Cullin-4 regulates Wingless and JNK signaling-mediated cell death in the Drosophila eye

Meghana Tare1,5, Ankita Sarkar1, Shimpri Bedi1, Madhuri Kango-Singh1,2,3 and Amit Singh*1,2,3,4

In all multicellular organisms, the fundamental processes of cell proliferation and cell death are crucial for organ growth and differentiation. A fine balance between control of cell death and cell survival is responsible for final organ shape and size during development. We used Drosophila eye model to identify genes involved in promoting growth and cell survival. The Drosophila adult eye contains 750–800 differentiated ommatidia, and develops from a sac-like epithelial structure called the imaginal disc housed in the larva. The ommatidia differentiate in the wake of a synchronous wave of retinal differentiation called the Morphogenetic Furrow (MF). The MF originates at the posterior eye margin, and the anterior movement of the MF. 2

Wingless (Wg) and c-Jun-amino-terminal-(NH2)-Kinase (JNK) signaling are ectopically induced in cul-4 mutant clones, and these signals co-localize with the dying cells. Modulating levels of Wg and JNK signaling by using agonists and antagonists of these pathways demonstrated that activation of Wg and JNK signaling enhances cul-4 mutant phenotype, whereas downregulation of Wg and JNK signaling rescues the cul-4 mutant phenotypes of reduced eye. Here we present evidences to demonstrate that cul-4 is involved in restricting Wg signaling and downregulation of JNK signaling-mediated cell death during early eye development. Overall, our studies provide insights into a novel role of cul-4 in promoting cell survival in the developing Drosophila eye.

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During organogenesis, regulation of conserved processes like cell proliferation, cell survival and cell death is crucial for organ growth and differentiation. A fine balance between control of cell death and cell survival is responsible for final organ shape and size during development. We used Drosophila eye model to identify genes involved in promoting growth and cell survival. The Drosophila adult eye contains 750–800 differentiated ommatidia, and develops from a sac-like epithelial structure called the imaginal disc housed in the larva. The ommatidia differentiate in the wake of a synchronous wave of retinal differentiation called the Morphogenetic Furrow (MF). The MF originates at the posterior eye margin, and the anterior movement of the MF. Wg, a secreted morphogen, initiates an intracellular signaling cascade by binding to its receptors Arrow (Arr) and Frizzled (Fz), which triggers downstream events to control the nuclear localization of the Drosophila beta-catenin Arm, and the spatial expression of Wg target genes.

In Drosophila eye, Wg is also known to induce the proapoptotic genes, head involution defective (hid), reaper (rpr) and grim (together referred as HRG), to trigger programmed cell death to remove extra cells from the periphery of the pupal retina. Further, aberrant signaling during development, e.g., abnormal Wg signaling, also causes stress-induced apoptosis.

The intrinsic caspase-dependent cell death involves activation of HRG, which are negatively regulated by Drosophila inhibitor of apoptosis (DIAPs). Inactivation of DIAP-1 can trigger cell death by the activation of cysteine proteases Dronc and Drice, the Drosophila homolog of Caspase-9 and Caspase-3, respectively. In Drosophila expression of baculo-virus protein, P35 can block caspase-dependent cell death. Besides Wg, activation of c-Jun amino-terminal (NH2) Kinase (JNK) signaling triggers cell

1Department of Biology, University of Dayton, Dayton, OH, USA; 2Premedical Program, University of Dayton, Dayton, OH, USA; 3Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton, Dayton, OH, USA and 4Center for Genomic Advocacy (TCGA), Indiana State University, Terre Haute, IN, USA
*Corresponding author: A Singh, Department of Biology and Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton, 300 College Park, Dayton, Ohio 45469-2320, USA. Tel: 937-229-2894; Fax: 937-229-2021; E-mail: asingh1@udayton.edu
Current address: Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA

