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Lobe and Serrate are required for cell survival during early eye development in *Drosophila*

Amit Singh^{1,*}, Xiao Shi¹ and Kwang-Wook Choi^{1,2,3,*}

Organogenesis involves an initial surge of cell proliferation, leading to differentiation. This is followed by cell death in order to remove extra cells. During early development, there is little or no cell death. However, there is a lack of information concerning the genes required for survival during the early cell-proliferation phase. Here, we show that *Lobe* (*L*) and the Notch (*N*) ligand *Serrate* (*Ser*), which are both involved in ventral eye growth, are required for cell survival in the early eye disc. We observed that the loss-of-ventral-eye phenotype in *L* or *Ser* mutants is due to the induction of cell death and the upregulation of secreted Wingless (*Wg*). This loss-of-ventral-eye phenotype can be rescued by (i) increasing the levels of cell death inhibitors, (ii) reducing the levels of Hid-Reaper-Grim complex, or (iii) reducing canonical *Wg* signaling components. Blocking Jun-N-terminal kinase (JNK) signaling, which can induce caspase-independent cell death, significantly rescued ventral eye loss in *L* or *Ser* mutants. However, blocking both caspase-dependent cell death and JNK signaling together showed stronger rescues of the *L*- or *Ser*-mutant eye at a 1.5-fold higher frequency. This suggests that *L* or *Ser* loss-of-function triggers both caspase-dependent and -independent cell death. Our studies thus identify a mechanism responsible for cell survival in the early eye.

KEY WORDS: *Drosophila* eye, Cell survival, Cell death, JNK Signaling, *Wg* Signaling, *Lobe*, *Serrate*

INTRODUCTION

The developing eye of the fruit fly (*Drosophila melanogaster*) has been extensively used to study developmental patterning. The compound eye of *Drosophila*, comprised of approximately 800 ommatidia, or unit eyes, develops from an epithelial bi-layer structure called the imaginal disc. Differentiation in the eye is marked by a wave of apical indentation of the disc referred to as the morphogenetic furrow (MF), which is initiated in the posterior margin of the eye disc and proceeds anteriorly (Ready et al., 1976; Wolff and Ready, 1993). Prior to furrow initiation, it is important to establish the dorsal and ventral compartments for the growth of early eye discs (for a review, see Singh et al., 2005b). Recent studies suggest that the ground or default state of the entire early eye imaginal primordium is ventral, and that the onset of expression of the dorsal selector gene *pannier* (*pnr*), establishes the dorso-ventral (DV) lineage in the eye (Maurel-Zaffran and Treisman, 2000; Singh and Choi, 2003).

L and *Ser* regulate ventral eye growth (Singh et al., 2005b). *L* acts upstream of *Ser*, a Notch (*N*) ligand in the ventral eye (Chern and Choi, 2002; Singh et al., 2005b). Loss of *L* or *Ser* (hereafter *L/Ser*) function exhibits distinct mutant phenotypes depending on the stage of DV eye patterning. Early loss-of-function (LOF) of *L/Ser*, when the entire eye field is in the default ventral state, results in the loss of the entire eye. However, LOF of *L/Ser* after the onset of *pnr* expression, during second larval instar of development, results in the loss of only the ventral half of the eye (Singh and Choi, 2003; Singh et al., 2005b). This suggests that ventral-specific cells are selectively lost from the developing eye of *L/Ser* mutants by a currently unknown mechanism. One possible mechanism for the loss of eye pattern in *L/Ser* mutants may be due to the induction of cell death in the ventral cells.

In the *Drosophila* eye, active caspases execute apoptosis (Song et al., 1997; Fraser et al., 1997; Meier et al., 2000; Hays et al., 2002; Yu et al., 2002). Inhibitor of apoptosis proteins (IAPs), a highly conserved class of proteins, negatively regulate caspase activity (Miller, 1999; Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000; Wilson et al., 2002). The *Drosophila* pro-apoptotic genes *wrinkled* [also known as *head involution defect* (*hid*), and hereafter referred to as *hid*], *reaper* (*rpr*) and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996) in turn negatively regulate IAPs. These genes encode the members of Hid-Reaper-Grim (HRG) complex, which binds to and inactivates IAPs (Ryoo et al., 2002; Yoo et al., 2002; Holley et al., 2002). The overexpression of the baculovirus protein P35 can block caspase-dependent cell death (Hay et al., 1994). However, there are also some caspase-independent cell-death pathways. For example, cell death induced by extrinsic signals, such as UV-irradiation, causes DNA damage and consequently triggers P53-dependent cell death (Brodsky et al., 2000; Ollmann et al., 2000).

In the growing discs, apoptosis can be induced by a variety of stimuli, such as inappropriate levels of morphogens or extracellular signaling (Mehlen et al., 2005). Interestingly, the morphogens, such as Decapentaplegic (*Dpp*; a homolog of transforming growth factor- β) and Wingless (*Wg*; a Wnt homolog protein), that are required for early developmental steps, cause cell death when ectopically induced in a developing wing imaginal disc (Adachi-Yamada et al., 1999; Ryoo et al., 2004). During eye development, one of the many functions of *Wg* signaling is to induce apoptosis by activating the expression of *hid*, *rpr* and *grim* in ommatidia at the periphery of the eye during the pupal stage (Lin et al., 2004; Cordero et al., 2004).

When secreted morphogens attain inappropriate levels in the wing disc, activation of the c-Jun N-terminal kinases (JNKs) of the mitogen-activated protein-kinase super family occurs (Adachi-Yamada et al., 1999). This JNK-signal activation leads to caspase-3 activation, which induces cell death to eliminate cells with aberrant morphogen signal and to correct the morphogen gradient (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002). Caspase-3 plays a central role in many types of

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apoptosis, whereas JNK activation elicits a limited group of apoptotic events (Davis, 2000). In some cases, JNK signaling can induce caspase-independent cell death. Thus, the JNK-signaling pathway regulates apoptosis as well as other fundamental cell behaviors such as differentiation and morphogenesis (Adachi-Yamada and O'Connor, 2004; Stronach, 2005). In *Drosophila*, JNK signaling has a core signaling module consisting of Hemipterous (Hep, a JNK kinase), Basket (Bsk, a JNK) and Jun (Stronach, 2005). JNK signaling is manifested by the expression levels of *puckered* (*puc*), a gene encoding a dual-specificity phosphatase that forms a negative feedback loop by downregulating the activity of JNK (Martin-Blanco et al., 1998; Adachi-Yamada et al., 1999). Despite the known function of JNK signaling in morphogenetic cell death, its role in apoptosis during early eye imaginal disc development is not known.

During early eye development, there is a spurt of cell proliferation that allows the developing eye to attain a threshold cell number to initiate differentiation. As there is normally little or no cell death observed during early eye development, it will be important to understand the mechanism of cell survival during this stage. Therefore, there is a need to identify the genes required for cell survival during early eye development.

We propose that *L* and *Ser*, members of the N-signaling pathway, are required for cell survival during early eye development. We also present evidence that *L* and *Ser* promote cell survival through a mechanism that involves inactivation of the Wg signaling pathway. In the early eye, LOF of *L/Ser* results in the upregulation of Wg and the induction of caspase-dependent cell death. Our results demonstrate that, during the second larval instar stage, a time window prior to the initiation of retinal differentiation, the ventral cells are preferentially sensitive to Wg-dependent cell death. Wg is known to induce JNK signaling in the wing. In the eye, blocking the JNK signaling pathway can significantly rescue the *L/Ser*-mutant phenotype. Lastly, we found that the *L/Ser*-mutant phenotype observed in the eye is a result of the cumulative effect of the induction of the JNK-signaling pathway and of caspase-dependent cell death. Thus, we have identified a cell-survival mechanism required for early eye development.

