ERRATUM

Notch activity in neural cells triggered by a mutant allele with altered glycosylation

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Several errors in this article were not corrected before going to press.

The name of the second author is Liang Lei.

On p. 2830, two fly strains were incorrectly referenced. The text should read: *armlacZ* transgenes (Vincent et al., 1994); and *md0.5-Lacz* (Cooper and Bray, 1999).

On p. 2831, two antibodies were incorrectly referenced. The text should read: guinea pig anti-Senseless (Nolo et al., 2000); and rabbit anti-CM1 (Srinivasan et al., 1998).

We apologise to readers and the authors for these mistakes.

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Notch activity in neural cells triggered by a mutant allele with altered glycosylation

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SUMMARY

The receptor protein Notch is inactive in neural precursor cells despite neighboring cells expressing ligands. We investigated specification of the R8 neural photoreceptor cells that initiate differentiation of each *Drosophila* ommatidium. The ligand Delta was required in R8 cells themselves, consistent with a lateral inhibitor function for Delta. By contrast, Delta expressed in cells adjacent to R8 could not activate Notch in R8 cells. The *split* mutation of *Notch* was found to activate signaling in R8 precursor cells, blocking differentiation and leading to altered development and neural cell death. *split* did not affect other, inductive functions of Notch. The Ile578→Thr578 substitution responsible for the *split* mutation introduced a new site for *O*-fucosylation on EGF repeat 14 of the Notch extracellular domain. The *O*-fucose monosaccharide did not require extension by Fringe to confer the phenotype. Our results suggest functional differences between Notch in neural and non-neural cells. R8 precursor cells are protected from lateral inhibition by Delta. The protection is affected by modifications of a particular EGF repeat in the Notch extracellular domain. These results suggest that the pattern of neurogenesis is determined by blocking Notch signaling, as well as by activating Notch signaling.

Key words: Notch, Delta, Fringe, *O*-fucose, *Drosophila* eye, Neurogenesis, Lateral inhibition

INTRODUCTION

During development, neural cells often arise separated from one another by ectodermal cells. Spacing of neural cells implies a lateral inhibitory signal that prevents neural specification by neighboring cells. This idea predicts that if prospective neural cells were ablated the inhibitory signal would be lost and a neighboring cell would be released to take neural fate. The prediction has been confirmed for grasshopper embryogenesis. It proved impossible to eliminate identified neuroblasts by ablation of single cells from the proneural regions. Neural fate was always taken by one of the cells in the equivalence group (Doe and Goodman, 1985).

The receptor protein Notch (N) appears to be the receptor for the lateral inhibitory signal (Artavanis-Tsakonas et al., 1999). N is required cell autonomously to suppress neural fate, and is sufficient to block neural fate specification when activated in all the cells (Hoppe and Greenspan, 1990; Heitzler and Simpson, 1991; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). These results imply that N activity must remain low in the cells that take neural fate.

The transmembrane protein Delta (Dl) is a ligand for N and is required to inhibit neural fate (Kopczynski et al., 1988; Lehmann et al., 1981; Vassin et al., 1987). Dl is required cell nonautonomously and is thought to encode the lateral inhibitory signal (Fehon et al., 1990; Heitzler and Simpson, 1991). Such a view predicts that Dl expression should be required in the neural cell to signal to other cells, and that if Dl in the non-neural cells was able to activate N in neural precursor cells, neural fate specification would be prevented by non-neural Dl overexpression. Neither of these predictions has yet been tested directly.

One way that N activity could be restricted to some cells would be if N was not expressed in the future neural cells, or if Dl was expressed in the neural cells only. Such reciprocal expression has been reported for the anchor cell equivalence group in the nematode *C. elegans* (Wilkinson et al., 1994). By contrast, in *Drosophila*, N and Dl are expressed homogeneously in both neural and non-neural cells (Baker, 2000). One model proposes that N or Dl are modified or associated with other molecules so that one or both proteins becomes active in only a subset of the locations where they are expressed. Alternatively, it has been suggested that homogenous Dl expression reflects spatially uniform mutual inhibitory signaling, to which non-neural cells make the same contribution as do neural precursor cells (Muskavitch, 1994). In support of the idea that non-neural cells also signal, Dl suppresses neurogenesis in some tissues that lack any neural precursor cells (Parks and Muskavitch, 1993). Mutual inhibition would require some other mechanism to release each neural precursor cell from receiving the homogenous N-activating signals.

N and Dl function in many developmental processes in addition to neural fate specification. For example Dl activation of N is important in the induction of the dorsoventral boundary during wing development (Doherty et al., 1996; Irvine, 1999), in the induction of proneural development in the morphogenetic furrow of the developing eye imaginal disc (Baker and Yu, 1997; Baonza and Freeman, 2001; Li and Baker, 2001), in preventing the recruitment of supernumerary photoreceptor cells to the ommatidia of the developing eye (Cagan and Ready, 1989; Sun and Artavanis-Tsakonas, 1996), in specifying the difference between the R3 and R4 photoreceptor cells of each ommatidium (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999), and in specifying the difference between R7 and R1 or R6 photoreceptor cells of each ommatidium (Cooper and Bray, 2000; Tomlinson and Struhl, 2001). In these inductions, ectopic expression of Dl leads to ectopic activation of N, as predicted if the expression pattern of Dl determines the spatial pattern of normal induction. One qualification is that during wing development Dl activity largely depends on modification of N by the glycosyltransferase Fringe to extend *O*-fucose glycans (Bruckner et al., 2000; Moloney et al., 2000a). Because Fringe is only expressed dorsally, ectopic Dl activates N predominantly in cells of the dorsal compartment of the developing wing (Fleming et al., 1997; Irvine, 1999; Panin et al., 1997). This provides at least one precedent for differential activity of modified N proteins.

We report that mosaic analysis supports the lateral inhibition model of Dl function more than mutual inhibition. This leads us to hypothesize that either N or Dl proteins must be differentially active within the R8 proneural group. We present evidence that R8 cell precursors do not normally respond to Dl, despite expressing N. As one approach to investigating this, we have examined a particular N mutant allele called *split*, which affects eye and bristle development to a greater degree than other aspects of N function. We discover that the *split* mutation renders R8 precursors sensitive to Dl, leading to N activity within the R8 cell, and that the consequences of such neural N signaling include defective specification, differentiation and survival both of R8 cells and of other retinal cells that depend on R8 via other signaling pathways. We show that the amino acid substitution responsible for the *spl* phenotype introduces a site for *O*-fucosylation into EGF repeat 14 of the N extracellular domain, and that although this glycan is a substrate for the glycosyltransferase Fringe, extension by Fringe is not necessary for N activity. We propose that the spatial pattern of N activity in wild type may be determined by interactions that prevent N activity as much as by interactions that activate N.

MATERIALS AND METHODS

Plasmid constructions

The 13th, 14th and 15th EGF repeats of N were PCR amplified from the pMTN plasmid (Fehon et al., 1991) or from a pMTspl plasmid (a gift from S. Artavanis-Tsakonas). Primers were GAAGATCTTGCC-AATCGAATCCCTGC (the *Bgl*II site is underlined) and CCG-CTCGAGTTCGTTGATCTGCTT (the *Xho*I site is underlined). PCR products were digested by *Bgl*II and *Xho*I, and then ligated into the *Bgl*II and *Xho*I sites of the expression vector pMT/BiP/V5-HisA (Invitrogen). The pMT13-15EGFN and pMT13-15EGFspl plasmids were verified by sequencing. *Msc*I *Pme*I fragments of pMT13-15EGFN and pMT1-15EGFspl were transferred into the *Eco*RV and *Pme*I sites of the expression vector pAc5.1/V5-HisA (Invitrogen). The final constructs were named pAc13-15EGFN and pAc13-15EGFspl. The orientation of insertion was verified by double enzyme digestion using *Kpn*I and *Xba*I. The protein encoded by pAc13-15EGFN includes the Bip signal sequence, two amino acids (RS) introduced to generate the construct, amino acids 530 to 641 from the N protein, the V5 tag and the 6His tag. The protein made by pAc13-15EGFspl is the same as above except that the Ile corresponding to residue 578 of wild type N is mutated to Thr.

