THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 17, pp. 15556–15564, April 29, 2011


# The NAB-Brk Signal Bifurcates at JNK to Independently Induce Apoptosis and Compensatory Proliferation*[S]

Received for publication, October 18, 2010, and in revised form, January 27, 2011 Published, JBC Papers in Press, March 8, 2011, DOI 10.1074/jbc.M110.193235

**Yaron Suissa**[‡1], **Oren Ziv**[‡1], **Tama Dinur**[‡], **Eli Arama**[§], **and Offer Gerlitz**[‡2]

*From* [‡]*Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel-Canada, Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel and the* [§]*Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel*

**Apoptosis operates to eliminate damaged or potentially dangerous cells. This loss is often compensated by extra proliferation of neighboring cells. Studies in *Drosophila* imaginal discs suggest that the signal for the additional growth emanates from the dying cells. In particular, it was suggested that the initiator caspase Dronc mediates compensatory proliferation (CP) through Dp53 in wing discs. However, the exact mechanism that governs this CP remained poorly understood. We have previously shown that elimination of misspecified cells due to reduced Dpp signaling is achieved by the interaction of the co-repressor NAB with the transcriptional repressor Brk, which in turn induces Jun N-terminal kinase-dependent apoptosis. Here, we performed a systematic *in vivo* loss- and gain-of-function analysis to study NAB-induced death and CP. Our findings indicate that the NAB primary signal activates JNK, which in turn transmits two independent signals. One triggers apoptosis through the pro-apoptotic proteins Reaper and Hid, which in turn promote activation of caspases by the apoptosome components Ark and Dronc. The other signal induces CP in a manner that is independent of the death signal, Dronc, or Dp53. Once induced, the apoptotic pathway further activates a CP response. Our data suggest that JNK is the candidate factor that differentiates between apoptosis that involves CP and apoptosis that does not.**

During development, apoptosis shapes structures by removing excess cells (1). Apoptosis also has an important non-developmental role of cellular proofreading: it is used to eliminate misspecified or damaged cells. In this context, apoptosis is associated with compensatory proliferation (CP),[3] a mechanism that replaces eliminated cells through stimulation of proliferation, which contributes to maintaining tissue homeostasis. Recent studies in *Drosophila* indicate that the signal for CP emanates from the dying cells themselves, which signal to the neighboring cells to extra proliferate to maintain tissue homeostasis and allow for proper development (2, 3). More specifically, the initiator caspase-9 homolog Dronc, a major death effector in the fly, was suggested to play an important role in promoting CP of surrounding cells (2, 4, 5). How Dronc induces CP is still unclear. One interesting candidate is the Jun N-terminal kinase signaling pathway, which was found to be activated in the dying cells and required for secretion of the mitogenic factors Wingless (Wg) and Decapentaplegic (Dpp) and stimulation of compensatory growth (3). Dronc was also shown to activate Wg and growth in response to cellular damage through activation of *Drosophila* p53 (Dp53) (5). Nevertheless, the exact interplay between JNK signaling, the apoptotic machinery, and compensatory growth has not been directly addressed.

Dpp, a member of the TGF$\beta$ superfamily in *Drosophila*, functions in the wing primordium (wing imaginal disc (WID)) as a long-range morphogen to specify cell fates in a concentration-dependent manner. Much of the transcriptional output of Dpp is mediated through repression of the transcriptional repressor Brinker (Brk), which functions as a negative regulator of Dpp targets. Because Dpp also provides growth and survival cues, impaired Dpp signaling results in accumulation of Brk, which in turn triggers apoptosis through activation of the JNK pathway (6–8). However, the mechanisms that execute this apoptotic pathway downstream of JNK were poorly investigated. Importantly, elimination of cells with impaired Dpp signaling in the developing wing does not cause aberrations in the adult wing (7), indicating that cell loss is compensated for by extra proliferation. The signal that drives this CP remained to be discovered. We recently identified the transcription co-regulator NAB as an effector of Dpp signaling in the WID (9). NAB is not required for Dpp-dependent patterning in the developing wing but acts as a Brk co-repressor to eliminate cells with impaired Dpp signaling. This cell loss requires the induction of the JNK pathway, which in turn triggers effector caspase activation and apoptosis (9).

Here, we performed a systematic *in vivo* loss- and gain-of-function analysis to study NAB-induced death and CP. We show that the NAB primary signal activates JNK, which in turn bifurcates to transmit two independent signals. One signaling branch triggers apoptosis through induction of two pro-apoptotic proteins, Reaper (Rpr) and Hid. This leads to a reduction in the levels of the major *Drosophila* inhibitor of apoptosis protein, Diap1, and activation of the apoptosome components Ark and Dronc, which then activate the effector caspases and cell

* This work was supported by Israel Science Foundation Grant 869/04, the Israel Cancer Research Foundation, and Abisch-Frenkel Foundation Grant 0304624.
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed. Tel.: 972-2-675-7528; Fax: 972-2-675-7379; E-mail: offerg@ekmd.huji.ac.il.
[3] The abbreviations used are: CP, compensatory proliferation; WID, wing imaginal disc; UAS, upstream activation sequence; MARCM, mosaic analysis with a repressible cell marker; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LOF, loss-of-function.

death. The other JNK-dependent signaling branch induces CP even in the absence of Rpr, Hid, Dronc, and Dp53, demonstrating for the first time that the decision for CP is made upstream of induction of the core apoptotic pathway. Finally, consistent with previous observations, we found that the apoptotic pathway (apparently through Dronc) reinforces the CP response in a positive feedback loop. Our data suggest that the decision of whether or not to link apoptosis with growth is made at the level of JNK upstream of the death signal.

## EXPERIMENTAL PROCEDURES

*Fly Strains*—The following strains were used in this study: Df(3L)H99, *Dredd*$^{L23}$, *Dredd*$^{F64}$, *rpr* Df(3L)XR38 (gift from K. White), *hid* P[PZ]w$^{05014}$, *dark*$^{L46}$, UAS-*flp*, UAS-*p35*, UAS-*lacZ*, UAS-*p53-DN*, UAS-*puc*, EP-*NAB*, EP-*diap1*, ubi-GFP,FRT42D, *puc-lacZ*$^{E69}$, UAS-RNAi-*dronc* (NIG 8091-R1), UAS-RNAi-*grim* (VDRC 39689), and UAS-RNAi-*brk* (VDRC 2919). Transgenes were expressed using the *GAL4*/UAS binary system with drivers *hh-GAL4* and *apterous* (*ap*)-*GAL4.*

*Generation of Flp-out and Loss-of-function Clones*—We generated overexpressing Flp-out clones using the *act>CD2>GAL4* cassette, recombined to a UAS-GFP construct for the detection of the clones. Larvae were subjected to a 37 °C heat shock for 10 min. Unless noted otherwise, larvae were dissected at 48 h after clone induction.