MATERIALS AND METHODS

Stocks

Fly stocks used are described in Flybase (<http://flybase.bio.indiana.edu>). We used *y w eyFLP* (Newsome et al., 2000), *y w; L^{rev6-3} FRT42D/CyO*, *L²/CyO*, *L^{si}*, (Chern and Choi, 2002; Singh and Choi, 2003), *UAS-Ser^{DN}* (Hukriede et al., 1997), *wg¹* (Couso and Martinez-Arias, 1994), *wg^{CX3}*, *wg^{CX4}*, *UAS-wg* (Azpiroz and Morata, 1998), *wg^{IL114}* (Nusslein-Volhard et al., 1984), *wg-LacZ/CyO* (Kassis et al., 1992), *UAS-sgg^{S9A}*, *ey-GAL4* (Hazelett et al., 1998), *UAS-arm* (Zecca et al., 1996), *UAS-dTCF^{DN}* (van de Wetering et al., 1997), *puc^{E69}*, *UAS-puc* (Martin-Blanco et al., 1998), *hep^{r75}/FM7* (Glise et al., 1995), *bsk² FRT40/CyO*, *UAS-bsk* (Boutros et al., 1998), *UAS-bsk^{DN}* (Adachi-Yamada et al., 1999), *UAS-DJun^{aspv7}* (Treier et al., 1995), *UAS-p53* and *UAS-p53^{DN}* (Brodsky et al., 2000; Ollmann et al., 2000), *UAS-P35* (Hay et al., 1994), *UAS-diap1* (Lisi et al., 2000), and *Df(3L)H99/TM6B* (Abbott and Lengyel, 1991). We used a GAL4/UAS system for targeted misexpression studies (Brand and Perrimon, 1993). All GAL4/UAS crosses were performed at 18, 25 and 29°C, unless specified, to sample different induction levels.

Genetic mosaic analysis

LOF clones were generated using the FLP/FRT system of mitotic recombination (Xu and Rubin, 1993). To generate LOF clones of *L* in eye, *eyFLP; FRT42D ubi-GFP* females were crossed to *L^{rev6-3} FRT42D* males. MARCM clones were generated (Lee and Luo, 1999) by crossing *yw, hs-FLP, tub-GAL4, UAS-GFP-6xMYC-NLS; tubP-GAL80, FRT42/CyO* virgins to *L^{rev6-3} FRT42D; UAS-sgg^{S9A}* or *L^{rev} FRT42D; UAS-dTCF^{DN}* males.

Immunohistochemistry

Eye-antenna discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al., 2002). Antibodies used were mouse rabbit anti-β-galactosidase (1:200) (Cappel); chicken anti-GFP (1:200; Upstate Biotechnology); rat anti-Elav (1:100); mouse 22C10 (1:20); mouse anti-Wg (1:20) (Developmental Studies Hybridoma Bank); rabbit anti-*Drosophila* Ice (Drice) (a gift from B. Hay, California Institute of Technology, CA, USA); and rabbit anti-Dlg (a gift from K. Cho, Baylor College of Medicine, Houston, TX, USA). Secondary antibodies (Jackson Laboratories) used were goat anti-rat IgG conjugated with Cy5 (1:200); donkey anti-rabbit IgG conjugated to Cy3 (1:250) or donkey anti-mouse IgG conjugated to FITC (1:200); and donkey anti-chicken IgG conjugated to FITC.

Detection of cell death

Apoptosis was detected by TUNEL assays. Eye-antennal discs, after secondary-antibody staining (Singh et al., 2002), were blocked in 10% normal goat serum in phosphate buffered saline with 0.2% Triton X-100 (PBT) and labeled for TUNEL assays using a cell-death detection kit from Roche Diagnostics.

Temperature shift regimen

Eggs were collected from a synchronous culture for a period of 2 hours. Each egg collection was divided into several batches in different vials. These independent batches of cultures were reared at 16.5°C, except for a single shift to 29°C in a 24-hour time window during different periods of development spanning from first instar to the late third instar of larval development. After the incubation at 29°C, these cultures were returned to 16.5°C for the latter part of development.

RESULTS

Loss of *L/Ser* causes induction of cell death in the eye imaginal disc

To understand the mechanism by which the preferential elimination of ventral eye cells takes place in *L/Ser* mutants, we tested whether cell numbers are reduced because of cell-proliferation arrest or by the induction of cell death. BrdU labeling (de Nooij et al., 1996) and the expression of the cell-proliferation marker phospho-Histone H3, was not significantly affected in LOF clones of *L^{rev6-3} (L^{rev})*, a null

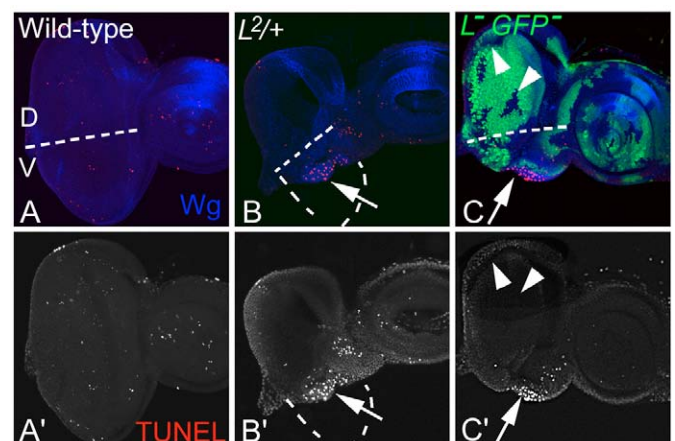


Fig. 1. Loss of *L* function causes selective induction of cell death in the ventral eye. (A,A') Wild-type eye imaginal disc showing a few randomly distributed TUNEL-positive nuclei of dying cells. (B,B') *L*-mutant eye disc (*L²/+*), showing TUNEL-positive ventral eye cells nuclei (arrow). (B,B') Semicircular dashed line marks the lost boundary of the ventral eye field. (C,C') LOF clones of *L* show TUNEL-positive cells nuclei in the ventral eye (arrow). The cells lacking *L* gene function in the dorsal half of the eye are not affected (arrowheads). Straight dashed lines in A-C indicate the approximate midline – the border between the dorsal (D) and ventral (V) eye.

allele of *L* (Chern and Choi, 2002), and *L*²/*+*-mutant eye discs (data not shown). *L*², a dominant-negative allele of *L*, shows loss of ventral eye in the *L*²/*+* background in 100% of flies (Fig. 4A,B) (Singh and Choi, 2003; Singh et al., 2005a). We overexpressed

cyclin A, cyclin B and cyclin E to accelerate cell-cycle progression in the *L*²/*+*-mutant background and did not find any significant rescues (data not shown). These results suggested that the cell-proliferation arrest may not play a significant role in the loss-of-ventral-eye phenotype in *L* mutation.

To test the possibility that cell death plays a role in the loss of the ventral eye, we employed the TUNEL assay, which marks the nuclei of dying cells. In the eye imaginal discs of wild-type flies, only rarely are a few nuclei TUNEL positive, suggesting that the majority of the cells are not undergoing cell death (Fig. 1A,A'). However, in the eye discs of *L*²/*+* mutants, TUNEL-positive nuclei were observed predominantly on the ventral eye margin (Fig. 1B,B'), but not in the dorsal half of the eye. We confirmed this result by TUNEL assays in the LOF clones of *L*^{rev} in the eye disc. As most of the ventral eye imaginal disc containing *L*^{rev} clones were eliminated, we were only able to see the TUNEL-positive nuclei near the ventral margin of the LOF clones (Fig. 1C,C'). However, the nuclei of *L*-mutant cells in the dorsal half of the eye, marked by the loss of GFP reporter, were not TUNEL positive (Fig. 1C,C').

