Development 125, 1361-1369 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV7612

Twist and Notch negatively regulate adult muscle differentiation in *Drosophila*

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Accepted 9 February; published on WWW 18 March 1998

SUMMARY

Twist is required in *Drosophila* embryogenesis for mesodermal specification and cell-fate choice. We have examined the role of Twist and Notch during adult indirect flight muscle development. Reduction in levels of Twist leads to abnormal myogenesis. Notch reduction causes a similar mutant phenotype and reduces Twist levels. Conversely, persistent expression, in myoblasts, of activated Notch causes continued *twist* expression and failure of differentiation as assayed by myosin expression. The gain-of-function phenotype of *Notch* is very similar to that seen upon persistent *twist* expression. These results point to a relationship between Notch function and *twist* regulation during indirect flight muscle development and show that decline in Twist levels is a requirement for the differentiation of these muscles, unlike the somatic muscles of the embryo.

Key words: Notch, Twist, muscle, *Drosophila*, cell fate

INTRODUCTION

In *Drosophila*, the mesoderm is specified by genes that allot fates along the dorsal-ventral axis and results in activation of *twist (twi)* (Thisse et al., 1987a,b, 1988). *twi* expression is retained in adult muscle progenitors set aside during embryogenesis but declines in embryonic muscle progenitors as they fuse to form muscle (Bate, 1990, 1991). *twi*-expressing adult myoblasts proliferate in the larva and contribute to adult muscles during metamorphosis and as in the embryo, *twi* expression is shut-off as differentiation begins (Currie and Bate, 1991; Fernandes et al., 1991).

twi down-regulation during differentiation suggests that its function could be required to maintain myoblasts in an undifferentiated state. In a systematic analysis, Baylies and Bate (1996) showed that this hypothesis is not valid in the embryo. Persistent *twi* expression, at high levels using the GAL4/UAS system (Brand and Perrimon, 1994) had no effect on the development of somatic muscles. Baylies and Bate (1996) also showed that Twi behaved like a 'myogenic switch'. *twi* mis-expression in the ectoderm caused these cells to express muscle marker genes and, in the mesoderm, Twi levels help determine cell-fate choices between somatic and visceral mesoderm.

Vertebrate *twi* expression and function show similarities with *Drosophila* but there are significant differences. During murine myogenesis, *twi* expression is initially observed all over the epithelial somite (Wolf et al., 1991; Stoetzel et al., 1995; Fuchtbauer et al., 1995). Subsequently, Twi is excluded from the myotome upon initiation of skeletal myogenesis. Expression of *twi* in myogenic cell lines prevents the onset of muscle differentiation (Hebrok et al.,1994), a result different from that seen in the *Drosophila* embryo (Baylies and Bate, 1996).

We have examined the regulation and function of *twi* during development of the adult flight muscles. We show that decline in Twi levels coincides with the onset of expression, in indirect flight muscle (IFMs) progenitors, of the *erect-wing* (*ewg*) encoded transcription factor (DeSimone and White, 1993) and that *twi* regulation involves *Notch* signalling. Both reduction and constitutive activation of *Notch* affect the development of the IFMs but not the closely related direct flight muscles (DFMs). These effects on differentiation are very similar to that seen in hypomorphic-conditional-*twi* mutant flies and in animals with persistent *twi* expression respectively. High levels of Twi in adult myoblasts during larval life does not affect larval development, but persistent high levels of Twi in these cells during pupal development affects IFMs, but not DFMs. Myosin Heavy Chain (MHC) levels are reduced and the muscles degenerate. Our results suggest a regulatory mechanism for *twi* expression in myogenesis and demonstrate that persistent *twi* expression can, as in mouse, inhibit muscle differentiation in adult *Drosophila*.

MATERIALS AND METHODS

Strains

Canton-S was used as wild-type. N^{ts1}(Shellenbarger and Mohler, 1975, 1978) is a conditional *Notch* allele. N^{ts1} animals were grown at the permissive temperature, 22°C, and shifted to the non-permissive temperature, 31.5°C, for varying intervals during pupation. twi^{ry50} and twi^{v50} are homozygous lethal, viable in *trans* combination at 18°C but show a *twi*-phenotype when grown above 29°C (Thisse et al., 1987b). The *MHC-lacZ* (Hess et al., 1989) reporter gene is expressed in all muscles and *twi-lacZ* (Thisse et al., 1991), in all myoblasts. An X-chromosome *UAS-twi* transgenic strain was a gift from M. K. Baylies and M. Bate (Cambridge University, UK). The *UAS-twi*

transgene in this strain was mobilised and for the experiments mentioned here, a second chromosome insert was used. The *UAS-activated Notch (UAS-N-intra)* line was a gift from Masahiro Go and S. Artavanis-Tsakonas (Yale, USA). N-intra is a truncated form of the Notch receptor and is constitutively active (Rebay et al., 1993). The *1151 GAL4* driver was obtained from L. S. Shashidhara (CCMB, India). In the third larval instar, expression is seen in myoblasts on the wing and leg imaginal discs (Fig. 4A), and in nerve associated myoblasts (Fig. 4B). The adepithelial cells on the wing imaginal disc give rise to the dorsal mesothoracic muscles, the IFMs and the DFMs (Lawrence, 1982). The *1151* driver continues to be expressed in the pupa as myoblasts fuse to form muscles (Fig. 4C) and also in the adult IFMs and DFMs. There is no expression seen in larval muscles (Fig. 4B) that function as templates for DLM formation though a low transient expression is seen in almost all embryonic muscles (not shown). This does not appear to have any effects on embryonic muscle development when activated-*Notch*, *twi* or several other control UAS constructs such as *UAS-Ubx*, *UAS-Scr*, *UAS-Antp and UAS-p21* (a gift from I. Hariharan, MGH Cancer Centre, USA) are expressed with the *1151* driver (Roy and VijayRaghavan, 1997, Roy and VijayRaghavan, unpublished observations). These latter UAS-constructs cause lethality when expressed ubiquitously in the embryonic mesoderm or muscle but when expressed with the *1151* driver allow survival to pupal or adult stages and the examination of adult muscle development (Roy and VijayRaghavan, 1997, Roy and VijayRaghavan, unpublished data).

