with an MHC-GFP reporter. mute1281 muscles are significantly thinner and have abnormal morphologies. Many are detached and rounded (asterisk), eventually forming blebs (L, arrow). Some are extremely thin (L, arrowhead). (M) Schematic representation of larval and embryonic somatic muscles. (N) Schematic representation of UAS-mute constructs used to rescue mute1281. Numbers indicate length (as the number of amino acids). The percentage of embryos rescued is indicated. (O-R) Dorsal muscles of stage 16 embryos labelled with anti-MHC. All constructs are expressed in mute1281/Df embryos using 24B GAL4 at 25°C. UAS-mute-L rescues the muscle wasting defect (O) to a level comparable to that observed in WT embryos (E). UAS-mute-S fails to rescue the muscle defect, as shown by the persistence of thin muscles (P, arrows). UAS-mute-LΔC is able to rescue the muscle defect (Q). UAS-muteΔC,Atro fails to rescue the muscle defect (R). Similar results were obtained with multiple independent transgenic lines. Anterior is left, dorsal is up. Vertical white bars demarcate segment boundaries. Scale bar: 20 μm.
Late differentiation is defective in mute\textsuperscript{1281}

In order to determine if the late muscle-wasting phenotype is caused by defects in early or late myogenic events, we tested stages of myogenesis from early specification to late differentiation. The overall numbers of muscle progenitors in the early mesoderm was unaffected, as shown by the expression of Mef2 (supplementary material Fig. S2A,B) and there was no significant difference in FC numbers using the enhancer trap line, rp298-lacZ (supplementary material Fig. S2C,D). The number of FCMs was similar to that of the WT, as shown by the expression of D-Titin (also known as sallimus) (supplementary material Fig. S2E,F). Also, muscle specification was unaffected as shown by the expression of Even skipped (Eve) in the muscle nuclei at stage 13 (supplementary material Fig. S2G,H). Similar results were obtained using the D01 marker, Kruppel (data not shown).

We next examined whether late specification and differentiation were affected. Even as muscles started to lose their mass at stage 16, Eve expression was sustained and there was no significant decrease in nuclear number. A small fraction (one in seven DA1 muscles) of abnormally shaped muscles showed a more drastic reduction in Eve-positive nuclei (Fig. 3A,B, arrow). Importantly, the expression of Mef2 was reduced in the nuclei of mature mute\textsuperscript{1281/Df} myotubes (Fig. 3C,D). As myogenesis proceeds, muscles extend and attach to specialised epithelial cells, the tendon cells. D-Titin labels the mature myotube and is enriched at sites of muscle attachment at late embryonic stages. D-Titin was unevenly expressed in the cytoplasm (Fig. 3E,F, arrowheads) and reduced at the myotube attachment sites in mute\textsuperscript{1281/Df} embryos (Fig. 3E,F, arrows). The reporter line, Kettin-GFP, another attachment-site marker, also showed a reduction in expression at muscle attachment sites (Fig. 3G,H, arrows). We did not observe any defects in the general organisation of the epithelium when muscles were thinner than normal (data not shown).

Thus, the specification of myoblasts and muscles is unaffected in mute\textsuperscript{1281}. The thinning muscles continue to express myotube specification markers. Hence the loss in muscle mass is due to neither defective myoblast specification nor the inability to maintain the expression of specification genes in myonuclei. Late differentiation is affected as observed by a reduction in Mef2 expression and attachment site markers concurrently with detached muscles. It is possible that these defects result in the inability of mature muscles to maintain their integrity.

mute\textsuperscript{1281} muscles do not undergo apoptosis

Previously it has been shown that apoptotic markers are upregulated in degenerating muscles (Nguyen et al., 2007). TUNEL stainings and anti-caspase 3 antibodies, apoptotic markers, labelled cells in both WT and mute\textsuperscript{1281/Df} embryos, below the plane of the myotubes. These are probably fusion-competent myoblasts, given their location (data not shown and supplementary material Fig. S3A,B, arrowheads). In addition, we also tested whether the muscle-wasting defect was rescued by removing grim, reaper (rpr) and head involution defective (hid), key regulators of apoptosis in Drosophila, using the chromosomal deficiency H99. Double mutants homozygous for mute\textsuperscript{1281} and H99 continued to show thin muscles characteristic of mute\textsuperscript{1281/Df} (supplementary material Fig. S3B,C,D). This suggests that the muscle-wasting defect is not due to apoptosis in the myotubes.

