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dUev1a modulates TNF-JNK mediated tumor progression and cell death in *Drosophila*

Xianjue Ma [1], Lixia Yang [1], Yang Yang, Maoquan Li, Wenzhe Li *, Lei Xue *

Department of Interventional Radiology, Shanghai 10th People's Hospital, Shanghai Key Laboratory of Signaling and Disease Research, School of Life Science and Technology, Tongji University, Shanghai 200092, China

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ABSTRACT

Loss of cell polarity cooperates with oncogenic Ras to induce JNK-dependent tumor growth and invasion. To identify additional genes that modulate tumor progression, we have performed a genetic screen in *Drosophila* and found that loss of dUev1a, the ortholog of mammalian Uev1, suppressed $lgl^{-/-}/Ras^{V12}$ induced JNK-mediated tumor growth and invasion. Furthermore, loss of dUev1a suppressed TNF ortholog Eiger-induced JNK-mediated cell invasion and cell death. Finally, dUev1a cooperated with Bendless to activate JNK signaling through dTRAF2. Together, our data indicate that *dUev1a* encodes an essential component of the evolutionary conserved TNF–JNK signaling pathway that modulates tumor progression and cell death in metazoan.

Introduction

Tumor metastasis, rather than the primary tumor arises from malignant lesions, is the main cause of mortality from cancer (Valastyan and Weinberg, 2011). Although extensive progress has been made toward understanding the nature of tumor development, a large number of genes that affect tumor metastasis remained unknown. *Drosophila melanogaster*, with its reduced genome redundancy as well as the ease of genetic manipulation, has become an excellent model organism to address tumor formation and progression (Miles et al., 2011). A number of mutations, including the cell polarity genes *scribble* (*scrib*), *lethal giant larvae* (*lgl*) and *disc large* (*dlg*), have been shown to cause excessive growth that results in tumor production (Bilder, 2004; Hariharan and Bilder, 2006). Loss of these genes could cooperate with oncogenic Ras (Ras^{V12}) to drive tumor growth and invasion *in vivo* (Brumby and Richardson, 2003; Pagliarini and Xu, 2003).

The c-Jun N-terminal Kinase (JNK) pathway is evolutionarily conserved from fly to human, and has emerged as an essential signaling pathway to regulate a wide range of biological activities, including cell death, proliferation and migration (Davis, 2000). In *Drosophila*, the JNK pathway is activated by Eiger (Egr), the ortholog of tumor necrosis factor (TNF) (Igaki et al., 2002; Moreno et al., 2002). Previous genetic studies have identified several novel components of the Egr-JNK signaling pathway and substantially improved our understandings of the molecular and genetic regulation of this pathway *in vivo* (Igaki, 2009). Recently, Egr-JNK signaling was shown to play an important role in modulating $Ras^{V12}/scrib^{-/-}$ triggered tumor metastasis (Cordero et al., 2010; Igaki et al., 2006).

Uev (ubiquitin-conjugating enzyme variant) encodes a conserved protein family that resembles the ubiquitin-conjugating enzyme in both sequence and structure but lacking the conserved cysteine residue essential for the conjugation and transfer of ubiquitin to protein substrates (Broomfield et al., 1998; Sancho et al., 1998). Four members of the Uev protein family, including Mms2, Uev1 (also known as CROC-1), TSG101 and UEV3, have been identified in human (Kloor et al., 2002; Palencia et al., 2006; Xiao et al., 1998). Mms2 and Uev1 have been shown to form a stable E2 complex with Ubc13 (Andersen et al., 2005; Deng et al., 2000; Pelzer et al., 2009), yet they are involved in different cellular functions. While Ubc13–Mms2 complex is required for DNA damage response (Hoege et al., 2002; Li et al., 2002), Ubc13–Uev1 complex is involved in IκB/NF-κB signaling-mediated cell survival (Deng et al., 2000; Syed et al., 2006). In addition, Ubc13 was found to be required for TNF-induced TRAF2 ubiquitination and JNK activation (Shi and Kehrl, 2003). Since Uev1 was the only known Uev to bind Ubc13 at that time (Deng et al., 2000), a similar role in TNF–JNK signaling was proposed for Uev1 (Shi and Kehrl, 2003). However, shortly afterwards, MMs2 was also shown to bind Ubc13 and form a E2 complex (Andersen et al., 2005), hence, it has remained unknown which E2 partner, Uev1 or Mms2, or probably another Uev protein, is required for Ubc13 to modulate TNF–JNK signaling pathway.

* Corresponding authors. Fax: +86 21 65985407.
E-mail addresses: lwz@tongji.edu.cn (W. Li), lei.xue@tongji.edu.cn (L. Xue).
[1] These authors contribute equally.

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dUev1a, the *Drosophila* ortholog of Uev1, plays essential roles in regulating IMD pathway-mediated innate immunity and NOPO-mediated genomic integrity, by forming an E2 complex with its heterodimer partner Bendless (Ben) (Merkle et al., 2009; Zhou et al., 2005), the *Drosophil*a ortholog of Ubc13 (Oh et al., 1994). Our recent work demonstrated that Ben and dUev1a are also required for NOPO induced cell death in *Drosophila* (Ma et al., 2012).

In this report, we identified dUev1a as a crucial regulator of $Ras^{V12}/lgl^{-/-}$ induced JNK-mediated tumor growth and invasion, as well as Egr-triggered JNK activation and cell death in development. Our genetic epistasis analysis demonstrated that dUev1a cooperates with Ben to modulate JNK signaling upstream of dTRAF2. Taken together, our data indicated that dUev1a constitutes an essential component of the evolutionary conserved TNF–JNK signaling pathway that modulates tumor progression and cell death in *Drosophila*.

Materials and methods

Drosophila strains and generation of clones

All stocks were raised on standard *Drosophila* media and crosses were performed at 25 °C unless otherwise indicated. Fluorescently labeled invasive tumors were produced in the eye discs as previously described (Pagliarini and Xu, 2003) using following strains: *y*,*w*, *ey*-Flp; *tub*-Gal80, FRT40A; *Act* > y^+ > Gal4, *UAS*-GFP and lgl^4 FRT40A *UAS*-Ras^{V12}. Additional strains including w^{1118}, *tub*-Gal4, *GMR*-Gal4, *sev*-Gla4, *ptc*-Gla4, *pnr*-Gla4, *Df*(3*L*)*Exel6104*, *Df*(3*L*)*Exel6105*, $dUev1a^{DG14805}$ were obtained from Bloomington Stock Center; *UAS-dUev1a-IR*, *UAS-lgl-IR*, *UAS-scrib-IR* were obtained from VDRC; *UAS*-Egr (Igaki et al., 2002; Moreno et al., 2002), *reaper*-lacZ (Nordstrom et al., 1996), *UAS*-Hep^{CA} (Kanda et al., 2002), *UAS*-dTAK1, *UAS*-Puc, puc^{E69} (Xue et al., 2007), *UAS*-Ben^{T8} (Uthaman et al., 2008), *UAS*-dUev1a (Ma et al., 2012) were previously described.

