Development 133, 3305-3315 (2006) doi:10.1242/dev.02495

The *Drosophila* caspase Ice is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process

Israel Muro[1,*], Deborah L. Berry[2], Jun R. Huh[1], Chun Hong Chen[1], Haixia Huang[1], Soon Ji Yoo[3], Ming Guo[4], Eric H. Baehrecke[2] and Bruce A. Hay[1]

Caspase family proteases play important roles in the regulation of apoptotic cell death. Initiator caspases are activated in response to death stimuli, and they transduce and amplify these signals by cleaving and thereby activating effector caspases. In *Drosophila*, the initiator caspase Nc (previously Dronc) cleaves and activates two short-prodomain caspases, Dcp-1 and Ice (previously Drice), suggesting these as candidate effectors of Nc killing activity. *dcp-1*-null mutants are healthy and possess few defects in normally occurring cell death. To explore roles for Ice in cell death, we generated and characterized an *Ice* null mutant. Animals lacking *Ice* show a number of defects in cell death, including those that occur during embryonic development, as well as during formation of adult eyes, arista and wings. *Ice* mutants exhibit subtle defects in the destruction of larval tissues, and do not prevent destruction of salivary glands during metamorphosis. Cells from *Ice* animals are also markedly resistant to several stresses, including X-irradiation and inhibition of protein synthesis. Mutations in *Ice* also suppress cell death that is induced by expression of Rpr, Wrinkled (previously Hid) and Grim. These observations demonstrate that Ice plays an important non-redundant role as a cell death effector. Finally, we demonstrate that Ice participates in, but is not absolutely required for, the non-apoptotic process of spermatid differentiation.

KEY WORDS: *Drosophila*, Ice, Apoptosis

INTRODUCTION

Programmed cell death, or apoptosis, is a genetically encoded form of cell elimination that results in the orderly death and phagocytic removal of excess, damaged or dangerous cells during development and in the adult (Baehrecke, 2002; Benedict et al., 2002; Green and Evan, 2002; James and Green, 2004; Opferman and Korsmeyer, 2003). Caspase family proteases are the central executioners for most genetically encoded cell deaths in animals (Degterev et al., 2003). In caspase-dependent death, signals arising from specific cellular compartments, such as the plasma membrane or mitochondria, promote the activation of adaptor proteins that recruit long N-terminal prodomain-containing initiator caspases into macromolecular complexes in which caspase activation occurs. Once activated, initiator caspases can cleave and thereby activate short prodomain effector caspases. These cleave a number of target proteins, bringing about death and ultimately phagocytosis of the cell (Degterev et al., 2003; Hay et al., 2004). The *Drosophila* genome encodes seven caspases. Three of these, Nc (Dorstyn et al., 1999a), Strica (Doumanis et al., 2001) and Dredd (Chen et al., 1998), contain long prodomains characteristic of initiator caspases. A large body of evidence implicates Nc as an important cell death activator (reviewed by Hay and Guo, 2006).

[1]Division of Biology, MC 156-29, California Institute of Technology, Pasadena, CA 91125, USA. [2]Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, MD 20742, USA. [3]Department of Biology, Kyung Hee Institute of Age-related and Brain Diseases, Kyung Hee University, Seoul, 130-701, Korea. [4]Department of Neurology, Brain Research Institute, The David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA.

*Author for correspondence (e-mail: imuro@caltech.edu)

Accepted 5 June 2006

Nc contains an N-terminal CARD (caspase recruitment domain) motif, as does the mammalian cell death caspase caspase 9, and the *C. elegans* caspase CED-3. Homotypic interactions between the CARD in caspase 9 and a similar motif in the cytoplasmic adaptor Apaf1, in the presence of cytoplasmic cytochrome c and dATP, results in caspase 9 recruitment into a multimeric complex known as the apoptosome. Caspase activation occurs in the apoptosome and activated caspase 9 then cleaves and activates effector caspases such as caspase 3. CED-3 activation in *C. elegans* is also mediated by interactions with an Apaf1-like molecule known as CED-4 (reviewed by Yan and Shi, 2005). *Drosophila* encodes one Apaf1 homolog, Ark (also known as Hac-1 or dAPAF-1) (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Ark binds Nc, and decreasing or eliminating *ark* expression results in defects in cell death that are thought to be *Nc*-dependent (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999; Igaki et al., 2002; Muro et al., 2002; Zimmermann et al., 2002; Muro et al., 2004; Akdemir et al., 2006; Mills et al., 2006; Srivastava et al., 2006).

Caspase activity is inhibited by members of the inhibitor of apoptosis (IAP) family of proteins through several different mechanisms (reviewed by Clem, 2001; Hay, 2000; Vaux and Silke, 2005; Hay and Guo, 2006). Expression of the *Drosophila* IAP Thread (Th; previously Diap1) inhibits caspase-dependent cell death (Hay et al., 1995), and is essential for the survival of many otherwise healthy cells (Goyal et al., 2000; Hay et al., 1995; Lisi et al., 2000; Wang et al., 1999). Importantly, the death of healthy cells in response to loss of Th can be inhibited by removal of Ark or Nc (Igaki et al., 2002; Muro et al., 2002; Rodriguez et al., 2002; Zimmermann et al., 2002). These observations suggest that Ark-dependent activation of Nc occurs constitutively, and that Th is required continuously to inhibit this activity and the activity of caspases activated by Nc. In one major pathway, caspase-dependent cell death is initiated by increased expression or release from a

sequestering environment of proteins such as Reaper (Rpr) (White et al., 1994), Head involution defective (W; previously Hid) (Grether et al., 1995), Grim (Chen et al., 1996), Sickle (Skl) (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002) and Jafrac2 (Tenev et al., 2002). These proteins disrupt Th-caspase interactions, liberating Nc and Nc target caspases from inhibition by Th, thereby initiating apoptosis.

An important unresolved issue in the context of the above-described pathway is the identity of the effector caspase(s) that cleaves the cellular substrates that actually bring about cell destruction. That such caspases must exist is indicated by the fact that although expression of the baculovirus caspase inhibitor p35 inhibits many cell deaths in *Drosophila*, at least some of which are known to be Nc-dependent, it does not inhibit Nc (Hay et al., 1994; Hawkins et al., 2000; Meier et al., 2000; Martin and Baehrecke, 2004). Thus, the activation of Nc, in the absence of other p35-sensitive caspases, is insufficient to bring about cell death. The *Drosophila* genome encodes four short prodomain caspases that are candidate effectors: Damm (Harvey et al., 2001), Decay (Dorstyn et al., 1999b), Dcp-1 (Song et al., 1997) and Ice (Fraser and Evan, 1997). Little is known about the roles that Damm and Decay play in cell death. By contrast, Dcp-1 and Ice share a high degree of homology with each other, and are most homologous among the *Drosophila* caspases to the mammalian death effector caspases caspase 3, caspase 6 and caspase 7. In addition, they are both expressed broadly throughout development (Arbeitman et al., 2002), inhibited by p35 (Hawkins et al., 1999; Wang et al., 1999), and cleaved and activated by Nc (Hawkins et al., 2000; Meier et al., 2000; Muro et al., 2002). However, although *dcp-1*-null mutants do show mild defects in starvation-induced cell death during oogenesis (Laundrie et al., 2003), they are otherwise quite healthy. This stands in contrast to *Nc* mutants, which show many defects in developmental cell death (Chew et al., 2004; Daish et al., 2004; Waldhuber et al., 2005; Xu et al., 2005), and have a very low rate of survival to adulthood (Xu et al., 2005). By contrast, several observations suggest that Ice may play an important role as a cell death effector. First, depletion of Ice from S2 cells inhibits apoptotic events in response to a variety of stimuli (Fraser et al., 1997; Muro et al., 2002; Muro et al., 2004). In addition, antibodies that recognize the Nc-cleaved, and therefore activated, version of Ice, label dying cells during development (Yoo et al., 2002; Yu et al., 2002), as well as cells exposed to a variety of apoptotic stimuli (cf. Huh et al., 2004a; Perez-Garijo et al., 2004).

To explore roles of *Ice* as a cell death effector, we generated a null mutation for the *Ice* locus, $Ice^{\Delta 1}$. $Ice^{\Delta 1}$ animals show defects in some, but not all apoptotic cell deaths, $Ice^{\Delta 1}$ animals also show defects in a non-apoptotic process: spermatid individualization.

