Notch signaling patterns *Drosophila* mesodermal segments by regulating the bHLH transcription factor *twist*

Alexis Tapanes-Castillo and Mary K. Baylies*

Program in Developmental Biology, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, Weill Graduate School of Medical Sciences at Cornell University, 1275 York Avenue, New York, NY 10021, USA

*Author for correspondence (e-mail: m-baylies@ski.mskcc.org)

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Summary

One of the first steps in embryonic mesodermal differentiation is allocation of cells to particular tissue fates. In *Drosophila*, this process of mesodermal subdivision requires regulation of the bHLH transcription factor Twist. During subdivision, Twist expression is modulated into stripes of low and high levels within each mesodermal segment. High Twist levels direct cells to the body wall muscle fate, whereas low levels are permissive for gut muscle and fat body fate. We show that Su(H)-mediated Notch signaling represses Twist expression during subdivision and thus plays a critical role in patterning mesodermal segments. Our work demonstrates that Notch acts as a transcriptional switch on mesodermal target genes, and it suggests that Notch/Su(H) directly regulates *twist*, as well as indirectly regulating *twist* by activating proteins that repress Twist. We propose that Notch signaling targets two distinct 'Repressors of twist' – the proteins encoded by the *Enhancer of split* complex [*E(spl)-C*] and the HLH gene *extra machrochaetae* (*emc*). Hence, the patterning of *Drosophila* mesodermal segments relies on Notch signaling changing the activities of a network of bHLH transcriptional regulators, which, in turn, control mesodermal cell fate. Since this same cassette of Notch, Su(H) and bHLH regulators is active during vertebrate mesodermal segmentation and/or subdivision, our work suggests a conserved mechanism for Notch in early mesodermal patterning.

Key words: *Drosophila*, Mesoderm, Muscle, Subdivision, Signaling, Transcriptional regulation, *Notch*, *Suppressor of Hairless*, bHLH, *twist*, *daughterless*, *extra machrochaetae*, *Enhancer of split*

Introduction

Early in vertebrate and invertebrate development, uncommitted mesodermal cells are patterned into repetitive segments and allocated to specific tissue fates. In *Drosophila*, this process of segmentation and patterning first involves partitioning the mesoderm into segmentally repeated blocks of cells (Campos-Ortega and Hartenstein, 1985). Then each mesodermal segment is further subdivided into four domains: two across the anterior–posterior axis and two across the dorsal–ventral axis. Depending on their position, cells are assigned a specific tissue fate: dorsal anterior, visceral mesoderm (gut muscle); ventral anterior, fat body or mesodermal glia; dorsal, heart; and posterior, somatic muscle (body wall muscle) (Azpiazu et al., 1996; Carmena et al., 2002; Riechmann et al., 1997; Ward and Skeath, 2000; Zhou et al., 1997).

Essential to the process of *Drosophila* mesoderm subdivision and patterning is the regulation of the bHLH transcription factor Twist (Baylies and Bate, 1996). Twist is initially required for mesoderm specification. It is expressed at high levels in all mesodermal cells through the activity of the NFKB homologue, Dorsal, and the bHLH protein, Daughterless (Castanon et al., 2001; Jiang et al., 1991; Leptin, 1991; Simpson, 1983; Thisse et al., 1991). Following gastrulation, a segmentally repeated pattern of Twist expression forms along the anterior–posterior axis of the embryo, subdividing each mesodermal segment into a low and high Twist domain. Cells located in the high Twist domain develop into somatic muscles and heart, whereas cells located in the low Twist domain differentiate into visceral muscle, fat body, heart and mesodermal glia (Baylies and Bate, 1996; Borkowski et al., 1995). High Twist levels are required for somatic myogenesis, and they inhibit the differentiation of other mesodermal tissue fates, such as the visceral mesoderm and fat body (Baylies and Bate, 1996). While it is known that Wingless and Hedgehog signaling modulate Twist expression, through the pair-rule genes *sloppy-paired* (*slp*) and *even-skipped* (*eve*), respectively (Azpiazu et al., 1996; Lee and Frasch, 2000; Riechmann et al., 1997), Twist regulation during mesoderm subdivision and patterning is not fully understood.

Recently, genetic data implicated the Notch signaling pathway in early somatic myogenesis (Brennan et al., 1999). Following mesodermal subdivision, somatic myogenesis proceeds within the high Twist domain. Wingless signaling leads to the specification of groups of equipotent myoblasts, which express the gene *lethal of scute* (Carmena et al., 1995; Carmena et al., 1998). While all cells within an equivalence group have the potential to develop into a muscle progenitor, lateral inhibition, mediated by Notch signaling, leads to the selection of one progenitor per group (Bate et al., 1993; Carmena et al., 2002; Corbin et al., 1991). Analysis of Notch and Wingless signaling double mutants revealed that in addition to its later role in lateral inhibition, Notch activity

represses somatic development concurrently or prior to Wingless signaling and equivalence group formation, possibly during the time of Twist modulation (Brennan et al., 1999).

Classical Notch signaling is activated by the DSL (Delta and Serrate in *Drosophila* and vertebrates; Lag-2 in *C. elegans*) ligand family and is mediated by the CSL (CBF1/RBP-JK in vertebrates; Suppressor of Hairless [Su(H)] in *Drosophila*; Lag-1 in *C. elegans*) transcription factor family (Artavanis-Tsakonas et al., 1999). A transcriptional switch model has been put forward to describe Notch target gene regulation (Bray and Furriols, 2001; Hsieh et al., 1996; Klein et al., 2000). In the absence of Notch signaling, default repression by Su(H) prevents transcription (Barolo and Posakony, 2002; Barolo et al., 2002). Su(H) binds specific enhancer sequences, recruits co-repressors, such as Hairless, and represses transcription (Barolo et al., 2002; Furriols and Bray, 2000; Klein et al., 2000; Morel et al., 2001). Upon ligand binding, the Notch intracellular domain, N^{icd}, is released from the cell membrane and translocates into the nucleus (Kidd et al., 1998; Struhl and Adachi, 1998). N^{icd} then associates with Su(H) and alleviates Su(H)-mediated repression, for example by displacing co-repressors. Depending on the specific enhancer and the particular combinations of transactivators present in the cell, Notch target genes are proposed to have different requirements for Su(H) and N^{icd} (Bray and Furriols, 2001; Klein et al., 2000). N^{icd} instructive enhancers additionally require N^{icd} to serve as a coactivator for Su(H) and activate transcription. N^{icd} permissive enhancers solely require N^{icd} to alleviate the repression caused by Su(H). Once the enhancer is de-repressed, Su(H) and/or the other bound transactivators promote transcription.

In this paper, we demonstrate that Notch signaling plays a critical role in mesoderm subdivision prior to its well-established role in lateral inhibition. Proper modulation of Twist into low and high expression domains requires Notch signaling. By focusing on how Notch and Su(H) regulate Twist, we unraveled the molecular mechanism that Notch utilizes to regulate a single target gene: (1) Notch acts as a transcriptional switch that converts Su(H) from a repressor into an activator; and (2) Notch/Su(H) regulate *twist* directly, as well as indirectly, by activating proteins that repress *twist*. We hypothesize that these 'Repressors of Twist' are the transcriptional repressors of the *Enhancer of split complex* [*E(spl)-C*] and the HLH protein Extra machrochaetae (Emc) which dimerizes and inhibits the activity of Daughterless, a bHLH transcription factor required for high levels of *twist* (Castanon et al., 2001). Our work underscores the complexity of Notch/Su(H) bHLH regulation in the early *Drosophila* embryo and suggests a mechanism for the analogous process of somite formation and patterning in vertebrate embryos.

Materials and methods

Drosophila stocks

Notch and *Su(H)* germline clones (GLCs) were generated using the dominant female-sterile/flippase (FLP) system (Chou and Perrimon, 1996). *Df(1)N*^{81k1}, *v*, [*FRT101w*$^{+}$]/*FM7c*, *ftz-lacZ* and *C(1)DX/w*, *ovo*^{D1}, [*FRT101w*$^{+}$]/*Y; FLP*^{38} flies were used to produce embryos lacking maternally contributed and zygotically expressed Notch, N^{null} (Brennan et al., 1999). *Su(H)*^{del47} *FRT40A P[l(2)35Bg*$^{+}$*]/CyO, ftz-lacZ* and *w*^{118}*; Su(H)*^{del47}*/CyO, ftz-lacZ* flies, in addition to *P{ry*$^{+t7.2}$*=hsFLP}1, w*^{118}*;Adv*^{1}*/CyO* and *P{w*$^{+mC}$*=ovo*$^{D1-18}$*}, P{ry*$^{+t7.2}$*=neoFRT}40A/Dp(?;2) bw*^{D}*, S*^{1} *wg*$^{Sp-1}$ *Ms(2)M*^{1} *bw*^{D}*/CyO* flies, were used to produce embryos lacking maternally contributed and zygotically expressed *Su(H), Su(H)*^{null} (Morel and Schweisguth, 2000).

The GAL4/UAS system (Brand and Perrimon, 1993) was used to express *Notch* and *Su(H)* constructs. Females carrying *twist-GAL4* on both the X and the second chromosomes {2X *twist-GAL4*} (Baylies et al., 1995) were crossed to males carrying constitutively active forms of Notch or Su(H): UAS-*N*^{intra} (Lieber et al., 1993) or UAS-*Su(H)-VP16* (Kidd et al., 1998). N^{intra} encodes the intracellular domain of Notch (N^{icd}) that is released upon Notch cleavage. Su(H)-VP16 is a Su(H)/VP-16 activation domain fusion protein. The VP16 activation domain inhibits the repressive activity of Su(H) and promotes transcriptional activation. Similar results were obtained with UAS-*N*^{intra} and UAS-*Su(H)-VP16* utilizing *twi-GAL4; Dmef2-GAL4* (data not shown). 2X *twist-GAL4* was additionally used to drive expression of UAS-*Su(H)* (Kidd et al., 1998).

