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The intracellular deletions of DELTA and SERRATE define dominant negative forms of the *Drosophila* Notch ligands

Xin Sun and Spyros Artavanis-Tsakonas

Howard Hughes Medical Institute and Department of Cell Biology and Biology, Boyer Center for Molecular Medicine, Yale University, 295 Congress Avenue, New Haven, Connecticut 06536-0812, USA

SUMMARY

We examined the function of the intracellular domains of the two known *Drosophila* Notch ligands, Delta and Serrate, by expressing wild-type and mutant forms in the developing *Drosophila* eye under the *sevenless* promoter. The expression of intracellularly truncated forms of either *Delta* (*sev-DlTM*) or *Serrate* (*sev-SerTM*) leads to extra photoreceptor phenotypes, similar to the eye phenotypes associated with loss-of-function mutations of either *Notch* or *Delta*. Consistent with the notion that the truncated ligands reduce Notch signalling activity, the eye phenotypes of *sev-DlTM* and *sev-SerTM* are enhanced by loss-of-function mutations in the *Notch* pathway elements, *Notch*, *Delta*, *mastermind*, *deltex* and *groucho,* but are suppressed by a duplication of *Delta* or mutations in *Hairless*, a negative regulator of the pathway. These observations were extended to the molecular level by demonstrating that the expression of *Enhancer of split mδ*, a target of Notch signalling, is down-regulated by the truncated ligands highly expressed in neighbouring cells. We conclude that the truncated ligands act as antagonists of Notch signalling.

Key words: cell signalling, Notch, Delta, Serrate, Notch signalling, Notch antagonist, ligand, *Drosophila*

Introduction

The Notch signalling pathway defines an evolutionarily conserved cell interaction mechanism that controls diverse cell-fate choices (Artavanis-Tsakonas et al., 1995). The receptor in this pathway is encoded by the *Notch* (*N*) gene and is a transmembrane protein bearing tandem Epidermal Growth Factor (EGF)-like repeats in its extracellular domain and CDC10 repeats in its intracellular domain (Wharton et al., 1985). In *Drosophila melanogaster*, genetic analysis has revealed several additional components of the *N* pathway including two ligands Delta(DL) and Serrate(SER), as well as cytoplasmic and nuclear proteins that cooperate to send extracellular signals to the nucleus (reviewed by Artavanis-Tsakonas et al., 1995). Studies on the developmental role of N signalling suggest that N does not transmit specific inductive differentiation signals. Rather, it controls the competence of immature cells to respond to specific differentiation signals (Coffman et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Fortini et al., 1993).

Both of the known *Drosophila* N ligands are transmembrane proteins (Vässin et al., 1987; Kopczynski et al., 1988; Fleming et al., 1990; Thomas et al., 1991). Previous studies have shown that DL and SER can exert their actions by interacting with specific EGF-like repeats of N expressed on adjacent cells (Fehon et al., 1990; Rebay et al., 1991). In addition to DL and Ser, genetic and molecular studies have identified a family of structurally related N ligands in vertebrates and *C. elegans* (Tax et al., 1994; Mello et al., 1994; Henderson et al., 1994; Henrique et al., 1995; Chitnis et al., 1995; Lindsell et al., 1995). All of these ligands are transmembrane proteins with extracellular domains containing a variable number of EGF-like repeats and a second conserved cysteine-rich motif, referred to as the DSL (Delta-Serrate-Lag-2) region (Tax et al., 1994). It has been suggested that the DSL region is important for ligand function (Muskavitch, 1994; Fitzgerald and Greenwald, 1995). In contrast, none of the intracellular domains of the putative N ligands display any significant sequence similarity. Replacement of most of the intracellular domain of LAG-2, a *C. elegans* lin-12 ligand, with a β-galactosidase fusion protein has no discernible effect on LAG-2 function (Henderson et al., 1994; Fitzgerald and Greenwald, 1995). In contrast, however, experiments with *Xenopus* involving a DL-like ligand (X-Delta-1) have suggested that the intracellular domain is required for normal function (Chitnis et al., 1995).

We have further investigated the function of the *Drosophila* N ligands, DL and SER. These two ligands have different developmental expression patterns and mutant phenotypes (Fleming et al., 1990; Vässin et al., 1987; Thomas et al., 1991; Kooh et al., 1993). Nevertheless, they share the DSL motif and interact physically with the same region of N (Rebay et al., 1991). Also, the neurogenic phenotype of a *Dl* null allele is suppressed by the ectopic expression of *Ser,* indicating that *Ser* can substitute for *Dl* function at least to some extent (Gu et al., 1995).

The involvement of N signalling in *Drosophila* eye development has been well established by both mutant analyses and over-expression studies (Cagan and Ready, 1989; Fortini et al., 1993; Parks et al., 1995; Baker et al., 1995). We decided to evaluate the

action of ectopic ligand expression in the eye by expressing full length and truncated forms of *Dl* and *Ser* under the *sevenless(sev)* promoter. The resulting phenotypes were characterized in wild-type and *N* pathway mutant backgrounds. Our data suggest that the intracellularly truncated forms of the ligands behave as dominant-negative proteins in an apparently non-cell autonomous manner. This observation was extended by monitoring the levels of *E(spl) mδ*, a gene whose expression reflects the activation status of the N receptor (Jennings et al., 1994, 1995). We conclude that the presence of the intracellular domains is essential for proper N ligand function in *Drosophila* eye.

MATERIALS AND METHODS

Fly strains

Ser mosaic clones were generated using $Ser^{rev2\text{-}11}/TM3$ flies. $Ser^{rev2\text{-}11}$ is an amorphic allele with an inversion breakpoint which maps within the extracellular domain of SER (Fleming et al., 1990). All transgenic flies were generated in a w^{1118} background. The fly strains used in the *sev-DlTM* interaction study are: $N^{54l9}/FM6$; $H^{1}/TM6B$ and $Dp(3;3)bxd^{110}/TM6C$. The generation of the *sev-*N^{nucl} transgenic flies has been described by Fortini et al. (1993).

Mosaic analysis

Mitotic clones of $Ser^{rev2\text{-}11}$ were generated using the FLP/FRT system as described by Xu and Rubin (1993). A $Ser^{rev2\text{-}11}$ mutation was recombined onto a third chromosome containing a FRT site at position 82B. The *w; 82B-FRT,* $Ser^{rev2\text{-}11}/TM6B$ males were crossed to 82-2πmF females as described by Xu and Rubin (1993). 48 hours after egg laying, the first instar progeny were heat shocked twice at 37°C for 1 hour, with a 1 hour interval at 25°C. Adult progeny were scored for eye clones.

Constructs for germline transformation

The *sev*-ligand plasmids were constructed in several steps. The *pHSS7* vector (kindly provided by the Rubin laboratory) containing the *sevenless* promoter was modified to contain a unique *Eco*RI site in the polylinker region. Full length *Dl* cDNA from *pMtDl* (Fehon et al., 1990) was inserted into the *Eco*RI site in the modified *sevenless* promoter vector. From this construct, a fragment containing the promoter, the cDNA, and the polyA sequences was inserted in the correct orientation into the *Not*I site of a modified *pCasper3* vector. This modified vector contains an *Eco*RI-*Pst*I fragment with 3 tandem copies of the *sevenless* enhancer (Bowtell et al., 1991). We termed the resulting construct *psev-Dl*. The *Dl* cDNA region was removed from *psev-Dl* as an *Eco*RI fragment and the resulting vector, *pESCasper*, was used for the cloning of the five other ligand cDNAs as described below.