Mute is a nuclear protein

Antibodies were generated against the N- (anti-Mute-L, aa 3-164) and C-termini (anti-Mute-LS, aa 1093-1291) of the mute coding sequence and used to study Mute expression pattern in embryos and larvae. Anti-Mute-L, which recognises the N-terminus of Mute-L, detects a nuclear protein that is expressed as a prominent nuclear focus in all cells of the embryo. A single nuclear focus was clearly observed in WT somatic muscles (Fig. 4A, arrow), which was undetectable in mute\textsuperscript{1281/Df} embryos (Fig. 4E, arrow). A similar pattern was detected using anti-Mute-LS antibodies, which recognise the C-terminus that is common to both Mute-L and Mute-S (Fig. 4B, arrow). Expression levels were drastically reduced in mute\textsuperscript{1281/Df} embryos (Fig. 4E, arrow). It is probable that this weak expression is that of Mute-S, which is also nuclear but is insufficient for normal function. The anti-Mute-L and anti-Mute-LS-positive foci overlapped in both muscle (Fig. 4C, arrow) and non muscle (Fig. 4C, arrowhead) nuclei. Protein expression in the mutant was restored by rescue with UAS-mute-L expressed under 24B-GAL4, which drives expression in muscle and tendon cells (Fig. 4G, arrows indicate muscle, arrowhead indicates tendon cell). The diffuse nuclear stain is probably due to overexpression of the protein.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mute1281_embryos.pdf}
\caption{Late muscle differentiation is defective in mute\textsuperscript{1281} embryos. (A,C,E,G) WT and (B,D,F,H) mute\textsuperscript{1281/Df} late stage 16 embryos. Dorsal muscles labelled with anti-MHC (red; A-D, G-H) or D-Titin (red; E,F). (A,B) Mature DA1 myotubes continue to express Eve (outlined). Occasionally abnormally shaped muscles show a reduction in Eve-positive nuclei (B, arrow). (C,D) Mef2 (green) expression is reduced in mute\textsuperscript{1281/Df} (D) compared with WT (C) embryos. (E,F) The enrichment of D-Titin at attachment sites is uneven (E, F, arrowheads). (G,H) Kettin-GFP (green) at the attachment sites is markedly reduced in mute\textsuperscript{1281} embryos (G,H). Scale bar: 20\,\mu m.}
\end{figure}
To assess whether Mute associates with DNA, we tested its localisation in the larger salivary gland nuclei of WT third instar larvae. We found that Mute was expressed at a single locus associated with the large polytene chromosomes (Fig. 4D, arrowhead). Thus, the antibodies generated against Mute label a specific nuclear organelle or body and Mute appears to associate with a specific chromosomal locus.

Mute is a novel component of the histone locus body

The nuclear expression of Mute was reminiscent of the localisation patterns of components of two nuclear bodies, the Cajal body (CB) and the histone locus body (HLB). In order to determine whether Mute was a component of either of these nuclear bodies, we investigated whether Mute colocalised with markers for these nuclear structures, such as Lsm10 and Lsm11, components of the HLB (Liu et al., 2006). We found that Mute colocalised with both Lsm10 and Lsm11 in the somatic muscles (Fig. 4I-K and 4N-P, arrows) as well as other cells of the embryo (not shown). Colocalisation was also apparent in salivary gland nuclei (Fig. 4L,Q, arrows). The localisation of Lsm10 and Lsm11 foci are present in mute^{+1281/Df} somatic muscles (arrows) (H) HeLa cells labelled with anti-FLASH (red), anti-Mute-LS (green) and Hoechst (blue). Mute colocalises with FLASH (arrows). Scale bars: 10 μm.

