Current Biology, Vol. 14, 1262–1266, July 27, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.06.015

Compensatory Proliferation Induced by Cell Death in the *Drosophila* Wing Disc Requires Activity of the Apical Cell Death Caspase Dronc in a Nonapoptotic Role

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Summary

Achieving proper organ size requires a balance between proliferation and cell death. For example, at least 40%–60% of cells in the *Drosophila* wing disc can be lost, yet these discs go on to give rise to normal-looking adult wings as a result of compensatory proliferation [1–3]. The signals that drive this proliferation are unknown. One intriguing possibility is that they derive, at least in part, from the dying cells. To explore this hypothesis, we activated cell death signaling in specific populations of cells in the developing wing but prevented these cells from dying through expression of the baculovirus p35 protein, which inhibits the activity of effector caspases that mediate apoptosis [4]. This allowed us to uncouple the activation steps of apoptosis from death itself. Here we report that stimulation of cell death signaling in the wing disc—in the absence of cell death—results in increased proliferation and ectopic expression of Wingless, a known mitogen in the wing. Activation of the apical cell death caspase Dronc is necessary and sufficient to drive both of these processes. Our results demonstrate an unanticipated function, the nonautonomous induction of proliferation, of an apical cell death caspase. This activity is likely to contribute to tissue homeostasis by promoting local compensatory proliferation in response to cell death. We speculate that dying cells may communicate cell fate or behavior instructions to their neighbors in other contexts as well.

Results and Discussion

In the fly, expression of the cell death activator head involution defective (Hid) promotes caspase activation and cell death in many contexts [5], including the wing [4, 6]. We characterized wing discs expressing Hid and p35 under the control of the *engrailed* promoter, which drives expression in the posterior wing compartment. As noted previously [4], cells expressing Hid and p35 show decreased levels of the Inhibitor of apoptosis (IAP) family caspase inhibitor DIAP1 and high levels of activation of the cell death effector caspase Drice. However, these cells do not die because expression of p35 inhibits the activity (but not the activation) of effector caspases such as Drice [4]. We monitored cell proliferation in wing discs by using anti-phosphohistone H3 antibodies, which label cells in M phase. Wing discs that express p35 alone in the posterior compartment showed a general low level of proliferation in both anterior and posterior compartments (Figures 1A and 1B). In contrast, wing discs that express Hid and p35 under *engrailed* control consistently showed a 2- to 4-fold increase in proliferation in the posterior compartment (Figures 1C and 1D; Figure S1 in the Supplemental Data available with this article online).

How are Hid-dependent cell death signals transduced to promote cell proliferation? Multiple IAPs, including DIAP1, can participate in various signal transduction pathways, in addition to functioning as caspase inhibitors [7–9]. To determine if the Hid-dependent increase in proliferation required the loss of DIAP1, we decreased DIAP1 levels in the absence of other apical death signals by using RNA interference (RNAi) in the presence of p35. Double-stranded RNA corresponding to DIAP1 (DIAP1-RNAi) and p35 were expressed under the control of the *patched* promoter, which drives expression in a stripe at the anterior-posterior compartment border. Expression of p35 alone did not cause a decrease in DIAP1 [4] (data not shown) or result in increased levels of proliferation within the domain of *patched* expression (Figures 2A and 2B). Expression of DIAP1 RNAi and p35 under *patched* control resulted in a dramatic decrease in DIAP1 protein levels (Figures 2C and 2D), but again there was no increase in proliferation (Figures 2E and 2F). These observations (as well as observations presented in Figures 3 and 4) argue that Hid and/or molecules activated by Hid, but not the loss of DIAP1 (which may however facilitate the activation or activity of these other molecules), are required to induce cell proliferation.