Abbreviations: Cul-4, Cullin-4; Wg, Wingless; JNK, c-Jun amino-terminal (NH2) Kinase; MF, Morphogenetic Furrow; Arm, Armadillo; dTCF, Drosophila T cell factor; Arr, Arrow; Fz, Frizzled; Sgg, Shaggy; Hid, head involution defective; Rpr, reaper; HRG, hid, reaper, and grim; DIAP1, Drosophila inhibitor of Apoptosis; PCD, programmed cell death; Cas-9, caspase-9; Cas-3, Caspase-3; TNF, Tumor necrosis factor; Eg1, Egger; Wgn, Wengen; Tak1, TGF-β; activated kinase 1; Hep, hemipterus; Bsk, basket; Bskδ, basket dominant negative; Puc, puckered; L, Lobe; Ey, eyeless; UAS, Upstream Activator Sequence; Cl, cell lethal; B, bifid; DV, Dorso-ventral; GFP, Green Fluorescent Protein; FLP, Flippase; ACF, after clone formation; RNAi, RNA interference; N, Notch; Hh, Hedgehog; Neu, Neuralized; Mib, Mind Bomb; Yki, yorkie; Cul-3, Cullin-3; RNF, Ring finger proteins; NIG, National Institute of Genetics; Twi, twist; FRT, Flippase recombination targets; AEL, after egg laying; Dlg, disc large; j, beta; Elav, embryonic lethal, abnormal vision; IgG, Immunoglobulin G; FITC, Fluorescein isothiocyanate; Cy3, Cyanines 3 dye; Cy5, Cyanines 5 dye
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Cullin-family E3 ubiquitin ligases. Earlier studies showed the *cul-4* gene belongs to an evolutionary conserved class of proteins required for cell survival during early eye development. The *cul-4* mutant exhibits reduced eye phenotype in larval eye disc (Figure 1c) and adults (Figure 1d). Misexpression of *cul-4* using Gal4/UAS approach resulted in the rescue of *L*2-reduced eye phenotype (Figures 1e and f). Misexpression of *cul-4* does not affect the eye size (not shown) suggesting that *cul-4* may not promote cell proliferation.

We analyzed loss-of-function phenotype of *cul-4* to understand its role during eye development.

**Loss-of-function of *cul-4* fail to survive and exhibit reduced eye phenotype.** We generated *cul-4* loss-of-function clones by cell lethal (cl) approach, which results in an eye disc comprising of nearly 80% mutant cells due to elimination of the wild-type twin spot by a cl mutation. Loss-of-function clones of *cul-4* alleles (Figure 2a) like *cul-4*ExG1 (Figure 2c) or *cul-4*J11 (Figures 2d and e) resulted in a small eye phenotype as compared with the wild-type eye (Figure 2b). It is to be noted that both *cul-4*J11 and *cul-4*ExG1 loss-of-function phenotypes were similar in the eye. Downregulation of *cul-4* expression in the dorso-ventral (DV) margins of developing eye disc by using bi-Gal4 driver (Figure 2t; bi-GFP) resulted in reduction of eye field on DV margins (Figures 2g and h, arrows). It suggests that there is no domain constraint in *cul-4* function in the eye. It is possible that reduced eye phenotype may be due to induction of cell death. To test this, we used ey-Flipase (ey-Flip) to induce somatic ‘twins’ using Ubi-GFP (1XGFP), where *cul-4−/−* mutant cells (GFP-negative) were adjacent to their wild-type twin spot (2XGFP). However, we found that only the wild-type twin clone (2XGFP) and the heterozygous cells (express 1XGFP) could be identified. However, we found wild-type twin clones (2XGFP) but no *cul-4* mutant clones (GFP-negative) in the third instar eye disc (not shown), suggesting that the *cul-4* mutant cells failed to survive.

