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Lobe mediates Notch signaling to control domain-specific growth in the *Drosophila* eye disc

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SUMMARY

Notch (N) activation at the dorsoventral (DV) boundary of the *Drosophila* eye is required for early eye primordium growth. Despite the apparent DV mirror symmetry, some mutations cause a preferential loss of the ventral domain, suggesting that the growth of individual domains is asymmetrically regulated. We show that the *Lobe* (*L*) gene is required non-autonomously for ventral growth but not dorsal growth, and that it mediates the proliferative effect of midline N signaling in a ventral-specific manner. *L* encodes a novel protein with a conserved domain. Loss of *L* suppresses the overproliferation phenotype of constitutive N activation in the ventral, but not in the dorsal eye, and gain of *L* rescues ventral tissue loss in *N* mutant background. Furthermore, *L* is necessary and sufficient for the ventral expression of a N ligand, Serrate (Ser), which affects ventral growth. Our data suggest that the control of ventral *Ser* expression by *L* represents a molecular mechanism that governs asymmetrical eye growth.

Key words: Notch signaling, Dorsoventral patterning, Growth control, Imaginal disc development, *Drosophila*

INTRODUCTION

The *Drosophila* eye is consisted of approximately 800 ommatidia and is subdivided into symmetrical dorsal and ventral domains. Its larval precursor, the eye imaginal disc, grows by cell division, increasing in number from about 130 cells at the end of the first instar to 9700 cells at the third instar (Wolff and Ready, 1993). Signaling events that occur at the DV boundary are required to coordinate this extensive amount of growth.

Dorsal and ventral domains are distinguished by the asymmetrical expression of dorsal-specific *wingless* (*wg*) and ventral-specific *decapentaplegic* (*dpp*) in early eye disc (Cho et al., 2000). Dorsal *wg* activates the dorsal-specific *iroquois* (*iro*) gene complex whose products restrict *fringe* (*fng*) expression to the ventral domain (Heberlein et al., 1998; Cho and Choi, 1998; Dominguez and de Celis, 1998; Yang et al., 1999; Maurrel-Zaffran and Treisman, 2000). Opposing fng^+ and fng^- cells across the DV boundary establish the molecular mechanism that functionally defines the DV boundary. Fng promotes ventral cells along the DV boundary to express a N ligand, Ser, which in turn initiates a Ser-N-Delta (Dl) positive-feedback loop that activates N signaling (Kim et al., 1995; Huppert et al., 1997). Furthermore, Fng inhibits the Ser-dependent N activation in the rest of the ventral domain and potentiates N-Dl interactions at the DV boundary (Fleming et al., 1997; Panin et al., 1997). It does so by directly binding N (Ju et al., 2000) and enzymatically modifying N (Bruckner et al., 2000; Munro and Freeman, 2000). Thus, although N receptors are ubiquitously present in the eye disc, only those at the DV boundary are activated.

N activation at the DV boundary is required for the subsequent eye growth. Loss of *N* results in small-eye or no-eye (Cagan and Ready, 1995; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998), and *N* gain-of-function induces non-autonomous overgrowth (de Celis et al., 1998; Go et al., 1998). The non-autonomous effect of midline N activation suggests the presence of a diffusible growth factor that is emitted from the DV boundary, but the identity of this factor and its downstream mediators remain unknown.

To better understand how midline N activation is coupled to the subsequent eye growth, we studied a *Drosophila* mutant with a small eye, *L*. The gene was first reported in 1925 and has been commonly used as a second chromosome dominant marker (Morgan et al., 1925). However, mechanisms that underlie its growth defect are little understood. We report the cloning of *L* and characterization of its functions. Remarkably, *L* functions are domain specific. *L* mediates the proliferative effect of N signaling only in the ventral domain, and loss of *L* specifically abolishes the ventral eye growth without affecting dorsal eye development. We also show that ventral-specific N ligand, Ser, plays dual functions in eye development. In addition to its role in DV boundary formation, *Ser* is required for the growth of the ventral eye disc, and its ventral expression is regulated by *L*. We propose that the eye disc is partitioned into dorsal, medial and ventral domains, the growth of which is independently regulated.

MATERIALS AND METHODS

Fly stocks

We used *UAS-Ser^{DN}* (R. Fleming), *UAS-fng*, *UAS-N^{intra}*, *UAS-N^{DN}*, *Ser-lacZ*, *Ser$^{rev2-11}$FRT82B* (S. Artavanis-Tsakonas), *ey-GAL4*, *dpp-GAL4*, *N$^{264-43}$*, *UAS-mirr*, *L^{1}*, *L^{2}*, *L^{4}*, *L^{5}*, *L^{si}*, *L^{rv5}*=In(2LR) 26F; 51F2-51A8, *L^{D}*, *phyl^{221}* (B. J. Dickson), *cpsf* mutants (H. W. Brock), *Asx^{1}* and *Asx^{P1}* (D. Sinclair), *l(2)03563*, and two deficiency chromosomes that uncover *L* [*Df(2R)trix* 51A1-6; 51B6 and *Df(2R)L4* 51A2; 52A12-B1], which have been described by FlyBase.

Cloning of *L* and production of *L* antibody

Overlapping cosmids covering the genomic region between *Asx* and *phyl* were isolated from a genomic cosmid library (Tamkun et al., 1992). Additional cosmids covering the 51A region were obtained from the European *Drosophila* Genome Project. Standard germline transformations were carried out to generate transgenic lines that rescued *L* mutations (Rubin and Spradling, 1992). To generate antibody, cDNA coding the C-terminal 370 amino acids of L protein was cloned into pGEX vector (Invitrogen) and the fusion protein was used to immunize mice at Caltech monoclonal facility (Ou et al., 1993). Sequences were deposited with GenBank – Accession Number AF522076.