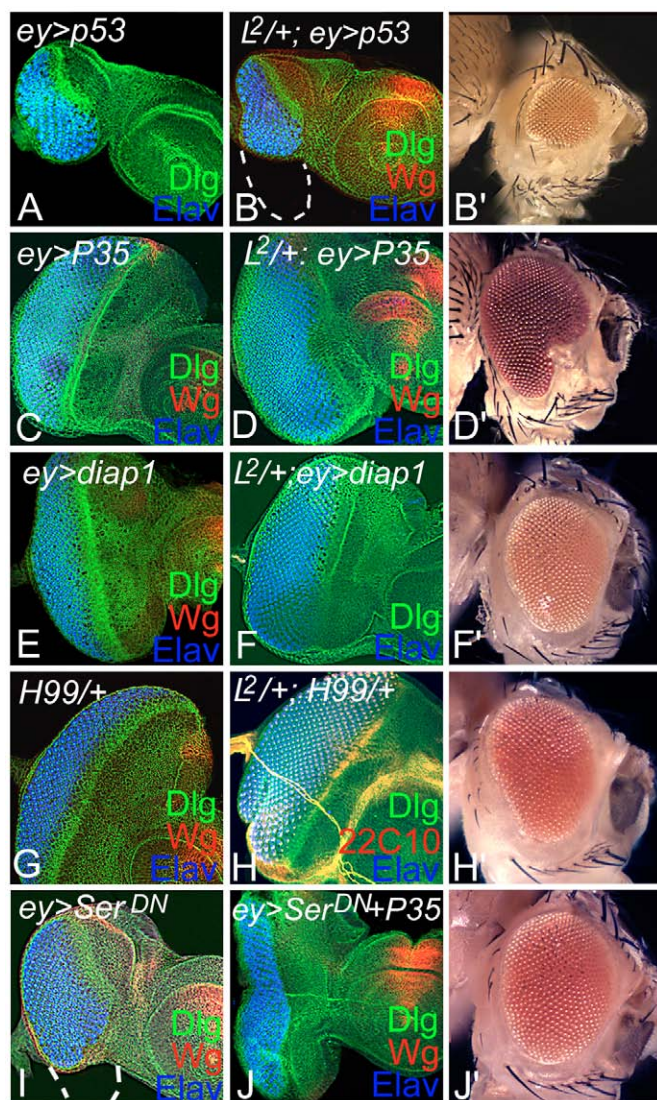


Fig. 2. Cell death in *L*/*Ser*-mutant cells is p53-independent and caspase-dependent. (A-B') Overexpression of p53 (A) in the wild-type eye using *ey*-GAL4 (*ey*>*p53*) results in a small eye and, in the *L*²/*+*-mutant eye (*L*²/*+*; *ey*>*p53*; B,B'), does not affect the loss-of-ventral-eye phenotype. Dashed line marks the lost boundary of the eye field. (C-D') Blocking caspase-dependent cell death by overexpression of baculovirus P35 in the wild-type eye (C) does not affect eye size whereas, in the *L*²/*+*-mutant background (*L*²/*+*; *ey*>*p53*), this can significantly rescue the loss-of-ventral-eye phenotype in the eye imaginal disc (D) and adult eye (D'). (E-H') Overexpression of *diap1* (*ey*>*diap1*; E) or a reduction in the levels of the Hid-Reaper-Grim complex by using deficiency H99 (G) in wild-type eye does not affect eye size, whereas, in *L*²/*+*-mutant backgrounds (F,F',H,H'), these can rescue the loss-of-ventral-eye phenotype. (I) Overexpression of dominant-negative *Ser* (*ey*>*Ser*^{DN}) results in loss of ventral eye (Singh and Choi, 2003). Dashed line marks the lost boundary of the eye field. (J,J') Blocking caspase-dependent cell death can significantly rescue the loss-of-ventral-eye phenotype of dominant-negative *Ser* overexpression (*ey*>*Ser*^{DN}+*P35*), as seen in eye disc (J) and adult eye (J'). Markers for immunostaining are shown in color labels.

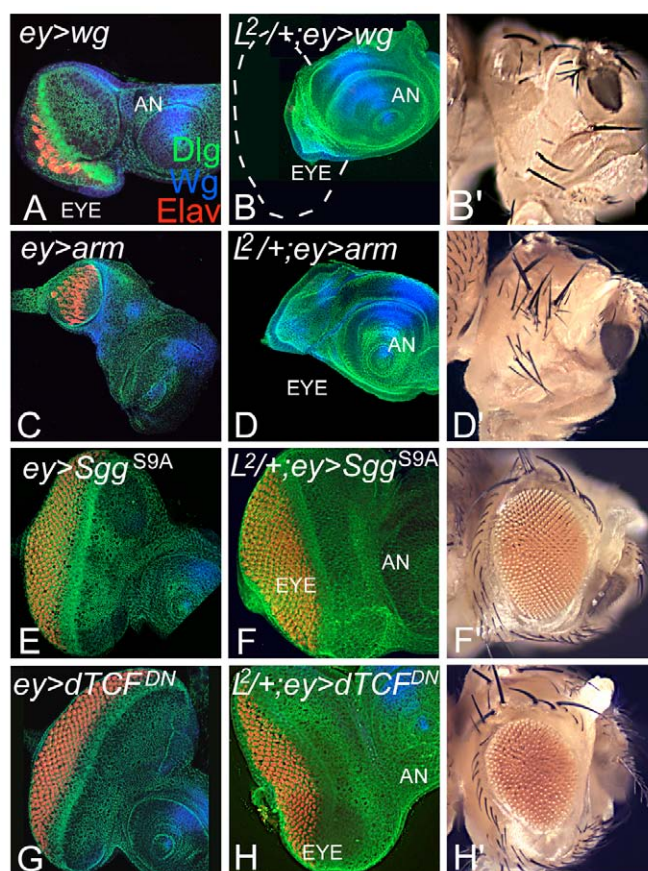


Fig. 3. Canonical Wg signaling pathway affects the *L*-mutant phenotype. (A-D') Increasing levels of canonical Wg signaling in the eye by overexpressing Wg (A) or Arm (C) results in small eyes. However, in the *L*²/*+*-mutant background, overexpression of Wg (B,B') or Arm (D,D') results in the enhancement of loss-of-ventral-eye to a no-eye phenotype. (E-H') Reducing Wg signaling by overexpressing *Sgg* (E) or dTCF^{DN} (G) in wild-type eye does not affect eye size. However, in the *L*²/*+* background, overexpression of *Sgg* (F,F') or dTCF^{DN} (H,H') strongly suppresses the loss-of-ventral-eye phenotype to near that of wild-type eye. Dashed line marks the lost boundary of the eye field. AN, antenna.

Table 1. Effect of signaling pathways on *L*-mutant phenotype of ventral eye loss

	Genotype	Strong suppression (%)	Strong enhancement (%)
Control	<i>L</i> ² /+	0 (0/1000+)	0 (0/1000+)
Cell-death genes	<i>L</i> ² /+; <i>ey>P35</i>	40 (33/82)	
	<i>L</i> ² /+; <i>ey>diap1</i>	27 (12/44)	
	<i>L</i> ² /+; <i>H99</i> /+	38 (21/55)	
Wg pathway	<i>L</i> ² /+; <i>ey>wg</i>		92 (23/25)
	<i>L</i> ² /+; <i>ey>arm</i>		74 (29/36)
	<i>L</i> ² /+; <i>ey>dsh</i>		70 (31/44)
	<i>L</i> ² /+; <i>ey>Sgg</i> ^{S9A}	26 (9/35)	
	<i>L</i> ² /+; <i>ey>dTCF</i> ^{DN}	23 (7/34)	
	<i>L</i> ² ; <i>wg</i> ¹ /+	35 (19/54)	
JNK pathway	<i>L</i> ² /+; <i>ey>puc</i>	36 (20/56)	
	<i>hep</i> ^{r75} /+; <i>L</i> ² /+	32 (23/73)	
	<i>L</i> ² /+; <i>ey>bsk</i> ^{DN}	34 (21/62)	
	<i>L</i> ² /+; <i>ey>bsk</i> ^{Act}		39 (26/67)
	<i>L</i> ² /+; <i>ey>Jun</i>		36 (19/53)
	<i>L</i> ² /+; <i>ey>puc+P35</i>	69 (49/71)	

In these genetic tests, the flies showing strong suppression (75-100% of wild-type eye size) or enhancement (0-25% of wild-type eye size) could be easily scored. The majority of the remaining flies also showed a range of mild suppression (or enhancement), but some weak phenotypes were too subtle to categorize. To avoid subjective characterization of these phenotypes, only the strong phenotypes were scored for a minimum estimate of genetic interactions.