Genotypes of flies used in article

Fig. 1. (A, A′, and E) *y w*, *ey*-Flp/+; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/+
(B, B′, and F) *y w*, *ey*-Flp/+; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/*UAS-dUev1a-IR*
(C and C′) *y w*, *ey*-Flp/+; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/$dUev1a^{DG14805}$
(D and D′) *y w*, *ey*-Flp/+; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > $^+$ > Gal4, *UAS*-GFP/*UAS*-Puc
Fig. 2 (A) *ptc*-Gal4 *UAS*-GFP/+
(B) *ptc*-Gal4 *UAS*-GFP/+; *UAS-scrib-IR*/+
(C) *ptc*-Gal4 *UAS*-GFP/+; *UAS-scrib-IR*/*UAS-dUv1a-IR*
(D) *ptc*-Gal4, *UAS*-GFP/*UAS*-Egr
(E) *ptc*-Gal4 *UAS*-GFP/UAS-Egr; *UAS-dUv1a-IR*/+
Fig. 3 (A and J) *GMR*-Gal4/+
(B) *GMR*-Gal4 *UAS*-Egr/+
(C) *GMR*-Gal4 *UAS*-Egr/+; *Df*(3*L*)*Exel6104*/+
(D) *GMR*-Gal4 *UAS*-Egr/+; *Df*(3*L*)*Exel6105*/+
(E) *GMR*-Gal4 *UAS*-Egr/+; $dUev1a^{DG14805}$/+
(F) *GMR*-Gal4 *UAS*-Egr/+; *Df*(3*L*)*Exel6104*/*Df*(3*L*)*Exel6105*
(G) *GMR*-Gal4 *UAS*-Egr/+; $dUev1a^{DG14805}$/*Df*(3*L*)*Exel6104*
(H and K) *GMR*-Gal4 *UAS*-Egr/*UAS*-GFP
(I and L) *GMR*-Gal4 *UAS*-Egr/+; *UAS-dUev1a-IR*/+
(M) *GMR*-Gal4/+; *reaper*-LacZ/+
(N) *GMR*-Gal4 *UAS*-Egr/+; *reaper*-LacZ/*UAS*-GFP
(O) *GMR*-Gal4 *UAS*-Egr/+; *reaper*-LacZ/*UAS-dUevia-IR*
Fig. 4 (A and D) *pnr*-Gal4/+
(B and E) *UAS*-Egr/+; *pnr*-Gal4/+
(C and F) *UAS*-Egr/+; *pnr*-Gal4/*UAS-dUev1a-IR*
(G and J) *sd*-Gal4/+
(H, K) *sd*-Gal4/+; *UAS*-Egr/+
(I and L) *sd*-Gal4/+; *UAS*-Egr/+; *UAS-dUev1a-IR*/+
Fig. 5 (A) *GMR*-Gal4/puc^{E69}
(B) *GMR*-Gal4 *UAS*-Egr/+; puc^{E69}/*UAS*-GFP
(C) *GMR*-Gal4 *UAS*-Egr/+; $dUev1a^{DG14805}$/puc^{E69}
(D) *GMR*-Gal4 *UAS*-Egr/+; puc^{E69}/*UAS-dUev1a-IR*
(E) *ptc*-Gal4/+; puc^{E69}/+
(F) *ptc*-Gal4 *UAS*-Egr/+; puc^{E69}/*UAS*-GFP
(G) *ptc*-Gal4 *UAS*-Egr/+; $dUev1a^{DG14805}$/puc^{E69}
(H) *ptc*-Gal4 *UAS*-Egr/+; puc^{E69}/*UAS-dUev1a-IR*
Fig. 6 (A) *GMR*-Gal4 *UAS*-Hep^{CA}/+
(B) *GMR*-Gal4 *UAS*-Hep^{CA}/*UAS-dUev1a-IR*
(C) *UAS*-Hep/+; *pnr*-Gal4/+
(D) *UAS*-Hep/+; *pnr*-Gal4/*UAS-dUev1a-IR*
(E) *sev* > dTAK1/+
(F) *sev* > dTAK1/*UAS-dUev1a-IR*
Fig. 7 (A) *ptc*-Gal4 *UAS*-Ben/+; puc^{E69}/+
(B) *ptc*-Gal4/+; puc^{E69}/*UAS*-dUev1a
(C) *ptc*-Gal4 *UAS*-Ben/+; puc^{E69}/*UAS*-dUev1a
(D) *ptc*-gal4 *UAS*-Hep/+; puc^{E69}/+
(E and E′) *ptc*-Gal4/*UAS*-Ben
(F and F′) *ptc*-Gal4/+; *UAS-dUev1a*/+
(G and G′) *ptc*-Gal4/*UAS*-Ben; *UAS*-dUev1a/+
(H and H′) *ptc*-gal4/*UAS*-Hep
(I, I′) *ptc*-Gal4/*UAS*-Ben; *UAS-dUev1a*/*UAS-dTRAF2-IR*
(J and J′) *ptc*-Gal4/*UAS*-Ben; *UAS*-dUev1a/*UAS-dTAK1-IR*
(K) *GMR*-Gal4/*UAS*-Ben; *UAS*-dUev1a/+
(L) *GMR*-Gal4/+; *UAS*-dTRAF2/+
(M) *GMR*-Gal4/*UAS*-Ben; *UAS*-dUev1a/*UAS*-dTRAF2

Immunohistochemistry

Antibody staining of imaginal discs was performed as previously described (Igaki et al., 2006). The following antibodies were used: rabbit anti-phospho-JNK (1:200, Calbiochem), mouse anti-MMP1 (1:100, Developmental Studies Hybridoma Bank). Secondary antibodies were anti-rabbit-Alexa (1:1000, Cell Signaling and Technology) and anti-mouse-Cy3 (1:500, Jackson ImmunoResearch).

X-gal staining. Eye and wing discs were dissected from 3rd instar larvae in PBST and stained for β galactosidase activity as described (Xue and Noll, 2000).

AO staining. Eye and wing discs were dissected from 3rd instar larvae in PBST and incubated in 1×10^{-5} M acridine orange for 5 min at room temperature prior to imaging.

qRT-PCR. Fifty freshly eclosed flies' heads or 20 pupaes were collected from indicated genotypes. Total RNA was isolated using TRIzol (Invitrogen), and RT-PCR was performed as previously described (Ma et al., 2012).

Primers for *d Uev 1 a* : Sense5′ – ATCGGTCCGCCTAGAACACC – 3′
Antisense 5′ – CATCTGGCCAACATCTGAACTG – 3′
Primers for *actin5c* : Sense 5′ – AAGTTGCTGCTCTGGTTGTC – 3′
Antisense 5′ – GGGTACTTCAGGGTGAGGATA – 3′

Results and discussion

dUev1a is required for JNK-mediated tumor growth and invasion

As previously described, expression of oncogenic Ras (Ras^{V12}) in *lgl* mutant cells in eye-antenna discs using the *eyFLP*/MARCM system resulted in tumor-like over-growths (Fig. 1A), with invasive migration into the ventral nerve cord (VNC) of the central nervous system (Fig. 1A′) (Igaki et al., 2006; Pagliarini and Xu, 2003; Uhlirova and Bohmann, 2006). The mutant animals kept growing