We generated mutant clones using Flp-mediated mitotic recombination and identified them by the loss of GFP. Clones were induced with *hh-GAL4/*UAS-*flp.* Genotypes of dissected larvae were as follows: *hs-flp*, *dark*$^{L46}$, Y+,FRT42D/ubi-GFP,FRT42D; and *hh-GAL4*, UAS-*flp*/EP-*NAB*, UAS-*lacZ*.

For MARCM experiments, genotypes of dissected larvae were as follows: *sd-GAL4/hs-flp*;;*tubP-GAL80* FRT80/EP-*NAB*, H99, FRT80. Clones were induced by heat shock for 60 min at 37 °C. Larvae were dissected at 72 and 96 h after clone induction.

*Immunohistochemistry*—Imaginal discs from late wandering stage larvae were fixed and stained by standard techniques. The specific primary antibodies used were as follows: mouse anti-β-gal (1:1000; Promega), rat anti-NAB (1:1000), rabbit anti-cleaved caspase-3 (1:40; Cell Signaling Technology), rabbit anti-Diap1 (1:150; a gift from Paul D. Friesen), rabbit anti-phosphohistone H3 (1:1000, Upstate), and mouse anti-Wg (4D4; Hybridoma Bank). TUNEL staining was carried out as described (18). Images were taken on a TE2000-E confocal microscope (Nikon) using a ×20 objective.

*Quantification of WID Size and Caspase-3 Activation*—Using Adobe Photoshop, we measured the average pixel area (per disc) in which caspase-3 was activated by overexpressing NAB on the background of *hid*, *rpr*, or H99 heterozygous flies or together with Diap1 overexpression. The measured values were normalized to caspase-3 activation in discs overexpressing NAB. Using ImageJ, we measured the size of the WID pouch overexpressing NAB on an H99 loss-of-function (LOF) background or with p35 normalized to the control.

*Anti-NAB Antibody Production*—To generate the anti-NAB antibody, two rats were immunized with a GST fusion of NAB (amino acids 1–284). After three immunizations, the rats were bled, and sera were tested on imaginal discs. The two sera gave rise to the same expression pattern. We confirmed that the antibody recognized NAB by immunolabeling *act>CD2>GAL4/*EP-*NAB* wing discs. The expression patterns revealed by the antibodies were identical to those obtained with *NAB*$^{SH143}$.

## RESULTS

*NAB-induced Apoptosis Is Mediated by the Activators of Apoptosis Rpr and Hid*—We recently demonstrated that *Drosophila* NAB acts as a co-repressor together with Brk to induce apoptosis in cells with impaired Dpp signaling (9). Indeed, overexpression of NAB in the posterior compartment of the *Drosophila* WID caused massive cell death detected by both TUNEL assay (Fig. 1, *A* and *B*) and active effector caspase expression (Fig. 1, *C* and *D*), and this cell death was blocked either by *brk* loss of function (9) or knockdown (Fig. 1, *M* and *N*). Furthermore, most of the somatic clones that overexpress NAB were eliminated from the WID 48 h after their induction (Fig. 1, compare *E* and *F* with *G* and *H*), indicating that these cells are dying rapidly. Finally, we found that this death was JNK-dependent, as NAB overexpression activated a downstream target of this pathway called Puckered (Puc) (Fig. 1, *I* and *J*), and ectopic expression of Puc, an inhibitor of this pathway, blocked NAB-induced apoptosis (Fig. 1, *K* and *L*). Non-autonomous JNK and caspase-3 activation was frequently seen around the border of NAB-overexpressing clones (see Fig. 3, *A* and *B*) (9). This phenomenon was attributed to local interactions between misspecified/dying cells and their neighbors. (Adachi-Yamada and O'Connor term this phenomenon "morphogenetic apoptosis" (10)).

To further explore the role of the canonical apoptotic pathway in NAB-dependent apoptosis, we first examined the involvement of the RHG protein family. In *Drosophila*, Rpr, Hid, and Grim (RHG) proteins are key activators of the apoptotic machinery (11). NAB-induced apoptosis in the dorsal compartment of the WID (using the *ap-GAL4* driver) was virtually abolished upon removal of one copy of each of these three genes using the small genomic deletion Df(3L)H99 (Fig. 2, compare *A* and *B* with *C* and *D*). To identify which of the RHG proteins is required for NAB-induced apoptosis, we tested individual mutants. Whereas removal of one copy of *rpr* (+/Df(3L)XR38) or *hid* (+/P[PZ]w$^{05014}$) significantly attenuated the level of NAB-induced apoptosis (Fig. 2, *E–H* and *O*), no change was observed upon knockdown of *grim* expression (Fig. 2, *I* and *J*). We conclude that NAB-induced apoptosis is meditated by both Rpr and Hid.

*NAB Apoptotic Signal Triggers Reduction in Diap1 Protein Levels*—Diap1 is a potent inhibitor of caspases required for the survival of somatic cells in *Drosophila* (12–14). In the absence of apoptotic signal, Diap1 inhibits caspase activity through binding and ubiquitination of caspases (15, 16). Upon apoptosis induction, the RHG proteins bind Diap1, disrupting its interaction with caspases and stimulating its autoubiquitination and degradation (12, 14, 17, 18). Consistently, coexpression of Diap1 and NAB in the WID abolished effector caspase activation and apoptosis (Fig. 2, *K* and *L*). Moreover, overexpression of NAB in the dorsal compartment of the WID resulted in a reduction in the level of the Diap1 protein (Fig. 2, *M* and *N*).

