Corrigendum

Scabrous and Gp150 are endosomal proteins that regulate Notch activity

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Figure 5 of this paper illustrates the distribution of Sca protein and of several deletions of Sca after heatshock-induced expression in the pupal retina of *Drosophila*. Owing to confusion between two plasmids in N.E.B.'s laboratory, the preparation labelled ScaΔ41-514 in Fig. 5G was, in fact, expressing a different protein, ScaΔ319-463. When the experiment was repeated using the correct genotypes, we found that whereas full-length Sca associated with the lattice of pigment cells that express the Notch protein at high levels, ScaΔ41-514 associated predominantly with other cells. The results indicate that the N-terminal 514 amino acids of Sca mediate colocalization with Notch, not the Fibrinogen-Related Domain contained within amino acids 515-774. We apologize to readers for this mistake, which does not affect the major conclusions of the paper. A revised version of Fig. 5 and its legend are shown below.

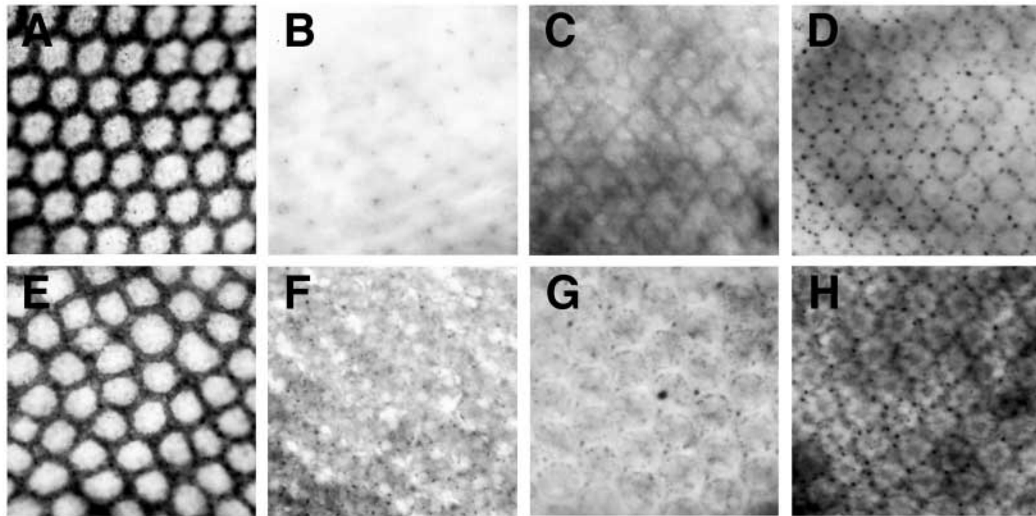

Fig. 5. Sca and N association in pupal retina. Immunohistochemistry was used to label proteins in pupal retinas. (A,E) N protein in wild type (A) or in *gp1503*/*gp1504* (E). (B-D,F-H) Sca protein. (B) Sca is absent from wild-type retinas until weak expression begins in a single sensory organ lineage between each ommatidium. (C) Twenty minutes after heatshock-induced expression, intracellular Sca protein is distributed homogenously. (D) Sixty minutes after heatshock-induced expression, Sca protein is concentrated in particles within the pigment cell lattice that also expresses N (compare with A). (F) Sixty minutes after heatshock-induced expression, Sca protein is not concentrated in particular cells from *gp1503*/*gp1504* retinas. (G) Sixty minutes after heatshock-induced expression, ScaΔ41-514 is concentrated in cells that lack N expression, the converse of the pattern seen with full-length Sca (compare with D). (H) Sixty minutes after heatshock-induced expression, ScaΔ513-773 shows some concentration in N-expressing cells, although reduced compared with that of full-length Sca (compare with D).

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Scabrous and Gp150 are endosomal proteins that regulate Notch activity

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SUMMARY

Notch and Delta are required for lateral inhibition during eye development. They prevent a tenfold excess in R8 photoreceptor cell specification. Mutations in two other genes, Scabrous and Gp150, result in more modestly increased R8 specification. Their roles in Notch signaling have been unclear. Both *sca* and *gp150* are required for ectopic Notch activity that occurs in the *split* mutant. Similar phenotypes showed that *sca* and *gp150* genes act in a common pathway. Gp150 was required for all activities of Sca, including inhibition of Notch activity and association with Notch-expressing cells that occur when Sca is ectopically expressed. Mosaic analysis found that the *gp150* and *sca* genes were required in different cells from one another. Gp150 concentrated Sca protein in late endosomes. A model is proposed in which endosomal Sca and Gp150 promote Notch activation in response to Delta, by regulating acquisition of insensitivity to Delta in a subset of cells.

Key words: Notch, *Drosophila* eye, Scabrous, Gp150, Endosome

INTRODUCTION

The receptor protein Notch (N) has two transmembrane ligands encoded by the *Delta* (*Dl*) and *Serrate* (*Ser*) genes (Artavanis-Tsakonas et al., 1999; Fehon et al., 1990; Rebay et al., 1991). Ligand binding somehow promotes cleavage of the extracellular domain of N, in turn promoting proteolytic release of the intracellular domain by the γ-secretase. The released intracellular domain then enters the nucleus to activate target genes (Mumm and Kopan, 2000; Weinmaster, 2000). At the molecular level, it is not yet clear how ligand binding triggers progressive proteolysis of the N protein. At the developmental level it is uncertain how N activity becomes restricted to a subset of proneural cells, while N is inactive in adjacent cells that are destined for neural fate. Neither the distribution of N nor the distribution of Dl seems able to account for this, as both are homogenous during specification of the neural cells (Baker, 2000).

The accompanying paper describes a Notch mutation that specifically elevates Notch activity in the neural cells. The *split* mutation alters glycosylation of the N extracellular domain and leads to inappropriate N activity within R8 precursor cells in the developing eye (Li et al., 2003). This suggests that factors specifically regulating the inactivity of N in neural cells contribute to the spatial pattern of neurogenesis.

Genetic studies have identified several genes whose mutations interact with the *split* allele (Brand and Campos-Ortega, 1990). One gene has been reported where deletion of a single allele is sufficient to suppress the *spl* phenotype. This gene encodes the secreted protein Scabrous (Baker et al., 1990). In addition in the homozygous absence of *sca*, the *spl* mutation has no detectable effect, i.e. *spl* mutant and wild-type N behave indistinguishably. Conversely duplications of *sca* enhance the *spl* phenotype (Rabinow and Birchler, 1990). These results indicate that activity of N in neural cells depends critically on *sca*. By contrast, none of the well-known components of N signaling behave as such dose-sensitive genetic modifiers of *spl*. Special alleles of *Dl* were also recovered as dominant *spl* suppressors (Brand and Campos-Ortega, 1990), consistent with the finding that in *spl* the N activity in neural cells is ligand dependent (Li et al., 2003).

