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Notch pathway repression by *vestigial* is required to promote indirect flight muscle differentiation in *Drosophila melanogaster*

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Abstract

Drosophila dorsal longitudinal muscles develop during metamorphosis by fusion of myoblasts with larval templates. It has been shown that both *vestigial* and *Notch* are crucial for correct formation of these muscles. We investigated the relationship between *vestigial* and the Notch pathway during this process. Using *Enhancer of Split Region Transcript m6* gene expression as a reporter of Notch pathway activity, we were able to demonstrate that this pathway is only active in myoblasts. Moreover, close examination of the cellular location of several of the main actors of the N pathway (*Notch*, *Delta*, *neuralized*, *Serrate*, *Mind bomb1* and *fringe*) during dorsal longitudinal muscle development enabled us to find that Notch receptor can play multiple roles in adult myogenesis. We report that the locations of the two Notch ligands (Delta and Serrate) are different. Interestingly, we found that *fringe*, which encodes a glycosyltransferase that modifies the affinity of the Notch receptor for its ligands, is expressed in muscle fibers and in a subset of myoblasts. In addition, we demonstrate that *fringe* expression is essential for Notch pathway inhibition and muscle differentiation. Lastly, we report that, in *vestigial* mutants, *fringe* expression is lost, and when *fringe* is overexpressed, a significant rescue of indirect flight muscle degeneration is obtained. Altogether, our data show that a vestigial-differentiating function is achieved through the inhibition of the Notch pathway.
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Keywords: Myogenesis; Notch pathway; *Vestigial*; Dorsal longitudinal muscles

Introduction

During *Drosophila* development, two waves of myogenesis occur. The first, during embryogenesis, gives rise to the larval musculature, and the second, during pupation, leads to the formation of body wall, leg and flight muscles. Embryonic myogenesis has been extensively studied and has brought new insight into the processes generating differentiated muscles from undetermined mesodermal cells (for review, see Baylies et al., 1998). Adult myogenesis has been less studied, but flight muscles have emerged as a suitable model for the study of muscular differentiation. Flight muscles are located in the thorax and are subdivided into two distinct classes: the direct flight muscles (DFMs) involved in changing orientation of the wing and indirect flight muscles (IFMs) contributing to flight by deformation of the thorax. These muscles are formed from precursors selected during embryogenesis that proliferate during the three larval stages on the notal part of the wing imaginal disc. These cells are named adepithelial cells as long as they remain associated with the wing disc (Bate et al., 1991). The DFMs are small tubular muscles that arise from the fusion of the most proximal adepithelial cells that express high levels of *cut* (*ct*) (Sudarsan et al., 2001). The IFMs, which represent the most prominent muscles of the thorax, have a striated structure very close to that of vertebrate muscles. They are constituted of 6 dorsal longitudinal muscles (DLMs) and 7 dorsal ventral muscles (DVMs) per hemi segment (Crossley, 1978). The adepithelial cells involved in their formation show specific expression of the Vestigial (VG) transcription factor and a low level of CT (Sudarsan et al., 2001). Development of each category of IFMs is based on two different developmental strategies. DVMs are formed de novo, whereas, to form, DLMs use persistent larval muscles as a scaffold. At the onset of metamorphosis, all larval muscle fibers histolyze except for a subset of thoracic muscles, the larval oblique muscles (LOMs;

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three LOMs by hemi segment). About 8 h after puparium formation (APF), adepithelial cells have migrated from the imaginal discs to surround the three LOMs and fusion begins. Between 14 and 20 h APF, the three larval templates (LOMs) vacuolate and split into six DLMs (Fernandes et al., 1991). The fusion between developing DLMs and myoblasts is achieved at 36 h APF. Myofibers differentiate and increase in size to complete the formation of the adult pattern (Fernandes et al., 1991).

Myogenesis is a complex process which includes proliferation of myoblasts, their migration and physical alignment, and finally their differentiation. The differentiation of myoblasts results in both profound morphological and biochemical changes. Morphologically, myoblasts fuse to form multinucleated myotubes. Biochemically, myogenic differentiation is characterized by a coordinate induction of genes encoding the proteins necessary for the operating functions of muscle, such as myosin and sarcomeric actin, and a repression of the genes involved in myoblast determination and proliferation. In *Drosophila*, as in vertebrates, it has been shown that activation of the Notch (N) pathway inhibits myogenic differentiation (Anant et al., 1998; Nofziger et al., 1999; Shawber et al., 1996). The N pathway is an evolutionarily conserved cell–cell signaling pathway that plays a critical role in tissue development in many organisms. The signal receiving cell expresses the transmembrane N receptor, and the signal sending cell expresses the N transmembrane ligands, Serrate (SER) and/or Delta (DL). When N binds to its ligand, it undergoes a series of proteolytic cleavages leading to the release of the N intracellular domain (N^I). N^I is relocalized in the nucleus where it interacts with the Suppressor of Hairless factor (Su(H)) to activate the targets of the N pathway (for review on N pathway, see Lai, 2004; Mumm and Kopan, 2000; Schweisguth, 2004). Several other factors have been involved in N signaling such as Fringe (FNG), a glycosyltransferase that enhances N-DL interactions by modifying N (Bruckner et al., 2000; Moloney et al., 2000; Panin et al., 1997), and E3 ubiquitin ligases which positively regulate the N pathway by promoting the endocytosis of the ligands (Le Borgne et al., 2005a).

During *Drosophila* adult myogenesis, it has been shown by Anant et al. that both reduction and constitutive activation of *N* affect the development of IFMs but not the closely related DFMs (1998). Moreover, the authors demonstrate that the expression of the transcription factor TWIST (TWI), a myogenic inhibitory factor, is under the control of N in swarming myoblasts about 24 h APF. Ectopic expression of N^I in developing fibers leads to ectopic expression of *twi* and to muscle degeneration, and thermosensitive mutants of the *N* receptor show a decrease in *myosin heavy chain* (*mhc*) expression level (Anant et al., 1998). It was then proposed that N and TWI act as muscle differentiation inhibitors and that N signaling could play a direct role in maintaining myoblasts in an undifferentiated state until they are correctly positioned for differentiation (Anant et al., 1998). In a previous study, we have reported that flies that are homozygous for vg^{null} mutation present specific degenerations of the IFMs. We have shown that degenerating fibers exhibit ectopic expression of TWI and that these fibers lose the expression of the differentiation marker *Actin88F* (Bernard et al., 2003). These data strongly suggest that muscular differentiation is impaired in vg^{null} mutants. With regard to the similarity of phenotypes, we investigated *vg-N* interactions during *Drosophila* IFM formation.

So far, studies involving the Notch (N) pathway in IFM development have been based on genetic experiments modulating N receptor activity (Anant et al., 1998). No experiments have as yet been performed to determine whether core components of this pathway are expressed during this process. We therefore began our investigation of *vg-N* relationships by examining the expression and the cellular location of several components of this pathway during DLM development. In this paper, we describe the location of *Dl*, *N*, *fng*, as well as the E3 ubiquitin ligases *neuralized* (*neur*) and *mind bomb 1* (*mib*1) during DLM development. We report that the spatial patterns of the two N ligands (DL and SER) are different during DLM formation. Then we demonstrate that the N pathway is active only in the myoblasts. Interestingly, *fng*, which encodes a glycosyltransferase that modifies the affinity of the N receptor for its ligands, is expressed in differentiated muscles and in a few myoblasts. In vg^{null} mutants, *fng* expression is not detected, and an ectopic expression of N targets is observed in developing DLMs. When *fng* is overexpressed, in vg^{null} mutants, a significant rescue is obtained, showing that VG's main function in adult myogenesis is to activate *fng*, which inhibits the N pathway and allows a correct differentiation of DLMs.

Material and methods

Fly stocks

The vg^{null} strain was generated in our laboratory (Paumard-Rigal et al., 1998; Zider et al., 1998). *MHC-LacZ* (Myosin Heavy Chain promoter), *Duf-LacZ* and *Enhancer of Split Region Transcript m6-GFP* strains are respectively described in Hess et al. (1989), Nose et al. (1998), and Lai et al. (2000b). The *neur^{P72}-GAL4* and *UAS-H2B::YFP* lines are described in Bellaiche et al. (2001). *UAS-CD8::GFP* strain is described in Lee and Luo (1999). *1151-GAL4* has been described in Anant et al. (1998). The *fng^{35UZ-1}* strain comes from S. Cohen's laboratory. The *UAS-fng* and *N^{ts1}* strains were described in Rauskolb and Irvine (1999) and Shellenbarger and Mohler (1978), respectively, and were obtained from the Bloomington *Drosophila* stock center (strains #5831 and 2533, respectively). *UAS-N^I* strain is described in Go et al. (1998).

