Development 128, 4489-4500 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV7909

Drosophila Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development

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Accepted 24 August 2001

SUMMARY

A hallmark of mature skeletal muscles is the presence of multinucleate muscle fibers. In *Drosophila*, the formation of muscle syncytia requires the cooperative participation of two types of myoblasts, founder cells and fusion-competent myoblasts. We show that a newly identified gene, *lame duck* (*lmd*), has an essential regulatory role in the specification and function of fusion-competent myoblasts. Embryos that lack *lmd* function show a loss of expression of two key differentiation and fusion genes, *Mef2* and *sticks-and-stones*, in fusion-competent myoblasts and are completely devoid of multinucleate muscle fibers. By contrast, founder cells are specified and retain their capability to differentiate into mononucleate muscle cells. *lmd* encodes a novel member of the Gli superfamily of transcription factors and is expressed in fusion-competent myoblasts and their precursors in a Wingless- and Notch-dependent manner. The activity of the Lmd protein appears to be additionally controlled by its differential cytoplasmic versus nuclear localization. Results from an independent molecular screen for binding factors to a myoblast-specific *Mef2* enhancer further demonstrate that Lmd is a direct transcriptional regulator of *Mef2* in fusion-competent myoblasts.

Key words: *Drosophila*, Lmd, Muscle specification, *Mef2*, Differentiation, Myoblast fusion

INTRODUCTION

The formation of the larval body wall musculature in *Drosophila* results from the sequential implementation of highly regulated genetic programs in the mesoderm during embryogenesis. The molecular mechanisms and components that mediate some of these regulatory events have been identified. Importantly, it has been established that these regulatory events lead to the generation of two functionally distinct types of myoblasts in the somatic muscle lineage: muscle founders and fusion-competent myoblasts (Bate, 1990; Dohrmann et al., 1990). The phenotype of fusion mutants, such as *myoblast city*, has demonstrated that muscle founders have the necessary intrinsic information for complete differentiation (Rushton et al., 1995). Genetic and molecular studies have further shown that the function of a particular group of genes, designated as 'muscle identity' genes, is critical for specifying muscle founders and defining the characteristics of the corresponding muscles (Frasch, 1999). Specification of muscle founders and activation of identity genes in these cells have been found to be regulated by stepwise processes that require the concerted action of transcriptional regulators, such as Twist, Tinman, Lethal-of-scute and Sloppy-paired, and signaling events that involve Dpp, Wingless, Notch, EGF and FGF (Baylies et al., 1998; Frasch and Nguyen, 1999; Halfon et al., 2000; Lee and Frasch, 2000). In contrast to founder cells, fusion-competent myoblasts have been considered to be unspecified somatic mesodermal cells which acquire the identity of the founder cells with which they fuse. Nevertheless, fusion-competent myoblasts must also be endowed with a distinct genetic program that would enable them to develop and function as fusion-competent myoblasts. To date, little is known about the regulatory pathways that control the formation of these myoblasts and define their unique features.

After the specification phase, both types of myoblasts undergo characteristic differentiation events, including activation of muscle gene transcription and initiation of cell fusion. More recent ultrastructural studies have provided detailed morphological descriptions of the events that occur sequentially during myoblast fusion (Doberstein et al., 1997). Genetic studies have identified loss-of-function mutations that affect the cell fusion process at discrete stages (Paululat et al., 1999; Frasch and Leptin, 2000). An important aspect of myoblast fusion is the asymmetrical fusion between muscle founders and fusion-competent myoblasts during which both

types of myoblasts play active roles. This asymmetry implicated the existence of regulatory molecules that would be differentially expressed in the two populations of myoblasts. Recent studies have reported two new members of the Ig superfamily, *sticks-and-stones* (*sns*) and *dumbfounded* (*duf*), which are expressed exclusively in fusion-competent myoblasts and muscle founders, respectively (Bour et al., 2000; Ruiz-Gomez et al., 2000). The exact functions of *sns* and *duf* are not yet known but experimental evidence suggests that Duf serves as an attractant for fusion-competent myoblasts. Of note, the specific expression of Sns in fusion-competent myoblasts and the active participation of these cells in the fusion process underscore the existence of specific genetic programs that operate within this type of myoblast.

Among all mutations that are presently known to result in defects during the differentiation phase, *Mef2* is the only one that affects the entire somatic muscle differentiation process (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995; Lin et al., 1996). In the absence of *Mef2* function, muscle founders are specified but they do not undergo terminal differentiation and cell fusions that lead to the formation of multinucleate MHC-expressing muscle fibers are not observed. Genetic analysis of *Mef2* mutant embryos has also revealed that *Mef2* function is required in both types of myoblasts. In mutant embryos, neither the unfused founder cells nor the defective fusion-competent myoblasts express *Mhc* or *tropomyosin 1*.

In vivo analysis of the regulatory regions of the *Mef2* gene locus has partly revealed the molecular basis of regulated *Mef2* expression during embryogenesis (Cripps et al., 1998; Gajewski et al., 1998; Nguyen and Xu, 1998). In these studies, Twist and extrinsic signals provided by Dpp and Wg were identified as regulators of *Mef2* expression during early stages of development. Enhancer elements were also defined that drive *Mef2* expression differentially in fusion-competent myoblasts versus muscle founders. However, direct regulators of *Mef2* expression in fusion-competent myoblasts and founder cells during mid-embryogenesis when *Mef2* function is critically required for various aspects of somatic muscle differentiation have yet to be identified.

In this study, we describe the identification of the *lame duck* (*lmd*) gene as a novel regulator of somatic muscle specification and differentiation. Embryos lacking *lmd* function show a specific loss of *Mef2* and *sns* expression in fusion-competent myoblasts and an absence of multinucleate muscle fibers. The *lmd* gene encodes a new and distinct member of the Gli superfamily of transcription factors. *lmd* expression, which requires both Wg and Notch activities, is restricted to mesodermal cells that will become fusion-competent cells. Activation of MEF2 in fusion-competent myoblasts is associated with increased nuclear localized Lmd protein expression. Moreover, one-hybrid screening with a *Mef2* enhancer that is active in fusion-competent myoblasts provides molecular evidence that Lmd is a transcriptional regulator of *Mef2* expression in these cells.

MATERIALS AND METHODS

Drosophila stocks

lmd^1 and lmd^2 came from an EMS-induced screen for lethal mutations on the third chromosome which showed an aberrant Eve expression pattern in either the mesoderm or nervous system (J. B. S. and C. Q. Doe, unpublished) (Gisselbrecht et al., 1996). Stocks for recombination mapping (*ru h th st cu sr e ca*) and complementation testing were obtained from the Bloomington Stock Center or specific investigators: *Df(3R)hh-GW2* and *Df(3R)hh-EB6* (Mohler and Vani, 1992), *Df(3R)M95A* (Hales and Fuller, 1997), *E226* and *E1432* (Butler et al., 1997). Other stocks used included wg^{cx4} (Baker, 1988) and N^{5419} (Johansen et al., 1989).

Preparation of embryo DNA and sequence analysis of the *lmd*^{1} mutation

The *lmd* mutation was maintained over a 'blue' balancer to identify homozygous mutant embryos after staining with an antibody against β-gal. Embryos of the appropriate genotype were hand-picked into a solution of 10 mM Tris HCl, 1 mM EDTA, 25 mM NaCl and 200 μg/ml proteinase K. Proteinase K was inactivated before PCR amplification. Amplified products were purified and subjected directly to automated DNA sequencing. Specific primers were used to sequence all exons and exon-intron junctions. For confirmation, the fragment showing the sequence aberration was re-amplified from genomic DNA and re-sequenced.

In situ hybridization and immunocytochemistry of whole-mount embryos

In situ hybridization was carried out essentially as described (Tautz and Pfeifle, 1989), with the use of digoxigenin-labeled RNA probes (Roche Molecular Biochemicals). Immunocytochemistry was performed as described (Nguyen and Xu, 1998). The TSA Fluorescence system (NEN) was used for signal amplification as needed. Embryos were photographed with Nomarski DIC optics on an Olympus AX70 microscope with a 20× UPlan objective or analyzed on a Leica TCS 4D confocal microscope with a 40× or 100× objective.

