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Bendless modulates JNK-mediated cell death and migration in *Drosophila*

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The TNF–JNK pathway is a highly conserved signaling pathway that regulates a wide spectrum of biological processes including cell death and migration. To further delineate this pathway, we carried out a genetic screen for dominant modifiers of the cell death phenotype triggered by ectopic expression of Eiger (Egr), the *Drosophila* TNF ortholog. Here we show that Bendless (Ben), an E2 ubiquitin-conjugating enzyme, modulates Egr-induced JNK activation and cell death through dTRAF2. Furthermore, Ben physically interacts with dTRAF2 and regulates Egr-induced dTRAF2 polyubiquitination. Finally, Ben is required for JNK-dependent tumor progression, cell migration, oxidative stress resistance and longevity. Our results indicate that Ben constitutes an essential component of the evolutionarily conserved TNF–JNK pathway that modulates cell death and invasion, tumor progression, stress response and lifespan in metazoans.
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Tumor necrosis factor (TNF) is an important cytokine that regulates a variety of cellular processes including proliferation, differentiation and survival, and has been implicated in the pathogenesis of many diseases including cancer, insulin resistance and autoimmune disorders.[1] The c-Jun N-terminal kinase (JNK) is a key downstream mediator that, upon activation through the MAPK cascade, translocates to the nucleus where it phosphorylates and activates the transcription factors including AP-1.[2] This signaling pathway has been conserved in *Drosophila melanogaster*, in which the TNF ortholog Eiger (Egr) triggers the activation of Basket (Bsk), the *Drosophila* JNK, through the JNK kinase kinase dTAK1 and the JNK kinase Hemipterous (Hep).[3,4]

The Egr–JNK signaling pathway was originally reported as a crucial regulator of cell death in *Drosophila* development.[3,4] Further studies suggested that JNK signaling also has a vital role in modulating stress resistance and tumor longevity.[5,6] Recently, JNK signaling was shown to be upregulated by the loss of tumor suppressor genes, for example, *scrib* and *csk*, and be critically required for $Ras^{V12}/scrib^{-/-}$-triggered tumor growth and metastasis, as well as loss of *scrib* or *csk* induced invasive cell migration.[7–13]

Drosophila genetics has provided a powerful tool for dissecting conserved signaling pathways and their roles in development. Several novel components of the Egr–JNK signaling pathway, including Wengen (Wgn) and dTAB2, the *Drosophila* ortholog of TNFR and TAB2/3, respectively, have been isolated as dominant suppressor of Egr-triggered cell death.[14,15] Our previous work has shown that the TNF receptor-associated factor dTRAF2, which encodes an E3 ubiquitin ligase, regulates JNK-mediated tumor progression, cell death, oxidative stress resistance and longevity in *Drosophila*.[6,7] However, the E2 ubiquitin-conjugating enzyme that modulates dTRAF2 activity in this pathway has remained unknown.

bendless (*ben*) encodes an E2 ubiquitin-conjugating enzyme that has important roles in escape response,[16] axon guidance,[17,18] synaptic growth and maturation,[19] long-term memory,[20] innate immunity[21] and genomic integrity.[22] In this report, we performed a genetic screen and identified Ben as a novel component of the Egr–JNK pathway. Our genetic and biochemical analysis further indicated that Ben acts as the E2 enzyme for dTRAF2 that regulates JNK signaling-mediated tumor growth and invasion, cell death, oxidative stress resistance and longevity in *Drosophila*.

Results and Discussion

Loss of *ben* suppressed ectopic egr-induced cell death. To identify additional components of the TNF–JNK signaling pathway in *Drosophila*, we performed a deficiency screen to search for dominant modifiers of the *GMR*>Egr small eye phenotype (Figure 1b)[23,24] that results from ectopic Egr-induced JNK-mediated cell death.[3,4] Deletions