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HeLa cells that colocalised with FLASH (FLICE-associated huge protein; Fig. 4H, arrows). FLASH-positive nuclear bodies have been suggested to be similar to the *Drosophila* HLB and have been associated with histone transcription (Barcaroli et al., 2006; Bongiorno-Borbone et al., 2008). These results suggest that the epitope recognised by anti-Mute-LS might be conserved, and a structural or functional homologue of Mute might exist in vertebrates.

**mute regulates histone mRNA transcription**

It has previously been shown that mutations in components of the histone pre-mRNA processing machinery results in the production of aberrantly long mRNAs with polyadenylated tails because of the presence of cryptic polyadenylation signals downstream of the cleavage site in the histone gene (Godfrey et al., 2006; Godfrey et al., 2009; Lanzotti et al., 2002). Since *U720* and *Slbp15* mutants have previously been shown to accumulate high levels of misprocessed histone transcripts (Godfrey et al., 2006; Sullivan et al., 2001), we examined the relative changes in polyadenylated histone transcripts in *mute1281* versus *Slbp15* and *U720*. Although we found higher levels of poly(A) histone H3 (7.14±2.9 fold) and histone H4 (7.41±1.77 fold) mRNAs in *mute1281/Df* embryos at early stage 17 by quantitative RT-PCR (qPCR), the levels of misprocessed histone H3 and H4 transcripts were ~5 to 6-fold less than that observed in stage 17 *Slbp15/15* embryos and *U720/20* third instar larvae compared with stage matched WT controls (Fig. 5A). Interestingly, northern analysis showed higher levels of normal H3 and H4 transcripts in *mute1281/Df* embryos unlike *Slbp15* and *U720* mutants, which had high levels of mostly misprocessed transcripts (Fig. 5D), as has been reported previously (Godfrey et al., 2006; Sullivan et al., 2001). Similarly, qPCR using histone-specific primers for the reverse transcription, also showed significantly higher total levels of H3 and H4 transcripts, in *mute1281/Df* embryos [compare Fig. 5A poly(A) fraction to 5C-total transcript].

**Fig. 5. Histone pre-mRNAs are misprocessed and HP1 localisation is aberrant in **mute**1281**.

(A) Quantitative RT-PCR (qPCR) using oligo(dT) on total RNA extracted from early stage 17 embryos. All values on the y-axis are normalized against the ribosomal gene *rp49*. H3 and H4 poly(A) transcripts were higher by 7.14±2.9, 37.73±4.28, 41.51±5.65 and 7.41±1.77, 17.24±0.43, 18.91±2.4 fold in *mute1281/Df*, *Slbp15/15* and *U720* mutants, respectively (Student’s t-test *P*<0.05). Error bars show standard deviation from three biological replicates. (B) Western blot of acid-extracted histones and heterochromatin protein 1 (HP1) from early stage 17 embryos. 5 μg protein was loaded per lane. Levels of Ac H3K9 were lower (arrow) in *mute1281/Df* compared with WT embryos. (C) qPCR using histone-specific primers shows more total H3 and H4 transcripts in *mute1281/Df* compared with WT embryos, 5 μg protein was loaded per lane. Levels of Ac H3K9 were lower (arrow) in *mute1281/Df* compared with WT embryos. (D) Northern analysis on 1 μg total RNA shows the presence of misprocessed H3 and H4 in *Slbp15/15* and *U720* mutants and more total H3 and H4 transcripts in *mute1281/Df* embryos. (E-L) Dorsal muscles (outlined in H and L) of late stage 16 embryos labelled with Hoechst (blue), anti-HP1 (red) and anti-MHC (green). HP1 encircles the heterochromatin in WT muscles (F, arrow and inset). The distribution of HP1 is more punctate than circular in *mute1281/Df* myonuclei (J, arrow and inset). In some cases the area encircled by HP1 is smaller (J, arrowhead). Scale bar: 10 μm.
To test whether levels of histone proteins were affected, histones extracted from early stage 17 embryos were analysed by western blotting. Surprisingly, we were unable to detect any differences in the overall levels of histones H3 and H4 (Fig. 5B). The modification of histone tails is known to affect higher order chromatin structure by affecting the stability and packaging of chromatin and also the transcriptional status of genes. During muscle differentiation there is large scale reorganisation of heterochromatin (Agarwal et al., 2007). Defects in establishing and maintaining heterochromatin has deleterious effects, often leading to disease (Delcude et al., 2009; Zhang and Adams, 2007). We, therefore, tested the levels of modified histones H3 and H4. We found that di- and trimethyl-H3-(Lys)9 (di/tri-met H3K9) levels were unaffected in mute1281/Df embryos. Levels of acetyl-H3-(Lys)9 (Ac H3K9) were slightly lower but we were unable to detect any muscle-specific differences by immunofluorescence (Fig. 5B, arrow and data not shown). The histone variant H2Av has been shown to regulate the formation of heterochromatin and to also function in the silencing of euchromatic genes (Swaminathan et al., 2005). The levels of H2Av were unaffected (Fig. 5B).

