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A subset of *Notch* functions during *Drosophila* eye development require *Su(H)* and the *E(spl)* gene complex

Petros Ligoxygakis[1], Sung-Yun Yu[2], Christos Delidakis[1] and Nicholas E. Baker[2,*]

[1]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, and Department of Biology, University of Crete, Box 1527, GR 71110 Heraklion, Greece
[2]Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
*Author for correspondence (e-mail: baker@aecom.yu.edu)

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SUMMARY

The Notch signalling pathway is involved in many processes where cell fate is decided. Previous work showed that *Notch* is required at successive steps during R8 specification in the *Drosophila* eye. Initially, *Notch* enhances *atonal* expression and promotes *atonal* function. After *atonal* autoregulation has been established, Notch signalling represses *atonal* expression during lateral specification. In this paper we investigate which known components of the Notch pathway are involved in each signalling process. Using clonal analysis we show that a ligand of Notch, Delta, is required along with Notch for both proneural enhancement and lateral specification, while the downstream components Suppressor-of-Hairless and Enhancer-of-Split are involved only in lateral specification. Our data point to a distinct signal transduction pathway during proneural enhancement by Notch. Using misexpression experiments we also show that particular Enhancer-of-split bHLH genes can differ greatly in their contribution to lateral specification.

Key words: *Drosophila* eye, Notch, Enhancer of split, Suppressor of Hairless, Atonal

INTRODUCTION

A signalling pathway involved in many cell fate decisions is centred around the transmembrane receptor encoded by the gene *Notch* (*N*). Several elements in the *Notch* signalling pathway have been identified (Artavanis-Tsakonas et al., 1995). In *Drosophila* these are encoded by, among others, the *Delta* (*Dl*), *Serrate* (*Ser*), *Suppressor of Hairless* (*Su(H)*) and *Enhancer of Split* (*E(spl*)) loci. *Dl* and *Ser* encode membrane-bound ligands of Notch. Su(H) acts as an intracellular transducer of the signal from the membrane to the nucleus. *E(spl)* is a nuclear target in the relay of the signal(Artavanis-Tsakonas et al., 1995). *E(spl)* is a complex locus, including seven genes that encode closely related transcription factors bearing a basic Helix-Loop-Helix (bHLH) motif (*m8*, *m7*, *m5*, *m3*, *mβ*, *mγ* and *mδ*), and two genes that encode non-bHLH proteins (*m4* and *groucho*; Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992; Schrons et al., 1992) (Fig. 1).

In many cases Notch signalling is thought to delay cell differentiation, keeping cells competent to respond to later inductive signals (Coffman et al., 1993; Fortini et al., 1993). Lateral specification, exemplified by embryonic neuroblast commitment, is an example. During lateral specification an individual cell is singled out from a group of equipotent cells and follows a different developmental fate. Notch does not signal in this committed cell, whereas the remaining cells remain uncommitted as a result of Notch signalling (Lieber et al., 1993; Struhl et al., 1993; Jennings et al., 1994).

In addition to delaying differentiation, recent studies reveal examples of inductive Notch signalling. Activation of Notch induces particular cell fates at the developing wing margin (Kim et al., 1995; Couso et al., 1995; Doherty et al., 1996). The different types of N signalling can share signal transduction components. Both lateral specification and inductive signalling at the wing margin require the *Su(H)* signal transducer, but at the wing margin target genes that act positively to promote cell fates are activated, in addition to the repressors of the *E(spl)* gene complex (*E(spl)*-C) (de Celis et al., 1996a,b; Kim et al., 1996). Although the *E(spl)*-C is expressed at the wing margin, little function has yet been found for this expression (de Celis et al., 1996a,b).

Here we investigate the signal transducers and target genes involved in Notch signalling in the eye imaginal disc. The adult eye of the fly contains 800 ommatidia (unit eyes) arranged in a two-dimensional lattice. Each ommatidium includes eight photoreceptor neurons and twelve accessory cells arranged in a stereotypical pattern, and is founded by a single R8 photoreceptor cell, which induces most of the other cell types (Wolff and Ready, 1993; Tio and Moses, 1997). Expression of the proneural gene *atonal (ato)* is autonomously required for R8 determination (Jarman et al., 1994).

Notch signalling serves dual roles in the specification of R8 cells (Baker and Yu, 1997). As expected, *Notch*-dependent

lateral specification occurs to restrict *ato* expression to dispersed single cells that become R8 photoreceptor precursors. In an additional earlier process, *Notch* enhances *ato* expression and function as *ato* transcription becomes autoregulatory and independent of prepattern signals. The initial enhancement of *ato* expression occurs in many cells, but once *ato* autoregulation is established, lateral specification starts to limit *ato* expression to R8 precursor cells. Thus Notch signalling is required at successive steps during R8 specification, initially to promote neural potential and later to suppress it through lateral specification. Consequently the phenotype of loss of *Notch* gene function varies with time. If *Notch (N)* function is removed conditionally once *ato* expression has been enhanced, supernumerary R8 cells differentiate because lateral specification is affected. If *N* function is absent from the outset, such as in a clone of cells lacking *N*, little R8 specification can occur. For this reason clones of *N* null mutant cells in the eye disc almost completely lack neural differentiation, contrasting with the neurogenic phenotype of null mutant embryos (Baker and Yu, 1997) (Fig. 2).

Although Notch signalling is involved in many cell fate decisions during development, the dual role in R8 specification is unusual in that both roles affect the same cell-fate decision in the same cells. The two processes occur consecutively without any clear gap between them. In this study we investigated whether components of the pathway are involved in both processes and whether the *E(spl)* genes serve as the last step in the relay of the signals. We started this work with two hypotheses in mind. In the first, by analogy with the wing margin, proneural enhancement of *ato* would require *Su(H)*, but *E(spl)* genes would only be needed to mediate lateral specification. Our second hypothesis was that both processes could involve *E(spl)* genes, with different combinations of E(spl) proteins playing distinct roles. For example, some of the bHLH genes, or *groucho*, could be involved in lateral specification, and others in proneural enhancement. Our data argue against both of these hypotheses. We show that Notch and Delta participate in both proneural enhancement and lateral specification, but that *Su(H)* and *E(spl)* are only required for lateral specification, not for proneural enhancement. It follows that the proneural function of *Notch* is mediated through a distinct signal transduction route.