Dronc is an apical caspase that mediates Hid-dependent cell death, as well as that initiated by other death activators [10–13]. Dronc and several mammalian apical cell death caspases have also been shown to participate in several nonapoptotic processes [14, 15]. To explore roles for Dronc in mediating Hid-induced proliferation in the wing, we expressed a dominant-negative version of Dronc ($Dronc^{C318S}$) [10, 15] along with Hid and p35 under *engrailed* control (Figure 3A). DIAP1 levels were still decreased (Figure 3B), as with expression of Hid and p35 [4]. However, increased cell proliferation in the posterior compartment was no longer observed (Figures 3C, 3D, and S1). These results demonstrate that Dronc activity is required to mediate Hid-dependent proliferation. Further support for this conclusion comes from an examination of adult wings. Wings from flies expressing p35 under *engrailed* control appeared normal (Figure 3E), whereas wings from flies expressing Hid and p35 under *engrailed* control had an expanded posterior compartment, consistent with the observation of increased proliferation in this domain during larval development (Figures 3G and 3H). In contrast, wings from flies that expressed $Dronc^{C318S}$ as well Hid and p35 under *en-*

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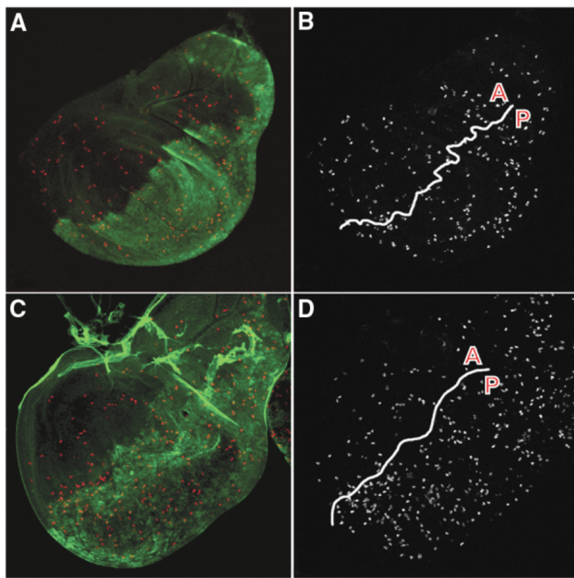

Figure 1. Activation of Hid-Dependent Cell Death Signaling, in the Absence of Cell Death, Induces Cell Proliferation

Confocal images of wing discs from third-instar larvae of various genotypes. Posterior is to the right. (A) Wing disc from a third-instar larva of genotype UAS::p35/*engrailed* Gal4 (en::Gal4), stained with anti-phosphohistone H3 (red) and anti-p35 (green). (B) The same wing disc with anti-phosphohistone H3 staining in white. (C) Wing disc from a third-instar larva of genotype UAS::Hid, UAS::p35/*engrailed* Gal4 (en::Gal4), stained with anti-Hid (green) and anti-phosphohistone H3 (red). (D) The same wing disc with anti-phosphohistone H3 staining in white. In panels (B) and (D), a white line indicates the anterior-posterior compartment boundary. Anti-phosphohistone H3 staining, which labels cells in M phase of the cell cycle, is increased in the posterior compartment of Hid- and p35-expressing discs (C and D), but not those that express p35 alone (A and B).