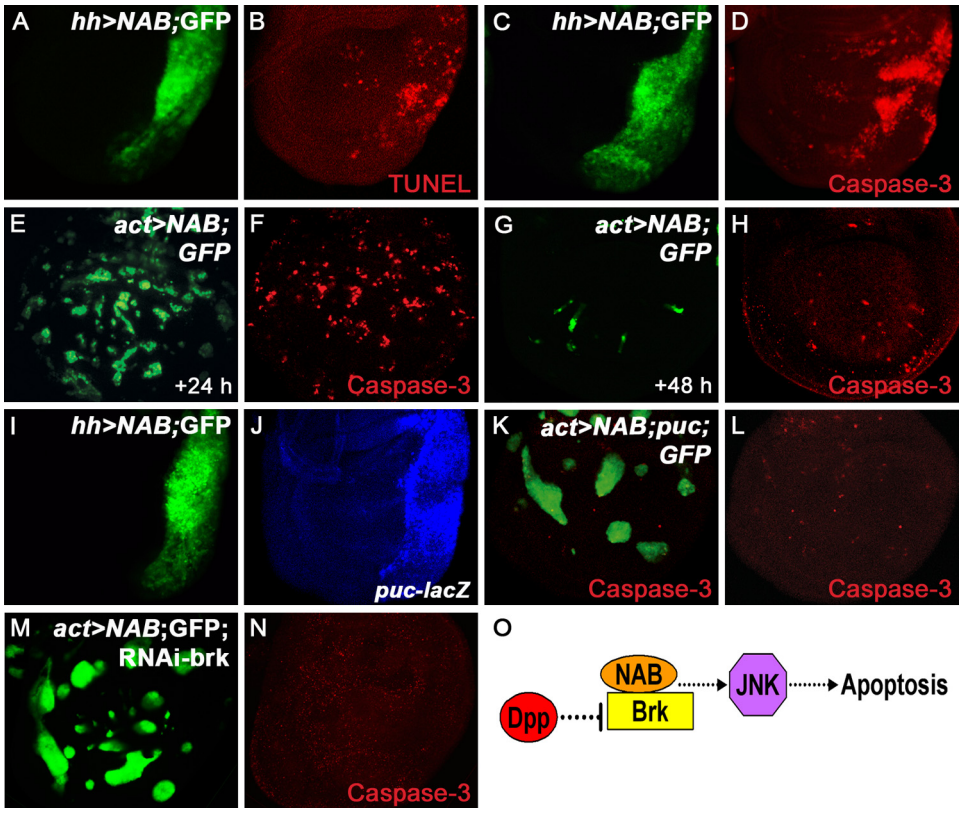


FIGURE 1. **NAB-induced apoptotic cell elimination is Brk-dependent and is associated with and caused by activation of the JNK pathway.** *A–D*, overexpression of NAB in the posterior compartment using the *hh-GAL4* driver (marked by GFP, *green*; *A* and *C*) led to apoptosis as visualized by TUNEL staining (*red*; *B*) and activated caspase-3 (*red*; *D*). *E–H*, NAB-overexpressing clones were apoptotically eliminated from the *Drosophila* wing disc. Clones overexpressing NAB 24 (*E*) and 48 (*G*) h after induction (*green*) up-regulated activated caspase-3 (*red*; *F* and *H*). *I–L*, NAB-dependent cell elimination was associated with and caused by activation of the JNK pathway. *I* and *J*, *hh-GAL4*-driven NAB overexpression in the posterior wing compartment (marked by GFP, *green*; *I*) activated the JNK pathway (*puc-lacZ*, *blue*; *J*). *K* and *L*, overexpression of both NAB and Puc (*green*; *K*) abolished caspase-3 activation (*red*; *L*). *M* and *N*, NAB requires Brk to induce cell death. Clones overexpressing both NAB and *brk* RNAi (*green*; *M*) showed no caspase-3 activation (*red*; *N*). *O*, the model shows that the Dpp survival signal acts through repression of Brk. Impaired Dpp signaling up-regulates Brk, which, together with NAB, activates JNK-mediated apoptosis.

Collectively, these results suggest that NAB induces apoptosis through induction of Rpr and Hid, which in turn stimulate Diap1 degradation and subsequent caspase activation.

*Apoptosome Components Ark and Dronc Are Required for NAB-induced Cell Death*—Inactivation of the main initiator caspase-9 homolog Dronc blocks most of the developmental and stress-induced apoptosis in *Drosophila* (19–21). Consistently, we found that RNAi-mediated knockdown of *dronc* abolished most, if not all, effector caspase activation associated with NAB overexpression (Fig. 2, *P* and *Q*). Dronc activation requires the adaptor protein Ark to assemble an apoptosome-like complex (22, 23). We therefore tested the effect of Ark removal on NAB-induced effector caspase activation using the Flp/FRT system to generate mutant cells homozygous for *ark* LOF ($ark^{L46}/ark^{L46}$) in a NAB-overexpressing background. Indeed, NAB-induced effector caspase activation was absent in *ark* mutant clones (Fig. 2, *R* and *S*). Finally, we found that loss of the initiator-like caspase Dredd did not affect effector caspase activation associated with NAB overexpression (supplemental Fig. 1). This result is consistent with the idea that Dredd is not essential for apoptosis in most of the cell death paradigms in *Drosophila* but is required for the antibacterial immune response (24–26). We conclude that NAB activity triggers apoptosis through the pro-apoptotic proteins Rpr and Hid, which in turn reduce Diap1 protein levels, allowing Dronc activation by Ark and subsequent activation of the effector caspases.

*NAB Induces JNK-dependent CP and Wg Expression*—The local induction of apoptosis in imaginal discs is often accompanied by CP of neighboring cells to maintain tissue homeostasis. Overexpression of the effector caspase inhibitor p35 to block the execution of apoptosis in dying cells (that are normally quickly removed) enables a direct analysis of the molecular events underlying CP. Dying cells that are kept alive (undead cells) using p35 exhibit strong non-autonomous growth-stimulating activity (3, 27) that is associated with and depends on activation of JNK. In some of the undead cells, JNK signaling induces secretion of the mitogenic factors Wg and Dpp (3, 27). Up-regulation of JNK activity and Wg expression was also required for regenerative growth in response to various tissue damages (28–31). Importantly, secretion of Wg and Dpp by apoptotic cells was shown to be dispensable for CP induced by x-ray (32), suggesting that JNK signaling induces several mitogenic signals that are redundant for CP.

NAB triggers cell elimination through activation of the JNK signaling pathway (Fig. 1, *I–L*, and 3, *A* and *B*) (9). To examine autonomous and non-autonomous effects on proliferation and gene expression associated with NAB-induced cell death, we generated clones of cells coexpressing both NAB and p35 in the

