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The effector caspases *drICE* and *dcp-1* have partially overlapping functions in the apoptotic pathway in *Drosophila*

D Xu[1,2], Y Wang[1,2], R Willecke[1], Z Chen[1], T Ding[1,2] and A Bergmann[*,1,2]

[1] Department of Biochemistry and Molecular Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 1000, Houston, TX 77030, USA
[2] The Genes and Development Graduate Program (http://www.mdanderson.org/genedev)
[*] Corresponding author: A Bergmann, Department of Biochemistry and Molecular Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 1000, Houston, TX 77030, USA. Tel: +1-713-834-6294; Fax: +1-713-834-6291; E-mail: abergman@mdanderson.org

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Abstract

Caspases are essential components of the apoptotic machinery in both vertebrates and invertebrates. Here, we report the isolation of a mutant allele of the *Drosophila* effector caspase *drICE* as a strong suppressor of *hid*- (head involution defective-) induced apoptosis. This mutant was used to determine the apoptotic role of *drICE*. Our data are consistent with an important function of *drICE* for developmental and irradiation-induced cell death. Epistatic analysis suggests that *drICE* acts genetically downstream of *Drosophila* inhibitor of apoptosis protein 1 (Diap1). However, although cell death is significantly reduced in *drICE* mutants in all assays, it is not completely blocked. A double-mutant analysis between *drICE* and death caspase-1 (*dcp-1*), another effector caspase, reveals that some cells (type I) strictly require *drICE* for apoptosis, whereas other cells (type II) require either *drICE* or *dcp-1*. Thus, these data demonstrate a barely appreciated complexity in the apoptotic pathway, and are consistent with current models about effector caspase regulation in both vertebrates and invertebrates.
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Abbreviations: AO, acridine orange; APF, after pupariation formation; Ark, Apaf-1-related killer; CARD, caspase activation and recruitment domain; Dcp-1, death caspase-1; Diap1, *Drosophila* inhibitor of apoptosis protein 1; DrICE, *Drosophila* ICE; Dronc, *Drosophila* Nedd-2-like caspase; *ey*, *eyeless*; FLP, Flippase; FRT, Flippase recombination target; *GheF*, *GMR-hid ey-Flp*; GMR, glass multimer reporter; *hid*, head involution defective; IAP, inhibitor of apoptosis proteins; MG, midline glia; RHG, Reaper Hid Grim; RNAi, RNA interference; su, suppressor; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling

Introduction

Programmed cell death or apoptosis is an essential physiological process required for normal development of metazoan organisms and tissue homeostasis. The key mediators of cell death are caspases, a highly specialized class of Cysproteases. Caspases are produced as inactive zymogens. During apoptosis, activation of caspases involves proteolytic processing, cleaving off an N-terminal prodomain, and generating the large and the small catalytic subunits (reviewed in Ref.[1]).

Two classes of caspases have been defined based on the length of the prodomain. Initiator caspases contain long prodomains that harbor regulatory motifs such as the caspase activation and recruitment domain (CARD) in the prodomain of Caspase-9.[1] These regulatory motifs serve as binding sites for upstream apoptotic signaling factors. For example, 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 multisubunit complex, where it undergoes autoprocessing and activation.[1] Once activated, Caspase-9 cleaves and activates the effector Caspase-3, which is characterized by the presence of a short prodomain. Effector caspases execute the cell death process by cleaving a large number of cellular proteins, triggering the morphological events leading to apoptotic cell death.

Caspases are negatively regulated by inhibitor of apoptosis proteins (IAPs). IAPs bind to processed caspases and inhibit them (recently reviewed in Ref.[2]). Thus, IAPs provide the last line of defense against inappropriate caspase activity. In apoptotic cells, IAP antagonists such as the *Drosophila* Reaper, Hid (Head involution defective), and Grim (RHG) proteins (recently reviewed in ref.[3]) displace IAPs directly from caspases[4,5] which are then released from IAP inhibition, and induce apoptosis. In addition, Reaper, Hid and Grim also promote proteolytic degradation of *Drosophila* IAP1 (Diap1).[3]

The *Drosophila* genome contains a total of seven caspase genes, three of which encode putative initiator caspases (*Drosophila* Nedd-2 like Caspase (Dronc), Dredd and Strica), whereas the remaining four are putative effector caspases (*Drosophila* ICE (DrICE), death caspase-1 (Dcp-1), Decay and Damm) (reviewed in Ref.[1,6]). Mutations in these caspase genes that would allow their genetic characterization have been described for *dredd*, *dcp-1* and *dronc*. However, the available evidence suggests that *dredd* is not an apoptotic caspase, but instead appears to have a fundamental role in innate immunity (reviewed in Ref.[1]). Homozygous *dcp-1* mutants are viable and fertile.[7] The only cell death phenotype reported for *dcp-1* is lack of germline cell death during

mid-oogenesis in response to nutrient deprivation.[7] *dronc* is the only caspase gene described so far whose mutations display a clear apoptotic phenotype. Genetic inactivation of *dronc* blocks most developmental cell death during embryogenesis, imaginal disc development and metamorphosis.[8–11] Dronc is functionally similar to human Caspase-9 because it contains a CARD motif in the prodomain,[12] and interacts with *Drosophila* Apaf-1-related killer (Ark), also known as Dark, Hac-1 and D-Apaf-1 (reviewed in Ref.[3]).

It has not been genetically determined whether any of the effector caspases in *Drosophila* are apoptotic (with the exception of *dcp-1* during oogenesis).[7] However, several observations suggest that *drICE* is an important component of the apoptotic machinery in *Drosophila*. First, overexpression of *drICE* sensitizes *Drosophila* S2 cells to apoptosis.[13] Immunodepletion of DrICE in S2 cells reduces the ability of cycloheximide and *reaper* expression to induce apoptotic morphology.[14] Furthermore, silencing of DrICE by RNA interference (RNAi) blocks S2 cell apoptosis.[15] These findings suggest that DrICE is required for apoptosis in S2 cells. Second, *drICE* expression is induced by the insect hormone ecdysone, which stimulates apoptosis during metamorphosis.[16] Active DrICE was also found during cell death in both mid- and late oogenesis.[17] Third, the initiator caspase Dronc can cleave and activate DrICE *in vitro*.[18,19] DrICE also cleaves Dronc,[15] and it has been proposed that this cleavage constitutes a caspase amplification loop.[16] Fourth, Diap1, which is essential for cellular survival,[2,3] can inhibit DrICE through direct physical interactions.[4,5,20,21] These observations imply, but do not prove, that *drICE* encodes an important component of the apoptotic machinery in *Drosophila*. Thus, to clarify the role of *drICE* for developmental apoptosis, analysis of mutations in the endogenous gene are necessary.

In this study, we describe the isolation and genetic characterization of an EMS-induced allele of the effector caspase *drICE*. This mutant is characterized by reduced levels of developmental and irradiation-induced cell death, and contains additional cells. We show that the strong apoptotic phenotype of *diap1* mutants is partially suppressed by *drICE* inactivation, suggesting that inappropriate activation of DrICE contributes to the *diap1* mutant phenotype, and that *drICE* acts genetically downstream of *diap1*. However, even though the *drICE* mutant reduces developmental apoptosis, it does not completely block it. We show that *drICE* and *dcp-1* share a partially overlapping function in such a way that some cells (type I) strictly require *drICE* for apoptosis, whereas other cells (type II) are more flexible and die either through *drICE* or *dcp-1*.