Isolation of *L* alleles

P-element mobilization was carried out by crossing *P1201(ry^{+})/+; ry^{506}[Δ2-3 ry^{+}] Sb/+* mosaic male with *L^{si}/L^{si}; ry* females. *L^{si}*/+ flies have an anterior nick in the eye or wild-type eyes and *L^{si}/Df(2R)trix* flies have half-eyes. We selected *P1201*/ L^{si}* flies that had half-eyes and established multiple lines (*indicates a new insertion). P-element insertion site was then determined by sequencing the flanking genomic DNA isolated from inverse PCR. The *P1201* line used for local transposition was reported to be homozygous lethal and have a P insertion at base 162 of CG10109, but it unexpectedly complements the deficiency chromosome of the region, *Df(2R)trix* (Spradling et al., 1999). We had re-identified the P insertion site of *P1201* to be within the first intron, at 956 bp from the exon/intron junction.

L loss-of-function alleles were isolated from a screen for revertant of the *L^{D}* dominant half-eye phenotype. *L^{D}*/+ male flies were irradiated with 4000 rads γ-ray and crossed to wild-type females. Four revertants were isolated from a screen of approximately 40,000 progenies. One of which, *L^{rev6-3}*, failed to complement *Df(2R)trix* or *L* alleles but complemented multiple alleles of *Asx*, *cpsf* and *l(2)03563*, known mutations adjacent to *L*. Hetero-allelic combination of *L^{rev6-3}* and other eye-specific *L* alleles generated ventral-reduced eyes that were indistinguishable from those of eye-specific *L* alleles over *Df(2R)trix*.

Mosaic analysis

yw hsFLP122; L^{rev6-3} FRT42D/arm-lacZ FRT42D and *yw hsFLP122; Ser$^{rev2-11}$FRT82B/arm-lacZ FRT82B* larvae were heat shocked at 37°C for 1 hour either at 24 hours after egg laying (AEL), during the first instar stage or 48 hours AEL (during the second instar stage), and dissected after a 24 hour interval or at the wandering larvae stage. Mutant clones were identified by the absence of β-galactosidase protein.

Misexpression experiments

UAS-L construct was made by cloning full-length *L* cDNA into the transformation vector *pUAST* (Pirrota, 1988). To generate clones of cells ectopically expressing L or Ser^{DN}, first instar larvae from the cross between *UAS-L* (or other *UAS* flies); *UAS-lacZ* males and *yw P[Actin>CD2>GAL4; w]; hs-Flp,MKRS/TM6Tb* females were heat shocked at 37°C for 1 hour at 0 or 24 hour after hatching. The larvae were dissected either 24 hours after the heat shock or at the wandering larval stage, in order to visualize early first instar clones and late clones. Expression clones were identified by the presence of β-galactosidase protein.

Histochemistry

Dissected imaginal discs were stained with either diaminobenzidine (DAB) or immunofluorescent markers as described (Choi et al., 1996). Primary antibodies used were mouse anti-L (1:20), mouse anti-β-galactosidase (1:250; Promega), rabbit anti-β-galactosidase (1:200; Cappel), rabbit anti-Dlg (1:1000; K. Cho), guinea pig anti-Dlg (P. Bryant), rabbit anti-Ser (1:20; E. Knust) and mouse anti-Elav. Secondary antibodies were horse radish peroxidase-conjugated anti-rabbit IgG, anti-rabbit fluorescein isothocyanate (FITC) and anti-mouse CY3 (Jackson Laboratory). Fluorescent images were taken with Zeiss LSM laser-scanning confocal microscope.

RESULTS

L is required for ventral-specific growth during early eye disc development

With variable severities and penetrances, all *L* alleles examined (*L^{1}*, *L^{2}*, *L^{4}*, *L^{5}* and *L^{si}*) as heterozygotes exhibit a nick near the anterior midline of the eye, and the overall size of the eye is slightly reduced. As homozygotes, eye size is greatly reduced, primarily in the ventral domain. *L^{si}* allele typifies the observed phenotypes with the highest penetrance. Homozygous *L^{si}* animals show a preferential loss of the ventral eye with complete penetrance (Fig. 1B), and ~70% of *L^{si}*/+ animals have an anterior nick in one or both eyes (Fig. 2G). The preferential loss of the ventral eye is also apparent in the eye imaginal disc morphology (Fig. 1D). Importantly, as homozygous *L^{si}* mutants are viable and its half-eye mutant phenotype is indistinguishable from that of *L^{si}* over deficiency chromosomes, this suggests that *L^{si}* is a strong eye-specific allele that minimally affects the development of other tissues.

To assess the extent of ventral eye loss in *L^{si}* homozygotes, we used an enhancer trap line, *mirr^{B1-12}* (*mirr-lacZ*), which has a dorsal-specific expression of *white* (*w*) reporter gene (Fig. 1E) (Choi et al., 1996). In *w$^{–}$*; *L^{si}/L^{si}*; *mirr^{B1-12}*/+ flies, the overall eye size was reduced, and all but one or two rows of remaining ommatidia were *w^{+}*, suggesting that most, if not all, of the ommatidia were dorsal (Fig. 1F). The dorsal polarity was confirmed in adult-eye sections (data not shown). Eye imaginal discs from staged *w$^{–}$*; *L^{si}/L^{si}*; *mirr^{B1-12}*/+ larvae showed ventral domain reduction starting at early second instar (Fig. 1H), indicating that *L* functions are required for early eye development.

