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DOI:
10.1038/s41419-019-1862-0

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Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Li, M, Sun, S, Priest, J, Bi, X & Fan, Y 2019, 'Characterization of TNF-induced cell death in Drosophila reveals caspase- and JNK-dependent necrosis and its role in tumor suppression', Cell death & disease, vol. 10, no. 8, 613. https://doi.org/10.1038/s41419-019-1862-0

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Checked for eligibility: 04/09/2019

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Characterization of TNF-induced cell death in *Drosophila* reveals caspase- and JNK-dependent necrosis and its role in tumor suppression

**Mingli Li¹, Shiyao Sun¹, Jessica Priest¹, Xiaolin Bi² and Yun Fan¹**

**Abstract**

Tumor-necrosis factor (TNF) and its superfamily members are pleiotropic cytokines. Activation of TNF can lead to distinct cellular outcomes including inflammation, cell survival, and different forms of cell death, such as apoptosis and necrosis in a context-dependent manner. However, our understanding of what determines the versatile functions of TNF is far from complete. Here, we examined the molecular mechanisms that distinguish the forms of cell death induced by Eiger (Egr), the sole homolog of TNF in *Drosophila*. We show that expression of Egr in the developing *Drosophila* eye simultaneously induces apoptosis and apoptosis-independent developmental defects indicated by cellular disorganization, both of which rely on the c-Jun N-terminal kinase (JNK) signaling activity. Intriguingly, when effector caspases DrICE and Dcp-1 are defective or inhibited, expression of Egr triggers necrosis which is characterized by loss of cell membrane integrity, translucent cytoplasm, and aggregation of cellular organelles. Moreover, such Egr-induced necrosis depends on the catalytic activity of the initiator caspase Dronc and the input from JNK signaling but is independent of their roles in apoptosis. Further mosaic analysis with mutants of *scribble* (*scrib*), an evolutionarily conserved tumor suppressor gene regulating cell polarity, suggests that Egr/JNK-mediated apoptosis and necrosis establish a two-layered defense system to inhibit the oncogenic growth of *scrib* mutant cells. Together, we have identified caspase- and JNK-dependent mechanisms underlying Egr-induced apoptosis versus necrosis and their fail-safe roles in tumor suppression in an intact organism in vivo.

**Introduction**

Apoptosis is a major form of programmed cell death critical for development and damage response. The key factors driving apoptosis are caspases, a family of cysteine proteases. These apoptotic caspases can further group into initiator and effector caspases. The initiator caspases, once activated, cleave and activate the effector caspases leading to apoptosis. Unlike apoptosis, necrosis has long been considered as an uncontrolled form of cell death. However, recent studies have revealed that certain types of necrosis are molecularly regulated. Intriguingly, our growing understanding of apoptosis and regulated necrosis has unveiled multiple molecular interplays between them. One example of such molecules is the tumor-necrosis factor (TNF) superfamily, a group of cytokines which was initially discovered because of its antitumor activity. Binding of TNF to their receptors promotes recruitment of various death-inducing protein complexes depending on the context. A key component common in these complexes is caspase-8, an initiator caspase. Once activated, caspase-8 cleaves and activates effector caspases, such as caspase-3 and −7, to trigger

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apotosis. Notably, caspase-8 also cleaves and inactivates two receptor-interacting serine/threonine-protein kinases (RIPks), RIPK1, and RIPK3, which mediate activation of necroptosis, a form of regulated necrosis. Therefore, when Caspase-8 is deficient or inhibited, activation of TNF induces necrosis via RIPK1 and RIPK3.

In addition to cell death, functions of TNF family members have been revealed in immunity, inflammation, cell survival, and proliferation. Many of these functions are mediated by pleiotropic molecules, such as the Nuclear Factor-kB (NF-kB) transcription factor, the c-Jun N-terminal kinase (JNK), and the p38 mitogen-activated protein kinase (p38-MAPK). It is therefore not surprising that dysregulated TNF signaling is associated with numerous pathological conditions including cancer, inflammation, and host defense, tissue growth, and regeneration in a context.