Su(H)^{null} embryos that express N^{intra} panmesodermally were created by recombining *twist-GAL4* onto the *Su(H)*^{del47} chromosome. *Su(H)*^{del47}*, twist-GAL4/CyO, ftz-lacZ* males were then crossed to females carrying *Su(H)*^{del47}; UAS-*N*^{intra} GLCs.

Notch deletion constructs, constitutively active forms of Notch and Su(H), and 2X UAS-*emc* (gift of M. Ruiz-Gomez) were expressed in *N*^{null} embryos with one copy of *twist-GAL4*. Females producing *Df(1)N*^{81k1}; *twist*-GAL4 GLCs were crossed to males carrying *FM7c*, *ftz-lacZ* and one of the following constructs: UAS-*FLN*, UAS-*N*^{intra}, UAS-*FLNΔcdc10*, UAS-*FLNΔ10-12* (Zecchini et al., 1999), UAS-*Su(H)-VP16*, or two copies of UAS-*emc* {2X UAS-*emc*}. *FLN* encodes the full length Notch receptor. The Notch protein encoded by *FLNΔcdc10* lacks the RAM-23 domain and the cdc10/ankyrin repeats, while the Notch protein encoded by *FLNΔ10-12* contains a deletion in the extracellular domain that removes EGF-like repeats 10-12.

Transgenic lines carrying 1428*twist-GFP* (Cox, 2004; Thisse et al., 1991), and 1428*twist*$^{mutSu(H)}$*-GFP* were generated by injection of *yw* embryos as previously described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Four 1428*twist-GFP* and two 1428*twist*$^{mutSu(H)}$*-GFP* independent transformant lines were obtained, mapped, expanded into homozygous stocks and analyzed. 2X *twist-GAL4,* and in additional experiments, *twist-GAL4; Dmef2-GAL4* (data not shown), were utilized to drive UAS-*N*^{intra} and UAS-*Su(H)-VP16* in wild-type and mutated reporter construct backgrounds.

Two *E(spl)-C* deficiency strains were analyzed: *Df(3R)E(spl)*^{R1} and *Df(3R)E(spl)*$^{b32.2}$*, P[gro*$^{+}$*]* (gifts of A. Martinez-Arias). *Df(3R)E(spl)*^{R1} deletes all *E(spl)-C* genes, including *groucho* (*gro*) (de Celis, 1991; Knust et al., 1987). *Df(3R)E(spl)*$^{b32.2}$ deletes all *E(spl)-C* genes, except for *gro*. However, while *Df(3R)E(spl)*$^{b32.2}$ leaves the *gro* coding region intact, its disruption of *gro*'s 5′ noncoding region partially affects *gro* function (Schrons et al., 1992). *gro* function is restored in *Df(3R)E(spl)*$^{b32.2}$*, P[gro*$^{+}$*]* flies, which carry a wild-type *groucho* allele (Heitzler et al., 1996). *twist-GAL4; Dmef2-GAL4* (at 29°C) and/or 2X *twist-GAL4,* in an otherwise wild-type or sensitized *twi*^{ID96} (null *twist* allele) heterozygous background, were used to drive the following UAS-*E(spl)-C* constructs: UAS-*m2*, UAS-*m3*, UAS-*m4,* UAS-*m5*, UAS-*m7,* UAS-*m8*, and UAS-*mα* (gifts of C. Delidakis, J. W. Posakony, S. Bray, and A. Preiss).

2X *twist-GAL4* was employed to drive expression of UAS-*da* (Castanon et al., 2001), UAS-*da-da*, two copies of UAS-*emc* (Baonza et al., 2000), and UAS-*da-da*; UAS-*emc*. In an additional experiment, UAS-*da* was expressed with *twist-GAL4; Dmef2-GAL4* at 29°C to increase *da* expression. Transgenic UAS-*da-da* flies were generated by injection of *yw* embryos as previously described (Castanon et al., 2001).

Embryos carrying the following *emc* loss-of-function alleles were analyzed: *emc*^{1}, *emc*^{ip15}, and *emc*^{E12} (Cubas et al., 1994). *emc*^{1} and *emc*^{ip15} are recessive lethal hypomorphs. The *emc*^{E12} deficiency is recessive lethal; it removes 10 chromosomal bands, including the *emc* locus. To minimize the effect of maternal inheritance, mutant embryos were obtained from heterozygous *emc*^{E12} females that were crossed to heterozygous *emc*^{1}, *emc*^{ip15}, or *emc*^{E12} males.

In addition to the above strains, wild-type Oregon-R and *da* maternal/zygotic mutant embryos were examined. Maternal and zygotic Da levels were reduced with the temperature sensitive da^1 allele: permissive at 18°C, lethal at 25°C (Castanon et al., 2001).

All crosses were conducted at 25°C unless otherwise noted.

Plasmid construction

A 1428 base pair *twist* regulatory region (1428*twist*) was PCR amplified from a *pBS* plasmid containing a minimal *twist* promoter, a 3141 base pair insert of sequence that lies upstream of the *twist* ORF (Cox, 2004; Thisse et al., 1991). Primers 5′GCTCTAGAGCGA-CCAATAGTTTAAG3′ and 5′CGGGATCCCTTGGTGATCTTGC-TTGG3′ containing an Xba and BamHI restriction site, respectively, amplified the region we termed 1428*twist*. 1428*twist* was then subcloned as a Xba-BamHI fragment into the *pH-Stinger* transformation vector upstream of nuclear enhanced *GFP* (Barolo et al., 2000a).

Sequence analysis, using MacVector, of 1428*twist* identified one site (TGTGGGAA) matching the YRTGDGAD consensus Su(H) binding sequence (Barolo et al., 2000b). Using site-directed mutagenesis (Promega, USA, Gene Editor), the conserved Su(H) binding site was mutated to TTCTATCC. The mutation was verified by sequencing. Following the same procedures described for 1428*twist*, the mutated 1428 base pair *twist* regulatory region [$1428twist^{mutSu(H)}$] was subcloned into *pH-Stinger*.

To create the Da-Da tethered dimer, *da* cDNA (provided by M. Caudy) and a *pcDNA3* plasmid containing a 16 amino acid Gly/Ser rich flexible polypeptide linker were used (Castanon et al., 2001; Markus, 2000; Neuhold and Wold, 1993). *da* cDNA was cloned in frame on either side of the flexible linker so that translation results in a Da homodimer. For P-element transformation, *da-da* was subcloned into *pUAST* (Brand and Perrimon, 1993).

Immunocytochemistry and imaging

Embryos for immunocytochemistry were fixed following standard techniques for whole mounts (Wieschaus and Nüsslein-Volhard, 1986). The following antibodies were used: anti-Twist (1:5000; gift of S. Roth), anti-Emc (1:1000; gift of Y. N. Jan), anti-Da (1/50; gift of C. Cronmiller), anti-β-galactosidase (1:2000; Promega, USA), and anti-GFP (1:250 with glutaraldehyde treatment; Abcam ab6556). Double staining with anti-β-galactosidase was performed to identify embryos carrying *lacZ* marked chromosomes. Biotinylated secondary antibodies were utilized in combination with the Vector Elite ABC Kit (Vector Laboratories, USA). Embryos were embedded in Araldite. Images were captured using Nomarski optics on an Axiocam digital camera (Zeiss). Lateral views of whole embryos are shown at 40X magnification, close-ups at 63X. Anterior is left. Embryos were staged according to Campos-Ortega and Hartenstein (1985). Since the neurogenic phenotype of Notch signaling mutants disrupts the mesodermal layer, all embryo pictures (mutant, transgenics, and wild type) are a merge of several mesodermal sections. Sections were photographed with Axiovision and merged together using Adobe Photoshop. Different focal planes were also merged in the pictures of embryos stained with anti-Emc so that both the ectoderm and mesoderm are visualized.

Results

Notch repression of Twist is required to form low and high Twist domains

Notch is ubiquitously expressed in the mesoderm throughout gastrulation and subdivision (Fehon et al., 1991; Kidd et al., 1989). Genetic experiments suggested that Notch plays an early role in mesoderm development, prior to its well-characterized function in lateral inhibition. This novel Notch activity represses somatic muscle development prior to, or at the time of, equivalence group formation (Brennan et al., 1999). During these early stages, Twist is a key regulator of mesoderm and somatic fate. Hence, we investigated whether Notch regulates Twist.