Muscle preparation

Dissection of pupae was performed as previously described (Fernandes et al., 1991). Adult flies were fixed in 4% paraformaldehyde overnight. Thoraces were cut sagittally, mounted in glycerol and viewed under polarized light.

Immunochemistry

Tissues were fixed for 1 h in 4% paraformaldehyde, washed three times in PBT (phosphate saline buffer, 0.3% triton X100) and incubated for 1 h in PBT-NGS (PBT, 4% normal goat serum) at 4°C. Samples were incubated overnight in a PBT-NGS antibody solution at 4°C. Samples were washed three times in PBT for 10 min. For fluorescence detection, samples were then incubated with fluorescently labeled secondary antibody for 2 h, washed three times in PBT and mounted in Citifluor (Citifluor Ltd., London, England). anti-VG, anti-TWI, anti-

MIB1 and anti-SER antibodies were generous gifts from S. Carroll, S. Roth, F. Schweisguth and K. Irvine, respectively, and were used at a 1:200, a 1:5000, a 1:200 and a 1:2000 dilution, respectively. Mouse anti-GFP (used at 1:200 dilution) and rabbit anti-GFP (used at 1:1000 dilution) antibodies were purchased from Roche (Penzberg, Germany) and Molecular Probes (Foster City, USA), respectively. Rabbit anti-βGAL antibody was purchased from Cappel (Durham, NC, USA) and used at a 1:1000 dilution. Mouse anti-βGAL, anti-CT, and anti-N^I antibodies are supernatant purchased from the Developmental Studies Hybridoma Bank (DSHB) and were used at a 1:200 dilution. Anti-DL is a concentrate serum also purchased from DSHB and used at a 1:1000 dilution. Preparations were observed with a Leica TCS-SP confocal microscope.

Results

N is expressed in myoblasts and in muscles fibers

We first looked for the N receptor expression in 8 h APF *Dumbfounded-LacZ* (*Duf-LacZ*) pupae (Figs. 1A–C). In this strain, βGAL was visualized in the nuclei of LOMs, which serve as templates for DLM formation (Dutta et al., 2004), as revealed by anti-βGAL detection (Fig. 1A, asterisks). Anti-N labeling showed that N is expressed in myoblasts (arrowheads) and in muscle fibers (arrows) (Fig. 1B; merge in C). N expression persists during the development as shown by immunodetection experiments performed in 21 h APF *MHC-LacZ* pupae (Figs. 1D–F). In *MHC-LacZ* strain, βGAL was expressed in developing fibers according to *MHC* expression pattern (Fig. 1D, asterisks). Anti-N labeling allowed the detection of N in myoblasts between developing fibers (Fig. 1E, m; merge in F). It was difficult, however, to know whether N is expressed at the muscle fiber membranes or not. To address this question, we used an *1151-GAL4*; *UAS-CD8*: :*GFP* strain. In this strain, GFP-tagged transmembrane CD8 protein is expressed in muscles and myoblasts and makes it possible to reveal cell

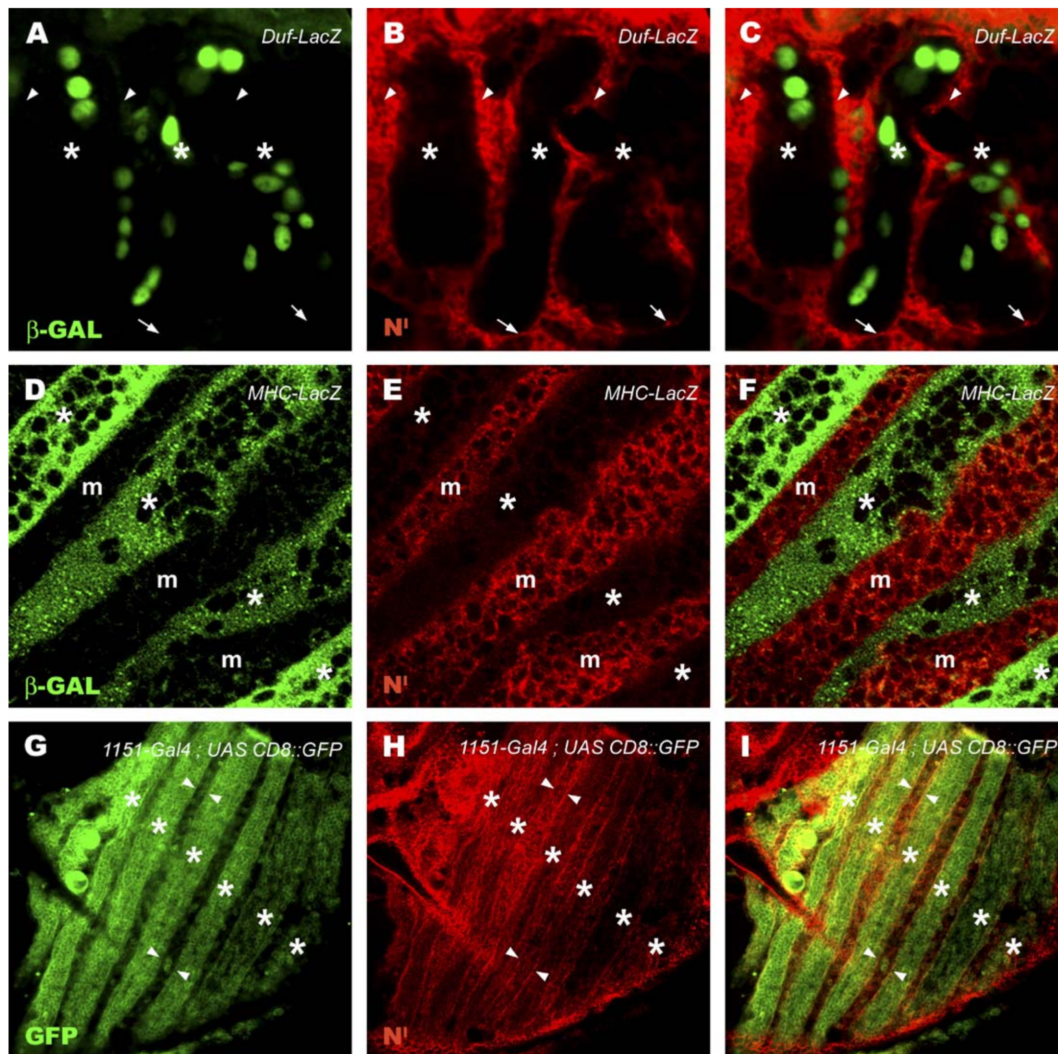

Fig. 1. Expression of the N receptor in developing DLMs. In all panels, asterisks indicate the location of muscle fibers. In 8 h APF *Duf-LacZ* pupae (A–C), LOMs were located by βGal staining (A). N expression (B) is visualized on myoblasts (arrowheads) and muscle membranes (arrows). In 21 h APF *MHC-LacZ* pupae (D–F), βGal labeling reveals developing muscle fibers (D) and anti-N^I antibodies (E) show an expression of the N receptor at the membrane surface of myoblasts (m). In the *1151-gal4; UAS-mCD8*: :*GFP* strain (G–I), GFP (G) is tagged to membranes. Merge in panel I reveals that the N receptor (H) is also localized on membrane muscles (arrowheads).