On the key components in the apoptosis pathway. Loss of Egr induces apoptosis or nonapoptotic cell death, we first sought to identify a marker specifically recognizing activated effector caspases, e.g., the cleaved DrICE and Dcp-1, in Drosophila because antibodies recognizing the cleaved human caspase-3 are not specific to these proteins. A recently developed cleaved Dcp-1 (Asp216) antibody (referred to as cDcp1) from Cell Signaling Technology is a polyclonal antibody raised against the large 22 kDa fragment of cleaved Dcp-1. Although this antibody has been increasingly used to label apoptosis in Drosophila, a detailed characterization of its specificity has not been performed. To address this, we used GMR-hid, a transgene activating apoptosis in the eye. In late 3rd instar larval eye disks, compared with wild type, cDcp1-labeling was observed in dcp-1 null mutants (Fig. 1e). Because DrICE and Dcp-1 share similar sequences cleaved by Dronc, it is possible that cDcp1 also recognizes the cleaved DrICE. Consistent with this idea, cDcp1 detects a relatively low level of proteins, presumably the cleaved DrICE, in dcp-1 null mutants (Fig. 1e).

Results
The cleaved Dcp-1 antibody is a specific marker for activated effector caspases DrICE and Dcp-1 in Drosophila
To determine whether GMR > Egr induces apoptosis or nonapoptotic cell death, we first sought to identify a marker specifically recognizing activated effector caspases, e.g., the cleaved DrICE and Dcp-1, in Drosophila because antibodies recognizing the cleaved human caspase-3 are not specific to these proteins. A recently developed cleaved Dcp-1 (Asp216) antibody (referred to as cDcp1) from Cell Signaling Technology is a polyclonal antibody raised against the large 22 kDa fragment of cleaved Dcp-1. Although this antibody has been increasingly used to label apoptosis in Drosophila, a detailed characterization of its specificity has not been performed. To address this, we used GMR-hid, a transgene activating apoptosis in the eye. In late 3rd instar larval eye disks, compared with wild type, cDcp1-labeling was observed in dcp-1 null mutants (Fig. 1e). Because DrICE and Dcp-1 share similar sequences cleaved by Dronc, it is possible that cDcp1 also recognizes the cleaved DrICE. Consistent with this idea, cDcp1 detects a relatively low level of proteins, presumably the cleaved DrICE, in dcp-1 null mutants (Fig. 1e).

Expression of Eiger induces strong apoptosis through the canonical apoptosis pathway and the pro-apoptotic gene hid
It has been reported that GMR > egr activates JNK resulting in an eye ablation phenotype (compare 2b to 2a), which can be suppressed by RNAi knockdown of egr (Fig. 2c) or expression of puckered (puc) (Fig. 2d), a negative regulator of JNK. To assess whether GMR > egr induces apoptosis, we used the cDcp1 antibody. Compared with wild type, a strong wave of cDcp1-labeling was observed in GMR > egr disks (Fig. 2f). To confirm it is apoptosis, we performed genetic analyses on the key components in the apoptosis pathway. Loss of Dronc, the major initiator caspase mediating apoptosis in Drosophila, or expression of P35, an inhibitor of activated DrICE and Dcp-1 (ref. 26), completely blocks the cDcp1 signals in GMR > egr (Fig. 2g, h). Consistently,
GMR > egr-induced eye ablation phenotype is suppressed in dronc null mutants (compare Fig. 2m to 2b). Moreover, expression of a RING domain-deleted (therefore stabilized) form of Diap1 (BIR), an apoptosis inhibitor acting upstream of Dronc37, also suppresses GMR > egr small eyes (compare Fig. 2l to 2b). Therefore, GMR > egr triggers massive apoptosis in the developing Drosophila eye.

To identify which proapoptotic genes mediate GMR > egr-induced apoptosis, we examined expression of hid and rpr by using their reporters38. Compared with the control, expression of hid, but not rpr, significantly increases in GMR > egr (Supplementary Fig. S1). Moreover, loss of rpr in rpr87/XR38, a combination of a deletion39 and a null mutant of rpr (ref.40), does not affect GMR > egr-induced apoptosis (Fig. 2j). In contrast, such apoptosis is lost in hid mutant clones (Fig. 2i–i`). Consistent with these results, the hid mutant clones, but not rpr mutants, suppress GMR > egr-induced small eyes (Fig. 2k, n). Taken together, expression of Egr activates apoptosis through the proapoptotic gene hid in the developing Drosophila eye.

Expression of Eiger induces developmental defects independent of apoptosis

Notably, unlike expression of puc (Fig. 2d), loss of dronc or expression of Diap1 (GMR > BIR) does not completely restore the GMR > egr eyes back to normal. These eyes show glassy appearance suggesting defects in their ommatidial patterning (Fig. 2l, m). In contrast, expression of a dominant negative form of bsk (bskDN, bsk = Drosophila JNK) or hemizygous mutants of Tak1 (Tak12527, null mutant41), an upstream kinase of JNK, almost completely rescue GMR > egr-induced adult eye defects including the glassy eye appearance (Fig. 3a, b). Therefore, GMR > egr also induces apoptosis-independent, but JNK-dependent, developmental defects (Fig. 3c).