For the construction of *psev-DlTM*, a PCR fragment was generated which included nucleotides 1850 (*Bst*EII site) to 2024 (nucleotides and amino acids numbers as described by Kopczynski et al., 1988). A stop codon and a *Eco*RI restriction site were added at the 3′ end of this PCR fragment. The resulting *Bst*EII-*Eco*RI fragment was used to replace the 1 kb *Bst*EII-*Eco*RI fragment in *psev-Dl*, creating a premature stop after amino acid 630.

For the *psev-Ser* construct, a PCR fragment was generated including nucleotides 4073 (*Sfi*I site) to 4656 (end of the open reading frame, described by Fleming et al., 1990). A 33 nucleotide sequence coding the *VSV-G* tag (Kreis, 1986) was added to the 3′ end of the PCR fragment, together with a stop codon and an *Eco*RI site. The *Sfi*I-*Eco*RI fragment was used to replace the equivalent *Sfi*I-*Eco*RI sequence in the *pMtSer* plasmid (Rebay et al., 1991). The *VSV-G*-tagged full length *Ser* cDNA was then inserted into the *sevenless* cassette vector *pESCasper* as an *Eco*RI-*Eco*RI fragment.

For the *psev-SerTM* construct, a PCR fragment including nucleotides 4073 (*Sfi*I site) to 4249 was generated. A *VSV-G* tag sequence, a stop codon and an *Eco*RI site were added at the 3′ end of this PCR product. The *Sfi*I-*Eco*RI fragment was used to replace the 0.6 kb *Sfi*I-*Eco*RI fragment in the *psev-Ser* construct (see above). The resulting *Ser-TM* cDNA encodes *Ser* coding sequence with amino acid 1269 fused to a VSV-G tag before a premature stop codon. The nucleotides and amino acids numbers of *Dl* and *Ser* are as described by Kopczynski et al. (1988) and Fleming et al. (1990).

The membrane attached intracellular *Dl* (*DlTMIC*) cDNA was obtained when three fragments were ligated together. The 5′ *Eco*RI-*Bam*HI fragment contains the translation initiation site, the signal sequence and the transmembrane sequences from N (K. Matsuno). The middle *Bam*HI-*Bam*HI fragment is a PCR product of intracellular *Dl* from nucleotides 1991 to 2631. The 3′ fragment is a *Bam*HI-*Not*I fragment encoding three tandem repeats of the 11 amino acid myc tag and a stop codon at the 3′ end (K. Matsuno). An *Eco*RI site was added 3′ to the *Not*I site. The resulting *Eco*RI-*Eco*RI fragment was inserted into *pESCasper* to create *psev-DlTMIC*.

For soluble intracellular *Dl* (*DlICS*) cDNA, two fragments were ligated. The 5′ *Eco*RI-*Bam*HI fragment is a PCR product containing the CAAC site for translational initiation followed by the *Dl* intracellular sequence from nucleotides 1991 to 2631. The 3′ fragment is the *Bam*HI-*Not*I piece for myc tags and stop codon. An *Eco*RI site was added 3′ and the resulting *Eco*RI-*Eco*RI fragment was inserted into *pESCasper* vector to create *psev-DlICS*.

Germline transformation

Germline transformation was performed using standard procedures described by Spradling (1986). All six *sevenless* constructs (1.2 mg/ml) were injected with helper plasmid Δ2-3 (0.6 mg/ml) into w^{1118} embryos.

Scanning electron microscopy (SEM) and sectioning of adult eyes

For SEM, adult flies were dehydrated sequentially in 25%, 50%, 75% and 100% ethanol, for at least 12 hours in each step. The 100% dehydration was repeated three times. The preparation was then subjected to critical point drying before being mounted on stubs and viewed on an ISI-SS40 scanning electron microscope.

Plastic sections were prepared and examined as described by Carthew and Rubin (1990).

Antibody staining of third instar larvae eye discs

Eye discs were dissected and stained as described by Gaul et al. (1992). The antibody for DL is described by Kooh et al. (1993) and Fehon et al. (1991). The antibody for the VSV-G tag was provided by Dr Thomas Kreis through Dr Ira Mellman's lab and is described by Kreis (1986). Anti-E(spl) mδ antibody 174 was provided by Dr Sarah Bray and is described by Jennings et al. (1994). The ELAV antibody was provided by Dr Gerald Rubin and is described by Rabinow and Birchler (1990). The mouse anti-myc monoclonal antibody MYC1-9E10.2 was obtained from the American Type Culture Collection. The anti-Bar antibody was kindly provided by Dr Tetsuya Kojima and is described by Higashijima et al. (1992).

RESULTS

In wild-type third instar larval eye discs, *N* is expressed ubiquitously while DL is detected transiently in differentiating cells (Fehon et al., 1991; Kooh et al., 1993). Studies using temperature sensitive alleles of *N* and *Dl* show that both genes are required for proper differentiation of all cell types in the eye including the photoreceptors (Cagan and Ready, 1989; Parks et al., 1995; Baker and Zitron, 1995). Transient reduction of either *N* or *Dl* in third instar larvae results in extra photoreceptor cells. However, mosaic eye clones of the amorphic

allele $Ser^{rev2\text{-}11}$ (Fleming et al., 1990) appear wild type, suggesting that *Ser* activity is not essential for proper eye development (Fig. 1A,B).

For all the experiments described below, wild-type and mutant *N* ligand expression was driven by the *sevenless(sev)* promoter cassette (Bowtell et al., 1991; Basler et al., 1989; Fortini et al., 1992). This promoter expresses at high levels in the R3, R4 and R7 photoreceptor precursors, cone cell precursors, the mystery cells, and, if at all, at low levels in R1 and R6 precursors (Tomlinson et al., 1987; Banerjee et al., 1987; Bowtell et al., 1989).

In our analyses, we focused our attention on two distinct phases of *sev* driven expression: an early phase, involving expression in R3, R4, R1, R6 precursors and mystery cells, and a late phase, involving expression in R7 and the cone cell precursors. See Fig. 2 for a schematic description of the *sev* promoter activity.

The expression of *Dl* and *Ser* under the *sev* promoter

To create the truncated *Dl* (*DlTM*) and truncated *Ser* (*SerTM*) constructs used in our experiments, stop codons were inserted 10-25 amino acids downstream of the transmembrane domains (Fig. 3). Both full length and truncated forms of *Ser* carry a *VSV-G* epitope tag at the C terminus (Kreis, 1986).