To mutate Thr540 in the 13th EGF repeat of pAc13-15EGFN and pAc13-15EGFspl into I540, the site-directed mutagenesis kit (Clontech) was used. The oligonucleotides used were: p-CCTGAA-CGATGGAATTTGCCACGACAAGATC (to mutate Thr540 into Ile540) and p-GTGACTGGTGAATACTCAACCAAGTC (to mutate the *Sca*I site for selecting). Products were verified by sequencing.

Cell culture and transfection

Drosophila melanogaster Schneider cells were kept at 25°C in Shields and Sang M3 insect medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and penicillin (50 U/ml)-streptomycin (50 mg/ml) (Gibco). Cells were transfected using lipofectin (Lee et al., 1996). The three EGF repeat proteins were purified from cell media using ProbondTM resin (Invitrogen).

Labeling EGF fragments of N

After elution of EGF polypeptides from metal chelating beads, the buffer from 100-400 μl eluant was exchanged with Glyco buffer (50 mM HEPES pH 7.0, 140 mM NaCl, 10 mM $MnCl_2$, 0.2% Tween-20) by concentration in Centricon filter units, dilution into 400 μl Glyco buffer, and reconcentration to 20 μl. Labeling reactions were conducted by incubating this 20 μl EGF polypeptide with 20 μl [^{14}C]UDP-GlcNAc (25 μCi/ml, AP Biotech), 5 μl purified Fringe:His6 (0.1 μg/μl) (Moloney et al., 2000a) and 5 μl Glyco buffer at 25°C for 2 hours. The reaction mixture was then boiled in SDS-PAGE sample buffer and run on two parallel gels. One gel was subject to western blotting, using Mouse anti V5-HRP (Invitrogen) for detection. The other gel was subject to Fluorography, with Amplify (AP Biotech) for signal enhancement.

Fly strains

Fly strains are as follows.

The *spl* mutation was obtained from R. Cagan.

UAS-N (Seugnet et al., 1997)

UAS-mδ (Ligoxygakis et al., 1999)

UASN$^{\Delta E}$B5a and UASN$^{\Delta E}$B37D were gifts from E. Giniger.

fng^{13} (Irvine and Wieschaus, 1994)

The U*AS-Dl* insertions will be described in more detail elsewhere (Y.L. and N.B., unpublished).

G109-68 (White and Jarman, 2000)

GMRGal4 (Freeman, 1996)

armlacZ transgenes [number 133 from Vincent (Vincent et al., 1994)]

md0.5-Lacz [number 181 from Cooper (Cooper and Bray, 1999)].

Clones were induced by heat shock FLP-mediated recombination of larvae heterozygous for mutants linked to appropriate FRT chromosomes and FRT [*arm-lacZ*] chromosomes (Golic, 1991; Xu and Rubin, 1993). Fly stocks were maintained on standard cornmeal-agar medium at 25°C. Sections of adult retinas were prepared as described (Baker et al., 1990).

Antibodies

Antibody staining was performed as described (Li and Baker, 2001). Monoclonal antibodies specific for β-galactosidase (mAb40-1a) and Elav (rat mAb7E8A10) were obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa,

Department of Biological Sciences, Iowa City IA52242, USA under contract N01-HD-7-3263 from the NICHD. Other antisera were guinea pig anti-Senseless [number 173 from Nolo et al. (Nolo et al., 2000)], rabbit anti-CM1 [number 202 from Srinivasan et al. (Srinivasan et al., 1998)], rabbit anti-Boss (Kramer et al., 1991) and monoclonal anti-Dl (Parks et al., 1995). Secondary antibodies include HRP-, Cy2- or Cy3-conjugated antisera from Jackson Immunoresearch.

RESULTS

Dl encodes a signal for lateral inhibition during R8 specification

One interpretation of homogenous expression patterns of Dl and N during neural specification is that these genes inhibit neural specification equally in all the proneural cells. Such 'mutual inhibition' would be overcome in presumptive neural cells by other mechanisms (Muskavitch, 1994). Mutual inhibition predicts different spatial requirements for Dl function from lateral inhibition. If Dl encodes a lateral inhibitory signal, then Dl should be required in the neural precursor cell. Regardless of how many cells might initially express Dl protein, the cell that finally takes neural fate must express Dl in order to ensure inhibition of its neighbors. If this cell were unable to express Dl, lateral inhibition would be lost just as though the cell had been ablated, and another nearby cell would take the neural fate in its stead. According to the mutual inhibition theory, however, all the proneural cells that express Dl are contributing to inhibition of the entire equivalence group. When the single proneural cell escapes inhibition by some mechanism, the other cells continue to participate in inhibiting one another. This theory suggests Dl expression in the neural precursor cell is no more important than Dl expression in the other cells, and a single neural precursor is just as likely to result from a Dl mutant cell as from a cell next to a Dl mutant cell.

Although cell nonautonomy of Dl function is well established, the focus of Dl function has never been mapped precisely within proneural groups to determine whether normal patterning can occur when a single neural precursor cell is mutant for *Dl*. It has been reported that single *Dl* mutant cells transplanted into wild-type host embryos can take neural fates, consistent with the mutual inhibition model, but in these experiments the transplanted cells may not all integrate into proneural regions (Technau and Campos-Ortega, 1986). In the case of thoracic microchaete bristles a modest bias against neural specification by cells with lower *Dl* gene dose, and increased levels of Dl signal from ectopic microchaete together support a lateral inhibition model for this class of epidermal sense organ (Heitzler and Simpson, 1991; Heitzler and Simpson, 1993).

The ideal experiment of removing *Dl* function from single cells and determining their fate is difficult to achieve by mitotic recombination because of perdurance. A single recombinant cell that has lost the *Dl* gene may not lose Dl mRNA and protein immediately. A suitable opportunity arises during *Drosophila* eye development because of regulation of both Dl expression and cell cycle progression (Fig. 1A). Founding R8 photoreceptor neurons are specified during an extended G1 arrest of the cell cycle (Wolff and Ready, 1993). Loss of the *Dl* gene by mitotic recombination would have to occur at or before the preceding mitosis, anterior to the morphogenetic furrow. Dl protein levels drop below the threshold of detection before the G1 arrest, and Dl protein that appears during R8 specification is the product of new transcription which would be absent from a recombinant cell mutant for *Dl* (Parks et al., 1995; Baker and Yu, 1998). Thus, any genetically *Dl* mutant R8 cell must have undergone R8 specification in the absence of both the *Dl* gene and its products.

Mitotic recombination was induced late in larval development to generate *Dl*-null mutant cells ahead of the morphogenetic furrow. Such cells give rise to clones of single or small numbers of *Dl* mutant cells that lose all Dl product prior to R8 specification. The resulting adult eyes were sectioned, and the cellular contribution of *Dl* mutant cells to ommatidium development recorded (Fig. 1B,C). In the majority of cases, presence of *Dl* mutant cells in mosaic regions was associated with changes in the number of photoreceptor cells. Both ommatidia with too many neural cells and ommatidia with too few were observed. Neither category was analyzed in detail. More rarely, ommatidia containing one or more *Dl* mutant cells differentiated eight photoreceptor cells in the normal arrangement. Such cases

