INTRODUCTION

Metazoan development relies on two broad categories of mechanisms for specifying cell fate: autonomous, in which fate is determined by factors inherited by, or expressed in, the cell itself; and conditional, in which fate is determined by external factors, particularly cell-cell signaling. The cell lineage that gives rise to Drosophila adult mechanosensory organs (Fig. 1) is well known for its elegant combination of these two modes of fate specification (Hartenstein and Posakony, 1990; Posakony, 1994; Rhyu et al., 1994; Frise et al., 1996; Guo et al., 1996). At each of several precursor cell divisions in this lineage, the two daughter cells signal to each other via the Notch pathway. The fate of one daughter is specified by this signal. The other daughter inherits the Notch pathway antagonist Numb, asymmetrically segregated from the precursor cell. This renders the second daughter immune to the reciprocal Notch signal, ensuring that it adopts the alternative, Notch-independent, cell fate. The fly sensory organ lineage thus embodies a universal strategy for generating cell fate asymmetry during development.

In this lineage, the fates of two of the precursor cells (pIIa and pIIb) are specified by Notch signaling (Fig. 1, blue arrowheads). It is essential, therefore, that these two cells do not inherit substantial amounts of Numb from their respective mother cells. However, each must make Numb to distribute to its own Notch-independent daughter cell. The solution to this regulatory problem has been a lingering question (Rhyu et al., 1994).

SUMMARY

The Notch cell-cell signaling pathway is used extensively in cell fate specification during metazoan development. In many cell lineages, the conditional role of Notch signaling is integrated with the autonomous action of the Numb protein, a Notch pathway antagonist. During sensory bristle development, precursor cells segregate Numb asymmetrically to one of their progeny cells, rendering it unresponsive to reciprocal Notch signaling between the two daughters. This ensures that one daughter adopts a Notch-independent, and the other a Notch-dependent, cell fate. In a genome-wide survey for potential Notch pathway targets, the second intron of the numb gene was found to contain a statistically significant cluster of binding sites for Suppressor of Hairless, the transducing transcription factor for the pathway. We show that this region contains a Notch-responsive cis-regulatory module that directs numb transcription in the pIIa and pIIb cells of the bristle lineage. These are the two precursor cells that do not inherit Numb, yet must make Numb to segregate to one daughter during their own division. Our findings reveal a new mechanism by which conditional and autonomous modes of fate specification are integrated within cell lineages.

KEY WORDS: Asymmetric cell division, Notch signaling, numb, Suppressor of Hairless, Default repression, Cis-regulatory module, Drosophila
numb796 is a strong loss-of-function allele induced by EMS mutagenesis (Buescher et al., 1998). Df(2L)30A-C is an X-ray-induced deficiency lacking DNA from chromosomal region 30A3-C5, which overlaps the numb locus (30B3-35) (Uemura et al., 1989; Tweedie et al., 2009). Lacking DNA from chromosomal region 30A3-C5, which overlaps the numb locus (30B3-35) (Uemura et al., 1989; Tweedie et al., 2009). Lacking DNA from chromosomal region 30A3-C5, which overlaps the numb locus (30B3-35) (Uemura et al., 1989; Tweedie et al., 2009). Lacking DNA from chromosomal region 30A3-C5, which overlaps the numb locus (30B3-35) (Uemura et al., 1989; Tweedie et al., 2009).

Reporter gene constructs

Fragments tested in reporter constructs were amplified by PCR on genomic DNA templates; primer sequences are supplied in Table S1 in the supplementary material. Binding site mutants were created by overlap-extension PCR (Ho et al., 1989); see Table S1 in the supplementary material for mutagenesis primer sequences. The CD2 fragment and all mutant variants thereof were PCR cloned from the Celera (Alameda, CA, USA) genomic sequence; lowercase, bases added for cloning purposes) and their 5' I site (both underlined) were used to clone a 19.5 kb SmaI/PmeI fragment containing the entire numb noncoding sequence (lowercase, bases added for cloning purposes) and cloned into pCR2.1-TOPO, and subsequently digested from BAC clone RP98-6D23 (Malicki et al., 1993) using the flanking NotI sites (external primers: 5'-gtgcccagatgtgcagat-3'. reverse 5'-gcggccgc-tt-3'. internal primers: 5'-ttttgcagatatttaataagattataggtgtaa-aatccttaaatgctgcaa-3', 5'-aagtttttaaagttttctacatcataataattttatgctgcaa-3'. This 1.6 kb fragment was cloned into pMel/SacI sites into a 5.3 kb pMel/SacII fragment contained in the above-mentioned 358 bp fragment. The 19.5 kb fragment was then cloned into CaSpeR(NotI) (Malicki et al., 1993) using the flanking NotI sites. In clones used for both wild-type and CD2 deletion rescue constructs, the Drd/Smal fragment insertion occurred in the reverse orientation, yielding a fully functional rescue transgene, with a small portion of 5' noncoding sequence inserted downstream of the genomic sequence, which should have no effect on the outcome of our experiments.