Heat shocks

White prepupae (0 hours APF) at 22°C were collected on moist filter paper in a Petri-dish and transferred to a 31.5°C incubator for different intervals. To correlate developmental stages after heat shocks with that at 25°C, *MHC-lacZ* pupae were grown either entirely at 22°C or at 31.5°C for different intervals and dissected. IFM development was compared to that at 25°C. We found that the rate of development at 22°C was 0.75 times that at 25°C and that at 31.5°C approximately 1.3 times that at 25°C.

Dissections and mounting

Staged, heat-shocked pupae were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS and later stained with X-GAL (Fernandes et al., 1991) or with appropriate antibodies. Flies were cut sagitally to view IFMs, dehydrated in an alcohol series, cleared in methyl salicylate, mounted in Canada balsam and viewed under polarized light. To view the DFMs, the DLMs and in some cases the DVMs were dissected out for greater clarity. Pupal preparations were mounted in 70% glycerol or in GelTol (Immunon, USA).

Antibody staining

Anti-Twi and mAb22C10 stainings were done according to the protocols described by Fernandes and VijayRaghavan (1991). Twi antibody was a gift from Siegfried Roth (Max Planck Institute, Tubingen, Germany) and mAb22C10 from Seymour Benzer (Caltech, USA). Anti-Ewg (a gift from Kalpana White, Brandeis University, USA) staining was done as described by DeSimone et al. (1995). For confocal microscopy, anti-rabbit secondary was conjugated to rhodamine to mark anti-Ewg or anti-Twi primary antibodies. Anti-mouse secondary conjugated to FITC was used against anti-β-galactosidase. Confocal analysis was done on a Bio-Rad Model 600 confocal microscope.

RESULTS

Background: IFM and DFM development

The development, innervation, differentiation and segmental properties of the IFMs have been well investigated (Fernandes et al., 1991; Fernandes and VijayRaghavan, 1993; Fernandes et al., 1996; Roy et al., 1997; Roy and VijayRaghavan, 1997). Early during pupal development three dorsal muscles in each mesothoracic hemisegment escape histolysis and serve as templates for the formation of one group of IFMs, the dorsal longitudinal muscles (DLMs; El Shatoury, 1956; Costello and Wyman, 1986; Fernandes et al., 1991)(Fig. 1A-C). Another group of IFMs, the dorsoventral muscles (DVMs) are also derived from progenitors on the wing imaginal disc. However, the formation of these muscles takes place by the de novo fusion of myoblasts at appropriate epidermal sites without the use of larval templates (Fig. 1D). Apart from the IFMs, there are other muscles, the DFMs (Fig. 1E), also derived from wing-imaginal disc-located progenitors (Lawrence, 1982). Although the IFMs and DFMs are clonally related and share progenitors at least till the late third larval instar (Lawrence 1982), they differentiate into very different muscle types in terms of molecular markers, anatomy and physiology (Bate, 1993).

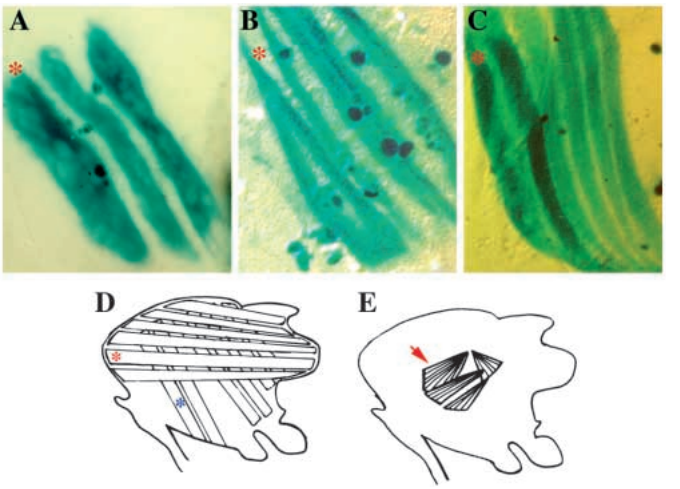

Fig. 1. Development and schematic representation of IFMs and DFMs in the adult fly. (A-C) Development of the DLMs revealed by the expression of *MHC-lacZ* transgene in muscles. A single hemithorax is shown in all cases. (A) Un-histolysed larval muscles, at 12 hours. (B) At 16 hours APF, the larval muscles have started splitting longitudinally to form templates for DLM development. (C) DLMs at 24 hours APF. Splitting and formation of the six DLM units is seen by 20 hours APF. The red asterisk marks the dorsal-most larval template in A and B, and the dorsal-most DLM in C. (D) Schematic representation of the IFMs, shown in a sagittal view of a hemithorax. The six DLMs are along the anteroposterior axis. A single DLM is marked here by a red asterisk. The seven DVMs are arranged in three bundles and run dorsoventrally. The blue asterisk marks the anterior-most DVM bundle. (E) The DFMs lie close to the lateral body wall of the mesothorax behind the DVMs. There are four muscles, 51, 52, 53 and 54 (see Bate 1993 for a detailed description of muscle position and nomenclature). DFM 51 is shown by a red arrow. In A-C, the dorsal midline is to the left and anterior is to the top. In D,E anterior is to the left and the dorsal, top.

twi expression declines as *ewg* expression begins in IFM progenitors

We have shown earlier that *ewg* is first expressed in IFM development as myoblasts begin to fuse to form these muscles (DeSimone et al., 1995). We now demonstrate that *ewg*

