Caspase levels and execution efficiencies determine the apoptotic potential of the cell

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Essentially, all metazoan cells can undergo apoptosis, but some cells are more sensitive than others to apoptotic stimuli. To date, it is unclear what determines the apoptotic potential of the cell. We set up an in vivo system for monitoring and comparing the activity levels of the two main effector caspases in Drosophila melanogaster, Drice and Dcp-1. Both caspases were activated by the apoptosome after irradiation. However, whereas each caspase alone could induce apoptosis, Drice was a more effective inducer of apoptosis than Dcp-1, which instead had a role in establishing the rate of cell death. These functional differences are attributed to their intrinsic properties rather than merely their tissue specificities. Significantly, the levels of the procaspases are directly proportional to their activity levels and play a key role in determining the cell’s sensitivity to apoptosis. Finally, we provide evidence for the existence of a cellular execution threshold of caspase activity, which must be reached to induce apoptosis.

Introduction

Proteases are a large group of enzymes that can cleave proteins during a multitude of physiological reactions in all organisms (Hooper, 2002). A unique group of cysteine proteases called caspases functions to execute the apoptotic cell death program (Yuan et al., 1993; Thornberry and Lazebnik, 1998; Song et al., 2000). Caspases are synthesized as inactive zymogens (or pro-enzymes) and work in a controlled proteolytic cascade to activate themselves and one another (Riedl and Shi, 2004; Salvesen and Riedl, 2008). Initiator caspases are generally activated through dimerization, facilitated at multiprotein complexes such as the apoptosome (cytochrome c-Apaf-1–caspase-9; Zou et al., 1997; Rodriguez and Lazebnik, 1999; Salvesen and Riedl, 2008). Once activated, initiator caspases cleave and activate effector caspases, such as caspase-3 and -7 (Boatright and Salvesen, 2003; Riedl and Shi, 2004), which in turn cleave a variety of cellular protein substrates, ultimately leading to apoptosis (Lüthi and Martin, 2007; Dix et al., 2008; Mahrus et al., 2008). Caspases are also regulated by inhibitory proteins, such as the inhibitor of apoptosis proteins, which can bind to and inhibit caspases in both insects and mammals (Goyal et al., 2000; Vaux and Silke, 2005; Orme and Meier, 2009). In addition to the efforts invested in revealing and understanding these global pathways of caspase regulation during apoptosis, other studies imply that caspase activation is not necessarily an all-or-nothing process (Hoeppner et al., 2001; Reddien et al., 2001; Ribeiro et al., 2007). Indeed, low, transient, or subcellularly restricted levels of caspase activity have recently reported to promote vital cellular processes (Koto et al., 2009; Kaplan et al., 2010; Li et al., 2010; Schoenmann et al., 2010). Furthermore, different cells, under varying physiological and pathological conditions, often display substantial differences in their sensitivity to apoptotic stimuli, which may reflect distinct apoptotic potentials. However, it is still poorly understood why restrictive levels of caspase activity fail to induce apoptosis and what factors determine the apoptotic potential of the cell. The Drosophila melanogaster genome encodes seven distinct caspases, out of which the initiator caspase-9 ortholog Drone and the effector caspase-3 ortholog Drice are the major apoptotic caspases (Kumar, 2007; Cooper et al., 2009; Xu et al., 2009). Mutations in these caspases cause pleiotropic defects in developmental cell death and stress-induced apoptosis as well as the apoptosis-like process of spermatid individualization (Chew et al., 2004; Daish et al., 2004; Waldhuber et al., 2005; Xu et al., 2005, 2006; Arama et al., 2006; Muro et al., 2006). In contrast, only minor physiological roles in apoptosis have been demonstrated for the initiator-like atypical caspase Strica/Dream and
Drice is more efficient than Dcp-1 in processing this reporter and an endogenous protein substrate. The apoptosome components, Dronc and its adaptor protein Ark (the Apaf-1 homolog), constitute the initiator activity required for Drice and Dcp-1 activation, which can be blocked by expression of caspase inhibitory proteins. Importantly, in a series of genetic and transgenesis studies, we demonstrate that whereas both Drice and Dcp-1 can induce apoptosis, Drice can execute apoptosis more effectively than Dcp-1. Dcp-1, on the other hand, functions to fine tune the rate of cell death, at least in part through further activation of Drice in a positive amplification loop. Building on the differential efficiencies of these caspases to induce apoptosis, we show that the apoptotic potential of the cell is significantly affected by the total level of caspase activity, which in turn is directly proportional to the levels of the procaspases and their ratios. Finally, we demonstrate that the total level of caspase activity must reach a critical threshold in order for the cell to undergo apoptosis; short of that threshold, apoptosis cannot occur, and the cell will recover.

**Results**

Detection of effector caspase-like DEVDase activity during apoptosis in vivo

To monitor effector caspase activity in vivo, we took advantage of a genetic reporter previously designed for detection of caspase activity during the vital process of dendritic pruning in *Drosophila* sensory neurons (Williams et al., 2006; Schoenmann et al., 2010). This reporter, dubbed *CD8::PARP::Venus (CPV)*, encodes an the effector-like caspases Dcp-1 and Decay (Laundrie et al., 2003; Leulier et al., 2006; Muro et al., 2006; Xu et al., 2006; Baum et al., 2007; Denton et al., 2009). The caspase-8–like initiator Dredd appears not to be involved in cell death but rather in the innate immune response (Leulier et al., 2000; Stoven et al., 2003), whereas no role in apoptosis has been thus far demonstrated for the effector-like caspase Damm/Daydream (Harvey et al., 2001; Leulier et al., 2006). It is unclear why these caspases display different roles in apoptosis. For example, although Dcp-1 is highly homologous to Drice (67% identity; Song et al., 2000) and loss of *dcp-1* can aggravate *drice* mutant phenotypes (Leulier et al., 2006; Muro et al., 2006; Xu et al., 2006), *dcp-1* mutant flies are quite healthy, displaying only mild defects during starvation-induced autophagy and cell death in midoogenesis (Laundrie et al., 2003; Hou et al., 2008). Likewise, in mammals, knocking out caspase-3 causes decreased apoptosis and pleiotropic morphological defects, whereas caspase-7 knockout mice exhibit only mild antiapoptotic defects (Kuida et al., 1996; Woo et al., 1998; Houde et al., 2004). Whether these functional differences are the consequence of tissue specificity, distinct cellular levels of activity, or different execution efficiencies remains to be investigated.