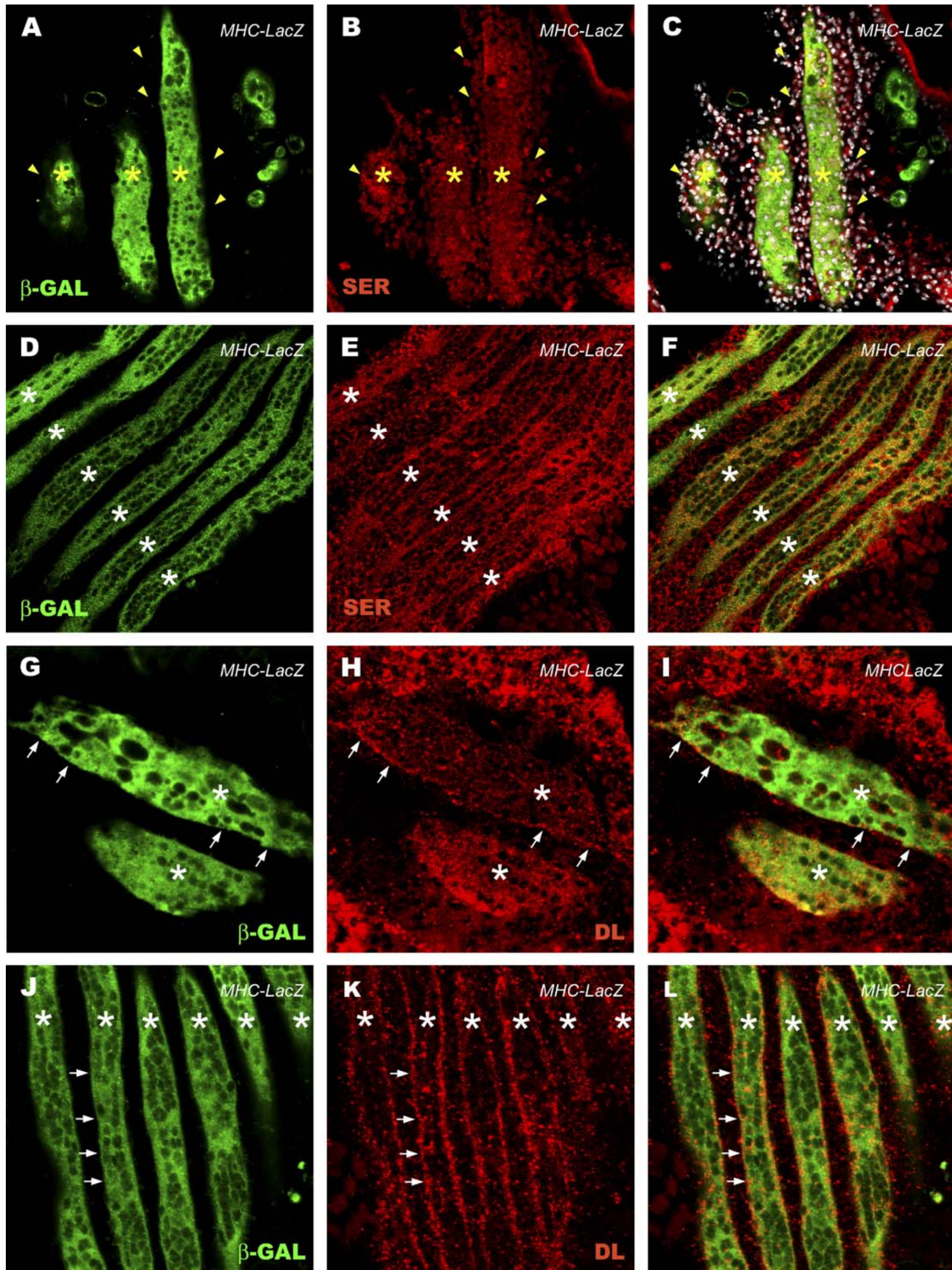

Fig. 2. Expression of N ligands during DLM formation. In all panels, asterisks indicate the location of muscle fibers. In 10 h APF (A–C, G–I) and 21 h APF pupae (D–F, J–L) of *MHC-LacZ* strain (A–L), βGal antibodies reveal muscles fibers (A, D, G, J). Antibodies against SER show that the SER ligand (B, E) is present at the membranes of swarming myoblasts (arrowheads) and muscles fibers (asterisks). By 10 h APF (A, C), all swarming myoblasts express SER, as shown by a DAPI staining (in white, on merge I). Antibodies against DL (H, K) show that the DL ligand is present at the membrane surface of developing muscles (arrows).

membranes (Fig. 1G asterisks: muscles). Anti-N labeling showed that N is expressed at muscle membranes (Fig. 1H, arrow, merge in I) and at myoblast membranes (Figs. 1G–I, arrowheads). Thus, between 8 h APF and 30 h APF, N is detected at the membranes of both myoblasts and muscles fibers.

DL and SER are differentially expressed during DLMs development

In *Drosophila*, two transmembrane proteins can activate the N receptor: DL and SER. During *Drosophila* development, DL and SER have distinct functions, possibly not because they have

distinct signaling properties but probably because their expression patterns are non-overlapping. In the sensory bristle lineages, DL and SER have been proposed to have redundant functions (Zeng et al., 1998). Furthermore, DL and SER appear to be interchangeable; an overexpression of *Ser* can partially rescue the *Dl* neurogenic phenotype (Gu et al., 1995) and, conversely, ectopic expression of *Dl* can partially rescue the *Ser* wing phenotype (Klein and Arias, 1998). A recent study in vertebrates, however, shows that DL and SER vertebrate homologs can have different signaling activities (Amsen et al., 2004).

When we looked for SER expression, we did not detect any expression before 10 h APF. In 10 h APF (Figs. 2A–C) and 21 h APF (Figs. 2D–F) *MHC-LacZ* pupae, however, anti-SER antibodies (Figs. 2B, E) revealed the presence of SER in both developing fibers (asterisks), located by anti-βGAL labeling (Figs. 2A, D), and swarming myoblasts (arrowheads) (merge in Figs. 2C, F).

During DLM development, no DL expression was found at 8 h APF, and we started to detect DL expression at 10 h APF. In 10 h APF pupae of the *MHC-LacZ* strain (Figs. 2G–I), anti-βGAL labeling revealed small developing fibers (Fig. 2G, asterisks) that had begun to fuse with surrounding myoblasts. DL expression detected by anti-DL antibodies was restricted to developing DLMs (Fig. 2H arrows; merge in 2I). At 21 h APF in the *MHC-LacZ* strain (Figs. 2J–L), muscles labeled by anti-βGAL antibodies (Fig. 2J, asterisks) showed a strong DL labeling at the membrane of developing fibers (Fig. 2K, merge in L, arrows). Thus, it appears that both N ligands are expressed during DLM formation after 10 h APF. Nevertheless, their expressions differ spatially; DL is specifically expressed at the membrane of developing DLMs, whereas SER is expressed in both myoblasts and muscles.

Neur is only expressed in the developing muscle fibers

Numerous studies have emphasized the role of E3-ubiquitin ligases in activation and regulation of the N pathway (for review, see Le Borgne et al., 2005a). It has been shown that internalization of ligands in signal sending cells is necessary for the activation of the pathway in receiving cells (Le Borgne and Schweisguth, 2003a; Le Borgne et al., 2005b; Seugnet et al., 1997). In *Drosophila*, two E3-ubiquitin ligases are known to be responsible for internalization of DL and SER. Neuralized (NEUR) was the first to be described, and it has been reported that NEUR positively regulates the N pathway by promoting the endocytosis and degradation of DL (Lai et al., 2001; Pavlopoulos et al., 2001; Le Borgne and Schweisguth, 2003b). Recently, Mind bomb 1 (MIB1), a second E3-ubiquitin ligase, was reported to ubiquitinate both N ligands (Lai et al., 2005; Le Borgne et al., 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005).

We looked for MIB1 expression during DLM development, using anti-MIB1 antibodies. No expression of MIB1 was detectable in 8 h APF animals. MIB1 expression was first visualized at 10 h APF and persisted during DLM formation. In the *MHC-LacZ* strain, 10 h APF pupae (Figs. 3A–C) showed that MIB1 (Fig. 3B) was strongly expressed in myoblasts (arrowheads), and a faint expression was detected in muscle fibers (asterisks) that were located by anti-βGAL labeling (Fig. 3A). Likewise, in 21 h APF pupae (Figs. 3D–F), MIB1 expression (Fig. 3E) was clearly observed at the membrane of swarming myoblasts (arrowheads), whereas developing DLMs showed a reduced expression (asterisks).

At 8 h APF, *neur* expression was monitored using the *neur^{P72}* GAL4 enhancer trap driving the *H2B::YFP* transgene in a *Duf-LacZ* background (Figs. 3G–I, asterisks). *H2B::YFP* was observed in the larval nuclei (Fig. 3H, red) as revealed by the co-localization with βGAL labeling (Fig. 3G, merge in I). Conversely, no expression was found in myoblasts labeled by TWI antibodies (Fig. 3H, blue). Similarly, in a *MHC-LacZ* background, 21 h APF pupae showed no *H2B::YFP* expression in swarming myoblasts where TWI was expressed (Fig. 3K, m). *neur^{P72}* expression was only found in developing fibers (Fig. 3K, asterisks). Muscular expression was confirmed by *MHC-LacZ* labeling (Fig. 3J, asterisks; merge in L). A similar expression pattern of *neur* was found with the A101 (*neur-LacZ*) enhancer trap (data not shown). Muscular *neur* expression associated with the presence of DL at DLM membranes strongly suggests that muscle fibers can be considered to be signal sending cells that activate a DL-N pathway in signal receiving cells.

The Notch pathway is active in myoblasts

The N pathway is essential for the proper patterning and development of most tissue in all metazoan organisms. This signaling pathway regulates directly or indirectly the expression of many genes during *Drosophila* development. In order to test N activity during DLM development, we looked for a direct and specific target of this pathway. There are two large families of direct N target genes that are clustered in two genomic regions, named the Enhancer of split-Complex (E(spl)-C) and the Bearded Complex. Together, these complexes contain seven basic helix–loop–helix repressor encoding genes and ten Bearded genes, all of which regulate N signaling (Artavanis-Tsakonas et al., 1999). In every process in which the N pathway is involved, some of these genes are expressed, but each gene has a specific pattern of expression.