**Results**

**Gain-of-function of *cul-4* rescues reduced eye mutant phenotype.** In comparison with the wild-type larval eye disc and the adult eye (Figures 1a and b), *L*2 mutant exhibits reduced eye phenotype in larval eye disc (Figure 1c) and adults (Figure 1d). Misexpression of *cul-4* using Gal4/UAS approach resulted in the rescued *L*2-reduced eye phenotype (Figures 1e and f). Misexpression of *cul-4* does not affect the eye size (not shown) suggesting that *cul-4* may not promote cell proliferation. We analyzed loss-of-function phenotype of *cul-4* to understand its role during eye development.
performed a 'twin spot' analyses in the heat-shock-Flippase (hs-FLP)-induced clones, to test survival profiles of cul-4 mutant cells. The heat shock was administered in the first instar larva and the resultant clones were examined in the second (24 h after clone formation (ACF)) and third instar (48–72 h after clone formation) disc to determine whether or not these clones could survive. Very small clones were detected at second instar stage (within 24 h after clone formation). However, within 48 h after clone formation, the mutant clones were lost. In comparison with the wild-type clones (Figure 3a), the mutant clones generated at later time points (second instar (48 h) or early third instar (60 h)) and examined at late third instar stage (within 24 h after clone formation), showed smaller cul-4−/− clones (Figures 3b and c; clone boundary marked by red dotted lines). These cul-4−/− clones failed to survive until 120 h of development. Quantification of clone size showed that cul-4 mutant clones are significantly smaller than their wild-type twin clones (Figure 3c), suggesting that cul-4 mutant clones either fail to survive or are slow growing compared with wild-type cells. We tested if cul-4 mutant cells are eliminated by cell death using TUNEL labeling. 11,17 Wild-type eye disc showed few TUNEL-positive dying cells (Figures 3d and d′), whereas eye disc lacking cul-4 function in the entire eye disc (cul-4−/−, Figures 3e and e′) or on DV margins (bi> cul-4RNAi, Figures 3f and f′) showed a threefold increase in TUNEL-positive cells suggesting that cul-4 mutant cells are eliminated by cell death.

**cul-4 prevents cell death in the developing eye.** We therefore, tested if Drosophila Caspases- Dronc and Drice activation is part of the mechanism. In the eye disc, cul-4JJ11 loss-of-function clones generated by 'cell-lethal' clonal approach,39 exhibited robust induction of activated Caspase-3* (Cas-3*) and a signaling molecule Wg (Figures 4a and a′). Similarly, in semi-quantitative western blots, Dronc levels were nearly two fold higher in cul-4 mutant as compared with the wild-type eye disc (Figure 4b). Thus, both Dronc and Drice are induced in cul-4 mutant cells. Misexpression of baculo-virus P35 in the cul-4 loss-of-function clones (cul-4JJ11−/−, eyP35) resulted in a significant rescue of the reduced eye phenotype (Figures 4c and c′). In cul-4 loss-of-function background reducing level of proapoptotic genes using H99 deficiency43 (cul-4JJ11−/−; H99−/−) resulted in significant rescue of the cul-4 mutant phenotype (Figures 4e and e′). It suggests that activation of caspases triggers apoptosis in cul-4 mutant cells. Aberrant signaling from key developmental and...
signaling pathways, like Wg/Wnt, can induce apoptosis to prevent defective development.\textsuperscript{10,14}

cul-4 represses Wg levels in the developing eye. In the third-instar stage, Wg is expressed along the antero-lateral margins of the wild-type eye disc (Figures 5a and a'). Reducing cul-4 function on DV margin of eye disc using bi-Gal4 driver (bi\textgreater cul-4\textsuperscript{RNAi}) resulted in strong ectopic induction of Wg in DV domain of the eye (Figures 5b and b'; white arrows). Loss-of-function clones of cul-4 using the cul-4\textsuperscript{ExG1\textsuperscript{−3}} (Figures 5c and c') and cul-4\textsuperscript{J11J11} (Figures 5d and d') alleles showed a robust ectopic induction of Wg in the eye disc (Figures 5c', white arrows). Wg levels were significantly upregulated in semi-quantitative western blots on total protein isolated from eye imaginal discs from wild-type, and cul-4\textsuperscript{ExG1\textsuperscript{−3}} and cul-4\textsuperscript{J11J11} (Figure 5e). These data suggest that cul-4 may downregulate Wg in the eye imaginal disc. Misexpression of wg on DV margins of eye disc bi\textgreater wg results in ectopic wg transcription suggesting that wg is a target of Wg pathway in developing eye (Supplementary Fig. S1). We then tested if aberrant Wg signaling is responsible for cul-4 mutant phenotypes.

