The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in *Drosophila*

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Summary

The initiator caspase Dronc is the only *Drosophila* caspase that contains a caspase activation and recruitment domain (CARD). Although Dronc has been implicated as an important effector of apoptosis, the genetic function of *dronc* in normal development is unclear because *dronc* mutants have not been available. In an EMS mutagenesis screen, we isolated four point mutations in *dronc* that recessively suppress the eye ablation phenotype caused by eye-specific overexpression of *hid*. Homozygous mutant *dronc* animals die during pupal stages; however, at a low frequency we obtained homozygous adult escapers. These escapers have additional cells in the eye and wings that are less transparent and slightly curved down. We determined that this is due to lack of apoptosis. Our analyses of *dronc* mutant embryos suggest that *dronc* is essential for most apoptotic cell death during *Drosophila* development, but they also imply the existence of a *dronc*-independent cell death pathway. We also constructed double mutant flies for *dronc* and the apoptosis inhibitor *diap1*. *dronc* mutants can rescue the ovarian degeneration phenotype caused by *diap1* mutations, confirming that *dronc* acts genetically downstream of *diap1*.

Key words: Dronc (Nc), CARD, Caspase, Apoptosis, Cell death, *Drosophila*, Diap1

Introduction

Programmed cell death, or apoptosis, is an important feature of metazoan development and is crucial for tissue homeostasis. It helps sculpt our bodies, removes unwanted cells or cells that are no longer needed, and eliminates cells that are potentially dangerous for the organism (Baehrecke, 2002). In addition, many human conditions are associated with altered rates of cell death, including cancer, autoimmune diseases, and neurodegenerative disorders (Thompson, 1995). Thus, to improve our knowledge about these conditions and to develop methods to treat them, a thorough understanding of the underlying apoptotic mechanisms is crucial.

Molecular genetic studies performed in the last 10 to 15 years revealed that the basic principles of regulation and execution of apoptosis are conserved. Genetic studies in *Caenorhabditis elegans* have implicated caspases as central to the apoptotic program (Yuan et al., 1993). Caspases are a highly specialized class of cysteine proteases that cleave target proteins specifically after Asp residues. At least 11 human caspases are known, while the *Drosophila* genome contains seven caspase genes (Salvesen and Abrams, 2004). Caspases are synthesized as catalytically inactive zymogens, the activation of which is tightly controlled and involves both positive and negative input (for reviews, see Danial and Korsmeyer, 2004; Salvesen and Abrams, 2004). Activation occurs through proteolytic processing, generating a large and small subunit that then form a tetramer containing two large and two small subunits (Danial and Korsmeyer, 2004). Caspases are negatively regulated by inhibitor of apoptosis proteins (IAPs), a highly conserved class of proteins with members in all eukaryotic species (Miller, 1999). IAPs directly bind to and inhibit caspases. Thus, IAPs represent the last line of defense for a cell against apoptotic stimuli.

Two classes of caspases have been defined based on the length of the prodomain. Initiator caspases such as Caspase 9 contain long prodomains that harbor regulatory motifs such as the caspase activation and recruitment domain (CARD). Through homotypic interactions of the CARD motif of Caspase 9 with the CARD motif of Apaf-1, Caspase 9 is recruited into the apoptosome, a large multi-subunit complex, where it undergoes autoprocessing and activation (Danial and Korsmeyer, 2004). Once activated, Caspase 9 cleaves and activates effector caspases (Caspase 3, -6 and -7), which are characterized by the presence of short prodomains. Effector caspases execute the cell death process by cleaving a large number of cellular proteins (Danial and Korsmeyer, 2004).

The *Drosophila* genome contains a total of seven caspase genes, three of which encode putative initiator caspases [Dronc (Nedd2-like caspase – FlyBase), Dredd and Strica (Dream – FlyBase)], whereas the remaining four are putative effector caspases [DrICE (Ice – FlyBase), DCP-1, Decay and Damm] (reviewed by Kumar and Doumanis, 2000; Salvesen and Abrams, 2004). Dronc is the only *Drosophila* caspase that carries in its prodomain a CARD motif (Dorstyn et al., 1999),

which interacts with the CARD of Dark (Ark – FlyBase), the *Drosophila* Apaf-1 homolog, also known as D-Apaf-1 or Hac-1 (Rodriguez et al., 1999; Kanuka et al., 1999; Zhou et al., 1999b). In this respect, Dronc is functionally most similar to human Caspase 9. Consistent with its function as an initiator caspase, Dronc can cleave and activate the effector caspase DrICE in vitro (Hawkins et al., 2000). *dronc* is ubiquitously expressed throughout development and is a target of the insect hormone ecdysone, which stimulates increased *dronc* expression during metamorphosis (Dorstyn et al., 1999). Several observations suggest that *dronc* is an important component of the apoptotic machinery in *Drosophila*. Overexpression of *dronc* in the developing fly eye induces cell death and tissue loss (Meier et al., 2000; Quinn et al., 2000). Dominant negative constructs and RNA interference experiments support a role for *dronc* in developmental cell death (Meier et al., 2000; Quinn et al., 2000). However, without mutations in the endogenous gene, a definitive role of *dronc* in developmental apoptosis cannot be defined.

Like Caspase 9, Dronc is subject to negative regulation by IAPs, in particular *Drosophila* IAP1 [Diap1 (Thread – FlyBase) (Meier et al., 2000)]. Diap1 is characterized by two tandem repeats of approximately 70 amino acids each, known as the Baculovirus IAP Repeat (BIR; for a review, see Deveraux and Reed, 1999), and one C-terminally located RING domain. The BIR domains are required for binding and inhibiting caspases (Zachariou et al., 2003). The RING domain has been shown to encode an E3 ubiquitin ligase (Yang et al., 2000). Ubiquitin ligases mediate the transfer of ubiquitin from E2 conjugating enzymes to target proteins that are subsequently degraded by the 26S proteasome (Joazeiro and Weissman, 2000). Target proteins of Diap1/RING-mediated ubiquitination include Dronc in the absence of apoptotic signals (Wilson et al., 2002) and Diap1 itself in the presence of apoptotic signals (Ryoo et al., 2002; Yoo et al., 2002; Bergmann et al., 2003). Loss-of-function *diap1* mutations cause a dramatic cell death phenotype, in which nearly every cell in mutant embryos is apoptotic, suggesting an essential genetic role for *diap1* in cellular survival (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). This phenotype is presumably caused by inappropriate activation of caspases (Meier et al., 2000; Rodriguez et al., 2002).