To delete the CD2 enhancer, overlap-extension PCR (Ho et al., 1989) was used to generate a 1.6 kb fragment that overlapped endogenous Pmel and SacI sites (external primers: 5'-GAATTCAGATATTCGCAGATGG-3', 5'-TAAGCTTATCTGCTTGATGCGA-3', internal primers: 5'-TTTTCGACTTAAATATGTATATAGCTGTGAA-3', 5'-AAGTTTAAAGTTTCTACATCATAATTATTTATGCTGCAA-3'). This 1.6 kb fragment was cloned via Pmel/SacI sites into a 5.3 kb Pmel/SacII fragment contained in pH-Stinger (Barolo et al., 2000). Next, the 5.3 kb Pmel/SacII fragment harboring a deletion of the CD2 enhancer was cloned into the wild-type numb rescue fragment contained in pCR2.1-TOPO, and subsequently inserted into CaSpeR(NotI). Transgenic fly lines carrying either the wild-type (numbRCwt) or CD2 deletion (numbRCdel) rescue constructs were generated by Genetic Services (Cambridge, MA, USA).
variant. Curly+ progeny were collected and scored for the ‘double socket’ phenotype at macrochaete positions along the dorsal head and thorax, in postorbital bristle rows and at bristle positions around the circumference of the T1 femur. Three independent insertion lines were analyzed for each rescue construct variant; the statistical significance of phenotypic differences between the variant groups was evaluated by the Mann-Whitney U test.

To evaluate pIIIb daughter cell fates, the same cross was performed except that CyO, Kr-GAL4 and UAS-GFP balancers were substituted and rescue construct lines wt-A and 2del-B were used. Pupae lacking Kr>GFP expression were dissected and stained at 24-30 hours APF. For each genotype, confocal images of the microchaete field from five nota were collected. Only positions for which a Su(H)-positive cell(s) could be easily matched with a Pros/Pros or Pros/Elav pair were marked and counted.

Transgene rescue of the numb mutant phenotype in mosaic clones

To assay phenotypic rescue in numb mutant clones, females of the genotype y w Ubx-FLP; FRT40A were crossed to males of the genotype y numb2 ck FRT40A/CyO; numbRCX/Sb, where X is either ‘wt’ or ‘2del’. Flies not carrying the CyO balancer were sorted for presence of the rescue construct (numbRC). Because of the dark eye color provided by the Ubx-FLP transgene, Sh was used to identify flies that did not receive the rescue construct chromosome, and annotated as ‘no RC’. Sh+ flies were assumed to contain a copy of the rescue construct. Forty macrochaete bristle positions on the dorsal head and thorax per fly were assayed for the ck mutant phenotype, indicating numb mutant territories, and the bristle phenotype in these territories was scored as shown in Table S2 in the supplementary material.

Immunohistochemistry

Staining of pupal nota was performed on animals incubated at 25°C and dissected at timed stages measured in hours APF. Timed pupae were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) with 0.1% Triton X-100 in PBS. For experiments using the anti-Hamlet antibody, the Triton X-100 concentration in the fixative solution was raised to 0.3% to increase tissue permeability. Before both primary and secondary antibody incubations, samples were blocked for 1 hour in a 1:10 solution of western blocking reagent (Roche) in PBS with 0.1% Triton X-100. Stained tissues were imaged on a Leica confocal microscope.