Similar results were seen with *Ser* (see Fig. S1 in the supplementary material). These results suggest that *L* and *Ser* are required for the survival of ventral eye cells.

Loss-of-ventral-eye in *L*/*Ser* mutants is p53-independent and caspase-dependent

Cell death can be caused by cellular insults by both extrinsic and intrinsic mechanisms. Cell death may occur because of the induction of p53 in response to DNA damage (Brodsky et al., 2000; Ollmann et al., 2000). Overexpression of a *Drosophila* p53 homolog (hereafter p53) in the eye, by *eyeless* (*ey*)-*GAL4* (*ey>p53*) results in reduced eyes (Fig. 2A). In the *L*²/+ mutant eye, overexpression of p53 (*L*²/+; *ey>p53*) did not enhance the *L*²/+ mutant phenotype of ventral eye loss (Fig. 2B,B'). We verified our results by

overexpressing dominant-negative p53, *UAS-p53*^{DN}, in the eye (*L*²/+; *ey>p53*^{DN}) and found that the *L*²/+ mutant phenotype was not rescued (data not shown). Our results suggest that cell death from the loss of *L* gene function is p53-independent.

We then tested whether the cell death observed because of the loss of *L* function was caspase-dependent by determining the effects of altering the level of caspase-pathway gene functions in the *L*²/+ background. Overexpression of the baculovirus P35 in the *Drosophila* eye (*ey>P35*) selectively blocks caspase-dependent cell death (Hay et al., 1994), and resulted in eye discs that resemble those of wild-type flies in size (Fig. 2C). Overexpression of P35 in the eye of *L*-mutants (*L*²/+; *ey>P35*) restored the loss-of-ventral-eye phenotype to more than three-quarters of the wild-type eye size in 40% of the flies (Fig. 2D,D', Table 1). In addition, a range of weaker

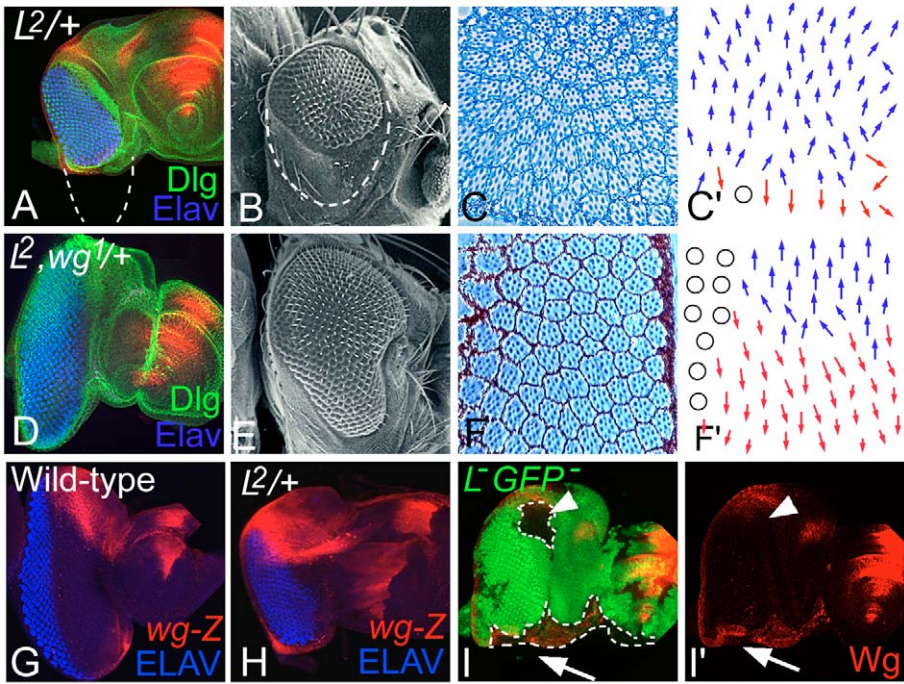


Fig. 4. Wg acts antagonistically to *L*. Loss of *L* (*L*²/+) results in preferential elimination of the ventral eye cells, as seen in eye disc (A), adult eye (B) and adult eye sections (C,C'). The loss-of-ventral-eye phenotype of *L*²/+ mutants is restored in the *wg*-mutant background (*L*²; *wg*¹/Cyo), as seen in the eye disc (D) and adult eye (E). Sections of *L*²; *wg*¹/+ fly eye show restoration of the ventral eye-specific ommatidia (F,F'). In C' and F', circles represent ommatidia with uncertain polarity, and blue and red arrows indicate dorsal and ventral polarity, respectively. In third instar eye disc, *wg-lacZ* is strongly expressed on both dorsal and ventral polar margins (Royet and Finkelstein, 1997). (G) In the *L*²/+ mutant background, *wg-lacZ* is ectopically induced on the ventral posterior margin of the eye. (H-I') LOF clones of *L*^{rev}, marked by loss of the GFP reporter, in the ventral eye show ectopic induction of Wg (arrow). The size of the ventral eye is smaller, suggesting that some of the ventral eye cells are lost. There is no effect on Wg in LOF clones of *L* in the dorsal eye (marked by dashed line, arrowhead).

rescue phenotypes (partial rescue of $L^2/+$ ventral eye loss) was observed in the remaining 60% of $L^2/+$; $ey>P35$ flies. Overexpression of *diap1* (also known as *thread* – Flybase), an inhibitor of apoptosis, in the L -mutant background ($L^2/+$; $ey>diap1$) rescued the $L^2/+$ -mutant phenotype (Fig. 2F,F'). The frequency of strong rescues (i.e. more than three-quarters of the wild-type eye size) was 27% (Table 1), and a range of intermediate and weaker rescues were observed in the remaining progeny. The $ey>diap1$ control flies showed wild-type eye size (Fig. 2E).

Most instances of cell death analyzed in *Drosophila* require the function of the HRG-complex members, encoded by *hid*, *reaper* and *grim* (Bergmann et al., 1998; Bergmann et al., 2003). We tested the role of the HRG complex in the cell death caused by loss of L gene function. When the gene dosage for HRG was reduced to half of that observed in wild type in the $L^2/+$ background ($L^2/+$; $H99/+$) using the *Df(3L)H99* deficiency, which uncovers all three genes (White et al., 1994), the $L^2/+$ phenotype of loss-of-ventral-eye was rescued to near wild type in the eye imaginal disc and in the eyes of 38% of adult flies (Fig. 2H,H', Table 1). The control $H99/+$ eye discs were normal in size (Fig. 2G). We verified our results using other L mutations [L^{rev} and L^{si} , a hypomorph allele of L (Chern and Choi, 2002)] to rule out any allele-specific interactions (data not shown). These results suggest that members of the HRG complex are required for the L -mutant phenotype of loss-of-ventral-eye.

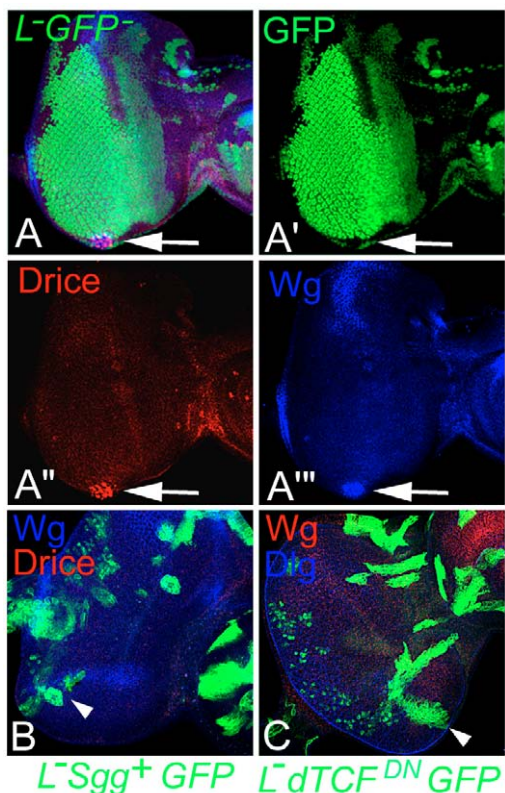


Fig. 5. Cell death in the L -mutant eye is due to ectopic induction of Wg. (A–A''') The LOF clones of L on the ventral eye margin (A, arrow), posterior to MF and marked by the loss of GFP reporter (A', arrow), show ectopic induction of Drice (A'', arrow), coincident with the ectopic induction of Wg (A''', arrow). **(B,C)** LOF clones of L , generated by the MARCM approach, are positively marked by the GFP reporter and accompanied by the overexpression of Sgg^{39A} (B) or $dTCF^{DN}$ (C) to abolish Wg signaling in the eye. Notice that LOF clones of L in the ventral eye can no longer eliminate eye pattern (arrowheads).