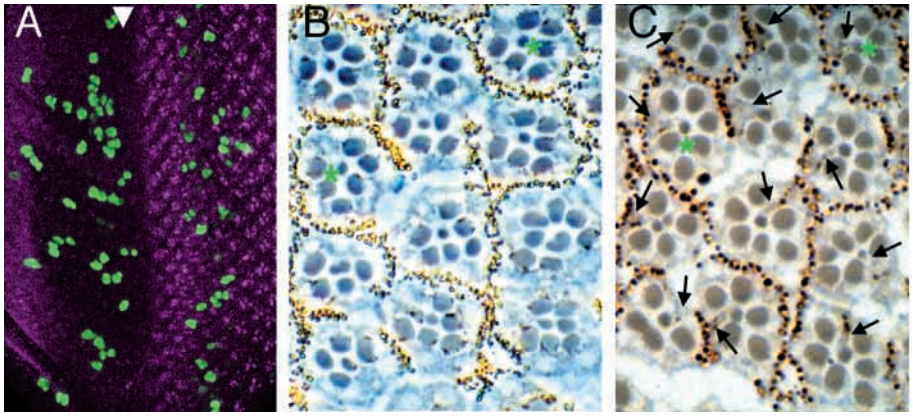

Fig. 1. Mosaic analysis with *Dl*. (A) Eye imaginal disc (anterior towards the left). Dl protein (magenta) accumulates in the differentiating region posterior to the morphogenetic furrow (arrowhead). Mitotic cells (labeling for phosphorylated histone H3 in green) are absent from the morphogenetic furrow region where the cell cycle is arrested. R8 cells are always specified from cells born anterior to the morphogenetic furrow when Dl protein is undetectable. (B) Phase-contrast micrograph of an eye section through the apical R7 level. Photoreceptor cells homozygous mutant for *Dl* are detected by absence of dark pigment granules at the rhabdomere base. Two ommatidia in this section lack any *Dl* mutant photoreceptor cells (green stars). All the other ommatidia contain one or more mutant cells. A variety of developmental defects are seen in these ommatidia. (C) Bright-field micrograph through the same eye at the basal R8 level. Every R8 cell is pigmented and therefore wild type for *Dl* (arrows).

were scored and the individual *Dl* mutant cells identified from position and morphology. Eighty-eight *Dl* mutant photoreceptor cells were identified that had not altered ommatidial construction. Their identities are summarized in Table 1. Each of the R1-R7 photoreceptor cells was able to contribute to a normally constructed ommatidium without the *Dl* gene. As reported by others, loss of *Dl* function from R3 led to exchange of R3 and R4 fates and chiral reversal, and simultaneous loss of *Dl* function from R1 and R6 led R7 to adopt R1/6-like morphology (Cooper and Bray, 1999; Cooper and Bray, 2000; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999; Tomlinson and Struhl, 2001). R4 was the most common Dl^- cell because of the adoption of R4 fate by *Dl* cells that otherwise would have become R3. Dl^- R2 and R5 cells were recovered rarely, suggesting that ommatidia with mutant R2 or R5 cells are unlikely to develop normally. We have not investigated the role of *Dl* in R2 and R5 further. It may be to inhibit nearby unspecified cells from recruitment to photoreceptor cell fate (Sun and Artavanis-Tsakonas, 1996) (L. Yang and N.B., unpublished). By contrast, no *Dl* mutant R8 cell was ever observed in a normal ommatidium. R8 is the only photoreceptor cell for which this was true, consistent with the notion that the role of R8 is never taken by a cell unable to express Dl.

Previous studies of eye development in *Dl* mutants, or of large clones of *Dl* mutant cells, show that excess R8 cells are specified in the wholesale absence of *Dl* (Baker and Zitron, 1995; Parks et al., 1995; Baker and Yu, 1997). Thus, *Dl* mutant cells can initiate R8 differentiation. The failure of single *Dl* mutant cells to take R8 fate must be due to the presence of nearby *Dl*/+ cells that inhibit R8 specification on the part of the *Dl* mutant cells. The simplest interpretation is that *Dl* encodes a signal sent from R8 to prevent multiple cells taking R8 fate. The data exclude the mutual inhibition model that *Dl* functions equally among the cells in which it is expressed, because *Dl* was more important in the R8 cell than in the neighboring R2, R3, R4 and R5 cells. The data do not exclude more complicated models in which *Dl* is required to release R8 precursors from mutual inhibition, as well as for mutual inhibition itself.

Table 1. Dl^- cells in mosaic ommatidia with normal complements of photoreceptor cells

R1	R2	R3	R4	R5	R6	R7	R8	Total
7	3	2*	50†	3	11	12‡	0	88

**Dl* is required in R3 cells only if the neighboring R4 cell is Dl^+. Two Dl^- R3 cells were observed in ommatidia where R4 was also Dl^-.

†The total includes R4 cells in chirally reversed ommatidia that were otherwise constructed normally.

‡The total includes R7 cells that adopted R1/6 morphology in ommatidia that were otherwise constructed normally.

R8 was unique in that *Dl* function is absolutely required, unlike the R2/3/4/5 cells that are nearby during R8 selection. Frequency of Dl^- R8 cells (0/88) was significantly lower than 3/88 seen for R2 or R5 cells (95% confidence limit; binomial distribution).

Based on the higher recovery of Dl^- R4 cells and R7 cells, it is possible that that *Dl* expressed in each of the other photoreceptor cells may play some role in normal photoreceptor recruitment (see text). It is unlikely that possible sibling relationships of other R cells to R8 is sufficient to explain their reduced frequency in normal ommatidia, because in this experiment many Dl^- clones include only one cell.

Dl does not activate N in R8 precursor cells

If Dl acts a lateral inhibitor from the R8 cell despite apparently homogenous expression, perhaps Dl protein expressed in cells neighboring the R8 precursor is less able to activate N in the R8 precursor than Dl protein in the R8 precursor is able to activate N in the neighboring cells. We determined the consequences of ectopic Dl expression to test this model. UAS-Dl transgenes were expressed posterior to the morphogenetic furrow using the GMR:Gal4 driver. Ectopic N activation at this stage causes loss of R8 cells and prevents differentiation of other neural cell types (Baker et al., 1996) (see below). Ectopic Dl expression led to abnormal, rough eyes in the adult (Fig. 2A,B). Labeling imaginal discs with neural-specific antibodies showed missing photoreceptor cells (data not shown), but R8-specific markers revealed no change in the number or distribution of R8 cells (Fig. 2C,D). R8 cells are eliminated when Ser or intracellular effectors of N signaling are expressed by GMR-Gal4 (not shown). We examined many different UAS-Dl insertion lines conferring varying levels of Dl function without observing effects on R8 specification, even though the strongest lines were associated with ectopic Dl expression levels higher than those of the endogenous protein. Gal4 lines that drive UAS:Dl expression earlier in eye development do not eliminate R8 cells either (Baonza and Freeman, 2001; Li and Baker, 2001) (Y.L. and N.E.B., unpublished). Because the same UAS-Dl transgenes did activate N during wing development, leading to formation of ectopic wing margin (Lee et al., 2000), did activate N when expressed anterior to the morphogenetic furrow, leading to accelerated furrow

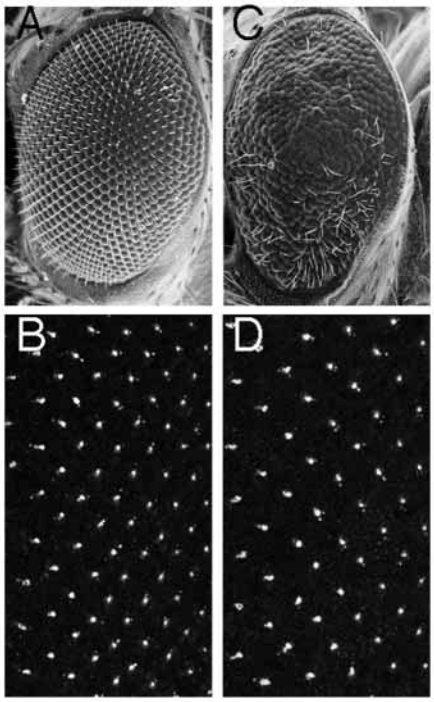

Fig. 2. *Dl* overexpression. (A,C) Adult eyes (anterior towards the left). (B,D) Details of eye imaginal discs labeled for the R8-specific protein Boss. (A,B) The wild-type pattern of ommatidia (A) and R8 cells (B). (C,D) Similar numbers of ommatidia and R8 cells in the GMR>Dl genotype (the adult eye is abnormal because of developmental defects that occur after R8 specification).

progression (Li and Baker, 2001), and also repressed neurogenesis of photoreceptor neurons other than R8 cells (data not shown), we conclude that N in R8 precursor cells is particularly insensitive to activation by Dl.