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Abbreviations: TNF, tumor necrosis factor; JNK, c-Jun N-terminal Kinase; MAPK, mitogen-activated protein kinases; GMR, glass multiple reporter; MF, morphogenetic furrow; MARCM, mosaic analysis with a repressible cell marker; ey, eyeless; VNC, ventral nerve cord; MMP1, matrix metalloproteinase 1; A/P, anterior/posterior
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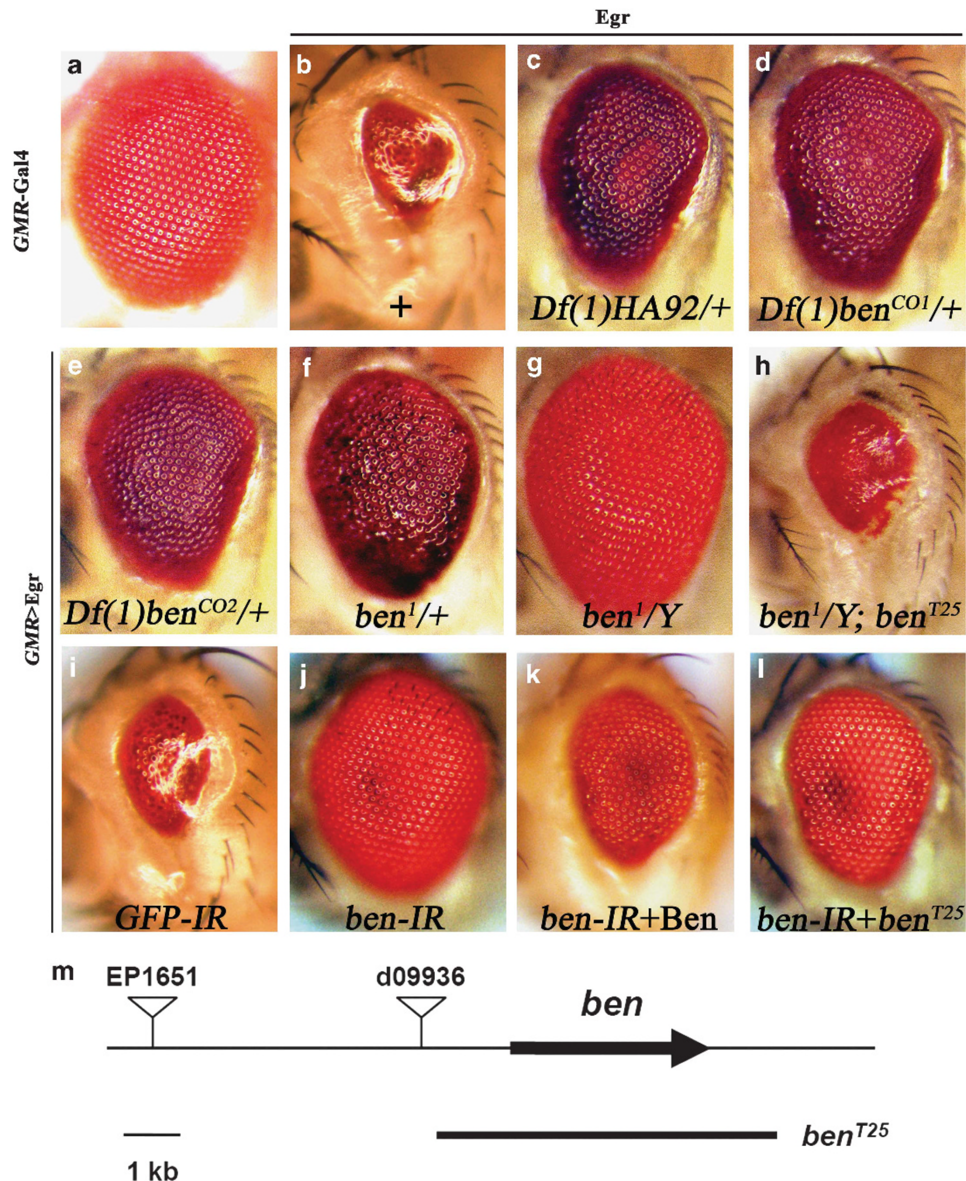

Figure 1 Ben regulates Egr-induced cell death in *Drosophila*. (**a**–**l**) Light micrographs of *Drosophila* adult eyes are shown. Compared with the controls (**a**, *GMR*-Gal4/+), *GMR*>Egr triggers cell death and produces a small eye phenotype (**b**, *UAS*-Egr/+; *GMR*-Gal4/+). This phenotype is suppressed partially by *Df(1)HA92* (**c**, *Df(1)HA92/X*; *UAS*-Egr/+; *GMR*-Gal4/+), *Df(1)ben^{CO1}* (**d**, *Df(1)ben^{CO1}/X*; *UAS*-Egr/+; *GMR*-Gal4/+) or *Df(1)ben^{CO2}* (**e**, *Df(1)ben^{CO2}/X*; *UAS*-Egr/+; *GMR*-Gal4/+), or heterozygosity of *ben^1* (**f**, *ben^1/X*; *UAS*-Egr/+; *GMR*-Gal4/+), and fully by hemizygosity of *ben^1* (**g**, *ben^1/Y*; *UAS*-Egr/+; *GMR*-Gal4/+) or expression of a *ben* RNAi (**j**, *UAS*-Egr/+; *GMR*-Gal4/*UAS-ben-IR*) but not a GFP RNAi (**i**, *UAS*-Egr/*UAS-GFP-IR*; *GMR*-Gal4/+). The suppression of *GMR*>Egr eye phenotype can be reversed by *ben^{T25}* (**h**, *ben^1/Y*; *UAS*-Egr/*ben^{T25}*; *GMR*-Gal4/*ben^{T25}*; **l**, *UAS*-Egr/*ben^{T25}*; *GMR*-Gal4/*UAS-ben-IR*) or expression of Ben (**k**, *UAS*-Egr/*UAS*-Ben; *GMR*-Gal4/*UAS-ben-IR*). (**m**) The *ben* gene, *ben^{T25}* transgene and two P-elements, EP1651 and d09936, used for ectopic Ben expression are indicated

uncovering the canonical Egr–JNK pathway components, for example, *wengen* (*wgn*, encoding an Egr receptor),[14] *dTRAF2*,[6] *dTAK1*,[25,26] *hep*[27] and *basket* (*bsk*, encoding the *Drosophila* JNK)[28] were isolated as suppressors in our screen, indicating that the screen was effective. One of the suppressors was mapped cytologically between 12C6 and 12D3, a region uncovered by three deficiencies, *Df(1)HA92*, *Df(1)ben^{CO1}* and *Df(1)ben^{CO2}*, that partially suppressed the *GMR*>Egr phenotype (Figures 1c–e). This region contains multiple genes including *ben*, which is located in 12D2, as predicted by the *Drosophila* genome project.[29] Further experiments showed that the *GMR*>Egr eye phenotype was partially suppressed in heterozygous *ben^1*/+ females (Figure 1f) and fully suppressed in hemizygous *ben^1*/Y males (Figure 1g), which could be reverted by *ben^{T25}* (Figure 1h), a genomic rescue fragment for *ben* (Figure 1m).[18] Furthermore, expression of a *ben* RNAi[30,31] but not a *GFP* RNAi strongly suppressed the *GMR*>Egr eye phenotype (Figures 1i and j). The suppression by *ben* RNAi was partially released by *ben^{T25}* (Figure 1l), or expression of Ben (Figure 1k), confirming the efficacy and specificity of the *ben* RNAi. Together, these results indicate that Ben is absolutely required for ectopic Egr-induced cell death in eye development.