During DV patterning of the eye, *Ser* acts downstream of L (Chern and Choi, 2002). Dominant-negative *Ser* (Ser^{DN}) is commonly used to sample the *Ser* LOF phenotypes in the eye (Kumar and Moses, 2000; Singh and Choi, 2003). Overexpression of Ser^{DN} in the eye by an *ey-GAL4* driver ($ey>Ser^{DN}$) resulted in the loss of the ventral-half of the eye (Fig. 2I). We tested whether the *Ser* LOF phenotype of loss-of-ventral-eye is due to the induction of caspase-dependent cell death. Overexpression of P35 in the Ser^{DN} background ($ey>Ser^{DN}+P35$) significantly rescued the loss of the ventral-half of the eye imaginal disc and the adult eye (Fig. 2J,J') in 32% (22/69) of flies, whereas remaining flies showed weaker rescues. As the frequency of strong rescues by blocking caspase-dependent cell death was less than 40%, it suggested that caspase-dependent cell death is one of the major, but not the sole, reason for the loss-of-ventral-eye in L/Ser mutants.

wg acts antagonistically to L gene function

Ectopic upregulation of Wg in the wing is known to induce cell death (Adachi-Yamada et al., 1999). In a genetic screen, we identified Shaggy (*Sgg*), an antagonist of Wg Signaling (Hazelett et al., 1998; Heslip et al., 1997), as the modifier of the L -mutant phenotype (Singh et al., 2005a). To test if the loss of L results in the abnormal induction of Wg signal and thereby causes cell death, we overexpressed the members of the Wg-signaling pathway to increase or decrease the levels of Wg signaling and sampled its effect on the L -mutant phenotype. Overexpression of *wg* in the eye of $L^2/+$ mutants ($L^2/+$; $ey>wg$) completely eliminated the eye field in the eye imaginal disc and in adult the eye (Fig. 3B,B', Table 1). Overexpression of *wg* in the eye ($ey>wg$) suppressed eye development (Fig. 3A) (Lee and Treisman, 2001), but the phenotypes are much weaker than $L^2/+$; $ey>wg$. Increasing the levels of Armadillo (*Arm*) in the wild-type eye ($ey>arm$) resulted in small eyes (Fig. 3C), whereas, in the $L^2/+$ background, $L^2/+$; $ey>arm$ eliminated the entire eye field (Fig. 3D,D'). Similar phenotypes were observed upon overexpression of Dishevelled (*Dsh*) (Table 1). Thus, increasing levels of canonical Wg signaling can enhance the L -mutant phenotype.

We blocked Wg signaling by overexpressing antagonists of the Wg signaling pathway. Overexpression of *Sgg* in the eye ($ey>Sgg$) does not affect the eye size (Fig. 3E) (Singh et al., 2002), whereas, in the eye of $L^2/+$ mutants ($L^2/+$; $ey>Sgg$), overexpression significantly rescued the ventral eye loss in 26% of flies (Fig. 3F,F', Table 1). In the remaining progeny, weaker rescue phenotypes were seen. The transcription factor TCF is the downstream target of Wg signaling, and is inhibited by overexpression of the N-terminal deleted dominant-negative form of TCF ($dTCF^{DN}$) (van de Wetering et al., 1997). Overexpression of $dTCF^{DN}$ in the eye ($ey>dTCF^{DN}$) does not affect eye size (Fig. 3G) (Singh et al., 2002), whereas in the eye of $L^2/+$ mutants ($L^2/+$; $ey>dTCF^{DN}$), it significantly rescued the phenotype of the loss of ventral eye in 23% of flies (Fig. 3H,H', Table 1). Thus, reducing levels of Wg signaling can rescue the L -mutant phenotype. Similar results were seen in eye discs in which *Ser* function was abolished along with increased or decreased levels of Wg signaling (see Fig. S1 in the supplementary material).

L is required for the repression of Wg expression in the ventral eye

Our results demonstrate that Wg signaling acts antagonistically to L function in the ventral eye and raise the possibility that L may act upstream to Wg signal transduction. Therefore, we tested genetic interaction between L and *wg*. Loss of L results in preferential loss-of-ventral-eye pattern in the eye disc (Fig. 4A) and adult eye (Fig. 4B) (Singh and Choi, 2003). The sections of the $L^2/+$ -mutant adult eye

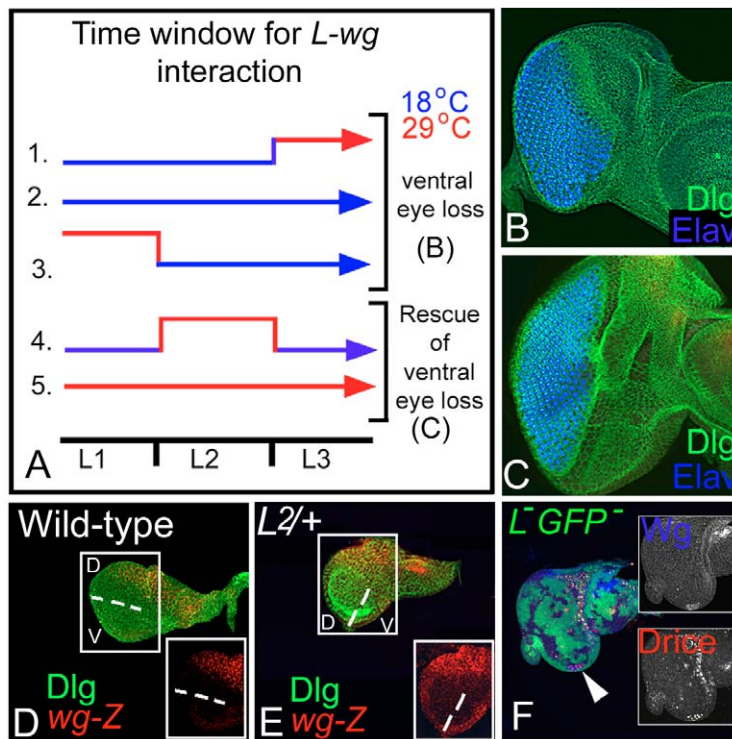


Fig. 6. *L* is involved in the repression of *wg* during second larval instar. (A–C) Abolishing *wg* function in different time windows using a temperature-sensitive *wg^{IL114}* allele (details in Materials and methods) shows that second instar of larval development (L2) is crucial for L-Wg interaction. (B) When *wg* function was abolished during first or third instar, there was no significant rescue of the L-mutant phenotype. (C) When *wg* function was abolished during second instar, loss-of-ventral-eye phenotype of *L^{2/+}* mutants was significantly rescued to near that of wild-type eye. (D) In second instar wild-type larval eye disc, *wg-lacZ* is expressed strongly on the dorsal margin. (E) In the *L^{2/+}*-mutant background, a strong induction of *wg-lacZ* reporter expression is seen all along the ventral polar margin. Dashed lines in D,E mark the boundary between the dorsal- and the ventral-half of the eye. (D,E) Inset shows *wg-lacZ* expression (red) in a separate channel in second instar eye imaginal discs. (F) In the second instar eye disc, LOF clones of *L* cause ectopic induction of Wg and Drice in the ventral eye (arrowhead). Separate channels are shown in inset.

showed selective loss of the ventral ommatidial clusters (Fig. 4C,C'). When we generated recombinant chromosome harboring both *L* and *wg* mutations (*L², wg¹/CyO*), the loss-of-ventral-eye was strongly rescued to near wild-type eye size in 35% of flies (Fig. 4D,E, Table 1). In adult eye sections of the double mutant fly (*L², wg¹/CyO*), we confirmed that ommatidia in the rescued ventral eye (Fig. 4E) are indeed of the ventral polarity (Fig. 4F,F'). Similar results were found using other alleles of *L* (*L^{si}* and *L^{rev}*) and *wg* (*wg^{CX3}* and *wg^{CX4}*). This suggests that the *L*-mutant phenotype in the ventral eye is probably caused by ectopic induction of Wg expression.