Results

Isolation and identification of a *drICE* mutant

Recently, we described GheF (*GMR-hid ey-FLP*) screening method which allows to identify mutants in genes required for *hid*-induced cell death.[10] The GheF method takes advantage of the eye-ablation phenotype caused by expression of *hid* under eye-specific GMR-enhancer control (*GMR-hid*; Figure 1b). During GheF screening, suppressors of *GMR-hid* (*su(GMR-hid)*) are identified in homozygous mutant eye clones obtained by *ey-FLP/FRT*-mediated recombination in otherwise heterozygous animals (for further technical details see Xu *et al*., 2005).[10]

The *drICE* locus maps to cytological position 99C1 of the polytene map on the right arm of chromosome 3 (3R). Thus, to isolate mutants in *drICE*, we carried out an EMS mutagenesis screen using the GheF method for 3R (see Materials and

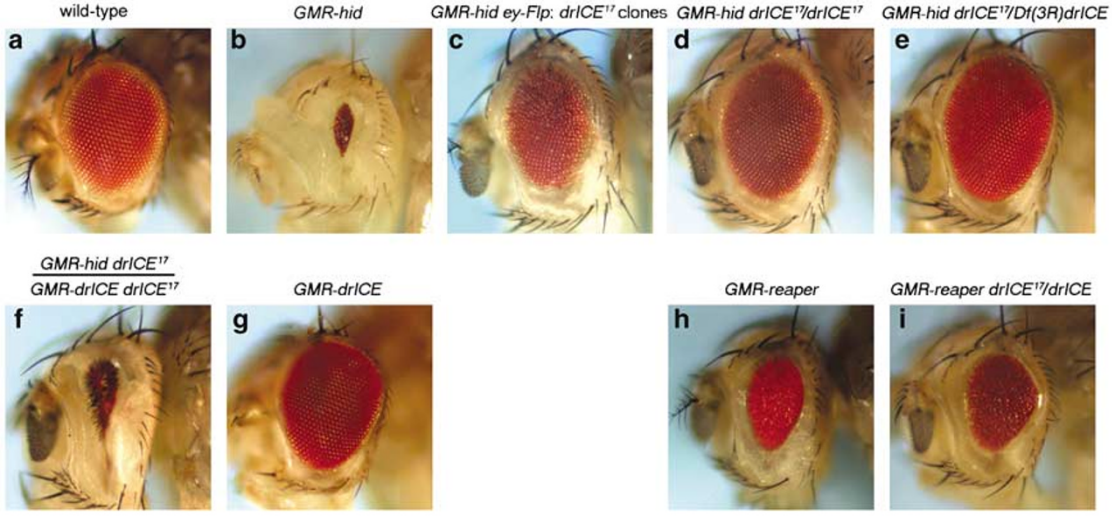

Figure 1 Isolation of *drICE*^{17} as strong suppressor of *GMR-hid*. (**a**) Wild-type eye. (**b**) *GMR-hid*-induced eye-ablation phenotype. (**c**) Suppression of *GMR-hid* in *ey-Flp*-induced *drICE*^{17} clones. Exact genotype: *GheF*; FRT82B *drICE*^{17}/FRT82B P[w^+]. (**d**) Suppression of *GMR-hid* in homozygous *drICE*^{17} background. Exact genotype: *GMR-hid drICE*^{17}/*drICE*^{17}. (**e**) Suppression of *GMR-hid* in *trans*-heterozygous *drICE*^{17} over *Df(3R)drICE* mutant background. Exact genotype: *GMR-hid drICE*^{17}/*Df(3R)drICE*. (**f**) Transgenic rescue of the *GMR-hid*-induced eye-ablation phenotype in *drICE*^{17} mutant background by a *GMR-drICE* transgene. Exact genotype: *GMR-hid drICE*^{17}/*GMR-drICE drICE*^{17}. (**g**) *GMR-drICE* does not cause an eye-ablation phenotype on its own. (**h**) Eye-ablation phenotype caused by *GMR-reaper*. Exact genotype: *CyO*, 2x *GMR-reaper*/+. (**i**) Weak suppression of *GMR-reaper* by *drICE*^{17}. Exact genotype: *CyO*, *2x GMR-reaper*, *drICE*^{17}/*drICE*^{17}

Methods). The strongest suppressor of *GMR-hid* obtained in the screen, *su(GMR-hid)17*, was chosen for further characterization. In *ey-FLP*/*FRT* clones, *su(GMR-hid)17* suppresses *GMR-hid* strongly (Figure 1c). *su(GMR-hid)17* is homozygous viable (see also below), and homozygously suppresses *GMR-hid* even stronger (Figure 1d), restoring the eye back to wild-type size (Figure 1a).

To identify the gene mutant in *su*(*GMR-hid*)*17*, we mapped the mutation to the distal tip of 3R by P-element mapping. In parallel to the above described EMS screen, a small deficiency deleting cytological range 99B3/B8–99C2/C4 on the polytene map was isolated by X-ray irradiation (see Materials and Methods). This deficiency, referred to as *Df*(*3R*)*drICE*, over *su*(*GMR-hid*)*17* strongly suppresses *GMR-hid* (Figure 1e). Thus, this analysis suggests that *su*(*GMR-hid*)*17* maps to the cytological range 99B3/B8–99C2/C4. None of the genes residing in this cytological range have been implicated in apoptosis, with the exception of *drICE* which maps to 99C1. Thus, we tested whether a *GMR-drICE* transgene[22] can restore the small eye phenotype of *GMR-hid* in homozygous *su*(*GMR-hid*)*17* animals. This was found to be the case (Figure 1f). *GMR-drICE* does not display a small eye phenotype on its own[22] (Figure 1g). These findings establish that the suppression of *GMR-hid* by *su*(*GMR-hid*)*17* is caused by genetic inactivation of *drICE*. This is also confirmed by DNA sequencing analysis and immunoblotting (see next section). Therefore, we refer to *su*(*GMR-hid*)*17* from now on as $drICE^{17}$. The rescue of *GMR-hid* by $drICE^{17}$/*Df*(*3R*)*drICE* is slightly better than the one of homozygous $drICE^{17}$ animals (Figure 1d and e) suggesting that $drICE^{17}$ is a very strong hypomorphic allele, but not a null allele.

We also determined whether $drICE^{17}$ could suppress the *GMR-reaper*-induced small eye phenotype. The *GMR-reaper* eye-ablation phenotype is weaker compared to *GMR-hid* (Figure 1h). Surprisingly, although homozygous $drICE^{17}$ animals do suppress *GMR-reaper* (Figure 1i), the suppression is significantly weaker compared to the suppression of *GMR-hid* (Figure 1d). The weak suppression of *GMR-reaper* could reflect an unanticipated complexity of the apoptotic process, or could be allele specific for $drICE^{17}$. Additional *drICE* mutants are necessary to distinguish between these possibilities.

In summary, this analysis identifies a mutation in the *drICE* gene and provides evidence that $drICE^{+}$ is genetically required for *GMR-hid*-induced cell death. Homozygous $drICE^{17}$ adults are viable, and carry wings that appear less transparent compared to wild type (data not shown). This wing phenotype which is difficult to illustrate in photographs, appears to be characteristic for mutants of cell death genes as it has been previously observed in *hid*, *ark* and *dronc* mutants.[10] Although $drICE^{17}$ mutant animals are homozygous viable, they were not obtained at mendelian ratios. Only about 1/3 of the expected progeny ($\sim$500 offspring scored) was found relative to controls ($drICE^{17}$/TM3). A similar semilethality was observed for $drICE^{17}$/*Df*(*drICE*) animals. The lethal phase of those homozygous individuals which die occurs during embryogenesis without detectable phenotype. In addition, it is difficult to keep $drICE^{17}$ flies in a homozygous condition. They appear to be semisterile, especially the males, but we have not characterized this phenotype in detail.

$drICE^{17}$ encodes for an unstable protein

DNA sequencing reveals one single base pair change in the $drICE^{17}$ open reading frame changing Asn116 to Tyr. Asn116 lies in a well conserved domain of the large subunit of DrICE (Figure 2a). This residue is conserved in all invertebrate caspases and in some mammalian caspases including Caspase-6 from mouse and even human Caspase-9, an initiator caspase (Figure 2a). In mouse and human Caspase-3 and Caspase-7, this position is occupied by a semiconserved Asp residue (Figure 2a). Immunoblot analysis showed that $drICE^{17}$ encodes for an unstable protein (Figure 2b). We quantified that $<5\%$ of the wild-type levels of DrICE protein are detectable in immunoblots of $drICE^{17}$ mutant embryos.