Wg signaling pathway alters the cul-4 mutant phenotype. Arm translocates to the nucleus in response to Wg signaling and binds with the transcription factor dTCF (LET/TCF family
protein) to turn on the transcription of Wg target genes (Figure 6a). In western blots a two fold increase in Arm protein levels was observed in cul-4 mutant eye discs as compared with wild-type (Figure 6b). We tested if modulating Wg signaling levels can affect the cul-4 mutant phenotype. In the eye imaginal discs, activation of Wg signaling by misexpression of wg (ey>wg) (Figure 6c) or arm (ey>arm) (Figure 6f) resulted in reduced eye phenotypes. Misexpression of P35 can significantly reduce the number of dying cell nuclei in cul-4 loss-of-function eye disc as compared with wild-type.

Wg signaling by misexpression of a constitutive active form of Shaggy/Zeste-White-3/GSK-3 (Sgg), a negative regulator of the Wg signaling pathway (ey>sgg) (Figure 6i), or dominant negative form of TCF (ey>dTCFDN) (Figure 6i) does not affect the size of the eye field. However, in cul-4 loss-of-function background, misexpression of sgg (cul-4JJ11−/−, ey>sgg) (Figures 6e and k) or dTCFDN (cul-4JJ11−/−, ey>dTCFDN) (Figures 6m and n) resulted in a significant rescue of the cul-4 loss-of-function phenotype to a near wild-type eye. It suggests that cul-4 is involved in downregulation of Wg signaling in the eye. JNK is known to
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work in conjunction with Wg in multiple contexts including correction of morphogen gradient discontinuities,26 and differential levels of JNK signaling are associated with cell survival.26,28

cul-4 prevents JNK-mediated cell death in the developing eye. We tested if JNK pathway is associated with the cul-4 loss-of-function phenotypes (Figure 7). We tested JNK levels in cul-4 loss-of-function background using puc (puc-lacZ) the downstream target, which serves as the functional readout for JNK pathway activation.30 In wild-type eye disc, puc is expressed in differentiated photoreceptor neurons of eye disc (Figure 7a). In cul-4 loss-of-function background, ectopic induction of puc was seen in the eye as well antenna disc (Figures 7b and 7b'), suggesting that JNK signaling is activated in cul-4 mutant cells. To confirm, we checked levels of p-JNK, a reporter for activated JNK signaling, in western blots and found significant upregulation of p-JNK levels in cul-4 mutant cells. Wg band staining intensity calculated by Image-J using bi-4, ey-2, and sgg clones in the wild-type eye disc and (d) In the developing eye disc, strongly enhanced the reduced eye phenotype (Figures 7e and e'). The reduced eye phenotype of cul-4 misexpression of activated Jun (junaspv7') in the eye disc, strongly enhanced the reduced eye to a ‘no-eye’ phenotype (Figures 7k and k'). Misexpression of junaspv7' in the eye disc results in a highly reduced eye field (Figure 7j). It suggests that loss of cul-4 leads to activation of JNK signaling in the eye.

To confirm that activation of Wg/JNK signaling pathway are both associated with the induction of cell death observed in cul-4 mutant cells, we monitored cell death using TUNEL assay when Wg (Figure 8) and JNK (Figure 9) levels are modulated in the wild-type, and in cul-4 mutant eye discs. We found that cell death is reduced when Wg or JNK signaling is downregulated in cul-4 mutant background. However, cell death is elevated when Wg/JNK signaling is activated. Thus, cul-4 may be involved in limiting JNK as well as Wg activation in the developing eye disc, and thereby promote cell survival during development.

Discussion

Cul-4, an E3 ligase, is involved in regulation of chromatin function through heterochromatin gene silencing, maintenance of genomic integrity by promoting the ubiquitylation

Figure 5   Wg is ectopically induced in cul-4 mutant background. Expression of Wg (blue) in (a,a') Wild-type, (b and b') bi>cul4RNAi, (cul-4 RNAi) is misexpressed on DV margin using bgal4, (c and c') cul-4eg1-3 and (d and d') cul-4 JJ11 loss-of-function clones. Note robust ectopic Wg (blue) expression on (b') DV margin (marked by white arrows) along with suppression of eye fate. (c and d) The reduced eye phenotype of cul-4 loss-of-function clones generated by cell-lethal approach is accompanied by ectopic upregulation of Wg (blue, marked by white arrow). (a'-d') Shown is the split channel of Wg expression. (e) In western blot analysis, the Wg protein levels are more than twofolds in eye discs with cul-4 loss-of-function clones as compared with wild-type eye disc. Wg band staining intensity calculated by Image-J