Using a transgenic reporter of caspase-3–like (DEVDase) activity (Williams et al., 2006), we set up an in vivo system for monitoring and comparing the activity levels and execution efficiencies of the two main effector caspases in *Drosophila*, Drice and Dcp-1, during apoptosis. We show that after irradiation-induced apoptosis, Drice and Dcp-1 are the only DEVDases that become activated in wing imaginal discs (WDs), although Drice is more efficient than Dcp-1 in processing this reporter and an endogenous protein substrate. The apoptosome components, Dronc and its adaptor protein Ark (the Apaf-1 homolog), constitute the initiator activity required for Drice and Dcp-1 activation, which can be blocked by expression of caspase inhibitory proteins. Importantly, in a series of genetic and transgenesis studies, we demonstrate that whereas both Drice and Dcp-1 can induce apoptosis, Drice can execute apoptosis more effectively than Dcp-1. Dcp-1, on the other hand, functions to fine tune the rate of cell death, at least in part through further activation of Drice in a positive amplification loop. Building on the differential efficiencies of these caspases to induce apoptosis, we show that the apoptotic potential of the cell is significantly affected by the total level of caspase activity, which in turn is directly proportional to the levels of the procaspases and their ratios. Finally, we demonstrate that the total level of caspase activity must reach a critical threshold in order for the cell to undergo apoptosis; short of that threshold, apoptosis cannot occur, and the cell will recover.
Artificial effector caspase substrate composed of a 40-aa-long fragment of the human poly(ADP-ribose) polymerase (PARP) protein (including the caspase-3 consensus site DEVD) flanked by the extracellular/transmembrane domain of the mouse CD8 protein at the N-terminal side and the YFP Venus at the C terminus (Fig. 1 A). The activity is detected by staining with the anticleaved human PARP antibody, whereas the fluorescence of the Venus protein indicates which cells express the reporter. The CPV reporter has not been characterized during apoptosis, and the exact caspases (or other proteases) that may cleave it have not been determined.

Therefore, we established a genetic assay for monitoring the CPV reporter during irradiation-induced apoptosis in Drosophila. The CPV was expressed in the pouch region of the WD using the spalt (sal)-Gal4 driver. Transgenic larvae were then γ-irradiated and subsequently allowed a 4-h recovery before their WDs were removed and stained to visualize cleaved human PARP. Whereas nonirradiated animals displayed almost no reporter processing activity, strong activity was detected after apoptosis induction (Fig. 1 B, a and b, respectively). Furthermore, coexpression of the apopptosome components Ark and Dronc, using the sal-Gal4 driver, also induced a high level of CPV processing activity (Fig. 1 B, c), consistent with the idea that this combination is a strong inducer of apoptosis (Shapiro et al., 2008).

Apoptosis is associated with massive proteolytic activity (Crawford and Wells, 2011). To validate that CPV is cleaved only (immediately) after the DEVD consensus site, we introduced a point mutation to this reporter, which changes the invariably conserved aspartic acid residue to glycine (DEVD to DEVG). Transgenic flies expressing this modified construct (dubbed CPVG) were treated as described above for the visualization of CPV processing activity. However, this mutation completely abrogated processing of the reporter (Fig. 1 B, d). Therefore, the proteolytic cleavage of CPV during apoptosis reflects DEVDase activity only.

The effector caspases Drice and Dcp-1 are both activated during γ-irradiation-induced apoptosis

Apoptosis is mainly mediated by caspases, but other proteases may also be involved in this process (Schrader et al., 2010). To test whether CPV is cleaved by effector caspases during apoptosis, the effector caspase inhibitor baculovirus gene p35 and the Drosophila caspase inhibitor gene diap1 were each expressed in the pouch region of WDs. After irradiation, CPV processing activity was completely blocked in the presence of p35 or largely suppressed in the presence of Diap1, suggesting that effector caspases are indeed responsible for this activity.
The apoptosome components Ark and Dronc are exclusively required for Drice and Dcp-1 activation after irradiation-induced apoptosis

The apoptosome components Ark and Dronc are associated with most of the developmental and stress-induced apoptotic events in Drosophila. To examine whether these apoptosome components are also required for Drice and Dcp-1 activation in our system, CPV processing activity was monitored in WDs from ark and dronc mutant flies. Indeed, most of the activity was strongly attenuated in these backgrounds, although some residual levels of activity remained (Fig. 3, A [a–c] and B). As both are null allelic backgrounds for their respective mutations, we asked whether this residual activity might be mediated by the initiator-like atypical caspase Strica, which has been suggested to be functionally redundant with Dronc in certain cellular paradigms (Baum et al., 2007). However, no significant attenuation of the activity was detected in the strica-null mutant (Fig. 3, A [d] and B). Moreover, WDs from dronc and strica double mutants exhibited similar levels of residual activity as those in the dronc mutant alone (Fig. 3, A [e] and B). An alternative possibility for the retained residual activity in the dronc and ark mutant backgrounds may be the persistence of maternal mRNA of these genes. Indeed, CPV processing activity was almost completely

Out of the four structurally related effector-like caspases in Drosophila, only Drice and Dcp-1 have been previously associated with apoptosis in vivo, although only drice mutants were shown to block irradiation-induced apoptosis in the WD (Muro et al., 2006; Xu et al., 2006). Therefore, we used a null allele of drice (drice; Fig. S1; see Fig. 6 E) to examine whether it is the only effector caspase responsible for the CPV processing activity in irradiated WDs. Unexpectedly, CPV processing activity persisted in WDs from irradiated drice mutants, suggesting the involvement of another effector caspase (Fig. 2, A [e] and B). Indeed, whereas a protein-null allele of dcp-1 (dcp-1pre; see Fig. 6 E) also failed to block the CPV processing activity in this system, this activity was almost completely abrogated in the double mutants (Fig. 2, A [d and f] and B). Therefore, Drice and Dcp-1 are both activated in WDs after irradiation-induced apoptosis. Notably, quantification of the processing activity in each of the mutants suggests that Dcp-1 activity is slightly lower than that of Drice (Fig. 2 B). However, comparative analysis of transcript expression by real-time quantitative PCR (RT-qPCR) indicates that both caspases are expressed at comparable levels in this tissue, suggesting that the Dcp-1 activity in this system may be less efficient at cleaving this reporter (Fig. S1).

The apoptosome components Ark and Dronc are exclusively required for Drice and Dcp-1 activation after irradiation-induced apoptosis.