MATERIALS AND METHODS

Isolation of a *Ice* null allele and generation of a rescue construct

We used transposon mobilization to generate the $Ice^{\Delta 1}$ excision allele from The P transposon line $EP^{GE28489}$ (Genexel, Seoul, Korea). $EP^{GE28489}$ is inserted at base 1711 with respect to the start of the *Ice* transcription unit. A genomic rescue fragment containing 2.4 kb of DNA flanking the Ice gene was generated using PCR from genomic DNA with the primers 5′ CGC CTC GAG CCT CTT TGA GAG TGT GAC CGT GCA TAA and 5′ CGC TCT AGA ACG ATC AGG GTC AGC CAA TGG CTG GAC. Products were digested with *Xho*I and *Xba*I and cloned into the P element transformation vector pCasper4 (Thummel and Pirotta, 1992). UAS-ark-RNAi and UAS-Ice-RNAi constructs were made by introducing 21 bp sequences complementary to the *ark*- or *Ice*-coding regions into one strand of the stem region of a 70 nucleotide fragment encoding the *Drosophila* microRNA mir-6.1. These fragments were cloned into the UASt vector (Brand and Perrimon, 1993). Details of these constructs will be provided elsewhere (C.H.C. and B.A.H., unpublished).

Fly stocks and genetics

All crosses and stocks were maintained at 25°C. The following fly stocks were used: *GMR-rpr* and *GMR-hid* (Hay et al., 1995), *GMR-grim* and *GMR-Nc* (Hawkins et al., 2000), *GMR-p35* (Hay et al., 1994), *GMR-GAL4-UAS-Th- RNAi* (Huh et al., 2004a), *en-GAL4, UAS-GFP* (Kimura et al., 2004), $dcp\text{-}1^{Prev1}$ (Laundrie et al., 2003) and *P[sli-1.0-lacZ]* (Wharton and Crews, 1993).

Fly viability determination

Third instar larvae were collected from each genotype and put into vials with fresh food at 25°C. They were followed for 7 days and viability was determined as the fraction of eclosed adults compared with the total number of third instar larvae. At least 200 third instar larvae were scored for each genotype.

Western blotting, Immunohistochemistry and TEM

For Western blotting, adult flies were lysed and processed as described previously (Huh et al., 2004b). Blots were probed with rabbit anti-full-length Ice sera (1:1000) and anti-tubulin (Sigma) at a dilution of 1:500. Embryos and wing discs were fixed and processed for anti-caspase and TUNEL staining as described (Yoo et al., 2002). Anti-active Ice (Yoo et al., 2002) and CM1 (Cell Signaling #9661) were used at 1:50, and the secondary anti-rabbit antibody (Molecular Probes) at 1:500. Similar conditions were used for adult testis staining. Third instar larvae were exposed to 2000 rads of X-irradiation using a Torex 120D X-ray inspection system (Astrophysics Research, Long Beach, CA). They were processed 4 hours after irradiation. Pupal eyes were fixed and stained with anti-Dlg (Developmental Studies Hybridoma Bank) at 1:300. The midline glia were visualized by anti-β-gal (Sigma Chemical Corp.) immunohistochemistry. TEM of adult testis was carried out as described previously (Huh et al., 2004b).

Pupal histology

Flies were maintained at 25°C and aged to 24 hours after puparium formation. For histology, whole pupae were fixed and processed as described previously (Martin and Baehrecke, 2004) for paraffin sectioning and light microscopy. The number of abnormal masses in the head and in the wing and leg discs was counted from every fifth histological section of the pupa. Sections were counted throughout the entire pupa and, owing to the size of the pupa, on average eight sections were counted for each pupa. The average number of masses per section was determined for each pupa and at least five different pupae were counted per genotype to determine the average number of masses per section for each genotype.

Ex vivo hemocyte analyses

Hemocyte analysis was performed essentially as previously described (Chew et al., 2004). Hemocytes were plated in Schneider's media with 10% FBS. Hemocytes were seeded for 1 hour and then washed with media and treated with 20 μg/ml of cycloheximide. Four hours after treatment, cells were visualized for cell membrane blebbing. The fraction of blebbing cells was used as a measure of apoptosis.

RESULTS

Generation of $Ice^{\Delta 1}$, a *Ice* null allele

To generate a *Ice* mutant, we mobilized the P transposon $EP^{GE28489}$, which is located within the 3′ UTR of the *Ice* transcription unit. One excision line, designated $Ice^{\Delta 1}$, lacks the entire *Ice*-coding region, but has no effect on the structure of the surrounding genes (Fig. 1A). We generated $Ice^{\Delta 1}$ flies that also carried a 2.4 kb fragment ($Ice^{+2.4}$) encompassing the *Ice* transcription unit ($Ice^{+2.4}$; $Ice^{\Delta 1}$ flies). As expected, $Ice^{\Delta 1}$ animals lacked detectable Ice protein, while $Ice^{+2.4}$; $Ice^{\Delta 1}$ flies expressed Ice at wild-type levels, as did flies that lacked *dcp-1* (Fig. 1B). In addition, all the phenotypes detailed below for $Ice^{\Delta 1}$ mutants were suppressed in the presence of $Ice^{+2.4}$, demonstrating that the *Ice* locus, and only the *Ice* locus, is altered in the $Ice^{\Delta 1}$ flies.

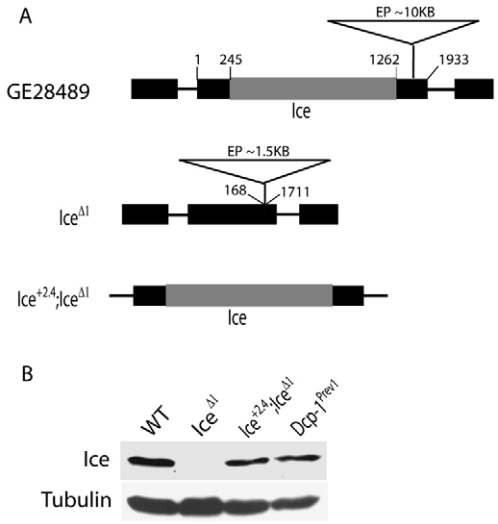

Fig. 1. *Ice*$^{\Delta 1}$ is a null allele of *Ice*. (**A**) Genomic organization of the *Ice* region. The P transposon element EP^{GE28489} is inserted at base 1711 in the Ice transcription unit. The *Ice*$^{\Delta 1}$ excision line removes the entire *Ice*-coding region, fusing small remaining fragments of the *Ice* 5′- and 3′-UTR sequences. A 1.5 kb fragment of EP^{GE28489} also remains. The gray region indicates the *Ice*-coding region; the black boxes indicate the UTR sequences of *Ice* and of two neighboring genes. Locations of the EP^{GE28489} element, the *Ice*$^{\Delta 1}$ deletion and the *Ice* genomic rescue construct (*Ice*$^{+2.4}$) are indicated to scale. (**B**) Western blot of adults of various genotypes probed with anti-Ice antibodies. The genotypes are wild type, *Ice*$^{\Delta 1}$, *Ice*$^{\Delta 1}$; *Ice+2.4* and *dcp-1*^{Prev1} (ΔDcp-1), a null allele of *dcp-1*.

Ice$^{\Delta 1}$ flies have decreased viability and pupae contain abnormal masses

Ice$^{\Delta 1}$ embryos and larvae showed normal levels of survival. However most animals (80%) died following puparium formation (Fig. 2A). *Ice*$^{\Delta 1}$ pupal lethality is due to loss of *Ice* as it was suppressed in the presence of the *Ice*$^{+2.4}$ rescue DNA. Survival during embryonic and larval stages, and pupal lethality, are not due to perdurance of maternally deposited protein, as a similar survival profile was observed for *Ice*$^{\Delta 1}$ animals derived from homozygous *Ice*$^{\Delta 1}$ mothers (data not shown). Flies lacking *dcp-1* (*dcp-1*^{Prev1}) (Laundrie et al., 2003) showed a modest decrease in survival compared with wild-type animals, whereas animals lacking Ice that were heterozygous for *dcp-1*^{Prev1} eclosed only rarely (~1%) (data not shown). Animals lacking *Ice* and *dcp-1* (*dcp-1*^{Prev1}*; Ice*$^{\Delta 1}$) all died prior to, or during, early pupal stages. Together, these observations suggest that *Ice* and *dcp-1* both contribute to pupal development, perhaps playing partially redundant roles.

