



Cite this article: Wu C, Chen C, Dai J, Zhang F, Chen Y, Li W, Pastor-Pareja JC, Xue L. 2015 Toll pathway modulates TNF-induced JNK-dependent cell death in *Drosophila*. *Open Biol.* **5**: 140171. <http://dx.doi.org/10.1098/rsob.140171>

Received: 24 September 2014

Accepted: 19 June 2015

Subject Area:

developmental biology/genetics

Keywords:

cell death, *Drosophila*, Eiger, c-Jun N-terminal kinase, Toll

Author for correspondence:

Lei Xue

e-mail: lei.xue@tongji.edu.cn

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsob.140171>.

Toll pathway modulates TNF-induced JNK-dependent cell death in *Drosophila*

Chenxi Wu¹, Changyan Chen¹, Jianli Dai³, Fan Zhang², Yujun Chen¹, Wenzhe Li¹, José Carlos Pastor-Pareja³ and Lei Xue¹

¹Institute of Intervention Vessel, Shanghai 10th People's Hospital, Shanghai Key Laboratory of Signaling and Disease Research, School of Life Science and Technology, and ²School of Life Science and Technology, Tongji University, 1239 Siping Road, Shanghai 200092, People's Republic of China
³School of Life Sciences, Tsinghua University, Medical Science Building, D224, Beijing 100084, People's Republic of China

Signalling networks that control the life or death of a cell are of central interest in modern biology. While the defined roles of the c-Jun N-terminal kinase (JNK) pathway in regulating cell death have been well-established, additional factors that modulate JNK-mediated cell death have yet to be fully elucidated. To identify novel regulators of JNK-dependent cell death, we performed a dominant-modifier screen in *Drosophila* and found that the Toll pathway participates in JNK-mediated cell death. Loss of Toll signalling suppresses ectopically and physiologically activated JNK signalling-induced cell death. Our epistasis analysis suggests that the Toll pathway acts as a downstream modulator for JNK-dependent cell death. In addition, gain of JNK signalling results in Toll pathway activation, revealed by stimulated transcription of *Drosomycin* (*Drs*) and increased cytoplasm-to-nucleus translocation of Dorsal. Furthermore, the Spätzle (*Spz*) family ligands for the Toll receptor are transcriptionally upregulated by activated JNK signalling in a non-cell-autonomous manner, providing a molecular mechanism for JNK-induced Toll pathway activation. Finally, gain of Toll signalling exacerbates JNK-mediated cell death and promotes cell death independent of caspases. Thus, we have identified another important function for the evolutionarily conserved Toll pathway, in addition to its well-studied roles in embryonic dorso-ventral patterning and innate immunity.

1. Background

The excellent work for studying the pivotal functions of the Toll/IL-1R pathway in innate immunity in *Drosophila* and mammals earned Jules A. Hoffmann and Bruce A. Beutler the Nobel Prize in Medicine in 2011. Toll was initially identified in *Drosophila* as a type I trans-membrane receptor required for establishing the dorsal-ventral polarity during embryonic development [1,2]. Other components of the Toll pathway, including Spätzle, Tube, Pelle, Cactus and Dorsal, were also characterized as crucial regulators of dorsal-ventral patterning [3-9]. Subsequently, the Toll signalling pathway was implicated in host resistance against fungal and Gram-positive bacterial infections [10-12], which triggers in the fat body the production of antimicrobial peptides (AMPs), among which the antifungal peptide *Drosomycin* (*Drs*) appears to be the principal target of the Toll pathway [10,13]. To activate the Toll pathway in development or immunity, the first step is to cleave the inactive precursor of the Toll receptor ligand Spätzle (*Spz*) [8,9]. Upon binding to the active *Spz* ligand, Toll receptor recruits Tube and the kinase Pelle through the adaptor protein myeloid differentiation primary response protein 88 (*MyD88*), to assemble a receptor-proximal oligomeric complex [14,15]. Activation of Pelle triggers the phosphorylation and degradation of *Drosophila* IκB factor Cactus, which sequesters Dorsal and Dif (Dorsal-related immunity factor), the NF-κB factors in *Drosophila*, in the cytoplasm [16]. Once Cactus is degraded in response to the signal, Dorsal and Dif translocate to the nucleus and activate the

transcription of target genes [17]. The innate immune system, which serves as the first-line defence against pathogen infection, appeared early in evolution and is highly conserved in metazoans [18,19]. To date, there are 10 Toll-like receptors (TLRs) in humans and 12 TLRs in mice, which all activate NF- κ B factors in a MyD88-dependent manner to induce a set of immune responses, such as inflammation [20,21]. Although the Toll/TLR pathway has been conserved in evolution, mammalian TLR signalling is not involved in development, whereas the *Drosophila* Toll pathway plays pivotal roles in both development and immunity [10,22,23].

The c-Jun N-terminal kinase (JNK) represents one subgroup of the MAP kinase (MAPK) family [24,25]. The JNK pathway has been evolutionarily conserved from fly to human, and is involved in the regulation of a wide range of cellular activities including proliferation, differentiation, migration and apoptosis [26,27]. In *Drosophila*, the tumour necrosis factor (TNF) orthologue Eiger (Egr) triggers cell death through its receptor Grindelwald (Grnd) [28] and the TNF receptor-associated factor 2 (dTRAF2), which in turn activates the conserved JNK cascade including the JNKK kinase dTAK1 (MAP3K), the JNK kinase hemipterous (Hep, MAP2K) and Basket (Bsk) that encodes the *Drosophila* JNK (MAPK) [29–35]. The activated JNK phosphorylates and activates transcription factors including the AP-1 family members Jun and Fos, which are encoded by the *jra* and *kayak* genes in *Drosophila*, respectively [36–39]. JNK signalling also activates another transcription factor forkhead box O (FoxO), which promotes cell death by upregulating the transcription of the pro-apoptotic gene *head involution defective* (*hid*) [36–39]. Although recent studies have identified additional components in this pathway [40–44], the regulating network centred on JNK in modulating cell death, as well as the underlying mechanisms, have not been fully elucidated.

Associations among the TNF/JNK pathway, immune signalling and cell death have been reported in *Drosophila* [45–48], yet the conclusions are controversial, and the underlying mechanisms remain elusive. Seong *et al.* [46] found low-dose radiation (LDR) induces both Toll signalling-mediated innate immunity and activation of the JNK pathway, yet a potential interaction between the Toll and JNK pathways was not investigated. In contrast to the Toll signalling that is activated by fungi and Gram-positive bacteria, the Immune Deficiency (*Imd*) pathway is predominantly activated by Gram-negative bacteria [49,50]. Bangi *et al.* [45] suggested that an *Imd*-dTab2-dTAK1-JNK signalling is involved in bacterial-induced invasion and dissemination of oncogenic hindgut cells. However, the Vidal laboratory reported that tumours trigger a systemic immune response through the Egr pathway, which upregulates Toll signalling in the fat body. This activation of Toll, in turn, is required to induce tumour cell death through haemocyte-derived Egr, whereas the *Imd* pathway is not implicated in this crosstalk [47]. Together, these studies suggest that the Toll pathway may interact with Egr–JNK signalling in regulating cell death during tumour development, yet the modulation mechanisms and a direct role of Toll signalling in cell death have not been documented.

In this study, we have characterized the Toll pathway as an essential modulator of Egr-induced JNK-mediated cell death in *Drosophila*. First, loss of Toll signalling blocks Egr-induced JNK-mediated cell death in eyes and wings. Second, the Toll pathway acts downstream of FoxO to modulate Egr-triggered JNK-mediated cell death. Third, gain of JNK signalling induces

Toll pathway activation, indicated by nuclear accumulation of Dorsal and transcriptional activation of *Drosomycin*. Furthermore, JNK signalling activates the Toll pathway through transcriptional upregulation of the Spz family ligands in a non-cell-autonomous manner. Finally, gain of Toll signalling promotes cell death and synergistically enhances Egr-triggered cell death. In conclusion, we have identified a previous unknown function of the Toll pathway in modulating TNF-induced JNK-dependent cell death, in addition to its well-established roles in dorsal–ventral patterning and immunity.

2. Results

2.1. Depletion of Toll signalling suppresses Egr-induced cell death in eye development

Ectopic expression of Egr in *Drosophila* eyes driven by *GMR-GAL4* leads to vastly reduced adult eye size (figure 1*b,c* and 2*c,d*) [29,30], resulting from extensive cell death posterior to the morphogenetic furrow (MF) in third-instar eye discs (figure 2*c',d'*), visualized by acridine orange (AO) staining that detects dying cells [51]. To identify additional regulators of Egr-triggered cell death, we performed a genetic screen for dominant modifiers of the *GMR>Egr* small-eye phenotype using the Bloomington *Drosophila* Stock Center Deficiency kit that covers more than 95% of the genome. Once a deficiency was found to enhance or suppress the *GMR>Egr* eye-ablation phenotype, additional overlapping deficiencies were used for fine-mapping of the candidate gene [40,42,52]. One of the suppressors was mapped cytologically within 97D2–97D4, a region uncovered by three overlapping deficiencies: *Df(3R)BSC496*, *Df(3R)BSC524* and *Df(3R)ED6255* (figure 1*a*). The *GMR>Egr* eye phenotype is partially suppressed by the three deficiencies (figure 1*e–g*), but not by the adjacent *Df(3R)ED6232* (figure 1*d*). The region of 97D2–97D4 contains six genes including *Toll* (figure 1*a*), the *Drosophila* homologue of TLRs, that encodes a type I trans-membrane receptor involved in the activation of NF- κ B in dorsal–ventral patterning and immunity [3,10,21]. Accordingly, the *GMR>Egr* eye phenotype is suppressed to a similar extent by deleting half of the dosage of *Toll* (figure 1*i*) or expressing a *Toll* RNAi (figure 2*h*). In addition, loss of *Toll* significantly suppressed *GMR>Egr*-triggered cell death in eye imaginal discs (figure 2*h',m*). Thus, the Toll receptor is required for Egr-triggered cell death in eye development.

From the same screen, we also identified *Df(2L)BSC294* as a suppressor of *GMR>Egr*-induced eye phenotype (figure 1*h*). This small deficiency uncovers 36C2–36C9, a region that harbours 15 genes including *dorsal* and *Dif* (figure 1*j*), both of which encode the *Drosophila* NF- κ B factor operating in the Toll pathway (figure 2*a*). Consistently, *GMR>Egr*-triggered cell death in eye discs and adult eye phenotype (figure 2*d,d'*) are partially inhibited by removing one copy of endogenous *Dif* (figure 2*g,g',m*), or RNAi-mediated knocking-down of *dorsal* or *Dif* (figure 2*k,k',l,l',m*), but not of *LacZ* (figure 2*e,e',m*), suggesting the involvement of NF- κ B in Egr-triggered cell death.