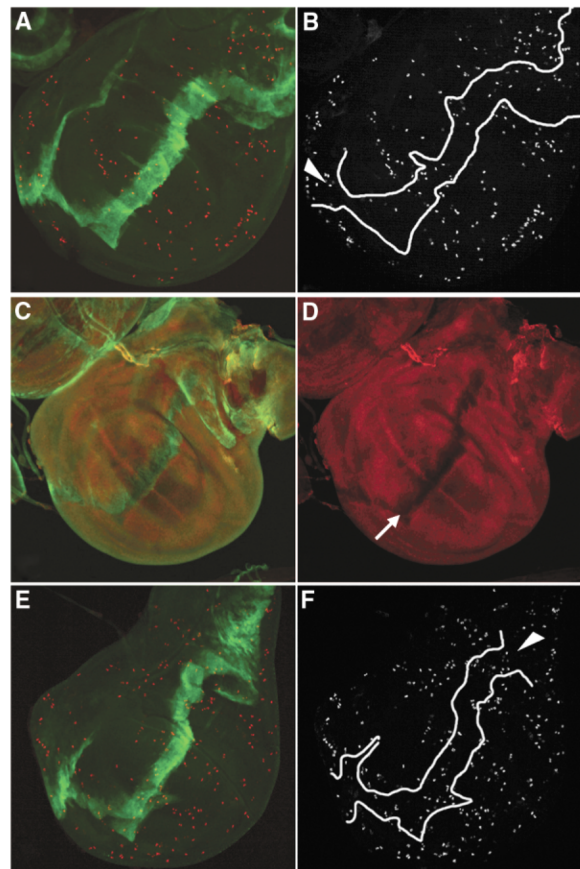

Figure 2. Loss of DIAP1, in the Presence of p35, Is Not Sufficient for Promoting Cell Proliferation in the Wing Disc

Confocal images of wing discs from third-instar larvae of various genotypes. Posterior is to the right. (A) Wing disc from a third-instar larva of genotype UAS::p35/*patched* Gal4 (ptc::Gal4), stained with anti-p35 (green) and anti-phosphohistone H3 (red). (B) The same wing disc with anti-phosphohistone H3 staining shown in white. (C) Wing disc of genotype UAS::DIAP1-RNAi, UAS::p35/ptc::Gal4 stained with anti-DIAP1 (red) and anti-p35 (green). (D) The same wing disc as in (C); only the anti-DIAP1 staining is shown. (E) Wing disc of genotype UAS::DIAP1-RNAi, UAS::p35/ptc::Gal4 stained with anti-p35 (green) and anti-phosphohistone H3 (red). (F) The wing disc in (E) with anti-phosphohistone H3 shown in white. In panels (B) and (F), the boundaries of the ptc::Gal4 expression domain are shown as a white line. Panels (A) and (B) show the pattern of proliferation in wing discs expressing p35 alone under *patched* control. Panels (C) and (D) show that expression of UAS::DIAP1-RNAi, UAS::p35 under *patched* control leads to the loss of DIAP1 (arrow) within the *patched* expression domain. Panels (E) and (F) show that expression of UAS::DIAP1-RNAi, UAS::p35 under *patched* control does not lead to an increase in proliferation within the ptc::Gal4 expression domain (arrowhead). Note that the wing discs in panels (C and D) are different from those in panels (E and F) because both anti-DIAP1 and anti-phospho-histone H3 are mouse monoclonal antibodies.

grailed control were normal in appearance (Figure 3F). We showed that activation of Dronc, in the absence of cell death, was sufficient to drive cell proliferation by following the fate of cells in wing discs from flies that expressed Dronc and p35 under *patched* or *engrailed* control. Dronc-expressing cells did not die, as evidenced by a lack of increased TUNEL staining in these cells (data not shown), and DIAP1 levels were if anything slightly increased in the regions with elevated Dronc (Figures 4A and 4B). These regions did, however, show increased cell proliferation (Figures 4C–4F).

Hid promotes cell death, at least in part by disrupting interactions between caspases and DIAP1 [16, 17] and through stimulation of DIAP1 degradation [4]. Loss of DIAP1 results in cell death [16, 18–25], and where it has been examined, this is mediated by Ark-dependent activation of Dronc, which cleaves and activates the downstream effector caspases Dcp-1 and Drice [10, 11, 20–23]. Together, these observations raise a question as to why expression of Hid or Dronc in the presence of p35, but not loss of DIAP1 in the presence of p35, resulted in increased proliferation. One possibility is that Hid promotes Dronc activation or activity through pathways other than simple elimination of the Dronc inhibitor DIAP1. In the context of such a model, Hid-dependent activation of Dronc would simply be more robust than that due to loss of DIAP1 in otherwise healthy cells. Alternatively, Hid may have activities that cooperate with Dronc (but are not sufficient by themselves; see Figures 3C and 3D) to promote proliferation. Regardless of the mechanisms by which Dronc is activated, our observations demonstrate that Dronc activity is both necessary and sufficient to stimulate cell proliferation in response to a signal that would normally induce cell death. Loss