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Effector caspases and the cell apoptotic potential  •  Fiorentin and Arama

abrogated in WDs from dronc^{I24I29} mutants, which also express an RNAi construct against dronc (Fig. 3, A [f] and B). Therefore, the Ark-Dronc apoptosome is exclusively required for Drice and Dcp-1 activation after irradiation-induced apoptosis.

**Dcp-1 activity is insufficient to induce apoptosis in WDs after irradiation**

Previous works have suggested that irradiation-induced apoptosis in WDs is blocked in drice mutants (Muro et al., 2006; Xu et al., 2006). Therefore, we asked whether the Dcp-1 activity detected in WDs after irradiation is dispensable for cell death. To address this, WDs were first costained with anticleaved PARP (anti-cPARP) and TUNEL to visualize CPV processing activity and dying cells, respectively. In addition, discs of the same genotypes were also labeled with the vital dye acridine orange (AO), which is known to detect apoptotic corpses in *Drosophila* (Arama and Steller, 2006). 4 h after 50 Gray (Gy) irradiation, effector caspase activity and cell death were dramatically induced in WDs from larvae treated as in Fig. 1 B. The specific genotypes indicated at the top relate to all the panels in a column. The first three columns are of the same imaginal disc. Note that caspase activity is confined to the reporter expression area, whereas cell death is detected in the entire WD. The open boxes group images of the same disc detected in different fluorescent channels. nonirrad, nonirradiated; wt, wild type. (B) WDs from irradiated larvae of the same genotypes as in A were stained with the vital dye AO to detect apoptotic corpses. (C) Similar WDs treated as in A and B and stained with the anticleaved caspase-3 antibody (cCasp. 3; lot no. 32; Cell Signalling Technology) to visualize the active forms of the effector caspases. Bars, 50 µm.

Figure 4. **Inactivation of Drice, but not Dcp-1, completely blocks irradiation-induced cell death.** (A) Visualization of the CPV expression domain (Venus), effector caspase activity (CPV processing), and cell death (TUNEL) in WDs from larvae treated as in Fig. 1 B. The specific genotypes indicated at the top relate to all the panels in a column. The first three columns are of the same imaginal disc. Note that caspase activity is confined to the reporter expression area, whereas cell death is detected in the entire WD. The open boxes group images of the same disc detected in different fluorescent channels. nonirrad, nonirradiated; wt, wild type. (B) WDs from irradiated larvae of the same genotypes as in A were stained with the vital dye AO to detect apoptotic corpses. (C) Similar WDs treated as in A and B and stained with the anticleaved caspase-3 antibody (cCasp. 3; lot no. 32; Cell Signalling Technology) to visualize the active forms of the effector caspases. Bars, 50 µm.
the UAS-CPV construct alone (control) or flies containing the driver construct sal-Gal4 and the UAS-CPV. Importantly, the rates of cell death were almost identical between the control and CPV-expressing WDs at both 2 and 4 h after 50 Gy irradiation, suggesting that at least under these conditions, this reporter does not significantly affect the processing of endogenous death-associated substrates by caspases (Fig. S2).

It has been recently suggested that the antigen cleaved caspase-3 antibody (also known as CM1) may reflect Drice activity in Drosophila, but the specific cleaved caspases identified by this antibody have not been determined (Fan and Bergmann, 2010). Furthermore, it has remained controversial whether this antibody can detect active Dcp-1 in vivo (Yu et al., 2002; Peterson et al., 2003; Muro et al., 2006). As the antigen cleaved caspase-3 antibody is widely used as a marker of caspase activation and apoptosis in Drosophila, we decided to examine four antibodies from two different sources raised against the same epitope (see also in Materials and methods). Interestingly, staining WDs with these antibodies revealed that they can recognize both active Drice (Fig. 4, C, d) and active Dcp-1 (Fig. 4, C, e) in vivo, and this staining was completely absent in the double mutants (Fig. 4, C, e).

Nevertheless, the specificity of these antibodies toward active Dcp-1 varied among the different antibodies, which in all cases were much more specific toward active Drice (Fig. S3).

Dcp-1 activity fine tunes the rate of cell death after irradiation

The idea that endogenous Dcp-1 activity cannot induce cell death in WDs after irradiation coupled with the fact that Drice activity alone is sufficient to induce apoptosis under the same conditions raised the question about the role of Dcp-1 in this system. One possibility is that Dcp-1 activity increases the total levels of effector activity in the cell, thus making cell death more efficient. To investigate this possibility, we quantified the levels of cell death in WDs from wild-type and dcp-1 and drice mutants at different time points after 50 Gy irradiation. In wild type, the number of dying cells started to accumulate ~1.5 h after irradiation; this number reached its peak after 3 h and plateaued in the following hours (Fig. 5, A [a] and B). Interestingly, although the drice mutants displayed the expected complete block in cell death, the dcp-1 mutants displayed a significant delay of ~1 h in the rate of cell death, reaching the wild-type plateau level 4 h after irradiation (Fig. 5, A [b] and B). To examine the correlation between the rates of cell death and caspase activity, we then quantified the levels of the CPV processing activities in these WDs. In accordance with the measured rates of cell death, both caspases became activated at ~1.5 h after irradiation (Fig. 5 C).

However, whereas Drice activity reached a peak and a plateau after 3 h, Dcp-1 activity reached that same plateau level ~4 h after irradiation, suggesting that Dcp-1 may be less efficient at cleaving the CPV reporter than Drice or that Drice is more efficiently activated than Dcp-1 (Fig. 5 C). We conclude that whereas Drice activity is the trigger of cell death in this system, Dcp-1 activity fine tunes the rate of this process.

One way by which Dcp-1 may contribute to the acceleration of Drice-triggered apoptosis may be through a positive amplification loop. According to this model, after apoptosis induction, the Ark-Dronc apoptosome activates Drice and Dcp-1, and, in turn, these effector caspases cleave death-associated substrates, whereas Dcp-1 may also cleave and further activate Drice. In support of this model, it has been previously shown that Dcp-1 can cleave Drice in a cell-free system, but not vice versa, similar to the way Drone cleaves Drice (Hawkins et al., 2000; Song et al., 2000). To test this possibility in vivo, we performed Western blot analysis on protein extracts from wild-type, drice, and dcp-1 mutant adult flies using an anti-Drice antibody. Basal cleavage of Drice is detected in wild-type flies but not in the dcp-1 mutants, suggesting that Dcp-1 may promote cleavage of Drice also in vivo, albeit the significance of this cleavage (nonclassical p10 and p20) is unclear (Fig. S4 A). To test whether Dcp-1 may indeed promote amplification of Drice activation in vivo, we used the antigen cleaved caspase-3 antibody to quantify the levels of active Drice in WDs from wild-type and dcp-1 mutants at different time points after 50 Gy irradiation. Interestingly, a significant delay of ~1 h is detected in the rate of Drice activation in the dcp-1 mutant versus wild type (Fig. S4 B). As the antigen cleaved caspase-3 antibody mostly detects active Drice in vivo (Fig. S3), this delay is mainly attributed to the lack of Drice activation by Dcp-1. Collectively, these findings provide evidence that Dcp-1 may function, at least in part, through activating Drice in a positive amplification loop.