To further characterize this nonapoptotic developmental defects, we examined cellular organization in the pupal eye disks, which are composed of well-patterned ommatidia (Fig. 3d, d`, ELAV labeling) and
interommatidial cells (Fig. 3d, d'). At 25 °C, developmental apoptosis occurs at 28 h after pupal formation (APF28h) to remove extra interommatidial cells in eye disks (Supplementary Fig. S2). In contrast, no apoptosis is observed at APF22h (Fig. 4a, a'). We therefore focused our analysis of GMR > egr on the stage of APF22h.
to avoid developmental apoptosis. Compared with wild-type eye disks in which ommatidia and interommatidial cells are well-patterned (Fig. 3d–d″), GMR > egr induces disorganization of these cells (Fig. 3e–e″) indicated by both increased interommatidial spacing (Fig. 3e″, arrows) and ommatidial fusion (Fig. 3e″, arrowheads). Such defects persist when apoptosis is inhibited in dronc mutants (Fig. 3g–g″) or by expression of Diap1 (Fig. 3i–i″). As controls, ommatidial organization is not affected by loss of dronc (Fig. 3f–f″) or expression of Diap1 (Fig. 3h–h″) alone. Moreover, GMR > egr-induced ommatidial organization defects are strongly suppressed by expression of bskON (Fig. 3j–j″) or in hemizygous mutants of Tak1 (Fig. 3k–k″). Altogether, these data suggest that expression of egr induces both apoptosis and nonapoptotic, but JNK-dependent, cellular disorganization in the developing eye (Fig. 3c).

Inhibition of effector caspases in GMR > egr induces necrosis

One intriguing observation is that, unlike dronc mutants or expression of Diap1, expression of P35, an inhibitor of effector caspases, cannot or only slightly suppresses the GMR > egr-induced eye ablation phenotype (compare Fig. 4d to Fig. 2b, quantified in Fig. 6f). One possible explanation is that P35 may not be sufficient to block GMR > egr-induced apoptosis because it is a pseudosubstrate of effector caspases. We examined this possibility. Compared with wild type (Fig. 4a, a′), GMR >