Fig. 2. A cluster of conserved Su(H) binding sites in the Drosophila numb gene. (A) Diagram of the numb locus and genomic DNA fragments used in this study. The diagram is drawn to scale, and shows the position of high-affinity Su(H) binding motifs (S). The upper transcript isoform is zygotically expressed; the lower isoform is maternally expressed (Uemura et al., 1989). Boxed area marks the cluster of five predicted Su(H) binding sites found by SCORE analysis (Rebeiz et al., 2002). Genomic DNA fragments used for numb mutant rescue experiments (numbRC) are indicated, as are fragments tested for enhancer activity in GFP reporter transgenes. Red boxes indicate numb coding sequences. (B) Electrophoretic mobility shift assay (EMSA) analysis of predicted Su(H) binding sites in the boxed region shown in A. Sites are numbered as in C. All five sites are efficiently bound in vitro by a purified GST-Su(H) fusion protein; the binding of a positive control Su(H) site [m4 S3 (+); S3 from the E(spl)m4 gene (Bailey and Posakony, 1995)] is shown for comparison. A single-base substitution in numb site S2 (S2mut, red) almost completely abolishes binding. (C) Patterns of evolutionary conservation of Su(H) binding sites in the identified cluster. Solid lines indicate site orthology, based on the presence of a conserved site motif in a comparable location and on shared sequences adjacent to individual sites. Dotted line connects a pair of site occurrences (10/10 match) in D. pseudoobscura and D. mojavensis only. Data for D. simulans, D. sechellia and D. persimilis are omitted for clarity; all five sites are conserved in all three of these species, except site 1b, which is absent in D. persimilis. See Fig. S1 in the supplementary material for sequence alignments. Diagrams in A and C were generated using the GenePalette software tool (Rebeiz and Posakony, 2004).
Primary antibodies used in this study were: mouse-anti-Cut [1:100; Developmental Studies Hybridoma Bank (DSHB)] (Blochlinger et al., 1990), mouse anti-Propero [1:10; DSHB] (Spana and Doe, 1995), guinea pig anti-Hamlet [1:1000] (Moore et al., 2002), guinea pig anti-Senseless [1:2000] (Nolo et al., 2000), guinea pig anti-Numb [1:2000] (O’Connor-Giles and Skeath, 2003), rabbit anti-Su(H) [1:1000; Santa Cruz Biotechnology], rabbit anti-CG3227 [1:2000], rabbit anti-GFP [1:500; Invitrogen] and rat anti-Elav [1:200; DSHB]. Secondary antibodies used (all from Invitrogen) were: Alexa 488-conjugated goat anti-mouse (1:1000), Alexa 647-conjugated goat anti-guinea pig (1:1000), Alexa 647-conjugated goat anti-rabbit (1:1000), Alexa 555-conjugated goat anti-rat (1:1000) and Alexa 488-conjugated chicken anti-rabbit (1:1000). Hoechst stain (1:1000; Invitrogen) was used as a DNA marker.

In situ hybridization
A 1.8 kb PCR-cloned genomic DNA fragment overlapping the last exon of numb was cloned into EcoRI and NotI sites of pBluescript SK I and used to transcribe a digoxigenin-labeled riboprobe (see Table S1 in the supplementary material for PCR primer sequences). In situ hybridization was performed as previously described (Reeves and Posakony, 2005) with the modification that instead of the normal 1:1500 dilution of 10 mg/ml proteinase K, a 1:25,000 dilution was used to protect protein epitopes for detection of the Cut protein by antibody stain (above) after the in situ hybridization step.

Mobility shift assays
Electrophoretic mobility shift assays (EMSAs) using purified GST-Su(H) were performed as described previously (Bailey and Posakony, 1995). Oligonucleotide probe sequences are listed in Table S1 in the supplementary material.

Scanning electron microscopy (SEM)
Adult flies were collected for SEM and prepared as described previously (Miller et al., 2009).

RESULTS
Experimental verification and evolutionary conservation of computationally identified Su(H) binding sites in numb
To obtain a preliminary assessment of the functionality of the putative Su(H) binding site cluster identified in Drosophila melanogaster numb by the SCORE computational method (Fig. 2A) (Rebez et al., 2002), we carried out two additional analyses: an electrophoretic mobility shift assay (EMSA) and phylogenetic footprinting (Fig. 2B,C).

The SCORE survey of the genome used a stringent definition of the DNA-binding specificity of Su(H), requiring a sequence that matches the motif YGTGDGAA (TGTGTGAA omitted). From previous studies of the interactions of Su(H) with its target genes, such sites are predicted to be bound by Su(H) protein with high affinity (Tun et al., 1994; Bailey and Posakony, 1995; Nellesen et al., 1999). Indeed, we found by EMSA that Su(H) efficiently bound all five predicted sites in the numb cluster in a sequence-specific manner (Fig. 2B).

Sequence comparisons revealed that, of the five Su(H) sites in the cluster (designated 1a, 1b, 2, 3 and 4), three (sites 2, 3 and 4) are precisely conserved in all 12 Drosophila species with sequenced genomes, with the exception of a single nucleotide change (CGTGGGA to CGTGAGA) in site 3 of D. virilis, which remains compatible with high-affinity binding by Su(H) (Fig. 2C; see Fig. S1 in the supplementary material for sequence alignments). Site 1a is exactly conserved in 10 of the 12 species, being absent only in the sister pair D. virilis and D. mojavensis.