In *Drosophila*, the genes *reaper*, *hid* (*wrinkled* – FlyBase) and *grim* are both necessary and sufficient for the induction of apoptosis (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Deletion of these genes, as seen in the *H99* deficiency, results in a complete lack of developmental cell death in *Drosophila* embryos (White et al., 1994). Overexpression of any of those genes in the fly eye using the eye-specific enhancer *GMR* (for example, *GMR-hid* or *GMR-reaper*) causes a severe eye ablation phenotype resulting from inappropriate apoptosis (Grether et al., 1995; White et al., 1996) (see also Fig. 2A,E). Subsequent genetic and biochemical analyses have shown that these genes induce apoptosis through direct inhibition of Diap1 (Wang et al., 1999; Goyal et al., 2000). In response to expression of *reaper* and *hid,* the RING domain of Diap1 changes its substrate specificity, self-ubiquitinates and induces its own degradation (Ryoo et al., 2002; Yoo et al., 2002; Bergmann et al., 2003). Caspases, most notably Dronc, are thus relieved from Diap1 inhibition and can induce apoptosis. In mammals, the factors Smac/DIABLO and HtrA2 are known to relieve caspase inhibition by IAPs (Du et al., 2000; Verhagen et al., 2000; Verhagen et al., 2002; Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002). These factors share with Reaper, Hid, and Grim a conserved N-terminus that is required for interaction with IAPs.

These studies indicate that Dronc is important for the induction of apoptosis. However, mutants that would allow us to determine the genetic requirement of *dronc* in apoptosis during development have not been available. Here we describe the isolation and genetic characterization of ethyl methane sulfonate (EMS)-induced point mutations in *dronc*. Our phenotypic analysis of these mutants in the wing, eye and embryo showed that *dronc* is essential for most developmental cell death. We also provide genetic evidence that *dronc* acts genetically downstream of *diap1*. However, although apoptosis was substantially reduced in our null mutants of *dronc*, it was not completely blocked, suggesting that some cells can undergo apoptosis independently of *dronc*.

Materials and methods

Identification of *dronc* mutant alleles

We obtained the *GMR-hid ey-FLP* (*GheF*) chromosome by meiotic recombination on the X chromosome. *ey-FLP*; *FRT80* males were treated with 25 mmol/l EMS in 5% sucrose solution for 24 hours. After recovery for 3 hours, they were mated to *GheF*; *FRT80* w^+ females, and incubated at 25°C (Fig. 1). From this cross, 45,000 F1 progeny were analyzed for suppression of the *GMR-hid*-induced small eye phenotype. Three *dronc* alleles were isolated as strong suppressors of *GMR-hid,* and one *dronc* allele was a medium-strong suppressor. A detailed description of the mutagenesis screen will be published elsewhere.

Fly stocks and genetics

The following fly stocks were used: $dronc^{I24}$, $dronc^{I29}$, $dronc^{2}$ and $dronc^{L32}$ (this study); $diap1^{6B}$ (Lisi et al., 2000); $diap1^{8}$ (Rodriguez et al., 2002); P[*sli-1.0-lacZ*] (Wharton and Crews, 1993); *UAS-proDronc* (Meier et al., 2000). The $dronc^{I24}$ $diap1^{8}$, $dronc^{L32}$ $diap1^{6B}$, $dronc^{I24}$ $diap1^{109.07}$ and $dronc^{I24}$ $diap1^{5}$ double mutants were obtained by meiotic recombination. The wild-type stocks used for comparison were Canton S and w^{1118}.

A recombinant chromosome containing *en-Gal4* and *UAS-GFP* transgenes (referred to as *en::GFP*), located on the second chromosome, was crossed into a $dronc^{I24}$/$dronc^{I29}$ trans-heterozygous mutant background, and GFP expression in the wing was monitored.

Mosaic eye clones were obtained from *ey-FLP*; $dronc^{I24}$ *FRT80*/*ubi-GFP FRT80* pupae and analyzed by anti-Discs large (Dlg) labeling.

For germline clone (GLC) analysis, $dronc^{I24}$ and $dronc^{I29}$ were recombined onto the FRT2A chromosome. GLC were induced by the DFS-FRT method as described (Chou et al., 1993; Chou and Perrimon, 1996).

To visualize midline glia (MG) cells, males of the genotype P[*sli-1.0-lacZ*]; $dronc^{I24}$/TM6B, *ubx-lacZ* were crossed to GLC $dronc^{I24}$ or $dronc^{I29}$ females.

Immunohistochemistry

TUNEL and immunohistochemistry were done as described (Goyal et al., 2000; Patel, 1994). CM1 (anti-cleaved Caspase 3) antibody was used at a dilution of 1:50, Elav antibody at dilution of 1:20, anti-Krüppel antibody at 1:50, and anti-Dlg antibody at 1:300. The MG was visualized by β-gal immunohistochemistry. Ovary dissections were done as described (Rodriguez et al., 2002).

Results

Isolation of *dronc* mutants

Eye-specific expression of *hid* under GMR promoter control (*GMR-hid*) results in an eye ablation phenotype (Fig. 2A) (Grether et al., 1995). The *GMR-hid* eye ablation phenotype has been used in dominant modifier screens to isolate mutants in genes that are directly or indirectly involved in the control and execution of *hid*-induced apoptosis (Bergmann et al., 1998; Kurada and White, 1998; Goyal et al., 2000; Ryoo et al., 2002). Notably, however, no mutations in any caspase gene, including *dronc,* were recovered in these screens (A.B., unpublished). A prerequisite for a dominant modifier screen is that a reduction in the dose of a gene by 50% must be sufficient to visibly modify the phenotype under study (Simon et al., 1991). Thus, we reasoned that this might not apply to *dronc*, and that a reduction of the gene dose of *dronc* by 100% (i.e. a homozygous mutant condition) might be necessary to visibly modify the *GMR-hid* phenotype.

We therefore developed a method that allows screening for recessive suppressors of *GMR-hid* in a homozygous mutant condition. However, we were uncertain as to whether *dronc* is an essential gene for development. If it were, homozygous *dronc* mutants would die, which would prevent us from screening modifications of the *GMR-hid* eye phenotype. Instead, we screened for suppressors of *GMR-hid* in homozygous mutant eye clones obtained by FLP/FRT-mediated recombination in otherwise heterozygous animals. Specifically, we used *ey-FLP* (Newsome et al., 2000) to express the FLP recombinase under *eyeless* (*ey*) enhancer control in the developing eye to induce homozygous mutant clones. We termed this approach the *GheF* method for *GMR-hid ey-FLP*. Because the *ey* enhancer used to express FLP is active before the *GMR* enhancer, the eye tissue is already mosaic for any induced mutation when *GMR* begins to drive *hid* expression. If a gene required for *GMR-hid*-induced apoptosis and eye ablation was mutagenized, the homozygous mutant clone cells would be resistant to the effects of *GMR-hid*. However, the twin-spot and heterozygous cells would contain either two or one functional copies of the mutagenized gene, respectively, and would be sensitive to *GMR-hid*-induced apoptosis. As a result, any surviving eye tissue in the adult organism would be homozygous for the mutagenized gene.