We checked whether the regulation of Wg levels by *L* is at the transcriptional level by using a *wg* reporter-gene construct (Kassis et al., 1992; Heberlein et al., 1998). During eye development, the reporter gene reliably represents Wg expression (Royet and Finkelstein, 1997). In the third instar wild-type eye disc, *wg-lacZ* is expressed in the dorsal, and weakly in ventral, polar margins, anterior to the MF (i.e. on the DV margins of the undifferentiated eye disc; Fig. 4G). In the *L^{2/+}*-mutant eye disc, the expression of the *wg* reporter is ectopically induced on the ventral eye margin posterior to the MF (Fig. 4H).

To test the possibility that *L* represses Wg expression only in the ventral eye, we generated LOF clones of *L^{rev}* by the genetic-mosaics approach (Xu and Rubin, 1993). The LOF clones of *L* do not show any phenotype in the dorsal eye, whereas clones in the ventral eye result in the loss of eye (Singh and Choi, 2003). Consistent with this observation, LOF clones of *L* in the ventral eye showed ectopic induction and upregulation of Wg expression on the posterior and lateral margin of the ventral eye, whereas dorsal eye clones did not show ectopic Wg induction (Fig. 4I,I').

Loss of *L/Ser* induces ectopic Wg and the downstream caspase Drice

Wg induces cell death to eliminate excess ommatidia at the periphery of the pupal eye (Lin et al., 2004; Cordero et al., 2004). To test whether Wg is responsible for the induction of cell death in the

eye mutant for *L/Ser*, we looked at the expression of the downstream effector caspase Drice and Wg in LOF clones of *L* in the eye. The LOF clone of *L* at the ventral margin of the eye disc, posterior to the morphogenetic furrow, showed ectopic induction of activated Drice caspase accompanied by the induction of Wg (Fig. 5A–A''). Similar results were observed with *Ser* (data not shown). Thus, during eye development, *L/Ser* function to prevent cell death, which is most probably induced by aberrant Wg signaling.

It can also be argued that changes in the eye size because of activation of Wg signaling could be due to changes in the pattern of differentiation or of proliferation rather than cell death. To rule out this possibility and to support that Wg signaling is directly responsible for the cell death of ventral eye cells in *L*-mutant eye discs, we blocked Wg signaling only in the cells lacking *L* gene function. We overexpressed Sgg (Fig. 5B) or dTCF^{DN} (Fig. 5C) in the *L^{rev}* clones by MARCM analysis and found that these LOF clones of *L* marked by GFP reporter expression in the ventral eye can no longer eliminate eye and cannot induce activated Drice in these clones. We counted 11 MARCM clones for Sgg overexpression in the ventral eye, and seven for dTCF^{DN} overexpression, and none showed the loss-of-ventral-eye phenotype.

L inactivates Wg during the second larval instar

To understand the physiological relevance of the genetic interaction between *L* and *wg*, we looked for the developmental time window for Wg to inhibit ventral eye growth. We employed a conditional mutant *wg^{IL114}* that encodes a temperature-sensitive Wg protein (Baker, 1988; Treisman and Rubin, 1995). The cultures harboring *L* and temperature-sensitive *wg^{IL114}* mutations were maintained at 16.5°C, the temperature at which mutant Wg protein is functional for most Wg activities (Baker, 1988). The cultures were shifted to the restrictive temperature of 29°C in a 24-hour time window during different stages of development (Fig. 6A). The rationale was to block Wg function in the *L^{2/+}* background and to

look for the time window in which reducing *wg* function can rescue the loss-of-ventral-eye phenotype. Based on sampling of the phenotypes from different batches, we found that reducing *wg* function during the second instar of larval development can significantly rescue the *L*-mutant phenotype of ventral eye loss (Fig. 6A,C). However, reducing *wg* function very early during the first instar or later during third instar of larval eye development had no significant effect on the *L*-mutant phenotype (Fig. 6A,B). These results suggest that *L* and *wg* act antagonistically during the second instar of larval eye development before the onset of retinal differentiation.

During the second instar of wild-type larval eye development, *wg* reporter (*wg-lacZ*) expression was enriched on the dorsal polar region of the eye (Fig. 6D). However, in the *L*^{2/+}-mutant eye disc at a similar developmental stage, there was strong ectopic induction of *wg-lacZ* expression along the ventral margin of the eye disc (Fig. 6E, inset). As the antagonistic relation between *L* and *wg* is important during the second instar of eye development, we tested whether LOF of *L* can induce cell death during the second instar of larval eye development. We found that activated Drice is ectopically induced in LOF clones of *L* in the ventral eye (Fig. 6F, inset), consistent with the ectopic induction of Drice in *L*-mutant clones shown in third instar eye discs (Fig. 5A).

L blocks ectopic induction of the JNK signaling pathway

Ectopic induction of *Wg* can induce JNK signaling in developing wing imaginal discs (Adachi-Yamada et al., 1999; Moreno et al., 2002). Therefore, we tested whether elimination of the ventral eye pattern in the eye of *L* mutants is affected by the induction of the JNK signaling pathway. The extent of activation of the *Drosophila* JNK pathway can be monitored by *puc-lacZ* expression (Adachi-Yamada et al., 1999). In the eye disc, *puc-lacZ* is expressed in the peripodial cells (Adachi-Yamada, 2002). In the *L*^{2/+} mutant eye disc, there was a strong induction of *puc-lacZ* on margins of the ventral eye (Fig. 7A). We also checked *puc-lacZ* expression in the LOF clones of *L*^{rev} and found that *puc-lacZ* was ectopically induced in the *L*-mutant cells near the ventral eye margin (Fig. 7B,B'; arrow). In the dorsal eye clones, *puc-lacZ* was not induced (Fig. 7B,B'; arrowhead). *Puc* downregulates JNK activity by a negative-feedback loop (Martin-Blanco et al., 1998; McEwen and Peifer, 2005). Thus, overexpression of *Puc* can be used to repress JNK activity. Overexpression of *Puc* alone (*ey>puc*) does not affect eye size (Fig. 7C). However, in the *L*^{2/+}-mutant background, *puc* overexpression (*L*^{2/+}; *ey>puc*) resulted in the rescue of the ventral eye loss in 36% of flies (Fig. 7D,D', Table 1).

Inhibition of JNK signaling by the use of a dominant-negative form of *Drosophila* JNK (*ey>bsk^{DN}*) resulted in wild-type eye (Fig. 7E), whereas in *L*^{2/+}; *ey>bsk^{DN}*, it rescued the loss-of-ventral-eye phenotype in 34% of flies (Fig. 7F, Table 1). Reducing the levels of *Drosophila* JNK kinase (DJNKK) encoded by *hep*, strongly rescued the *L*^{2/+}-mutant phenotype in 32% of *hep^{r75/+}*; *L*^{2/+} flies (Fig. 7G). Overexpression of the activated form of Bsk (*ey>bsk^{Act}*) alone resulted in eyes smaller than those of wild type (Fig. 7H). Consistently, when we increased the levels of Bsk by overexpressing the activated form of Bsk in the *L*^{2/+}-mutant background (*L*^{2/+}; *ey>bsk^{Act}*), the loss-of-ventral-eye phenotype was enhanced to near complete loss of eye in 39% of flies (Fig. 7I,I', Table 1). Finally, increasing JNK signaling by the overexpression of the activated form of *Drosophila* Jun (*L*^{2/+}; *ey>jun^{aspv7}*) resulted in the strong enhancement of the *L*^{2/+}-