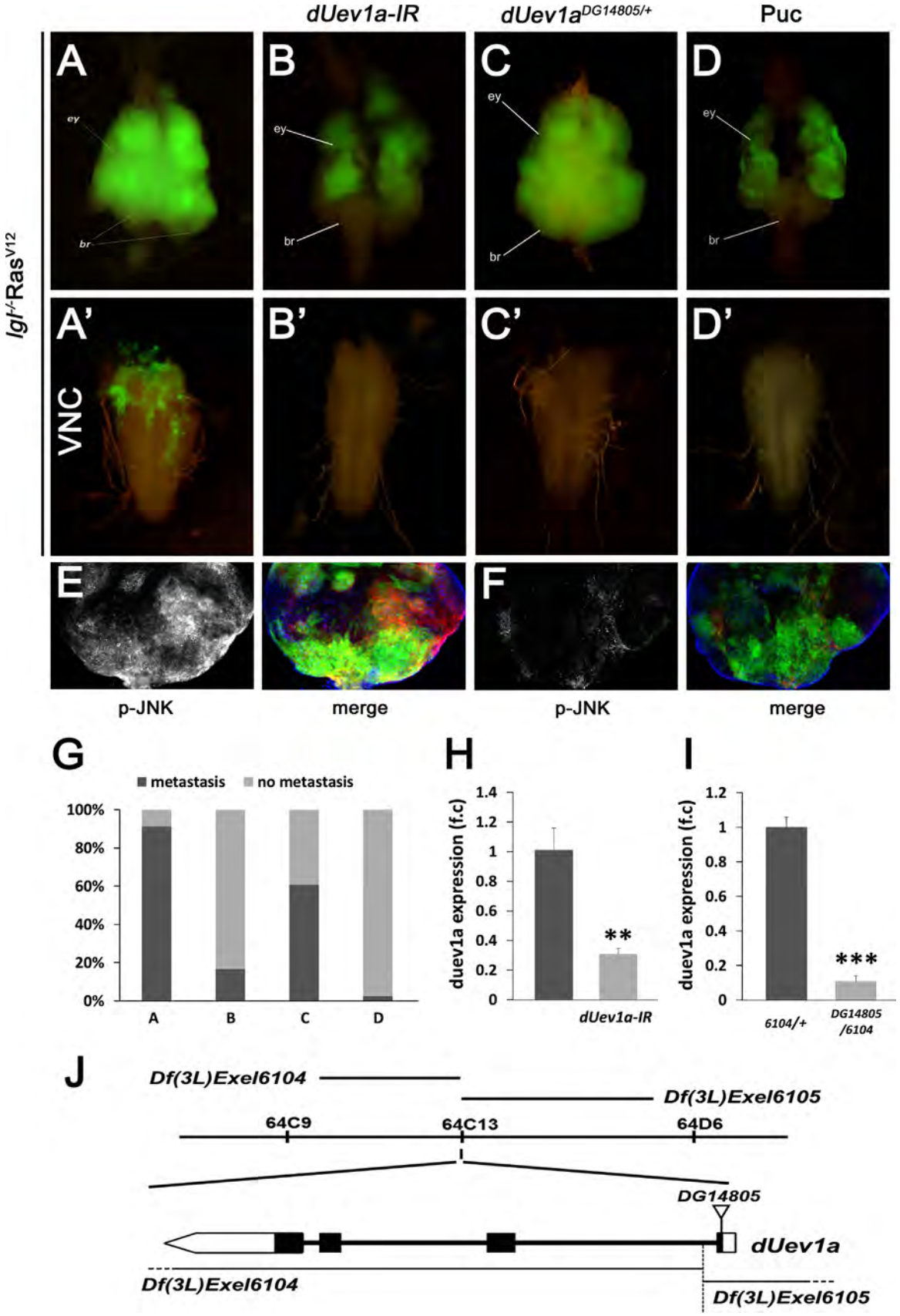

Fig. 1. dUev1a is required for *Ras–lgl* induced tumor progression and JNK activation. GFP-labeled clones of cells with indicated genotypes were created in the developing eye-antennal discs. $Ras^{V12}/lgl^{-/-}$ induced tumor growth (A, eye-antennal disc, ey, brain, br) and invasion to the VNC (A′) were strongly suppressed by expressing a *dUev1a RNAi* (B–B′) or Puc (D–D′), and partially suppressed by mutating one copy of endogenous *dUev1a* (C–C′). $Ras^{V12}/lgl^{-/-}$ induced JNK activation (E) were significantly suppressed by down regulation of *dUev1a* (F). (G) Quantification of larva showing invasion phenotype in A–D (A, 91% invasion penetrance, $n=68$; B, 12%, $n=45$; C, 62.5%, $n=32$; D, 2.5%, $n=40$). (H) Expression of a *dUev1a* RNAi driven by *GMR*-Gal4 significantly reduced *dUev1a* mRNA level in adult heads (right, *GMR*-Gal4/*UAS-dUev1a-IR*), as compared to that of control (left, *GMR*-Gal4/+). Values represent mean ± SEM. $P<0.01$, $n=50$. (I) Compared to the heterozygous *Df*(3L)*6014*/+ (left), *dUev1a* mRNA level is dramatically reduced in transheterozygous $dUev1a^{DG14805}/Df(3L)6014$ mutants (right). Values represent mean ± SEM. $P<0.001$, $n=20$. (J) A schematic depiction of the genomic region surrounding *dUev1a* locus. The intron–exon structure and the open reading frame (in black) of *dUev1a* are shown. The P-element *DG14805* and two deficiencies, *Df*(3L)*Exel6104* and *Df*(3L)*Exel6105*, are indicated.