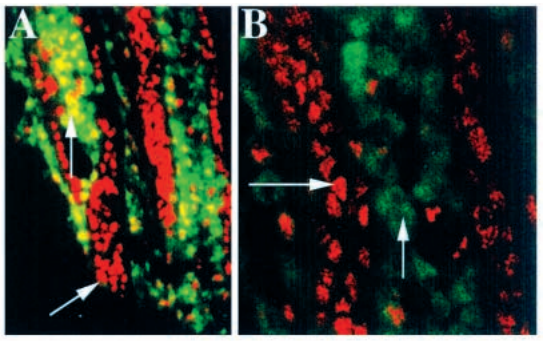

Fig. 2. Complementary expression pattern of *ewg* and *twi* during DLM formation. *twi* expression was monitored by immunoreactivity to β-galactosidase expressed from a *twi-lacZ* transgene, while *ewg* expression was monitored using antibodies specific to the Ewg protein. (A) There is hardly any overlap among *twi* expressing and *ewg*-expressing myoblasts (overlap areas are yellow, vertical arrow). *ewg* is expressed in myoblasts as they align along the larval templates before they fuse (red immunoreactivity). Also note the presence of Ewg-positive nuclei in the developing DLM syncitial myofibres (bottom arrow). No *twi* expression is detected in the developing myofibres (absence of green immunoreactivity). (B) A magnified optical section of A showing β-galactosidase expression from the *twi* promoter is cytoplasmically localised (green, vertical arrow) and antibodies to Ewg mark the nucleus of myoblasts (red, horizontal arrow in B).

expression is seen in IFM myoblasts just prior to their fusion at a time when *twi* expression is declining. Fig. 2 shows the developing DLMs at 16 hours APF. Twi expression (green) is seen in myoblasts present over the larval templates. The developing DLMs are visualised by the presence of Ewg-positive nuclei, aligned in rows, inside the fibre (red). Outside the fibre, myoblasts overlying the templates have started expressing Ewg while Twi expression has declined (red spots). Few myoblasts express both Twi and Ewg (yellow) and myoblasts still at a distance from the developing DLMs express only Twi (green). The timing of *ewg* expression and mutant phenotypes suggest that *ewg* acts after myoblast proliferation at the earliest stages of differentiation (DeSimone et al., 1995).

twi expression declines prematurely in N^{ts1} pupae

The function of the *Notch* locus has been demonstrated in several developmental pathways in flies and other animals (For review see Artavanis-Tsakonas, 1995). We examined the role of *Notch* in adult myogenesis by using a conditional allele, N^{ts1}. In wild-type muscles, expression of a *twi-lacZ* reporter gene is seen in differentiating IFMs although *twi* expression is itself absent in multinucleate cells. This expression of β-galactosidase in the developing fibre is due to the 'perdurance' of the enzyme synthesized in myoblasts prior to fusion. When N^{ts1} animals are shifted to non-permissive temperature during early pupal development β-galactosidase activity staining resulting from *twi-lacZ* reporter gene expression, is greatly reduced (Fig. 3A,C) in the developing DLMs. This result suggested the possibility that *Notch* function could be required for the maintenance, directly or indirectly, of *twi* expression till early during metamorphosis. Alternatively, the lowering of *twi-lacZ* expression could merely be a consequence of the possible failure of several myoblasts to fuse to the developing DLMs in N^{ts1} animals that were shifted to non-permissive temperatures

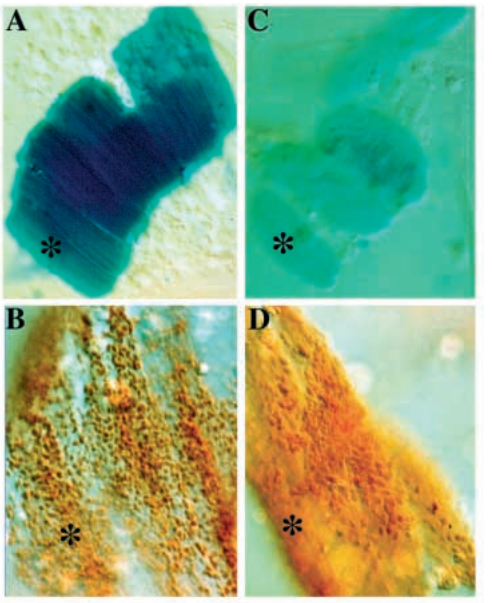

Fig. 3. *Notch* regulates *twi* expression. *twi* expression was examined by activity of a *twi-lacZ* transgene (A,C) and by immunostaining for Twi (B,D). (A) *twi-lacZ* expression in DLMs of a N^{ts1} heterozygote (i.e. wild type for IFM development) grown from 0-24 hours APF at the non-permissive temperature. At this stage (equivalent to 32 hours APF at 25°C) fusion is almost complete and few myoblasts are present near the muscles. Staining within the muscles is due to perdurance of β-galactosidase activity from the cytoplasm of fused myoblasts. (B) *Canton-S* pupa pulsed from 10-19 hours APF at the non-permissive temperature shows a large number of Twist-positive myoblasts. (C) N^{ts1} pupa under identical conditions as A shows very little *twi-lacZ* expression in the DLMs. (D) N^{ts1} pupa pulsed as in B shows myoblasts with almost no *twi* expression. Asterisks mark the dorsal-most DLM in all panels. Anterior is to the top and the dorsal midline to the left.

