Development 135, 849-857 (2008) doi:10.1242/dev.015529

Mind bomb 2, a founder myoblast-specific protein, regulates myoblast fusion and muscle stability

Marta Carrasco-Rando and Mar Ruiz-Gómez*

A fundamental step during *Drosophila* myogenesis is the specification of founder myoblasts (FMs). Founders possess the information required for the acquisition of muscle identity and for the execution of the myogenic programme, whereas fusion-competent myoblasts (FCMs) acquire this information after fusing to founders. Very little is known about genes that implement the execution of the myogenic programme. Here we characterise Mind bomb 2 (Mib2), a protein with putative E3 ubiquitin ligase activity that is exclusive of FMs and necessary for at least two distinct steps of the founder/myotube differentiation programme. Thus, in *mib2* mutants, the early process of myoblast fusion is compromised, as FMs undergo a reduced number of rounds of fusion with FCMs. At later stages, with the onset of muscle contraction, many muscles degenerate, display aberrant sarcomeric structure and detach from tendons. The fusion process requires intact E3-RING-finger domains of Mib2 (the putative catalytic sites), probably to eliminate the FCM-specific activator Lmd from nascent myotubes. However, these sites appear dispensable for muscle integrity. This, and the subcellular accumulation of Mib2 in Z and M bands of sarcomeres, plus its physical interaction with nonmuscle myosin (a Z-band-localised protein necessary for the formation of myofibrils), suggest a structural role for Mib2 in maintaining sarcomeric stability. We suggest that Mib2 acts sequentially in myoblast fusion and sarcomeric stability by two separable processes involving distinct functions of Mib2.

KEY WORDS: *mind bomb 2*, *Drosophila*, Founder myoblasts, Myoblast fusion, Myofibrillogenesis

INTRODUCTION

The functional unit of the muscular system is the skeletal muscle fibre, or myotube. In most animals myotubes are syncytial, originated by the fusion of myoblasts. In the *Drosophila* embryo, muscles arise from progenitors that are selected from a group of competent mesodermal cells by a mechanism of lateral inhibition mediated by the Notch pathway. Progenitors divide asymmetrically to produce two distinct founder myoblasts (FMs) or an FM and an adult muscle precursor (Carmena et al., 1998; Ruiz-Gómez and Bate, 1997), whereas the unselected mesodermal cells become fusion-competent myoblasts (FCMs) (Bate, 1993; Baylies et al., 1998). The activation of *lame duck* (*lmd*) in FCMs initiates the expression of genes exclusive to the FCM population, such as *sticks and stones* (*sns*), and maintains the expression of the myogenic differentiation gene *Drosophila Myocyte enhancing factor 2* (*Mef2*) (Duan et al., 2001; Ruiz-Gómez et al., 2002).

Individual muscle identity is defined very early during myogenesis in progenitors by specific combinations of transcriptional factors and other proteins that are inherited by FMs (Baylies et al., 1998). Thus, as FCMs fuse to FMs to generate myotubes, FCMs are reprogrammed to the exclusive genetic programme of the FM, which defines the characteristics of the final muscle, including size, choice of tendon sites and distinctive pattern of innervation. Under mutant conditions that block fusion, FMs are the sole myoblasts able to complete myogenesis, giving rise to mononucleated fibres that, otherwise, exhibit the same properties as the wild-type muscles. By contrast, FCMs initiate the expression of differentiation genes, such as *Mef2* and *Myosin heavy chain* (*Mhc*), but they fail to exhibit contractile capability, are unable to contact tendon cells or to be recognised as targets for innervation. FCMs die before the end of embryogenesis without completing the muscle terminal differentiation programme (Rushton et al., 1995).

All FMs share the expression of a set of genes that confer to them the general properties of this population. For example, *dumbfounded* (*duf*; also known as *kin of irre* – FlyBase), a member of the immunoglobulin superfamily, enables FMs to attract FCMs to their vicinity and thus nucleate the fusion process (Ruiz-Gómez et al., 2000; Strunkelnberg et al., 2001). The cytoplasmic protein Rolling pebbles (Rols), which is recruited to the membrane by interaction with Duf, acts as an adaptor between events taking place at the membrane during the fusion process and the cytoskeleton, through its interaction with two other proteins, Myoblast city (Mbc) and Sallimus (Sls; also known as D-titin) (Chen and Olson, 2001; Menon and Chia, 2001). Similarly, all founders express the α2 position-specific Integrin subunit PS2, which is required to maintain the integrity of apodemes and stabilise the binding of muscles to tendon cells via the extracellular matrix (Brown, 1994). However, the genes that control other properties of FMs, such as their unique ability to complete myogenesis in the absence of fusion, are unknown. For instance, although all myoblasts express genes encoding myofibrillar proteins [i.e. Myosin, Kettin (also known as Sallimus – FlyBase), Tropomyosin], only FMs are capable to build functional sarcomeres.

To identify additional genes conferring FM-specific characteristics, we searched for genes with expression restricted to the founder population. Here we describe *mind bomb 2* (*mib2*), a gene that encodes a founder-specific modular protein containing two RING-finger domains with putative E3 ubiquitin ligase activity. Mib2 performs separable functions during myogenesis. Thus, Mib2 is necessary to complete myoblast fusion, a function that requires the E3-RING-finger domain. Mib2 is also required to maintain muscle integrity, as its absence leads to loss of sarcomeric structure, in both larval and adult muscles, and muscle detachment from tendons.

Centro de Biología Molecular Severo Ochoa, CSIC and UAM, Universidad Autónoma de Madrid, 28049, Madrid, Spain.

*Author for correspondence (e-mail: mruiz@cbm.uam.es)

Accepted 17 December 2007

MATERIALS AND METHODS

Drosophila stocks

The following stocks were used: *Df(2L)Exel8039*, *l(2)37Be*^{1}, *CG17492*^{KG10508} (Bloomington Stock Center), *Mhc–τ-GFP*, *kettin-GFP*, *UAS-Dmib* (Le Borgne et al., 2005), *rP298*, *24B-GAL4*, *Mef2-GAL4*, *1151-GAL4* (Dutta et al., 2004), *duf-GAL4* and *sns-GAL4* (Stute et al., 2006). *Df(2L)mib2*^{r14} was obtained as an imprecise excision of the *P* element in *CG17492*^{KG10508} (Hamilton and Zinn, 1994). *UAS-SKE* and *UAS-mib2* were obtained after insertion of human *SKE* cDNA (Takeuchi et al., 2005) and a synthetic *mib2* cDNA, respectively (details will be provided under request), into pUAST and transformation into *yw* embryos. To generate the *UAS-RNAi-mib2* construct a fragment of 503 bp obtained by PCR on *mib2* cDNA using primers 5′-CAACGATGCCAACAAAGTGC and 5′-GCAGA-GCTGAATCACCTTCC was used to make intron-spliced hairpin RNA according to Nagel et al. (Nagel et al., 2002). *UAS-mib2-C935S-C1020S* was generated by replacement of cysteines C935 and C1020 for serines (performed according to a modification of the Quick Change Mutagenesis method, Stratagene). Mutants were identified by the absence of embryonic *lacZ* expression or GFP fluorescence.

Antibody production

A bacterial 6×His-tagged protein containing amino acids 494 to 795 of Mib2 was obtained by cloning into the expression vector pQE-31 (Qiagen), following the recommended protocol for purification under denaturing conditions using Ni-NTA Agarose columns. The 6×His-tagged Mib2 protein was used to inoculate guinea pigs following standard protocols.