The *split* mutant allele is specific for neural cells

Our results suggest that different forms or complexes of N or its ligands might be present in R8 cells. N in R8 cells might be unable to respond to Dl, or only the Dl in R8 cells might be able to activate N on neighboring cells. If this hypothesis was correct, then particular mutations in relevant domains of N or its ligands might affect R8 specification more than N functions in other tissues where all cells can respond to ligands. We have focused on a mutation called *split* that corresponds to a substitution of Thr for Ile578 in EGF repeat 14 of the N extracellular domain (Hartley et al., 1987; Kelley et al., 1987). The *spl* mutation recessively affects eye and bristle formation but not wing margin formation, even though the wing margin is normally the most sensitive tissue to reduction in N function. Unlike R8 specification, wing margin induction is sensitive to ectopic Dl expression (Doherty et al., 1996).

The *spl* mutant eyes are smaller, have reduced numbers of ommatidia, and frequently lack ommatidial cells (Cagan and Ready, 1989). The *spl* mutation was reported not to affect specification of bristle precursor cells but altered their differentiation, causing both missing and duplicated external bristle shafts (Lees and Waddington, 1942).

Previously, mosaic analysis determined that in the eye the *spl* phenotype depended on the genotype of R8 cells. Mosaic ommatidia with *spl* mutant R8 cells developed normally only 40% of the time (Baker et al., 1990). As N is normally inactive in R8 precursor cells, the mosaic analysis indicates inappropriate N activity in R8 cells. The *spl* phenotype was investigated further to determine the nature of the N activity.

As described previously, the smaller eyes of *spl* mutants are associated both with fewer ommatidia and with ommatidia containing less than the normal complement of differentiated cells (Cagan and Ready, 1989; Campos-Ortega and Knust, 1990). When molecular markers for R8 specification are examined, fewer R8 cells were seen, with greater separation than in wild type (Baker et al., 1990; Nagel and Preiss, 1999) (Fig. 3A,B). In addition, we noticed that the expression level of R8 genes varied within individual cells. One example is the nuclear protein Senseless, which is required for proper R8 differentiation (Frankfort et al., 2001). Whereas in wild type each R8 cell expresses a uniform level of the Senseless protein appropriate for its developmental age, in the *spl* mutant Senseless expression levels varied between normal and much lower levels, as though some cells were adopting R8 fate less successfully than others (Fig. 3A,B). In addition, the proneural groups from which R8 cells emerge frequently contained fewer cells and lower levels of Senseless than in wild type (Fig. 3A,B).

Next we tested the role of cell death in the *spl* phenotype. Fewer than one cells dies per ommatidium in wild-type eye imaginal discs (Wolff and Ready, 1991; Yu et al., 2002) (Fig. 3A). Cell death was elevated in *spl* eye discs (Fig. 3B). In order to determine the identity of the dying cells, *spl*, GMRp35 eye discs were examined. GMRp35 prevents eye disc cells death but caspase zymogen cleavage continues in the protected cells and can be detected with an antibody (Hay et al., 1994; Yu et al., 2002). Caspase activation occurred in undifferentiated cells surrounding the *spl* ommatidia, in cells that would be

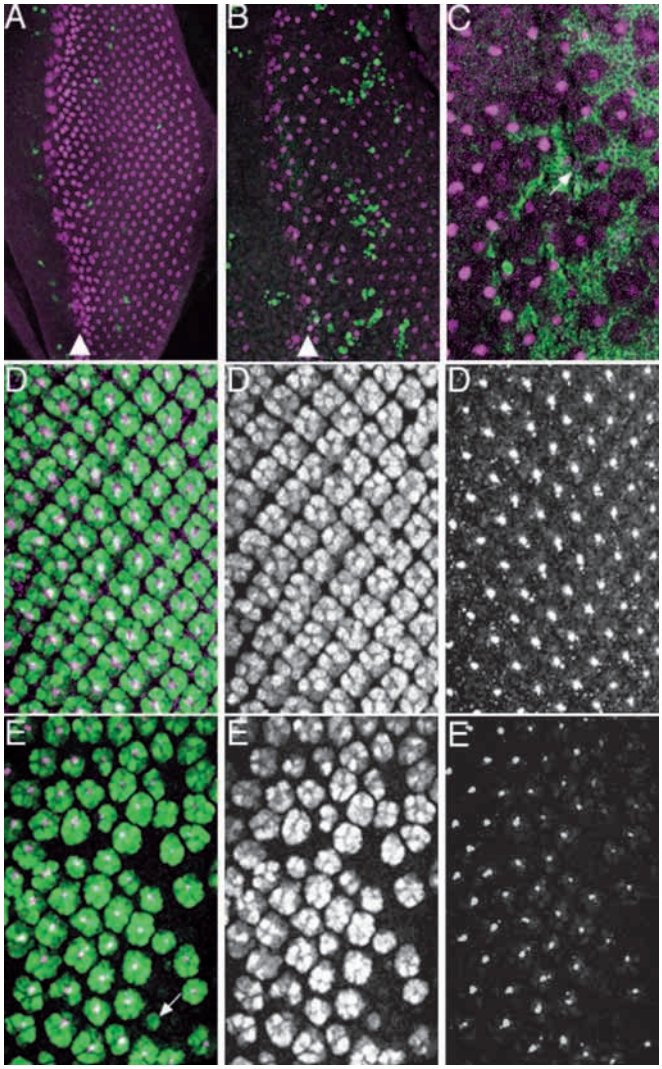

Fig. 3. Defective development in *spl* mutants. (A) Wild-type eye disc labeled for Senseless protein (magenta) and apoptotic cells with activated caspases (green). Posterior to column 0 in the morphogenetic furrow, Senseless labels one R8 cell in each ommatidium. Anterior to column 0, Senseless is expressed in a single column of 'intermediate groups', clusters of six to ten cells from which each R8 cell emerges. (B) *spl* mutant eye disc labeled as in A. Anterior to column 0, the intermediate groups contain fewer cells often expressing Senseless at lower levels than wild type. Posterior to column 0 many R8 cells are missing and those remaining express Senseless at varying levels. In the posterior of the eye disc cell death is elevated compared with wild type. (C) Detail from *spl*; GMRp35 eye disc. Cell death is prevented but caspase activation still occurs. Arrowhead indicates caspase activation in an R8 cell. R8 expression of Senseless is variable despite the blockade to cell death. (D) Wild-type eye disc labeled for the photoreceptor marker ELAV (green; left and middle) and R8-specific marker Boss (magenta; left and right). Note the consistent level of Boss expression. (E) *spl*; GMRp35 labeled as in D. Note the inconsistent Boss expression levels and variable numbers of photoreceptor cells. Arrow indicates a one-cell ommatidium lacking any Boss-expressing R8 cell.

differentiating photoreceptor cells in wild type and in some R8 precursor cells (Fig. 3C).

If cell death was the primary effect of *spl*, then preventing cell death would rescue the spl phenotype. We found, however, that many R8 cells were missing in *spl*; *GMRp35* eye discs, similar to *spl* (Fig. 3D,E). Other photoreceptor cells were also missing from many of the ommatidia. In a few cases, we observed ommatidia where the R8 cells were absent but other photoreceptor cell types present (Fig. 3D,E). As all other R cells depend on R8 for recruitment (Jarman et al., 1994), this should not be observed unless some R8 precursor cells stop differentiating after recruiting other R cells. Taken together, these results indicate that the *spl* mutation causes the failure to specify and maintain R8 cells and other photoreceptor cells. In addition, a proportion of R8 cells, other photoreceptor cells and unspecified cells undergo apoptosis.