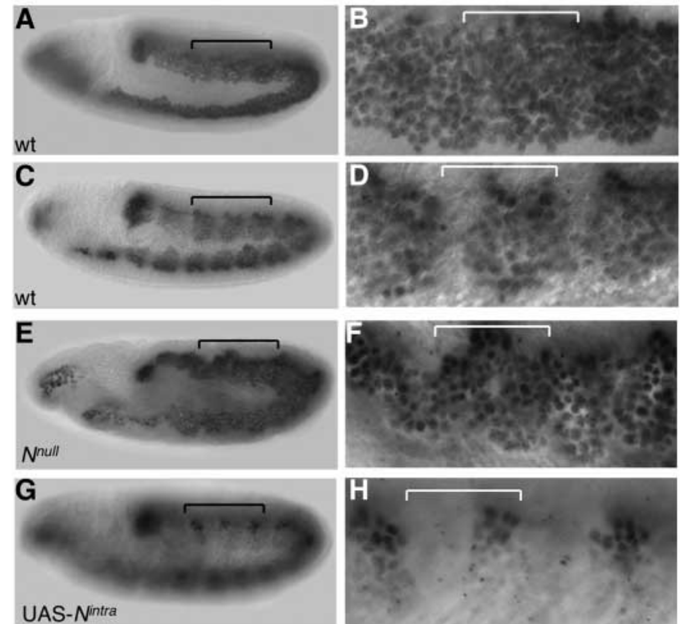

Fig. 1. Notch represses Twist expression. Lateral views of embryos stained with anti-Twist. (A,C,E,G) Whole-mount embryos. In this and all the following figures, the black bracket denotes the mesodermal segments shown at higher magnification in (B,D,F,H). (B,D,F,H) Corresponding close-ups of each embryo in (A,C,E,G). In this and all the following figures, the white bracket demarcates one mesodermal segment. (A,B) Wild-type (wt) stage 9 embryo expresses Twist at high levels uniformly throughout its mesoderm. (C,D) Wt stage 10 embryo exhibits a modulated Twist pattern along its anterior–posterior axis. Each segment consists of a low and high Twist domain. (E,F) N^{null} stage 10 embryo maintains high Twist expression throughout its mesoderm. Rather than modulating Twist levels, N^{null} mutants display uniform high Twist expression pattern characteristic of wt stage 9 embryos. (G,H) UAS-N^{intra} stage 10 embryo has fewer high Twist expressing cells than wt.

Twist is expressed in all mesodermal cells at high levels throughout gastrulation. However, during mesoderm subdivision, Twist expression is modulated. The distinctive uniform high Twist expression pattern seen when gastrulation is complete (stage 9) changes into a segmented pattern of low and high Twist domains, so that at stage 10, each mesodermal segment consists of a low and high Twist domain (Fig. 1A-D).

Notch null (N^{null}) embryos, lacking both maternally contributed and zygotically expressed *Notch*, fail to modulate Twist expression into low and high domains at stage 10, resulting in maintained uniform high Twist levels (Fig. 1E,F). The maintenance of high Twist levels during subdivision has drastic consequences for the subsequent development of mesodermal tissues (Baylies and Bate, 1996); for example, N^{null} embryos fail to set aside the proper number of visceral mesoderm progenitor cells (Baylies and Bate, 1996; Lawrence et al., 2001; Rusconi and Corbin, 1999). Panmesodermal expression of a constitutively activated form of Notch (N^{intra}) had the opposite effect when compared to complete loss of

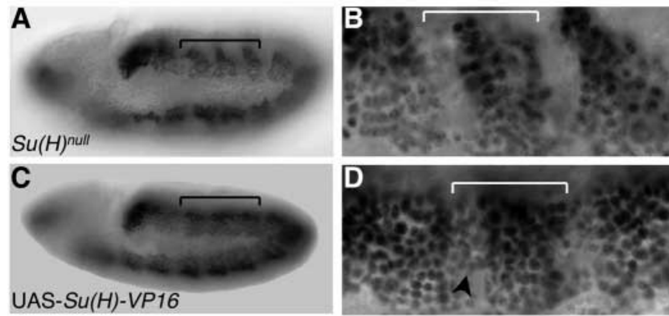

Fig. 2. Twist expression in *Su(H)* loss- and gain-of-function embryos. (A,C) Stage 10 whole-mount embryos stained with anti-Twist. (B,D) Corresponding close-ups of embryos in A,C. (A,B) *Su(H)^{null}* mutant, like wild type, modulates Twist into low and high domains. (C,D) UAS-*Su(H)-VP16* embryo ectopically expresses high Twist levels. Black arrowhead points to cells, located in what should be the low Twist domain, that express high amounts of Twist compared to wild type.

Notch function: fewer cells express high Twist levels (Fig. 1G,H). This reduced Twist expression contributes to severe defects in the somatic musculature (Baylies and Bate, 1996; Fuerstenberg and Giniger, 1998). Together, loss- and gain-of-function experiments revealed that Notch is required to repress Twist expression at stage 10.

Su(H) regulates Twist differently from Notch

To establish the mechanism by which Notch represses Twist expression, we investigated how Su(H), the only identified transcriptional effector of Notch signaling, affects Twist. Twist expression was examined in *Su(H)^{null}* mutant embryos derived from *Su(H)^{del47}* germline clones. In sharp contrast to *N^{null}* mutants, *Su(H)^{null}* mutant embryos modulate Twist levels properly and exhibit the low and high Twist pattern characteristic of wild-type embryos at stage 10 (Fig. 2A,B; compare with Fig. 1E,F). In addition, Su(H) gain of function was analyzed. Panmesodermal expression of a constitutively transactivating form of Su(H), Su(H)-VP16, resulted in expanded high Twist domains. UAS-*Su(H)-VP16* embryos ectopically expressed high levels of Twist in presumptive low Twist domains (Fig. 2C,D; compare with wild type in Fig. 1C,D). This result contrasted with the repressed Twist expression seen in embryos that panmesodermally express N^{intra} (compare with Fig. 1G,H). Panmesodermal expression of UAS-*Su(H)*, which simply increased the amount of wild type Su(H), did not affect Twist expression (data not shown). Taken together, the disparities between the phenotypes of *N^{null}* mutants versus *Su(H)^{null}* mutants and UAS-*N^{intra}* versus UAS-*Su(H)-VP16* embryos indicated that Notch and Su(H) regulate Twist differently.

Su(H)-mediated Notch signaling regulates Twist

Differences between *Notch* and *Su(H)* phenotypes and gene regulation have previously been reported in a variety of invertebrate and vertebrate systems (Barolo et al., 2000b; Brennan et al., 1999; Furriols and Bray, 2000; Hsieh et al., 1996; Klein et al., 2000; Koelzer and Klein, 2003; Ligoxygakis et al., 1998; Morel and Schweisguth, 2000; Ordentlich et al., 1998; Rusconi and Corbin, 1998; Shawber et al., 1996). These results were explained by two non-exclusive models: (1) Notch signals through a Su(H)-independent pathway and (2) Notch acts as a transcriptional switch that alleviates Su(H)-mediated repression; this switch can convert Su(H) from a repressor into an activator. We next investigated which mechanism Notch uses to regulate Twist.

First we analyzed whether Notch requires Su(H) to repress Twist by expressing N^{intra} panmesodermally in *Su(H)^{null}* mutant embryos [*Su(H)^{null};* UAS-*N^{intra}*]. We expected that if Notch signals through an Su(H)-independent pathway, Twist would still be repressed by UAS-N^{intra} in the *Su(H)^{null}* background. Interestingly, Twist is not repressed in *Su(H)^{null};* UAS-*N^{intra}* embryos. Unlike UAS-*N^{intra}* embryos, which have few cells that express Twist at high levels, *Su(H)^{null};* UAS-*N^{intra}* mutant embryos, similarly to *Su(H)^{null}* mutant embryos, exhibit a 'wild-type-like' Twist pattern (compare Fig. 3A,B with Fig. 1G,H). This result indicated that N^{intra} requires Su(H) to repress Twist. Furthermore, it strongly suggested that Twist is not regulated by Su(H)-independent Notch signaling at subdivision.

Thus, we considered the transcriptional switch model. We reasoned that if Notch regulates Twist through a transcriptional switch that converts Su(H) from a transcriptional repressor into an activator, then the *N^{null}* phenotype would be caused by Su(H) constitutively acting as a repressor. Consequently, we examined whether the constitutively activating form of Su(H) [Su(H)-VP16] could rescue Twist modulation in *N^{null}* embryos.

As a control, we first tested whether panmesodermal transgene expression could restore wild-type-like Twist expression in *N^{null}* embryos. Panmesodermal expression of a full-length Notch construct (UAS-*FLN*) rescued the Twist phenotype of *N^{null}* embryos. Instead of the uniform high Twist levels characteristic of *N^{null}* mutant embryos, low and high Twist domains were observed in *N^{null}*; UAS-*FLN* embryos (compare Fig. 3C,D with Fig. 1E,F). Similarly, panmesodermal expression of N^{intra} restored Twist modulation. *N^{null}*; UAS-*N^{intra}* embryos exhibited low and high Twist domains; as expected, UAS-*N^{intra}* repressed Twist more strongly than UAS-*FLN* (Fig. 3E,F).

In addition, we assessed whether panmesodermal expression of a Notch protein that lacks its Su(H) interaction domain (*FLNΔcdc10*) would rescue Twist modulation. *FLNΔcdc10* is a full-length *Notch* transgene that carries an intracellular deletion that removes the RAM23 domain and cdc10 repeats, both of which have been shown to bind Su(H) (Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1997). Published work has also shown that cdc10 repeats are required for Notch signal transduction (Lieber et al., 1993). In contrast to what was seen with FLN, FLNΔcdc10 did not rescue Twist modulation in *N^{null}* embryos. *N^{null}*; UAS-*FLNΔcdc10* embryos maintained Twist at uniform high levels throughout the mesoderm at stage 10 (Fig. 3G,H). This finding strengthens our conclusion that Notch requires Su(H) to repress Twist.