$drICE^{17}$ mutants exhibit reduced developmental cell death

The $drICE^{17}$ mutant was isolated as a strong suppressor of *GMR-hid* (Figure 1). Furthermore, immunoblot analysis showed that $drICE^{17}$ encodes for an unstable protein. These observations suggest that $drICE^{17}$ represents a loss-of-function allele. To determine the genetic requirement of *drICE* for normal developmental cell death, we analyzed $drICE^{17}$ mutant embryos by acridine orange (AO), terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) and anticleaved Caspase-3 (referred to as Caspase-3*) labelings. AO stains specifically dying cells. Compared to wild-type embryos, AO-positive cell death is significantly reduced in $drICE^{17}$ mutants (Figure 3a and b). Similar observations were made using TUNEL, an alternative method to label dying cells (Figure 3c and d). Thus, consistent with its postulated role as effector caspase, *drICE* is required for developmental cell death. However, even though cell death is reduced in $drICE^{17}$ mutants, it is not completely blocked (Figure 3b and d). Because $drICE^{17}$ is not a defined

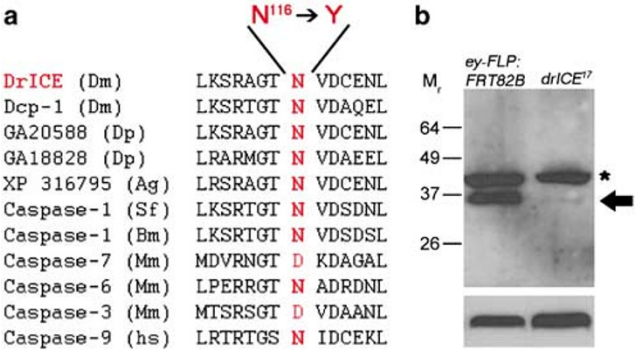

Figure 2 $drICE^{17}$ encodes for an unstable protein. (**a**) Alignment of the amino-acid residues surrounding Asn116 in DrICE and various effector caspases in insects and mammals, and human Caspase-9. $drICE^{17}$ carries a mutation in Asn116, changing it to Tyr. Dm, *Drosophila melanogaster*; Dp, *Drosophila pseudoobscura*; Ag, *Anopheles gambiae*; Sf, *Spodoptera frugiperda*; Bm, *Bombyx mori*; Mm, *Mus musculus*; Hs, *Homo sapiens*. (**b**) Embryonic extract obtained from homozygous *ey-Flp*; *FRT82B* (the stock used for mutagenesis) and $drICE^{17}$ mutant flies were analyzed by immunoblotting using an antibody raised against the prodomain of DrICE (upper panel). Lower panel is the same blot probed with antiactin antibody as loading control. The arrow indicates full-length DrICE, the asterisk an unspecific protein

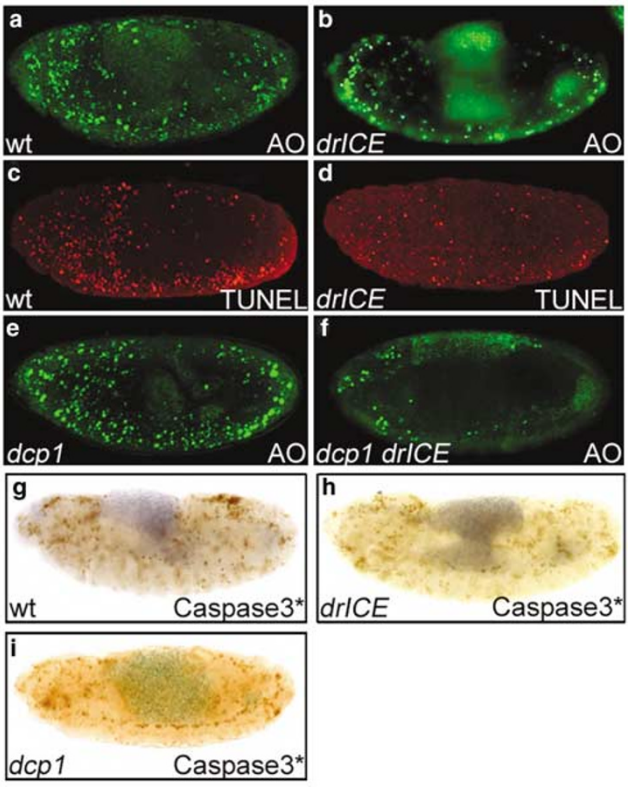

Figure 3 Acridine orange (AO), TUNEL and anticleaved caspase-3 labelings. (**a**) Wild-type embryo (stage 13) labeled with AO. (**b**) Homozygous *drICE*^{17} mutant embryo (stage 13) labeled with AO. This embryo was obtained from a cross of homozygous *drICE*^{17} males and females to remove the maternal contribution. (**c**) Wild-type embryo (stage 13) labeled with TUNEL. (**d**) Homozygous *drICE*^{17} mutant embryo (stage 13) labeled for TUNEL. This embryo was obtained from a cross of homozygous *drICE*^{17} males and females to remove the maternal contribution. (**e**) Homozygous *dcp-1*^{Prev1} mutant embryo (stage 13) labeled for AO. This embryo was obtained from a cross of homozygous males and females to remove the maternal contribution. (**f**) *dcp-1*^{Prev1}*drICE*^{17} double-mutant embryo (stage 13) labeled for AO. This embryo was obtained by induction of germline clones as described in Materials and Methods. (**g**) Wild-type embryo (stage 13) labeled with anticleaved caspase-3 antibody. (**h**) Homozygous *drICE*^{17} embryo (stage 13) labeled with anticleaved caspase-3 (Caspase-3*) antibody. The labeling pattern is not appreciately altered compared to wild-type (**g**). (**i**) Homozygous *dcp-1*^{Prev1} mutant embryo (stage 13) labeled with Caspase-3* antibody. This embryo was obtained from a cross of homozygous males and females to remove the maternal contribution

null allele, the incomplete block of developmental apoptosis could be due to partial activity of *drICE*^{17}; or, *drICE* is functionally redundant with another effector caspase. One candidate for an alternative effector caspase is *dcp-1* (see Song *et al.*[23]) which is most similar to *drICE* at the sequence level (57% identical).[22] Deletion of *dcp-1*, using *dcp-1*^{Prev1} a protein null allele,[7] does not or only weakly affect the global embryonic cell death pattern (Figure 3e), consistent with previous reports.[7] However, *dcp-1*^{Prev1} *drICE*^{17} double-mutant embryos (see Materials and Methods) display significantly reduced levels of embryonic apoptosis compared to *drICE*^{17} single mutants, and contain only a few dying cells (Figure 3f and b). This residual apoptosis might be the result of weak activity of *drICE*^{17}, or may imply that a third effector caspase (Damm or Decay) is required for embryonic cell death. In any case, this analysis establishes that *drICE* and *dcp-1* have overlapping functions in the apoptotic pathway in *Drosophila* embryos (see Discussion).