RNA probes were generated using a *lmd* cDNA or *sns* subclone (nucleotides 4981-6200). Antibodies were used as follows: rabbit anti-β-gal (1:3000, ICN), mouse anti-β-gal (1:2500, Sigma), anti-MEF2 (1:750) (Bour et al., 1995), anti-MHC (1:8, gift from D. Kiehart), anti-Kruppel (1:400, gift from D. Kosman), anti-Lamin (1:10, gift from M. Frasch), anti-Lmd (1:250, this study), and biotinylated (1:200, Vector Labs) and fluorescent (1:100, Jackson ImmunoResearch) secondary antibodies.

Generation of *lacZ* reporter gene constructs and germline transformation

Deletion constructs, I-$E_{D5\text{-}DelA}$ to I-$E_{D5\text{-}DelF}$, were generated with the ExSite PCR-based site-directed mutagenesis kit (Stratagene). The 35 bp internal deletions in enhancer I-E_{D5} were substituted by a *Hin*dIII restriction site. I-$E_{D5\text{-}mt1}$ to I-$E_{D5\text{-}mt4}$ constructs were similarly generated, except that the 10 bp mutations therein are transition-type of substitutions. All fragments were transferred to pCaSpeR-hs43-β-gal and germline transformation was performed as described (Nguyen and Xu, 1998). For each construct, three to five independent lines were examined for reproducible patterns of expression.

Yeast one-hybrid screening

The one-hybrid system (Clontech) was used to isolate specific DNA binding factors. Multimers (5 copies) of the [C/D]* region from enhancer I-E_{D5} or sequences from an unrelated enhancer T2 were generated by ligating the relevant oligos, which contain sequences of interest and an *Ava*I site for cloning in a unidirectional manner:

[C/D]*: 5′-TCGGGGAAATTACCTACGCAGCGTTTACAAAAA-CATCATCGGCGGAGGGCAGTGG-3′

T2: 5′-TCGGGTTTTCCGAGTCGAAATCACTTGAGCTGAA-CTGAACTTCAATTGCTTTTTTTTTCGGGGCC-3′

Multimers were cloned upstream of HIS3 and *lacZ* reporter genes. The modified reporter constructs were integrated into YM4271 yeast cells, and the double reporter gene yeast cells were transformed with

a 0- to 21-hour-old *Drosophila* embryo cDNA library (Clontech). Transformed cells were plated under His-free conditions (with 45 mM of 3-aminotriazole to suppress basal HIS3 activity) to select for colonies in which AD/*Drosophila* hybrid proteins were capable of binding to the [C/D]* target. For verification, His-expressing colonies were assayed for *lacZ* activity. Plasmid DNA was recovered from all His-positive/*lacZ*-positive colonies and transformed into yeast cells with T2 target reporter constructs to test for target specificity.

Truncated *lmd* constructs were generated by PCR amplification of the relevant regions and cloned into pGADT7 (Clontech). All clones were verified by sequencing.

DNA-binding assays

The *lmd* cDNA clone was used as template in the T7 in vitro transcription translation coupled reticulocyte lysate system (Promega) with the addition of 50 μM $ZnCl_2$. Standard DNA binding reactions (10 μl) contained ~0.5 ng of γ^{32}P-labeled probe, 1 μg of poly dI-dC, 1-5 μl of translated product and specific competitor DNAs in a buffer of 75 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 50 μM $ZnCl_2$ and 6% glycerol. The complexes were resolved on native 5% bis-acrylamide/polyacrylamide (1:29)/0.25× TBE gels.

RESULTS

lmd mutant embryos show a complete absence of multinucleate muscle fibers

Two mutant alleles, *lmd*^{1} and *lmd*^{2}, were identified based upon their disrupted MEF2 expression in a collection of lines with lethal EMS-induced mutations on the third chromosome (see Materials and Methods). To assess the degree of muscle differentiation in *lmd* mutants, we examined homozygous *lmd*^{1} and *lmd*^{2} embryos, as well as embryos trans-heterozygous for *lmd*^{1} and *Df(3R)M95A*, a deficiency that deletes the entire *lmd* gene locus (data not shown), for MEF2 and MHC expression. This analysis revealed that embryos homozygous for either *lmd*^{1} or *lmd*^{2} exhibit identical phenotype as *lmd*^{1}*/Df(3R)M95A* embryos shown in Fig. 1, indicating that both *lmd* alleles are genetically null mutations. In *lmd* mutant embryos, the early pan-mesodermal *Mef2* expression pattern is normal (data not shown). However, we observe specific defects within the somatic muscle lineage starting from late stage 12. In particular, we find that MEF2 expression is significantly reduced in somatic myoblasts (Fig. 1A-D) although its expression in cardioblasts is not affected (Fig. 1C,D). Expression of markers of visceral mesoderm specification and differentiation, such as MEF2, Bagpipe (Bap) and Fasciclin III (FasIII), is also not affected (data not shown). Consistent with the reduced MEF2 expression in the somatic mesoderm, *lmd* mutant embryos do not exhibit multinucleate MHC-expressing muscle fibers. Only mononucleate, elongated MHC-positive muscle cells are detectable in the mutant embryos (Fig. 1E-H). As observed for MEF2, MHC expression is not affected in the cardiac and gut musculatures (Fig. 1G,H; and data not shown). Gut constrictions also do not appear to be defective (data not shown). These observations indicate that *lmd* function is critical for somatic myogenesis, while its function appears dispensible for cardiac and visceral muscle development.

To address the possibility that reduced MEF2 expression in *lmd* mutant embryos reflects a general loss of somatic myoblasts, we crossed a *twist*-dependent *Mef2-lacZ* enhancer line (Nguyen and Xu, 1998) that generates β-gal protein in the somatic muscle lineage, which perdures until late stage 13, into the *lmd*^{1} mutant background. Wild-type and mutant embryos harboring this *Mef2* enhancer insertion were double-labeled with antibodies against β-gal and MEF2. *lmd* mutant embryos

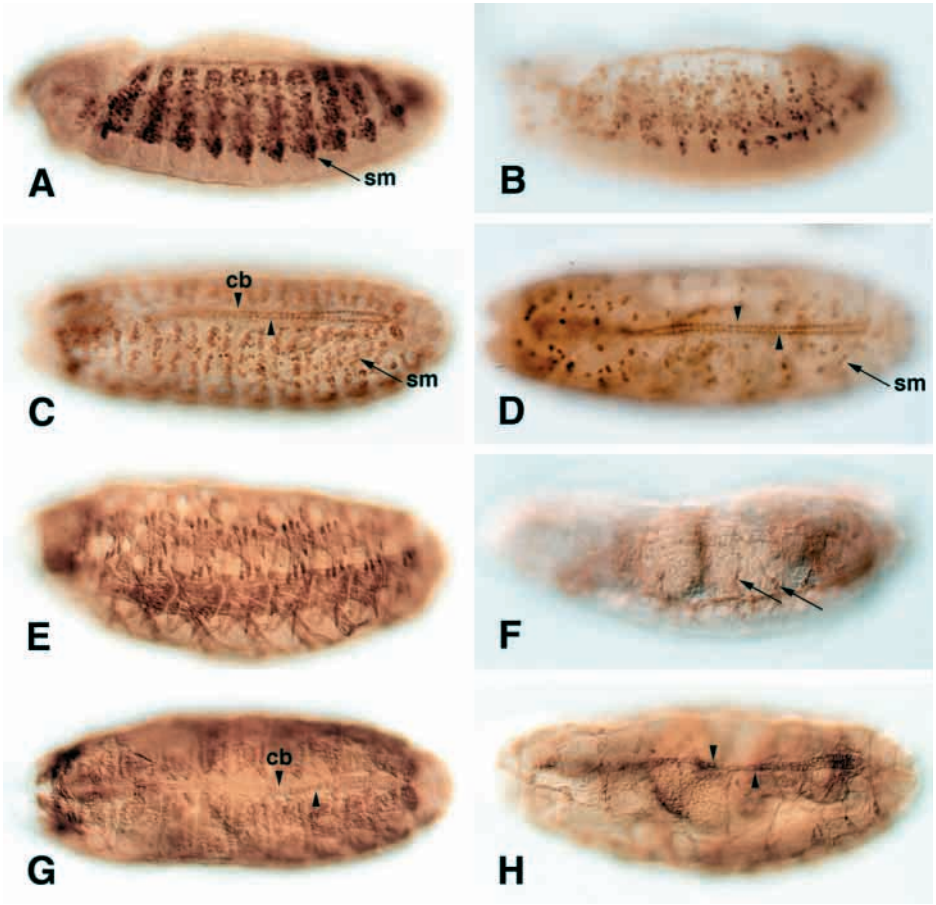

Fig. 1. *lame duck* (*lmd*) mutant embryos exhibit severe defects within the somatic muscle lineage. Wild-type and mutant embryos (trans-heterozygous for *lmd*^{1} and *Df(3R)M95A*)were stained with an antibody against MEF2 (A-D) or MHC (E-H). (A,B) Lateral views of stage 13 embryos. When compared with control embryo (A), mutant embryo shows a dramatic reduction in MEF2 expression in somatic mesodermal cells. (C,D) Dorsal views of stage 16 embryos with normal MEF2 expression in cardioblasts (cb; arrowheads) but a reduced number of MEF2-positive somatic muscle nuclei (arrows in D). (E,F) Multinucleate MHC-positive fibers are present in late stage 15 control embryo (E) while only elongated, mononucleate MHC-expressing muscle cells are detected in mutant embryo (arrows in F). (G,H) MHC expression in cardioblasts and gut muscles is normal.

