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ORIGINAL ARTICLE

NOPO modulates Egr-induced JNK-independent cell death in *Drosophila*

Xianjue Ma[1], Jiuhong Huang[1], Lixia Yang[1], Yang Yang[1], Wenzhe Li[1], Lei Xue[1]

[1]*Shanghai Key Laboratory for Signaling and Diseases, School of Life Science and Technology, Tongji University, 1239 Siping Road, Shanghai 200092, China*

Tumor necrosis factor (TNF) family ligands play essential roles in regulating a variety of cellular processes including proliferation, differentiation and survival. Expression of *Drosophila* TNF ortholog Eiger (Egr) induces JNK-dependent cell death, while the roles of caspases in this process remain elusive. To further delineate the Egr-triggered cell death pathway, we performed a genetic screen to identify dominant modifiers of the Egr-induced cell death phenotype. Here we report that Egr elicits a caspase-mediated cell death pathway independent of JNK signaling. Furthermore, we show NOPO, the *Drosophila* ortholog of TRIP (TRAF interacting protein) encoding an E3 ubiquitin ligase, modulates Egr-induced Caspase-mediated cell death through transcriptional activation of pro-apoptotic genes *reaper* and *hid*. Finally, we found Bendless and dUEV1a, an ubiquitin-conjugating E2 enzyme complex, regulates NOPO-triggered cell death. Our results indicate that the Ben-dUEV1a complex constitutes a molecular switch that bifurcates the Egr-induced cell death signaling into two pathways mediated by JNK and caspases respectively.

***Keywords*:** cTNF; JNK; NOPO; caspase; cell death

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Introduction

Tumor necrosis factor (TNF) family ligands are type II transmembrane proteins that play key roles in regulating a variety of physiological processes including cell proliferation, differentiation, survival and immunity [1-3]. Mammalian studies revealed that TNF stimulates activation of nuclear factor-κB (NF-κB), caspase, c-Jun N-terminal kinase (JNK) and p38 signaling. Upon phosphorylation by the mitogen-activated protein (MAP) kinase cascade, JNK translocates into the nucleus, phosphorylates and activates transcription factors such as Jun and Fos, and finally leads to caspase-mediated apoptosis [4, 5]. This signaling pathway is highly conserved in *Drosophila*, in which the TNF orthlog Eiger (Egr) triggers cell death through its receptor Wengen (Wgn), the TNF receptor-associated factor 2 (dTRAF2), the JNKK kinase dTAK1, the JNK kinase Hemipterous (Hep) and Basket (Bsk) that encodes the *Drosophila* JNK [6-8]. Although considerable progress has been made to characterize the molecular mechanism underlying cell death induced by TNF-JNK signaling [9], whether caspases are involved in this process has remained controversial [6, 7].

TRIP (TRAF interacting protein) encodes a RING domain-containing E3 ubiquitin ligase [10] that negatively regulates NF-κB activation through physical interaction with the tumor suppressor CYLD or Syk *in vitro* [11, 12]. Targeted disruption of TRIP in mice, or loss-of-function mutation of its *Drosophila* ortholog no poles (NOPO), resulted in early embryonic lethality [13, 14], suggesting TRIP/NOPO plays important roles in embryonic development. Recently, it was shown that NOPO physically interacts with the ubiquitin E2 complex consisting of Bendless (Ben)-dUEV1A heterodimer and regulates genomic integrity in *Drosophila* [14].

In this study, we performed a genetic screen for dominant modifiers of the Egr-triggered cell death phenotype, and found that Egr induced a caspase-dependent but JNK-independent cell death pathway. Furthermore, we showed that NOPO modulated Egr-induced caspase activation and cell death through up-regulation of the proapoptotic genes *reaper* (*rpr*) and *head involution*

Correspondence: Lei Xue
Tel: +86-21-65985407; Fax: +86-21-65985407
E-mail: lei.xue@tongji.edu.cn
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defective (*hid*). Finally, we demonstrated that the Ben-dUEV1A E2 complex modulated NOPO activity in the cell death signaling pathway. These data, together with previous results, suggest that the Ben-dUEV1A E2 complex selects dTRAF2 or NOPO as its ubiquitin E3 ligase partner and switches Egr-induced cell death signaling into two independent downstream pathways, one mediated by dTRAF2-JNK and another by NOPO-caspases.