The expression of wild-type and mutant forms of the N ligands was first tested under the inducible *Drosophila* metallothionein promoter in S2 cells. Using anti-DL or anti-VSV-G antibodies, we detected expression from all four cDNAs in S2

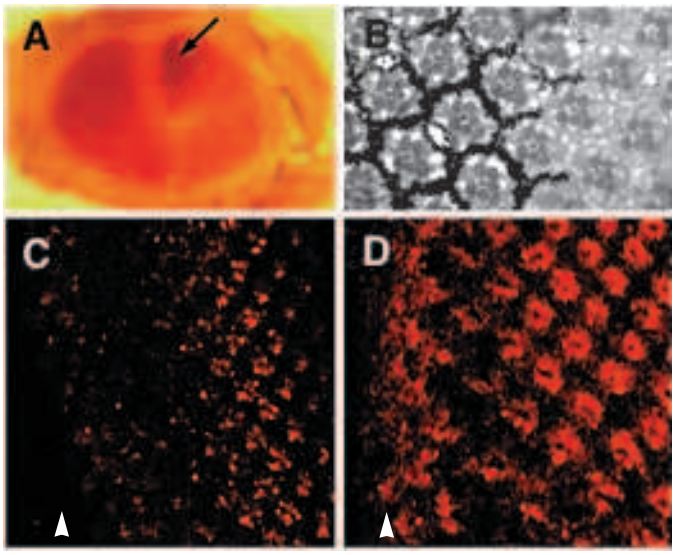

Fig. 1. Phenotype of *Serrate* mosaic clones and transgene expression under the *sevenless* promoter. (A,B) Eye clones showing the phenotype produced by the amorphic allele $Ser^{rev2\text{-}11}$. Clones are marked with the pigment marker *white*. (A) An adult eye containing a *w/w;* $Ser^{rev2\text{-}11}/Ser^{rev2\text{-}11}$ clone. The arrow points to the wild-type twin spot. The ommatidial structure appears normal in all the examined $Ser^{rev2\text{-}11}$ clones. (B) Tangential sections (2 μm) through the apical retina of a $Ser^{rev2\text{-}11}$ clone. The field shows part of the *Ser* mutant clone (right) next to its twin spot (left). The number and position of the rhabdomeres is wild type in the *Ser* mutant clone. (C,D) Magnified view of third instar larval eye discs stained with anti-DL monoclonal antibody 9B directed against the EGF-like repeats of DL (Kooh et al., 1993). The morphogenetic furrow is marked by arrowheads; posterior is to the right. (C) A wild-type eye disc showing the punctate vesicle staining of endogenous DL in photoreceptor and cone cell precursors. (D) A *sev-Dl* eye disc showing the typical *sevenless* cassette expression pattern: the butterfly shaped R3 and R4 precursors near the furrow and the ring shaped staining of the cone cell precursors (Tomlinson et al., 1987). Note that the staining is less punctate than the endogenous *Dl*. The expression pattern of the other three constructs are similar to *sev-Dl* (data not shown).

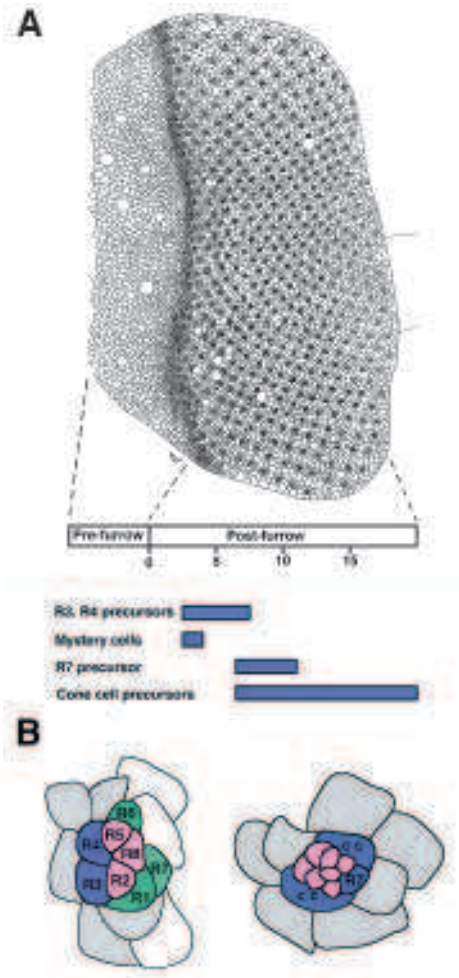

Fig. 2. Diagram of the cellular architecture and the *sevenless* expression pattern of a third instar larval eye disc. Posterior is to the right. (A) A diagram of a wild-type third instar eye disc (taken from Wolff and Ready, 1993). The dark stripe represents the cell clustering in the morphogenetic furrow. Cells start differentiating posterior to the furrow. The row numbers for ommatidial clusters are marked under the post-furrow box. Below, the blue boxes indicate the approximate time window and the cell types in which the *sev* cassette is highly active. (B) Diagrams illustrating two typical ommatidial clusters during early (left) and late (right) period of *sevenless* expression. Blue cells exhibit *sevenless* promoter activity. Pink cells have already adopted their developmental fate. Green cells represent photoreceptor precursors that have not yet differentiated. Stippled cells are in contact with the *sevenless* active cells. A subset of the photoreceptor cells and the cone cells (cc) are indicated. The left diagram illustrates a cluster in which the mystery cells have already been expelled from the precluster and only R3 and R4 precursors are strongly expressing the *sevenless* cassette (rows 4-6 posterior to the furrow). The diagram on the right represents an ommatidial cluster in which the *sev* promoter is active in the R7 precursor as well as the anterior and posterior cone cell precursors (rows 8-10).

cells upon $CuSO_4$ induction (data not shown). In addition, using confocal microscopy, we could clearly detect the signals on the cell surface indicating proper sorting and membrane insertion of the proteins. The coding sequences were then cloned into the *sevenless* promoter cassette and transgenic lines carrying each construct were isolated: 9 independent lines for full length *Dl*, termed *sev-Dl*; 3 independent lines for truncated *Dl*, termed *sev-DlTM*; 6 independent lines for full length *Ser*, termed *sev-Ser* and 8 independent lines for truncated *Ser*, termed *sev-SerTM*.

The expression of the cDNAs under the *sevenless* promoter was examined using anti-DL or anti-VSV-G antibodies. In the cases of all four constructs, staining of third instar larval eye discs revealed high levels of surface staining in the characteristic *sevenless* expression pattern. While the anti-DL antibody does not discriminate between the endogenous DL and that expressed by the transgene, the transgenic *Dl* expression is clearly discernible since it differs qualitatively and quantitatively from the endogenous gene (Fig. 1C,D). The *sev*-driven expression is seen at high levels in R3, R4, R7, cone cell precursors and mystery cells, whereas endogenous DL is detected at lower levels in all differentiating cells. Endogenous *Dl* is expressed in 90% of the cells within the furrow while *sev* driven *Dl* expression starts in preclusters three rows posterior to the furrow. Finally, while endogenous *Dl* is detected predominantly in vesicles near the apical surface of the cells, in *sev-Dl* and *sev-DlTM* discs, overexpression of *Dl* results in a broader distribution of the protein throughout the apical and lateral membranes (Parks et al., 1995; Fig. 1D and data not shown).

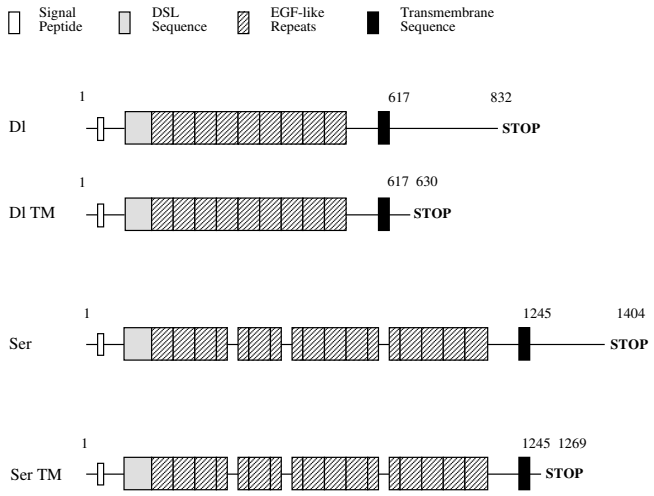

Fig. 3. Schematic diagram of the *Dl* and *Ser* constructs. The various sequence motifs are indicated by shaded boxes and defined at the top of the figure. Numbers refer to the amino acids of the *Drosophila* DL and SER sequences as described previously (Kopczynski et al., 1988; Fleming et al., 1990). *Dl* and *Ser* encode structurally related transmembrane molecules that contain Epidermal Growth Factor (EGF)-like repeats in their extracellular domains as well as the so-called DSL motif, a region of shared homology with other N ligands (Tax et al., 1994). In DLTM, a stop codon was inserted after amino acid 630, creating an intracellular deletion from amino acids 631 to 832. In SERTM, a stop codon was inserted after amino acid 1269, creating a deletion from amino acids 1270 to 1404. Both of the *Ser* constructs contain *VSV-G* epitope tags at the very C terminus of the proteins. Each of the four constructs was inserted into a vector carrying the *sevenless* gene regulatory region including the promoter and three tandem enhancer elements (see Materials and Methods).

