The Hh pathway promotes cell apoptosis through Ci-Rdx-Diap1 axis

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Apoptosis is a strictly coordinated process to eliminate superfluous or damaged cells, and its deregulation leads to birth defects and various human diseases. The regulatory mechanism underlying apoptosis still remains incompletely understood. To identify novel components in apoptosis, we carry out a modifier screen and find that the Hh pathway aggravates Hid-induced apoptosis. In addition, we reveal that the Hh pathway triggers apoptosis through its transcriptional target gene rdx, which encodes an E3 ubiquitin ligase. Rdx physically binds Diap1 to promote its K63-linked polyubiquitination, culminating in attenuating Diap1−Dronc interaction without affecting Diap1 stability. Together, our findings unexpectedly uncover the oncogenic Hh pathway is able to promote apoptosis through Ci-Rdx-Diap1 module, raising a concern to choose Hh pathway inhibitors as anti-tumor drugs.

Introduction

Multicellular organisms keep homeostasis through a balance between cell proliferation and cell apoptosis. In embryonic development, apoptosis removes unnecessary cells to coordinate organogenesis. In adult tissues, apoptosis could eliminate senescent cells to maintain homeostasis. When the organism undergoes external stimuli, including ultraviolet light and reactive oxygen species, damaged cells are also cleared by apoptosis [1]. Therefore, apoptosis plays important role in both physiological and pathological conditions. Recently, increasing studies have shown that abnormal regulation of apoptosis leads to a variety of human diseases, such as tumors [2] and neurodegenerative diseases [3]. Exploring the mechanism underlying apoptosis is helpful to discover novel drug targets for the treatment of apoptosis-related diseases.

An evolutionarily conserved process during apoptosis is the sequential activation of several caspases, which trigger apoptotic cell death by cleaving many structural and regulatory proteins [4]. As a matter of fact, the activation of caspase cascade is tightly monitored due to a family of anti-apoptotic proteins, termed inhibitor of apoptosis proteins (IAPs) [5]. IAPs are first identified as baculoviral proteins that block the defensive apoptosis of insect cells after infection [6]. In addition, many IAPs comprise a carboxy-terminal RING domain and function as E3 ubiquitin ligases to ubiquitinate pro-apoptotic proteins, including caspases [7]. In fruit flies, Drosophila IAP-1 (Diap1) prevents cells from apoptosis through ubiquitinating and subsequent destabilizing Drosophila Nedd2-like caspase (Dronc), the initiator caspase ortholog to human Caspase-9 [8, 9]. In cells that undergo apoptosis, the anti-apoptotic activity of Diap1 is suppressed by upstream antagonists, including head involution defective (Hid), Reaper (Rpr), and Grim [5]. These three proteins negatively regulate Diap1 through distinct mechanisms, either by decreasing Diap1 level or by disrupting Diap1−Dronc interaction [10, 11]. Furthermore, Diap1 protein could be degraded by N-end rule pathway [12]. The E3 ligase Ubr3 enhances Diap1 activity though promoting Diap1-Dronc association, without affecting the ubiquitination of Diap1 [13]. In conclusion, Diap1 is a key modulator for cell death, and its activity should be strictly controlled by multiple mechanisms to avoid unfitted apoptosis.