In situ hybridisation and immunohistochemistry

Whole-mount in situ hybridisation with digoxigenin-labelled EST LD36078 RNA probe and immunocytochemistry were performed as described previously (San-Martín et al., 2001). The following primary antibodies were used: anti-muscle Myosin (Kiehart and Feghali, 1986), anti-Eve, anti-Runt (Kosman et al., 1998), anti-Rols (Menon and Chia, 2001), anti-Kz (Machado and Andrew, 2000), anti-Connectin (Meadows et al., 1994), anti-Lmd (Duan et al., 2001), anti-Tm (MAC141, Babraham Tech), anti-cleaved Caspase 3 (also known as Decay – FlyBase) (Cell signaling Tech), anti-GFP (Molecular Probes) and anti-β-galactosidase (Cappel).

Electron and confocal microscopic analysis

Electron microscopic analyses were carried out according to Beall and Fyrberg, 1991 (Beall and Fyrberg, 1991). Samples were observed in a Jem1010 (JEOL) instrument working at 80 kV. Fluorescent preparations were scanned using confocal microscopes MicroRadiance (BioRad) and LSM510 META (Zeiss) and images were analysed using the software Zeiss LSM Image or LaserSharp and Adobe Photoshop 7.0. In most cases the images correspond to *z*-projections of series of confocal sections.

Protein blot and co-immunoprecipitation

Protein extracts from adult thoraces and co-immunoprecipitations with anti-Mib2 and anti-GFP (using 300-500 μg of protein) were performed following standard protocols with minor modifications (Sambrook et al., 1989). Primary antibodies used for immunoblots were anti-nonmuscle myosin (Kiehart and Feghali, 1986) and anti-GFP (clones 7.1 and 13.1, Roche).

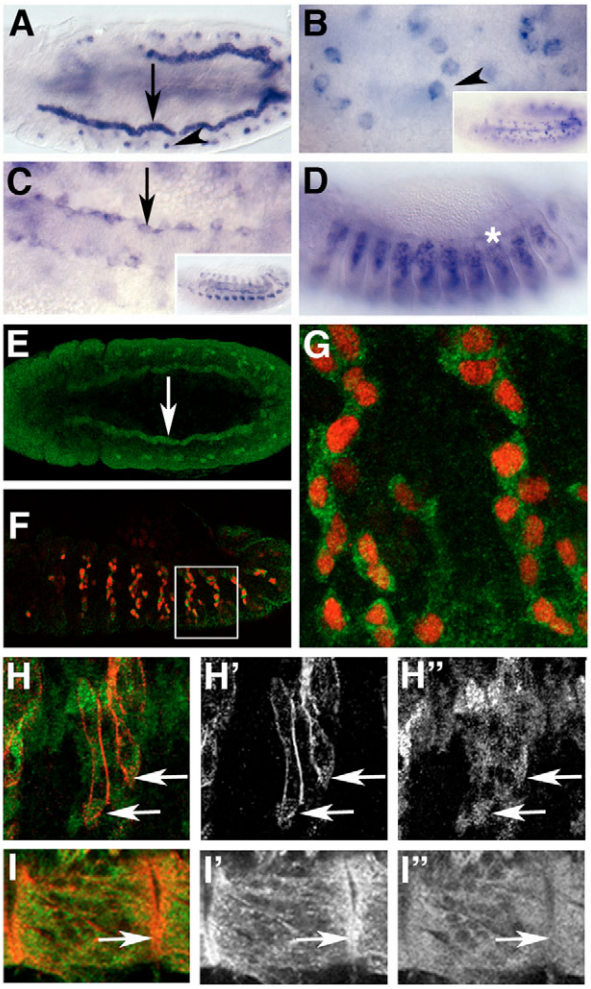

Fig. 1. Embryonic expression of *mib2* in *Drosophila*. (**A-D**) *mib2* RNA in situ hybridisations to wild-type embryos. (A,B) In stage 11 embryos, *mib2* is expressed in founders of the somatic (arrowheads) and circular visceral (arrow) muscles. (C) Detail of a late stage 12 embryo (inset). *mib2* expression occurs in founders of the longitudinal visceral muscles (arrow). (D) At stage 14, *mib2* expression is maintained in muscle precursors and is also detected in cardioblasts (asterisk). (**E-G**) Mib2 (green) accumulates in founders of circular visceral muscles (E, arrow) and in somatic founders, revealed by anti-β-gal accumulating in the nuclei (red) in *rP298-lacZ* embryos (F, enlarged in G). (**H-I″**) Mib2 (green) accumulates in the cytoplasm but not in Connectin-labelled membranes (red, arrows in H-H″). Mib2 is not enriched at muscle attachments (anti-Kettin, red, arrows in I-I″). See Fig. 3 for a diagram of the muscle pattern. In all embryos, anterior is to the left and dorsal to the top, except for the ventral view in E.

RESULTS

Muscle progenitors and founders express *mib2*

mib2 is one of the few identified genes with a mesodermal expression restricted to FMs (http://fruitfly.berkely.edu). Zygotic expression, as detected by hybridisation in situ to *mib2* mRNA, starts at stage 6 in ectoderm and mesoderm, declines at stage 9 (not shown) and reappears at stage 11 at high levels in the founders of the circular visceral mesoderm (arrow, Fig. 1A) and in the progenitors and FMs of the somatic mesoderm (arrowheads, Fig. 1A,B). By late stage 12, *mib2* mRNA is also detectable in the longitudinal visceral FMs (arrow, Fig. 1C). Expression is maintained in muscle precursors, as they increase in size by fusion (Fig. 1D), and appears at stage 14 in cardioblasts (asterisk, Fig. 1D). At postembryonic stages, *mib2* is ubiquitously expressed in imaginal discs (not shown).

We generated a specific antibody against the Mib2 protein (Fig. 1E). Using the *duf-lacZ* line rP298 as a nuclear marker for FMs, we observed co-expression in all FMs of the somatic and visceral mesoderm (Fig. 1F,G). Double stainings with antibodies to Connectin and Kettin (used to reveal myotube membranes and muscle attachments sites, respectively), showed that Mib2 accumulates in the cytoplasm (Fig. 1H-H″) and that it is not concentrated at the attachment sites (Fig. 1I-I″). We did not detect Mib2 in FCMs by double staining with the Sns marker (not shown).

Physical organisation of the *mib2* locus

mib2 encodes a modular protein that contains, at the amino-terminus, a 'ZZ-zinc-binding' domain (Ponting et al., 1996), which is flanked on either side by a 'mib/herc2' domain (Fig. 2A). At the carboxy-terminus, Mib2 has two 'RING-finger' motifs that fulfil the consensus for RING domains with catalytic E3 ubiquitin ligase activity (Fig. 2B) (Joazeiro and Weissman, 2000). The central part of the protein bears seven 'ankyrin repeats', normally implicated in protein-protein interactions. This molecular organisation is similar to that of two E3 ubiquitin ligases that regulate Notch signalling: namely, *Drosophila* Mind bomb (Mib1) and human skeletrophin (SKE; also known as MIB2 – Human Gene Nomenclature Database) (Lai et al., 2005; Le Borgne et al., 2005; Takeuchi et al., 2005). The similarity of the conserved domains is higher with SKE (51%) than with Mib1 (44%, Fig. 2B).