In order to test for other detectable changes in the heterochromatin of myonuclei, we studied the localisation of the non-histone protein, Heterochromatin protein 1 (HP1). HP1 functions as a gene repressor and localises to heterochromatic regions via interaction with methylated H3K9 and has recently been shown to localise to transcriptionally active regions as well (Ayyanathan et al., 2003). We found that in WT muscles HP1 appeared to surround the heterochromatin and was present as a ring (Fig. 5E-H, arrows and inset). Interestingly this pattern was reduced to a punctate distribution in many of the thin muscles in mute1281/Df embryos (Fig. 5I-L, arrows and inset). Some muscles did show the circular HP1 stain but the area encircled appeared to be much smaller compared with WT (Fig. 5FJ, arrowhead). The overall levels of HP1 were unaffected in mute1281/Df embryos (Fig. 5B).

Thus, mute appears to regulate histone gene expression, possibly by functioning as a transcriptional repressor. The higher amounts of normal histone transcripts in mute1281 does not affect the overall levels of histone proteins. The localization of HP1 is affected in mute1281, which suggests that mute may play a role in the organisation of heterochromatin. It is possible that by some, as yet unknown, mechanism, or some other function of mute the stability of chromatin is affected in terminally differentiated muscle cells.

**Muscle genes are misregulated in mute1281 embryos**

The loss in muscle mass and change in HP1 localisation observed in mute1281 embryos might be concurrent with the misregulation of muscle-specific genes. In order to test this, muscle genes expressed late in embryonic development (i.e. early stage 17) were chosen for analysis by qPCR, in both WT and mute1281/Df embryos. This data is summarised in Table S1 in supplementary material. We quantified the levels of some Mef2 targets and found that the levels of Myosin alkali light chain 1 (Mlc1), Myosin alkali light chain 2 (Mlc2), Muscle LIM protein 60A (Mlp60A) and Mhc were lower in the mutant (Fig. 6A; P<0.1). Concurrently, we also observed that levels of MHC protein were lower. This could be attributed to the reduced muscle mass in the mutant (Fig. 6B, arrow; quantified in Fig. 6C). There was no significant difference in the transcript levels of actin57B (act57B; Fig. 6A), CG6972, was significantly upregulated by approximately threefold compared with WT (Fig. 6A; P<0.1). CG6972, an uncharacterised gene, has been proposed to be positively regulated by Mef2 and is expressed specifically in the somatic muscles (Elgar et al., 2008; Sandmann et al., 2006) but has also been shown to be upregulated in response to hypertrophy-induced muscular degeneration (Montana and Littleton, 2006). It is possible that the higher levels of CG6972 observed in mute1281 is due to the muscle-wasting defect.

We also examined the expression levels of the RNA binding protein How that is expressed in both somatic muscles and tendon cells and is required during terminal differentiation, when myotubes begin to attach to their epidermal attachment sites (Baehrke, 1997). How transcript levels were ~20% lower in mute1281/Df compared with WT embryos (Fig. 6A; 0.79±0.05 fold; P<0.05). The how locus encodes two isoforms, the long isoform [how(L)], which is expressed throughout embryonic development, and the short [how(S)], which is expressed only at later stages of embryogenesis (Nabel-Rosen et al., 1999). We further tested the
protein levels of How. The levels of How(L) were comparable to that observed in WT but the levels of How(S) were significantly lower in $mute^{1281/Dy}$ embryos (Fig. 6B, arrowhead; quantified in C). how has been shown to regulate muscle activity as well as tendon cell differentiation. It is known that levels of How(S) are upregulated in tendon cells during terminal differentiation (Nabel-Rosen et al., 1999). It is possible that in $mute^{1281}$, How(S) expression in both muscles and tendon cells is affected.