The finding that Dcp-1 activity makes cell death more efficient prompted us to explore other conditions in which Drice activity alone may be suboptimal. By gradually decreasing the dose of γ-irradiation, the rate of cell death was reduced accordingly. At 4 h after an irradiation dose of 20 Gy, the rate of cell death in the wild type was reduced to about half that observed after a dose of 50 Gy (compare D [a] and E with B in Fig. 5). Under these conditions, the rate of cell death in the dcp-1 mutants (i.e., Drice activity only) was further reduced by 50% compared with wild type (Fig. 5, D and E). In contrast, the level of Drice activity alone at 4 h after 50 Gy irradiation could induce a similar level of cell death as that in wild type (albeit in a delay; Fig. 5 B). Therefore, the role of Dcp-1 activity in tuning the rate of cell death may be more significant when facing relatively weaker stresses.

Dcp-1 can induce cell death but less efficiently than Drice

The fact that Dcp-1 activity cannot induce apoptosis in irradiated WDs raised the question of whether it is capable of inducing cell death at all. To answer this question, we generated transgenic fly lines expressing either the full-length drice or dcp-1 genes at identical levels. For this, the complete ORFs of drice and dcp-1 were each subcloned under the same regulatory regions (promoter 5’ and 3’ untranslated regions [UTRs]) of the drice gene, generating the drice:drice and drice:dcp-1 constructs, respectively (Fig. 6 A). Each construct was then targeted to the same genomic point using the qC31 integrase system, thus ensuring identical expression levels of these transgenes. By crossing these transgenes to the drice and dcp-1 double mutant, we validated that both can induce CPV processing activity after irradiation (Fig. 6 B, e and f). Next, we asked whether these transgenes could restore irradiation-induced cell
death in the *drice* mutants (where Dcp-1 is the only endogenous source of effector activity), in *drice* mutants that also lack one allele of *dcp-1*, and in the double mutants. Interestingly, under these conditions, both transgenes were able to induce cell death in the *drice* mutants, as detected by TUNEL labeling (Fig. 6 B, a and b), but only *drice:drice* could induce cell death in the *drice* mutants that are also heterozygous for the *dcp-1* allele (Fig. 6 B, c and d) and in the double mutants (Fig. 6 B, g and h). Of note, the same transgenes were also able to reverse the lethality of the *drice* mutant flies (see in the text for Fig. 7), indicating that the TUNEL-labeled WD cells are bona fide dying cells. Therefore, Dcp-1 has the potential to induce cell death by itself, but it is less efficient in doing so than Drice.

Procasps levels and ratios affect cellular sensitivity to apoptosis by determining whether and at what rate caspase activity levels may reach a critical execution threshold

Next, we assessed the differences in the abilities of the two caspases to induce apoptosis by quantifying the levels of cell death induced by each transgene. Consistent with the idea that Drice is more efficient in inducing apoptosis than Dcp-1, *drice:drice* induced more than twice as many cell death events than *drice: dcp-1* in the background of the *drice* mutant (Fig. 6 C, I). Further reduction of one or two copies of endogenous *dcp-1* in this mutant abolished the ability of *drice:dcp-1*, but not *drice:drice*, to induce cell death, implying the existence of a threshold effect (Fig. 6 C, II). Finally, in the double mutant background, only *drice:drice* could induce cell death but in a more reduced rate than in the single *drice* mutant (Fig. 6 C, III).

In living cells, Diap1 binds to and inhibits the activation of both Drice and Dcp-1, whereas upon induction of apoptosis, Reaper family proteins bind to Diap1, relieving its inhibition of caspases (Ditzel et al., 2008; Steller, 2008). Therefore, we hypothesized that reducing the level of *diap1* may lower the execution threshold of the cell. To test this, a *diap1*-RNAi transgene was expressed in the pouch region of the WD in the background of the *drice* mutant. After larval irradiation, these discs displayed dramatic induction of cell death in the pouch region but not in the surrounding tissue (Fig. 6 D). Therefore, reducing the levels of *diap1* elevates the levels of the
Drice and Dcp-1 display distinct efficiencies in cleaving a protein substrate in vivo

A plausible reason for the relatively low efficiency of Dcp-1 to induce apoptosis is that it may cleave death-associated substrates less effectively than Drice. One such in vitro target of Drice and Dcp-1 is the Drosophila B-type lamin, lamin Dmo (Fraser and Evan, 1997; Song et al., 2000). To test whether Drice and Dcp-1 can also cleave this protein in vivo, Western blot analysis was performed on extracts prepared from wild-type, dcp-1, and drice mutant WDs both before and 5–7 h after irradiation. Using a specific antibody for lamin Dmo, cleavage of this protein was detected in all of the examined genotypes after irradiation, although this activity was dramatically reduced when drice was inactive (i.e., Dcp-1 activity only; Fig. 6 E).

Furthermore, in accordance with the ability of transgenic activated Dcp-1 in the cell, leading to apoptosis. Finally, to test the possibility that Diap1 may be a stronger inhibitor of Dcp-1 than Drice, the diap1-RNAi transgene was expressed in the pouch region of WDs in either the drice or dcp-1 mutants, and the WDs were tested for cell death induction as a result of spontaneous activation of the caspases (without irradiation). However, cell death was only detected in the pouch area of the dcp-1 mutant but not the drice mutant, which is consistent with the idea that the differences in the execution efficiencies of Drice and Dcp-1 are not the consequence of differential inhibition of these caspases by Diap1 (Fig. S5). Collectively, these results demonstrate that the levels of the proeffector caspases can tip the scales between cell survival and death and that it must go over a critical threshold to induce apoptosis.
Dcp-1 to rescue cell death after irradiation in the \textit{drice} mutant, expression of transgenic Dcp-1 (on top of the endogenous Dcp-1 in the \textit{drice} mutant) also increased the cleavage of lamin Dmo (Fig. 6 E). Therefore, Dcp-1 is less efficient than Drice at cleaving both the artificial CPV reporter and the death-associated subrate lamin Dmo in vivo.

