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# The *muscleblind* Gene Participates in the Organization of Z-Bands and Epidermal Attachments of *Drosophila* Muscles and Is Regulated by *Dmef2*

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**We report the embryonic phenotype of *muscleblind* (*mbl*), a recently described *Drosophila* gene involved in terminal differentiation of adult ommatidia. *mbl* is a nuclear protein expressed late in the embryo in pharyngeal, visceral, and somatic muscles, the ventral nerve cord, and the larval photoreceptor system. All three *mbl* alleles studied exhibit a lethal phenotype and die as stage 17 embryos or first instar larvae. These larvae are partially paralyzed, show a characteristically contracted abdomen, and lack striation of muscles. Our analysis of the somatic musculature shows that the pattern of muscles is established correctly, and they form morphologically normal synapses. Ultrastructural analysis, however, reveals two defects in the terminal differentiation of the muscles: inability to differentiate Z-bands in the sarcomeric apparatus and reduction of extracellular tendon matrix at attachment sites to the epidermis. Failure to differentiate both structures could explain the partial paralysis and contracted abdomen phenotype. Analysis of *mbl* expression in embryos that are either mutant for *Dmef2* or ectopically express *Dmef2* places *mbl* downstream of *Dmef2* function in the myogenic differentiation program. *mbl*, therefore, may act as a critical element in the execution of two *Dmef2*-dependent processes in the terminal differentiation of muscles.** © 1998 Academic Press

***Key Words: muscleblind;* Z-bands; muscle attachments; myogenesis; *Drosophila.***

## INTRODUCTION

Skeletal muscle fibers are highly specialized cells formed during development by the fusion of myoblasts. These fibers form in reproducible patterns, have a prominent intracellular filament system (the contractile apparatus), interact selectively with other tissues (i.e. the body skeleton and the nervous system), and contribute to the formation and function of cell junctions such as the neuromuscular junction (NMJ). The development of such complex cells and their arrangement into a functional pattern requires a coordinated gene regulatory cascade.

In *Drosophila,* specification of somatic muscles, the invertebrate counterpart of skeletal muscle fibers, begins by the end of gastrulation when uniformly high levels of Twist become modulated in a segmentally repeated fashion. High

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levels of Twist propel cells into somatic myogenesis whereas low levels of Twist allow for formation of other mesodermal tissues (Baylies and Bate, 1996). Subsequent genetic interactions involving proneural and neurogenic genes and signals from the overlying ectoderm, lead to the precise specification of muscle founder cells within the high Twist domain (Corbin *et al.,* 1991; Bate *et al.,* 1993; Baylies *et al.,* 1995; Carmena *et al.,* 1995). Each muscle founder cell gives rise to one individual muscle that inserts at particular points on the epidermis and establishes contacts with specific motorneurons (Bate, 1990; Rushton *et al.,* 1995). Acquisition of these individual properties depends on regulatory genes such as *Krüppel, S59* and *apterous* which are expressed in subsets of body wall muscles (Bate, 1993). Mutations in *Krüppel,* for example, affect the shape and innervation pattern of particular muscles. These misspecified cells in *Krüppel* mutants, however, can still carry out a differentiation program and acquire their functional properties as muscles (Ruiz-Gómez *et al.,* 1997). *Drosophila* myogenesis, therefore, can be understood as two distinguishable processes, a general differentiation program that leads to ubiquitous muscle traits such as the contractile apparatus, and a specific pathway that conditions the general differentiation to produce distinctly different muscles.

General muscle differentiation requires the construction of three critical structures: the contractile apparatus that generates force, the muscle attachments that transmit the force onto the body skeleton, and the NMJs that control contraction. So far, little is known regarding the genetic mechanisms that control and coordinate assembly of these structures in time and space. One key regulator of muscle differentiation is *Dmef2*, a member of the MEF2 family of transcription factors implicated in activation of muscle specific gene expression in *Drosophila* and vertebrates (Taylor, 1995; Molkentin and Olson, 1996). Analysis of *Drosophila* embryos which are null for *Dmef2* function shows that although specification of muscle founder cells and reception of specific innervation is essentially correct, myoblast fusion, assembly of the contractile apparatus and formation of junctions at muscle attachments and synapses at nerve muscle contacts fail (Bour *et al.,* 1995; Lilly *et al.,* 1995; Prokop *et al.,* 1996). Accordingly, expression of structural components of the muscles such as Myosin and Tropomyosin I is severely reduced in *Dmef2* mutants (Bour *et al.,* 1995; Lilly *et al.,* 1995; Lin *et al.,* 1996). Interestingly, genetic data suggest that the various *Dmef2* dependent properties of differentiated muscles are regulated independently from each other. For example, *myoblast city* mutant embryos lack myoblast fusion while other *Dmef2*-dependent myogenic differentiation features develop normally, such as neuromuscular synapse formation or Myosin expression (Rushton *et al.,* 1995; Prokop *et al.,* 1996). Similarly, junction formation at muscle attachments and assembly of the contractile apparatus (Z-bands) is affected in embryos lacking PS Integrins, but myoblast fusion, Myosin expression and neuromuscular synapse formation are unaffected (Volk *et al.,* 1990; Brown, 1993; Prokop *et al.,* 1998). Hence, although *Dmef2* is required as a generic element for muscle differentiation, a working hypothesis consistent with the published data is that *Dmef2* carries out its function by activating a variety of parallel, independent pathways (e.g. those involving *myoblast city* or PS integrins), each of which contributes certain properties to the differentiating muscle.

Here, we provide evidence in support of the existence of such independent pathways in the execution of the *Dmef2* dependent properties of muscles. *mbl* encodes several protein isoforms containing a common N-terminus and at least one copy of a $Cys_3His$ motif, similar to the TIS11/NUP475/TTP zinc finger family of proteins. Studies in the *Drosophila* eye have shown that *mbl* participates in the terminal differentiation of the ommatidial photoreceptors (Begemann *et al.,* 1997). We report that Mbl is localized in nuclei of embryonic pharyngeal, visceral and somatic muscles, in the larval photoreceptor system, and in cells within the central nervous system (CNS). We show that muscle expression of *mbl* is regulated by *Dmef2.* In addition, *mbl* function is required for two *Dmef2* dependent features of differentiating muscles, formation of Z-bands and tendons at muscle attachment sites. Our data are consistent with *mbl* being a regulatory factor required for terminal muscle differentiation acting downstream of *Dmef2.*

## MATERIALS AND METHODS

### *Drosophila* Stocks

We used the mutant fly stocks *Dmef2*$^{22.21}$ (Bour *et al.,* 1995), *mbl*$^{E27}$ and *mbl*$^{E2}$ (Begemann *et al.,* 1997), and *mbl*$^{E16}$, which is an imprecise lethal excision from the P-element insertion l(2)01038, approximately 4 kb downstream of exon 3 (Spradling *et al.,* 1995; Begemann *et al.,* 1997). Transgenic fly stocks carrying the *daughterless-GAL4* (Wodarz *et al.,* 1995), *engrailed*-GAL4 (generous gift from A. Brand) and the *UAS-Dmef2* fusion genes (Bour *et al.,* 1995) were used for ectopic expression of Dmef2 in the epidermis. Mutant stocks were balanced over *CyO* marked with *wg*$^{en11}$, which gives $\beta$-Galactosidase expression in the pattern of *wingless* in the non-mutant embryos (Kassis *et al.,* 1992).