Finally, the fifth site (1b) shows conservation only among members of the melanogaster subgroup. This site lies only eight bp away from site 1a, a distance that is likely to preclude simultaneous occupancy of both sites. We have previously observed similar evolutionary instability of adjacent Su(H) sites (Castro et al., 2005). Overall, the phylogenetic footprinting analysis indicated that four of the five sites in the numb cluster have withstood selection pressure for 40-60 million years, playing an functional role for these motifs.

Enhancer activity of the Su(H) binding site cluster region in numb
To test directly whether the Su(H) binding site cluster in the numb intron identifies one or more functional cis-regulatory modules, we incorporated into a GFP reporter transgene a 2.6 kb genomic DNA fragment bearing all five of the Su(H) sites identified in silico (see Fig. 2A). In multiple independent transgenic lines carrying this construct, we observed GFP expression in the embryonic CNS and PNS (data not shown); in the CNS, optic lobes, retinal field and Johnston’s organ primordium of late third-instar larvae (data not shown); and in pupal-stage precursor cells for the microchaete bristles of the notum (Fig. 3A). We next tested the activity of two smaller fragments from this 2.6 kb region: CD1, bearing sites 1a, 1b and 2; and CD2, containing sites 3 and 4 (see Fig. 2A). The 387 bp CD1 fragment directed expression in the embryonic CNS (see Fig. S2 in the supplementary material), but displayed no activity in the microchaete field (Fig. 3B). However, the CD2 fragment, spanning 682 bp and containing two conserved Su(H) binding sites, directed strong GFP expression in developing microchaetes (Fig. 3C). Like the 2.6 kb fragment, it was also active in the embryonic CNS (see Fig. S2 in the supplementary material), the retinal field, the larval brain and the Johnston’s organ primordium of the eye-antenna disc (data not shown). We conclude that the SCORE method has indeed identified regions of the numb gene that display transcriptional regulatory activity in vivo.

Cell-type specificity of the CD2 cis-regulatory module during bristle development
To define the cell-type specificity of the numb CD2 enhancer activity in the bristle lineage (Fig. 3K), we performed a detailed analysis of GFP accumulation from the reporter transgene in pupal nota at 16-18 hours APF. We used a monoclonal antibody against the Cut protein to fluorescently label the nuclei of all cells in developing microchaetes (Blochlinger et al., 1993).

No GFP was detected in the sense organ precursor (SOP), even when its division was imminent (Fig. 3D). At the two-cell stage of the lineage, however, many developing bristle organs expressed nuclear GFP in the posterior daughter of the SOP, the pIIa cell (Fig. 3E). Other two-cell positions displayed no GFP, consistent with a lag between the birth of the pIIa cell and the appearance of detectable GFP fluorescence. Because we never observed GFP in the anterior cell of a two-cell pair (pIIb), we conclude that reporter gene transcription is first stimulated in pIIa and not in the SOP. Fig. 3F shows a later-stage two-cell position, in which the pIIb cell was about to divide. Here, the intensity of GFP in pIIa was increased in comparison to the early two-cell position shown in Fig. 3E. By contrast, even as pIIb was dividing, no GFP was detectable in this cell (Fig. 3F). Fig. 3G shows a three-cell position, in which pIIb had already divided to yield its pIIib and pIIb sib daughters, and the posterior, GFP-positive pIIa cell was about to divide, as shown by diffuse fluorescence of both GFP and the anti-Cut antibody. In the four-cell position shown in Fig. 3H, the pIIa cell had divided,
and both of its progeny, the presumptive socket and shaft cells, exhibited what is likely to be inherited GFP fluorescence, though we cannot rule out the possibility that the CD2 enhancer continued to be active in one or both of these cells. Also at the four-cell stage, an anterior Cut-positive cell had also begun accumulating GFP (Fig. 3H). Based on its anterior location, size and apical disposition, this cell is putatively pIIIb. The transcription factor Hamlet (Ham) is first expressed in the microchaete lineage in pIIIb, and has been shown to be a crucial regulator of the fates of its progeny (Moore et al., 2004). Staining pupal nota at 17 hours APF with anti-Ham antibody confirmed that the newly GFP-positive cell was indeed pIIIb (Fig. 3J). Finally, once pIIIb divides, GFP is inherited by its progeny, the presumptive sheath cell and neuron (though, again, we cannot exclude continued enhancer activity in these cells). Thus, when the microchaete lineage divisions had been completed, a total of four GFP-positive cells were observed (Fig. 3I), representing the initial activation of the numb CD2 enhancer in the pIIa and pIIIb cells, and (at least) the perdurance of GFP in their postmitotic progeny. It is also clear from our analysis that the pIIIb sib cell and its precursors within the lineage (pIIb, SOP) do not activate the CD2 enhancer, as no GFP was ever observed in these cells. The cells that did activate novel transcription directed by the enhancer (pIIa and pIIIb) were Notch-responsive cells, consistent with the presence of two conserved Su(H) binding sites in the CD2 fragment.