The molecular role of Scabrous in the Notch pathway is not yet clear (Baker, 2000; Justice and Jan, 2002). Mutations of *sca* cause defects in the spacing and number of sensory mother cells in the epidermis and of R8 precursor cells in the retina, two founder cell types for adult peripheral nervous system (Mlodzik et al., 1990). The *sca* mutations act cell non-autonomously. Because N acts cell autonomously in the specification of these same cell types it was suggested that *sca* encoded an extracellular ligand for the receptor protein N (Baker et al., 1990). This hypothesis proved difficult to confirm, however, as *sca* mutations affected only a subset of Notch functions, had weaker effects than N null mutations, and as no direct interaction between the Sca and N proteins was demonstrated (Baker and Zitron, 1995; Lee and Baker, 1996). More recently, other ideas have been proposed: that Sca acts to scaffold N to the extracellular matrix to downregulate N activity (Powell et al., 2001), acts to preserve epithelial structure within proneural regions and so enhance function of other N ligands (Renaud and Simpson, 2001), or acts

independently of N to arrest ommatidial rotation (Chou and Chien, 2002).

Other findings strongly suggest that Sca and N proteins are closely associated in vivo. When Sca is overexpressed in the developing wing, N activity and specification of the wing margin are prevented, even though wing margin specification is independent of Sca function in the wild type. Sca protein appears to prevent Dl from activating of N in this ectopic expression assay. The results strongly suggest that Sca protein targets N signaling, although not defining the exact role of Sca in normal development (Lee et al., 2000). In other experiments, Powell et al. (Powell et al., 2001) reported that when ectopically expressed in pupal retina, Sca protein was preferentially stabilized in cells expressing N and that such stability depended on EGF repeats 19-26 of the N extracellular domain. Dl and Ser signal through EGF repeats 10-12 (de Celis et al., 1993; Rebay et al., 1991). The association with Sca occurred independently of N signaling activity (Powell et al., 2001). Chemical crosslinking of *Drosophila* embryos detected Sca protein in a complex with N, consistent with a close association between the proteins in vivo. Sca protein also appeared to stabilize N protein on the surface of tissue culture cells (Powell et al., 2001). It remains uncertain whether the interaction is direct or mediated by other proteins, or where in the cell it occurs.

Another gene required for proper eye and bristle patterning has recently been described. Mutations at the Gp150 locus cause defects in ommatidial development and cuticular bristle development that are similar to those seen in *sca* homozygotes (Fetchko et al., 2002). Gp150 protein was originally isolated biochemically as a phosphoprotein target of the receptor tyrosine phosphatase DPTP10D (Tian and Zinn, 1994; Fashena and Zinn, 1997). Recent work shows that Gp150 is located in endosomes and interacts with the Notch pathway (Fetchko et al., 2002).

We have explored the relationship of Sca and Gp150. We find that Gp150 is required for neural Notch activity in the *spl* mutant, and conclude that the Sca and Gp150 proteins must act in a common pathway, with Gp150 acting downstream in cells that respond to secreted Sca protein. Gp150 is required for all Sca activities yet identified, including those of ectopic expression and association with Notch in vivo. Sca is localized to endosomes along with Gp150. We propose that an endosomal pathway downregulates N activity in neural cells, and that Sca and Gp150 oppose this pathway to permit N activity in a subset of non-neural cells. Accordingly, Sca and Gp150 activate N indirectly, via effects on N downregulation.

MATERIALS AND METHODS

Drosophila strains

Strains used were as follows.

sca^{BP2} and *sca*^{OB7} (Baker et al., 1990; Mlodzik et al., 1990)
gp150^{1}, *gp150*^{2}, *gp150*^{3,} *gp150*^{4} (Fetchko et al., 2002)
spl (Lindsley and Zimm, 1992)
l (2)k08316 and *l (2)k00212* (Torok et al., 1993)
UASGFP-Rab7 (Entchev et al., 2000)
UASsca and *HSsca* (Ellis et al., 1994)
UASgp150 (this work)
dppGal4 (Staehling-Hampton et al., 1994)
tubGal4 (Lee and Luo, 1999)
FRT42 armlacZ (Vincent et al., 1994)

HSscaΔ 41-514 and *HSscaΔ513-773* transformants were obtained by transferring the corresponding gene sequences from pUAST plasmids described previously (Lee et al., 1998) into the pCasperHS vector and transforming *Drosophila* using standard procedures (Rubin and Spradling, 1982; Steller and Pirrotta, 1986).

Drosophila genetics

Fly stocks were maintained on standard cornmeal-agar medium. Crosses were performed at 25°C. Genetic mosaics were obtained by heat shock induction of FLP recombinase as described, using recombinant chromsomes carrying p[FRT] insertions FRT40, FRT42 or FRT82 as appropriate (Golic, 1991; Xu and Rubin, 1993).

For the ectopic expression of Sca in pupae, white prepupae were collected and aged 36 hours at 25°C prior to heat shock. Heat shock was at 35°C for 2 or 3 minutes for *HSsca* transformants, 36°C for 2 minutes for the *HSscaΔ513-773* transformant, and 34°C for 2 minutes for the *HSscaΔ 41-514* transformant.

Histology and immunochemistry

Sections of adult retinas were prepared as described (Tomlinson and Ready, 1987).

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 IA 52242, USA under contract N01-HD-7-3263 from the NICHD, and used as described (Li and Baker, 2001). Other antisera were rabbit guinea pig anti-Senseless (Nolo et al., 2000), rat anti-DE-cadherin (Oda et al., 1994), mouse and rabbit anti-Scabrous (Lee et al., 1996) and guinea pig anti-Hrs (Lloyd et al., 2002), mouse anti-Gp150 (Fetchko et al., 2002), and rabbit anti-GFP (Santa Cruz Biotechnology). Secondary antibodies were HRP-, Cy2- and Cy3-conjugated antisera from Jackson Immunoresearch or FITC- and Texas Red-conjugated antisera from Vector Laboratories.

Adult wing wholemounts were prepared and (where necessary) pharate adult wings expanded as described (Couso and Martinez Arias, 1994).