Ectopic expression of ligands causes rough eye phenotypes

All four transgenes cause rough eye phenotypes as determined by the macroscopic examination of several independent transgenic lines (Fig. 4A-J). However, both the severity and the underlying cellular abnormalities associated with the expression of each transgene vary.

The *sev-Dl* transgenic lines exhibit very mild rough eyes (Fig. 4B,G). Under SEM, we can see that apart from the occasional irregular ommatidial shape, *sev-Dl* eyes are missing interommatidial bristles (Fig. 4G). A more severe but similar phenotype is seen when full length *Ser* is expressed under the *sev* promoter (Fig. 4D,I). Since interommatidial bristle fates are adopted during the pupal stage (Wolff and Ready, 1993), a time period when the *sev* promoter activity is not well characterized, we could not explain the link between *sev* driven ligand expression and the 'bald eye' phenotype. We note that similar eye phenotypes have been observed when *wingless* is expressed under the *sev* promoter (Cadigan and Nusse, 1995), as well as in some loss-of-function mutations of *Hairless*, a negative regulator of the N pathway (Bang et al., 1991). The phenotypes associated with the *sev* driven expression of the truncated ligands are shown in Fig. 4C,E,H,J. The *sev-DlTM* eyes show severe roughness with multiple bristles and occasional 'blueberry' ommatidia, a phenotype that has been associated with the loss of cone cells (Kimmel et al., 1990; Moffat et al., 1992). Similarly, *sev-SerTM* eyes show severe roughness with collapsed ommatidia in the middle of the eye (Fig. 4E,J).

Abnormalities in different ommatidial cell types at different developmental stages can result in rough eyes. Given the *sev* expression profile, the rough eye phenotype is likely to be caused by abnormalities in distinct ommatidial cell types. We have therefore examined the cellular phenotypes by sectioning adult eyes, focusing our attention on the photoreceptors.

Eyes from multiple transgenic lines were sectioned for each construct. Both *sev-Dl* and *sev-Ser* transgenics have abnormal numbers of photoreceptors in approximately 60% of their ommatidia. The expression of either of the two truncated forms caused a more severe rough eye phenotype in which approximately 90% of the ommatidia are abnormal (Table 1). The photoreceptor phenotype in *sev-Ser* eyes is not very informative since 58% of the abnormal ommatidia contained extra photoreceptors while 42% contained fewer. However, the other three constructs gave more consistent phenotypes. In *sev-Dl* eyes, 88% of the abnormal ommatidia had fewer outer (i.e. R1 and R6) photoreceptors than wild type whereas in *sev-DlTM* and *sev-SerTM* eyes, the majority of the abnormal ommatidia (81% and 72%, respectively) contained extra photoreceptor cells.

Ectopic *Dl* expression interferes with neighbouring cell fate choices

In order to gain further insight into the basis of the abnormal photoreceptor phenotypes, we examined the neuronal fates of individual ommatidial clusters in the developing eye. We were particularly interested in examining the consequences of the early *sev*-ligand expression in R3 and R4 precursors since the cell contacts at this stage are well defined. We stained third instar larval eye discs with the neuron-specific anti-ELAV antibody, which recognizes the nuclei of all cells that have adopted a neuronal, i.e., photoreceptor fate (Rabinow and Birchler, 1990).

Table 1. Photoreceptor phenotypes in transgenic files

Constructs	Total ommatidia scored	Abnormal ommatidia	Abnormal ommatidia with fewer photoreceptors	Abnormal ommatidia with extra photoreceptors
sev-Dl	469	326 (69%)	286 (88%)	40 (12%)
sev-DlTM	333	272 (81%)	52 (19%)	220 (81%)
sev-Ser	639	392 (61%)	166 (42%)	226 (58%)
Sev-SerTM	413	381 (92%)	107 (28%)	274 (72%)

In wild-type discs, eight or nine rows posterior to the morphogenetic furrow, seven ELAV-positive cells can be recognized in each ommatidial cluster (Wolff and Ready, 1993). The nuclei of the photoreceptor precursors occupy different planes: three nuclei (corresponding to the R8, R2 and R5 nuclei) occupy a lower plane and four nuclei (corresponding to the R1, R6, R3 and R4 nuclei) are placed more apically (Fig. 5A).

Fig. 5B shows a *sev-Dl*-expressing eye disc stained with anti-ELAV antibody focused on the apical plane. Instead of the four nuclei expected in a wild-type ommatidium, often only three ELAV-positive cells could be detected. Typically, we saw one of the equatorial nuclei missing while the two polar ones were present. The polar nuclei belong to the R3 and R4 precursors, while the missing nucleus belongs to either R1 or R6 precursors. We confirmed this by staining the *sev-Dl* discs with an anti-Bar antibody, which recognizes the R1 and R6 nuclei, or the anti-rough antibody, which recognizes R2, R5, R3 and R4 nuclei (Higashijima et al, 1992, Kimmel et al, 1990). An example is shown in Fig. 5. We found that in *sev-Dl* discs, many ommatidial clusters have either none or only one R1 or R6 cell (Fig. 5D-F). This suggests that the R1 and R6 fates are either affected by the low level expression of *sev-Dl* in these cells, or by the non-autonomous action of the transgene expressed at high levels in the neighbouring R3 and R4 precursors. In this respect, it is worth noting that the high level expression of the *sev-Dl* transgene in R3 and R4 does not seem to affect the fates of R3 and R4 photoreceptors.