Finally, we found that panmesodermal expression of the constitutively transactivating form of Su(H), Su(H)-VP16, rescued Twist modulation in *N^{null}* embryos. Su(H)-VP16 repressed Twist expression in *N^{null}* mutant embryos such that low and high Twist expression domains were restored (Fig. 3 I,J). This result was consistent with our finding that Notch signals through Su(H) to regulate Twist. It also supported our hypothesis that the *N^{null}* Twist phenotype results from the loss

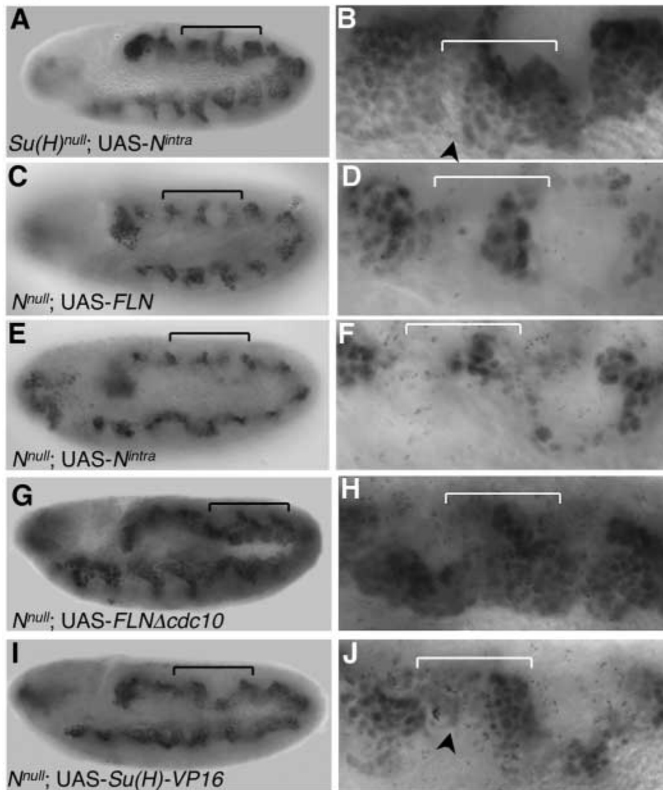

Fig. 3. Su(H) is required for Notch to repress Twist. (A,C,E,G,I) Lateral views of stage 10 whole-mount embryos stained with anti-Twist. (B,D,F,H,J) Corresponding close-ups of embryos in A,C,E,G,I. (A,B) $Su(H)^{null}$; UAS-N^{intra} embryo has a wild-type-like Twist pattern, similar to $Su(H)^{null}$. Low and high Twist domains are seen along the anterior–posterior axis; Twist is not strongly repressed as in UAS-N^{intra} embryos (see Fig. 1). (C,D) N^{null}; UAS-*FLN* embryo exhibits low and high Twist domains. FLN rescues the Twist pattern of N^{null} embryos (see Fig. 1). (E,F) N^{null}; UAS-N^{intra} embryo has low and high Twist domains. N^{intra} rescues the Twist pattern, but represses Twist expression compared to FLN. (G,H) N^{null}; UAS-*FLNΔcdc10* maintains uniform high Twist expression, similar to N^{null} embryos. FLNΔcdc10 does not rescue the Twist pattern. (I,J) N^{null}; UAS-*Su(H)-VP16* embryo no longer maintains uniform high Twist expression. Low and high Twist domains are observed, but high Twist domains appear slightly expanded. Black arrowhead points to cells in the low Twist domain that express higher levels of Twist than wild type. Su(H)-VP16 rescues the Twist pattern but not as strongly as FLN and N^{intra}.

of a transcriptional switch that converts Su(H) from a constitutive repressor into an activator. However, the simple model that Su(H) acts only on the *twist* promoter – first as a repressor and then upon Notch signaling as an activator – implies that Su(H)-VP16, as seen in Fig. 2C,D, should activate *twist* transcription. However, the rescue experiment showing that Su(H)-VP16 is capable of repressing Twist (Fig. 3I,J) suggested that Su(H) affects Twist by activating a gene that represses *twist*. This paradox can be resolved by the hypothesis that Su(H) can regulate the *twist* gene both directly and indirectly.

Lastly, the rescue experiments also suggested that the ability of UAS-*Su(H)-VP16* to repress Twist is not as strong as that of UAS-*FLN* and UAS-N^{intra}. Compared to N^{null}; UAS-*FLN* and N^{null}; UAS-N^{intra} embryos, N^{null}; UAS-*Su(H)-VP16* embryos exhibit higher Twist expression (Fig. 3). Although this may reflect variations in transgene expression, incomplete rescue by UAS-*Su(H)-VP16* was also consistent with the finding that UAS-*Su(H)-VP16* can activate, as well as repress, Twist.

Taking all our data together, we concluded that Su(H)-mediated Notch signaling regulates Twist. We proposed that Notch signaling acts as a transcriptional switch that alleviates Su(H)-mediated repression and converts Su(H) from a transcriptional repressor into a transcriptional activator. Furthermore, these results suggested that Su(H) could affect Twist expression through a multi-layered mechanism that includes direct, as well as indirect, transcriptional regulation.

Notch/Su(H) regulation of a minimal *twist* promoter

To explore the transcriptional mechanism that Notch and Su(H) utilize to affect Twist expression, we conducted promoter analysis. We uncovered a 1428-bp region of the *twist* promoter (1428*twist*), which lies immediately upstream of the transcriptional start site, that faithfully drives GFP reporter gene expression in a wild-type Twist pattern through mid-embryogenesis (Cox, 2004; Thisse et al., 1991). At stage 10, 1428*twist* embryos modulated GFP into low and high expression domains along the anterior–posterior axis (Fig. 4A,B).

In vivo, this minimal *twist* promoter responded to Notch signaling. For example, in a manner analogous to the endogenous *twist* gene, the GFP reporter was repressed by panmesodermal N^{intra} expression (Fig. 4C,D). 1428t*wist*;UAS-N^{intra} embryos exhibited narrower high GFP expression domains than 1428*twist* embryos. However, the effect of N^{intra} on the GFP reporter was not as dramatic as its effect on endogenous Twist expression (see Fig. 1G,H). It is likely that additional regulatory sequences, which are located outside 1428*twist,* contribute to Notch's regulation of the *twist* gene. Additionally, it is possible that some of Notch's effects on Twist are post-transcriptional and hence not reflected in this reporter assay. Nevertheless, since Notch exerted an effect on 1428*twist,* we utilized the promoter construct to further understand how Notch signaling regulates Twist expression.

1428*twist* contains only one site (TGTGGGAA) that matches the YRTGDGAD Su(H)-binding consensus sequence (Barolo et al., 2000b). Published gel shift experiments have shown that Su(H) binds oligonucleotides containing this GTGGGAA core sequence with high affinity (Morel and Schweisguth, 2000). Hence, it is likely that Su(H) strongly binds 1428*twist* in vivo.

To test how the Notch signaling pathway regulates *twist* modulation during subdivision, we mutated the conserved Su(H) site on the 1428*twist* promoter [1428$twist^{mutSu(H)}$] and cloned the mutated promoter upstream of a *GFP* reporter gene. We had two expectations: (1) if Su(H) binds the *twist* promoter and represses transcription until Notch signaling acts as a transcriptional switch that converts Su(H) into an activator, Su(H) site mutation should cause the 1428*twist* promoter to be de-repressed; and (2) if Notch signaling also represses *twist* indirectly, as suggested by our genetic experiments, Su(H) site mutation should not abolish Notch repression and modulation of the 1428*twist* reporter – another site should be employed. Hence, rather than exhibiting a N^{null}-like phenotype and uniformly maintaining high GFP levels throughout the

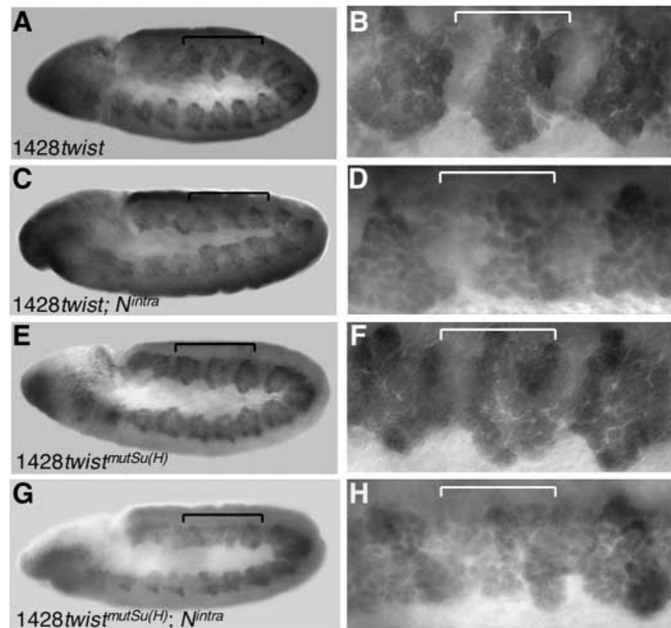

Fig. 4. Analysis of Notch/Su(H) regulation of a minimal *twist* promoter. Lateral views of stage 10 embryos stained with anti-GFP. (A,C,E,G) Whole-mount embryos. (B,D,F,H) Corresponding close-ups of embryos in A,C,E,G. (A,B) 1428*twist* embryo exhibits low and high GFP domains. Notice that high domains appear chevron-shaped. (C,D) 1428*twist*; UAS-N^{intra} embryo has narrower high GFP domains than 1428*twist* embryos. GFP expression is repressed so that high GFP domains appear triangle-like in 1428*twist*; UAS-N^{intra} embryos. (E,F) 1428$twist^{mutSu(H)}$ embryo displays a modulated pattern of low and high GFP domains. Uniform high GFP expression is not maintained throughout the mesoderm. Additionally, compared with 1428*twist,* high GFP domains appear to be slightly expanded. (G,H) 1428$twist^{mutSu(H)}$; UAS-N^{intra} embryo looks different from the three embryos shown above. GFP expression in presumptive high GFP domains, especially laterally, is repressed compared with 1428*twist* embryos, so 1428$twist^{mutSu(H)}$; UAS-N^{intra} high GFP domains appear most similar to those seen in 1428*twist*; UAS-N^{intra} embryos. However, at the same time, compared with 1428*twist* embryos, some cells in the presumptive low GFP domains of 1428$twist^{mutSu(H)}$; UAS-N^{intra} embryos express GFP at high levels; this phenotype is most similar to that of 1428$twist^{mutSu(H)}$ embryos.

mesoderm, we expected 1428$twist^{mutSu(H)}$ embryos to display a modulated low/high GFP pattern. This indirect mode of *twist* repression is consistent with the classic model of Notch signaling in which Notch stimulates Su(H) to activate direct targets, such as *Enhancer of split complex* [*E(spl)-C*] genes, which in turn repress *achaete-scute* (*ac-sc*) complex genes.