Next, we extended our curiosity to other components of the Toll/NF- κ B pathway (figure 2*a*). We found that both the small eye phenotype and increased cell death induced by *GMR>Egr* were notably inhibited by RNAi-mediated knocking-down of *tube* or *pelle* (figure 2*i,i',j,j',m*). Taken

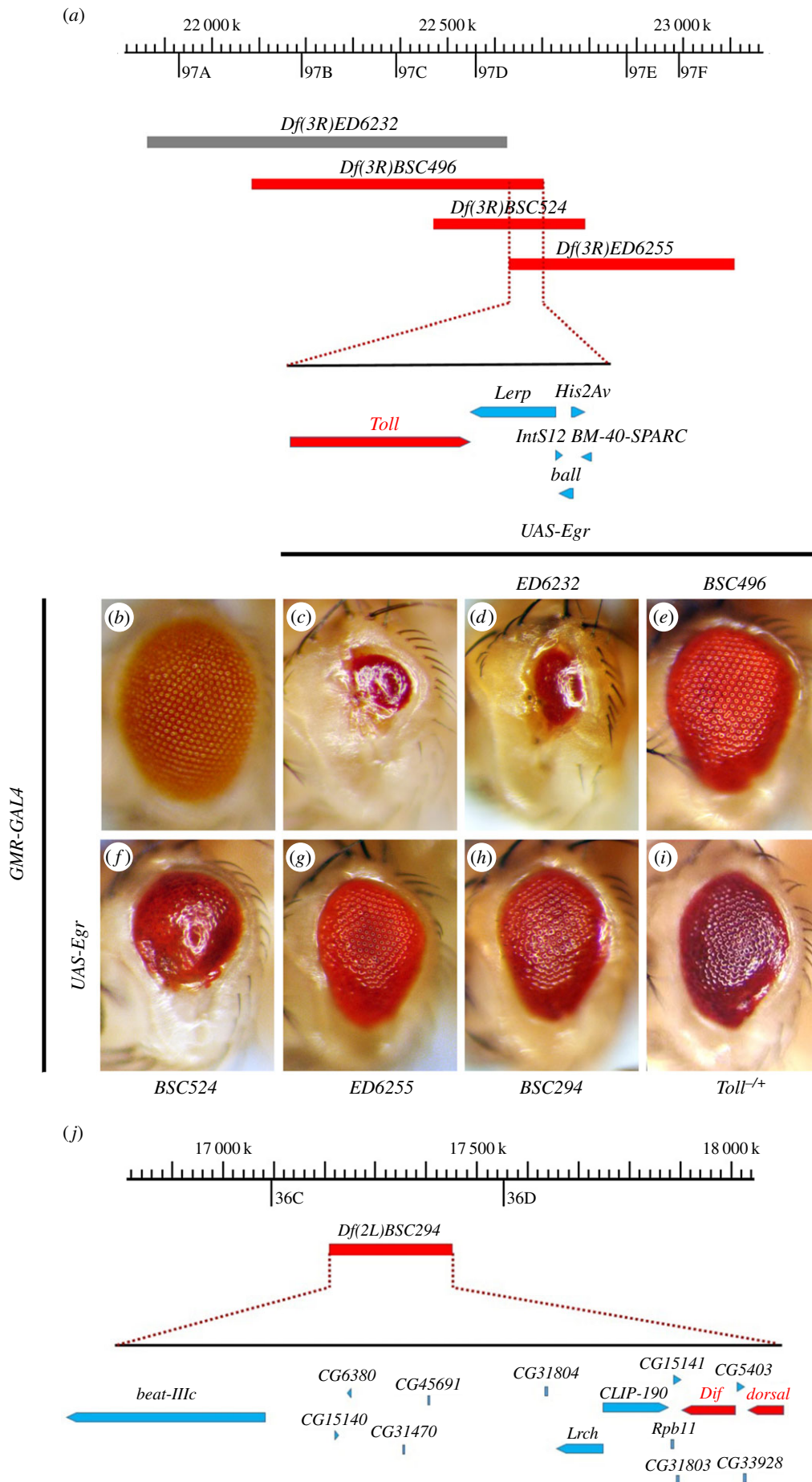


Figure 1. A genetic screen for dominant modifiers of *GMR*>*Egr*-induced eye-ablation phenotype. (a and j) Schematic of the genomic region surrounding *Toll* or *Dif* and *dorsal* locus. The deleted regions uncovered by deficiencies are indicated in grey (no effect) or red (suppressor). (b–i) Light micrographs of *Drosophila* adult eyes are shown. Compared with *GMR-GAL4* control (b), *GMR*>*Egr*-triggered small eye phenotype (c) remains unaffected by *Df(3R)ED6232* (d), but is partially inhibited by deficiencies *Df(3R)BSC496* (e), *Df(3R)BSC524* (f), *Df(3R)ED6255* (g), *Df(3R)BSC294* (h) or *Toll*³ mutation (i). See the electronic supplementary material for detailed genotypes.

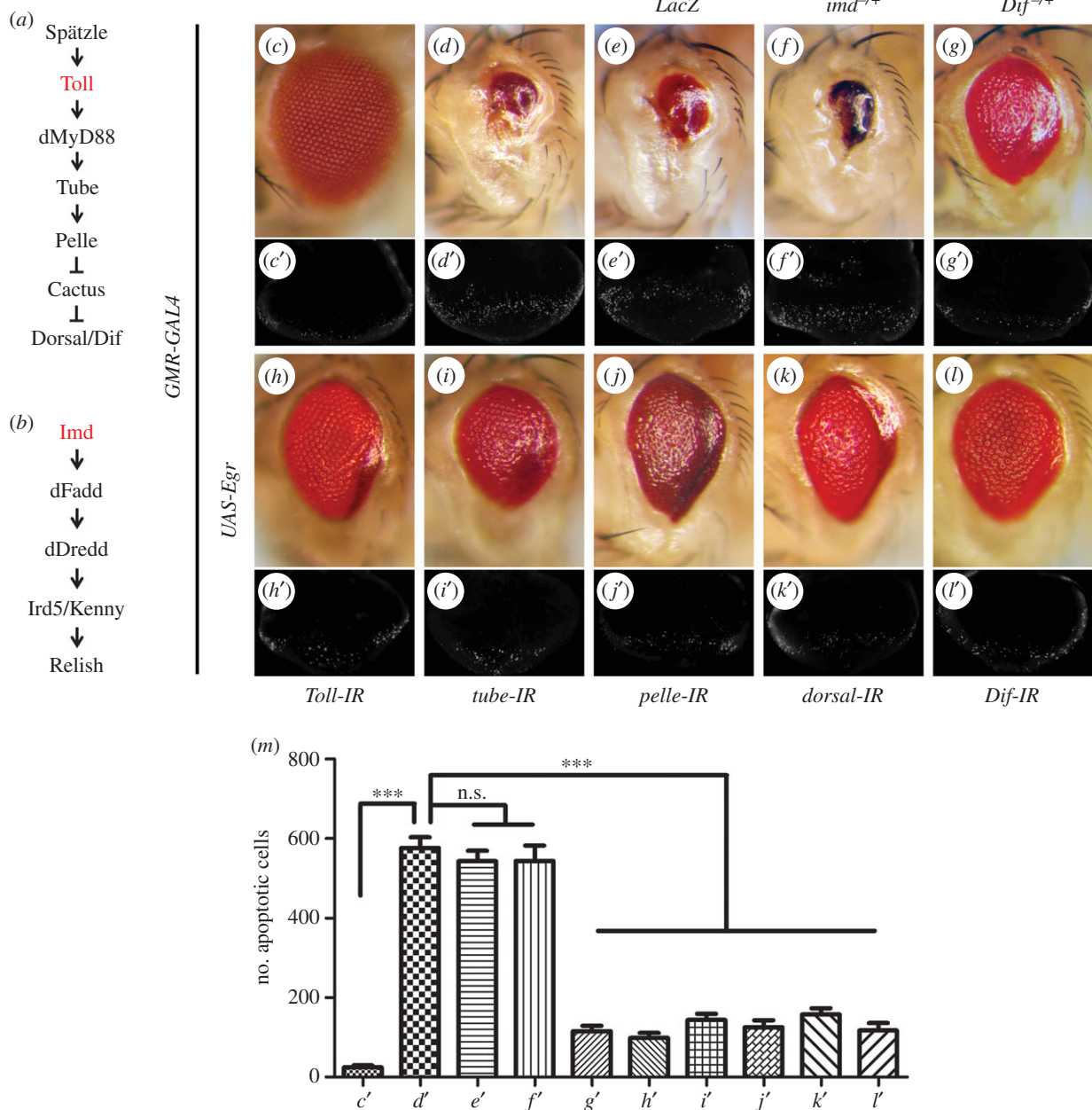


Figure 2. Loss of Toll signalling inhibits *GMR>Egr*-triggered cell death. (a,b) Diagrams for the key components of Toll and Imd signalling. Light micrographs of *Drosophila* adult eyes (c–l) and fluorescence micrographs of third-instar larval eye discs (c'–l') are shown. Compared with control (c and c'), *GMR>Egr*-induced small eye phenotype (d) and cell death in eye discs (d') remain unaffected by expression of LacZ (e and e') or mutation in *imd* (f and f'), but are partially suppressed by mutation in *Dif* (g and g') or RNAi-mediated downregulation of Toll pathway components: *Toll*, *tube*, *pelle*, *dorsal* and *Dif* (h–l and h'–l'). (m) Statistical analysis of cell death in eye discs shown in (c'–l'). Average number of dying cells labelled by AO staining are counted. Error bars indicate standard deviation. One-way ANOVA with Bonferroni multiple comparison test was used to compute *p*-values, significance was indicated with asterisks (***) ($p < 0.001$, $n = 10$ in each group); n.s., not significant. See the electronic supplementary material for detailed genotypes.

together, these data imply that the Toll pathway plays an essential role in *Egr*-triggered cell death.

Both Toll and Imd pathways are implicated in *Drosophila* innate immune response against microbial infection. The Toll pathway is activated primarily by Gram-positive bacteria and fungi while the Imd pathway responds mainly to Gram-negative bacteria infection (figure 2b) [13,53]. As *Egr*-triggered cell death depends on JNK signalling [29,30], which is also involved in the immune response of the Imd pathway [54,55], we examined a potential role of the Imd pathway in *Egr*-induced cell death. We found that *GMR>Egr*-induced cell death phenotypes were not suppressed by knocking-down *imd* or *relish* (electronic supplementary material, figure

S1a–c), or deleting one or two copies of the endogenous *imd* (figure 2f,f',m; electronic supplementary material, figure S1d,f), or homozygous mutation of *relish* (electronic supplementary material, figure S1g,i), as well as expression of LacZ (electronic supplementary material, figure S1e,h), suggesting the Imd pathway is dispensable for *Egr*-induced cell death.

2.2. Loss of Toll signalling suppresses JNK-mediated cell death in eye development

Egr-triggered cell death is mainly mediated by JNK signalling [29,30]. To genetically map the epistatic relationship between

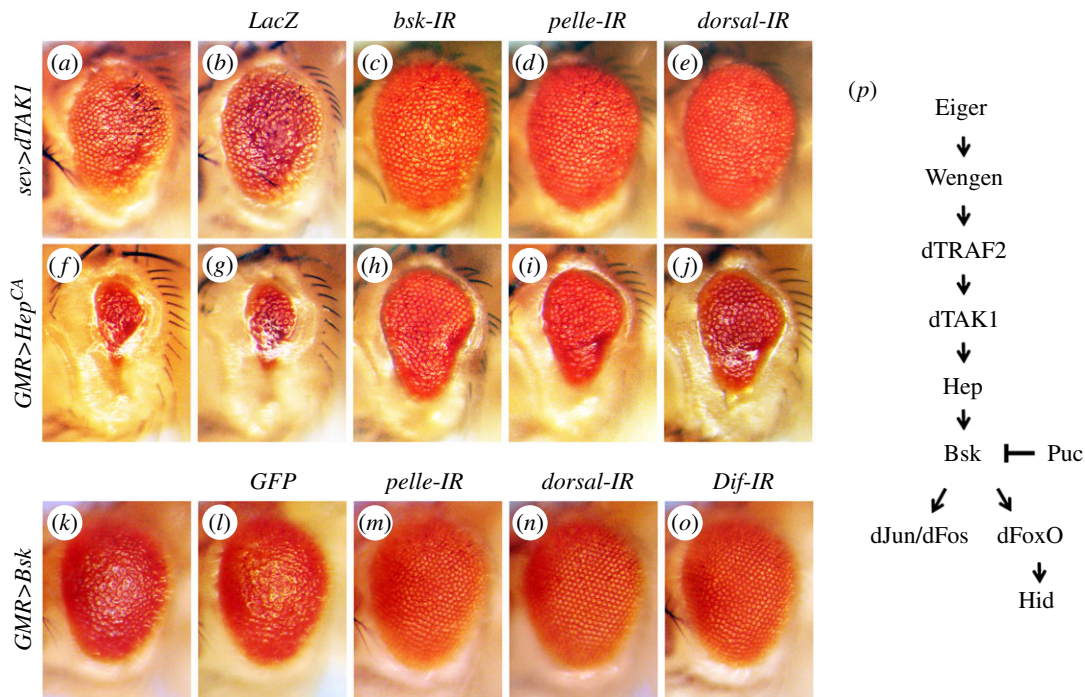


Figure 3. Toll signalling acts downstream of JNK in eye development. (a–o) Light micrographs showing *Drosophila* adult eyes. The small and rough eye phenotype resulting from ectopic expression of dTAK1 (a) or Hep^{CA} (f) is suppressed partially by RNAi-mediated knocking-down of *bsk* (c and h), *pelle* (d and i) or *dorsal* (e and j), but not of *LacZ* (b and g). The rough eye phenotype produced by *GMR>Bsk* (k) is obviously suppressed by RNAi-mediated inactivation of Toll pathway components: *pelle*, *dorsal* and *Dif* (m–o), but not by the expression of GFP (l). (p) A diagram for the key components of the Egr–JNK pathway. See the electronic supplementary material for detailed genotypes.

the Toll pathway and JNK cascade (figure 3p), we first examined the genetic interplays between the Toll pathway and dTAK1 (JNKKK) or Hep (JNKK) in the eye. As expression of dTAK1 driven by *GMR-GAL4* (*GMR>dTAK1*) results in pupa lethality [31], probably caused by the leaky expression of *GMR-GAL4* in other tissues including brain, wing and leg discs [56] we used *sev-GAL4*, another eye specific driver, to express dTAK1 in the eye. Eye specific expression of dTAK1 driven by *sevenless* (*sev*)-*GAL4* or a constitutive activated form of Hep (Hep^{CA}) driven by *GMR-GAL4* induces JNK-mediated cell death and generates rough eyes with reduced size (figure 3a,f) [31,42]. Both phenotypes are considerably suppressed by knocking-down *pelle* or *dorsal* (figure 3d,e,i,j). In the meantime, expression of *LacZ* (figure 3b,g) and a *bsk* RNAi (figure 3c,h) were included as a negative and positive control, respectively. Moreover, ectopic expression of Bsk (*Drosophila* JNK) under the control of *GMR-GAL4* also produces a small and rough eye phenotype (figure 3k), which is clearly suppressed by knockdown Toll signalling components (figure 3m–o), but not the expression of GFP (figure 3l). Taken together, these data suggest that the Toll pathway acts downstream of JNK in modulating cell death.

