Lateral inhibition in proneural clusters: cis-regulatory logic and default repression by Suppressor of Hairless

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This paper is dedicated to the memory of Prof. José A. Campos-Ortega (1940-2004).

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Summary

Lateral inhibition, wherein a single cell signals to its neighbors to prevent them from adopting its own fate, is the best-known setting for cell-cell communication via the Notch (N) pathway. During peripheral neurogenesis in *Drosophila*, sensory organ precursor (SOP) cells arise within proneural clusters (PNCs), small groups of cells endowed with SOP fate potential by their expression of proneural transcriptional activators. SOPs use N signaling to activate in neighboring PNC cells the expression of multiple genes that inhibit the SOP fate. These genes respond transcriptionally to direct regulation by both the proneural proteins and the N pathway transcription factor Suppressor of Hairless [Su(H)], and their activation is generally highly asymmetric; i.e. only in the inhibited (non-SOP) cells of the PNC, and not in SOPs. We show that the substantially higher proneural protein levels in the SOP put this cell at risk of inappropriately activating the SOP-inhibitory genes, even without input from N-activated Su(H). We demonstrate that this is prevented by direct 'default' repression of these genes by Su(H), acting through the same binding sites it uses for activation in non-SOPs. We show that de-repression of even a single N pathway target gene in the SOP can extinguish the SOP cell fate. Finally, we define crucial roles for the adaptor protein Hairless and the co-repressors Groucho and CtBP in conferring repressive activity on Su(H) in the SOP. Our work elucidates the regulatory logic by which N signaling and the proneural proteins cooperate to create the neural precursor/epidermal cell fate distinction during lateral inhibition.

Key words: Cell-cell signaling, Default repression, Co-repressors, Neural precursor specification, Cell fate, Cis-regulatory logic, *Drosophila*

Introduction

Cell-cell signaling through the Notch (N) receptor is the central mechanism underlying a large variety of conditional cell fate decisions during bilaterian development (for a review, see Lai, 2004). N signaling is particularly effective for establishing binary cell fate distinctions between two or more adjacent or nearby cells, which occurs in three general settings (Bray, 1998): lateral inhibition, wherein a single cell inhibits surrounding cells from adopting the same fate as itself; binary cell fate decisions between sister cells in asymmetric cell divisions; and communication between rows of juxtaposed cells in which each row adopts a distinct fate. The canonical N pathway involves two interacting cells: Delta (Dl) or Serrate (Ser) ligand on the surface of the sending cell binds N receptor on the responding cell, inducing proteolytic cleavage and nuclear translocation of the N intracellular domain (N^{IC}), which then complexes with the ubiquitously expressed transcription factor Suppressor of Hairless [Su(H)] and the co-factor Mastermind (Mam) to activate N target genes. Prior to its activation by N^{IC}, Su(H) is bound to target genes in a complex with co-repressor proteins; i.e. it is by default a repressor (Barolo et al., 2002; Barolo et al., 2000b; Furriols and Bray, 2001; Hsieh et al., 1996; Hsieh and Hayward, 1995; Morel et al., 2001; Morel and Schweisguth, 2000).

The capacity of N signaling to specify diverse cell fates in diverse developmental contexts depends on the ability of the pathway to activate in each setting the appropriate subset of its target genes (reviewed by Barolo and Posakony, 2002). This specificity is founded in turn on the cis-regulatory apparatus of the targets and on the use of regionally expressed 'local activators', which function cooperatively with Su(H) to effect target gene activation. A cell that responds to the N-mediated signal and expresses one or more local activators will activate only those target genes that include binding sites for both the local activator(s) and Su(H).

Lateral inhibition is the developmental setting classically associated with N pathway function (Lehmann et al., 1983; Poulson, 1967). Although it is also used in the *Drosophila* mesoderm for specification of muscle progenitor cells (Carmena et al., 1995; Corbin et al., 1991), lateral inhibition has been most comprehensively studied in the context of neurogenesis in the ectoderm. Here, local expression of proneural genes, which encode basic helix-loop-helix (bHLH) transcriptional activators, confers on small groups of cells

called proneural clusters (PNCs) the potential to adopt a neural precursor cell fate (Cubas et al., 1991; Skeath and Carroll, 1991). A single cell in the PNC, which displays the highest level of proneural protein accumulation, stably adopts the neural fate and laterally inhibits the remaining cells via N-mediated signaling, remanding them to an epidermal fate (Cabrera, 1990; Doe and Goodman, 1985; Hartenstein and Posakony, 1990; Simpson, 1990).

Lateral inhibitory signaling in *Drosophila* PNCs directly elicits the expression of multiple genes located in the *Enhancer of split* Complex [E(spl)-C] (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995), which are collectively required for inhibition of the neural precursor cell fate (Delidakis et al., 1991; Nagel et al., 2000; Schrons et al., 1992). These genes encode proteins belonging to one of two families: bHLH transcriptional repressors and Bearded (Brd) family proteins (Lai et al., 2000b). Each E(spl)-C gene is associated with a discrete enhancer module that includes high-affinity binding sites for the proneural proteins Achaete (Ac) and Scute (Sc), and for Su(H) (Nellesen et al., 1999). These sites constitute a cis-regulatory 'code' for expression specifically in PNCs; the proneural proteins serve as the local activators that cooperate with N-activated Su(H) to trigger robust transcription of E(spl)-C genes.

Consistent with their function in antagonizing the neural precursor cell fate, expression of both bHLH repressor and Brd family genes of the E(spl)-C is generally excluded from the committed precursor (Jennings et al., 1994; Jennings et al., 1995; Kramatschek and Campos-Ortega, 1994; Lai et al., 2000b; Nolo et al., 2000; Zaffran and Frasch, 2000). Although logical, this asymmetry is also somewhat paradoxical, given the elevated levels of the proneural proteins in the neural precursor cell. Earlier work with reporter genes revealed that the exclusion is transcriptionally based (Kramatschek and Campos-Ortega, 1994; Lai et al., 2000b; Nolo et al., 2000), but the underlying mechanism was not established. On the basis of genetic experiments, Koelzer and Klein (Koelzer and Klein, 2003) proposed that a repressive activity of Su(H) might be responsible. We demonstrate explicitly that direct transcriptional repression by Su(H) lies at the heart of the exclusion phenomenon.

The setting for these studies is the PNCs of the wing imaginal disc, from which sensory organ precursor (SOP) cells of the adult peripheral nervous system (PNS) arise. Using enhancer-reporter transgenes that are active specifically in the inhibited (non-SOP) cells of PNCs, we have found by mutational analysis that Su(H) binding sites are crucial not only for direct activation of E(spl)-C genes in non-SOP cells but also for their direct repression in SOPs. Loss of this repression results in ectopic expression of the reporter genes in SOPs, which is dependent on the integrity of proneural protein binding sites. The developmental importance of direct Su(H)-mediated repression of E(spl)-C genes in the SOP is demonstrated by our observation that de-repression of a single bHLH repressor gene [owing to mutations in its Su(H) binding sites] can result in loss of the SOP cell fate. Both loss- and gain-of-function experiments establish essential roles for the adaptor protein Hairless (H) and the transcriptional co-repressor proteins Groucho (Gro) and C-terminal binding protein (CtBP) in the repression activity of Su(H) in the SOP. Our results constitute the first evidence for direct transcriptional repression by Su(H) in the most classic setting for N signaling, and reveal how this repression serves to protect the fate of neural precursor cells during lateral inhibition.

Materials and methods

Fly stocks

H^{E31} is a null allele (Schweisguth and Posakony, 1994), gro^{E73} is a genetically null allele (Preiss et al., 1988), gro^{E48} is a strong hypomorphic or null allele (Preiss et al., 1988), and *A101* is a *lacZ*-expressing enhancer trap transposon insertion in the *neuralized* gene (Bellen et al., 1989), used here as an SOP-specific marker [see FlyBase (flybase.bio.indiana.edu) for details and additional references].

Gene diagrams

Gene diagrams in Figs 1-3 were created using the GenePalette software tool (Rebeiz and Posakony, 2004) (www.genepalette.org).