RESULTS

gp150 is a required for ectopic N signaling in the *spl* allele

Large screens for mutations suppressing *spl* previously identified *sca* as the only gene known where deletion of one allele was sufficient to suppress the *spl* phenotype, emphasizing the specificity of this genetic property (Brand and Campos-Ortega, 1990). We tested whether *gp150* mutants might also suppress *spl*. Fig. 1 shows that loss of one copy of *gp150* reverts the *spl* phenotype close to wild-type appearance. Notably, *spl* is not modified by loss of one copy of other components of the Notch pathway. We do not know why *gp150* mutations were not recovered as *spl* suppressors in prior genetic screens. We note, however, that *gp150* homozygotes appear less robust than *sca* homozygotes, and that *gp150* adults are often recovered at less than expected frequencies (Fetchko et al., 2002). Given that *spl* shows inappropriate N activity in neural cells (Li et al., 2003), this identifies *sca* and *gp150* as two genes required for N activity in neural cells.

gp150 and *sca* function in the same pathway

gp150 mutants have defects in sensory bristles and eye development resembling those caused by *sca* mutations. During eye development, the first manifestations of either

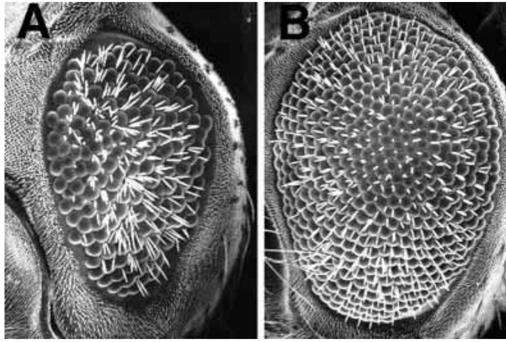

Fig. 1. Scanning electron micrographs of adult eyes. (A) *spl*/Y. (B) *spl*/Y; *gp150*^{2}/+. Haploinsufficiency for *gp150* restores almost normal development to *spl* mutant eyes. Mild aberrations in facet arrangement and some bristle duplications remain.

mutant are improper spacing of the 'intermediate groups' of proneural cells, and irregular spacing and variable number of R8 cells that emerge from them (Baker et al., 1990; Fetchko et al., 2002). The *gp150* phenotype appears to be autonomous in mosiacs. Possible nonautonomous defects are sometime seen in *sca* clones (Fig. 2A,B). Both *gp150* and *sca* mutant phenotypes are sensitive to the dose of the *N* and *Dl* genes, especially *Dl* (Hu et al., 1995; Fetchko et al., 2002).

Phenotypic similarities suggested that Gp150 and Sca proteins might be required for the same process. If this were the case, we would expect *sca gp150* double mutants to show the same phenotype, because it would make no difference whether the process was disabled by one mutation or by two. By contrast, if *sca* and *gp150* were each required for independent aspects of eye and bristle development, we would expect the double mutant phenotype to be more extreme than either single mutant.

Comparison of *sca*, *gp150* and *sca gp150* mutant eyes revealed only minor differences in internal or external structures, consistent with the model that Sca and Gp150 were required for the same process. In all three genotypes, the spacing of ommatidia and number of photoreceptor cells were abnormal (Fig. 2C-F; data not shown). Adult eyes from the double mutant were also similar to *sca* (Fig. 2G-J). These findings support the notion that *gp150* and *sca* affect a common process, at least in patterning the development of the *Drosophila* eye.

Gp150 may have some additional functions to Sca, as *gp150* homozygotes show reduced adult viability in comparison with *sca* homozygotes, and subtle defects in wing venations that are not seen in *sca* null mutations (Fetchko et al., 2002).

Assessing whether Sca can affect neural patterning independently of Gp150 requires identification of the *gp150*-null phenotype. The *gp150*^{1} or *gp150*^{2} alleles cause eye defects comparable with that of *sca* nulls, but we consistently observe the *gp150*^{3} or *gp150*^{4} homozygous phenotypes to be slightly weaker. Neither *gp150*^{3} nor *gp150*^{4} encodes detectable protein, and *gp150*^{4} contains an early stop codon (Fig. 2H,I) (the *gp150*^{3} open reading frame is unchanged although the protein is not expressed) (Fetchko et al., 2002). To determine the nature of the *gp150*^{1} and *gp150*^{2} mutations, regions of the *gp150* gene were PCR amplified. Smaller products were recovered compared with the controls (Fig. 3A). Sequence analysis revealed an identical 1305 bp deletion in both *gp150*^{1} and *gp150*^{2} alleles, covering a region of exon 5 and extending into exon 6 resulting in a stop codon at amino acid position 623 of the gp150-coding region (Fig. 3B). The identical