2.3. Toll signalling modulates JNK-mediated cell death in wing development

To investigate whether the Toll pathway modulates JNK-mediated cell death in other cellular contexts, we activated JNK signalling in distinct regions of the wing disc. Expression of Egr or Hep along the anterior/posterior (A/P) compartment boundary driven by *ptc-GAL4* results in cell death and produces a loss of anterior cross vein (ACV) phenotype (figure 4a,b,h,n) [42,57], which mimics the phenotype

generated by expressing the cell death gene *grim* (*ptc>Grim + Tub-GAL80^{ts}*; electronic supplementary material, figure S2). This phenotype is recapitulated by Toll expression (figure 4m), and suppressed by expressing a dominant-negative form of Bsk (Bsk^{DN}) (figure 4d,j) or RNAi-mediated inactivation of Toll pathway components (figure 4e–g,k,l), but not by expressing *LacZ* (figure 4c,i).

Furthermore, expression of Hep in the wing pouch driven by *Scalloped* (*Sd*)-*GAL4* triggers strong cell death (electronic supplementary material, figure S3) that results in severely reduced larval wing disc (electronic supplementary material, figure S4g,h) and adult wing blade (electronic supplementary material, figure S4a,b,f). Both phenotypes are significantly suppressed by expressing Bsk^{DN} or downregulation of Toll signalling (electronic supplementary material, figure S4d–f,j,k), but not by expressing GFP (electronic supplementary material, figure S4c,f,i), suggesting the Toll pathway modulates JNK-mediated cell death in a non-tissue-specific manner.

2.4. Toll signalling acts downstream of FoxO to modulates JNK-mediated cell death

In *Drosophila*, the AP-1 family members Jun and Fos, and the forkhead box factor FoxO act downstream of JNK as the transcription factors to mediate cell death [36–39]. To further delineate the interaction between JNK and Toll signalling, we ectopically expressed the three transcription factors in the developing wing by *Sd-GAL4*. Expression of dFoxO (*Sd>dFoxO*) triggered severe cell death and generated a small wing phenotype (figure 5a,d,i) [58], whereas expression of Jun (*Sd>dJun*) or Fos (*Sd>dFos*) did not produce any discernible defects in adult wings (figure 5b,c,i). Furthermore, *Sd>dFoxO*-induced wing defect could be partially

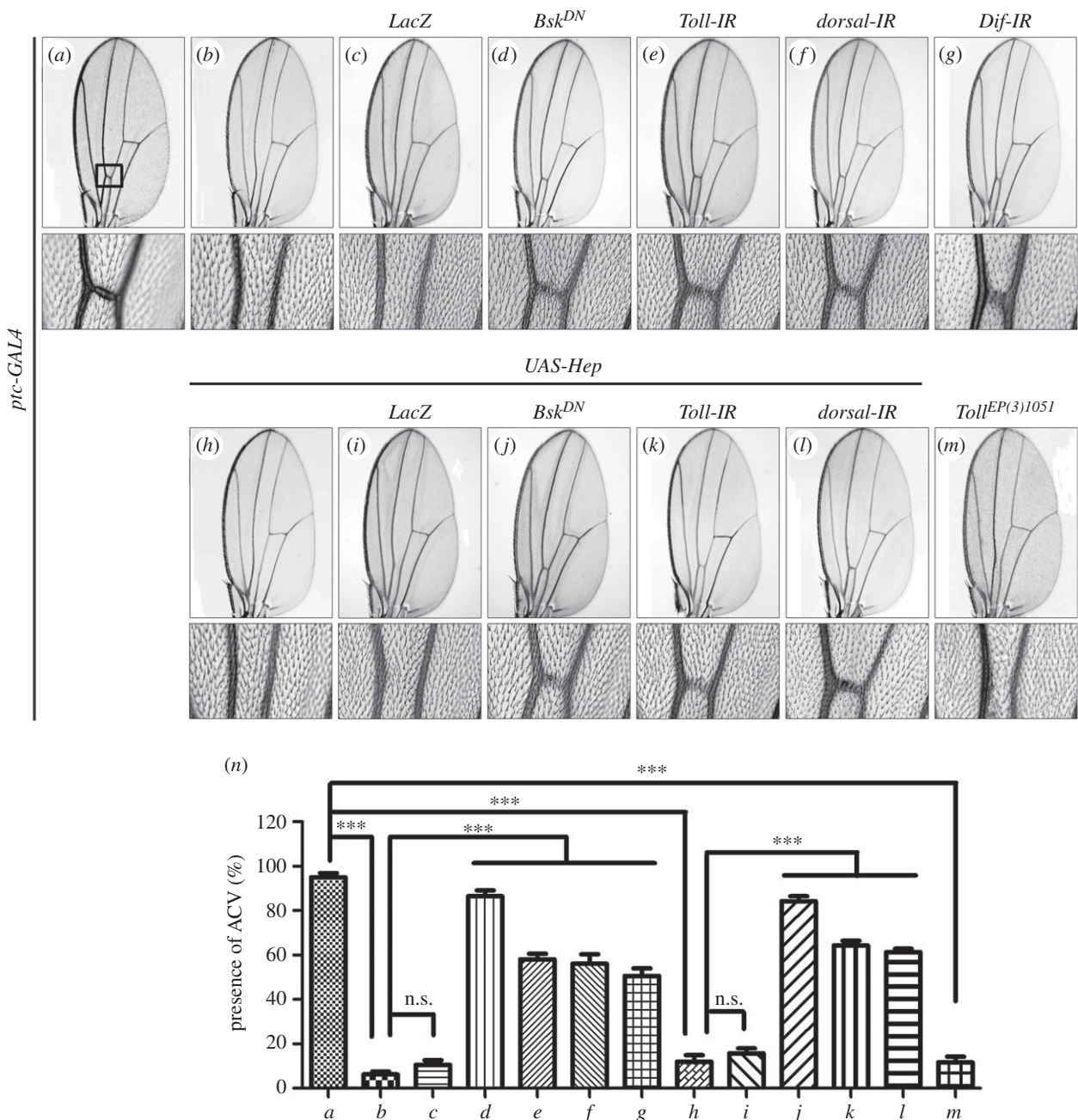


Figure 4. Toll signalling acts downstream of Hep in wing development. Light micrographs of *Drosophila* adult wings (a–m). Compared with *ptc-GAL4* control (a), ectopic expression of *Egr^w* (b) or Hep (h) or Toll (m) driven by *ptc-GAL4* generates loss of ACV phenotype. This phenotype, produced by *ptc>Egr^w* (b) or *ptc>Hep* (h), is strongly suppressed by a dominant-negative form of Bsk (d and j) or by depletion of Toll signal (e–g, k and l), but not that of LacZ (c and i). The lower panels show high magnification view of the boxed area in upper panels (a–m). (n) Quantification of the ACV phenotype as shown in panels (a–m) (for each genotype, $n = 20$). Error bars indicates standard deviation. One-way ANOVA with Bonferroni multiple comparison test was used to compute p -values, significance was indicated with asterisks (***) $p < 0.001$; n.s., not significant. See the electronic supplementary material for detailed genotypes.

suppressed by knocking-down Toll signalling (figure 5f–i), but remained unaffected by the expression of GFP (figure 5e,i), implying that Toll signalling acts downstream of dFoxO to modulates JNK-mediated cell death in *Drosophila*.

2.5. Toll pathway is dispensable for caspases-mediated cell death

Previous studies have suggested that Egr–JNK signalling is able to trigger two types of cell death: the apoptotic cell

death (caspases-dependent) and the non-apoptotic cell death (caspases-independent) [29,30,44]. In *Drosophila*, apoptotic cell death is induced by upregulation of three pro-apoptotic genes, *rpr*, *hid* and *grim*, and is mediated by activation of caspases [59]. Consistent with previous study [60], we found that activation of TNF–JNK signalling in the wing pouch by *Sd>Hep* upregulates the transcription of *hid*, visualized by X-gal staining of *hid-LacZ* reporter (figure 5o,p). In addition, *Sd>Hep*-induced small wing phenotype is partially rescued by the deficiency *Df(3L)H99* that deletes *rpr*, *hid* and *grim*, or by expressing the inhibitor