We conducted an EMS mutagenesis screen using the *GheF* method to isolate mutations in the *dronc* gene (Fig. 1). Because *dronc* maps to the left arm of the third chromosome (3L), we selected for mutations on this chromosome arm using FRT80, which is specific for 3L (Fig. 1). *dronc* is the only caspase known to map to 3L, so we expected to identify only *dronc* mutants in this screen. Among 45,000 F1 progeny screened, four mutations were isolated that suppressed the *GMR-hid* eye ablation phenotype. These mutations were subsequently confirmed to be mutant alleles of *dronc* (see next section). Three of them, $dronc^{I24}$, $dronc^{I29}$ and $dronc^{2}$, rescued the *GMR-hid* eye ablation phenotype almost entirely in *ey-FLP*/*FRT*-induced clones (Fig. 2B). The fourth allele, $dronc^{L32}$, was weaker and suppressed the *GMR-hid*-eye phenotype partially to medium size (Fig. 2C). It is important to note that the *dronc* mutants suppressed *GMR-hid* only in homozygous mutant clones. In a heterozygous condition, even a null allele ($dronc^{I24}$) did not modify the *GMR-hid* phenotype (Fig. 2D).

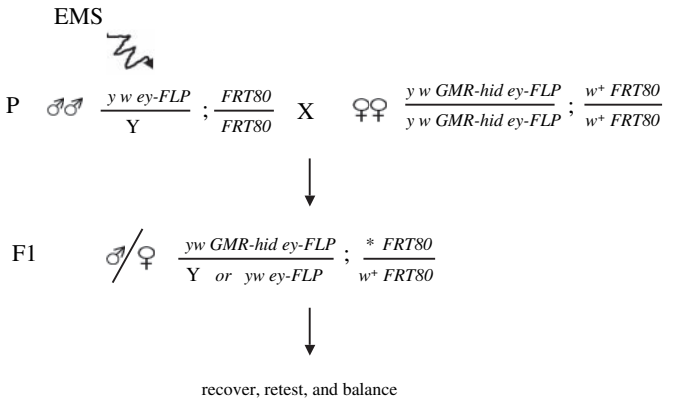

Fig. 1. The *GheF* screening method. Males of the indicated genotype were treated with the chemical mutagen EMS as described in Materials and methods. The mutagenized males were mated to the *GMR-hid ey-FLP* (*GheF*), *FRT80* tester females. F1 offspring of this cross were screened for a modification, usually a suppression, of the *GMR-hid* eye ablation phenotype. Suppressor mutants were recovered, retested and established as balanced stocks. Only recessive suppressors were maintained; dominant suppressors were discarded.

This finding is consistent with our assumption that a 50% reduction in the gene dose of *dronc* is not sufficient to visibly modify *GMR-hid* and provides an explanation for why *dronc* alleles were not recovered in the dominant modifier screen (see above). The *dronc* mutants also suppressed the *GMR-reaper*-induced small eye phenotype (Fig. 2E-G).

In summary, we isolated four *dronc* alleles as strong or medium-strength suppressors of *GMR-hid* in *ey-FLP*/*FRT*-induced clones. Our genetic analysis shows that *dronc* mutants recessively rescued the effect of *GMR-hid* and *GMR-reaper* expression in the eye, suggesting that $dronc^{+}$ is genetically required for *GMR-hid*- and *GMR-reaper*-induced apoptosis.

Molecular analysis of the *dronc* alleles

Inter se complementation studies indicated that the four suppressor mutations of *GMR-hid* all affected the same genetic function. These mutations were semi-lethal when carried in trans to each other. Usually, they died during pupal stages; however, at a low rate (less than 10% of the expected progeny), homozygous mutant escaper flies of the strong alleles could be recovered. These escapers are characterized by an abnormal wing phenotype (see below, Fig. 4), a weak rough eye phenotype (not shown) and a short life span. They died 2-3 days after eclosion.

We confirmed that these suppressors were *dronc* mutants in four ways. First, the *Df(3L)AC1* deficiency deleting *dronc*, among other genes, failed to complement the phenotypes (see above) caused by the suppressor mutants. Second, the mutations failed to complement a P-element revertant that deletes *dronc* and one neighboring gene (kindly provided by K. White). Third, expression of a $dronc^{+}$ transgene under *GMR* control partially restored the eye ablation phenotype of *GMR-hid* in $dronc^{I29}$ clones (Fig. 2H). Fourth, by DNA sequence analysis, we identified missense and nonsense mutations in the *dronc* gene of these mutants (Fig. 3). $dronc^{I24}$, $dronc^{I29}$ and $dronc^{2}$ contain premature stop codons at positions 28, 53 and 325, respectively. These results suggest that $dronc^{I24}$ and