We have found that *Enhancer of split region transcript m6* (*E (spl)-m6*) is expressed during IFM formation. *E(spl)m6* encodes a non-bHLH protein related to Bearded proteins and is encoded by the *Enhancer of Split* Locus. It was already reported that *E (spl)m6* is a direct target of the N pathway that is expressed in a subset of adepithelial cells (Lai et al., 2000a). When we looked at *E(spl)m6-GFP* reporter gene expression in 8 h APF *Duf-LacZ* pupae (Figs. 4A–C), we observed GFP expression (Fig. 4A) in some myoblasts swarming around the LOMs revealed by βGAL expression (Fig. 4B, merge in C). At 21 h APF *E(spl) m6-GFP* reporter gene was expressed in swarming myoblasts (Fig. 4D) revealed by a TWI labeling (Fig. 4E, merge in F). No labeling was found in developing fibers (Figs. 4D–F, asterisks).

These findings suggest that the N pathway is active in myoblasts and inactive in developing fibers. We had, however, to verify that *E(spl)m6-GFP* is a N pathway target in swarming myoblasts like in adepithelial cells, as well as whether the N

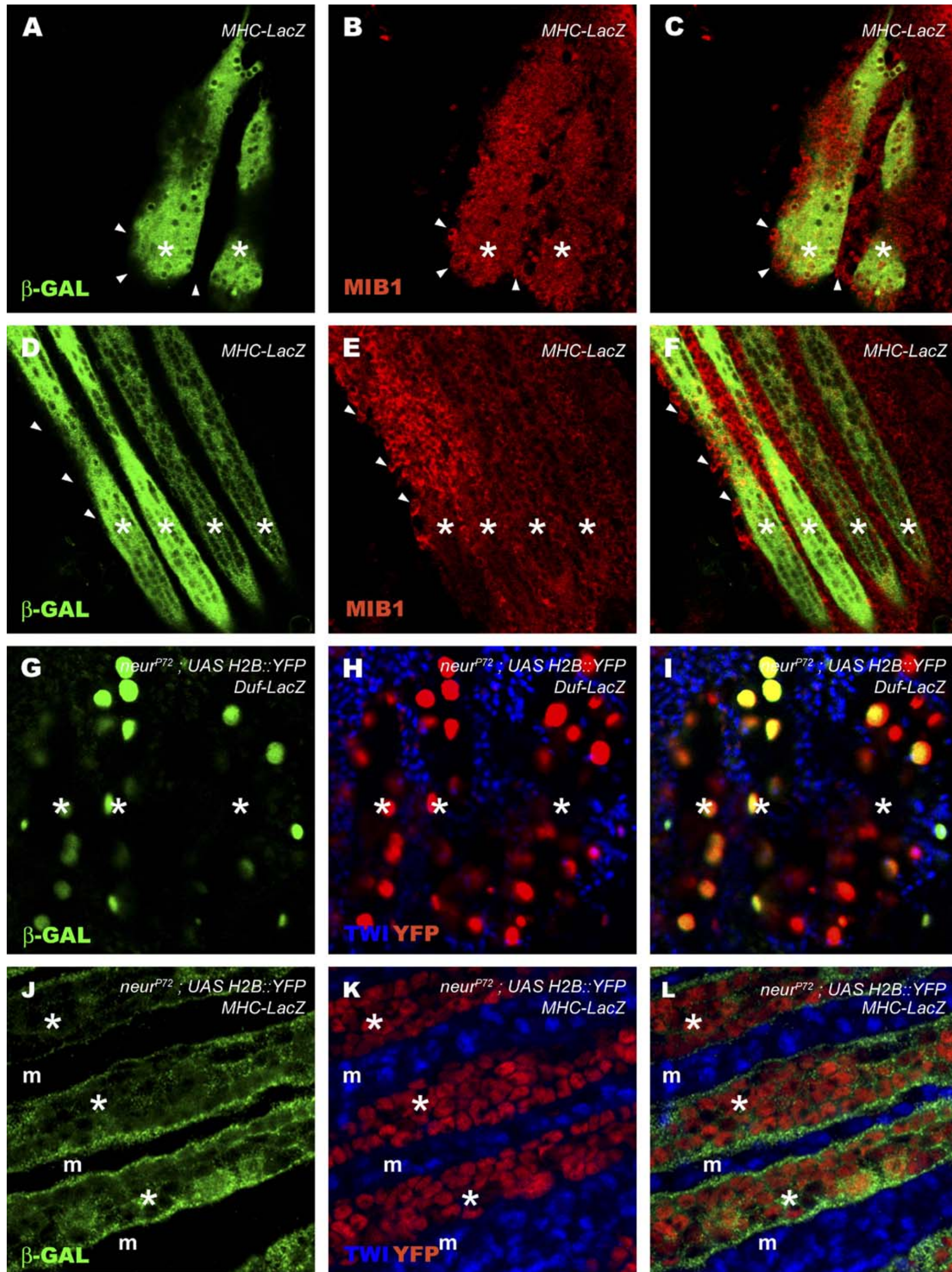

Fig. 3. *neur* and MIB1 expressions during DLM development. In all panels, asterisks indicate the location of muscle fibers. In 10 h APF (A–C) and 21 h APF (D–F) *MHC-LacZ* pupae, βGal antibodies revealed muscles fibers (A–D) and anti-MIB1 antibodies (B–E) show the presence of MIB1 protein in myoblasts (arrowheads) and developing muscles. In order to look at *neur* expression by 8 h APF (G–I), we crossed the $neur^{P72}$; *UAS H2B*: :*YFP* strain with *Duf-LacZ* strain. Pupae of the progeny stained with βGAL (G) and TWI (H, blue staining) antibodies revealed that *neur* expression (H, red staining) is restricted to muscle fibers (merge in panel I). The fact that not all the nuclei of LOMs appear to express βGAL is due to confocal Z sections. To look at *neur* expression by 21 h APF (J–L), we similarly crossed the $neur^{P72}$; *UAS H2B*: :*YFP* strain with the *MHC-LacZ* strain. At this stage, *neur* expression (K, red staining) is still restricted to muscle fibers located by βGAL labeling (J). Anti-TWI staining (K, blue staining) confirms that no *neur* expression is visualized in myoblasts (m).

pathway can activate *E(spl)m6-GFP* in developing muscles. Thus, we looked for *E(spl)m6-GFP* expression in N^{ts1} genetic context (Figs. 4G–J). Since *twi* is not expressed in N^{ts1} genetic context (Anant et al., 1998), it could not be used to label myoblasts. To bypass this problem, we used DAPI to label all nuclei (Fig. 4I) associated with a *MHC-LacZ* labeling to detect muscle fibers (Fig. 4G). Overlay of the two labelings enabled us to distinguish myoblast nuclei (arrowheads, merge in Fig. 4J) from muscle nuclei (asterisks, merge in Fig. 4J). No *E(spl)m6-GFP* expression was detected in myoblasts at restrictive