We obtained a novel *mib2* allele, $Df(2L)mib2^{r14}$, by imprecise excision of the *P* element of $CG17492^{KG10508}$, which generated a deletion of 3042 nucleotides. This removed two exons and the initiator ATG (Fig. 2A). Complementation analysis of $Df(2L)mib2^{r14}$ and the existing $l(2)37Be^{1}$ allele, a member of the *l(2)37Be* complementation group (Stathakis et al., 1995) that maps at 37B10-11 (http://www.flybase.org; Fig. 2A), indicated that *mib2* and *l(2)37Be* were the same gene. Therefore, we renamed the $l(2)37Be^{1}$ as $mib2^{1}$. Molecular analysis of $mib2^{1}$ revealed a C-to-T transition that changes residue Gln377 to an amber nonsense codon that should eliminate the ankyrin repeats and the catalytic RING-finger motifs. Comparison of the phenotypes of embryos homozygous for *Df(2L)Exel8039* (that removes *mib2* and adjacent genes), $mib2^{1}$ and $Df(2L)mib2^{r14}$, and in pair-wise combinations between them and *Df(2L)Exel8039*, suggested that all were null alleles, as their phenotypes were indistinguishable. Congruently, we failed to detect Mib2 protein in mutant embryos homozygous for each of these *mib2* alleles (not shown).

mib2 is required for proper myoblast fusion and muscle stability

We first analysed the specification of FMs by comparing the distinct patterns of expression of markers like *eve* (Fig. 3D,E; for three additional markers, see Fig. S1 in the supplementary material) in the individually identifiable nascent muscles of wild-type and $mib2^{1}$ embryos. No differences were observed (except in the number of nuclei present in the muscles), which indicated that all FMs segregated and were correctly specified. Numbers of FCMs, as determined by expression of *sns*, were essentially unmodified (see Fig. S1 in the supplementary material). All adult muscle precursors were also present (for expression of *twist*, see Fig. S1 in the supplementary material).

By contrast, myoblast fusion was clearly affected in mutant embryos. Muscles were smaller, and unfused myoblasts were still present at stage 16, when fusion should be completed (analysed by myosin staining; arrowheads, Fig. 3A-C). Quantification of the number of *eve*-expressing nuclei incorporated into the dorsal acute 1 (DA1) muscle at stages 14 and 15 showed a 31% and 40% decrease, respectively, under several *mib2* mutant conditions (Fig. 3D-F).

In stage 16 embryos, myosin staining also revealed other phenotypes: namely, the lack of gut constrictions, probably caused by defects in the visceral muscles, and the absence of some muscles, which was usually associated with the presence of myospheres (arrows in Fig. 3B; see Fig. S2B in the supplementary material) (Estrada et al., 2006). As this defect suggested muscle detachment, we examined muscle development in living embryos. To visualise muscles, the *Mhc-τ-GFP* chromosome was introduced into $mib2^{1}$ embryos. Initially, all muscles were present and made correct contacts with tendons (Fig. 3H, compare with 3G). However, when contractions started (stage 16) (Broadie and Bate, 1993), muscles begun to detach and form myospheres (Fig. 3I arrow and arrowhead). Subsequently, most muscles were

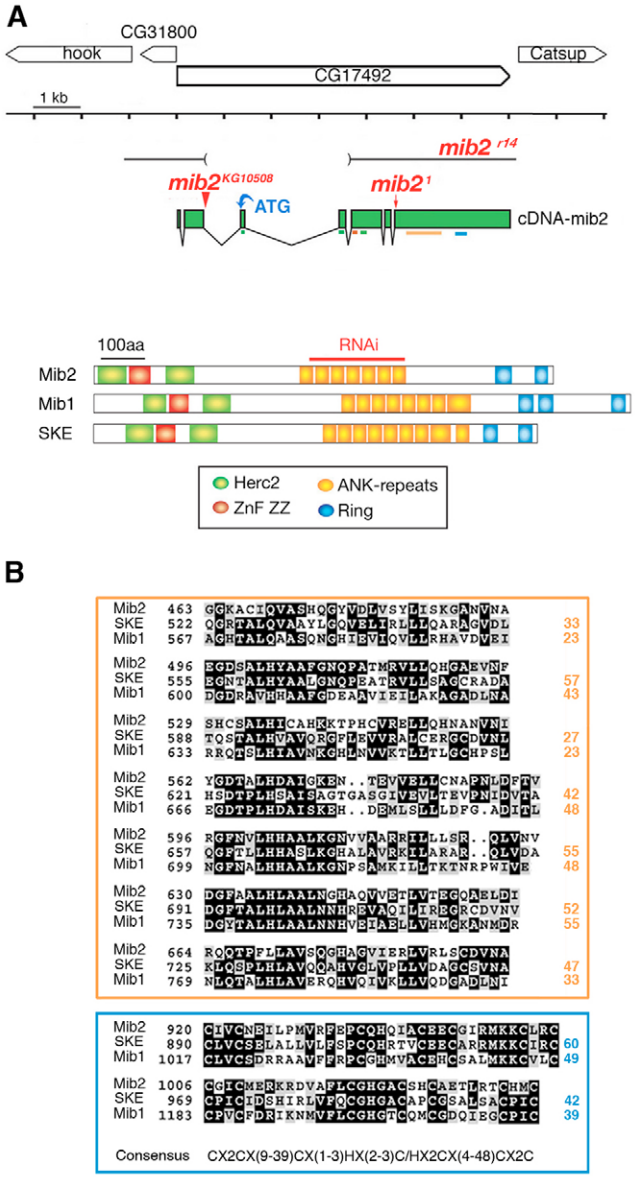

Fig. 2. Organisation of the *mib2* locus and the Mib2 protein. (**A**) The *Drosophila mib2* locus, as summarised by the BDGP and our sequence analysis of *mib2* alleles and cDNA. The horizontal line represents genomic DNA; the white boxes represent genes mapping in the 37B region; the green boxes represent exons of *mib2*. Positions of *P*-element insertion in $mib2^{KG10508}$ (red arrowhead), nonsense codon in $mib2^{1}$ (red arrow) and $mib2^{r14}$ deletion (bracket) are indicated. Below the cDNA, coloured lines indicate the extent of Mib2 domains. Modular organisation of Mib2 protein (1049 amino acids) and comparison with Mib1 and SKE. The red line represents the region of *mib2* sequence used to make the RNAi construct. (**B**) Alignment of ankyrin (framed in orange) and RING (framed in blue) domains of Mib2, SKE and Mib1 proteins. Percentage identity between Mib2 and SKE or MIb1 is shown in the column on the right. Consensus sequence for the RING-finger motif is indicated below the blue box.