Interestingly, we also found that levels of CG11658, the predicted fly homologue of vertebrate atrogin-1, an F-box protein and proposed ubiquitinylation enzyme, were ~30% higher (Fig. 6A, $P=0.17$). cDNA microarrays have previously shown that $atrogin-1$ levels are upregulated specifically in skeletal muscles when they undergo atrophy (Gomes et al., 2001). CG11658 is 26% identical to mouse $atrogin-1$ which in turn is 96% identical to human Atrogin-1 (Gomes et al., 2001).

These results show that the expression of a subset of important muscle-specific genes are affected in $mute^{1281}$. We speculate that even minor changes in gene expression might have cumulative effects on muscle differentiation. Misregulation of these and other untested genes, might be due to changes in chromatin organisation.

**Other processing mutants also display defective muscles**

Mutations in Slbp, U7 snRNA, Lsm10 and Lsm11 have been shown to affect the cell cycle and oogenesis in Drosophila (Godfrey et al., 2006; Godfrey et al., 2009; Sullivan et al., 2001). Thus far, none of these genes have been investigated for a possible role in muscle morphology. We analysed the somatic muscles of $Slbp^{15}$ mutant embryos at late stage 16 and $Lsm11^{c02047}$ mutant third instar larvae. Homozygous $Slbp^{15}$ embryos do not display any defects in their musculature (Fig. 7A), but interestingly, in the absence of one copy of $mute^{1281}$, $Slbp^{15/15}$ over half of this genotype ($n=9/15$) had somatic muscles that appeared significantly thinner than those of WT (compare Fig. 7A, dorsal muscles outlined).

Some muscles were detached (Fig. 7B, arrow) while others appeared to be missing (Fig. 7B, asterisk), similar to the $mute^{1281}$ phenotype. Thus, $mute$ appears to genetically interact with $Slbp$ to affect the somatic muscles.

There was no observable defect in the somatic muscles of $Lsm11^{c02047}$ homozygous mutant embryos. Hence we analysed their somatic muscles at the third instar larval stage since they die as non pharate pupae (Godfrey et al., 2009). There was a loss in muscle striations in 66.66% ($n=4/6$) of the $Lsm11^{c02047}$ homozygous mutant larvae compared with the regularly spaced striations in WT larval muscles (Fig. 7C,D), although the number of muscles affected and the extent of the area affected within a muscle were highly variable.

Thus, other processing factors and components of the HLB appear to regulate the terminal differentiation and integrity of myotubes to various extents. The observed lethality in these mutants might be due to defects in tissues that have not yet been analysed.

**Discussion**

Through a genetic screen for regulators of muscle development we have identified $mute$, the function of which appears to be required for the maintenance of muscle mass and integrity. We have attempted to characterize the muscle phenotype when the $mute$ locus is disrupted. Although early myogenic events are unaffected, terminal differentiation of muscle and/or tendon cell is affected. Terminal differentiation of muscles involves the expression of structural proteins and the attachment of muscles to tendon cells, through the coordinated action of several embryonic genes, including $Mef2$, resulting in a functional contractile larval muscle. Reciprocal signalling between the mesoderm and the overlying ectoderm is essential, as muscle growth cones seek out their proper attachment sites (Bate, 1992). This leads to tendon cell differentiation and successful myotube attachment (Becker et al., 1997). Vein protein secreted by the muscles upregulates the tendon cell differentiation factor How(S), through the EGF signalling pathway (Nabel-Rosen et al., 1999). We have shown that levels of How(S) is significantly reduced in $mute^{1281}$ cells. It is possible that How is affected in both muscle and tendon cells or the muscles do not secrete sufficient Vein. This could have drastic consequences, as has been shown in embryonic lethal $mute$ mutants that have defects in muscle migration and attachment (Baehrecke, 1997). Also, Kettin and D-Titin are significantly reduced at attachment sites, signifying defects in muscle and/or tendon cells in $mute^{1281}$ embryos. Kettin and D-Titin are essential in organising myofilaments into highly ordered arrays and they also interact with several factors at the attachment sites. $D-titin$ mutants lack visible striations, the muscles are thin and F-actin is disorganised (Zhang et al., 2000). Earlier defects in $mute^{1281}$ embryos could not be investigated because of a possible maternal contribution of $mute$ and the absence of egg laying in germline clones. It is possible that $mute$ also functions in aspects of oogenesis similar to what has been shown for other components of the histone pre-mRNA processing machinery, $Slbp$ and U7 (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001).

