**Drosophila mind bomb2 is required for maintaining muscle integrity and survival**

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We report that the *Drosophila mind bomb2* (*mib2*) gene is a novel regulator of muscle development. Unlike its paralogue, *mib1*, zygotic expression of *mib2* is restricted to somatic and visceral muscle progenitors, and their respective differentiated musculatures. We demonstrate that in embryos that lack functional Mib2, muscle detachment is observed beginning in mid stage 15 and progresses rapidly, culminating in catastrophic degeneration and loss of most somatic muscles by stage 17. Notably, the degenerating muscles are positive for apoptosis markers, and inhibition of apoptosis in muscles prevents to a significant degree the muscle defects. Rescue experiments with Mib1 and Neuralized show further that these E3 ubiquitin ligases are not capable of ameliorating the muscle mutant phenotype of *mib2*. Our data suggest strongly that *mib2* is involved in a novel Notch- and integrin-independent pathway that maintains the integrity of fully differentiated muscles and prevents their apoptotic degeneration.

**Introduction**

In *Drosophila*, the larval somatic (skeletal) musculature arises from the fusion of two distinct types of myoblasts, the founders and fusion-competent cells (for review see Beckett and Baylies, 2006). Subsequent differentiation programs, including activation of muscle-specific gene expression and asymmetrical cell fusion between the differentially marked founders and fusion-competent myoblasts, are required for the generation of syncytial muscle fibers. Maturation of these syncytia into functional muscle fibers involve additional events, including pathfinding processes and the formation of attachments to the tendon cells, as well as the establishment of neuromuscular junctions (for review see Volk, 1999; Schnorrer and Dickson, 2004). The functional characterization of integrins and downstream effectors of integrin signals has underscored the importance of this pathway in establishing muscle attachment sites (for review see Bokel and Brown, 2002). However, the molecular basis for many other aspects of morphogenesis and maintenance of the mature muscles is still poorly defined.

Herein, we present a functional characterization of *Drosophila mind bomb2* (*mib2*), which shares structural similarities with its paralogue *mib1*. Unlike *mib1*, *mib2* is prominently expressed in muscle progenitors and differentiated musculatures. We show that loss of *mib2* activity leads to muscle detachment and massive muscle degeneration. We also demonstrate that *mib2* functions in a novel integrin- and Notch-independent manner to maintain the integrity of the mature somatic musculature.

**Results and discussion**

**Characterization of the Drosophila mind bomb2 gene product**

*Drosophila mind bomb2* (*mib2*) encodes a 1,050-aa acid protein with several notable features (Fig. 1 A). A ZZ zinc finger domain within the N-terminal portion is flanked by two regions that share homology with HERC2, a protein that may function in protein trafficking and degradation pathways (Lehman et al., 1998; Ji et al., 1999). The ZZ domain, characterized by Cys-X2-Cys and Asp-Tyr-Asp-Leu motifs, is found in a small number of proteins, including some transcriptional adaptor proteins and Dystrophin/Utrophin. Our data suggest strongly that *mib2* is involved in a novel Notch- and integrin-independent pathway that maintains the integrity of fully differentiated muscles and prevents their apoptotic degeneration.
while RING fingers proteins are known to participate in protein–protein interactions in the ubiquitination pathway (Joazeiro and Weissman, 2000). The presence of these various domains suggests that Mib2 functions as an adaptor-type of protein and/or as a component of a ubiquitination pathway.

The Mib2 protein is conserved during evolution. *Drosophila* Mib2 and its murine orthologue display a similar structural organization and considerable degree of amino acid conservation within all the aforementioned domains (Fig. 1 A; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708135/DC1). When compared with Mib2 proteins across species, *Drosophila* Mib1, an E3 ubiquitin ligase that has been shown to be important in Notch signaling (Itoh et al., 2003; Lai et al., 2005; Le Borgne et al., 2005; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005), shows a lower level of homology in most of these domains, indicating that Mib2 is a paralogue of Mib1. In addition, the Mib2 proteins have only two RING finger domains while the Mib1 proteins have three.