Results and Discussion

Egr triggers two independent cell death pathways

Ectopic expression of Egr, the *Drosophila* TNF ortholog, in the developing eye (*GMR*>Egr) triggered cell death and produced a small eye phenotype (Figure 1A and 1B) [6, 7]. Previous studies showed that this phenotype was suppressed when JNK signaling was blocked, suggesting that Egr-induced cell death depends on JNK activation [6, 7]. Consistent with this hypothesis, expression of a constitutive active form of Hep, the *Drosophila* JNK kinase, in the developing eye (*GMR*>Hep^{CA}) induced JNK-mediated cell death and produced a similar small eye phenotype (Figure 1H) [7]. However, we found that the small eye phenotype induced by *GMR*>Egr (Figure 1B), but not that by *GMR*>Hep^{CA} (Figure 1H), was partially suppressed by the deficiency *Df(3L)H99* that deletes three proapoptotic genes, *rpr*, *hid* and *grim* (Figure 1C and 1I), or co-expression of the inhibitor of apoptosis protein DIAP1 (Figure 1D and 1J) or a dominant negative form of the caspase-9 homolog Drosophila Nedd-2-like caspase (DRONC) (Figure 1E and 1K). In addition, expression of Egr, but not Hep^{CA}, activated *hid* (Figure 1F and 1L) and *rpr* (Figure 1G and 1M) transcription in third instar larval eye discs. The *rpr* gene was also activated in hemizygous dTAK1 males or when Puc was co-expressed (Supplementary information, Figure S2F-S2I),