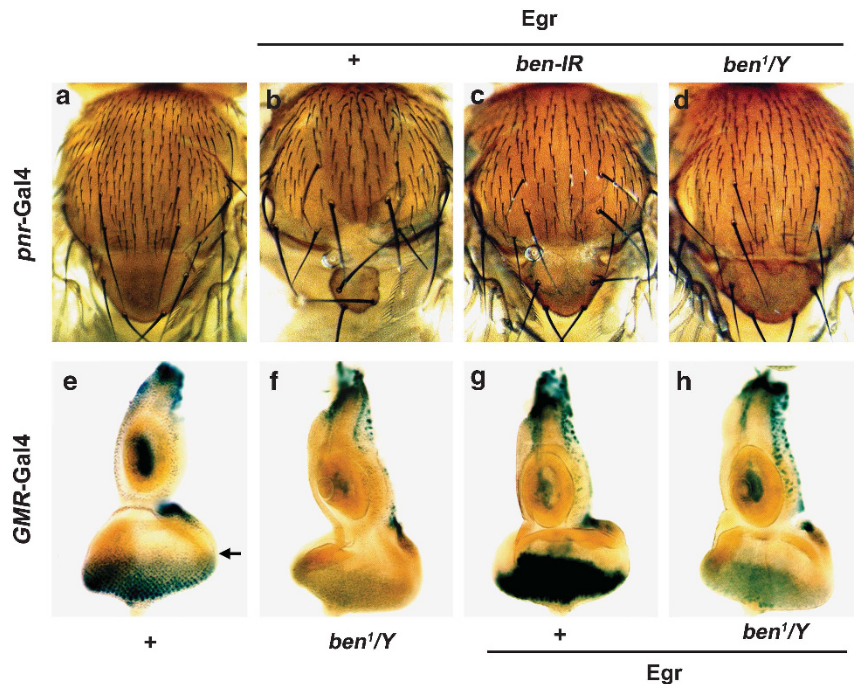

Figure 2 *ben* is required for endogenous JNK signaling. (**a**–**d**) Light micrographs of *Drosophila* adult thorax are shown. Compared with wild type (**a**, *pnr*-Gal4/ +), expression of Egr under the control of *pnr* promoter produces a small scutellum phenotype (**b**, *UAS*-Egr/ + ; *pnr*-Gal4/ +). The *pnr*> *Egr* phenotype is suppressed strongly by a *ben* RNAi (**c**, *UAS*-Egr/ + ; *pnr*-Gal4/*UAS*-*ben*-*IR*) and fully in *ben* mutants (**d**, ben^1/Y; *UAS*-Egr/ + ; *pnr-Gal4*/ +). (**e**–**h**) X-Gal staining of 3rd instar larval eye discs is shown. The weak endogenous *puc* expression is detected after extensive staining (**e**, *GMR*-Gal4/puc^{E69}). The *puc* expression posterior to the MF (arrow) is significantly reduced in *ben* mutants (**f**, ben^1/Y; *GMR*-Gal4/puc^{E69}), whereas the *puc* expression in disc edge are not affected. *GMR*> Egr induces *puc* expression posterior to the morphogenetic furrow (**g**, *UAS*-Egr/ + ; *GMR*-Gal4/puc^{E69}), which is strongly suppressed in *ben* mutants (**h**, ben^1/Y; *UAS*-Egr/ + ; *GMR*-Gal4/puc^{E69}). Staining was performed at 37 °C for 12 h (**e** and **f**) or at room temperature for 2 h (**g** and **h**)

To examine the role of Ben in Egr-induced cell death in another developmental context, we characterized the genetic interaction between Ben and Egr in the developing thorax. Ectopic Egr expression in the dorsal thorax driven by *pannier*–Gal4 (*pnr*–Gal4) induced JNK-dependent cell death and produced a small scutellum phenotype (Figure 2b).[3,24] This phenotype was suppressed by RNAi downregulation (Figure 2c) or mutation (Figure 2d) of *ben*, suggesting Ben is generally required for ectopic Egr-induced cell death in *Drosophila* development.

***ben* is required for endogenous JNK signaling.** To investigate the physiological role of *ben* in modulating JNK signaling, we examined *puc*-LacZ expression, an *in vivo* read-out of JNK activity,[32] in third-instar eye discs. Previous study has shown that *puc* expression posterior to the morphogenetic furrow (MF, arrow in Figure 2e) depends on endogenous Egr function.[3] We found that such a expression pattern was significantly reduced in *ben* mutants (Figure 2f). In contrast, *puc* expression in the disc margin, which is independent of the Egr signaling,[3] remained unaffected (Figures 2e and f). Besides, *GMR*>Egr sturdily activated *puc* transcription posterior to the MF (Figure 2g),[3] which was considerably suppressed by loss of *ben* (Figure 2h). Together, these data suggest that Ben is physiologically required for both endogenous and ectopic Egr-induced JNK activation.

Ben regulates JNK signaling through dTRAF2. To genetically map Ben in the Egr–JNK pathway, we examined the genetic interaction between Ben and dTAK1 or Hep in the developing eyes. Expression of dTAK1 or a constitutive active form of Hep (Hep^{CA}) under the control of the *sevenless* (*sev*) promoter (*sev*>dTAK1 and *sev*>Hep^{CA}) trigged extensive cell death in larval eye discs and produced rough eyes with reduced sizes (Figures 3a and c).[6,25,26] These phenotypes were suppressed by reducing *bsk* expression[23] but remained unaffected in *ben* mutants (Figures 3b and d), suggesting that Ben acts upstream of dTAK1 and Hep in the Egr–JNK signaling pathway.