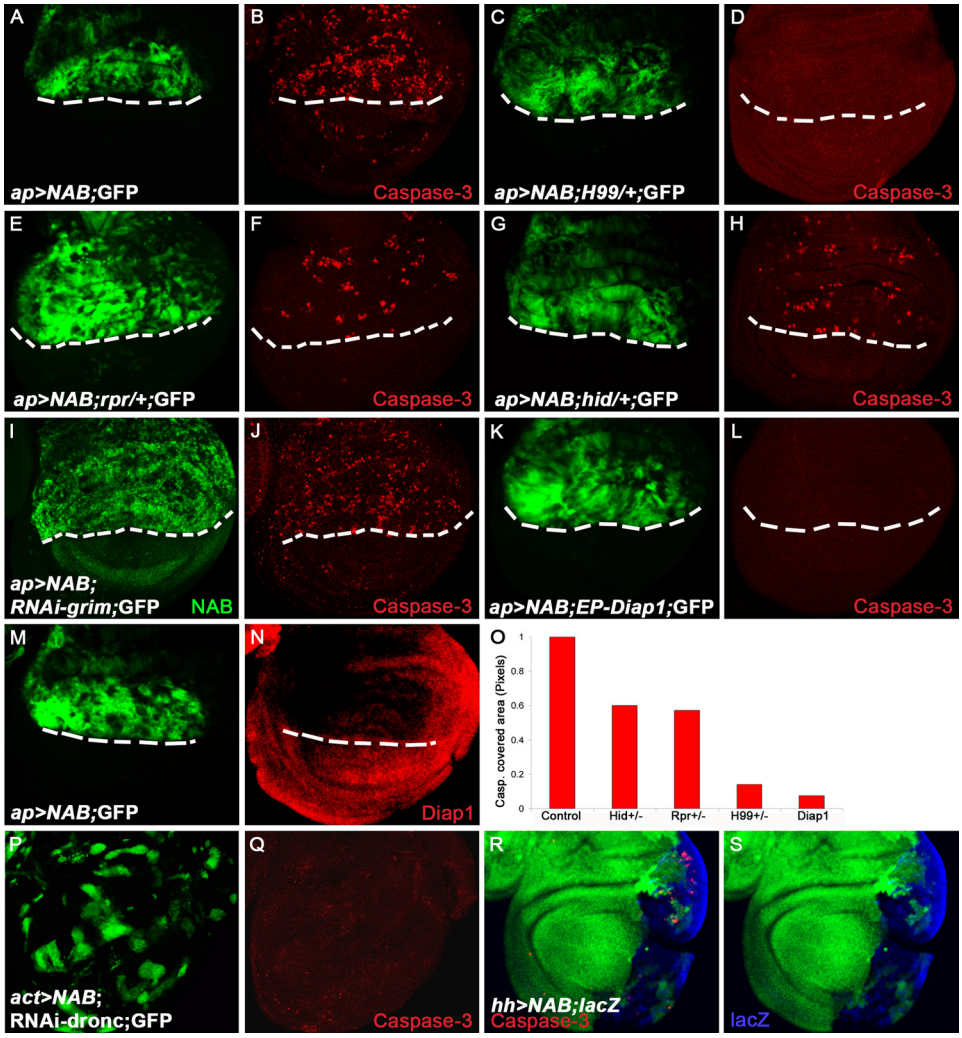


FIGURE 2. **NAB-induced cell death is mediated through multiple activators of apoptosis that trigger Diap1 protein degradation.** The *dashed lines* (*A–N*) mark the dorsal-ventral boundary. *A* and *B*, *ap-GAL4*-driven NAB overexpression in the dorsal wing compartment (*green*; *A*) led to apoptosis as visualized by activated caspase-3 (*red*; *B*). *C–J*, NAB requires Rpr and Hid but not Grim to induce cell death activation. *C* and *D*, NAB overexpression in the dorsal wing compartment (*green*; *C*) in flies heterozygous for the deficiency H99 (Df(3L)H99) showed no caspase-3 activation (*red*; *D*). *E–H*, NAB overexpression in the dorsal compartment in flies heterozygous for either *Rpr* (*green*; *E*) or *Hid* (*green*; *G*) showed modest caspase-3 activation (*red*; *F* and *H*, respectively, and compare with *B*). *I* and *J*, overexpression of NAB, together with RNAi-mediated knockdown of *grim* (*green*; *I*) in the dorsal compartment (using *ap-GAL4*) had no effect on caspase-3 activation levels (compare *J* with *B*). *K* and *L*, co-overexpression of Diap1 and NAB in the dorsal compartment of the wing disc abolished caspase-3 activation. *ap-GAL4*-driven NAB and Diap1 expression (*green*; *K*) abolished caspase-3 activation (*red*; *L*). *M* and *N*, Diap1 protein levels were reduced in response to NAB overexpression. *ap-GAL4*-driven NAB overexpression (*green*; *M*) triggered a reduction in Diap1 protein levels (*red*; *N*). *O*, relative quantification of NAB-induced caspase-3 activation in the dorsal compartment. Shown is the average area covered by activated caspase-3 (*Casp.*) of NAB overexpression together with *hid*, *rpr*, or H99 heterozygous flies or Diap1 overexpression relative to NAB overexpression only (*Control*). *P–S*, the apoptosome components Dark and Dronc are required for NAB-induced cell death. *P* and *Q*, RNAi-mediated knockdown of *dronc* expression in clones overexpressing NAB (*green*; *P*) virtually abolished caspase-3 activation (*red*; *Q*). *R* and *S*, NAB-induced cell death was absent in mutant clones of *dark*. *dark* LOF clones were generated in the posterior compartment using the *hh-GAL4* driver (marked by the loss of GFP; *R* and *S*) in the background of NAB overexpression (marked by *lacZ*, *blue*; *R* and *S*). Caspase-3 activation induced by NAB was nearly abolished (*red*; *R*) in the large *dark* LOF clones.

WID. Consistent with previous studies, we found that, in this context, NAB-overexpressing undead cells exhibited a strong non-autonomous proliferation stimulatory effect, as evident by WID overgrowth (Fig. 3, compare *C* with *D*, and see quantification in Fig. 4*C*). Ectopic up-regulation of Wg was frequently associated with NAB-overexpressing undead cells (Fig. 3, *E* and *F*). Also in this context, we observed non-autonomous up-regulation of Wg, likely due to non-autonomous activation of JNK signaling. Importantly, activation of JNK and occasionally up-regulation of Wg were also observed in the doomed cells overexpressing NAB without p35 (Fig. 3, *A*, *B*, *G*, and *H*), indicating that these effects are not merely secondary consequences of the "undead" state.

Next, we examined whether JNK activation is involved in up-regulation of Wg and CP. Coexpression of NAB and Puc, a phosphatase that negatively regulates JNK (33), blocked both Wg induction and overgrowth (Fig. 3, *I* and *J*). NAB has been previously shown to restrict Wg expression during proximodistal patterning of the WID (34). Our data show that the NAB-Brk-dependent signal activates JNK, which in turn induces compensatory growth and Wg expression.

*NAB Induces Tissue Growth Independently of the Death Signal*—Recent work showed that CP requires Dronc activity in undead cells (2). Likewise, JNK activity is required for the CP response associated with undead cells (3). Interestingly, coexpression of Dronc and p35 induces JNK activation and abnor-