### Immunohistochemical Methods

Immunocytochemical staining of embryos was carried out following standard techniques for whole mounts (Rushton *et al.,* 1995) or for flat preparations (Broadie and Bate, 1993). To detect the *mbl* proteins, we used a polyclonal antibody generated against the Mbl A isoform (rabbit; 1:300; Begemann *et al.,* 1997). As all Mbl isoforms share the amino termini, this antiserum should detect all four *mbl* proteins. Additionally, we used: 1) Anti-Myosin (rabbit, 1:1000; Kiehart and Feghali, 1986), 2) anti-Synaptotagmin (rabbit, 1:1000; Littleton *et al.,* 1993), 3) anti-Kettin (rat, 1:250; flat preparations were treated with 1 mg/ml CollagenaseIV for 30 seconds; Lakey *et al.,* 1993), 4) anti-$\beta_{PS}$ Integrin ascites (1:200 and 1:500; Brower *et al.,* 1984), 5) anti-Tiggrin (mouse, 1:500; Fogerty *et al.,* 1994), and 6) anti-$\beta$-Galactosidase (rabbit, 1:9000; Cappel). Biotinylated secondary antibodies were used in combination with the Vectastain Elite ABC kit (Vector Laboratories, CA). Specimens were embedded in Araldite. Flat preparations of late stage 17 embryos were dehy-

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drated and covered by Araldite on a slide, then cut off and embedded under a cover slip. Images were captured using the camera model DXC-970MD (Sony); alternatively, negatives were scanned with a Nikon Scan LS1000. Different focal planes were combined into one picture using Adobe Photoshop software.

### *In Situ Hybridization Methods*

*In situ* hybridization of embryos from the *daughterless-GAL4* × *UAS-Dmef2* cross and wild type embryos was done as described (Tautz and Pfeifle, 1989) using the *mblB* cDNA as probe (Begemann *et al.,* 1997). To assay the *mbl* transcription in the *Dmef2* mutant background, an antibody staining and *in situ* hybridization double labeling method was used (Taylor *et al.,* 1995). Homozygous mutant embryos were identified by the absence of β-Galactosidase staining.

### *Electron Microscopy*

Hourly egg lays were kept at 25°C until hatching. Unhatched embryos with contracted bodies were injected with 5% glutaraldehyde in 50 mM phosphate buffer, pH 7-7.2. Tips of injected specimens were cut off with a razor blade splinter, followed by 1 hour post-fixation in 2.5% glutaraldehyde in 50 mM phosphate buffer, brief wash in 50 mM phosphate buffer, post fixation for 1 hour in 1% osmium in $dH_2O$, wash in $dH_2O$ for 5 minutes, 30 minutes incubation in 2% aqueous solution of uranyl acetate, dehydration in alcohol and transfer to Araldite. Serial sections of 30-50 nm (silvergrey) thickness were obtained on a Reichert-Jung Ultracut, transferred to formvar-covered carbon-coated slot grids (Galey and Nilsson, 1966), post-stained with lead citrate for 5-10 minutes, and analyzed on a Jeol 200CX. Frontal serial thin sections were taken from abdominal segments at the anterior border of the denticle belts (indirect muscle attachments) or about 10-15 μm behind that region (direct muscle attachments). Oblique longitudinal sections of embryos were cut from the dorsolateral surface.

## RESULTS

### *muscleblind Is Expressed in the Visceral and Somatic Musculature of the Embryo*

We analyzed the embryonic pattern of *mbl* expression by *in situ* hybridization (not shown) and by antibody staining with an antiserum detecting all four *mbl* protein isoforms (see Materials and Methods; Begemann *et al.,* 1997). Both methods reveal expression of *mbl* in the same tissues, although appearance of protein is slightly delayed compared to RNA, probably due to the size of the transcription unit (>100 kb; Begemann *et al.,* 1997). The *mbl* protein is always detected in the nucleus, suggesting that it might play a gene regulatory role as proposed for other members of the $Cys_3His$ family of proteins (Fig. 1I; discussed in Begemann *et al.,* 1997).

Both *in situ* hybridization and antibody staining of wild type *Drosophila* embryos reveal expression of *mbl* in the ectoderm and mesoderm. Ectodermal expression is restricted to a segmentally repeated pattern of cells in the CNS and to Bolwig's organ, which contains the larval photoreceptors (Fig. 1G,H; Lee *et al.,* 1991). Expression in Bolwig's organ might reflect a role for *mbl* in the differentiation of larval photoreceptors, comparable to its function in adult photoreceptors (Begemann *et al.,* 1997). Conspicuous expression of *mbl* protein starts at late stage 11 in the mesoderm underneath the stomodeal invagination and continues as a barely visible signal throughout the rest of mesoderm (Fig. 1A). *mbl* becomes more abundant during germ band retraction after the mesoderm has subdivided into different derivatives (Bate, 1993). By the end of this stage, *mbl* expression is found throughout the somatic mesoderm (Fig. 1B). At stage 16, *mbl* expression is clearly restricted to a subset of mesodermal derivatives: It is not detected in heart (Fig. 1E), gonads, fat body, hemocytes or the dorsal midline cells above the ventral nerve cord (not shown). However, strong expression of *mbl* during late development occurs in the alary, pharyngeal, visceral and somatic musculature (Fig. 1C,D,F,H and not shown). This ubiquitous late expression of *mbl* in most muscle types, and particularly in the somatic musculature, is typical of genes involved in terminal muscle differentiation, like the genes coding for Myosin, PS2 Integrin or glutamate receptor (Kiehart and Feghali, 1986; Brown, 1993; Currie *et al.,* 1995).

### *muscleblind Mutant Embryos Have Normal Muscle Patterns and NMJs, but They Are Partially Paralyzed*

To uncover potential functions of *mbl* during myogenesis, we analyzed mutant embryos homozygous for the three strong alleles, $mbl^{E16}$(see Materials and Methods), $mbl^{E2}$ and $mbl^{E27}$ (Begemann *et al.,* 1997). Mutant individuals carrying any of these *mbl* alleles die as stage 17 embryos or during hatching and show no obvious defects in the formation of somatic and visceral muscles (Figs. 2A-D). Occasionally, a few muscles are absent in the homozygous mutant embryos, but no correlation with specific muscles has been found (not shown). Thus, *mbl* function is not essential for early muscle development and muscle specification.

However, at the end of embryogenesis $mbl^{E27}$, $mbl^{E2}$ or $mbl^{E16}$ homozygous or transheterozygous embryos are severely paralyzed and only twitching movements can be observed, and their segments are strongly contracted, especially in the abdominal region (Fig. 2E,F). Several defects in the differentiation of somatic muscles could lead to paralysis in the absence of *mbl*: (1) failure to differentiate a NMJ, (2) defects in the differentiation of the muscle-epidermis attachments, or (3) impairment in the contractile apparatus. We investigate these possibilities below.

Antibody stainings of *mbl* mutant embryos at late stage 17 with synapse-specific anti-Synaptotagmin antibodies (Littleton *et al.,* 1993) show that muscles bear NMJs which form normal swellings, called boutons (Fig. 3A,B). At the ultrastructural level, neuromuscular contacts in *mbl* mutant embryos show all morphological features characteristic of wild type contacts, i.e. normal embedding of the neuronal bouton into the muscle, a typical array of material in the extracellular gap, and normal synapses with presynaptic