To further study the wild-type function of *L*, we generated clones of *L$^{–}$* cells by mitotic recombination (Xu and Rubin, 1993), using a loss-of-function allele, *L^{rev6-3}* (see Materials and Methods). Homozygous *L^{rev6-3}* embryos with no detectable L protein expression fail to complete germ-band retraction and show no cuticle formation. *L^{rev6-3}* clones caused distinct eye phenotypes depending on the time of clone induction and the location of the clones. In one scheme, clones were induced at the first instar stage. In the resulting third instar eye discs that contained ventral *L^{rev6-3}* clones, the ventral eye disc was greatly reduced, and to a large extent the *L^{rev6-3}*/+ and +/+ tissue disappeared together with the *L^{rev6-3}/L^{rev6-3}* tissue (Fig. 1I). By contrast, dorsal clones of considerable size did not cause obvious size reduction in the dorsal eye, nor did the mutant clones significantly affect the ensuing photoreceptor differentiation and polarity determination (Fig. 1I). Consistent with the eye disc phenotype, adult mosaic eyes had a relatively

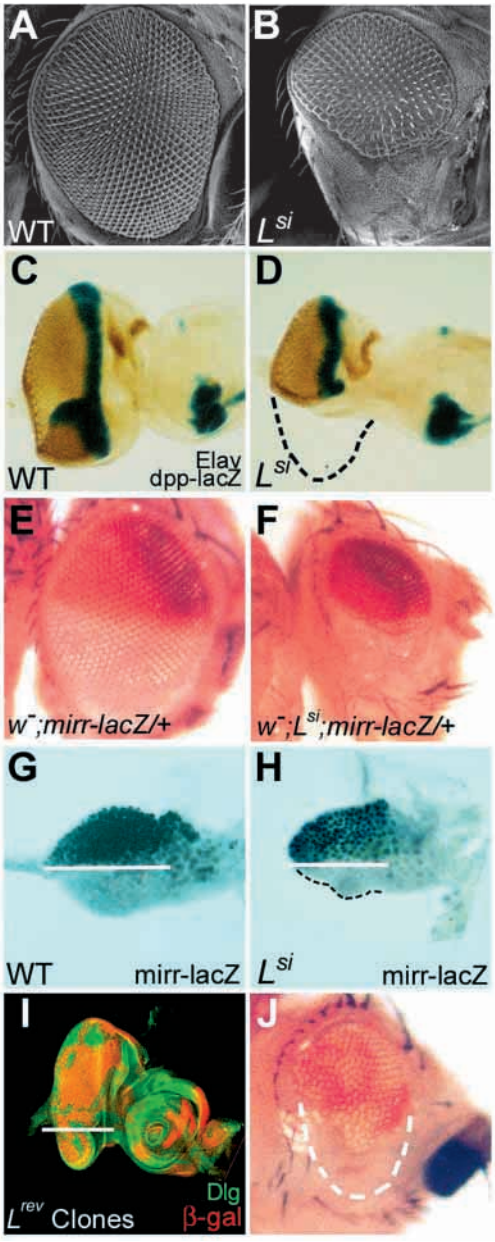

Fig. 1. Loss of ventral eye in *L* mutants. (A,B) Wild-type and L^{si} homozygote adult eyes by scanning electron microscopy. (C,D) Wild-type and L^{si} third instar eye discs. The morphogenetic furrow and photoreceptor clusters were respectively marked with *dpp-lacZ* reporter staining (blue) and neural marker Elav (brown). Furrow progression and Elav expression were normal within the remaining eye disc. (E) *mirr-lacZ* enhancer trap line, $mirr^{B1\text{-}12}$. (F) w^{-}; L^{si}; $mirr^{B1\text{-}12}$/+ flies have mostly w^{+} ommatidia. (G) Wild-type, second instar eye disc stained with anti-β-gal to label *mirr-lacZ*$^{+}$ dorsal cells (brown). (H) In L^{si} second instar eye disc, *mirr-lacZ*$^{+}$ dorsal domain is considerably larger than the ventral domain (outlined by the broken line). (I) L^{-} mosaic eyes disc generated with $L^{rev6\text{-}3}$ allele. Clones were visualized by the absence of the *lacZ* marker (red). Ventral, but not dorsal domain of the eye disc was greatly reduced. A cell junction marker, Dlg (green), was used to outline cells in the disc. (J) Adult mosaic eye showed intact dorsal eye and cuticle-replaced ventral eye. In all figures, white lines mark the DV boundary and broken lines mark the putative ventral domain. Dorsal is upwards and anterior towards the right.

normal appearing dorsal domain, while most of the ventral region was replaced by the cuticle (Fig. 1J). In related experiments, mitotic recombination induced at late second and third instar stages generated multiple clones in both dorsal and ventral domains but did not result in any obvious eye defects (data not shown), suggesting an early, transient requirement of *L*. All together, studies of L^{si} phenotype and $L^{rev6\text{-}3}$ clonal phenotype indicate that *L* is non-autonomously required for the ventral eye growth but not so in the dorsal, and its functions are required during early stages of eye development.

L mediates N signaling in the ventral eye

Previous studies have shown that N activation at the DV boundary is vital for eye disc growth (Papayannopoulos et al., 1998; Cho and Choi, 1998; Dominguez and de Celis, 1998). As *L* is required specifically for ventral growth, it raises the possibility that *L* may mediate the proliferative effect of midline N signaling in the ventral eye. We used the Gal4-UAS system (Brand and Perrimon, 1993) to test this hypothesis. Overexpression of a constitutively active *N* (N^{intra}) (Fortini et al., 1993) by the *dpp-Gal4* driver, which drives expression along the posterior edge of the eye disc, caused gross overgrowth of the eye in both dorsal and ventral domains (compare wild type in Fig. 2A with 2B). Reducing *L* gene dose strongly suppressed the ventral overgrowth but had much less of an effect on dorsal overgrowth (Fig. 2D). This ventral-specific suppression of N gain-of-function phenotype suggests that *L* acts downstream of *N*.

In contrast to *N*-induced overgrowth, eliminating N signaling by expressing a dominant-negative form of *N* (N^{DN}) (Klein et al., 1997) using the *eyeless (ey)-Gal4* driver consistently resulted in small-eye or no-eye (Fig. 2E). *ey-Gal4* drives Gal4 expression in early eye discs and anterior to the furrow in the third instar discs. Co-expression of *L* and N^{DN} partially suppressed this N^{DN} overexpression phenotype in the ventral domain: ventral eye was selectively restored in close to 20% of *ey-Gal4/UAS-*N^{DN} *UAS-L* animals (19/86 animals; Fig. 2F). The size of the restored ventral eye was either smaller or equal to the reduced dorsal eye, and in no instances did we detect ventral tissue without the presence of at least some dorsal tissue. The presence of residual dorsal eye indicates that N^{DN} overexpression may not completely eliminate endogenous N functions. It also suggests that N activity, even at a low level, is a prerequisite for *L* to induce ventral proliferation.