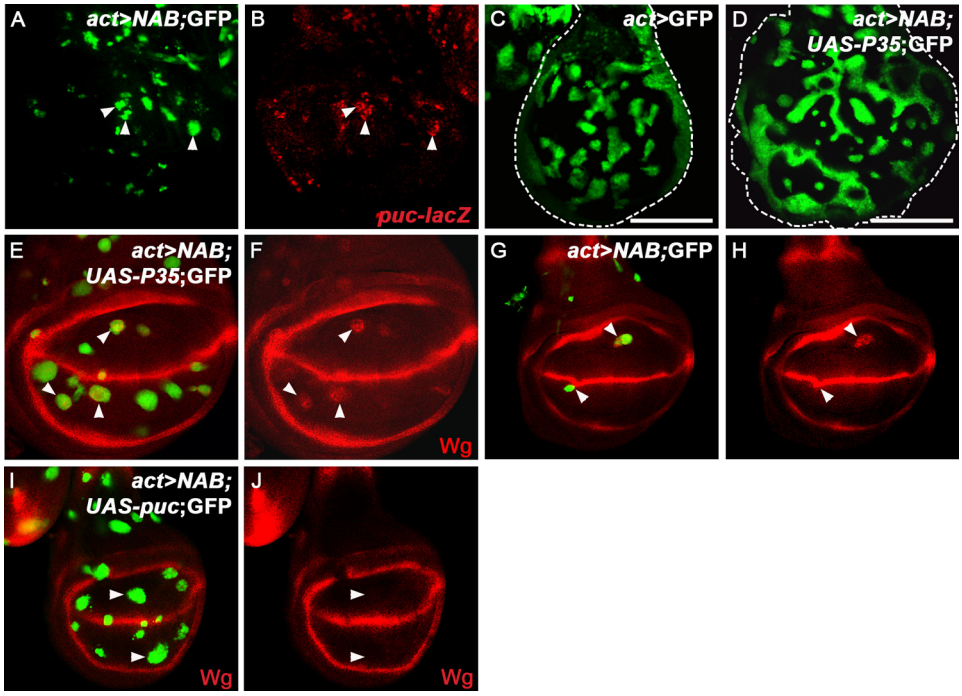


FIGURE 3. **NAB induces Wg expression and CP through the JNK pathway.** *A–H*, NAB overexpression induced Wg expression, JNK activation, and non-autonomous proliferation. *A* and *B*, clones overexpressing NAB (*green*; *A*) up-regulated *puc-lacZ* expression (*red*; *B*). *C* and *D*, WIDs with clones co-overexpressing both NAB and p35 exhibited a strong non-autonomous proliferation effect (compare wing disc size in *C* and *D* and see quantification in Fig. 4C). *Scale bars* = 100 $\mu$m. *E* and *F*, Wg expression was ectopically up-regulated (*red*; *E* and *F*) in clones co-overexpressing both NAB and p35 (*green*; *E*). *G* and *H*, Wg expression (*red*; *G* and *H*) was up-regulated in clones overexpressing NAB (*green*; *G*). *I* and *J*, NAB-induced Wg up-regulation was mediated via the JNK pathway. Coexpression of Puc and NAB (*green*; *I*) blocked both Wg induction (*red*; *I* and *J*) and non-autonomous overgrowth stimulation (compare wing discs size in *J* and *F*).

mal overgrowth (4, 5), indicating that activation of JNK signaling in undead cells is a downstream event of Dronc activation and thus occurs after the induction of apoptosis. On the other hand, various intrinsic and extrinsic stimuli activate JNK signaling (35), which in turn induces caspase-dependent apoptosis through *rpr* or *hid* (36, 37), indicting that, in these contexts, JNK functions upstream of the activator of apoptosis and the core cell death machinery.

Our findings indicate that NAB induces both apoptosis and CP through activation of the JNK signaling pathway. We therefore wished to determine whether NAB-dependent CP is secondary to its death signal. Taking advantage of the fact that removal of one copy of the RHG genes significantly suppressed effector caspase activation induced by NAB (Fig. 2, *C* and *D*, and Fig. 4, *D* and *E*), we generated clones of cells overexpressing NAB in the H99 heterozygous background (Fig. 4, *D–K*). NAB-overexpressing cells exhibited strong JNK activity detected by the *puc-lacZ* reporter (Fig. 4, *F* and *G*). Also in this context, non-autonomous JNK activation was seen around the NAB-overexpressing clones. Surprisingly, many of these cells also expressed high levels of Wg (Fig. 4, *H* and *I*) and stimulated a strong non-autonomous growth of neighboring tissue, as evident by disc overgrowth (Fig. 4, *D–K*, and supplemental Fig. 2, *A*, *B*, and *E*) and staining for phosphohistone H3 (Fig. 4, compare *J* and *K* with *A* and *B*).

To completely avoid autonomous death signaling, we used the MARCM technique to generate homozygous H99 LOF clones overexpressing NAB. Wg expression was up-regulated and CP still occurred in this experimental setting, albeit to a lesser extent (Fig. 4, *C* and *L–O*). We occasionally observed effector caspase activation in the cells bordering the NAB-overexpressing undead cells (Fig. 4*N*). The non-autonomous caspase activation is likely to result from local interactions between NAB-overexpressing cells and their neighbors. Taken together, these results imply that NAB induces CP and Wg expression independently of the apoptotic signal and that the levels of these effects increase upon apoptosis induction.

Although the exact mechanisms by which Dronc stimulates Wg expression and CP remain largely unclear, activation of the JNK pathway downstream of Dronc emerges as an important step in this process (4, 5). To directly examine whether Dronc is required for NAB-induced JNK activation and CP, we generated NAB-overexpressing clones with RNAi-mediated knockdown of *dronc*. These cells displayed strong JNK activity (Fig. 4, *P* and *Q*), which was associated with robust extra tissue growth (Fig. 4, *P–S*, and supplemental Fig. 2, *C–E*), indicating that activation of JNK by NAB is independent of Dronc. Although *dronc* RNAi efficiently suppressed NAB-induced effector caspase activation, CP and up-regulation of Wg still occurred (Fig. 4, *R* and *S*). Similarly, overexpression of Diap1, which inhibits Dronc activity (16), failed to suppress NAB-induced Wg expression and tissue overgrowth (Fig. 4, *T* and *U*, and supplemental Fig. 2, *E–H*). We conclude that the NAB signal bifurcates at the level of JNK to independently activate the apoptotic and CP pathways. However, once the apoptotic pathway is activated, it further promotes JNK-dependent compensatory growth.

*Dp53 Is Not Required for NAB-induced Cell Death or CP*—Dp53 was shown to mediate apoptosis induced by ionizing irradiation (38). Whereas the mechanism by which Dp53 activates