At stage 10, 1428$twist^{mutSu(H)}$ embryos modulate GFP into low and high domains (Fig. 4E,F). In addition, high GFP domains appear slightly expanded when compared with 1428*twist* embryos, a result consistent with de-repression of the *twist* promoter. These data suggested that normally, Su(H) binds its consensus site on 1428*twist* and represses transcription until Notch signals. However, since 1428$twist^{mutSu(H)}$ embryos still modulate GFP, we concluded that a Notch/Su(H) regulated non-Su(H) site is also required to repress *twist* and create a modulated pattern. This result probably explains why the 1428$twist^{mutSu(H)}$ promoter is only mildly de-repressed; the indirect repressive activity of Notch inhibits strong de-repression. In sum, these findings, combined with our earlier genetic data, provide evidence for a direct effect of Su(H) on the *twist* promoter, as well as an indirect effect of Notch signaling that represses *twist*.

To gather further support for this conclusion, we examined how panmesodermal expression of N^{intra} affects the 1428$twist^{mutSu(H)}$ promoter. Since we hypothesized that Notch indirectly represses Twist, we predicted that N^{intra} would repress GFP expression, despite the elimination of the only Su(H) binding site in the *twist* promoter. Indeed, the GFP pattern seen in 1428$twist^{mutSu(H)}$; UAS-N^{intra} embryos revealed that N^{intra} can repress the activity of the 1428$twist^{mutSu(H)}$ promoter (Fig. 4G,H). GFP expression in presumptive high GFP domains, especially laterally, was repressed compared with 1428*twist* embryos. However, at the same time, some cells in presumptive low GFP domains expressed higher amounts of GFP, suggesting that the *GFP* reporter was de-repressed in presumptive low GFP domains. As noted above, this de-repression was probably caused by the removal of Su(H) mediated repression of *twist*. Thus, the abnormal GFP pattern of 1428$twist^{mutSu(H)}$; UAS-N^{intra} embryos appeared to be a combination of indirect N^{intra} repression and Su(H) de-repression of the 1428*twist* promoter. Panmesodermal expression of UAS-Su(H)-VP16 in a 1428$twist^{mutSu(H)}$ background resulted in a phenotype similar to that seen in 1428$twist^{mutSu(H)}$; UAS-N^{intra} embryos (data not shown).

Taken together, promoter and genetic analyses indicated that, in addition to the conserved Su(H) site in the *twist* promoter, an additional, non-Su(H) site is involved in Notch-mediated *twist* repression. We suggest that this non-Su(H) site is the binding site of a Notch/Su(H) regulated gene that represses *twist*, called '*repressor of twist*'. We had four expectations of a '*repressor of twist*': (1) it would be regulated by Notch signaling; (2) it would act as a transcriptional repressor; (3) 'Repressor of twist' would be expressed in the early mesoderm just before or at the time of apparent Twist modulation; and (4) it would impinge on the *twist* promoter, either by directly binding to specific sequences or by affecting the activity of bound factors. Two types of candidate genes emerged as possible '*repressors of twist*' based on these qualifications – *Enhancer of Split* complex [*E(spl)-C*] genes and *extra machrochaetae* (*emc*).

Notch represses Twist indirectly through E(spl)

E(spl)-C encodes 7 bHLH proteins (*m3*, *m5*, *m7*, *m8*, *m*β, *m*γ and *m*δ) and six non-bHLH proteins – *m1*, *m2*, *m4*, *m6*, *m*α, and *groucho* (Knust et al., 1987). Expression of *E(spl)* complex genes is regulated by the classical Notch signaling pathway. In loss-of-function *Notch* mutant embryos, members of the complex show no detectable expression, indicating that Notch is required for activation of these genes (Furriols and Bray, 2000; Jennings et al., 1994). In loss-of-function *Su(H)* mutant backgrounds, the expression of *m4*, *m8* and *m*α in the wing (Bailey and Posakony, 1995; Koelzer and Klein, 2003) and *m2* in germline clone embryos (Wurmbach et al., 1999) is upregulated, indicating that these genes are repressed by Su(H) in the absence of Notch signaling.

E(spl) bHLH proteins are Notch-regulated transcriptional repressors. Yeast-two hybrid experiments showed that they can homodimerize as well as heterodimerize with each other

(Alifragis et al., 1997). E(spl) bHLHs can directly and indirectly repress transcription. They directly bind promoters, recruit co-repressors, and repress transcription (Oellers et al., 1994). In addition, they interact with other promoter-bound bHLH proteins to indirectly repress transcription (Giagtzoglou et al., 2003). In vitro, E(spl) bHLH homodimers have been shown to bind canonical E boxes (CANNTG, preferably of the class B-type CACGTG), N boxes (CACNAG) and Hairy sites (CACGCG) (Jennings et al., 1999). 1428*twist* contains a consensus E box (CAGTTG), four 'N box-like' (CANNAG) motifs, and seven 'hairy-like' (CANNCG) motifs.

At stage 10, four E(spl) bHLHs – *m3*, *m5*, *m8* and *m7* – are expressed throughout the mesoderm at uniform low levels (Knust et al., 1987). Four non-bHLH *E(spl)-C* genes are also expressed in the early mesoderm, prior to stage 11: *m2*, *m4*, *m*α and *groucho* (Knust et al., 1987; Wurmbach et al., 1999). M2 is a novel Notch-regulated protein; M4 and *M*α are Notch-regulated Bearded-like proteins. Lastly, Groucho is a ubiquitously expressed transcriptional co-repressor (Paroush et al., 1994). It interacts with E(spl) bHLHs as well as other transcriptional regulators including Runt, Hairy, Dorsal, TCF, and Hairless, all of which function in the early embryo (Aronson et al., 1997; Barolo et al., 2002; Cavallo et al., 1998; Dubnicoff et al., 1997; Flores-Saaib et al., 2001; Levanon et al., 1998; Paroush et al., 1994; Roose et al., 1998).

Since the *E(spl)-C* genes fulfill our four requirements for a possible Notch-regulated '*repressor of twist*', we analyzed Twist expression in *E(spl)* mutant embryos. Two sets of embryos were analyzed: embryos carrying a deficiency that deletes the entire *E(spl)-C* locus, including the co-repressor *groucho* (*gro*) [*Df(3R)E(spl)*]; and embryos carrying a deficiency that removes the entire *E(spl)-C* but carries a transgene that restores wild-type *gro* function {*Df(3R)E(spl), P[gro$^+$]*}. These embryos were compared to ascertain the contribution of the entire *E(spl)-C* with and without *groucho*.

At stage 10, *Df(3R)E(spl)* mutant embryos maintained uniform high Twist expression throughout the mesoderm (Fig. 5A,B). Like N^{null} mutants, *Df(3R)E(spl)* mutant embryos did not modulate Twist into low and high domains. In a similar, albeit less severe, manner, *Df(3R)E(spl), P[gro$^+$]* mutants ectopically expressed high levels of Twist (Fig. 5C,D). Cells, located in what should be the low Twist domain, expressed higher amounts of Twist than wild type.

Taken together, these results indicated that E(spl) proteins – probably the mesodermally expressed *E(spl)* genes *m2*, *m3*, *m4*, *m5*, *m7*, *m8* and/or *m*α – repress Twist at stage 10. Since removing zygotic *groucho* expression exacerbates the Twist phenotype, our findings also demonstrate that Groucho-mediated repression is critical for Twist modulation into low and high domains.

To ascertain the effect that individual *E(spl)-C* genes and bHLH versus non-bHLH E(spl) proteins have on Twist, we conducted gain-of-function analysis. Panmesodermal expression of UAS-*m2*, UAS-*m3*, UAS-*m4*, UAS-*m5*, UAS-*m7*, UAS-*m8* or UAS-*m*α did not affect Twist expression; all embryos exhibited a wild-type-like Twist pattern (data not shown, see Materials and methods). These results revealed that overexpression of individual mesodermal *E(spl)-C* genes is not sufficient to repress Twist. Perhaps, in the embryo, a combination of several E(spl)-C proteins, bHLH and/or non-bHLH, are required to repress Twist. It is also possible that E(spl)-C proteins work in concert with another factor, a non-E(spl) protein, to repress Twist.

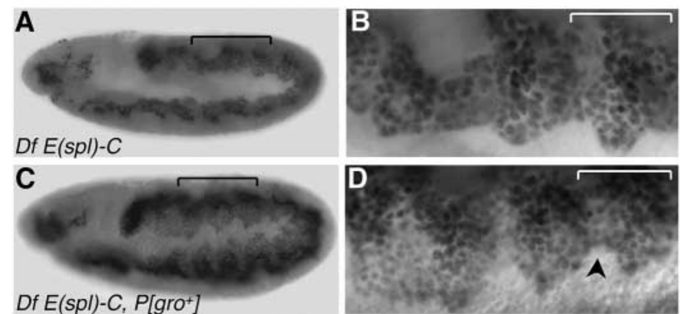

Fig. 5. *E(spl)-C* locus regulates Twist modulation into low and high domains. (A-D) Lateral views of stage 10 embryos stained with anti-Twist: (A,C) whole-mount embryo, (B,D) corresponding close-ups of embryos in (A,C). (A,B) *Df E(spl)-C* mutant maintains high Twist expression uniformly throughout its mesoderm, similar to N^{null} embryos. (C,D) *Df E(spl)-C, P[gro$^+$]* mutant has expanded high Twist domains compared with wild type. Black arrowhead indicates cells, located in a presumptive low Twist domain, expressing higher levels of Twist than wild type.