Given that the requirement of *L* functions is early and transient, the suppression by *L* of N^{DN} phenotype may be specific to undifferentiated cells. That is indeed the case. We overexpressed N^{DN} using *GMR-Gal4* that induces Gal4 expression in all cells posterior to the furrow. *GMR-Gal4/UAS-*N^{DN} animals showed a rough eye phenotype with a relatively normal eye size, and the eye roughness was not suppressed by overexpression of *L* in *GMR-Gal4/UAS-*N^{DN} *UAS-L* animals (data not shown).

If *L* and *N* act in the same pathway, transheterozygous mutations of these two genes may result in enhanced phenotypes. L^{si}/+ flies have nicks at the anterior edge of the eye (Fig. 2G), but the defect is not so severe to result in half-eyes. Loss of one copy of *N* does not cause visible eye defects

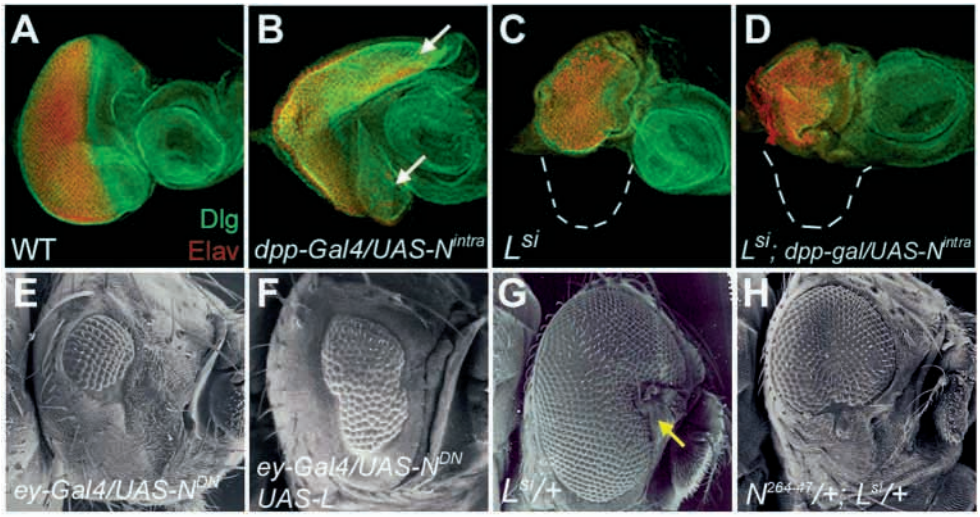

Fig. 2. *L* mediates N signaling in the ventral eye. (A) Wild-type, third instar disc double-stained with anti-Elav (red) and anti-Dlg (green). (B) *UAS-N^{intra}/dpp-Gal4* caused overgrowth in dorsal and ventral domains (arrows). (C) *L^{si}* mutant showed a loss of the ventral domain. (D) Loss of *L* suppressed *UAS-N^{intra}/dpp-Gal4* phenotype in the ventral domain. The putative ventral domain is outlined. Dorsal overgrowth persisted, such that the tissue had a crumpled, uneven appearance. (E) *UAS-N^{DN}/ey-GAL4* caused a general loss of tissue. (F) Co-expression of *UAS-L* and *UAS-N^{DN}* specifically restored the ventral eye. (G) *L^{si}/+* adult eye showed a characteristic anterior notch (yellow arrow). (H) Removing a copy of *N* in *L^{si}/+* animals increased the severity of the *L* eye phenotype.

(Grimwade et al., 1985). Transheterozygote *N$^{264-47}$/+*; *L^{si}/+* adults, however, had half-eyes in one or both eyes with approximately 50% penetrance (Fig. 2H). We had also observed similar enhancement of *L* phenotype by mutations in *Enhancer of split*, a major downstream effector of N signaling (data not shown). In summary, genetic interactions between *L* and *N* support the hypothesis that *L* mediates the proliferative effect of N signaling specifically in the ventral domain.

L regulates *Ser* expression in the eye disc

It is likely that *L* interacts with other ventral-specific genes, and one candidate gene is *Ser*, a N ligand, whose expression is ventrally enriched in the wild-type first instar eye disc (Fig. 3A) (Cho et al., 2000). *Ser* is required for eye development as *Ser* loss-of-function mutants have small eyes (Speicher et al., 1994; Go et al., 1998). To understand possible regulatory relations between *L* and *Ser*, we first examined *Ser* expression in first instar eye discs from *L^{si}* homozygotes. In these eye discs, ventral *Ser* expression was greatly reduced, but notably the expression along the DV boundary was not affected (Fig. 3B, bracket). We used both a *Ser-lacZ* enhancer trap line and an anti-Ser antibody to detect *Ser* expression in these *L^{si}* homozygotes eye discs, and similar *Ser* expression patterns were observed (data not shown). This observation suggests that *L* might promote ventral *Ser* expression except in regions near the DV boundary. In addition, as *L^{si}* homozygotes eventually lose its ventral eye, loss of ventral *Ser* expression in these mutants suggests that *Ser* may positively regulate ventral eye growth.