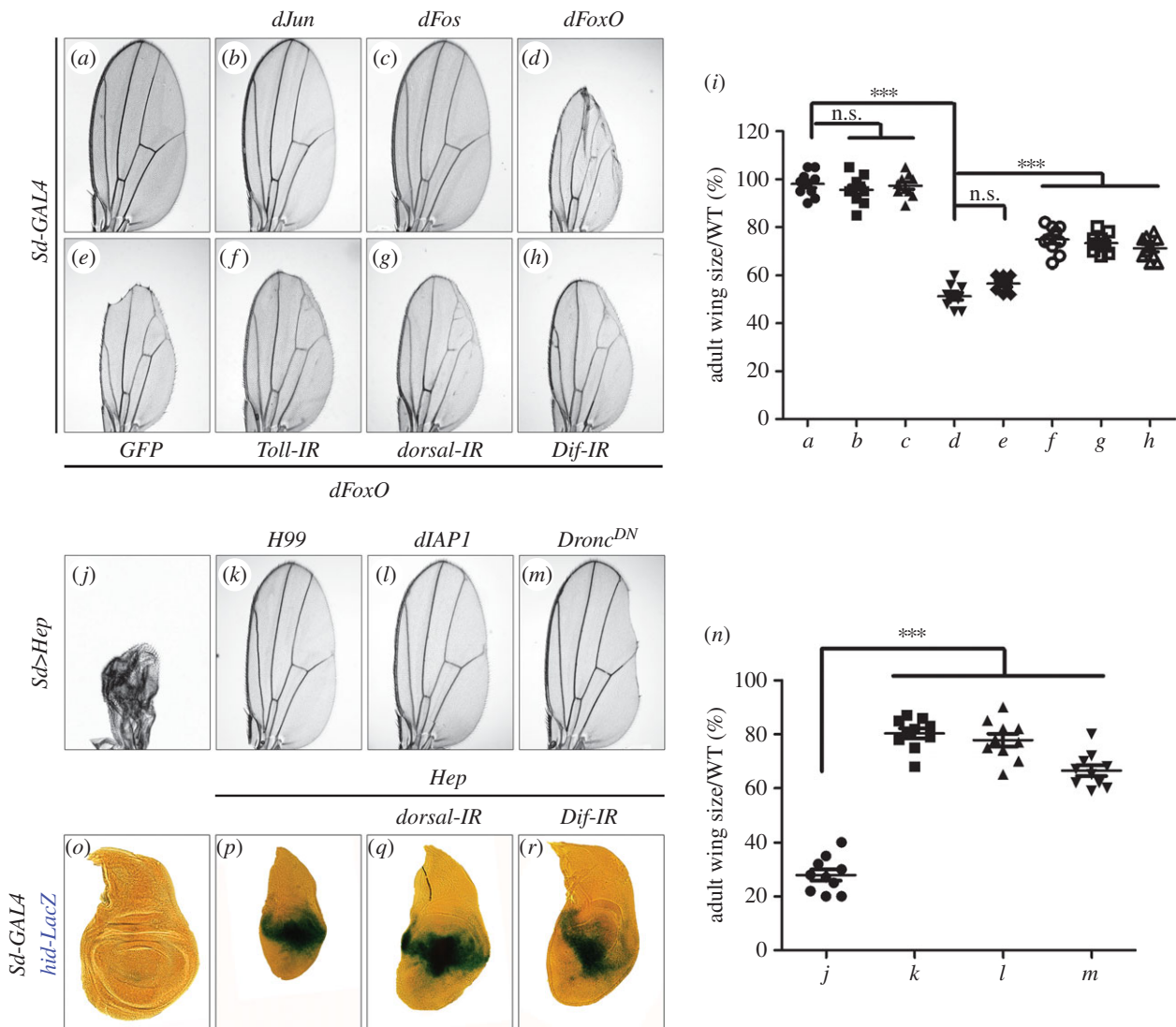


Figure 5. Toll signalling acts downstream of FoxO and mediates caspases-independent cell death. (a–h and j–m) Light micrographs of *Drosophila* adult wings are shown. Compared with *Sd-GAL4* control (a), ectopic expression of dFoxO results in an evident reduction in wing size (d), which is suppressed significantly by loss of Toll signalling (f–h), but not that of GFP (e). Expression of dJun or dFos driven by *Sd-GAL4* does not produce any visible defects (b and c). The small wing phenotype induced by *Sd>Hep* (j) is strongly suppressed by *Df(3L)H99* that deletes one copy of the apoptotic genes *reaper*, *hid* and *grim* (k), the expression of DIAP1 (l) or *Dronc^{DN}* (m). (i and n) Quantifications of adult wing size/wild-type (WT) ratio shown in figures a–h and j–m, respectively ($n = 10$). One-way ANOVA with Bonferroni multiple comparison test was used to compute p -values, significance was indicated with asterisks (***) ($p < 0.001$); n.s., not significant. (o–r) X-Gal staining of a *hid-LacZ* reporter in wing discs. Compared with the control (o), expression of *Hep* in the wing pouch driven by *Sd-GAL4* induces *hid* transcription (p), which cannot be suppressed by knocking-down *dorsal* or *Dif* (q and r). See the electronic supplementary material for detailed genotypes.

of apoptosis protein DIAP1 or a dominant-negative form of Dronc (*Drosophila* caspase-9) (figure 5j–n) [59,61,62], suggesting TNF–JNK signalling could induce apoptotic cell death in wing development.

To test whether the Toll pathway is involved in JNK-mediated caspases-dependent cell death, we knocked-down Toll signalling and found that loss of the Toll pathway could partially suppress *Sd>Hep*-induced size reduction, but not the transcriptional upregulation of *hid* in the wing disc (figure 5o–r). Moreover, ectopic expression of Hid in *Drosophila* eyes driven by *GMR-GAL4* triggers caspases-mediated cell death and produces small adult eye phenotype (electronic supplementary material, figure S5a), which cannot be rescued by depletion of the Toll signalling pathway (electronic supplementary material, figure S5b and c). Hence, we conclude that the Toll pathway is dispensable for caspases-mediated cell death.

2.6. Toll signalling modulates physiological JNK signalling-mediated cell death

To investigate whether the Toll pathway is involved in the physiological role of JNK signalling in cell death, we knocked-down *puc*, a negative regulator of JNK activity (figure 3p) [63], along the A/P compartment boundary under the control of *ptc-GAL4* (*ptc>puc-IR*). We observed robust cell death in third-instar larval wing discs and loss of ACV in adult wings (figure 6a,h,o,p). Both phenotypes are fully suppressed by expressing the dominant-negative Bsk (figure 6c,j,o,p), but remain unaffected by expressing LacZ, which serves as a negative control (figure 6b,i,o,p), suggesting loss-of-*puc*-triggered cell death depends on JNK. Furthermore, *ptc>puc-IR*-induced cell death and loss-of-ACV phenotypes are suppressed by RNAi-mediated inactivation of Toll pathway components, e.g. *Toll*, *tube*, *dorsal* or *Dif* (figure 6d–g, k–p), indicating that the Toll

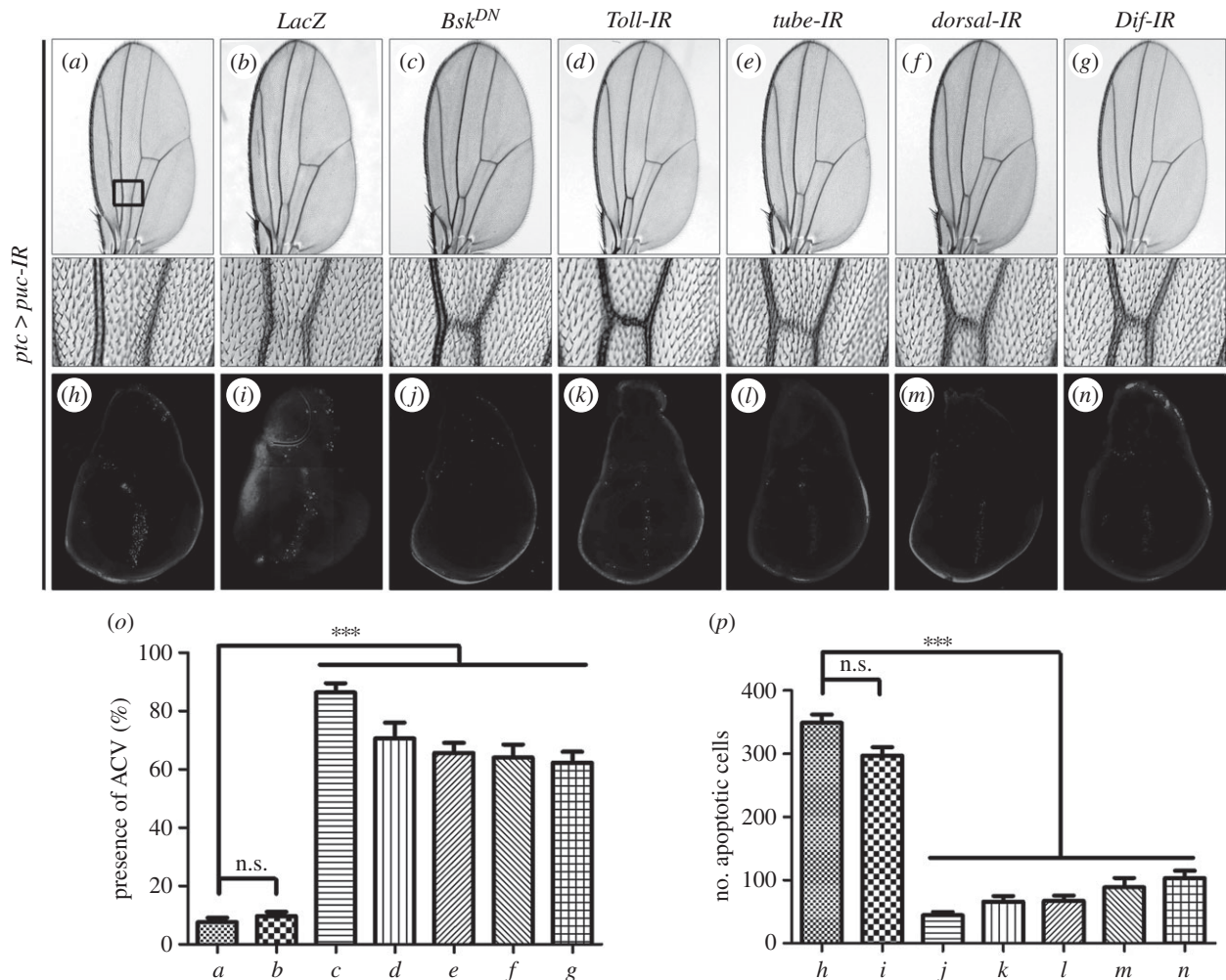


Figure 6. Loss of Toll signalling suppresses physiological JNK-induced cell death. Light micrographs of *Drosophila* adult wings (a–g) and fluorescence micrographs of third-instar larval wing discs (h–n) are shown. RNAi-mediated downregulation of *puc* along the A/P compartment boundary by *ptc-GAL4* produces the loss-of-ACV phenotype in adult wings (a), which results from strong cell death in larval wing discs (h). Both phenotypes depends on endogenous JNK (c and j) and the Toll pathway (d–g and k–n), but not on LacZ (b and i). The bottom panels show high magnification views of the boxed area in upper panels (a–g). (o and p) Statistical analysis of ACV phenotype ($n = 20$ for each genotype) and cell death in wing discs ($n = 10$) as shown in figures a–g and h–n respectively. Error bars indicate standard deviation. One-way ANOVA with Bonferroni multiple comparison test was used to compute p -values, significance was indicated with asterisks (***) $p < 0.001$; n.s., not significant. See the electronic supplementary material for detailed genotypes.

pathway is involved in the physiological function of JNK signalling in cell death.

2.7. JNK signalling promotes Toll pathway activation

It is known that a fungal infection could trigger the activation of the Toll pathway, which leads to the induction of antifungal peptide *Drs* in the fat body as its principal target [10,13]. To determine whether JNK signalling is sufficient to elicit Toll pathway activation, we examined the expression of *Drs* by a previously described *Drs-GFP* reporter [64], and found that expression of dTAK1 driven by the fat body specific *Cg-GAL4* resulted in elevated expression of *Drs-GFP* (figure 7a,b). Furthermore, knocking-down *puc* in the fat body also triggers *Drs-GFP* expression (figure 7c). Hence, both ectopic and physiological JNK activation could induce the transcriptional upregulation of *Drs*, a primary target gene of the Toll pathway.

Our previous data indicated that the Imd pathway is not implicated in TNF/JNK-mediated cell death (figure 2f,f,m and electronic supplementary material, figure S1). Consistent with these data, activation of JNK signalling did not induce

the expression of *Diptericin* (*Dipt*) (electronic supplementary material, figure S6), a reporter of Imd pathway activity [65–68].

In the non-signalling condition, the I- κ B orthologue Cactus retains the NF- κ B factor Dorsal in the cytoplasm, inhibiting its nuclear localization and transcription factor activity. Upon activation of Toll signalling, Dorsal is released from Cactus and translocates to the nucleus [69]. To monitor the Toll pathway activity directly, we examined the expression level and subcellular localization of Dorsal *in vivo* by anti-Dorsal staining. Activation of JNK signalling by dTAK1 expression in third-instar eye discs driven by *GMR-GAL4* resulted in elevated Dorsal expression posterior to the MF (electronic supplementary material, figure S7). As eye disc cells are too small to distinguish the subcellular distribution of Dorsal protein, we induced ectopic dTAK1-expressing clones in the fat body and examined the nuclear–cytoplasmic shuttling of endogenous Dorsal protein. While Dorsal is mainly localized to the cytoplasm in control cells, it is accumulated in the nuclei and periphery in cells expressing high levels of dTAK1 (figure 7d,d'). Consistently, we observed increased nuclear accumulation of Dorsal in all fat body cells when