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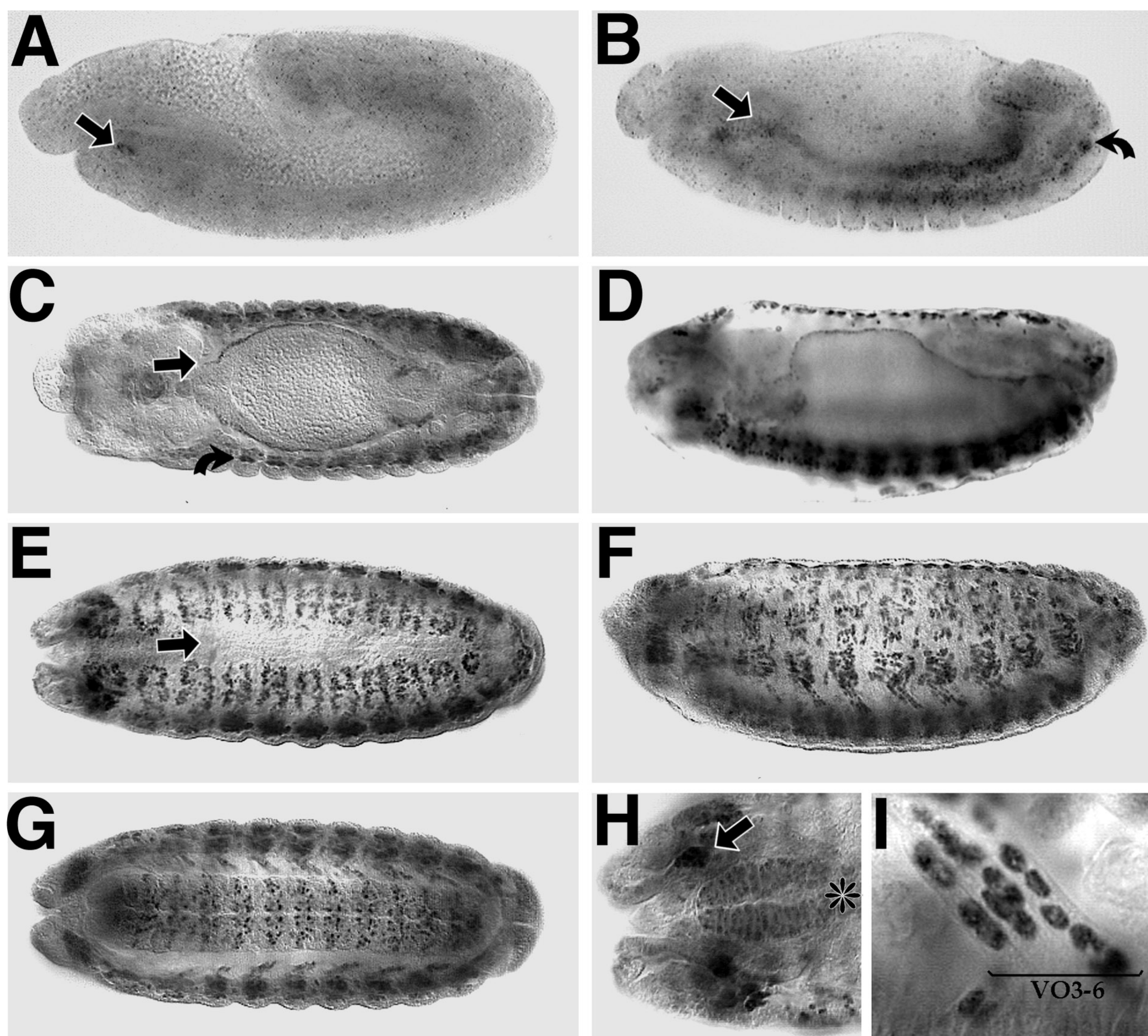


**FIG. 1.** *mbl* protein is nuclear and present in somatic, visceral, and pharyngeal musculature. Wild-type embryos were stained with an antiserum against *Drosophila mbl* protein (Begemann *et al.,* 1997). In all figures, embryos are oriented with anterior to the left, dorsal up, and stages given according to Campos-Ortega and Hartenstein (1985). (A) Lateral view, stage 11 embryo with Mbl expression in the cephalic mesoderm (arrow) and a barely detectable signal in the remainder of mesoderm. (B, C) Mbl expression restricted to visceral (arrow) and somatic mesoderm (bent arrow) in a lateral view of a late germ-band retracting embryo (B) and a dorsal view of a late stage 13 embryo (C). (D) Ventral–lateral view of an embryo at stage 15 showing expression in visceral and somatic musculature and CNS (out of focus). (E) Dorsal view, stage 16 embryo. Mbl expression is observed in repeating nuclear clusters of fused somatic mesodermal cells but not in the heart precursor cells (arrow). (F) Lateral view, stage 16 embryo. Mbl-positive clusters of nuclei in locations corresponding to all syncitial fibers of differentiating somatic muscles are shown. (G) Ventral view, stage 16 embryo. Mbl expression is also detected in the CNS in repeated clusters of cells. (H) High magnification of the embryo in E showing the expression of Mbl in pharyngeal muscles (asterisk) and Bolwig's organ (arrow). (I) High magnification of VO3-6 muscles showing individual nuclei expressing Mbl.

densities and clustered vesicles (Fig. 3C,D). Thus, NMJs in *mbl* mutant embryos can assemble normally.

### *muscleblind* Function Is Required for Tendon Matrix Deposition at Muscle Attachments

At the end of embryogenesis, wild type muscles connect to the epidermal cells via either direct or indirect muscle attachments (Figs. 4A,C; Prokop *et al.,* 1998). Direct muscle attachments, where muscle tips and epidermal cells stay in close contact and form connecting hemiadherens junctions (e.g. at muscles LT1-4; see Bate, 1993 for nomenclature; Tepass and Hartenstein, 1994), are not affected in *mbl* mutant embryos (Figs. 4A,B). Indirect muscle attachments are formed at the segment border (e.g. muscles VL1-4), where many muscle tips converge onto a limited amount of epider-

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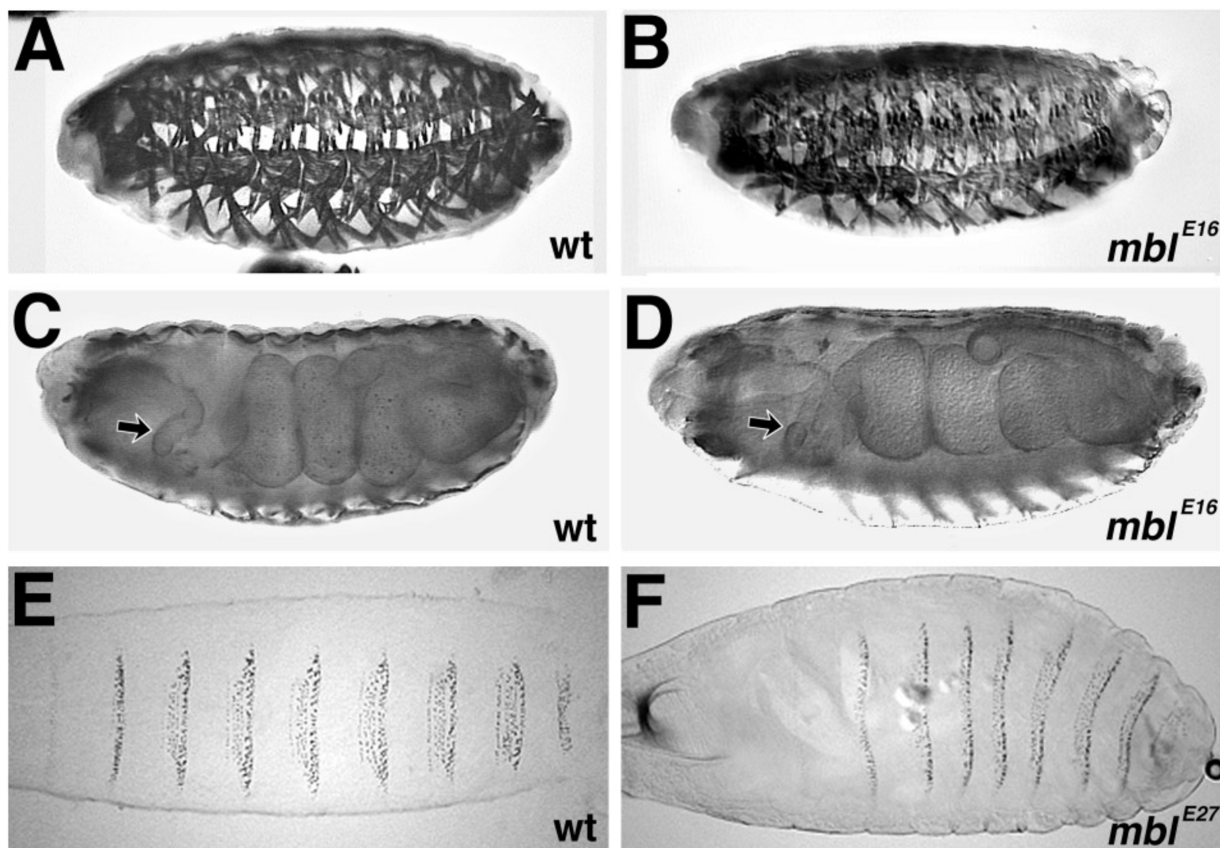