Transgene construction

E(spl)mα-RFP and *E(spl)mα-GFP* reporter constructs were prepared by cloning a 1.0 kb *Bam*HI-*Xho*I genomic DNA fragment (–1083 to –71) from the *E(spl)mα* gene into the multiple cloning site (MCS) of the pRed H-Stinger and pGreen H-Stinger insulated P element transformation vectors (Barolo et al., 2000a; Barolo et al., 2004), respectively. An orthologous fragment from *D. virilis E(spl)mα* was cloned into pGreen H-Stinger as a *Bam*HI-*Xho*I restriction fragment, to make *Dv E(spl)mα-GFP*. *E(spl)m8-GFP* was constructed by cloning a 1.1 kb genomic *Eco*RI-*Xho*I DNA fragment (–1174 to –72) into the MCS of pGreen H-Stinger.

An *E(spl)m8* transgene was constructed by replacing the *lacZ* reporter gene in the insulated pPelican vector (Barolo et al., 2000a) with a 2.4 kb genomic *Spe*I-*Kpn*I DNA fragment (–1174 to +1243).

An *E(spl)mα-Gal4* driver was constructed by cloning the 1.0 kb genomic DNA sequence described above into the MCS of the H-GAL4 vector as an *Eco*RI-*Bam*HI fragment. H-GAL4 is based on the CaSpeR P-element transformation vector (Pirrotta, 1988) and consists of the GAL4-coding sequence downstream of the *Hsp70* minimal (–43) promoter and upstream of a fragment containing the *Hsp70* polyadenylation signal and site; a region of the Pelican/Stinger MCS (Barolo et al., 2000a) is immediately upstream of the promoter. Proper expression of *E(spl)mα-Gal4* in non-SOPs was verified by crossing to flies carrying *UAS-Stinger* (Barolo et al., 2000a).

Wild-type and mutant *UAS-H* misexpression constructs were cloned into the pUAST vector (Brand and Perrimon, 1993). *UAS-H* and *UAS-HΔC* were described previously; a stop codon placed after codon 1070 in *UAS-HΔC* eliminates the seven C-terminal amino acids (PLNLSKH), which constitute the CtBP-binding motif (Barolo et al., 2002). *UAS-H[Gm]* encodes a H protein in which the Gro-binding motif YSIHSLLG is changed to AAAHSAAG; this abolishes the in vitro interaction between the two proteins (see Barolo et al., 2002). *UAS-H[Gm]ΔC* expresses a H protein that lacks both motifs.

Transcription factor binding site mutations were made using the Transformer (Clontech) or Chameleon (Stratagene) mutagenesis kits. The E-box proneural protein binding site GCAGGTG was changed to GAAGCTT (Van Doren et al., 1992); Su(H) sites of the form YGTGRGAA were changed to YGTGRCAA (Bailey and Posakony, 1995); these mutations abolish binding of the respective proteins in vitro.

Cloning and sequencing of *E(spl)mα* orthologs

A 40-mer primer containing *D. melanogaster* sequence that included both the S_2 Su(H) site and the E box of *E(spl)mα* was used to recover orthologous upstream sequence from libraries of *D. virilis* or *D. hydei* genomic restriction fragments that had been ligated to pBlueScript IIKS (Stratagene) using the RAGE (Rapid Amplification of Genomic

DNA Ends) method (Mizobuchi and Frohman, 1993). Additional rounds of RAGE were used to obtain complete *E(spl)mα* PNC cis-regulatory modules from *D. hydei* and *D. virilis*. *D. pseudoobscura* sequence was obtained from the Human Genome Sequencing Center at Baylor College of Medicine (www.hgsc.bcm.tmc.edu/projects/drosophila/).

Bristle scoring

To measure the phenotypic effect of relieving *E(spl)m8* from direct repression by Su(H), 50 flies (25 females and 25 males) of the following genotypes were scored at early adulthood for missing macrochaete bristles at 20 positions each: w^{1118}; w^{1118} homozygous for the wild-type *E(spl)m8* transgene (six lines); or w^{1118} homozygous for the mutant *E(spl)m8 Sm* transgene (10 lines).

To measure the SOP fate-promoting activity of wild-type and mutant forms of H, both left and right orbital regions of 25 adult females of each genotype were scored for supernumerary bristles. All genotypes carried one copy of *E(spl)mα-Gal4*, driving expression of one copy of *UAS-H*, *UAS-HΔC*, *UAS-H[Gm]* or *UAS-H[Gm]ΔC*. Two different insertions were tested for each *UAS* construct.

Antibody staining

Wing imaginal discs were dissected from late third-instar larvae, fixed in 4% paraformaldehyde in PBS for 30 minutes, washed, and incubated with anti-Hnt (monoclonal, Developmental Studies Hybridoma Bank), anti-Sens (polyclonal; gift of H. Bellen), or anti-β-galactosidase (monoclonal, Roche; gift of W. McGinnis) primary antibody followed by Cy3 (Jackson Labs; for β-galactosidase) or Alexa 647 (Molecular Probes; for Hnt and Sens) secondary antibody. Pupal notum in Fig. 1 was dissected and stained at 14 hours APF.

Confocal microscopy

A Leica TCS SP2 microscope (equipped with Leica Confocal Software v2.5; Leica Microsystems) was used for confocal imaging. Images in Figs 1-5 are average projections of stacks taken along the apicobasal axis at 1 μm increments. *Z*-axis range was delimited to collect the full signal from all fluorophores. Fluorophores were excited separately at 488 nm (GFP), 543 nm (RFP, Cy3) or 633 nm (Alexa 647); emissions were collected at 490-530 nm (GFP), 630-710 nm (RFP, Cy3) or 640-740 nm (Alexa 647). All GFP signals were collected at the same gain.

De-repression of *E(spl)mα-GFP* expression in SOPs

Wing discs were dissected from late third-instar larvae and stained with anti-β-galactosidase antibody to detect *A101*-positive SOPs. Using constant excitation and collection parameters, expression of one copy of *E(spl)mα-GFP* was scored by confocal microscopy in 16 discs at each of seven SOP positions: ANWP, ANP, PNP, APA, PSA, PDC and PSC (see figure legends for abbreviations). Four genotypes were analyzed: (1) w^{1118}; *E(spl)mα-GFP/+; A101/+ +*; (2) w^{1118}; *E(spl)mα-GFP/+; A101/*H^{E31} *+*; (3) w^{1118}; *E(spl)mα-GFP/+; A101/+* gro^{E48}; and (4) w^{1118}; *E(spl)mα-GFP/+; A101/*H^{E31} gro^{E48}. Only six out of 16 discs from larvae of the last genotype included PSA SOPs that were present to score.

Results

A cis-regulatory module upstream of *E(spl)mα* drives strong PNC expression but is directly repressed by Su(H) in SOPs

We have reported previously that a 1.1 kb genomic DNA fragment that includes the promoter and proximal upstream region of *E(spl)mα* drives strong reporter gene expression in the PNCs of late third-instar imaginal discs, but that this expression is excluded from SOPs (Lai et al., 2000b). We have found that a 1.0 kb subfragment lacking the *E(spl)mα* promoter confers this same expression pattern on a heterologous promoter, thus defining a discrete PNC-specific cis-regulatory module for the gene (Fig. 1A, construct 1, Fig. 1B-D). We were especially interested in the mechanistic basis of the striking specificity of activation of the module; i.e. only in the inhibited (non-SOP) cells of the PNC (Fig. 1D).

Activation of N-regulated genes of the E(spl)-C in imaginal disc PNCs makes use of a combination of Su(H)-binding sites and binding sites for the proneural proteins Ac and Sc (Bailey and Posakony, 1995; Nellesen et al., 1999; Singson et al., 1994). Consistent with this 'Su(H) plus proneural' cis-regulatory code, the *E(spl)mα* module includes five high-affinity Su(H) sites and a single high-affinity proneural site (Fig. 1A, construct 1) (Lai et al., 2000a; Nellesen et al., 1999). We examined the effects on reporter gene activity of mutating only the proneural site (*Em*, Fig. 1A, construct 2), only the five Su(H) sites (*Sm*, Fig. 1A, construct 3) or all six sites (*EmSm*, Fig. 1A, construct 4). We first observed that the integrity of the 'E box' proneural protein binding site is strictly required for detectable reporter expression in nearly all wing disc PNCs; residual expression is observed along the entire wing margin and in a very small subset of PNCs (Fig. 1E). This result demonstrates that, as for other N pathway target genes, the proneural proteins make an essential input as direct transcriptional activators of *E(spl)mα* in PNCs.