We induced *L* null clones to further test the hypothesis that *L* is a region-specific, positive regulator of *Ser* expression in first instar eye discs. *L^{rev6-3}* clones altered *Ser* expression in a position-dependent way. Ventral *L*$^{-}$ clones away from the DV boundary showed decreased *Ser* expression within the clone (four clones scored; Fig. 3D-F, white arrow), but clones near the posterior DV boundary had no such effects (three clones scored; Fig. 3D-F, yellow arrow). The effect of *L*$^{-}$ cells on *Ser* expression appeared to be restricted within the clone as *Ser* expression outside of the

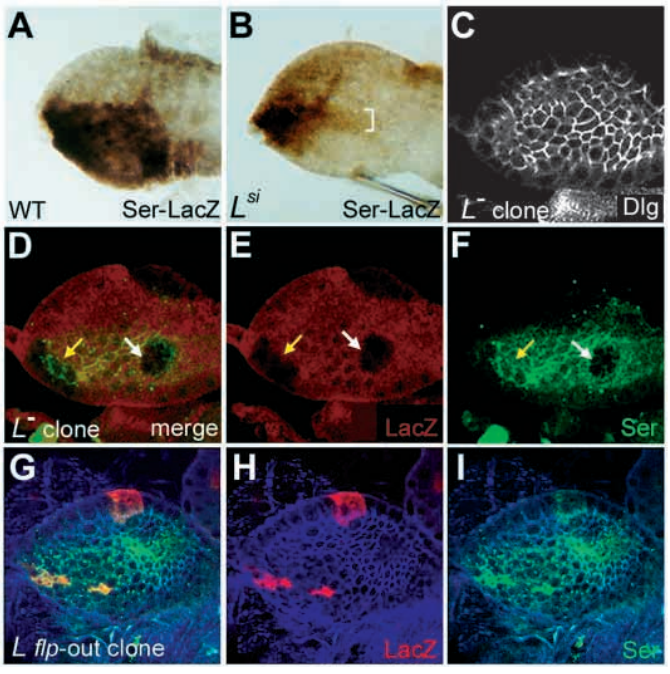

Fig. 3. *L* regulates *Ser* expression. (A) In wild-type first instar eye disc, *Ser* was expressed in the ventral domain and at the DV boundary. (B) In *L^{si}* first instar eye disc, ventral *Ser* expression was lost but posterior midline *Ser* expression (bracket) persisted. (C) Dlg marked cell boundaries of the eye disc shown in (D-F). (D) Composite of E,F. Ventral *L^{rev6-3}* clone in first instar eye disc showed a decreased *Ser* expression within the clone (white arrow). However, *L^{rev6-3}* clone near the posterior DV boundary did not affect *Ser* expression (yellow arrow). There was also a dorsal anterior clone present. (E) *L^{rev6-3}* clones were marked by the absence of *lacZ* (red). (F) Anti-Ser antibody detected Ser protein expression. (G) Composite of H,I. *L flp*-out clones (red in H) upregulated *Ser* expression (green in I) in both dorsal and ventral domains of the first instar eye disc. Dlg staining (blue) marks disc cells.

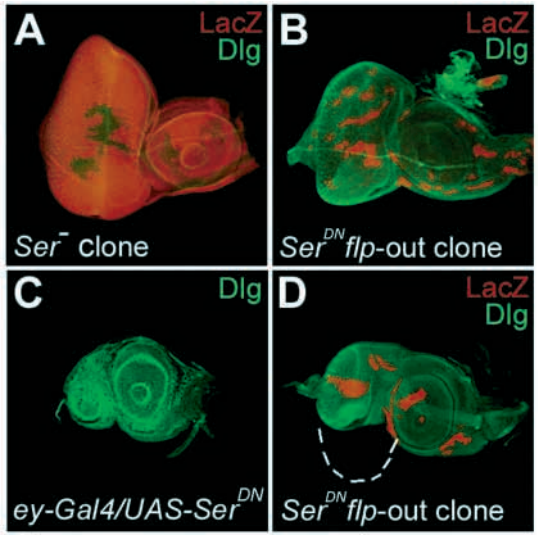

Fig. 4. Loss of *Ser* affects ventral growth. (A) *Ser*$^{-}$ clones, marked by the absence of *lacZ* (red), did not affect eye size or photoreceptor cluster formation in either domain. (B) An eye disc that contains *Ser*^{DN} *flp*-out clones. Overexpression of Ser^{DN} decreased ventral cell viabilities such that only small ventral clones were observed. By contrast, there were more dorsal clones and they were considerably larger. (C) Overexpression of Ser^{DN} by the *ey-Gal4* driver abolished eye development. (D) *Ser*^{DN} *flp*-out clones caused a preferential loss of the ventral domain, similar to *L*.

clone was not visibly affected (Fig. 3D-F, white arrow). To test if *L* positively regulates *Ser* expression, we used the *flp*-out system (Pignoni and Zipursky, 1997) to generate clones of cells that overexpress *L*. In dorsal and ventral domains of first instar eye discs, *L flp*-out clones either induced or upregulated *Ser* expression, respectively (Fig. 3G-I). In summary, *L* is crucial in maintaining ventral *Ser* expression levels.

Loss of *Ser* affects ventral eye disc growth

To understand the role of *Ser* in eye growth, *Ser*-null (*Ser*$^{rev2\text{-}11}$) mutant clones were examined in eye discs and adult eyes. We did not find obvious defects in the size of the eye disc or photoreceptor differentiation (Fig. 4A), consistent with previous reports (Sun and Artavanis-Tsakonas, 1996; Papayannopoulos et al., 1998). This lack of *Ser*-null clone phenotype implies that either the Ser protein is diffusible, or *Ser* is functionally redundant. In order to remove more of the wild-type *Ser* functions, we generated *flp*-out clones expressing a diffusible, truncated form of *Ser* (*Ser*^{DN}). Ser^{DN} consists of Ser extracellular domain but lacks the transmembrane domain and is capable of antagonizing wild-type *Ser* functions (Hukriede et al., 1997).

Eye discs that contained *Ser*^{DN} *flp*-out clones were variably reduced. Three kinds of phenotypes were observed and might be attributed to slight variations in the timing of clone inductions and the location of the clones:

(1) In 19 eye discs that contained 52 dorsal clones, there were only two small ventral clones found (one such eye disc is shown in Fig. 4B). This suggests that while the expression of Ser^{DN} had little effects on dorsal and medial cell viabilities, ventral cells expressing Ser^{DN} failed to proliferate and/or died.