**FIG. 2.** Myosin heavy chain expression and cuticle preparations in wild-type and *mbl* mutant embryos. (A) Wild-type, lateral view stage 16 embryo showing the normal pattern of body wall muscles. (B) Same stage $mbl^{E16}$ homozygous embryo. The pattern of somatic musculature is essentially unaffected in the mutant. (C) Wild-type, lateral view stage 16 embryo showing the morphology of the visceral musculature (arrow). (D) $mbl^{E16}$ homozygous embryo at the same stage. The overall morphology of the gut (arrow) and time of appearance of the constrictions is indistinguishable from wild type. (E) First instar wild-type larva showing the normal pattern of ventral cuticle belts. (F) Same stage $mbl^{E16}$ mutant larva (unhatched embryo dechorionated) showing an essentially normal pattern of denticle belts and severe contraction in the abdominal segments.

mis. At indirect muscle attachments, muscle tips and epidermal cells are connected indirectly via tendon matrix (TM), an accumulation of extracellular matrix (ECM) specific to these sites that acts as a flexible spacer allowing numerous muscle tips to attach to a limited epidermal surface (Fig. 4C; Prokop *et al.,* 1998). In *mbl* mutant embryos, the extracellular TM is severely reduced, forcing the muscles to compete for epidermal surface at the segment border (Fig. 4D). Hence, indirect muscle attachments do not assemble properly and this defect may contribute to the contracted appearance and severe paralysis of late stage 17 *mbl* mutant embryos.

Given the effect on muscle attachments, we investigated the expression of two muscle attachment specific proteins in *mbl* mutant embryos, PS-Integrins, which are concentrated at muscle tips and are essential for maintenance of muscle attachments, and Tiggrin, a hemocyte and fat body derived extracellular component (Brown, 1993; Fogerty *et al.,* 1994). At late stage 17, both wild type and $mbl^{E27}$ mutant embryos show strong $\beta_P$**S** Integrin expression at all muscle tips and do not reveal any obvious defect at muscle attachments (not shown). Anti-Tiggrin antibody stainings (Fogerty *et al.,* 1994) also did not reveal obvious differences between wild type, $mbl^{E27}$ and $mbl^{E2}$ mutant embryos, consistent with the fact that a certain amount of TM can still be seen at the ultrastructural level. This remaining TM connects normally to muscle and epidermal surfaces via tendon hemiadherens junctions (Figs. 4E,F; Prokop *et al.,* 1997). We therefore suspect that reduction of TM in *mbl* mutant embryos is not due to failure of muscles to anchor to the matrix properly, but that components of the TM are muscle derived and require *mbl* function. This interpretation is consistent with the finding that *mbl* is not expressed in hemocytes, fat body, or epidermis, three further sources for ECM components, amongst them Tiggrin (Fessler and Fessler, 1989; Fogerty *et al.,* 1994; Murugasu-Oei *et al.,* 1995).

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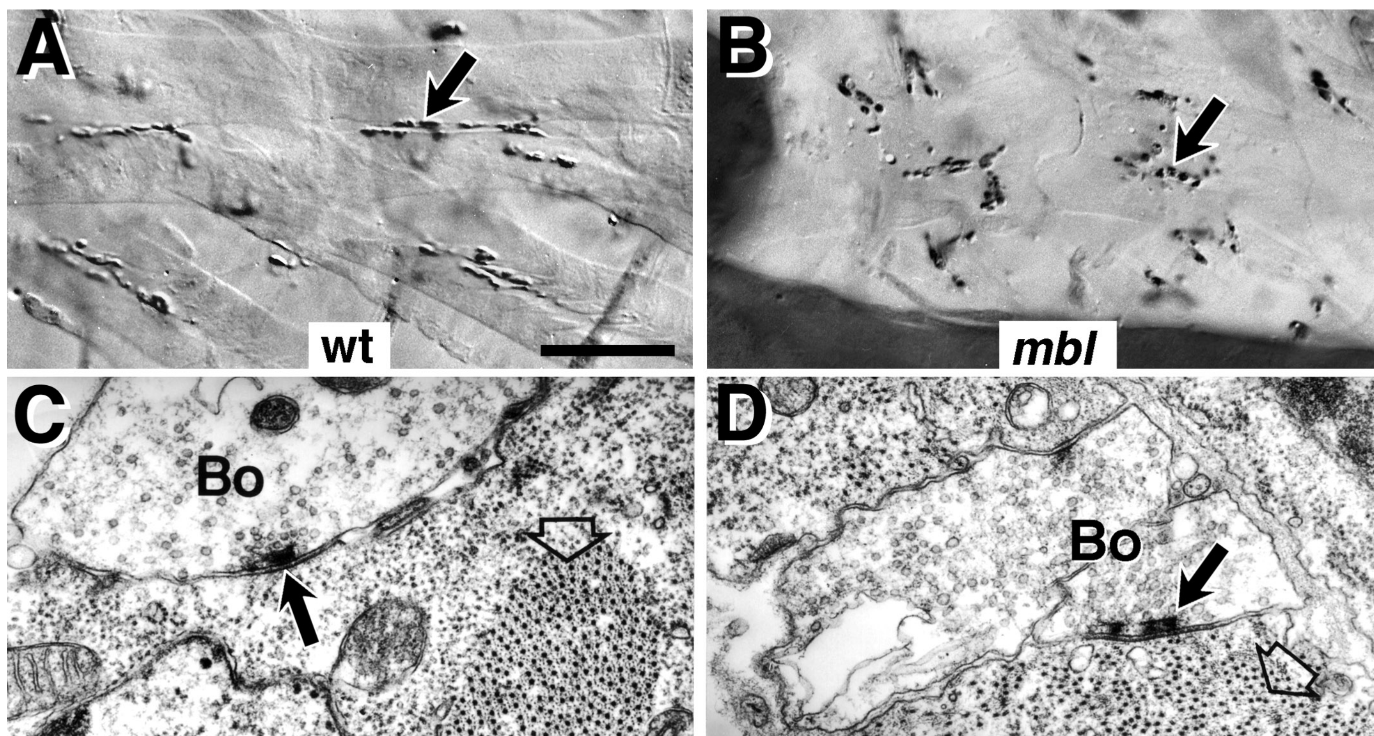


**FIG. 3.** *mbl* mutant NMJs are morphologically normal. (A, C) At wild type NMJ's motorneuronal termini form boutons, which can be identified by anti-synaptotagmin staining (arrow in A). (C) At the ultrastructural level the presynaptic boutons (Bo) form synapses (arrow in C; see Prokop *et al.,* 1996) with the postsynaptic muscle (open arrow indicates myofilaments). (B, D) In *mbl* mutant embryos, synaptotagmin appears in the right locations (arrow in B; note that muscles are contracted and therefore shape changes occur) and normal synapses are formed (arrow in D). Contractile filaments are less well ordered in *mbl* mutant embryos compared to wild type (open arrows). All specimens are late stage 17. Scale bar 20 $\mu$m in A and B and 560 nm in C and D.