*mib2* is highly expressed in visceral and somatic mesodermal cells

Maternally derived *mib2* transcripts are detected prominently in the fertilized egg (Fig. 1 B). Zygotic expression is first observed at low levels pannemoderally, and beginning at stage 11, high levels of expression appear in progenitors of somatic and visceral muscles (Fig. 1 C) and persist in the differentiated muscles of late stage embryos (Fig. 1, D and E; and unpublished data). *mib2* is not detectable in cardiomyocytes. Co-localization of *mib2* RNA (cytoplasmic) and LacZ protein (nuclear) in embryos derived from the rP298 enhancer trap line (Nose et al., 1998), which carries a P-LacZ insertion within the *dumbfoundered (duf)* gene that is active in all founders (Ruiz-Gomez et al., 2000), confirmed that *mib2*...
expression is specific for founder myoblasts (Fig. 1 F). Accordingly, mib2 is not detected in Lame duck (Lmd)–positive fusion-competent cells (Duan et al., 2001; Fig. 1 G). Mib2 protein expression is identical to that of mib2 mRNA and appears to be in the cytoplasm of founder cells (Fig. 1, H–J). In contrast, mib1 expression is not detectable in mesodermal cells (unpublished data).

Identification of mib2 mutant alleles

Genetic and molecular analysis in the vicinity of the 37B10 locus identified the lethal complementation group l(2)37Be as a likely candidate for mib2 (CG17492; see Materials and methods). We obtained the two extant alleles, l(2)37Be1 and l(2)37Be4, for further analysis. Sequence analysis of the protein-coding exons showed that the mib2 gene on the l(2)37Be1 mutant chromosome contains a nucleotide change (C to T) that converts Gln377 to a Thr, which is then followed by a nonsense codon (Fig. 1 A and Fig. S1). On the l(2)37Be4 chromosome, a two-base pair deletion converts Asn587 to a Thr, which is then followed by a nonsense codon. As shown below, expression of wild-type mib2 in l(2)37Be1 mutant embryos can rescue the observed muscle phenotype. We conclude that the l(2)37Be1 and l(2)37Be4 alleles correspond to bona fide mib2 mutations and henceforth designate these alleles as mib21 and mib24, respectively. Based upon our analysis, the mutant Mib21 protein lacks all Ankyrin repeats and RING fingers while the mutant Mib24 protein lacks the RING fingers but retains four out of the predicted eight Ankyrin repeats.

mib2 mutant embryos exhibit detached muscles during later stages of embryogenesis

To assess the consequence of loss of mib2 function on muscle development, we stained wild-type and mutant embryos with an antibody against Myosin to visualize the muscle pattern. We focused more on the mib21 allele because the molecular nature of this mutation suggests that it is a stronger mutant allele. As compared with wild-type embryos, stage 15 mutant embryos (derived from mib21 germline clones and zygotically mib21/Df(2L)TW130, termed “mib2/m&z”), which lack both maternal and zygotic mib2 activity, have a well-developed somatic muscle pattern that is characterized by a massive number of detached muscles (Fig. 2 E and F; orange-yellow signals) occurs before it detaches and shrinks after stage 16 (G–H). Occasional red signals come from longitudinal gut muscles.

Figure 2. mib2 is required for maintaining the attachment and integrity of somatic muscles. Muscle phenotypes were analyzed in embryos that were derived from homozygous mib21 mutant germ line clones and have the zygotic genotype of mib21/Df(2)TW130 (except for embryo in D, which is zygotically mib21/SM6 wg-lacZ). Embryos in E–H also carry P5053A-Gal4 and UAS-lacZ on the Df(2)TW130 chromosome to highlight muscle 12 [V11]. Color codes for probes are indicated on the left. (A) At stage 15, a few somatic muscles lacking functional Mib2 are detached and rounded up (arrowheads), whereas the majority of muscles are still normal. (B) At stage 16, most somatic muscles (except for some LT and DO muscles, arrows) are rounded up and decreased in size (arrowheads). (C) At stage 17, only small remnants of rounded somatic muscle syncytia remain. (D) Normal somatic muscle pattern in germ line–derived embryo, which is zygotically heterozygous for mib2, shows that maternal products are not required in the presence of functional zygotic mib2. (E–H) Normal attachment and morphogenesis of muscle 12 (E and F; orange-yellow signals) occurs before it detaches and shrinks after stage 16 (G–H). Occasional red signals come from longitudinal gut muscles.