Figure 6   Activation of Wg pathway results in the cul-4 mutant’s reduced eye phenotype. (a) Cartoon showing Wg signaling pathway. (b) In the western blot performed by using protein extracts prepared from the wild-type and cul-4 mutant eye disc, the Armadillo (Arm) (a downstream cytoplasmic target of Wg signaling), protein levels are enhanced (twofold) in cul-4 loss-of-function background as compared with the wild-type (WT) control. Activation of Wg signaling by misexpression of (d and e) wg (cul-4−/−; ey>wg), (g and h) arm (cul-4−/−; ey>arm) results in enhancement of loss-of-function phenotype of cul-4 as seen in (d and g) eye disc as well as (e and h) adult eye. (c and f) Misexpression of (c) wg (ey>wg) and (f) arm (ey>arm) results in small eye. Blocking Wg signaling by misexpression of negative regulators/antagonists like (j and k) sgg arms (cul-4−/−; ey>s9g arms) and (m and n) dTCPF (cul-4−/−; ey>dTCPF) suppresses the reduced eye phenotype of cul-4 loss-of-function to near normal as seen in (j and m) eye disc and (k and n) adult eye. Misexpression of (i) sgg (ey>s9g arms) and (l) dTCPF (ey>dTCPF) results in normal eye sizes
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and degradation of key cell cycle regulators. A number of ligases work in concert with the signaling pathways (Notch (N), Hedgehog (Hh), Wg and so on) for regulating gene expression. For example, Slimb, is involved in regulating Wg and Hh signaling during eye development. Neuralized (Neu) and Mind Bomb (Mib), are E3 ligases that are components of N signaling pathway; and are required for Drosophila eye development. Recently other functions for E3 ligases are being recognized. For example, DIAP1 regulates Dronc/Hid caspases, and is transcriptionally regulated by yorkie (yki) for survival function. DIAP-1 in turn, is regulated by Cul-3 in the developing eye to regulate apoptosis. Our studies provide evidences for a new function for cul-4 in cell survival during eye development.

Homozygous larvae of some cul-4 alleles are larval lethal that can survive until early third instar and produce smaller imaginal discs than wild-type discs at comparable developmental age. These phenotypes were attributed to problems with cell division. Our twin spot analysis revealed an interesting result that cul-4 mutant tissues in the developing eye imaginal disc failed to survive (Figure 2), and are eliminated by activation of caspases (Figure 3). Generating cul-4 mutant clones by using multiple approaches (for example, eyeless and heat-shock flippase) validated that...
cul-4 mutant cells failed to survive when generated in early embryonic or larval stages. Blocking caspase-mediated cell death led to significant rescue of reduced eye phenotypes of cul-4 loss-of-function (Figures 4c and d), supporting a role for cul-4 in cell survival.

We tested several cell signaling pathways in cul-4 loss-of-function background and found aberrant activation of Wg and JNK signaling (Figure 10). Wg is required for patterning, growth regulation and cell survival in multiple tissues including the eye discs. Ectopic induction of Wg induces cell death.\(^9,10,11,55\) We found that cells lacking cul-4 function also undergo cell death and they express high levels of Wg. Arm, the nuclear effector of the Wg signaling pathway, is a target of E3 ubiquitin ligase-mediated degradation.\(^56\) Loss-of-function phenotype of cul-4 mutants could be modified by modulating the levels of canonical Wg signaling (Figures 6 and 7). Our data suggests that cul-4 may downregulate Wg signaling in the eye to promote cell survival in the eye disc. Since the cul-4 mutant phenotype was not completely rescued by blocking Wg signaling, we also tested the JNK signaling in the cul-4 mutant clones. The possibility of indirect consequences responsible for the mutant phenotype can be refuted because these phenotypes can be rescued by blocking Wg as well as JNK-mediated cell death. We found that Wg levels were affected when JNK signaling was modulated in cul-4 mutant background (Figure 10a–c). However, the converse did not show effect on phospho-JNK levels (Figure 10d–f). Our studies generate insights into genetic mechanisms that regulate cell survival.
survival during normal development by demonstrating the role of \textit{cul-4} in preventing inappropriate upregulation of Wg and JNK signaling in the developing \textit{Drosophila} eye during early stages (Figure 10). A recent study showed that loss of Godzilla, a member of the RNF family of membrane-anchored E3 ubiquitin ligases regulates Wg levels on the basolateral surface of the tissues through dynamin-dependent endocytosis from the apical surface and subsequent trafficking from early apical endosomes to the basolateral surface. Our studies also generate mechanistic insights into genetic mechanisms that regulate cell survival during normal development.