In conclusion, published work from several labs has demonstrated that Notch signaling transcriptionally regulates *E(spl)-C* genes. Based on our loss-of-function data, we suggest that one aspect of the mechanism employed by Notch to indirectly repress *twist* involves direct Notch regulation of *E(spl)-C* genes.

twist regulation by Extra machrochaetae (Emc) and Daughterless (Da) activity

In the *Drosophila* wing and eye, Notch signaling regulates *emc* transcription (Baonza et al., 2000; Baonza and Freeman, 2001). In the embryonic mesoderm, Emc is expressed uniformly during gastrulation until stage 10. Embryos carrying strong hypomorphic *emc* alleles showed a variety of mesodermal phenotypes, including muscle losses and aberrant muscle attachments, as well as misregulation of Twist expression (Cubas et al., 1994). Emc contains an HLH domain but not a basic domain (Garrell and Modolell, 1990). Thus, while it can dimerize with bHLH proteins, Emc cannot bind DNA. Consequently, Emc acts as a dominant negative; the formation of inactive Emc/bHLH heterodimers inhibits bHLH transcriptional activity.

Emc genetically interacts with the bHLH protein Daughterless, Da (Ellis et al., 1990). In-vitro gel shift experiments demonstrated that Emc heterodimerizes with Da with high affinity; this interaction prevents Da from binding canonical CANNTG E boxes, such as the one found on 1428*twist*, and activating transcription (Van Doren et al., 1991). Emc does not form dimers with Twist nor any of the seven E(spl) bHLH transcription factors; the proteins have poor affinity for one another (Alifragis et al., 1997) (Kass and Baylies, unpublished). Thus in-vitro and in-vivo data suggest that Emc exerts its effects in vivo by inhibiting Da dimerization (Ellis et al., 1990; Van Doren et al., 1991).

Da is ubiquitously expressed throughout development (Cronmiller and Cummings, 1993) and required to maintain uniform high Twist expression throughout the mesoderm

during gastrulation (Castanon et al., 2001). While Notch signaling components genetically interact with *da* (Cummings and Cronmiller, 1994; Smith et al., 2002), they have not been reported to transcriptionally regulate Da (Smith and Cronmiller, 2001). *N* and *Su(H)* mutant embryos show no discernible effect on Da expression through mid-embryogenesis (data not shown). Based on these Emc and Da data, we investigated whether Emc is also a Notch-regulated '*repressor of twist*', acting via Da to control Twist levels. We first examined the effect of Da and, particularly, the effect of Da dimerization on Twist regulation in the early embryo.

Loss of Da in early embryos reduces Twist expression, indicating that Da is required for high levels of Twist (Castanon 2001) (Fig. 6A,B). Thus, we next asked whether increasing Da levels ectopically activates high Twist expression. Different amounts of Da were expressed utilizing different conditions and panmesodermal *GAL4* lines. All combinations resulted in stage 10 embryos that ectopically expressed high levels of Twist; cells located in presumptive low Twist domains expressed high amounts of Twist, a phenotype resembling that of N^{null} embryos (Fig. 6C-F). However, the strength of the *GAL4* driver used to express UAS-*da* affected the severity of the phenotype. For example, embryos that ectopically expressed a lower level of Da had fewer ectopic cells that expressed high Twist levels (Fig. 6E,F) than embryos that ectopically expressed a higher level of Da (Fig. 6C,D). Since Emc can dimerize with Da and compete with other proteins for Da monomers, we asked whether the milder *da* overexpression phenotype was caused by high Emc levels in the early embryo (Cubas et al., 1994). We hypothesized that under milder Da overexpression conditions, endogenous Emc interfered with Da dimerization and impaired the ability of Da to activate *twist* expression.

To minimize these potential Da/Emc heterodimer effects on Twist, we examined if linked Da homodimers that were panmesodermally expressed utilizing the weaker GAL4 condition could fully increase Twist expression, similar to that seen in N^{null} embryos and in embryos in which UAS-*da* was ectopically expressed with the stronger driver. Linked Da dimers were created by physically tethering two Da proteins by a flexible glycine–serine polylinker. As a result of this linkage, the local concentration of Da increases, and the formation of the linked dimer is favored over dimers formed between Da and endogenous proteins, and in our case, Emc. This 'tethered' dimer strategy has been successfully employed by several groups to determine the function of bHLH homodimers and heterodimers in vivo and in vitro, most recently in *Drosophila* to uncover the function of Twist–Twist homodimers and Twist–Daughterless heterodimers (Castanon et al., 2001; Markus, 2000; Neuhold and Wold, 1993). Embryos expressing the tethered Da homodimer construct (*da–da*) maintained uniform high Twist expression at stage 10 (Fig. 6G,H); this strongly resembled Twist expression in N^{null} mutant embryos. As expected by our use of the tethering strategy, expressing more Emc in the mesoderm was unable to suppress the effects of Da–Da overexpression. UAS-*da-da*; UAS-*emc* embryos maintained Twist at uniform high levels at stage 10 (data not shown). Thus both loss-and-gain-of-Da experiments indicated that Da is a critical regulator of Twist in the early mesoderm and that inhibition of Da activity is required for proper Twist modulation. Emc, which is expressed

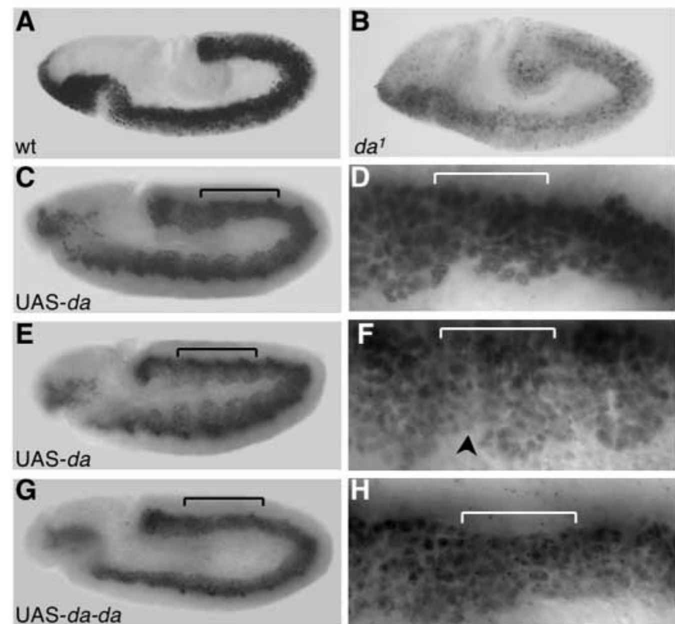

Fig. 6. Da activity regulates Twist expression. Lateral views of stage 8 (A,B) and stage 10 (C-H) embryos stained with anti-Twist. (A,B,C,E,G) Whole-mount embryos. (D,F,H) Corresponding close-ups of embryos in C,E,G. (A) Wild-type (wt) stage 8 embryo expresses Twist uniformly at high levels throughout the mesoderm. (B) da^1 embryo, a mutant with reduced maternal and zygotic Da levels, expresses Twist at low levels at stage 8, as well as at later stages (data not shown) (Castanon et al., 2001). (C,D) UAS-*da* embryo, panmesodermally expressing high levels of Da, ectopically expresses high levels of Twist, similar to N^{null} mutants. (E,F) UAS-*da* embryo, panmesodermally expressing lower levels of Da than the embryo in C-D, ectopically expresses high levels of Twist, but shows a milder phenotype than the embryo in C-D. Black arrowhead indicates cells, located in what should be the low Twist domain, that express higher amounts of Twist than wild type. (G,H) UAS-*da-da* embryo, panmesodermally expressing the linked Da transgene under the same GAL4 conditions as the embryo in E-F, maintains uniform high Twist expression throughout its mesoderm similar to N^{null} mutants (see Fig. 1E,F).

at high levels in the early mesoderm and has been shown to genetically and biochemically interact with Da, provided a mechanism for inhibiting Da activity.

Since Emc expression is upregulated by Notch in the wing and eye (Baonza et al., 2000; Baonza and Freeman, 2001), we next analyzed the effect of Notch on mesodermal Emc expression. In wild-type embryos, Emc is uniformly expressed throughout the mesoderm prior to stage 10; at stage 11, Emc is strongly expressed around ectodermal tracheal pits but absent or expressed at low levels in the mesoderm (Fig. 7A,B). Panmesodermal N^{intra} expression resulted in ectopic Emc expression. The phenotype was especially apparent at stage 11, when UAS-N^{intra} embryos displayed strong mesodermal Emc expression (Fig. 7C,D). This suggested that Notch positively regulates Emc expression. However, like wild-type embryos, N^{null} mutants expressed Emc at uniform levels throughout the mesoderm prior to stage 10. Similar effects on Emc levels were found in $Su(H)^{null}$ embryos (data not shown). We caution, however, that anti-Emc staining and in-situ analysis employing a probe complementary to *emc* cDNA (data not shown) may