MATERIALS AND METHODS

Drosophila strains and mutations

The *FRT82 Dl Ser* chromosome was derived by standard genetic crosses from the Dl^{rev10} and Ser^{RX106} strains described previously (Baker and Yu, 1997). *FRT82* $Df(3R)gro^{b32.2}$ and *FRT82* $Df(3R)E(spl)^{BX22}$ chromosomes were described previously (Heitzler et al., 1996; Treisman et al., 1997), as was $Su(H)^{SF8}$ *FRT40* (Schweisguth, 1995). For chromosome arm 3R the LacZ marker was p[construct D]96A (Tio and Moses, 1997). For chromosome 2L the LacZ marker was *armLacZ.2L* (Vincent et al., 1994); a *hs-π-myc* marker was also used (Xu and Rubin, 1993). The *hsFLP1* transgene was used (Xu and Rubin, 1993). The *UAS-E(spl)bHLH* transgenes were prepared as described previously (de Celis et al., 1996a), and the h^{H10} Gal4 driver has been described before (Ellis et al., 1994). We have observed weaker phenotypes towards the posterior of the eye disc using h^{H10} to express these and many other genes, so this may be a property of the h^{H10} driver. Whereas h^{H10}/+; *UAS-mδ*/+ flies had mild rough eyes, similar combinations with *m3, m5, m8, mβ* or *mγ* were phenotypically wild type. Only the *mδ, m5* and *mβ* genotypes have been examined as double homozygotes.

Mosaic induction

Clones were induced by heat-shocking larvae (1 hour, 37-38°C) of the following genotypes: (1) *hsFLP*/+; *FRT82 p[construct D]96A*/*FRT82* Dl^{rev10} *e* Ser^{RX106}; (2) *hsFLP*/+; *FRT82 p[construct D]96A*/*FRT82* kar^2 ry^{506} $P[gro^+ry^+]$ $Df(3R)gro^{b32.2}$; (3) *hsFLP*/+; *FRT82 p[construct D]96A*/*FRT82* $Df(3R)E(spl)^{BX22}$; (4) *hsFLP1*/+; *armLacZ.2L FRT40*/$Su(H)^{SF8}$ *FRT40*.

Immunohistochemistry

Antibody stainings were performed as described (Baker and Yu, 1997). Rabbit anti-Boss antibody was kindly provided by H. Kramer (Kramer et al., 1991) and rabbit anti-Atonal antibody by A. Jarman and Y.-N. Jan (Jarman et al., 1994). Monoclonal antibodies specific for β-galactosidase (mAb40-1a) and Elav protein (mAb7E8A10) were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA, under contract N01-HD-7-3263 from the NICHD. These monoclonal antibodies were developed by J. R. Sanes and G. M. Rubin, respectively. Secondary antibodies were obtained from Jackson Immunoresearch. HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were used as well as Cy3-conjugated goat anti-mouse and FITC- or Cy2-conjugated goat anti-rabbit and goat anti-rat.

Scanning electron microscopy was performed as described (Kimmel et al., 1990).

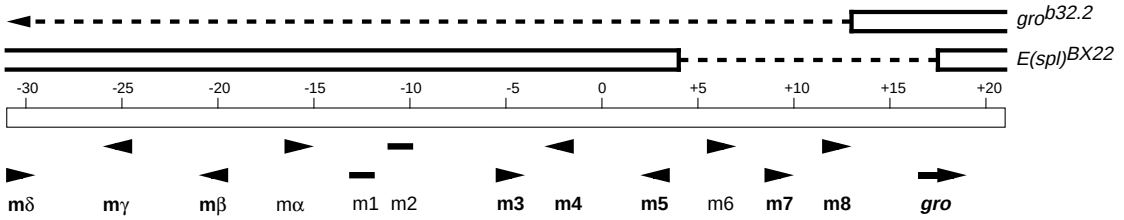

Fig. 1. Organization of the *E(spl)* complex. The *E(spl)*-C, shown with distal to the right, contains seven genes encoding bHLH genes, shown in bold type beneath the scale (*mδ, mγ, mβ, m3, m5, m7* and *m8*) and two functionally related genes of distinct sequence, the co-repressor gene *groucho* (*gro*) and the *m4* locus. *N* signalling mediated by *Su(H)* is required for eye disc expression of at least *mδ, mγ, mβ, m7* and *m8*, although in the cases of *m8* and *mγ* some features of the expression pattern are retained in *Su(H)* or *N* mutant cells (Bailey and Posakony, 1995; de Celis et al., 1996a,b; Baker and Yu, 1997). Dashed lines above the scale indicate the extent of deficiencies for the locus, $Df(3R)gro^{b32.2}$ deleting all the bHLH genes but not *groucho* (which is nevertheless affected), whereas *Df(3R)BX22* affects *groucho* and *m5, m7* and *m8* (Delidakis et al., 1992; Schrons et al., 1992; C. Delidakis and A. Preiss, unpublished data).

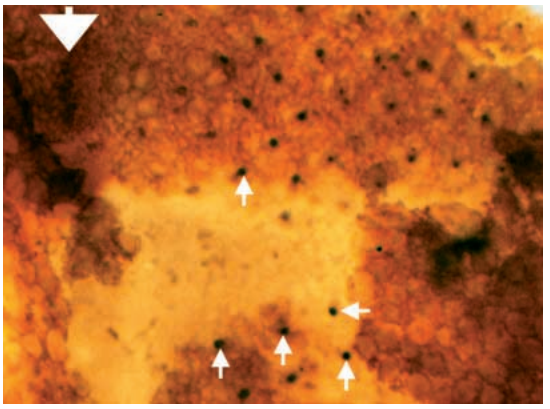

Fig. 2. Eye development in the absence of Notch. A segment of an eye imaginal disc mosaic for *N* is shown. Anterior is to the left in this and subsequent figures. Notch protein is stained brown, so the mutant clone is identified from loss of Notch immunoreactivity. The R8-specific Boss protein is stained blue-black. The mutant clone lacks differentiating R8 cells. Several R8 cells differentiating from *N*+ cells at the clone boundary are shown by white arrows. Two of these (lower right) lie within small clusters of N^+ cells surrounded by N^- cells. The position of the morphogenetic furrow is shown by the large arrowhead.