Cell death is extensive during pupal stages, in which larval tissues are eliminated and replaced with adult-specific structures (reviewed by Robertson, 1936; Baehrecke, 2003). Prominent among the larval tissues eliminated are salivary glands, midgut and body wall muscles. Homozygous *Ice*$^{\Delta 1}$ and *Ice*$^{\Delta 1}$/+ wild-type control pupae were aged to 24 hours after puparium formation, a time when the death of larval structures is well advanced, embedded in paraffin wax, sectioned and stained for examination of cell death defects (Fig. 2). In sections of control *Ice*$^{\Delta 1}$/+ pupae, salivary glands, midgut and larval muscle had undergone degeneration, as previously described (Jiang et al., 1997; Lee et al., 2002) (Fig. 2B). Most (73%) homozygous *Ice*$^{\Delta 1}$ pupae appeared similar to controls 24 hours after puparium formation, with larval salivary glands, midguts and muscles having largely undergone degeneration (Fig. 2C). Only a few remaining fragments of salivary glands and larval muscle were still present (Fig. 2C). A small number of homozygous *Ice*$^{\Delta 1}$ pupae (27%) were developmentally delayed, and either arrested development prior to head eversion, or head everted and possessed defects in head and nervous system morphology (Fig. 2D). Although these delayed mutant pupae had larval salivary glands, midgut and muscles, we presume that the persistence of these tissues is a consequence of their arrested development (the basis of which is unknown). Almost all double mutant *dcp-1*^{Prev1}*; Ice*$^{\Delta 1}$ arrested development prior to head eversion and were not characterized further. All of the *Ice*$^{\Delta 1}$ mutant pupae possessed many abnormal masses in the head, abdomen, wing disc and leg disc (~30 per pupa when compared with one for wild type) (Fig. 2C,E-G, see Table S1 in the supplementary material). Interestingly, the frequency of masses was increased almost two-fold in *Ice*$^{\Delta 1}$ animals that had been subject to X-irradiation during larval stages (see Fig. S1 and Table S1 in the supplementary material). Although further studies are required to determine the identity of these abnormal masses (see Discussion), they are specific to mutant pupae.

Ice$^{\Delta 1}$ adults display a number of defects

Homozygous *Ice*$^{\Delta 1}$ adults showed several phenotypes suggestive of decreased cell death during development. The arista is a feather-like structure derived from the third antennal segment. It consists of a central core of epidermal cells and a series of lateral branches. The number of lateral branches is regulated by apoptotic cell death (Cullen and McCall, 2004; He and Adler, 2001). Thus, adults homozygous for the tissue-specific Th loss-of-function allele *th*^{1} lack essentially all lateral branches, a phenotype that is suppressed by mutations in *ark* (Cullen and McCall, 2004). By contrast, flies mutant for *hid*, an inhibitor of Th, have extra lateral branches (Cullen and McCall, 2004). As shown in Fig. 3, the arista of *Ice*$^{\Delta 1}$ flies (Fig. 3B) has a much thicker central shaft and many more lateral branches than does the arista from wild-type flies (Fig. 3A). In addition, removal of *Ice* suppressed the loss of lateral shafts seen in *th*^{1} flies (Fig. 3C,D). Finally, although the arista appeared wild type in *dcp-1*^{Prev1} flies (data not shown), adult *Ice*$^{\Delta 1}$ flies heterozygous for *dcp-1*^{Prev1} had an increased number of lateral branches when compared with *Ice*$^{\Delta 1}$ alone (Fig. 3E).

The adult male terminalia, which derives from the genital imaginal disc, undergoes a 360° clockwise rotation during development (Gleichauf, 1936; Adam et al., 2003). Mutations in *hid* (Abbott and Lengyel, 1991), as well as overexpression of the baculovirus caspase inhibitor p35 (Macias et al., 2004), give rise to adults in which complete rotation fails to occur. This results in adults in which the genitalia and analia are mislocalized with respect to the abdomen. Approximately 50% of *Ice*$^{\Delta 1}$ males showed a similar phenotype (Fig. 3F,G), as did all *Ice*$^{\Delta 1}$ males heterozygous for *dcp-1*^{Prev1} (*n*=30). Finally, we noted that more than 50% of *Ice*$^{\Delta 1}$ adults had an open ‘scar’ of varying severity along the dorsal midline (Fig. 3I; compare with wild type, Fig. 3H). The tissue appeared thin, white and fragile, with some flies possessing holes or tears in this area that were associated with leakage of hemolymph. The integument of the adult abdomen is derived from four groups of abdominal histoblast nest cells that are present at characteristic positions in each segment of the larval abdomen. Following pupariation, histoblasts replace larval epidermal cells, which undergo apoptosis and are phagocytosed by hemocytes (Madhavan and Madhavan, 1980). We speculate that loss of *Ice* compromises some aspect of the process of cell replacement, histoblast nest fusion or cell differentiation.

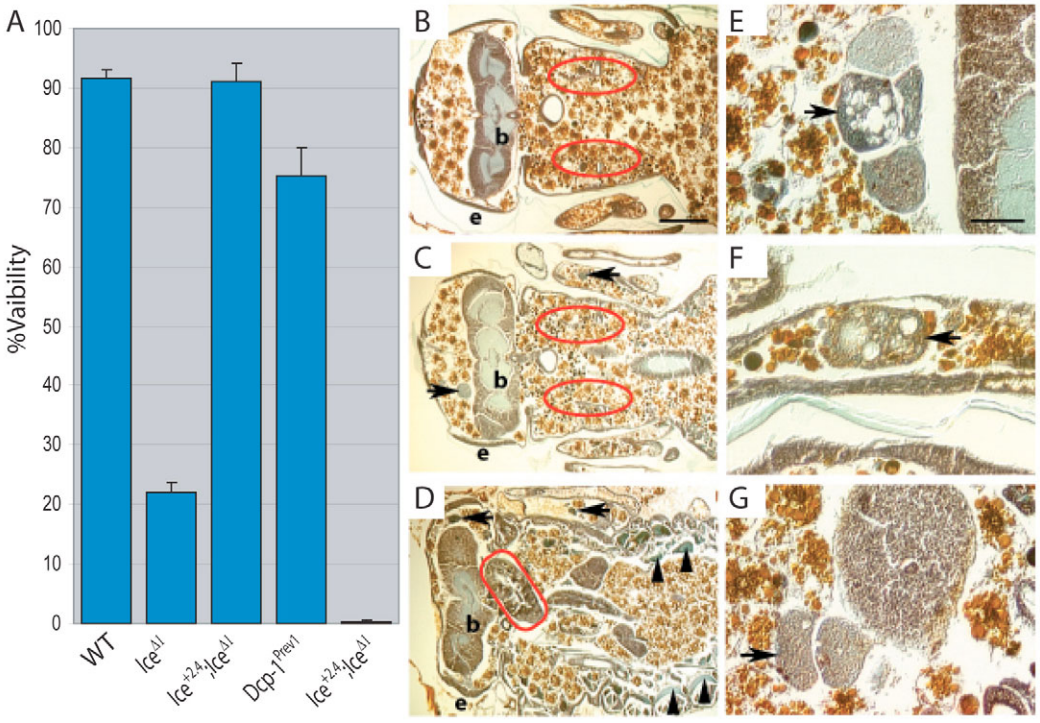

Fig. 2. *Ice* is required for normal pupal development. (**A**) The percent viability to adulthood for wandering third instar larvae of several different genotypes is shown: wild type, $Ice^{\Delta 1}$, $Ice^{+2.4;}$ $Ice^{\Delta 1}$, $dcp\text{-}1^{Prev1}$ and $dcp\text{-}1^{Prev1}$; $Ice^{\Delta}1$. (**B-G**) Paraffin sections of pupae aged 24 hours after puparium formation (apf). (B) In control pupae, salivary glands and larval muscle have both disappeared and the larval mid-gut has degenerated, as previously described (Lee and Baehrecke, 2001). (C) Seventy-three percent of $Ice^{\Delta 1}$ pupae develop normally. By 24 hours apf, only a few remaining small degraded fragments of salivary glands and larval muscles are observed and the mid-gut is normal. (D) Twenty-seven percent of $Ice^{\Delta 1}$ pupae were arrested in development. Of those that arrested, one-third did not undergo head eversion and arrested prior to the commencement of salivary gland cell death. The remaining pupae appear to have arrested 1 or 2 hours after head eversion, and thus the salivary glands and larval muscle are not degraded, and the larval gut has failed to condense properly. $Ice^{\Delta 1}$ pupae have abnormal masses in the head (C,E), wing and leg discs (C,F), and abdomen (G). Arrows indicate abnormal masses; arrowheads indicate muscle; red circles are placed around salivary glands, salivary gland fragments and regions where degraded salivary glands would be if they had failed to die. Animals homozygous for $Ice^{\Delta 1}$ lack zygotic but not maternal *Ice*. Scale bars: 200 μm in B-D; 40 μm in E-G.