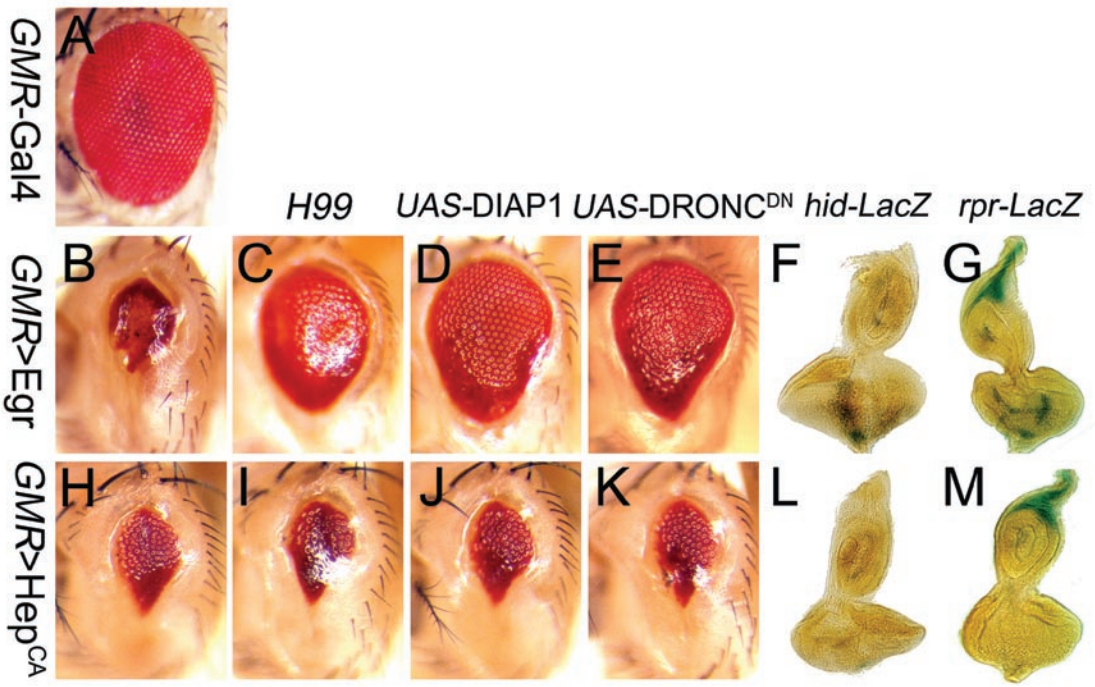

Figure 1 Egr elicits a JNK-independent apoptotic pathway in *Drosophila*. **(A-E)** Compared to wild type (**A**, *GMR*-Gal4/+), *GMR*>Egr triggered a cell death phenotype (**B**, *UAS*-Egr/+; *GMR*-Gal4/+) that could be partially suppressed by removing one copy of the genes *hid*, *reaper* and *grim* using *Df(3L)H99* (**C**, *UAS*-Egr/+; *GMR*-Gal4/*Df(3L)H99*), expression of DIAP1 (**D**, *UAS*-Egr/+; *GMR*-Gal4/*UAS*-DIAP1) or a dominant negative form of DRONC (**E**, *UAS*-Egr/*UAS*-DRONC^{DN}; *GMR*-Gal4/ *UAS*-DRONC^{DN}). **(F-G)** Expression of Egr in developing eye induces *hid* and *reaper* expression. *hid* (**F**, *GMR*-Gal4 *UAS*-Egr/*W*^{05014}) and *reaper* (**G**, *rpr*-lacZ/+; *GMR*-Gal4 *UAS*-Egr/+) expression were detected posterior to the morphological furrow in *GMR*>Egr eye discs. **(H-K)** *GMR*>Hep^{CA}-triggered apoptosis in the eye (**H**, *GMR*-Gal4 *UAS*-Hep^{CA}/+) cannot be rescued by *Df(3L)H99* (**I**, *GMR*-Gal4 *UAS*-Hep^{CA}/*Df(3L)H99*), expression of DIAP1 (**J**, *GMR*-Gal4 *UAS*-Hep^{CA}/*UAS*-DIAP1) or a dominant negative form of DRONC (**K**, *GMR*-Gal4 *UAS*-Hep^{CA}/*UAS*-DRONC^{DN}). **(L-M)** Expression of *hid* (L, *GMR*-Gal4 *UAS*-Hep^{CA}/*W*^{05014}) and *reaper* (**M**, *rpr*-lacZ/+; *GMR*-Gal4 *UAS*-Hep^{CA}/+) are not detected posterior to the morphological furrow in *GMR*>Hep^{CA} eye discs. For all the genetic interaction experiments, over 50 adult eyes and wings, 30 eye and wing discs were examined for each genotype, and the results have been highly consistent. A representative picture of each genotype is shown in this and following figures.

suggesting that the JNK pathway is dispensable for Egr-triggered rpr activation. Finally, Egr-induced cell death was not completely suppressed in hemizygous dTRAF2 or dTAK1 mutant males (Supplementary information, Figure S2B and S2C), or by the co-expression of Bsk^{DN} or Puc (Supplementary information, Figure S2D and S2E), suggesting that the dTRAF2-dTAK1-Hep-Bsk pathway is not the sole downstream mediator of Egr-induced cell death. Taken together, these results indicate that Egr induces cell death through two independent pathways, one mediated by JNK signaling and another by caspase activation.

nopo is required for Egr-induced cell death

To investigate the genetic mechanism underlying Egr-induced caspase activation, we performed a whole genome deficiency screen using the Bloomington deficiency kit to search for dominant modifiers of the *GMR*>Egr small eye phenotype (Figure 2C). One of the suppressors was mapped cytologically between 55B12 and 55C1, a region uncovered by two overlapping deficiencies, *Df(2R)Exel7153* and *Df(2R)BSC337* (Figure 2A). Incorporating such deficiency into GMR>Egr flies mildly suppressed the small eye phenotype (Figure 2E and 2F), while *Df(2R)ED3485*, an adjacent deficiency