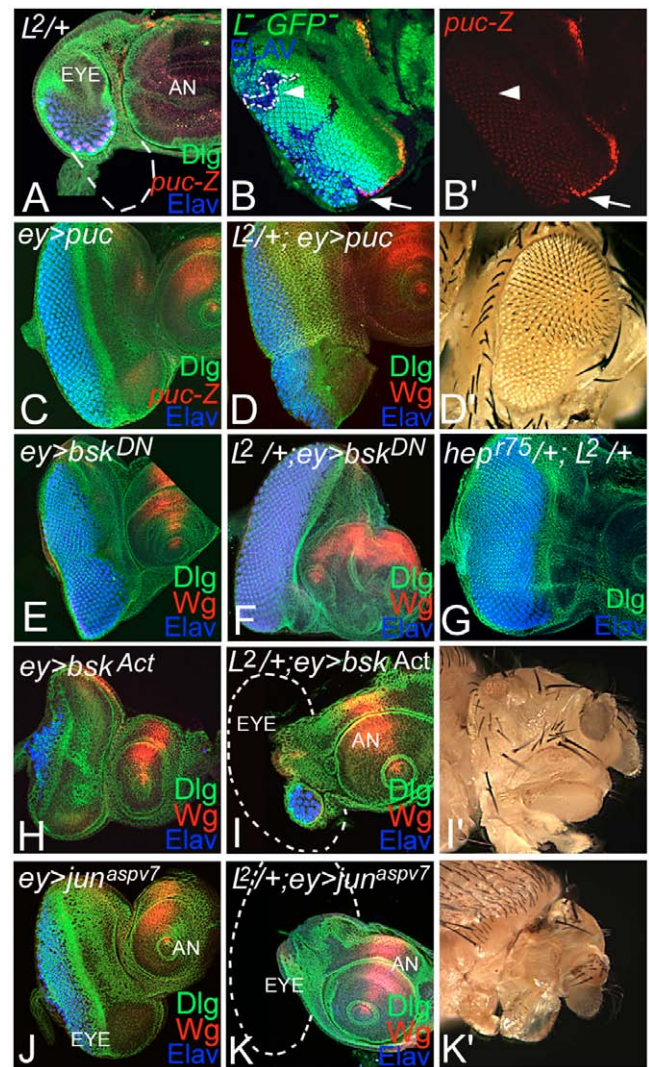


Fig. 7. Loss of *L* gene function results in activation of the JNK signaling pathway. (A) In *L*^{2/+} eye disc, *puc-lacZ*, a reporter for JNK signaling pathway activation, is ectopically induced on the ventral eye margin. (B,B') In LOF clones of *L*, ectopic induction of *puc-lacZ* was observed in only the ventral eye margin (arrows). In the dorsal eye clones, *puc-lacZ* was not induced (arrowheads). (C-D') Blocking JNK signaling by overexpression of *puc* (Martin-Blanco et al., 1998) in the eye does not affect eye size (C), whereas, in *L*^{2/+}-mutant eyes, it rescues the ventral eye loss in the eye imaginal disc (D) and adult eye (D'). (E) Overexpression of a dominant-negative form of Bsk (*Bsk^{DN}*) alone in the eye served as control. (F,G) Reducing levels of JNK signaling by overexpressing *Bsk^{DN}* (F) or by using a heterozygous background of a null mutant of *hep* (*hep/+*; *L*^{2/+}; G) rescues the *L*-mutant eye phenotype. (H-I') Increasing levels of JNK signaling by overexpressing the activated form of Bsk (*Bsk^{Act}*) in wild-type eye (H) results in small eye but, in *L*^{2/+}-mutant eye (I,I'), it causes severe enhancement of the loss-of-ventral-eye phenotype. (J-K') Overexpression of the activated form of *Drosophila* Jun in the wild-type (J) does not affect the eye. However, overexpression of *Drosophila* Jun strongly enhances the *L*^{2/+}-mutant phenotype (K,K') in the eye. Dashed lines mark the lost boundary of the eye field. AN, Antenna.

mutant phenotype to a very small eye or no eye in 36% of flies (Fig. 7K,K', Table 1), whereas overexpression of *Drosophila* Jun alone (*ey>jun^{aspv7}*) resulted in weaker eye reduction (Fig. 7J). Our results demonstrate that the ventral eye cells lacking *L* gene function ectopically induce the JNK signaling pathway.

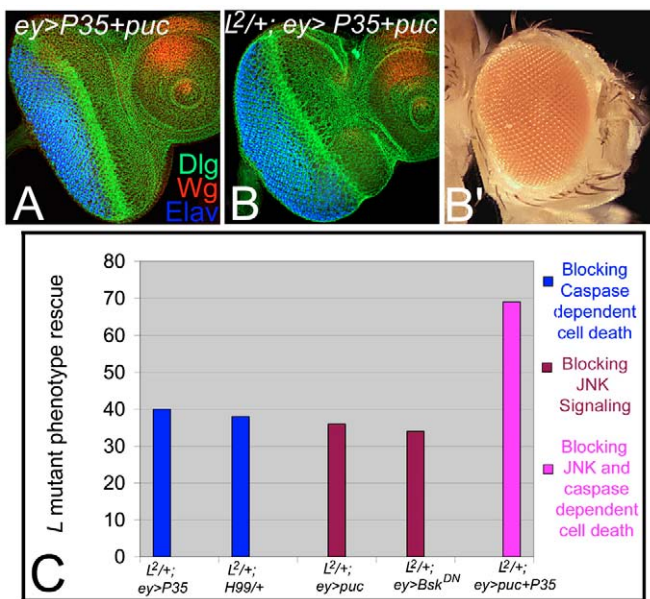


Fig. 8. *L*-mutant phenotype is dependent on activation of both JNK signaling and caspase-dependent cell death. Blocking caspase-dependent cell death and the JNK signaling pathway in the wild-type eye ($ey>P35+puc$) (A) have no significant effect on eye development, whereas, in the $L^2/+$ mutant ($L^2/+; ey>P35+puc$) it results in the strong rescue of the loss-of-ventral-eye phenotype in the eye disc (B) and adult eye (B'). (C) Graph showing more than 1.5-fold increases in the frequency of rescue of the *L*-mutant phenotype by blocking both caspase-dependent cell death and JNK signaling as compared with blocking either one of them alone.

***L*-mutant phenotype depends on both JNK signaling and caspase-dependent cell death**

We found that blocking JNK signaling or caspase-dependent cell death individually did not completely rescue the *L*-mutant eye and strong rescues were seen in less than 40% of total progeny. JNK signaling is known to induce caspase-independent cell death. To test the possibility that the cell death observed in the *L*-mutant background results from the cumulative outcome of both caspase-independent/JNK-signaling mediated cell death and caspase-dependent cell death, we blocked both JNK signaling and caspase-dependent cell death together in the *L*-mutant background. Overexpression of P35 and Puc ($ey>P35+puc$), which blocks caspase-dependent cell death and JNK-signaling, respectively, results in wild-type eye (Fig. 8A). Overexpression of P35 and Puc in the $L^2/+$ -mutant eye ($L^2/+; ey>P35+puc$) rescued the ventral eye loss to a near wild-type eye (Fig. 8B,B') in 69% of flies and pharates (Table 1). There was a greater than 1.5-fold increase in the frequency of strong rescues seen in these mutants than by blocking caspase-dependent cell death or JNK signaling alone. Our results suggest that *L* blocks cell death by preventing caspase induction, as well as by activating JNK signaling.

DISCUSSION

During development, cell survival, growth, proliferation and differentiation define the final shape and size of the organ. We have used *Drosophila* eye to identify the genes required for cell survival and to sustain early growth. Previously, we have shown that ventral is the ground state of the entire early larval eye primordium (Singh and Choi, 2003), and that *L/Ser* are required for development of the

ventral eye (Chern and Choi, 2002; Singh and Choi, 2003; Singh et al., 2005a; Singh et al., 2005b). During early eye development, little or no cell death is observed. At the mid-pupal stage, programmed cell death plays an important role in the selective removal of a large number of excess undifferentiated cells in the interommatidial space that fail to be recruited to the ommatidia (Miller and Cagan, 1998; Rusconi et al., 2000). During this time window of pupal development, EGFR and Ras act as survival cues (Silver and Rebay, 2005; Bergmann et al., 2002; Yang and Baker, 2003). Surprisingly, there is little information concerning genes responsible for cell survival during early larval eye imaginal disc development. Our studies show that, during early eye development, *L* and *Ser* are required for the survival of ventral eye cells. We found that one of the major reasons for the elimination of the ventral eye cells in *L/Ser* mutants is due to the induction of caspase-dependent cell death. We found that *L*- and *Ser*-mutant phenotypes can be rescued by blocking inappropriate induction of (i) Wg, (ii) JNK-signaling-mediated cell death and (iii) caspase-dependent cell death.