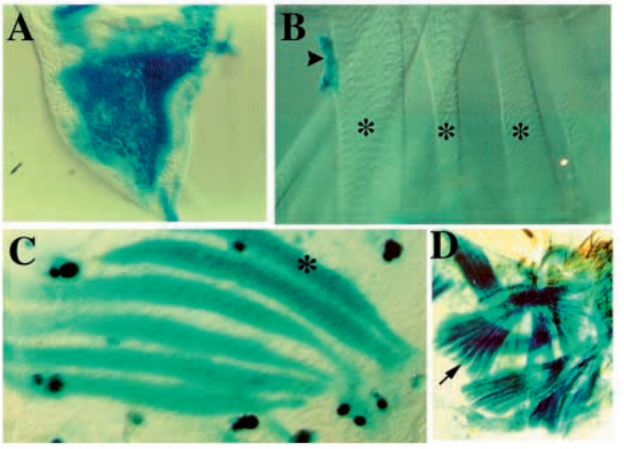

Fig. 4. Expression pattern of the *1151 GAL 4* driver visualised by β-galactosidase expression from a *UAS-lacZ* transgene. *1151* expresses in (A) adepithelial cells, (B) in nerve associated myoblasts (arrowhead). It however does not express in DOMs 1, 2 and 3 (asterisks) which in the pupa serve as templates for DLM formation. In C expression is seen in the IFMs of a 24 hours APF pupa (the dorsal-most DLM is indicated by an asterisk). This expression continues in the adult IFMs. (D) *1151* expression in the DFMs. DFM 51 is marked by an arrow.

during pupation. To examine the relationship between *Notch* function and *twi* expression we used antibodies specific to Twi to examine myoblasts in *Notch* mutant animals. As can be seen by Twi antibody staining on N^{ts1} animals (Fig. 3B,D) pulsed between 10-19 hours APF, Twi immunoreactivity is drastically lowered compared to wild-type animals at the corresponding developmental stage.

twi expression persists in *N-intra* pupae

The lowering of *twi* expression upon reduction of *Notch* function led us to ask if constitutive activation of *Notch* can cause elevation of Twi levels. We used the GAL4-UAS system (Brand and Perrimon, 1994) for this purpose. The *1151* driver described earlier (Roy and VijayRaghavan, 1997) was used to drive the expression of *UAS-N-intra* in disc associated myoblasts throughout larval and pupal development (Fig. 4). The *1151* driver is not detectably expressed in the thoracic epidermis or its progenitor cells on the wing disc (Fig. 4A; Roy and VijayRaghavan, 1997). In wild-type pupae, *twi* expression is not seen within the muscle (Fernandes et al., 1991) and this is documented again in optical sections (Fig. 5A-C). Expression of *N-intra* in developing adult thoracic muscles using *1151*, however, results in Twi expression continuing within the DLMs (Fig. 5D). Thus, expression of activated *Notch* in adult myoblasts does not eliminate fusion but Twi fails to be down-regulated. The phenotypes of *Notch* mutant animals in both loss- and gain-of-function situations is described below.

Notch is required for Indirect Flight Muscle development

To examine the role of *Notch* in flight muscle development more closely, animals carrying the temperature sensitive allele, N^{ts1}, were shifted to the non-permissive temperature between 0-18 hours APF. Pupae dissected soon after, showed that the process of splitting of the three larval templates for the DLMs had not begun (Fig. 6A). Depletion of wing disc myoblasts prevents splitting and differentiation of the larval templates (Roy and VijayRaghavan, unpublished data) suggesting that myoblast fusion is required for DLM formation. However, some myoblast fusion must occur in *Notch* mutant animals because the DLMs differentiate as three 'un-split' fibres (Fig. 6B). The DVMs in such pupae are sometimes mis-aligned and attach to each other or to the DLMs (not shown). Several tests were done to establish the minimum time window required to observe an effect of *Notch* reduction on IFM development. We find that IFMs visualized at 24 hours APF after a brief pulse between 9-16 hours APF at the non-permissive temperature showed that the larval templates do not split (Fig 6B). When N^{ts1} pupae, were grown at permissive temperature after the above pulse, the adults that emerged had three DLMs and not six (not shown).

N^{ts1} pupae pulsed between 9-18 hours APF were examined at 24 hours APF with the monoclonal antibody mAb 22C10 which marks motor innervation and also developing adult muscle (Fujita et al., 1982; Fernandes and VijayRaghavan, 1993). A large number of spindle-shaped cells (average=45, n=14 hemithoraces) over the 3 unsplit DLMs were seen in a N^{ts1} pupa (Fig. 6D). Such cells are very rarely seen in heat shocked control animals (average=2, n=8 hemithoraces, an example is shown in Fig. 6C). The extended processes of these spindle-shaped cells suggest that they could be prematurely differentiating myoblasts. Adult N^{ts1} mutant animals raised to non-permissive temperature during pupal development show normal DFMs.

Constitutive expression of *N-intra* in the mesoderm affects IFMs but not DFMs

We used *1151* to drive *UAS-N-intra* in the developing flight muscles. *1151* (Materials and methods, also Roy and VijayRaghavan, 1997) expresses in disc-associated myoblasts and expression continues through pupal development in all the muscles derived from these myoblasts. When activated *Notch* is expressed under the control of this GAL4 driver, both the DLMs and DVMs are missing (Fig. 7B). To determine in more detail the effect, upon differentiation, of activated *Notch* we examined the expression of *Myosin heavy chain* (*MHC)* and indirect flight muscle actin. *MHC* gene expression during wild-type IFM development at 24 hours APF, as seen with a specific antibody, is shown in Fig. 8A. When *N-intra* is expressed persistently during IFM development, MHC levels are considerably lowered (Fig. 8B). A similar lowering of levels of IFM-specific actin, assayed by *Actin 88F-lacZ* expression, is observed (data not shown). However, DFMs, which derive from the same pool of disc-associated myoblasts as IFMs, are not noticeably affected by activated Notch (Fig. 7B), although the GAL4 driver is expressed at high levels in DFMs (Fig. 4D) and in their progenitor myoblasts (Fig. 4A).

Twi reduction affects IFM development in a manner similar to that seen when Notch function is reduced

The effect of Notch activation and reduction on *twi*, and the phenotypes of *Notch* mutants led us to examine the effects of *twi* mutant combinations on flight muscle development. Null mutations in the *twi* gene cause embryonic lethality when homozygous, since the gene product is required for ventral furrow formation (Thisse et al., 1987a,b, 1988). However, a heteroallelic combination of two *twi* mutations, twi^{ry50}/twi^{v50}, is viable and shows a temperature-sensitive loss of *twi* function (Thisse et al., 1987b). Animals of the genotype twi^{v50}/twi^{ry50} were grown at permissive temperature till the second larval instar and then raised to non-permissive temperature for the rest of their development. Such a treatment did not affect larval development. However, the development of IFMs was affected. In particular, the DLMs were severely affected and only three fibres are seen (Fig. 7C). DFM development is not significantly affected though individual muscles are slightly thinner that in the wild type (Fig. 7D).