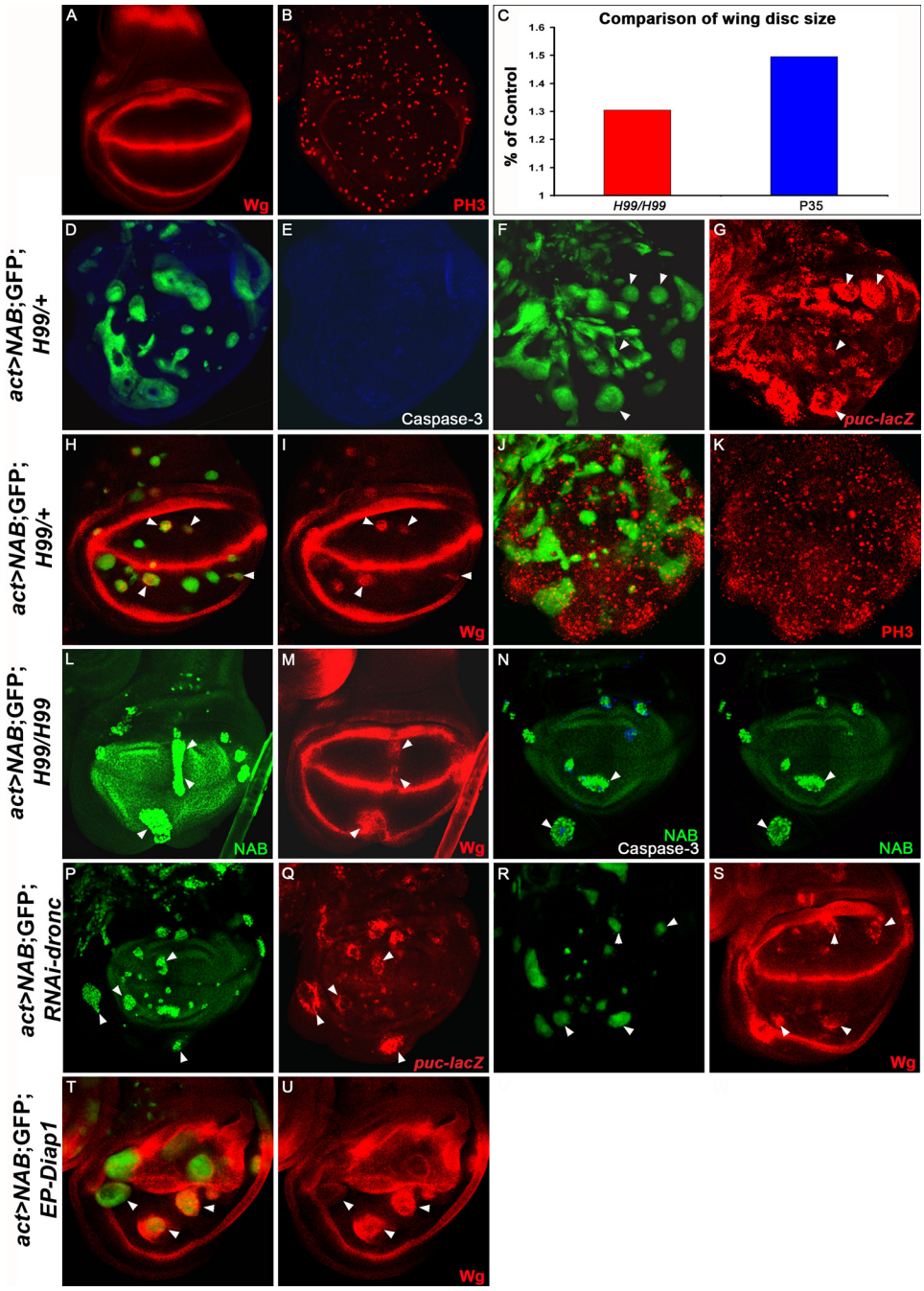


FIGURE 4. **NAB induces CP and Wg expression through the JNK pathway independently of the death signal.** *A*, WT wing disc stained for Wg (*red*). *B*, WT wing disc stained for phosphohistone H3 (*PH3*; *red*). C, comparison of sizes of wing discs overexpressing NAB on an H99 LOF background (*red bar*; $n = 13$) and with p35 (*blue bar*; $n = 16$) relative to the WT ($p < 0.001$). *D–K*, clones of cells overexpressing NAB in H99 heterozygous flies (*green*; *D*, *F*, *H*, and *J*) showed significantly reduced caspase-3 activity (*blue*; *D* and *E*), strong *puc-lacZ* expression (*red*; *F* and *G*), Wg up-regulation (*red*; *H* and *I*), and phosphohistone H3 staining (*red*; *J* and *K*). L–*O*, NAB induced CP and Wg expression in H99 LOF clones. Clones overexpressing NAB and homozygous for H99 (using the MARCM technique; stained for NAB, *green*; *L*, *N*, and *O*) exhibited non-autonomous growth, Wg up-regulation (*red*; *M*), and caspase-3 activation mainly in neighboring cells (*blue*; *N*). *P–S*, *dronc* knockdown did not block the NAB-induced non-autonomous growth effect on neighboring tissue. Clones coexpressing NAB and *dronc* RNAi (*green*; *P* and *R*) exhibited up-regulated *puc-lacZ* (*red*; *Q*), Wg expression (*red*; *S*), and a strong non-autonomous proliferation effect. *T* and *U*, coexpression of both NAB and Diap1 resulted in elevated Wg expression and tissue overgrowth. Clones coexpressing both NAB and Diap1 (*green*; *T*) exhibited up-regulated Wg expression (*red*; *T* and *U*).

apoptosis is still unclear, it may involve direct induction of expression of the pro-apoptotic genes *hid* and *rpr* (38). On the other hand, recent work has placed Dp53 downstream of Dronc in the regulation of ectopic Wg expression and the subsequent CP response (5). We therefore wished to investigate the possible role of Dp53 in the apoptotic and compensatory growth response associated with NAB-JNK signaling. To that end, we used a dominant-negative form of Dp53 (D259H) that was shown to readily protect imaginal disc cells from radiation-induced apoptosis (39). We found that apoptotic cell elimination induced by NAB was not affected by coexpression of dominant-negative Dp53 (Fig. 5, *A–C*). We then generated clones of

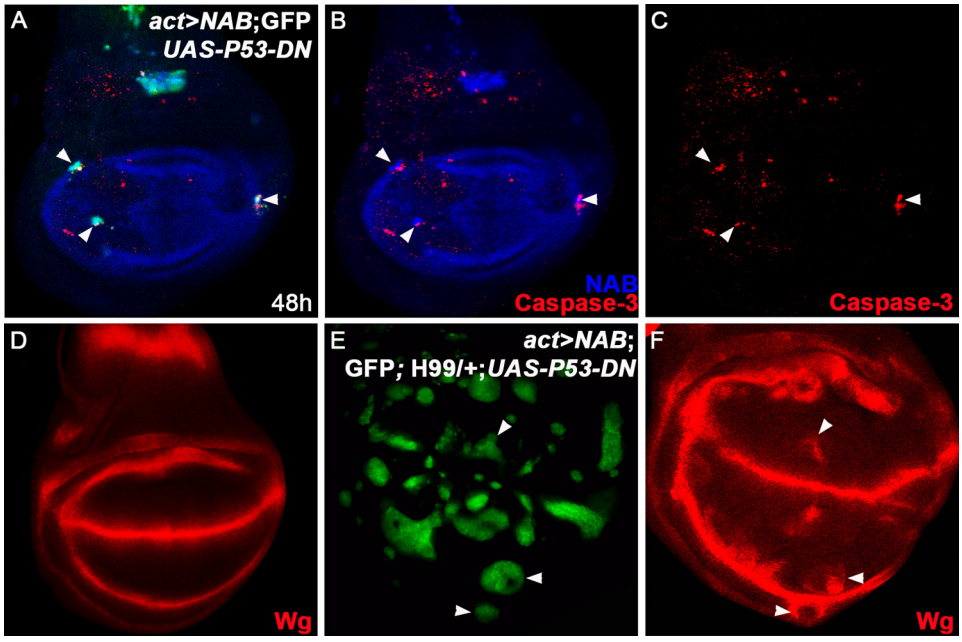


FIGURE 5. **Dp53 is not required for NAB-induced apoptotic cell elimination.** *A* and *B*, clones overexpressing NAB (*blue*; *A* and *B*) and the dominant-negative (*DN*) form of Dp53 (*green*; *A*) were eliminated from the wing disc through caspase-3 activation (*red*; *B* and *C*, *arrowheads*). *D–F*, Dp53 is not required for NAB-induced CP. Clones overexpressing NAB and the dominant-negative form of Dp53 (*green*; *E*) in an H99 heterozygous background showed ectopic Wg expression (*red*; *E* and *F*) and tissue overgrowth (compared with the WT WID in *C*).

cells coexpressing both NAB and dominant-negative Dp53 in H99 heterozygous flies. These cells exhibited ectopic Wg expression and tissue overgrowth similar to those seen with *dronc* RNAi (Fig. 5, *E* and *F*), suggesting that Dp53 is not essential for NAB-JNK-induced CP.