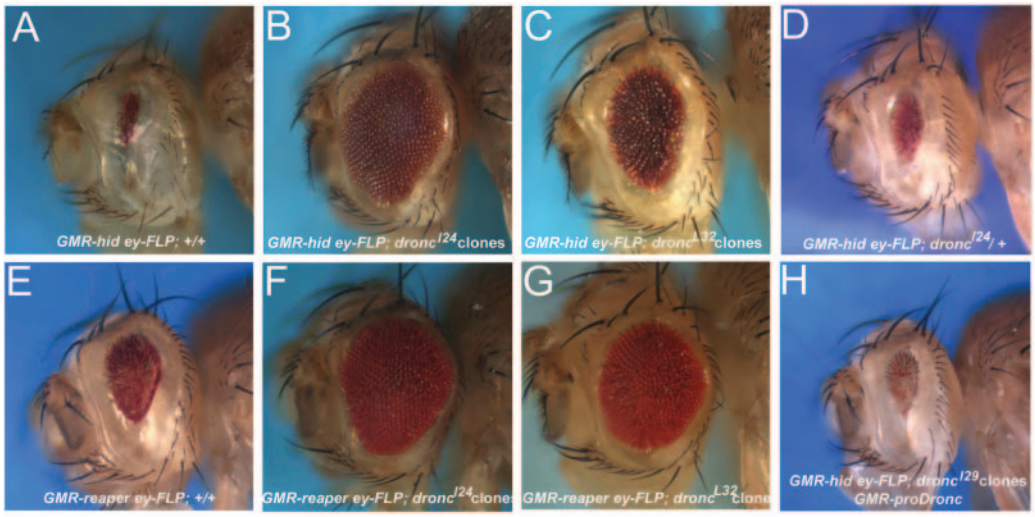

Fig. 2. *dronc* mutants suppress the *GMR-hid*- and *GMR-reaper*-induced small-eye phenotype in recessive clones. (A) The unsuppressed *GMR-hid ey-FLP* (*GheF*) eye ablation phenotype. (B) Suppression of the *GheF* phenotype in *ey-FLP*/*FRT*-induced clones of *dronc*^{I24}. The exact genotype of this fly is *GheF*/*w*; *dronc*^{I24} *FRT80*/*w*$^{+}$ *FRT80*. This *dronc* allele gives rise to strong suppression of *GMR-hid* and is molecularly a null allele. Similar results were obtained for *dronc*^{I29} and *dronc*^{2}. Despite the fact that mutant clones are phenotypically *w*$^{-}$ (see Fig. 1), these flies still produce red eye pigment because the *GMR-hid* transgene is marked with *w*$^{+}$ (not indicated in Fig. 1). (C) Suppression of the *GheF* phenotype in *ey-FLP*/*FRT*-induced clones of *dronc*^{L32}. The exact genotype of this fly is *GheF*/*w*; *dronc*^{L32} *FRT80*/*w*$^{+}$ *FRT80*. This *dronc* allele gives rise to medium-strong suppression of *GMR-hid*, and is thus a hypomorphic allele. (D) *dronc* mutants fail to dominantly modify the *GMR-hid* eye ablation phenotype. The genotype of this fly is *GheF*/*w*; *dronc*^{I24} *FRT80*/+. (E) The unsuppressed *GMR-reaper ey-FLP* eye ablation phenotype. Genotype of this fly: *ey-FLP*/*w*; *CyO, 2xGMR-reaper*/+. (F,G) Suppression of *GMR-reaper* in *ey-FLP*/*FRT*-induced clones of *dronc*^{I24} (F) and *dronc*^{L32} (G). Genotypes: *ey-FLP*/*w*; *CyO, 2xGMR-reaper*/+; *dronc*^{I24} *FRT80*/*w*$^{+}$ *FRT80* (F) and *ey-FLP*/*w*; *CyO, 2xGMR-reaper*/+; *dronc*^{L32} *FRT80*/*w*$^{+}$ *FRT80*. (H) Partial restoration of the *GMR-hid* eye ablation phenotype in *dronc* mutant clones by a *dronc*$^{+}$ transgene. The genotype of this fly is *GheF*/*w*; *GMR-Gal4*/+; *dronc*^{I29} *FRT80 UAS-proDronc*/*w*$^{+}$ *FRT80*.

dronc^{I29} do not produce any functional Dronc protein, because the catalytic large and small subunits do not form.

The *dronc*^{2} allele contained a mutation that introduced a stop codon at position 325, exactly where the large and small subunits of Dronc are separated by caspase cleavage during activation (Dorstyn et al., 1999). Thus, in this mutant the large subunit of Dronc was intact, whereas the small subunit was completely missing. Because *dronc*^{2} was isolated as a very strong suppressor of *GMR-hid*, it is likely to encode a null allele of *dronc*, suggesting that the small subunit lacking in this mutant is essential for *dronc*$^{+}$ function. Recent data by Muro et al. (Muro et al., 2004) indicate that auto-processing at Glu352, but not at Asp324, is crucial for Dronc activation. However, even in this case, the *dronc*^{2} mutation completely deleted the small subunit and even part of the large subunit, again providing evidence that it is genetically a null allele of *dronc*.

dronc^{L32} behaves genetically as a hypomorph (Fig. 2C,G), changing Leu25 in the CARD domain to Glu. Leu25 is a conserved residue in the CARDs of Caspase 9, Caspase 2, Apaf-1 and Ced-4. Interestingly, structural analyses of the CARD/CARD interaction between human Caspase 9 and Apaf-1 showed that the equivalent residue in human Caspase 9, Leu16, is not directly involved in the interaction between the two CARDs (Qin et al., 1999; Zhou et al., 1999a). Nevertheless, the fact that this residue is conserved in various CARD motifs and that its mutation results in partial loss of function suggests that it is important for appropriate CARD activity.

In summary, this analysis suggests that the isolated suppressor mutations of *GMR-hid*-induced eye phenotypes represent *dronc* alleles. With respect to *dronc*^{I24}, *dronc*^{I29} and *dronc*^{2}, our genetic and molecular analyses suggest that they are complete loss-of-function alleles, whereas *dronc*^{L32} is a hypomorphic allele.

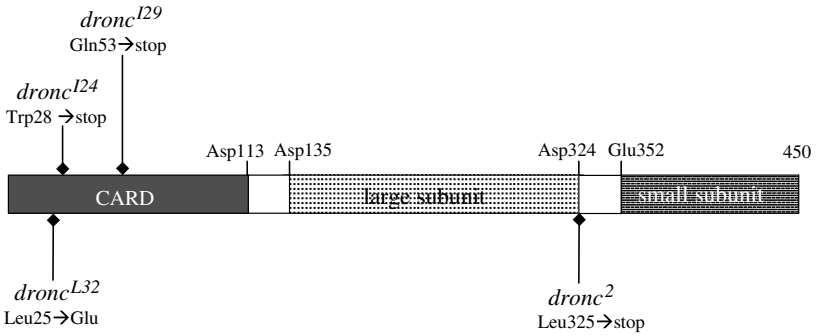

Fig. 3. Location of point mutations in the *dronc* gene. The domain structure of Dronc with CARD, large and small subunits is depicted. Essential residues for maturation of Dronc are indicated. Asp113, Asp135, Asp324, and Glu352 are putative caspase cleavage sites. The molecular lesions of the isolated *dronc* mutations are shown.

dronc mutants have an abnormal wing phenotype and extra cells in the eye

As mentioned above, most homozygous *dronc* mutant animals died during pupal stages. However, at a low rate (<10%), homozygous escapers, even of the null alleles (*dronc*^{I24} and *dronc*^{I29}), did eclose. These flies were characterized by a short life span (they died within 3 days of eclosion), an abnormal wing phenotype (Fig. 4A,B) and a rough eye (data not shown). The mutant wing phenotype, although difficult to illustrate in photographs, is readily scored under the dissecting microscope using low magnification (8-10×). The mutant wing appeared opaque by comparison to wild type,