Persistent expression of *twi* in the developing flight muscles affects IFM but not DFM development

The expression pattern of *twi* during wild-type development suggested that continued expression of the gene could prevent differentiation. To test this we expressed a *UAS-twi* transgene under the control of the 1151-GAL4 driver described above. Fig. 9A and B show the expression of *ewg* in wild-type animals at 24 hours APF. When *twi* is expressed in a persistent manner using the 1151-GAL4 driver, IFM development is aborted. Although *ewg* expression is seen at 24 hours APF in such animals, the larval templates fail to split and eventually degenerate (Fig. 9C,D). It appears that a substantial part of the *ewg* expression is in unfused myoblasts, although it is not

possible to unequivocally state that no fusion has occurred. This is in contrast to the wild type at this stage where a substantial part of *ewg* expression is already inside the developing fibres (Figs 2, 9A,B). In order to examine further the effect on muscle differentiation of persistent Twi expression we studied the expression of *Actin 88F-lacZ* (not shown) and *MHC*. As in the situation where *N-intra* is persistently expressed during IFM development, persistent *twi* expression results in lowering of MHC levels in the IFMs (Fig. 8C). Curiously, some elongated myoblasts with long processes are seen over the IFMs. They express high levels of MHC (Fig. 8C inset). Ectopic MHC expressing multinucleate myotubes are also seen in this region. In the mature animals that develop upon persistent expression of *twi* in the flight muscles, DLMs and DVMs are absent, while DFMs are present and appear normal (Fig. 9E,F).

DISCUSSION

Recent studies on the mechanisms underlying specification of muscle identity and differentiation have suggested similarities and differences between *Drosophila* and vertebrates (Michelson, 1996; Spicer et al., 1996; Baylies and Bate, 1996) and even within individual organisms. The Twi protein is an example of this. In vertebrates, Twi regulates skeletal muscle differentiation negatively. In cell transfection studies, continued expression of murine Twi impaired the ability of myogenic cells to differentiate (Hebrok et al., 1994) as assayed by prevention of myoblast fusion and lowered induction of myogenic markers. Although Twi can negatively regulate MEF-2, a positive regulator of muscle differentiation, in mouse cells as well as in flies (Spicer et al., 1996; Nyugen et al., 1994; Lilly et al., 1994; Taylor et al., 1995), dissimilarities exist. In the developing *Drosophila* embryo (Baylies and Bate, 1996), Twi is not only involved in cell-fate choice between somatic and visceral mesoderm in a dose-dependent manner, but *twi* mis-expression in the ectoderm can give this germ layer mesodermal properties such as the expression of a muscle-specific gene. However, prolonged *twi* expression at high levels does not prevent differentiation of somatic muscle although, during normal muscle development, *twi* expression declines at differentiation.

Our observations on *twi* function in the developing flight muscles lead us to suggest that *twi* repression could be a requirement for the initiation of muscle differentiation in some muscles of the fly. Persistent *twi* expression aborts the development of these muscles and markers of differentiation such as myosin are greatly reduced (Fig. 8). Thus, in these muscles at least, Twi functions in a manner similar to that observed for murine Twist.

twi expression during IFM development declines as muscle differentiation begins (Fernandes et al., 1991). We have shown in this study that Ewg, a transcription factor required for indirect flight muscle differentiation (DeSimone et al., 1995) begins to be expressed as *twi* expression declines (Fig. 2). It is important to note here that *ewg* expression is seen in myoblasts, overlying the larval templates, prior to fusion. In addition expression is subsequently seen inside the fibre. *ewg* encodes a regulatory factor and not a structural component of the muscle. Thus, the onset of regulatory events that lead to differentiation, just prior to myoblast fusion, is strongly correlated with the lowering of *twi* expression in these myoblasts. Very few myoblasts express both *twi* and *ewg*. Is Twi a negative regulator of *ewg* expression? Extant reagents - a conditional *twi* allelic combination and *ewg* regulatory sequence information, do not provide an unequivocal answer to this. However, when *twi* is persistently expressed during adult flight muscle development, *ewg* expression is still seen, indicating that the mere presence of Twi is not sufficient to prevent the onset of *ewg* expression.

Temperature-sensitive *twi* mutant animals grown at the non-permissive temperature from the second larval instar appear not to be affected until pupal development, at which time the formation of the IFMs is severely affected. In such animals, the development of the DFMs appears normal. The interpretation of the phenotype from conditional loss-of-function mutants must be tempered by some unknowns. Although the conditional allelic combination appears to exhibit a null phenotype in the embryo, it is possible that the effect of raising larvae and pupae to the non-permissive temperature effective for embryogenesis does not result in an adult 'null' phenotype. Given this caveat, our results from the *twi* temperature-sensitive mutant experiment suggest that Twi function is not required for larval life, during and after the second instar. However, Twi is required during pupal development for the development of the IFMs, but not the DFMs. Does removal of *twi* function result in premature muscle differentiation? The defects seen in *twi* mutant animals are at the earliest stages of DLM development when myoblasts fuse to the larval templates. This is consistent with the interpretation that reduction of Twi levels could have caused premature differentiation and thus have left fewer myoblasts that are correctly positioned to contribute to DLM development. This interpretation would also be consistent with the *Notch* loss-of-function data.