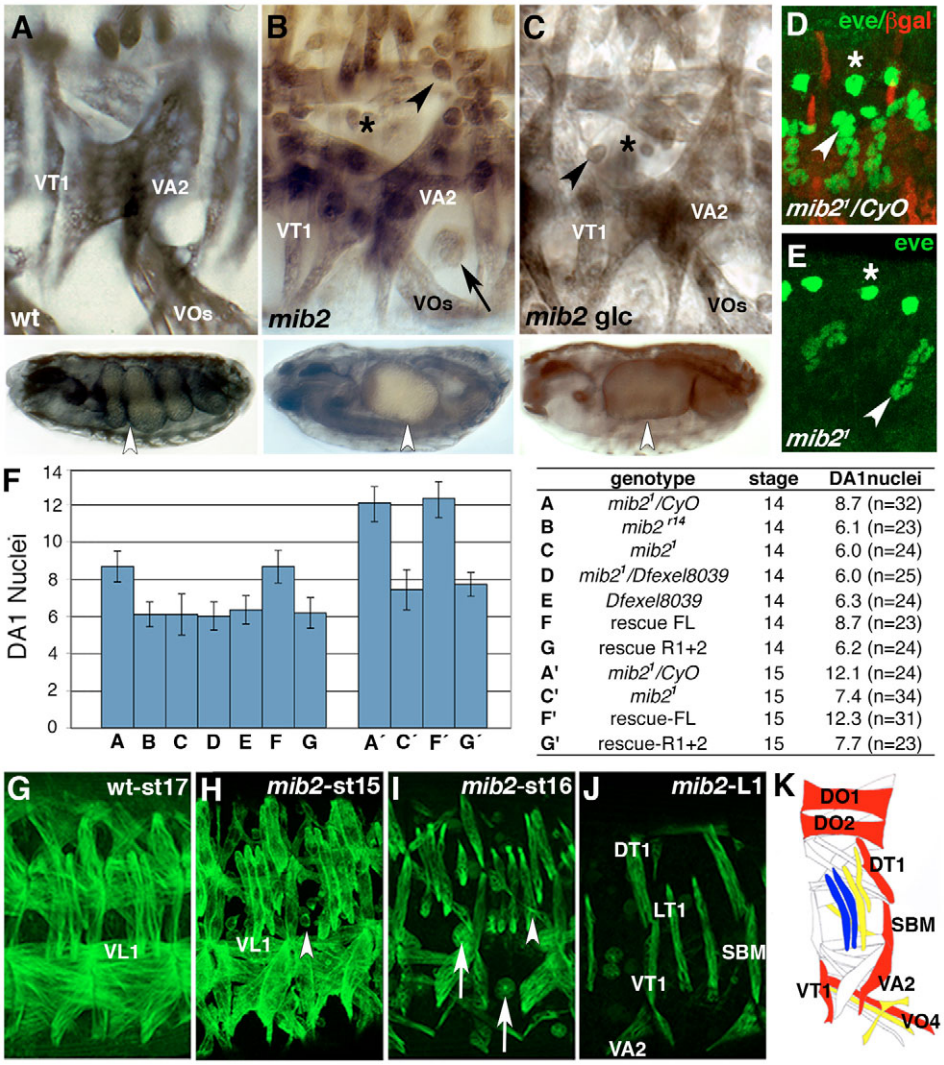

Fig. 3. Embryonic loss-of-function phenotypes of *mib2* alleles. (**A-C**) Lateral views of the ventral region of stage 16 wild-type (A), *mib2*^{1} (B) and *mib2*^{1} (C) germline clone *Drosophila* embryos stained with anti-myosin (A,B) or anti-Tropomyosin (C). Positions of VA2, VT1 and VO muscles are indicated. Note the presence of unfused myoblasts (black arrowheads in B,C), the absence of muscles in the VL region (asterisks in B,C, and muscle scheme in Fig. 4G), and presence of myospheres (arrow, B). Whole-embryo pictures (below) are focused on the gut. White arrowheads point to the position of the first midgut constriction (A) and to the unfolded midgut (B,C). (**D**,**E**) Dorsal views focused on the DA1 muscle of *mib2*^{1}/*CyO* and *mib2*^{1} stage 15 embryos, respectively. Arrowheads point to Eve-labelled nuclei (green). Asterisks mark nuclei of pericardial cells. (**F**) Number of *eve*-expressing nuclei in DA1 muscles of embryos of the indicated genotype (*n*, number of hemisegments quantified). (**G-J**) Progressive degeneration of muscles in *mib2*^{1} embryos. Muscles were revealed by *Mhc-τ-GFP* (G,I,J) and by anti-Tm staining (H). At stage 15 (H), all muscles are present and attached to the apodemes (arrowhead, unfused myoblast). At stage 16 (I), some muscle attachments are becoming thinner (arrowhead), while others, many in the VL region, have detached (arrows). In L1 larvae, most muscles are missing (J). (**K**) Scheme indicating percentages of specific muscles that remain attached to apodemes in *mib2*^{1} first instar larvae: red (80-100%), blue (50-80%), yellow (10-25%) and white (<10%) (*n*=50 abdominal hemisegments; 2 to 5 examined).

affected, although some of them were most resistant to detachment (Fig. 3K). Approximately one-third of individuals died before hatching, while the remaining ones reached first larval instar. These died shortly afterwards with most of the muscles missing (Fig. 3J and see Fig. S3A,B in the supplementary material). Essentially the same phenotype was observed in mutant embryos derived from *mib2*^{1} germline clones (Fig. 3C), which indicated that there is no major contribution of maternally delivered Mib2 to the zygotic function.

We next examined whether the fine structure of muscles was affected by the loss of Mib2. We used the reporter chromosome *kettin-GFP* as a marker for sarcomeric Z bands and focused on the persistent dorsal oblique (DO) muscles of stage 17 *mib2*^{1} embryos. These bands were clearly affected, and whereas some muscles still maintained a certain alignment of Z bands in sarcomeres (that resembled the repetitive banding pattern of wild-type muscles; Fig. 4A,B, arrow), other DO muscles lacked any sign of regularity (Fig. 4B, arrowhead). Muscle contraction occurred only in muscles that maintained Z banding regularity, although contractions were erratic in frequency and extension (not shown). Fracture of Z bands was also observed in electron micrographs of mutant larval muscles (not shown). We conclude that absence of *mib2* permits sarcomeric assembly, but leads to defective sarcomeric structure.

Rescue of the *mib2*^{1} phenotype

Forced expression of *UAS-mib2* in the mesoderm (*24B-GAL4* driver) or exclusively in FMs (*duf-GAL4* driver) rescued the somatic and visceral mesodermal defects of *mib2*^{1} embryos (Fig. 4D,D′ and see Fig. S2A-SA″ in the supplementary material). (Similar overexpression in a wild-type background did not produce obvious defects and individuals survived up to pupal stages.) Neither *UAS-SKE* nor *UAS-mib1* was able to rescue any aspect of the *mib2*^{1} embryonic phenotype (not shown), which suggested that the closely related E3 ubiquitin ligases Mib1 and SKE are not functional homologues of Mib2.

The Mib2 RING-finger domains are required for myoblast fusion and are dispensable for muscle integrity

We next examined whether the RING-finger domains of Mib2, putatively responsible for an E3 ubiquitin ligase activity, were necessary for the function of this protein in myogenesis. We prepared *UAS-mib2-C935S-C1020S* by replacing the third conserved cysteine in each Mib2 RING domain by serine, a substitution shown to abrogate ubiquitin ligase activity in other RING-finger E3 proteins with minimal modification of tertiary structure (Takeuchi et al., 2005). *UAS-mib2-C935S-C1020S* did not rescue the fusion defect of *mib2*^{1} embryos, as they displayed unfused myoblasts (black arrowhead, Fig.