**Increasing Dcp-1 levels can compensate for the loss of \textit{drice} during developmental cell death**

Cell death plays a major role during metamorphosis of the fly by removing many larval structures, thus paving the way for the generation of new adult tissues. Indeed, whereas \textit{drice} mutants survive to the third instar larval stage, most of them die during pupal development (Muro et al., 2006; Xu et al., 2006). As transgenic Dcp-1 could restore irradiation-induced cell death in the \textit{drice} mutants (albeit in a reduced rate than transgenic Drice), we asked whether it can also compensate for the loss of Drice during pupal development. Therefore, we evaluated the ability of these transgenes to increase the rate of larva-to-adult fly survival in different combinations of \textit{drice} and \textit{dcp-1} mutants. As shown in Figs. 7 and 8 C, only ~20–30% of the \textit{drice} mutants survived to adulthood, whereas the survival rate of the \textit{dcp-1} mutants was similar to that of wild type. Further loss of one or two copies of endogenous \textit{dcp-1} in the background of the \textit{drice} mutants significantly reduced the survival rate or caused complete pupal lethality, respectively (Fig. 7). Importantly, all of the allelic combinations that restored irradiation-induced cell death, such as the transgenic Drice or Dcp-1 in the \textit{drice} mutant background or transgenic Drice in the double mutants (Fig. 6 C), could also reverse pupal lethality and restore wild-type levels of survival, irrespective of the differences in the rates of cell death (Fig. 7). On the other hand, inability to restore irradiation-induced cell death (e.g., \textit{drice:dcp-1} in the double mutant background; Fig. 6 C) fully correlated with a failure of these transgenes to reverse fly lethality (Fig. 7). Therefore, Drice is also a more effective executioner caspase than Dcp-1 during developmental cell death.

**Distinct tissue specificities of Drice and Dcp-1 also affect their differential requirements during developmental cell death**

RT-qPCR analysis (Fig. S1) and microarray data and EST tissue information deposited in public domains suggested that \textit{drice} expression levels are much higher than \textit{dcp-1} levels in almost all tissues investigated (unpublished data). To investigate the contribution of tissue specificities to the differences between the roles of the two caspases during developmental cell death, we generated new transgenes that contain the coding regions of \textit{drice} and \textit{dcp-1}, this time under the regulatory elements of \textit{dcp-1} (Fig. 8 A). Similar to the \textit{drice:drice} and \textit{dcp-1:drice} transgenes and in agreement with the findings that the two caspases are expressed at similar levels in WDs (Fig. S1), the \textit{dcp-1:drice} and \textit{dcp-1:dcp-1} transgenes were also able to restore irradiation-induced cell death in WDs of the \textit{drice} mutants, with \textit{dcp-1:drice} being more effective than \textit{dcp-1:dcp-1} (Fig. 8, B [a and b] and C). Furthermore, this similarity is extended to the double mutant backgrounds, showing that the \textit{dcp-1:drice} transgene, but not the \textit{dcp-1:dcp-1}, could restore irradiation-induced cell death in \textit{drice} mutant flies that also contain either one or two copies of the \textit{dcp-1} mutant allele (Fig. 8, B [c–h] and C). Therefore, the fact that a different promoter gives almost identical results reinforces our findings that Dcp-1 is a less efficient inducer of cell death than Drice and further demonstrates the existence of a threshold effect.

However, as opposed to \textit{drice:drice} and \textit{dcp-1:drice} transgenes, the \textit{dcp-1:drice} transgene only partially rescued pupal lethality of the \textit{drice} mutants, whereas the \textit{dcp-1:dcp-1} transgene failed to rescue this lethality and instead further aggravated this phenotype (compare Fig. 8 D with Fig. 7). These results suggest that \textit{dcp-1} is either not expressed or expressed at very low levels in at least some of the tissues where Drice activity is required during pupal development. In addition, Dcp-1 may also be uniquely expressed in some other tissues where high Dcp-1 activity, but not Drice activity, is deleterious to the cells. Altogether, these results suggest that the differential requirement for Drice and Dcp-1 during developmental cell death is also the consequence of tissue specificities.

**Discussion**

**The threshold effect of the effector caspase activity**

One of the most important questions in the fields of developmental apoptosis and cancer biology is what makes different cells more resistant or more sensitive to apoptotic stimuli. In the present study, we uncovered two factors important for establishing the overall apoptotic potential of the cell: the levels of the pro-effector caspases and their execution efficiencies. We showed that the \textit{Drosophila} effector caspases Drice and Dcp-1 differ in their competencies to induce apoptosis in vivo, with Drice being more effective than Dcp-1 (also illustrated in Fig. 9 A). Comparative transgenesis experiments indicate that these differences are the consequence of distinct intrinsic properties of...
likely because the metabolic processes that normally sustain the cell overwhelm the catabolic processes, allowing for recovery and survival (Fig. 9 B). Interestingly, in an extreme case of apoptosis regulation at the level of procaspase expression, the onset of cell death in the tail-spiked cell of *Caenorhabditis elegans* was reported to be induced by an increase in the expression level of the caspase-encoding gene *ced-3* (Maurer et al., 2007); however, whether or not this is caused by a threshold effect is still unclear.

How does the level of effector caspase activity affect the rate of cell death? The length of the time between the apoptotic stimulus and the point when the cell becomes apoptotic varies between different cell types and distinct apoptotic stimuli. It is believed that this time period mainly reflects the time it takes for the cells to process and transduce the apoptotic signal and eventually translate it to the action of the caspases (Pluquet and Hainaut, 2001; Hellwig et al., 2008). However, our work suggests that the levels of effector caspase activity also vary between different cells as a consequence of the levels and ratios of the two main proeffector caspases, and this factor plays an important role in determining the time length between the activation of caspases and the eventual death of the cell, where high levels of effector activity shorten that period, thus increasing the rate of cell death. A plausible explanation for this correlation is that the two caspases. Importantly, these experiments further demonstrate that cellular lethality is exerted only when caspase activity reaches a critical threshold. Short of that lethal level, cells fail to induce apoptosis, whereas surpassing that level increases the rate of cell death, thus further sensitizing the cells to apoptosis (see the model in Fig. 9 B and also below).