![Fig. 3 GMR > egr induces nonapoptotic, but JNK-dependent, cellular disorganization in the Drosophila eye. a, b Adult eye images. Expression of a dominant negative form of bsk (bskDN, bsk = Drosophila JNK) (a) or a hemizygous mutant of Tak1 (Tak1−/−, b), an upstream kinase of JNK, almost completely suppresses GMR > egr-induced eye ablation phenotype. (c) A diagram showing that JNK signaling induced by Egr can lead to both apoptosis and nonapoptotic defects in the developing eye. APF22h pupal eye disks labeled with a cellular membrane marker Dlg (green in d–k and d′–k′) and a neuronal marker ELAV (red in d–k and d′–k′). In wild-type disks (d–d′), ommatidia (each is composed of eight photoreceptor neurons), as indicated by ELAV, and interommatidial cells, as indicated by Dlg, are well-patterned. In contrast, defective cellular organization was observed in GMR > egr disks (e–e″). Examples of these defects such as ommatidial fusion (arrowheads in e″, g″, i″) and increased interommatidial spacing (arrows in e″, g″, i″) are highlighted. dronc null mutants (f–f″, dronc−/−) or expression of a stabilized form of Diap1 (h–h″, GMR-BIR) neither alter the ommatidial patterning in wild-type eye disks (compare f–f″ and h–h″ to d–d″) nor suppress the irregular ommatidial organization in GMR > egr disks (compare g–g″ and i–i″ to e–e″). In contrast, expression of a dominant-negative form of JNK (bskDN, j–j″) or a hemizygous mutant of Tak1 (k–k″) strongly suppresses the cellular disorganization induced by GMR > egr.](image-url)
Diap1, expression of P35 in adult eyes (compare Fig. 4d to Fig. 2l, m). This is not due to any artefacts induced by P35 itself because double RNAi knockdown of drICE and dcp-1 are inhibited (APF22h pupal disks labeled with cDcp1 (green in a, b, c and gray in d, e, f) and ELAV (red in a, b, c), a neuronal marker. At this stage, no apoptotic cells were detected in wild-type disks (cDcp1, a). Ommatidia are also well-patterned (ELAV, a). In contrast, strong apoptosis was detected at APF22h in GMR > egr eye disks (cDcp1, b and b′). Cellular disorganization indicated by increased interommatidial spacing (arrows, b) and ommatidial fusion (arrowheads, b) was observed. Although GMR > egr-induced apoptosis is almost completely blocked by expression of P35 (cDcp1, c and c′), the irregular ommatidial organization (c, arrows and arrowheads) is not suppressed. d–f Adult eye images. Expression of P35 (GMR-p35, d), RNAi knockdown of drICE and dcp-1 (e), or drICE null mutants (f) do not or only slightly suppress GMR > egr-induced eye ablation phenotype (compare 4d, e, f to 2b). APF22h pupal eye disks labeled with Propidium Iodide (PI, green in g, h, i and gray in g′, h′, i′) and Hoechst (red in g, h, i and gray in g′, h′, i′). In GMR > egr disks, PI detects a background level of signals (arrows, g′–g′′) which often do not co-localize with the Hoechst labeling, a nucleus marker. In contrast, expression of P35 (GMR > egr/GMR-p35) results in a strong increase of PI-positive nuclei, majority of which are also Hoechst-positive (arrowheads, h–h′). Suppression of these PI signals in dronc heterozygous mutants can be reversed by expression of a wild-type dronc transgene (arrowheads, h′–h″). Asterisks indicate irregular cellular spacing caused by expression of Egr in the corresponding eye disks. j Quantification of PI-positive cell numbers in APF22h pupal eye disks of various genetic backgrounds as indicated. One-way ANOVA with Bonferroni multiple comparison test was used to compute p-values. Asterisks indicate statistically significant changes (***p < 0.0001). A background low level of PI-labeling was observed in both wild type and GMR > egr disks. This low PI-labeling in GMR > egr is not increased in dronc mutants or by expression of a stabilized form of Diap1 (GMR-DRi). The PI-labeling is also low in GMR-p35 disks. In contrast, strong PI-labeling was observed in GMR > egr/GMR-p35 disks. This PI-labeling is largely suppressed in dronc heterozygous mutants (GMR > egr/GMR-p35, dronc+/–). In this background, further expression of a wild-type form of Dronc (GMR > egr-dronc+/+GMR-p35, dronc+/–) but not a catalytic site-mutated form of Dronc (GMR > egr-droncC318A/GMR-p35, dronc+/–), is sufficient to restore the PI signals egr induces massive apoptosis and defective ommatidial patterning in pupal eye disks at APF22h (Fig. 4b, b′). Expression of P35 is sufficient to block apoptosis (Fig. 4c, c′). However, unlike dronc mutants or expression of Diap1, expression of P35 in GMR > egr still results in small adult eyes (compare Fig. 4d to Fig. 2l, m). This is not due to any artefacts induced by P35 itself because double RNAi knockdown of drICE and dcp-1 or drICE mutants do not suppress, or even enhance, GMR > egr-induced eye ablation phenotype (Fig. 4e, f). These RNAi lines and mutants are functional because they can suppress GMR-hid-induced apoptosis (Supplementary Fig. S3). These suggest that inhibition of effector caspases, particularly DrICE, in GMR > egr may induce another form of cell death while it suppresses apoptosis.

To further characterize this nonapoptotic cell death, we performed labeling with propidium iodide (PI), a dye enters a cell and binds to its DNA when the cell membrane is
disrupted. Because loss of membrane integrity is a hallmark of necrosis, PI has been used to label necrotic cells (Supplementary Fig. S4a,b). Interestingly, no PI-positive cells were detected in late 3rd instar larval GMR\textgreater egr disks with or without expression of P35 (Supplementary Fig. S4c, d). We then examined a later pupal stage (APF22h). In GMR\textgreater egr, a very low level of PI-labeling was observed (Fig. 4g, g'). Notably, most of these PI signals are unspecific because they do not co-localize with a DNA marker Hoechst (Fig. 4g, g''), suggesting that they specifically label membrane-compromised cells. As a control, expression of P35 alone does not induce PI-labeling (Fig. 4j). Altogether, these data suggest that Egr may induce necrosis when apoptosis is blocked.