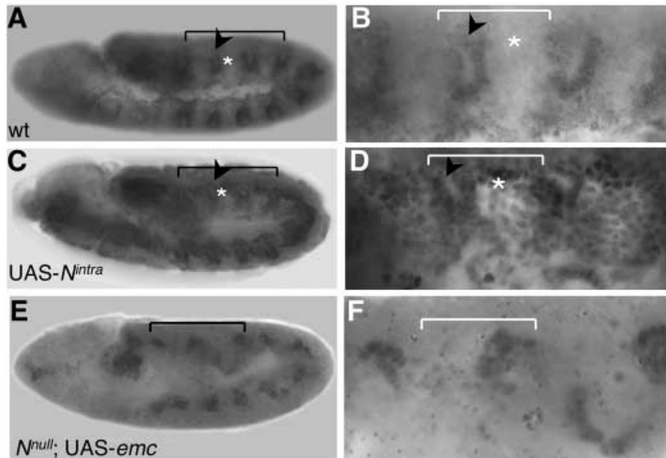

Fig. 7. Notch represses Twist by regulating Emc activity. (A,C,E) Whole-mount embryos. (B,D,F) Corresponding close-ups of embryos in (A,C,E). (A-D) Lateral views of stage 11 embryos stained with anti-Emc. (A,B) Wild-type (wt) embryo shows strong Emc expression around its ectodermal tracheal pits (black arrowheads) and little or no mesodermal Emc expression (white asterisks). (C,D) UAS-N^{intra} embryo expresses Emc both around its tracheal pits (black arrowheads) and throughout its mesoderm (white asterisks). (E,F) Lateral views of a stage 10 N^{null}; UAS-*emc* embryo stained with anti-Twist. Emc represses Twist in N^{null} mutants such that Twist is expressed in low and high domains; compare with Fig. 1E,F and Fig. 3C-J.

not be sensitive enough to detect a uniform slight decrease in Emc expression during stages 9/10. Hence, while early mesodermal Emc expression does not absolutely require Notch, our data demonstrated that Notch signaling is able to upregulate Emc expression.

We further explored the connection between Notch and Emc by addressing whether Emc could rescue the Twist phenotype of N^{null} mutant embryos. We reasoned that since we measured a detectable difference in Emc levels upon Notch activation, then increasing Emc might rescue the effects of loss of Notch. A panmesodermal driver was used to express UAS-*emc* in N^{null} mutant embryos. We found that N^{null}; UAS-*emc* mutant embryos modulated Twist into low and high domains; uniform high Twist expression was not maintained as in N^{null} embryos (Fig. 7E-F, compare with Fig. 1E,F and Fig. 3C-J). On its own, this result revealed that Emc overexpression represses Twist. While these data were consistent with the hypothesis that Emc acts downstream of Notch to regulate Twist expression, this experiment does not definitively place Emc as a downstream Notch target, and it does not rule out the possibility that Emc (via Da) modulates Twist through a parallel, Notch-independent pathway. However, taken together with our finding that Notch upregulated Emc expression, the rescue experiment data suggested that Notch might repress Twist by increasing Emc activity, through transcriptional and/or post-transcriptional regulation.

Lastly, we looked at Twist expression in *emc* loss-of-function mutants. Emc is expressed in the ovary, maternally inherited by the embryo, and expressed throughout the gastrulating mesoderm. Since strong *emc* alleles are cell lethal (Cubas et al., 1994), and *emc* plays a role in oogenesis (J. C. Adam and D. J. Montell, unpublished), we did not generate embryos that completely lack *emc*. We attempted to reduce the effect of maternally contributed *emc* by analyzing embryos obtained from females heterozygous for a deficiency that removes the *emc* locus emc^{E12} (Cubas et al., 1994). Embryos were obtained from emc^{E12} heterozygous females that had been crossed to males heterozygous for the following *emc* recessive lethal alleles: emc^{1}, emc^{ip15} or emc^{E12}. Stage 10 Twist expression appeared wild-type-like in all *emc* mutants examined (data not shown). These experiments indicated that the reduced zygotic Emc activity and/or maternally loaded Emc found in these embryos are sufficient for early Twist expression. Nevertheless, these data do not rule out the hypothesis that Emc regulates Twist modulation.

Taken together, the findings that Notch activated Emc expression and that Emc rescued N^{null} embryos lead us to favor the model that Emc – transcription and/or post-transcriptional activity – is regulated by Notch signaling. We propose that Notch signaling represses Twist expression, through the E(spl)-C proteins, as well as by increasing Emc activity, which inhibits Da from transcriptionally activating *twist*.

Discussion

Analysis of *Notch* mutant embryos revealed that Notch signaling is essential for Twist regulation at mesodermal subdivision. However, comparison of *Notch* and *Su(H)* mutant embryos indicated that Notch regulates Twist differently from Su(H). At stage 10, uniform high Twist expression was maintained in N^{null} mutants; by contrast, $Su(H)^{null}$ mutants have a wild-type-like Twist pattern. Furthermore, while constitutive activation of Notch repressed Twist expression at stage 10, constitutive expression of a transactivating form of Su(H) [Su(H)-VP16] increased Twist expression. Despite these differences, double mutant analysis and rescue experiments demonstrated that Notch requires Su(H) to repress Twist. Moreover, further rescue experiments showed that Notch signaling acts as a transcriptional switch, which alleviates Su(H)-mediated repression and promotes transcription. In addition, genetics, combined with promoter analysis, suggested that Notch and Su(H) have multiple inputs into *twist*. Notch/Su(H) signaling both directly activates *twist* and indirectly represses *twist* expression by activating proteins that repress Twist. Finally, our data indicate that Notch targets two distinct ‘Repressors of twist’ – *E(spl)-C* genes and Emc. We propose that Notch signaling activates expression of *E(spl)-C* genes, which then act directly on the *twist* promoter to repress transcription. Since removing *groucho* enhances the phenotype of the *E(spl)-C* mutant embryos, we suggest that the co-repressor, Groucho, acts with E(spl)-C proteins and the Hairless/Su(H) repressive complex to mediate direct repression of *twist*. Our second ‘Repressor of twist’, Emc, mediates repression of Twist in an alternative fashion. We hypothesize Emc activity inhibits dimerization of Da with itself or another bHLH protein. This, in turn, prevents Da from binding DNA and activating *twist* transcription. Since Emc is expressed in the embryo prior to stage 10, it is likely that the transition from uniform high Twist expression to a modulated Twist pattern involves Emc inhibition of Da activity at stage 9. In conclusion, our work uncovered how Notch signaling impacts a network of mesodermal genes, and specifically Twist expression. Given that Notch signaling directs cell fate decisions in many

Drosophila embryonic and adult tissues and that Notch regulates Twist in adult flight muscles (Anant et al., 1998), these data may suggest a more universal mode of Notch regulation.

Models of Notch target gene regulation

The distinct mesodermal phenotypes of *Notch* and *Su(H)* mutants can be explained by Notch acting as a transcriptional switch. This aspect of Notch signaling has been described in other systems (Bray and Furriols, 2001; Hsieh et al., 1996; Klein et al., 2000), and the early *Drosophila* mesoderm appears no different in this regard. However, our data suggested that there was more to the phenotypes; that is, additional layers of Notch regulation in the transcriptional control of one gene.

Genetic experiments, as well as promoter analysis, raised the hypothesis that Notch signaling regulates *twist* directly, as well as indirectly by activating expression of a '*repressor of twist*' (Fig. 8A). This indirect repression of *twist* concurred with the role of Notch in activating *E(spl)* transcriptional repressors. Moreover, a mechanism involving direct and indirect regulation was consistent with *Su(H)* mutant phenotypes. In $Su(H)^{null}$ embryos, neither *twist* nor *repressor of twist* (for example, *emc*) are repressed. The de-repression of both genes at the same time resulted in Twist expression appearing 'wild-type-like'. When a constitutively activating form of Su(H) was expressed, both *twist* and *repressor of twist* were activated. In these embryos, high Twist domains were expanded, but uniform high Twist expression was not observed because *repressor of twist* was expressed.

However, simple direct and indirect regulation [through *emc* and *E(spl)-C* genes] by Notch still does not fully explain the phenotypes of *Notch* mutants. Based on the model shown in Fig. 8A, both *twist* and *repressor of twist* should be repressed in N^{null} embryos because Su(H) will remain in its repressor state. While the N^{null} phenotype was consistent with *repressor of twist* being repressed, *twist* was still strongly expressed. Additionally, based on Fig. 8A, constitutive Notch activation should cause both *twist* and *repressor of twist* to be expressed. Consequently, N^{intra} was expected to cause a phenotype similar to that caused by Su(H)-VP16. Contrary to these predictions, panmesodermal expression of N^{intra} repressed Twist, consistent with only *repressor of twist* being strongly expressed. Taken together, these results suggested that at stage 10, the *twist* promoter is less receptive to Notch/Su(H) activation than to Notch/Su(H) repression. As a result, constitutive activation of Notch represses *twist*, while loss of Notch activates *twist* ectopically.

While Notch signaling has the ability to activate *twist*, Notch/Su(H) signaling ultimately leads to repression of *twist* at stage 10. This predominance of repression can be explained in two ways: (1) direct Notch activation of the *twist* promoter is overpowered by Notch activated *repressors of twist*; and (2) a *repressor of twist* gene, such as *E(spl)*, is more responsive to Notch/Su(H) activation than *twist*. These ideas are discussed below in light of our results.

The first model proposes that while Notch signaling might directly promote both *twist* and *repressor of twist* activation, *repressors of twist* might suppress an increase in *twist* transcription. Our data suggested that Notch regulates multiple *repressors of twist*, including *E(spl)-C* genes and Emc. On the *twist* promoter, these multiple repressors could overwhelm Su(H) activation. Hence, *twist* would be transcriptionally repressed rather than activated. In Su(H)-VP16 embryos, the constitutive activating ability of Su(H) on the *twist* promoter might inhibit some of this repression. Consequently, Twist is ectopically expressed at high levels.