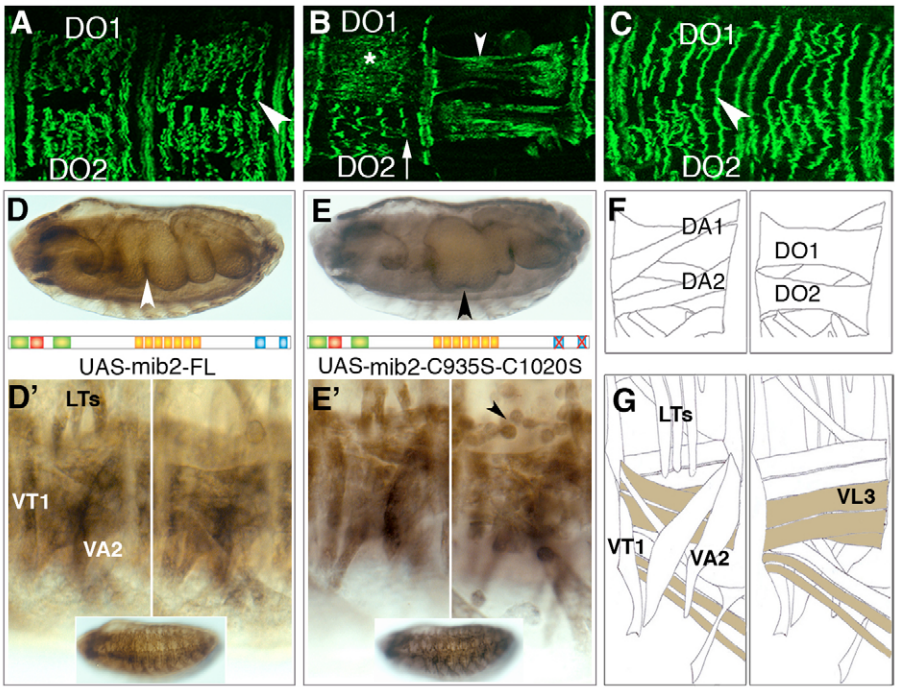

Fig. 4. Embryonic rescue of *mib2* phenotypes and ultrastructure organisation of *mib2* larval muscles. (**A-C**) Dorsal region of stage 17 *kettin-GFP* (A), *mib2*^{1}*; kettin-GFP* (B) and *mib2*^{1}*; kettin-GFP* rescued with *UAS-mib2-C935S-C1020S* (C) embryos. Note the alignment of Z bands in the DO1/2 muscles in A and C (arrowheads), and the distorted (arrow), irregular (asterisk) or absent (arrowhead) bands in B. Images in A and C also show the Z bands of the immediately overlying DA1/2 muscles (see F for dorsal muscle scheme). (**D-E′**) Mesodermal phenotype of stage 16 *mib2*^{1} embryos (anti-myosin stained), expressing *UAS-mib2-FL* (D,D′) and *UAS-mib2-C935S-C1020S* (E,E′) (*24BGAL4* driver). Gut constrictions (D,E) and external (D′,E′, left) and internal (D′,E′, right) views of the ventral muscles schematised in G are shown. Mib2-FL completely rescues the mutant phenotype (D,D′, compare with Fig. 3A,B). Mib2-C935S-C1020S rescues all embryonic phenotypes except the fusion defect, indicated by the presence of unfused myoblasts (arrowhead in E′) and number of DA1 nuclei (Fig. 3F). Some embryos also failed to complete the first gut constriction (arrowhead, E), a phenotype common to most fusion mutants. (**F**) Scheme indicating dorsal muscles. (**G**) Scheme indicating ventral muscles (brown) missing in Fig. 3B,C.

4E′). Moreover, the number of *eve*-expressing nuclei in DA1 of the rescued embryos was the same as in *mib2* mutant embryos (Fig. 3F). However, this Mib2 variant completely rescued the detachment phenotype (Fig. 4E′), the sarcomeric organisation (Fig. 4C) and, to a large extent, the visceral mesoderm defects, as all but the first gut constriction were formed (Fig. 4E). Hence, the RING-finger domains, and consequently the putative ubiquitin ligase activity of Mib2, appear essential for myoblast fusion, but they are largely dispensable for other functions of Mib2.

The FCMs-specific regulator Lmd accumulates in FMs devoid of Mib2

In many instances, ubiquitination of target proteins by E3 ligases labels them for degradation or for changes in cellular localisation (reviewed by Welchman et al., 2005). As the above experiments suggested that the putative E3 ligase activity of Mib2 is important for myoblast fusion, we explored whether the fusion phenotypes associated with the absence of Mib2 were related to modifications in the accumulation of proteins required for fusion. Two obvious founder-specific candidates were Duf and Rols (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001; Ruiz-Gómez et al., 2000). However, we did not detect changes in the accumulation or subcellular localisation of either of them in *mib2*^{1} embryos (not shown). Next, we overexpressed two copies of *UAS-mib2* in the mesoderm using a strong GAL4 line, *Mef2-GAL4*. This caused a severe defect in myoblast fusion (compare Fig. 3A with Fig. 5A,A′) that required the presence of intact RING domains (Fig. 5C,C′) but was not associated with changes in the membrane localisation of Duf and Rols in FMs (Fig. 5D). Furthermore, the resulting mini-myotubes elongated properly and were able to contract under these conditions (not shown).

By contrast, this overexpression of *UAS-mib2* induced early massive death of FCMs (Fig. 5E,F). This suggested that the failure to fuse was due to a deleterious effect of Mib2 on FCMs. To verify this, we overexpressed Mib2 exclusively in FMs (*duf-GAL4*) or FCMs (*sns-GAL4*). Interference with fusion was associated only with overexpression in FCMs (Fig. 5B,B′). This suggested an interference with an FCM-specific factor. Lmd was an obvious candidate as it is a regulator of the FCMs differentiation programme. We reasoned that Mib2 might be important in FMs to keep Lmd inoperative after fusion, when muscle precursors are loaded with Lmd brought in as FCMs fuse with founders. We explored this by examining the distribution of Lmd in wild-type and *mib2*^{1} embryos. Lmd was absent from wild-type muscle precursors (Fig. 5G,J), but it was present in precursors (Fig. 5H) and mature muscles (Fig. 5K) of mutant embryos. Thus, Mib2 is required to remove Lmd from developing myotubes. The persistence of Lmd could be the basis of the fusion defects observed in *mib2* mutants, as forced expression of Lmd in FMs interferes with fusion (Duan and Nguyen, 2006).

Mib2 accumulates in sarcomeres and is required for their stability

The dispensability of the Mib2 RING-finger domains for muscle stability points to a possible structural role of Mib2 in regulating muscle integrity. Due to the difficulty in performing structural