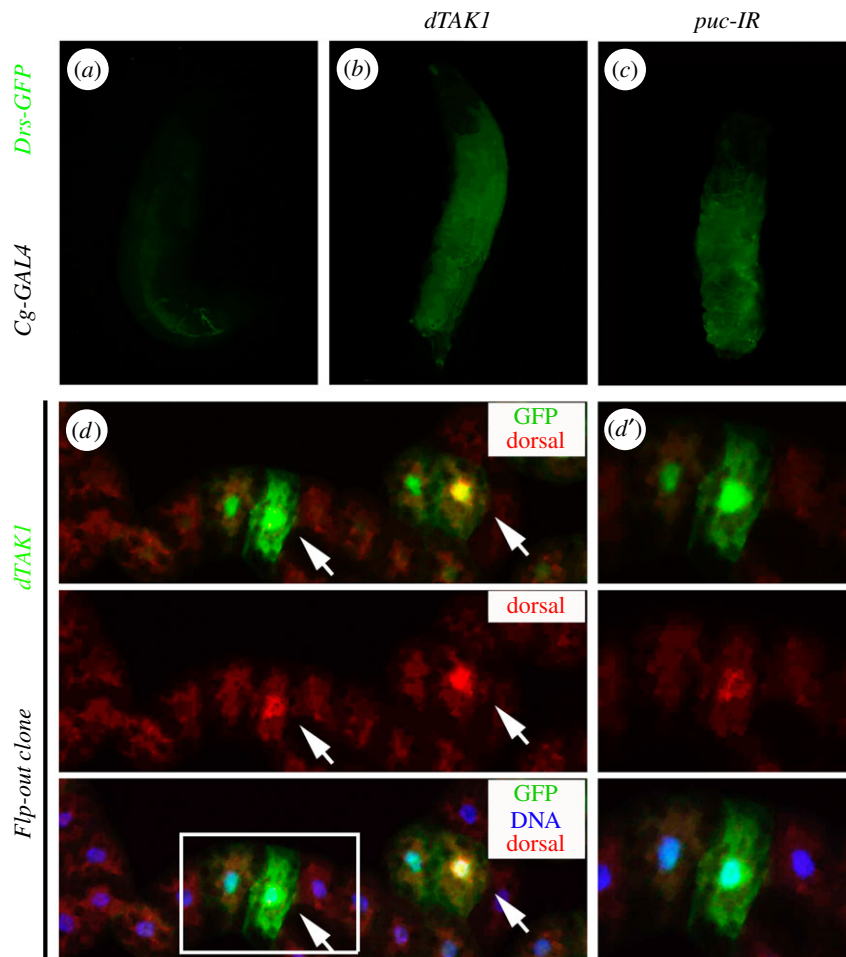


Figure 7. Gain of JNK signalling promotes *Drs* expression and dorsal nuclear localization. (a–c) Fluorescent microscope images showing third-instar larvae. Compared with control (a), expression of *dTAK1* (b) or RNAi inactivation of *puc* (c) in fat body upregulates *Drs-GFP* expression. (d) Fluorescent microscope images showing fat body dissected from third-instar larvae stained with anti-dorsal (red). *dTAK1*-expressing clones were tagged by GFP (green) and induced for 1 h by heat shock at 37°C and recovered for 24 h at 25°C. Nuclei were labelled with DAPI (blue). Endogenous Dorsal protein displays nuclear localization in cells expressing high level of *dTAK1* (arrows). (d') shows high magnification views of the boxed area in (d). DAPI, 4, 6-diamidino-2-phenylindole. See the electronic supplementary material for detailed genotypes.

JNK signalling was activated by *Cg-GAL4*-driven expression of *Egr* or *puc* RNAi (electronic supplementary material, figure S8). These results imply that gain of JNK signalling is sufficient to trigger the activation of the Toll pathway.

Intriguingly, we found that gain of JNK signalling-triggered Toll pathway activation, visualized by stimulated *Drs-GFP* expression and increased Dorsal nuclear localization, could not be suppressed by expressing caspases inhibitor P35 (electronic supplementary material, figure S9a–c), implying that activation of Toll signalling by activated JNK is caspases independent.

2.8. Gain of JNK signalling upregulates the transcription of *Spz* family ligands

The gene *spz* encodes the *Drosophila* ligand for Toll receptor that activates the Toll pathway in embryonic development and the innate immune response [8,9,70]. As JNK signalling triggers Toll pathway activation and depletion of Toll receptor suppresses JNK-induced cell death, we postulated that JNK signalling might operate upstream of Toll receptor, for instance, by regulating the transcription of *Spz*. To test this hypothesis, we activated JNK signalling in the adult eye

(*GMR>Egr*), extracted total mRNA from the heads and performed a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. In support of our assumption, *Spz* transcription was upregulated more than three-fold upon JNK activation (figure 8a). *Spz* homologues, referred to as *Spz2* to *Spz6*, have been identified in the *Drosophila* genome by an iterative searching method [71]. They share a characteristic intron–exon structure with the prototype *spz* gene, and could execute a similar or redundant function as *Spz* by binding to the Toll receptors [71]. Intriguingly, activation of JNK signalling is able to upregulate the transcription of all five *Spz* homologues, with *Spz2* level increased by more than 20-fold, as analysed by qRT-PCR assay (figure 8a).

To confirm the qRT-PCR data *in vivo*, we examined the transcription of *Spz6* with a *Spz6-GFP* reporter strain [72]. Elevated *Spz6-GFP* expression was noted posterior to MF in third-instar larval eye discs when JNK signalling was activated by ectopic expression of *Egr*, *dTAK1* or *Hep*, driven by *GMR-GAL4* whose expression pattern was visualized by mRFP (figure 8b–f), suggesting that gain of JNK signalling could induce Toll pathway activation through transcriptional upregulation of *Spz* family ligands. Consistent with our observation that induction of the Toll pathway by activated JNK signalling is caspase independent (electronic

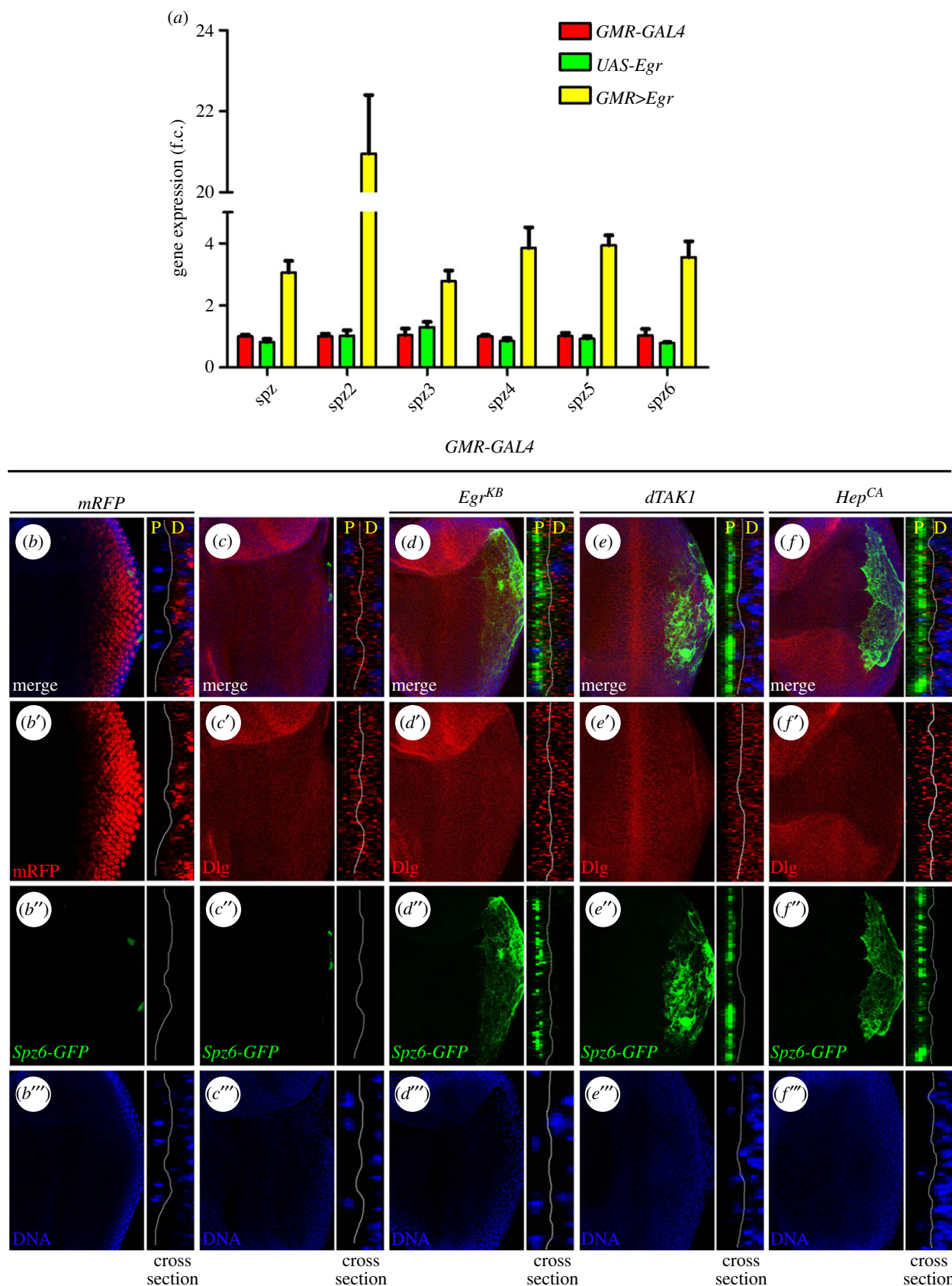


Figure 8. Gain of JNK signalling upregulates the expression of Spz family ligands. (a) Histogram showing the levels of *Spz1–6* mRNAs measured by quantitative RT-PCR. Total RNA of *Drosophila* adult eyes was extracted and normalized for cDNA synthesis. Error bars represent standard deviation from three independent experiments. (b–f) Fluorescence micrographs of *Drosophila* third-instar larval eye discs are shown. Compared with the GMR-GAL4 control (c), *Spz6-GFP* expression was evidently increased by expression of Egr (d), dTAK1 (e) or Hep (f), mRFP marking the GMR-GAL4 expression region (b). The right panels show views of vertical cross sections corresponding to the left panels (b–f). Nuclei were labelled with DAPI (blue), cell membranes were stained by anti-Dlg antibody (red). Imaging of prepared samples was conducted by a Leica confocal microscope (Leica SP5). See the electronic supplementary material for detailed genotypes.

supplementary material, figure S9a–c), the increased *Spz6-GFP* expression was not suppressed by expressing the caspases inhibitor P35 (electronic supplementary material,

figure S9d,e), but was suppressed by the expression of Bsk^{DN} that served as a positive control (electronic supplementary material, figure S9f).

While we found that *Spz6-GFP* expression is upregulated by JNK signalling in third-instar larval eye discs (figure 8*b–f*), we are not sure this activation is cell autonomous or non-cell autonomous. As previous study has shown that Spz ligands produced by haemocytes could induce the activation of the Toll/NF- κ B pathway in the fat body non-cell autonomously [73], we wondered whether the *Spz6-GFP* expressing cells are haemocytes attached to the eye disc. To test this possibility, we performed antibody staining against the haemocyte marker NimC1 [74]. We observed numerous haemocytes associated with control eye discs (electronic supplementary material, figure S9*g*), yet the haemocyte number remained unchanged upon activation of JNK signalling by *GMR>Egr* or *GMR>dTAK1* (electronic supplementary material, figure S9*h* and *i*). In the meantime, we found that the region attached by haemocytes does not overlap with that of *Spz6-GFP* expression (figure 8*d–f* and electronic supplementary material, figure S9*g–i*). Together, these data suggest that *Spz6-GFP* expressing cells induced by JNK signalling are not haemocytes.

Anatomically, the eye imaginal disc, with the sac-like two-layered structure, comprises a columnar cell monolayer (named disc proper, DP) covered by a squamous epithelium known as the peripodial membrane (PM) [75]. As the communications between the two distinct cell layers are important for concerted growth and patterning of the disc during development [76,77], we examined the vertical cross section of eye imaginal discs to investigate whether the *Spz6-GFP* expressing cells are DP or PM cells. Indeed, we observed two opposing cell layers, PM (P) and DP (D), in the control discs (figure 8*c*), with *GMR-GAL4* expression, marked by mFRP, specifically located within the DP cells (figure 8*b*). When JNK signalling was activated by ectopic expression of *Egr*, *dTAK1* or *Hep* driven by *GMR-GAL4* in the DP cells, *Spz6-GFP* expression was significantly increased in the PM cells (figure 8*d–f*). These results suggest that Spz ligands are induced by activated JNK signalling in a non-cell-autonomous manner in eye discs.