Consistent with its role in regulating JNK signaling, ectopic expression of Ben in developing eyes produced small and rough eyes (Figure 3e), which were suppressed by loss of *dTRAF2* (Figure 3g) or *dTAK1* (Figure 3h) but not that of *wgn* (Figure 3f). Furthermore, Ben expression in the dorsal thorax driven by *pnr-GAL4* resulted in a small scutellum phenotype (Figure 3i), which was also suppressed by loss of *dTRAF2* (Figure 3k) or *dTAK1* (Figure 3l) but not that of *wgn* (Figure 3j). Collectively, these data indicate that Ben acts upstream of dTRAF2 and downstream of Wgn in modulating JNK signaling.

Ben physically binds dTRAF2 and promotes dTRAF2 polyubiquitination. Ben encodes an E2 ubiquitin-conjugating enzyme that has been conserved from yeast to mammals.[33,34] Our genetic data suggested that Ben acts upstream of dTRAF2, which encodes a RING finger-containing E3 ubiquitin-ligating enzyme. To test whether Ben could physically interact with dTRAF2, we examined the

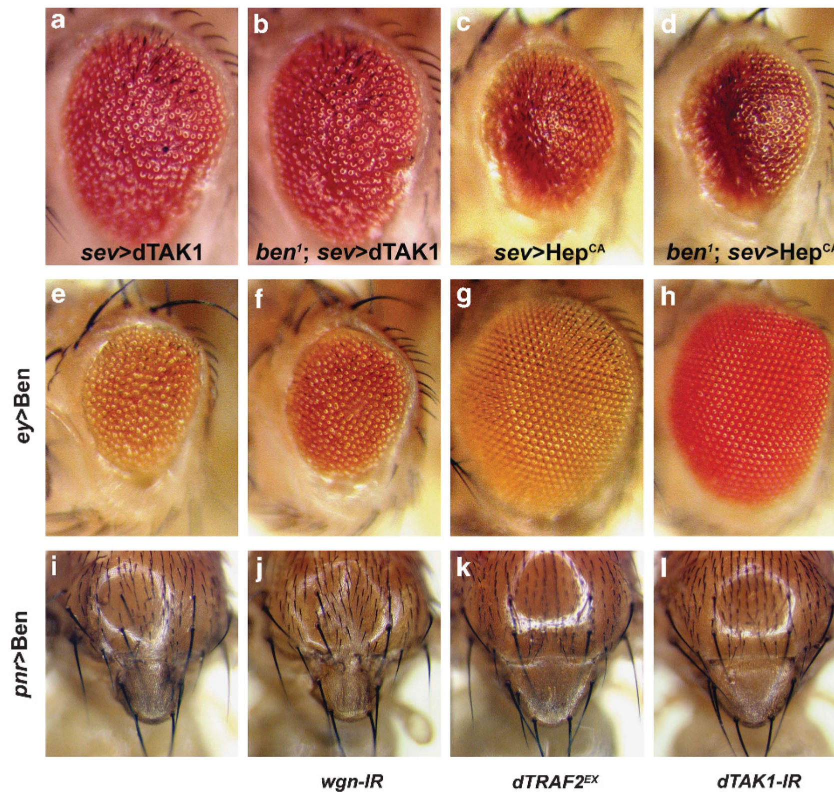

Figure 3 Ben regulates JNK signaling through dTRAF2. Genetic epistatic analysis of Ben in Egr-JNK pathway. (**a**–**h**) Light micrographs of *Drosophila* adult eyes are shown. Expression of dTAK1 (**a**, *sev*-Gal4 *UAS*-dTAK1/+) or a constitutive active form of Hep (**c**, *sev*-Gal4 *UAS*-Hep^{CA}/+) in developing eyes under the control of *sev* promoter induces apoptosis and generates rough eyes with reduced size. These phenotypes are not suppressed in *ben* mutants (**b**, *ben^{1}*; *sev*-Gal4 *UAS*-dTAK1/+ and **d**, *ben^{1}*; *sev*-Gal4 *UAS*-Hep^{CA}/+). Expression of Ben in developing eyes under the control of *ey* promoter induces apoptosis and generates rough eyes with reduced size (**e**, *ben^{d09936}*/Y; *ey*-Gal4/+). This phenotype is suppressed in *dTRAF2* mutants (**g**, *dTRAF2^{EX1} ben^{d09936}*/Y; *ey*-Gal4/+) or by the expression of a *dTAK1* RNAi (**h**, *ben^{d09936}*/Y; *ey*-Gal4/*UAS-dTAK1-IR*) but not by that of *wgn* (**f**, *ben^{d09936}*/Y; *ey*-Gal4/*UAS-Wgn-IR*). (**i**–**l**) Light micrographs of *Drosophila* adult thorax are shown. Expression of Ben under the control of *pnr* promoter produces a small scutellum phenotype (**i**, *ben^{EP1651}*/Y; *pnr*-Gal4/+). The *pnr*> *Ben* phenotype is suppressed in *dTRAF2* mutants (**k**, *dTRAF2^{EX1} ben^{EP1651}*/Y; *pnr*-Gal4/+) or by a *dTAK1* RNAi (**l**, *ben^{EP1651}*/Y; *pnr*-Gal4/*UAS-dTAK1-IR*) but not by that of *wgn* (**j**, *ben^{EP1651}*/Y; *pnr*-Gal4/*UAS-wgn-IR*)