The phenotypes resulting from persistent *twi* expression are also revealing about the mechanisms that could operate during flight muscle development. Persistent and high levels of *twi* expression in wing-disc associated myoblasts during larval life has no apparent effect on larval development. But persistent *twi* expression affects IFM differentiation in a manner very similar to persistent expression of activated Notch: the larval templates degenerate and often fail to split (Fig. 9C,D) suggesting that very early events in myogenesis such as fusion are affected. However, Ewg, a marker for the onset of differentiation is detected in this situation. Since muscle development is in the process of being aborted in animals with persistent *twi* expression at the stage when Ewg is initiated, it is not easy to determine whether levels of Ewg are reduced or not. It is clear though that the expression of markers of differentiation such as myosin (Fig. 8) and IFM-specific actin are reduced. In addition to its effect on muscle differentiation, continued expression of Twi causes myoblasts to ectopically differentiate and express high levels of MHC. A possible explanation of these results is that persistent expression of *twi* does delay differentiation; eventually, however, unfused myoblasts may differentiate at ectopic locations as the long *MHC* expressing cells shown in Fig. 8C. This raises the question as to what happens during normal development to unfused myoblasts. It is likely that unfused myoblasts die during normal development and the ectopic differentiation seen upon persistent *twi* expression may

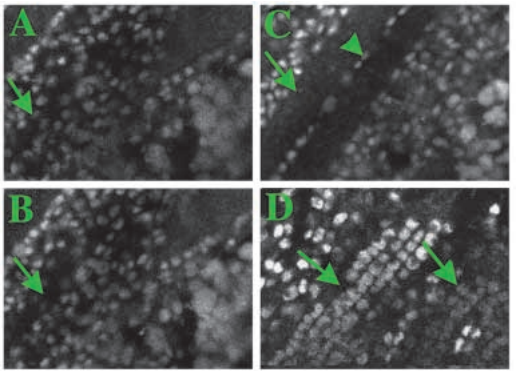

Fig. 5. Confocal images of myoblasts that give rise to the IFMs, labelled with anti-Twi rhodamine. (A-C) *twi* expression in myoblasts of a *Canton-S* pupa at 21 hours APF. (A) A superficial section shows only Twi expressing myoblasts but not the underlying DLMs. The area beneath which a DLM is present is marked by an arrow. (B) A deeper optical section shows the presence of a DLM (arrow) in that area. Note the absence of *twi* expression in the muscle. (C) A still deeper section clearly shows two DLMs (arrows) separated by a row of unfused *twi*-expressing myoblasts (arrowhead). (D) A similar optical section as in C of a *1151; UAS-N-intra* pupa. *twi* expression is seen both outside the DLMs in the unfused myoblasts and within the DLMs in neat rows of fused myoblast nuclei (arrows).

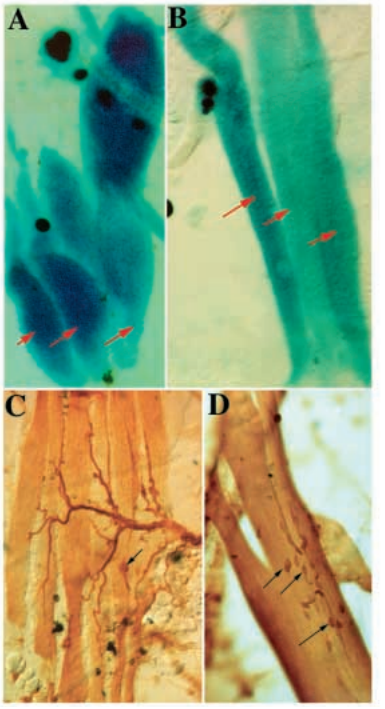

Fig. 6. IFM development in N^{ts1} pupae. (A,B) N^{ts1} animals carrying a *MHC-lacZ* transgene were staged at 22°C and shifted to the non-permissive temperature, 31.5°C for varying times. (See Materials and Methods for comparison of growth rates at various temperatures.) (A) DLMs (red arrows) after a 0-18 hours APF (equivalent to 24 hours APF at 25°C) pulse at the non-permissive temperature. (B) DLMs at 24 hours APF (equivalent to 21 hours APF at 25°C) after growing at the non-permissive temperature from 9-16 hours APF. This period encompasses, in wild type at 25°C, the stage just prior to splitting of the larval muscle templates to a few hours into splitting. Note that there are only three DLMs (red arrows) because the larval templates have not split. (C,D) mAb 22C10 staining of N^{ts1} pupae at 24 hours APF. mAb22C10 marks motor innervation and differentiating adult muscles. (C) *Canton-S* pupa at 24 hours APF (equivalent to 24 hours APF at 25°C) grown at 31.5°C between 9-18 hours APF has six DLMs. Note the presence of a single spindle-shaped myoblast (arrow) over the DLMs (average=2, n=8 hemithoraces). (D) N^{ts1} pupa at 24 hours APF grown under conditions similar to the *Canton-S* pupa in C has three DLMs. The number of spindle-shaped myoblasts (arrows) over the DLMs is far greater than seen in C (average=45, n=14 hemithoraces). In all panels anterior is to the top, the dorsal midline is to the left.

be suggestive of interference with cell-death programs, a hypothesis which though tantalising, needs substantial investigation. In addition, when *twi* expression persists, the development of DFMs is apparently not significantly perturbed, a result similar to that seen upon reduction of *twi* function. It could be argued that the lack of a DFM phenotype in conditional *twi* mutant animals could be because of remnant *twi* function in the allelic combination used. However, we know that the GAL4 driver used is expressed at high levels in the developing direct flight muscles (Fig. 4D). Therefore, the presence of DFMs when *twi* is expressed persistently during direct flight muscle development very likely reflects the fact that these muscles are relatively refractive to manipulations of Twi levels. We discuss this in relation to other manipulations of DFMs later in this section.

