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

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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* (*eme*). 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; Carmen 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; Carmen 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; Carmen 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 vertebrates; Suppressor of Hairless [Su(H)] in Drosophila and vertebrates; Lag-2 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, Nintra, is released from the cell membrane and translocates into the nucleus (Kidd et al., 1998; Struhl and Adachi, 1998). Nintra 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 Nintra (Bray and Furriols, 2001; Klein et al., 2000). Nintra instructive enhancers additionally require Nintra to serve as a coactivator for Su(H) and activate transcription. Nintra permissive enhancers solely require Nintra 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 maerhoelaeae (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/flipper (FLP) system (Chou and Perrimon, 1996). Df(2)1null, y, FRT101w, FVM7c, ftz-lacZ and C(1)DXw, yw01, [FRT101w]1; Y; FLP10w/Y; FLP10w flies were used to produce embryos lacking maternally contributed and zygotically expressed Su(H), Su(H)nullo (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-Nnull (Lieber et al., 1993) or UAS-Su(H)-VP16 (Kidd et al., 1998). Nnull encodes the intracellular domain of Notch (Nintra) that is released upon Notch cleavage. Su(H)-VP16 is a Su(H)/VP16 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-Nnull and UAS-Su(H)-VP16 utilizing twist-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)nullo embryos that express Nnull pamesodermonally were created by recombining twist-GAL4 onto the Su(H)nullo chromosome. Using Df(3R)E(spl) bw D, S1 wg Sp–1 Ms(2)M 1 bw D/CyO, ftz-lacZ; UAS-Su(H) del47 FRT40A P[l(2)35Bg + ]/CyO, ftz-lacZ females were used to produce embryos lacking maternally contributed and zygotically expressed Notch, Su(H), and 2X UAS-enc (gift of M. Ruiz-Gomez) were expressed in Nnull embryos with one copy of twist-GAL4. Females producing Df[1]Nnullki; twist-GAL4 GLCs were crossed to males carrying FM7c, ftz-lacZ and one of the following constructs: UAS-FLN, UAS-Nnull, UAS-FLNadecl0, UAS-FLNIO-12 (Zucchin et al., 1999), Su(H)-VP16, or two copies of UAS-enc [2X UAS-enc]. FLN encodes the full length Notch receptor. The Notch protein encoded by FLNadecl0 lacks the RAM-3 domain and the cdcl0/ankyrin repeats, while the Notch protein encoded by FLNIO-12 contains a deletion in the extracellular domain that removes EGF-like repeats 10-12.

Transgenic lines carrying 1428twist-GFP ( Cox, 2004; Thiss et al., 1991), and 1428twistnull/h-GFP were generated by injection of yw embryos as previously described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Four 1428twist-GAL4 and two 1428twistnull/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-Nnull 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)1 and Df[3R]E(spl)12, P[gro+1] (gifts of A. Martinez-Arias). Df[3R]E(spl)12 deletes all E(spl)-C genes, including groucho (gro) ( de Celis, 1991; Knust et al., 1987). Df[3R]E(spl)12 deletes all E(spl)-C genes, except for gro. However, while Df[3R]E(spl)12 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)12, P[gro+1] 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 twistnull (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-m9 (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-enc (Baona et al., 2000), and UAS-da-da; UAS-enc. 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: emc1, emc915, and emc12, (Cubas et al., 1994). emc1 and emc915 are recessive lethal hypomorphs. The emc12 deficiency is recessive lethal; it removes 10 chromatosomal bands, including the emc locus. To minimize the effect of maternal inheritance, mutant embryos were obtained from heterozygous emc12 females that were crossed to heterozygous emc1, emc915, or emc12 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 da1 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 (1428twist) was PCR amplified from a pHStinger 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-CCAATAGTTAAAG3’ and 5’CGGAGTCCCTTGTATCTTGCG-TTGCG3’ containing an Xba and BamHI restriction site, respectively, amplified the region we termed 1428twist. 1428twist 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 1428twist identified one site (TGTGGGAA) matching the YRTGDGAD consensus Su(H) binding site (Barolo et al., 2000b). Using site-directed mutagenesis (Promega, USA, Gene Editor), the conserved Su(H) binding site was mutated to TTCTA TCC. The mutation was verified by sequencing. Following the same procedures described for 1428twist, the mutated 1428 base pair twist regulatory region [1428twistmutSu(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 Axioscan 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.