To further confirm this, we performed the Transmission Electron Microscopy (TEM) analysis on GMR\textgreater egr/GMR-p35 pupal eye disks. Compared with the wild-type control (Fig. 5a), cells with typical apoptotic features, such as high-electron-density chromatin condensation and apoptotic bodies were frequently observed in GMR\textgreater egr disks (Fig. 5b, b'). In contrast, cells with typical necrotic features were observed in GMR\textgreater egr/GMR-p35 disks (Fig. 5c-c''). These cells have translucent cytoplasm, mal-shaped or unidentifiable nuclei, and aggregation of cellular organelles such as endoplasmic reticulum, which are characteristics of necrotic cell death\textsuperscript{40,49}. Therefore, necrosis is induced in GMR\textgreater egr/GMR-p35 pupal eye disks.

The initiator caspase Dronc mediates Eiger-induced necrosis

To determine how necrosis is activated when effector caspases are inhibited, we first examined PI-labeling in GMR\textgreater egr disks in a background of dronc mutants or with expression of the stabilized Diap1 (GMR-BIR). This is because both of them suppress GMR\textgreater egr-induced apoptosis upstream of effector caspases (Fig. 2l, m). Only a background level of PI-labeling was detected under these conditions (Fig. 4j). Strikingly, loss of one copy of dronc strongly suppresses the PI-labeling induced in GMR\textgreater egr/GMR-p35 (Fig. 4j). However, it only weakly affects GMR\textgreater egr-induced apoptosis (Fig. 6a, a') hence the eye ablation phenotype (Fig. 6b, h). Consistent with these observations, dronc heterozygous mutants strongly suppress the GMR\textgreater egr/GMR-p35 small eyes (Fig. 6c, h). Therefore, Dronc is a key component mediating necrosis when GMR\textgreater egr-induced apoptosis is inhibited.

It is known that Dronc, the initiator caspase, cleaves its downstream effector caspases, e.g., DrICE and Dcp-1, to activate apoptosis in Drosophila\textsuperscript{33,50}. We thus examined whether the catalytic activity of Dronc is required for necrosis induced in GMR\textgreater egr/GMR-p35. To do this,
either a wild-type Dronc (dronc\textsuperscript{wt}) or a catalytic site-mutated form of Dronc (dronc\textsuperscript{C318A}) transgene was used\textsuperscript{51}. As expected, expression of the wild-type Dronc in GMR\textsuperscript{}>egr/GMR-p35; dronc\textsuperscript{−/−} animals restores PI-positive necrosis (Fig. 4i–i′′, quantified in 4j) and, consequently, the eye ablation phenotype (Fig. 6d, h). In contrast, expression of dronc\textsuperscript{C318A} in the same background does not have such effects (Figs. 4i and 6e, h). As controls, expression of dronc\textsuperscript{wt} or dronc\textsuperscript{C318A} in a similar background without expression of egr does not result in small eyes (Fig. 6f, g). Notably, an eye pigmentation defect was observed when dronc\textsuperscript{wt} is overexpressed (Fig. 6f), consistent with what has been previously reported\textsuperscript{33,50}. Altogether, these data suggest that the catalytic activity of Dronc is required for Egr-induced necrosis.

JNK signaling is required for Eiger- and Dronc-induced necrosis

Unlike GMR\textsuperscript{>egr}, the eye ablation phenotype induced by expression of hid, e.g., GMR-hid, can be suppressed by expression of P35 (ref.\textsuperscript{26}) or reduction of DrICE and Dcp-1 (Supplementary Fig. S3). Therefore, factor(s) other than inhibition of apoptosis is required for induction of necrosis in GMR\textsuperscript{>egr/GMR-p35}. Because Egr activates the JNK signaling upstream of hid-mediated apoptosis, we examined whether there is a nonapoptotic input from JNK contributing to induction of necrosis when apoptosis
(See legend on next page.)
is blocked. Hypomorphic mutants of bsk, hep, MKK4 and Tak1, genes encoding various kinases in the JNK pathway\textsuperscript{52}, were used. We observed that heterozygosity of these mutants can only moderately suppress the GMR > egr-induced eye ablation phenotype (compare Fig. 7a–d to Fig. 2b). In contrast, GMR > egr/GMR-p35-induced small eyes are strongly suppressed by heterozygous mutants of bsk (e), MKK4\textsuperscript{G680} (g) and Tak1\textsuperscript{f1} (h), but not hep (f) (compared with Fig. 4d). Late 3rd instar larval eye disks labeled with Propidium Iodide (PI). Compared with expression of a wild-type form of Dronc (GMR > dronc\textsuperscript{wt}+/−, b), co-expression of Dronc and P35 (GMR > dronc/GMR-p35) induces PI-positive necrosis (arrowhead, j). In contrast, PI-labeling is not observed when a catalytic site-mutated form of Dronc is expressed instead (GMR > dronc\textsuperscript{C318A}/GMR-p35, k). Loss of one copy of bsk (bsk\textsuperscript{1−/−}) strongly suppresses necrosis induced in GMR > dronc\textsuperscript{wt}/GMR-p35 (l). Quantification of PI-positive cell numbers in late 3rd instar larval eye disks of various genetic backgrounds as indicated. One-way ANOVA with Bonferroni multiple comparison test was used to compute p-values. Asterisks indicate statistically significant changes (***, p < 0.0001). Loss of one copy of Tak1 (Tak1\textsuperscript{1+/−}) or a Tak1 null mutant (Tak1\textsuperscript{−/−}) strongly suppresses necrosis induced in GMR > dronc\textsuperscript{wt}/GMR-p35. A diagram showing comparable molecular mechanisms of regulated necrosis in Drosophila and mammals. The Drosophila TNF (Egr), similar to its mammalian counterparts, has multiple context-dependent functions including induction of necrosis when apoptosis is blocked. In mammals, necrosis can occur when inhibition of caspase-8 on RIPK1 and RIPK3 is removed. JNK contributes to both apoptosis and necrosis. While in Drosophila, effector caspases DrICE and Dcp-1 inhibit Egr-induced necrosis. Once this inhibition is removed, the initiator caspase Dronc can activate necrosis with an additional input(s) from JNK signaling. Key factors that mediate this necrosis downstream of caspases and JNK are currently unknown (indicated by the question mark). Moreover, energy metabolism regulators have been implicated in regulation of Egr/TNF-induced signaling responses although their exact roles remain to be determined (see “Discussion”).