Mutation of the five Su(H)-binding sites in the *E(spl)mα* PNC module (*Sm*, Fig. 1A, construct 3) yields a dramatic alteration in the spatial pattern of its activity (Fig. 1F-H). Reporter gene expression in non-SOP cells is drastically reduced or eliminated, and strong ectopic expression is now observed in SOPs (Fig. 1F-G). Direct comparison of the wild-type (RFP) and *Sm* (GFP) reporter transgenes in the same disc emphasizes the stark contrast in their specificities (Fig. 1H). This finding indicates, first, that Su(H) has an essential role as a direct transcriptional activator of *E(spl)mα* in the N-responsive non-SOPs, and, second, that it acts as a direct transcriptional repressor of the gene in SOPs.

Finally, we observed that mutation of the proneural protein binding site in addition to the Su(H)-binding sites (*EmSm*, Fig. 1A, construct 4) abolishes detectable PNC expression of the reporter gene (Fig. 1I). Most importantly, this result shows that both the residual non-SOP and the ectopic SOP expression of the *Sm* mutant (Fig. 1F) is strictly dependent on direct proneural input. It also indicates that the residual activity of the *Em* proneural site mutant along the wing margin and in a few PNCs (Fig. 1E) requires direct input from Su(H).

The bHLH repressor gene *E(spl)m8* is also subject to direct transcriptional repression by Su(H) in SOPs

The bHLH repressor-encoding genes of the E(spl)-C, exemplified by *E(spl)* itself [referred to hereafter as *E(spl)m8* to distinguish it readily from *E(spl)mα*], likewise make use of a 'Su(H) plus proneural' cis-regulatory code for their activation in PNCs during lateral inhibition (Fig. 2A, construct 1) (Bailey and Posakony, 1995; Nellesen et al., 1999; Singson et al., 1994). We sought to determine whether direct repression by Su(H) in SOPs applies as well to this class of N pathway target genes. We found that a 1.1 kb genomic DNA fragment from immediately upstream of *E(spl)m8* (Fig. 2A, construct 2) confers PNC-specific expression on a heterologous promoter-

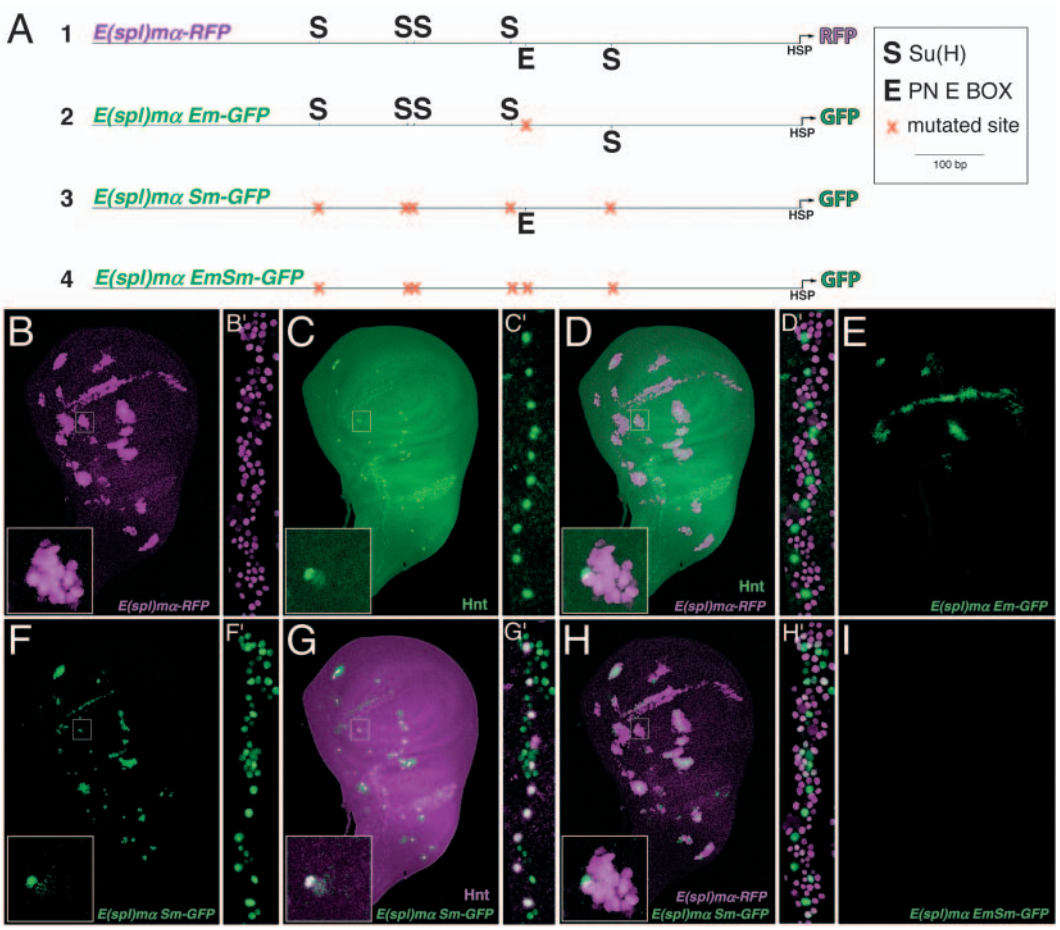

Fig. 1. The *E(spl)mα* PNC enhancer drives strong expression in non-SOPs but is directly repressed by Su(H) in SOPs. (A) Diagram of reporter constructs in which wild-type and mutant versions of the *E(spl)mα* PNC cis-regulatory module drive expression of GFP or RFP via a minimal *Hsp70* promoter. Positions of the five Su(H)-binding sites (S) and single proneural (PN) protein-binding site (E box) are indicated; mutant sites are indicated by a red cross. (1) Wild-type module; (2) module with E box mutated (*Em*); (3) Su(H)-binding sites mutated (*Sm*); (4) E box and Su(H) sites mutated (*EmSm*). (B-D,F-H) Single wing disc from a late third-instar larva carrying one copy each of *E(spl)mα-RFP* and *E(spl)mα Sm-GFP* and stained with anti-Hindsight (Hnt) antibody (C) to mark SOPs. Insets show higher-magnification views of the boxed region of the wing disc. (B′-D′,F′-H′) Microchaete row (adjacent to the dorsal midline) on the notum of a single pupa of the same genotype 14 hours after puparium formation (APF), also stained with anti-Hnt (C′). (B,B′) RFP signal driven by the wild-type module is strong in most non-SOP cells but minimal or absent in SOPs (B,D,B′,D′). (E) Mutation of the E box (*Em*) causes severe loss of PNC expression; expression is retained at the wing margin and in a small subset of PNC cells. (F,G,F′,G′) Mutation of the Su(H)-binding sites (*Sm*) abolishes or severely lowers reporter expression in non-SOP cells of the PNCs but causes strong ectopic expression in SOPs. (H,H′) Overlay of B (B′) and F (F′) highlights dramatic change in the pattern of reporter gene activity when Su(H) sites are mutated. (I) Mutation of both the E box and the Su(H) sites (*EmSm*) demonstrates that ectopic SOP expression of the *Sm* mutant (F) is dependent on direct input from the proneural proteins.

reporter construct in late third-instar wing discs (Fig. 2B). Expression is also observed along the wing margin (see also Bailey and Posakony, 1995). As with *E(spl)mα*, double labeling (using anti-Hnt to mark SOPs) reveals that the PNC activity of this fragment is predominantly in non-SOPs and excluded from SOPs (Fig. 2B-D). Mutation of the three Su(H) binding sites (*Sm*, Fig. 2A, construct 3) abolishes most non-SOP expression (Fig. 2E) and yields strong ectopic expression in SOPs (Fig. 2E-G). We conclude that Su(H) normally acts as a direct repressor of both the Brd family genes and the bHLH repressor genes of the E(spl)-C (see Lai et al., 2000b) in SOPs of the adult PNS.