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). Twist null (Nnull) 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, Nnull 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 intrat) had the opposite effect when compared to complete loss of

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) Nnull stage 10 embryo maintains high Twist expression throughout its mesoderm. Rather than modulating Twist levels, Nnull mutants display uniform high Twist expression pattern characteristic of wt stage 9 embryos. (G,H) UAS-Nintrat stage 10 embryo has fewer high Twist expressing cells than wt.
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; Furiolos and Bray, 2000; Hsieh 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 Nintra panmesodermally in Su(H)\textsuperscript{null} mutant embryos [Su(H)\textsuperscript{null}, UAS-Nintra]. We expected that if Notch signals through an Su(H)-independent pathway, Twist would still be repressed by UAS-Nintra in the Su(H)\textsuperscript{null} background. Interestingly, Twist is not repressed in Su(H)\textsuperscript{null}, UAS-Nintra embryos. Unlike UAS-Nintra embryos, which have few cells that express Twist at high levels, Su(H)\textsuperscript{null}, UAS-Nintra mutant embryos, similarly to Su(H)\textsuperscript{null} mutant embryos, exhibit a ‘wild-type-like’ Twist pattern (compare Fig. 3A,B with Fig. 1G,H). This result indicated that Nintra 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\textsuperscript{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\textsuperscript{null} embryos.

As a control, we first tested whether panmesodermal transgene expression could restore wild-type-like Twist expression in N\textsuperscript{null} embryos. Panmesodermal expression of a full-length Notch construct (UAS-FLN) rescued the Twist phenotype of N\textsuperscript{null} embryos. Instead of the uniform high Twist levels characteristic of N\textsuperscript{null} mutant embryos, low and high Twist domains were observed in N\textsuperscript{null}, UAS-FLN embryos (compare Fig. 3C,D with Fig. 1E,F). Similarly, panmesodermal expression of Nintra restored Twist modulation. N\textsuperscript{null}, UAS-Nintra embryos exhibited low and high Twist domains; as expected, UAS-Nintra 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 (FLN\textsuperscript{Δcdc10}) would rescue Twist modulation. FLN\textsuperscript{Δcdc10} 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; Matsumo 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, FLN\textsuperscript{Δcdc10} did not rescue Twist modulation in N\textsuperscript{null} embryos. N\textsuperscript{null}; UAS-FLN\textsuperscript{Δcdc10} 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\textsuperscript{null} embryos. Su(H)-VP16 repressed Twist expression in N\textsuperscript{null} mutant embryos such that low and high Twist expression domains were restored (Fig. 3 IJ). This result was consistent with our finding that Notch signals through Su(H) to regulate Twist. It also supported our hypothesis that the N\textsuperscript{null} Twist phenotype results from the loss of Su(H) expression.
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\textsuperscript{intra}. Compared to N\textsuperscript{null}, UAS-FLN and UAS-N\textsuperscript{intra} embryos, N\textsuperscript{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\textit{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; Thissie et al., 1991). At stage 10, 1428\textit{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\textsuperscript{intra} expression (Fig. 4C,D). 1428\textit{twist};UAS-N\textsuperscript{intra} embryos exhibited narrower high GFP expression domains than 1428\textit{twist} embryos. However, the effect of N\textsuperscript{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\textit{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\textit{twist}, we utilized the promoter construct to further understand how Notch signaling regulates Twist expression.