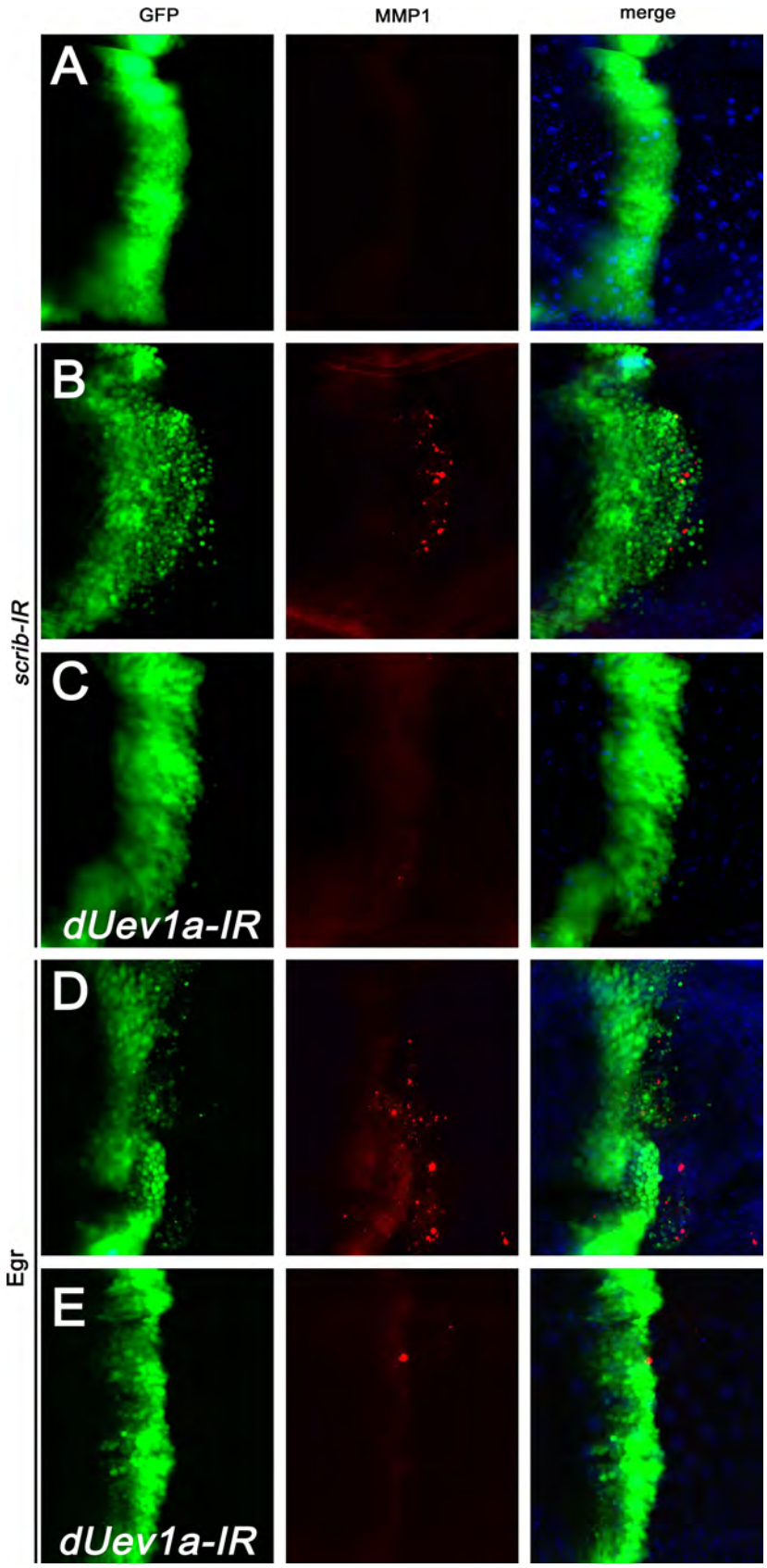

Fig. 2. dUev1a is required for JNK-mediated MMP1 expression and cell invasion. Fluorescence micrographs of wing discs are shown. Compare to the control (A), elevated MMP1 expression and cell invasion induced by down-regulation of *scrib* (B) or ectopic Egr expression (D) were suppressed by loss of *dUev1a* (C and E).

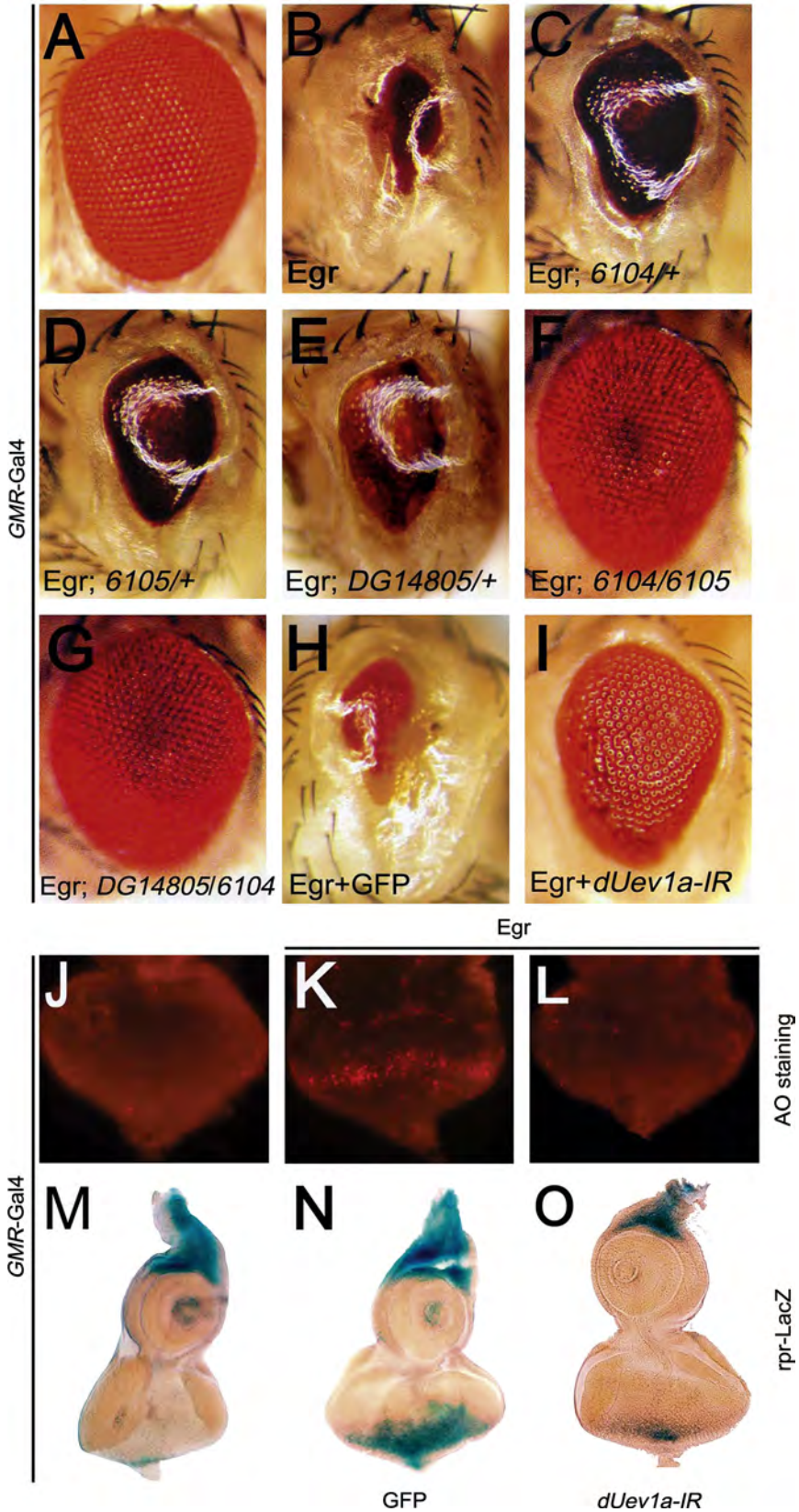

Fig. 3. dUev1a regulates Egr-induced cell death and *rpr* activation in developing eyes. (A–I) Light micrographs of *Drosophila* adult eyes are shown. Compared to the *GMR*-Gal4 control (A), the *GMR* > Egr small eye phenotype (B) is suppressed partially in heterozygous *Df*(3L)*Exel6104*/+ (C), *Df*(3L)*Exel6105*/+ (D) and $dUev1a^{DG14805}$/+ (E) flies, and fully suppressed in trans-heterozygous *Df*(3L)*Exel6104*/*Df*(3L)*Exel6105* (F) and $dUev1a^{DG14805}$/*Df*(3L)*Exel6104* (G) mutants. The *GMR* > Egr small eye phenotype remained unchanged by expressing an *UAS*-GFP transgene (H), but was partially suppressed by expressing an *UAS*-*dUev1a* RNAi (I). (J–L) *dUev1a* is required for Egr-induced cell death in developing eye. Compared to the *GMR*-Gal4 control (J), *GMR* > Egr-triggered cell death posterior to MF (K) was suppressed by the expression of a *dUev1a* RNAi (L), as indicated by acridine orange staining in 3rd instar larval eye discs. (M–O) dUev1a is required for Egr-induced *rpr* activation in developing eye. Compared to the *GMR*-Gal4 control 3rd instar larval eye disc (M), *GMR* > Egr induced strong *rpr* activation posterior to the MF (N), which was significantly inhibited by the expression of a *dUev1a* RNAi (O).