exhibit a comparable amount of β-gal-marked somatic mesoderm as wild-type embryos. However, the majority of β-gal-positive somatic mesodermal cells fail to express MEF2, suggesting that *lmd* function is needed for activating *Mef2* expression in a particular subset of myoblasts (Fig. 2A,B).

Founder cells are not affected in *lmd* mutant embryos

The presence of MHC-positive muscle cells in *lmd* mutant embryos suggested that founder cells are not affected and are capable of differentiating into mononucleate mini muscles. To examine this possibility, we crossed an enhancer trap insertion in the *duf* locus, rP298-*lacZ*, which marks a large number of founder cells (Nose et al., 1998; Ruiz-Gomez et al., 2000) into the lmd^1 background. Wild-type and mutant embryos with this enhancer construct were double-labeled for MEF2 and *lacZ* expression. In a wild-type background, we observe both MEF2-positive/*lacZ*-positive founders and MEF2-positive/*lacZ*-negative fusion-competent myoblasts (Fig. 2C). By contrast, MEF2-positive/*lacZ*-negative fusion-competent myoblasts are absent in *lmd* mutant embryos, while MEF2-positive/*lacZ*-positive founder cells are similar in number and position to those observed in wild-type embryos (Fig. 2D). These data suggest that loss of *lmd* function results in a specific loss of *Mef2* expression in fusion-competent myoblasts.

To analyze the development of founder cells and fusion-competent myoblasts in greater detail, we followed the expression of *Kruppel* (*Kr*) and *sticks-and-stone* (*sns*) in wild-type and mutant lmd^1 embryos. *Kr* marks a subset of founder cells (Ruiz-Gomez et al., 1997), whereas *sns* is a marker for fusion-competent myoblasts and encodes an Ig-type protein that is essential for the fusion process (Bour et al., 2000). Until late stage 12, the number and position of Kr-positive founders are approximately normal in mutant embryos when compared with wild-type embryos (data not shown). However, after stage 12, wild-type embryos show an increase of Kr-positive nuclei as a result of myoblast fusion, while *lmd* mutant embryos fail to show a similar increase (Fig. 2E,F). Examination with other founder markers, such as Nau and Lb (Michelson et al., 1990; Jagla et al., 1997), yielded similar results (data not shown). Thus, founder cells in *lmd* mutant embryos do not appear to undergo cell fusion as observed in wild-type embryos. Significantly, *sns* expression in fusion-competent myoblasts is completely abolished in *lmd* mutant embryos (Fig. 2G,H). Only residual expression is observed in cells in positions corresponding to garland cells (which function as nephrocytes) (Rizki, 1978). Together, these results strongly suggest that *lmd* is required for proper specification and development of fusion-competent myoblasts. During this process, *lmd* is essential for activating the expression of *Mef2* and *sns*, two genes that regulate myoblast fusion.

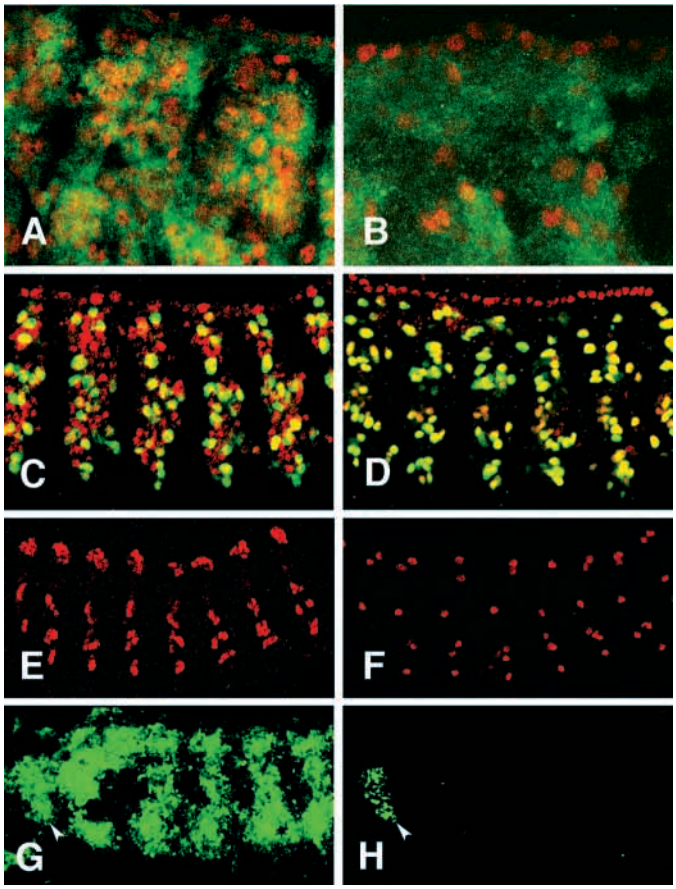

Fig. 2. *lmd* mutant phenotype involves loss of *Mef2* and *sns* expression specifically in fusion-competent myoblasts. Late stage 13 wild-type and lmd^1 mutant embryos, both of which also carry a *twist*-dependent *Mef2-lacZ* insertion (A,B) (Nguyen and Xu, 1998) or the enhancer trap insertion rP298-*lacZ* (C,D) (Nose et al., 1998) were double-stained with antibodies against MEF2 and *lacZ*, and analyzed by confocal microscopy. Control embryo (A) exhibits coincident nuclear MEF2 (red) and cytoplasmic β-gal (green) expression in somatic myoblasts and cardioblasts at the dorsal margin while mutant embryo (B) shows a significant number of *lacZ*-positive somatic myoblasts that do not exhibit nuclear MEF2 expression. Control embryo (C) shows founders that are rP298-*lacZ* positive/MEF2 positive (yellow signals) and fusion-competent myoblasts that are only MEF2-positive (red), while mutant embryo (D) exhibits MEF2 expression only in *lacZ*-positive founders and cardioblasts. (E,F) Embryos were stained with an anti-Kr antibody. Kr-positive multinucleate muscle precursors in control embryo (E) are equivalent in position and number to Kr-positive mononucleate muscle precursors in lmd^1 mutant embryo (F). (G,H) Embryos were hybridized with a digoxigenin-labeled *sns* RNA probe. In *lmd* mutant embryo, *sns* expression in fusion-competent myoblasts is completely abolished. Residual *sns* expression is in presumed garland cells (arrowheads).

lmd encodes a novel member of the Gli superfamily of Zn-finger type of transcription factors

We initially mapped *lmd* between *ebony* and *claret*. Complementation tests with deficiencies further localized *lmd* to the region defined by the distal and proximal breakpoints of *E226* and *Df(3R)hh-GW2*, respectively (data not shown). The candidate region, demarcated by the 3′ ends of the *klg* and *hh* genes, was examined for potential transcripts by in situ hybridization. A ~1.3 kb genomic fragment detected RNA expression exclusively in mesodermal cells between late stage 11 and early stage 14 (data not shown), and encoded sequences for a protein with homology to a novel Zn-finger type of transcription factor. This information was used to identify a group of EST clones (LD47926, LD22708, LD23050, LD34514, LD39035) from the Berkeley