What are the signals that control the down-regulation of *twi* during normal development? Flies carrying a temperature-sensitive allele at the *Notch* locus show a flight muscle phenotype strikingly similar to *twi* mutant animals when grown during early pupal development at non-permissive temperature. Here too, IFMs are affected but DFMs are not (and here too, it can be argued that the absence of effect on DFMs is due to perdurance of wild-type *Notch* function or because developing DFMs may have a very different temperature sensitive period). However, persistent expression of an activated Notch protein using the 1151-GAL4 driver also affects the development of the IFMs but not the DFMs. Thus *Notch* loss- and gain-of-function animals show similar effects (Figs 6 and 7) as *twi* loss- and gain-of-function animals on flight muscle development (Figs 7 and 9). In addition, *twi-lacZ* reporter gene expression and Twi protein levels are significantly lowered in N^{ts1} animals (Fig. 3), and persistent *twi* expression is seen when activated *Notch* is expressed constitutively during flight muscle development (Fig. 5).

There are at least two explanations for these results that are consistent with known functions of Notch. Notch could function in the development of adult muscle, in a manner similar to that in the embryo, by its involvement in choice of a founder cell by ‘lateral inhibition’ (Corbin et al., 1991; Bate et al., 1993; Baker and Schubiger, 1996). In this a ‘founder cell’ is chosen from a pool of myoblasts and the Notch receptor functions in other myoblasts to receive signals that inhibit these myoblasts from becoming founder cells. Founder cells, identified in embryonic muscle development (Rushton et al., 1995), prefigure the developing muscle fibre and are the earliest detectable event in muscle differentiation in the embryo. Thus, in this model of Notch function, similar to that well-characterized in neurogenesis (Simpson, 1990), loss of

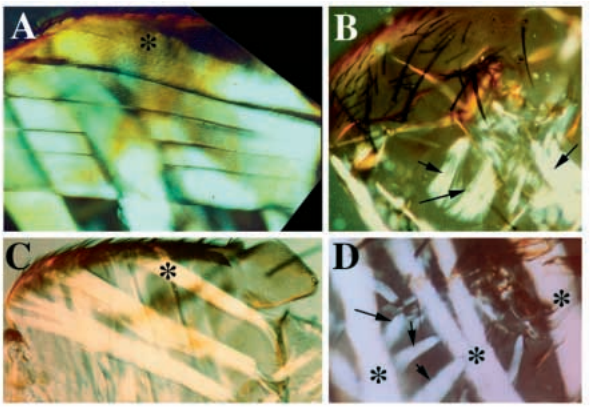

Fig. 7. Expression of activated *Notch* in myoblasts prevents differentiation of the IFMs but apparently not the DFMs. (A) *Canton-S* hemithorax showing IFMs. (B) *1151* driven expression of *N-intra* causes complete loss of the IFMs. Compare with A. Note however that expression of activated *Notch* in the DFMs does not drastically affect their development, though DFM 54 appears thinner than normal. Arrows mark DFMs 51 and 53. (C) Flies harbouring a temperature-sensitive combination of *twi* alleles were grown at the non-permissive temperature from the second larval instar till eclosion. Such flies have just three DLMs. (D) DFMs (51, 53 and 54 are marked by arrows) appear normal. In A,C, asterisks mark the dorsal most DLM. In D, asterisks mark DVMs. In all panels anterior is to the left and dorsal to the top.

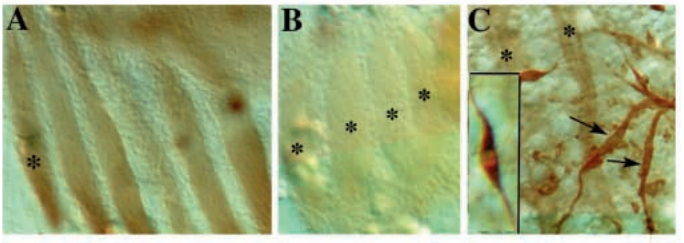

Fig. 8. Myosin levels in the IFMs are lowered by constitutive expression of activated *Notch* and *twi*. Antibodies to MHC were used to visualise IFMs of 24 hours APF pupae. (A) *MHC* expression in the DLMs of a *Canton-S* pupa. (B) *MHC* expression is lowered in the IFMs of a *1151; UAS-N-intra* pupa. (C) *MHC* expression is lowered in the IFMs of a*1151; UAS-twi* pupa. Note the presence of long, ectopically differentiated myoblasts and myotubes (arrows) expressing high levels of MHC. Inset is a single myoblast with long processes expressing MHC. In all panels anterior is to the top and the dorsal midline is to the left. Asterisks mark the DLMs.