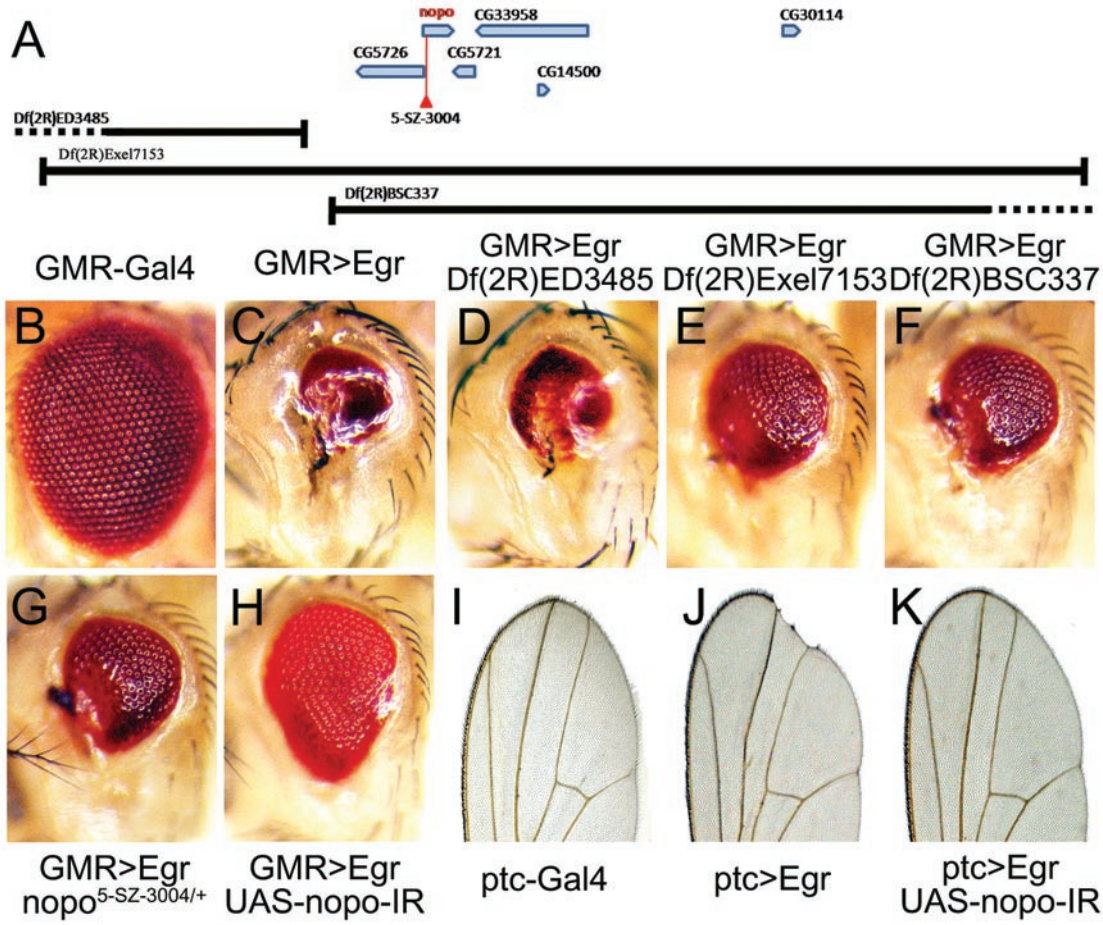

Figure 2 NOPO regulates Egr-induced cell death. **(A)** A schematic depiction of the genomic region surrounding the *nopo* locus. The P element *5-SZ-3004* and three deficiencies *Df(2R)ED3485*, *Df(2R)Exel7153* and *Df(2R)BSC33* are indicated. **(B-H)** Light micrographs of *Drosophila* adult eyes are shown. Compared to wild type (**B**, *GMR*-Gal4/+), the *GMR*>Egr small eye phenotype (**C**, *UAS*-Egr/+; *GMR*-Gal4/+) remains unaffected by *Df(2R)ED3485* (**D**, *UAS*-Egr/+; *GMR*-Gal4/*Df(2R)ED3485*), but is partially suppressed by deficiency *Df(2R)Exel7153* (**E**, *UAS*-Egr/+; *GMR*-Gal4/*Df(2R)Exel7153*) or *Df(2R)BSC337* (**F**, *UAS*-Egr/+; *GMR*-Gal4/*Df(2R)BSC337*) that deletes genes including *nopo*. This phenotype is also suppressed in heterozygous *nopo* mutant (**G**, *UAS*-Egr/nopo$^{5\text{-}SZ\text{-}3004}$; *GMR*-Gal4/+) or by expression of a *nopo* RNAi (**H**, *UAS*-Egr/+; *GMR*-Gal4/*UAS-nopo-IR*). **(I-K)** Light micrographs of *Drosophila* adult wings are shown. Compared to wild-type wing (**I**, *ptc*-Gal4/+), *ptc*>Egr-induced notch phenotype along the wing margin (**J**, *ptc*-Gal4 *UAS*-Egr/+) is suppressed by expression of a *nopo* RNAi (**K**, *ptc*-Gal4 *UAS*-Egr/ *UAS-nopo-IR*).