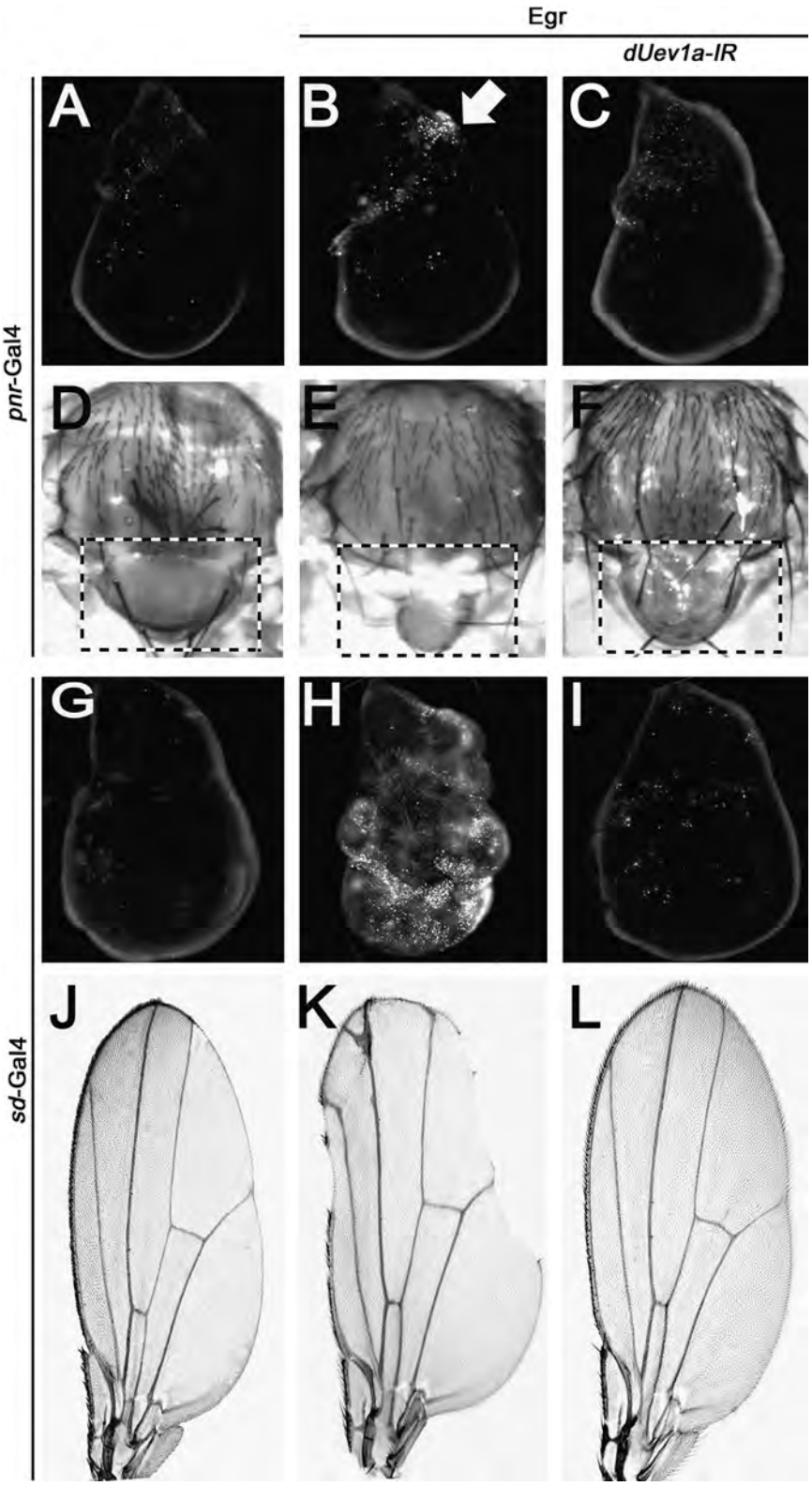

Fig. 4. dUev1a is required for Egr-induced cell death in thorax and wing. (A–F) dUev1a is required for Egr-induced cell death in developing thorax. Compared to the wild type control (A and D), expression of Egr under the control of *pnr* promoter triggered cell death in the notum area of 3rd instar larval wing discs (arrow in B) and produced a small scutellum phenotype in adults (E), both of which were suppressed by the expression of a *dUev1a* RNAi (C and F). (G–L) dUev1a is required for Egr-induced cell death in developing wing. Compared to the wild type control (G and J), ectopic Egr expression driven by *sd* promoter triggered extensive cell death in 3rd instar larval wing discs (H) and resulted in loss of wing margin tissue in adults (K), both phenotypes were suppressed by the expression of a *dUev1a* RNAi (I and L).

as oversized larvae with huge tumors in the head region and died before pupation (data not shown). The Egr-JNK signaling has been reported to play a pivotal role in modulating the tumor growth and invasion behavior induced by such oncogenic cooperation (Cordero et al., 2010; Igaki et al., 2006). Consistently, we found strong activation of JNK in $Ras^{V12}/lgl^{-/-}$ clones, as

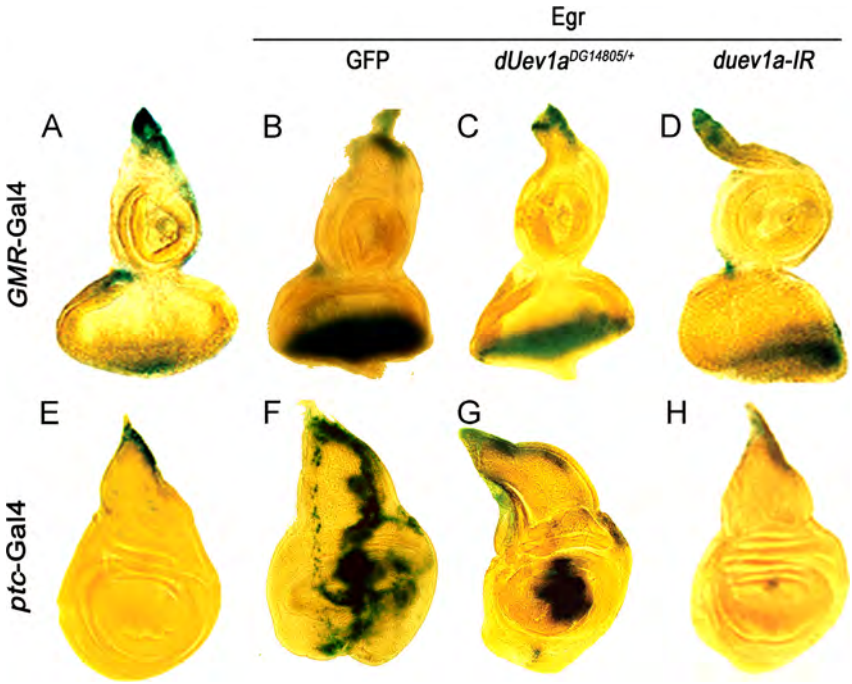

Fig. 5. dUev1a is required for Egr-induced JNK activation. (A–D) Egr-induced JNK activation in developing eye requires dUev1a activity. Compare to the *GMR*-Gal4 control (A), *GMR* > Egr induced strong *puc* expression posterior to MF (B) was suppressed by mutating one copy of endogenous *dUev1a* (C) or expressing a *dUev1a* RNAi (D). (F–J) Egr-induced JNK activation in developing wing requires dUev1a activity. Compare to the *ptc*-Gal4 control (E), *ptc* > Egr induced strong *puc* expression along the anterior posterior boundary of wing disc (F) was dramatically reduced by mutating one copy of endogenous *dUev1a* (G) or expressing a *dUev1a* RNAi (H).

detected by JNK phosphorylation (Fig. 1E), while inactivation of JNK signaling by expressing the JNK phosphatase Puc dramatically suppressed $Ras^{V12}/lgl^{-/-}$ tumor growth (Fig. 1D) and invasion (Fig. 1D′ and G), and rescued the animals to pupal stage (data not shown).