(2) The most severely affected eyes were rudimentary, probably owing to clones near the DV boundary that disrupted the wild-type *Ser* functions in DV boundary formation (Papayannopoulos et al., 1998; Cho and Choi, 1998; Dominguez and de Celis, 1998). Similar phenotype was also observed in *ey-Gal4/UAS-Ser*^{DN} eye discs that lacked a DV boundary (Fig. 4C) (Kumar and Moses, 2001).

(3) In some cases, a preferential reduction of the ventral eye occurred (Fig. 4D), resulting in a phenotype that was strikingly similar to that of *L*$^{-}$ mosaic clones (Fig. 1I,J).

Taken together with the regulatory relations between *L* and *Ser*, these results suggest that loss of ventral *Ser* expression probably contributes to *L* ventral eye loss.

Cloning and expression of *L*

L was placed in the 51A2-B1 region based on the breakpoints of *Df(2R)L4*, a chromosomal deficiency uncovering *L* (Baker and Ridge, 1980). Multiple overlapping cosmids spanning the 150 kb *L* region were isolated, and genetically transformed lines containing these rescue constructs were generated (see Materials and Methods). One of these lines, cosD3, contains ~25 kb genomic DNA and was able to rescue eye phenotypes of the eye-specific *L*^{si} allele, reducing both severity and penetrance of mutant phenotypes (Fig. 5A, Table 1). The rescue of *L*^{si}/+ eye phenotypes by increasing wild-type copies of the gene suggests that *L*^{si} mutation is dominant negative in nature (Table 1). cosD3 also rescued the recessive lethality of *L*^{P17} allele (see below).

The rescue of *L* associated phenotypes and lethality by cosD3 identifies the genomic region that encodes the *L* gene. Furthermore, a 8.5kb *Eco*RI fragment of cosD3 detected a restriction polymorphism on the Southern blot of an *L* inversion allele, *L*^{rv5} (Baker and Ridge, 1980) (data not shown). Using this 8.5 kb fragment as a probe, multiple overlapping cDNA clones from an embryo cDNA library were isolated. Sequence analysis of multiple cDNA clones revealed a 1.6 kb open reading frame (ORF) and 5′ and 3′ untranslated sequences of 308 and 565 bp, respectively. The ORF agrees with the CG10109 sequence deposited by the *Drosophila* Genome Project. A representative 2.6 kb cDNA probe detected a single transcript of similar size on a northern blot made from larvae of all stages, suggesting that it represents the full-length transcript (Fig. 5D).

To verify that CG10109 is indeed the *L* gene, we identified the P-element insertion site of *L*^{P17} (see Materials and Methods). *L*^{P17} is recessive embryonic lethal and is phenotypically allelic to *L* alleles. *L*^{P17}/*L*^{si} animals showed loss of the ventral eye that is indistinguishable from *L*^{si}/*L*^{si} half-eye phenotype. Approximately 40% of *L*^{P17}/+ heterozygotes had defects at the anterior edge of the eye, similar to *L*^{si}/+ heterozygotes. Precise excision of the P-element restored the wild-type eye as well as viability, demonstrating that it was responsible for the mutant phenotypes. The P-element of *L*^{P17} was inserted 163 bp downstream to the start codon in the first exon of CG10109 (Fig. 5A,B). The recessive lethality of *L*^{P17} was partially rescued by cosD3: the viability of *L*^{P17}/*L*^{P17}; cosD3/+ animals was 80% less than the *L*^{P17}/+; cosD3/+ animals. Finally, full-length *UAS-L* cDNA construct under the control of *hs-Gal4* driver was able to rescue lethality associated with the *L*^{P17} allele when given daily heat shock throughout embryonic and larval stages. The rescued animal restored the eye fully (~70% of flies) or partially (30% of flies). The rescue