In addition to the above-noted defects, $Ice^{\Delta 1}$ flies also displayed several phenotypes, in the wing and eye, which could be directly attributed to defects in cell death. Epidermal cells that make up the adult *Drosophila* wing undergo death within the first hour after eclosion (Kimura et al., 2004). When this death fails to occur, the wing appears opaque when compared with wild type, and sometimes contains trapped fluid. An opaque wing phenotype has also been reported for *hid* (Abbott and Lengyel, 1991), *ark* (Rodriguez et al., 1999) and *Nc* (Xu et al., 2005) mutants. Cell death in the adult wing can be visualized in living animals that express a nuclear-localized green fluorescent protein. Live cells show GFP fluorescence, while dead cells do not. Importantly, these deaths are inhibited – and thus GFP fluorescence retained – in cells that express baculovirus p35 (Kimura et al., 2004), or that are mutant for *Nc* (Xu et al., 2005). All $Ice^{\Delta 1}$ flies have opaque wings, which sometimes contain trapped fluid or never fully extend (data not shown). We used en-GAL4 and UAS-GFP (en::GFP) to visualize cell death in wild-type and $Ice^{\Delta 1}$ backgrounds. In wild-type adults less than 1 hour after eclosion, GFP was seen throughout the posterior compartment, the region in which en::GFP is expressed (Fig. 4A). By 2 hours post-eclosion GFP fluorescence was largely absent from posterior compartment (Fig. 4B). By contrast, in $Ice^{\Delta 1}$ animals, GFP fluorescence could be observed in the posterior compartment for greater than 24 hours (Fig. 4C).

Cell death also plays an important role in eye development (reviewed by Baker Brachmann and Cagan, 2003), serving to eliminate excess 2° and 3° pigment cells that surround the ommatidia (Cagan and Ready, 1989; Wolff and Ready, 1991). Cell death in the pupal retina requires *hid* (Kurada and White, 1998; Yu et al., 2002; Cordero et al., 2004) and *Nc* (Xu et al., 2005), and is inhibited by expression of p35 or Th (Hay et al., 1995; Hay et al., 1994). To determine if $Ice^{\Delta 1}$ flies had excess inter-ommatidial cells, we stained 40-hour-old pupal retina with an antibody to Discs large (Dlg), a membrane protein that allows the visualization of cell borders and the determination of the number of inter-ommatidial cells. In wild-type retinas at this stage, cell death has eliminated excess interommatidial cells, and each ommatidia is surrounded by six 2° pigment cells that define the faces of a hexagon, and 3° pigment cells and bristles, located at alternate vertices (Fig. 4D). Pupal eyes from $dcp\text{-}1^{Prev1}$ flies appeared wild type (data not shown). By contrast, $Ice^{\Delta 1}$ retinae contained on average three additional interommatidial cells per ommatidia (Fig. 4E).

Ice is important for cell death during embryogenesis

Cell death is extensive during embryogenesis and is regulated by the RHG family of proteins (White et al., 1994; Grether et al., 1995; Zhou et al., 1995; Huh and Hay, 2002), Nc (Quinn et al., 2000; Chew

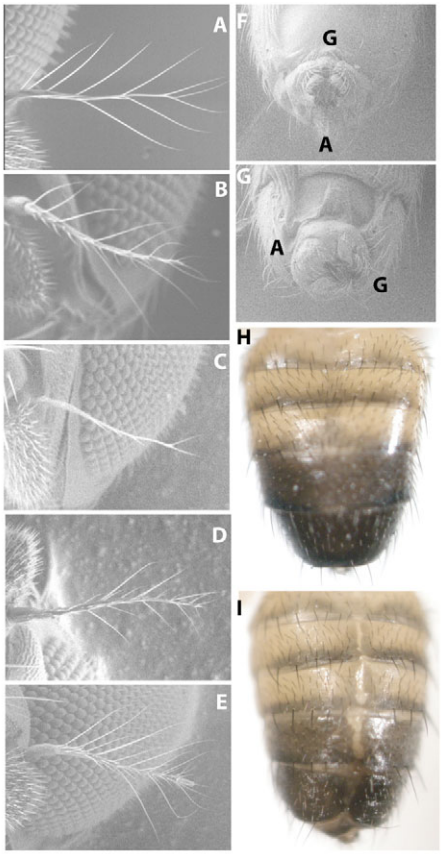

Fig. 3. Adult *Ice$^{\Delta 1}$* show multiple defects suggestive of defects in developmental cell death. (**A-E**) SEM images of arista of different genotypes. (A) Wild type, (B) *Ice$^{\Delta 1}$*, (C) *th^{1}*, (D) *th^{1}*, *Ice$^{\Delta 1}$* and (E) *dcp-1^{Prev1}*/+; *Ice$^{\Delta 1}$*. (**F**) Ventral view of the abdomen of a wild-type male, The genitalia (G) and analia (A) are marked. (**G**) Similar view of the abdomen of a *Ice$^{\Delta 1}$* adult. Genitalia are markedly rotated. (**H**) Dorsal view of the abdomen of a wild-type adult male. (**I**) Similar view of the abdomen of a *Ice$^{\Delta 1}$* adult male, showing a pale line that runs along the dorsal abdominal midline. Animals homozygous for *Ice$^{\Delta 1}$* lack zygotic but not maternal *Ice*.

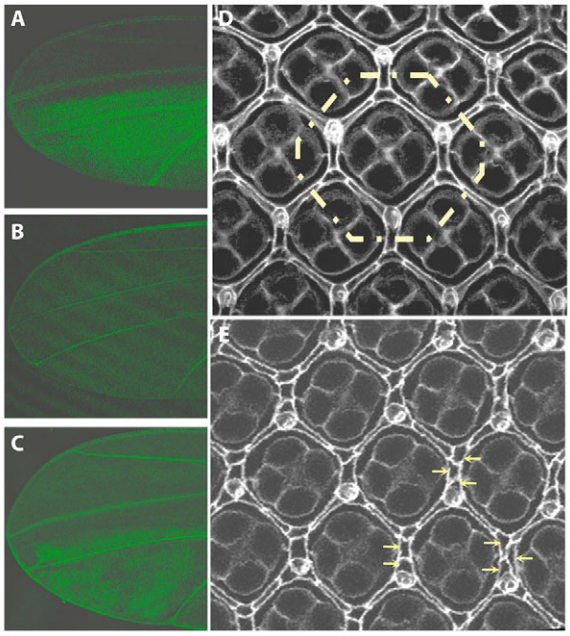

Fig. 4. Ice is required for normally occurring cell death in the adult wing and pupal retina. (**A**) en::GFP expression in the wing of a wild-type adult (eclosed for less than 1 hour). (**B**) en::GFP expression in the wing of a wild-type adult (eclosed for 2 hours). (**C**) en::GFP expression in the wing of a *Ice$^{\Delta 1}$* adult (eclosed for 24 hours). (**D**,**E**) Forty-hour pupal eyes stained with anti-Dlg. (D) Wild type; (E) *Ice$^{\Delta 1}$*. The area in which the number of interommatidial cells was counted for each genotype is indicated in D by the broken line. Several regions with extra cells are indicated with arrows in the mutant (E). Animals homozygous for *Ice$^{\Delta 1}$* lack zygotic but not maternal *Ice*.

et al., 2004; Daish et al., 2004; Xu et al., 2005) and Th (Goyal et al., 2000; Lisi et al., 2000; Wang et al., 1999). These deaths are also sensitive to the expression of baculovirus p35, suggesting important roles for one or more effector caspases (Hay et al., 1994; Zhou et al., 1997). To explore roles for *Ice* in normally occurring cell death during embryogenesis, we characterized wild-type embryos and embryos lacking both maternal and zygotic *Ice* using the TUNEL assay, which labels fragmented DNA within dying cells. Dying cells were present throughout the wild-type stage 14 embryo, and were particularly prevalent in the head region (Fig. 5A). *Ice$^{\Delta 1}$* embryos showed a modest reduction in TUNEL labeling (Fig. 5B) that was restored to wild-type levels in the presence of the genomic rescue construct (Fig. 5C).