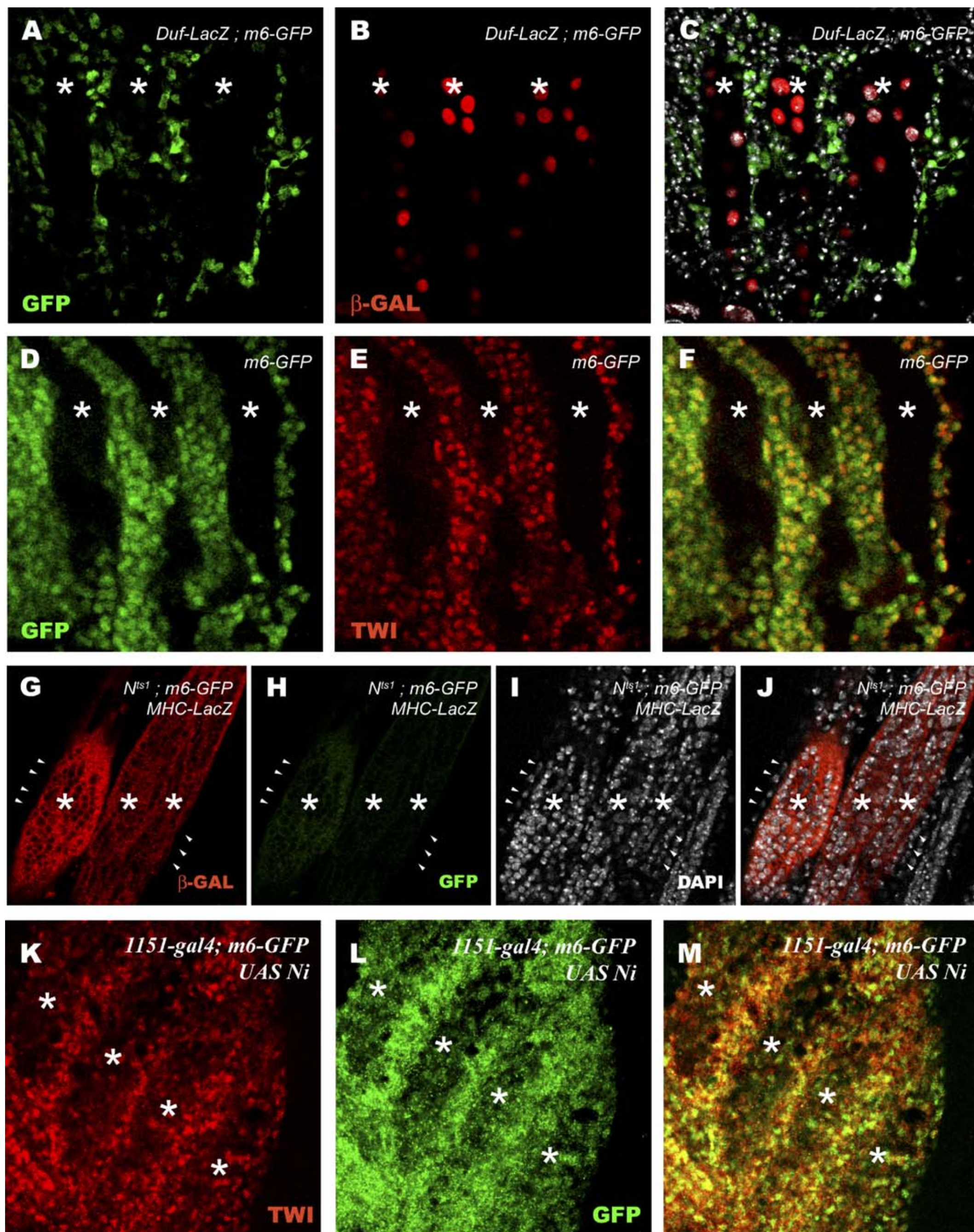

Fig. 4. *E(spl)m6* is a target of the N pathway and is specifically expressed in swarming myoblasts. In all panels, asterisks indicate the location of muscle fibers. To look at *E(spl)m6* expression in 8 h APF pupae (A–C), we crossed the *E(spl)m6-GFP* strain with the *Duf-LacZ* strain. In 8 h APF pupae of the progeny, staining with βGal antibodies (B) locates LOMs, and anti-GFP antibodies (A) revealed the expression of *E(spl)m6-GFP* transgene in swarming myoblasts only. DAPI labeling (in white, on merge in panel C) show that at the 8 h APF *E(spl)m6-GFP* transgene is not expressed in all myoblasts. In 21 h APF pupae of *E(spl)m6-GFP* strain (D–F), anti-TWI antibodies (E) show that transgene expression (D) is only detectable in myoblasts surrounding developing fibers. Merge in panel F reveals that all TWI-expressing cells are GFP positives. In N^{ts1}; *MHC-LacZ* pupae grown under restrictive conditions (G–J), anti-βGAL labeling reveals muscle fibers (G, asterisks). DAPI staining shows nuclei (I). Nuclei outside the developing fibers (arrowheads, merge in panel J) belong to swarming myoblasts. No *E(spl)m6-GFP* expression (H) is detectable in myoblasts (arrowheads) or developing fibers (asterisks), confirming that *E(spl)m6* is a target of the N pathway in swarming myoblasts. When N^I is overexpressed in *1151-Gal4*, *UAS-N^I*, *E(spl)m6-GFP* flies, staining with TWI (K) and GFP (L) antibodies reveals ectopic *E(spl)m6-GFP* expression in muscle fibers, as well as TWI. Merge in panel M.

temperature (Fig. 4H, arrowheads) confirming that *E(spl)m6-GFP* is a N pathway target in swarming myoblasts. We next verified that ectopic activation of the N pathway in developing fibers could lead to ectopic expression of *E(spl)m6-GFP*. We overexpressed N^I in myoblasts and developing fibers using the *1151-GAL4* driver. As previously described, an ectopic expression of TWI can be detected in developing fibers (Fig. 4K). Moreover, an ectopic expression of *E(spl)m6-GFP* was observed in fibers (Fig. 4L, merge in M). Thus, ectopic activation of the N pathway in developing fibers leads to ectopic

activation of *E(spl)m6-GFP*. These results clearly demonstrate that the N pathway is activated in myoblasts and not in developing fibers.

fng is expressed in a subset of myoblasts

We found that both N ligands are expressed during DLM formation, and that activity of the N pathway is detected only in swarming myoblasts. Moreover, it has been shown that activation of the N receptor by its ligands is influenced by glycosyltransferases that participate in the synthesis of *O*-linked fucose glycans attached to its extra-cellular domain (reviewed in Haines and Irvine, 2003). *O*-linked fucose is a substrate for β1,3-*N*-acetylglucosaminyltransferases encoded by *fringe* (*fng*) gene. Glycosylation by Fringe exerts a positive influence on DL-N signaling but a negative influence on SER-N signaling (Bruckner et al., 2000; Moloney et al., 2000).

We subsequently looked for *fng* expression, using the $fng^{35UZ\text{-}1}$ reporter. In 8 h APF pupae, *fng* expression (Fig. 5A) was detected in a subset of swarming myoblasts (arrowheads) as revealed by TWI labeling (Fig. 5B, merge in C). Similarly, in 21 h APF $fng^{35UZ\text{-}1}$ pupae (Figs. 5D–F), anti-βGAL antibodies revealed *fng* expression (Fig. 5D) in only a small proportion of swarming myoblasts detected by anti-TWI antibodies (Fig. 5E, merge in F) and in muscles fibers (asterisks). Muscular expression of *fng* became detectable from 16 h APF (data not shown). Interestingly, *fng*-expressing myoblasts seems to be very close to the DL-expressing developing fibers (Figs. 5D–F, arrowheads), suggesting that these myoblasts will shortly fuse and thus differentiate soon. Since the N pathway is known to inhibit differentiation, we wanted to find out whether N activity differed in *fng*-expressing and non-expressing myoblasts. We, therefore, looked to see whether or not *fng* expression was correlated with differential expression of *E(spl)m6-GFP* transgene. In 21 h APF *fng-LacZ*, *E(spl)m6-GFP* pupae (Figs. 5G–I, region boxed is magnified in Figs. 5G′–I′), βGAL labeling (Figs. 5G, G′) revealed that *fng*-expressing myoblasts showed no expression (white arrowhead) or a reduced expression (yellow arrowhead) of GFP (Figs. 5H, H′) compared to *fng* non-expressing myoblasts (arrows) (merge Figs. 5I, I′). Thus, it is likely that *fng* represses *E(spl)m6* expression.

VG represses the N pathway in developing DLMs

A previous study has shown that ectopic activation of the N pathway in developing fibers leads to ectopic expression of *twi* (Anant et al., 1998). Moreover, we have previously shown that loss of *vg* induces an ectopic reactivation of *twi* in developing fibers after 24 h APF (Bernard et al., 2003). Thus, we propose that absence of VG in developing fibers could lead to N pathway ectopic activation and finally to TWI expression. To address these questions, we looked for *E(spl)m6-GFP* reporter gene expression in vg^{null} developing fibers. No ectopic expression of *E(spl)m6-GFP* was seen in developing fibers of vg^{null} pupae about 24 h APF (data not shown). In 30 h APF vg^{+} developing fibers, *MHC-LacZ* transgene allowed the visualization of developing fibers (Fig. 6A, asterisks). *E(spl)m6-GFP* was exclusively expressed in swarming myoblasts at this stage (Fig. 6B, merge in C). In 30 h APF vg^{null} pupae, developing fibers, located by *MHC-LacZ* expression (Fig. 6D, asterisks), ectopically expressed the *E(spl)m6-GFP* reporter gene (Fig. 6E, merge in F). Thus, since the absence of VG in developing DLMs leads to N pathway activation about 30 h APF, we conclude that VG represses the N pathway in developing DLMs.

fng is responsible for repression of the N pathway in developing fibers

In order to understand the mechanisms leading to N derepression in vg^{null} developing DLMs, we first looked for N actor expression in DLMs of vg^{null} homozygous fly. N, DL, SER expressions did not change compared to vg^{+} controls (data not shown). On the other hand, *fng* expression monitored by the $fng^{35UZ\text{-}1}$ reporter gene was completely lost during DLM formation. In 21 h APF vg^{null}/vg^{null} pupae, anti-βGAL antibodies did not reveal any expression of *fng* (Fig. 7A) either in muscle fibers (asterisks) or in swarming myoblasts located by TWI (Fig. 7B, merge in C). This result shows that VG is required for *fng* expression during DLM development. We then decided to overexpress FNG in myoblasts and developing fibers of homozygous vg^{null} pupae using the *1151-GAL4* driver and looked for *E(spl)m6-GFP* in developing muscles at 30 h APF (Figs. 7D–F). Fibers and myoblasts were visualized by an anti-CT labeling (Fig. 7D, asterisk for muscles). No ectopic expression of *E(spl)m6-GFP* was seen in developing fibers (Fig. 7E, asterisks, merge in F), showing that FNG expression is sufficient to repress the N pathway in developing fibers, even in the absence of VG. We subsequently looked at adults overexpressing FNG in vg^{null} genetic context according to the *1151-GAL4* driver (Figs. 7G–H). A significant rescue of muscle degeneration phenotype was observed (Fig. 7H) compared to controls (no FNG overexpression, Fig. 7G). Thus, FNG repression of the N pathway in developing DLMs is a key feature of their development: absence of *fng* expression leads to N derepression and finally to muscle degeneration. Thus, it appears that the main function of VG is to activate *fng*, either directly or indirectly, to allow correct development of DLMs.