Role of Wg in *L/Ser* function in the eye

In animal tissues, Wg is required to drive developmental patterning. Wg is produced in a restricted area and is distributed either by diffusion or by transport to generate a concentration gradient throughout the tissue to induce proper differentiation (Tabata, 2001; Adachi-Yamada and O'Connor, 2004). In the developing wing imaginal disc, Wg has also been shown to promote growth. By contrast, abnormal expression of Wg or Dpp triggers aberrant differentiation signals that result in the induction of apoptotic cell death in the wing disc (Adachi-Yamada et al., 1999). However, it is difficult to directly extrapolate results from the wing disc to the eye disc because of organ-specific functions of Wg.

In the eye, Wg has complex functions at different stages of development: (i) prior to eye differentiation, Wg is involved in growth and in the establishment of the dorsal eye fate (McNeill et al., 1998; Maurel-Zaffaran and Treisman, 2000); (ii) during eye differentiation, initiation of the morphogenetic furrow by *hedgehog* (*hh*) is restricted to the posterior margin by the presence of Wg, which represses *hh* and *dpp* at the lateral eye margins (Ma and Moses, 1995; Treisman and Rubin, 1995; Dominguez and Hafen, 1997); and (iii) in the pupal stage, Wg is responsible for inducing apoptosis by activating the expression of *hid*, *rpr* and *grim* in ommatidia at the periphery of the eye (Lin et al., 2004; Cordero et al., 2004).

We found that, during early eye development, *L* and *Ser* are required to repress Wg signaling in the ventral eye disc. Our genetic-interaction studies demonstrate that Wg expression is ectopically induced in the *L*-mutant background (Figs 3-6). Here, we propose a model in which *L* and *Ser* downregulate the level of Wg activity and expression in the eye. Loss-of-function of *L/Ser*, induce higher levels of Wg, which is coincident with the elimination of the ventral eye pattern by ectopic induction of caspase-dependent cell death (Fig. 9). Because blocking caspase-dependent cell death in *L/Ser*-mutant backgrounds results in striking but incomplete rescues of the loss-of-ventral-eye phenotypes, it suggests that *L/Ser*-mutant eye phenotypes are not solely due to the induction of caspase-dependent cell death. It is possible that ectopic upregulation of Wg signaling in the LOF of *L/Ser* causes abnormal induction of JNK signaling or that *L/Ser* LOF can induce JNK signaling independently (Fig. 9). Upregulation of JNK signaling can also induce caspase-independent cell death. It is possible that the loss of *L/Ser* can result in cell death caused by both caspase-dependent and caspase-independent mechanism. This may be one of the underlying developmental

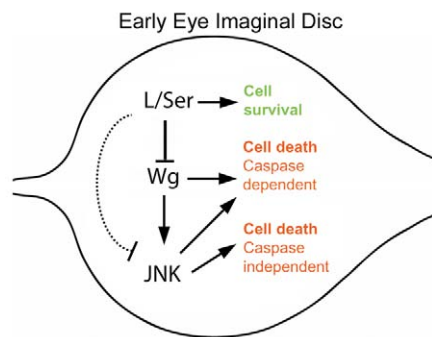


Fig. 9. Model for *L* and *Ser* function in cell survival during early eye development. *L* and *Ser* are required for cell survival during early eye development by preventing the ectopic induction of Wg- and JNK-signaling pathways in the ventral eye or in the entire eye disc prior to the establishment of the DV pattern. *L* and *Ser* can inhibit the JNK signaling pathway indirectly by repressing Wg signaling. It is also possible that *L* and *Ser* directly block JNK signaling. JNK-signaling activation can induce both caspase-dependent and -independent cell death. Thus, *L* and *Ser* promote cell survival by blocking both JNK-signaling-mediated and caspase-dependent cell death.

mechanisms for the early cell-survival function of *L* and *Ser* (Fig. 9). However, there can be several other interesting possibilities. Other studies have shown that JNK can be activated downstream of *rpr*, and that it affects the extent of *rpr*-induced cell death (Kuranaga et al., 2002). Also, *wg* can be induced downstream of *hid* and *diap1* (Ryoo et al., 2004; Huh et al., 2004). Therefore, one can suggest an alternative model where a low level of apoptosis induced by *hid*, *rpr* and *grim* is augmented by a secondary activation of JNK and Wg, which ultimately results in eye ablation.

***L/Ser*-mutant cells undergo cell death and lack compensatory growth**

Many animal tissues counter cell death, induced in response to injury, by triggering compensatory cell proliferation in the neighboring cells. It has been reported in flies that apoptotic or dying cells actively signal to induce compensatory proliferation in neighboring cells to maintain tissue homeostasis (Huh et al., 2004; Ryoo et al., 2004; Perez-Garizo et al., 2005). In cellular injury, *Diap1* is inhibited, whereas the JNK pathway and Wg/Dpp are induced in the apoptotic cells. Secretion of these factors stimulates the growth or proliferation in competent neighboring cells and leads to compensatory proliferation (Ryoo et al., 2004). In other scenarios in which morphogenetic cell death occurs, altered levels of morphogenetic signals give rise to abnormal cell types, which are frequently removed by activating apoptotic signals. Morphogenetic cell death is one of the strategies employed by the developing field to correct its morphogen gradient by eliminating cells with abnormal levels of morphogen by inducing the JNK-signaling pathway (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2004).

Cell death caused by loss of *L/Ser* function results in the induction of both JNK- and Wg-signaling pathways. However, the outcome is different from that seen in compensatory proliferation or morphogen-gradient correction. Instead of compensatory growth in neighboring cells, LOF of *L/Ser* triggers ectopic signaling, which can be neither corrected nor compensated for. As a consequence, the affected tissue, in this case the ventral half of the eye discs, cannot be rescued. It results in the loss of the ventral or entire eye

field, depending upon the domain of function of these survival factors. Our results demonstrate that one of the essential roles of *L* and *Ser* is their requirement for the survival of early proliferating cells in the eye.

Role of Notch signaling in cell survival in the early eye

During development, N signaling is involved in many processes, including cell-fate commitment, cell-fate specification and cell adhesion (Schweisguth, 2004). In the *Drosophila* eye, N signaling plays important roles in compound eye morphogenesis, such as DV patterning, cell proliferation and differentiation in the eye. However, N signaling has not been shown to promote cell survival during early eye development. Our studies raise the possibility of the role of the Ser-N pathway in cell survival during early eye development. Earlier, the extremely reduced or complete loss of eye field in N mutant eye disc was interpreted as being caused by a loss of proliferation. Our data raises another possibility: that N signaling may be playing an important role in cell survival.

The compound eye of *Drosophila* shares similarities with the vertebrate eye. There is conservation at the level of genetic machinery, as well as in the processes of differentiation (Hartenstein and Reh, 2002; Peters, 2002). Thus, the information generated in *Drosophila* can be extrapolated to higher organisms (Bier, 2005). As Wnt signaling induces programmed cell death in patterning the vasculature of the vertebrate eye (Lobov et al., 2005), it will be important to study what molecules prevent Wg signaling from inducing cell death during early eye development. Mutations in *Jagged1*, the human homolog of *Ser*, is known to cause autosomal-dominant developmental disorder, called Alagille's syndrome, which also affects eye development (Alagille et al., 1987; Li et al., 1997). Hence, it would be interesting to study what roles N pathway genes play in cell survival during early eye development and in the early onset of retinal diseases.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/23/4771/DC1>

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