Numerous studies have shown the role of ubiquitin-mediated proteolysis in a broad array of cellular processes like defects in organogenesis, growth, differentiation, metabolism and aging in all organisms. Abnormal protein homeostasis underlies various disorders ranging from growth defects to neurodegenerative disorders. Our studies introduced new role of \textit{cul-4} in cell survival in the developing \textit{Drosophila} eye. Since \textit{Drosophila} serves as an excellent model to study
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Figure 10  
cul-4 promotes cell survival by regulating Wg and JNK signaling in the developing eye. (a-c) Wg expression in (a) wild-type, (b) cul-4<sup>113/113</sup>; ey>jun<sup>emo457</sup>, and (c) cul-4<sup>113/113</sup>; ey>puc eye imaginal disc. Note that Wg levels are upregulated when JNK signaling is activated, whereas Wg levels are not much affected when JNK signaling is downregulated. p-JNK expression in (a) wild-type, (b) cul-4<sup>113/113</sup>; ey>wg and (c) cul-4<sup>113/113</sup>; ey>dTCFDN eye imaginal disc. Note that pJNK levels are not affected. (g) During eye development cul-4 is involved in (i) downregulating Wg signaling and (ii) inhibiting JNK signaling to promote cell survival. Activated JNK signaling can trigger Wg induction.

development and human disease. These studies may shed light on understanding genetic basis of neurodegenerative orders in future.

Materials and Methods
Fly stocks. The fly stocks used are described on Flybase (http://flybase.bio.indiana.edu). Cul-4 stocks used are EP 2518 (UAS-cul-4), cul-4 RNAi lines 8711 and 8711-R1 (from NIG). The N-terminal deletion mutants used were cul-4<sup>ExG1</sup> and cul-4<sup>ExG3</sup> function clones of Twin spot analysis. We used and 8711-R1 (from NIG). The N-terminal deletion mutants used were targeted misexpression studies using wandering third-instar larvae and stained following the standard protocol. Immunohistochemistry. function clones marked by the absence of GFP expression. cul-4 laying (AEL) at 37 °C for 50 min in order to induce loss-of-function clones. Eye discs were maintained at 25 °C. The cultures were heat shocked at 24 and 48 h after egg larval development stages. Egg laying were collected from synchronous cultures. Other stocks include Canton-S, fly-FLP<sup>LS</sup>/CyO<sup>7,Ey</sup> GMR>GFP, UAS ARM<sup>25</sup> and UAS DJunaspv<sup>7</sup> ( gift from Kwang Wook Choi), rabbit anti-caspase-3* (1:200) and rabbit Phospho-SAPK/JNK (Cell Signaling Thr183/Tyr185) (81E11) (Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies were rat anti-Elav (1:100), mouse anti-Wg (1:50), mouse anti-βgalactosidase (1:200) (Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, USA). Cell Death and Disease.

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TUNEL assays. Apoptotic cell death was assayed using TUNEL assays in the mutant clones generated via twin spot analysis and cell lethal approach. Eye discs, after secondary antibody staining, were blocked in 10% Normal Goat Serum in Phosphate Buffered Saline with 0.2% Triton X-100.TUNEL assays were done using the Cell-death Detection Kit from Roche Diagnostics following the standardized protocol. The TUNEL-positive nuclei were counted from five eye imaginal discs for each genotype using Image-J and statistical analysis was performed using Microsoft Excel 2013. The P-values were calculated and the error bars represent Standard Deviation. Adult eye imaging. Adult Drosophila eye images were taken using a Zeiss Apotome Imager.Z1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The flies were prepared by freezing them at ~20 °C for ~2 h. The legs and wings of the flies were removed and flies were mounted on a dissection needle, and the fly was positioned on a glass slide using mounting putty. Images were captured by using extended depth of focus function of the Axiovision software version 4.6.3 (Carl Zeiss Microscopy GmbH, Jena, Germany) by compiling the individual stacks from the Z-sectioning approach. The final images and figures were prepared using Adobe Photoshop CS4 software.