To identify additional genes essential for tumor progression, we performed a genetic screen for mutants and UAS-RNAi lines, and found that loss of *dUev1a* by expressing a RNAi, which significantly reduced mRNA level of *dUev1a* (Fig. 1H), dramatically suppressed $Ras^{V12}/lgl^{-/-}$ triggered tumor growth (Fig. 1B), invasion (Fig. 1B′ and G) and JNK activation (Fig. 1F), and rescued the mutant animals to pupal stage (data not shown). Consistent with this observation, $Ras^{V12}/lgl^{-/-}$ triggered tumor invasion was also suppressed in heterozygous $dUev1a^{DG14805}$/+ larva (Fig. 1C, C′ and G). $dUev1a^{DG14805}$ is likely a strong or null allele of *dUev1a*, since it has a P-element inserted 6 amino acids after the ATG translation start codon (Fig. 1J), which not only truncates most of the dUev1a protein, but also significantly reduces *dUev1a* transcription (Fig. 1I). In addition, $dUev1a^{DG14805}/Df$(3L)*Exel6104*, $dUev1a^{DG14805}/Df$(3L)*Exel6105* or *Df*(3L)*Exel6104*/*Df*(3L)*Exel6105* transheterozygous mutants died at late pupa stage, which could be rescued to adulthood by ubiquitous expression of dUev1a driven by a *tub*-Gal4 driver (data not shown). The two deficiencies are adjacent to each other, and the only gene disrupted in both deficiencies is *dUev1a* (Fig. 1J). Together, these data suggest that *dUev1a* encodes a crucial regulator of tumor growth and invasion, most likely through its modulation of JNK signaling.

dUev1a is required for JNK dependent MMP1 expression and cell invasion

The epithelia of the *Drosophila* larval wing imaginal disc have recently been utilized as another *in vivo* model to study cell invasion and metastasis (Vidal et al., 2006). RNAi down-regulation of cell polarity genes, *e.g. dlg*, in the wing disc resulted in JNK- dependent cell invasion (Cordero et al., 2010), which is partly mediated by transcriptional up-regulation of matrix metalloprotease 1 (MMP1) that is essential for basement membrane degradation (Beaucher et al., 2007; Deryugina and Quigley, 2006; Srivastava et al., 2007; Uhlirova and Bohmann, 2006). Consistently, down regulation of the cell polarity gene *scrib* under the *ptc* promoter along the anterior/posterior (A/P) compartment boundary (*ptc* > *scrib-IR*) produced a JNK-dependent invasion-like phenotype (Cordero et al., 2010), in which cells delaminated and migrated away toward the posterior part accompanied with increased expression of MMP1 (Fig. 2A and B). We found that loss of *dUev1a* significantly suppressed this epithelial migration phenotype as well as the up-regulation of MMP1 (Fig. 2C), indicating that *dUev1a* is required for JNK-dependent MMP1 expression and cell invasion induced by loss of cell polarity gene.

TNF is known for its evolutionarily conserved “tumor promoting” function in both *Drosophila* and mammals (Cordero et al., 2010; Moore et al., 1999). Consistently, ectopic expression of Egr driven by the *ptc* promoter resulted in cell invasion from the A/P boundary to the posterior compartment and up-regulation of MMP1 (Fig. 2D). We found that both phenotypes were significantly suppressed by down regulation of *dUev1a* (Fig. 2E). Based on the above results, we hypothesized that dUev1a modulates tumor progression and cell invasion through interfering the Egr-JNK signaling.

dUev1a is required for Egr-induced cell death in the developing eye

To further characterize the genetic interaction between dUev1a and Egr-JNK signaling, we examined the role of dUev1a in Egr-induced JNK-mediated cell death in the developing eye. Ectopic expression of Egr driven by *GMR*-Gal4 (*GMR* > Egr) triggers JNK-mediated cell death and produces a small eye phenotype (Fig. 3A and B) (Igaki et al., 2002; Moreno et al., 2002). We found that this small eye phenotype was partially suppressed in heterozygous *Df(3L)Exel6104*/+, *Df(3L)Exel6105*/+ or $dUev1a^{DG14805}$/+ animals (Fig. 3C–E), and was fully suppressed in transheterozygous *Df(3L)Exel6104/Df(3L)Exel6105* or $dUev1a^{DG14805}/Df$(3L)*Exel6104* mutants