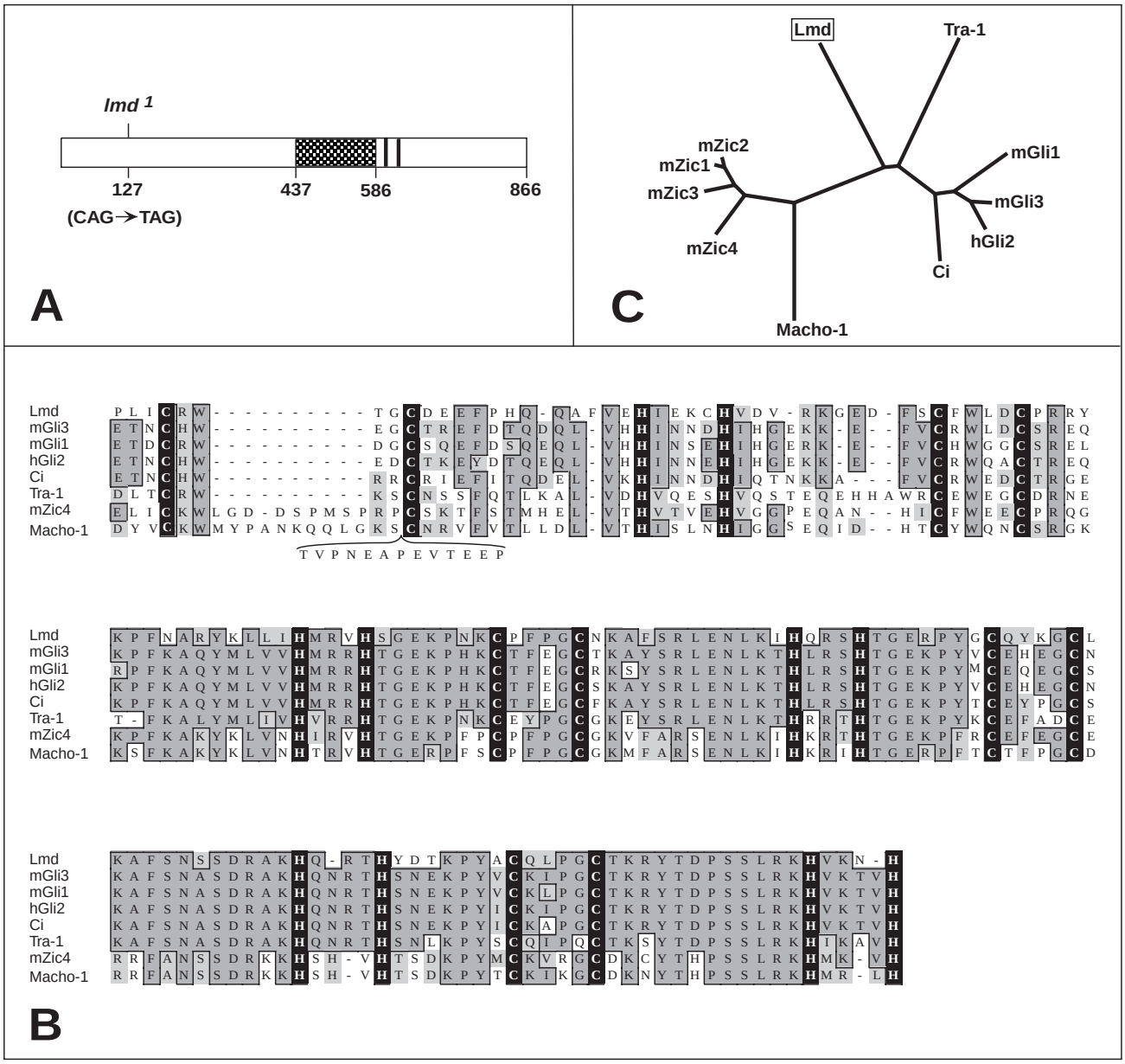

Fig. 3. Lmd is a C_2H_2-type of Zn-finger protein with homology to Gli family members. (A) Diagram of the predicted Lmd protein. Checked and black boxes denote the conserved Zn-finger domain and putative phosphorylation sites, respectively. Position of the mutation in *lmd*^{1} (nucleotide change from C to T, converting the Gln residue at position 127 to a nonsense residue) is indicated. (B) Sequence comparison of the Zn-finger domain of Lmd and representatives of the Gli superfamily was done using the Clustal W program: Lmd (Accession Number, AY032609); mouse Gli3 (Accession Number, Q61602; mouse Gli1 (Accession Number, BAA85004); human Gli2 (Accession Number, P10070); *Drosophila* Ci (Accession Number, A38926); *C. elegans* Tra-1 (Accession Number, P34708); mouse Zic4 (Accession Number, Q61467); ascidian Macho-1 (Accession Number, BAB19958). The Cys and His residues of each finger are highlighted in black. Amino acid residues that are identical or similar in at least 50% of the aligned sequences at a particular position are boxed in dark or light gray, respectively. (C) Phylogenetic tree of Lmd and representatives of the Gli superfamily, based upon Zn-finger domains shown in B. Programs Clustal X and TREEVIEW (Page, 1996) were used to generate the tree that displays the possible way in which the protein sequences may have evolved.

Drosophila Genome Project for further analysis. Further sequencing of these clones showed that they correspond to overlapping cDNAs, and all contain the coding sequences present in the 1.3 kb genomic fragment. This analysis also revealed that the reported partial sequence of the 'K' gene (Casal and Leptin, 1996) is included in these cDNA clones. The sequence of the longest clone, LD47926, predicted an open reading frame (ORF) of 866 amino acids, flanked by 5′ and 3′ untranslated regions of 248 nucleotides and 333 nucleotides, respectively. The predicted ORF encodes a C_2H_2-type of Zn-finger protein, which shares sequence homology within the Zn-finger domain with proteins belonging to the Gli superfamily (Fig. 3A). The observed homology between Lmd and members of the Gli superfamily does not extend beyond the Zn-finger domain.

To determine whether this novel Zn-finger protein is in fact

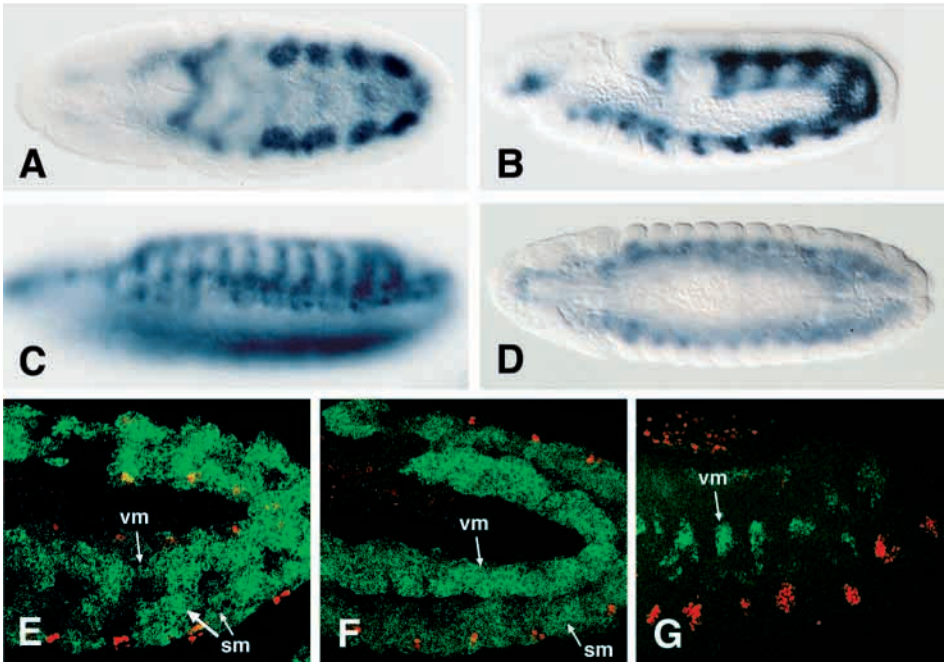

Fig. 4. *lmd* gene expression in somatic mesodermal cells is severely affected in *wg* and abolished in *N* mutant embryos. Embryos were hybridized with a *lmd* RNA probe (A-D). (A) Dorsal view of late stage 11 embryo with expression in patches of visceral mesoderm. (B) Lateral view of late stage 12 embryos with expression in both somatic and visceral mesodermal layers. (C,D) Stage 13 embryos show decreasing expression in somatic mesodermal cells. Expression is no longer detectable in visceral mesodermal cells. (E-G) Embryos were hybridized to a *lmd* RNA probe (green) and stained with anti-Eve antibody (red), followed by confocal microscopy. When compared with control embryo (E), wg^{cx4} mutant embryo (F) shows a dramatic decrease in *lmd* expression in somatic mesodermal cells in dorsolateral and lateral regions while ventrally located cells (sm; arrow) are not strongly affected. Visceral mesoderm (vm; arrow) is expanded in mutant embryo. In N^{5419} mutant embryo (G), *lmd* expression is abolished in all somatic mesodermal cells. Visceral mesoderm is reduced in mutant embryo.

encoded by the *lmd* locus, we sequenced the entire ORF of the Zn-finger protein in wild-type and lmd^{1} mutant embryos. This analysis showed that the Zn-finger protein contains a nucleotide change (C to T) that converts the Gln residue at amino acid 127 to a nonsense codon on the lmd^{1} mutant chromosome (Fig. 3A). We conclude that *lmd* encodes a Zn-finger protein of the Gli superfamily. Of note, the truncation of the mutant Lmd^{1} polypeptide is upstream of the putative DNA-binding domain, which is consistent with our identification of lmd^{1} as a genetically null mutation.