The evolutionarily conserved Hedgehog (Hh) pathway plays important roles in physiological and pathological processes, such as embryogenesis, cell fate determination, tissue damage repair, stem cell maintenance, and tumorigenesis [14]. Inactivation of the Hh pathway leads to developmental defect, while its hyperactivation causes several human cancers [15, 16]. The Drosophila hh gene encodes a diffusible ligand, which activates the pathway through binding its receptor Patched (Ptc) with the assist of co-receptors including Ihog/Boi [17, 18]. Ptc inhibits the cell surface accumulation and subsequent activation of Smoothened (Smo), an indispensable transducer for the Hh pathway [19]. Hh ligand is able to bind Ptc to relieve its inhibitory effect on Smo possibly through Smo phosphorylation and deubiquitination, culminating in the Hh pathway activation [20, 21]. During Hh signaling transduction, the transcriptional factor Cubitus interruptus (Ci) is a critical executor [22]. In the absence of Hh ligand, Ci is sequestered in the cytoplasm by the microtubule-associated protein Costal2 (Cos2) with the assist of the scaffold Rack1 [23]. In the presence of Hh ligand, Ci dissociates from Ci-Rack1-Cos2 complex and enters the nucleus to turn on the expression target genes [23]. Among Ci target genes, roadkill (rdx) encodes an E3 ligase to promote Cullin3 (Cul3)-mediated protein ubiquitination [24]. To date, several...
studies have shown that the Hh pathway is able to inhibit cell death via upregulating the anti-apoptotic gene Bcl2 in human tumor cells, providing Hh pathway inhibitors as proapoptotic drugs for tumor treatment [25, 26]. Although Drosophila genome encodes two orthologs of Bcl2, buffy and debcl, they do not play an obvious role in apoptosis [27]. Thus, it is still unclear whether and how the Hh pathway regulates apoptosis in Drosophila.

To find novel regulators in Drosophila apoptosis, we carried out a genetic screen and identified the Hh pathway as a positive regulator of apoptosis. Knockdown of ci effectively suppressed Hid-induced apoptosis and small eyes, while overexpression of ci or its upstream smo showed opposite results. Moreover, Ci aggravated Hid-induced apoptosis through its transcriptional target gene rdx, since the loss of rdx phenocopied ci knockdown. Biochemical analyses revealed that Rdx interacted with Diap1 through its N-terminal MATH domain. We also identified two matched recognition motifs in Diap1 responsible for binding Rdx. Interestingly, Rdx was unable to affect Diap1 protein stability. Furthermore, we found that Rdx promoted K63-linked polyubiquitination on Diap1, and decreased Diap1–DrnC interaction, culminating in inhibition of Diap1 activity. Taken together, our study uncovered an unexpected role of the Hh pathway in apoptosis, and raised a concern to choose Hh pathway inhibitors as anti-tumor drugs.

RESULTS

The Hh pathway is a positive regulator for Hid-induced apoptosis

To explore novel regulators of apoptosis, we established a modifier screening, in which the pro-apoptotic gene hid was overexpressed in Drosophila eyes using the eye-specific glass multimer repeat (GMR) promoter to induce massive cell death. Small eyes of GMR-hid (Fig. 1a) provided a sensitive background for subsequent screening, since partially rescued eyes are readily noticeable. Compared to the control (Fig. 1b), ectopic expression of the well-known anti-apoptotic baculovirus P35 protein almost restored the eye of GMR-hid to wild-type size (Fig. 1c), suggesting that the small eye of GMR-hid was indeed caused by excessive apoptosis. Next, we expressed transgenic RNAi lines to identify suppressors of the small eye. From this screening, we found that knockdown of ci apparently increased the eye size (Fig. 1d, g). In contrast, overexpression of ci decreased the eye size under GMR-hid background (Fig. 1e, g). Given that Ci is the unique transcriptional factor of the Hh pathway, we wanted to examine whether the pathway is involved in modulating Hid-induced apoptosis. Similar to Ci, overexpression of the upstream component Smo also reduced GMR-hid eyes (Fig. 1f, g). Since the Hh pathway regulates cell proliferation in Drosophila [28], we sought to test whether Ci controls GMR-hid eye size through cell proliferation. Compared with the control disc (Fig. 2a, d), neither ci knockdown (Fig. 2b, e) nor ci overexpression (Fig. 2c, f) influenced the level of phosphor-histone H3 (PH3), which is a marker for cell division [29]. In contrast, knockdown of ci decreased (Fig. 2e, f, h), while overexpression of ci elevated apoptosis under GMR-hid background (Fig. 2e, g, h), suggesting that the Hh pathway promotes Hid-induced cell death.