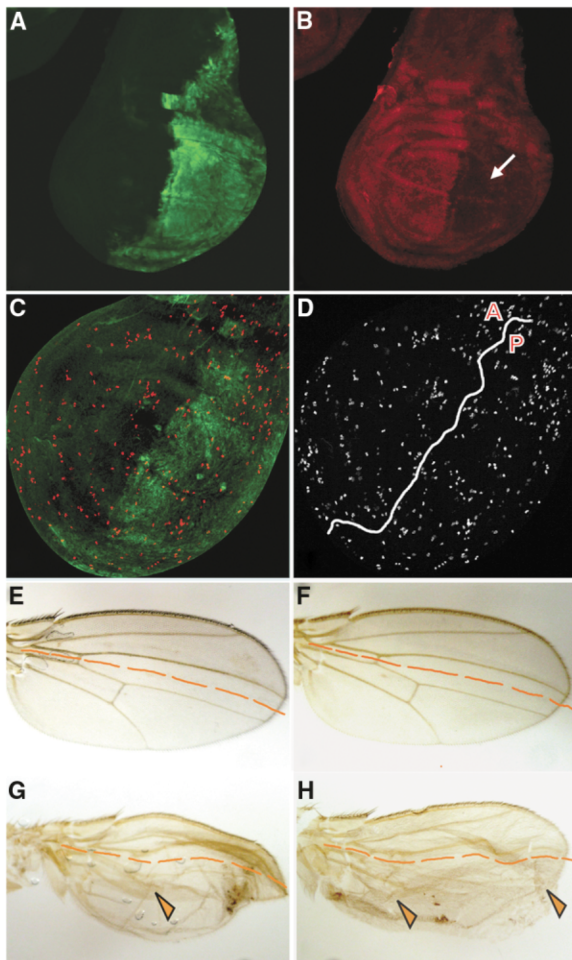

Figure 3. Dronc Activity Is Necessary for Hid-Dependent Stimulation of Proliferation in the Wing Disc

Confocal images of wing discs from third-instar larvae (A–D) and adult wings (E–H) of various genotypes. (A and B) Wing disc of genotype UAS::Dronc^{C318S}; UAS::Hid, UAS::p35/eng::Gal4 stained with anti-Dronc (green) (A) and anti-DIAP1 (red) (B). (C) Wing disc of genotype UAS::Dronc^{C318S}; UAS::Hid, UAS::p35/eng::Gal4 stained with anti-Hid (green) and anti-phosphohistone H3 (red). (D) The wing disc in (C) with anti-phosphohistone H3 in white. The anterior-posterior compartment boundary is indicated by the white line. Wing discs that express Hid and p35 under *engrailed* control show a dramatic decrease in DIAP1 levels in the posterior compartment of the wing disc [4]. Coexpression of Dronc^{C318S} with Hid and p35 under *engrailed* control (A) still led to a dramatic decrease in DIAP1 levels in the posterior compartment (arrow) (B). A wing disc of genotype UAS::Dronc^{C318S}; UAS::Hid, UAS::p35/eng::Gal4 showed no increase in proliferation in the posterior compartment relative to the anterior compartment (C and D). The wing disc in panels (A) and (B) is different from the one shown in panels (C) and (D). Anti-Dronc staining is used for marking the anterior-posterior compartment boundary in (A) and (B). Anti-Hid staining is used to mark the boundary in (C) and (D). (E) Adult wing of genotype UAS::p35/eng::Gal4. (F) Adult wing of genotype UAS::Dronc^{C318S}; UAS::Hid, UAS::p35/eng::Gal4. (G and H) Adult wings of genotype UAS::Hid, UAS::p35/eng::Gal4. Expression of p35 alone in the posterior wing compartment results in the formation of a normal adult wing (E). Expression of Hid and P35 in the posterior compartment resulted in an expansion of this compartment (G and H). This expansion is associated with the presence of extra tissue folds (several of which are indicated with the arrowhead). Coexpression of the dominant-negative form of Dronc, Dronc^{C318S}, with Hid and p35 in the posterior wing compartment suppressed the overgrowth phenotypes associated with Hid expression (F).