Intracellular truncations of *Dl* and *Ser* result in dominant negative ligands

The extra photoreceptor phenotype associated with the expression of *sev-DlTM* and *sev-SerTM* could reflect either the recruitment of extra cells into neuronal cell fates in larval stages or the fusion of differentiated ommatidia in the pupal stages (Wolff and Ready, 1993). Staining of third instar larval eye discs with anti-ELAV antibodies revealed that both *sev-DlTM* and *sev-SerTM* transgenic discs have more ELAV-positive cells in each ommatidial cluster than do wild-type controls (Fig. 5C). During the early *sev* expression, we have detected extra ELAV-positive nuclei in the position known to be occupied by mystery cells (data not shown), indicating that the misspecification of non-neuronal precursors into neuronal cells contributes to the extra photoreceptor phenotype in adult eyes. At later stages of development in *sev-DlTM* or *sev-SerTM* discs, since the morphology of the resulting ommatidial clusters are often severely distorted, it is

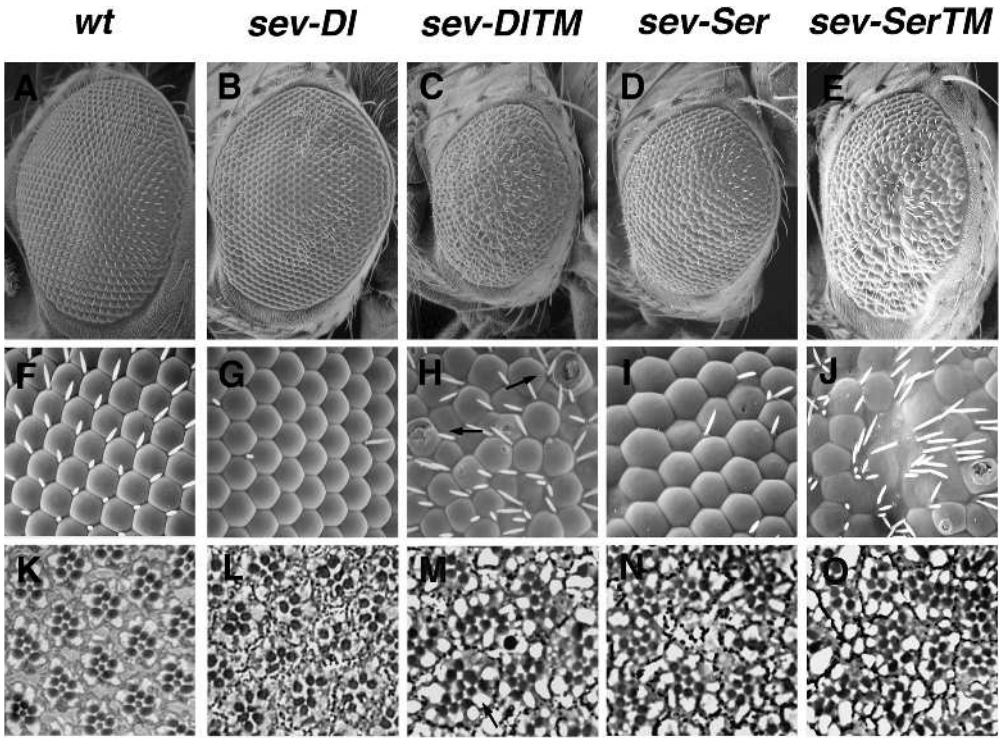

Fig. 4. Adult eye phenotypes of transgenic animals. Posterior is to the right in all panels. Scanning electron micrograph (SEM) of adult eyes: *Canton S* (A,F), *sev-Dl* (B,G), *sev-DlTM* (C,H), *sev-Ser* (D,I), and *sev-SerTM* (E,J) (F-J are magnified views of A-E, respectively). All transgenic lines shown carry two copies of the corresponding constructs. The *sev-Dl* eye shows a mild irregular ommatidial phenotype and the absence of many interommatidial bristles. The *sev-DlTM* eye shows very irregular ommatidial arrays with multiple bristles and, occasionally, the 'blueberry' phenotype (arrows), which has been associated with loss of cone cells (Moffat et al., 1992; Kimmel et al., 1990). The *sev-Ser* eye shows bristle loss in addition to irregular ommatidial arrays. The *sev-SerTM* eye shows severe roughness with collapsed ommatidia in the middle of the eye. (K-O) Tangential sections (1 μm) through the apical retina of adult eyes. (K) Wild-type eyes contain 8 rhabdomeres per ommatidium. In this apical section, 7 rhabdomeres are visible: a small R7 (inner type) rhabdomere is surrounded by the R1-6 (outer type) rhabdomeres. (L) A *sev-Dl* eye showing fewer rhabdomeres in each ommatidium. (M) A *sev-DlTM* eye showing extra rhabdomeres. The arrow points to an ommatidium with 11 rhabdomeres. As many as 13 rhabdomeres have been seen in one ommatidium (data not shown). (N) A *sev-Ser* eye showing ommatidia with both extra and fewer rhabdomeres. (O) A *sev-SerTM* eye showing extra rhabdomeres.

not possible to determine accurately the position and hence the origin of all the extra ELAV-positive cells. Therefore, from phenotype analysis, we could not conclude that the misspecification of cell fates is directly due to the non-autonomous action of the transgenes on surrounding undifferentiated cells.

Cell fate changes similar to those caused by our truncated transgenes can be elicited by inactivating the *N* pathway using loss-of-function, temperature sensitive mutations of either *Dl* or *N* at the late third instar larval stage, the same time period when the *sevenless* promoter is active (Parks et al., 1995; Cagan and Ready, 1989). This suggests that the expression of the truncated ligands results in the inactivation of the *N* pathway. Both *sev-DlTM* and *sev-SerTM* may thus behave as dominant negative mutations by interfering with *N* activation. Consistent with this interpretation is the finding that both the external roughness and the extra photoreceptor phenotypes of *sev-DlTM* eyes are almost completely suppressed by one copy of *Dl* duplication (Fig. 6C and data not shown). In addition, the rough eye phenotype is enhanced by loss-of-function alleles of *N, Dl, mastermind, deltex groucho,* but suppressed by mutations in *Hairless*, a negative regulator of the *N* pathway (Fig. 6 and data not shown, Bang et al., 1992; Brou et al., 1994). Similar genetic interactions were observed using *sev-SerTM* flies.

E(spl)mδ expression is suppressed by intracellularly truncated ligands

We investigated in more detail whether the truncated ligands can inactivate the *N* pathway in adjacent cells by examining the consequences of *sev-DlTM* and *sev-SerTM* expression on the activity of *E(spl)mδ*. It has been shown that the expression of *E(spl)mδ*, an HLH nuclear protein, is regulated by *N* activity in the *Drosophila* embryos and wing imaginal discs (Jennings et al., 1994; Jennings et al., 1995).

In wild-type eye discs, the *mδ* protein is first detected in the nuclei of cell clusters in the morphogenetic furrow (Fig. 7A,D). Its expression subsides in the mitotic wave and resumes more posteriorly in basal nuclei that belong to undifferentiated cells (Wolff and Ready, 1993). To examine whether *mδ* expression in eye discs depends on N signalling, we determined the mδ pattern in *sev-N^{nucl}* discs which express an activated form of N under the *sev* promoter (Fortini et al., 1993). We focused our attention on cone cell precursors since their identification is unambiguous given the characteristic shape of their nuclei. In wild-type discs, *mδ* is not detected in cone cell precursors. In contrast, in *sev-N^{nucl}* discs, *mδ* protein is clearly detected in cone cell precursors as a result of the ectopic expression of activated N in those cells (Fig. 7B,E). Thus, *mδ* expression can be up-regulated by the activation of N in eye discs.

We detect no obvious changes in *mδ* expression in *sev-Dl* eye discs (data not shown). Fig. 7C,F shows that in *sev-DlTM* or *sev-SerTM* discs, *mδ* expression is maintained in the furrow, a region where the *sev* promoter is inactive. In contrast, in the region following the mitotic wave where the *sev* promoter is active, *mδ* expression is largely suppressed in basal nuclei cells. Many of these cells, which represent undifferentiated precursors, are adjacent to the cells that express the *sev* promoter. This suggests that the truncated ligands are capable of inactivating the N receptor on the adjacent cells, consequently suppressing *mδ* expression. These observations further support the notion that *DlTM* and *SerTM* act as dominant negative mutations and demonstrate that the existence of the intracellular regions of the membrane bound ligands are required for normal N signalling.