subcellular localization of Ben and dTRAF2 in the developing eyes. Immunostaining of third-instar eye discs showed colocalization of Ben and dTRAF2 on the cell membrane posterior to MF (Figures 4a–f). In addition, co-immunoprecipitation experiment confirmed that ectopically expressed Flag-dTRAF2 could physically interact with endogenous (Figure 4g, lane 2) or ectopically expressed Ben (Figure 4g, lane 3) *in vivo*. As a negative control, the endogenous Ben protein could not be precipitated by anti-Flag M2 affinity gel in the absence of Flag-dTRAF2 (Figure 4g, lane 1). Parkin (Park) encodes another RING finger-containing E3 ubiquitin-ligating enzyme. We found that Flag-tagged Park (Park-Flag) failed to precipitated endogenous (Figure 4h, lane 2) or ectopically expressed Ben (Figure 4h, lane 3), further confirming that the physical interaction between Ben and dTRAF2 is specific. Previous studies have shown that TNF signaling induces K63-linked polyubiquitination of TRAF2/6.[35] This polyubiquitination does not target TRAF proteins for proteasome-dependent degradation, but instead serves as a signal to activate downstream pathway.[36] In agreement with the mammalian data, we found that ectopic Egr expression stimulated dTRAF2 polyubiquitination (Figure 4i, lane 3, upper) but did not affect the stability of dTRAF2 protein (Figure 4i, lane 3, middle). Loss of Ben blocked Egr-induced polyubiquitination of dTRAF2 (Figure 4i, lane 4), whereas ectopic Ben expression induced dTRAF2 polyubiquitination without affecting its protein stability (Figure 4i, land 5). Thus, Ben is both necessary and sufficient for Egr-induced polyubiquitination of dTRAF2.

***ben* regulates JNK-dependent oxidative stress resistance and lifespan.** The JNK signaling pathway has been implicated in regulating the oxidative stress resistance and normal lifespan in *Drosophila*.[5,6] Consistent with its role in modulating JNK signaling, *ben* mutants displayed a significantly compromised resistance against treatment of paraquat (Figure 5a), an herbicide that induces the formation of reactive oxygen species,[37] and shortened mean and maximal lifespan (Figure 5b). Both defects were rescued by *ben^{T25}* (Figures 5a and b), confirming that loss of *ben* function is responsible for the reduced oxidative stress resistance and shortened lifespan in *ben* mutants. We found that expression of the JNK kinase Hep in the nervous system (*elav*>Hep) fully rescued the compromised oxidative stress tolerance defect and shortened lifespan in *ben* mutants (Figures 5a and b), suggesting that Ben modulates these physiological functions through JNK signaling.