A more detailed comparison of the Zn-finger domain from Lmd and representatives of the Gli superfamily, such as vertebrate Gli proteins, *Drosophila* Ci, *C. elegans* Tra-1, mouse Zic4 protein and ascidian Macho-1 indicated that Lmd bears strongest homology to Ci/Gli proteins (Fig. 3B,C). Although a high degree of sequence identity exists throughout the Zn-finger domain among the Ci/Gli proteins, identity between Lmd and Ci/Gli proteins is restricted to the third, fourth and fifth fingers, and a high level of divergence exists in the first and part of the second fingers. Thus, we propose to classify Lmd as a new and distinct member of the Gli superfamily.

lmd expression is restricted to mesodermal cells and requires both Wg and Notch

To assess *lmd* expression during embryogenesis, embryos were hybridized with a digoxigenin-labeled *lmd* probe. *lmd* RNA transcripts are first detectable at late stage 11 in repeating patches of visceral mesoderm, corresponding to Bap-positive cells (Fig. 4A; and data not shown). Prominent expression is then observed in somatic and visceral mesodermal cells throughout stage 12 (Fig. 4B). During stage 13, lower levels of *lmd* expression persist in repeating groups of somatic mesodermal cells, whereas expression in the visceral mesoderm is no longer detectable (Fig. 4C,D). *lmd* expression is abolished in somatic mesodermal cells before cell fusion and is never detectable in muscle fibers. Its expression is also never detected in heart progenitors (data not shown). The Lmd protein expression pattern, obtained with an antibody against the N-terminal portion of the protein, is identical to its RNA profile (data not shown).

We also examined *lmd* expression in *wg* and *N* mutant embryos to determine the relative position of *lmd* within the genetic hierarchy that controls somatic muscle specification. In wg^{cx4} mutant embryos, *lmd* RNA expression is not detectable in dorsolateral and lateral somatic mesodermal cells although there is residual expression in cells located in the ventral region (Fig. 4E,F). Thus, activation of *lmd* expression is mediated via *wg*-dependent and *wg*-independent pathways. Significantly, *lmd* expression in the somatic mesoderm is completely abolished in N^{5419} mutant embryos (Fig. 4G), indicating that activation of *lmd* expression in presumed fusion-competent myoblasts requires active Notch signaling. By contrast, founder cell formation is promoted in the absence of Notch function (Corbin et al., 1991; Bate et al., 1993).

High levels of Lmd expression in fusion-competent myoblasts

We used confocal microscopy to determine precisely the cell type within the somatic mesoderm in which Lmd is expressed. Embryos derived from the rP298-*lacZ* line were triple-stained with antibodies against Lmd, MEF2 and β-gal. As noted above, rP298 drives *lacZ* expression in all founder cells. There is extensive co-expression of Lmd and MEF2 (Fig. 5A,B), but only within *lacZ*-negative fusion-competent myoblasts, whereas MEF2-positive/*lacZ*-positive founder cells are Lmd negative or express Lmd at extremely low levels (hollow arrowheads in Fig. 5A-D). These results indicate that Lmd is expressed highly in fusion-competent myoblasts and at barely

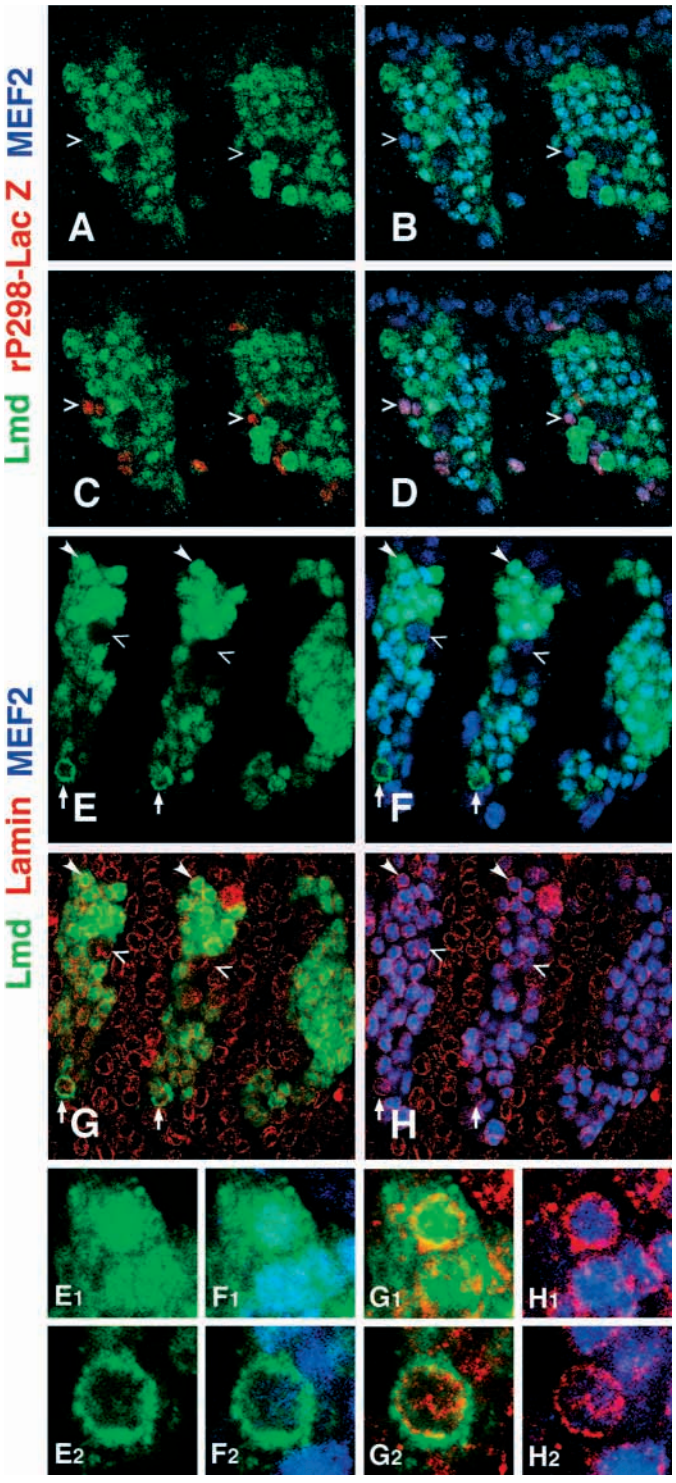

Fig. 5. Lmd protein expression is high in fusion-competent myoblasts and not exclusively nuclear. (A-D) Two segments of a representative rP298-*lacZ* embryo that was triple-stained with antibodies against Lmd, MEF2 and β-gal, and analyzed by confocal microscopy. Different channel combinations of the same scan are shown. Co-expression of Lmd (green) and MEF2 (blue) is observed in *lacZ*-negative fusion-competent myoblasts (green/turquoise signals in A,B). Hollow arrowheads identify representative *lacZ*-positive (red) founders that express MEF2 (red/pink signals in C,D). A very low level of Lmd expression is observed in these founders (hollow arrowheads in A). (E-H) Three segments of a representative late stage 12 embryo that was triple-stained with antibodies against Lmd, MEF2 and nuclear lamin. Different channel combinations of the same scan are shown. Cy3-labeled lamin (red) demarcates the nuclear envelope. Lmd expression (green) is observed in both the cytoplasm and nucleus (arrowheads in E,G), whereas MEF2 expression (blue) is strictly nuclear (arrowheads in H); see also high magnification views in E_1-H_1. Co-expression of nuclear Lmd and MEF2 is observed in fusion-competent myoblasts (green/turquoise signals in F). Arrows identify representative myoblasts with exclusively cytoplasmic Lmd and no MEF2 expression; see also high magnification views in E_2-H_2. Hollow arrowheads identify MEF2-positive founder cells that lack Lmd expression.

detectable, or undetectable (hollow arrowheads in Fig. 5E,F), levels in founder cells.