The Hh pathway promotes apoptosis through Rdx

The Hh pathway exerts biological function through its transcriptional targets. Well-documented target genes of Hh signaling include knot (kn), decapentaplegic (dpp), patched (ptc), engrailed (en) and roadkill (rdx) [30, 31]. Our previous study has demonstrated that overexpression of rdx produces shriveled wings, possibly due to apoptosis [32]. Thus, we focused on rdx in following studies. First, we employed a rdx-lacZ reporter [33], which the lacZ coding sequence was inserted downstream of rdx promoter, to monitor rdx expression. Compared with the control eye disc (Fig. 3a), overexpression of ci substantially increased rdx-lacZ expression (Fig. 3b). We further showed that Ci also activated rdx-lacZ expression in the wing disc (Fig. 3c), suggesting that rdx is a bona fide transcriptional target of Ci.

To test whether Rdx is involved in regulating Hid-induced apoptosis, we modulated Rdx level under GMR-hid background and analyzed eye sizes. Compared with GMR-hid control (Fig. 3d, e), knockdown of rdx enlarged the eye (Fig. 3e, f), whereas rdx overexpression reduced the eye (Fig. 3f, g). In addition, we used a null allele of rdx, rdx

Δ

, to validate the result. Similar to rdx RNAi, the eye of GMR-hid was increased in rdx

Δ

 heterozygote background (Fig. 3h, i). We could not delete two copies of rdx due to rdx

Δ

 homozygote was embryonic lethal [33]. Rdx is a Cul3-based E3 ligase, which recruits substrates to Cul3 for ubiquitination [34]. Cul3 acts as a scaffold to bridge E2 ubiquitin-conjugating enzymes and E3 ligases [35]. Rdx protein contains a 3box domain responsible for its interaction with Cul3 [32]. Deletion of 3box domain abolishes Rdx-Cul3 association and its E3 ligase activity [32]. We found that Rdx

Δ

box failed to decrease the eye size of GMR-hid (Fig. 3g, h), indicating Rdx E3 ligase activity is...
indispensable for its regulation on apoptosis. Taken together, these findings suggest that the Hh promotes Hid-induced apoptosis through Rdx.

**Rdx interacts with Diap1**

Previous studies have clearly elucidated that Hid induces apoptosis through inhibiting Diap1 [36], and Diap1 overexpression could totally restore GMR-hid to wild-type eye size [37], suggesting that Diap1 is important for Hid-caused apoptosis. Since our above results showed Rdx promotes apoptosis in an E3 ligase-dependent manner, we speculated that Rdx binds Diap1 to accelerate its ubiquitination and proteasome-mediated degradation. The co-immunoprecipitation (co-IP) assay indeed showed the interaction between Rdx and Diap1 (Fig. 4a, b). Rdx is comprised of a MATH domain in its N-terminus and a BTB domain in its C-terminus (Fig. 4c). To determine which domain on Rdx is responsible for its interaction with Diap1, we generated two truncated forms of Rdx, which exclusively contained MATH or BTB domain (Fig. 4c). The co-IP assay showed that Flag-tagged Diap1 protein only pulled down Rdx-MATH (Fig. 4d). Reciprocally, Rdx-MATH, not Rdx-BTB interacted with Diap1 (Fig. 4e), together suggesting that Rdx binds Diap1 through its N-terminal MATH domain.

The previous study has demonstrated that Speckle Type POZ Protein (SPOP), the mammalian counterpart of Rdx, recognizes a conserved degron named as SPOP binding consensus (SBC) [38]. Through examining the protein sequence of Diap1, we found two putative SBCs: EESSS (termed degron 1) and SGSTS (termed degron 2) (Fig. 4f). To test which SBC is required for Diap1-Rdx