RESULTS

Previous work established an autonomous, proneural function for *Notch*, which is required to enhance the expression and function of *ato* as eye differentiation begins (Baker and Yu, 1997). In contrast to the neurogenic phenotype seen in *N* null mutant embryos, *N* mutant clones in the eye disc lacked most neural differentiation, demonstrating an *N* requirement preceding lateral specification (Fig. 2). The Notch ligand Dl was nonautonomously required, so that the proneural enhancement was rescued in some *Dl* mutant cells by nearby cells wild type for *Dl*. Another ligand encoded by the *Serrate* gene appeared not to be required for eye differentiation (Sun and Artavanis-Tsakonas, 1996; Baker and Yu, 1997).

Delta-Serrate double mutant clones

The ligand encoded by *Ser* is also expressed in the eye disc (Baker and Yu, 1997). Because *Dl* and *Ser* function redundantly in some tissues and can regulate each other's expression, it was necessary to examine cells mutant for both genes to determine the effects of losing Notch ligands completely (Micchelli et al., 1997; Panin et al., 1997). FLP-mediated recombination was used to induce clones of cells homozygous null for both ligands. Phenotypically, clones of $Dl^- Ser^-$ mutant cells resembled those previously described for *Dl* alone. Neural differentiation was prevented as in *N* clones, except that near the clone margins nonautonomy was observed (Fig. 3). Another marker that labels all photoreceptor neurons, the Elav protein, was also examined, with similar results (not shown). Where neural differentiation was rescued near the clone margins, excessive numbers of R8 cells were observed (Fig. 3). This reflects rescue of the proneural function of Notch but insufficiency of signal during lateral specification, and was observed before for *Dl* mutant clones (Baker and Yu, 1997).

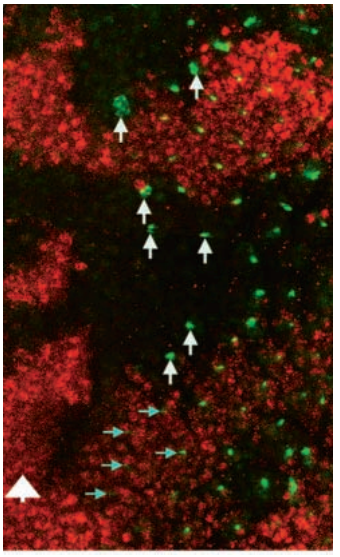

Fig. 3. Eye development in the absence of Dl and Ser. Cells wild type for the *Dl* and *Ser* loci express the β-galactosidase marker (red). Expression of the R8-specific Boss protein is in green. Anterior is to the left and the large arrowhead indicates the morphogenetic furrow. Some single R8 cells differentiating in wild-type territories are indicated by blue arrows. R8 differentiation (and differentiation of other neurons) does not occur within $Dl^- Ser^-$ clones, except near the boundaries where clusters of multiple R8 cells are seen (white arrows). Within each cluster the apical profiles of individual cells become hard to see as adjacent R8 cells become increasingly tightly bunched together posterior to the furrow. Many of the clusters contain 3-4 Boss-expressing cells.

Adjacent R8 cells bunch very tightly together at their apices. Larger R8 cell clusters seen in other genotypes are often associated with deep folds in the epithelium. In summary, no role could be identified for *Ser*, even in the simultaneous absence of *Dl*.

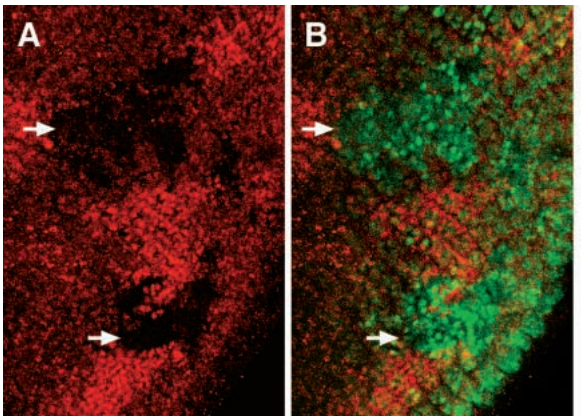

Fig. 4. Eye development in the absence of *E(spl)*-C. (A) β-galactosidase expression (red) marks cells with one or two copies of the wild-type *E(spl)*-C. Unlabelled cells are homozygous for $Df(3R)gro^{b32.2}$. (B) Specimen from A merged with image of Elav expression (green). Homozygous *E(spl)* mutant cells are neurogenic in phenotype as most or all *E(spl)* mutant cells differentiate as neurons.

The requirement for *E(spl)*-C during neurogenesis in the eye imaginal disc

E(spl) bHLH genes have been shown to be transcriptionally activated as a direct consequence of Notch signalling and, along with the corepressor protein Groucho, to mediate inhibition of proneural genes in the nucleus (Jennings et al., 1994; Paroush et al., 1994; Oellers et al., 1994; Dawson et al., 1995; Jarriault et al., 1995; Nakao and Campos-Ortega, 1996; Fisher et al., 1996; Giebel and Campos-Ortega, 1997). At the wing margin *E(spl)* genes are activated by *N*, but this expression appears to be dispensable for wing margin formation (de Celis et al., 1996a,b). In the eye, *Notch*-dependent expression of $m\delta$ and $m\gamma$ accompanies repression of *ato* expression, suggesting that at least these two of the *E(spl)* bHLH genes contribute to R8 patterning during lateral specification (Baker et al., 1996; Dokucu et al., 1996). In addition, $m\delta$ and perhaps $m\gamma$ are also transiently expressed prior to lateral specification (Baker et al., 1996), and the *m7*, *m8* and $m\beta$ genes are transcribed in distinct patterns that remain uncharacterized in detail for lack of specific antibodies (de Celis et al., 1996a). Thus particular E(spl) bHLH proteins might mediate proneural Notch signalling as well as or instead of lateral specification.