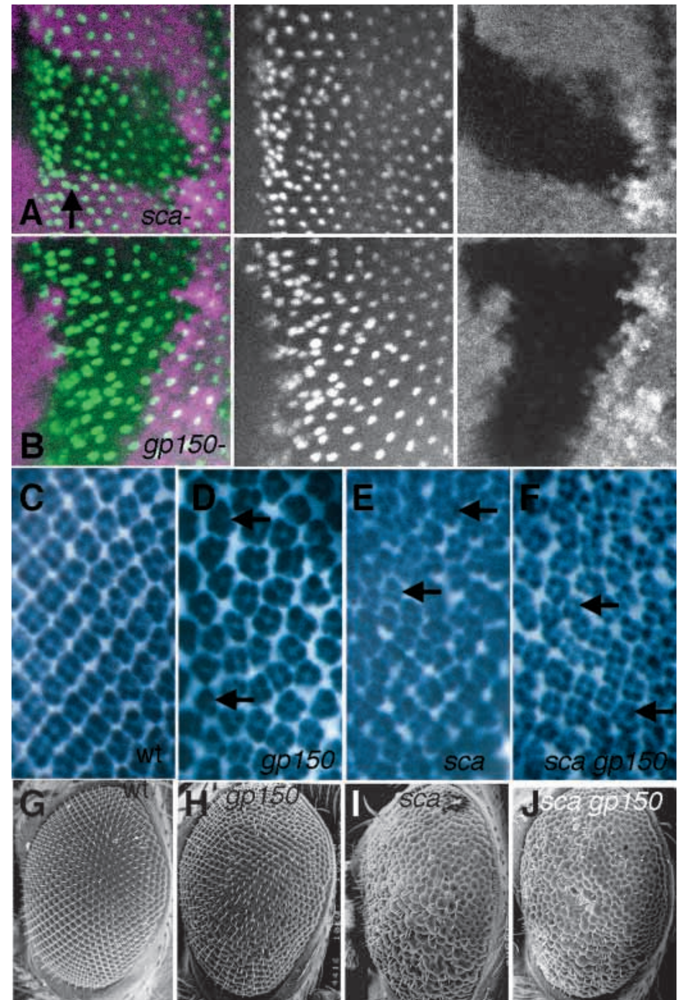

Fig. 2. Eye development in the absence of *sca* or *gp150*, or both. Cells mutant for *sca*^{OB7} are shown in A; cells mutant for *gp150*^{2} are shown in B. Homozygous mutant cells were identified by lack of β-galactosidase expression (magenta). R8 patterning is revealed by Senseless antibody staining (green). Separate Senseless and β-galactosidase channels are shown in the middle and right columns, respectively, of each merged color image shown on the left. In the *sca* mutant clone (A), the spacing of Senseless-expressing cells was irregular and there were extra R8 cells. The *gp150* mutant clone is similar (B). (C-F) Photoreceptor differentiation is shown by blue staining for ELAV antigen. In the wild-type (C) ommatidia are spaced regularly and each ommatidium consists of eight photoreceptor cells. (D) In *gp150*^{3}/*gp150*^{4} spacing of the ommatidia was abnormal and some ommatidia had fewer or more photoreceptor cells (arrows). (E) *sca*^{BP2} showed similar defects in slightly stronger form. (F) *sca gp150* double mutants resemble *sca*. (G-J) SEM of adult eyes. (G) Wild type. (H) The *gp150*^{3}/*gp150*^{4} mutant eye is rough. There are bigger or smaller ommatidia and extra bristles. (I) The *sca*^{BP2} mutant eye also contains bigger or smaller ommatidia, and extra bristles. (J) *sca*^{BP2} *gp150* ^{3}/*gp150*^{4} double mutant eyes resemble the *sca* single mutant.

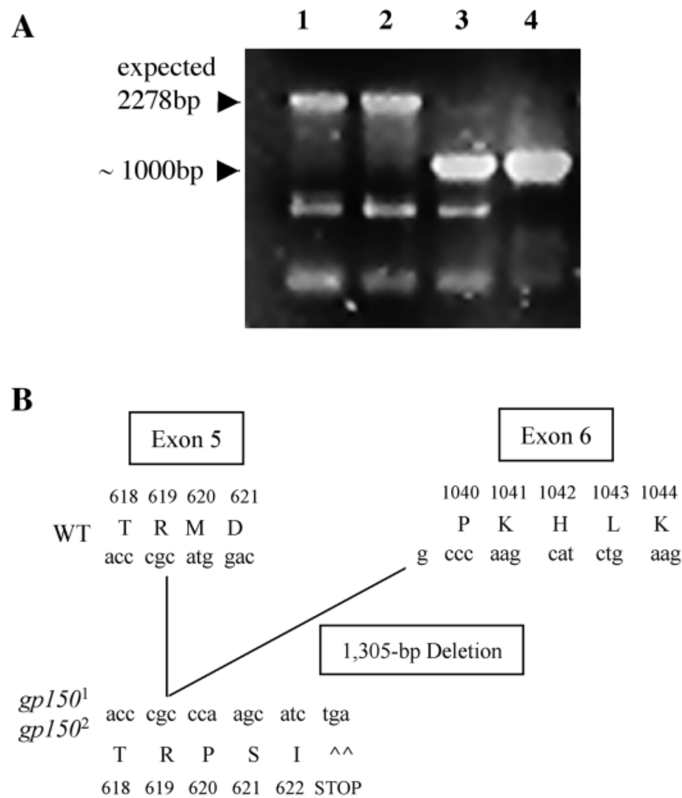

Fig. 3. Molecular defects of *gp150*^{1} and *gp150*^{2}. (A) PCR amplification revealed smaller genomic sequences from *gp150* mutations than from wild type. Lane 1, *l (2)k08316/Df (2R)02311*; lane 2, *l (2)k00212/Df (2R)02311*; lane 3, *gp1502/Df (2R)02311*; lane 4, *gp1501/Df (2R)02311*. All four mutant chromosomes were isolated from the same screen (Torok et al., 1993). Both *l (2)k08316 and l (2)k00212* were used as positive controls as these two lethal mutations are located outside of the deleted region in *Df (2R)02311*. The *gp150* gene was mapped within the deleted region in *Df (2R)02311* (Fetchko et al., 2002). (B) Sequence analysis detected a deletion extending from exon 5 into exon 6 of the *gp150* gene. The deleted chromosome was predicted to encode a truncated protein (see text for details).

changes suggest that *gp150*^{1} and *gp150*^{2} may be reisolates of the same mutation. Both mutants replace the C-terminal region (amino acids 620-1051) of the Gp150 protein by a proline-serine-isoleucine peptide. These results are consistent with the observation that a ~90 kDa protein product was detected in *gp150*^{2} mutant tissues (Fetchko et al., 2002). It is possible that *gp150*^{2} reflects the null phenotype for the gene. If *gp150*^{2} is dominant-negative and *gp150*^{3} and *gp150*^{4} represent the null phenotype, then slightly more severe eye defects in *sca* would suggest that some aspect of *sca* function can occur in the absence of *gp150*.

Gp150 is required for Sca activities

If Sca and Gp150 act in the same process, gp150 might be required for Sca function, or vice versa. To investigate this, we turned to ectopic expression experiments. Ectopic Sca expression can inhibit N signaling in the wing margin (Lee et al., 2000). UAS-gp150 transgenic flies were prepared and the dppGal4 transgene used to drive gp150 expression in a stripe across the wing, as described previously for Sca. Like Sca, targeted gp150 expression led to loss of wing margin, similar to that seen in *N*/+ heterozygotes (Fig. 4A-C). As gp150 protein is already present in the developing wing because of expression from the endogenous gene, we presume that elevated expression levels are responsible for defective wing development. Co-expression of Sca and gp150 increased the penetrance and expressivity of the wing defects (Fig. 4D). To test whether gp150 was required for Sca function, Sca was expressed ectopically in a gp150 mutant background. In the absence of gp150, wings developed normally, and appeared unaffected by targeted Sca expression (Fig. 4E). Even heterozygosity for *gp150* was sufficient to render wings insensitive to ectopic Sca expression (data not shown). Thus, gp150 was required for Sca function, at least in this ectopic expression assay. In converse experiments, gp150 was overexpressed in a *sca* mutant genotype. Targeted gp150 expression continued to affect wing margin development in the absence of *sca* function (Fig. 4F), although the penetrance was lower (data not shown). Thus, Sca was not essential for gp150 to affect development, at least in this wing overexpression assay.