Western blot analysis. Protein samples were prepared from third-instar eye-antennal imaginal discs of different cul-4 mutants dissected in ice-cold PBS. Samples were transferred to sample buffer containing SDS-(i)mercaptoethanol, boiled for 10 min, stored in ~80 °C. Protein samples were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in blocking solution (AMRESCO LLC, Solon, OH, USA) and incubated with primary antibody. The antibodies used were anti-mouse Wg (1:100) (DSHB); anti-mouse arm (1:2000) (DSHB), anti-rabbit p-JNK (1:2000) (Cell signaling Technologies), anti-rabbit Caspase-9 (1:1000) (Cell signaling Technologies) or anti-mouse tubulin (1:5000) (Sigma-Aldrich Corp., St. Louis, MO, USA). Secondary antibodies were horseradish peroxidaseconjugated goat anti-rabbit IgG, and the signal was detected using super-signal chemiluminescence substrate (Pierce Biotechnology, Thermo-fisher Scientific, Rockford, IL, USA).

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions

MT performed majority of the experiments and the data analysis. AS performed experiments. SB performed experiments. MK-S did the data analysis and the manuscript writing. AS developed the concept and did the data analysis and manuscript writing.

1. Ready DF, Hanson TE, Benzer S. Development of the Drosophila retina, a neurocrystalline lattice. Dev Biol 1976; 53: 217–240.
2. Ma C, Moses K. Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing Drosophila compound eye. Development 2002; 129: 2379–2440.
3. Tare M, Puli OR, and, Singh A. Molecular genetic mechanisms of axial patterning: mechanistic insights into generation of axes in the developing eye. In: Singh A, and Kango-Singh M (eds). Molecular Genetics of Axial Patterning, Growth and Disease in the Drosophila Eye. Springer: New York, 2013, pp 37–75.
4. Tisselre J, Rubin GM. Wingless inhibits morphogenetic furrow movement in the Drosophila eye disc. Development 1995; 121: 3519–3527.
5. Aberle H, Baurer A, Stappert J, Kispert A, Kemler R. Beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 1997; 16: 3797–3804.
6. Archbold HC, Yang YX, Chen L, Cadigan KM. How do they do Wnt they do?: regulation of planar cell polarity in the Wnt/beta-catenin pathway. Acta Physiol (Oxf) 2011; 204: 74–108.
7. Swarn P, Verheyen EM. Drosophila homeodomain-interacting protein kinase inhibits the Ski-Cut-Fbox-E3 ligase complex to dually promote Wingless and Hedgehog signaling. Proc Natl Acad Sci USA 2011; 108: 9887–9892.
8. Cordero J, Jassim O, Bas S, Cagan R. A role for wingless in an early pupal cell death event that contributes to patterning the Drosophila eye. Mech Dev 2004; 121: 1523–1530.
9. Lin HV, Rogulja A, Cadigan KM. Wingless enables ommatidia from the edge of the developing eye through activation of apoptosis. Development 2004; 131: 2409–2418.
10. Ryoo HD, Giorec T, Steller H. Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. Dev Cell 2004; 7: 491–501.
11. Singh A, Shi X, Choi KW. Lobe and Serrate are required for cell survival during early eye development in Drosophila. Development 2006; 133: 4771–4781.
12. Singh A, Lim J, Choi K-W. Dorso-ventral boundary is required for organizing growth and dorso-ventral patterning of the Drosophila planar polarity. Planar Cell Polarization during Planar Cell Polarity in the Drosophila Eye. In: Mlodzik M (ed). Genes Dev 1997; 11: 387–393.
13. Singh A, T are M, Puli OR, Kango-Singh M. A glimpse into dorso-ventral patterning of the developing eye disc. Dev Dyn 2005; 234: 1089–1097.