**Discussion**

Our findings in this study suggest an analogy between Drosophila and mammals in regulation of TNF-induced apoptosis and necrosis (Fig. 7n). Caspases play critical roles in these processes. In mammals, caspase-8, an initiator caspase, exerts a permissive role on TNF-induced apoptosis but an inhibitory role on necrosis. Here, we report that both apoptosis and necrosis induced by Egr,
Fig. 8 Egr/JNK-mediated necrosis restricts oncogenic growth of scrib mutant cells. a–c Late 3rd instar larval eye disks with GFP-positive scrib mutant (scrib<sup>−/−</sup>, a), scrib<sup>−/−</sup>-p35 (b) or scrib<sup>−/−</sup>-p35-bsk<sup>ΔN</sup> (c) clones in an otherwise wild-type background. These disks are labeled with Propidium iodide (PI, red in a–c and gray in a′–c′). Compared with scrib mutant clones (a, a′), PI-positive necrosis is induced in scrib<sup>−/−</sup>-p35 clones (arrows, b, b′). This necrosis is suppressed by inhibition of JNK via expression of bsk<sup>ΔN</sup> (c, c′). d, e Adult eye images. scrib mutant clones only cause a mild rough-eye phenotype (d). In contrast, necrotic patches (arrow, e) are observed in over 90% of scrib<sup>−/−</sup>-p35 mosaic eyes (n = 44). f scrib<sup>−/−</sup>-p35-bsk<sup>ΔN</sup> mosaic animals are pupal lethal. g Quantification of the clone/disk size ratio in late 3rd instar larval eye disks with various genetic backgrounds as indicated. The ratio is a comparison of the total clone size in each eye disk to the full disk size. One-way ANOVA with Bonferroni multiple comparison test was used to compute p-values. Asterisks indicate statistically significant changes (** < 0.01, *** < 0.001). Compared with wild-type clones which occupy an average of 40% of the whole eye disk, scrib mutant (scrib<sup>−/−</sup>) clones are much smaller with an average of 8% coverage on the disk. Expression of P35 in scrib<sup>−/−</sup> clones (scrib<sup>−/−</sup>-p35) moderately increases their sizes leading to an average disk coverage of 28%. Further expression of bsk<sup>ΔN</sup> in these clones results in their massive overgrowth and increases the clone/disk ratio to an average of 78%.