We have observed generally similar results misexpressing Sca and Gp150 in other tissues. For example, eyGal4 driven gp150 or Sca expression give a small eye phenotype (data not shown). The phenotype resembles loss of N function as seen when eyGal4 was used to express UAS-fng or UAS-N^{ECD} (a

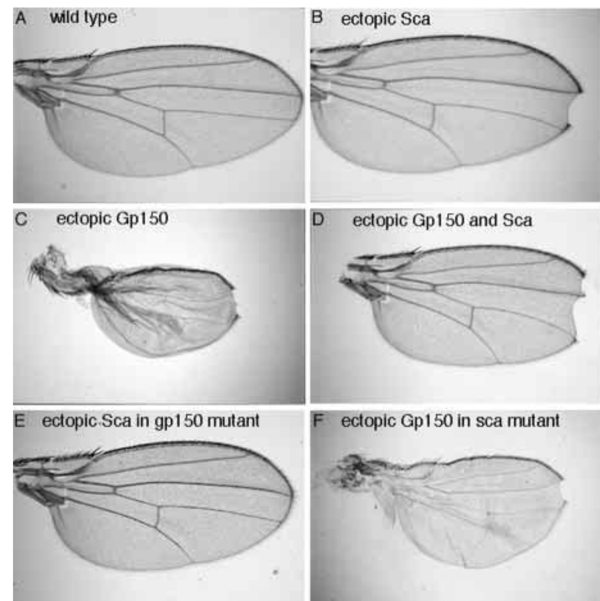

Fig. 4. Epistasis results from misexpression and mutants. Sca or Gp150 were targeted to wing regions anterior to the AP compartment boundary using the dppGal4 driver. In C and F, wings that are dissected from dead pharate adults and expanded artificially (Couso and Martinez Arias, 1994) have a rounded appearance because of the procedure rather than the genotype. (A) Wild-type wing. (B) The *dppGal4*/*UAS sca* wing has a nicked wing margin (Lee et al., 2000). (C) *UAS gp150 /UAS gp150; dppGal4*/+ is similar. (D) *UAS gp150/*+; *dppGal4*/*UAS sca* is more severe despite fewer doses of *UAS gp150*. (E) Normal wing from *gp150*^{3}/*gp150*^{4}; *dppGal4*/*UAS sca* fly. (F) Nicked wing from *sca*^{BP2}/*sca*^{BP2}; *dppGal4*/*UAS gp150* fly.

dominant-negative N construct) (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The effects of eyGal4>Sca or eyGal4>gp150 were enhanced by reduction in Dl or N gene dose, and suppressed by mutation of endogenous *sca* or *gp150* or co-expression of UAS-Dl or UAS-N^{ICD}. Thus, we conclude that ectopic Sca or Gp150 expression can inhibit N in multiple tissues.

Gp150 and Sca are required in different cells

To explore how gp150 was required for *sca* function, we sought to identify the cells in which gp150 was required using mosaic analysis. Mosaic analysis using *sca* mutations showed that the likelihood of normal ommatidial assembly was reduced unless the R8 cell was genetically wild type for *sca*, consistent with a nonautonomous role for *sca* in lateral inhibition (Baker et al., 1990). Previous mosaic analysis with *gp150* provided only limited data for R8 cells (Fetchko et al., 2002). Sections were cut through eyes containing *gp150*^{3} homozygous clones, and 90 ommatidia that were phenotypically normal scored (Table 1). No specific photoreceptor cell type was found to be important for *gp150* function, and ommatidia with R8 cells mutant for *gp150* developed normally with the same probability as ommatidia with R8 cells wild type for the *gp150* locus. Similar results were obtained from a smaller number of *gp150*^{1} and *gp150*^{2} mosaics (Table 1). The mosaic results show that *gp150* is not required in the same cells as *sca*, at least during eye development. They would be consistent with *gp150* function in cells that take many fates other than R8, so that no requirement is detected in any specific ommatidial cell. The data rule out the model that Gp150 is required for Sca protein synthesis, but are consistent with Gp150 being required for the localization or reception of Sca by other cells.

Gp150 and the Sca fibrinogen-related domain are required for colocalization with Notch

One piece of evidence that Sca interacts with N comes from colocalization studies in the pupal retina (Powell et al., 2001). Notch protein distribution is unusually asymmetric in pupal retina, being excluded from the differentiating ommatidia but expressed in the surrounding pigment cell lattice (Fehon et al., 1991) (Fig. 5A). Sca expressed transiently and uniformly from the hsp70 promoter accumulates in N-expressing cells, implying an interaction of some kind between the proteins (Powell et al., 2001).

Table 1. Mutant cells as a % of normally constructed mosaic ommatidia

Genotype	Cell R1	R2	R3	R4	R5	R6	R7	R8	Number scored
w	48	55	52	41	47	41	44	50	229*
sca	44	44	46	47	43	42	45	21	591*
defu^{3}	44	57	50	53	56	39	56	48	90
defu$^{1/2}$	37	58	55	61	56	30	45	52	23

*These data are from Baker et al. (Baker et al., 1990).