Both the architecture and the activity of the *E(spl)mα* PNC cis-regulatory module are evolutionarily conserved

Our results with mutant enhancer-reporter constructs define crucial roles for both proneural protein and Su(H)-binding sites in generating the non-SOP-only expression patterns of *E(spl)mα* and *E(spl)m8* in wing disc PNCs. We would expect, then, that these sites should be conserved in orthologous PNC cis-regulatory modules from other *Drosophila* species, and, further, that such modules should function appropriately when introduced into *D. melanogaster*. We first compared and aligned the upstream sequence regions corresponding to the *E(spl)mα* PNC module from four species, *D. melanogaster*, *D. pseudoobscura*, *D. hydei* and *D. virilis* (Fig. 3A). Consistent with the established phylogenetic relationships between these species (Beverley and Wilson, 1984; Russo et al., 1995), we found that the *D. melanogaster* and *D. pseudoobscura* sequences are overall more related to each other than to the *D. hydei* and *D. virilis* sequences, and vice versa. Of particular note is the stability, over 40-60 million years, of the number, spacing and exact sequences of the Su(H) and proneural protein binding sites (Fig. 3A,B). With a single exception, all of these sites are precisely conserved in sequence, as is the spacing between the S_2 site and the proneural site (Fig. 3B). We proceeded to test whether the activity of the *E(spl)mα* cis-regulatory module in PNCs is likewise evolutionarily conserved (Fig. 3C). Fig. 3C, part 1 shows that the *D. virilis* version of the *E(spl)mα* enhancer fragment drives reporter gene expression in the *D.*

melanogaster wing disc in a pattern that largely recapitulates the activity of the *D. melanogaster* module (compare with Fig. 1B-D); specifically, we observe strong activity in the non-SOP cells of PNCs but little or none in SOPs (Fig. 3C, parts 2-4). Thus, the binding site composition, architecture and in vivo function of the *E(spl)mα* PNC cis-regulatory module are all evolutionarily conserved, and hence clearly subject to strong selection.

Loss of Su(H)-mediated repression of *E(spl)m8* can extinguish the SOP cell fate

The results described thus far support the conclusion that direct

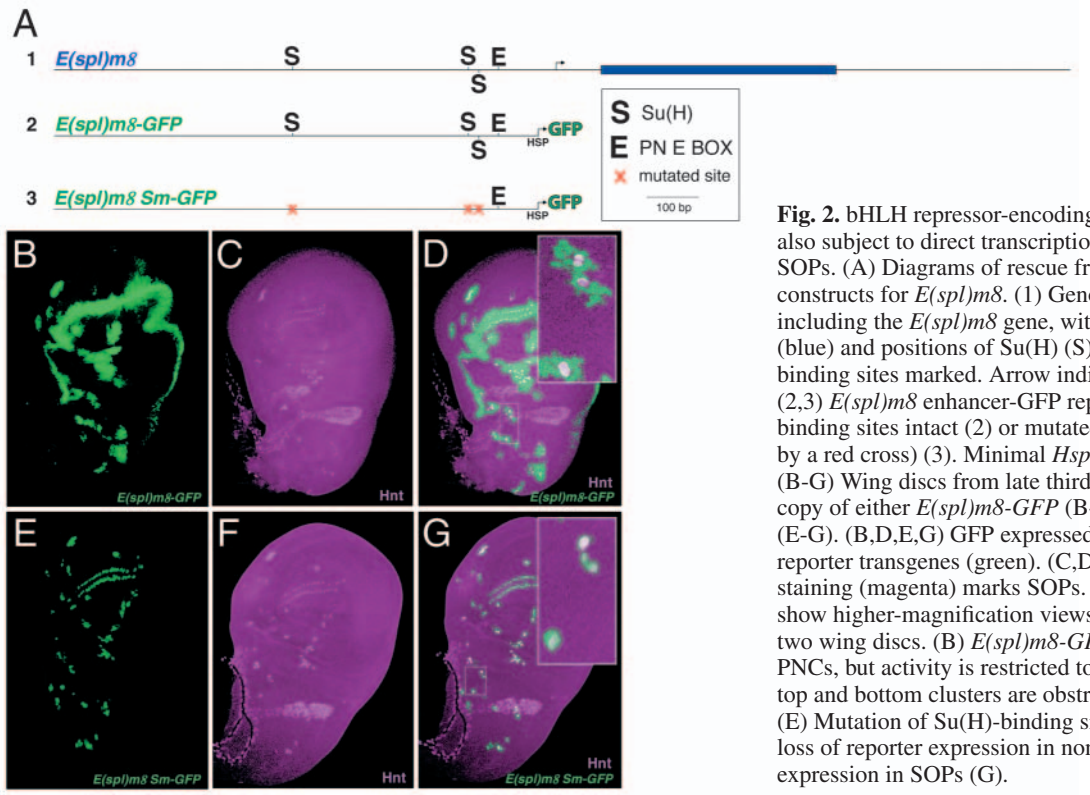

Fig. 2. bHLH repressor-encoding genes of the E(spl)-C are also subject to direct transcriptional repression by Su(H) in SOPs. (A) Diagrams of rescue fragment and enhancer-reporter constructs for *E(spl)m8*. (1) Genomic DNA fragment including the *E(spl)m8* gene, with protein coding sequence (blue) and positions of Su(H) (S) and proneural protein (E) binding sites marked. Arrow indicates transcription start site. (2,3) *E(spl)m8* enhancer-GFP reporter constructs with Su(H)-binding sites intact (2) or mutated (*Sm*; mutant sites indicated by a red cross) (3). Minimal *Hsp70* promoter is shown. (B-G) Wing discs from late third-instar larvae carrying one copy of either *E(spl)m8-GFP* (B-D) or *E(spl)m8 Sm-GFP* (E-G). (B,D,E,G) GFP expressed from *E(spl)m8* enhancer-reporter transgenes (green). (C,D,F,G) Anti-Hnt antibody staining (magenta) marks SOPs. Insets in merged D and G show higher-magnification views of the boxed regions of the two wing discs. (B) *E(spl)m8-GFP* is strongly expressed in PNCs, but activity is restricted to non-SOPs (D; inset: SOPs in top and bottom clusters are obstructed by non-SOPs). (E) Mutation of Su(H)-binding sites causes severe or complete loss of reporter expression in non-SOPs, but strong ectopic expression in SOPs (G).

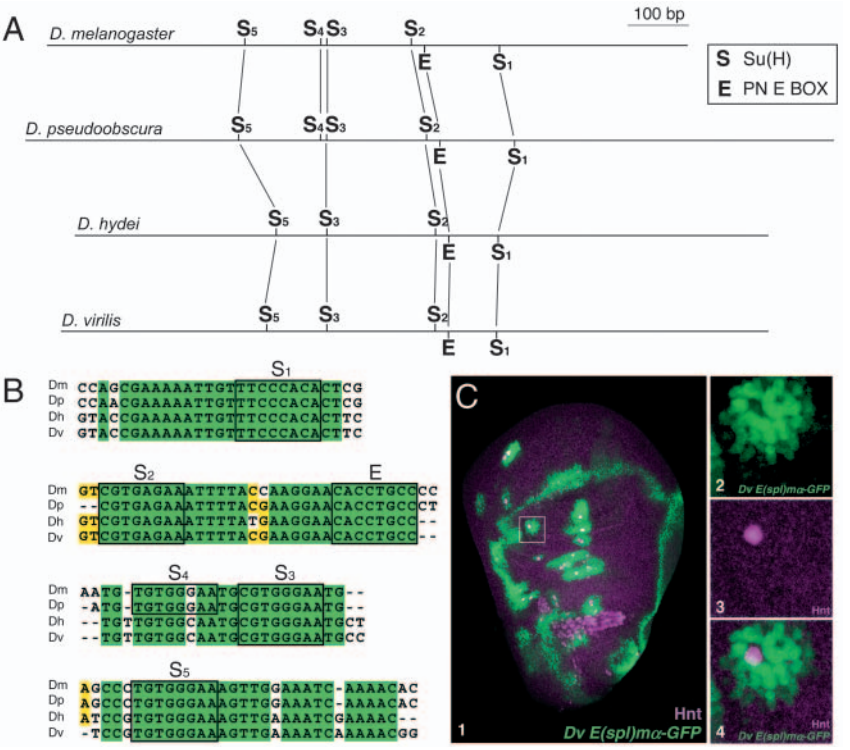

Fig. 3. Conservation of sequence, organization and activity of the *E(spl)mα* PNC cis-regulatory module. (A) Alignment of orthologous genomic DNA segments corresponding to the *E(spl)mα* PNC enhancer from *D. melanogaster*, *D. pseudoobscura*, *D. hydei* and *D. virilis*. Orthologous Su(H) (S) and proneural protein (E)-binding sites are connected by gray lines. (B) Nucleotide sequence alignments showing strong conservation (highlighted in green and yellow) in regions of the enhancer that contain Su(H) and proneural protein-binding sites (boxed). With the exception of S_4, the sequence of each binding site is precisely conserved, as is the spacing between S_2 and the E box. (C) The *D. virilis E(spl)mα* module drives strong GFP expression (green) in PNCs of the late third-instar wing disc of *D. melanogaster* that is excluded from SOPs, similar to the *D. melanogaster* module (see Fig. 1B-D). Boxed region in 1 is shown at higher magnification on right (2-4); anti-Hnt staining (magenta) marks SOPs (1,3,4).

