Apoptosis operates to eliminate damaged or potentially dangerous cells. This loss is often compensated by extraproliferation 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 through Dp53 in wing discs. However, the exact mechanism that governs this compensatory proliferation 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 JNK-dependent apoptosis. Here we performed a systematic in vivo loss- and gain-of-function analysis to study NAB-induced death and compensatory proliferation. 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 compensatory proliferation in a manner that is independent of the death signal, Dronc or Dp53. Once induced, the apoptotic pathway further activates compensatory proliferation response. Our data suggest that JNK is the candidate factor that differentiates between apoptosis that involves compensatory proliferation and apoptosis which does not.

Dpp, a member of the TGFβ 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 Dpp’s transcriptional output is mediated through repression of the transcriptional repressor Brinker (Brk) that functions as a negative regulator of Dpp targets. Since Dpp also provides growth and survival cues, impaired Dpp signaling results in accumulation of Brk, which in turn triggers apoptosis through cellular proofreading: it is used to eliminate misspecified or damaged cells. In this context, apoptosis is associated with compensatory proliferation (CP), 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 that signal to the neighboring cells to extraproliferate in order 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 (JNK) signaling pathway that 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.

During development, apoptosis shapes structures by removing excess cells (1). Apoptosis also has an important non developmental role of
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 compensatory proliferation 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 the induction of two pro-apoptotic proteins Reaper (Rpr) and Hid. This leads to a reduction in the level of JNK upstream of the death signal. 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 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-* Df(3L)H99, Dredd and Dredd. rpr: Df(3L)XR38 (gift from K. White), hid: (P[<br>](P{PZ}w<sub>1050</sub>), Dark<sup>L46</sup>, UAS-flp, UAS-P35, UAS-LacZ, UAS-P53-DN, UAS-puc, EP-NAB, EP-Diap1, ubi-GFP, FRT42D, puc-lacZ<sup>E69</sup>, 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 the following drivers: hh-Gal4, 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 minutes. Unless written otherwise, larvae were dissected at 48 hours after clone induction (ACI).

We generated mutant clones using Flp-mediated mitotic recombination and identified them by the loss of the GFP. Clones were induced with hh-Gal4/UAS-flp. Genotypes of dissected larvae were as follows: hs-flp; Dark<sup>L46</sup>, Y+, FRT42D/ ubi-GFP FRT42D; hh-Gal4, UAS-flp/EP-NAB, UAS-lacZ. For MARCM experiments genotypes of dissected larvae were as follows: sd-Gal4/hs-flp; tub-P-Gal80 FRT80/EP-NAB, H99, FRT80. Clones were induced by heat shock for 60min at 37°C. Larvae were dissected at 72 and 96 hrs after clone induction.

**Immunohistochemistry**—Imaginal discs from late wandering (LW) stage larvae were fixed and stained by standard techniques. The specific primary antibodies used were: mouse anti-β-gal (1:1000; Promega), rat anti-NAB (1:1000), rabbit anti-cleaved Caspase-3 (1:40; Cell Signaling Tech.), rabbit anti-Diap1 (1:150, gift from Paul D. Friesen), rabbit anti-phosphohistone H3 (1:1000, Upstate (Millipore)), mouse anti-Wg (4D4, Hybridoma bank), TUNEL staining as described in Yoo et al., 2002. Images were taken on a TE2000-E confocal microscope (Nikon) using a 20X 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 either 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 size of WID pouch overexpressing NAB on a H99 LOF background or with P35 normalized to control.

**NAB Antibody production**—To generate the NAB antibody, two rats were immunized with a GST fusionc of Nab1-284aa. After three
immunizations, the rats were bled and sera 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 the NabSH143.

RESULTS

NAB induced apoptosis is mediated by the activators of apoptosis Rpr and Hid. We recently demonstrated that the 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 wing imaginal disc (WID) caused massive cell death detected by both TUNEL assay and active effector caspase expression (Fig. 1A,B and 1C,D, respectively), and this cell death is blocked either by brk loss of function (9) or knockdown (Fig. 1M,N). Furthermore, most of the somatic clones that overexpress NAB were eliminated from the WID 48 h after their induction, indicating that these cells are dying rapidly (compare Fig. 1E,F with Fig. 1G,H). Finally, we found that this death is JNK dependent, as NAB overexpression activates a downstream target of this pathway called Puckered (Puc) and ectopic expression of Puc, an inhibitor of this pathway, blocked NAB-induced apoptosis (Fig. 1I,J and 1K,L, respectively). Non-autonomous JNK and Caspase 3 activation is frequently seen around the border of NAB-overexpressing clones (Fig. 3A-B and (9)). This phenomenon was attributed to local interactions between mis-specified/dying cells and their neighbors (Adachi-Yamada and O’Connor denote this phenomenon ‘Morphogenetic Apoptosis’ (10)).