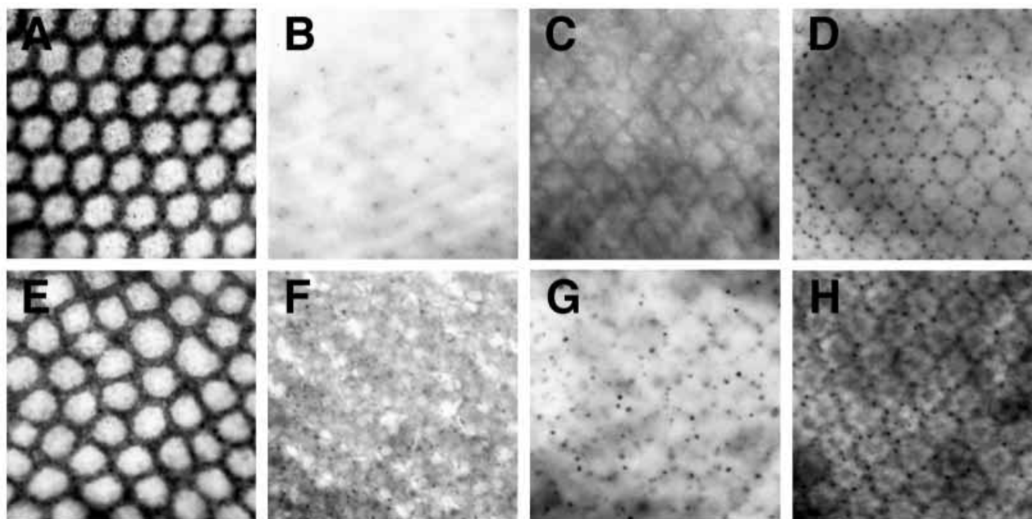

Fig. 5. Sca and N association in pupal retina. Immunohistochemistry was used to label proteins in pupal retinas. (A,E) N protein in wild type (A) and *gp150*^{3}/*gp150*^{4} (E). (B-H) Sca protein. (B) Sca is absent from wild-type retinas until weak expression begins in a single sensory organ lineage between each ommatidium. (C) Twenty minutes after heatshock-induced expression, intracellular Sca protein is distributed homogenously. (D) Sixty minutes after heatshock-induced expression, Sca protein is concentrated in particles within the pigment cell lattice that also expresses N (compare with A). (F) Sixty minutes after heatshock-induced expression, Sca protein is not concentrated in particular cells from *gp150*^{3}/*gp150*^{4} retinas. (G) Sixty minutes after heatshock-induced expression, ScaΔ41-514 shows concentration in N-expressing cells, although reduced compared with that of full-length Sca (compare with D). (H) Sixty minutes after heatshock-induced expression, ScaΔ513-773 shows little concentration in N-expressing cells (some of the labelling seen is of bristle precursor cells, which normally begin Sca expression at around this time, e.g. B).

To test whether Gp150 was required for Sca to associate with N, Sca was expressed in pupal retinas from *gp150* mutants. Prior to heat shock, pupal retinas from HS-sca transgenic flies lack Sca protein until specification of interommatidial bristle precursors begins (Fig. 5B). Within 20 minutes of mild heat shock newly synthesized Sca protein was cytoplasmic and uniformly distributed (Fig. 5C). As Sca protein was secreted and decayed, protein transiently accumulated in the Notch-expressing pigment cell lattice, usually between 40-80 minutes after heat shock (Fig. 5D). Notch protein was still expressed in the pigment cell lattice of *gp150* mutants, but heat-shock induced Sca protein showed no accumulation in these cells (Fig. 5E,F). Thus, *gp150* was required for Sca to accumulate in N-expressing cells in this assay.

Sca deletion proteins were used to investigate further how Sca associates with N. Sca comprises an N-terminal coiled-coil, previously found to be sufficient for *sca* function, and the C-terminal fibrinogen related domain (FReD) that increases the activity of the protein (Lee et al., 1998). Flies transgenic for truncated Sca proteins under control of the heat shock promoter were prepared. Neither the ScaΔ41-514 protein encoding the FReD nor the N-terminal sequences encoded by ScaΔ513-773 accumulated in N-expressing cells to the same degree as did full-length Sca (Fig. 5G,H). There seemed to be more accumulation with the ScaΔ41-514 protein, as if the FReD made more contribution to Sca accumulating in N-expressing cells (Fig. 5G).

Gp150 and Sca colocalize in late endosomes

Gp150 is located in endosomes where it may interact with endocytosed extracellular proteins (Fetchko et al., 2002). We sought to determine whether Sca protein was also found in endosomes. Although Sca is quantitatively secreted from tissue culture cells, antibodies detect Sca protein only within cells in epithelial tissues (Lee et al., 1996). Immunoelectron microscopy studies located Sca within large intracellular vesicles (Baker and Zitron, 1995). There is evidence that at least some such vesicles contain endocytosed Sca (Lee et al., 1996).

Double labelling using markers for particular parts of the endocytic pathway were examined by confocal microscopy. One such marker was Rab7, which associates with the cytoplasmic face of late endosomes (Entchev et al., 2000). In tubGal4>rab7-GFP eye discs, most of the Sca protein detected by confocal microscopy was located in late endosomes surrounded by rab7-GFP (Fig. 6A). A second marker was HRS, a protein found in early endosomes and required for maturation of endosomes into multivesicular bodies (Komada et al., 1997; Lloyd et al., 2002). HRS and Sca protein distributions did not overlap in eye disc cells, showing that Sca is not stably retained in early endosomes (Fig. 6B). Gp150 protein also overlaps with rab7-GFP, although Gp150 was found separately from GFP-rab7 in addition, perhaps in other parts of the endosome pathway (Fig. 6C). Further double labelling showed directly that Gp150 is present in the same late endosomes that were the major location of Sca protein (Fig. 6D).

The distribution of Sca protein was altered in clones of cells lacking *gp150* (Fig. 6E). The largest and most intensely labeled intracellular bodies were absent, although lower Sca levels were still detected. Similar results were observed in *gp150* mutant eye discs (data not shown). We do not know whether this change corresponds to lower Sca levels within the endosomes, or absence of Sca from the endosomal compartment but presence of lower levels at other locations. In any case, Gp150 is present in the same late endosomes as Sca and partly responsible for Sca concentration or stability there.