1428\textit{twist} contains only one site (TGTTGGAAA) 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 GTGGAAA core sequence with high affinity (Moe and Schweisguth, 2000). Hence, it is likely that Su(H) strongly binds 1428\textit{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\textit{twist} promoter [1428\textit{twist}\textsuperscript{mutSUH}] 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, Su(H) site mutation should cause the 1428\textit{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\textit{twist} reporter – another site should be employed. Hence, rather than exhibiting a N\textsuperscript{null}-like phenotype and uniformly maintaining high GFP levels throughout the
1428twist

A

1428twist

B

1428twist; N\textsuperscript{tra}

C

1428twist\textsuperscript{mutSu(H)}

D

1428twist\textsuperscript{mutSu(H)}; N\textsuperscript{tra}

E

1428twist\textsuperscript{mutSu(H)}

F

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) 1428twist embryo exhibits low and high GFP domains. Notice that high domains appear chevron-shaped. (C,D) 1428twist; UAS-N\textsuperscript{tra} embryo has narrower high GFP domains than 1428twist embryos. GFP expression is repressed so that high GFP domains appear triangle-like in 1428twist; UAS-N\textsuperscript{tra} embryos. (E,F) 1428twist\textsuperscript{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 1428twist, high GFP domains appear to be slightly expanded. (G,H) 1428twist\textsuperscript{mutSu(H)}; UAS-N\textsuperscript{tra} embryo looks different from the three embryos shown above. GFP expression in presumptive high GFP domains, especially laterally, is repressed compared with 1428twist embryos, so 1428twist\textsuperscript{mutSu(H)}; UAS-N\textsuperscript{tra} high GFP domains appear most similar to those seen in 1428twist; UAS-N\textsuperscript{tra} embryos. However, at the same time, compared with 1428twist embryos, some cells in the presumptive low GFP domains of 1428twist\textsuperscript{mutSu(H)}; UAS-N\textsuperscript{tra} embryos express GFP at high levels; this phenotype is most similar to that of 1428twist\textsuperscript{mutSu(H)} embryos.

Notch represses Twist indirectly through E(spl)

E(spl)-C encodes 7 bHLH proteins (m3, m5, m7, m8, mB, mγ, and mδ) and six non-bHLH proteins – m1, m2, m4, m6, mα, 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 mα in the wing (Bailey and Posakony, 1995; Koelzer and Klein, 2003) and m2 in germline clone embryos (Wurmback 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 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\textsuperscript{tra} affects the 1428twist\textsuperscript{mutSu(H)} promoter. Since we hypothesized that Notch indirectly represses Twist, we predicted that N\textsuperscript{tra} would repress GFP expression, despite the elimination of the only Su(H) binding site in the twist promoter. Indeed, the GFP pattern seen in 1428twist\textsuperscript{mutSu(H)}; UAS-N\textsuperscript{tra} embryos revealed that N\textsuperscript{tra} can repress the activity of the 1428twist\textsuperscript{mutSu(H)} promoter (Fig. 4G,H). GFP expression in presumptive high GFP domains, especially laterally, was repressed compared with 1428twist 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 1428twist\textsuperscript{mutSu(H)}; UAS-N\textsuperscript{tra} embryos appeared to be a combination of indirect N\textsuperscript{tra} repression and Su(H) de-repression of the 1428twist promoter. Panmesodermal expression of UAS-Su(H)-VP16 in a 1428twist\textsuperscript{mutSu(H)} background resulted in a phenotype similar to that seen in 1428twist\textsuperscript{mutSu(H)}; UAS-N\textsuperscript{tra} 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).
(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 (Giajztoglu et al., 2003). In vitro, E(spl) bHLH homodimers have been shown to bind canonical E boxes (CANNTG, preferably of the class B-type CACGTTG), N boxes (CANCAG) and Hairy sites (CACCCTG) (Jennings et al., 1999). 1428twist contains a consensus E box (CAGTGT), four ‘N box-like’ (CANCAG) motifs, and seven ‘hair-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, mα and gro (Knust et al., 1987; Wurmbach et al., 1999). M2 is a novel Notch-regulated protein; M4 and Mα are Notch-regulated Bearded-like proteins. Lastly, Grocho 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-Saiaib 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 removes the entire E(spl)-C locus, including the co-repressor gro (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 grocho.