Elevating N signaling in R8 cells mimics the *spl* phenotype

If N signaling in the R8 cells is the basis of the *spl* phenotype, ectopic activation of N signaling in the wild-type R8 cells should mimic *spl*. The UAS/Gal4 target gene expression system was used to elevate N signaling only in R8 cells. The Gal4 driver G109-68 was used to express the N intracellular domain specifically in R8 cells (White and Jarman, 2000). R8 cells were missing or expressed lower levels of the R8 marker Boss (Fig. 4A,B). Despite the R8 specific expression, other photoreceptor cells were also absent, cell death was elevated and the adult eyes were small and rough (Fig. 4A,B; data not shown). The phenotype was similar to that of the *spl* mutation, but stronger (Fig. 4C). Similar defects were obtained with a range of lower penetrances when G109-68 was used to drive R8 expression of N intracellular domain from a weaker UAS insertion line (Fig. 4D), R8 expression of full-length N (Fig. 4E) or R8 expression of the N target gene *E(spl)-mδ* (Fig. 4F). These eye discs closely resembled those from *spl* mutants (Fig. 4C). These results show that N activity in R8 cells reduces the neural differentiation and survival of other ommatidial cells as a secondary consequence of abnormal R8 development. They bolster the conclusion from mosaic analysis, that all aspects of the *spl* mutant phenotype depend on N activity in R8 cells, and suggest that such activity is mediated by N intracellular domain and E(spl) expression in the same way as canonical N signaling. We were unable to detect expression of N target genes from the E(spl)-C in R8 cells from the *spl* mutant using antibodies (data not shown). Low level or transient expression might be effective, however, as it is also difficult to detect E(spl) and Ato or Sens proteins in the same cells during N signaling in wild type (Baker et al., 1996; Dokucu et al., 1996). Interestingly, prolonged expression and stability of the E(spl) m8 protein enhances the *spl* phenotype in the $E(spl)^D$ mutant (Tietze et al., 1992)

R8 cells respond to Dl in the *spl* mutant

A possible explanation for N activation in R8 cells of the *spl* mutant is that the mutation renders R8 cells sensitive to Dl, whereas in wild type the N in R8 cells is somehow protected from activation by Dl. If this was the case, we would predict that increased levels of *Dl* expression would enhance the *spl* mutant phenotype. *Dl* was overexpressed in the *spl* mutant background to test this model. R8 differentiation was greatly reduced when GMRGal4 was used to drive Dl expression in *spl* mutants (Fig. 5A,B). The number of ommatidia in adult eyes were counted to quantify this result. Compared with wild-type eyes that contained 740±44 ommatidia (Fig. 1A), 493±103 ommatidia were seen in *spl* (Fig. 5C) and only 235±19 in *spl*, GMR>Dl (Fig. 5D). These results show that in *spl* mutants, R8 cells remain sensitive to Dl behind the morphogenetic furrow.

The effects of spl are specific for lateral inhibition

Other processes that require N function were examined to determine whether the *spl* mutation elevates N activity specifically in R8 cells or generally wherever N is expressed.

Anterior to the morphogenetic furrow, N signaling enhances proneural gene function to promote neurogenesis. By activating N, Dl relieves the baseline repression function of Su (H) protein and reduces levels of two proteins, hairy and extramacrochaete, that reduce proneural gene function (Baonza

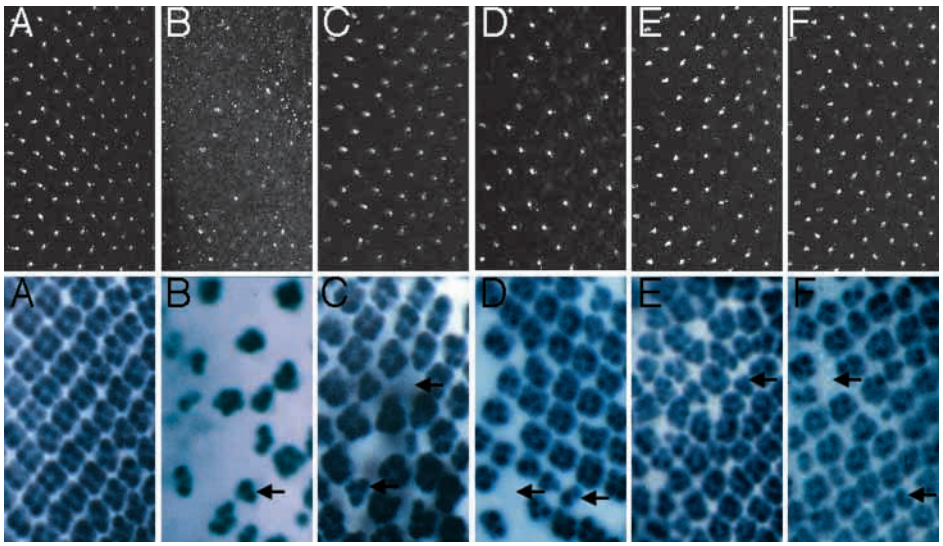

Fig. 4. Targeted activation of N in R8. Arrows indicate missing ommatidia or ommatidia with too few photoreceptor cells. (A) Wild-type pattern of R8 cells labeled for Boss (top) and all photoreceptors labeled for ELAV (bottom). (B) N intracellular domain targeted to R8 with the 109-68 Gal4 line eliminates most R8 cells and other photoreceptor cells also (*NΔEB37D*). (C) Boss labeling of the *spl* mutant shows defects in R8 patterning (top). Elav labeling (bottom) shows both missing and incomplete ommatidia (arrows). (D) R8 and other defects comparable to *spl* seen when 109-68 targets R8 expression of N intracellular domain from a more weakly-expressing transgene insertion (NΔEB5A). (E) R8 and other defects comparable to *spl* seen when 109-68 targets R8 expression of full-length N. (F) R8 and other defects comparable to *spl* seen when 109-68 targets R8 expression of the N target gene *E (spl)mδ*.

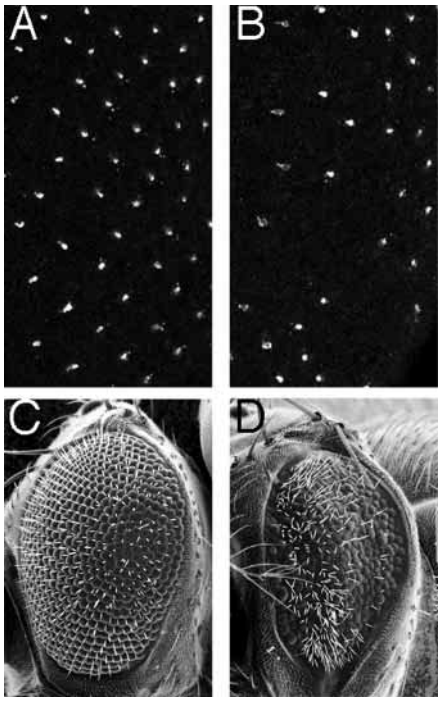

Fig. 5. R8 cells are sensitive to Dl in the *spl* mutant. (A,B) Boss labeling of R8 cells in *spl* mutant eye discs. (C,D) SEMs of adult eyes from *spl* mutants. (B,D) R8 cell number, adult eye size and number of ommatidia are significantly reduced by elevated Dl expression targeted posterior to the morphogenetic furrow by GMR Gal4. Compare with the insensitivity of R8 cells to targeted Dl expression in the absence of the *spl* mutation (Fig. 2).