Given that Lmd is a Gli-related protein, we examined in detail the subcellular distribution of Lmd protein expression. Wild-type late stage 12 embryos were triple-stained with antibodies against Lmd, MEF2 and nuclear lamin. Lmd expression is detected in both the nucleus and cytoplasm (arrowheads in Fig. 5E-G; see also $5E_1$-G_1 for high magnification views) of a large number of myoblasts whereas MEF2 expression is restricted to the nucleus of these same cells (Fig. 5F-H; see also $5F_1$-H_1 for high magnification views). The majority of these myoblasts appear, however, to have elevated levels of Lmd in their nuclei when compared with their cytoplasm. Interestingly, exclusively cytoplasmic Lmd expression is observed in some myoblasts and these do not express MEF2 (arrows in Fig. 5E-H; see also $5E_2$-H_2 for high magnification views). An apparent absence of Lmd expression is also seen in some MEF2-positive myoblasts which are presumably founder cells (hollow arrowheads in Fig. 5E-H). Furthermore, myoblasts from each of these different categories are found in stereotyped positions within each segment, suggesting that both the intracellular localization and expression of Lmd may correlate the distinct specification and differentiation state of a particular cell. Taken together, the subcellular localization data and results from a parallel study, which are presented below, indicate that *Mef2* activation in fusion-competent myoblasts requires nuclear-localized Lmd.

Lmd is a direct upstream regulator of MEF2 expression in fusion-competent myoblasts

We had previously identified multiple enhancers that mediate regulated *Mef2* expression during embryogenesis (Nguyen and Xu, 1998). Among them is the enhancer I-E, which drives *Mef2* expression in fusion-competent myoblasts. The phenotype of *lmd* mutant embryos suggested that *lmd* may be activating *Mef2* in fusion-competent myoblasts via enhancer I-E. Wild-type and *lmd* mutant embryos, carrying the enhancer I-E construct, were double-labeled for MEF2 and *lacZ* expression. In the wild-type background, *lacZ* expression is detected in a large number of MEF2-positive somatic myoblasts (Fig. 6A,B). By contrast, there is a complete absence of *lacZ* expression in *lmd* mutant background (Fig. 6C,D). Activation of two other somatic muscle enhancers, II-E and III-F, which drive *Mef2* expression in founder cells and muscle fibers, respectively, is not affected in mutant embryos (data not shown).

Functional dissection of enhancer I-E has also identified a

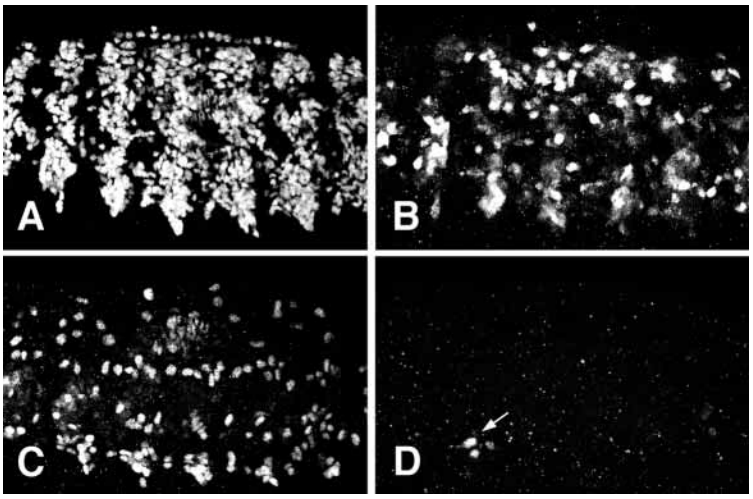

Fig. 6. Somatic myoblast enhancer I-E is not activated in *lmd* mutant embryos. Early stage 13 wild-type and *lmd*^{1} mutant embryos, both of which also carry the construct I-E were double-labeled for MEF2 and β-gal, and analyzed by confocal microscopy. (A,B) Control embryo shows MEF2 expression (A) in somatic myoblasts and cardioblasts at the dorsal margin and *lacZ* expression (B), directed by enhancer I-E, in somatic (fusion-competent) myoblasts. (C,D) *lmd*^{1} mutant embryo shows reduced MEF2 expression (C) in the somatic mesoderm and a complete absence of *lacZ* expression (D) in somatic myoblasts. Arrow denotes ectopic *lacZ* expression in non-mesodermal cells.

170 bp subfragment, I-E$_{D5}$, which is still active in somatic myoblasts (data not shown). Further analysis with deletion reporter gene constructs, each of which contained a small internal deletion within enhancer I-E$_{D5}$, defined the essential [C/D]* region (Fig. 7A). Notably, robust *lacZ* expression levels are obtained with I-E$_{D5}$, while a nearly complete absence of *lacZ* expression is observed with I-E$_{D5\text{-}DelD}$ (compare Fig. 7E with 7F). *lacZ* expression is slightly reduced with the overlapping I-E$_{D5\text{-}DelC}$ construct (data not shown). Moreover, a multimerized construct consisting of five copies of the [C/D]* region can direct *lacZ* expression comparably to I-E$_{D5}$ (Fig. 7G). Thus, the sequences within [C/D]* are both necessary and sufficient to direct expression in fusion-competent myoblasts.

To identify factors that bind specifically to [C/D]*, we undertook a yeast one-hybrid screen, using the multimerized [C/D]* region as target (see Materials and Methods). From this molecular screen, we obtained 37 His-positive/*lacZ*-positive cDNA fusion clones that encode proteins which bind the [C/D]* region. Twelve of the 37 clones encode truncated versions of the Lmd protein which can be grouped into four classes based upon the position of their N-terminal end (Fig. 7C). The encoded polypeptides in all 12 clones include the Zn-finger domain. To ascertain that the Zn-finger domain is responsible for specific target recognition, we tested in yeast cells constructs that encode defined portions of the protein. Indeed, constructs that include the Zn-finger domain are capable of activating robust levels of His and *lacZ* expression whereas those that span the N- or C-terminal region of Lmd, flanking the Zn-finger domain, are not able to activate His expression (Fig. 7C).

We performed standard DNA-binding assays to confirm that Lmd can bind specifically to enhancer I-E$_{D5}$. In the presence of in vitro-translated Lmd protein, a slower-migrating protein-DNA complex is observed with γ^{32}P-labeled I-E$_{D5}$ fragment (Fig. 7D, lanes 1-3). Formation of this complex is specifically competed by an excess amount of cold I-E$_{D5}$ DNA fragment but not by cold III-F$_{7}$, an unrelated DNA fragment of similar length (lanes 4-5).

Sequence analysis of enhancer I-E$_{D5}$ did not reveal any sequence elements that conform to the canonical binding site for Ci/Gli proteins (Aza-Blanc and Kornberg, 1999), indicating that Lmd recognizes a novel DNA sequence motif. To attempt to define the binding site, we tested in vivo four other mutated I-E$_{D5}$ derivatives (I-E$_{D5\text{-}mt1}$, I-E$_{D5\text{-}mt2}$, I-E$_{D5\text{-}mt3}$, I-E$_{D5\text{-}mt4}$), each of which contains a 10 bp block of substitutions (Fig. 7C). Normal levels of activation of reporter gene expression in somatic myoblasts are observed with I-E$_{D5\text{-}mt3}$ (data not shown) and I-E$_{D5\text{-}mt4}$ (Fig. 7J). By contrast, dramatically reduced levels of reporter gene expression are observed with I-E$_{D5\text{-}mt1}$ and I-E$_{D5\text{-}mt2}$ (Fig. 7H,I). These results, together with additional in vitro binding and competition data (not shown), indicate that the functional binding site of Lmd is within the sequence TTACCTACGCAGCGTTTACA.