Clones of cells deleted for portions of the *E(spl)* complex were used to define its role more precisely. The $E(spl)^{b32.2}$ deficiency deletes all seven bHLH genes and *m4* (Fig. 1; Schrons et al., 1992). Partial *gro* function was supplemented in our experiment by a linked gro^+ transgene (see Materials and methods). $E(spl)^{b32.2}$ gro^+ homozygous cells displayed a cell autonomous neurogenic phenotype quite unlike that of *N* or *Dl* mutant clones. Antibodies against Boss or Elav proteins each labelled a much greater number of cells within the clone than in the surrounding wild-type tissue (Fig. 4, and data not shown). Some clones were difficult to photograph because neurogenic regions often seem to fold in on themselves and crease the eye disc. Because neurogenesis can still occur, it appears that the proneural function of *Notch* can proceed without any *E(spl)*-C bHLH genes, whereas *N* function in lateral specification is severely impaired.

As recently reported (Treisman et al., 1997), we find that clones of cells homozygous for $E(spl)^{BX22}$ are also neurogenic in phenotype (data not shown). $E(spl)^{BX22}$ affects *gro* and the bHLH genes *m5*, *m7* and *m8* (Fig. 1). It follows that *gro* is also dispensable for the proneural function of *Notch*, although it is probably required in lateral specification.

Forced expression of E(spl) bHLH proteins

Forced expression experiments were performed to define

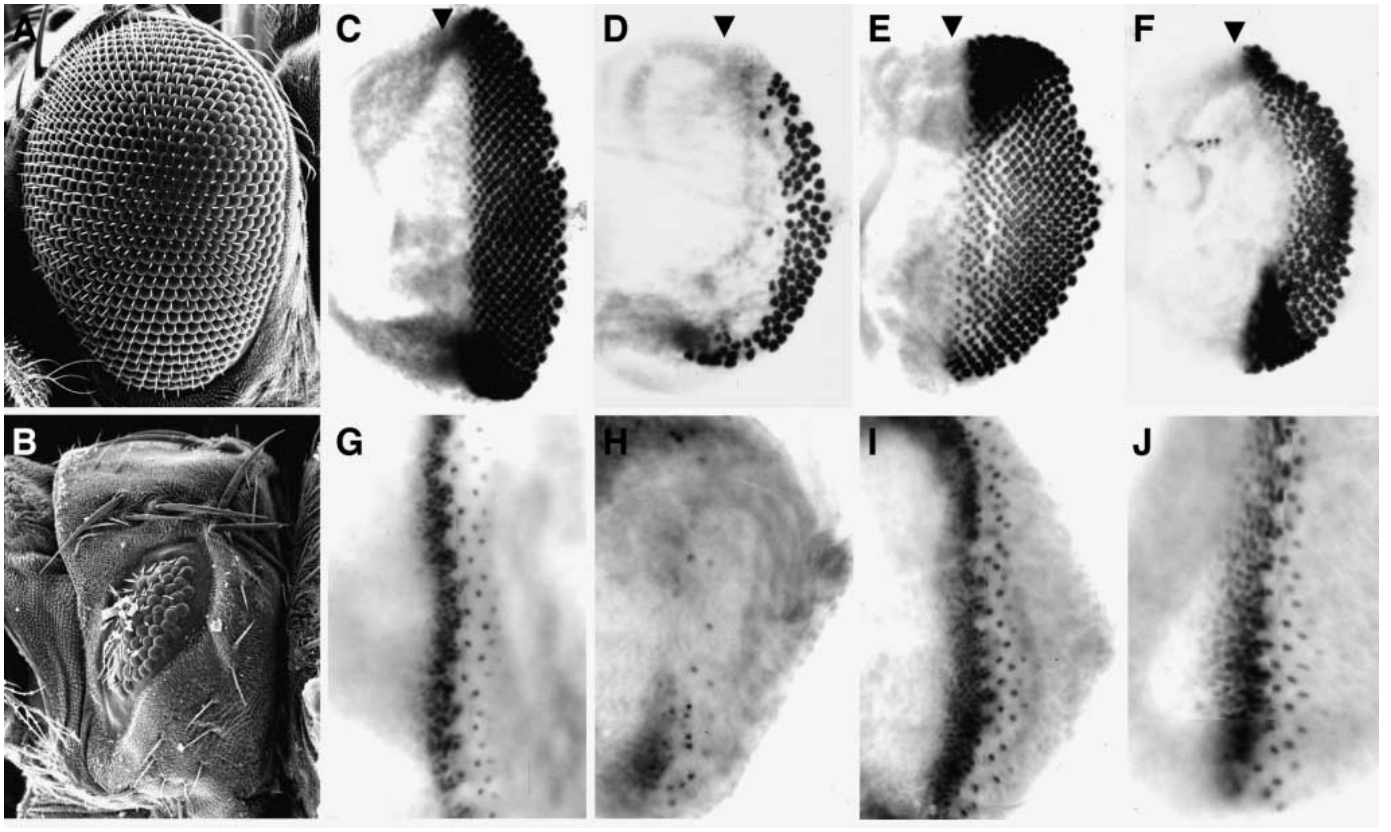

Fig. 5. Forced expression of E(spl) bHLH proteins. (A) Scanning electron micrograph of the surface of a wild-type eye. (B) In comparison, the eye is much smaller in h^{H10}/*UASmδ* flies. (C) Neural differentiation in wild-type eye disc, revealed by labelling for ELAV protein. (D) In h^{H10}/*UASmδ* many fewer ommatidia differentiate. Most are located close to the posterior eye margin. Weaker posterior effects are the rule with the h^{H10} driver (see Materials and methods). (E) Nearly normal numbers of ommatidia differentiate in h^{H10}/*UASm5* eye discs. (F) Neural differentiation in h^{H10}/*UASmβ* eye discs is more normal than for *mδ* (compare with D). (G) The normal evolution of ato protein expression in a wild-type eye disc. (H) In h^{H10}/*UASmδ* ato expression is greatly suppressed. (I) h^{H10}/*UASm5* αto expression resembles wild type (compare with G). (J) In h^{H10}/*UASmβ* ato expression resembles wild type (compare with G). Arrowheads in C-F, position of morphogenic furrow.

further the role of particular bHLH proteins. The *hairy^{H10}* enhancer trap was used to drive GAL4-dependent transgene expression anterior to and within the morphogenetic furrow. Fig. 5B shows the double homozygote for both *h^{H10}* and *UASmδ*, expressing mδ protein during the requirement for ato. The eyes contained few facets and were greatly reduced in size. Eye imaginal discs contained few ommatidia (Fig. 5D). The defect was associated with reduction or absence of *ato* expression in the morphogenetic furrow (Fig. 5H). These findings indicate that mδ protein is capable of repressing *ato* expression, as occurs during lateral specification.