14. Chen P, Nordstrom W, Gish B, Abrams JM. Grim, a novel cell death gene in Drosophila. Genes Dev 1994; 8: 1694–1708.
15. White K, Grether ME, Abrams JM, Cycle H. Hid, Rpr and Grim negatively regulate cell death pathways and stress-induced apoptosis. Dev Dyn 2000; 219: 379–387.
16. Chen P, Nordstrom W, Gish B, Abrams JM. Grim, a novel cell death gene in Drosophila. Nature 1994; 369: 783–789.
17. de la Cruz AF, T ran V, Shibutani ST, Bravo MJ, Nagarajan S et al. Control of endogenous and induced cell death by ERF and CRLR/CRD receptor tyrosine kinases. Nature 2001; 409: 123–133.
18. Singh A, Choi KW. Essential for most, but not all, developmental cell death in Drosophila. Development 2005; 132: 2125–2134.
19. Hay BA, Wolff T, Rubin GM. Expression of baculovirus P35 prevents cell death in Drosophila. Genes Dev 1994; 8: 1201–1212.
20. Adachi-Yamada T, Fujimura-Kamada K, Nishida Y, Matsumoto K. Distortion of proximal/distal information causes JNK-dependent apoptosis in Drosophila wing. Nature 1999; 400: 166–169.
21. Chew SK, Akdemir F, Chen P, Lu WJ, Mills K, Daish T. Drosophila caspase DRONC is required for specific developmental cell death. Cell Cycle 2013; 12: 2175–2182.
22. Braun S, Garcia JF, Rowley M, Rougemeille M, Shankar S, Madhani HD. The Cull4-Ddb1 (Cdt1) ubiquitin ligase inhibits invasion of a boundary-associated antisense factor into Drosophila eye. Cell 2003; 113: 379–391.
58. Varshavsky A. The ubiquitin system, an immense realm. Annu Rev Biochem 2012; 81: 167–176.
59. Dennissen FJ, Khodj N, van Leeuwen FW. The ubiquitin proteasome system in neurodegenerative diseases: culprit, accomplice or victim? Prog Neurobiol 2012; 96: 190–207.
60. Singh A, Irvine KD. Drosophila as a model for understanding development and disease. Dev Dyn 2012; 241: 1–2.
61. Zhang J, Liu M, Su Y, Du J, Zhu AJ. A targeted in vivo RNAi screen reveals deubiquitinases as new regulators of Notch signaling. G3 (Bethesda) 2012; 2: 1563–1575.
62. Singh A, Kango-Singh M, Sun YH. Eye suppression, a novel function of teashirt, requires Wingless signaling. Development 2002; 129: 4271–4280.
63. Kassis JA, Noll E, VanSickle EP, Odenwald WF, Perrimon N. Altering the insertional specificity of a Drosophila transposable element. Proc Natl Acad Sci USA 1992; 89: 1919–1923.
64. Azpiazu N, Morata G. Functional and regulatory interactions between Hox and extradenticle genes. Genes Dev 1998; 12: 261–273.
65. Hazelett DJ, Bourouis M, Waldorf U, Treisman JE. decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. Development 1996; 125: 3741–3751.
66. Zecca M, Basler K, Struhl G. Direct and long-range action of a wingless morphogen gradient. Cell 1996; 87: 833–844.
67. Treier M, Bohmann D, Mittelbronn M. JUN cooperates with the ETS domain protein pointed to induce photoreceptor R7 fate in the Drosophila eye. Cell 1995; 83: 753–760.
68. Blair SS. Genetic mosaic techniques for studying Drosophila development. Development 2003; 130: 5065–5072.
69. Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult Drosophila tissues. Development 1993; 117: 1223–1237.
70. Steffensmeier AM, Tare M, Puli OR, Modi R, Nainaparampil J, Kango-Singh M et al. Novel neuroprotective function of apical-basal polarity gene crumbs in amyloid beta 42 (abeta42) mediated neurodegeneration. PloS One 2013; 8: e78717.
71. Fan Y, Bergmann A. Multiple Mechanisms Modulate Distinct Cellular Susceptibilities toward Apoptosis in the Developing Drosophila Eye. Dev Cell 2014; 30: 48–60.

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