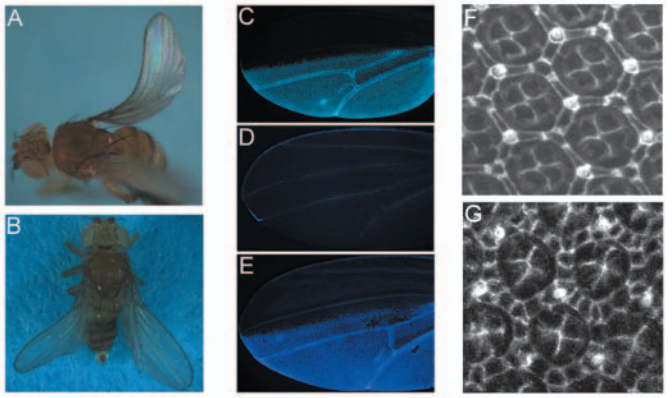

Fig. 4. *dronc* is essential for cell death in wing and eye development. (A,B) Abnormal wing phenotype of homozygous adult *dronc* mutants. The wings are less transparent and curved. The held-out wing in A is occasionally observed and not typical. Genotype: (A) $dronc^{I24}/dronc^{I24}$; (B) $dronc^{I29}/dronc^{I29}$. (C) *en::GFP* expression in a wing of a freshly eclosed wild-type male (less than 1 hour old). (D) *en::GFP* expression in a wing of a 24-hour-old wild-type male. No GFP expression is detectable. (E) *en::GFP* expression in a wing of a 24-hour-old $dronc^{I24}/dronc^{I29}$ male. GFP expression is still detectable. (F) Anti-Dlg labeling in a wild-type pupal retina disc to visualize cell outline. (G) Anti-Dlg expression in pupal retina of a $dronc^{I24}$ mutant clone. Additional inter-ommatidial cells are present, suggestive of lack of apoptosis.

occasionally contained trapped fluid, and was curved downward (Fig. 4A,B). This phenotype is similar to the wing phenotype seen in *dark* and *hid* mutant flies (Rodriguez et al., 1999; Abbott and Lengyel, 1991). Because these genes are involved in cell death, the wing phenotype seems likely to be the result of decreased cell death.

It has been recently shown that a wave of cell death occurs in the wing within the first hour after eclosion. Kimura et al. (Kimura et al., 2004) used *en*-Gal4 and UAS-*GFP* transgenes (referred to as *en::GFP*) to drive expression of GFP as a marker in the posterior compartment of the wing to analyze this cell death (Fig. 4C). The majority of *en::GFP*-positive cells are removed within one hour after eclosion in wild-type wings (Kimura et al., 2004). Co-expression of the caspase inhibitor P35 blocks the removal of *en::GFP*-expressing cells, suggesting that it is the result of a Caspase-driven cell death process in wild-type wings (Kimura et al., 2004). We examined whether this wave of cell death occurs in *dronc* mutants. By contrast to wild type, *en::GFP* expression was still detectable in wings of 24-hour-old *dronc* mutants (Fig. 4D,E). These data suggest that the persistence of *en::GFP* is the result of loss of developmental apoptosis in *dronc* mutant wings. Thus, lack of apoptosis probably contributes to the abnormal wing phenotype of *dronc* mutants.

Homozygous *dronc* mutant flies also exhibit a mild rough eye phenotype (data not shown). Using an antibody against the Discs large (Dlg) protein to visualize cell outlines, we determined that mid-pupal (50 hours) retinae of *dronc* mutants contained on average three additional inter-ommatidial cells (Fig. 4G). These cells usually die in wild-type retinae (Cagan and Ready, 1989; Wolff and Ready, 1991) (Fig. 4F), suggesting that $dronc^{+}$ is genetically required for developmental cell death in the retina. However, this result is contradictory to a recent study by Chew et al. (Chew et al., 2004), which reported fine patterning defects in the developing eye of *dronc* mutants rather than defects in cell death. However, the authors came to this conclusion by using photoreceptor markers to analyze *dronc* mutant eye discs (Chew et al., 2004). However, photoreceptors are not known to undergo developmental cell death. Furthermore, the *dronc* mutant by Chew et al. (Chew et al., 2004) is derived from an imprecise P-element excision, which also affects a neighboring gene, *CG6685*. Thus, the reported fine patterning defect may be due to inactivation of *CG6685*, and not of *dronc*.

In summary, this analysis provides strong evidence that $dronc^{+}$ is genetically involved in cell death during imaginal disc development of the eye and the wing in the fly.

dronc is essential for apoptosis during embryogenesis

We analyzed the genetic requirement of *dronc* for apoptosis during embryogenesis. Because of the large maternal contribution, *dronc* mutant animals survived embryogenesis and most of them died during pupal stages. The few homozygous escapers died within the first 3 days after eclosion; homozygous females were sterile and could not provide embryos for analysis. To analyze the genetic requirement of *dronc* for cell death during embryogenesis, we removed the maternal contribution by inducing germ line clones (GLC) in otherwise heterozygous females (Chou et al., 1993; Chou and Perrimon, 1996) (see Materials and methods). Embryos obtained from GLCs of the null mutants $dronc^{I24}$ and $dronc^{I29}$ were embryonic lethal if they were also zygotically mutant for *dronc*. These embryos exhibited a head defect similar to that of *hid* mutants (Abbott and Lengyel, 1991) (data not shown). Notably, the maternal loss of *dronc* was paternally rescuable; that is, $dronc^{+}$ provided by the father's sperm was sufficient to rescue the embryonic phenotypes due to maternal loss of *dronc*. Paternally rescued animals obtained from *dronc* GLC gave rise to normal and fertile adult flies (data not shown).

We performed TUNEL assays to confirm the possibility that maternally and zygotically mutant *dronc* embryos are embryonic lethal because they lack apoptosis. TUNEL detects DNA fragmentation, a hallmark of apoptosis (Wyllie, 1980; Gavrieli et al., 1992). Compared with wild-type embryos, the number of TUNEL-positive cells was substantially reduced during embryogenesis in both $dronc^{I24}$ and $dronc^{I29}$ embryos (Fig. 5A-C). We also analyzed whether downstream caspases such as DrICE were activated in *dronc* mutants. The CM1 antibody has been shown to detect only the activated form of DrICE (Yu et al., 2002). Compared with wild-type embryos, CM1 labeling of both $dronc^{I24}$ and $dronc^{I29}$ embryos was substantially reduced (Fig. 5D-F). Thus, consistent with its identity as an initiator caspase, $Dronc^{+}$ is required for activation of downstream caspases including DrICE. However, even though TUNEL-positive cell death is significantly reduced in $dronc^{-}$ embryos, it is not completely blocked. This finding suggests that $dronc^{+}$ is not required for all embryonic cell death and that a *dronc*-independent cell death pathway exists in the embryo (see Discussion).