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To analyze the temporal progression and cause of the observed muscle phenotype, we recombined P5053A-Gal4, which is a common marker for muscle 12 (or VL1) development (Ritzenthaler et al., 2000; Swan et al., 2004), and UAS-LacZ onto the mib2 chromosome. Wild-type and mutant embryos were double-labeled for Tropomyosin and LacZ expression. At late stage 14, the somatic musculature of mib2 mutant (m&z) embryos, including muscle 12, which is in the final stages of establishing its normal attachments, looks normal (Fig. 2 E). A low degree of muscle detachment becomes detectable at stage 15, although muscle 12 does not seem to be affected immediately, suggesting some differences in susceptibility to loss of mib2 function among the various muscles (Fig. 2 F). However, massive muscle detachments and degeneration, which include muscle 12, occur by stage 16 (Fig. 2 G and H). In the aggregate, this analysis indicates that mib2 function is not needed for the formation of somatic muscles and their initial attachment to tendon cells, but rather it is required at late embryogenesis for maintaining the attachments and the integrity of the mature musculature.

Loss of mib2 function does not interfere with the localization of known muscle attachment components

Because of the muscle detachment phenotype, we examined whether loss of mib2 function disrupts the localization of integrin signaling components that are known to establish stable muscle/tendon attachments (for review see Bokel and Brown, 2002). In mib2 (m&z) embryos, αPS2 integrin localizes normally to the attachment sites within the tips of muscle 12 before and during the early stages of their detachment (compare Fig. 3, A and B with Fig. 3 D). Hence, the gradual disappearance of localized αPS2 integrin during later stages (Fig. 3 C) is presumably a consequence of the muscle detachments and deterioration rather than a cause of the detachment. Likewise, all other integrin pathway components examined, including Talin (Fig. 3, E and F), Pinch (not depicted), ILK (Fig. 3, G and H), and Tyr397-phosphorylated FAK (Fig. S3, C and D) are initially localized normally within the muscle ends near the attachment sites in the absence of mib2 activity. Several components on the epidermal side of the attachments were also unaffected (unpublished data). These observations argue against a function of mib2 in establishing stable muscle attachments via integrin signaling components unless there is a yet unknown parallel pathway to ILK that is affected.

Absence of mib2 function triggers apoptosis in muscles

As shown in Fig. 4 A, Mef2-positive nuclei are still present in the large rounded muscles of zygotic (z) mib2 mutant embryos at stage 16, whereas they are absent in the rounded muscles that are severely decreased in size. Because cell shrinkage and chromatin deterioration are hallmarks of apoptosis, we used TUNEL staining to detect apoptotic cells in mib2 mutant embryos. Indeed, the detaching muscles in mib2 (z) embryos are positive for the apoptotic marker (Fig. 4 B), whereas heterozygous control embryos only show apoptotic signals in the CNS and other nonmuscle tissues (Fig. 4 C). Of note, the detached muscles in myospheroid (mys) mutant embryos, which lack functional β-integrin at their attachment sites, do not show any significant apoptotic signals and do not shrink, indicating that apoptosis is
not an automatic consequence of muscle detachment (Fig. 4 D). Hence, we propose that the muscle detachment in \textit{mib2} mutants is a consequence of apoptotic events in these muscles.