(Figure 2A), failed to do so (Figure 2D). This region contains six genes including *nopo* (Figure 2A), the *Drosophila* ortholog of TRIP encoding an E3 ubiquitin ligase [14], as predicted by the *Drosophila* genome project [15]. Consistently, we found mutation of endogenous *nopo* (Figure 2G and Supplementary information, Figure S3) or expression of a *nopo* RNAi (Figure 2H) partially suppressed the small eye phenotype of *GMR*>Egr, but not that of *GMR*>Hep^{CA} (Supplementary information, Figure S4), suggesting *nopo* modulates Egr-induced cell death in parallel or upstream of Hep. The knockdown effect of *nopo* RNAi was verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Supplementary information, Figure S5A) and its ability to suppress the *GMR*>NOPO eye phenotype (Supplementary information, Figure S5B-S5D).

We further characterized the genetic interaction between *nopo* and Egr in the developing wing. Ectopic expression of Egr along the anterior posterior compartment boundary of wing disc produces a notch phenotype in adult wing margin (Figure 2I and 2J). This phenotype was fully suppressed by the RNAi of *nopo* (Figure 2K). Taken together, these data suggest that *nopo* is required for Egr-induced cell death in *Drosophila*.

NOPO induces caspase-mediated cell death independently of JNK

To investigate the role of NOPO in Egr-induced cell death signaling, we expressed NOPO in the developing eye or wing under the control of *GMR* (*GMR*>NOPO) or scalloped (*sd*) (*sd*>NOPO) promoter. *GMR*>NOPO produced a small and rough eye phenotype (Figure 3A and 3B), resulting from extensive cell death posterior to the morphogenetic furrow in third instar eye discs (Figure 3C and 3D), as shown by acridine orange (AO) staining, a dye that detects dying cells [16]. Consistently, *sd*>NOPO triggered strong apoptosis along the dorsal ventral compartment boundary in wing discs (Figure 3G and 3H), and generated a small wing phenotype missing most of the wing margin tissue (Figure 3E and 3F). We also checked additional Gal4 drivers expressed in the wing discs, and found that MS1096>NOPO produced a similar but milder phenotype (Supplementary information, Figure S6B and S6E), whereas NOPO expression under the *engrailed* (*en*) promoter, which drives NOPO expression exclusively in the posterior compartment (Supplementary information, Figure S6F'), induced apoptosis in the posterior area of wing discs (Supplementary information, Figure S6F) and resulted in a significant reduction of the posterior/anterior compartment ratio in adult wings (Supplementary information, Figure S6C and S6G).

We further used the *GMR*>NOPO eye phenotype (Figure 3B) to characterize the genetic interaction between NOPO and the two signaling pathways, namely JNK and caspases, that mediate Egr-induced cell death. We found the *GMR*>NOPO phenotype was not suppressed by the expression of a dominant negative form of dTAK1 (Figure 3I), a *hep* RNAi (Figure 3J), a dominant negative form of Bsk that encodes the *Drosophila* JNK (Figure 3K) or the Bsk inhibitor Puckered (Puc) (Figure 3L). On the other hand, we observed significant rescue of the *GMR*>NOPO defective eye phenotype upon overexpression of DIAP1, an inhibitor for initiator caspase activity (Figure 3M) [17], a dominant negative form of DRONC (Figure 3N), or p35 (Figure 3O), a viral protein that inhibits downstream effector caspases [18]. Taken together, these results demonstrate that NOPO regulates the caspases-mediated, but JNK-independent cell death signaling pathway.

Since p53 also induces caspases-mediated apoptosis in *Drosophila* [19], we tested whether p53 is required for NOPO-induced cell death. To this end, the *GMR*>NOPO eye phenotype was not suppressed by the expression of p53^{259H} and p53^{CT} (Supplementary information, Figure S7), two dominant negative forms of p53 [19, 20], indicating that NOPO modulates cell death independent of p53 activity.

NOPO activates rpr and hid expression

Developmental cell death in *Drosophila* is mostly mediated by three closely linked pro-apoptotic genes *rpr*, *hid* and *grim*, which encode small proteins that inactivate DIAP1 [21, 22]. We found the *GMR*>NOPO eye phenotype (Figure 3) was significantly suppressed in flies heterozygous for the deficiency *Df(3L)H99* (Figure 3P), which simultaneously removes the three pro-apoptotic genes, suggesting their roles in NOPO-induced cell death. Consistent with this result, ectopic NOPO expression induced *rpr* and *hid* transcription in larval eye and wing discs (Figure 3Q-3T), and *rpr* expression in adult eyes, as revealed by qRT-PCR (Supplementary information, Figure S8). Thus, we conclude that *nopo* modulates Egr-induced caspase activation through transcriptional up-regulation of *rpr* and *hid*.