The intracellular domain of *Delta*

To further explore the possible function of the intracellular domains of the N ligands, we expressed both a soluble form (*DlICS*) and a membrane attached form (*DlTMIC*) of the intracellular DL under the *sevenless* promoter (Fig. 8A). 29 *sev-DlICS* and four *sev-DlTMIC* transgenic lines were generated and analyzed. Our results show that, although both cDNAs were expressed at high levels under the *sevenless* promoter, no eye phenotypes were observed (Fig. 8B-D). The photoreceptors in these transgenics appear wild type (Fig. 8E). Furthermore, they do not interact genetically with *sev-Dl* or with mutations in *N* and *Dl* (data not shown). Therefore, the intracellular domain of DL does not show any activity when it is expressed under the *sevenless* promoter.

The expression studies involving the *sev-DlICS* construct

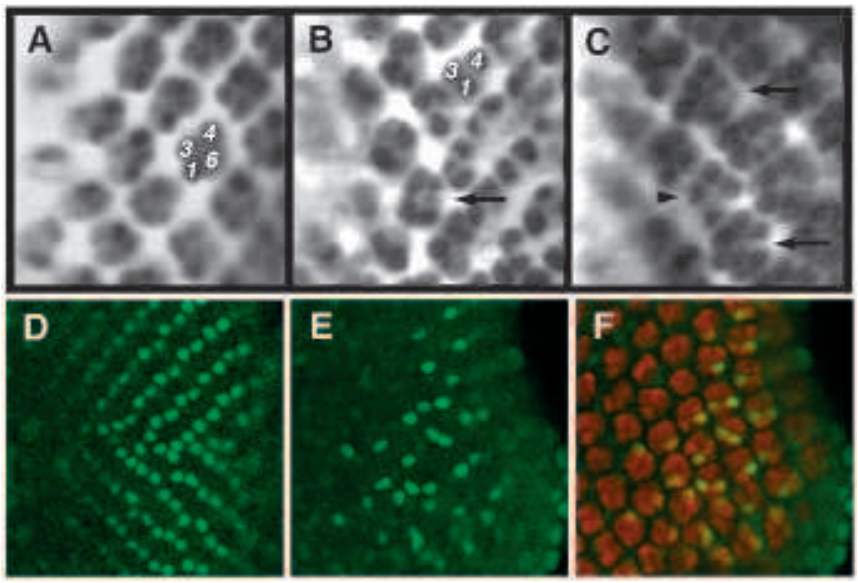

Fig. 5. Anti-ELAV staining and anti-Bar staining of third instar larval eye discs. Apical views are shown. In each panel, posterior is to the right, the morphogenetic furrow is just to the left. The equator is below in A-C,E,F. (A) Magnified view of a *w^{1118}* eye disc. One cluster is labeled to show the nuclei of the R3, R4, R1 and R6 precursors. (B) In the magnified view of a *sev-Dl* disc, clusters with fewer *ELAV*-positive nuclei are seen. The nuclei of one of the clusters are labeled. The arrow points to the tip of a R8 nucleus which is still visible as it moves out of the apical focal plane. (C) Magnified view of a *sev-DlTM* disc. Arrows point to clusters with extra ELAV-positive nuclei. The arrowhead points to a cluster with the normal number of ELAV-positive nuclei. (D) Anti-Bar staining of a *w^{1118}* disc. R1 and R6 precursor nuclei are seen in regular spaced pairs 45° to the equator which goes horizontally through the middle of the panel. (E) Anti-Bar staining pattern of a *sev-Dl* disc. Fewer and irregularly spaced Bar-positive nuclei are present. (F) The same *sev-Dl* eye disc showing both the anti-Bar (green) and the anti-ELAV (red) staining pattern. Pairs of Bar-positive nuclei are missing from many ommatidial clusters.

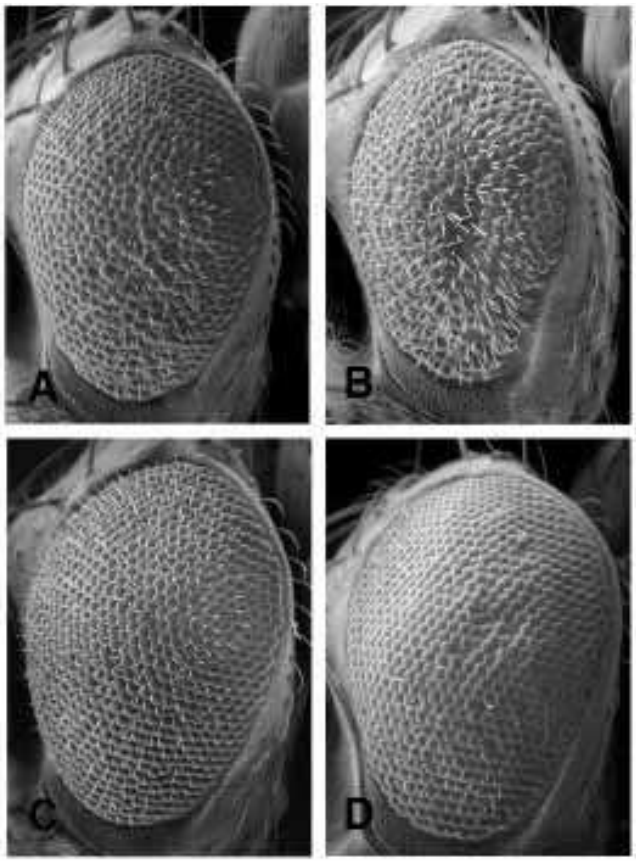

Fig. 6. Genetic interactions between *sev-DlTM* flies and mutations in the *N* pathway. Similar interactions were seen with *sev-SerTM* flies (data not shown). SEM of: (A) *sev-DlTM*/+ eye; (B) N^{54l9}/+; *sev-DlTM*/+ eye; (C) *sev-DlTM*/+; $Dp(3;3)bxd^{110}$/+ eye; (D) *sev-DlTM*/+; H^{1}/+ eye. Both *sev-DlTM* and *sev-SerTM* rough eye phenotypes are enhanced by loss-of-function mutations in *N, Dl, mastermind, deltex* and *groucho* (data not shown), but suppressed by a duplication of *Dl* or by a loss-of-function mutation in *Hairless*, a negative regulator of the pathway. Although neither *sev-DlTM* nor H^{1} show interommatidial bristle loss, double-heterozygotes show extensive 'baldness' (D) reminiscent of the *sev-Dl* mutant phenotype (Fig. 4B).

lead us to an unexpected observation. Cells expressing the soluble form of intracellular DL show this peptide in the nucleus. This is observed when this cDNA is expressed either under the metallothionein promoter in S2 cells (data not shown) or under the *sevenless* promoter in the eye (Fig. 8B). An amino acid sequence C-terminal of the DL transmembrane domain matches the consensus nuclear localization signal sequence (Fig. 8F, Dingwall and Laskey, 1991). Similar sequences can also be identified in JAGGED, a vertebrate N ligand (Lindsell et al, 1995). However, the putative nuclear localization sequences in DL and JAGGED are not conserved in any other N ligands and may indeed only reflect the clustering of charged residues often seen after the transmembrane domain. In *Drosophila*, we do not know whether the intracellular domain of endogenous DL is ever found in the nucleus, as we do not have antibodies specific for this part of the molecule.