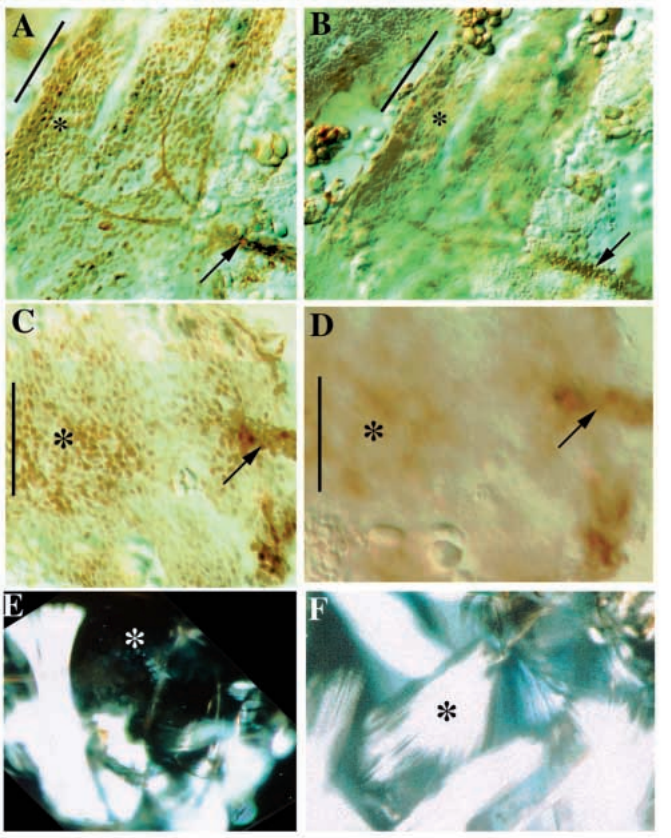

Fig. 9. Persistent expression of *twi* during flight muscle development prevents differentiation of the IFMs but does not affect the development of the DFMs. (A) A 24 hours APF pupal preparation showing *ewg* expression in the myoblasts that give rise to the IFMs. The asterisk marks the position of myoblasts that are lying directly over the two dorsal-most developing IFM fibres. (B) A deeper optical section of the same preparation as in A, showing the developing six IFM myofibres. One of the dorsal fibres is indicated by the asterisk. (C) *ewg* expression in a *1151; UAS-twi* pupa at 24 hours APF. The asterisk marks the myoblasts lying over the dorsal-most larval muscle. (D) A deeper optical section of the same preparation as in C, showing the complete absence of any developing myofibres. Compare this figure with B above. In *1151; UAS-twi* animals, the larval muscles do not split and the IFMs degenerate completely. In A-D, the dorsal midline is indicated by the bar, and the arrow indicates the developing innervation. (E) Adult hemithorax of a *1151; UAS-twi* fly showing almost complete absence of DLMs (asterisk) and DVMs. (F) Adult hemithorax of a *1151; UAS-twi* fly showing lack of discernible phenotype in the DFMs. One of the DFMs (51) is indicated by the asterisk. In E and F, anterior is to the right, and dorsal is top.

Notch function will result in 'more' founder cells and *twi* expression will decrease as a result of the onset of differentiation. Conversely, persistent expression of activated Notch will result in myoblasts being unable to take on a founder cell fate. Consequently *twi* expression persists. Experiments by Ruiz-Gomez and Bate (1997) have shown that Notch is required for the segregation of twi-expressing adult muscle progenitors in the embryo.

In the second model Notch signaling could play a direct role in maintaining the un-differentiated state till myoblasts are correctly positioned to receive appropriate environmental signals to differentiate. Similar roles for Notch during *Drosophila* compound eye development (Fortini et al., 1993) *Xenopus* (Dorsky et al., 1995) and chick (Austin et al., 1995, Henrique et al., 1997) eye development have been suggested. Thus, in the second model, Notch signalling maintains the undifferentiated state and reduction of Notch function results in the reduction of Twi levels, since differentiation begins as Notch function declines. Conversely, expression of activated Notch maintains the cells in an undifferentiated state, and *twi* expression persists as an indirect consequence or as a direct effect of Notch signalling.

In favour of Notch acting to select a founder cell, is the observation of a large number of spindle-shaped cells. Thus, if

loss of Notch function in the adult were to result in an excess of founder cells, these spindle-shaped cells could mark events consequent to this. However, if loss of Notch function were to lead to premature differentiation, the spindle-shaped cells could well represent premature differentiation events. Persistent expression of activated Notch affects the DVMs more severely than the DLMs (all three DVM groups seldom form). However occasional formation of a DVM is initiated as seen by IFM *actin-lacZ* expression. This suggests that though segregation of DVM founders do occur, high levels of activated Notch interfere with the process**.** Until markers for founder cells in adult myogenesis are identified, it will be difficult to distinguish between these possibilities. In the embryo, *Notch* loss-of-function results in an excess of cells that express markers for founder cells (Corbin et al., 1991; Bate et al., 1993; Baker and Schubiger, 1996) and expression of activated Notch in the mesoderm prevents myoblast fusion, though some muscles are unaffected (Baker and Schubiger, 1996).

An intriguing result obtained by persistent expression of activated *Notch* and *twi* is the significant difference in effects on very closely related muscles, the IFMs and the DFMs. It demonstrates how very closely related muscles can develop in very different ways. Supporting evidence comes from muscle development in the 'four-winged-fly' in which the T3 ectoderm is homeotically transformed towards a T2-like phenotype. Of the T2 set of thoracic muscles, DFMs develop normally beneath the transformed T3, however IFM development is severely deranged (Fernandes et al.,1994). In addition, DFMs, in otherwise wild-type animals, show no effect of ectopic expression of the homeotic selector genes *Antennapedia* or *Sex-combs-reduced* whereas the IFMs fail to develop in this situation (Roy et al., 1997 and S. Roy, unpublished observations). DFMs and IFMs are affected very differently by perturbations in levels of expression of various patterning genes. It is likely that developing DFMs respond to different cues possibly originating from other tissues like the overlying epidermis or the nervous system. Transplantation experiments (VijayRaghavan et al., 1996; Roy and VijayRaghavan, 1997) in the pupa and experiments by Baker and Schubiger (1995) in the embryo show that the ectoderm does influence the expression of muscle specific genes. For IFM development, these cues result in the *Notch* pathway being involved in differentiation. Mosaic analysis suggests that Notch receptor function is required autonomously in the mesoderm at this stage (S. Anant, unpublished observations). A mosaic analysis of the function of candidate ligands for the Notch receptor will reveal if the *Notch* phenotype is in response to signaling from the ectoderm, or if another signaling pathway is involved. Factors that regulate Notch activity in the embryonic segregation of adult myoblasts might also function at later stages during development to delineate IFM myoblasts from DFM myoblasts (Ruiz-Gomez and Bate, 1997). The investigation of how differences arise within the dorsal muscle set of the adult thorax is now tractable and promises to be of general value.

We thank Mary Baylies and Mike Bate for the *UAS-twi* strain and Masahiro Go and S. Artavanis-Tsakonas for the *UAS-N-intra* strain. Stimulating discussion with Mike Bate, Bill Chia and Veronica Rodrigues are gratefully acknowledged and we thank Veronica Rodrigues and Bill Chia for comments on the manuscript. We also thank the referees for their very constructive suggestions which have contributed substantially to improving the quality of this study. This work was supported by the Department of Science and Technology, Government of India, a Human Frontier Science Program grant and funds from NCBS.

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