### *muscleblind* Function Is Required for Z-Band Formation

We next investigated whether the partial paralysis of *mbl* mutant embryos is due to defects in the contractile apparatus of the musculature. At the ultrastructural level, the wild type contractile apparatus is composed of a dark A-band (containing intermingled thin and thick filaments) flanked by translucent I-bands (containing only thin filaments). Adjacent I-bands are separated from each other by electron dense Z-bands (Figs. 5A,C,E; Hardie, 1980). This regular organization of the contractile apparatus confers birefringent properties to the somatic muscles which, under polarizing light, show striation. In *mbl* mutant embryos, this striation is not apparent (not shown).

Ultrastructural analysis reveals that thick and thin filaments are correctly oriented in the A-bands of *mbl* mutant muscles. However, they appear less ordered and less densely packed than in wild type muscles and I-bands are always absent (Figs. 5B,D,F). Such a loss of I-bands occurs naturally when a wild type muscle is supercontracted (Hardie, 1980). Thus, muscles in *mbl* mutant embryos might be arrested in a supercontracted state, causing the body contraction observed in living *mbl* mutant embryos. Finally, the mesh-like electron dense matrix of Z-bands is completely absent and, instead, only spindle-like concentrations of dark thin fibers can be seen (Figs. 5D,F). The spindle-like fiber accumulations found in *mbl* mutant muscles might exist in normal Z-bands, however, they would be obscured by the mesh-like matrix, which interconnects and thus aligns them into one transverse line. Loss of this alignment due to lack of the mesh-like matrix in *mbl* mutant Z-bands might cause misalignment of sarcomeres and thus promote loss of both I-bands and striation under polarized light in addition to hypercontraction.

Kettin is a Z-band component expressed in somatic, visceral and pharyngeal muscles from approximately embryonic stage 13 onwards and stays evenly distributed (slightly concentrated at muscle tips) within the muscles until stage 17, when it arranges into regular transverse stripes (Fig. 5G). In *mbl* mutant embryos, Kettin is expressed in the musculature, however, it fails to arrange into stripes at stage 17 (Fig. 5H). Thus, *mbl* function is not required for Kettin expression but for its assembly into Z-bands.

### *Dmef2* Regulates *muscleblind* Where They Are Coexpressed

In *Dmef2* mutant embryos most aspects of terminal muscle differentiation fail, including formation of the contrac-

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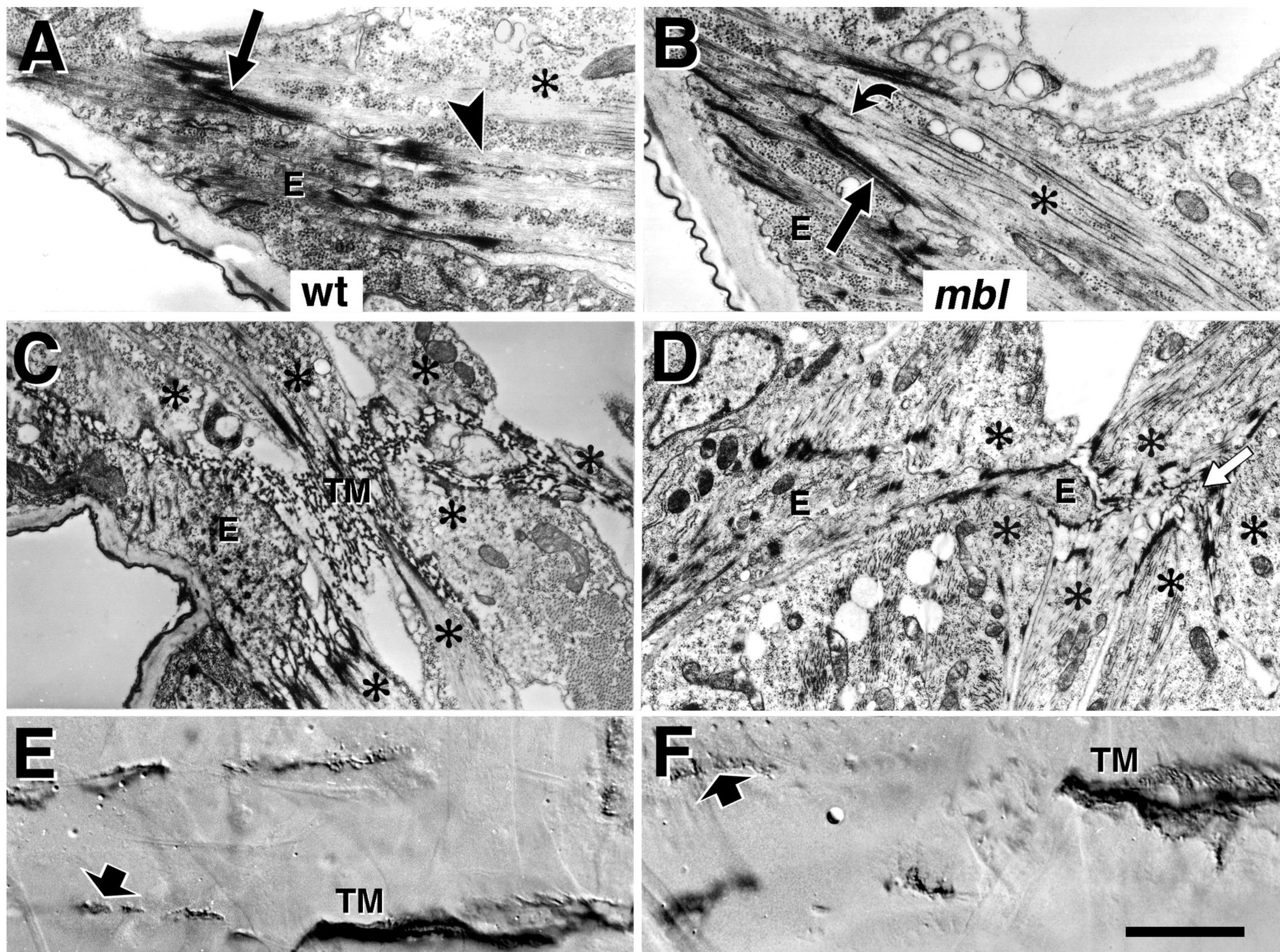


**FIG. 4.** TM is reduced at indirect muscle attachments in *mbl* mutant embryos. Muscles (*), epidermal cells (E), and tendon matrix (TM). (A) At wild-type direct muscle attachments the muscles attach to epidermal cells via connecting hemiadherens junctions (long black arrow; Prokop *et al.,* 1998) to which, in the muscle, thin filaments attach usually forming an I-band (arrowhead). (B) In *mbl* mutant embryos, connecting hemiadherens junctions appear normal (long black arrow), but thick filaments reach to the muscle tip (bent arrow), most likely due to hypercontraction. (C) At indirect muscle attachments numerous muscle tips are connected to few epidermal cells via TM. (D) At *mbl* mutant indirect muscle attachments, TM (white arrow) is severely reduced, and epidermal cells bulge into the body cavity, staying in close contact with muscles. (E, F) Antibody staining detects the ECM component Tiggrin at direct muscle attachments (flat arrows indicate ventral tips of VO muscles) and indirect muscle attachments (TM indicates area of VL muscle attachments) in both wild-type (E) and *mbl* mutant embryos (F). All specimens are late stage 17. Scale bar corresponds to 1 $\mu$m in A and B to 1.6 $\mu$m in C and D and 27 $\mu$m in E and F.

tile apparatus and TM deposition (Bour *et al.,* 1995; Lilly *et al.,* 1995; Prokop *et al.,* 1996 and A.P. observations). Given that *mbl* participates in at least two aspects of terminal differentiation of muscles, we examined *mbl* expression in embryos lacking *Dmef2* function by *in situ* hybridization (not shown) and with anti-Mbl antibodies (Fig. 6C). *mbl* expression in the CNS and Bolwig's organ is unaffected in *Dmef2* mutant embryos (Fig. 6C and not shown). As *Dmef2* is not expressed in these tissues at this point of development, they can be used as a positive control for the *mbl* staining. In contrast, all *mbl* positive muscle derivatives also express *Dmef2* (Lilly *et al.,* 1994; Bour *et al.,* 1995; Lilly *et al.,* 1995; Taylor *et al.,* 1995) and can therefore reveal a potential regulatory dependence. *Dmef2* mutant embryos at late stage 14 show strongly reduced *mbl* expression in somatic myoblasts and in the visceral and pharyngeal mesoderm (Fig. 6C and not shown). This reduction of *mbl* expression is not caused by cell death. *Dmef2* mutant myoblasts enter apoptosis late in embryogenesis (stage 15) due to their failure to differentiate properly (Bour *et al.,* 1995; Ranganay-