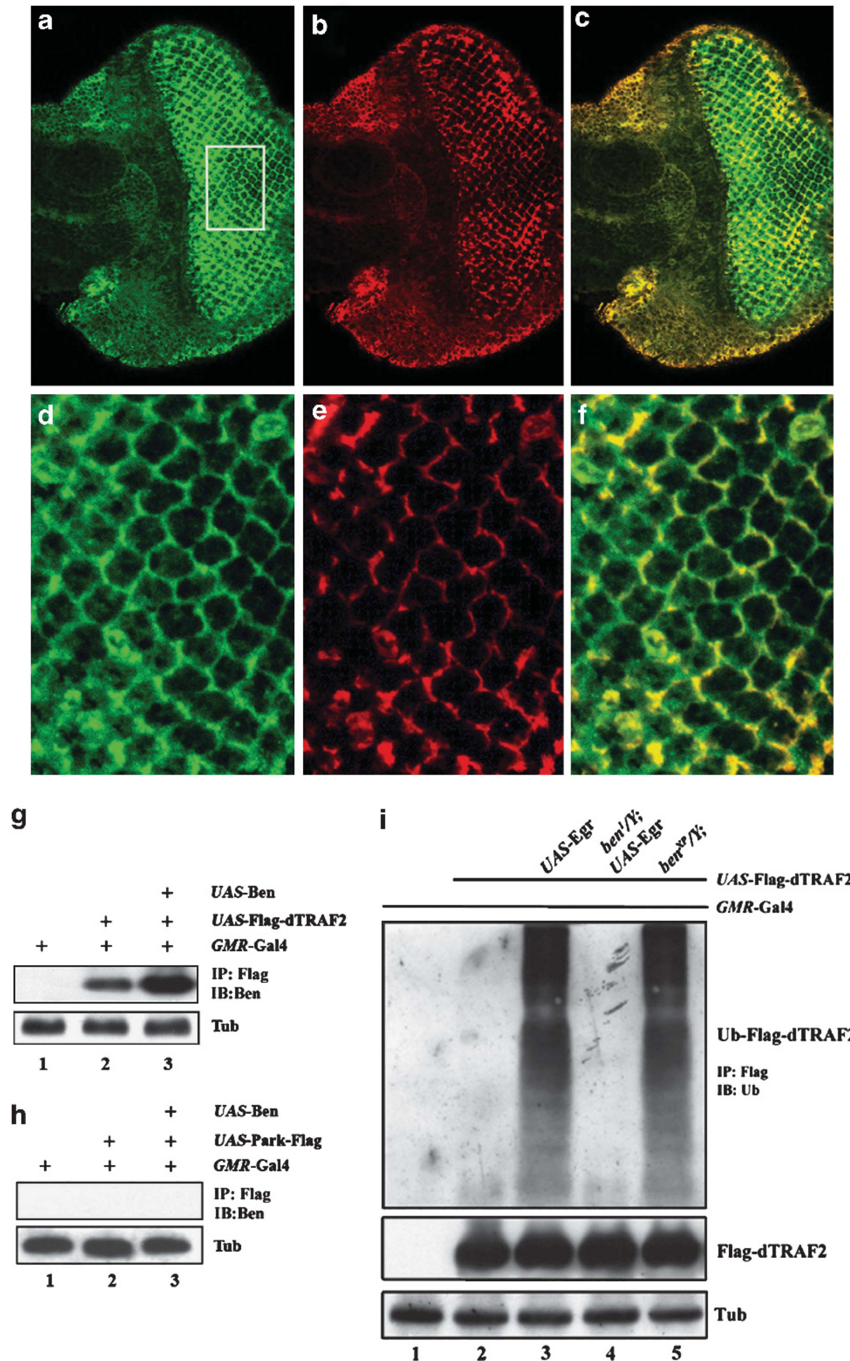

Figure 4 Ben binds to dTRAF2 and regulates dTRAF2 polyubiquitination. (**a**–**f**) Confocal images of *Drosophila* 3rd instar eye discs are shown. Ben (**a** and **d**) and dTRAF2 (**b** and **e**) co-localized to the cell membrane. (**c** and **f**) are merged pictures. (**d**–**f**) are high magnification of **a**–**c** (boxed area in **a**). (**g**) Ben physically interacts with Flag-dTRAF2 *in vivo*. Endogenous (lane 2, *GMR*-Gal4 *UAS*-Flag-dTRAF2/+) and ectopically expressed (lane 3, *UAS*-Ben; *GMR*-Gal4 *UAS*-Flag-dTRAF2/+) Ben binds to Flag-dTRAF2 driven by *GMR*-Gal4 in immunoprecipitation assay. *GMR*-Gal4 alone served as the negative control (lane 1, *GMR*-Gal4/+). IP, anti-Flag; IB detection, anti-Ben. β-tubulin served as a loading control (bottom). (**h**) Ben fails to interact with Park-Flag *in vivo*. Endogenous (lane 2, *GMR*-Gal4 *UAS*-Park-Flag/+) and ectopically expressed (lane 3, *UAS*-Ben; *GMR*-Gal4 *UAS*-Park-Flag/+) Ben does not bind to Park-Flag driven by *GMR*-Gal4 in immunoprecipitation assay. *GMR*-Gal4 alone served as the negative control (lane1, *GMR*-Gal4/+). IP, anti-Flag; IB detection, anti-Ben. β-tubulin served as a loading control (bottom). (**i**). The expression (middle) and polyubiquitination of Flag-dTRAF2 driven by *GMR*-Gal4. While dTRAF2 protein level remains constant (lane 2–5), the weak polyubiquitination of dTRAF2 (lane 2, *GMR*-Gal4 *UAS*-Flag-dTRAF2/+) is significantly enhanced by the expression of Egr (lane 3, *UAS-Egr*/+; *GMR*-Gal4 *UAS*-Flag-dTRAF2/+) or Ben (lane 5, ben^{d09936}; *GMR*-Gal4 *UAS*-Flag-dTRAF2/+). Loss of *ben* blocks the Egr- induced dTRAF2 polyubiquitination (lane 4, ben^{1}; *UAS*-Egr/+; *GMR*-Gal4 *UAS*-Flag-dTRAF2/+). *GMR*-Gal4 alone serves as a negative control (lane 1, *GMR*-Gal4/+). β-tubulin was used as a loading control (bottom). Data are representative of three independent experiments

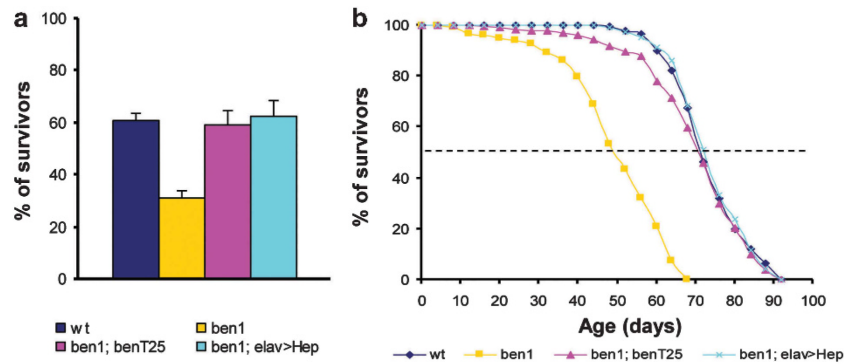

Figure 5 *ben* regulates JNK-dependent oxidative stress resistance and lifespan in *Drosophila*. (**a**) Loss of *ben* compromises oxidative stress resistance. Three-day-old flies were fed with 15 mM paraquat in 5% sucrose for 24 h, and their survival rates were measured. *ben* mutant males (ben1: w^{1118} ben^1/Y) showed significant reduction in survival rate as compared with wild-type males (wt: w^{1118}/Y). This reduction was fully rescued by ben^{T25} (ben1; benT25: w^{1118} ben^1/Y; ben^{T25}) or neural expression of Hep (ben1; elav > Hep: w^{1118} ben^1/Y; *UAS*-Hep^{WT}/ + ; *elav*-Gal4/ +). (**b**) Loss of *ben* reduces lifespan. *ben* mutant males show significant reduction of median and maximal lifespan as compared with wild-type males. This reduction could be rescued by ben^{T25} or neural expression of Hep. Survival of three independent cohorts was monitored over time. Cohort sizes and *P*-values are as follows: wt, $n = 197$; ben1, $n = 230$; ben1; benT25, $n = 295$; ben1; elav > Hep, $n = 260$; $P < 0.01$