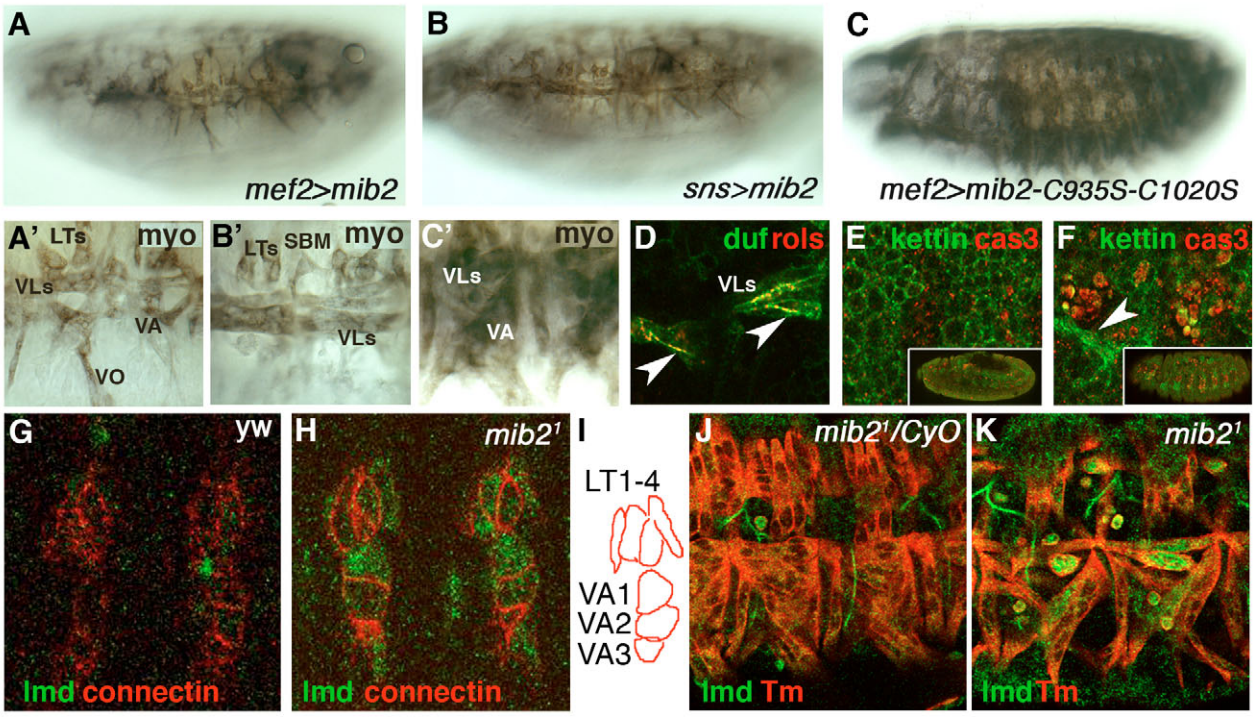

Fig. 5. Effects of Mib2 mis-expression in FCMs and its requirement in FMs to eliminate Lmd from myotubes. In all embryos, the lateral-ventral region is shown and anterior is to the left. (**A**) *Mef2GAL4*; 2x*UAS-mib2*, (**B**) *snsGAL4*; 2x*UAS-mib2* and (**C**) *Mef2GAL4*; 2x*UAS-mib2-C935S-C1020S Drosophila* embryos. (**A′**,**B′**,**C′**) Higher magnification views of corresponding embryos. Note the presence in A′ and B′ (but not in C′) of thin (mono/bi-nucleated) myotubes (anti-myosin staining). (**D-F**) *Mef2GAL4*; 2x*UAS-mib2* embryos. (D) Duf (green) and Rols (red) co-localise to the membrane of mini-myotubes (arrowheads) induced by overexpression of Mib2-FL. (E,F) Overexpression promotes death (activated-Caspase 3 expression, red) of FCMs (Kettin labelling, green) starting at stage 12 (E) and most prevalent at stage 14 (F). Mini-myotubes did not express activated-Caspase 3 (arrowhead in F). (**G**,**H**) Late stage 13 *yw* and *mib2*^{1} embryos, respectively, stained with anti-Connectin (red) and anti-Lmd (green). (**I**) Schematic representation of late stage 13 Connectin-expressing precursors. (**J**,**K**) Stage 16 *mib2*^{1}*/CyO* and *mib2*^{1} embryos stained with anti-Tm (red) and anti-Lmd (green). Lmd is not detected in early precursors (G) or late myotubes (J) of wild-type embryos, but it is not eliminated in *mib2*^{1} embryos (H,K).

analysis on *mib2* larval muscles, we turned our attention to adult muscles. Mib2 was present in myofibrils isolated from thoracic muscles. It accumulated preferentially in the Z bands (co-expression with Kettin, Fig. 6A,A‴) and at lower levels in the M bands (absence of phalloidin Fig. 6A,A″). Next, we examined the requirement for Mib2 during adult myogenesis by using a *UAS-RNAi-mib2* transgene. After confirmation of its ability to attenuate *mib2* function (see Fig. S3A-C,E in the supplementary material), we expressed two copies of *UAS-RNAi-mib2* in the precursors of adult muscles (*1151-GAL4*, see Fig. S3D-D″ in the supplementary material). The indirect flight muscles (IFM) of newly emerged flies (before acquisition of flight ability) presented poorly defined M bands, and the Z bands were less dense than those of the wild type and, occasionally, of zigzagging shape (Fig. 6B-C′). Flies 2-3 days old remained flightless and unable to jump, and their thoraces displayed empty cavities (see Fig. S3G in the supplementary material) due to loss of muscle mass. Their remaining IFMs lacked myofibrils (although both thick and thin filaments were present) and only possessed remnants of electron-dense material, which resembled Z bands (Fig. 6D,D′). The tergal depressor muscles of the trochanter (TDT) were similarly affected (M. Carrasco-Rando, PhD thesis, Universidad Autónoma de Madrid, 2005). We conclude that depletion of Mib2 disrupts myofibrillar organisation and affects sarcomere assembly to some an extent. Similarly to embryonic muscles, the defects worsen with age and probably with muscle use. This structural role of Mib2 was further evidenced by the ability of this protein to interact with Spaghetti-squash (Sqh) and Zipper, the regulatory light chain and the heavy chain of nonmuscle myosin (Fig. 6E-G), a component of muscle sarcomeres that plays a fundamental role in myofibrillogenesis during embryogenesis (Bloor and Kiehart, 2001).

DISCUSSION

During *Drosophila* myogenesis, founder myoblasts constitute an essential population that inherits the information to seed a specific muscle and to execute the myogenic programme. Thus, in the absence of fusion, only FMs are able to differentiate into mature myotubes that assemble contractile proteins into functional sarcomeres and receive the innervation required to control the motile function (Rushton et al., 1995). The nature of the genes responsible for regulating many of the properties exclusive to founders has remained elusive, in particular those necessary to implement the terminal differentiation programme. We searched for these genes using the criterion of FM-restricted expression, and identified and characterised *mib2* as such a candidate gene. Two recent genome-wide analyses have also found *mib2* expression enriched in the FM population (Artero et al., 2003; Estrada et al., 2006).

mib2 encodes a modular protein very similar to human SKE and *Drosophila* Mib1, two ubiquitin ligases that act as positive regulators of the N signalling pathway by targeting N ligands for

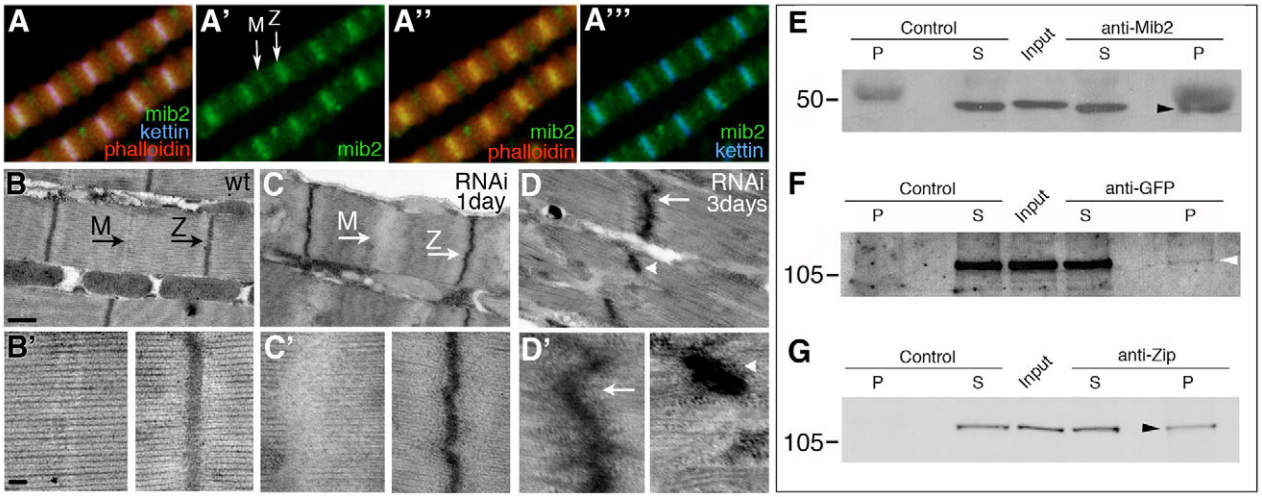

Fig. 6. Mib2 localisation and functional analysis in *Drosophila* adult muscle. (**A-A‴**) Mib2 (green) accumulates at the Z band (Kettin staining, blue) of myofibrils and to lesser an extent in the M band (absence of actin). (**B-D′**) Electron micrographs of longitudinal sections of IFM from 3-day-old wild-type (B,B′), 1-day-old (C,C′) and 3-day-old (D,D′) *1151-GAL4/UAS-RNAi-mib2* adults. IFMs of *1151-GAL4/UAS-RNAi-mib2* young adults show poorly defined M bands and irregular Z disks (C′), compared with the wild type (B′). Three-day-old *1151-GAL4/UAS-RNAi-mib2* IFMs lack sarcomeres and display remains of dense material that probably corresponds to distorted and split Z bands (D′, arrow and arrowhead, respectively). (**E-G**) Co-immunoprecipitation experiments showing a physical interaction between Mib2 and nonmuscle myosin. Protein extracts from *sqh-GFP* thoraces were immunoprecipitated with anti-Mib2 (E), anti-GFP (F) or anti-nonmuscle Mhc (G) antibodies, followed by immunoblotting with anti-GFP (E) or anti-Mib2 (F,G). Note the presence of bands of 47 kDa corresponding to Sqh-GFP (arrowhead in E) and 115 kDa corresponding to Mib2 (arrowheads in F,G) in the experimental and their absence in the control P lanes. In E, the weak bands detected in P lanes correspond to immunoglobulins. Input, immunoprecipitation reaction samples; P, immunoprecipitated pellets; S, supernatants. Scale bars: 0.5 μm in B; 0.1 μm in B′.