Interest in whether apoptosis and necrosis cooperate to prevent tumorigenesis in mammals. Another intriguing observation in our study is that expression of the full-length wild-type Dronc is sufficient to induce necrosis when apoptosis is blocked. Although the full-length Dronc is enzymatically inactive under physiological conditions, its overexpression triggers a moderate level of apoptosis (Supplementary Fig. S5b), which is probably due to spontaneous activation of the full-length Dronc. Suppression of this apoptosis by P35 results in a switch from apoptosis to necrosis (Fig. 7). Interestingly, although expression of the proapoptotic genes such as hid activates Dronc and apoptosis, it does not seem to trigger necrosis when apoptosis is blocked. This suggests that activation of Dronc alone is not sufficient to induce necrosis. It has recently been reported that the subcellular localization of Dronc determines its apoptotic versus nonapoptotic functions. Therefore, overexpression of Dronc may allow its protein level in a particular subcellular domain to exceed a threshold thus trigger necrosis. This may not occur in GMR-hid because it activates but does not induce expression of Dronc (Supplementary Fig. S5i). Notably, the catalytic activity of Dronc is required for
both Dronc- and Egr-induced necrosis. Hence, identification of the nonapoptotic substrates of Dronc may provide critical information to understand how Dronc mediates necrosis.

What are other factors that coordinate the inputs from caspases and JNK to differentiate apoptosis versus necrosis? This is an important question that remains largely open. In mammals, caspase-8 cleaves and inactivates RIPK1 and RIPK3 therefore inhibits TNF-induced necroptosis. However, no homologs of RIPK1 and RIPK3 have been reported in Drosophila. Notably, RIPK3 mediates TNF-induced necrosis at least partially via its role in regulation of energy metabolism and reactive oxygen species (ROS) production. The concept of metabolic checkpoints has been proposed to describe the complex roles of metabolism in regulation of cell death including necrosis. Intriguingly, a recent study in Drosophila also revealed roles of the energy metabolism pathways in regulation of GMR > egr-induced eye ablation phenotype. As we show in this study, expression of Egr can lead to distinct outcomes including apoptosis, nonapoptotic developmental defects and necrosis in a context dependent manner (Fig. 7n). It is therefore interesting to see whether the metabolism pathways regulate Egr-induced necrosis and, if yes, how they interact with caspases and JNK in this process. Furthermore, necrosis-like cell death in Drosophila has also been reported in the stressed larval brains, the developmental male testes and female ovaries. In these forms of necrosis, effector caspases and the catalytic activity of Dronc are not required. Hence, future research is needed to understand how different types of necrosis are regulated at the molecular level. What’s more, a series of intracellular events specific to necrosis, such as mitochondrial dysfunction, ATP depletion and increased cytosolic Ca^{2+}, appear to be conserved in multiple organisms. Therefore, studies on various necrosis models in Drosophila will likely provide insights into understanding the molecular regulation of necrosis.

Material and methods

Drosophila genetics

Genetic crosses for all experiments were reared at 25°C. GMR-GAL4 UAS-egr (GMR > egr) (ref. 19), GMR-hid (ref. 19), dcp-1RNAi (ref. 34), drICE (ref. 35), dronc (ref. 36), rpr (ref. 37), XR38 (ref. 38), hid(F5014) (ref. 39), GMR-BIR (ref. 40), GMR-p35 (ref. 40), hid(2-10)–lacZ (ref. 40), rpr(XRE–lacZ) (ref. 40), sev > GluC (ref. 41), M KK4 (ref. 42), UAS-droncwt and UAS-droncC138A (ref. 43), UAS-bakRNAi (on Chr.III) (ref. 44), scrib1 and scrib2 (refs 45, 46) were obtained from the NIG-Fly stock center. UAS-egrRNAi (108814) was obtained from the VDRC stock center.

Mosaic analysis

For mosaic analysis with hid mutant clones, larvae of the following genotype were analyzed at the late 3rd instar larval stage: ey–FLP/+; GMR > egr/+; FRT80B/hid(F5014) FRT80B. For mosaic analyses with wild-type control, scrib mutant (scrib–/–), scrib–/––p35 or scrib–/––p35-bakDN clones, the following genotypes were used: (1) ey–FLP/+; act > y+> GAL4 UAS-GFP/+; FRT82B/FRT82B tub–GAL80; (2) ey–FLP/+; act > y+> GAL4 UAS-GFP/+; FRT82B scrib1/FRT82B tub–GAL80; (3) ey–FLP/+; act > y+> GAL4 UAS-GFP/UAS-p35; FRT82B scrib1/FRT82B tub–GAL80; (4) ey–FLP/+; act > y+> GAL4 UAS-GFP/UAS-p35; FRT82B scrib1 UAS-bakDN/FRT82B tub–GAL80. Similar mosaic analyses with scrib2 mutants showed comparable results.