The mechanism of muscle degeneration in $mute^{1281}$ mutants is unknown. It has been suggested that the gross loss of muscle mass during vertebrate skeletal muscle atrophy is largely mediated by the ubiquitin-proteasome system or autophagy-lysosome pathway (Schwartz, 2008). $atrogin-1$, a ubiquitin ligase has been shown to be upregulated in several models of skeletal muscle atrophy (Glass, 2003; Gomes et al., 2001). CG11658 has
been proposed by sequence homology to be the Drosophila Atrogin-1 homologue (Gomes et al., 2001). It is interesting that we observe an upregulation of CG11658 transcripts in mute1281 embryos and it is thus tempting to speculate that the ubiquitin proteasome pathway might be activated in the atrophying muscles of mute1281.

We have shown that Mute colocalises with Lsm10 and Lsm11, two components of the Drosophila HLB, but does not appear to be required for their localization. The presence of misprocessed histone transcripts in mute mutants is unlikely to be the cause of the late embryonic lethality and muscle degeneration since Slbp13 and U720 mutants have been shown to have an approximate five- to six-fold higher level of misprocessed histone transcripts compared with mute1281, yet Slbp13 mutants show visible muscle defects only when in combination with mute1281 and U7 mutants are viable though sterile (Godfrey et al., 2006). Interestingly, we find an overall higher level of normal histone transcripts in mute mutants, an observation that has not been reported for the other characterised histone-processing mutants such as Slbp, U7, Lsm10 and Lsm11. Mute might function in a manner similar to the abnormal oocyte (abo) gene product, which localises to the histone gene cluster and is a negative regulator of histone gene expression (Berloco et al., 2001). These could lead to tissue-specific changes in histone protein levels, which are difficult to detect. Given the proximity of the HLB to the histone gene cluster it is possible that transcriptional and post-transcriptional activities are closely associated and that the HLB serves as an integrated machinery for histone gene expression. These and possibly other undiscovered functions of mute could be responsible for the observed embryonic lethality and muscle defects.

Mute is predicted to have PAH repeats, a SANT-DNA-binding domain and an Atrophin-1-like region which have been implicated in the association with, and the function of, chromatin remodelling complexes and the control of gene expression (Boyer et al., 2002; Boyer et al., 2004; Heinz et al., 1997; Nagy et al., 1997), although we have shown that the predicted SANT-DNA-binding domain is not required for maintenance of muscle integrity. The modification of histone tails has been implicated in muscle differentiation (McKinsey et al., 2002; Yahil et al., 2006) and deacetylation of histones has been implicated in gene silencing (Park et al., 1999). The defects in mute1281 mutants are observed during the late stages of development when most cells, including myoblasts, have exited the cell cycle. Muscles form a unique tissue in that they are formed by the fusion of differentiated myoblasts. Upon fusion, FCM nuclei are entrained by the FC nuclei to express FC specific genes (Baylies et al., 1998; Beckett and Baylies, 2006). Hence nuclear re-programming is critical in the formation of the mature multinucleated muscle. Differentiation and fusion of C2C12 myocytes is accompanied by remarkable reorganisation of constitutive heterochromatin domains (Shen et al., 2003; Terranova et al., 2005). Non-histone proteins such as HP1 also function in cell differentiation by associating with transcriptional repressors thus providing a mechanism for widespread silencing of gene expression (Lechner et al., 2000; McKinsey et al., 2000). We have shown that the presentation of HP1 is aberrant in the thinner muscles of mute1281 embryos. Most studies have focused on the role of HP1 in the organisation of heterochromatin, but more recently it has been implicated in the active transcription of euchromatic cell cycle genes (De Lucia et al., 2005). Interestingly it has also been shown that the association of HP1 gamma increases vertebrate myoblasts differentiate to form myotubes, possibly to stabilise transcriptional repression during differentiation (Agarwal et al., 2007).