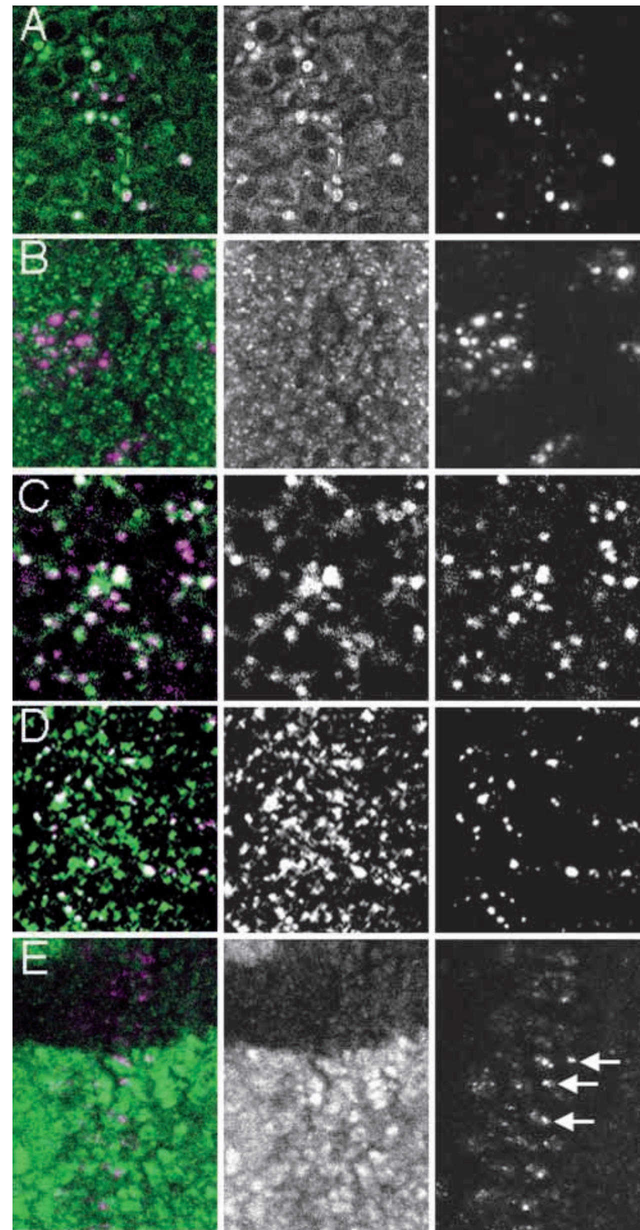

Fig. 6. Sca and Gp150 in endosomes. (A) A single confocal plane. (B-E) Confocal projections of eye discs [merged images in left column, Rab7-GFP (green) and Sca (magenta) in middle and right columns]. (A) Rab7-GFP (green) and Sca (magenta). All large Sca particles are surrounded by Rab7-GFP. Rab7-GFP also labels endosomes lacking Sca protein. (B) HRS (green) and Sca (magenta). Sca particles do not colocalize with HRS, even when the component planes of this confocal projection are examined. (C) GFP-Rab7 (green) and Gp150 (magenta). Most Gp150 is surrounded by GFP-Rab7. (D) Gp150 (green) and Sca (magenta). All large Sca particles colocalize with Gp150. (E) Absence of β-galactosidase (green) indicates cells homozygous for *gp150*^{2}. Sca protein in magenta. There is less Sca protein in large particle in the absence of Gp150.

DISCUSSION

The identification of *gp150* as a locus with a similar mutant phenotype to *sca* and likewise required for the *spl* phenotype helps define a novel genetic pathway regulating neural fate specification. The accompanying paper reports that the *split* mutant phenotype is due to inappropriate activation of N signal transduction in neural precursor cells, where N would normally become inactive, not because of an effect on the non-neuronal cells where most N activity occurs (Li et al., 2003). The Sca or Gp150 genes are essential for the *spl* mutant activity of N, but less important for normal N activity in non-neural cells.

We suggest that neural cells in the *spl* mutant mimic a subset of non-neural cells that approach neural fate in wild-type development, and that Sca and Gp150 chiefly contribute to N signaling in such cells. We propose that during lateral inhibition to select neural precursor cells, activation of N signaling is only one part of the story. Inactivation of N signaling in cells taking the neural fate is also required. We suggest that neural cells in which N is inactive have passed through a transient stage in which a low level of incipient N signaling is a normal occurrence prior to neural determination (Fig. 7). In our model, Sca and Gp150 normally function to sustain N activity in potential neural cells (or to block or delay N inactivation in potential neural cells). Accordingly, Sca and Gp150 increase N signaling by the same mechanism both in wild-type cells on the verge of neural specification and in *spl* mutant cells struggling to maintain N inactivity. This model predicts that absence of Sca or Gp150 could lead to N inactivity in too many cells and specification of extra neural precursor cells. This is consistent with the *sca* and *gp150* mutant phenotypes. Our model is consistent with the presence of Sca and Gp150 in endosomes, as it posits that they regulate inactive N molecules, not the process of N activation that occurs at the cell surface.