transcriptional repression by Su(H) is required to prevent inappropriate expression of N pathway target genes in SOPs. This raises the question of the developmental significance of such repression, particularly for the SOP cell fate. To investigate, we designed an assay based on previous observations that strong over- or mis-expression of either *E(spl)m5* (another bHLH repressor gene) or *E(spl)m8* leads to bristle loss in adult flies, the cellular basis of which is loss of SOPs (Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995). We anticipated that a wild-type *E(spl)m8* transgene might have minimal phenotypic effects (see Lai et al., 1998) because it would be expressed normally in non-SOP cells (reinforcing their commitment to the epidermal fate) and repressed normally in SOPs; thus, bristle development would be largely unaffected. By contrast, we hypothesized that a mutant transgene not subject to direct repression by Su(H) might yield *E(spl)m8* activity in the SOP sufficient to affect this development of the cell.

We compared the bristle patterns of w^{1118} adults carrying two copies of either a wild-type *E(spl)m8* transgene (Fig. 2A, construct 1) or the same transgene with its Su(H)-binding sites mutated [*E(spl)m8 Sm*]. We consider this a very stringent assay of the requirement for Su(H)-mediated repression in the SOP for two reasons. First, we are testing the effects of de-repressing a single N pathway target gene, although there are several other such genes (both bHLH repressor and Brd family) residing in the E(spl)-C alone. Second, the level of ectopic *E(spl)m8* expression generated by a de-repressed genomic DNA transgene is expected to be much lower than that achieved by a *UAS-E(spl)m8* construct activated by strong GAL4 drivers, as in the prior studies (Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995). We found that whereas flies carrying the wild-type *E(spl)m8* transgene display only very mild bristle loss (Fig. 4A,E), flies carrying *E(spl)m8 Sm* exhibit a significantly more severe bristle-loss phenotype (P=0.002; Fig. 4B,E). Staining of late third-instar wing discs with anti-Senseless (Sens) antibody to visualize SOPs showed that this bristle loss was due to a failure of SOP specification (Fig. 4D,D′; compare with Fig. 4C,C′). We conclude that loss of direct Su(H)-mediated repression of a single N pathway target gene can be sufficient to extinguish the SOP fate, thus altering the adult bristle pattern.

Reducing the dosage of *H* and *gro* de-represses *E(spl)mα* reporter gene expression in SOPs

Su(H) is known to act as a transcriptional repressor in another context during sensory organ development; namely, the socket/shaft sister cell fate decision in the bristle lineage (Barolo et al., 2000b). Auto-repression of *Su(H)* is necessary to prevent inappropriate high-level activation of the gene in the shaft cell, which in turn can cause this cell (which does not respond to N signaling) to adopt the N-responsive socket cell fate. The biochemical basis of transcriptional repression by Su(H) has been studied in some detail in this setting (Barolo et al., 2002). Specifically, the Hairless (H) protein has been shown to act as an adaptor that recruits the transcriptional co-repressor proteins Gro and CtBP to Su(H), thus conferring repressive activity.

Earlier work can be interpreted to suggest that a similar protein complex might mediate repression by Su(H) in the SOP. At several macrochaete and many microchaete positions on the adult fly, simultaneous reduction of the doses of *H* and *gro* in an otherwise wild-type background leads to significant bristle loss (A.G. Bang, PhD thesis, University of California San Diego, 1993) (Barolo et al., 2002; Price et al., 1997); we show now that this is due to a failure of commitment to the SOP cell fate (Bang et al., 1991) (see Fig. S1 in the supplementary material). In light of the results described here, a plausible interpretation of these findings is that H and Gro are normally part of a repressive Su(H)-containing complex in

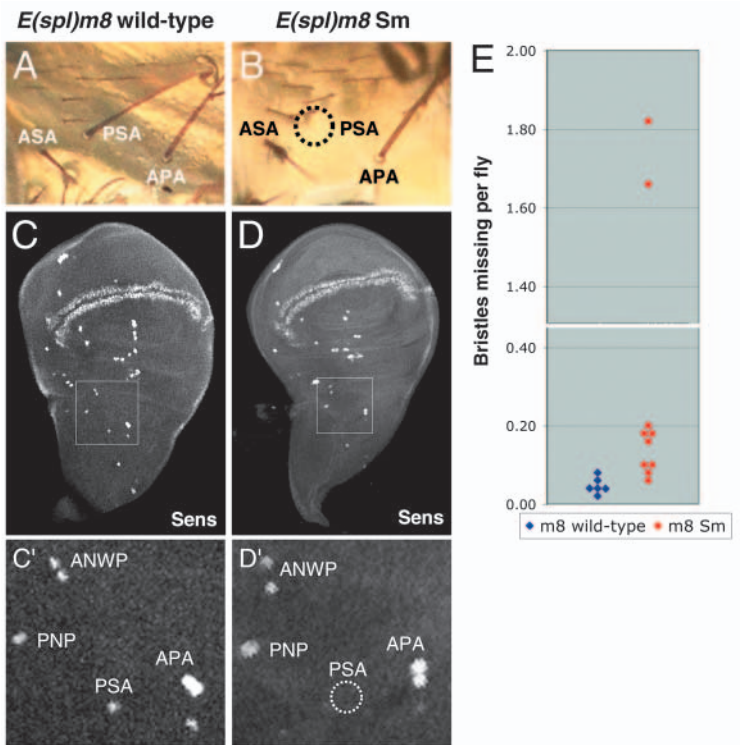

Fig. 4. Failure of direct repression of *E(spl)m8* by Su(H) can extinguish the SOP cell fate. (A) Part of notum region of an adult fly carrying two copies of an *E(spl)m8* genomic DNA transgene (see Fig. 2A, construct 1), showing a normal pattern of adult mechanosensory bristles. Labeled macrochaetes: ASA, anterior supraalar; PSA, posterior supraalar; APA, anterior postalar. (B) Same region from a fly carrying two copies of the *E(spl)m8 Sm* transgene [Su(H) binding sites mutated], showing loss of the PSA bristle (broken circle). (C,C′,D,D′) The cellular basis of bristle loss caused by the *E(spl)m8 Sm* transgene is failure of SOP specification. (C,C′) Same genotype as A; (D,D′) same genotype as B. (C,D) Late third-instar wing discs stained with anti-Sens antibody to mark SOPs. (C′,D′) Higher-magnification views of the regions boxed in C and D, respectively. Labeled SOPs: PNP, posterior notopleural macrochaete; ANWP, campaniform sensilla of the anterior notal wing process. Broken circle in D′ indicates position of missing PSA SOP. The presence of both ANWP SOPs indicates that both discs are at a stage late enough to observe the PSA SOP in wild-type discs. (E) Frequency of bristle loss is significantly greater in *E(spl)m8 Sm* homozygous flies (red) than in flies homozygous for the wild-type *E(spl)m8* transgene (blue). Mann-Whitney U test: U=2, P=0.002.

the SOP, and that reduction of their doses sufficiently compromises the repressive activity as to partially de-repress N pathway target genes like *E(spl)m8*, leading to failure of SOP specification. As a test of this model, we thought it might be possible to detect such de-repression of a suitable reporter gene; Fig. 5 shows that this expectation is borne out. Late third-instar wing discs from wild-type larvae (Fig. 5A,C-E,I) or larvae heterozygous for null alleles of either *H* or *gro* (not shown) only rarely exhibit detectable activity of an *E(spl)mα-GFP* reporter transgene in SOPs. By contrast, we found that wing discs from larvae doubly heterozygous for null alleles of both *H* and *gro* (H^{E31} gro^{E48}/+ +) show substantial frequencies of ectopic GFP expression in SOPs (Fig. 5B,F-I). Moreover, the SOP expression observed in the double heterozygotes is considerably stronger than that detected rarely in a wild-type background (Fig. 5I). These results demonstrate that normal levels of *H* and *gro* activity are required for the Su(H)-dependent repression of N pathway target genes in SOPs, and are consistent with the participation of a Su(H)-H-Gro-containing protein complex in this repression (Barolo et al., 2002).