To further explore the role of the canonical apoptosis 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 apterous (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 (Compare Fig. 2A-B with 2C-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]w05014) significantly attenuated the level of NAB induced apoptosis (Fig. 2E-H and 2O), no change was observed upon knockdown of grim expression (Fig. 2I-J). We conclude that, NAB-induced apoptosis is mediated 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 auto-ubiquitination and degradation (12,14,17,18). Consistently, co-expression of Diap1 and NAB in the WID abolished effector caspase activation and apoptosis (Fig. 2K-L). Moreover, overexpression of NAB in the dorsal compartment of the WID resulted in a reduction in the level of the Diap1 protein (Fig. 2M-N). 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 induces 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. 2P-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 loss of function (LOF) (Ark^{L46}/A^{Ark}) in a NAB overexpression background. Indeed, NAB-induced effector caspase activation was absent in Ark mutant clones (Fig. 2R-S). Finally, we found that loss of the initiator-like caspase Dredd did not affect effector caspase activation associated with NAB overexpression (Fig. S1). This result is
consistent with the idea that Dredd is not essential for apoptosis in most of the cell death paradigms in Drosophila, but rather required for 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 level, allowing Dronc activation by Ark and subsequent activation of the effector caspases.

**NAB induces JNK-dependent compensatory proliferation and Wingless 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), enabled 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 the Jun N-terminal Kinase (JNK). In some of the undead cells JNK signaling induces secretion of the mitogenic factors Wg and Dpp (3,27). Upregulation of JNK activity and Wg expression are 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 (9) (Fig. 1I-L and 3A-B). To examine autonomous and non-autonomous effects on proliferation and gene expression associated with NAB induced cell death, we generated clones of cells co-expressing both NAB and P35 in the WID. Consistent with previous studies, we found that in this context, NAB-undead cells exhibited a strong non-autonomous proliferation stimulatory effect, as evident by WID overgrowth (compare Fig. 3C with 3D and see quantification in Fig. 4C). Ectopic upregulation of Wg was frequently associated with NAB-undead cells (Fig. 3E-F). Also in this context, we observed non-autonomous upregulation of Wg, likely due to non-autonomous activation of JNK signaling. Importantly, activation of JNK and occasionally upregulation of Wg were also observed in the doomed cells overexpressing NAB without P35, indicating that these effects are not merely secondary consequences of the ‘undead’ state (Fig. 3A-B and Fig. 3G-H).

Next, we examined whether JNK activation is involved in upregulation of Wg and CP. Co-expression of NAB and Puckered (Puc), a phosphatase that negatively regulates JNK (33), blocked both Wg induction and overgrowth (Fig. 3I-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 independent 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, co-expression of Dronc and P35 induces JNK activation and abnormal 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 reaper or hid (36,37), indicating 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. 2C-D and Fig. 4D-E); we generated clones of cells overexpressing NAB in H99 heterozygous background (Fig. 4D-K). NAB overexpressing cells exhibited strong JNK activity detected by the puc-lacZ reporter (Fig. 4F-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. 4H-I) and stimulated a strong non-autonomous growth of neighboring
tissue, as evident by disc overgrowth (Fig. 4D-K and Fig. S2A-B and S2E) and staining for phospho-histone H3 (PH3) (compare Fig. 4J-K to 4A-B).

To completely avoid autonomous death signaling, we used the MARCM technique to generate homozygous H99 LOF clones overexpressing NAB. Wg expression was upregulated and CP still occurred in this experimental setting, albeit to a lesser extent (Fig. 4C and 4L-O). We occasionally observed effector caspase activation in the cells bordering the NAB ‘undead cells’ (Fig. 4N). The nonautonomous 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.