Midline glia (MG) are a particularly well characterized group of identifiable cells in which cell death occurs during embryogenesis. Approximately 10 MGs are initially generated per segment. These cells function to ensheath and separate commisural axon tracts. Subsequently, many of them die, such that by about stage 17 of embryogenesis only three MG remain per segment (Klambt et al., 1991; Sonnenfeld and Jacobs, 1995; Zhou et al., 1995). These deaths require the activity of the RHG family of proteins (Zhou et al., 1995), *Nc* (Xu et al., 2005) and p35-sensitive caspase activity (Zhou et al., 1997). We used the slit-*lacZ* enhancer trap line P[sli-1.0-lacZ], which is expressed in MG, as a marker for their fate (Wharton and Crews, 1993). Stage 17 IceΔ1 mutants contained on average six MGs per segment (Fig. 5E), whereas wild-type embryos expectedly contained three (Fig. 5D). As with the TUNEL staining above, wild-type levels of cell death were restored in IceΔ1 embryos that carried the genomic rescue construct (Fig. 5F). The level of cell death inhibition seen in the IceΔ1 mutant is significant, but somewhat lower than that seen in embryos that lack Nc or the RHG genes. In these mutant backgrounds, an average of 10 MGs are observed, indicating the presence of little or no MG cell death (Xu et al., 2005; Zhou et al., 1995). It is possible that *dcp-1* works with *Ice* to bring about MG cell death. However, the lethality of *dcp-1^{Prev1}*; *Ice$^{\Delta 1}$* flies prevented us from testing this hypothesis directly.

Ice is required for cell death induced by X-irradiation and inhibition of protein synthesis

A number of stresses activate apoptosis in *Drosophila*. These include X-irradiation and inhibition of protein synthesis. These deaths require *Nc* (Chew et al., 2004; Daish et al., 2004; Muro et al., 2002; Xu et al., 2005; Zimmermann et al., 2002) and are sensitive to expression of baculovirus p35 (Hay et al., 1994), again suggesting the importance of one or more p35-sensitive effector caspases. To explore the role of *Ice* in stress-induced apoptosis, we examined wing discs from third instar larvae exposed to X-irradiation. Wing discs from untreated wild-type larvae showed low levels of apoptosis

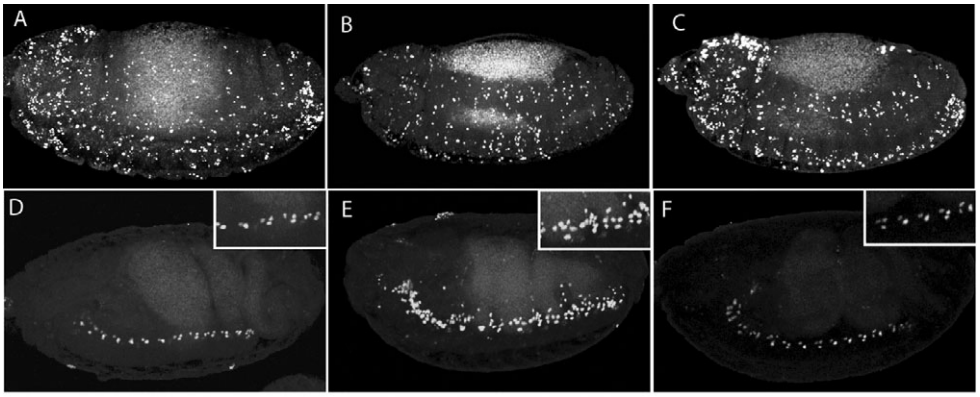

Fig. 5. *Ice* is required for some normally occurring cell death during embryogenesis. (**A-C**) TUNEL stains of stage 14 embryos of different genotypes. (A) Wild type; (B) $Ice^{\Delta 1}$; (C) $Ice^{+2.4}$; $Ice^{\Delta 1}$. (**D-F**) Stage 17 embryos of different genotypes, each of which carries the P[sli-1.0-lacZ] enhancer trap line, which is expressed in midline glia. (D) Wild type, (E) $Ice^{\Delta 1}$ and (F) $Ice^{+2.4}$; $Ice^{\Delta 1}$. Embryos homozygous for $Ice^{\Delta 1}$ lack maternal and zygotic *Ice*.

(Fig. 6A), which was greatly increased following irradiation (Fig. 6B). Wing discs from untreated $Ice^{\Delta 1}$ larvae also showed low levels of apoptosis (Fig. 6C). However, unlike the case with wild type, irradiation did not result in an increase in cell death (Fig. 6D), and cell death in response to X-irradiation was restored when $Ice^{\Delta 1}$ animals also carried the $Ice^{+2.4}$ rescue construct (Fig. 6E). Finally, loss of *dcp-1* resulted in little or no inhibition of apoptosis in response to X-irradiation (Fig. 6F).

Inhibition of protein synthesis through addition of cyclohexamide induces apoptosis in *Drosophila* S2 cells (Fraser and Evan, 1997; Muro et al., 2002), a cell line with characteristics of hemocytes (Eschalier, 1997). Cyclohexamide-induced cell death requires *Nc* and *dark* (Muro et al., 2002; Zimmermann et al., 2002; Chew et al., 2004; Akdemir et al., 2006), and is hypothesized to be due, at least in part, to the differential loss of Th, which has a short half life ($t_{1/2}$=30 minutes) when compared with its caspase targets ($t_{1/2}$=3 hours) (Holley et al., 2002; Yoo et al., 2002). To determine if *Ice* plays a role in bringing about cell death in response to protein synthesis inhibition, we collected hemocytes from wild-type, $Ice^{\Delta 1}$ and $Ice^{+2.4}$; $Ice^{\Delta 1}$ third instar larvae, and exposed them to cyclohexamide. Cyclohexamide-treated hemocytes from wild-type and $Ice^{+2.4}$; $Ice^{\Delta 1}$ animals essentially all died within 4 hours (>95%), as assayed by cell fragmentation. By contrast, hemocytes from $Ice^{\Delta 1}$ animals showed only very low levels of cell death (~5%), comparable with that seen in untreated cultures (Fig. 6G).

Ice is required for RHG-induced apoptosis

As discussed above, RHG family proteins promote cell death, at least in part, by disrupting interactions between Th and Nc, and/or caspases activated by Nc. The deaths they induce are powerfully suppressed by expression of baculovirus p35 (Chen et al., 1996; Grether et al., 1995; Hay et al., 1995; White et al., 1996), highlighting the importance of p35-sensitive caspase activity for their action. To explore roles for *Ice* as an effector of RHG protein function, we introduced flies that expressed *rpr* (*GMR-rpr*), *hid* (*GMR-hid*) or *grim* (*GMR-grim*) under the control of the eye-specific GMR promoter, into the $Ice^{\Delta 1}$ background. *GMR-rpr*, *GMR-hid* and *GMR-grim* flies, in an otherwise wild-type background, have small eyes owing to increased cell death (Fig. 7A-C). These phenotypes were not significantly suppressed in $Ice^{\Delta 1}$ heterozygotes (data not shown), but they were dramatically suppressed in the absence of *Ice* (Fig. 7F-H). However, in the case of *hid* the suppression was not complete (Fig. 7G). W-dependent cell death in the eye is completely blocked by expression of p35 (Grether et al., 1995). Together these observations suggest that although *Ice* is an important effector caspase for RHG protein function, at least in the case of *hid*, and perhaps for other RHG proteins as well, additional p35-sensitive caspases participate in cell killing. A similar point emerges from observations with flies in which Th levels were decreased directly in the eye, using GMR-driven expression of double-stranded RNA corresponding to Th (*GMR-GAL4-UAS-Th-*