To test this proposal further, we blocked apoptosis through forced expression of the caspase inhibitor p35 in muscle founders and their derived muscles. Blocking apoptosis in muscles of \textit{mib2} \textit{(z)} mutant embryos leads to a significant reduction of muscle detachment and deterioration at early stage 16 (compare Fig. 4 F with Fig. 4 B; no effects are seen with analogous expression of p35 in a wild-type background [Fig. 4 E]). At late stage 16, some muscle degeneration still occurs in the p35-overexpressing mutant embryos, as evidenced by the slightly larger number of rounded muscles with decreased sizes and missing muscle fibers, although it is much less severe than in \textit{mib2} \textit{(z)} mutants without blocked apoptosis (Fig. 4 G). A large number of muscle fibers are still present at stage 17 in these apoptosis-blocked \textit{mib2} \textit{(z)} mutant embryos (unpublished data). Notably, the muscle degeneration phenotype is rescued in \textit{mib2} \textit{(z)}
mutant embryos that are also homozygous for Df3R(H99), which deletes the apoptosis inducers reaper, hid, and grim, (Fig. 4 H). In this background the majority of the muscles appear normal until at least late stage 16, although we do not know whether they change their morphology after cuticle formation. Together, these observations suggest that muscle detachment and degeneration in mib2 mutants are largely a consequence of triggered apoptosis.

Ubiquitin ligases in the Notch pathway cannot substitute for Mib2 during muscle development

Because RING fingers are implicated in protein ubiquitination, we sought to test whether the RING fingers of Mib2 are required for its activity and whether Mib1 and Neuralized (Neur), E3 ubiquitin ligases that have been shown to be modulators of the Notch pathway by ubiquitinating Delta (Lai and Rubin, 2001; Pavlopoulos et al., 2001; Lai et al., 2005; Le Borgne et al., 2005; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005), could substitute for Mib2. Overexpression of full-length Mib2 in muscle founders and the derived muscles of wild-type embryos does not affect the pattern and stability of the muscles, although there is an increased number of unfused myoblasts at late stages (Fig. 5 A). In the mib2 mutant background, forced expression of full-length Mib2 leads to essentially complete rescue of the muscle detachment and deterioration phenotype (compare Fig. 5 B with Fig. 5 D), although an excessive number of unfused myoblasts is also evident. Notably, forced expression of a Mib2 version lacking both RING fingers (Mib2ΔRF; see Fig. 1 A) in the mutant background also allowed a significant, albeit incomplete, rescue of the muscle defects. In these embryos there is only occasional detachment of muscles and very few signs of apoptotic decay, although the muscles sometimes appear shorter and thicker as compared with normal muscles (compare Fig. 5 C with Fig. 5, A and D). Analogous overexpression of this mutated Mib2 version in a wild-type background does not have any effects on muscles (unpublished data). Based upon the significant degree of rescue with Mib2ΔRF, we conclude that the RING fingers have a less prominent role in promoting muscle integrity as compared with the other domains, and that ubiquitination may not be the main activity of Mib2 that is required for muscle development.

In sharp contrast to full-length Mib2 and Mib2ΔRF, Mib1 and Neur are not able to confer any rescuing activity under similar experimental conditions (compare Fig. 5, E and F with Fig. 5 D), suggesting that Mib2 possesses important targets that are different from those of Mib1 and Neur, and that blocked Notch signaling is not the cause of the observed muscle defects in mib2 mutant embryos. This latter point is underscored by our results from experiments with the Nts allele, which never yield any embryos with muscule phenotypes that are similar to those of mib2 mutants (unpublished data; see also Fuerstenberg and Giniger, 1998). However, we do not exclude the possibility that Mib2 can act in the Notch pathway in other contexts, such as in post-embryonic tissues, which we have not yet examined. It has been shown in cell culture that vertebrate Mib2 is capable of ubiquitinating...
Delta and Jagged, and Mib2 was also identified as a binding partner of *Drosophila* Delta in yeast two-hybrid screenings (Formstecher et al., 2005; Koo et al., 2005; Takeuchi et al., 2005; Zhang et al., 2006).