The anticleaved Caspase-3 antibody (Caspase-3*; Cell Signaling Technology) was raised against a peptide N-terminal to Asp175 in the large subunit of human Caspase-3. It is thought that this antibody recognizes its epitope only after cleavage and activation of Caspase-3. In uncleaved Caspase-3 the epitope is buried inside the protein inaccessible to the antibody. Interestingly, immunolabeling of *Drosophila* embryos using this antibody mimics the known AO and TUNEL pattern (Figure 3g). Furthermore, labeling with this antibody is dependent on the RHG genes *reaper*, *hid* and *grim*, as homozygous embryos deficient for the RHG genes (*Df*(*3L*)*H99*) fail to give a staining signal (data not shown). *diap1* mutant embryos which are characterized by a strong apoptotic phenotype[3,24] (Figure 7a) show increased labeling (data not shown). These observations suggest that the Caspase-3* antibody crossreacts with a *Drosophila* protein that becomes activated during apoptotic cell death. Such an apoptotic protein might be the initiator caspase Dronc or an effector caspase acting downstream of Dronc, because the Caspase-3* antibody shows reduced immunoreactivity in *dronc* mutants.[10] Another antibody, termed CM1, was raised against the same peptide and was shown to crossreact with cleaved DrICE in immunoblots.[25] It is not clear whether Caspase-3* antibody also crossreacts with cleaved DrICE. To address this question, we analyzed *drICE*^{17} mutant embryos with Caspase-3* antibody. However, compared to wild type, the level of immunoreactivity is not significantly altered in *drICE*^{17} embryos (Figure 3h), and surprisingly does not match the AO- and TUNEL patterns of *drICE*^{17} mutants (Figure 3b and d). As we showed in Figure 2b, that *drICE*^{17} produces an unstable protein with $<5\%$ of the total amount of DrICE protein left, it is unlikely that the Caspase-3* antibody specifically recognizes cleaved DrICE. We also tested the Caspase-3* antibody on homozygous *dcp-1*^{Prev1} embryos which lack Dcp-1 protein.[7] However, the immunolabeling is indistinguishable from that of wild-type embryos (Figure 3i). Thus, this antibody does not appear to bind to either DrICE or Dcp-1, or it may recognize multiple epitopes (see Discussion).

The data presented in Figure 3 provide strong evidence that *drICE*$^{+}$ is genetically required for developmental cell death. We therefore determined the consequences of reduced rates of cell death at the cellular level in *drICE*^{17} mutants. The best-characterized apoptotic model during *Drosophila* embryogenesis is the development of the midline glia (MG) in the central nervous system.[26] The MG are transient cells during embryogenesis and are required for the separation and ensheathment of commissural axon tracts.[26] 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 (Figure 4a and c).[26] The reduction in the number of MG cells is dependent on *reaper*, *hid*, *grim*, *ark* and *dronc*. In homozygous *H99* (deleting *reaper*, *hid* and *grim*),[27] *ark*[28] and *dronc*[8,10] mutant embryos, the MG cells fail to die by apoptosis. We determined the fate of MG cells in *drICE*^{17} mutants. At stage 17, *drICE*^{17} mutant embryos

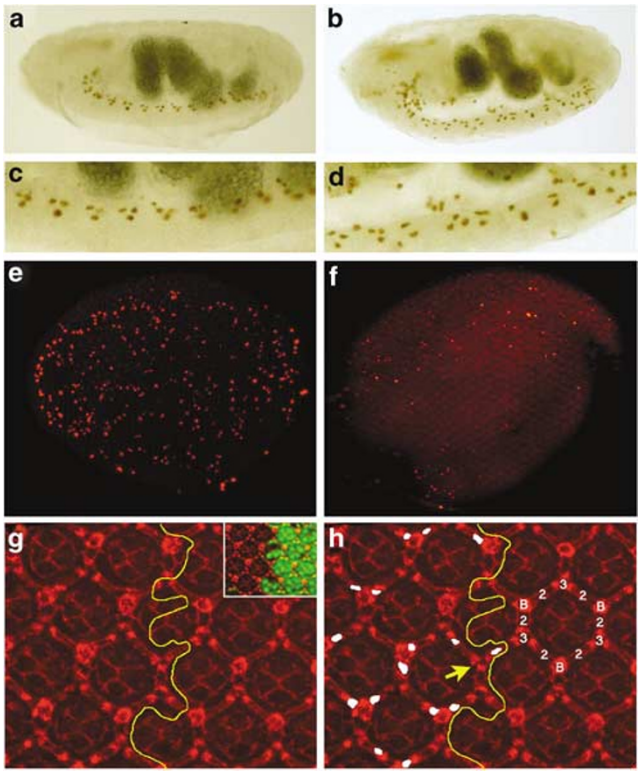

Figure 4 *drICE*^{17} mutants contain additional cells. (**a**) The midline glia (MG) of a stage 17 wild-type embryos visualized by a *P[sli-1.0]lacZ* reporter transgene. (**b**) The MG of stage 17 *drICE*^{17} embryos contains additional cells. (**c**) Enlargement of the ventral nerve cord of the wild-type embryo in (**a**). (**d**) Enlargement of the ventral nerve cord of the *drICE*^{17} mutant in (**b**). (**e**) Whole mount of a wild-type pupal eye disc 26 h APF labeled for TUNEL. (**f**) Whole mount of a *drICE*^{17} mutant eye disc 26 h APF labeled for TUNEL. The global cell death pattern is reduced compared to (**e**). (**g** and **h**) IOC survival in *drICE*^{17} mutant clones. (**g**) Overview of a Dlg-labeled (red) *drICE*^{17} mosaic eye disc 42 h APF to visualize the weak disorganization of the mutant lattice. The yellow line marks the clonal boundary. The inset shows the *drICE*^{17} clone marked by absence of GFP (green). (**h**) Shows the same field as (**g**). The interommatidial cell (IOC) cluster is composed of six secondary (2), three tertiary (3) and three bristle cells (B). The yellow line marks the clonal boundary. Extra IOCs in the *drICE*^{17} clone are marked in white. The yellow arrow points to a rare patterning defect in which a bristle cell is replaced by a tertiary pigment cell

contain on average more than twice the number of MG cells ($\sim$80–100; $n=5$) compared to wild-type ($\sim$40; $n=5$) (Figure 4b and d). This number is similar to *hid* mutant embryos,[27] but is significantly less compared to *H99* and *dronc* mutant embryos which contain on average 140–160 MG cells.[10,27] Thus, *drICE* is required for some, but not all, MG cell death.

Another very well-characterized cellular model system to study developmental cell death in *Drosophila* is the fate of interommatidial cells (IOCs) in the developing compound eye which is composed of approximately 750 individual unit eyes, called ommatidia.[29] Individual ommatidia are positioned within a hexagonal lattice of shared pigment cells and mechanosensory bristles.[29] Formation of the ommatidial core, composed of eight photoreceptors, four cone cells and two primary pigment cells, is completed 24 h after pupariation formation (APF), leaving an excess of undifferentiated cells in the interommatidial space.[29] The final step of ommatidial development is the differentiation of the IOCs into secondary (2°) and tertiary (3°) pigment cells (Figure 4h), and the elimination of excess IOCs by *hid*-dependent cell death in order to refine the hexagonal pigment cell lattice.[25,29,30] By 42 h APF, IOC differentiation and cell death is completed bringing individual ommatidia into register within the lattice.

IOC apoptosis throughout the eye disc is highest between 26 and 28 h APF[29] (Figure 4e). Thus, we tested whether *drICE*^{17} mutants change the global pattern of cell death by TUNEL labeling. Similar to *drICE*^{17} mutant embryos, the global pattern of TUNEL-positive cell death is significantly reduced, but not completely blocked (Figure 4f). Although we have been unable to test whether *dcp-1* accounts for the remaining death in *drICE*^{17} discs, it is likely that this is the case based on our findings in embryos.