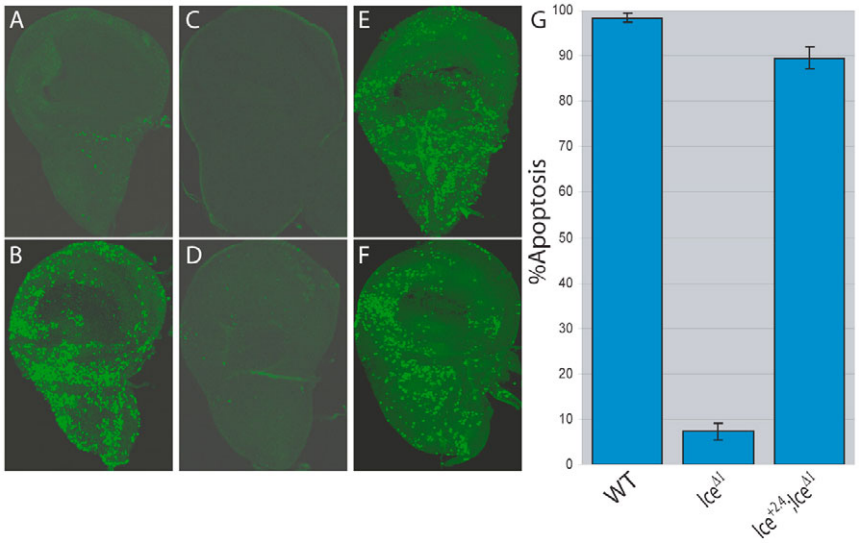

Fig. 6. *Ice* is required for cell death induced by X-irradiation and inhibition of protein synthesis. (**A-F**) TUNEL-stained third instar wing discs of various genotypes. (A) Untreated wild type; (B) X-irradiated wild type; (C) untreated $Ice^{\Delta 1}$; (D) X-irradiated $Ice^{\Delta 1}$; (E) X-irradiated $Ice^{+2.4}$; $Ice^{\Delta 1}$; (F) X-irradiated $dcp\text{-}1^{Prev1}$. (**G**) Wild-type hemocytes exposed to cyclohexamide died within 4 hours. Removal of zygotic Ice ($Ice^{\Delta 1}$)prevented almost all this death, which was restored in the presence of a wild-type *Ice* rescue transgene ($Ice^{\Delta 1}$,$Ice^{+2.4}$). Animals homozygous for $Ice^{\Delta 1}$ lack zygotic but not maternal *Ice*. Animals homozygous for $dcp\text{-}1^{Prev1}$ lack both maternal and zygotic *dcp-1*.

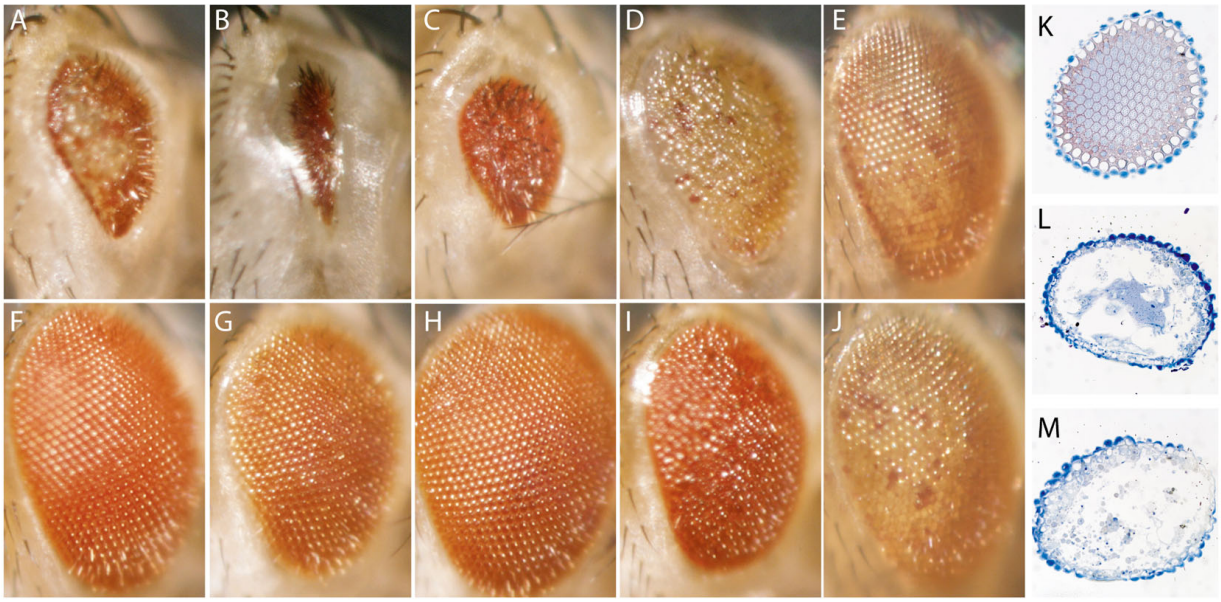

Fig. 7. *Ice* is important for cell death induced by *rpr*, *hid*, *grim* and loss of *Th*, but not *Nc* overexpression. (**A-J**) Adult eyes of various genotypes: (A) *GMR-rpr*; (B) *GMR-hid*; (C) *GMR-grim*; (D) *GMR-Th-RNAi*; (E) *GMR-Nc*; (F) *GMR-rpr*; $Ice^{\Delta 1}$; (G) *GMR-hid*;$Ice^{\Delta 1}$; (H) *GMR-grim*; $Ice^{\Delta 1}$; (I) *GMR-Th-RNAi*; $Ice^{\Delta 1}$; (J) *GMR-Nc*; $Ice^{\Delta 1}$. (**K-M**) Tangential sections through adult eyes of various genotypes: (K) wild type; (L) *GMR-Nc*; (M) *GMR-Nc*, $Ice^{\Delta 1}$. Pigment cells (red) and photoreceptors (blue) form a regular array in the wild-type adult eye (K). These cells are largely absent in the eyes of flies expressing *Nc*, in the presence (L) or absence (M) of *Ice*. Animals homozygous for $Ice^{\Delta 1}$ lack zygotic but not maternal *Ice*.

RNAi) (Huh et al., 2004a). *GMR-GAL4-UAS-Th-RNAi* flies have moderately small eyes, with an extensive loss of pigment (Fig. 7D). This phenotype was only partly suppressed in the absence of *Ice* (Fig. 7I). Finally, although many of the cell deaths discussed above are dependent on *Nc*, retinal degeneration induced by direct *Nc* overexpression in the eye (*GMR-Nc*) is not suppressed by p35 (Hawkins et al., 2000; Meier et al., 2000). These observations suggest that high level expression of *Nc* leads to cleavage of inappropriate substrates. Consistent with this hypothesis, removal of *Ice* was not associated with significant suppression of GMR-*Nc*-dependent retinal degeneration (Fig. 7E,J-M).

Ice participates in a non-apoptotic process, spermatid individualization

Spermatazoa are generated and mature within a germline syncytium. Differentiation of haploid syncytial spermatids requires that each spermatid become encapsulated by an independent plasma membrane, a process known as individualization (reviewed by Lindsley and Tokuyasu, 1980). This last process does not require cell death, but several lines of evidence suggest that it does use a number of pro-apoptotic components of the cell death machinery in non-apoptotic roles. These include *ark*, *hid*, *Nc*, *dcp-1*, *fadd*, and the immune-responsive caspase *dredd*, as well as p35-depdendent caspase activity (Arama et al., 2003; Huh et al., 2004b; Arama et al., 2005). To explore roles for *Ice* in spermatogenesis we examined testes from $Ice^{\Delta 1}$ mutants. As noted above, $Ice^{\Delta 1}$ males are fertile. However, thin sections from $Ice^{\Delta 1}$ testis showed a partial failure in individualization in almost all cysts (Fig. 8B,C, compare with wild-type cyst in Fig. 8A). These genetic experiments, using a *Ice*-null allele, demonstrate that *Ice* participates in, but is not absolutely essential for, the process of spermatid individualization.