DISCUSSION

We have presented genetic and molecular evidence that *lmd* function is required for the specification and development of fusion-competent myoblasts. Specified muscle founders and mononucleate muscle cells are present in *lmd* mutant embryos, indicating that *lmd* function is not critical for founder cells. By contrast, *lmd* expression marks fusion-competent myoblasts and its function is required for activating *Mef2* and *sns* in these cells. Cellular localization and molecular studies further show that *Mef2* is a transcriptional target of *lmd* in fusion-competent myoblasts. Together, the data demonstrate that not only founder cells but also fusion-competent myoblasts are the products of active specification programs.

Lmd functions in fusion-competent myoblasts

The cellular distribution of the Lmd protein is consistent with its mutant phenotype. The expression of *lmd* in somatic mesodermal cells between late stage 11 and early stage 14 is compatible with the phenotype of *lmd* mutant embryos in which loss of *Mef2* expression in fusion-competent myoblasts is first detected at late stage 12 and *sns* is never activated. Prominent levels of Lmd protein are detected in fusion-competent myoblasts, whereas extremely low, or undetectable, levels of Lmd expression are observed in rP298-*lacZ* positive founder cells. Although we can not rule out conclusively the possibility that the very low levels in founder cells could be functionally important, the presence of specified muscle founders in *lmd* mutant embryos which express *Mef2* and can differentiate into elongated MHC-expressing muscle cells

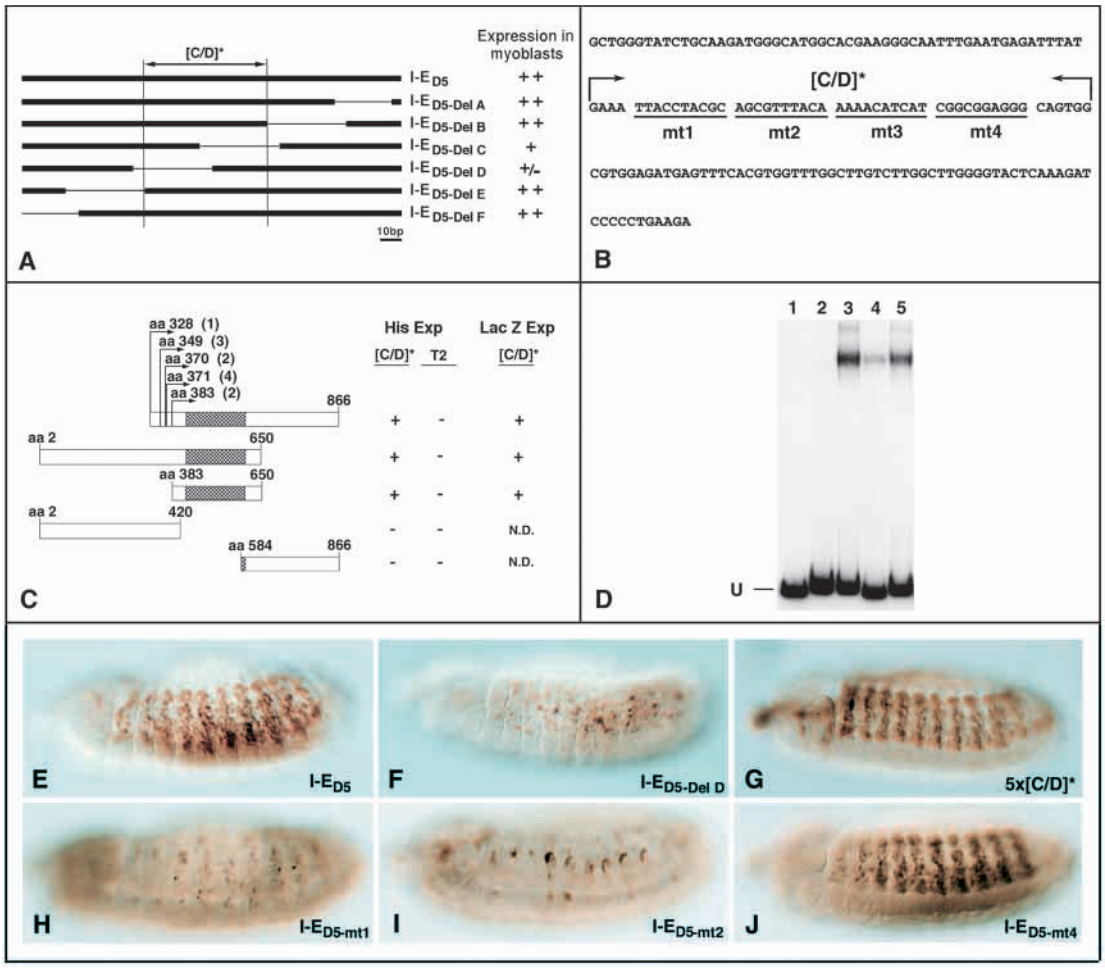

Fig. 7. Molecular screen with minimal sequences from myoblast enhancer I-E_{D5} identifies Lmd as the DNA-binding factor. (A) Diagram of deletion constructs, each of which has an internal deletion (thin line) within the 170 bp myoblast enhancer I-E_{D5}. Vertical lines bracket the endpoints of the essential [C/D]* region. (B) Sequence of enhancer I-E_{D5}. Arrows demarcate the [C/D]* region and mutated sequences in constructs I-$E_{D5\text{-}mt1}$, I-$E_{D5\text{-}mt2}$, I-$E_{D5\text{-}mt3}$ and I-$E_{D5\text{-}mt4}$ are underlined. (C) Diagram of the 12 cDNA clones encoding partial Lmd proteins from the one-hybrid screen with [C/D]* as a specific target. Unrelated target T2 was used to check for specificity. The number of clones recovered for each type is in parentheses. Lmd derivatives used to localize the DNA binding domain are also shown. The checked box denotes the Zn-finger domain. Activation of His and *lacZ* expression was monitored; N.D. denotes not determined. (D) DNA binding assays with in vitro translated Lmd protein and γ^{32}P-labeled I-E_{D5} probe, in the absence or presence of 50× molar excess of cold specific competitor DNA. Free probe is marked as (U). Lane 1, probe alone; Lane 2, lysate without DNA template; Lane 3, Lmd protein lysate; Lane 4, Lmd + cold I-E_{D5} DNA; Lane 5, Lmd + cold unrelated III-F_7 DNA. (E-J) Transgenic embryos were stained with an anti-β-gal antibody. Robust levels of *lacZ* expression are seen in somatic myoblasts of embryo with I-E_{D5} construct (E), whereas embryo with I-$E_{D5\text{-}DelD}$ shows a nearly complete loss of expression (F). Construct 5×[C/D]* drives expression in myoblasts similarly to parental I-E_{D5} (compare E with G). Embryo with I-$E_{D5\text{-}mt1}$ (H) or I-$E_{D5\text{-}mt2}$ (I) exhibits dramatic loss of reporter gene expression in somatic myoblasts, while embryo with I-$E_{D5\text{-}mt3}$ (data not shown) or I-$E_{D5\text{-}mt4}$ (J) shows normal levels of expression. Ectopic expression in I-$E_{D5\text{-}mt2}$ embryo is not in somatic myoblasts.

support the conclusion that *lmd* function is dispensible in founder cells.

Our data indicate that *lmd* activity is needed transiently during embryogenesis to trigger specific genetic programs that are critical for the generation of a fully functional somatic musculature. These *lmd*-dependent programs, which include activation of *Mef2* and *sns*, are essential for proper specification of fusion-competent myoblasts and their subsequent differentiation, which includes cell fusion. The phenotype of *Mef2*-deficient embryos, in which cell fusion is not observed, underscores the importance of *Mef2* function in fusion-competent myoblasts (Bour et al., 1995). However, it is currently not known whether *Mef2* is needed to activate fusion-specific genes or to promote this process in an indirect manner by activating genes that generate a suitable milieu for cell fusion. The *sns* gene, which is expressed exclusively in fusion-competent myoblasts independently of *Mef2*, has been shown to be required for myoblast fusion (Bour et al., 2000). Whether its functional importance is limited to being an adhesion-type of molecule or includes a potential role in signaling between myoblasts remains to be determined. In the aggregate, our findings support the notion that fusion-competent myoblasts are subject to a unique determination and differentiation program and are not simply products of a default state of cells that fail to become muscle founders. Lmd appears to be a key regulator in establishing this program. Therefore, it will be

important to identify additional targets of *lmd* that are essential for the generation of functional fusion-competent myoblasts.