numb transcript accumulation in Notch-responsive cells of the bristle lineage

The foregoing analysis of the activity of the CD2 enhancer fragment suggests that numb might be subject to selective transcriptional activation in the Notch-responsive precursor cells of the bristle lineage. To investigate this question, we carried out
in situ hybridization assays on pupal nota at 16 hours APF, when most microchaete organs were at the two- to three-cell stage of bristle development (Fig. 4). We found that, at this time, evenly spaced cells or cell clusters specifically accumulated elevated levels of \textit{numb} transcript (Fig. 4A; see Fig. S3 in the supplementary material). The spacing of these strongly expressing cells was reminiscent of the microchaete pattern in the notum. \textit{numb} transcript was also observed at low levels across the epidermal field (Fig. 4A; see Fig. S3 in the supplementary material).

To assess whether the strongly expressing cells belong to the microchaete lineage, the in situ-hybridized tissue was also labeled fluorescently with anti-Cut antibody. Cut-positive SOP positions showed only the low epidermal levels of \textit{numb} transcript accumulation. However, a number of two-cell positions displayed strong \textit{numb} transcript signals in the more posterior Cut-labeled cell, which is pIIa (Fig. 4B–B"). In a subset of three-cell positions, a small apical anterior Cut-positive nucleus was seen to be surrounded by cytoplasmic \textit{numb} transcript (Fig. 4C–C"). Based on the size and location of this cell, we interpret it to be pIIb. Therefore, strong transcript accumulation from the endogenous \textit{numb} gene was observed in the pIIa cell and probably the pIIb cell, but not in the SOP or pIIb cells. Our expression analysis thus shows that elevated \textit{numb} transcript levels appear in the bristle lineage in a pattern consistent with the activity of the CD2 enhancer fragment, i.e. in those precursor cells with Notch-specified fates.

\textbf{Default repression by Su(H) is required for the normal pattern of \textit{numb} CD2 enhancer activity}

The presence of two highly conserved Su(H) binding sites within the \textit{numb} CD2 enhancer fragment suggests that the specific activation of the enhancer in response to Notch signaling in pIIa and pIIb is dependent on Su(H). Introduction of single-base mutations (YGTGDGAA to YGTGDCAA) into both of the Su(H) binding sites resulted in the appearance of robust ectopic reporter gene expression in pIIb, without severely affecting its expression in pIIa (Fig. 5). This result suggests that the transcriptional activation function of Su(H) does not provide the principal contribution to the activity of the enhancer in pIIa; instead, expression is evidently driven largely by other activators bound to the module. Significantly, however, the experiment also reveals that Su(H) does indeed act as a ‘default repressor’ of the CD2 enhancer (Barolo and Posakony, 2002). This means that, immediately following the SOP division, Su(H) in its repressive mode would act to prevent CD2 activation in both pIIa and pIIb. The repressive state would persist in pIIb, but would be relieved in pIIa by the Notch signaling event that specifies the pIIa fate, thus permitting the enhancer to drive \textit{numb} transcription specifically in the latter cell.

\textbf{Activation of the \textit{numb} CD2 enhancer module depends on relief of Su(H)-mediated repression by Notch signaling}

The data presented thus far are consistent with the model that \textit{numb} transcription is stimulated by the CD2 enhancer in response to the asymmetric Notch signaling events that specify the fates of the pIIa and pIIb precursor cells. We sought to test directly whether Notch signaling is necessary for activation of the CD2 enhancer module.