The Gro- and CtBP-binding motifs of H are required to promote the SOP fate

We have reported previously that broad overexpression of *H* (including in proneural clusters) during lateral inhibition causes a 'neurogenic' phenotype; that is, the appearance of supernumerary bristles surrounding normal bristles (Bang and Posakony, 1992). This phenotype is readily understood in light of the model described above; namely, that H normally serves to recruit Gro and CtBP to Su(H) for its repressive activity in the SOP. Overexpression of H in the N-responsive non-SOP cells of the PNC would be expected to elevate their levels of the repressive form of Su(H), causing repression of N pathway target genes that would normally be activated by the Su(H)-N^{IC}-Mam complex. This in turn would result in a partial failure of lateral inhibition and the commitment of additional cells in the PNC to the SOP fate, giving rise to ectopic bristles in the adult (Bang and Posakony, 1992; Morel et al., 2001).

A key prediction of the model is that the ability of H to bind Gro (via the motif YSIHSLLG) (Barolo et al., 2002) and CtBP (via the motif PLNLSKH) (Barolo et al., 2002; Morel et al., 2001) should be required for the SOP fate-promoting

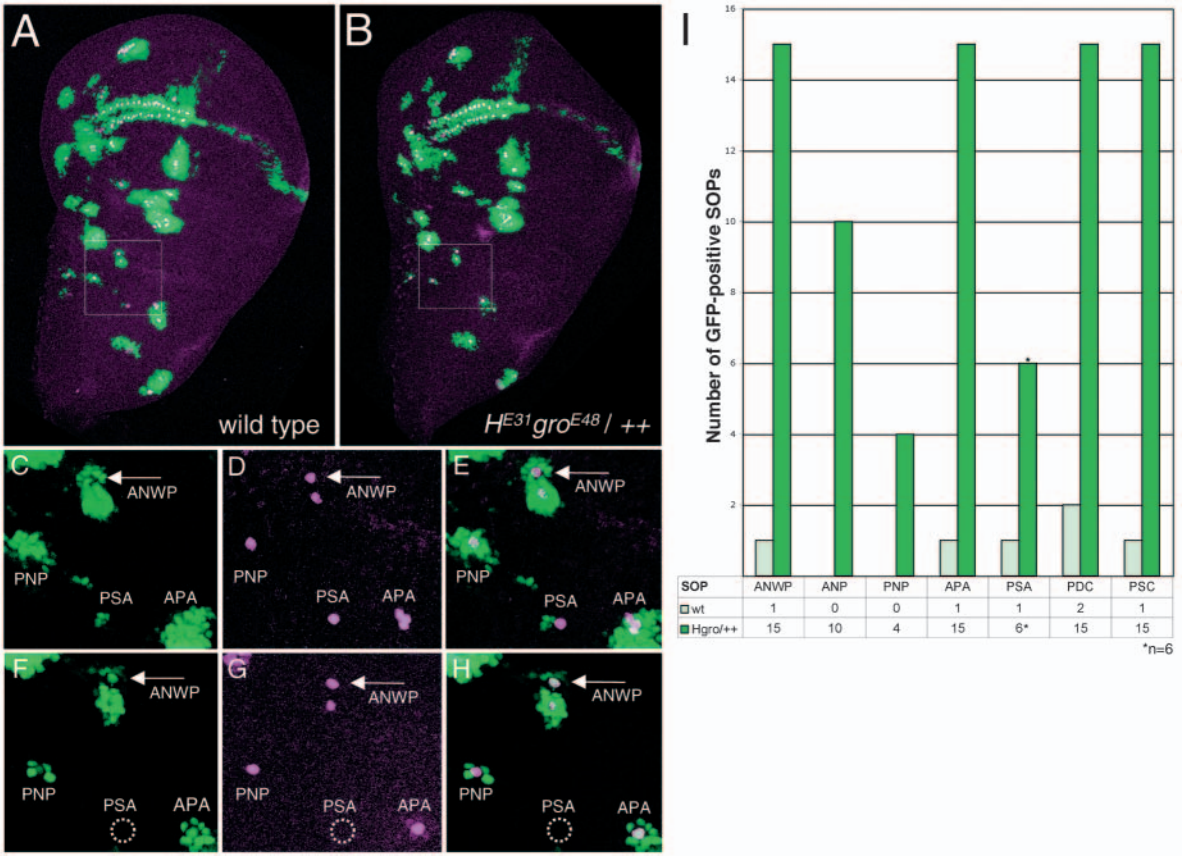

Fig. 5. Reducing the dose of *H* and *gro* de-represses expression of the *E(spl)mα* PNC reporter in SOPs. (A-H) Wing discs from late third-instar larvae carrying one copy of *E(spl)mα-GFP* (green) in either a w^{1118}; *A101*/+ + (A,C-E) or w^{1118}; *A101*/H^{E31} gro^{E48} (B,F-H) background. (A,B,D,E,G,H) Antibody staining of β-galactosidase expressed from the *A101* enhancer trap insertion (magenta) marks SOPs. (C-E) Higher magnification of boxed region in A, showing exclusion of GFP signal from SOPs (arrows). ANWP, campaniform sensilla of the anterior notal wing process; APA, anterior postalar; PSA, osterior supraalar; PNP, posterior notopleural macrochaete. (F-H) Higher magnification of boxed region in B, showing ectopic GFP expression in SOPs (arrows). Reporter expression in non-SOPs is often reduced in this background. (I) Ectopic *E(spl)mα-GFP* expression in SOPs is observed at much greater frequencies and intensities in w^{1118}; *A101*/H^{E31} gro^{E48} discs (dark green) than in w^{1118}; *A101*/+ + discs (light green). ANP, anterior notopleural; PDC, posterior dorsocentral; PSC, posterior scutellar. Only six discs (instead of 16) could be scored for GFP expression at the PSA position in the H^{E31} gro^{E48}/+ + background, as the PSA SOP is often missing in this genotype (broken circle in F-H).

activity of H. We tested this prediction by using an *E(spl)mα* GAL4 driver to express different forms of H specifically in the non-SOP cells of the PNCs. The orbital region of the adult fly head is a particularly favorable territory in which to assay the production of supernumerary bristles by H overexpression (Fig. 6A,B). Expression of a wild-type *UAS-H* transgene results in the appearance of an average of approximately four ectopic bristles in the orbital region (Fig. 6B,C). This activity is significantly impaired by mutating either the Gro recruitment motif (*UAS-H[Gm]*) or the CtBP-binding motif (*UAS-HΔC*) (Fig. 6C), suggesting that both co-repressors make a functional contribution. Loss of both motifs (*UAS-H[Gm]ΔC*) essentially abolishes the capacity of H to promote ectopic bristle development in this assay (Fig. 6C). Our results are strongly consistent with the interpretation that the SOP cell's requirement for *H* activity (Bang et al., 1995; Bang et al., 1991) is based on the recruitment by H of Gro and CtBP to confer repressive activity on Su(H), thus preventing inappropriate expression of inhibitory N pathway target genes.

Discussion

Direct transcriptional repression of N pathway target genes by Su(H) in the SOP

We have shown here that discrete transcriptional cis-regulatory modules, bearing binding sites for both Su(H) and the proneural proteins, direct the non-SOP-only expression pattern of E(spl)-C genes in PNCs. Mutation of the Su(H) sites in these modules results in an inversion of this pattern of activity, including both the loss of most non-SOP expression and the appearance of strong ectopic expression in SOPs. These observations reveal a dual role for Su(H) in the PNC: as a direct, N-activated transcriptional activator of E(spl)-C genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) in non-SOP cells, and as a direct transcriptional repressor of the same genes in the SOP (Fig. 7A).