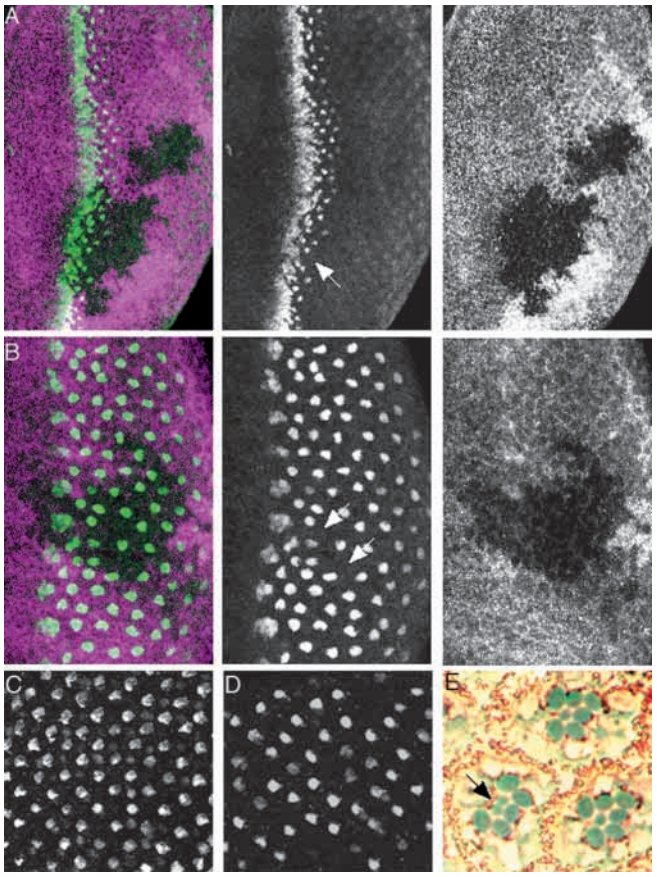

Fig. 6. Some Notch functions are unaffected by *spl*. (A) Clone of *spl* mutant cells revealed by absence of β-galactosidase (magenta; left and right). Atonal expression (green; left and middle) initiates similarly in *spl* mutant and wild-type territories. Maintenance of Atonal in R8 cells is defective in *spl* mutant territories (arrow). (B) Clone of *spl* mutant cells revealed by absence of β-galactosidase (magenta; left and right). Senseless expression (green; left and right) initiates similarly in *spl* mutant and wild-type territories. Maintenance of Senseless in R8 cells is defective in *spl* mutant territories (arrows). (C) *mδ0.5-lacZ* reporter expression predominantly in R4 cells of wild-type retina. (D) *mδ0.5-lacZ* reporter expression only in R4 cells of *spl* mutant retina. Expression is absent from some ommatidia where R4 cells may be affected. (E) Sections through *spl* mutant eyes reveal ommatidia containing multiple cells of R7-like morphology (arrow). This aspect of the *spl* phenotype is not cell autonomous in mosaics (not shown).

and Freeman, 2001; Li and Baker, 2001). Loss of proneural enhancement is associated with reduced levels of the proneural protein atonal and with gaps in the proneural intermediate groups (Baker and Yu, 1997). Atonal and Senseless expression are reduced in *spl* mutant eye discs, perhaps indicating an effect of *spl* on proneural enhancement (Nagel and Preiss, 1999) (Fig. 3C). Alternatively, *spl* might affect Atonal expression nonautonomously, through signals such as Hh, Dpp or Sca that diffuse anteriorly from differentiating cells to regulate Atonal expression (Curtiss and Mlodzik, 2000; Greenwood and Struhl, 1999; Lee et al., 1996). These signals may be affected in *spl* mutants where the cells that produce them cells differentiate abnormally and die.

Cell autonomous and nonautonomous features of the *spl* phenotype were distinguished to identify direct and indirect effects of the *spl* mutation. Unlike *spl* homozygotes, we found that atonal levels were normal in intermediate groups in homozygous *spl* clones, although the number of R8 cell precursors was reduced posterior to the furrow (Fig. 6A). In addition, levels of Senseless, a target genes whose expression reflects levels of Ato function were also normal in intermediate groups. As seen with Atonal, fewer than normal R8 precursor cells expressed Senseless posterior to the morphogenetic furrow (Fig. 6B). These results show that spl autonomously affects the specification and differentiation of R8 cells, but has no autonomous effect on proneural intermediate groups. We find no evidence that *spl* affects N activity during proneural enhancement and attribute the non-autonomous effect on intermediate groups seen in eye discs wholly mutant for *spl* to defective induction of Ato by posterior-to-anterior signals.

After R8 specification, Dl activates N to promote R4 specification by one member of the R3/R4 equivalence group within each ommatidium (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). If Spl elevated N signaling inappropriately in R3 cells, transformation of R3 to R4 cell fates would be observed in *spl* mutant ommatidia. No ommatidium with multiple R4 cells has been observed in sections through 141 *spl* mutant ommatidia. The expression of the *md0.5-LacZ* reporter also suggested normal R4 specification in *spl* mutant eye discs. This transgene reports N activation during R4 specification (Cooper and Bray, 1999). In

the wild type, *md0.5-LacZ* expression is elevated in R4 in the posterior of the eye discs (Fig. 6C). In the spl mutation, *md0.5-lacZ* expression resembled wild type; no additional cells were seen with the higher level *lacZ* expression typical of R4 precursors (Fig. 6D). These results provide no evidence for elevated N activity during R3/R4 specification.

After R4 specification, N promotes R7 specification within an R7 equivalence group that also produces R1 and R6 cells (Cooper and Bray, 2000; Tomlinson and Struhl, 2001). If Spl elevates N activity during R7 specification, R1 and R6 cells will be transformed into R7 cell fate. In this case multiple R7 cells should be found in *spl* mutant ommatidia. In sections through the adult retinas of *spl* mutants, cells that had the morphology of ectopic R7 cells were seen in 19/141 ommatidia examined (Fig. 6E) (Cagan et al., 1992). If these cells were R1/6 cells transformed by elevated N activity, we would expect cell autonomous transformation of R1 or R6 by *spl* in genetic mosaics. Out of 233 ommatidia mosaic for *spl*/*spl* and *spl*/+ cells, one showed a *spl* mutant cell in the R6 position that had R7-like morphology. By contrast, 205 of the mosaic ommatidia were constructed completely normally and contained 98 R1 cells and 94 R6 cells that were genetically *spl*/*spl*. These results indicate that R7-like morphology of cells in *spl* mutants does not result from cell autonomous effects on R1 or R6. An alternative possibility is that extra R7-like cells result from indirect effects of *spl* mutations on receptor tyrosine kinase signaling. In addition to N, R7 specification also requires activation of Sevenless and EGFR by ligands expressed from R8 and other cells. Ectopic activation of these receptors can transform R3, R4 and non-neuronal cone cells into R7 (Freeman, 1996; Tio et al., 1994; Zipursky and Rubin, 1994). In any case, our results provide no evidence of elevated N activity in *spl* mutant R1/6 cells.

In summary, we see no evidence for elevated N activity in three examples of inductive N function during eye development, consistent with the notion that the *spl* mutation is relatively specific for the inactive N protein in R8 precursor cells.

spl leads to an extra *O*-fucosylation site

The *spl* mutation is caused by a missense mutation affecting EGF repeat 14 of the extracellular domain, replacing Ile578 with a Thr. As others have noted, Thr is the consensus amino acid present at this position in 16 of the 36 EGF repeats from N (Hartley et al., 1987; Kelley et al., 1987). Another four EGF repeats have Ser at the corresponding position. It has therefore been unclear why the Ile578Thr substitution should mutate N function. One possibility is that Thr578 introduces a glycosylation site, and recently O-fucosylation has been identified as a novel modification of EGF repeat proteins including N (Moloney et al., 2000b).

The *O*-fucose modification is specifically found on epidermal growth factor-like repeats (Harris and Spellman, 1993). A consensus sequence for *O*-fucosylation derived from comparison of blood clotting proteins is C_2XXGGS/TC_3, where C_2 and C_3 are the second and third conserved cysteine in the EGF repeat, and X represents any amino acid (Wang and Spellman, 1998). Site-directed mutagenesis has shown that Gly at the –1 and –2 positions are not essential for fucosylation (Wang and Spellman, 1998). The corresponding EGF repeat 14 sequence is $C_2RNRGIC_3$ from wild type, and $C_2RNRGTC_3$ from *spl*, raising the possibility of *O*-fucosylation of EGF repeat 14 on the split mutant protein.