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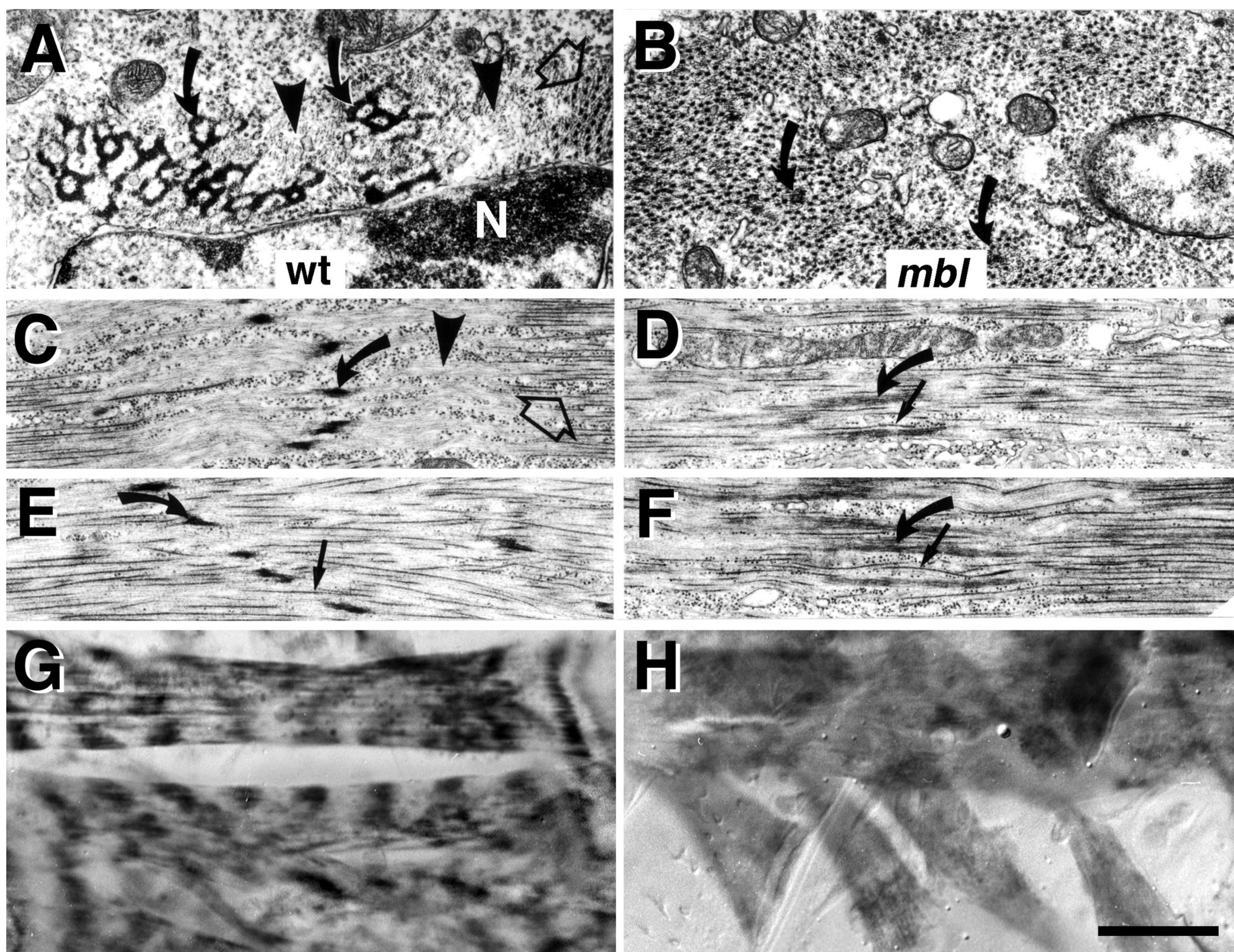


**FIG. 5.** Z-bands and I-bands are absent in *mbl* mutant muscles. (A) Transverse sections through wild-type muscles show A-bands (open arrow; compare Fig. 4C) with a densely packed pattern of thick filaments surrounded by thin filaments, electron dense reticulate Z-bands (bent arrows), and I-bands with only thin filaments (arrowheads); N indicates the nucleus. (B) *mbl* mutant embryos lack the I-band and electron dense matrix of Z-band; spot-shaped stippled areas (bent arrows) might represent remaining Z-band components. (C–F) Longitudinal muscle sections. (C) Relaxed wild-type muscle shows A-band with thick filaments (open arrow), I-band with only thin filaments (arrowhead), and Z-band in the form of electron dense spindles (bent arrow). (E) Contracted wild-type muscle shows Z-band (bent arrow), but no I-band; instead, thick filaments penetrate the Z-band (small black arrow). In $mbl^{E2}$ (D) and $mbl^{E27}$ mutant muscles (F) accumulations of electron dense thin filaments (bent arrows; corresponding to stippled areas in B) most likely represent components of the Z-band; the I-band is absent and thick filaments occur adjacent to potential Z-band structures (small arrows in D and F), comparable to hypercontracted wild-type muscle (small arrows in C). (G and H) Antibody stainings for the Z-band component Kettin reveal a banding pattern in the wild-type (G), but homogeneous distribution in *mbl* mutant muscles (H). All specimens are late stage 17. Scale bar corresponds to 1 $\mu$m in A to F and to 23 $\mu$m in G and H.

akulu *et al.,* 1995). However, we detect reduction in *mbl* expression before stage 15, and no invading macrophages, which are typically involved in the cell death process, can be seen at that stage (not shown).

The reduction in Mbl staining is also not due to myoblasts failing to fuse in the absence of *Dmef2*. As a control, we analyzed Mbl expression in *myoblast city* mutant embryos, in which myoblast fusion fails but other aspects of muscle differentiation occur normally (Rushton *et al.,* 1995; Prokop *et al.,* 1996). *myoblast city* mutant embryos show no reduction in the level of Mbl expression at similar stages (Fig. 6B). Therefore, Mbl expression in the mesoderm requires *Dmef2* function. Some Mbl expression, however, remains even in the null allele $Dmef2^{21.21}$, comparable to the levels