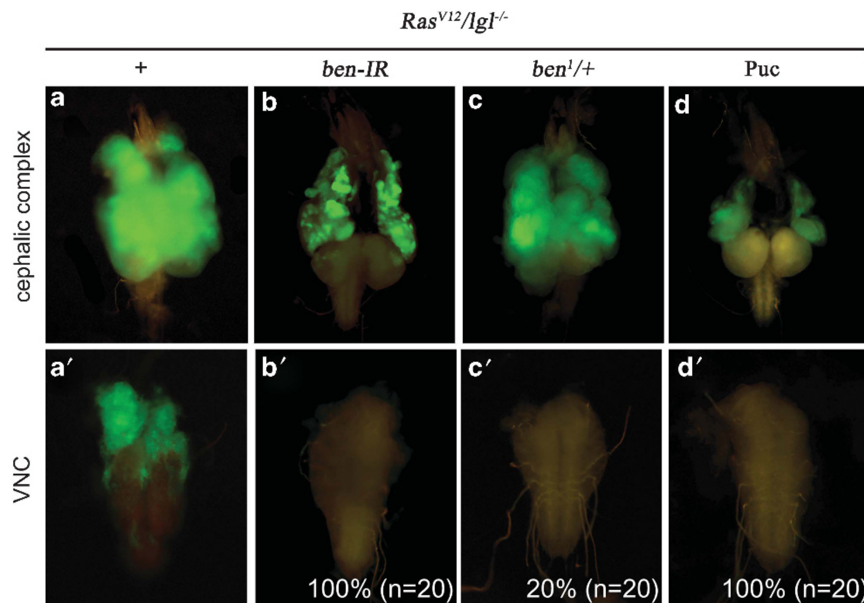

Figure 6 *ben* is required for *Ras-lgl* induced tumor growth and invasion. GFP-labeled clones of cells with indicated genotypes were created in developing eye-antennal discs. $Ras^{V12}/lgl^{-/-}$ induced tumor growth (**a**) and invasion to the VNC (**a**′) were suppressed by loss of *ben* (**b** and **c**) or expressing of Puc (**d**). (**a**) *y,w*, *ey*-Flp/ + ; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/ + . (**b**) *y,w*, *ey*-Flp/ + ; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/*UAS-ben-IR*. (**c**) *y,w*, *ey*-Flp/ben^1; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/ + . (**d**) *y,w*, *ey*-Flp/ + ; *tub*-Gal80, FRT40A/lgl^4 FRT40A *UAS*-Ras^{V12}; *Act* > y^+ > Gal4, *UAS*-GFP/*UAS*-Puc

***ben* is required for *Ras-lgl*-induced tumor growth and invasion.** As previously reported, expression of oncogenic *Ras* in *lgl* mutant cells ($Ras^{V12}/lgl^{-/-}$) in eye-antennal discs using the *ey-FLP*/MARCM system resulted in tumor-like growth (Figure 6a) with invasive migration into the ventral nerve cord (VNC) of the central nerve system (Figure 6a′).[7,9,13,38] The Egr–JNK signaling has been implicated as an important mediator of this oncogenic cooperation-induced tumor progression.[7,8] Consistently, we found that inactivation of JNK signaling by expression of the JNK phosphatase Puc could dramatically suppress the growth and invasion behavior of $Ras^{V12}/lgl^{-/-}$ tumors (Figures 6d–d′).

To investigate whether *ben* is also involved in $Ras^{V12}/lgl^{-/-}$-triggered tumor progression, we knocked down *ben* expression in tumor cells by expressing the *ben* RNAi or introduced the $Ras^{V12}/lgl^{-/-}$ tumor into heterozygous ben^1 mutants. We found that RNAi downregulation of *ben* phenocopied inactivation of JNK signaling, in which both tumor growth (Figure 6b) and invasion to the VNC (Figure 6b′) were significantly suppressed. Consistent with a weak suppression of the *GMR* > Egr eye phenotype (Figure 1f),