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**Fig. 2** Ci promotes Hid-induced apoptosis. All eye imaginal disks shown in this figure were oriented with anterior on the left. **a** A control eye disc was stained with PH3 antibody to mark proliferative cells. **b** Knockdown of Ci did not affect PH3 signals under GMR-hid background. **c** Overexpression of Ci did not regulate cell proliferation. **d** Quantification analyses the PH3-positive cells of a–c (n ≥ 6). **e** A control eye disc was stained to show Ci (green) and Cas3 (white). **f** Knockdown of Ci decreased apoptosis. **g** Overexpression of Ci promoted apoptosis. **h** Quantification analyses the Cas3-positive cells of e–g (n ≥ 6). Scale bars: 50 μm for all eye disks.
Fig. 3  The Hh pathway regulates apoptosis through Rdx. a A control eye disc was stained to show GFP (green), Ci (blue), and rdx-lacZ (red). Of note, rdx-lacZ expresses in the posterior region of the eye disc. b Overexpression of ci activated rdx-lacZ expression in the eye disc. c Ci was able to turn on rdx-lacZ expression in the wing disc. Compared to the control eye (d), knockdown of rdx elevated GMR-hid eye (e), while overexpression of rdx decreased GMR-hid eye (f). g Overexpression of rdx-Δ3box failed to affect GMR-hid eye. h Deletion one copy of endogenous rdx increased GMR-hid eye. i Quantification analyses of d–h eye sizes (n = 15). Scale bars: 50 μm for all disks and 200 μm for all adult eyes.
interaction, we constructed three $\text{Diap1}$ mutants with the replacement of each or both degrons by $\text{AAAAA}$. Mutation of degron 1 (M1) or degron 2 (M2) effectively diminished the interaction between $\text{Diap1}$ and $\text{Rdx-MATH}$, while mutation of both degrons (M1/2) totally abolished this interaction (Fig. 4g, h). These results demonstrate that Rdx binds two SBCs on $\text{Diap1}$ protein through its N-terminal MATH domain.

Rdx does not affect the stability of $\text{Diap1}$ protein

Since the above studies reveal that the E3 ligase Rdx binds $\text{Diap1}$, it is necessary to test whether Rdx promotes $\text{Diap1}$ degradation. Compared with $\text{diap1}$ expression alone (Fig. 5a), co-expression of $\text{rdx}$ was unable to decrease $\text{Diap1}$ protein in the wing disc (Fig. 5b). To validate this result, we generated a $\text{tub-Myc-diap1}$ transgenic fly, which drives Myc-tagged $\text{diap1}$ expression using $\text{tubulin}$ promoter. Immunostaining with anti-Myc antibody showed that $\text{tub-Myc-diap1}$ widely expressed in the wing disc (Fig. 5c), consistent with the expression pattern of $\text{tubulin}$ promoter. Overexpression of $\text{rdx}$ apparently decreased its well-known substrate $\text{Ci}$, but without affecting $\text{Myc-Diap1}$ level (Fig. 5d). Furthermore, we extracted protein of wing disks for western blot (WB) assay, and also revealed that overexpression of $\text{rdx}$ did not change $\text{Myc-Diap1}$ level (Fig. 5e). Overall, the results together suggest that Rdx binds $\text{Diap1}$, but does not promote $\text{Diap1}$ degradation.

Rdx promotes K63-linked ubiquitination of $\text{Diap1}$

The above results demonstrate that the E3 ligase activity is important for Rdx to promote Hid-induced apoptosis, since deletion of $\text{3box}$ abolishes its function. In addition, Rdx shows specific binding to $\text{Diap1}$. We next sought to determine whether Rdx accelerates ubiquitin modification of $\text{Diap1}$ protein. The cell-based ubiquitination assay revealed that Rdx indeed enhanced $\text{Diap1}$ ubiquitination (Fig. 6a).

For ubiquitination, the first ubiquitin (Ub) is covalently attached to the lysine residue (K) of the substrate [39]. The following Ub is attached to one of the lysine residues of the previous Ub to form polyubiquitin chain [39]. Ub contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), which can be used to create inter-Ub linkages during polyubiquitin chain formation [40]. It is well known that substrates with the different linkage of polyubiquitin chains have distinct fates. For instance, K33-linked polyubiquitination is involved in modulating protein trafficking [41]. K48-linked polyubiquitination generally targets proteins for proteasomal degradation [42]. K63-linked polyubiquitin modification usually plays a non-degradative role, instead regulates protein localization and protein–protein interaction [43, 44]. To examine which type of ubiquitination occurs on $\text{Diap1}$ protein, we employed several Ub mutants, in whom one of the seven Ks was substituted by arginine (R). The results showed that Rdx failed to promote $\text{Diap1}$ ubiquitination when Ub-K63R was used (Fig. 6b). On the other hand, Ub-K0, in which all Ks are replaced by Rs, could totally abolish Rdx-induced $\text{Diap1}$ ubiquitination (Fig. 6c). However, Ub-K63, which only harbor one K on 63, had a similar effect as wild-type Ub on Rdx-induced $\text{Diap1}$ ubiquitination (Fig. 6c). To reinforce this result, we chose Ub-K63 and Ub-K48 antibodies to distinguish K63-linked and K48-linked polyubiquitin chains. The co-IP assays showed that Rdx exclusively enhanced Ub-K63 signal, not Ub-K48 signal, suggesting that Rdx promotes K63-linked polyubiquitination on $\text{Diap1}$ protein (Fig. 6d). Besides,
Δ3box did not influence Diap1 ubiquitination (Fig. 6d), further proving that Rdx increases Diap1 ubiquitination through Rdx-Cul3 E3 ligase.