Immunohistochemistry

Pupal or larval disks were dissected, fixed (with 4% paraformaldehyde for 30 min at room temperature), and then labeled with antibodies using standard protocols as described. Primary antibodies used were rabbit anti-dCdc1 (the cleaved Dcp-1 antibody, 1:500, Cell Signaling), rabbit anti-phospho-JNK (pJNK, 1:200, Calbiochem), rat anti-ELAV, mouse anti-Dlg, mouse anti-Fab fragments conjugated to Alex488, 555, or 647 (all 1:50, DHSB). To generate the rabbit anti-Dronc antibody, the full-length Dronc cDNA was amplified by PCR with primers 5′-CGGGATCCATGGAGCGGCCGGAGCT3′ and 5′-CGGAATTCCTATTCCGGATCCTCATGCGTGAGGATGTC3′. It was then cloned into BamHI and EcoRI sites in pET-28a (Novagen) to produce a His-tagged recombinant protein. The purified recombinant protein was then injected into rabbits to generate a polyclonal antibody. The antiserum was subsequently affinity purified. Secondary antibodies were goat Fab fragments conjugated to Alexa488, 555, or 647 (all 1:1000) from Molecular Probes.

Propidium iodum (PI), Hoechst labeling and TUNEL

For PI and Hoechst double labeling on pupal disks, freshly dissected disks were incubated in dark with 4 μM PI (Sigma-Aldrich) and 16 μM Hoechst (ThermoFisher) in Schneider’s media for 1 h at room temperature. The disks were then fixed with 4% paraformaldehyde for 20 min at room temperature followed by gentle washes as previously described. For PI labeling alone, larval or pupal eye disks were incubated with 15 μM PI for 10 min at room temperature followed by fixation (with 4% paraformaldehyde for 20 min at room temperature) and gentle washes as previously described. PI labeling can also be observed and scored without fixation. For TUNEL (terminal deoxynucleotide transferase-mediated dUTP

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end labeling), dissected and fixed (with 4% paraformaldehyde for 20 min at room temperature) disks were incubated in 100 mM Na-citrate with 0.1% Triton X-100 for 30 min at 65 °C, followed by detection of dying cells using an in situ cell death detection kit (Roche).

**Imaging and statistical analysis**

Fluorescent eye disk images were taken with either a Zeiss or Leica confocal microscope. Adult fly eye images were taken using a Zeiss stereomicroscope equipped with an AxioCam ICC1 camera. For statistical analysis of PI-labeling (Fig. 4 and 7m), at least 20 pupal or larval disks from each genotype were used for counting the number of PI-positive cells. For quantification of adult eye size (Fig. 6h), the “histogram” function in Adobe Photoshop CS was used to measure ten representative adult eyes of each genotype. For quantification of the clone/disk size ratio in late 3rd instar mosaic eye disks (Fig. 8g), the sizes of GFP-positive clones and the whole eye disk were measured using the “histogram” function in Adobe Photoshop CS before the ratio is calculated for each disk. Ten representative mosaic disks of each genotype were used. For all quantifications, the statistical significance was evaluated through a one-way ANOVA with Bonferroni multiple comparison test using GraphPad Prism.

**Transmission electron microscopy (TEM)**

Freshly dissected pupal eye–brain complexes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 45 min followed by a secondary fixation of 1% Osmium Tetroxide for 1 h at room temperature. The samples were then washed with the buffer (10 × 5 min) and dehydrated in ascending concentrations of ethanol before they were embedded in epoxy resin. Sections (90 nm) were prepared and stained with uranyl acetate and lead citrate followed by examination using a JEOL 1200EX electron microscope fitted with a tungsten filament. Images were acquired through a GATAN MultiScan camera.

**Acknowledgements**

We would like to thank Paul Stanley and Theresa Morris at the Centre for Electron Microscopy in the University of Birmingham for their assistance on TEM. The confocal imaging was carried out with support of the Birmingham Advanced Light Microscopy (BALM) facility. We thank Kai Liu for advice on PI-labeling. We are grateful to Konrad Basler, Andreas Bergmann, Lei Liu, Pascal Meier, Mirka Uhlířová, the Bloomington Stock Center, the NIG-Fly stock center in Japan, the VDRC stock center in Vienna and the Developmental Studies Hybridoma Bank (DSHB) in Iowa for fly stocks and reagents. M.L. is supported by the China Scholarship Council (CSC)-Birmingham joint PhD program. Y.F. is supported by Grant BB/MT01880/1 from the Biotechnology and Biological Sciences Research Council (BBSRC) UK and the Marie Curie Career Integration Grant (CIG) 6319846 from the European Union’s Seventh Framework Program (FP7).

**Conflict of interest**

The authors declare that they have no conflict of interest.
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