In summary, Mib2 appears to have a unique and Notch-independent role in “protecting” differentiated body wall muscles from entering apoptosis, undergoing detachment, and being subject to degradation. We speculate that Mib2 is required in a yet undefined pathway for the establishment of specific functional features of the sarcomeres or other structures of the myofibers. In this context, it is interesting to note that mouse Mib2 (also known as skeletrophin) was identified as a binding partner of α-actin and is expressed in skeletal muscles (Takeuchi et al., 2003). The disruption of these unknown structural and functional features in the absence of *mib2* activity could become detrimental upon stimulation of contractility and trigger entry into apoptosis. Apoptotic degradation of multiple components of the muscle fibers could first result in detachment because the contractile force renders muscle attachment more sensitive to disruptions, and leads to the degradation of the entire syncitia. The identity of the functional target(s) of *mib2* in muscles is currently unknown, as the expression of all markers examined to date, including founder cell markers (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200708135/DC1), muscle attachment proteins (Fig. 3), and differentiation markers (Fig. 3; Fig. 4; and unpublished data), is unaffected in *mib2* mutant embryos. Future studies, including the identification of interaction partners or mutations in other genes with similar phenotypes, will help to elucidate the pathway in which Mib2 acts to protect muscle integrity. Whether this pathway is involved in preventing skeletal muscle atrophies in which caspase-3 activation contributes to the breakdown of actomyosin complexes of myofibrils (Du et al., 2004) could also be explored.

### Materials and methods

**Drosophila stocks**

*mibs*1 ([Digan et al., 1986], UAS-p35 [made by Bruce Hay, CalTech, Pasadena, CA; Zhou et al., 1997]), P5053A+Gal4 ([Lopez, 1998], P[neoFR]40A ([Xu and Rubin, 1993], UAS-lacZ [Bud-1; Brand and Perrimon, 1993]), CyO w*111*,LacZ ([Kassis et al., 1992], SM6 eve-LacZ8.0 ([Panzer et al., 1992]), TM2 P[Gal80]C40.15S ([Bellen et al., 1989], Df(3R)1TJW30 ([Wright et al., 1976], and the alleles (2)37Be1 and (2)37Be8 ([Stathakis et al., 1995]), which were induced by EMS and EMS plus γ-rays, respectively, were obtained from the Bloomingtom *Drosophila* stock center. Other fly stocks used include: *mib1* ([Panzer et al., 1992], UAS-mib1-3 ([Zervas et al., 2001]). To generate *UAS-mib2* and *UAS-mib2ARF* transgenic lines, we subcloned the regions coding for amino acids 1-1,050 and amino acids 1-907, respectively, from the EST clone LP14687 (obtained from the Berkeley *Drosophila* Genome Project/BDGP) into the pUAST vector and injected the resulting constructs into *Drosophila* embryos by using standard protocols. Multiple independent insertions were obtained and analyzed for each construct.

**Identification of *mib2* mutants**

The *mib2* gene maps genetically at 37B10, a genomic region that was characterized genetically and molecularly in the context of the *Dopa decarboxylase* gene (Stathakis et al., 1995). By comparing the data of Stathakis et al. (1995) with those from the BDGP, we determined that *mib2* is uncovered by the overlapping deficiencies Df(2L)UOD15, Df(2L)H4UC1, and Df(2)ITW130 ([Wright et al., 1976]). Based upon additional molecular and genetic mapping data, we identified a complementation group, (2)37Be, of originally five embryonic lethal alleles as the most likely candidate for the *mib2* gene ([Stathakis et al., 1995; Adams et al., 2000]). The supporting evidence includes: (1) genomic rescue experiments done by Stathakis et al. (1995) with a construct, which according to our analysis contains *mib2* and a neighboring gene called *catup* ([1/237Be]C, rescued the lethality of (1/2)37Be and *catup* mutations; and (2) one allele, (1/2)37B*, which no longer exists, was shown to be associated with an ~800-bp deletion of sequences that we now have identified as being part of the *Drosophila* *mib2* gene.

**Sequencing of *mib2* from wild-type and mutant alleles**

EST clone LP14687 was fully sequenced and the derived *mib2* ORF was identical to that of CG17492. For allele sequencing, the alleles were balanced with a “blue balancer.” Fixed embryos were stained with an antibody against β-galactosidase, and homozygous (1/2)37Be* or (1/2)37Be8 mutant embryos were identified by the absence of LacZ expression from the “blue balancer.” Hand-picked embryos of the appropriate genotype were incubated in a solution of 10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, and 200 μg/ml proteinase K. Amplified products were purified and subjected directly to automated sequencing. Specific primers were used for sequencing all exons and exon-intron boundaries. For confirmation, the fragment that contained a protein sequence aberration was reamplified from genomic DNA and resequenced. The mouse *Mib2* sequence data are based on our sequencing of the cdna clone IMAGE:6517673.