Discussion

A previous study stated that the N pathway is required to maintain *twi* expression in swarming myoblasts and inhibit muscle differentiation (Anant et al., 1998). These authors proposed two models to explain N function during DLM development. The first model proposed was based on a mechanism of ‘lateral inhibition’, where a ‘founder cell’ is chosen from a pool of myoblasts and the N receptor functions in other myoblasts to receive signals that inhibit these myoblasts from becoming founder cells. The recent description of a ‘founder cell’ marker in adult myogenesis, the *duf* gene, allowed VijayRaghavan and coworkers to test this model. It was challenged by the demonstration that selection of *duf*-

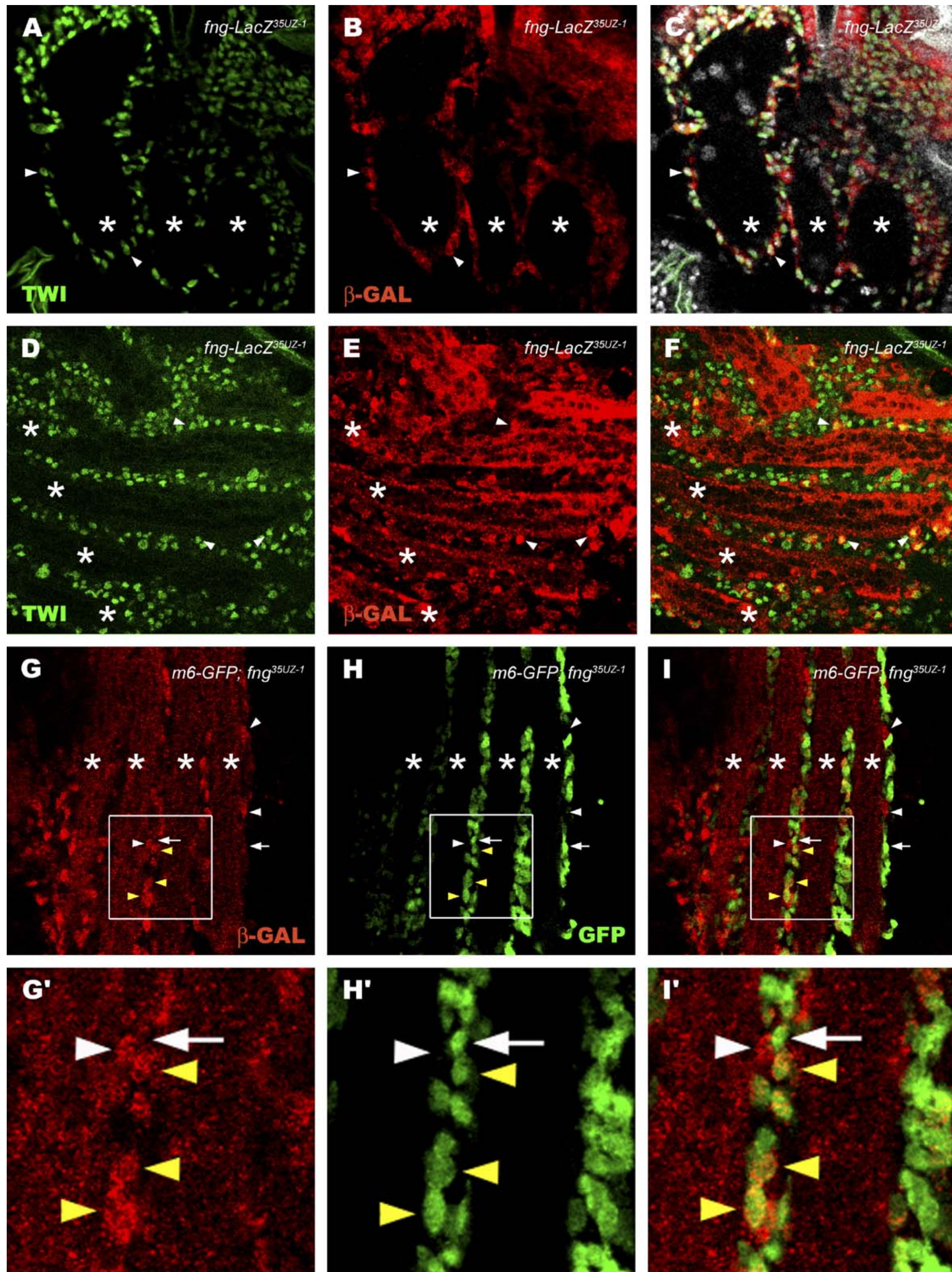

Fig. 5. *fng* expression is restricted to a subset of myoblasts. In all panels, asterisks indicate the location of muscle fibers. In order to characterize *fng* expression during DLM formation, we used the *fng*$^{35UZ-1}$ reporter gene. In 8 h APF (A–C), anti-βGal antibodies show *fng* expression (B) in myoblasts only. Anti-TWI labeling (A) reveals that only a subset of TWI-expressing myoblasts is *fng*-positive (arrowheads) (merge in panel C). In 21 h APF *fng*$^{35UZ-1}$ pupae, staining with βGal (D) and TWI (E) antibodies shows *fng* expression in muscles and in some swarming myoblasts, as revealed by co-localization of TWI (green) and βGAL (red) staining (arrowheads) in the merged picture (F). To examine the correlation between *E(spl)m6* and *fng* expression, we crossed *fng*$^{UZ35-1}$ and *E(spl)m6-GFP* strains. In the progeny, 21 h APF pupae anti-GFP (H) and βGAL (G) antibodies show that *fng*-expressing myoblasts show either reduced (yellow arrowheads) or no expression (white arrowheads) of *E (spl)m6-GFP* transgene, compare to *fng* non-expressing myoblasts (arrows) (merge in panel I). Panels G′–I′ are a magnification of the region boxed in panels G–I.