Our model suggests two slightly different routes for the

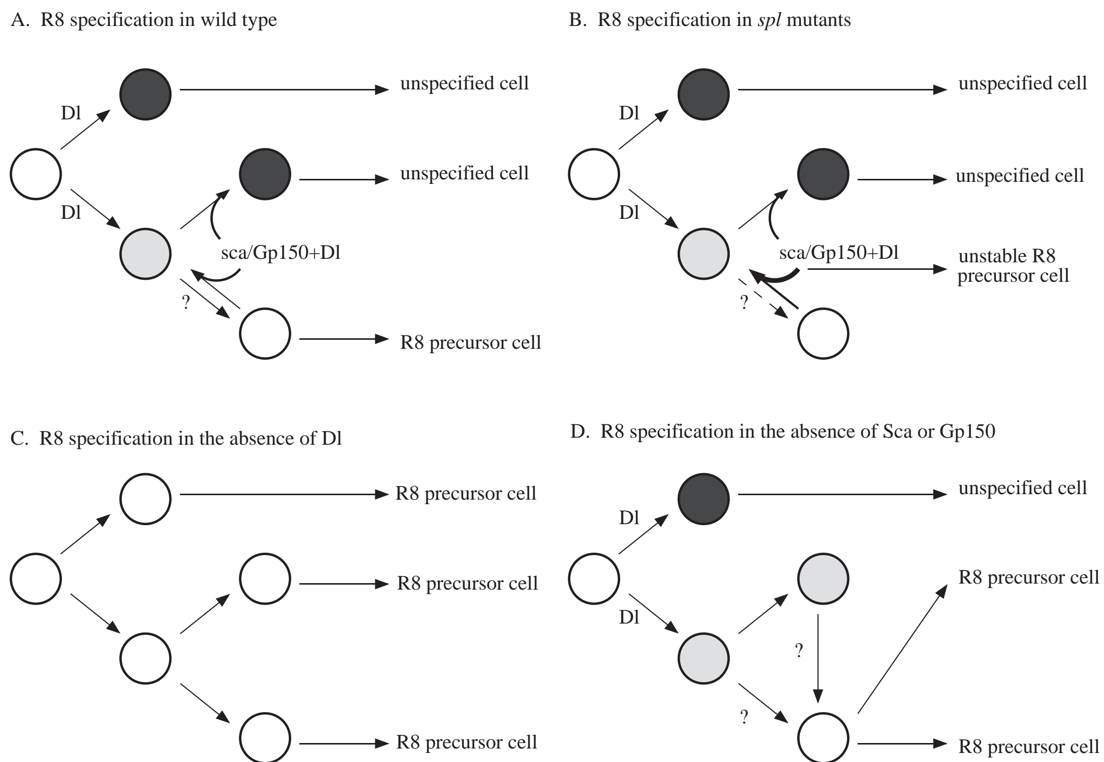

Fig. 7. Two-step model for R8 specification. (A) Our model for lateral inhibition during R8 specification proposes both activation of N in cells inhibited from R8 fate and inactivation of N in cells taking R8 fate. Notch signaling is indicated by shading. Darker shading corresponds to more intense Notch activity. The model proposes two routes for adopting non-R8 fate. In some cells, N activation by Dl is sufficient to block R8 specification. In other cells, less N activation occurs, leading to an intermediate state in which R8 specification is possible if N becomes insensitive to activation by Dl, and inhibition of R8 fate is possible if further activation of N by Dl occurs. The mechanism of desensitization is not known but might involve EGF repeat 14 of the N extracellular domain (Li et al., 2003). Sca and Gp150 promote sensitivity to Dl in such cells (or antagonize the block to Dl), allowing some cells in this intermediate state to activate N and avoid R8 specification. (B) R8 cells retain sensitivity to Dl in the *spl* mutant allele of N (Li et al., 2003). Such activity depends on Sca and Gp150, and is interpreted here as a shift in the equilibrium away from R8 specification back towards the intermediate state. Persistent N signaling in differentiating R8 cells is the cause of abnormalities in *spl* mutants (Li et al., 2003). (C) In the absence of Dl, no lateral inhibition occur and all cells competent to do so take R8 fate. (D) In the absence of Sca or Gp150, cells in the intermediate state tend to lose Dl sensitivity and take R8 fate. Two distributions of cells seem most at risk of acquiring R8 fate this way. One is cells closest to the actual R8 precursor and which have the highest probability of replacing it. The other would be cells distant from neural precursors, where the influence of Dl might be weakest and the level of N signaling lowest. In fact these are the locations where ectopic neural cells arise in *sca* or *gp150* mutants. Supernumerary R8 and bristle precursors propose both adjacent to normal precursors and at a distance from them (Baker and Zitron, 1995). We propose that these cells have greatest need of Sca and Gp150 to promote N activity and prevent protection from Dl.

inhibition of neural fate by N. In some cells, activation of N by Dl is sufficient (Fig. 7). As a by-product of the protection of future neural cells from Dl, there appear to be other cells that are also at risk for protection from Dl. By antagonizing protection, Sca and Gp150 promote N activity in such cells and prevent too many cells taking neural fate.

The current data focuses attention on possible roles of endosomes in N signaling. Both Sca and Gp150 proteins are found predominantly in endosomes, where Gp150 is required for Sca location or stability, and for Sca function. This suggests that Sca and Gp150 promote N function, or prevent N inactivation, through an effect on endosomes. Gp150 is thought to be transported to late endosomes directly from the Golgi (Fetchko et al., 2002). Sca is thought to reach late endosomes after uptake from outside the cell, because in cultured cells all the Sca is secreted (Lee et al., 1996). Several studies indicate that Sca can be taken up into other cells in vivo (Chou and Chien, 2002; Lee et al., 1996). Notably, the subcellular distribution of Sca proteins shows little dependence on dynamin function, suggesting a dynamin-independent mode of uptake (Chou and Chien, 2002) (Y.L., unpublished).

The pathway of N activation in which ligands trigger proteolytic cleavages to release the intracellular domain is thought to occur at the cell surface, and none of these reactions is thought to involve endosomes (Chung and Struhl, 2001; Lopez-Schier and St Johnston, 2002). N activation by trans-endocytosis of the N extracellular domain has been proposed, but this involves endosomes in the signal sending cell, which is not where mosaic analysis finds Gp150 to be required (Parks et al., 2000). Endocytosis has been proposed both to downregulate N activity and to promote N activity by removing inactive and inhibitory forms of both N and its ligands from the cell surface (Berdnik et al., 2002; Seugnet et al., 1997). Although our data are probably consistent with previous models for Sca function in increasing the sensitivity or range of N signaling (Baker and Zitron, 1995; Renaud and Simpson, 2001), both the idea that *sca* and *gp150* are most important in cells where N signaling would otherwise be downregulated, and the location of their products away from the cell surface supports the view that these proteins specifically affect a downregulatory mechanism, rather than acting directly on N activation. As the ectopic N activity in the *spl* mutant depends on *Dl* (Li et al., 2003), we infer that *sca* and *gp150* promote ligand-dependent N activation.

Several new models can be proposed. One model is that either before or after Dl binding, endocytosis reduces the amount of surface N available for activation. Sca and Gp150 might antagonize such endocytosis, or permit endocytosed N to be activated, either by permitting γ-secretase to act on endocytosed intermediates or by their return to the cell surface. A second model incorporates the observation that in addition to activating N signaling on neighboring cells, N ligands can ‘cis-inactivate’ N signaling in the same cell (Doherty et al., 1996; Doherty et al., 1997; Jacobsen et al., 1998; Klein et al., 1997; Micchelli et al., 1997). Protection of neural cells from N activation by Dl might reflect an increased cis-inactivation in neural cells. In this model, Sca and Gp150 would antagonize cis-inactivation, e.g. by removing Dl or N from cis-inactivatory interactions at the cell surface or in endosomes. Interestingly, Dl is also present in Gp150-positive vesicles. Elevated intracellular Dl levels have been observed in *gp150* mutants, suggesting that intracellular Dl may antagonize N signaling (Fetchko et al., 2002).

One problem for these models is that changes in the cell surface levels of N or Dl have not been detected during the selection of neural cells. It remains possible that there are changes in subsets of the detectable N or Dl proteins that are somehow particularly important for signaling. It is interesting to note that endocytosis is also implicated in N regulation within neural stem cell lineages. Asymmetric divisions during sensory organ lineages deliver Numb protein to particular daughter cells, where Numb then inhibits N signaling through binding to N and to α-adaptin, an adaptor for endocytosis via clathrin-coated pits. Although presumed to promote N endocytosis, numb and α-adaptin result in no detectable reduction in N protein levels despite blocking N activity (Berdnik et al., 2002). In nematodes, endocytosis has been proposed to permit downregulation of the N-homolog *lin-12* by Ras (Shaye and Greenwald, 2002). Perhaps endosomes provide an environment where N signaling components are neither degraded nor removed permanently from the cell surface, but rerouted or modified to change their signaling properties.

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