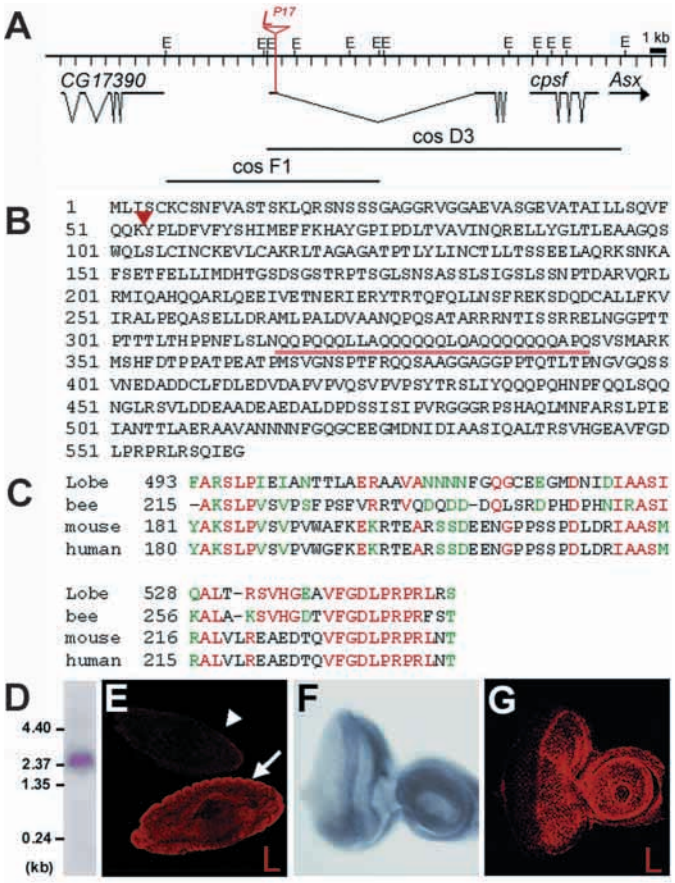

Fig. 5. *L* is a novel protein with preferential expression anterior to the furrow. (A) Overlapping cosmids spanning the 51A2-B1 regions were isolated. Cos D3, but not cos F7, rescued the *L* phenotype and the lethality of L^{P17} allele, which has a P-element inserted in the first exon. E: *Eco*RI site. (B) The putative L protein contains a poly-glutamine rich region (underlined). Arrowhead indicates the P-element insertion site of L^{P17}. (C) Sequences of the C terminus of *L* and its related proteins over a span of 67 amino acids: bee (BI509118) (43% identical; 63% positive), mouse (AK003638) and human (BC007416) (37% identical; 53% positive). In the last 30 amino acids, 56% are identical. The red letters indicate identical amino acids and green ones indicate similar amino acids. (D) Full-length *L* cDNA detected a single band on a northern blot of total RNA extracted from larvae of all stages. (E) Embryo of $L^{rev6\text{-}3}/L^{rev6\text{-}3}$ (arrowhead), a loss-of-function allele, has no detectable level of L protein (red). In $L^{rev6\text{-}3}/+$ embryo (arrow) the protein was readily detected. The genotype of the embryos was distinguished with green fluorescent protein marker (not shown). (F,G) In third instar eye/antenna discs, *L* transcripts, detected by RNA in situ hybridization (F), and L protein, detected by antibody (G), are most intense in the outer antenna ring and in the eye disc anterior to the furrow

of lethality and eye defects by *L* genomic DNA and cDNA positively identifies CG10109 as the *L* gene.

The putative L protein is 562 amino acids long and contains a poly-glutamine rich region and a C terminus that shares significant sequence similarities with novel insect, mouse and human proteins of unknown functions (Fig. 5C). A polyclonal antibody was generated, and it detected a single band of ~60 kDa on a western blot prepared from third instar eye imaginal discs (data not shown). Furthermore, this antibody revealed no

Table 1. cosD3 partially rescues L^{si} phenotype*

Genotype	Phenotype
$L^{si}/+$	66% Anterior nick (in one or both eyes) 34% Normal
$L^{si}/+$; *P{w⁺-cosD3}/+*	21% Anterior nick 79% Normal
L^{si}/L^{si}	100% Half eye
L^{si}/L^{si}; *P{w⁺-cosD3}/+*	54% Half eye 40% Intermediate phenotype 6% Normal
L^{si}/L^{si}; *P{w⁺-cosD3}/ P{w⁺-cosD3}*	16% Half eye 31% Intermediate phenotype 53% Normal

*For each genotype, at least 100 flies were scored to determine the percentage of individual phenotypes.

detectable level of L protein expression in null animals (Fig. 5E). To examine *L* expression pattern, *L* transcript and protein were detected by mRNA in situ hybridization and by the polyclonal antibody. *L* is ubiquitously expressed in first instar eye discs (data not shown). In the third instar disc, the RNA transcripts were detected in the antenna disc and in undifferentiated cells anterior to the furrow in the eye disc (Fig. 5F). By comparison, the protein could be readily detected in the antenna and anterior to the furrow, but a much lower level of expression is also present posterior to the furrow (Fig. 5G).

DISCUSSION

N promotes growth via *L* in the *Drosophila* eye

The role of N signaling in growth is well documented in many organisms (for a review, see Artavanis-Tsakonas et al., 1999). For example, constitutive N activation in *C. elegans* causes germline tumor formation (Berry et al., 1997), and in humans, expression of human *Ser* homolog, *JAG1* (jagged 1), induces the expansion of hematopoietic stem cells (Varnum-Finney et al., 1998; Karanu et al., 2000). In *Drosophila*, N activation at domain boundaries is required for the growth of wing and eye imaginal discs. In developing wing discs, activated N along the DV boundary upregulates the expression of Wg, which in turn induces wing outgrowth (Diaz-Benjumea and Cohen, 1995). In the eye disc, the proliferative effect of N is also likely to be mediated by diffusible factors emitted from midline, although their identities are not known at this time.

We have presented genetic evidence that *L* could be a component of an intracellular pathway that transduces N signaling in cells of the ventral eye (Fig. 2). The conserved *L* sequences suggest that its mammalian counterparts may play similar functions in mediating N signaling, although the precise function of the conserved domain is not known. The striking domain specificity of *L*-mediated growth may be the result of various mechanisms. It is possible that signaling molecules sent to the ventral domain are different from the ones sent to the dorsal. Signaling molecules may be selectively sequestered in one domain but not the other, as in the case of *dpp* signaling in the wing disc (Teleman and Cohen, 2000). *L* may cooperate with other ventral-specific genes to transduce

the N signaling, or the expression of *L* may be transiently ventral specific in the early eye disc. More than one mechanism may contribute to the domain specificity of *L* functions.

Role of *Ser* and *L* in asymmetrical eye growth

Previous experiments have shown that growth of neighboring, symmetrical domains may be independently controlled. In *Drosophila* wing discs, increased expression of *hedgehog* along the anteroposterior boundary causes anterior wing overgrowth but has no effects on the posterior wing (Tabata and Kornberg, 1994; Johnson et al., 1995); ubiquitous overexpression of *Ser* increases the ventral wing tissue but not the dorsal (Speicher et al., 1994). Additionally, there are other *Drosophila* eye mutants that show preferential reductions of the ventral eye, such as *wg* mutants (Ma and Moses, 1995) and *dpp* mutants (Treisman and Rubin, 1995). However, dominant mutation *Rough eye* suppresses these 'furrow stop' mutant phenotypes of *dpp* and *wg* but not the *L* phenotype (Heberlein et al., 1993), suggesting fundamental differences in nature and function between *L* and furrow stop mutants.