We used three different assays to analyze the consequences of lack of apoptosis at a cellular level in *dronc* mutants. The best-characterized apoptotic model in *Drosophila*

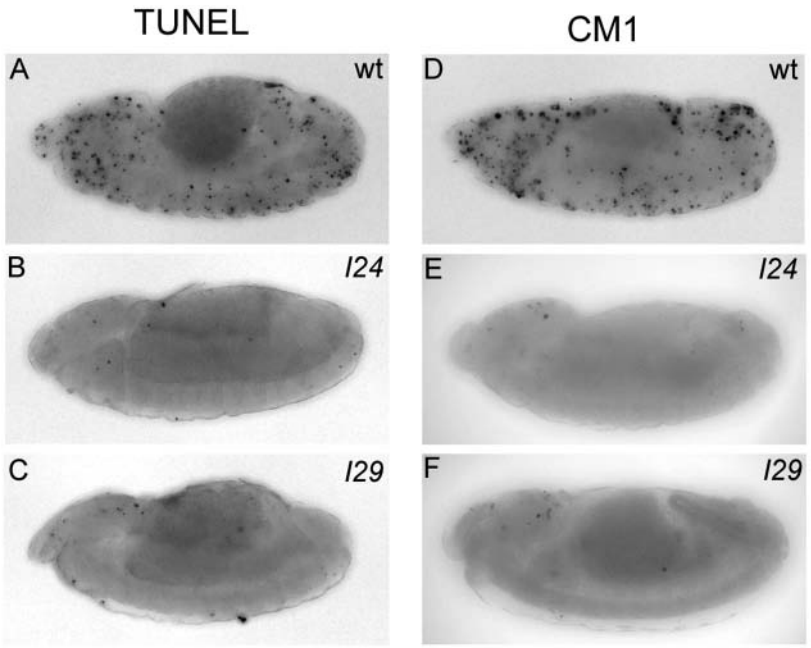

Fig. 5. Cell death analysis in wild-type and *dronc* mutant embryos. (A,D) Wild-type (wt) embryos stained with TUNEL (A) and CM1 antibody (D). CM1 labels activated DrICE (Yu et al., 2002). (B-F) Maternally and zygotically mutant $dronc^{I24}$ (B,E) and $dronc^{I29}$ (C,F) embryos labeled with TUNEL (B,C) and CM1 antibody (E,F). Despite the strong reduction in labeling signal, there were still a few TUNEL- and CM1-positive cells in *dronc* mutant embryos.

embryogenesis is the development of the midline glia (MG) in the central nervous system (Klämbt et al., 1991). The MG are transient cells during embryogenesis and are required for the separation and ensheathment of commissural axon tracts (Klämbt et al., 1991). At stage 13 of embryogenesis, about 10 MG cells per segment have been generated. Subsequent to the establishment of commissure morphology, a subset of the MG cells undergo apoptosis, leaving about three ensheathing MG cells per segment by the end of embryogenesis at stage 17 (Klämbt et al., 1991, Sonnenfeld and Jacobs, 1995; Zhou et al., 1995). The reduction in the number of MG cells is dependent on the cell death genes in the *H99* deficiency: *reaper*, *hid* and *grim*. In homozygous *H99* mutant embryos, the MG cells fail to die by apoptosis (Zhou et al., 1995; Zhou et al., 1997; Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997). We determined the fate of MG cells in *dronc* mutants. At stage 17, *dronc* mutant embryos contained additional MG cells compared with wild type (Fig. 6A,B). On average, *dronc* mutant embryos contain approximately 10 MG cells per segment at stage 17 (Fig. 6B). This rate of MG cell survival is similar to that seen in other apoptotically deficient backgrounds, such as *H99* and *dark* (Zhou et al., 1995; Sonnenfeld and Jacobs, 1995; Rodriguez et al., 2002). Thus, $dronc^{+}$ is essential for MG apoptosis.

We also used an antibody to the Krüppel protein to label a subset of cells in the late central nervous system (CNS) of the embryo. In *H99* mutants, this antibody stained two to three times as many cells in the CNS compared with wild type (White et al., 1994). Consistently, the antibody detected more cells in *dronc* mutant embryos, and there was a general enlargement of the CNS (Fig. 6C,D). Because apoptosis is very prevalent in the CNS of *Drosophila* embryos (Abrams et al., 1993), we conclude that the enlarged CNS in *dronc* mutants is due to loss of apoptosis.

In addition, we used the Elav antibody to visualize neurons in both the CNS and the PNS, in particular the mechanosensory chordotonal organs. We found that *dronc* mutants contain on average about three additional neurons in each chordotonal cell cluster compared with wild type (Fig. 6E,F). We also found examples of unidentified neurons, which are increased in number in *dronc* mutants compared with wild type (data not shown).

Taken together, these data support the notion that $dronc^{+}$ is essential for embryonic cell death. Thus, the *dronc* gene is genetically required for cell death in embryogenesis.

Analysis of *dronc diap1* double mutants

Diap1 is an essential inhibitor of apoptosis during *Drosophila* embryogenesis. *diap1* mutant embryos die during early embryonic development due to massive inappropriate apoptosis (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). Because *diap1* and *dronc* mutants have opposite phenotypes, and because their gene products directly interact with each other, it was proposed that Diap1 acts as an inhibitor of Dronc (Meier et al., 2000; Chai et al., 2003). To determine the genetic relationship between *dronc* and *diap1*, we analyzed the phenotype of double mutants of these genes. One allelic combination of weak *diap1* alleles ($diap1^{6B}/diap1^{8}$) generates viable, but sterile, females due to ovarian atrophy (Rodriguez et al., 2002) (Fig. 7A,C). We used this phenotype to analyze the genetic relationship between *diap1* and *dronc*. In a double-mutant combination with *dronc* ($dronc^{I24}$ $diap1^{8}/dronc^{L32}$ $diap1^{6B}$), the ovarian atrophy phenotype of $diap1^{6B}/diap1^{8}$ females was partially reversed. The size of the egg chamber was significantly enlarged compared with that of $diap1^{6B}/diap1^{8}$ single mutants (Fig. 7B,C). This finding suggests that *dronc* mutations are able to rescue the *diap1* phenotype, and places *dronc* genetically downstream of *diap1* (see also Discussion). Despite this rescue, the double-mutant females still did not produce functional embryos and were sterile (data not shown).