Analyses in *C. elegans* prompted the suggestion that the intracellular domains of the N ligands perform a simple multimerization function (Henderson et al., 1994). We have attempted to address this issue by examining the involvement of the DL intracellular domain in homotypic interactions using the yeast two hybrid system (Zervos et al., 1993), an approach that has been successfully used to characterize protein interactions in the N pathway (Matsuno et al., 1995). Using constructs involving the entire DL intracellular domain, we did not detect any evidence of homotypic interactions (data not shown).

DISCUSSION

The development of an ommatidium depends on regulative events which control successive cell fate choices leading to the formation of a mature ommatidial unit. In third instar eye discs, the entire developmental progression of cells can be followed as they are gradually recruited into ommatidial units (Wolff and Ready, 1993). Mutational analyses have implicated the N

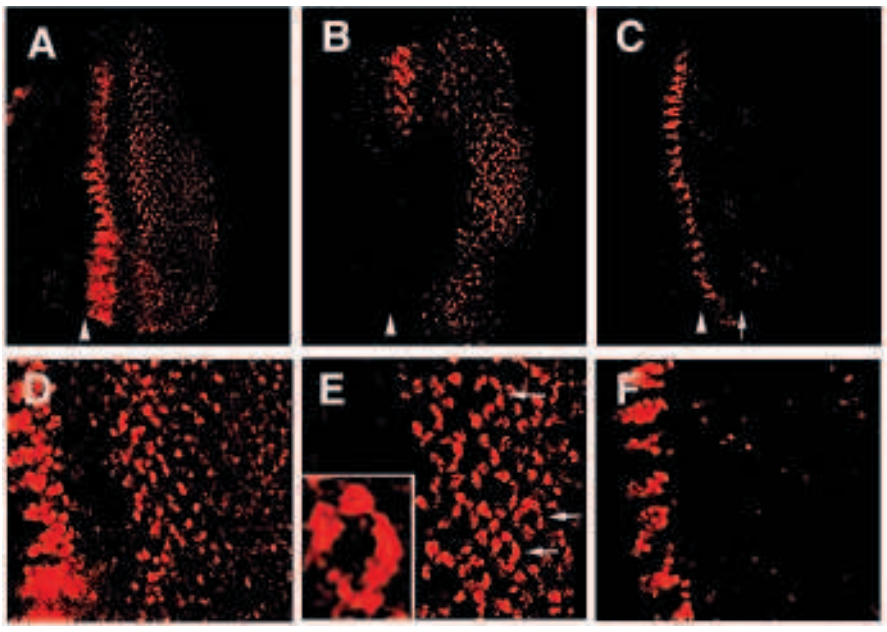

Fig. 7. *E(spl) mδ* antibody staining of third instar eye discs. Discs were stained with monoclonal antibody m174 raised against the *E(spl) mδ* protein. Posterior is to the right in all panels and the morphogenetic furrow is marked by arrowheads in A-C. D-F are corresponding magnified views of A-C. (A,D) A w^{1118} disc. Nuclear staining is detected in cell clusters in the furrow and more posteriorly in undifferentiated cells. Note that there is no staining in the mitotically active cells just posterior to the furrow. (B,E) A *sev-*N^{nucl} disc. As this disc is not flat, the top of the panel is focused on the basal plane showing that the endogenous furrow and basal nuclei express *mδ*. The middle of the panel is focused on the cone cell nuclei which occupy a more apical plane. The unique kidney shapes of the cone cell precursor nuclei allow their unambiguous identification (arrows in E). The insert shows a cluster with cone cell nuclei expressing *mδ*. (C,F) A *sev-DlTM* disc. The arrow marks the anterior border of *sevenless* promoter expression. The expression in the furrow remains while the expression in undifferentiated basal nuclei cells is suppressed.

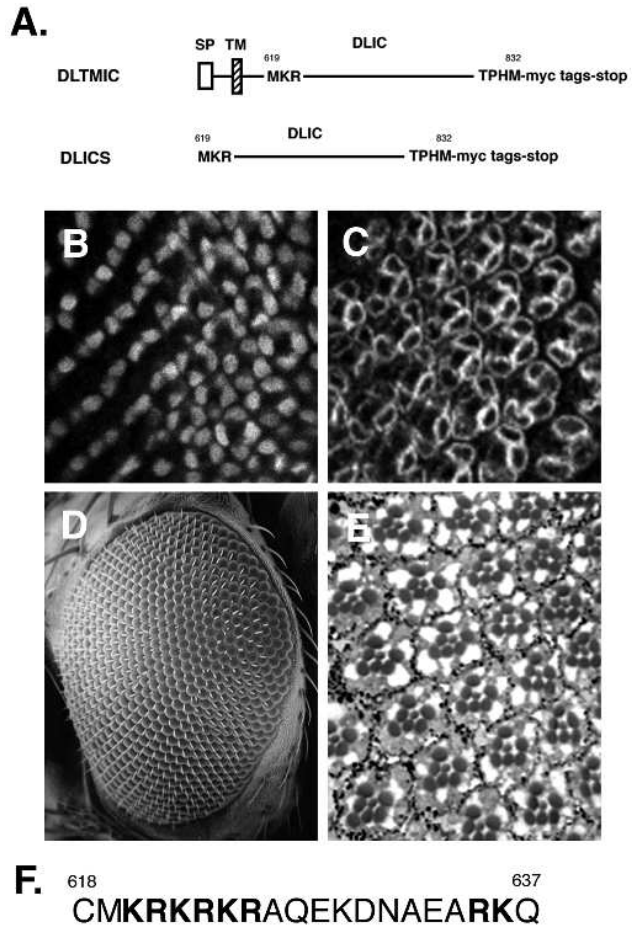

Fig. 8. Expressions and phenotypes of *sev-DlICS* and *sev-DlTMIC* transgenics. (A) Schematic drawings of the soluble (*DlICS*) and membrane attached (*DlTMIC*) protein product. The signal peptide (SP) and transmembrane domain (TM) in DLTMIC are from N. The numbers above are amino acid numbers of the DL sequence. Both constructs contain three tandem repeats of myc tags before the stop codon. (B,C) *sev-DlIC* expression in third instar eye discs detected by anti-myc antibody. Anterior is to the left. (B) A *sev-DlICS* disc. Nuclear staining shown in R3, R4, R7 precursors and cone cells. (C) A *sev-DlTMIC* disc. Cone cell staining is shown. (D) A *sev-DlTMIC* eye. No mutant phenotype was observed. (E) A section through a *sev-DlTMIC* adult eye. The photoreceptor structure is normal. (F) The putative nuclear localization sequence in the intracellular domain of DL. The numbers above are amino acid numbers of DL sequence (Kopczynski et al., 1988). The sequence shown is immediately after the transmembrane region as defined by hydrophobicity plots. The amino acids in bold are those that match both the consensus of the SV40 nuclear targeting sequence (stretch of at least 5 basic amino acids) and an inverted bipartite nuclear targeting sequence (two basic amino acids, a spacer of any ten amino acids and a basic cluster in which three of the next five amino acids must be basic; Dingwall and Laskey, 1991).

pathway in every step of ommatidial differentiation. Thus, we decided to examine the function of both *Drosophila* N ligands, DL and Ser, in the developing eye. The cell-specific *sev* promoter cassette used here allows us to evaluate the consequences of over-expressing N ligands in defined ommatidial cell precursors.