Several genetic studies have highlighted the regulatory role of Mef2 throughout muscle development. Subtle changes in Mef2 transcripts result in stringent regulation of muscle genes (Elgar et al., 2008; Sandmann et al., 2006). We have shown that Mef2 transcript and protein is downregulated in mute1281 embryos. Mute might regulate the expression of genes such as Mef2 through its action on heterochromatin, perhaps on Mef2 regulatory regions. It is possible that a large array of untested muscle and tendon genes are mis-regulated. We speculate that even minor changes in the levels of these transcripts could lead to sufficient misregulation of muscle and tendon proteins (e.g. How), resulting in cumulative deleterious effects on processes of terminal muscle differentiation in the embryo, as we have shown here in the case of muscle attachment.

To our knowledge, this is the first report implicating a histone gene regulatory factor in the maintenance of muscle integrity. Perhaps the identification of mute can provide some insight into the role of the HLB in differentiated tissue. It could also be used to investigate the regulatory machinery of histone expression by studying mute-interacting molecules. It may well be that mute has other gene regulatory functions possibly through the organisation of heterochromatin, that might also contribute to the severe muscle degeneration. Interestingly we also find a Mute-positive nuclear body in HeLa cells that colocalises with FLASH, suggesting evolutionary conservation. Mutations in survival motor neuron (smn), a CB marker, and a factor implicated in snRNP biogenesis (Fischer et al., 1997; Liu et al., 1997) leads to spinal muscular atrophy in humans. It is possible that factors involved in the assembly of processing factors or more directly in histone transcription, might have functions in tissues such as muscle that undergo extensive chromatin reorganisation because of cell fusion.

**Materials and Methods**

**Plasmids and cloning**

pUAST-Mute-S was generated from the cDNA clone RE27864 (Drosophila Genomic Resource Centre). pUAST-Mute-L was generated by ligating a 2.7 kb fragment from RE27864 to a 3.7 kb genomic DNA fragment. pUAST-Mute-L and pUAST-Mute-ΔC were generated by introducing a stop codon at position 5038 and 2356 of the coding sequence, respectively.

**Drosophila strains**

All crosses were maintained at 25°C. Stocks used were: yw, MHC-tau-GFP (E. Chen and E. Olson), P(GawB)NP1160 (Bloomington), Df H99 (Bloomington), Kettin-GFP (Mar Ruiz-Gomez), U720, Slbp13, Lsm119202w (Godfrey et al., 2006; Godfrey et al., 2009; Sullivan et al., 2001), rp298-lacZ (A. Nose), rp298-Ga14 and 2B-Gal4 (Zeffran et al., 1997), Df(2R)Exel 6065 (Bloomington). Homozygous mutants were identified by the absence of β-galactosidase or GFP. Transgenic flies were generated as described previously (Spradling, 1986). Rescue tests were performed using single copies of the UAS transgene and 2B-Gal4 or rp298-Gal4 in mute1281 mutant embryos at 25°C. Results are representative of two independent insertions for each transgene.

**Genetic screen**

Wild-type males were mutagenised with EMS. ~4000 stocks carrying the mutagenised chromosome over a Cy-Actin-GFP balancer were established. F2 embryos of ~2000 embryonic lethal stocks were analysed for muscle defects using anti-MHC.

**Antibody generation**

Regions between aa 3-164 and 1093-1291 were used to generate the cDNA clone RE27864. Anti-HLA-DR antibody was generated using single copies of the UAS transgene and 2B-Gal4 or rp298-Gal4 in mute1281 mutant embryos at 25°C. Results are representative of two independent insertions for each transgene.
rabbit anti-caspase 3 (Cell Signaling Technology) rabbit anti-Mef2 (Bruce Patterson, guinea pig anti-MEF2C, thin, mouse anti-Lamin (Abcam), mouse anti-Lamin (DSHB), rabbit anti-Lsn10 and anti-Lsn11 (J. Gall), mouse anti-HPI (DSHB), mouse anti-Dlg (DSHB) and Hoechst 3342 (Invitrogen). Secondary antibodies were conjugated to Cy3 (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488 (Molecular Probes). Samples were analysed under a Zeiss LSM 5 Exciter confocal microscope.