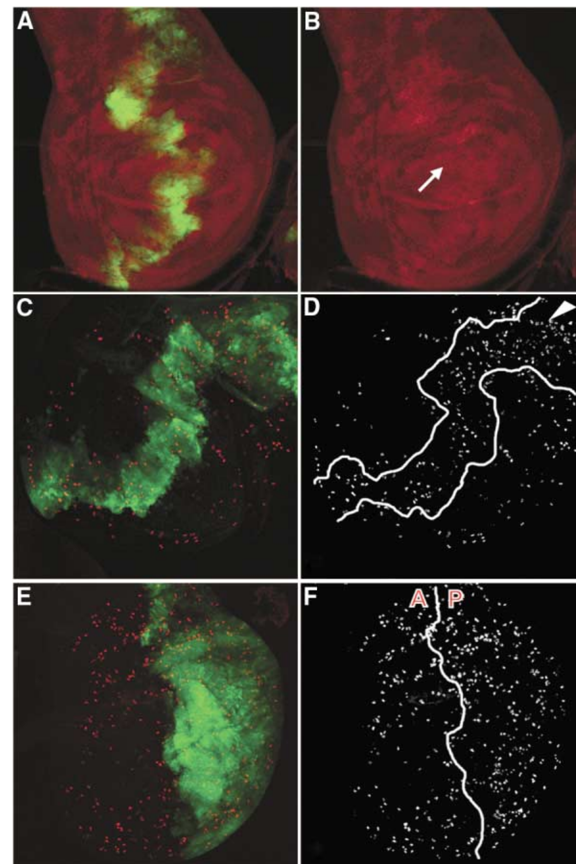

Figure 4. Dronc Activation Is Sufficient to Stimulate Proliferation in the Wing Disc

Confocal images of wing discs from third-instar larvae of various genotypes. Dronc expression was visualized with GFP (green) (see Supplemental Experimental Procedures for details). (A) Wing disc of genotype UAS::Dronc; UAS::p35/ptc::Gal4 stained with anti-DIAP1 (red). (B) Disc in (A) with only anti-DIAP1 staining shown. (C) Wing disc of genotype UAS::Dronc; UAS::p35/ptc::Gal4 stained with anti-phosphohistone H3 (red). (D) Disc in (C) with anti-phosphohistone H3 shown in white. The ptc-expressing domain lies within the white lines. (E) Wing disc of genotype UAS::Dronc; UAS::p35/en::Gal4 stained with anti-phosphohistone H3 (red). (F) Disc in (E) with anti-phosphohistone H3 shown in white. The white line indicates the anterior-posterior compartment border.

of DIAP1 is not necessary, as shown in Figures 3 and 4. However, it is worth noting that loss of DIAP1 is likely to be sufficient in wild-type discs, in which Dronc activation leads to activation of downstream caspases, which may themselves promote further Dronc activation and, ultimately, apoptosis. In our system, we have simply dampened any such feedback pathways through expression of p35.