**Rescue (and overexpression) experiments**

For rescue and overexpression experiments the following stocks were generated and used: UAS-mib2full Length[Fl1] UAS-mib2ARF[RF2], rP294-Gal4; mib2+/SM6, eve-LacZ8.0. mib2+/SM6, eve-LacZ8.0; UAS-mib2-1. mib2+/SM6, eve-LacZ8.0; UAS-mib2ARF-2. mib2+/CyO, w1118, LacZ, UAS-mib1-3. mib2+/SM6, eve-LacZ8.0; UAS-p35. mib2+/SM6, eve-LacZ8.0; Df3R)H99/TM2. P[Gal80]C40.15S. mib2+/SM6, eve-LacZ8.0; UAS-neur14.2.

**Generation of *Drosophila* Mib2 antibodies**

pET30-Mib2(COOH) was generated by cloning the PCR fragment that code for amino acids 650-1,038 of Mib2 in frame with the 6xHis tag of *E. coli* and purified with Ni2+-affinity chromatography under denaturing conditions (QiAGEN). Antiserum production in guinea pigs was done by Covance Research Products and affinity purification was performed against bacterially expressed 8-tag (Novagen)/Mib2 fusion protein.

**In situ RNA hybridization and immunocytochemistry of whole-mount embryos**

The embryonic CG17492 mRNA expression was first described in the BDGP in situ hybridization database ([Tomancak et al., 2002]). We confirmed and extended these expression data with the use of a digoxigenin-labeled *mib2* RNA probe that was generated by using the LP14687 cdna clone and published protocols (Tautz and Pfeifle, 1989). Embryos were photographed with Nomarski DIC optics on a microscope (Ax70; Olympus) with a 20× Uplan Fl/0.5 NA objective and a color camera (5.0 RTV; QImaging). Images were acquired with QImaging software and processed with Adobe Photoshop.

Immunocytochemistry was performed essentially as described (Reim et al., 2003) and the TSA amplification system was used as needed. Cy3 and FITC were used as fluorochromes. Embryo stainings were analyzed on a confocal microscope (TCSSP 4D; Leica) with a HC Plan Apo20×/0.7 NA and a HCX Plan Apo40×/0.75–1.25 NA oil objective at 20°C. Generally, Z-scans were taken at 1-1.5 μm steps and four to eight Z-scans were merged via maximum projection using the Leica TCS software package or Adobe Photoshop CS. Except for the final adjustment of contrast and brightness with Adobe Photoshop CS, no other processing of the imaging was performed.

Antibodies were used as follows: mouse anti-β-galactosidase ([1:100] developed by J. Sanes, Washington University, St. Louis. MO, and obtained through DSIB, Iowa University, Iowa City, IA), rabbit anti-β-galactosidase ([1:1,500] ICBM, Chicago, IL), anti-α-Tubulin ([1:200], Babraham Lab), mouse anti-Myosin ([1:200], Kiehart and Feghali, 1986), rabbit anti-Mef2 ([1:700], Bour et al., 1993), rat anti-Mps5 ([1:10], TSA; Wilcox et al., 1991), rabbit and mouse anti-Talin ([1:200], TSA, and 1:20, TSA, respectively; Brown et al., 2002), rabbit anti-FinCh ([1:15,000], TSA; Clark et al., 2003), rabbit anti-FAK([pY397] ([1:300], Biocare International); and rabbit anti-β-GFP ([1:100,000], TSA, Molecular Probes). Biotinylated ([1:200], Vector Laboratories) and fluorescent ([1:100], Jackson ImmunoResearch Laboratories) secondary.
antibodies were also used. The Apoptag kit [Intergen] was used for detecting apoptotic cells as described in Reim et al. (2003).

Online supplemental material

Figure S1 shows protein sequence alignment of D. mel. Mib2 with D. mel Mib1 and mouse Mib2. Figure S2 shows absence of any effects of mib2 mutation on muscle founder marker expression. Figure S3 shows gut phenotype and phospho-FAK staining in mib2 mutant embryos. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708135/DC1.

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