## DISCUSSION

In multicellular organisms, apoptotic removal of aberrant or damaged cells is often compensated by extra proliferation of neighboring cells to allow for proper development. Recent studies in *Drosophila* indicated that CP is triggered by the initiator caspase Dronc through Dp53 during the execution of cell death. In this context, transcriptional activation of Dp53 by Dronc causes ectopic expression of Wg and CP (5). Wg induction and stimulation of compensatory growth also require activation of JNK (3), which can be induced by Dronc overexpression (4). However, the roles of JNK in the Dronc-dependent compensatory growth response and the relations between Dp53 and JNK in this context have not been directly addressed.

We have recently shown that NAB acts as a co-repressor that interacts with Brk to apoptotically eliminate cells with impaired Dpp signaling through activation of the JNK signaling pathway (9). Here, we studied NAB-induced death and CP responses with respect to JNK, Dronc, and Dp53. Our results indicate that the NAB-Brk signal first activates JNK, which in turn transmits two independent signals. One stimulates compensatory growth. The other signal triggers apoptosis through the pro-apoptotic proteins Rpr and Hid, which in turn reduce Diap1 protein levels and release the apoptosome components Dronc and Ark to activate effector caspases (Fig. 2). The idea that the NAB-JNK primary signal acts upstream of the death signaling is consistent with previous studies showing that JNK

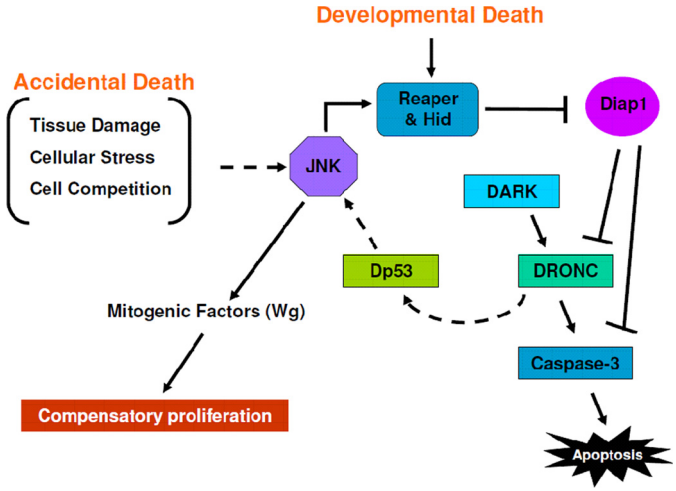


FIGURE 6. **Accidental death induces JNK signaling, which simultaneously activates apoptosis and CP.** Accidental death mediates JNK activation, which triggers, amplifies, and sustains both death and CP responses. CP is enhanced by a positive feedback loop with Dronc and the apoptosis-inducing factors Hid and Rpr. This regulatory loop between JNK, Dronc, Hid, and Rpr impinges on both the apoptotic and growth responses. Similar to the situation in which a low Dpp signal results in NAB-Brk-dependent JNK activation (shown in Fig. 1), other cellular stresses and extra/intracellular signals simultaneously activate cell death and CP through JNK.

triggers apoptosis by inducing the transcriptional activation of both *rpr* and *hid* (36, 37). Therefore, in contrast to the previous notion that CP is initiated after the induction of apoptosis, we now show that JNK signaling triggers apoptosis and growth in parallel and that JNK activation, but not the induction of apoptosis, is a prerequisite for a compensatory growth.

Similar to previous studies (3–5), our results also support a central role for the apoptotic pathway (probably through Dronc) in stimulation of compensatory growth (Fig. 6). However, it appears that the apoptotic signal is not the initiator of

this response but rather acts to further activate the growth response associated with NAB-JNK signaling (Fig. 4). Collectively, these data provide a model in which JNK amplifies and sustains the CP responses through a positive feedback loop with the core apoptotic pathway (Fig. 6).

A recent study demonstrated that ectopic expression of Dronc and p35 results in elevation of Dp53 RNA and that Dp53 mutants suppress Dronc-induced Wg up-regulation and the overgrowth phenotype in imaginal discs. These results indicate that Dronc is both necessary and sufficient to induce p53-dependent overgrowth in imaginal discs (5). We therefore investigated how JNK signaling and p53 are integrated. We found that NAB induces both apoptotic cell elimination and compensatory growth in the presence of dominant-negative Dp53. This implies that Dp53 is not essential for NAB-JNK-induced CP. Interestingly, expression of Dp53 was shown to activate JNK, whereas loss of Dp53 prevented radiation-induced JNK activation (36), indicating that JNK acts downstream of Dp53 in response to cellular stress. On the basis of these findings, we propose that, in the positive regulatory loop between JNK, Dronc, and the activator of apoptosis, Dp53 may function downstream of Dronc to activate JNK (Fig. 6).

CP is necessary to restore tissue homeostasis following removal of misspecified cells and apoptosis induced by cellular stress or tissue damage (non-developmental apoptosis). However, CP must not be induced by developmentally programmed apoptosis because it is required to maintain tissue homeostasis and to shape organ structure by removing extra cells (40). How the CP response is linked to one form of apoptosis and not the other is a fundamental question in the understanding of growth regulation and tissue homeostasis. The NAB-Brk signal serves as a paradigm for non-developmental apoptosis coupled with CP, which, similar to other cellular stresses and extra/intracellular signals, activates JNK-mediated cell death. We propose that signaling through the JNK pathway is the common denominator that regulates CP in non-developmental cell death contexts. Consistent with this notion, JNK signaling does not seem to play a significant role in the induction of developmental apoptosis, as JNK inactivation in imaginal discs results in viable flies with no discernable phenotypic consequences (36). Conversely, non-developmental forms of apoptosis associated with compensatory growth are commonly triggered by JNK signaling (35).