In contrast to its critical role in fusion-competent myoblasts, the function of *lmd* in visceral mesodermal cells is not clear. We have noted that loss of *lmd* expression does not affect the expression of genes that are involved in visceral mesoderm specification and differentiation, such as *bap*, *Mef2*, *FasIII* and *Mhc*. In addition, *sns* is expressed in *lmd* mutant embryos albeit at reduced levels (data not shown). Thus, it appears that *lmd* function in the visceral mesoderm could be partially compensated by other gene(s) and that loss of *lmd* activity results only in subtle defects that remain to be defined.

lmd is a transcriptional regulator of *Mef2*

Previous studies demonstrated that *Mef2* expression within the somatic muscle lineage is controlled by a modular-type of regulation, suggesting that specific activators exist that differentially exert regulatory effects on the various somatic muscle enhancers (Cripps et al., 1998; Gajewski et al., 1998; Nguyen and Xu, 1998). *lmd* is identified in the present study as a direct upstream regulator of *Mef2* expression in fusion-competent myoblasts. As discussed earlier, a notable feature of the *lmd* mutant phenotype is the loss of *Mef2* expression in fusion-competent myoblasts beginning at mid-embryogenesis, whereas the earlier ubiquitous Twist-dependent *Mef2* expression in the forming mesoderm is not affected. Muscle founders also express *Mef2* normally and are capable of differentiating into mononucleate muscle cells. These observations suggest that, after the disappearance of Twist and other unknown early regulators of *Mef2*, *lmd* activity is required to activate *Mef2* expression in the fusion-competent myoblasts. Indeed, direct and independent support for *lmd* as a direct transcriptional regulator of *Mef2* in these myoblasts was derived from our yeast one-hybrid screen. In this unbiased approach, Lmd was identified as a DNA binding factor that can activate the particular enhancer I-E_{D5}, which directs *Mef2* expression in fusion-competent myoblasts. Based upon the present data, *Mef2* expression in founder cells must require yet unknown regulators that would function primarily through the two distinct founder cell enhancers that were previously defined.

Modes of regulation of *lmd* expression and activity

Our observations suggest that the development of fusion-competent myoblasts is regulated in a two-step process. The first step involves the activation of *lmd* transcription, which provides cells with the potential to become fusion competent, and the second step promotes nuclear translocation of the Lmd protein, which allows Lmd to make the cells functional for fusion.

We have shown that *lmd* expression in fusion-competent myoblasts is regulated by *Notch* and *wg*, as well as through *wg*-independent pathways. The regulation by *wg* is reminiscent of the *wg*-dependent formation of the majority of S59- and Nautilus-expressing muscle founders and the *wg*-independence of a subset of them (Baylies et al., 1995; Ranganayakulu et al., 1996). These observations suggest a coordinate regulation of both founder and fusion-competent myoblasts through Wg-dependent events. By contrast, Notch signaling has a reciprocal effect on the expression of regulatory genes in prospective muscle progenitors (which will form founders) and fusion-competent cells and, as a consequence, on the formation of these two types of myoblasts. Previous studies have established that the formation of muscle progenitors requires the absence of Notch signaling and loss of Notch function leads to increased numbers of muscle founders (Corbin et al., 1991; Bate et al., 1993). Conversely, we have found that Notch function is essential for *lmd* expression and hence the formation of fusion-competent myoblasts. This result also explains the reported Notch dependence of *sns*, a downstream gene of *lmd* (Bour et al., 2000). Interestingly, we have observed that *E(spl)* function is also required for *lmd* activation (data not shown), thus suggesting that *E(spl)* could function as an activator of *lmd* or that it allows *lmd* transcription by downregulating a repressor in precursors of fusion-competent cells. Altogether, it appears that *lmd* may be the first example of a regulatory gene that is turned on by Notch in cells that fail to be singled out from a pre-cluster and that serves to specify cell identity, which in this particular case is that of fusion-competent cells.

The nuclear/cytoplasmic distribution of the Lmd protein is reminiscent of the related *Drosophila* Ci and vertebrate Gli proteins, which are effectors of Hh signaling. The function of Ci/Gli proteins as transcriptional activators or repressors has been shown to be regulated by protein proteolysis, subcellular localization and levels (Aza-Blanc and Kornberg, 1999; Matise and Joyner, 1999; Ruiz i Altaba, 1999). Our analysis suggests that some of the post-transcriptional events described for *ci*/*Gli* gene products could also contribute to the regulation of Lmd activity. High resolution analysis showed that MEF2 expression is correlated with elevated levels of nuclear-localized Lmd protein whereas exclusive cytoplasmic-localized Lmd expression is correlated with an absence of MEF2 expression. These observations suggest that subcellular localization and, by analogy to Ci/Gli proteins, regulated processing of the Lmd protein may be required for activating *Mef2* and other target genes. The presence of two putative PKA phosphorylation sites in the C-terminal region of the Lmd protein invokes the possibility that phosphorylation could have a regulatory role, as with Ci/Gli proteins (Chen et al., 1998; Price and Kalderon, 1999; Wang and Holmgren, 2000). However, it does not appear that *hh* is needed for regulating Lmd activity because relatively well-developed muscle fibers are present in mutant embryos in which Hh activity has been removed during the relevant stages (Park et al., 1996). Nevertheless, if modulation of Lmd activity were to involve cell-cell communication through other pathways, then this could provide a mechanism to coordinate the final stage of development of the fusion-competent myoblasts with that of neighboring muscle founders.

Lmd defines a new family within the Gli superfamily of transcription factors

Although the high degree of sequence identity within the Zn-finger domain and the spacing of the Cys and His residues puts Lmd closest to Ci/Gli proteins, several notable differences exist. First, Lmd has no additional homology outside of the Zn-finger domain, as observed among Ci/Gli proteins (Matise and Joyner, 1999). Second, there is a striking divergence between Lmd and Ci/Gli proteins in the first and part of the second finger, although the terminal three fingers are highly conserved. Third, our in vivo data indicate that Lmd recognizes a novel

sequence, suggesting an involvement of the first two fingers in DNA-binding specificity. This would contrast with Gli proteins, in which binding has been shown to be mediated through the two C-terminal fingers (Pavletich and Pabo, 1993). Fourth, *ci*/*Gli* genes have important roles in a variety of Hh-dependent patterning events during *Drosophila* development, and patterning of the neural ectoderm and somites in vertebrates (Aza-Blanc and Kornberg, 1999; Matise and Joyner, 1999; Borycki et al., 2000). By contrast, Lmd appears to function only within the mesoderm and to regulate specification and differentiation events. This mesoderm-restricted feature is shared with *macho-1*, a Zic-related gene that was shown to encode an mRNA that functions as a localized determinant of muscle fate in ascidians (Nishida and Sawada, 2001).

Taken together, these features identify Lmd as the first representative of a new type of protein family within the Gli superfamily of transcription factors. Our results indicate that *lmd* function in the specification of fusion-competent myoblasts requires Wingless and Notch signaling for its initial expression and yet unknown signals for its transition into the nucleus. Nuclear Lmd then activates a spectrum of downstream genes, including the *Mef2* and *sns* genes, which have critical roles in the development and functioning of fusion-competent myoblasts. Given the critical role of *lmd* in myogenesis, it will be interesting to identify vertebrate homologs of *lmd* to determine whether analogous mechanisms of muscle cell specification and development have been conserved during evolution.

We thank N. Baker, M. Fuller, J. Hiromi, J. Mohler and A. Nose for fly stocks; S. Abmayr, M. Frasch, K. Jagla, D. Kiehart, D. Kosman and J. Reinitz for antibodies; S. Zaffran for advice on DNA binding assays, and M. Silvestro for technical assistance. H. T. N. thanks M. Frasch for advice on confocal microscopy and critical reading of the manuscript. This work has been supported by grants to H. T. N from the NSF (IBN-9905105) and NIH (AR-46281). J. B. S is supported by the NSF (IBN-0077727) and NIH (NS-35670). H. T. N dedicates this paper to the memory of Ruth M. Gubits, a dear friend and colleague who died on March 18, 2000.

Note added in proof

lame duck is the same gene as *gleeful*, which was recently isolated in DNA microarray experiments for twist target genes (Furlong et al., 2001).

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