Discussion

The *GheF* method presented in this paper uses clonal induction by FLP/FRT-mediated mitotic recombination to screen for recessive suppressors or enhancers of the *GMR-hid*-induced eye ablation phenotype. This type of screening has several advantages and disadvantages over traditional dominant modifier screens. It allows the generation of homozygous mutant tissue in specific tissues such as the eye, while the remainder of the animal is heterozygous for the induced mutation. This is particularly useful in conditions where the homozygous mutation causes lethality, thus preventing screening of adult structures such as the eye. Moreover, many genes are expressed at levels beyond their genetic requirement. Mutations in these genes often cannot be recovered in

traditional modifier screens, whereas FLP/FRT-induced clones allow their recovery. However, a clear disadvantage of FLP/FRT-mediated screening is the fact that the five major chromosome arms of the *Drosophila* genome have to be screened separately. To test the feasibility of the *GheF* screening method, we conducted a pilot screen for chromosome arm 3L to isolate mutations in the gene encoding *dronc*.

Using *GheF* screening, we isolated four EMS-induced point mutations of the initiator caspase *dronc* in *Drosophila*, demonstrating feasibility of the *GheF* screening method. Our genetic characterization of these mutants in the wing, eye and embryo is consistent with an essential role for *dronc*$^{+}$ in developmental cell death. The importance of caspases for programmed cell death was first revealed in genetic studies in *C. elegans* (Yuan et al., 1993), and later confirmed by targeted gene disruptions in mice (Kuida et al., 1996; Kuida et al., 1998). In *Drosophila*, the first report implicating caspases as important mediators of programmed cell death took advantage of the universal caspase inhibitor P35. In P35-overexpressing animals, cell death is significantly reduced (Hay et al., 1994). More recently, dominant-negative constructs of cloned caspases and RNAi experiments further supported the involvement of caspases in the cell death response in *Drosophila* (Meier et al., 2000; Quinn et al., 2000).

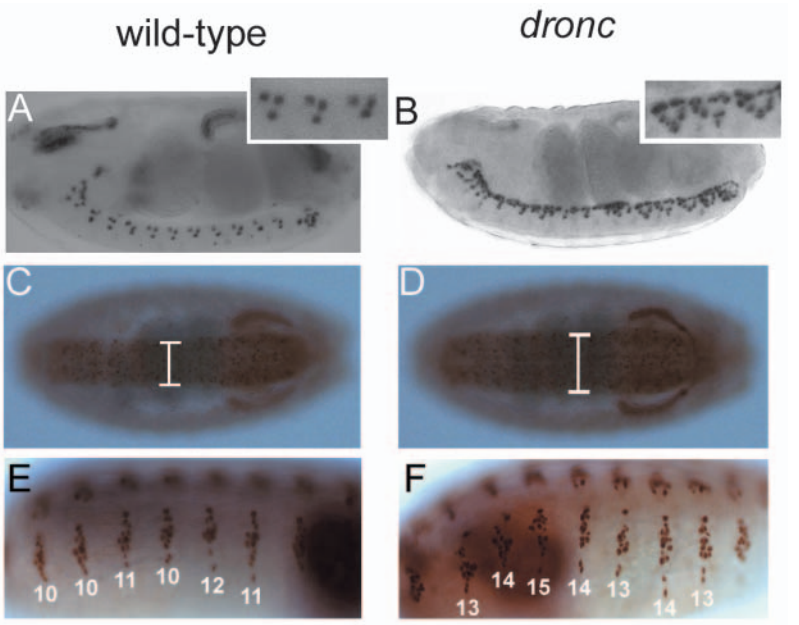

Fig. 6. *dronc* mutant embryos contain additional cells. (A,C,E) Wild-type embryos stained for the midline glia (A), Krüppel (C), and Elav (E). (B,D,F) Maternally and zygotically *dronc*^{I29} mutant embryos stained for midline glia (B), Krüppel (D) and Elav (F). Similar results were obtained for *dronc*^{I24} embryos (data not shown). (C,D) Ventral views of the CNS. Note the enlarged band in D compared with C (white bar). The numbers in E and F indicate the number of chordotonal cells in each cluster.

The *Drosophila* genome encodes seven caspase genes (Kumar and Doumanis, 2000; Salvesen and Abrams, 2004). Despite considerable efforts in multiple mutagenesis screens, mutations in any of the *Drosophila* caspases have not been reported. The only exception is *dredd* (Chen et al., 1998); however, *dredd* does not appear to be an apoptotic caspase, as *dredd* mutations do not affect the global cell death pattern. Instead, genetic analysis has established that *dredd* has a fundamental role in innate immunity (Leulier et al., 2000). Mutations in the effector caspase *dcp-1* have also been reported (McCall and Steller, 1998). However, it was recently found that *dcp-1* lies embedded in an intron of another gene, called *pita*, and the reported phenotypes are due to combined inactivation of both *pita* and *dcp-1* (Laundrie et al., 2003).

In this study we show that a 50% reduction in the gene dose of *dronc* is not sufficient to modify the *GMR-hid* phenotype. This result is in contrast to previous reports that interpreted the dominant suppression of *GMR-hid* by the *dronc* deficiency *Df(3L)AC1* as evidence that *dronc* is the underlying cause of this suppression (Meier et al., 2000; Quinn et al., 2000). However, in addition to *dronc*, *Df(3L)AC1* deletes a number of other genes including *gap1*, which is a known suppressor of *GMR-hid* (Bergmann et al., 1998). Because the *dronc* mutants we isolated failed to dominantly modify *GMR-hid* (Fig. 2D), the authors of the aforementioned reports scored the suppression by *Df(3L)AC1* due to the absence of *gap1* rather than *dronc*.

Interestingly, the fact that a 50% reduction of *dronc* is insufficient to dominantly modify *GMR-hid* suggests that *dronc* is produced in excess over its genetic requirement. A similar conclusion can be made about *dark*, mutations of which modify *GMR-hid* and *GMR-reaper* only in homozygous mutants (Rodriguez et al., 1999; Kanuka et al., 1999). This conclusion is also consistent with the large maternal supply of *dronc* provided by the mother to the oocyte (see below). Because caspases including *dronc* are synthesized as inactive zymogens, which rely on association with scaffolding proteins such as Apaf-1/Dark or proteolytic processing for activation, the cell can afford to produce excessive amounts of these potentially dangerous proteins without damaging consequences.

Most homozygous *dronc* mutant animals die at pupal stages. However, embryos obtained from *dronc* GLCs are embryonic lethal. This suggests that the maternal contribution compensates for the loss of zygotic *dronc* until pupal stages, at which time the maternal contribution is depleted and most animals die. However, a few homozygous animals survive and hatch as adults, presumably because the maternal stores lasted slightly longer in these flies than in others. These escaper flies are characterized by an abnormal wing phenotype. We determined that lack of cell death contributes to this phenotype. They also exhibit a rough eye phenotype due to additional inter-ommatidial cells. However, homozygous *dronc* escapers live for only 2 to 3 days after eclosion. It is not clear why they die, but the fact that they do suggests that *dronc* might also have important functions for adult survival.