To investigate the relevance between K63-linked polyubiquitination and Hid-induced apoptosis, we generated three transgenic flies to express Ub-K48R, Ub-K63R, or Ub-K0 respectively. Compared with control eyes, Ub-K63R and Ub-K0 enlarged GMR-hid eyes, whereas Ub-K48R did not (Fig. 6e), indicating that K63-linked polyubiquitination possibly inhibits Diap1 activity. It is worth noting that although overexpression of Ub-K63R indeed enlarged GMR-hid eyes, the enlargement was not significant, likely due to endogenous wild-type Ub.

Rdx represses Diap1–Dronc interaction

Previous studies have revealed that K63-linked polyubiquitin chains could serve as a scaffold to regulate protein complex formation [45]. We hypothesized that Rdx-mediated K63-linked polyubiquitination likely influence the interaction of Diap1 with Dronc, a critical target of Diap1. Reciprocal co-IP assays showed that an interaction exists between Diap1 and Dronc (Fig. 7a, b). Consistent to previous studies, Diap1 was capable of decreasing Dronc protein in a dose-dependent manner (Fig. 7c). Contrarily, we found that Rdx could stabilize Dronc protein (Fig. 7d). The co-IP results revealed that Rdx attenuated Diap1–Dronc interaction (Fig. 7e). In addition, Rdx was able to trigger apoptosis, while Rdx-Δ3box failed to do so (Fig. 7f). Taken together, these results suggest that the E3 ligase Rdx promotes K63-linked polyubiquitination of Diap1 to attenuate Diap1–Dronc interaction, culminating Dronc stabilization and apoptosis (Fig. 7g).

DISCUSSION

Apoptosis is a process of programmed cell death that helps to clear away unwanted or dangerous cells. Under normal physiological conditions, apoptosis is maintained at a low level to avoid unfitted cell death. In fact, staining wild-type wing and eye disks with active-caspase3 antibody show weak signals [46, 47]. Therefore, it is challenging to identify negative regulators of apoptosis using RNAi-mediated genetic screening under the normal physiological background. To overcome this difficulty, we developed a modifier screening in this study. Overexpression of the pro-apoptotic hid using GMR promoter produces small eyes due to excessive apoptosis. We conducted RNAi-mediated screening under GMR-hid background to identify which RNAi could enlarge the eye size. Through unbiased screening, we found that ci RNAi apparently increased GMR-hid eye. In contrast, overexpression of ci or its upstream smo decreased the eye size, together suggesting that the Hh pathway is able to promote Hid-induced apoptosis. Next, we showed that the E3 ligase Rdx could mimic Ci to elevate apoptosis. Given rdx is a transcriptional target of Hh signaling, we proposed that the Hh pathway accelerates apoptosis through Rdx. In addition, we demonstrated that Rdx bound Diap1 promotes K63-linked polyubiquitination of Diap1, without affecting Diap1 stability. Finally, we revealed Rdx suppressed Diap1–Dronc...
interaction. Taken together, our findings uncover a Hh-Ci-Rdx axis promoting apoptosis through inhibiting Diap1-mediated Dronc degradation.