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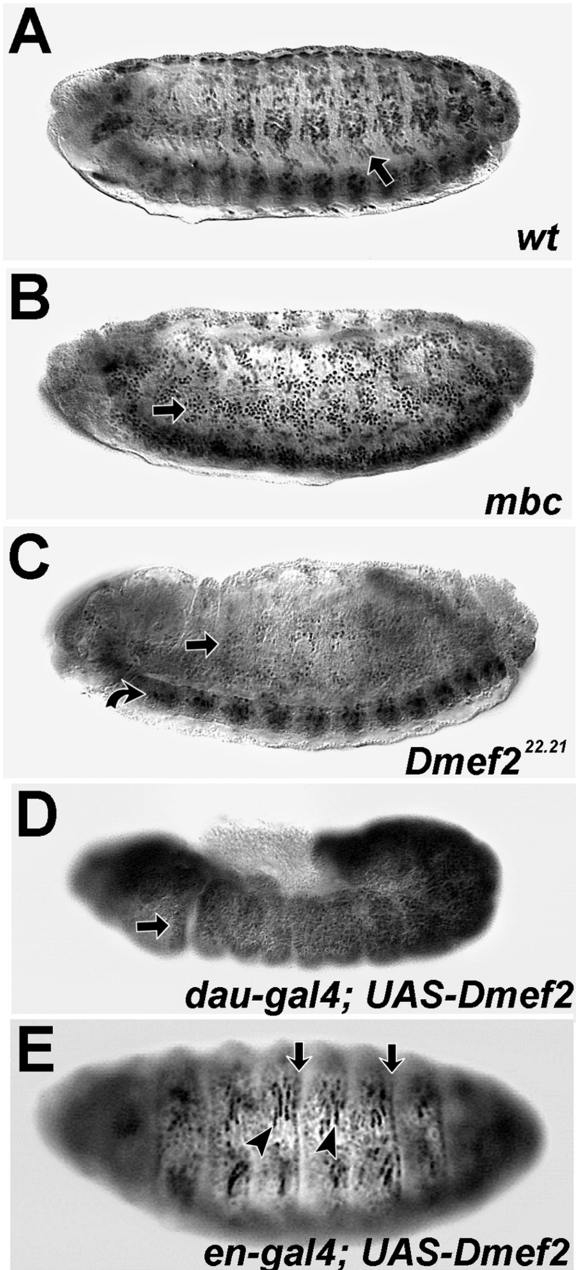


**FIG. 6.** *mbl* expression in different genetic backgrounds. All views are lateral. (A) Wild-type, stage 15 embryo showing the normal arrangement of muscle fibers. (B) Stage 15, homozygous *myoblast city* mutant embryo characterized by the absence of fusion among myoblasts (Rushton *et al.*, 1995). Mbl expression is normal in the unfused myoblasts. (C) Late stage 14, homozygous *Dmef2*$^{22.21}$ mutant embryo (Bour *et al.*, 1995) showing approximately normal levels of Mbl expression in the CNS (bent arrow) and a severe reduction in the levels in cells of somatic musculature (arrow). The visceral mesoderm and pharyngeal muscles show a similar reduction. (D) *daughterless-GAL4* driven expression of *UAS-Dmef2* (Bour *et al.*, 1995) in the epidermis of embryos leads to the ectopic expression of Mbl throughout this tissue (arrow). (E) *engrailed*-GAL4 (en-gal4) driven expression of the same UAS construct in the posterior epidermal cells of each embryonic segment leads to the ectopic expression of Mbl in this tissue (arrows). Arrowheads designate the normal expression of Mbl in the lateral muscles which are in the same optical focal plane.

of Myosin and Tropomyosin I expression which remain in *Dmef2* null mutant embryos (Lin *et al.*, 1996; Prokop *et al.*, 1996). These observations suggest that there are other regulators of Mbl, Myosin or Tropomyosin I, in agreement with the current model that MEF2 family members cooperate with other transcription factors such as bHLH-containing proteins to regulate gene expression (Molkentin and Olson, 1996).

To test the regulation of *mbl* by *Dmef2* further, we used the Gal4 targeted expression system (Brand and Perrimon, 1993) to express *Dmef2* ectopically in the embryonic epidermis. *mbl* is never detected in the epidermis in wild type embryos. Cuticle preparations of *mbl* homozygous embryos show no obvious defects, suggesting that *mbl* function is not required in this tissue (Figs. 2E,F and not shown). *In situ* hybridization and antibody staining of embryos with ectopic epidermal *Dmef2* in the *daughterless* or *engrailed* expression pattern clearly show induction of *mbl* in this tissue (Figs. 6D,E and not shown). Thus, normal expression of *mbl* in the mesoderm is dependent on *Dmef2* and ectopic *Dmef2* expression is sufficient, at least in the epidermis, to induce ectopic *mbl* expression. Similar regulation by *Dmef2* has been demonstrated for other terminal differentiation genes like Tropomyosin I, $\beta_3$-Tubulin, and $\alpha_{PS2}$ Integrin (Lin *et al.*, 1997). Interestingly, Kettin expression (which is unaffected in *mbl* mutant embryos) is also reduced in *Dmef2* mutant embryos and can be ectopically induced in the epidermis by misexpression of Dmef2 (not shown), indicating that Z-band formation is not regulated by *mbl* alone but requires other *Dmef2* dependent factors.

## DISCUSSION

### *muscleblind Function Is Required for Two Ultrastructural Features of Late Muscle Differentiation*

Mutations in *mbl* have no obvious effects during early phases of muscle formation and specification. However, our analysis of *mbl* mutant embryos has identified two ultrastructural defects: absence of electron dense material at the Z-bands and reduction in TM at indirect muscle attachments.

*mbl* mutant muscles lack reticular matrix at the Z-band. In wild type embryos, this reticular matrix contains Kettin (Lakey *et al.*, 1993), and accordingly, Kettin fails to localize properly in *mbl* mutant muscles, either due to direct dependence on *mbl* or secondarily due to loss of other Z-band components. Components that are potentially dependent on *mbl* function are Laker, $\alpha$-Actinin, and a variety of yet uncharacterized proteins (Saide *et al.*, 1989; Lakey

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et al., 1990; Fyrberg *et al.,* 1990; Sparrow *et al.,* 1991; Lakey *et al.,* 1993; Cripps *et al.,* 1994). Like Z-bands, the hemiadherens junctions at muscle tips contain dense material that anchors thin filaments (Tepass and Hartenstein, 1994; Prokop *et al.,* 1998). However, the hemiadherens junctions are not affected in *mbl* mutant embryos, revealing a qualitative or developmental difference between the two kinds of densities.

*mbl* mutant embryos also lack TM at the indirect muscle attachments. Known ECM components at embryonic muscle attachments are produced in hemocytes and fat body (e.g. Collagen IV, Tiggrin), in epidermal cells (Masquerade) and in muscles (M-spondin) (Fessler and Fessler, 1989; LeParco *et al.,* 1989; Fogerty *et al.,* 1994; Murugasu-Oei *et al.,* 1995; Umemiya *et al.,* 1997). The restriction of *mbl* expression to the somatic muscles suggests that *mbl* dependent TM is released from the muscles. Such components could be Collagens (e.g. Collagen II), as suggested from analyses of muscle attachments in cockroach, or M-Spondin (Hagiopan, 1970; Umemiya *et al.,* 1997). The assembly of other components of TM, which are not muscle-derived, appears normal in *mbl* mutant embryos, as we demonstrate for Tiggrin.

At the light microscopic level, *mbl* mutant embryos exhibit loss of muscle striation under polarizing microscopy, hypercontraction of muscles, body contraction, severe partial paralysis and late embryonic (stage 17) or early larval lethality. The absence of Z-bands is likely the reason for loss of muscle striation. Likewise, the occurrence of hypercontraction may be a secondary defect due to the aberrant sarcomeric structure. However, *Act88F*$^{E93K}$ mutant flight muscles, which also lack Z-bands, are not hypercontracted (Sparrow *et al.,* 1991) and would argue against this possibility. Finally, hypercontraction of muscles and disorder of the contractile apparatus, along with the reduction of TM, could cause the severe partial paralysis and thus early lethality. Furthermore, it still remains a possibility that central neurons or NMJs might not function normally in *mbl* mutant embryos and thus contribute to the paralysis and lethal phenotype.

### *How Does muscleblind Regulate the Formation of Z-Bands and Tendon Matrix?*

The *mbl* protein is localized in the nuclei and, depending on the protein isoform, contains one or two copies of a $Cys_3His$ zinc finger motif found in the TIS11/NUP475/TTP protein family. Members of this family have been implicated in gene transcription, but also in posttranscriptional processing and turnover of messenger RNA (discussed in Begemann *et al.,* 1997). Which genes might be targets of *mbl*?