Despite the fact that endogenous cell death was significantly reduced in *dronc* mutants, it is not completely blocked, even for the putative null alleles. Using TUNEL and CM1 antibody labeling as two independent cell death assays, we consistently detected a few cells that underwent cell death in *dronc* mutants. This observation suggests that *dronc* is not required for all embryonic cell death. This is in contrast to the *H99* deficiency,

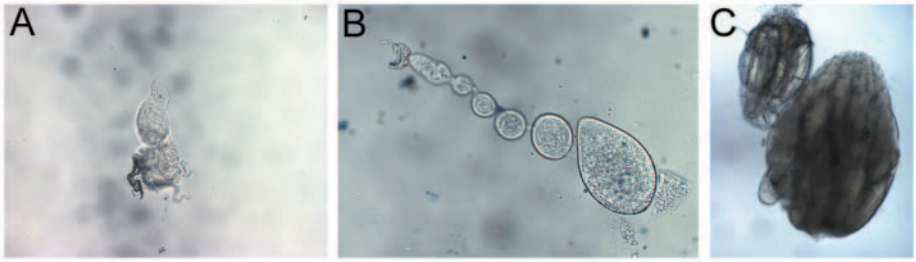

Fig. 7. Ovarian atrophy caused by weak *diap1* mutations is rescued by *dronc*. (A) The $diap1^{6B}/diap1^{8}$ mutant ovariole is poorly developed and atrophied. (B) By comparison, the $dronc^{I24}$ $diap1^{8}/dronc^{L32}$ $diap1^{6B}$ double-mutant ovariole shows improved differentiation of nurse cells and advanced maturation of the oocyte. (C) This improvement is visible in a global view of single mutant (left) and double mutant (right) ovaries.

in which *reaper*, *hid* and *grim* are deleted. Homozygous *H99* embryos completely lack developmental apoptosis (White et al., 1994). These observations suggest that the *H99* genes can induce at least a few apoptotic deaths independently of *dronc*. The nature of this *dronc*-independent pathway is not known. However, *dredd*, which encodes an initiator caspase most similar to human Caspase 8 (Chen et al., 1998), is a good candidate to mediate *dronc*-independent cell death. Although *dredd* mutants do not change the global cell death pattern visibly (Leulier et al., 2000), it is still possible that a few cells are dependent on $dredd^{+}$ function for apoptosis. In addition to Dronc and Dredd, a third potential initiator caspase is encoded by the *strica* gene. Strica bears an unusual N-terminal prodomain that does not contain any of the known interaction motifs (Doumanis et al., 2001). Overexpression of *strica* causes cell death, but mutants are not available for analysis of the role of *strica* in developmental cell death. Finally, it is possible that an unknown mechanism leads to *dronc*-independent cell death. Identification of the cells that die in a *dronc*-independent manner and development of sensitive cell death assays will be required to address this issue in the future. We are also using the *GheF* screening method to identify genes involved in the *dronc*-independent cell death pathway.

dronc is epistatic to *diap1* in the ovary

Based on binding studies in vitro and overexpression studies in vivo, a model has emerged that predicts Diap1 to be an important negative regulator of Dronc (Meier et al., 2000; Hawkins et al., 2000; Quinn et al., 2000; Muro et al., 2002). However, because of the lack of *dronc* mutants, the genetic relationship between *dronc* and *diap1* was unknown. We addressed the genetic relationship between *dronc* and *diap1* in the female ovary.

There are at least two different phenotypes associated with the loss of *diap1* function in the ovary. The first is an ovary degeneration phenotype generated by combination of two viable *diap1* alleles in trans to each other, $diap1^{6B}$ and $diap1^{8}$ (Rodriguez et al., 2002). Removing *dronc* in $diap1^{6B}/diap1^{8}$ mutant females strongly suppresses the ovarian degeneration phenotype (Fig. 7), demonstrating a strong genetic interaction between *dronc* and *diap1*. The second phenotype, described recently by Geisbrecht and Montell (Geisbrecht and Montell, 2004), involves border cell migration defects due to an apoptosis-independent role of *diap1*. The two phenotypes in the ovary are independent of each other, because the $diap1^{6B}$ allele that alters one key residue in the RING domain does not display border cell migration defects, suggesting that the RING domain is not required for the non-apoptotic function of Diap1 (Geisbrecht and Montell, 2004). RING domain mutants of *diap1* have been shown to display a strong apoptotic phenotype in the embryo (Lisi et al., 2000), implying that the degeneration phenotype of $diap1^{6B}/diap1^{8}$ mutant ovaries is likely to be the consequence of excessive apoptosis. Therefore, the rescue of the ovary degeneration phenotype in the *dronc diap1* double mutants appears to result from suppression of apoptosis, as it is clearly not related to border cell migration. Furthermore, the rescue strongly suggests that *dronc* acts genetically downstream of *diap1*.

However, we also wanted to analyze the genetic relationship between *dronc* and *diap1* in a better-characterized apoptotic setting, such as early in embryonic development. At this stage, strong *diap1* mutants show extensive TUNEL-positive nuclei and inappropriate caspase activation, resulting in developmental arrest and organismal death shortly after gastrulation (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). Ideally, we wished to analyze this embryonic cell death phenotype of *diap1* mutants in the absence of *dronc* function; that is, in a *dronc diap1* double mutant. Unfortunately, despite the ovarian rescue of $diap1^{6B}/diap1^{8}$ mutants by removal of *dronc* function, the *dronc diap1* double mutant females were still sterile and did not produce embryos that would have allowed us to analyze the embryonic cell death phenotype of *dronc diap1* mutants.

We therefore attempted to address this problem in GLCs. Both *dronc* and *diap1* map to the left arm of chromosome 3. Thus, we induced GLCs that were double mutant for *dronc* and *diap1*. We used two different *diap1* alleles, $diap1^{109.07}$ and $diap1^{5}$, which both behave genetically as null alleles (Lisi et al., 2000). Unfortunately, females with double mutant GLCs were sterile, and we did not recover embryos for phenotypic analysis. We are currently designing alternative methods to address this issue.

In summary, we have isolated and characterized four mutant alleles of *dronc*. At least two, but probably three, of them are complete loss-of-function alleles. These mutants lack most developmental cell death, suggesting that *dronc* is required for most cell death. However, a few cells die in a *dronc*-independent manner. Future studies will identify the nature of the *dronc*-independent pathway and clarify the genetic relationship between *dronc* and *diap1*.

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Note added in proof

While this paper was in preparation, two additional studies appeared that reported similar results about *dronc* mutants (Chew et al., 2004; Daish et al., 2004).

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