Bendless and dUEV1a regulate NOPO-induced apoptosis

NOPO has been found to interact with Ben, the *Drosophila* ortholog of Ubc13, in the yeast two-hybrid experiments [14, 23], and it is postulated that Ben could associate with dUEV1a to form a heterodimer that functions as an E2 ubiquitin-conjugating enzyme [24]. Recent reports showed that Ben-dUEV1a and NOPO form an E2-E3 ubiquitination complex that regulates genomic integrity during early embryogenesis [14]. In support with the physical interaction data between Ben-dUEV1a and

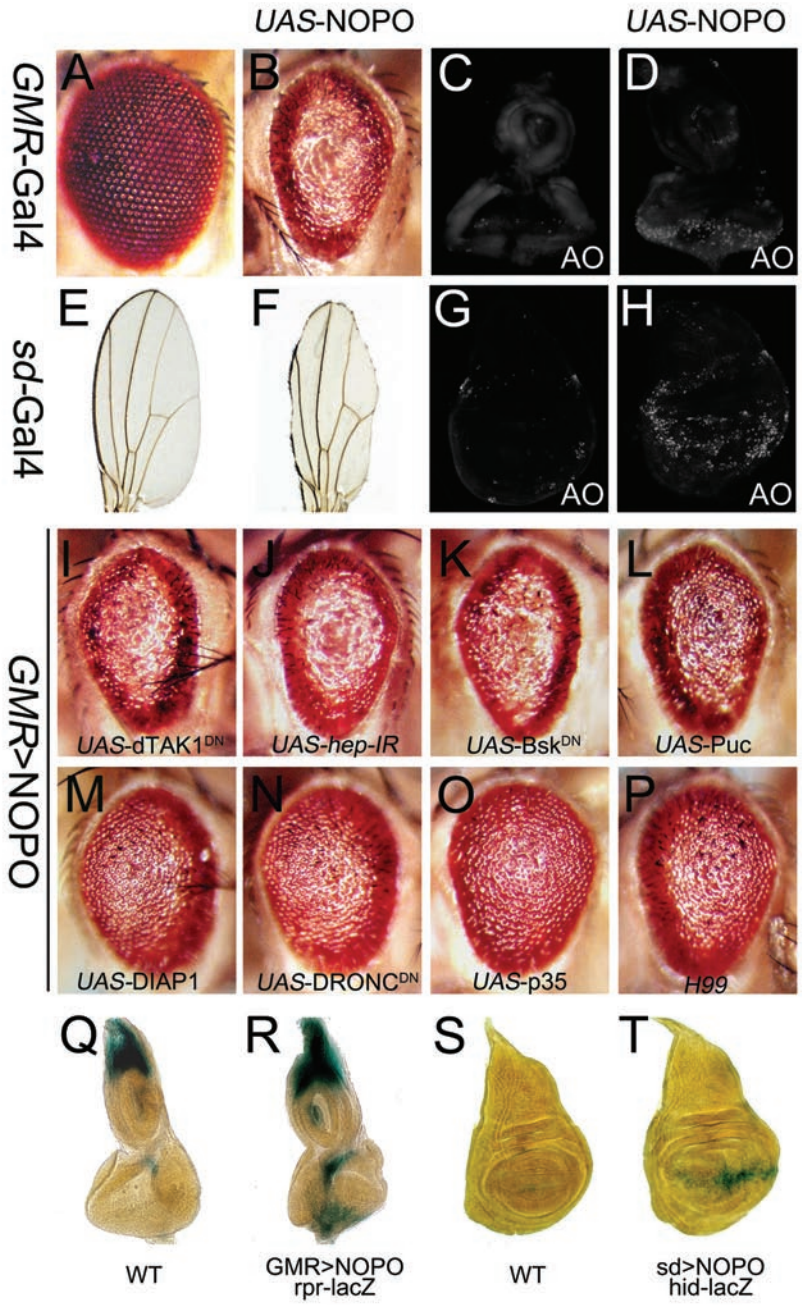

Figure 3 Ectopic NOPO expression induces caspase-dependent but JNK-independent cell death. **(A-H)** Ectopic expression of NOPO induces cell death. Compared to wild-type eyes (**A**, *GMR*-Gal4/+) or wings (**E**, *sd*-Gal4/+), NOPO expression driven by *GMR*-Gal4 or *sd*-Gal4 produces small and rough eyes (**B**, *UAS*-NOPO/+; *GMR*-Gal4/+) or small wings missing most of the margin tissue (**F**, *sd*-Gla4/*UAS*-NOPO), which are resulted from increased cell death indicated by acridine orange staining, in third instar larval eye discs (**C**, *GMR*-Gal4/+; and **D**, *UAS*-NOPO/+; *GMR*-Gal4/+) or wing discs (**G**, *sd*-Gal4/+; and H, *sd*-Gla4/*UAS*-NOPO).**(I-P)** The *GMR*>NOPO phenotype is caspase-dependent but JNK-independent. The *GMR*>NOPO eye phenotype could not be suppressed by the expression of a dominant negative form of dTAK1 (**I**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-dTAK1^{DN}), or a *hep* RNAi (**J**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS-hep-IR*), or a dominant negative form of Bsk (**K**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-Bsk^{DN}), or Puc (**L**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-puc), but suppressed by the expression of DIAP1 (**M**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-DIAP1), or a dominant negative form of DRONC (**N**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-DRONC^{DN}), or p35 (**O**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-p35), or removing one copy of *reaper*, *hid* and *grim* (**P**, *UAS*-NOPO/+; *GMR*-Gal4/*Df(3L)H99*). **(Q-T)** Ectopic NOPO expression induces *reaper* and *hid* transcription. X-Gal staining of a *reaper*-LacZ **(Q**, **R)** and a *hid*-LacZ **(S**, **T)** reporter in wild-type **(Q**, **S)** or NOPO-expressing **(R**, **T)** imaginal discs are shown. Genotypes: **Q**, *rpr*-lacZ/+; *GMR*-Gal4/+; **R**, *UAS*-NOPO/+; *rpr*-lacZ/+; *GMR*-Gal4/+; **S**, *sd*-Gal4/W^{05014}; **T**, *UAS*-NOPO/+; *sd*-Gal4/W^{05014}.