2.9. Gain of Toll signalling aggravates Egr-induced cell death in eye development

To further characterize the role of the Toll pathway in cell death, we upregulated Toll signalling in the developing eye by expressing Toll, Pelle, Dorsal, Dif, or an RNAi of *cactus*, the unique *Drosophila* orthologue of κ B that operates as a negative regulator of the Toll pathway [78]. Compared with the controls or expression of LacZ (figure 9*a,a',b,b'* and *k*), enhanced Toll signalling results in mild but significant cell death in the eye discs (figure 9*c'–e',k* and electronic supplementary material, figure S10*a–d*) and produces rough eyes in adults (figure 9*c–e* and electronic supplementary material, figure S10*e* and *i*), suggesting that activation of the Toll pathway is sufficient to induce cell death. To confirm the activation of Toll signalling, we expressed Toll, Pelle and *cactus-IR* in the fat body, and monitored the expression of *Drs* by *Drs-GFP* [64], the recognized Toll pathway reporter [10,13]. We found that the activation of Toll signalling induced by Toll^{10B} (an activated form of Toll) or *cactus-IR* is much stronger than that induced by Pelle (electronic supplementary material, figure S11). We next questioned whether elevated Toll signalling could boost Egr-induced cell death. For this

purpose, a weak *UAS-Egr* line (*UAS-Egr^w*) [29] was expressed in the eye, which caused limited cell death in third-instar eye discs and adult eyes (figure 9*f,f',k*). A synergistic enhancement in cell death and eye size reduction was observed when *Egr^w* was co-expressed with Toll, Pelle or the *cactus* RNAi, but not with LacZ (figure 9*g–j,g',j',k*), suggesting that gain of Toll signalling exacerbates Egr-triggered cell death.

2.10. Toll signalling-triggered cell death is independent of JNK, caspase or necroptosis

While the Toll/NF- κ B pathway is able to promote cell death, the underlying mechanisms remains largely unknown. We found that expression of Bsk^{DN}, or Puckered (Puc) encoding a JNK phosphatase that negatively regulates JNK kinase activity [63], exhibits no suppressive effect on the rough eye phenotypes induced by Dorsal or Dif expression (electronic supplementary material, figure S10*e–l*), implying that the JNK pathway is not involved in Toll pathway-induced cell death.

Our previous data have implied that the Toll signalling pathway is not required for caspases-mediated cell death (electronic supplementary material, figure S5). To further investigate the relationship between Toll pathway-induced cell death and caspases, we firstly checked antibody staining for the activated form of Caspase-3 (CC-3), a read-out of the initiator caspase Dronc (*Drosophila* caspase-9) activity [79], and found that gain of Toll signalling by over-expressing Dorsal or Dif did not result in increased caspases activity (electronic supplementary material, figure S12*a–d*). Consistently, we also observed that the rough and small eye phenotypes induced by Dorsal or Dif expression remain unaffected by blocking caspase signalling (electronic supplementary material, figure S12*e–h,k–n*). Together, these data indicated that the Toll pathway triggers caspase-independent cell death.

Necroptosis/programmed necrosis, which is a RIP1/RIP3-dependent caspase-independent programmed necrotic cell death, plays essential roles in development [80–84], and has been implicated in a variety of pathological progresses, including tumourigenesis, metastasis, inflammation and liver diseases [85–87]. To investigate whether cell death induced by the Toll/NF- κ B pathway is mediated by necroptosis, we depleted dTRAF2, the key component of necroptosis machinery [88,89]. We found that mutation or RNAi-mediated inactivation of *dTRAF2* has no effect on the rough eye phenotypes of *GMR>Dorsal* or *GMR>Dif* (electronic supplementary material, figure S12*i,j,o,p*), indicating that Toll/NF- κ B pathway-triggered cell death is independent of necroptosis.

3. Discussion

The Toll pathway has been implicated in embryonic dorsal–ventral patterning and innate immunity in *Drosophila*. In this study, we demonstrate that the Toll pathway modulates JNK-mediated cell death *in vivo*, adding a distinct but vital role to this well-characterized signalling pathway, and further show that JNK signalling triggers Toll pathway activation through transcriptional upregulation of Spz family members encoding the ligands for Toll receptor in a non-cell-autonomous manner (figure 9*l*). In the light of their conserved function from fly to human, the implication of the *Drosophila* Toll pathway in JNK signalling-mediated cell death shall provide a

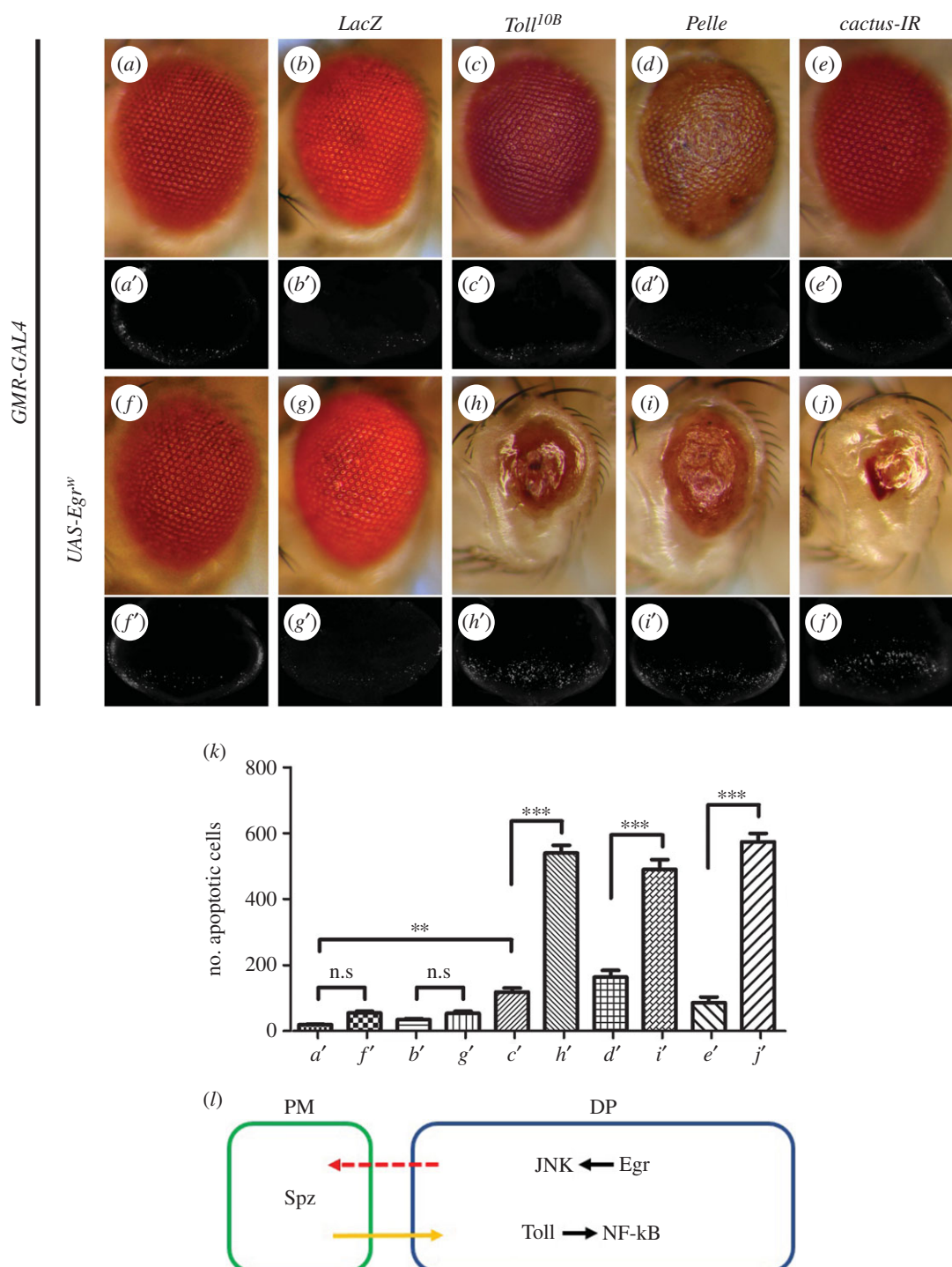


Figure 9. Gain of Toll signalling enhances *GMR>Egr*-induced cell death. Light micrographs of *Drosophila* adult eyes (a–j) and fluorescence micrographs of third-instar larval eye discs (a'–j') are shown. Compared with control (a and a'), expression of *Toll*^{10B}, *Pelle*, a *cactus* RNAi or a weak *Egr* line (*Egr*^W) promotes little or mild cell death in eye discs and adult eyes (c–f and c'–f'). Co-expression of *Egr*^W with *Toll*^{10B}, *Pelle* or a *cactus* RNAi displays synergistic enhancement of cell death in eye discs and adult eyes (h–j and h'–j'). As a negative control, expression of *LacZ* neither triggers cell death by itself (b and b') nor aggravates *Egr*^W-induced cell death (g and g'). (k) Statistical analysis of cell death in eye discs shown in (a'–j'). Average number of dying cells labelled by AO staining are counted. Error bars indicate standard deviation. One-way ANOVA with Bonferroni multiple comparison test was used to compute *p*-values, significance was indicated with asterisks (***p* < 0.01, ****p* < 0.001, *n* = 10 in each group); n.s., not significant. (l) A model for the role of the Spz/Toll/NF-κB pathway in modulating Egr-triggered JNK-mediated cell death. See the electronic supplementary material for detailed genotypes.

novel connection for the crosstalk between IL-1R and JNK signalling in cell death in mammals.

We found that loss of Spz, either by mutation or RNAi inactivation, failed to suppress *GMR-Egr*-triggered cell death (data not shown), suggest other Spz family ligands may play redundant function in modulating cell death. Consistent with this interpretation, Spz2 was shown to play redundant function with other Spz ligands and regulate cell

death in CNS [90]. As there is no mutant allele available for other *spz* genes at the moment, it is not feasible to determine whether all or a specific set of Spz molecules are required for JNK-induced Toll pathway activation. Further investigation will be required to clarify this matter.

Recent study reported that the Toll pathway could be activated by danger signals released by apoptosis-deficient cells in a non-cell-autonomous manner [91]. Consistently, we

found that elevated JNK signalling triggers Toll pathway activation independent of caspase-mediated apoptosis (electronic supplementary material, figure S9a–f). Furthermore, we provide compelling evidence for a molecular mechanism of this activation: activated JNK signalling in the columnar disc (DP) cells induces elevated expression of *Spz* family ligands in the PM cells (figure 8b–f), which in turn activates the Toll/NF- κ B pathway in DP cells (figure 9l). Thus, our data reveal a functional interplay between the DP cells and the PM cells in eye discs. However, the mechanism by which JNK signalling triggers the expression of *Spz* ligands non-cell autonomously remains elusive.

A recent study suggested that the Toll/NF- κ B pathway promotes JNK-independent cell death by upregulating the expression of pro-apoptotic gene *rpr* in the loser cells of wing discs during cell competition [92]. However, we have provided evidence indicating that Toll pathway-mediated cell death is mostly caspase-independent: (i) loss of Toll signalling does not suppress *Sd>Hep* induced transcriptional upregulation of *hid* in the wing disc (figure 5o–r); (ii) loss of Toll signalling does not affect *GMR>Hid* induced small eye phenotype (electronic supplementary material, figure S5); (iii) *GMR>Dorsal* or *GMR>Dif*-induced cell death in third-instar larval eye discs (electronic supplementary material, figure S10a–d) is independent of caspase activation (electronic supplementary material, figure S12a–c) and (iv) *GMR>Dorsal* and *GMR>Dif*-induced small eye phenotypes are not suppressed by blocking caspase signalling (electronic supplementary material, figure S12e–h,k–n). In addition, we found that Toll pathway-triggered cell death in the eye is independent of JNK or necroptosis (electronic supplementary material, figure S10e–l; S12i, j, o, p). Thus, activated Toll signalling may promote cell death via distinct mechanisms in a context-dependent manner.

4. Material and methods

4.1. Fly strains

Flies were kept on a cornmeal and agar medium at 25°C according to standard protocols. *Drosophila* strains used include: *Toll^{r3}*, *Toll^{r4}*, *Dif¹*, *spz²*, *spz³*, *imd¹*, *relish^{E38}*, *UAS-Toll-IR* (31044, 41477 and 35628), *UAS-pelle-IR* (34733 and 35577), *Spz6-GFP* (23305), *UAS-mGFP* (32197), *UAS-mRFP* (32218), *UAS-Dorsal* (9319), *UAS-Dif* (22201), *UAS-dJun* (7216), *UAS-dFos* (7213), *UAS-dFoxO*, *UAS-DIAP1*, *UAS-Bsk*, *Df(3L)H99*, *Cg-GAL4* (7011), *Tub-GAL80^{ts}* (7017), *Drs-GFP*, *Dipt-LacZ* (55707), *yw¹¹¹⁸ hs-Flp; act>y + >GAL4 UAS-GFP*, these and the deficiency kit were obtained from Bloomington *Drosophila* stock centre. *UAS-puc-IR* (3018 and 3019), *UAS-dorsal-IR* (45996 and 45998), *UAS-Dif-IR* (30578 and 30579) and *UAS-relish-IR* (49413 and 49414) were obtained from Vienna *Drosophila* RNAi centre. *UAS-tube-IR* (105520R1 and 10520R3), *UAS-imd-IR* (5576R1 and 5576R2), *UAS-cactus-IR* (5848R1 and 5848R3) and *UAS-bsk-IR* (5680R2) were obtained from Fly Stocks of National Institute of Genetics (NIG). *GMR-GAL4* [56], *Sd-GAL4*, *ptc-GAL4* [57], *UAS-Toll^{10B}* [93], *Toll^{EP(3)1051}* [94], *UAS-Egr*, *UAS-Egr^W* [29], *UAS-Egr^{KB}* [30], *UAS-Bsk^{DN}* [31], *UAS-Puc*, *sev-GAL4*, *UAS-dTAK1*, *UAS-Hep^{CA}*, *UAS-Hep*, *UAS-Grim^{M146}*, *UAS-Hid^{M137}*, *UAS-Imd.SN^{F32}*, *UAS-Dronc^{DN}* [29], *UAS-P35*, *UAS-GFP* [41,42,52], *hid-LacZ* [40], *dTRAF2^{EX1.1}* and *UAS-dTRAF2-IR* were previously described.