Not all E(spl)bHLH proteins repressed *ato*. Fig. 5 shows that eye disc patterning occurred almost normally in *h^{H10}/h^{H10};UASm5/UASm5* homozygotes and in *h^{H10}/h^{H10}; UASmβ/UASmβ* homozygotes. Both *h^{H10}/h^{H10}; UASm5/UASm5* homozygotes and *h^{H10}/h^{H10};UASmβ/UASmβ* homozygotes died as pupae without differentiating adult structures, whereas *h^{H10}/h^{H10};UASmδ/UASmδ* homozygotes survived to pharate adulthood and produced occasional adult escapers. Because of the earlier lethality due to *m5* or *mβ* expression it is difficult to attribute the lack of effect on eye development to lower expression levels than for *mδ*, and we instead conclude that the mδ protein is qualitatively distinct from m5 and mβ proteins in its ability to inhibit *ato* expression.

Role of Su(H)

Recent studies have identified Su(H) as a common component in Notch signal transduction pathways. Ligand binding (Delta or Serrate) to Notch activates Su(H), which can shuttle between the cytoplasm and the nucleus and act as a transcription factor. Activated Su(H) turns on a number of downstream target genes mediating Notch signalling in lateral specification or inductive processes (Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995; Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995; Kim et al., 1996; Eastman et al., 1997). Since the *E(spl)*-C was not required for proneural *N* signalling in the eye it was possible that other effector genes were transcribed in response to Su(H) activation, as happens at the wing margin.

In order to investigate the role of Su(H), clones of cells homozygous for an apparent null allele of *Su(H)* were generated by FLP-mediated recombination. In the eye imaginal disc *Su(H)$^{-}$* mutant cells were associated cell autonomously with neural hypertrophy (Fig. 6). Many of the ectopic neural cells were R8 photoreceptors, based on expression of the R8-specific protein BOSS (Fig. 6C,D). It appeared that, like the *E(spl)*-C, *Su(H)* was required for lateral specification but not for R8 differentiation. To confirm this conclusion *ato* expression was examined. In wild type, initial broad expression of ato protein is replaced by R8-specific expression that persists for 6-8 hours (3-4 columns of ommatidia) and then fades (Jarman et al., 1994; Fig. 5G). Fig. 6E-J shows that whereas *ato* expression begins normally in *Su(H)* mutant cells, *ato* expression is maintained in many more R8 cells than in wild type, indicating failure of lateral specification. Expression of ato then fades from *Su(H)* mutant R8 cells at the same time as from wild-type cells. Thus, like the *E(spl)-C*, *Su(H)* is required for lateral specification but not for the proneural function of *Notch* in the retina.

Interestingly, although many extra R8 precursors form in *Su(H)* mutant clones, not all *Su(H)* mutant cells maintain ato expression or subsequently express the R8-specific Boss protein. Instead clusters of R8-like cells often seem interspersed with non-R8 neurons (Fig. 6D,J). As noted by Jarman et al. (1995), ato expression in wild type first becomes patterned into regular 'intermediate groups' of about ten ato-expressing cells before resolving to individual R8 precursors. Our results support previous conclusions that initial spacing of intermediate groups is not part of the *N*-dependent lateral specification process (Baker and Zitron, 1995; Lee et al., 1996), and so does not depend on *E(spl)* or *Su(H)*.

DISCUSSION

For many of the developmental decisions mediated by Notch, the signal transduction pathway is known in outline. Activated Su(H) protein transcribes target genes, which include bHLH proteins from the E(spl)-C during lateral specification, or other positive factors during inductive processes (Artavanis-Tsakonas et al., 1995; Lewis, 1996). Two successive roles of *N* signaling have been described during R8 cell specification in the developing eye. First, *N* is required for the full level of *ato* expression and function, so that neural differentiation fails in *N* null mutant clones. Later *N* signalling represses *ato* expression during lateral specification. Extra R8 cells are produced if *N* is inhibited during lateral specification with a temperature-sensitive allele (Baker and Yu, 1997). We have now determined the respective roles of the *Su(H)* locus and E(spl)-C. Our major conclusion is that while lateral specification of the R8 cells requires *Su(H)* and *E(spl)*, earlier proneural signalling requires neither and must differ from both lateral specification and induction by using other signal transduction components whose identity is not yet known.

E(spl) genes mediate lateral specification but not proneural enhancement

The phenotype of *E(spl)* mutant cells is dramatically different from that of *N* mutant clones (Fig. 4). *E(spl)* mutant cells show an autonomous neurogenic phenotype in which nearly all cells differentiate as neurons, many of which are R8 photoreceptor cells. This showed that *E(spl)*-C was not required for neural differentiation and cannot be essential for the proneural enhancement for which *N* and *Dl* are required. This conclusion applies to all the *E(spl)* bHLH genes, *gro* and *m4*, even although at least some of these genes seem to be expressed in response to the proneural *N* signalling (Baker and Yu, 1997; Baker et al., 1996). However, lateral specification failed in *E(spl)* clones, showing that some of the bHLH genes and probably *gro* are required for lateral specification of R8 cells. This is consistent with the characterization of E(spl) bHLH proteins and gro as repressors that inhibit proneural gene expression and function in other parts of the nervous system (Jennings et al., 1994; Paroush et al., 1994; Oellers et al., 1994; Dawson et al., 1995; Jarriault et al., 1995; Nakao and Campos-Ortega, 1996; Fisher et al., 1996; Giebel and Campos-Ortega, 1997).