The levels of the proeffector caspases affect the sensitivity of the cell to apoptotic stimuli

Our experiments demonstrate that the level of activity of an effector caspase is directly proportionate to the level of expression of the respective inactive zymogene. Therefore, in addition to the established posttranslational regulatory mechanisms of caspases, which are crucial for the initiation of apoptosis, the expression levels of the proeffector caspases are an important factor in determining the sensitivity of the cells to apoptotic stimuli. This sensitivity is reflected in the rate by which the total level of effector activity meets the execution threshold of the cell. The higher the levels of the procaspases, the faster the cells reach that execution threshold level and the more sensitive the cells are to apoptotic stimuli (illustrated in Fig. 9 B). However, if the total levels of procaspases are too low, the activity rate may fail to reach that particular threshold, likely because the metabolic processes that normally sustain the cell overwhelm the catabolic processes, allowing for recovery and survival (Fig. 9 B). Interestingly, in an extreme case of apoptosis regulation at the level of procaspase expression, the onset of cell death in the tail-spiked cell of *Caenorhabditis elegans* was reported to be induced by an increase in the expression level of the caspase-encoding gene *ced-3* (Maurer et al., 2007); however, whether or not this is caused by a threshold effect is still unclear.

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is that in order for the cell to start dying, a critical mass of death-associated substrates must be cleaved by the effector caspases, such that the more caspase molecules available, the faster this mass is cleaved. To date, ∼1,000 cellular proteins are thought to be cleaved by effector caspases during apoptosis, many of which are believed to gain old or new activities (Lüthi and Martin, 2007; Dix et al., 2008; Mahrus et al., 2008). However, it is still unknown what caspase substrates are critical in apoptosis and what levels of cleaved substrates are required for induction of apoptosis. Future approaches to study that critical subset may further help in understanding how precisely the level of caspase activity affects the rate of cell death.

**The Drosophila effector caspases differ in their abilities to induce apoptosis in vivo**

We have presented several lines of evidence demonstrating that Drice is more efficient in inducing apoptosis than Dcp-1. First, despite the fact that both Drice and Dcp-1 are normally expressed at similar levels in the WD, only Drice activity could induce apoptosis after irradiation. Second, when expressed at similar levels in the WD, only Drice activity could induce apoptosis after irradiation. Second, although when expressed under the drice regulatory regions both of the procaspase transgenes could induce apoptosis after irradiation, activated Drice has the ability to induce apoptosis in this setup. Another possibility is that Dronc may cleave and activate Drice more efficiently than Dcp-1. In support of this idea, recombinant Dronc was reported to only poorly process Dcp-1, as compared with Drice, in a cell-free system (Hawkins et al., 2000). Consistently, cleavage of the endogenous Drice was more prominent than that of endogenous Dcp-1 in irradiated WDs (unpublished data). However, an alternative or additional mechanism is that Drice may be more efficient than Dcp-1 at cleaving some of the more critical death-associated substrates. Indeed, Drice was able to process both the CPV reporter and an endogenous death-associated substrate, lamin Dmo, more efficiently than Dcp-1 in irradiated WDs. Whereas both possibilities support a model in which Drice and Dcp-1 function in a similar manner to induce apoptosis (i.e., possessing overlapping repertoires of death-associated protein substrates) but with different efficiencies, our data additionally suggest that these caspases may also have some distinct protein substrates. The findings that when expressed under the regulatory regions of dcp-1 transgenic Drice partially rescued the survival of the drice mutants while the Dcp-1 transgene further increased their lethality imply that Dcp-1 may also cleave some distinct protein substrates. This is further supported by the notion that Dcp-1 can cleave Drice, whereas Drice cannot cleave Dce or Dcp-1 (see also below).

Therefore, Drice and Dcp-1 may differ in their activation efficiencies by Dronc, their ability to effectively cleave similar critical death-associated substrates, and their repertoires of distinct substrates.

**The coarse and fine modes of effector activity control**

Both Drosophila and mammals possess major and minor effector caspases. Our work suggests that having both coarse (Drice; major) and fine (Dcp-1; minor) modes of effector activity control may allow the cells more flexibility in determining their apoptotic potential by setting their optimal execution threshold and rate of cell death. The finding that the rate of cell death in the absence of dcp-1 was dramatically reduced after a relatively weak stress (i.e., 20-Gy irradiation) as compared with a stronger stress (i.e., a 50-Gy dose) suggests that under conditions resembling the normal situation in the wild, the minor caspase plays a more prominent role in determining whether or not the effector activity will even meet the execution threshold. In higher stresses, however, there is no need for a cell to rely on Dcp-1 activity to reach the execution threshold, as its activity is obliterated by the high activity of Drice. Interestingly, in addition to ced-3, which is essential for apoptosis in C. elegans (Yuan et al., 1993), the worm genome also contains other caspase genes, but no documented roles in when the procaspase transgenes were expressed under the dcp-1 regulatory regions.

What are the reasons for the differences in the in vivo execution efficiencies of Drice and Dcp-1? One idea is that Diap1 may inhibit Dcp-1 more efficiently than Drice. However, this possibility is unlikely, as reduction of the same endogenous levels of Diap1 led to spontaneous induction of cell death only in the dcp-1 mutant and not the drice mutant, demonstrating that despite similar levels of Diap1-free endogenous caspases, only activated Drice has the ability to induce apoptosis in this setup. Another possibility is that Dronc may cleave and activate Drice more efficiently than Dcp-1. In support of this idea, recombinant Dronc was reported to only poorly process Dcp-1, as compared with Drice, in a cell-free system (Hawkins et al., 2000). Consistently, cleavage of the endogenous Drice was more prominent than that of endogenous Dcp-1 in irradiated WDs (unpublished data). However, an alternative or additional mechanism is that Drice may be more efficient than Dcp-1 at cleaving some of the more critical death-associated substrates. Indeed, Drice was able to process both the CPV reporter and an endogenous death-associated substrate, lamin Dmo, more efficiently than Dcp-1 in irradiated WDs. Whereas both possibilities support a model in which Drice and Dcp-1 function in a similar manner to induce apoptosis (i.e., possessing overlapping repertoires of death-associated protein substrates) but with different efficiencies, our data additionally suggest that these caspases may also have some distinct protein substrates. The findings that when expressed under the regulatory regions of dcp-1 transgenic Drice partially rescued the survival of the drice mutants while the Dcp-1 transgene further increased their lethality imply that Dcp-1 may also cleave some distinct protein substrates. This is further supported by the notion that Dcp-1 can cleave Drice, whereas Drice cannot cleave Dce or Dcp-1 (see also below).