At stage 10, Df(3R)E(spl) mutant embryos maintained uniform high Twist expression throughout the mesoderm (Fig. 5A,B). Like Nnull 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 mesodernally expressed E(spl) genes m2, m3, m4, m5, m7, m8 and/or mα – repress Twist at stage 10. Since removing zygotic grocho expression exacerbates the Twist phenotype, our findings also demonstrate that Grocho-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. Pansmosdermal expression of UAS-m2, UAS-m3, UAS-m4, UAS-m5, UAS-m7, UAS-m8 or UAS-mα 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.

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 1428twist, 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 (Crommiler 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 \textit{‘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\textsuperscript{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 \textit{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\textsuperscript{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 \textit{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\textsuperscript{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 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\textsuperscript{null} expression resulted in ectopic Emc expression. The phenotype was especially apparent at stage 11, when UAS-N\textsuperscript{null} embryos displayed strong mesodermal Emc expression (Fig. 7C,D). This suggested that Notch positively regulates Emc expression. However, like wild-type embryos, N\textsuperscript{null} mutants expressed Emc at uniform levels throughout the mesoderm prior to stage 10. Similar effects on Emc levels were found in Su(H)\textsuperscript{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
Notch regulates early Twist levels

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-\textit{N}^{null} embryo expresses Emc both around its tracheal pits (black arrowheads) and throughout its mesoderm (white asterisks). (E,F) Lateral views of a stage 10 \textit{N}^{null}; UAS-\textit{emc} embryo stained with anti-Twist. Emc represses Twist in \textit{N}^{null} mutants such that Twist is expressed in low and high domains; compare with Fig. 1E,F and Fig. 3C-J.

Discussion

Analysis of \textit{Notch} mutant embryos revealed that Notch signaling is essential for Twist regulation at mesodermal subdivision. However, comparison of \textit{Notch} and \textit{Su(H)} mutant embryos indicated that Notch regulates Twist differently from \textit{Su(H)}. At stage 10, uniform high Twist expression was maintained in \textit{N}^{null} mutants; by contrast, \textit{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 \textit{Su(H)} [\textit{Su(H)}-VP16] increased Twist expression. Despite these differences, double mutant analysis and rescue experiments demonstrated that Notch requires \textit{Su(H)} to repress Twist. Moreover, further rescue experiments showed that Notch signaling acts as a transcriptional switch, which alleviates \textit{Su(H)}-mediated repression and promotes transcription. In addition, genetics, combined with promoter analysis, suggested that Notch and \textit{Su(H)} have multiple inputs into \textit{twist}. Notch/\textit{Su(H)} signaling both directly activates and indirectly represses \textit{twist} expression by activating proteins that repress Twist. Finally, our data indicate that Notch targets two distinct ‘Repressors of twist’ – \textit{E(spl)}-C proteins and Emc. We propose that Notch signaling activates expression of \textit{E(spl)}-C genes, which then act directly on the \textit{twist} promoter to repress transcription. Since removing \textit{groucho} enhances the phenotype of the \textit{E(spl)}-C mutant embryos, we suggest that the co-repressor, Groucho, acts with \textit{E(spl)}-C proteins and the Hairless/\textit{Su(H)} repressive complex to mediate direct repression of \textit{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 transcriptionally activating \textit{twist}.

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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 Nnull embryos because Su(H) will remain in its repressor state. While the Nnull 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, Nint was expected to cause a phenotype similar to that caused by Su(H)-VP16. Contrary to these predictions, panmesodermal expression of Nint 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.

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 (Nicd) first to alleviate Su(H)-mediated repression and then to serve as a coactivator for Su(H). Transcription of Notch permissive target genes requires Nicd 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\textsuperscript{intr} 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\textsuperscript{intr} 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\textsuperscript{null} and Su(H)\textsuperscript{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 germine requirement for Delta prevents germine 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\textsuperscript{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, En Gr., 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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References


Notch regulates early Twist levels