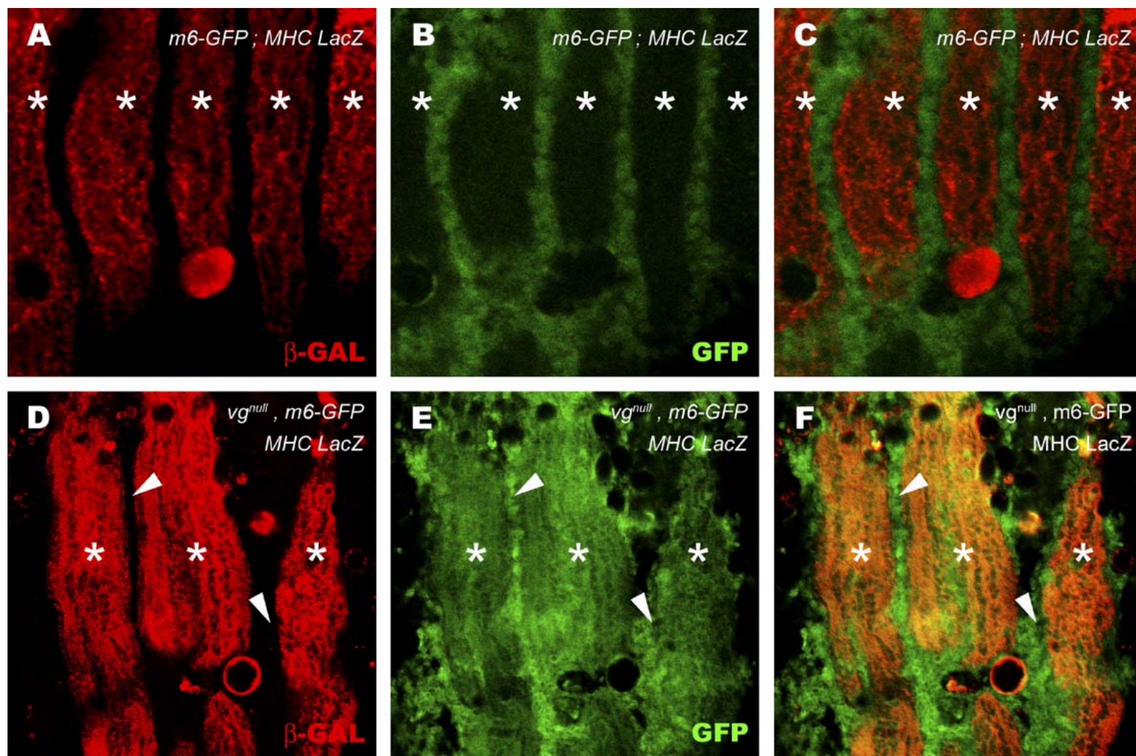

Fig. 6. *E(spl)m6* derepression in *vg*^{null} genetic context. In all panels, asterisks indicate the location of muscle fibers. In control 30 h APF *E(spl)m6-GFP*; *MHC-LacZ* pupae, staining with βGAL antibodies reveals developing fibers (A). *E(spl)m6-GFP* expression (B) is detectable in swarming myoblasts only (merge in panel C). In *vg*^{null} homozygous, *MHC-LacZ*, *E(spl)m6-GFP* 30 h APF pupae, anti-βGAL staining reveals developing fibers (D, asterisks). *E(spl)m6-GFP* is ectopically expressed in developing fibers (E, asterisks, merge in panel F), showing that the N pathway is ectopically activated in a *vg*^{null} genetic context.

expressing founders is not mediated by lateral inhibition during adult myogenesis (Dutta et al., 2004). The second model proposed by Anant et al. was that N signaling is directly involved in maintaining myoblasts in an undifferentiated state until they are correctly positioned to receive the appropriate environmental signals to differentiate. According to this model, in N^{ts} conditions, myoblasts express differentiation markers, but degeneration of adult muscles have also been reported (Anant et al., 1998), suggesting that the N receptor probably plays an additional role. The close examination of the cellular locations of several components of the N pathway during DLM development that we report in this paper, as well as the role of *fng*, enable us to complete this model and further suggest that during DLM formation N function is multiple.

Indeed, we have observed that the N receptor is expressed in myoblasts and in muscle fibers from 8 h APF and persists during development (Fig. 1). By 10 h APF, expression of the two known N ligands begins. DL is specifically expressed in developing fibers, whereas SER is expressed in both swarming myoblasts and developing fibers (Fig. 2). The signaling properties of these ligands have been demonstrated to require endocytosis (Le Borgne and Schweisguth, 2003a; Le Borgne et al., 2005b; Seugnet et al., 1997). Until today, two proteins have been shown to be involved in endocytosis and activation of N ligands: NEUR and MIB1 (Lai et al., 2001, 2005; Pavlopoulos et al., 2001; Le Borgne et al., 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). These proteins have similar molecular activities but different developmental functions in *Drosophila* (Lai et al., 2005; Le Borgne et al., 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). We found that during DLM development, *neur* is only expressed in developing muscles (Figs. 3G–L), whereas MIB1 is expressed in both myoblasts and developing fibers (Figs. 3A–F).

Thus, the nature of these locations, together with that of N ligand locations, strongly suggests that developing DLMs send a DL signal, and that a SER signal is sent by myoblasts and/or developing DLMs. When using the *E(spl)m6-GFP* reporter gene, we only detected an activity of the N pathway in myoblasts surrounding the muscle fibers. This observation is consistent with the fact that TWI expression in swarming myoblasts in under the control of the N pathway (Anant et al., 1998). When we expressed the intracellular part of the N receptor, using the *1151-GAL4* driver, we observed *E(spl)m6-GFP* expression in developing DLMs, showing that the absence of *E(spl)m6-GFP* muscular expression does indeed reflect an absence of N signaling in muscles. Lastly, when we looked for *fng* expression during DLM development, we observed that, at any given time, a subset of myoblasts-expressing *fng* is located in the vicinity of developing fibers-expressing DL. FNG is a glycosyltransferase that enhances DL-N signaling and inhibits SER-N signaling (Bruckner et al., 2000; Moloney et al., 2000). Thus, myoblasts that express *fng* are likely to be receiving cells for DL signaling sent by developing DLMs. Moreover, we report that *fng*-expressing myoblasts have either reduced or no expression of *E(spl)m6* (Figs. 5G–I), strongly suggesting that *E (spl)m6* is a target of SER-N signaling and is not activated when

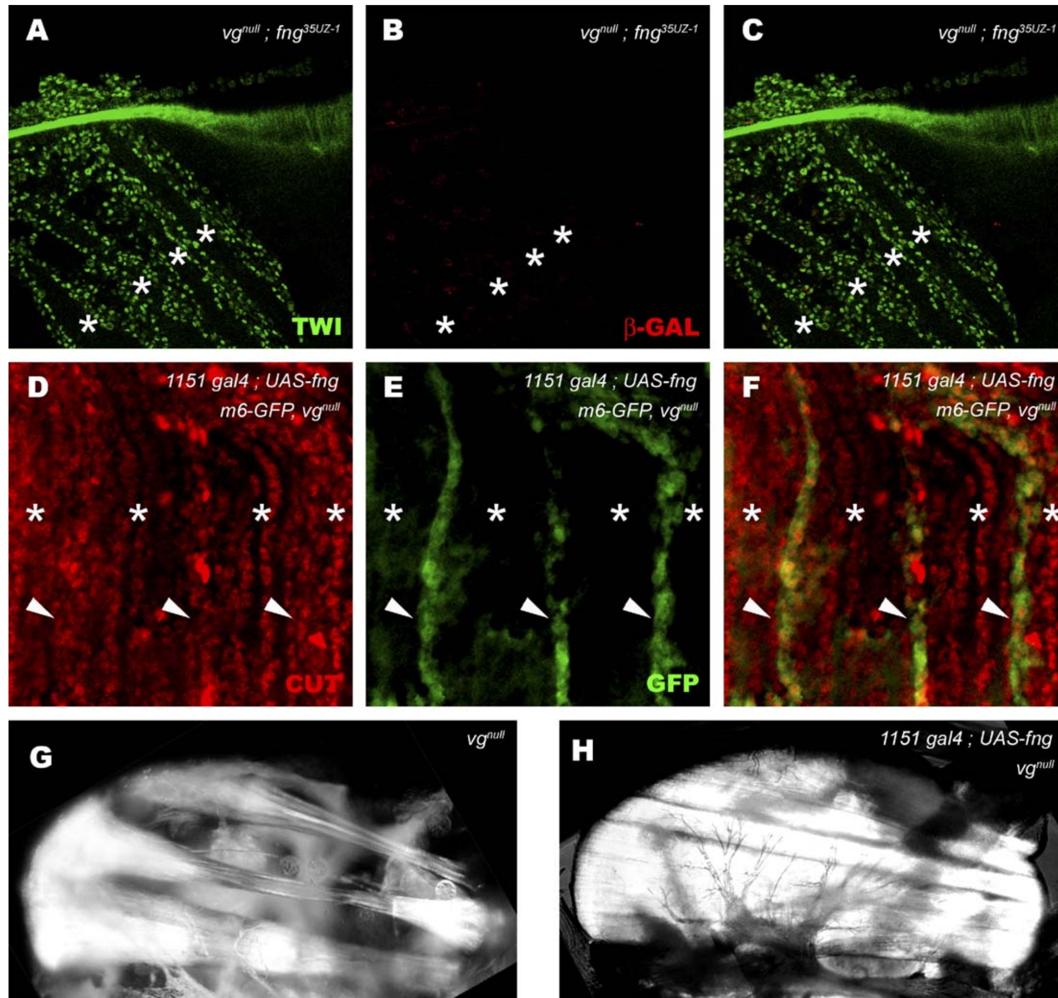

Fig. 7. *fng* involvement in muscle degeneration. vg^{null}/vg^{null}, fng^{35UZ-1} 21 h APF pupae stained with TWI (A) and βGal (B) antibodies show no fng^{35UZ-1} reporter gene expression (merge in panel C). In vg^{null} homozygous, *1151-GAL4*, *UAS-fng*, *E(spl)m6-GFP* 30 h APF pupae, CT is expressed in swarming myoblasts (arrowheads) and developing fibers (asterisks), as revealed by anti-CT labeling (D). *E(spl)m6-GFP* is still expressed in myoblasts (E, arrowheads) but is not expressed in developing DLMs (E, asterisks, merge in panel F). Adult thoraces of vg^{null}/vg^{null}, *1151-GAL4* show typical muscle degeneration (G). Adult thoraces of vg^{null}/vg^{null}, *1151-GAL4 UAS-fng* genotype show a significant rescue of muscle degeneration (H) compared to thoraces of vg^{null}/vg^{null} genotype (G).