Immunostaining of HeLa cells

HeLa cells were seeded onto polylysine-coated coverslips (Iwaki) and fixed in 3% paraformaldehyde. Primary antibodies used were: guinea pig anti-MUe-L5, rabbit anti-HPI (Santa Cruz Biotechnology, Inc.). Secondary antibodies and imaging as above.

Northern blotting

Total RNA was run on a formaldehyde-agarose gel. Northern blotting was performed as per the Roche-DiAG protocol. Probe was identical to the anti-Mu-e-L5 antigenic region, H3 and H4 coding regions.

Embryo collections

Two-hour embryo collections of yw and mused1281/H11032 were aged together at 25°C up to early embryonic stage 17. Homozygous mutant embryos were selected on the basis of the absence of Actin-GFP. 200 embryos of each genotype were used for acid extraction of histones. 200 embryos of each genotype were used for acid extraction of histones. pPCR and total protein extraction.

Acid extraction of histones

Histones were extracted in lysis buffer (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 15 mM PIPES pH 6.8, 0.8% Triton X-100, 0.1 mM Pefabloc and protease inhibitor cocktail (Roche). Homogenate was spun at 10,000 g for 20 minutes. Samples were incubated for 1 hour in 0.4 M H2SO4 and spun at 12,000 g for 5 minutes. Proteins were precipitated in acetone at −20°C. Samples were spun at 10,000 g for 15 minutes. Pellet was resuspended in 4 M urea. Total protein was quantified. 5 μg protein was loaded per well.

Western blotting

Samples were run on an SDS-PAGE gel and transferred onto a PVDF membrane (Immobilon-P, Millipore). Primary antibodies used were: mouse anti-MHC, rabbit anti-Actin (T. Volk), mouse anti-Tubulin (Sigma), rabbit anti-H3 (Santa Cruz Biotechnology), rabbit anti-H4 (Abcam), rabbit anti-actin-trim H3K9 (Abcam), rabbit anti-dimet H3K9 and rabbit anti-Ac H3K9 (Millipore), mouse anti-HPI (DSHB), rabbit anti-H2A (Robert Glaser). Secondary antibodies were anti-mouse or anti-rabbit HRP (Roche). Proteins were detected using Lumino, coomassie acid (Sigma) and Amersham Hyperfilm ECL.

Quantitative RT-PCR

Total RNA was extracted using the TriFast method. genomic DNA was removed with DNase I. Equal amounts of RNA were used for cDNA synthesis. first strand cDNA was synthesized using oligo(dT) or H3/H4-specific primers and SuperScript RT-III (Invitrogen). Samples were treated with RNase-H and used for qPCR. SYBR Green was used. Total RNA was run on a formaldehyde-agarose gel. Northern blotting was performed as per the Roche-DiAG protocol. Probe was identical to the anti-Mu-e-L5 antigenic region, H3 and H4 coding regions.

References


**mute** is required for muscle integrity


Table S1. Muscle genes are misregulated in *mute* embryos. Relative expression level compared to WT is indicated. All values are normalized against the ribosomal protein 49 (*rp49*).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change ± SE</th>
<th>Students t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dmef2</td>
<td>0.79 ± 0.08</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>how</td>
<td>0.79 ± 0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>CG6972</td>
<td>3.82 ± 0.28</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>mhc</td>
<td>0.78 ± 0.16</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>act57B</td>
<td>0.92 ± 0.02</td>
<td>P=0.2</td>
</tr>
<tr>
<td>mlp60A</td>
<td>0.67 ± 0.04</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>mlc1</td>
<td>0.78 ± 0.01</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>mlc2</td>
<td>0.75 ± 0.02</td>
<td>P&lt;0.1</td>
</tr>
<tr>
<td>CG11658</td>
<td>1.32 ± 0.15</td>
<td>P=0.17</td>
</tr>
</tbody>
</table>