It has been known for many years that cell death in the larval wing disc leads to compensatory proliferation [1–3]. We showed that activation of a Dronc-dependent death signal, even in the absence of death itself, also leads to proliferation. Based on these observations, we argue that dying cells provide, through a Dronc-dependent pathway, at least one component of the signals that stimulate compensatory proliferation in healthy neighbors. Further support for this hypothesis comes

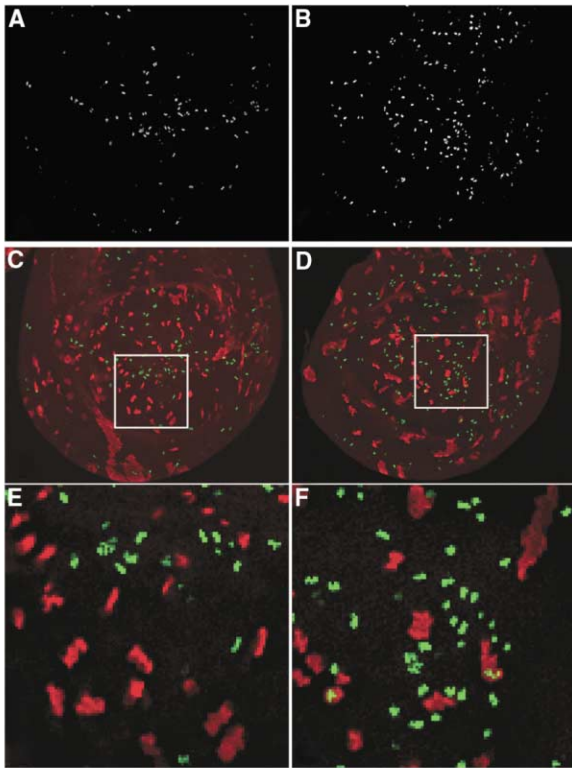

Figure 5. Activation of Hid-Dependent Cell Death Signaling in Clones Is Associated with Increased Total Disc Proliferation

Single-section confocal images of discs from third-instar larvae of various genotypes. Wing discs are stained with anti-phosphohistone-H3 (white or green) and anti-p35 (red). (A,C, and E) HS::FLP; UAS::p35/Act5C<CD2<Gal4. (B, D, and F) HS::FLP; UAS::p35, UAS::Hid^{Ala5}/Act5C<CD2<Gal4.

from examination of wing discs carrying clones of tissue expressing p35 alone (Figures 5A, 5C, and 5E), or Hid and p35 (Figures 5B, 5D, and 5F), in an otherwise wild-type background. As compared to discs expressing p35 alone, Discs with clones expressing Hid and p35 showed increased proliferation. Importantly, most of this proliferation occurred in cells outside the clones. Cells that express Hid and activate Dronc normally die. Therefore, an important prediction of this model is that apical death signals within the dying cell lead to the generation of signals that stimulate the proliferation of cells that neighbor dying cells. The secreted protein Wingless is a candidate for mediating at least some component of such a signal because it can drive proliferation in the wing disc (c.f. [24] and references therein). In fact, we observed a Dronc-dependent increase in Wingless expression in regions of the wing disc expressing Hid and p35, but not p35 alone (Figure S2). These results are consistent with the hypothesis that Wingless contributes to cell proliferation induced in response to death signaling. However, several points should be noted. (1) Wingless expression does not occur uniformly in areas expressing Hid and p35 (Figure S2). (2) Our results do not address the question of which cells express Wingless—cells with high-level Dronc activation or their neighbors. (3) The effects of Wingless on proliferation in the wing are complex. Proliferation is stimulated in some contexts [24], but inhibited in others [26]. (4) Roles for other pathways remain to be explored. Testing this hypothesis directly will require experiments that follow the consequences of manipulating *wingless* expression.