Therefore, Drice and Dcp-1 may differ in their activation efficiencies by Dronc, their ability to effectively cleave similar critical death-associated substrates, and their repertoires of distinct substrates.
apoptosis have been thus far assigned for these caspases (Shaham, 1998). Likewise, Drosophila and mammals also contain additional caspases, which do not appear to have a major role in apoptosis (Salvesen and Abrams, 2004). Therefore, it is attractive to consider that similar to Dcp-1, at least some of the additional caspases in these organisms may also function to fine tune the rate of cell death, providing maybe even a finer tuner.

Our RT-qPCR analysis suggests that the WD is an exceptional tissue in relation to the similar levels of dcp-1 and drice expression, as microarray data and EST tissue information deposited in public domains (FlyAtlas and FlyBase, respectively) suggest that drice expression levels are much higher than dcp-1 levels in almost all tissues examined (unpublished data). Thus, fine tuning the rate of cell death may be the main role of Dcp-1 in apoptosis. However, this is probably not the only role of Dcp-1, as this caspase appears to have more prominent roles in certain cellular paradigms. For example, Dcp-1 was reported to be essential for the sporadic and nutrient deprivation–induced germ-line cell death during midoogenesis, with Drice playing only a minor role, if any, in this system (Laundrie et al., 2003; Baum et al., 2007). Furthermore, genetic analyses revealed additional cellular paradigms in which the role of Dcp-1 in cell death is more prominent or that Dcp-1 inactivation significantly enhances drice mutant phenotypes (Leulier et al., 2006; Muro et al., 2006; Xu et al., 2006; Baum et al., 2007). Although it is largely unknown why different cells/tissues need to acquire distinct apoptotic potentials, it is not hard to envision that certain cells may need to be more resistant or more sensitive to apoptosis. For instance, cell types with low regeneration potential and with limited numbers, such as neurons, or cells with direct interface with the outside world and that are in higher risk of facing different stresses (e.g., eyes or sperm) may be more resistant to apoptosis and hence may express relatively low levels of Drice. On the other hand, dying cells that send signals to the surrounding tissue area (e.g., signals for compensatory proliferation) may need to express higher levels of Dcp-1 to fine tune their dying rate and thus control the level of the signal they send. Therefore, different cells in the organism may use distinct levels and ratios of Drice and Dcp-1 to optimize their sensitivity to apoptosis induction.

**Drice and Dcp-1 synergize their activities during apoptosis**

Our data also point to a synergistic effect of Drice and Dcp-1 on the overall effector activity levels in the cell. This effect is clearly detected when comparing the overall level of effector caspase activity in wild type (which reflects the combined activities of Drice and Dcp-1) and the sum of the single activity levels of Drice and Dcp-1 (measured in dcp-1 and drice mutant backgrounds, respectively). During the time window of 1–2 h after irradiation, wild-type activity levels were significantly higher than the simple summation of the levels in the mutants, implying the existence of a positive amplification loop of effector caspase activation. In a later window of time 3–4 h after irradiation, activity levels in wild type reached a peak and plateaued (Fig. 5 C). Because the levels of Drice activity alone also reached a similar peak and plateaued within the same time window, we attribute the formation of this plateau to either staining saturation or limited substrate availability but not the actual levels of caspase activity during that time frame. Interestingly, it has been previously shown that Dcp-1 can cleave Drice in a cell-free system but not vice versa, similar to the way Dronc cleaves Drice (Hawkins et al., 2000; Song et al., 2000). Likewise, we showed cleavage of Drice, but not Dcp-1, in extracts from wild-type adult flies, which was blocked in the dcp-1 mutants (Fig. S4 A). Moreover, monitoring the kinetics of Drice activation (using the anticleaved caspase-3 antibody) in wild-type and dcp-1 mutant WDs displayed a significant delay in the rate of Drice activation when Dcp-1 was lacking (Fig. S4 B). This delay cannot be attributed to merely the lack of staining of activated Dcp-1, as even if we were to assume that this antibody has identical specificities toward activated Drice and activated Dcp-1 (although, as aforementioned, this is not the case), it would be expected that without an amplification loop, the maximal contribution of activated Dcp-1 to the overall staining level shall not surpass that of activated Drice (i.e., a maximum of twofold increase). However, the lack of Dcp-1 caused much more than a twofold reduction in the levels of staining (i.e., 3–7 fold), indicating that this reduction is mainly a result of less activated Drice in the absence of Dcp-1. Altogether, these findings provide an indirect support to a model whereby Dcp-1 may, either directly or indirectly, promote further activation of Drice through a positive amplification loop (see also the model in Fig. 9 A). It remains to be determined whether this cleavage of Drice by Dcp-1 in vivo leads to activation of the former and whether this may account for the aforementioned synergistic effect.

In conclusion, we have demonstrated that cells can continue living in the presence of a considerable level of effector caspase activity, as long as this level does not reach a critical execution threshold. Current models of the conserved core apoptotic machinery between Drosophila and mammals commonly illustrate the effector caspase activation step as an all-or-nothing process (Fuchs and Steller, 2011). However, recent studies of caspase-dependent nonapoptotic cellular processes suggest that to avoid excessive caspase activation and apoptosis, effector caspase activity may be low or restricted in space and time in these cells (Feinstein-Rotkopf and Arama, 2009; Kaplan et al., 2010; Li et al., 2010). Furthermore, it is well established that cancer cells can escape cellular lethality by manipulating a variety of steps and components in the apoptotic machinery, ultimately affecting the activity levels of the effector caspases (Johnstone et al., 2002; Hanahan and Weinberg, 2011). The current study provides insight into why low or restricted caspase activity levels fail to induce apoptosis (i.e., the threshold effect) and suggests that the critical step of caspase activation is more complex than has been previously appreciated.