We first wanted to show that Su(H), as the transducing transcription factor for the Notch pathway, is required in trans for the cell type-specific activity of the enhancer, as implied by the Su(H)-binding-site-mutation experiment just described (Fig. 5). We used a pannier (\textit{pnr})-GAL4 driver to express a \textit{Su(H)} short-hairpin RNAi construct in the central region of the pupal notum; the flanking regions served as the control tissue (Fig. 6A). This treatment resulted in a complete ‘balding’ phenotype in the adult thorax (see Fig. S4 in the supplementary material) mimicking that observed in \textit{Su(H)}-mutant mosaic territories (Schweisguth, 1995), indicating its effectiveness in reducing \textit{Su(H)} gene activity.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{\textit{numb} transcript accumulates at higher levels in specific cells of the bristle lineage in \textit{Drosophila}. (A) At 16 hours after puparium formation (APF), elevated levels of transcript from the endogenous \textit{numb} gene accumulate at discrete positions within the microchaete field of the developing notum, against a background of low-level ubiquitous expression. Anterior is at the top; image is centered on the notum midline and shows two symmetrical microchaete rows. See Fig. S3 in the supplementary material for a lower-magnification view of the full notum. (B–B") \textit{numb} transcript (B’, B") accumulates specifically in the posterior pIIa cell, identified by anti-Cut staining of a developing microchaete at the two-cell stage (red in B and B’; see Fig. 3K for a lineage diagram). (C–C") \textit{numb} transcript accumulation (C’, C") in the pIIb cell, identified by anti-Cut staining of a developing microchaete at the three-cell stage (red in C and C’). The anterior pIIb cell has divided into the pIIb and pIIb sib cells. The larger pIIb cell accumulates \textit{numb} transcript, whereas the smaller, apical pIIb sib cell does not (C’, C’).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Mutation of the \textit{Su(H)} binding sites in the \textit{numb} CD2 enhancer yields ectopic activity in the Notch-independent pIIb cell. (A–B") Developing \textit{Drosophila} microchaetes at the two-cell stage, marked by Cut immunoreactivity (A; magenta in merge panels A’ and B’). The posterior pIIa cell and anterior pIIb cell are indicated. (A’, A") \textit{numb} CD2 enhancer-reporter transgene (GFP; green in A") is active only in Notch-dependent pIIa. (B’, B") \textit{numb} CD2 reporter gene bearing single-base mutations in its two \textit{Su(H)} binding sites (GFP; green in B") is active in both pIIa and pIIb.}
\end{figure}
At the two-cell stage, Su(H) RNAi caused both of the progeny of the SOP to adopt the pIIb fate, owing to a failure of Notch signal transduction in the pIIa cell (Fig. 6B,C). Nevertheless, as expected from the result presented in Fig. 5, both of these cells expressed GFP from the CD2 enhancer-reporter transgene (Fig. 6C′), as Su(H) was no longer able to repress the enhancer in either cell. This experiment also suggests that Su(H) does normally make some contribution to the activation of the enhancer in pIIa, as the Su(H) RNAi treatment appeared to reduce the level of GFP accumulation in this cell relative to that observed in the wild-type territory (compare Fig. 6B′ and 6C′). Later, following the division of pIIb [which yields three cells in wild-type territory and four cells in the Su(H) RNAi domain], GFP from the CD2 reporter appeared only in pIIa and pIIb at wild-type positions, but was expressed in all four cells (two ‘pIIIb’ and two pIIb sib cells) where Su(H) RNAi was active (Fig. 6D,E).

We directly investigated the role of Notch signaling in activating the CD2 enhancer by examining expression of the reporter gene in notum tissue of female pupae bearing the temperature-sensitive allele Nts1 in trans to a Notch null allele (N81k1/N81k1). Exposure of these animals to 37°C for two hours at the appropriate time caused a failure of the Notch signaling event that specifies the pIIa cell, yielding two-cell positions in which both daughters of the SOP have adopted the pIIb fate (Fig. 6F) (Hartenstein and Posakony, 1990; Posakony, 1994). Here, in contrast to what was observed when Su(H) activity was reduced (Fig. 6C′), neither cell expressed the CD2 reporter (Fig. 6F′). Combining the results presented in Figs 5 and 6, we conclude that Notch signaling is indeed required to activate the CD2 enhancer specifically in pIIa, and that it does this, in part, by relieving Su(H)-mediated repression. We confirmed the role of Notch signaling in CD2 enhancer activation in a separate experiment in which overexpression of Numb was used to abrogate Notch signaling activity throughout the sensory organ lineage (Reddy and Rodrigues, 1999a) (see Figs S5 and S6 in the supplementary material).

**The numb CD2 enhancer is required for proper specification of the shaft and neuron cell fates**

The specific activation of the numb CD2 enhancer in the pIIa and pIIb precursor cells of the bristle lineage suggested to us that it might have a role in specifying the fates of their Numb-inheriting progeny cells, the shaft cell and neuron, respectively. We tested this prediction by carrying out rescue experiments using genomic DNA transgenes containing the numb locus. We found that a 20 kb fragment that includes the proximal (zygotic) promoter and entire coding region, along with substantial amounts of 5′ and 3′ flanking sequence (Fig. 2A), efficiently rescued the lethality associated with two different numb loss-of-function genotypes (Tweedie et al., 2009). Moreover, adult mechanosensory organ development was almost completely normal in numb mutant flies bearing the wild-type rescue fragment (Fig. 7F; see Table S2 in the supplementary material). By contrast, mutant flies bearing a version of this construct in which the numb CD2 enhancer had been deleted (Fig. 2A) displayed a widespread ‘double socket’ phenotype, in which the shaft cell was transformed into a second socket cell (Fig. 7A-F; see Table S2 in the supplementary material). Similarly, pupal-stage nota of CD2 enhancer-deleted animals showed frequent transformation of the sensory neuron into a second sheath cell (Fig. 7G,H). These results demonstrate that the function of the CD2 enhancer module is required for the normal specification of the numb-dependent, Notch-independent shaft and neuron cell fates.