We were interested in addressing the issue of whether Su(H)-mediated repression of E(spl)-C genes in the SOP is important developmentally. Our experiments with wild-type and *Sm* versions of an *E(spl)m8* genomic DNA transgene demonstrate that it is (Fig. 7B). Failure to repress this single bHLH repressor gene is sufficient to extinguish the SOP fate (marked by Sens) at a frequency significantly greater than that observed with a repressible (wild-type) transgene. We have provided evidence here that the H protein is responsible for conferring repressive activity on Su(H) in the SOP, by recruiting the co-repressors Gro and CtBP (Fig. 7A). We suggest that the *H* null phenotype – widespread, irreversible loss of the SOP fate in an E(spl)-C-dependent manner (Bang et al., 1995; Bang et al., 1991) – offers the best indication of the developmental consequences of relieving Su(H)-mediated repression of all E(spl)-C genes in the SOP.

Consistent with the model proposed here, prior studies have shown that loss of *Su(H)* function in imaginal disc tissue has complex effects on gene expression in PNC cells. Lateral inhibition fails (Schweisguth, 1995; Schweisguth and Posakony, 1992), as Su(H) is not available to transduce the N signal by activating E(spl)-C genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Hence, the cells of the mutant PNC exhibit many of the characteristics of SOPs, such as high levels of Ac and *A2-6* (*scabrous*) expression (Schweisguth and Posakony, 1994), and expression of the early SOP markers *A101* (*neuralized*) and *A37* (*neuromusculin*) (Schweisguth and Posakony, 1992). They also display very high levels of Dl (Schweisguth and Posakony, 1994). However, *Su(H)* mutant PNC cells also express multiple E(spl)-C genes (Bailey and Posakony, 1995; Koelzer and Klein, 2003; Lai et al., 2000b; Nellesen et al., 1999), a characteristic of non-SOPs; this is due to activation by the high proneural levels prevailing

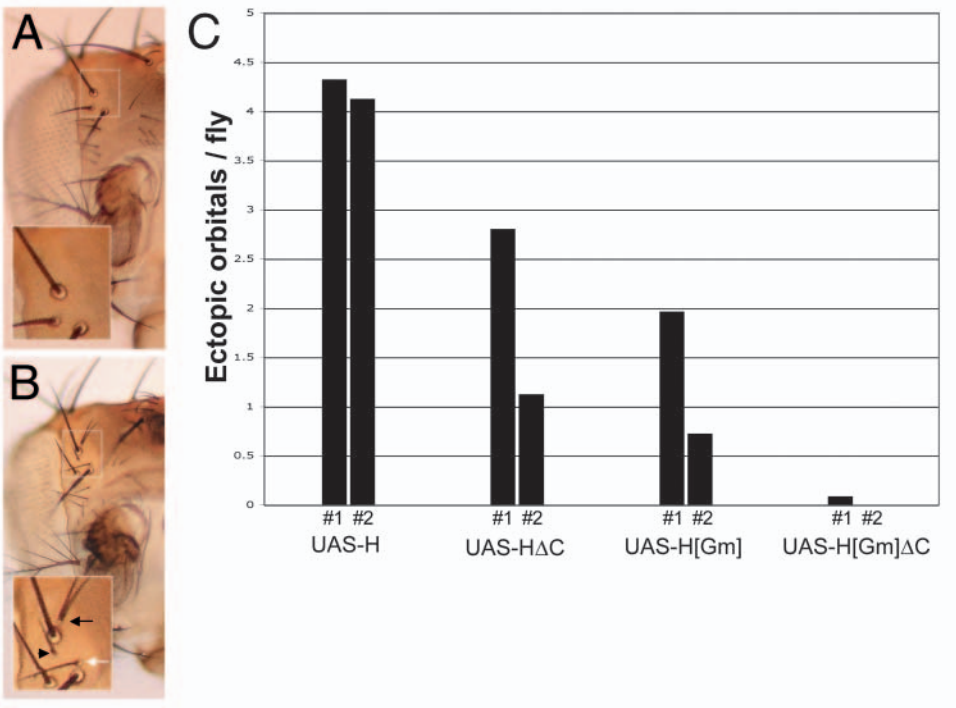

Fig. 6. The Gro- and CtBP-binding motifs of H are each required for its full activity in promoting the SOP cell fate. (A) Half of a wild-type fly head showing the three macrochaete bristles of the orbital region (boxed; magnified in inset). (B) Expressing wild-type H (via *UAS-H*) in non-SOPs with an *E(spl)mα-Gal4* driver converts some of these cells to SOPs, producing ectopic bristles that are either normal (white arrow) or display a 'double shaft' phenotype (black arrow) due to a cell fate conversion in the bristle lineage (Barolo et al., 2002) (black arrowhead indicates another double shaft broken during manipulation). (C) Frequency of supernumerary orbital bristles observed in adult flies carrying one copy of *E(spl)mα-Gal4* driving expression of wild-type H (one copy of *UAS-H*), H lacking its CtBP-binding motif (*UAS-HΔC*), H with its Gro-binding motif mutated (*UAS-H[Gm]*) or H lacking both motifs (*UAS-H[Gm]ΔC*). Two different insertions of each *UAS* construct were tested (#1, #2).

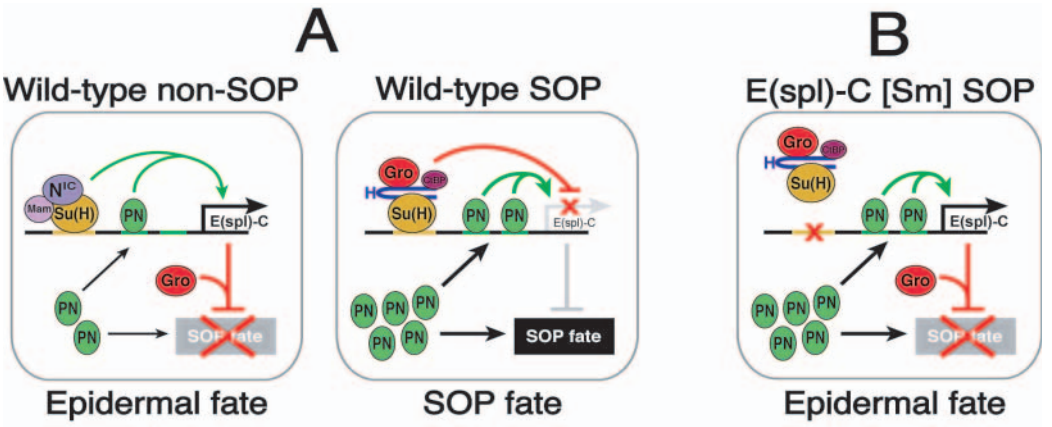

Fig. 7. Model of cell fate specification during lateral inhibition in PNCs. (A) In wild-type SOPs (right), which do not respond to the Dl/N signal, Su(H) is in its default repressive state, linked via H to the co-repressors Gro and CtBP. It is bound directly to high-affinity sites in SOP-inhibitory target genes in the E(spl)-C, and prevents activation of these genes by the high proneural protein levels prevailing in that cell. The proneural proteins are thus free to activate genes that promote the SOP fate. In wild-type non-SOPs (left), activation of the N receptor associates Su(H) with a transcriptional activation complex that includes N^{IC} and Mam. In this activated state, Su(H) synergizes with the proneural proteins to directly activate expression of the same SOP-inhibitory genes, committing the cell to an epidermal fate. (B) In the absence of direct repression by Su(H) [e.g. by mutation of Su(H)-binding sites (Sm)], SOP-inhibitory genes of the E(spl)-C are ectopically activated in the SOP by the high levels of proneural proteins, which can drive the cell to adopt an inappropriate epidermal fate. In this model, three key regulators – the proneural proteins, Su(H) and Gro – each have transcriptional regulatory activities that are oppositely directed, with respect to cell fate, in SOPs versus non-SOPs. All three function both to promote and to inhibit the SOP fate during lateral inhibition.

in these cells, in the absence of default repression by Su(H) (Bailey and Posakony, 1995) (this paper).