In order to test whether *spl* introduced an additional fucosylation site into N, sequences corresponding to parts of the N extracellular domain were expressed and purified from *Drosophila* Schneider line 2 cells. A region including the 13th, 14th and 15th EGF repeats flanked by V5 epitope and His6 tags was secreted from SL2 cells using the BiP signal peptide. EGF repeat 13 has a potential *O*-fucosylation site at Thr540. Thr540 was substituted with Ile in some constructs so that Thr578 would be the only possible site for *O*-fucosylation in the *spl*-derived EGF13-15 protein (Fig. 7A).

Four purified proteins (EGF13-15, EGF13-15T540I, EGF13-15I578T and EGF13-15T540I,I578T) were incubated with purified Fringe protein in the presence of UDP[3H]GlcNAc (Fig. 7B,C). As peptidyl-*O*-fucose and UDPGlcNAc are the substrates for the specific glycosyltransferase activity of Fringe protein, only *O*-fucosylated proteins are expected to be radiolabeled in this

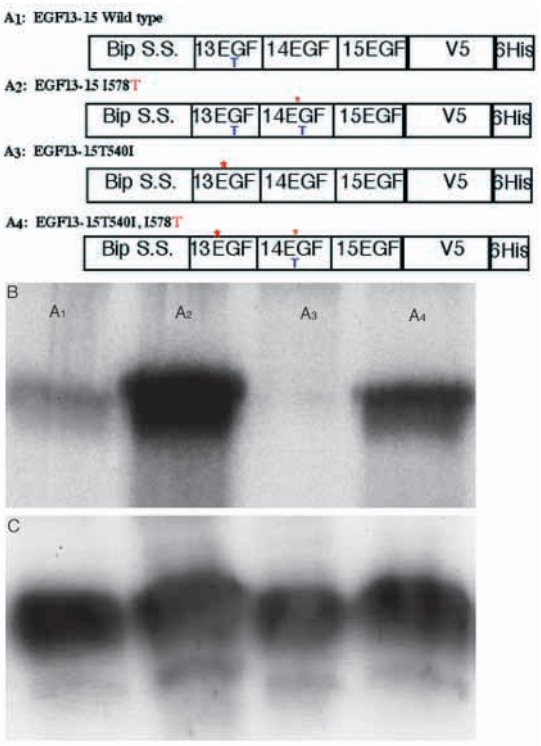

Fig. 7. Fucosylation of EGF repeats from wild-type and Fringe. (A) Proteins containing EGF repeats. Protein EGF13-15 includes EGF repeats 13-15 from wild type N, flanked by V5 and 6His tags. There is a potential *O*-fucosylation site in EGF repeat 13 (T540; blue T). Protein EGF13-15 I578T carries the Ile578 to Thr578 substitution (red T) from the *spl* allele. There are potential *O*-fucosylation sites in EGF repeat 13 and 14. Protein EGF13-15T540I resembles protein EGF13-15 with Thr540 substituted by Ile. This protein has no potential *O*-fucosylation site. Protein EGF13-15T540I,I578T is same as protein EGF13-15I578T, except that Thr540 is substituted into Ile. Ile578 is the only potential fucosylation site. (B) Autoradiographs of radiolabeled proteins after membrane transfer. EGF13-15T540I protein is not labeled (lane 3), unlike the other proteins. (C) Immunoblotting with anti-His_6 control for equal protein loading. Red asterisk indicates mutation.

assay (Moloney et al., 2000a). We found that while the EGF13-15T540I protein remained unlabeled, all three other proteins were radiolabeled, with EGF13-15I578T showing the highest incorporation (Fig. 7B). These results demonstrated that Thr540 of EGF repeat 13 and Thr578 of the *spl* mutant EGF repeat 14 are sites of *O*-fucosylation in *Drosophila* cells, and that both these sites are also in vitro substrates for Fringe.

The *spl* mutant phenotype does not depend on extension of *O*-fucose by Fringe

Several models can be proposed to account for the change in N activity in the *spl* mutant. One possibility is that EGF repeat 14 has a normal role in preventing N activation by Dl in R8 precursor cells from wild type. In this case, mutating EGF repeat 14 would interfere with the normal blocking function, allowing R8 cells to respond to Dl in the *spl* mutant. It is possible that *O*-fucosylation might contribute to inactivating EGF repeat 14, although it is also possible other mutations not altering glycosylation would have the same effect. Alternatively, *O*-fucosylation of EGF repeat14 might introduce a novel functional site on N that promotes N activity in R8 precursor cells. As extension of the *O*-fucose chain by Fringe increases N sensitivity to Dl during wing development, it is plausible that *fringe* might participate in the *spl* mutant eye also. In this case the *spl* mutant phenotype is expected to depend on *fng*.

The role of *fringe* was investigated by inducing clones of cells mutant for *fringe* in eye discs from wild type and from *spl* mutants. As reported previously, cells lacking *fringe* are defective in dorsoventral patterning, but R8 specification occurs almost normally, as evidenced by the Senseless pattern (Fig. 8A) (Cho and Choi, 1998; Papayannopoulos et al., 1998; Dominguez and de Celis, 1999). We do see occasional aberrations in R8 spacing pattern, however. In the *spl* mutant, many R8 precursors are absent or show reduced Senseless expression levels. These features of the *spl* mutant phenotype were slightly enhanced in *spl* mutant cells that were also mutant for *fringe*. In a sample of *fringe* clones in *spl* mutants, 72% of R8 cells showed reduced or absent Senseless expression, compared with 66% in control clones (Fig. 8B). There was no significant difference between clones in the dorsal or ventral parts of the eye. It is difficult to determine whether the enhancement is significant, as we did occasionally see subtle R8 spacing defects in *fng* clones in a background wild type for *spl*. In any case these data show that presence of the *O*-fucosylation site on EGF repeat 14 of N is sufficient to affect R8 development, without further extension of any carbohydrate chains by Fringe.

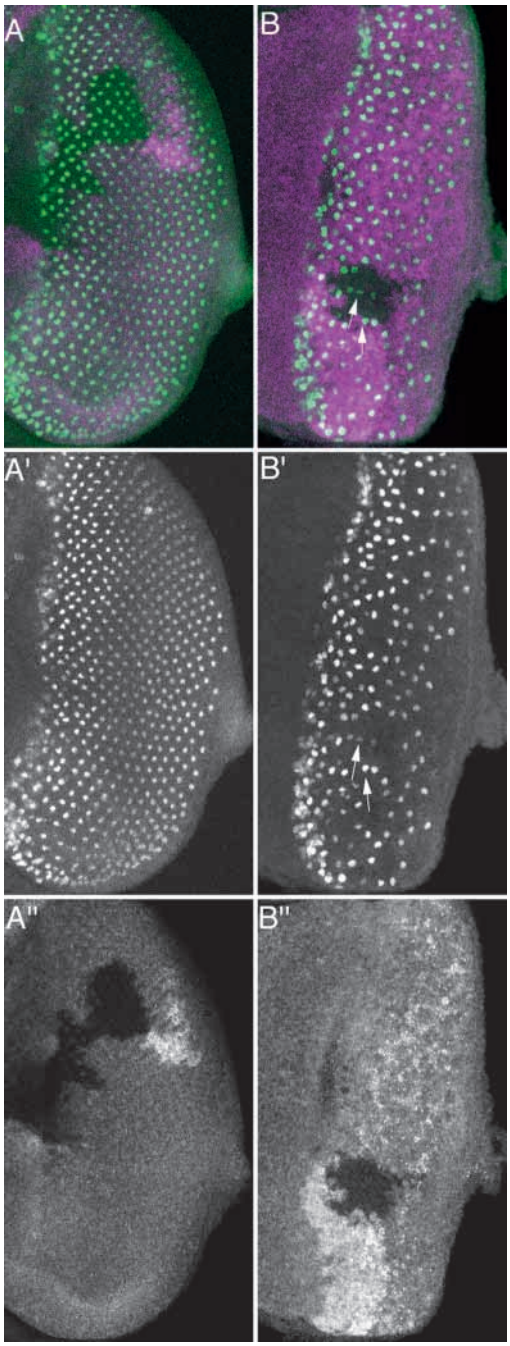

Fig. 8. The *spl* mutation affects R8 independent of Fringe. Clones of *fng* homozygous mutant cells were identified by a lack of β-galactosidase expression (magenta; A,A″,B,B″). R8 cells were labeled for Senseless protein (green; A,A′,B,B′). (A) Senseless expression was largely normal in the absence of *fng*. (B) The *spl* mutation reduced the number of R8 cells in *fng* mutant cells and in neighboring territories. As was typical in the *spl* mutant, the level of Senseless expression varied in cells that were also mutant for *fng* (arrows).