**Materials and methods**

**Fly strains and expression vectors**

Flies containing the sal-gal4 driver (provided by K. Basler, University of Zurich, Zurich, Switzerland) were crossed to flies with the reporter transgene CPV (Williams et al., 2006), and the progeny were used as wild-type controls. The fly mutant alleles used in this study are dronc<sup>24</sup> and dronc<sup>29</sup> (Xu et al., 2005), drice<sup>17</sup> (Muro et al., 2006), dcp-1<sup>prev</sup> (Laundrie et al., 2003),
The rescue transgenic lines drice:drice and drice:dcp-1 were generated as follows: first, a 564-bp fragment from the drice coding region encompassing its promoter and 5' UTR was PCR amplified using the forward primer 5'-GGCAATTGCCTCTTTGAGAGTGTGACCG-3' and reverse primer 5'-CCAAGATCTGGCATAGTTCTCCTTGAG-3' with added Mil6 and BgIII restriction sites, respectively. Second, a 1,090-bp fragment of the dcp-1 coding region was amplified from a cDNA clone (a gift from H. Steller) using the forward primer 5'-CAGGATCTCCAGTGTATGATAAC-3' and reverse primer 5'-GGCTTGGACCTCGAT-3' with added XbaI and BglII restriction sites. Both the promoter/5' UTR fragments and 3' UTR fragments were cloned into a sequential order into the EcoRI + BglII and NotI + Acc65I sites of the patb plasmid (a gift from J. Bischof, University of Zurich, Zurich, Switzerland), giving rise to the patb-drice-5'3' plasmid. The drice coding region was amplified from a cDNA clone (a gift from H. Steller) using the forward primer 5'-CAAGATCTTATGGACGC-3' and reverse primer 5'-GGGGGCGCTTACAAACC-GTCCGGCGTTGTG-3' with added BglII and PspOMI restriction sites and was subsequently respectively cloned into the BglII and NotI restriction sites of the patb-drice-5'3' plasmid. The dcp-1 coding region was amplified from a cDNA clone (a gift from H. Steller) using the forward primer 5'-CGGGGATCCATACGGCGTACAGC-3' and reverse primer 5'-CGGCGGCCGCGCTTACAGCAGTGCTGTAAC-3' with added XbaI and NotI restriction sites and was subsequently respectively cloned into the BamHI and NotI restriction sites of the patb-drice-5'3' plasmid. The two transgenic fly lines, drice:drice and drice:dcp-1, were generated using the pC31-mediated site-specific transgenesis technique, which allows insertion of transgenes into known sites of the Drosophila genome (Birchler et al., 2007; Fish et al., 2007). Specifically, these transgenes were inserted into the attP18 site on the X chromosome. Transcriptional expression of the transgenes was confirmed by RT-qPCR analysis on RNA from WDs of the two transgenic fly lines.

The rescue transgenic lines dcp-1:drice and dcp-1:dcp-1 were generated as follows: first, a 708-bp fragment from the dcp-1 gene encompassing its promoter and 5' UTR was amplified using the forward primer 5'-GCTGCTACCCGCTCCTC-3' and reverse primer 5'-GGCTGCTACCCGCTCCTC-3' with added Mil6 and BgIII restriction sites, respectively. Second, a 1,090-bp fragment of the dcp-1 coding region was amplified from a cDNA clone (a gift from H. Steller) using the forward primer 5'-CAAGATCTTATGGACGC-3' and reverse primer 5'-GGGGGCGCTTACAAACC-GTCCGGCGTTGTG-3' with added BglII and PspOMI restriction sites and was subsequently respectively cloned into the BglII and NotI restriction sites of the patb-drice-5'3' plasmid. The dcp-1 coding region was amplified from a cDNA clone (a gift from H. Steller) using the forward primer 5'-GCTGCTACCCGCTCCTC-3' and reverse primer 5'-GGCTGCTACCCGCTCCTC-3' with added XbaI and BglII restriction sites, respectively. Both the promoter/5' UTR and 3' UTR fragments were respectively cloned in a sequential order into the BamHI + EcoRI and XhoI + XbaI sites of the patb plasmid, giving rise to the patb-dcp-1-5'3' plasmid. The dcp-1 and dcp-2 coding regions were each amplified from the CDNA clones as described above but this time using primers with added NotI and SalII restriction sites. Each fragment was then cloned into the NotI and XbaI restriction sites in the patb-dcp-1-5'3' plasmid. These transgenes were inserted into the attP40 site on chromosome 2L using the pC31 system as before.

Quantification of images

Quantification of staining was performed by measuring the area of the positively stained pixels (i.e., cPARP or TUNEL) and dividing it in the area of the Venus expression for cPARP or the total disc area for TUNEL using the Imaged program (National Institutes of Health; Abramoff et al., 2004). Statistical analysis was performed using a one-way analysis of variance test followed by Fisher’s protected least significant difference posttest for multiple comparisons using the StatView Program (Abacus Concepts). For each experiment, 12–20 WDs were analyzed for each genotype or time point. Significance level was considered as either P < 0.05 or P < 0.001, as indicated above the bars in the figures.

Quantification of dce and dcp-1 mRNA expression levels

WDs from wild-type and mutants flies were dissected, and total RNA was extracted using the RNeasy Micro Kit (Qiagen). Reverse transcription was performed with 1 µg of total RNA. First-strand synthesis was performed using oligo(dT) primers (Promega) and SuperScript II Reverse Transcriptase (Promega) in the presence of RNase-free DNase to eliminate DNA contamination and Protector RNase inhibitor (Roche). Measurements were normalized to mitochondrial large-ribosomal RNA (MitRNA: forward 5'-AAAAAGAGGCGAGAGCGCTCAGGTG and reverse 5'-AAAAACACCTTGCGTACAC-3') or RP49 (Dmelt/Rpl32: forward 5'-GACATCGGCGCCACGACATAC-3' and reverse 5'-CCATTCGCGGCGCAGCTTACG-3'). The primers for dce mRNA were forward 5'-CCACTAAATATGGAAGAATACGC-3' and reverse 5'-GGCCGTCTACCCGCCTTCTC-3'. The primers for dcp-1 mRNA were forward 5'-ACCGGAGTCGACGGGCAAG-3' and reverse 5'-ACAAGAAGACGGCGAGG-3'. Products were amplified by the KAPA SYBR FAST quantitative PCR kit (Kapa Biosystems) using the LightCycler 480 quantitative PCR machine (Roche). Results of expression levels were calculated as the mean from three independent experiments with at least two biological repeats with two duplicates each.
Online supplemental material

Fig. S1 shows that drice and dcp-1 transcripts are expressed at comparable levels in WGS. Fig. S2 shows that the CPV reporter does not affect the rate of cell death. Fig. S3 shows that different anticleaved caspase-3 (CM1) antibodies detect both active Drice and Dcp-1, although they are more specific toward active Drice. Fig. S4 shows that Dcp-1 may further activate Drice in a positive amplification loop. Fig. S5 shows that Diap1 does not inhibit Drice and Dcp-1 with different efficiencies. 

Supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107133/DC1.

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