We also determined the number of IOCs in *drICE*^{17} mutant clones in discs 42 h APF. Using an antibody against the Discs-large (Dlg) protein to visualize membranes and thus cell outline, we found that ommatidia in *drICE*^{17} mutant clones 42 h APF contained on average 1.6 ± 0.75 S.E.M. additional IOCs (Figure 4g and h; marked in white; see Materials and Methods) which is approximately half the number of additional IOCs reported for *dronc* mutant ommatidia.[10] Thus, this analysis provides further support for a redundant function of effector caspases. Nevertheless, these data suggest that *drICE*$^{+}$ is genetically required for some IOC apoptosis in the developing retina. Interestingly, occasionally we also observe a patterning defect in which a bristle cell is replaced by a 3° pigment cell (Figure 4h, yellow arrow).

drICE^{17} partially protects against irradiation-induced cell death

Ionizing radiation induces apoptosis in both mammalian cells and in *Drosophila* embryos. Radiation-induced apoptosis requires the RHG genes, *ark*, and *dronc*. Thus, we determined whether *drICE* is required for irradiation-induced cell death. Wild-type and *drICE*^{17} mutant embryos were irradiated with 4000 rad. In wild-type embryos this treatment induces a strong apoptotic response (Figure 5a). However, compared to irradiated wild-type embryos, fewer apoptotic cells were consistently observed in *drICE*^{17} mutants (Figure 5b). Therefore, *drICE*^{17} partially protects against radiation-induced cell death.

drICE^{17} fails to suppress *GMR-dronc*- and *GMR-dcp-1*-induced eye phenotypes

Based on its domain structure, DrICE has been classified as an effector caspase.[6,13] Consistent with this notion is the observation that the initiator caspase Dronc cleaves and activates DrICE *in vitro*.[18,19] However, DrICE can also cleave Dronc *in vitro*,[15] and it was proposed that DrICE is required for amplification of Dronc processing in a caspase activation loop following the initial activation of Dronc.[15,16] We attempted to determine the genetic relationship between *dronc* and *drICE* by epistasis analysis. Expression of the full-length form of Dronc in third instar larval eye discs using the GMR enhancer (*GMR-pro-dronc*) causes induction of apoptosis[18,19,31] (Figure 6a). To determine the genetic relationship between

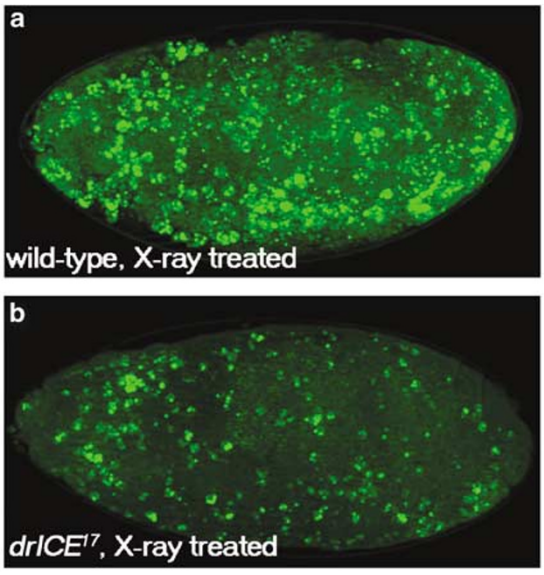

Figure 5 *drICE*^{17} partially protects against irradiation-induced cell death. Wild-type (**a**) and *drICE*^{17} (**b**) were exposed to X-ray irradiation (4000 rad), aged for 1.5 h and labeled with acridine orange. These embryos were X-ray treated at stage 9

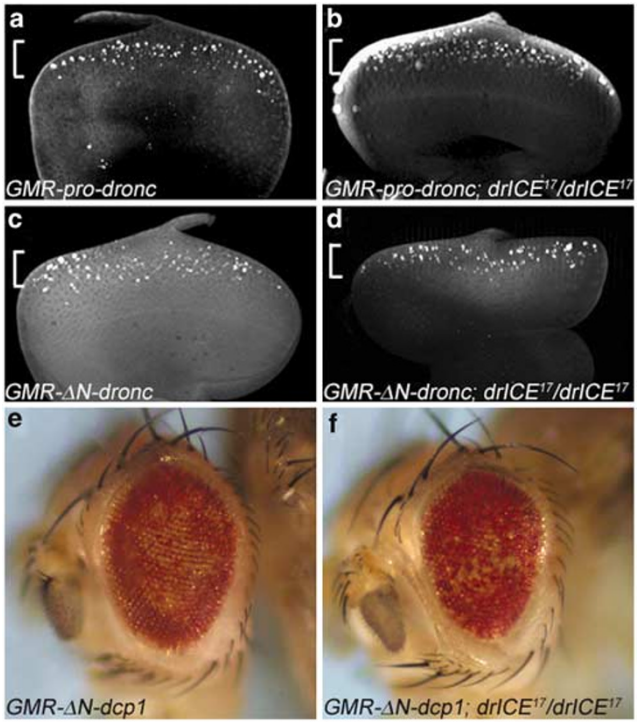

Figure 6 *drICE*^{17} fails to suppress *GMR-dronc*- and *GMR-dcp-1*-induced eye phenotypes. (**a**) Third instar *GMR-pro-Dronc* larval eye disc labeled by TUNEL. The brackets in **a**–**d** indicate the expression domain of the GMR-dronc transgenes, overlapping with TUNEL-positive apoptosis. (**b**) Third instar *GMR-pro-dronc* larval eye disc mutant for *drICE*^{17} labeled by TUNEL. There is no significant difference compared to (**a**). (**c**) Third instar *GMR-ΔN-dronc* larval eye disc labeled by TUNEL. (**d**) Third instar *GMR-ΔN-dronc* larval eye disc mutant for *drICE*^{17} labeled by TUNEL. There is no significant difference compared to (**c**). (**e**) The 'spotted' eye phenotype caused by *GMR-ΔN-dcp-1*. (**f**) Homozygous *drICE*^{17} flies do not suppress *GMR-ΔN-dcp-1*

dronc and *drICE*, we analyzed the apoptotic phenotype of *GMR-pro-dronc* in a *drICE*^{17} mutant background. Surprisingly, in homozygous *drICE*^{17} animals the apoptotic phenotype of *GMR-pro-dronc* in larval eye discs is not suppressed (Figure 6b). Similarly, the strong apoptotic phenotype caused by GMR-induced expression of a dominant active allele of Dronc which deletes the prodomain (*GMR-ΔN-dronc*)[18] is not significantly suppressed by *drICE*^{17} (Figure 6c and d). Consistently, the adult eye phenotype of *GMR-pro-dronc* and *GMR-ΔN-dronc* are not significantly rescued by *drICE*^{17} (data not shown). These observations suggest that overexpressed Dronc can induce apoptosis independently of *drICE* either through activation of other effector caspases, or it behaves as an effector caspase itself (see Discussion).

Dcp-1 is a candidate effector caspase activated by Dronc in parallel to DrICE.[7,22,23] *GMR-ΔN-dcp-1* which lacks the N-terminal prodomain gives rise to a 'spotted' eye phenotype[22] (Figure 6e). As expected, *drICE*^{17} mutants are unable to suppress this eye phenotype (Figure 6f), implying that *dcp-1* either acts downstream of *drICE*, or in parallel. Thus, this observation suggests that *dcp-1* can induce cell death independently of *drICE*.

drICE^{17} partially suppresses the apoptotic phenotype of *diap1* mutants

IAPs, most notably Diap1, are important regulators of apoptosis.[1,2] The *diap1*^{5} allele is a strong loss-of-function allele characterized by a dramatic apoptotic phenotype. Essentially every cell is TUNEL positive in these embryos[24] (Figure 7a) suggesting an essential function of Diap1 for cellular survival. There is overwhelming biochemical evidence that IAPs regulate apoptosis through inhibition of caspases.[1,2,5,18,21] Diap1 has been shown to be able to directly bind to and inhibit DrICE *in vitro*.[4,5,20,21] However, the functional significance of this interaction has never been genetically demonstrated. Thus, we determined whether Diap1 inhibits DrICE *in vivo* by double-mutant analysis. *diap1*^{5} *drICE*^{17} double-mutant embryos contain fewer apoptotic cells compared to *diap1*^{5} single-mutant embryos (Figure 7b). This finding suggests that Diap1 indeed regulates the apoptotic activity of DrICE. Furthermore, this analysis places *drICE* genetically downstream of *diap1*, consistent with the expectation. However, we note that the *diap1* mutant phenotype is only partially suppressed by *drICE*^{17}. The partial suppression can be explained either by the hypomorphic nature of *drICE*^{17} or by the activity of other effector caspases such as Dcp-1 which is also target of Diap1 inhibition.