degradation (Lai et al., 2005; Le Borgne et al., 2005; Takeuchi et al., 2005). Our results, however, ruled out a role for Mib2 in regulating N during myogenesis, as the processes of progenitor segregation and their subsequent asymmetric division, both dependent on N signalling (Corbin et al., 1991; Ruiz-Gómez and Bate, 1997), were not affected in *mib2*^{1} mutants. Moreover, the failure to rescue *mib2*^{1} phenotypes with SKE or Mib1 indicated that they were not functional homologues.

Functional analyses of *mib2* revealed that it is repeatedly required during myogenesis. Thus, loss-of-function mutants display correct specification of FMs (visceral and somatic), but they show early signs of faulty differentiation. The visceral muscles are unable to drive the formation of the gut constrictions. The somatic muscles undergo fewer rounds of fusion than do wild-type muscles, and although they attach to apodema and initiate the synthesis of sarcomeres, with the onset of muscle contractions muscles start detaching and sarcomers disintegrate. Adult muscles also require *mib2* for sarcomeric stability.

Mib2 RING fingers are necessary for correct myoblast fusion

To study the contribution of the putative E3 ubiquitin ligase activity of Mib2 to its function we prepared the modified variant *UAS-mib2-C935S-C1020S*, which should lack ligase activity because two conserved cysteines that bind the Zn^{2+} ions in the putative catalytic centre were replaced by serine (Lorick et al., 1999). Its mesodermal expression in *mib2*^{1} mutant embryos rescued the detachment phenotype and the sarcomeric defects, but not the myoblast fusion problem. This suggested that the RING-finger domains actually have enzymatic activity.

As Mib2 is an FM-restricted protein, the requirement of its RING domains for fusion pointed to Duf and Rols as targets for Mib2, as they are exclusive to FMs and are implicated in myoblast fusion. However, we failed to detect an effect on the stability or subcellular localisation of these crucial proteins, both in *mib2*^{1} mutants and under overexpression conditions that compromise fusion. Moreover, by means of overexpressions directed at either FMs or FCMs, we found that these overexpressions induced damage, including cell death, only in FCMs, but did not interfere with the survival or contractile capability of the FM-derived mini-myotubes. Based on these results, and on our finding that Lmd accumulates in *mib2*^{1} muscle precursors, we suggest that Mib2 is required in FMs to eliminate Minc/Lmd provided to nascent muscles by the fusing FCMs. The presence of the FCM-specific factor Lmd in FMs would interfere with normal myotube development, in agreement with a recent report showing that forced expression of Lmd in FMs induces fusion defects (Duan and Nguyen, 2006) similar to those observed in *mib2*^{1} mutant embryos.

Mib2 plays a structural role in muscle stability

As the presumed ligase activity of the RING-finger domains appear dispensable for the Mib2 functions related to muscle stability, we inferred that Mib2 might have a structural role in maintaining muscle attachments and sarcomeric stability. In the light of the similarity of the *mib2* detachment phenotype to that produced by the loss-of-function of Integrins (Brown, 1994), and the presence of ankyrin repeats in Mib2, we reasoned that Mib2 might mediate interactions with proteins involved in stabilising muscle attachments, such as Inflated, Integrin-linked kinase (Ilk) and Tensin (also known as Blistery – FlyBase). However, the absence of Mib2 did not influence their cellular localisations. Similarly, we did not detect changes in the expression or localisation of Alien and Stripe, two markers for tendon cells (M. Carrasco-Rando, PhD thesis, Universidad Autónoma de Madrid, 2005). By contrast, *mib2* mutant embryos showed extensively disrupted myofibrillar organisation at the ultrastructural level. In vivo observations (Kettin-GFP) revealed

that sarcomeric assembly proceeded almost normally, as imperfect Z bands were evident in stage 17 mutant muscles. These muscles could contract, but progressively the Z bands broke. The splitting of the Z bands was concomitant with a decrease of contraction frequency and ended up with the loss of contractile ability.

These observations are consistent with ultrastructural data obtained in adult muscles. Here again, the slight defects observed in muscles of newborn adults, before acquisition of flight ability, suggested that muscle assembly did not require Mib2; but the absence of striated myofibrils in older muscles points to a structural role of Mib2 in maintenance of muscle integrity. To our knowledge, this is the first phenotype of muscle decay described in *Drosophila*. It is noteworthy that Mib2-deficient muscles display signs of faulty differentiation when they still can contract, which suggests that muscle decay is a consequence of loss of sarcomeric integrity.

Mib2 localises to the sarcomeres of adult muscles and accumulates at the Z bands and, at lower levels, at the M bands. Furthermore, during embryogenesis it does not co-localise with Integrins at the muscle termini. Thus, detachments are probably a consequence of loss of sarcomeric structure. In this line of thinking, it should be stressed that Z bands and muscle termini function as transmitters of muscle tension during contraction. The *mib2* phenotypes and Mib2 localisation suggest a role of this protein as a cross-linker that helps to maintain Z band and muscle termini integrity. Many proteins have been identified at Z bands, alpha-actinin being one of the major components (reviewed by Sanger et al., 2005). So far, we have not found evidence for a physical interaction between Mib2 and alpha-actinin. However, we show an interaction in adults with nonmuscle myosin, recently identified as another component of embryonic Z bands (Bloor and Kiehart, 2001). This interaction is of interest, as it might be related to the unique ability of FMs, as opposed to FCMs, to synthesise stable sarcomeres, as nonmuscle myosin is required for the formation of striated myofibrils (Bloor and Kiehart, 2001). Moreover, during embryogenesis, the genes encoding two of the nonmuscle myosin chains (*zipper* and *spaghetti squash*) are in the fraction of genes with expression that is strongly enriched in FMs versus FCMs, similarly to *duf* and *rols*, two FM-specific genes (Estrada et al., 2006). These data support the idea that sarcomeres are synthesised only in FMs. In addition, the restricted localisation of Mib2 to FMs favours the idea that stabilisation of the assembled sarcomeres occurs exclusively in muscle precursors and myotubes. In this context, it is worth mentioning that although the closest human structural homologue of Mib2, skeletrophin, does not seem to play the same role during myogenesis, there is another E3 ubiquitin ligase, TRIM32, that has been linked to two forms of progressive skeletal muscle-wasting dystrophies (Kudryashova et al., 2005). TRIM32 binds to muscle myosin and is able to ubiquitinate actin. Although the molecular bases for these dystrophies are unknown, our findings highlight the importance that bifunctional ubiquitin ligases may have in the control of sarcomeric stability in both systems.