Koelzer and Klein (Koelzer and Klein, 2003) have shown that many *Su(H)* mutant PNC cells fail to express later markers of the SOP cell fate (Sens and Hnt), owing to one or more unidentified inhibitory functions encoded in the E(spl)-C. They concluded that this leads to an arrest of their development as SOPs. However, Schweisguth (Schweisguth, 1995) had shown that *Su(H)*-null clones induced in the first larval instar contain abundant neuronal progeny in the pupa, indicating that at least some proportion of *Su(H)* mutant PNC cells can function as neural precursors. Koelzer and Klein (Koelzer and Klein, 2003) suggested that Su(H) protein persists in *Su(H)* mutant clones induced in the first instar; however, no experimental support was offered, so it is unclear whether this would, by itself, resolve the discrepancy. Another possible explanation for the inconsistency is that some or many *Su(H)* mutant PNC cells may suffer only a transient delay, and not a permanent arrest, in their expression of Sens/Hnt and their execution of a precursor fate – perhaps owing to their very unusual state of gene expression (e.g. their very high levels of Ac) (Schweisguth and Posakony, 1994).

The above considerations lead us to conclude that *Su(H)* mutant PNC cells are an unsatisfactory surrogate for SOPs. In the present study, we have investigated instead the behavior of authentic SOPs, and have shown that this fate is indeed at risk under conditions in which direct 'default repression' of E(spl)-C genes by Su(H) is compromised. We find that several conditions that partially or completely relieve this repression [including the *Sm* mutation of an *E(spl)m8* transgene (this paper), a *H gro*/+ + double-heterozygote background (this paper) (Barolo et al., 2002) and a *H* loss-of-function genotype (Bang et al., 1995; Bang et al., 1991)] cause, to various extents, the loss of expression of even the earliest SOP markers and irreversible failure to execute the SOP fate.

Cis-regulatory logic: the 'Su(H) plus proneural' code in PNC expression of E(spl)-C genes

In earlier work, we have demonstrated that a 'Su(H) plus proneural' cis-regulatory code directs specific expression of E(spl)-C genes (both bHLH repressor and Brd family) in imaginal disc PNCs (Bailey and Posakony, 1995; Nellesen et al., 1999; Singson et al., 1994). The results reported here enlarge our understanding of the regulatory logic embodied in this code. Mutational analysis of the Su(H) and proneural protein-binding sites in the *E(spl)mα* PNC module demonstrates that each activator makes an essential contribution to the activity of the module in non-SOP cells of the PNC; indeed, we found that neither class of binding site is sufficient to activate detectable reporter gene expression in most non-SOPs. This is a classic example of 'cooperative activation' (Barolo and Posakony, 2002) by the combination of a signal-regulated factor [Su(H)] and a regionally expressed 'local activator' (Ac/Sc). The 'Su(H) plus proneural' cis-regulatory code is thus very effective in eliciting robust and specific expression of *E(spl)mα* in non-SOPs (Fig. 7A).

However, our work reveals that the use of this code puts the SOP at risk of inappropriately activating N pathway target genes, such as *E(spl)mα* and *E(spl)m8*, for a specific reason: the elevated level of proneural protein accumulation in SOPs (Cubas et al., 1991; Skeath and Carroll, 1991). We found that, in the absence of direct repression by Su(H), the proneural proteins can act through the single E box binding site in the *E(spl)mα* PNC module to drive strong ectopic reporter gene expression in the SOP – in contrast to their insufficiency in most non-SOPs. Thus, even in the absence of any activating contribution from Su(H), which is required in non-SOPs, E(spl)-C genes would respond to the high proneural levels in the SOP were it not for direct repression by Su(H) (Fig. 7B). The elegant logic of the 'Su(H) plus

Table 1. Default repression by Su(H) in *Drosophila* development

Developmental context	Gene(s)	Identified local activators	Identified members of repressive complex	References
Lateral inhibition in proneural clusters	*E(spl)mα*, *E(spl)m8*	Ac, Sc	H, Gro, CtBP	This paper; Barolo et al., 2002; Morel et al., 2001
Binary cell fate decisions in lineages	*Su(H)*	–	H, Gro, CtBP	Barolo et al., 2002; Barolo et al., 2000b
Boundary formation in tissues	*sim*	Dorsal, Twist	H	Morel et al., 2001; Morel and Schweisguth, 2000

proneural' code insures instead that only non-SOPs activate effective levels of the genes by which the N pathway inhibits the SOP cell fate.

Recently, Cave et al. (Cave et al., 2005) have reported that a specific configuration of Su(H)-binding sites known as the Suppressor of Hairless Paired Site (SPS) (Bailey and Posakony, 1995) is essential for transcriptional synergy between proneural proteins and Su(H) in driving specific expression in PNCs. The results reported here on transcriptional regulation of *E(spl)mα* contradict this conclusion. We have clearly demonstrated that the strong expression of *E(spl)mα* in the non-SOP cells of the PNC depends crucially on cooperation between proneural activators and Su(H), yet none of the Su(H) sites of this gene are in the SPS configuration. Thus, until the mechanistic basis for proneural/Su(H) synergy is more fully elucidated, we believe that the term 'Su(H) plus proneural' remains the most accurate and most general description of the PNC cis-regulatory code.

Default repression and N signaling in *Drosophila* development

Direct repression of E(spl)-C genes in the SOP during lateral inhibition is a conspicuous example of what we have termed 'default repression', a property of developmental signaling pathways whereby pathway target genes are repressed by a signal-regulated transcription factor in the absence of signaling (Barolo and Posakony, 2002). We proposed that default repression has evolved in order to prevent inappropriate (signal-independent) activation of pathway target genes in cells that express local activators but do not respond to the signal. Indeed, as discussed above, the SOP is in particular need of default repression because it is characterized (perhaps unusually) by elevated accumulation of the local activators for the PNC, the proneural proteins. That Su(H) can keep N pathway target genes off in SOPs even in the face of exceptionally high local activator levels (Fig. 7A) is testament to the efficacy of default repression as a regulatory strategy.

It is now clear that default repression by Su(H) is a crucial feature of the operation of the N pathway in all three of the developmental situations in which it is known to function (Table 1) (Bray, 1998): lateral inhibition (this paper), binary cell fate decisions in lineages (Barolo et al., 2002; Barolo et al., 2000b), and formation of tissue boundaries (Morel and Schweisguth, 2000). This conclusion is based on an analysis, in all three cases, of the consequences of mutating Su(H)-binding sites in one or more N pathway-activated genes (Table 1); we emphasize that attribution of a default repression activity to a signal-regulated transcription factor can be made only after such cis-regulatory experiments have been performed. It is likely that default repression by Su(H) is an integral part of N pathway function during *Drosophila* development.

A dual function of three key regulators during lateral inhibition in *Drosophila*

The studies presented here, when combined with earlier reports, illuminate a prominent feature of the transcriptional regulation of gene expression and cell fate during lateral inhibition in *Drosophila*. It is now clear that three key regulatory factors – the proneural proteins (Ac and Sc), Su(H) and Gro – each have dual, and oppositely directed, functions in the SOP versus the non-SOP cells of the PNC during lateral inhibition (Fig. 7A). The proneural proteins are strictly required for the SOP cell fate, at least in part because they directly activate genes that promote or execute this fate, such as *sens* (Jafar-Nejad et al., 2003), *phyllopod* (Pi et al., 2004) and *ac* itself (Van Doren et al., 1992). But as described earlier (Kramatschek and Campos-Ortega, 1994; Nellesen et al., 1999; Singson et al., 1994), proneural proteins also have a vital role in non-SOPs as direct activators of genes, including those of the E(spl)-C, that are involved in inhibiting the SOP fate. We have shown that Su(H) also has crucial, but opposing, functions in the SOP [as a direct default repressor of SOP-inhibitory E(spl)-C genes] and in the non-SOPs (as an essential direct activator of these same genes in response to N signaling) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Finally, we have presented evidence in this paper strongly supporting our hypothesis that Gro is likewise a 'double agent' during lateral inhibition (Barolo et al., 2002): in the non-SOPs, it serves as the co-repressor for the E(spl)-C bHLH repressor proteins to inhibit the SOP fate (its traditional function in the process) (Fisher and Caudy, 1998; Paroush et al., 1994), whereas in the SOP it partners with Su(H) via H to effect default repression and thus protect the SOP fate. The regulatory machinery underlying lateral inhibition is all the more elegant for its versatility and economy.

Note added in proof

Our sequences of the *E(sp1)mα* PNC enhancers from *D. hydei* and *D. virilis* have been deposited in GenBank; Accession Numbers DQ076189 and DQ076190, respectively.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/15/3333/DC1

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