In the simplest model, *mbl* could directly regulate genes encoding components of Z-band and TM, as mentioned above (Fig. 7A). Alternatively, *mbl* could have an indirect effect on those components by regulating genes involved in the recruitment, processing, modification or turnover of them. For example, *mbl* might regulate genes involved in the processing of ECM proteins (Fig. 7B). This would explain not only the reduction of TM but also the impairment of Z-band formation since Z-band formation has been shown to depend on extracellular factors such as serum or fibronectin *in vitro* (Volk *et al.,* 1990). Also, deficiency of the ECM component Collagen IV leads to absence of defined Z-bands, in addition to defects similar to *mbl* mutant embryos, i.e. paralysis and reduction in length of the body wall muscles (Borchiellini *et al.,* 1996).

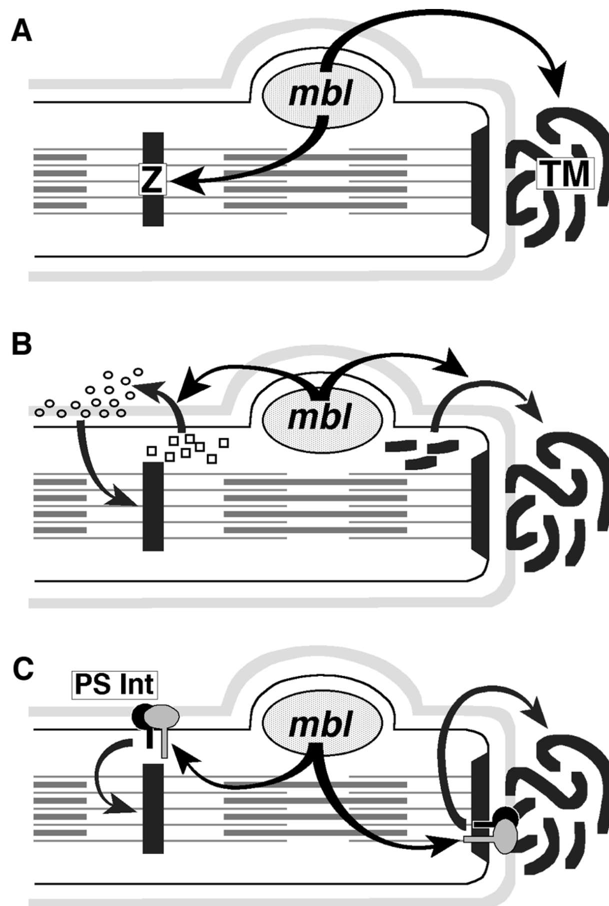


**FIG. 7.** Possible targets for *mbl* function. Mbl is nuclear (shaded circle) and participates in the differentiation of Z-bands (Z) and extracellular tendon matrix (TM) at indirect muscle attachments. Three hypotheses are illustrated. (A) *mbl* could directly activate genes encoding Z-band or TM components. Alternatively, *mbl* could regulate genes involved in the processing or recruitment of Z-band or TM components: for example, (B) genes involved in the processing of ECM components (small squares inside the cell and small circles associating with the gray basement membrane on the left; thick lines and TM on the right), which in turn are required for Z-band formation, or (C) genes required for PS integrin function (PS Int) which appear to be involved in TM and Z-band formation. A combination of these mechanisms is also possible, e.g., ECM signaling required for Z-band formation has been shown to be relayed by PS Integrins (Volk *et al.,* 1990). See text for further details. Arrows indicate putative functional relationships.

Another possibility is that *mbl* could participate in the

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differentiation of Z-bands and/or TM through the activation of other regulative genes. For example, several observations suggest that *mbl* could regulate genes involved in a PS integrin-mediated signaling process (Hynes, 1992; Schwartz *et al.,* 1995; Fig. 7C). First, *Drosophila* muscle cells require $\beta_{PS}$ subunit integrin *in vitro* and *in vivo* in order to assemble or stabilize formation of Z-bands (Volk *et al.,* 1990). Second, the *piopio* and *steamer duck* genes interact genetically with PS Integrin mutations, and the phenotypes of both are strikingly similar to *mbl* mutant embryos: their somatic muscles lack striation under polarized light, their abdomen is severely contracted and they are late embryonic or early larval lethal (Prout *et al.,* 1997). Third, besides its activity during late myogenesis, *mbl* is required for the differentiation of photoreceptor neurons: *mbl* mutant photoreceptors can form and be specified, but they do not differentiate normal rhabdomeres, the light harvesting structures (Begemann *et al.,* 1997). Retinal tissue mutant for the $\beta_{PS}$ or $\alpha_{PS1}$ Integrin subunits exhibits comparable defects in rhabdomere organization (Zusman *et al.,* 1993; Brower *et al.,* 1995; Longley and Ready, 1995). Fourth, PS Integrins also appear to be required for TM assembly (Newman and Wright, 1981), although Tiggrin can localize properly in the absence of PS Integrin function (Fogerty *et al.,* 1994), again resembling the TM phenotype of *mbl* mutant embryos. Therefore, it is conceivable that lack of *mbl* either upstream or downstream of PS integrins would disrupt the signaling pathway (not $\beta_{PS}$ integrin expression, which is unaffected in *mbl* mutant embryos) and bring about an impairment in the formation of Z-bands and possibly TM.

In summary, we have shown that *mbl* participates in Z-band and TM formation; however, the targets of *mbl* activity are not clear. On the one hand, *mbl* may regulate Z-band and TM components directly. On the other hand, *mbl* may regulate genes that act in the formation of one or both structures by indirect mechanisms, one possibility being through PS Integrins. Of course, these different models are not mutually exclusive.

### *muscleblind Is Regulated by Dmef2 and Participates in a Subset of Dmef2 Functions*

*Dmef2* null mutant embryos display a severe reduction in the amount of *mbl* protein in their musculature without any effect on expression in the CNS or Bolwig's organ. These results imply that *mbl* is under the control of at least two genetic systems: unknown transcriptional activators in the CNS and Bolwig's organ, and *Dmef2* in visceral, somatic and pharyngeal muscle derivatives. On the other hand, *Dmef2* is produced in cardial cells (Lilly *et al.,* 1994; Bour *et al.,* 1995; Lilly *et al.,* 1995; Taylor *et al.,* 1995), whereas Mbl has not been detected in this tissue in all stages studied. Kettin is also not detected in the cardioblasts suggesting that Z-bands might be different in these cells or that, besides *Dmef2,* regulatory factors in the heart cells are different from other muscles. One possibility is that specific repressors exist in the cardioblasts or necessary cofactors are lacking. Interestingly, we found that both Mbl and Kettin can be induced upon misexpression of *Dmef2* in the epidermis, suggesting that either repressors of *mbl* do not exist in the epidermis, or that appropriate coactivators are present as similarly pointed out by Lin *et al.* (1997) for Tropomyosin I.

*Dmef2* plays a pivotal role in the late differentiation process of muscles in *Drosophila* and is required for myoblast fusion, NMJ formation, muscle attachments and organization of the contractile apparatus (Bour *et al.,* 1995; Lilly *et al.,* 1995; Ranganayakulu *et al.,* 1995; Prokop *et al.,* 1996). A possible scenario is that the various *Dmef2* dependent properties of differentiated muscle are regulated independently by *Dmef2* dependent genes either alone or in conjunction with *Dmef2.* For example, the signaling molecule $\alpha_{PS2}$ Integrin has been shown to be regulated by *Dmef2* and also to be essential for a specific aspect of late muscle differentiation, the maintenance of muscle attachments (Brown, 1994; Ranganayakulu *et al.,* 1995; Prokop *et al.,* 1998). As shown here, *mbl* is likewise regulated by *Dmef2* and participates in the late differentiation of two muscle specific features, the TM and the Z-bands. Our results are consistent with the hypothesis that *Dmef2* function can be separated into independent genetic pathways and also shed light on the terminal muscle differentiation program by identifying a gene, *mbl,* which is required for the implementation of a subset of differentiated muscle properties.

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