In *Drosophila*, forced expression of positive components of the Hh pathway in the wing disc will induce overgrowth [33], indicating its ability to promote cell proliferation. However, the function of the Hh pathway in apoptosis is still unclear. Although it reported that the mammalian Hh pathway suppresses apoptosis via activating anti-apoptotic gene *Bcl2* in tumor cells, no evidence supports the Hh pathway is able to activate *Bcl2* orthologs in *Drosophila*. In addition, Buffy and Debcl, two counterparts of *Bcl2* in *Drosophila*, do not play a key role in apoptosis. In this study, we provided enough data to support that the Hh pathway promotes apoptosis through Rdx. Although Rdx is sufficient to trigger apoptosis, overexpression of *ci* does not decrease the wing disc size, possibly due to Ci activating pro-proliferative genes expression to mask Rdx’s effect. Consistently, overexpression of *ci* using *GMR-gal4* does not decrease the wild-type eye [33], suggesting that the Hh pathway plays its pro-apoptotic under *GMR-hid* background. It will be interesting to test whether the Hh pathway promotes apoptosis in *rpr* and *grim*-overexpressing backgrounds.

Rdx binds two SBCs of Diap1 through its N-terminal MATH domain, indicating Diap1 is a possible substrate of Rdx E3 ligase. The following biochemical assays confirm that Rdx promotes K63-linked ubiquitination of Diap1. Rdx does not affect Diap1 protein level, showing that Rdx regulates Diap1 in a degradation-independent manner. The previous study has shown that Rdx prefers to add K48-linked Ub chains on Ci, leading to proteasome-mediated Ci degradation [48]. These results indicate that Rdx could add distinct modes of Ub chains on substrates to achieve different regulations. Supportively, Rdx’s mammalian ortholog SPOP targets inverted formin2 (INF2) for polyubiquitination to control its subcellular localization, without influencing its degradation [49].

Human Spop protein shares about 80% amino acid sequence identity with *Drosophila* Rdx, and they show a high degree of functional similarity [33]. *Drosophila* phenotypes caused by Rdx deficiency could be rescued by Spop expression [33]. Recently, Spop has garnered more attention due to its important role in tumorigenesis. Many studies have shown that Spop is frequently mutated in several types of tumors, such as prostate cancer and glioma [50, 51]. Resisting cell death is one of the hallmarks of cancer [52]. It will be fruitful to test whether Spop regulates tumorigenesis through promoting apoptosis. Intriguingly, the exome sequencing using 112 human prostate tumor samples shows that most mutations of Spop localize on its MATH domain [51]. A possible explanation is the MATH domain mutation attenuates its interaction with substrates, including IAPs, to relieve the pro-apoptotic function of Spop in tumor cells.
MATERIALS AND METHODS

Fly stocks
Some stocks used in this study were kindly from Dr. Qing Zhang’s lab, including UAS-\(\text{rdx}\)-RNAi [33], UAS-HA-\(\text{rdx}\) [33], \(\text{rdx}\)-lacZ [33], UAS-Ub-K48R [32], UAS-Ub-K0 [32] and UAS-HA-\(\text{rdx}\)-Δ3box [32]. UAS-\(\text{ci}\) and \(\text{rdx}\)-Δ6 were gifted from Junzheng Zhang’s lab. UAS-\(\text{ci}\)-RNAi (NIG #2125R-1), UAS-P35 (BDSC #5072), UAS-\(\text{smo}\) (BDSC #44620), GMR-hid (BDSC #5771), Ay-gal4 (BDSC #4411), UAS-GFP (BDSC #1522), GMR-gal4 (BDSC #8605), MS1096 (BDSC #8860), UAS-Diap1 (BDSC #6657), Ap-\(\text{gal4}\) (BDSC #3041) were obtained from NIG or BDSC. The detailed information of these fly stocks has been described in Flybase database. The \(\text{tub}\)-\(\text{Myc}\)-\text{diap1} construct was made by cloning a full-length \text{diap1} cDNA downstream of the \(\alpha\)-\text{tubulin} promoter [53]. Then the \(\text{tub}\)-\(\text{Myc}\)-\text{diap1} construct was injected into \(\text{w}^{1118}\) \text{Drosophila} embryos according to the method described previously [21].