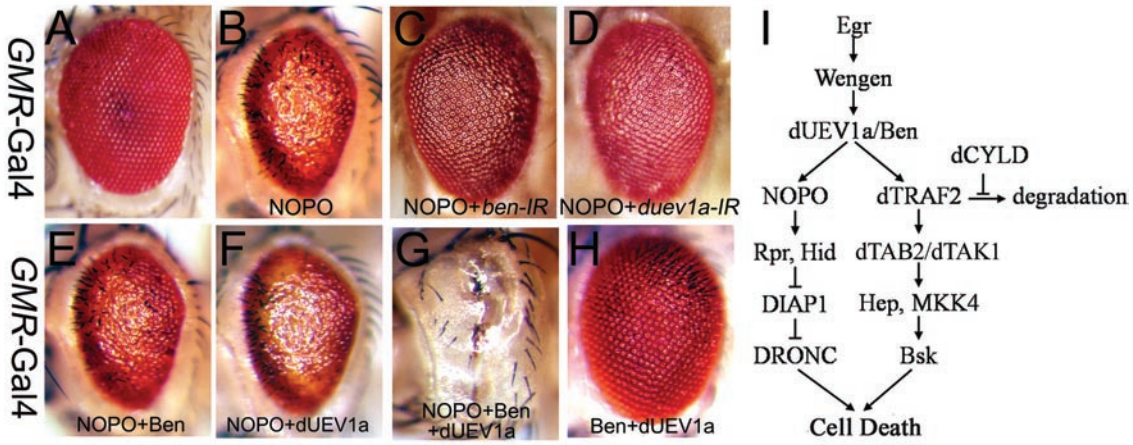

Figure 4 Bendless and dUEV1a regulate NOPO-induced apoptosis. **(A-D)** Light micrographs of *Drosophila* adult eyes are shown. Compared to the wild-type eye (**A**, *GMR*-Gal4/+), NOPO-induced cell death (**B**, *UAS*-NOPO/+; *GMR*-Gal4/+) could be suppressed by RNAi inactivation of *ben* (**C**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS-ben-IR*) or *duev1a* (**D**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS-duev1a-IR*). **(E-H)** Expression of either Ben (**E**, *UAS*-NOPO/+; *UAS*-Ben^{T8}/+; *GMR*-Gal4/+) or dUEV1a (**F**, *UAS*-NOPO/+; *GMR*-Gal4/*UAS*-dUEV1a) has no effect on the *GMR*>NOPO phenotype, co-expression of both, though appears normal by itself (**H**, *UAS*-Ben/+; *GMR*-Gal4/*UAS*-dUEV1a), significantly enhances the NOPO-induced cell death phenotype (**G**, *UAS*-NOPO/+; *UAS*-Ben/+; *GMR*-Gal4/*UAS*-dUEV1a). **(I)** A model for the role of NOPO in Egr-induced cell death signaling.

NOPO, we found that loss of *ben* or *duev1a* significantly suppressed the *GMR*>NOPO eye phenotype (Figure 4C, 4D and Supplementary information, Figure S9), suggesting the ectopic NOPO activity crucially depends on the E2 function of the endogenous Ben-dUEV1a complex. In addition, while expression of Ben or dUEV1a alone failed to enhance the *GMR*>NOPO eye phenotype (Figure 4E and 4F), co-expression of Ben and dUEV1a, which by itself did not lead to any obvious defect (Figure 4H), significantly enhanced the *GMR*>NOPO eye phenotype (Figure 4G), indicating that Ben and dUEV1a might function as a complex to regulate NOPO-induced cell death *in vivo*. Our unpublished results revealed that Ben and dUEV1a also regulate Egr-induced JNK activation through physical interaction with dTRAF2, another ubiquitin E3 ligase. Taken these data together, our genetic experiments demonstrate an essential role of NOPO in regulating