Cleavage-specific anti-Ice antibodies function as accurate reporters of Ice activation in wing disc cells induced to die in response to X-irradiation

We previously generated a polyclonal rabbit antiserum containing antibodies directed against the C terminus of the Ice p20 fragment (QRSQTETD) that is generated following cleavage by Nc (Yoo et al., 2002). These antibodies (anti-active-Ice) recognize versions of Ice that have been cleaved, and label dying cells in several different contexts (Yoo et al., 2002). Cleaved versions of Ice, but not Dcp-1, and dying cells in *Drosophila* are also recognized by antibodies present in a polyclonal rabbit antiserum (CM1) raised against a related peptide, that corresponds to the C-terminus of the mammalian caspase 3-cleaved p20 fragment (Yu et al., 2002).

To explore the origins of anti-active Ice staining in dying cells, we exposed wing discs from larvae of several different genetic backgrounds to X-irradiation and stained for TUNEL, anti-active Ice and CM1. Un-irradiated wild-type wing discs showed only occasional TUNEL, anti-active Ice or CM1-positive cells (data not shown). By contrast, wild-type discs exposed to X-irradiation showed high levels of TUNEL staining (Fig. 9A,C,D,F), and of active-Ice (Fig. 9B,C) and CM1 (Fig. 9E,F) staining. Wing discs from irradiated $Ice^{\Delta 1}$ animals showed essentially no TUNEL (Fig. 9G,I,J,L), anti-active Ice (Fig. 9H,I) or CM1 (Fig. 9K,L) staining. As an internal control, and to demonstrate that the loss of Ice was responsible for the lack of TUNEL and anti-active Ice and CM1 staining, we also examined irradiated wing discs from animals expressing a microRNA targeting *Ice* (en-Ice-RNAi) in the posterior wing compartment. Consistent with the results obtained with the $Ice^{\Delta 1}$ mutant, TUNEL (Fig. 9M,O,P,R), anti-active Ice (Fig. 9N,O) and CM1 (Fig. 9Q,R) staining were each largely absent, specifically in the posterior wing compartment. Together, these results argue that the anti-active Ice and CM1 staining observed in wing disc cells stimulated to die is *Ice* dependent. Although we cannot rule out the possibility that the epitope recognized

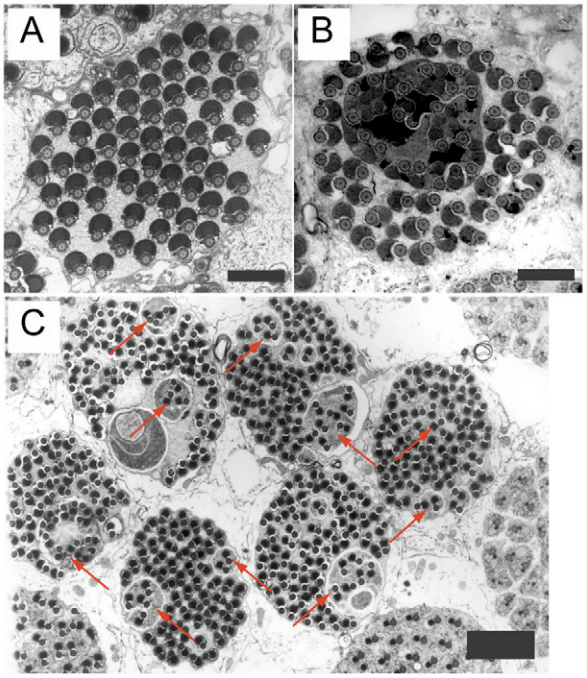

Fig. 8. *Ice* is important for spermatid individualization. (**A-C**) EM sections of elongated cysts of different genotypes: (A) wild type; (B) $Ice^{\Delta 1}$; (C) $Ice^{\Delta 1}$. Scale bars: 1 μm in A,B; 2.4 μm in C. Arrows in C indicate some regions within cysts in which individualization has failed. Animals homozygous for $Ice^{\Delta 1}$ lack maternal and zygotic *Ice*.

in dying cells is a product of Ice-dependent cleavage of substrates, the most parsimonious explanation is that this staining reflects recognition of cleaved Ice itself.

Cleavage-specific anti-Ice antibodies recognize Ice-independent epitopes in embryos and spermatogenesis

We also examined embryos with anti-active Ice and CM1 antibodies. Strong evidence that anti-active Ice and CM1 staining in embryos identifies dying cells comes from observations that antibody staining is essentially eliminated in embryos lacking *Nc* (Xu et al., 2005), or that are homozygous for the H99 deficiency (Yoo et al., 2002), which results in loss of almost all developmental death, and which removes *rpr*, *hid* and *grim*. However, to our surprise, and in contrast to our observations in wing discs, extensive anti-active Ice (Fig. 9T) and CM1 (Fig. 9V) staining was observed in embryos lacking both maternal and zygotic *Ice*. These observations suggest that in embryos anti-active Ice and CM1 recognize, in addition to activated Ice, other death-specific epitopes.

Elongated wild-type spermatids undergoing individualization also stain intensely with anti-active Ice and CM1 (Arama et al., 2003; Huh et al., 2004b) (Fig. 9W,Y). The observations presented in Fig. 8 demonstrate that Ice participates in normal spermatogenesis. To test the hypothesis that this staining represents the presence of activated Ice, we stained testes from $Ice^{\Delta 1}$ with anti-active Ice and CM1 sera. Both antisera (Ice, Fig. 9X; CM1, Fig. 9Z) labeled individualizing spermatids from mutant testis to the same extent as in wild type (Fig. 9W,Y), demonstrating that although these antibodies recognize dying cells very specifically in some contexts, they cannot be (primarily) labeling cleaved Ice in individualizing spermatids. Instead, this intense and specific staining must reflect the presence of an unknown individualization-specific epitope.

DISCUSSION

In mammals the effector caspases, caspase 3 and caspase 7, which are most closely related to Ice and Dcp-1, show a high level of functional redundancy or compensation. Thus, single mutants for either caspase are viable and healthy, while double mutants die before birth with many defects in apoptosis (Lakhani et al., 2006). By contrast, while loss of *dcp-1* has little effect on viability or apoptosis in most *Drosophila* tissues, loss of *Ice* results in many phenotypes attributable to, or suggestive of, defects in developmental or stress-induced apoptosis, indicating a non-redundant role for this caspase. Related phenotypes have recently been reported for flies carrying a missense mutation in *Ice* (Xu et al., 2006). All of the deaths we identify as being Ice dependent are also *Nc* dependent. However, our observations do not directly address the issue of whether the requirement we observe for *Ice* is achieved only through activation by *Nc*, or whether it also involves the activity of other proteases, such as Damm, Decay and Strica.

Only 20% of $Ice^{\Delta 1}$ third instar larvae survived to adulthood, with the rest dying during pupal development. Cell death plays a major role in pupal development, removing many larval structures, including gut, salivary glands, epidermis, muscle and neurons. Ice cannot be absolutely required for these deaths as a significant fraction of $Ice^{\Delta 1}$ animals survive to adulthood. However, defects suggestive of compromised cell death, such as persistent larval muscle and immature gut were often observed in $Ice^{\Delta 1}$ pupae. These were never seen in $Ice^{\Delta 1}$/+ pupae. We also observed large numbers of abnormal masses in $Ice^{\Delta 1}$ pupae. Similar masses were seen only very rarely in $Ice^{\Delta 1}$/+ animals. Interestingly, the frequency of these masses was increased in $Ice^{\Delta 1}$ animals that had been exposed to X-irradiation as first instar larvae. Although the origin of these structures is unknown, we speculate that they may arise as a consequence of defective cell death signaling. For example, in the larval wing disc, cells that are stressed, but prevented from dying, send signals to neighbors that promote their proliferation (Huh et al., 2004a; Perez-Garijo et al., 2004; Ryoo et al., 2004). Perhaps the masses represent cells or populations of cells that have failed to die and/or that have responded in some way to the prolonged presence of signals generated by undead or slowly dying cells that lacked *Ice*. If such signals exist in undead pupal cells, this may drive increased proliferation or other cell fate changes in surrounding cells that manifest themselves by the presence of these masses. A characterization of the origins and cell types that make up the masses will be required to address this hypothesis.