Discussion

The importance of caspases for programmed cell death was first revealed in genetic studies in *Caenorhabditis elegans*,[32] and later confirmed by targeted gene disruptions in mice.[33–35] In *Drosophila*, the first report implicating caspases as important mediators of programmed cell death took

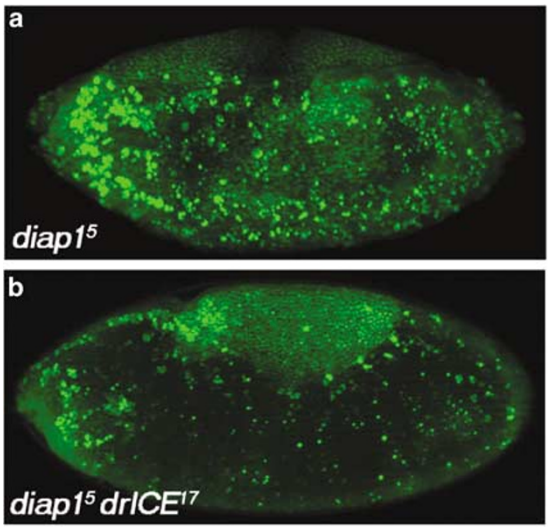

Figure 7 *drICE*^{17} suppresses the *diap1* mutant apoptotic phenotype. (**a**) *diap1*^{5} mutant embryos labeled with AO display a strong apoptotic phenotype. (**b**) *diap1*^{5} *drICE*^{17} double-mutant embryos strongly suppress the *diap1* phenotype

advantage of the universal caspase inhibitor P35. In P35-overexpressing animals, cell death is significantly reduced. More recently, dominant-negative constructs of cloned caspases and RNAi experiments further supported the involvement of caspases in the cell death response in *Drosophila*.[18,31] Finally, the availability of mutations in the initiator caspase *dronc* confirmed an essential role of caspases for developmental cell death.[8–11] Here, we report the isolation and characterization of a mutant in the effector caspase *drICE*. The phenotypic characterization of this mutant is consistent with a role of *drICE* for developmental and irradiation-induced cell death.

The *su*(*GMR-hid*)*17* mutant was isolated as a strong recessive suppressor of *GMR-hid* by GheF screening. We demonstrated in four ways that *su*(*GMR-hid*)*17* corresponds to a mutation in *drICE*. First, *su*(*GMR-hid*)*17* in trans to the deficiency *Df*(*3R*)*drICE*, which deletes 99B3/B8–99C2/C4 including *drICE*, strongly suppressed *GMR-hid* (Figure 1d) suggesting that *su*(*GMR-hid*)*17* maps to this cytological range. Second, expression of a *GMR-drICE* transgene restored the eye-ablation phenotype of *GMR-hid* in homozygous *su*(*GMR-hid*)*17* mutants (Figure 1f). Third, by DNA sequencing we identified a missense mutation in the *drICE* gene of *su*(*GMR-hid*)*17* changing the conserved Asn116 to Tyr. Finally, immunoblot analysis using an anti-DrICE antibody suggests that *su*(*GMR-hid*)*17* encodes for an unstable DrICE protein. Thus, these observations suggest that *su*(*GMR-hid*)*17* carries a mutation in *drICE*, and we referred to this mutant as *drICE*^{17}.

drICE^{17} carries a missense mutation in a conserved residue, changing Asn116 to Tyr, and thus, is not a defined null mutant. However, we have reasons to believe that *drICE*^{17} is a very strong hypomorphic allele. It is a strong suppressor of *GMR-hid* and produces $<5\%$ of the wild-type levels of DrICE protein. However, *drICE*^{17}/*Df*(*3R*)*drICE* mutants suppress the *GMR-hid* eye-ablation phenotype slightly better than homozygous *drICE*^{17} animals (Figure 1d and e) suggesting that *drICE*^{17} is a very strong, but not a null allele.

Partial redundancy between *drICE* and *dcp-1*

The phenotypic analysis of *drICE*^{17} in embryos and imaginal eye discs establishes that *drICE*$^{+}$ functions in developmental cell death. It is also partially required for irradiation-induced cell death and for establishment of the *diap1* mutant phenotype. However, the cell death phenotypes observed for *drICE*^{17} are weaker compared to the phenotypes reported for mutations of the initiator caspase *dronc* (see Daish *et al*.,[8] Chew *et al*.,[9] Xu *et al*.[10] and Waldhuber *et al*.[11]) suggesting that at least one additional effector caspase is required for apoptosis in *Drosophila*. One potential effector caspase which can compensate for the loss of *drICE* may be *dcp-1*.[23] Consistently, we showed by double-mutant analysis with *dcp-1*, that *drICE* and *dcp-1* have overlapping functions in the apoptotic pathway. The double-mutant phenotype is similar, although slightly weaker compared to the *dronc* null phenotype.[10] This slightly weaker phenotype could be caused by the hypomorphic nature of *drICE*^{17}, or alternatively by a third effector caspase such as Damm or Decay which may also be activated by Dronc.[6] In any case, this study demonstrates that effector caspases in *Drosophila* have overlapping functions.

However, it is interesting to note that *dcp-1* mutants display an apoptotic phenotype only in a double mutant with *drICE* (Figure 3f). In contrast, the *drICE* mutant has an apoptotic phenotype on its own. These observations suggest that Dcp-1 is not sufficient to induce apoptosis in those cells which survive in *drICE*^{17} mutants, but would otherwise die in wild-type embryos, implying that some cells strictly require DrICE for apoptosis independently of Dcp-1 (we refer to these cells as type I cells), and these cells survive in *drICE* mutants, whereas other cells (type II) require either DrICE or Dcp-1, and these cells still die in either *drICE* or *dcp-1* mutants, but survive in the double mutant. This model also explains why *drICE*^{17} mutants fail to suppress the *GMR-ΔN-dcp-1* eye phenotype. One example of a class I cell type are S2 cells. Immunodepletion of DrICE and gene silencing by RNAi results in block of apoptosis[14,15] suggesting that DrICE is the only effector caspase required for S2 apoptosis. How this partial redundancy is regulated and why type I cells can tolerate Dcp-1 is unclear. However, it reveals an unanticipated complexity in the apoptotic pathway in *Drosophila*. Similarly, analysis of genetic knockouts in mouse has revealed that Caspase-3 is essential in some cell types for apoptosis, but not in others, and that additional effector caspases can compensate for the loss of Caspase-3.[36]

Interestingly, labeling of *drICE*^{17} mutant embryos with cleaved caspase-3 (Caspase-3*) antibody produced a wild-type pattern. This is puzzling because the Caspase-3* pattern does not match the AO- and TUNEL patterns of *drICE*^{17} mutants (Figure 3). This observation suggests that Caspase-3* recognizes an epitope which is produced upstream of *drICE* such as the initiator caspase Dronc. We have previously shown that the immunoreactivity of the Caspase-3* antibody is strongly reduced in *dronc* mutants.[10] This observation implies, but does not prove, that the Caspase-3* antibody recognizes cleaved Dronc. However, it is also

possible that this antibody recognizes multiple apoptotic proteins including Dronc, effector caspases and even caspase substrates.