Su(H)-independent Notch signalling

At the developing wing margin, inductive Notch signalling can occur in the absence of *E(spl)*, but is still dependent on *Su(H)* to induce expression of other target genes. Our data showed

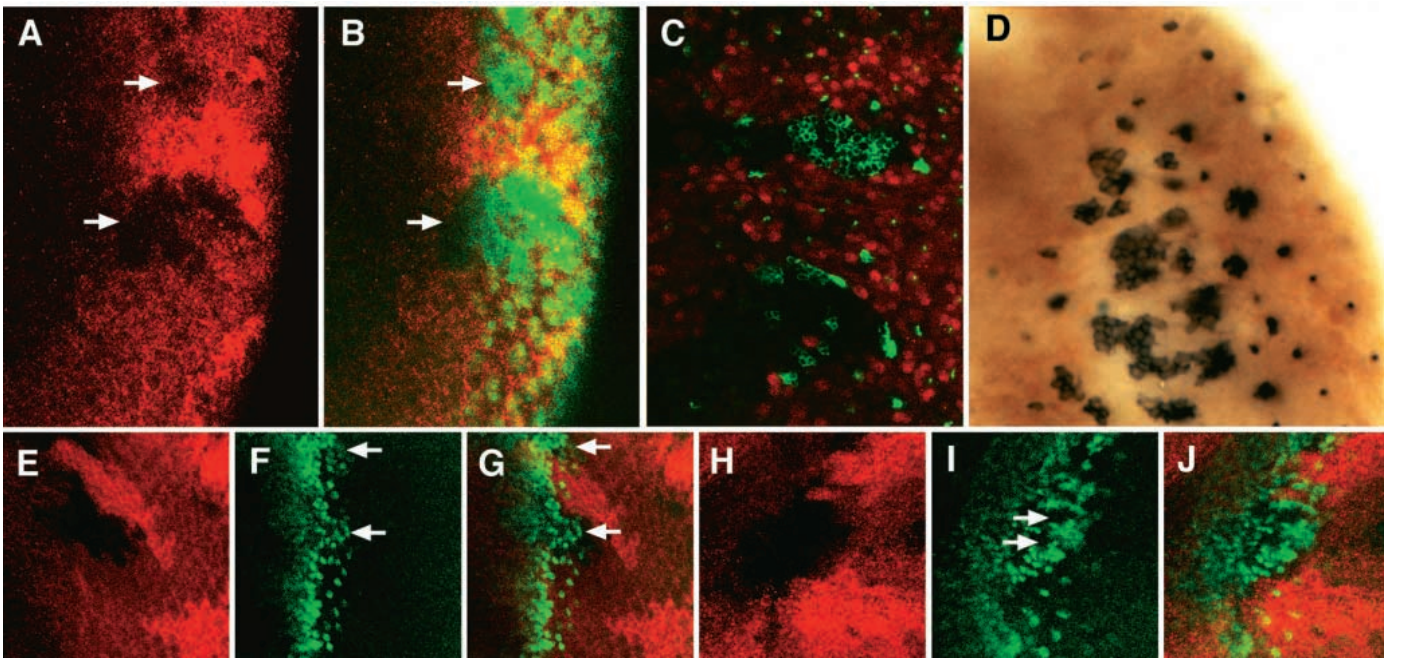

Fig. 6. Eye development in the absence of Su(H). (A) Arrows indicate *Su(H)* mutant cells, identified by lack of β-galactosidase expression (red). (B) Same image merged with Elav expression (green). *Su(H)* mutant cells show autonomous neural hypertrophy. (C) Boss expression (green) reveals clusters of extra R8 cells developing from *Su(H)* mutant cells lacking the β-galactosidase marker (red). Although most or all of the *Su(H)* mutant cells differentiate as neurons (see B), not all express the R8-specific Boss protein. (D) A second specimen in which *Su(H)*$^{-}$ cells lack a nuclear myc-epitope marker (brown). Boss expression (blue-black) shows a clear appearance of clusters of extra R8 cells within the *Su(H)* mutant region. (E-G) and (H-J) show two *Su(H)* mutant clones doubly labelled for *Su(H)* mutant cells, identified by lack of β-galactosidase expression (red; E,H), ato expression (green; F,I) and the merged images (G,J). In wild type, broad ato expression is replaced by R8-specific expression for 3-4 columns. In *Su(H)* mutant cells, excess cells continue ato expression (green), e.g. arrows in F and G. Note that some *Su(H)* mutant cells do not express ato (e.g. arrows in I and J), consistent with the clusters of Boss-expressing R8 cells seen at later stages (C and D).

that proneural enhancement in the eye differed and occurred independently of *Su(H)*. Later, *Su(H)* was required for lateral specification mediated by the *E(spl)*-C. In *Su(H)* mutant cells, *ato* expression was initiated normally but subsequently persisted in too many cells, leading to ectopic R8 cell differentiation and neural hypertrophy (Fig. 5). We showed previously that the proneural enhancement is mediated by the intracellular domain of Notch (Baker and Yu, 1997). Taken together these findings imply a distinct signaling mechanism downstream of Notch that permits proneural enhancement to occur independently of *Su(H)* function, and which also does not require the *E(spl)*-C bHLH genes or *gro*.

Several prior studies have given indications that *Su(H)*-independent signalling might occur. These have included studies of the *C. elegans* gene *lag-2*, a *Su(H)* homolog, which did not employ null mutations (Lambie and Kimble, 1991), and effects in several systems of misexpressing ankyrin-repeat portions of the Notch intracellular domain (Roehl and Kimble, 1993; Shawber et al., 1996; Matsuno et al., 1997). As this portion of Notch binds Su(H) poorly (Tamura et al., 1995), these misexpression effects might be *Su(H)*-independent. However, an additional interaction between Su(H) and the ankyrin-repeat region of the receptor has now been implicated in at least some of these examples (Fortini and Artavanis-Tsakonas, 1994; Roehl et al., 1996; Kato et al., 1997). Our data, and also a study of the role of Notch in regulating *single-minded* gene expression in the embryonic mesectoderm (Lecourtois and Schweisguth, 1995), show definitively through studies of loss-of-function mutations that there are functions of the wild-type Notch protein that occur in apparently *Su(H)* null mutant cells but not in *N* null mutant cells, and so must be independent of Su(H).