Both forms of apoptosis are mediated through the activators of apoptosis, but their activation is achieved in different ways. In non-developmental paradigms, apoptosis is activated by the JNK pathway, whereas in developmental contexts, apoptosis is activated by alternative signals. In the non-developmental context, the early activation of JNK, together with its later activation downstream of Dronc, causes strong and prolonged activation of the proliferative response. In accordance, when we blocked the death signal downstream of JNK and prevented the positive regulatory loop between JNK, Dronc, and the RHG proteins, we observed a reduction in the activation of extra growth associated with undead cells overexpressing NAB. On the other hand, in developmental contexts in which the death signal is not conveyed by JNK, Dronc-dependent activation of JNK is weaker and shorter; hence, death is induced without stimulating proliferation. Although our results propose that Dronc is not the initiator of the CP signal, the extra JNK activation induced by Dronc may be essential for the CP response in normal situations of damage-induced death. Indeed, it was recently shown that Dronc inactivation is sufficient to suppress CP in imaginal discs from irradiated animals (4, 5).

*Acknowledgments—We thank K. White, P. D. Friesen, K. Basler, L. Luo, the National Institute of Genetics Fly Stock Center (NIG), the Vienna Drosophila RNAi Center (VDRC), the Bloomington Drosophila Stock Center, the Developmental Studies Hybridoma Bank, and the Drosophila Genomics Resource Center (DGRC) for fly stocks, antibodies, and DNA clones and G. Deshpande and E. Ordan for insightful discussions and comments on the manuscript.*

## REFERENCES

1. Baehrecke, E. H. (2002) *Nat. Rev.* **3,** 779–787
2. Huh, J. R., Guo, M., and Hay, B. A. (2004) *Curr. Biol.* **14,** 1262–1266
3. Ryoo, H. D., Gorenc, T., and Steller, H. (2004) *Dev. Cell* **7,** 491–501
4. Kondo, S., Senoo-Matsuda, N., Hiromi, Y., and Miura, M. (2006) *Mol. Cell. Biol.* **26,** 7258–7268
5. Wells, B. S., Yoshida, E., and Johnston, L. A. (2006) *Curr. Biol.* **16,** 1606–1615
6. Adachi-Yamada, T., Fujimura-Kamada, K., Nishida, Y., and Matsumoto, K. (1999) *Nature* **400,** 166–169
7. Burke, R., and Basler, K. (1996) *Development* **122,** 2261–2269
8. Moreno, E., Basler, K., and Morata, G. (2002) *Nature* **416,** 755–759
9. Ziv, O., Suissa, Y., Neuman, H., Dinur, T., Geuking, P., Rhiner, C., Portela, M., Lolo, F., Moreno, E., and Gerlitz, O. (2009) *Development* **136,** 1137–1145
10. Adachi-Yamada, T., and O'Connor, M. B. (2002) *Dev. Biol.* **251,** 74–90
11. Steller, H. (2008) *Cell Death Differ.* **15,** 1132–1138
12. Goyal, L., McCall, K., Agapite, J., Hartwieg, E., and Steller, H. (2000) *EMBO J.* **19,** 589–597
13. Lisi, S., Mazzon, I., and White, K. (2000) *Genetics* **154,** 669–678
14. Wang, S. L., Hawkins, C. J., Yoo, S. J., Müller, H. A., and Hay, B. A. (1999) *Cell* **98,** 453–463
15. Ditzel, M., Broemer, M., Tenev, T., Bolduc, C., Lee, T. V., Rigbolt, K. T., Elliott, R., Zvelebil, M., Blagoev, B., Bergmann, A., and Meier, P. (2008) *Mol. Cell* **32,** 540–553
16. Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D. A., Agapite, J., Steller, H., and Meier, P. (2002) *Nat. Cell Biol.* **4,** 445–450
17. Ryoo, H. D., Bergmann, A., Gonen, H., Ciechanover, A., and Steller, H. (2002) *Nat. Cell Biol.* **4,** 432–438
18. Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M., Clem, R. J., Müller, H. A., and Hay, B. A. (2002) *Nat. Cell Biol.* **4,** 416–424
19. Chew, S. K., Akdemir, F., Chen, P., Lu, W. J., Mills, K., Daish, T., Kumar, S., Rodriguez, A., and Abrams, J. M. (2004) *Dev. Cell* **7,** 897–907
20. Daish, T. J., Mills, K., and Kumar, S. (2004) *Dev. Cell* **7,** 909–915
21. Xu, D., Li, Y., Arcaro, M., Lackey, M., and Bergmann, A. (2005) *Development* **132,** 2125–2134
22. Adrain, C., Brumatti, G., and Martin, S. J. (2006) *Trends Biochem. Sci.* **31,** 243–247
23. Shapiro, P. J., Hsu, H. H., Jung, H., Robbins, E. S., and Ryoo, H. D. (2008) *Nat. Cell Biol.* **10,** 1440–1446
24. Foley, E., and O'Farrell, P. H. (2004) *PLoS Biol.* **2,** E203
25. Leulier, F., Rodriguez, A., Khush, R. S., Abrams, J. M., and Lemaitre, B. (2000) *EMBO Rep.* **1,** 353–358
26. Stoven, S., Silverman, N., Junell, A., Hedengren-Olcott, M., Erturk, D., Engstrom, Y., Maniatis, T., and Hultmark, D. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 5991–5996
27. Pérez-Garijo, A., Martín, F. A., and Morata, G. (2004) *Development* **131,** 5591–5598
28. Bergantiños, C., Corominas, M., and Serras, F. (2010) *Development* **137,** 1169–1179

29. Bosch, M., Serras, F., Martín-Blanco, E., and Baguñà, J. (2005) *Dev. Biol.* **280,** 73–86
30. Mattila, J., Omelyanchuk, L., Kyttälä, S., Turunen, H., and Nokkala, S. (2005) *Int. J. Dev. Biol.* **49,** 391–399
31. Smith-Bolton, R. K., Worley, M. I., Kanda, H., and Hariharan, I. K. (2009) *Dev. Cell* **16,** 797–809
32. Pérez-Garijo, A., Shlevkov, E., and Morata, G. (2009) *Development* **136,** 1169–1177
33. Martín-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M., and Martinez-Arias, A. (1998) *Genes Dev.* **12,** 557–570
34. Terriente Félix, J., Magariños, M., and Díaz-Benjumea, F. J. (2007) *Development* **134,** 1845–1852
35. Igaki, T. (2009) *Apoptosis* **14,** 1021–1028
36. McEwen, D. G., and Peifer, M. (2005) *Development* **132,** 3935–3946
37. Moreno, E., Yan, M., and Basler, K. (2002) *Curr. Biol.* **12,** 1263–1268
38. Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M., and Abrams, J. M. (2000) *Cell* **101,** 103–113
39. Peters, M., DeLuca, C., Hirao, A., Stambolic, V., Potter, J., Zhou, L., Liepa, J., Snow, B., Arya, S., Wong, J., Bouchard, D., Binari, R., Manoukian, A. S., and Mak, T. W. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 11305–11310
40. Fan, Y., and Bergmann, A. (2008) *Trends Cell Biol.* **18,** 467–473