The genetic relationship between *diap1, dronc* and *drICE*

Mutations in *diap1* cause a dramatic apoptotic phenotype in early embryos.[3,24] We showed that *drICE*^{17} partially suppresses the apoptotic phenotype of *diap1* mutants suggesting that DrICE is regulated by Diap1 and acts genetically downstream of Diap1. However, it is unclear from this genetic analysis whether Diap1 directly inhibits DrICE, or whether the *diap1* mutant phenotype is caused by loss of inhibition of Dronc, which then activates DrICE. The available biochemical evidence suggests a combination of both. Binding studies *in vitro* have shown that Diap1 can be a negative regulator of DrICE.[4,5,20,21] However, Diap1 cannot inhibit DrICE until DrICE becomes activated and proteolytically removes the 20 N-terminal residues of Diap1,[5,37,38] which constitute an autoinhibitory domain for the function of Diap1.[5,37] These findings suggest that Dronc has to cleave and activate DrICE first before Diap1 can inhibit it. Dronc is also target of negative regulation by Diap1,[18] and we have previously shown that *dronc* suppresses the *diap1* mutant phenotype in the ovary, placing *dronc* downstream of *diap1*.[10] Thus, consistent with a previously proposed model,[37] Diap1 appears to inhibit primarily Dronc, whereas the inhibition of activated DrICE constitutes a minor activity of Diap1, which might be necessary to protect the cell against weak apoptotic signals or against inappropriately activated DrICE. Nevertheless, consistent with the role of DrICE as effector caspase, this analysis establishes that *drICE* acts genetically downstream of *diap1*.

The genetic relationship between *dronc* and *drICE* is less clear. *drICE*^{17} is unable to suppress apoptosis induced by *GMR-pro-dronc* and *GMR-ΔN-dronc*. This observation can be explained in several ways. First, *dronc* and *drICE* might act in independent pathways. However, this possibility is unlikely as both *dronc* and *drICE* mutants suppress *GMR-hid*, suggesting that they indeed do act in the same pathway. Second, Dronc might activate several effector caspases. Our data suggest that at least in embryos Dcp-1 is an alternative effector caspase which under the unphysiologically high concentration of *pro-Dronc* and *ΔN-Dronc* may be sufficiently active to compensate for the loss of *drICE*. Consistent with this scenario is our observation that *GMR-ΔN-dcp-1* is unaffected by *drICE*^{17} suggesting that *dcp-1* acts in parallel or downstream of *drICE*.

However, there is another possibility. The *GMR-pro-dronc* and *GMR-ΔN-dronc* eye phenotypes are insensitive to expression of the caspase inhibitor P35.[18,19] In contrast, Dcp-1 and DrICE can be inhibited by P35.[22] Thus, these observations suggest that the *GMR-dronc*-induced eye phenotypes are independent of Dcp-1 and DrICE. It is unclear how *GMR-pro-dronc* and *GMR-ΔN-dronc* induce apoptosis independently of Dcp-1 and DrICE. It is possible that the *dronc* transgenes induce the activation of another P35-insensitive effector caspase, or that overexpressed Dronc can also act as effector caspase. More experiments are needed to clarify these observations.

In summary, we have isolated a strong loss-of-function mutant in *drICE*. The phenotypic analysis is consistent with a role of *drICE* as an effector caspase. Our data establish that *drICE* and *dcp-1* function redundantly in some cells, whereas other cells strictly require *drICE* for apoptosis. Future studies will reveal how specificity is conferred in these paradigms.

Materials and Methods

Isolation and identification of *drICE*^{17}

ey-Flp; *FRT82B* males were starved for 12 h, followed by treatment with 25 mM EMS in 5% sucrose solution for 24 h. After recovery for 3 h, the mutagenized males were mated to *GheF*; *FRT82B* w^+ females and incubated at 25°C. 40 000 F1 progeny were screened for suppression of the *GMR-hid*-induced small eye phenotype. The strongest suppressor, *su*(*GMR-hid*)*17*, was selected for further analysis. *su*(*GMR-hid*)*17* was identified as a *drICE* allele by genetic tests described in the Results section and by DNA sequencing.

X-ray mutagenesis

Df(*3R*)*drICE* was obtained in the following manner: males carrying the P-element *l*(*3*)*05884* inserted in 99C1–2 were treated with X-ray, crossed to TM2,*ry*/TM6B,*ry* females, and F1 progeny was screened for loss of the eye color marker (ry^+) of *l*(*3*)*05884*. *l*(*3*)*05884* is a P-element insertion in the *ncd* gene, approximately 6 kb distal from the *drICE* locus. Loss of the eye color marker indicates that the P-element along with flanking genomic sequences has been deleted from the genome. Loss of the P-element was confirmed by PCR analysis. *Df*(*3R*)*drICE* has a proximal breakpoint between 99B3 and 99B8, because it complements *Dr* at 99B3, and fails to complement *ca* at 99B8. Its distal breakpoint lies between 99C2 (because it lacks the original P-element) and 99C4 (because it complements the lethality of *CG18041*^{EY04131} which maps to 99C4).

Fly stocks and genetics

The following mutant and transgenic fly stocks were used: *drICE*^{17} and *Df*(*3R*)*drICE* (this study); *dcp-1*^{Prev1} (see Laundrie *et al*.[7]); *diap1*^{5} (see Lisi *et al*.[24]); *UAS-pro-dronc* and *UAS-ΔN-dronc*;[18] *GMR-ΔN-dcp-1* and *GMR-drICE*;[22] *GheF*.[10] The wild-type stock used for comparison was the *ey-Flp*; *FRT82B* stock used for the mutagenesis.

The following stocks were obtained by meiotic recombination:
GMR-hid drICE^{17}
GMR-drICE drICE^{17}
GMR-ΔN-dcp-1 drICE^{17}
GMR-Gal4 drICE^{17}
UAS-pro-dronc drICE^{17}
GMR-Gal4 UAS-ΔN-dronc drICE^{17}
th^5*drICE*^{17}

For embryonic analysis, homozygous *drICE*^{17} males and females (unless otherwise noted) were crossed with each other to remove maternal and zygotic *drICE*. Double-mutant *diap1*^{5} *drICE*^{17} embryos were obtained by crossing males and females of *diap1*^{5} *drICE*^{17}/*drICE*^{17} genotypes. All embryos in these collections are phenotypically similar to *drICE*^{17} single mutants, suggesting that the *diap1* mutant phenotype is effectively suppressed.

Generation of *dcp-1 drICE* double-mutant embryos in germline clones (GLCs): *dcp-1 drICE* double mutants are almost completely lethal. Only four double homozygous adult flies were recovered in <1000 control flies. Such a small number of flies is impractical for embryonic analysis. However, homozygous *dcp-1*^{Prev1} flies in a heterozygous *drICE*^{17} mutant background are viable which enabled us to remove the maternal contribution of both *dcp-1* (see Song *et al.*[23]) and *drICE* (see Fraser and Evan[13]) by GLC analysis[39] to increase the number of double homozygous embryos lacking both maternal and zygotic *dcp-1* and *drICE*. Double-mutant GLC were obtained by crossing females of genotype *hs-FLP*; *dcp-1*^{Prev1}/*dcp-1*^{Prev1}; *FRT82B drICE*^{17}/*FRT82B* P[*ovo*^{D}] with males of genotype *dcp-1*^{Prev1}/*dcp-1*^{Prev1}; *drICE*^{17}/TM6B,*lacZ*. To induce GLCs, first instar larvae were heat shocked at 37°C for one hour.

To visualize the MG, males of the genotype P[*sli-1.0-lacZ*]; *drICE*^{17}/TM6B, *ubx-lacZ* were crossed to *drICE*^{17}/*drICE*^{17} females, and labeled by β-Gal immunohistochemistry.

For Dlg labelings, *drICE*^{17} mosaic pupal eye discs 42 h after pupariation were dissected and labeled with anti-Dlg antibody and GFP to mark the clones. Cell counting was done using criteria established by Cordero *et al.*[29] Ten hexagons corresponding to 20 ommatidia from four individuals each were analyzed.

Fly crosses were carried out under standard conditions at 25°C.

X-ray treatment of embryos

Embryos were treated with 4000 rad in a Nasatron X-ray machine with a Caesium^{137} source. After recovery the embryos were fixed and prepared for AO labeling.