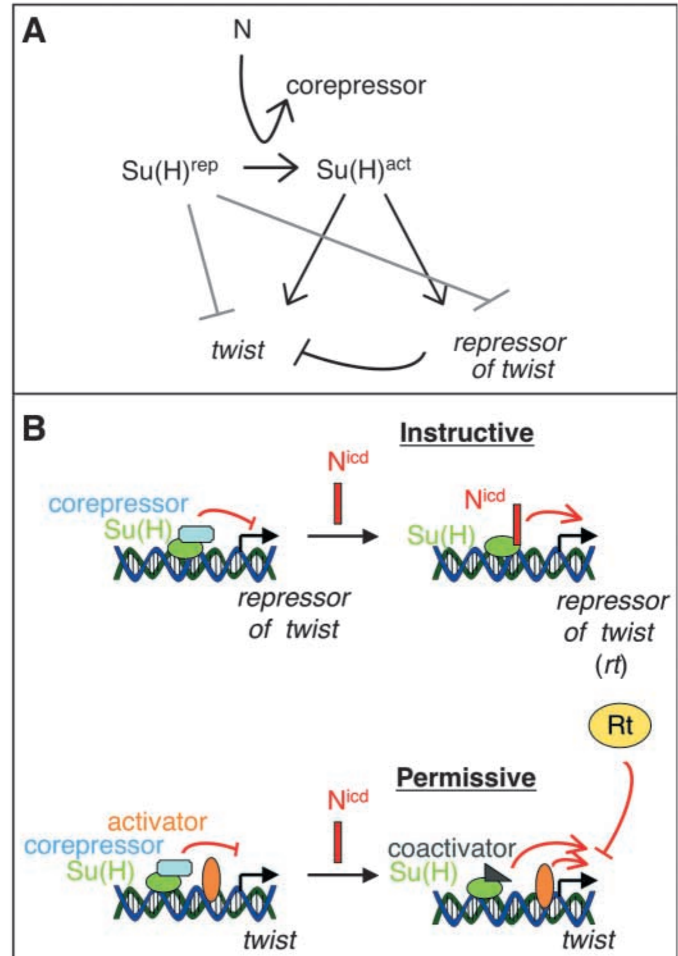

Fig. 8. Models of Notch target gene regulation. (A) Notch signaling has multiple inputs into *twist*; it regulates *twist* directly and indirectly, through the *repressor of twist* genes. Both modes of regulation require Notch to act as a transcriptional switch. In the absence of Notch signaling, Su(H) interacts with co-repressors [$Su(H)^{rep}$] to repress transcription of both *twist* and *repressor of twist*. Upon activation of the Notch receptor, the intracellular domain of Notch (N^{icd}) enters the nucleus and associates with Su(H). This interaction displaces co-repressors, de-represses Su(H) and allows Su(H) to serve as a transcriptional activator [$Su(H)^{act}$]. Hence, Notch signaling promotes transcription of both *twist* and *repressor of twist*. (B) Notch acts permissively on the *twist* gene, but instructively on a *repressor of twist* gene. *repressor of twist* [*E(spl)-C*] transcription requires N^{icd} to alleviate Su(H)-mediated repression and to serve as a coactivator for Su(H). *twist* transcription is dependent on N^{icd}, as well as other factors. N^{icd} is solely required to de-repress Su(H). Su(H) bound to other coactivators and/or other transcriptional activators is necessary for *twist* activation.

Our data are also consistent with the second model, which proposes that *twist* and a *repressor of twist* gene, such as *E(spl)*, respond differently to Notch activation. The reason for this

differential response is provided by the concept of Notch instructive and permissive genes (Bray and Furriols, 2001). Transcription of Notch instructive genes requires the intracellular domain of Notch (N^{icd}) first to alleviate Su(H)-mediated repression and then to serve as a coactivator for Su(H). Transcription of Notch permissive target genes requires N^{icd} to solely de-repress Su(H); Su(H) bound to other coactivators and/or other transcriptional activators are necessary for permissive gene activation (Fig. 8B). Since panmesodermal expression of N^{intra} does not activate *twist*, we conclude that simple de-repression of Su(H) is insufficient to activate *twist* expression and that other factors are required. Hence, Notch acts permissively on the *twist* promoter. By contrast, panmesodermal expression of N^{intra} is sufficient to activate a *repressor of twist*, resulting in the strong Twist repression shown in Fig. 1. As *E(spl)-C* genes have been categorized as Notch instructive target genes (Bray and Furriols, 2001; Klein et al., 2000), we suggest that *E(spl)-C* genes are the Notch instructive *repressor of twist* genes depicted in Fig. 8B. Although Notch can upregulate Emc expression, the inability to see a change in Emc expression in N^{null} and $Su(H)^{null}$ mutants suggests Emc is not a Notch instructive target gene. Thus, based on all of our work, we currently favor the instructive and permissive target gene regulation model.

Notch activation in the early mesoderm

In *Drosophila*, Notch signaling is activated by the Delta (Dl) and Serrate ligands. Delta is expressed throughout the mesoderm at late stage 9 and stage 10 (Kooh et al., 1993), while Serrate is not embryonically expressed until stage 11 (Thomas et al., 1991). While the germline requirement for Delta prevents germline clone embryos from being produced by recombination (Lopez-Schier and St Johnston, 2001), embryos lacking zygotically expressed *Dl* exhibited a wild-type-like Twist pattern (Tapanes-Castillo and Baylies, unpublished). In addition, expression of a full-length Notch protein missing the two EGF repeats critical for Dl binding (Lawrence et al., 2000; Lieber et al., 1992; Rebay et al., 1991), EGF repeats 11 and 12, rescued Twist modulation in N^{null} mutant embryos (Tapanes-Castillo and Baylies, unpublished). Thus Notch does not require EGF-like repeats 10-12 to repress Twist. This preliminary data suggested that Delta may use EGF-like repeats other than 10-12 to activate Notch (Martinez Arias et al., 2002). Alternatively, Notch may not be activated by canonical Delta signaling; a novel (non-DSL) ligand may activate Notch in the early mesoderm. Further experiments are required to evaluate whether the maternal component of Delta regulates Twist.

Notch's role in patterning *Drosophila* mesodermal segments – establishment of periodicity in Twist expression

While our work elucidates the molecular mechanism by which Notch represses Twist, we have yet to understand how Notch signaling establishes a segmentally repeated pattern of low and high Twist domains – that is, periodicity in Twist expression. We propose two models, consistent with our data, to describe how Notch signaling contributes to a modulated Twist pattern. Model I proposes that during the transition from a uniform to a modulated Twist pattern, Notch signaling represses *twist* only in presumptive low Twist domains. Transcriptional activators, such as Da, maintain high Twist expression in presumptive high Twist domains. While Notch signaling components such as Notch, Su(H), and Delta are expressed throughout the mesoderm at late stage 9 and stage 10, this model predicts that Notch signaling is simply not activated in presumptive high Twist domains. Model II proposes that during the transition in Twist expression, Notch signaling represses *twist* throughout the mesoderm, but Notch independent transcriptional activators antagonize Notch repression in what will become high Twist domains, thereby promoting the formation of high Twist domains. For example, transcriptional effectors of Notch signaling [such as Su(H) and E(spl)] and an ‘activator’ that is only expressed in presumptive high Twist domains may converge and compete on the *twist* promoter.

Consistent with model II, the segmentation gene *sloppy-paired* (*slp*) is a spatially regulated ‘high Twist domain’ activator. At stages 9-10, Slp is expressed in the mesoderm in transverse stripes that correspond to high Twist domains. Moreover, loss- and gain-of-function experiments indicate that Slp is required for high Twist expression at stage 10 (Lee and Frasch, 2000). No change in Slp expression is found in *Notch* and *Su(H)* mutant embryos through mid-embryogenesis, indicating that *slp* is not regulated by Notch signaling at these stages (Tapanes-Castillo and Baylies, unpublished). Mesodermal *slp* expression is activated by Wingless signaling; therefore, Wingless signaling is likely to alleviate Notch repression in high Twist domains. In the future, we wish to establish the mechanism through which Notch signaling is antagonized in high Twist domains. Slp and Notch effectors may converge on the *twist* promoter to regulate expression. Additionally, Wingless signaling components may directly regulate and/or inhibit Notch (Axelrod et al., 1996; Barolo et al., 2002; Couso and Martinez Arias, 1994; Foltz et al., 2002; Ramain et al., 2001; Strutt et al., 2002).

A conserved role for Notch in early mesodermal patterning

During vertebrate segmentation, mesodermal segments (called somites) are progressively segregated from a terminal undifferentiated growth zone called the presomitic mesoderm (Pourquie, 2000). Somites are then patterned though a process of subdivision, so that cells are allocated cells to distinct tissue fates (Saga and Takeda, 2001). First subdivision partitions each somite across the anterior–posterior axis into rostral and caudal halves. Later each somite is further subdivided across the dorsal–ventral axis into dermomyotome, which gives rise to dermis and skeletal muscle, and sclerotome, which develops into the axial skeleton. The Notch signal transduction pathway has been shown to play a central role in both somite segmentation and rostral/caudal subdivision (Jiang et al., 2000; Rawls et al., 2000; Saga and Takeda, 2001).

While Notch does not appear to be involved in fly segmentation, our work uncovers a previously uncharacterized role for Notch in the subdivision of *Drosophila* mesodermal segments. We show that Notch repression is required to subdivide each mesodermal segment into a low and high Twist domain. Hence, *Drosophila*, like vertebrates, utilizes Notch and bHLH regulators to subdivide the mesoderm and transform uncommitted mesoderm into patterned segments. Since the homologs and/or family members of the bHLH regulators

studied here – Twist, Emc, Da and E(spl) – are involved in vertebrate segmentation and/or somite subdivision (Rawls et al., 2000), it will be interesting to determine whether these proteins are regulated in vertebrates in a similar manner as they are regulated in the fly.

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