While 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. 4P-Q), which was associated with robust extra tissue growth (Fig. 4P-S and Fig. S2C-E), indicating that activation of JNK by NAB is independent of Dronc. Although Dronc RNAi efficiently suppressed NAB-induced effector caspase activation, CP and upregulation of Wg (Fig. 4R-S) still occurred. Similarly, overexpression of Diap1, which inhibits Dronc activity (16), failed to suppress NAB-induced Wg expression and tissue overgrowth (Fig. 4T-U and Fig. S2E-H). We conclude that NAB signal bifurcates at the level of JNK to independently activate the apoptotic and the 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 compensatory proliferation.** The *Drosophila* p53 (Dp53) was shown to mediate apoptosis-induced by ionizing irradiation (38). Whereas the mechanism by which Dp53 activates 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 (DN) form of Dp53 (D259H), which 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 co-expression of Dp53-DN (Fig. 5A-C). We then generated clones of cells co-expressing both NAB and Dp53-DN in H99 heterozygous flies. These cells exhibited ectopic Wg expression and tissue overgrowth similar to those seen with Dronc RNAi suggesting that Dp53 is not essential for NAB/JNK induced CP (Fig. 5E-F).

**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 requires activation of JNK (3), which can be induced by Dronc overexpression (4). However, the roles of JNK in 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 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. 4). The idea that the NAB/JNK primary signal acts upstream of the death signaling is consistent with previous studies showing that JNK triggers apoptosis by inducing the transcriptional activation of both \textit{rpr} and \textit{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 where 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 upregulation and 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 Dp53-DN. This implies that Dp53 is not essential for NAB/JNK induced CP. Interestingly, expression of Dp53 was shown to activate JNK, while 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 must not be induced by developmentally programmed apoptosis, since it is required to maintain tissue homeostasis and to shape organ structure by removing extra cells (40). How 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 a non-developmental apoptosis coupled with CP, which similar to other cellular stresses and extra/intra-cellular 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, while 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 where 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).
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REFERENCES


**FIGURE LEGENDS**

**Fig. 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, C) leads to apoptosis as visualized by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (red, B) and activated Caspase-3 (red, D). (E-H) NAB-overexpressing clones are apoptotically eliminated from the *Drosophila* wing disc. Clones overexpressing NAB 24 (E) and 48 (G) hours after induction (green) upregulate activated Caspase-3 (red, F, H). (I-L) NAB-dependent cell elimination is associated with, and caused by activation of the JNK pathway. (I-J) *hh-Gal4* driven NAB overexpression in the posterior wing compartment (marked by GFP, green, I) activates the JNK pathway (*puc-lacZ*, blue, J). (K-L) Overexpression of both NAB and Puc (K, green) abolishes Caspase-3 activation (L, red). (M-N) NAB requires Brk to induce cell death. Clones overexpressing both NAB and *brk-RNAi* (green, M) show no Caspase-3 activation (red, N). (O) A model showing that Dpp survival signal acts through repression of Brk. Impaired Dpp signaling upregulates Brk which together with NAB activates JNK-mediated apoptosis.

**Fig. 2.** NAB induced cell death is mediated through multiple activators of apoptosis which trigger Diap1 protein degradation. The dashed line (white, A-N) marks the dorsal-ventral boundary. (A-B) *ap-Gal4* driven NAB overexpression in the dorsal wing compartment (green, A) leads 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-D) NAB overexpression in the dorsal wing compartment (green, C) in flies heterozygous for the deficiency H99 (Df(3L)H99) show 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) show modest Caspase-3 activation (red, F and H respectively, compare to B). (I-J) Overexpression of NAB together with RNAi-mediated knockdown of grim (green, I) in the dorsal compartment (using *ap-Gal4*) have no effect on Caspase-3 activation levels (compare J to B). (K-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 (K, green) abolishes Caspase-3 activation (red, L). (M-N) Diap1 protein levels are reduced in response to NAB overexpression. *ap-Gal4* driven NAB overexpression (green, M) triggers reduction in Diap1 protein levels (red, N). (O) Relative quantification of NAB induced Caspase-3 activation in the dorsal compartment. Average area covered by activated Caspase-3 of NAB overexpression together with hid, rpr, 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-Q) RNAi-mediated knockdown of *dronc* expression in clones overexpressing NAB (green, P) virtually abolished Caspase-3 activation (red, Q). (R-S) NAB-induced cell death is absent in mutant clones of *dark*. *dark* loss-of-function 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 is nearly abolished (red, R) in the large *dark* loss-of-function clones.