How does Dronc activity stimulate proliferation? In general, caspases recognize tetrapeptide substrates in which the C-terminal P1 residue is an aspartate. Dronc is exceptional in this regard in that it also cleaves well after glutamate. The only known Dronc targets are itself and the effector caspases Dcp-1 and Drice [10, 11]. Dcp-1 and Drice are unlikely to mediate Dronc-dependent effects on proliferation because they are inhibited by p35 in our experiments. Screens with combinatorial peptide libraries indicate that although Dronc has distinct overall target sequence preferences, many different tetrapeptide substrates are acceptable [10]. In addition, caspases interact with their substrates only very transiently. These facts make target identification through bioinformatic or protein purification approaches problematic. Other approaches, such as genetic screens for enhancers and suppressors of phenotypes associated with ectopic Dronc expression in contexts in which death is inhibited by p35 (J.R.H. and B.A.H., unpublished data), may provide insight into this question.

Concluding Remarks

Altogether, our results demonstrate that activation of the apical caspase Dronc, which is important for many cell deaths in the fly, is necessary and sufficient to induce proliferation in cells of the developing wing in response to a cell death stimulus such as Hid expression. Caspase activity has also been shown to stimulate proliferation in B and T cells in mammals [14, 27]. In these cells, low levels of caspase activity are thought to function cell autonomously to cleave and modify the functions of key cell cycle regulators. In contrast, our results argue that Dronc, in its role as an apoptosis inducer, plays two distinct roles that link cell death and proliferation, but in different cells. In its primary role, Dronc transduces death signals in the cells in which it is activated. At the same time, it promotes the production by these cells of a signal that drives the proliferation of nearby cells. Together, these two Dronc functions provide a mechanism that couples ectopic cell death with the compensatory proliferation that is required to maintain tissue size homeostasis and allow normal development.

Paradoxically, Dronc's role as a component of such a homeostatic mechanism may also contribute to deregulation of tissue growth in some contexts. For example, it has recently been reported that cells expressing high levels of dMyc, the *Drosophila* homolog of the *myc* oncogene, are super-competitors. They survive and proliferate at the expense of neighboring cells, which are eliminated by apoptosis [6, 28]. This death is mediated at least in part by increased expression of Hid [6]. As demonstrated above, expression of Hid and the subsequent activation of Dronc in dying cells lead to the generation of signals that drive the proliferation of neighbors. Therefore, upregulation of dMyc may induce a positive-feedback cycle in which cells with increased dMyc levels promote the death of neighbors, which then send a signal back to the dMyc-overexpressing cells to further

stimulate their proliferation. To focus on one specific version of such a model, it will be interesting to determine if Dronc activation in dying cells results in changes in the levels of dMyc expression in neighboring cells.

Finally, we note that activation of Dronc-dependent cell death leads in many contexts to a decrease in total cell number (c.f. [10–12]). Thus, it is certainly not the case that activation of Dronc in dying cells provides a compensatory proliferation signal to neighbors in all contexts. Instead, it seems likely that cells in the wing disc, and probably those of other tissues going through a period of unpatterned growth, are primed to respond to Dronc activation with the production of signals that induce compensatory cell proliferation in neighbors that are themselves receptive to these signals. Given our observations, it is interesting to consider the possibility that apical caspases such as Dronc may function to regulate cell fate nonautonomously in other contexts as well. For example, one can easily imagine a situation in which activation of Dronc-dependent death in one group of cells results in the transmission of a signal that informs surrounding cells that a particular developmental event has been completed and/or provides instructions on what to do next. This idea is speculative, but one can easily test it by following the consequences of up- or downregulation of Dronc activity in specific genetic backgrounds, as described in this report.

Supplemental Data

Supplemental Experimental Procedures as well as two additional figures are available with this article online at http://www.current-biology.com/cgi/content/full/14/14/1262/DC1/.

Acknowledgments

We thank Sharad Kumar, Helena Richardson, Stephen Crews, Andreas Bergmann, and Ennio Giordano for fly stocks and vectors, as well as Melvin Simon for use of his confocal microscope. The Wingless antibodies developed by Stephen M. Cohen were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences at the University of Iowa, Iowa City.

Received: May 5, 2004
Revised: May 27, 2004
Accepted: May 27, 2004
Published online: June 3, 2004

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