Fig. 6. Response of the numb CD2 enhancer to perturbations in Notch signaling. (A–E′′′) CD2-GFP reporter gene expression (green) in the genotype w¹; CD2-GFP/+; pnr-GAL4/um-1; Su(H)shRNA. (A,A′) Wide-field view of pupal notum, with approximate boundary between GAL4-expressing and non-expressing territories denoted by a dashed line, as indicated above panel A. Positions enlarged in subsequent panels are labeled. (B–C′′′) GFP expression in progeny cells following the SOP division in either the GAL4-non-expressing (B–B′′′) or GAL4-expressing (C–C′′′) territory. (D–E′′′) GFP expression in progeny cells following the pIIb division in either the GAL4-non-expressing (D–D′′′) or GAL4-expressing (E–E′′′) territory. (F–F′′′) Lack of GFP expression at the two-cell stage in a N81k1/N81k1 background following temperature shift. Cells are marked by anti-Sens (blue in A-F) and anti-Pros (red in A-F) immunoreactivity. GFP is also absent from four-cell Pros-positive positions (data not shown). Arrows in B–C′′′ and F–F′′′ indicate cells adopting the pIIb fate. Arrowheads in D–E′′′ indicate cells adopting the pIIIb sib fate.
DISCUSSION
Successful computational identification of cis-regulatory modules based on binding site clustering

The transcriptional regulation of the numb gene has not previously received much attention because most experimental efforts have been focused on Numb protein localization, asymmetric segregation and function as a Notch pathway inhibitor. The motivation for the present study originated in a computational search of the fly genome for new Notch pathway target genes based on statistically significant clustering of Su(H) binding sites (Rebeiz et al., 2002). Although it has been suggested that homotypic site clustering is not a general property of cis-regulatory modules in Drosophila, and therefore that this parameter is of limited utility in computational prediction of enhancers (Li et al., 2007), the data that we have presented here and in other reports (Bailey and Posakony, 1995; Nellesen et al., 1999; Lai et al., 2000; Rebeiz et al., 2002; Castro et al., 2005) indicate that this approach can be quite effective in the case of Su(H) and other transcription factors. One beneficial feature of our SCORE method (Rebeiz et al., 2002) is the use of a largely unbiased window size (100-5000 bp) for the identification of statistically significant binding site clusters. This wide range allows the detection of local maxima that do not necessarily conform to the size expected for a canonical cis-regulatory module. Judging from the present study, the unbiased window-size approach might permit functional enhancer elements to be detected owing to the proximity of multiple enhancers with similar binding inputs. In any case, the SCORE technique successfully identified a functional cis-regulatory module within the ~50 kb of non-coding DNA within and surrounding numb.

Role of the Notch-activated numb CD2 enhancer in the specification of the shaft and neuron cell fates

We have shown here that a 20 kb genomic DNA fragment is capable of nearly complete phenotypic rescue of two different numb loss-of-function genotypes, and that deletion of the numb CD2 enhancer from this fragment results in widespread ‘double socket’ and ‘double sheath’ phenotypes, reflecting a failure to specify the numb-dependent neuron fate (H).
Given the high proportion of sensory organs in which the shaft and neuron cell fates are correctly specified in the absence of the CD2 enhancer, it seems clear that CD2 is not the only source of Numb for pIIa and pIIIb. We confirmed this inference directly by detecting Numb crescents in dividing pIIa cells in tissue lacking CD2 function, having first demonstrated that the numb796 allele is protein-null (see Fig. S7 in the supplementary material).

What might be the source of this additional Numb protein? It is, of course, possible that numb is served by a second enhancer module that also contributes to the transcriptional activation of the gene in pIIa and pIIIb in response to Notch signaling; there is substantial precedent for such ‘shadow’ or ‘secondary’ enhancers in insects (Hong et al., 2008; Frankel et al., 2010). However, it is very likely that the basal level of Numb protein that is detected in all cells in the epidermis (Rhyu et al., 1994) also accumulates in developing sensory organ cells, including pIIa and pIIIb, independently of the CD2 enhancer. This protein would presumably be segregated by the two precursor cells to their shaft and neuron daughter cells, respectively, and might suffice, in most cases, to inhibit Notch signaling in those cells.