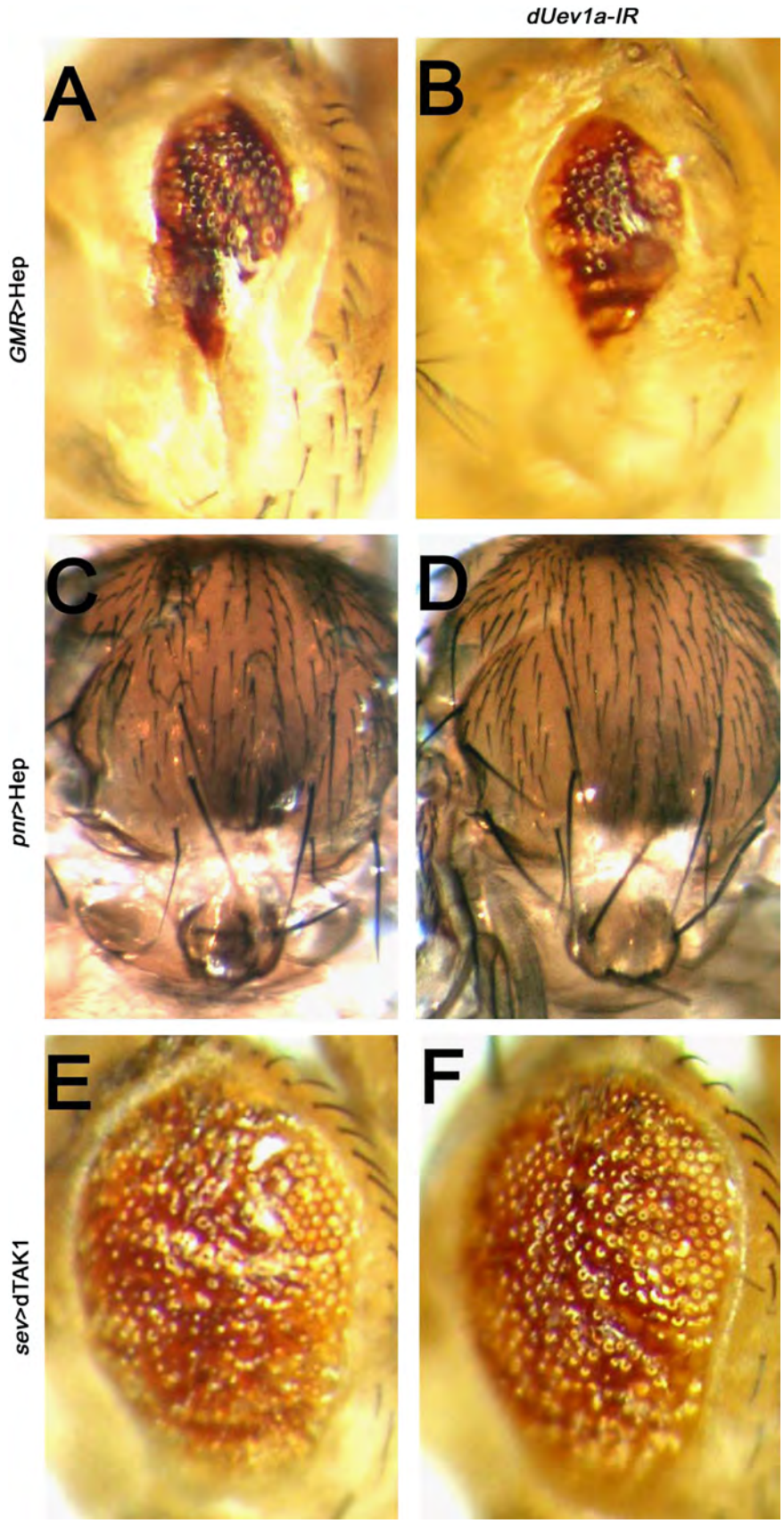

Fig. 6. dUev1a acts upstream of dTAK1 and Hep in Egr-JNK pathway. Light micrographs of *Drosophila* eyes (A, B, E, and F) or thorax (C and D) are shown. The small eye phenotype of *GMR* > Hep^{CA} (A), the small scutellum phenotype of *pnr* > Hep (C), or the rough eye of *sev* > dTAK1 (E) could not be suppressed by the expression of a *dUev1a* RNAi (B, D and F).

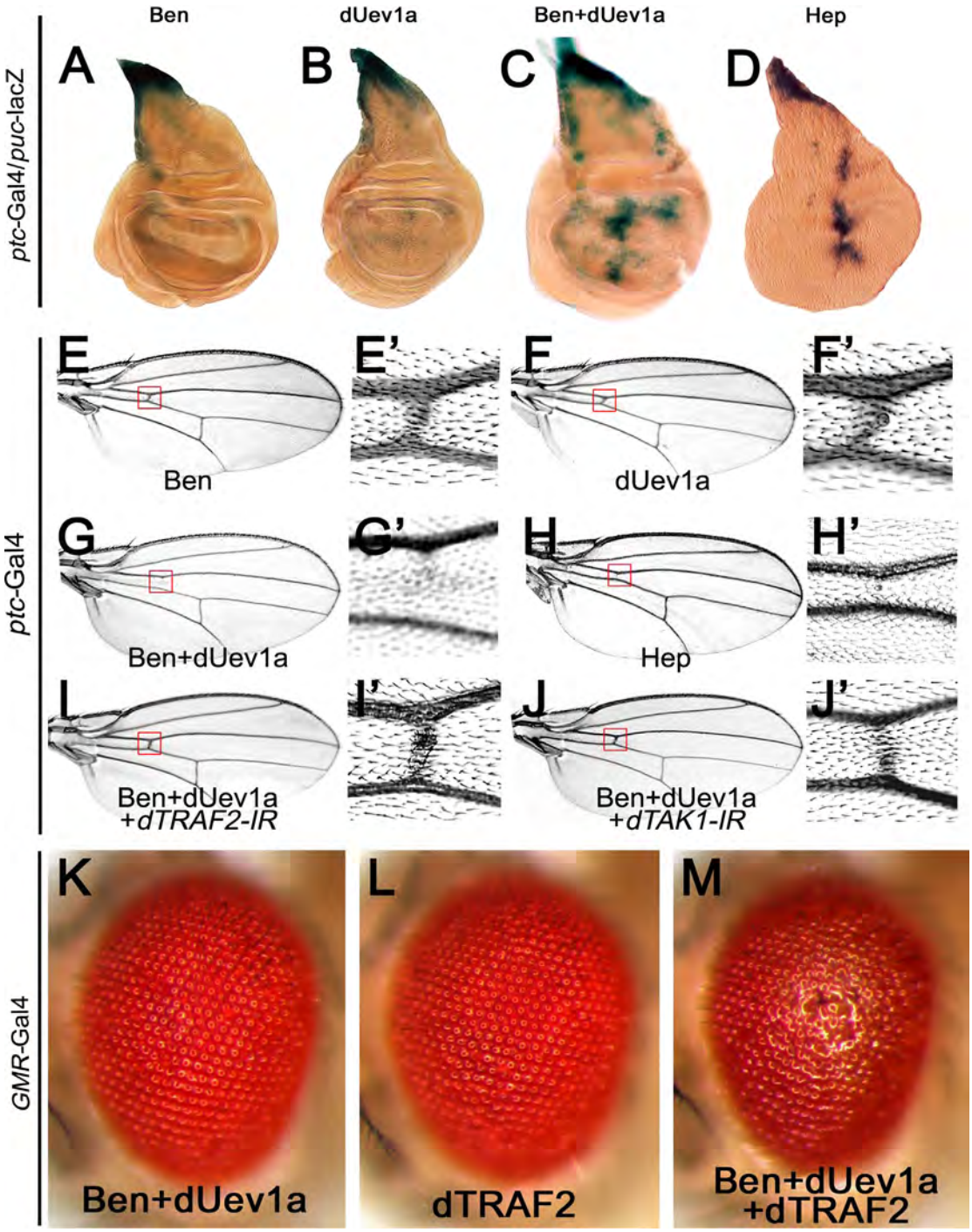

Fig. 7. dUev1a cooperates with Ben to activate JNK signaling. (A–D) Co-expression of dUev1a and Ben induced *puc* expression. Expression of either Ben (A) or dUev1a (B) along the A/P boundary under *ptc* promoter could not activate *puc* expression, while co-expression of Ben and dUev1a (C), or expression of Hep (D), induced *puc* expression as indicated by *puc*-LacZ staining. (E–J) Light micrographs of *Drosophila* adult wings are shown. Expression of either Ben (E and E′,) or dUev1a (F and F′) driven by *ptc*-Gal4 produced no obvious phenotype, while co-expression of Ben and dUev1a resulted in the loss of anterior cross vein (G and G′), which phenocopied that of JNK activation resulted from ectopic Hep expression (H and H′). This phenotype could be suppressed by the expression of a *dTRAF2* RNAi (I and I′) or a *dTAK1* RNAi (J and J′). (K–M) Light micrographs of *Drosophila* adult eyes are shown. Expression of Ben/dUev1a complex (K) or dTRAF2 (L) alone in developing eye produced no significant phenotype, while co-expression of Ben/dUev1a and dTRAF2 resulted in rough eyes with fused ommatidia (M).

(Fig. 3F and G), which died as pharate adults in the pupa case. Consistent with these results, the *GMR* > Egr eye phenotype was also suppressed by expressing a *UAS-dUev1a* RNAi transgene (Fig. 3I), but not a *UAS*-GFP (Fig. 3H). Together, these data indicate that *dUev1a* is absolutely required for *GMR* > Egr triggered small eye phenotype.