The postembryonic action of the *sev* promoter circumvents maternal effects associated with *N* and *Dl* in the embryo (Fehon et al., 1991; Kopczynski et al., 1988) and permits expression studies of proteins that may cause lethality of the developing organism. One disadvantage of this particular experimental approach stems from the difficulty in evaluating the *sev* action on relatively late cell fate decisions, e.g. R7 differentiation, especially when non-cell-autonomous events are involved. The cellular events that follow the late *sev* driven expression in R7 and cone cell precursors may result in scorable mutant phenotypes in the adult, they are often difficult to evaluate at a single cell level due to complex cell contacts. However, the early *sev* action provides an opportunity to evaluate the effects of proteins in a precisely defined and actively developing cellular milieu.

Since the consequences of over-expressing or ectopically expressing a particular protein in a cell may not necessarily reflect the wild-type function, one must interpret such studies with caution. However, *sev*-driven overexpression has proved to be quite informative in the analysis of the N pathway as well as of other signalling pathways, such as the Ras pathway (Zipursky and Rubin, 1994; Hafen et al., 1993). It is noteworthy that screens for modifiers of mutant N receptors expressed under the *sev* promoter have led to the identification of known elements of the pathway. For example, mutations in *Dl, mastermind*, *deltex* and *Suppresser of Hairless* were identified as modifiers of activated N expressed under the *sev* promoter (Fortini, M.E., Verheyen, E., Purcell, C.J. and Artavanis-Tsakonas, S., unpublished results).

Previous studies have shown that the *sev* driven expression of activated N in photoreceptor precursors blocks their differentiation (Fortini et al., 1993). For example, the early action of *sev-N^{nucl}* in the R3 and R4 precursors prevents them from adopting the R3 and R4 fates. Importantly, it was observed that the ability of these cells to differentiate into neurons has not been irreversibly lost. When the *sev* promoter activity subsides later in development (rows 7-8), the R3 and R4 precursors are capable of differentiating into photoreceptors. However, they do not differentiate into R3 and R4 but respond to a later signal to become R7 photoreceptors.

The *sev-Dl* phenotype we observed support and extend the above data. The overexpression of full length *Dl* appears to maintain the activity of the N receptor in R1 and R6, blocking their responses to neuronal differentiation signals. In contrast to the *sev-Dl* phenotype, the expression of the truncated forms of the ligands leads to additional photoreceptors. Normally, the precursor cells are blocked from differentiation as their N receptors are activated. In *sev-DlTM* and *sev-SerTM* discs, the N receptor is inactivated in these precursor cells, allowing them to adopt neuronal fates. It has been shown that the undifferentiated precursors can be misrouted into photoreceptor cell fates by inactivating N signalling using either *N* or *Dl* temperature-sensitive mutations (Cagan and Ready, 1989; Parks et al., 1995). Hence, the deletion of the intracellular domain of the ligands leads to a dominant negative behaviour, producing eye phenotypes that resemble those seen in loss-of-function *N* or *Dl* mutations. The fact that analogous results have been obtained by the expression of a similarly truncated form of DL during sensory organ precursor specification in the developing notum implies that the DL intracellular domain is required for ligand function in this context as well (T. R. Parody and M. A. T. Muskavitch, unpublished data).

While several studies have demonstrated that *Dl* function is necessary in eye development, mutational studies have failed to provide evidence that *Ser* is involved. However, overexpression studies in the embryo have shown that *Ser* can substitute for some aspects of *Dl* function, consistent with the notion that both act as ligands for the N receptor (Gu et al., 1995). Even though the neurogenic phenotype of *Dl* mutant embryos can be rescued by the ectopic expression of *Ser*, these embryos do not survive. Therefore, we do not know when or if *Ser* can fully substitute for *Dl* function, nor do we know whether they use identical downstream effectors. Notwithstanding the fact that the severity of the *sev-Dl* and *sev-Ser* phenotypes is not identical, our data suggest that *Ser* and *Dl* act in a similar way. In this context, it is noteworthy that cell adhesion assays involving ligand-expressing and receptor-expressing cells have raised the possibility that DL and SER may bind to N with different affinities (Rebay et al., 1991).

The conclusions drawn from the phenotypic analysis of *sev*-driven ligands were supported by analysis of *mδ* expression as a molecular parameter for *N* activation. In agreement with what has been observed in embryos and wing discs, we found that in eye discs, the expression of the *mδ* protein depends on the activation of the N receptors. In addition, normal *mδ* expression can be suppressed in precursors due to the expression of truncated ligands on their neighboring cells. These observations confirm the dominant-negative behaviour of the truncated ligands. Furthermore, they suggest that both *Dl* and *Ser* share a common transcriptional target. Although our data suggest that the truncated ligands suppress *mδ* expression in adjacent cells, we do not know if this is directly linked to the extra photoreceptor phenotype. Relevant to our observations is the finding that overexpression of *m5* and *m8*, which like *mδ* are members of the functionally redundant *E(spl)HLH* genes, inhibits bristle differentiation (Tata and Hartley, 1995). Also, in embryos, wing discs and eye discs, *mδ* protein is not detected in differentiating cells but is present in undifferentiated precursors (Jennings et al., 1994, 1995).

Functional studies in *Xenopus* examining the activity of a DL-like ligand have also indicated that the truncation of the intracellular domain results in a dominant-negative mutant (Chitnis et al., 1995). The molecular basis of how a truncated ligand inactivates N signalling is not clear, and it is also possible that a feedback mechanism is involved in producing such a phenotype (Wilkinson et al., 1994). Our preliminary experiments involving truncated DL or SER molecules expressed on S2 cells have shown that these cells can adhere to *N*-expressing cells (unpublished results). This suggests that the non-productive binding between truncated ligands and N in adjacent cells can contribute to the dominant-negative phenotype of *sev-DlTM* and *sev-SerTM*. It is also possible that the truncated ligands can interact with the endogenous ligands in the same cells, rendering them inactive for signalling in the eye, as was suggested by Muskavitch (1994).

In this regard, several other experimental observations warrant comment. It has been demonstrated that by replacing the intracellular domain of LAG-2 with β-galactosidase, the resulting chimera can rescue *lag-2* null mutations (Henderson et al., 1994, Fitzgerald and Greenwald, 1995). As it is known that β-galactosidase molecules can tetramerize (Zabin, 1982), this suggests that the specific amino acid sequence of the LAG-2 intracellular domain is not required for function, and that the intracellular domain may be replaced by a peptide fragment that mediates homotypic interactions. Consequently, the intracellular domains of the Notch ligands may perform a simple multimerization function that is important for signalling. Such an activity could explain the dominant negative behaviour of our *DlTM* and *SerTM* transgenes.

It could be argued that if the intracellular domain of DL is a simple template for homotypic interactions, then overexpression of this fragment in vivo would interfere with the function the endogenous ligand, causing mutant phenotypes. Our failure to observe such phenotypes does not support this argument, yet we cannot exclude the possibility that the subcellular localization of the mutant polypeptide in our transgenic eyes prevents their interactions with the endogenous ligand.

While the studies involving *Drosophila* or vertebrate N ligands have not fully elucidated the function of the intracellular domains, the existence of these domains is clearly important for their function. An attractive hypothesis is that the intracellular sequences maintain the extracellular domains in their proper conformation, similar to the function of the integrin intracellular sequences (O'Toole et al., 1994). However, Fitzgerald and Greenwald (1995) have shown that secreted forms of either LAG-2 or APX-1 consisting of only the extracellular domains posses signalling activity. Assuming that N ligands function the same way in different species, it seems that the membrane bound extracellular domains function differently from a soluble form. Our results demonstrate that the presence of the intracellular domains of the N ligands can modulate N signalling in the developing *Drosophila* eye.

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