Clonal study shows that *L* null clones have striking domineering non-autonomous effects, such that the viability of wild-type tissue immediately adjacent to *L* mutant clones are severely reduced (Fig. 1I). Nevertheless, this non-autonomous deleterious effect is limited to ventral domain, as clones abutting the DV boundary do not seem to affect dorsal cell viability. This domineering non-autonomous effect may be the result of interspersed *L* null clones disrupting the physical integrity of the imaginal disc epithelium, causing the disc to fall apart. It is also possible that *L* is redundant in the dorsal domain, thus loss of *L* can be compensated by another dorsal-specific gene. Another possibility that we favor incites a failure of the clone cells to send out a locally acting growth signal. As our data indicate that *L* is a regulator of *Ser* expression (Fig. 3), could Ser be this local-acting, diffusible factor?

Homozygous *Ser* mutants have small eyes, indicating that *Ser* is required for proper eye growth. However, removing *Ser* in clones of cells does not result in mutant eye phenotypes, as shown in this report (Fig. 4A) and others (Sun and Artavanis-Tsakonas, 1996; Papayannopoulos et al., 1998). These observations are consistent with Ser being a diffusible factor, but other possibilities exist.

First, there may be other functionally redundant *Ser*-like molecules, but no candidates have yet been identified. Second, in *Ser*-expressing cells, *Ser* may autonomously induce the expression of diffusible signaling molecules that act non-autonomously. Ligand and receptor interactions within the membrane of the same cell have been demonstrated in the case of *N* and *Dl*. *N* and *Dl* can physically associate within the membrane of a single cell (Fehon et al., 1990), and the expression level of *Dl* in a cell can modulate its own N response (Jacobsen et al., 1998). In this manner, *Ser*-*N* interaction may lead to the expression of diffusible factors that rescue clones of Ser^{-} cells. Third, the ability of adjacent wild-type cells to rescue Ser^{-} cells suggests that Ser protein may be diffusible. This is consistent with low levels of Ser expression that we observed in L^{-} clones; and in our anti-Ser antibody staining, we consistently observed intense, dotty cytoplasmic staining, possibly secretory vesicles (Fig. 3). However, the presence of secreted Ser is yet to be confirmed, even though diffusible Dl has been detected in *Drosophila* extract (Qi at al., 1999).

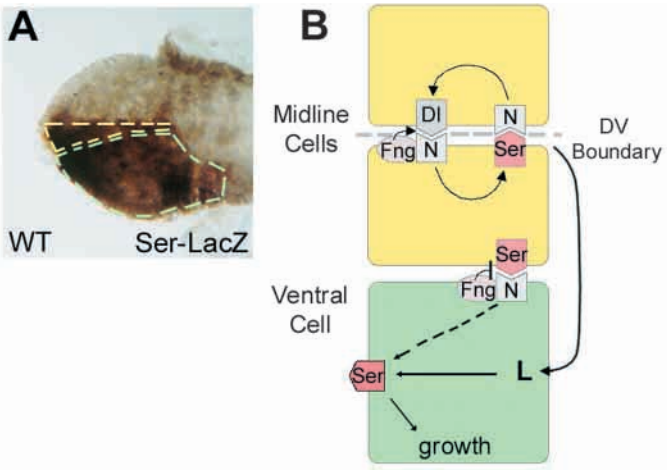

Fig. 6. *Drosophila* eye disc is partitioned into three domains. (A) Dorsal cells, ventral midline cells (yellow outline) and ventral cells (green outline) may develop independently. (B) Molecular interactions among N ligands and N in midline cells (yellow cells) result in the non-autonomous activation of *L*/*Ser* pathway to promote proliferation of the rest of the ventral domain (green cells).

Two distinct groups of cells control DV boundary formation and domain-specific proliferation

The domain-specificity of *L* phenotype indicates that the eye disc is partitioned, and the growth of individual domain is differentially regulated. Loss of the ventral eye in *L* mutants does not seem to affect DV boundary formation or the associated midline N activation, because disruptions of either of these events would result in abnormal dorsal growth. Additional data suggest that *L* does not affect the initial DV domain specification: (1) *L* is functionally downstream to N activation (Fig. 2); (2) *L* mutation does not affect *Ser* expression at the DV boundary (Fig. 3); and (3) domain-specific expression patterns of *dpp*, *fng* and *wg* are not affected in the first instar *L* mutant eye discs (data not shown).

Consistent with this model, we propose that in the seemingly homogenous *Ser*-expressing, first instar ventral domain, there are actually two distinct groups of *Ser*-expressing cells: ventral midline cells (Fig. 6A, yellow outline) abutting the dorsal midline cells, and the rest of the ventral cells (Fig. 6A, green outline). Their putative functions are different and their *Ser* expression is independently regulated. In the ventral midline cells, Ser is involved in setting up the DV boundary, and its expression is regulated by the Ser-N-Dl positive-feedback loop (Fig. 6B, yellow cells). The midline *Ser* expression can be further modified by Fng and Hedgehog, both of which can induce *Ser* expression only near the DV boundary but not elsewhere in the eye field (Papayannopoulos et al., 1998; Cho et al., 2000), emphasizing again the distinctiveness of these midline cells.

By comparison, in the rest of the ventral domain, *Ser* is directly involved in controlling local growth. Loss of *Ser* in the ventral domain causes ventral-specific growth defects similar to the loss of *L* (Figs 1, 4). *Ser* expression in the ventral domain may not be sustained by the Ser-N-Dl loop, as ventral Fng inhibits potential Ser-N interaction which is necessary to

initiate the positive feedback loop (broken line in Fig. 6B) (Papayannopoulos et al., 1998). Instead, ventral *Ser* expression is regulated by *L* (Fig. 3).

Our data suggest the eye primordium is partitioned into dorsal, midline and ventral domains with different gene expression and growth properties. It highlights the importance of local cellular context in interpreting signals released from the domain boundaries and shows that the growth of symmetrical domains may be asymmetrically regulated. Our model may also be applicable to the development of other imaginal discs as well as other developmental systems.

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