DISCUSSION

Our results indicate that N signaling in response to Dl is patterned in two distinct ways. In some situations, typified by induction of the wing margin, the expression pattern of Dl contributes to where N will be activated. N remains inactive where Dl is not expressed. In other cases, typified by lateral specification of R8 precursor cells during eye development, N and Dl are expressed homogeneously, and the pattern of N signaling depends on differential activity of the N and Dl proteins. Even though Dl is expressed homogeneously, it is essential in the cells taking R8 precursor fate. The requirement for Dl in the R8 precursor cannot be substituted by Dl expression in the other cells, even though together they contact

all of the cells that the R8 precursor contacts. This suggest that the interaction between Dl in cells selected for R8 precursor fate and N in other cells might be qualitatively different from any interaction between Dl on non-R8 cells and N in R8 precursor cells.

Altered sensitivity of neural cells to *Dl*

Inactivity of N in R8 precursor cells is not a passive event defined by absence of ligands, because even ubiquitous Dl overexpression fails to activate N in R8 precursor cells. By contrast, a recessive mutation, the *split* allele of N, now permits N to be activated by Dl in R8 precursor cells but has little or no effect on N signaling in many other contexts. The Dl protein in non-R8 cells is in an active form, because it can activate R8-cell N in the *spl* mutant.

The *spl* mutant affects development of many retinal cell types. There is an R8 cell deficit, many other retinal cells are missing, cell death is elevated and additional cells may take R7 fate. The initiation and maintenance of atonal expression is deficient even before R8 specification begins. Mosaic analysis demonstrates that all these defects depend on the genotype of R8 cells only. Therefore N is activated in *spl* mutant R8 cells. Other cells must be affected indirectly as a consequence of the abnormal R8 cells. In confirmation of this, activation of the N signal transduction pathway solely in R8 cells recapitulates the *spl* phenotype, including the effects on other cell types.

The notion that many cells might be affected indirectly in *spl* mutants is consistent with the role of R8 cells in founding each ommatidium. R8 cells initiate the cascade of EGF receptor-mediated inductions that recruit most of the retinal cell types, and are required for the survival of unspecified cells (Jarman et al., 1994; Jarman et al., 1995). The effectiveness with which R8 cells carry out these roles depends on the level of atonal expression in the R8 precursors (White and Jarman, 2000). Reduced atonal expression in the ato^2 mutant, which is defective in ato autoregulation, reduces recruitment of other cell fates because EGF receptor is activated in fewer surrounding cells. Elevating atonal expression by targeted expression in R8 using the G109-68 driver leads to activation of EGF receptor in more cells than normal and recruitment of excess outer photoreceptor cells (White and Jarman, 2000). Thus, losses of many other cells are an expected consequence of the reduced atonal expression that we demonstrate in *spl* mutant R8 cells.

In addition to producing ligands for the EGF receptor, R8 and other photoreceptor cells also secrete Hh, the primary signal moving the morphogenetic furrow across the eye disc (Ma et al., 1993). Altering atonal levels in R8 has further phenotypic effects through altered Hh signaling (White and Jarman, 2000). We propose that defective Hh signaling is the likely explanation of non-autonomous effects of *spl* on the initiation of atonal expression in the morphogenetic furrow.

The *spl* mutation also affects differentiation of sensory bristles in the epidermis (Lees and Waddington, 1942). As in R8 cells in the eye, sensory organ precursor cells are specified by lateral inhibition but not inhibited by ectopic Dl expression (Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991) (Y.L. and N.E.B., unpublished). N signaling is important in cell fate specification within the lineage of cells descended from sensory organ precursors (Hartenstein and Posakony, 1990; Zeng et al., 1998). It is plausible that aberrant N signaling might be responsible for bristle defects in *spl* mutants, although we have not examined this directly.

EGF repeat modification in wild-type and mutant N

The substitution of Thr for Ile578 in the *spl* mutation has been known for some time (Hartley et al., 1987; Kelley et al., 1987). Here, we show that the *spl* mutation introduces a site for O-fucosylation into EGF repeat 14 of the N extracellular domain. This site is fucosylated in SL2 cells and provides a substrate for the further action of Fringe.

Comparisons of *O*-fucosylation sites on clotting factors identified a consensus sequence, C_2XXGGS/TC_3 (Wang and Spellman, 1998). Similar sequences are found in eleven EGF repeats of N, although little is known about which EGF repeats are actually modified in vivo (Moloney et al., 2000b). However, site-directed mutagenesis of Factor IX and other proteins indicated that Gly residues at the –1 and –2 positions of the consensus were not essential for fucosylation (Panin et al., 2002; Wang and Spellman, 1998). This raises the possibility that some of the other EGF repeats that contain C_2XXXXS/TC_3 sequences might be fucosylated. Indeed EGF repeat 25, which contains C_2QNGAS/TC_3 is fucosylated by *Drosophila* SL2 cells and a substrate for Fringe (Panin et al., 2002). We report here that SL2 cells fucosylate the sequence $C_2RNRGTC_3$ in the *spl* mutant EGF repeat 14 and the sequence $C_2LNDGTC_3$ in wild-type EGF repeat 13. In light of these results, it seems possible that many of the 22 N EGF repeats that contain C_2XXXXS/TC_3 sequences might be fucosylated. These include the sequence $C_2QNEGSC_3$ in EGF repeat 12, required for Dl to bind and activate N (Rebay et al., 1991; de Celis et al., 1993). It is important to note that the efficiency of *O*-fucosylation at all these sites is unknown, as well as the efficiency with which *O*-fucose is extended by Fringe, so that it is possible that even within the same cell individual N molecules may carry different combinations of *O*-fucose and of extended *O*-fucose glycans.

During eye development, *fng* mutants have little direct effect on R8 specification. In addition, *fng* was not required for the *spl* mutant phenotype. This means that N function during R8 specification is little affected by any extension of *O*-fucose chains that occurs, unlike N function during wing development. It is possible that *O*-fucose monosaccharides affect N function during eye development, with or without modification to polysaccharide forms. Consistent with this interpretation, *O*-fucosylation has been found to be important for many aspects of N function, including others not dependent on Fringe (Okajima and Irvine, 2002).

Taken together, our studies suggest that introduction of an *O*-fucosylation site into EGF repeat 14 confers sensitivity to Dl on N expressed in R8 precursors, but has little effect on N activity in many other cells. One interpretation is that additional *O*-fucosylation of N increases sensitivity to ligand, so that N activation occurs in R8 precursors. Our finding that in the wild type R8 cells are insensitive to Dl also suggest another possibility: that EGF repeat 14 has a normal function inhibiting signaling, and that this function is disrupted by *O*-fucosylation. These two models cannot be distinguished definitively on the basis of current data. The model that EGF repeat 14 has a normal function blocking N signaling in R8 cells is supported by the recessive genetics of the *spl* mutation, however, because in heterozygous cells that contain wild-type

and *O*-fucosylated EGF repeat 14, the wild-type protein continues to maintain N inactivity in R8 cells. As EGF repeat 12, which is essential for many aspects of N signaling, contains a potential *O*-fucosylation site, one very simplistic hypothesis is that whereas *O*-fucosylated EGF repeats promote N activity, during lateral inhibition EGF repeats lacking this modification inhibit N activity. We suggest that during lateral inhibition of neural cells the spatial pattern of N activity is determined by insensitivity of presumptive neural cells to N ligands, and that such insensitivity is regulated by modifications or interactions of EGF repeats on the N extracellular domain.

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