We are grateful to S. Prieto for helping with the production of the anti-Mib2 antibody and to M. Guerra from the Electron Microscopy Service of the CBMSO for technical assistance. We thank J. Modolell, S. Campuzano, N. Azpiazu and J. F. de Celis for constructive criticisms on the manuscript. We are grateful to D. Kiehart, D. Kosman, H. Nguyen, W. Chia, R. Renkawitz-Pohl and the Bloomington Stock Center for contributing antisera and flies and to T. Takeuchi for the *SKE* cDNA. This work was supported by grants BMC2003-05056 and BFU2006-07372 from MEC to M. Ruiz-Gómez and LSHG-CT-2004-511978 from the EC to the Network of Excellence MYORES. Predoctoral fellowship from MCyT to M. Carrasco-Rando and an institutional grant from Fundación Ramón Areces to the CBMSO are acknowledged.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/849/DC1

Note added in proof

After submission of this work, Nguyen et al. (Nguyen et al., 2007) reported a requirement of *Drosophila mib2* for embryonic muscle integrity and survival.

References

Artero, R., Furlong, E. E., Beckett, K., Scott, M. P. and Baylies, M. (2003). Notch and Ras signaling pathway effector genes expressed in fusion competent and founder cells during Drosophila myogenesis. *Development* **130**, 6257-6272.

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 1013-1090. Cold Spring Harbor: CHS Laboratory Press.

Baylies, M. K., Bate, M. and Ruiz-Gómez, M. (1998). Myogenesis: a view from Drosophila. *Cell* **93**, 921-927.

Beall, C. J. and Fyrberg, E. (1991). Muscle abnormalities in Drosophila melanogaster heldup mutants are caused by missing or aberrant troponin-I isoforms. *J. Cell Biol.* **114**, 941-951.

Bloor, J. W. and Kiehart, D. P. (2001). zipper Nonmuscle myosin-II functions downstream of PS2 integrin in Drosophila myogenesis and is necessary for myofibril formation. *Dev. Biol.* **239**, 215-228.

Broadie, K. S. and Bate, M. (1993). Development of the embryonic neuromuscular synapse of Drosophila melanogaster. *J. Neurosci.* **13**, 144-166.

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

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F. and Chia, W. (1998). Inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. *Genes Dev.* **12**, 304-315.

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

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the Drosophila neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Duan, H. and Nguyen, H. T. (2006). Distinct posttranscriptional mechanisms regulate the activity of the Zn finger transcription factor lame duck during Drosophila myogenesis. *Mol. Cell. Biol.* **26**, 1414-1423.

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

Dutta, D., Anant, S., Ruiz-Gómez, M., Bate, M. and VijayRaghavan, K. (2004). Founder myoblasts and fibre number during adult myogenesis in Drosophila. *Development* **131**, 3761-3772.

Estrada, B., Choe, S. E., Gisselbrecht, S. S., Michaud, S., Raj, L., Busser, B. W., Halfon, M. S., Church, G. M. and Michelson, A. M. (2006). An integrated strategy for analyzing the unique developmental programs of different myoblast subtypes. *PLoS Genet.* **2**, e16.

Hamilton, B. A. and Zinn, K. (1994). From clone to mutant gene. *Methods Cell Biol.* **44**, 81-94.

Joazeiro, C. A. and Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**, 549-552.

Kiehart, D. P. and Feghali, R. (1986). Cytoplasmic myosin from Drosophila melanogaster. *J. Cell Biol.* **103**, 1517-1525.

Kosman, D., Small, S. and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. *Dev. Genes Evol.* **208**, 290-294.

Kudryashova, E., Kudryashov, D., Kramerova, I. and Spencer, M. J. (2005). Trim32 is a ubiquitin ligase mutated in limb girdle muscular dystrophy type 2H that binds to skeletal muscle myosin and ubiquitinates actin. *J. Mol. Biol.* **354**, 413-424.

Lai, E. C., Roegiers, F., Qin, X., Jan, Y. N. and Rubin, G. M. (2005). The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. *Development* **132**, 2319-2332.

Le Borgne, R., Remaud, S., Hamel, S. and Schweisguth, F. (2005). Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. *PLoS Biol.* **3**, e96.

Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S. and Weissman, A. M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. USA* **96**, 11364-11369.

Machado, C. and Andrew, D. J. (2000). D-Titin: a giant protein with dual roles in chromosomes and muscles. *J. Cell Biol.* **151**, 639-652.

Meadows, L. A., Gell, D., Broadie, K., Gould, A. P. and White, R. A. (1994). The cell adhesion molecule, connectin, and the development of the Drosophila neuromuscular system. *J. Cell Sci.* **107**, 321-328.

Menon, S. D. and Chia, W. (2001). Drosophila rolling pebbles: a multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast attractant Dumbfounded. *Dev. Cell* **1**, 691-703.
Nagel, A. C., Maier, D. and Preiss, A. (2002). Green fluorescent protein as a convenient and versatile marker for studies on functional genomics in Drosophila. *Dev. Genes Evol.* **212**, 93-98.
Nguyen, H. T., Voza, F., Ezzeddine, N. and Frasch, M. (2007). *Drosophila mind bomb2* is required for maintaining muscle integrity and survival. *J. Cell Biol.* **179**, 219-227.
Ponting, C. P., Blake, D. J., Davies, K. E., Kendrick-Jones, J. and Winder, S. J. (1996). ZZ and TAZ: new putative zinc fingers in dystrophin and other proteins. *Trends Biochem. Sci.* **21**, 11-13.
Rau, A., Buttgereit, D., Holz, A., Fetter, R., Doberstein, S. K., Paululat, A., Staudt, N., Skeath, J., Michelson, A. M. and Renkawitz-Pohl, R. (2001). rolling pebbles (rols) is required in Drosophila muscle precursors for recruitment of myoblasts for fusion. *Development* **128**, 5061-5073.
Ruiz-Gómez, M. and Bate, M. (1997). Segregation of myogenic lineages in Drosophila requires numb. *Development* **124**, 4857-4866.
Ruiz-Gómez, M., Coutts, N., Price, A., Taylor, M. V. and Bate, M. (2000). Drosophila dumbfounded: a myoblast attractant essential for fusion. *Cell* **102**, 189-198.
Ruiz-Gómez, M., Coutts, N., Suster, M. L., Landgraf, M. and Bate, M. (2002). myoblasts incompetent encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in Drosophila. *Development* **129**, 133-141.
Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for Drosophila muscle development. *Development* **121**, 1979-1988.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
San-Martín, B., Ruiz-Gómez, M., Landgraf, M. and Bate, M. (2001). A distinct set of founders and fusion-competent myoblasts make visceral muscles in the Drosophila embryo. *Development* **128**, 3331-3338.
Sanger, J. W., Kang, S., Siebrands, C. C., Freeman, N., Du, A., Wang, J., Stout, A. L. and Sanger, J. M. (2005). How to build a myofibril. *J. Muscle Res. Cell Motil.* **26**, 343-354.
Stathakis, D. G., Pentz, E. S., Freeman, M. E., Kullman, J., Hankins, G. R., Pearlson, N. J. and Wright, T. R. (1995). The genetic and molecular organization of the Dopa decarboxylase gene cluster of Drosophila melanogaster. *Genetics* **141**, 629-655.
Strunkelnberg, M., Bonengel, B., Moda, L. M., Hertenstein, A., de Couet, H. G., Ramos, R. G. and Fischbach, K. F. (2001). rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila. *Development* **128**, 4229-4239.
Stute, C., Kesper, D., Holz, A., Buttgereit, D. and Renkawitz-Pohl, R. (2006). Establishment of cell type specific Gal4-driver lines for the mesoderm of Drosophila. *Drosoph. Inf. Serv.* **89**, 111-115.
Takeuchi, T., Adachi, Y. and Ohtsuki, Y. (2005). Skeletrophin, a novel ubiquitin ligase to the intracellular region of Jagged-2, is aberrantly expressed in multiple myeloma. *Am. J. Pathol.* **166**, 1817-1826.
Welchman, R. L., Gordon, C. and Mayer, R. J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat. Rev. Mol. Cell Biol.* **6**, 599-609.