DNA constructs
To generate \text{Myc-Diap1}, Flag-Diap1, Flag-Rdx, Myc-Rdx, HA-Ub, and Myc-Rdc constructs, we amplified the corresponding cDNA fragments using Vazyme DNA polymerase (P505), and inserted them into pcDNA3.1-Myc, pcMV-Flag, or pcMV-HA backbone vectors respectively. Truncated constructs including Myc-Rdx-MATH (aa1-179), Myc-Rdx-\text{BTB} (aa180-374), and Flag-Rdx-Δ3box (deletion aa299-330) were made by inserting the corresponding coding sequences into pcDNA3.1-Myc or pcMV-Flag vectors. Flag-Diap1-M1, Flag-Diap1-M2, Flag-Diap1-M1/2, HA-Ub-K0, HA-Ub-K48, HA-Ub-K63, HA-Ub-K6R, HA-Ub-K11R, HA-Ub-K27R, HA-Ub-K29R, HA-Ub-K33R, HA-Ub-K48R, and HA-Ub-K63R were made by PCR-based site-directed mutagenesis.

Immunostaining and confocal
Immunostaining of wing and eye disks was carried out according to our previous protocols [54]. Briefly, third-instar larvae were dissected in PBS and fixed in freshly made 4% formaldehyde in PBS at room temperature for 20 min, then washed three times with PBT (PBS supplemented with 0.1% Triton X-100). Larvae were incubated overnight with primary antibodies in PBT at 4 °C, then washed with PBT for three times and incubated with fluorophore-conjugated secondary antibodies for 2 h at room temperature. After washed for three times in PBT, disks were separated and mounted with 40% glycerol. Images were captured with Zeiss confocal microscope. Primary antibodies used in this study were shown as follows: rabbit anti-PH3 (1:100, Abcam); rat anti-\(\text{Ci}\) (1:50, DSHB); rabbit anti-\(\text{Cas3}\) (1:100, ABclonal); mouse anti-\(\beta\)-\text{Gal} (1:500, Santa Cruz); mouse anti-HA (1:200, Santa Cruz); mouse anti-Myc (1:200, Santa Cruz); rabbit anti-DrICE (1:100, CST) and rabbit anti-Diap1 (1:100) [55]. All secondary antibodies used in this study were bought from Jackson ImmunoResearch, and were diluted at 1:500.

Cell culture, transfection, and immunoblot
All cell-based assays in this study were carried out in 293T cells. 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco)
supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/ streptomycin (Sangon Biotech). Construction transfection was performed using PEI (Sigma) according to the manufacturer’s instructions [54]. Two days after transfection, cells were harvested to extract total protein for following co-IP and immunoblot (IB) according to our previous protocols [55]. The following antibodies were used for IP and IB: mouse anti-Flag (1:2000 for IB, Sigma); mouse anti-Myc (1:2000 for IB; Sigma, Santa Cruz); mouse anti-HA (1:2000 for IB); mouse anti-Actin (1:5000, Genscript); rabbit anti-ub-K63 (1:1000 for IB, ABclonal); rabbit anti-ub-K48 (1:1000 for IB, ABclonal); goat anti-mouse HRP (1:10000, Abmax) and goat anti-rabbit HRP (1:10,000, Abmax). The densities of IB bands were measured by Image J software.

Eye size quantification and statistical analysis

For eye size analysis, all groups were crossed at 25 °C. Photos of adult eyes were taken on female flies at the same magnification. Sizes of eyes were measured by Image J software. Statistical analyses were performed with GraphPad Prism software, using one-way ANOVA. All data were presented as means ± standard deviation (SD), and P < 0.05 was considered statistically significant. Where exact P-values are not shown, statistical significance is shown as with *P < 0.05, **P < 0.01, ***P < 0.001, and ns no significance.

DATA AVAILABILITY

The datasets used and analyzed in this study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
ZZ and MZ designed this study and provided financial support. BL, YD, and BS carried out the experiments and analyzed the results. BS provided financial support to make transgenic flies. ZZ, QL, and MZ wrote the article with the assist of all authors. All authors read and approved the final paper.

COMPETING INTERESTS
The authors declare no competing interests.

ETHICS
All experiments in this study do not use human or mouse samples.

ADDITIONAL INFORMATION
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