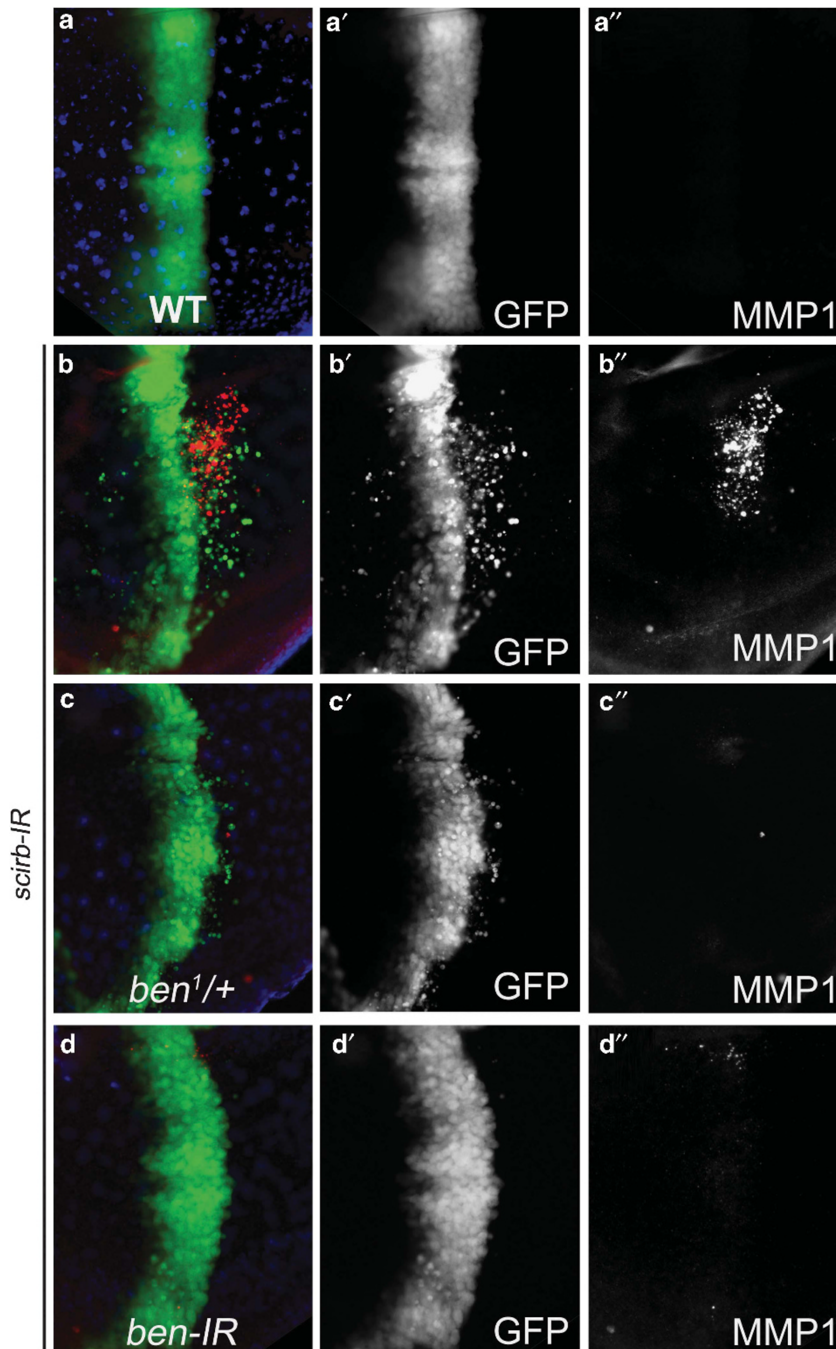

Figure 7 *ben* is required for JNK-mediated cell migration. (**a**–**d**) Fluorescence micrographs of wing discs are shown. Compared with the controls (**a** and **a**″, *ptc*-Gal4 *UAS*-GFP/ +), RNAi downregulation of *scrib* (**b**, *ptc*-Gal4 *UAS*-GFP/*UAS-scrib-IR*) induced cell migration (**b**′) and MMP1 upregulation (**b**″) were suppressed by mutation (**c**, ben^1/ + ; *ptc*-Gal4 *UAS*-GFP/*UAS-scrib-IR*) or RNAi downregulation (**d**, *ptc*-Gal4 *UAS*-GFP/*UAS-scrib-IR*; *UAS-ben-IR*/ +) of *ben*

heterozygosis of ben^1 mutation partially suppressed the tumor growth and invasion phenotype (Figures 6c–c′). Thus, *ben* is required for $Ras^{V12}/lgl^{-/-}$ triggered JNK signaling-mediated tumor growth and invasion.

***ben* is required for JNK-dependent cell invasion and MMP1 expression.** The epithelia of the *Drosophila* wing imaginal disc have been established as another *in vivo* model to study invasive cell migration.[10,39,40] It was shown that downregulation of cell polarity genes, for example, *disc large* (*dlg*), resulted in JNK-dependent cell invasion and upregulation of MMP1,[8] a matrix metalloprotease essential for basement membrane degradation.[38,41,42] Consistently, RNAi downregulation of the cell polarity gene *scrib* driven by *ptc*–Gal4 along the anterior/posterior (A/P) compartment boundary of wing imaginal discs (Figures 7a–a″) resulted in a JNK-dependent invasion-like phenotype,[8] in which cells delaminated and migrated away from the A/P boundary,

accompanied with increased expression of MMP1 (Figures 7b–b″). We found both the cell migration phenotype and MMP1 elevation were suppressed by mutation or RNAi downregulation of *ben* (Figures 7c–c″ and d–d″), confirming that *ben* is required for JNK-dependent cell invasion and MMP1 expression induced by loss of cell polarity gene.

Conclusions

We have identified Ben as a crucial regulator of JNK-dependent cell death and invasion, tumor progression, oxidative stress resistance and longevity in *Drosophila.* Our genetic epistasis and biochemical analysis established Ben as a novel component of the Egr–JNK signaling pathway via acting upstream of dTRAF2. Given the evolutionary conservation of the JNK pathway, the ortholog of Ben might have a similar role in modulating JNK-mediated cell death, tumor progression, stress response and longevity in other animals.

Materials and Methods

***Drosophila* strains and generation of clones.** Fluorescently labeled invasive tumors were produced in the eye discs as previously described[13] using the following strains: *y,w, ey*-Flp; *tub*-Gal80, FRT40A; $Act > y^{+} >$ Gal4, *UAS*-GFP (40A tester) and lgl^{4} FRT40A *UAS*-Ras^{V12} (40A tester). Additional strains including *elav*-Gal4, *GMR*-Gal4, *sev*-Gal4, *ey*-Gal4, *pnr*-Gal4 drivers and ben^{EP1651}, *UAS-GFP-IR* were obtained from Bloomington stock center; ben^{d09936} was obtained from Harvard Exelixis collection; ben^{143} and ben^{T25},[18] *UAS*-Ben,[19] *UAS-ben-IR*[30,31]; $DTRAF2^{ex1}$ and hep^{1},[27] bsk^{1},[28] puc^{E69},[44] *UAS*-Egr,[3] *UAS*-dTAK1,[26] *UAS*-Hep^{CA}, *UAS-Wgn-IR*,[14] *UAS-scrib-IR*, *UAS-dTAK1-IR*,[24] *UAS-dTRAF2-IR*, *UAS*-Puc and *UAS*-Flag-dTRAF2[6] were previously described.

Immunohistochemistry. Antibody staining of wing and eye imaginal discs was performed as previously described.[24] X-gal staining of eye discs was performed as described.[45]

Lifespan and oxidative stress resistance assay. Stocks were backcrossed with w^{1118} for more than eight generations, and lifespan and oxidative stress resistance were monitored at 25 °C as previously described.[5]

Immunoprecipitation and western blot. Heads were cut from freshly eclosed flies of indicated genotypes and homogenized in lysis buffer.[46] Immunoprecipitation, western blot, antibody staining and detection were performed as previously described.[46]

Conflict of Interest

The authors declare no conflict of interest.

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