N is glycosylated. Moreover, by 16 h APF, we started to detect a muscular expression of *fng*. We monitored *fng* expression using the *fng-LacZ*$^{35UZ-1}$ reporter gene, and thus, we cannot exclude that muscular expression could be a perdurance of βGAL protein after the fusion of *fng*-expressing myoblasts. Nevertheless, an overexpression of *fng* in the vg^{null} context is sufficient to repress the ectopic expression of *E(spl)m6* in developing fibers (Figs. 6D–F), strongly suggesting that *fng* is in part responsible for N pathway repression in muscles.

All together, these findings support the hypothesis that there are two N signaling pathways: a DL-N pathway with DLMs as sending cells and *fng*-expressing myoblasts as receiving cells and a SER-N pathway between *fng* non-expressing myoblasts and possibly between DLM fibers and *fng* non-expressing myoblasts. We propose that the SER-N pathway could be responsible for maintaining myoblasts in a state of proliferation and undifferentiation, whereas the DL-N pathway could regulate differentiation. Thus, the SER-N pathway would be responsible for the expression of specific myoblast markers such as *E(spl)m6* and TWI. Our results show that *fng* expression is necessary for correct DLM development. This function could be achieved by either simply inhibiting activation of the N receptor by SER or allowing a DL-N signaling that inhibits *E(spl)m6* expression. Nevertheless, when we overexpressed *fng* using the *1151-GAL4* driver, we still observed swarming myoblasts-expressing *E(spl)m6*, showing that *fng* expression alone is not sufficient to repress N pathway activity in swarming myoblasts and suggesting rather that DL-N signaling could be involved in this inhibition process. Thus, a DL-N pathway could induce a differentiation factor responsible for muscle differentiation. To complete the testing of this model, the role of each ligand needs to be examined by loss of function experiments, but the lethality of *Ser* and *Dl* mutants and the impossibility to generate mitotic clones in muscles make this difficult.

We have previously reported that vg^{null} flies exhibit specific IFM degenerations, and that degenerating DLMs show an ectopic expression of TWI and a loss of *Act88F* expression,

suggesting that in vg^{null} mutants an overexpression of the N pathway occurs (Bernard et al., 2003). In this paper, we report that, in a vg^{null} context, the ectopic expression of *E(spl)m6*, a direct target of N signaling, normally restricted to myoblasts, can be visualized in developing fibers (Figs. 6A–F). We show that this ectopic expression is associated with the lack of *fng* expression (Figs. 7A–C). Moreover, we show that an overexpression of *fng* in vg^{null} animals is sufficient to rescue the muscular phenotype of these animals (Figs. 7D–I). These data demonstrate that VG is necessary for *fng* expression during adult myogenesis. VG alone is not sufficient, however. First, VG is expressed in all swarming myoblasts, whereas *fng* expression is restricted to a subset of myoblasts, and secondly, overexpression of VG by means of the *1151-GAL4* driver did not induce a larger number of *fng*-expressing myoblasts compared to the wild type situation (data not shown). Nevertheless, taken together, our data show that VG is necessary for muscle differentiation during DLM development, since its absence is associated with overexpression of the N pathway and TWI, a myogenic inhibitory factor, and with the absence of *Act88F* expression, an isoform specific to muscle differentiation (Bernard et al., 2003; this work). Moreover, in this study, we show that the differentiation properties of VG are linked to an inhibition of the N pathway activity.

Another study, performed during chick limb myogenesis, has reported that *Delta1* is expressed in differentiating cells and triggers the N pathway in undifferentiated cells that express *Notch1* (Delfini et al., 2000). These locations are very similar to the N and DL locations found during DLM development. Moreover, we demonstrate that the N pathway is active in undifferentiated swarming myoblasts and must be inhibited in muscles to allow correct differentiation. Similarly, in vertebrates, activation of the N pathway inhibits myogenic differentiation (Delfini et al., 2000; Nofziger et al., 1999; Shawber et al., 1996). The mechanisms responsible for this inhibition, however, have not been clearly established and seem to be multiple. Indeed, ex vivo experiments have demonstrated that a canonical N signaling that leads to *E(spl)* homolog activation (i.e., Hes and Hey families of genes) is rapidly induced by N activation in C2C12 cells, and this induction is correlated with an inhibition of differentiation (Jarriault et al., 1998). N^I has also been shown to bind to MEF2C protein and to inhibit its cooperative activity with MyoD and myogenin necessary for the activation of myogenesis (Wilson-Rawls et al., 1999).

For a long time, *vg* has been considered to be the “wing selector gene”, but this concept has been recently revisited because this protein is required during adult myogenesis (Bernard et al., 2003; Sudarsan et al., 2001). Several studies performed in wing imaginal disc, however, have demonstrated that VG, which is devoid of a DNA-binding domain, forms a heterodimeric transcription factor with the protein encoded by *scalloped* (*sd*) (Halder et al., 1998; Simmonds et al., 1998; Vaudin et al., 1999). VG protein has little homology with any known proteins in *Drosophila*, except for a N-terminal domain resembling that of the protein Paired, but the domain of interaction between VG and SD seems to be conserved across evolution and has made it possible to describe several *vg* homologs in vertebrates *vestigial like 1*, *2*, and *4* (*vgll1*, *2*, and *4*) (Chen et al., 2004; Maeda et al., 2002; Mielcarek et al., 2002). In mouse, *vgll2* is expressed in skeletal muscles, whereas *vgll4* is expressed in cardiac muscle (Chen et al., 2004). Interestingly, VG and *Vgll2* (former *Vgl-2* and *VITO-1*) in their respective functions have multiple features in common. Indeed, *vg* is expressed in adult DLM, swarming myoblasts and developing fibers (Bernard et al., 2003), and *Vgll2* is expressed in chick skeletal muscles (Chen et al., 2004) as well as in C2C12 myoblasts and differentiated C2C12 myotubes (Mielcarek et al., 2002). Several sets of evidence have been reported which show that *Vgll2* is necessary for muscle differentiation. Antisense-Vgll2 morpholinos block Vgll2 and myosin expression in C2C12 cells (Chen et al., 2004). Lastly, co-expression of *Vgll2* and MyoD enhances Myo-D induced differentiation of 10T1/2 cells with a 7-fold increase in myosin heavy chain expression (Maeda et al., 2002). In *Drosophila*, flies homozygous for the vg^{null} mutation exhibit strong DLM degeneration and have no DVMs. Moreover, absence of *vg* is associated with a loss of *Actin88F* gene expression and an ectopic expression of TWI (Bernard et al., 2003). In this present paper, we confirm that *vg* plays a role in muscle differentiation and show that this role is achieved by regulating the N pathway. These data suggest that vertebrate myogenesis and DLM development share biochemical processes and genetic cascades to give rise to a muscle fiber. Thus, it should be interesting to test whether *vgll2* regulates the N pathway during vertebrate myogenesis.

SD is also expressed during DLM development (Bernard et al., 2003) and is related to the Transcriptional Enhancer Factor 1 (TEF1) family of proteins which are involved in muscle differentiation (Maeda et al., 2002). In vertebrates, these proteins were first identified as proteins that bind to MCAT elements (originally defined as a muscle-specific cytidine adenosine thymidine sequence) which are required for high levels of promoter activity in cardiac and skeletal muscle cells (Mar and Ordahl, 1988, 1990). Similar MCAT elements have been characterized in a large number of skeletal and cardiac muscle-specific promoters (Larkin et al., 1996). Moreover, it has been demonstrated that VGLL2 could physically interact with MEF2 and TEF1 proteins (Maeda et al., 2002). Interestingly, it was recently reported that the *Drosophila* MEF2 homolog is expressed, like *vg*, in both swarming myoblasts and developing DLMs (Baker et al., 2005). VGLL2 is able to activate TEF-1 and MEF2-dependent promoters in myotubes but not in myoblasts (Maeda et al., 2002). It will be interesting to find out whether VG, SD and MEF2 can physically interact and activate specific muscle targets to promote muscle differentiation. Among the various types of muscles in *Drosophila*, IFMs are unique with respect to their fibrillar organization and the specific expression of different isoforms of structural proteins (Crossley, 1978). Thus, VG functions in muscle differentiation might also be linked to a direct activation of these genes by the VG-SD heterodimeric transcription factor.

In conclusion, many lines of evidence suggest that the DLM differentiation process might be conserved between *Drosophila* and vertebrates and might be one of the basic principles of

striated muscle differentiation. Interestingly, IFMs are the only muscles in *Drosophila* that exhibit a sarcomeric organization with continuous Z-bands (Crossley, 1978). Therefore, the results obtained from studies of IFM differentiation should lead to a better understanding of the process of myogenesis in vertebrates.

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