Immunohistochemistry

TUNEL, AO and immunohistochemistry were carried out as described.[40] Anticleaved Caspase-3 antibody (Cell Signaling Technology) was used at a dilution of 1 : 50, β-Gal antibody (Promega) at dilution of 1 : 500, and anti-Dlg antibody (a kind gift of G Halder) at 1 : 2000. The MG was visualized by β-Gal immunohistochemistry. Fluorescent photography was carried out using a Zeiss Axio Imaginer Z1 with ApoTome technology.

Immunoblotting

Embryos were collected, decorionated and snap frozen in liquid nitrogen. Embryos were sonicated in Laemmli SDS loading buffer while being frozen. The equivalent of 20 lysed embryos was loaded per lane. Immunoblots were carried out using standard procedures and were probed with anti-DrICE antibodies (diluted 1 : 1000) raised against the prodomain of DrICE (provided by Andy Fraser). This antibody recognizes only the full-length form of DrICE.

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References

1. Salvesen GS and Abrams JM (2004) Caspase activation – stepping on the gas or releasing the brakes? Lessons from humans and flies. Oncogene 23: 2774–2784.
2. Vaux DL and Silke J (2005) IAPs, RINGs and ubiquitylation. Nat. Rev. Mol. Cell. Biol. 6: 287–297.
3. Cashio P, Lee TV and Bergmann A (2005) Genetic control of programmed cell death in *Drosophila melanogaster*. Semin. Cell Dev. Biol. 16: 225–235.
4. Zachariou A, Tenev T, Goyal L, Agapite J, Steller H and Meier P (2003) IAP-antagonists exhibit non-redundant modes of action through differential DIAP1 binding. EMBO J. 22: 6642–6652.
5. Yan N, Wu JW, Chai J, Li W and Shi Y (2004) Molecular mechanisms of DrICE inhibition by DIAP1 and removal of inhibition by Reaper, Hid and Grim. Nat. Struct. Mol. Biol. 11: 420–428.
6. Kumar S and Doumanis J (2000) The fly caspases. Cell Death Differ. 7: 1039–1044.
7. Laundrie B, Peterson JS, Baum JS, Chang JC, Fileppo D, Thompson SR and McCall K (2003) Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in *Drosophila*. Genetics 165: 1881–1888.
8. Daish TJ, Mills K and Kumar S (2004) *Drosophila* caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. Dev. Cell 7: 909–915.
9. Chew SK, Akdemir F, Chen P, Lu WJ, Mills K, Daish T, Kumar S, Rodriguez A and Abrams JM (2004) The apical caspase dronc governs programmed and unprogrammed cell death in *Drosophila*. Dev. Cell 7: 897–907.
10. Xu D, Li Y, Arcaro M, Lackey M and Bergmann A (2005) The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in *Drosophila*. Development 132: 2125–2134.
11. Waldhuber M, Emoto K and Petritsch C (2005) The *Drosophila* caspase DRONC is required for metamorphosis and cell death in response to irradiation and developmental signals. Mech. Dev. 122: 914–927.
12. Dorstyn L, Colussi PA, Quinn LM, Richardson H and Kumar S (1999) DRONC, an ecdysone-inducible *Drosophila* caspase. Proc. Natl. Acad. Sci. U.S.A. 96: 4307–4312.
13. Fraser AG and Evan GI (1997) Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. EMBO J. 16: 2805–2813.
14. Fraser AG, McCarthy NJ and Evan GI (1997) drICE is an essential caspase required for apoptotic activity in *Drosophila* cells. EMBO J. 16: 6192–6199.
15. Muro I, Monser K and Clem RJ (2004) Mechanism of Dronc activation in *Drosophila* cells. J. Cell Sci. 117: 5035–5041.
16. Kilpatrick ZE, Cakouros D and Kumar S (2005) Ecdysone-mediated up-regulation of the effector caspase DRICE is required for hormone-dependent apoptosis in *Drosophila* cells. J. Biol. Chem. 280: 11981–11986.
17. Peterson JS, Barkett M and McCall K (2003) Stage-specific regulation of caspase activity in *Drosophila* oogenesis. Dev. Biol. 260: 113–123.
18. Meier P, Silke J, Leevers SJ and Evan GI (2000) The *Drosophila* caspase DRONC is regulated by DIAP1. EMBO J. 19: 598–611.
19. Hawkins CJ, Yoo SJ, Peterson EP, Wang SL, Vernooy SY and Hay BA (2000) The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J. Biol. Chem. 275: 27084–27093.
20. Kaiser WJ, Vucic D and Miller LK (1998) The *Drosophila* inhibitor of apoptosis D-IAP1 suppresses cell death induced by the caspase drICE. FEBS Lett. 440: 243–248.
21. Tenev T, Zachariou A, Wilson R, Ditzel M and Meier P (2005) IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. Nat. Cell Biol. 7: 70–77.
22. Song Z, Guan B, Bergman A, Nicholson DW, Thornberry NA, Peterson EP and Steller H (2000) Biochemical and genetic interactions between *Drosophila* caspases and the proapoptotic genes rpr, hid, and grim. Mol. Cell. Biol. 20: 2907–2914.

23. Song Z, McCall K and Steller H (1997) DCP-1, a *Drosophila* cell death protease essential for development. Science 275: 536–540.
24. Lisi S, Mazzon I and White K (2000) Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. Genetics 154: 669–678.
25. Yu SY, Yoo SJ, Yang L, Zapata C, Srinivasan A, Hay BA and Baker NE (2002) A pathway of signals regulating effector and initiator caspases in the developing *Drosophila* eye. Development 129: 3269–3278.
26. Klambt C, Jacobs JR and Goodman CS (1991) The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. Cell 64: 801–815.
27. Zhou L, Schnitzler A, Agapite J, Schwartz LM, Steller H and Nambu JR (1997) Cooperative functions of the reaper and head involution defective genes in the programmed cell death of *Drosophila* central nervous system midline cells. Proc. Natl. Acad. Sci. U.S.A. 94: 5131–5136.
28. Rodriguez A, Chen P, Oliver H and Abrams JM (2002) Unrestrained caspase-dependent cell death caused by loss of Diap1 function requires the *Drosophila* Apaf-1 homolog, Dark. EMBO J. 21: 2189–2197.
29. Cordero J, Jassim O, Bao S and Cagan R (2004) A role for wingless in an early pupal cell death event that contributes to patterning the *Drosophila* eye. Mech. Dev. 121: 1523–1530.
30. Wolff T and Ready DF (1991) Cell death in normal and rough eye mutants of *Drosophila*. Development 113: 825–839.
31. Quinn LM, Dorstyn L, Mills K, Colussi PA, Chen P, Coombe M, Abrams J, Kumar S and Richardson H (2000) An essential role for the caspase dronc in developmentally programmed cell death in *Drosophila*. J. Biol. Chem. 275: 40416–40424.
32. Yuan J, Shaham S, Ledoux S, Ellis HM and Horvitz HR (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75: 641–652.
33. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW and Mak TW (1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 12: 806–819.
34. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P and Flavell RA (1998) Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. Cell 94: 325–337.
35. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P and Flavell RA (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384: 368–372.
36. Zheng TS, Hunot S, Kuida K, Momoi T, Srinivasan A, Nicholson DW, Lazebnik Y and Flavell RA (2000) Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. Nat. Med. 6: 1241–1247.
37. Yokokura T, Dresnek D, Huseinovic N, Lisi S, Abdelwahid E, Bangs P and White K (2004) Dissection of DIAP1 functional domains via a mutant replacement strategy. J. Biol. Chem. 279: 52603–52612.
38. Ditzel M, Wilson R, Tenev T, Zachariou A, Paul A, Deas E and Meier P (2003) Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. Nat. Cell Biol. 5: 467–473.
39. Chou TB and Perrimon N (1996) The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. Genetics 144: 1673–1679.
40. McCall K and Peterson JS (2004) Detection of apoptosis in *Drosophila*. Methods Mol. Biol. 282: 191–205.