4.2. Acridine orange staining

Eye and wing discs were dissected from third-instar larvae in 0.3% PBST (phosphate-buffered saline (PBS) + 0.3% Triton X-100) and incubated in 1×10^{-5} M AO for 5 min at room temperature prior to imaging as described [40].

4.3. X-gal staining

Wing discs were dissected from third-instar larvae in 0.1% PBST (PBS + 0.1% Triton X-100) and stained for β -galactosidase activity as described [95].

4.4. Immunohistochemistry

Fat body and eye discs dissected from third-instar larvae were collected in cold PBS, and fixed in 4% paraformaldehyde. After fixation, samples were washed three times in 0.3% PBST, blocked in 10% horse serum and stained with primary antibody overnight at 4°C. Samples were washed as previously described and subjected to secondary antibodies for 2 h. Primary antibodies used included mouse anti-dorsal (1:100, Cell Signaling Technology), mouse anti-Dlg (1:200, DSHB), mouse anti-NimC1 (1:200, kind gift of I. Ando) and rabbit anti-Cleaved Caspase-3 (1:200, Cell Signaling Technology). Secondary antibodies used were anti-mouse-Cy3 (1:1000, Jackson Immuno Research) and anti-rabbit-Cy3 (1:1000, Cell Signaling Technology).

4.5. Quantitative reverse transcription polymerase chain reaction

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 [96]. Primers for *Rp49* and *spz1–6* were kindly provided by Dr Ketu Mishra at Yale University.

5. Statistics

Results are presented as bar graphs or scatter plots created using GRAPHPAD PRISM 6.0. A combination of unpaired *t*-test and one-way ANOVA with Bonferroni's multiple comparison test was used to calculate statistical significance; *p*-values are included in the relevant figure legends.

Authors' contributions. C.W. and L.X. conceived and designed the experiments. C.W., C.C., J.D., F.Z., Y.C. and W.L. conducted the experiments. C.W. J.C.P.-P. and L.X. analysed the data, C.W. and L.X. wrote the manuscript. All authors gave final approval for publication.

Competing interests. The authors declare no conflict of interest.

Funding. This work was supported by the National Basic Research Program of China (973 Program) (2011CB943903), National Natural Science Foundation of China (31071294, 31171413, 31371490), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20120072110023) and Shanghai Committee of Science and Technology (09DZ2260100, 14JC1406000) to L.X., and National Nature Science Foundation of China (31371459), the Tsinghua Initiative Program (20131089281) and the 1000 Talents award to J.C.P.-P.

Acknowledgements. We thank Bloomington, VDRC and NIG stock centres for fly stocks, Dr Ketu Mishra (Yale University) for *spz1–6* primers, and members of the Xue laboratory, Margaret Ho and Ying Cao for discussion and critical comments.

- Gerttula S, Jin YS, Anderson KV. 1988 Zygotic expression and activity of the *Drosophila* Toll gene, a gene required maternally for embryonic dorsal–ventral pattern formation. *Genetics* **119**, 123–133.
- Hashimoto C, Hudson KL, Anderson KV. 1988 The Toll gene of *Drosophila*, required for dorsal–ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269–279. (doi:10.1016/0092-8674(88)90516-8)
- Steward R. 1987 Dorsal, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, *c-rel*. *Science* **238**, 692–694. (doi:10.1126/science.3118464)
- Roth S, Hiromi Y, Godt D, Nusslein-Volhard C. 1991 *cactus*, a maternal gene required for proper formation of the dorsoventral morphogen gradient in *Drosophila* embryos. *Development* **112**, 371–388.
- Letsou A, Alexander S, Orth K, Wasserman SA. 1991 Genetic and molecular characterization of *tube*, a *Drosophila* gene maternally required for embryonic dorsoventral polarity. *Proc. Natl Acad. Sci. USA* **88**, 810–814. (doi:10.1073/pnas.88.3.810)
- Hecht PM, Anderson KV. 1993 Genetic characterization of *tube* and *pelle*, genes required for signaling between Toll and dorsal in the specification of the dorsal-ventral pattern of the *Drosophila* embryo. *Genetics* **135**, 405–417.
- Shelton CA, Wasserman SA. 1993 *pelle* encodes a protein kinase required to establish dorsoventral polarity in the *Drosophila* embryo. *Cell* **72**, 515–525. (doi:10.1016/0092-8674(93)90071-W)
- Morisato D, Anderson KV. 1994 The *spatzle* gene encodes a component of the extracellular signaling pathway establishing the dorsal–ventral pattern of the *Drosophila* embryo. *Cell* **76**, 677–688. (doi:10.1016/0092-8674(94)90507-X)
- Schneider DS, Jin Y, Morisato D, Anderson KV. 1994 A processed form of the *Spatzle* protein defines dorsal–ventral polarity in the *Drosophila* embryo. *Development* **120**, 1243–1250.
- Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996 The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983. (doi:10.1016/S0092-8674(00)80172-5)
- Hetru C, Hoffmann JA. 2009 NF- κ B in the immune response of *Drosophila*. *Cold Spring Harb. Perspect. Biol.* **1**, a000232. (doi:10.1101/cshperspect.a000232)
- Aggarwal K, Silverman N. 2008 Positive and negative regulation of the *Drosophila* immune response. *BMB Rep.* **41**, 267–277. (doi:10.5483/BMBRep.2008.41.4.267)
- De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. 2002 The Toll and *lmd* pathways are the major regulators of the immune response in *Drosophila*. *EMBO J.* **21**, 2568–2579. (doi:10.1093/emboj/21.11.2568)
- Horng T, Medzhitov R. 2001 *Drosophila* MyD88 is an adapter in the Toll signaling pathway. *Proc. Natl Acad. Sci. USA* **98**, 12 654–12 658. (doi:10.1073/pnas.231471798)
- Sun H, Bristow BN, Qu G, Wasserman SA. 2002 A heterotrimeric death domain complex in Toll signaling. *Proc. Natl Acad. Sci. USA* **99**, 12 871–12 876. (doi:10.1073/pnas.202396399)
- Towb P, Bergmann A, Wasserman SA. 2001 The protein kinase *Pelle* mediates feedback regulation in the *Drosophila* Toll signaling pathway. *Development* **128**, 4729–4736.
- Wu LP, Anderson KV. 1998 Regulated nuclear import of Rel proteins in the *Drosophila* immune response. *Nature* **392**, 93–97. (doi:10.1038/32195)
- Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. 1999 Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318. (doi:10.1126/science.284.5418.1313)
- Silverman N, Maniatis T. 2001 NF- κ B signaling pathways in mammalian and insect innate immunity. *Genes Dev.* **15**, 2321–2342. (doi:10.1101/gad.909001)
- Barton GM, Medzhitov R. 2003 Toll-like receptor signaling pathways. *Science* **300**, 1524–1525. (doi:10.1126/science.1085536)
- Valanne S, Wang JH, Ramet M. 2011 The *Drosophila* Toll signaling pathway. *J. Immunol.* **186**, 649–656. (doi:10.4049/jimmunol.1002302)
- Belvin MP, Anderson KV. 1996 A conserved signaling pathway: the *Drosophila* Toll–Dorsal pathway. *Annu. Rev. Cell Dev. Biol.* **12**, 393–416. (doi:10.1146/annurev.cellbio.12.1.393)
- Kimbrell DA, Beutler B. 2001 The evolution and genetics of innate immunity. *Nat. Rev. Genet.* **2**, 256–267. (doi:10.1038/35066006)
- Davis RJ. 2000 Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239–252. (doi:10.1016/S0092-8674(00)00116-1)
- Weston CR, Davis RJ. 2002 The JNK signal transduction pathway. *Curr. Opin. Genet. Dev.* **12**, 14–21. (doi:10.1016/S0959-437X(01)00258-1)
- Weston CR, Davis RJ. 2007 The JNK signal transduction pathway. *Curr. Opin. Cell Biol.* **19**, 142–149. (doi:10.1016/j.ccb.2007.02.001)
- Dhanasekaran DN, Reddy EP. 2008 JNK signaling in apoptosis. *Oncogene* **27**, 6245–6251. (doi:10.1038/onc.2008.301)
- Andersen DS *et al.* 2015 The *Drosophila* TNF receptor Grindelwald couples loss of cell polarity and neoplastic growth. *Nature* **522**, 482–486. (doi:10.1038/nature14298)
- Igaki T *et al.* 2002 Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway. *EMBO J.* **21**, 3009–3018. (doi:10.1093/emboj/cdf306)
- Moreno E, Yan M, Basler K. 2002 Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the *Drosophila* homolog of the TNF superfamily. *Curr. Biol.* **12**, 1263–1268. (doi:10.1016/S0960-9822(02)00954-5)
- Xue L, Igaki T, Kuranaga E, Kanda H, Miura M, Xu T. 2007 Tumor suppressor CYLD regulates JNK-induced cell death in *Drosophila*. *Dev. Cell.* **13**, 446–454. (doi:10.1016/j.devcel.2007.07.012)
- Takatsu Y, Nakamura M, Stapleton M, Danos MC, Matsumoto K, O'Connor MB, Shibuya H, Ueno N. 2000 TAK1 participates in c-Jun N-terminal kinase signaling during *Drosophila* development. *Mol. Cell Biol.* **20**, 3015–3026. (doi:10.1128/MCB.20.9.3015-3026.2000)
- Glise B, Bourbon H, Noselli S. 1995 *hemipterous* encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. *Cell* **83**, 451–461. (doi:10.1016/0092-8674(95)90123-X)
- Kanda H, Igaki T, Kanuka H, Yagi T, Miura M. 2002 Wengen, a member of the *Drosophila* tumor necrosis factor receptor superfamily, is required for Eiger signaling. *J. Biol. Chem.* **277**, 28 372–28 735. (doi:10.1074/jbc.C200324200)
- Kaupilla S *et al.* 2003 Eiger and its receptor, Wengen, comprise a TNF-like system in *Drosophila*. *Oncogene* **22**, 4860–4867. (doi:10.1038/sj.onc.1206715)
- Luo X, Puig O, Hyun J, Bohmann D, Jasper H. 2007 Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation. *EMBO J.* **26**, 380–390. (doi:10.1038/sj.emboj.7601484)
- Kockel L, Homsy JG, Bohmann D. 2001 *Drosophila* AP-1: lessons from an invertebrate. *Oncogene* **20**, 2347–2364. (doi:10.1038/sj.onc.1204300)
- Accili D, Arden KC. 2004 FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* **117**, 421–426. (doi:10.1016/S0092-8674(04)00452-0)
- Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, Burgering BMT. 2004 FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J.* **23**, 4802–4812. (doi:10.1038/sj.emboj.7600476)
- Ma X, Huang J, Yang L, Yang Y, Li W, Xue L. 2012 NOPO modulates Egr-induced JNK-independent cell death in *Drosophila*. *Cell Res.* **22**, 425–431. (doi:10.1038/cr.2011.135)
- Ma X, Shao Y, Zheng H, Li M, Li W, Xue L. 2013 Src42A modulates tumor invasion and cell death via Ben/dUev1a-mediated JNK activation in *Drosophila*. *Cell Death Dis.* **4**, e864. (doi:10.1038/cddis.2013.392)
- Ma X, Yang L, Yang Y, Li M, Li W, Xue L. 2013 dUev1a modulates TNF-JNK mediated tumor progression and cell death in *Drosophila*. *Dev. Biol.* **380**, 211–221. (doi:10.1016/j.ydbio.2013.05.013)
- Geuking P, Narasimamurthy R, Basler K. 2005 A genetic screen targeting the tumor necrosis factor/Eiger signaling pathway: identification of *Drosophila* TAB2 as a functionally conserved component. *Genetics* **171**, 1683–1694. (doi:10.1534/genetics.105.045534)
- Kanda H, Igaki T, Okano H, Miura M. 2011 Conserved metabolic energy production pathways govern Eiger/TNF-induced nonapoptotic cell death.