Qualitative differences between E(spl) bHLH proteins

Although we did not find distinct proneural functions of any E(spl)-C genes, there was evidence for other differences between them, based on forced expression. We find that *mδ* represses *ato* expression and function much more efficiently than either *m5* or *mβ* do (Fig. 5). Because *m5* or *mβ* expression causes earlier lethality than *mδ* expression, it is difficult to account for this except by functional differences between distinct bHLH genes. Lack of point mutations affecting individual E(spl) bHLH proteins has led to the conclusion that these genes are redundant, but the gene complex has nevertheless been conserved through evolution (Schrons et al., 1992; Maier et al., 1993). It may be that the seven bHLH proteins show overlapping functions, not identical ones. The distinct expression patterns of individual genes suggest they may differ in function and so contribute to the specificity of *N* responses in particular tissues (de Celis et al., 1996a).

Taken together with expression studies that show mδ protein spatially replacing ato protein during lateral specification of R8 cell fate (Baker et al., 1996; Dokucu et al., 1996), our results

point to a major role for *mδ* in this particular instance of lateral specification. However, both *mδ* and *mγ*, which is expressed similarly in the eye, can be deleted from the genome without affecting eye development (The et al., 1997). At least one other E(spl) bHLH protein must be able to substitute. A candidate is m8, whose overexpression and mutation in the *E(spl)^D* mutation can suppress eye neurogenesis in the presence of the *N* mutant allele *spl* (Campos-Ortega and Knust, 1990).

Role of ligands

Both proneural *N* signalling and lateral specification require activation of *N* by *Dl* but no role can be demonstrated for *Ser*, even in the absence of *Dl*. Cells mutant for *Dl*, or for both *Dl* and *Ser*, fail to undergo neural differentiation although the phenotype is rescued nonautonomously near the boundary with wild-type cells (Fig. 2). Proneural signalling seems to be rescued over a greater range than lateral specification, so that neurogenic clusters of R8 cells can differentiate from cells where proneural enhancement occurred but lateral specification did not. Because of this greater range for proneural signalling, described previously for *Dl* mutant cells (Baker and Yu, 1997), we had considered the possibility of a relay in which signalling by *Dl* from wild-type cells activated *Ser* to transmit a proneural signal further into the clone, but the similar phenotype of *Dl,Ser* double mutant cells to that of *Dl* mutants alone rules out this model. The basis of the greater range for proneural signalling than lateral inhibition remains unknown.

Divergent signalling downstream of Notch

The independence of proneural enhancement from *Su(H)* and *E(spl)*-C may suggest explanations for how these two functions of *N* can occur sequentially in the same cells. It is possible that the unidentified proneural signalling pathway may act on *ato* more directly than *Su(H)*, which has first to activate *E(spl)* expression, so ensuring that proneural enhancement precedes lateral specification. Alternatively, the two pathways might be activated by different levels of *N* activation. Proneural signaling would be replaced by lateral specification when rising levels of *N* activation become sufficient to activate *Su(H)*. Finally, elevated *ato* expression might contribute directly to lateral inhibition, if *E(spl)* genes require both Su(H) and a proneural activator (ato) for transcription.

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REFERENCES

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signalling. *Science* **268**, 225-232.

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of Hairless directly activates transcription of *Enhancer of split* Complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.

Baker, N. E. and Yu, S. (1997). Proneural function of neurogenic genes in the developing *Drosophila* eye. *Curr. Biol.* **7**, 122-132.

Baker, N. E., Yu, S. and Han, D. (1996). Evolution of proneural atonal expression during distinct regulatory phases in the developing *Drosophila* eye. *Curr. Biol.* **6**, 1290-1301.

Baker, N. E. and Zitron, A. E. (1995). *Drosophila* eye development: *Notch* and *Delta* amplify a neurogenic pattern conferred on the morphogenetic furrow by *scabrous. Mech. Dev.* **49**, 173-189.

Campos-Ortega, J. A. and Knust, E. (1990). Defective ommatidial cell assembly leads to defective morphogenesis: a phenotypic analysis of the *E(spl)^D* mutation of *Drosophila melanogaster. Roux's Arch. Dev. Biol.* **198**, 286-294.

Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of Xotch diverts cellfate in Xenopus embryos. *Cell* **73**, 659-671.

Couso, J. P., Knust, E. and Martinez Arias, A. (1995). *Serrate* and *wingless* cooperate to induce *vestigial* gene expression and wing formationm in *Drosophila. Curr. Biol.* **5**, 1437-1448.

Dawson, S. R., Turner, D. L., Weintraub, H. and Parkhurst, S. M. (1995). Specificity for the Hairy/Enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. *Mol. Cell. Biol.* **15**, 6923-6931.

de Celis, J. F., de Celis, J., Ligoxygakis, p., Preiss, A., Delidakis, C. and Bray, S. (1996a). Functional relationships between Notch, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal development. *Development* **122**, 2719-2728.

de Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996b). Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-369.

Delidakis, C. and Artavanis-Tsakonas, S. (1992). The *Enhancer of split* [*E(spl)*] locus of *Drosophila* encodes seven independent helix-loop-helix proteins. *Proc. Nat. Acad. Sci. USA* **89**, 8731-8735.

Doherty, D., Feger, G., Younger-Sheperd, S., Jan, L. Y. and Jan, Y. N. (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation. *Genes Dev.* **10**, 421-434.

Dokucu, M. E., Zipursky, S. L. and Cagan, R. L. (1996). Atonal, Rough and the resolution of proneural clusters in the developing *Drosophila* retina. *Development* **122**, 4139-4147.

Eastman, D. S., Slee, R., Skoufos, E., Bangalore, L., Bray, S. and Delidakis, C. (1997). Activated Notch regulation of *Enhancer of split mγ* and *mδ* expression is mediated by Suppressor of Hairless. *Mol. Cell. Biol.* **17**, 5620-5628.

Ellis, M. C., Weber, U., Wiersdorff, V. and Mlodzik, M. (1994). Confrontation of *scabrous* expressing and non-expressing cells is essential for normal ommatidial spacing in the *Drosophila* eye. *Development* **120**, 1959-1969.

Fisher, A. L., Ohsako, S. and Caudy, M. (1996). The WRPW motif of the Hairy-related basic Helix-Loop-Helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol. Cell. Biol.* **16**, 2670-2677.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The Suppressor of Hairless protein participates in Notch signaling. *Cell* **79**, 273-282.

Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. *Nature* **365**, 555-557.

Giebel, B. and Campos-Ortega, J. A. (1997). Functional dissection of the *Drosophila* Enhancer-of-split protein, a suppressor of neurogenesis. *Proc. Nat. Acad. Sci. USA* **94**, 6250-6254.

Heitzler, P., Bourois, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* **122**, 161-171.

Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1994). *atonal* is the proneural gene for *Drosophila* photoreceptors. *Nature* **369**, 398-400.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-358.

Jennings, B., Preiss, A., Delidakis, C. and Bray, S. (1994). The Notch signalling pathway is required for *Enhancer of split* bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development* **120**, 3537-3548.

Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura-

Okazaki, S., Tamura, K. and T., H. (1997). Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development* **124**, 4133-4141.

Kim, J., Irvine, K. D. and Carroll, S. B. (1995). Cell recognition, signal induction and symmetrical gene activation at the dorsal-ventral boundary of the developing *Drosophila* wing. *Cell* **82**, 795-802.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, M., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila vestigial* gene. *Nature* **382**, 133-138.

Kimmel, B. E., Heberlein, U. and Rubin, G. M. (1990). The homeo domain protein rough is expressed in a subset of cells in the developing *Drosophila* eye where it can specify photoreceptor cell subtype. *Genes Dev.* **4**, 712-727.

Knust, E., Schrons, H., Grawe, F. and Campos-Ortaga, J. A. (1992). Seven genes of the *Enhancer of split* complex of *Drosophila melanogaster* encode helix-loop-helix proteins. *Genetics* **132**, 505-518.

Kramer, H., Cagan, R. L. and Zipursky, S. L. (1991). Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. *Nature* **352**, 207-212.

Lambie, E.J. and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* **112**, 231-240.

Lecourtois, M. and Schweisguth, F. (1995). The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the Enhancer of split Complex genes triggered by Notch signaling. *Genes Dev.* **9**, 2598-2608.

Lee, E.-C., Hu, X., Yu, S. Y. and Baker, N. E. (1996). The *scabrous* gene encodes a secreted glycoprotein dimer and regulates proneural development in *Drosophila* eyes. *Mol. Cell. Biol.* **16**, 1179-1188.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **6**, 3-10.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V. and Young, M. W. (1993). Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function of *Notch* in nuclei. *Genes Dev.* **7**, 1949-1965.

Maier, D., Marte, B. M., Schafer, W., Yu, Y. and Preiss, A. (1993). *Drosophila* evolution challenges postulated redundancy in the E(spl) gene complex. *Proc. Nat. Acad. Sci. USA* **90**, 5464-5468.

Matsuno, K., Go, M. J., Eastman, D. S. and Artavanis-Tsakonas, S. (1997). Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements. *Development* **124**, 4265-4273.

Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.

Nakao, K. and Campos-Ortega, J. A. (1996). Persistent expression of genes of the *Enhancer of split* complex suppresses neural development in Drosophila. *Neuron* **16**, 275-286.

Oellers, N., Dehio, M. and Knust, E. (1994). bHLH genes encoded by the *Enhancer of split* complex of Drosophila negatively interfere with transcriptional activation mediated by proneural genes. *Mol. Gen. Genet.* **244**, 465-473.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.

Paroush, Z., Finley, R. L. J., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* **79**, 805-815.

Roehl, H. and Kimble, J. (1993). Control of cell fate in *C. elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. *Nature* **364**, 632-635.

Roehl, H., Rosenberg, M., Blelloch, R. and Kimble, J. (1996). Roles of the RAM and ANK domains in signalling by the *C. elegans* GLP-1 receptor. *EMBO J.* **15,** 7002-7012.

Schrons, H., Knust, E. and Campos-Ortega, J. A. (1992). The *Enhancer of split* Complex and adjacent genes in the 96F region of *Drosophila melanogaster* are required for segregation of neural and epidermal progenitor cells. *Genetics* **132**, 481-503.

Schweisguth, F. (1995). *Suppressor of Hairless* is required for signal reception during lateral inhibition in the *Drosophila* pupal notum. *Development* **121**, 1875-1884.

Shawber, C., Nofziger, D., Hsieh, J. J.-D., Lindsell, C., Bögler, O., Hayward, D. and Weinmaster, G. (1996). Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development* **122**, 3765-3773.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the *lin-12* and *Notch* intracellular domains in vivo. *Cell* **74**, 331-345.

Sun, X. and Artavanis-Tsakonas, S. (1996). The intracellular deletions of DELTA and SERRATE define dominant negative forms of the *Drosophila* Notch ligands. *Development* **122**, 2465-2474.

Tamura, K., aniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T. and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J*k*/Su(H). *Curr. Biol.* **5**, 1416-1423.

The, I., Hannigan, G. E., Cowley, G. S., Reginald, S., Zhong, Y., Gusella, J. F., Hariharan, I. K. and Bernards, A. (1997). Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. *Science* **276**, 791-794.

Tio, M. and Moses, K. (1997). The *Drosophila* TGFalpha homolog Spitz acts in photoreceptor recruitment in the developing retina. *Development* **124**, 343-351.

Treisman, J. E., Luk, A., Rubin, G. M. and Heberlein, U. (1997). *eyelid* antagonizes *wingless* signaling during *Drosophila* development and has homology to the Bright family of DNA-binding proteins. *Genes Dev.* **11**, 1949-1962.

Vincent, J., Girdham, C. and O'Farrell, P. (1994). A cell-autonomous, ubiquitous marker for the analysis of *Drosophila* genetic mosaics. *Dev. Biol.* **164**, 328-331.

Wolff, T. and Ready, D. F. (1993). Pattern formation in the *Drosophila* retina. In *The Development of Drosophila melanogaster*, (ed. M. Bate and A. Martinez Arias), pp. 1277-1325: Cold Spring Harbor Laboratory Press.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in the developing and adult *Drosophila* tissues. *Development* **117**, 1223-1236.

Note added in proof

A Su(H)-independent function for N was also reported by Wang et al. (1997) *Development* **124**, 4435-4446.