**Fig. 3. NAB induces Wingless expression and CP through the JNK pathway.** (A-H) NAB overexpression induces Wingless expression, JNK activation and non-autonomous proliferation. (A-B) Clones overexpressing NAB (A, green) upregulate *puc-lacZ* expression (red, B). (C-D) WID with clones co-overexpressing both NAB and p35 exhibit strong non-autonomous proliferation effect (compare wing disc size between C and D and quantification in Fig. 4C; scale bar equals 100μm). (E-F) Wg expression is ectopically upregulated (red, E, F) in clones co-overexpressing both NAB and p35 (green, E). (G-H) Wg expression (red, G, H) is upregulated in clones overexpressing NAB (green, G). (I-J) NAB induced Wg upregulation is mediated via the JNK pathway. Co-expression of puc and NAB (green, I) blocked both Wg induction (red, I, J) and non-autonomous overgrowth stimulation (compare wing discs size between J and F).

**Fig. 4. NAB induces CP and Wg expression through the JNK pathway independent of the death signal.** (A) WT wing disc stained for Wg (red). (B) WT wing disc stained for PH3 (red). (C) Comparison of wing discs size overexpressing NAB on a H99 LOF background (red bar, n=13) or with P35 (blue bar, n=16) relative to WT (p<0.001). (D-K) Clones of cells overexpressing NAB in H99 heterozygous flies (green, D, F, H, J) show significantly reduced Caspase-3 activity (blue, D, E), strong *puc-lacZ* expression (red, F, G), Wg upregulation (red, H, I), and PH3 staining (red, J, K). (L-O) NAB induces 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, O) exhibit non-autonomous growth, Wg upregulation (red, M) and Caspase-3 activation mainly in neighboring cells (blue, N). (P-S) Dronc knockdown does not block NAB induced non-autonomous growth effect on neighboring tissue. Clones co-expressing NAB and RNAi-dronc (green, P, R) exhibit upregulated *puc-lacZ* (red, Q), Wg expression (red, S) and strong non-autonomous proliferation effect. (T-U) Co-expression of both NAB and Diap1 results in elevated Wg expression and a tissue overgrowth. Clones co-expressing both NAB and Diap1 (green, T) exhibit upregulated Wg expression (red, T, U).

**Fig. 5. Dp53 is not required for NAB induced apoptotic cell elimination.** Clones overexpressing NAB (blue, A, B) and the dominant negative form of Dp53 (green, A) are eliminated from the wing disc through Caspase-3 activation (red, B, 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 H99 heterozygous background show ectopic Wg expression (red, E, F) and tissue overgrowth (compared to WT WID, C).
Fig. 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 where low Dpp signal results in NAB/Brk dependent JNK activation (shown in Fig. 1), other cellular stresses and extra/intra-cellular signals simultaneously activate cell death and CP through JNK.

Fig. Sup1. Dredd is not required for NAB-induced apoptosis. Strong Caspase-3 activation (red, B) is observed in cells overexpressing NAB in the posterior compartment using hh-GAL4 driver (green, A) in flies homozygous for dredd loss of function.

Fig. Sup2. NAB induces CP and tissue overgrowth in the WID. (A-D) Arrowheads mark areas of wing pouch overgrowth. (A-B) Clones of cells overexpressing NAB in H99 heterozygous flies (green, A) exhibit a non-autonomous overgrowth effect on neighboring tissue in the wing pouch region; marked by the wing pouch marker NAB (red, B). (C-D) Clones co-expressing NAB and Drone-RNAi (green, C) exhibit non-autonomous overgrowth in the wing pouch region marked by NAB (red, C, D). (E-H) Clones co-expressing NAB and Diap1 (green, E, G) exhibit Wg upregulation (red, E, F) and non-autonomous WID overgrowth marked by fasciclin (red, G, H). Note the extra folds of the tissue next to the clones (arrowheads). (I) Comparison of wing discs size overexpressing NAB in a H99 heterozygous background (blue bar, n=15) or with Dronc-RNAi (red bar, n=16) relative to WT (p<0.001).
Figure 1
Figure 3
Figure 4
Figure 5

Figure 6
The NAB-Brk signal bifurcates at JNK to independently induce apoptosis and compensatory proliferation
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