What, then, would generate the need for the numb CD2 enhancer activity? Integrating all of our findings, we currently favor the following evolutionary scenario. Among the cells in the bristle lineage, the pIIa and pIIIb precursors face a unique challenge: because their own fates are specified by Notch signaling, it is crucial that they do not inherit Numb, yet each must make sufficient Numb to distribute asymmetrically to one of their progeny cells (Rhyu et al., 1994). In an ancestral sensory organ lineage, the ubiquitous basal level of Numb accumulation might have been adequate to supply the needs of pIIa and pIIIb. But, perhaps as the execution of the lineage became faster in some rapidly developing insects [the time from birth to division for pIIa and pIIIb is only 3–4 hours in Drosophila (Hartenstein and Posakony, 1989; Reddy and Rodrigues, 1999b)], Numb accumulation in these cells failed to meet the required threshold, resulting in unacceptably high failure rates in shaft cell and neuron specification. The emergence of the CD2 enhancer would then have offered the selective advantage of supplementing the basal Numb specifically in these two Notch-dependent precursor cells, without elevating the global activity of the gene. In this scenario, CD2 represents an evolutionary adaptation for ensuring the fidelity of two cell fate decisions during mechanosensory organ development.

**Integration of conditional and autonomous modes of cell fate specification**

The *Drosophila* external sensory organ lineage has stood for many years as an elegant example of the integration of conditional and autonomous mechanisms of cell fate specification (Posakony, 1994). The repeated use of a combination of bi-directional Notch signaling between sister cells and asymmetric segregation of the Notch pathway antagonist Numb is a highly effective strategy for ensuring the proper specification of cell fates in a succession of asymmetric cell divisions. This is particularly so because the orientation of the mitotic spindles and the segregation of Numb are tied to the planar polarity system (Bellaiche et al., 2001), such that the appropriate fate is assigned to the appropriate daughter with extremely high fidelity. The results reported here bring this Notch-Numb partnership full circle by demonstrating that a reciprocal regulatory linkage also exists: Notch signaling regulates *numb* (Fig. 8).

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**Fig. 8. Model for the Notch-stimulated activation of *numb* transcription in the pIIa precursor cell.** (A) As the Notch-independent SOP prepares to divide, it segregates Numb protein (red) in a crescent to its anterior side (right). Su(H) binds the CD2 enhancer module and represses it (OFF). (B) Immediately after the division of the SOP, pre-loaded transcriptional activator proteins (ACT) are bound to the CD2 enhancer in both daughter cells. Su(H) in its repressor mode keeps the enhancer OFF in both cells. The anterior daughter, the presumptive pIIb, has inherited Numb from the SOP (red ring). (C) Bi-directional Notch (N) signaling between the two daughter cells then specifies the pIIa fate (black horizontal arrow) and relieves ‘default repression’ of the CD2 enhancer by Su(H) in this cell, permitting the bound ACT proteins to activate *numb* transcription (ON) in partnership with activated Su(H). Inherited Numb protein in pIIb renders this cell unresponsive to the reciprocal Notch signal (gray horizontal arrow), and Su(H) continues to repress the enhancer. (D-F) Interpretation of experiments with the GFP reporter gene (see Figs 5, 6). (D) Mutation of the two binding sites in the CD2 module prevents repression by Su(H), leaving ACT-stimulated reporter activity in pIIa and permitting ectopic activation of the reporter in pIIb. (E) Loss of Su(H) gene function causes cell-autonomous failure of pIIa specification (so that both sisters adopt the pIIb fate) and activation of the wild-type CD2 reporter gene in both cells [due to loss of Su(H)-mediated repression]. (F) Loss of Notch function likewise causes cell-autonomous failure of pIIa specification, but the wild-type CD2 reporter remains off in both cells owing to continued repression by Su(H).
We have shown that, although Notch signaling is essential to the activation of the numb bristle enhancer, the transcriptional activation function of Su(H) is not strictly required for enhancer activity. Accordingly, we suggest that Notch signaling acts here in large part as a trigger, relieving Su(H)-mediated ‘default repression’ and permitting other activators bound to the enhancer to drive numb transcription (Fig. 8). Some or all of these activators are likely to be expressed in both pIIa and pIIb, as implied by the nearly equivalent level of reporter gene activity observed in the two cells when the Su(H) binding sites of the enhancer are mutated. We further suggest that this regulatory strategy is relevant to the question of timing raised earlier. Having Notch signaling act as a trigger for the action of a pre-assembled complex of other activators might help to ensure that the transcriptional response is very rapid, allowing sufficient numb mRNA to be accumulated and translated in pIIa and pIIb before they divide.

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References

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