Previous study has shown that *GMR* > Egr triggers extensive cell death posterior to the morphogenetic furrow in 3rd instar larval eye discs (Igaki et al., 2002), as shown by acridine orange (AO) staining (Fig. 3J and K). We found that loss of *dUev1a* significantly reduces the number of AO positive cells (Fig. 3L), indicating that *dUev1a* is required for Egr-induced cell death in the

developing eye. Furthermore, we found that transcription of *reaper* (*rpr*), a proapoptotic gene in *Drosophila* (White et al., 1996), was activated by *GMR* > Egr posterior to the MF in 3rd instar larval eye discs (Fig. 3M and N), and such activation was dramatically reduced by down-regulation of *dUev1a* (Fig. 3O). Taken together, these data suggest that *dUev1a* is critically required for Egr-induced *rpr* activation and cell death in the developing eye.

dUev1a is required for Egr-induced cell death in developing Scutella and wing

To further investigate the role of dUev1a in Egr-induced cell death, we characterized the genetic interaction between dUev1a and Egr in other tissues. Ectopic expression of Egr driven by *pnr*-GAL4 triggered cell death in the notum area of 3rd instar larval wing discs (Fig. 4A and B), and generated a small scutellum phenotype (Fig. 4D and E) (Xue et al., 2007), both of which were strongly suppressed by the loss of *dUev1a* (Fig. 4C and F). Furthermore, Egr expression driven by *sd*-Gal4 (*sd* > Egr) resulted in extensive cell death in 3rd instar larval wing discs (Fig. 4G and H), and loss of wing margin tissue in adults (Fig. 4J and K). We found that both phenotypes were considerably suppressed by down-regulation of *dUev1a* (Fig. 4I and L). Together, these results demonstrate that dUev1a is an essential downstream mediator of Egr-induced cell death in development.

dUev1a is required for Egr-induced JNK activation

Previous studies have shown that Egr-triggered cell death is mediated by JNK activation (Igaki et al., 2002; Moreno et al., 2002), which could be monitored by the expression of *puc*, a readout of JNK signaling *in vivo* (Agnes et al., 1999; Xue et al., 2007). To determine the role of dUev1a in modulating Egr-triggered JNK activation *in vivo*, we checked whether *dUev1a* is required for Egr-induced *puc* expression in 3rd instar larval discs. Compared to the control eye (Fig. 5A) or wing disc (Fig. 5E), ectopic expression of Egr posterior to MF in the eye disc (*GMR* > Egr) or along the anterior/posterior compartment boundary in the wing disc (*ptc* > Egr) induces strong *puc* expression (Fig. 5B and F). We found such expression patterns were partially suppressed when one copy of endogenous *dUev1a* gene was mutated (Fig. 5C and G), and were significantly reduced by RNAi down-regulation of *dUev1a* (Fig. 5D and H), suggesting that *dUev1a* is required for Egr-induced JNK activation *in vivo*.

dUev1a acts upstream of dTAK1 and hep in the TNF–JNK signaling pathway

The fact that loss of *dUev1a* suppresses Egr-induced JNK activation and cell death suggests that dUev1a is a novel component of the TNF–JNK signaling pathway. To genetically map dUev1a in the Egr-JNK signaling pathway, we performed genetic epistasis analysis between dUev1a and dTAK1 or Hemipterous (Hep). Expression of a constitutive activated form of Hep, the *Drosophila* JNK kinase, in the developing eye (*GMR* > Hep^{CA}) induces JNK-mediated cell death and produces a small eye phenotype (Fig. 6A). This phenotype could not be suppressed by the loss of *dUev1a* (Fig. 6B). Consistently, the small scutellum phenotype resulted from ectopic Hep expression driven by *pnr*-Gal4 (*pnr* > Hep) (Fig. 6C) could not be suppressed by down regulation of *dUev1a* (Fig. 6D). Furthermore, expression of dTAK1, the *Drosophila* JNK kinase (Mihaly et al., 2001; Takatsu et al., 2000), in the eyes (*sev* > dTAK1) produced rough eyes with reduced size (Fig. 6E), which was not suppressed by the loss of *dUev1a* (Fig. 6F). Taken together, these results indicate that dUev1a acts downstream of Egr but upstream of dTAK1 and Hep in the Egr-JNK signaling pathway.

dUev1a and Bendless cooperate to activate JNK signaling

dUev1a has been reported to associate with Bendless (Ben), the *Drosophila* ortholog of Ubc13 (Oh et al., 1994), to form a heterodimer that functions as an E2 ubiquitin-conjugating enzyme complex (Merkle et al., 2009; Zhou et al., 2005). In mammalian cells, the Uev1/Ubc13 complex is required to promote Lys63 ubiquitination-mediated activation of TRAF6 in the IκB/NF-κB signaling pathway (Deng et al., 2000). Our previous work has suggested that dTRAF2 is required for the TNF–JNK signaling (Xue et al., 2007). Based on these previous results, we propose that *dUev1a* may form a functional complex with Ben to activate JNK signaling through the E3 ubiquitin ligase dTRAF2. Ectopic expression of Hep driven by *ptc*-Gal4 (*ptc* > Hep) induces JNK activation along the A/P boundary of 3rd instar larval wing discs (Fig. 7D), which resulted in the loss of anterior cross vein (ACV) in adult wings (Fig. 7H and H′). Consistent with our hypothesis, ectopic expression of Ben (*ptc* > Ben) or dUev1a (*ptc* > dUev1a) alone failed to induce JNK activation along the A/P boundary in wing discs (Figs. 7A and B) and loss of ACV in adult wings (Fig. 7E, E′, F, and F′), whereas co-expression of Ben and dUev1a activated JNK signaling (Fig. 7C) and generated the loss of ACV phenotype in adult wings (Figs. 7G and G′). Furthermore, the loss of ACV phenotype induced by ectopic Ben and *dUev1a* expression could be suppressed by loss of *dTRAF2* (Fig. 7I and I′) or *dTAK1* (Fig. 7J and J′). Finally, although expression of Ben/dUev1a complex (Fig. 7K) or dTRAF2 (Fig. 7L) alone in the developing eye failed to produce any obvious phenotypes, co-expression of these three proteins resulted in rough eyes with fused ommatidia (Fig. 7M). Together, these data confirmed that dUev1a and Ben form a functional E2 complex that cooperates with the E3 ligase dTRAF2 to modulate the Egr-JNK signaling pathway in *Drosophila*.

We have identified dUev1a as a crucial regulator of tumor progression and cell death in *Drosophila.* Our genetic epistasis analysis established dUev1a as a novel component of the TNF–JNK signaling pathway that collaborates with Ben to act upstream of dTRAF2. Interestingly, the dUev1a/Ben complex was also reported to regulate NF-κB signaling in *Drosophila* (Zhou et al., 2005). However, dTRAF2 is not a downstream mediator of dUev1a/Ben in this pathway (Zhou et al., 2005), and the *Drosophila* TRAF protein involved in dUev1a/Ben/NF-κB signaling has remained unidentified. Thus, dUev1a/Ben complex modulates NF-κB and JNK signaling through different TRAF proteins. Consistent with our data, mammalian Uev1 was also identified as a trans-activator of *c-fos*, a crucial downstream target of JNK activation (Xiao et al., 1998). In addition, Uev1 is often up-regulated in human tumor cells (Syed et al., 2006; Xiao et al., 1998), indicating a potential pro-oncogenic function of Uev1. Further investigation is needed to clarify the roles of Uev1 in regulating mammalian tumor growth and invasion.

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