TNF-induced cell death, which is conserved from *Drosophila* to mammals. We propose a model that the Ben-dUEV1a E2 complex constitutes a molecular switch which, by choosing dTRAF2 or NOPO as its E3 partner, transduces Egr-induced cell death signaling through two independent pathways, one mediated by dTRAF2-JNK and another by NOPO-caspase (Figure 4I).

Materials and Methods

Drosophila strains

GMR-Gal4, *UAS*-DIAP1, *UAS*-p35, *GUS*-p53^{259H}, *GUS*-p53^{CT}, *Df(3L)H99*, *sd*-Gal4, *hid*-lacZ (w^{05014}) and the deficiency kit were obtained from Bloomington stock center, *UAS-nopo-IR*, *UAS-ben-IR*, *UAS-duev1a-IR* were obtained from Vienna stock center, $nopo^{5\text{-}SZ\text{-}3004}$ was obtained from Szeged Drosophila Stock Center. *UAS*-Egr, *UAS*-DRONC^{DN} [6], *reaper*-lacZ [25], *UAS*-Hep^{CA} [26], ben^{1} [27], *UAS*-NOPO, $nopo^{Exc142}$ [14], *ptc*-Gal4, *UAS*-dTAK1^{DN}, *UAS-hep-IR*, *UAS*-Bsk^{DN}, *UAS*-Puc, puc^{E69}, $dTRAF2^{EX1.1}$ [8], *UAS*-Ben^{T8} [28], $dTAK1^{l}$ [29] were previously described.

X-Gal staining

Eye and wing discs were dissected from third instar larvae in PBST and stained for β-galactosidase activity as described [30].

AO staining

Eye and wing discs were dissected from third instar larvae in PBST and incubated in 1×10^{-5} M AO for 5 min at room temperature prior to imaging.

qRT-PCR

Thirty adult heads were collected from freshly eclosed flies of indicated genotypes. Total RNA was isolated using TRIzol (Invitrogen), and RT-PCR was performed as previously described [31]. Primers for *reaper* are sense 5′-GAGCAGAAGGAGCAGACGAT-3′ and antisense 5′-CGATATTTGCCGGACTTTCT-3′, primers for n*opo* are sense 5'-CATCGTAAAATCGCCATCAGTCA-3' and antisense 5′-GCTGCTCCTGCAGTGAACCAAC-3′, primers for *actin5c* are sense 5′-AAGTTGCTGCTCTGGTTGTC-3′ and antisense 5′-GGGTACTTCAGGGTGAGGATA-3′

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)