- Proc. Natl Acad. Sci. USA* **108**, 18 977–18 982. (doi:10.1073/pnas.1103242108)
45. Bangi E, Pitsouli C, Rahme LG, Cagan R, Apidianakis Y. 2012 Immune response to bacteria induces dissemination of Ras-activated *Drosophila* hindgut cells. *EMBO Rep.* **13**, 569–576. (doi:10.1038/embor.2012.44)
 46. Seong KM *et al.* 2012 Low-dose radiation induces *Drosophila* innate immunity through Toll pathway activation. *J. Radiat. Res.* **53**, 242–249. (doi:10.1269/jrr.11170)
 47. Parisi F, Stefanatos RK, Strathdee K, Yu Y, Vidal M. 2014 Transformed epithelia trigger non-tissue-autonomous tumor suppressor response by adipocytes via activation of Toll and Eiger/TNF signaling. *Cell Rep.* **6**, 855–867. (doi:10.1016/j.celrep.2014.01.039)
 48. Ganesan S, Aggarwal K, Paquette N, Silverman N. 2011 NF- κ B/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. *Curr. Top. Microbiol. Immunol.* **349**, 25–60. (doi:10.1007/82_2010_107)
 49. Boutros M, Agaisse H, Perrimon N. 2002 Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev. Cell.* **3**, 711–722. (doi:10.1016/S1534-5807(02)00325-8)
 50. Lemaitre B, Hoffmann J. 2007 The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **25**, 697–743. (doi:10.1146/annurev.immunol.25.022106.141615)
 51. Abrams JM, White K, Fessler LI, Steller H. 1993 Programmed cell death during *Drosophila* embryogenesis. *Development* **117**, 29–43.
 52. Ma X, Li W, Yu H, Yang Y, Li M, Xue L, Xu T. 2014 Bendless modulates JNK-mediated cell death and migration in *Drosophila*. *Cell Death and Differ.* **21**, 407–415. (doi:10.1038/cdd.2013.154)
 53. Hoffmann JA, Reichhart JM. 2002 *Drosophila* innate immunity: an evolutionary perspective. *Nat. Immunol.* **3**, 121–126. (doi:10.1038/ni0202-121)
 54. Vidal S, Khush RS, Leulier F, Tzou P, Nakamura M, Lemaitre B. 2001 Mutations in the *Drosophila* *dTAK1* gene reveal a conserved function for MAPKKs in the control of rel/NF- κ B-dependent innate immune responses. *Genes Dev.* **15**, 1900–1912. (doi:10.1101/gad.203301)
 55. Delaney JR, Stoven S, Uvell H, Anderson KV, Engstrom Y, Mlodzik M. 2006 Cooperative control of *Drosophila* immune responses by the JNK and NF- κ B signaling pathways. *EMBO J.* **25**, 3068–3077. (doi:10.1038/sj.emboj.7601182)
 56. Li WZ, Li SL, Zheng HY, Zhang SP, Xue L. 2012 A broad expression profile of the GMR-GAL4 driver in *Drosophila melanogaster*. *Genet Mol Res.* **11**, 1997–2002. (doi:10.4238/2012.August.6.4)
 57. Wang X, Wang Z, Chen Y, Huang X, Hu Y, Zhang R, Ho MS, Xue L. 2014 FoxO mediates APP-induced AICD-dependent cell death. *Cell Death Dis.* **5**, e1233. (doi:10.1038/cddis.2014.196)
 58. Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, Radimerski T, Greenberg ME, Hafen E. 2003 The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J. Biol.* **2**, 20. (doi:10.1186/1475-4924-2-20)
 59. Steller H. 2008 Regulation of apoptosis in *Drosophila*. *Cell Death Differ.* **15**, 1132–1138. (doi:10.1038/cdd.2008.50)
 60. Shlevkov E, Morata G. 2012 A dp53/JNK-dependant feedback amplification loop is essential for the apoptotic response to stress in *Drosophila*. *Cell Death Differ.* **19**, 451–460. (doi:10.1038/cdd.2011.113)
 61. Dick SA, Megeney LA. 2013 Cell death proteins: an evolutionary role in cellular adaptation before the advent of apoptosis. *BioEssays* **35**, 974–983. (doi:10.1002/bies.201300052)
 62. Kumar S. 2007 Caspase function in programmed cell death. *Cell Death Differ.* **14**, 32–43. (doi:10.1038/sj.cdd.4402060)
 63. Martin-Blanco E, Gampel A, Ring J, Virdee K, Kirov N, Tolkovsky AM, Martinez-Arias A. 1998 *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev.* **12**, 557–570. (doi:10.1101/gad.12.4.557)
 64. Ferrandon D *et al.* 1998 A drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J.* **17**, 1217–1227. (doi:10.1093/emboj/17.5.1217)
 65. Dijkers PF, O'Farrell PH. 2007 *Drosophila* calcineurin promotes induction of innate immune responses. *Curr. Biol.* **17**, 2087–2093. (doi:10.1016/j.cub.2007.11.001)
 66. Kim T, Kim YJ. 2005 Overview of innate immunity in *Drosophila*. *J. Biochem. Mol. Biol.* **38**, 121–127. (doi:10.5483/BMBRep.2005.38.2.121)
 67. Tanji T, Ip YT. 2005 Regulators of the Toll and Imd pathways in the *Drosophila* innate immune response. *Trends Immunol.* **26**, 193–198. (doi:10.1016/j.it.2005.02.006)
 68. Myllymaki H, Valanne S, Ramet M. 2014 The *Drosophila* imd signaling pathway. *J. Immunol.* **192**, 3455–3462. (doi:10.4049/jimmunol.1303309)
 69. Lemaitre B *et al.* 1995 Functional analysis and regulation of nuclear import of dorsal during the immune response in *Drosophila*. *EMBO J.* **14**, 536–545.
 70. Arnot CJ, Gay NJ, Gangloff M. 2010 Molecular mechanism that induces activation of Spatzle, the ligand for the *Drosophila* Toll receptor. *J. Biol. Chem.* **285**, 19 502–19 509. (doi:10.1074/jbc.M109.098186)
 71. Parker JS, Mizuguchi K, Gay NJ. 2001 A family of proteins related to Spatzle, the toll receptor ligand, are encoded in the *Drosophila* genome. *Proteins* **45**, 71–80. (doi:10.1002/prot.1125)
 72. Bellen HJ *et al.* 2004 The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* **167**, 761–781. (doi:10.1534/genetics.104.026427)
 73. Shia AK, Glittenberg M, Thompson G, Weber AN, Reichhart JM, Ligoxygakis P. 2009 Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spatzle secreted by haemocytes. *J. Cell Sci.* **122**, 4505–4515. (doi:10.1242/jcs.049155)
 74. Kurucz E *et al.* 2007 Nimrod, a putative phagocytosis receptor with EGF repeats in *Drosophila* plasmatocytes. *Curr. Biol.* **17**, 649–654. (doi:10.1016/j.cub.2007.02.041)
 75. Haynie JL, Bryant PJ. 1986 Development of the eye-antenna imaginal disc and morphogenesis of the adult head in *Drosophila melanogaster*. *J. Exp. Zool.* **237**, 293–308. (doi:10.1002/jez.1402370302)
 76. Gibson MC, Schubiger G. 2000 Peripodial cells regulate proliferation and patterning of *Drosophila* imaginal discs. *Cell* **103**, 343–350. (doi:10.1016/S0092-8674(00)00125-2)
 77. Cho KO, Chern J, Izaddoost S, Choi KW. 2000 Novel signaling from the peripodial membrane is essential for eye disc patterning in *Drosophila*. *Cell* **103**, 331–342. (doi:10.1016/S0092-8674(00)00124-0)
 78. Fernandez NQ, Grosshans J, Goltz JS, Stein D. 2001 Separable and redundant regulatory determinants in Cactus mediate its dorsal group dependent degradation. *Development* **128**, 2963–2974.
 79. Rodriguez-Berriguete G *et al.* 2013 Clinical significance of both tumor and stromal expression of components of the IL-1 and TNF- α signaling pathways in prostate cancer. *Cytokine* **64**, 555–563. (doi:10.1016/j.cyto.2013.09.003)
 80. Linkermann A, Green DR. 2014 Necroptosis. *N. Engl. J. Med.* **370**, 455–465. (doi:10.1056/NEJMr1310050)
 81. Vanlangenakker N, Bertrand MJ, Bogaert P, Vandenebeebe P, Vanden Berghe T. 2011 TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. *Cell Death Dis.* **2**, e230. (doi:10.1038/cddis.2011.111)
 82. Holler N *et al.* 2000 Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat. Immunol.* **1**, 489–495. (doi:10.1038/82732)
 83. Degterev A *et al.* 2008 Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* **4**, 313–321. (doi:10.1038/nchembio.83)
 84. Christofferson DE, Yuan J. 2010 Necroptosis as an alternative form of programmed cell death. *Curr. Opin. Cell Biol.* **22**, 263–268. (doi:10.1016/j.ceb.2009.12.003)
 85. Su Z, Yang Z, Xu Y, Chen Y, Yu Q. 2015 Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol. Cancer* **14**, 48. (doi:10.1186/s12943-015-0321-5)
 86. Saeed WK, Jun DW. 2014 Necroptosis: an emerging type of cell death in liver diseases. *World J. Gastroenterol.* **20**, 12 526–12 532. (doi:10.3748/wjg.v20.i35.12526)
 87. Obata F *et al.* 2014 Necrosis-driven systemic immune response alters SAM metabolism through the FOXO-GNMT axis. *Cell Rep.* **7**, 821–833. (doi:10.1016/j.celrep.2014.03.046)
 88. Park SM, Yoon JB, Lee TH. 2004 Receptor interacting protein is ubiquitinated by cellular inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) in vitro. *FEBS Lett.* **566**, 51–56. (doi:10.1016/j.febslet.2004.04.021)
 89. Haas TL *et al.* 2009 Recruitment of the linear ubiquitin chain assembly complex stabilizes the

- TNF-R1 signaling complex and is required for TNF-mediated gene induction. *Mol. Cell.* **36**, 831–844. (doi:10.1016/j.molcel.2009.10.013)
90. Zhu B *et al.* 2008 *Drosophila* neurotrophins reveal a common mechanism for nervous system formation. *PLoS Biol.* **6**, e284. (doi:10.1371/journal.pbio.0060284)
91. Ming M, Obata F, Kuranaga E, Miura M. 2014 Persephone/Spatzle pathogen sensors mediate the activation of Toll receptor signaling in response to endogenous danger signals in apoptosis-deficient *Drosophila*. *J. Biol. Chem.* **289**, 7558–7568. (doi:10.1074/jbc.M113.543884)
92. Meyer SN *et al.* 2014 An ancient defense system eliminates unfit cells from developing tissues during cell competition. *Science* **346**, 1258236. (doi:10.1126/science.1258236)
93. Hu X, Yagi Y, Tanji T, Zhou S, Ip YT. 2004 Multimerization and interaction of Toll and Spatzle in *Drosophila*. *Proc. Natl Acad. Sci. USA* **101**, 9369–9374. (doi:10.1073/pnas.0307062101)
94. Pena-Rangel MT, Rodriguez I, Riesgo-Escovar JR. 2002 A misexpression study examining dorsal thorax formation in *Drosophila melanogaster*. *Genetics* **160**, 1035–1050.
95. Xue L, Noll M. 2000 *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. *Proc. Natl Acad. Sci. USA* **97**, 3272–3275. (doi:10.1073/pnas.97.7.3272)
96. Wang MC, Bohmann D, Jasper H. 2003 JNK signaling confers tolerance to oxidative stress and extends lifespan in *Drosophila*. *Dev. Cell.* **5**, 811–816. (doi:10.1016/S1534-5807(03)00323-X)