Although *Ice* has non-redundant roles as a death effector, several observations suggest that effector caspase redundancy and/or compensation does play important roles in *Drosophila*. As noted above, whereas most animals lacking *Ice* die during pupal stages, about 20% survive as fertile adults. This, in conjunction with the recent observation that *ark* mutants are completely pupal lethal (Akdemir et al., 2006; Mills et al., 2006; Srivastava et al., 2006), suggests that other (presumably caspase-dependent) pathways are important for bringing about cell death and phagocytosis of corpses. The fact that heterozygosity for $dcp\text{-}1^{Prev1}$ resulted in a further reduction in the survival of $Ice^{\Delta 1}$ pupae suggests that *dcp-1* might be a component of such a pathway. A similar conclusion is suggested by several observations of embryos and the larval salivary gland. We observed some decrease in cell death in embryos that lacked maternal and zygotic *Ice*, but many TUNEL-positive cells were still present. By contrast, embryos that lacked maternal and zygotic *Nc* showed very few TUNEL-positive cells (Xu et al., 2005). The stronger phenotype observed in embryos that lack *Nc* may reflect the fact that *Nc* itself cleaves targets that cooperate with *Ice* activity. Alternatively,

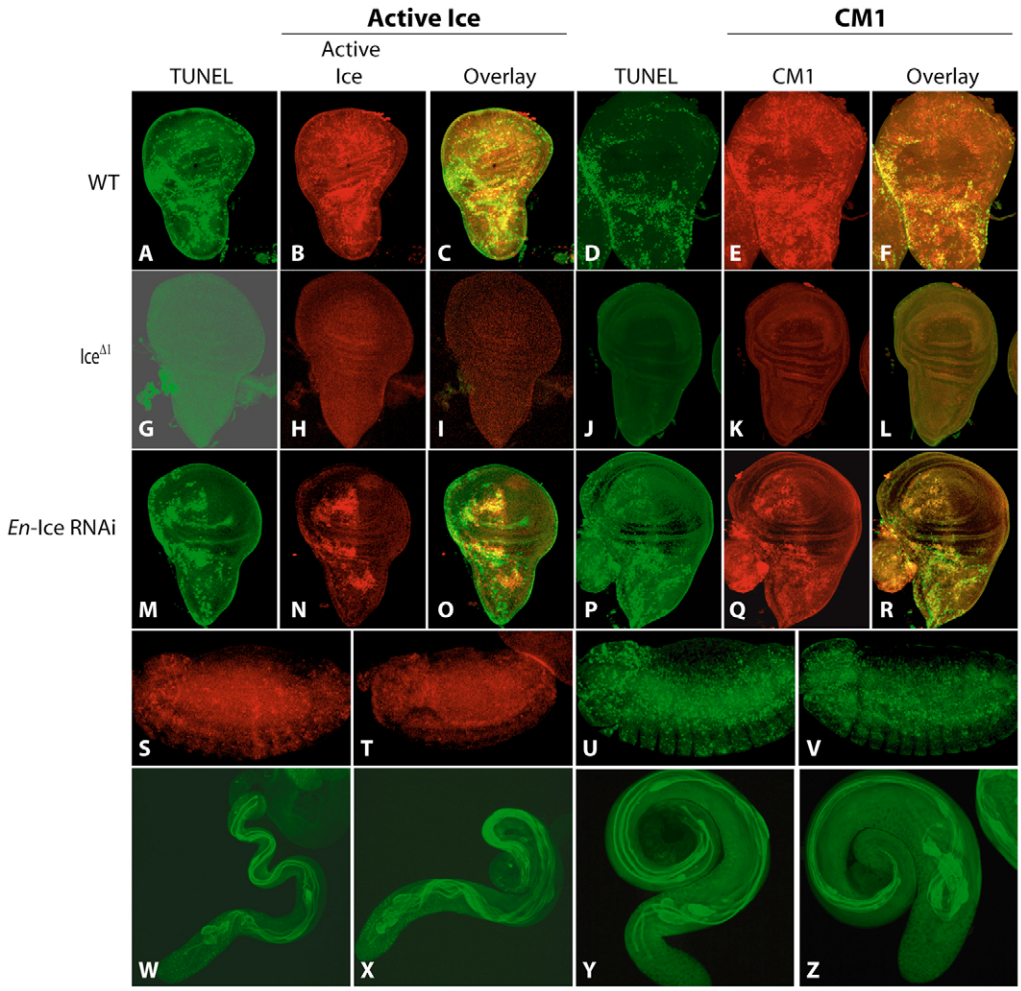

Fig. 9. Antibodies that recognize cleaved versions of Ice are accurate reporters of Ice activation in irradiated wing discs, but not in embryos and spermatids. (**A-R**) X-irradiated wing discs of various genotypes stained for TUNEL (A,C,D,F,G,I,J,L,M,O,P,R), anti-active Ice (B,C,H,I,N,O) or CM1 (E,F,K,L,Q,R). Genotypes are as indicated in the figure. Wild-type (**S**,**U**) or $Ice^{\Delta 1}$ (**T**,**V**) embryos stained with anti-active Ice (S,T) or CM1 (U,V). (**W-Z**) Wild-type (W,Y) or $Ice^{\Delta 1}$ testis stained with anti-active Ice (W,X) or CM1 (Y,Z).

and/or in addition, other *Nc*-dependent effector caspases such as Dcp-1 may be important. The fact that p35 is a potent inhibitor of cell death in the embryo is consistent with this latter possibility (Hay et al., 1994; Zhou et al., 1997). So, also, is our observation that anti-active Ice and CM1 recognize many cells in embryos lacking *Ice* (Fig. 7), but not embryos lacking *rpr*, *hid* and *grim*, or *Nc* (Yoo et al., 2002; Xu et al., 2005). The basis for the residual anti-active Ice and CM1 staining in $Ice^{\Delta 1}$ embryos is presently unknown. However, the fact that it requires upstream death activators suggests it may represent, in part, recognition of one or more cleaved caspase substrates with a sequence similar to that of cleaved Ice. Again, one likely candidate is Dcp-1. Embryos lacking *dcp-1* contain many anti-active Ice- and CM1-positive cells (data not shown). However, this is not unexpected as cleaved Ice also contributes to this staining. Mutants that remove both maternal and zygotic *Ice* and *dcp-1* will be needed to address the nature of this epitope. The above hypothesis is not inconsistent with our observation that anti-active Ice and CM1 act as accurate reporters of Ice activation in response to X-irradiation in wing discs. The irradiated wing disc may simply lack the unknown epitope-carrying protein(s) present in the embryo. Alternatively, X-irradiation may lead to the activation of a caspase cascade that does not promote the cleavage of the relevant protein(s), an issue that requires further exploration.

In the case of the larval salivary gland, cell death is also inhibited by expression of baculovirus p35 (Jiang et al., 1997; Lee and Baehrecke, 2001; Martin and Baehrecke, 2004), as well as by mutations in either *ark* (Mills et al., 2006; Akdemir et al., 2006) or *Nc* (Daish et al., 2004). However, as illustrated in Fig. 2, salivary gland death occurred in animals that lacked *Ice*. We cannot rule out the possibility that p35 has caspase-independent effects on salivary gland cell death. However, notwithstanding this possibility, a simple interpretation of our observations is that p35-sensitive caspases other than *Ice* contribute to salivary gland cell death. Dcp-1, for example, may contribute to these deaths, though it cannot be essential as $dcp\text{-}1^{Prev1}$ adults eclose at high frequency.

Finally, we identified a non-apoptotic role for *Ice* in spermatid individualization. Testes from $Ice^{\Delta 1}$ animals consistently showed defects in spermatid individualization similar to those seen when *ark*, *Nc* or *hid* activity was decreased (Huh et al., 2004b; Arama et al., 2005), with some spermatids failing to undergo individualization. However, other spermatids developed normally, and males lacking *Ice* are fertile. In the present work, we show that that CM1 and anti-active Ice staining of individualizing spermatids does not reflect the presence of active Ice, as high levels of spermatid-specific staining are observed in the complete absence of this protein. This staining is also not eliminated when Nc is downregulated through expression of a dominant-negative protein, or eliminated through mutation (Huh et al., 2004b; Arama et al., 2005). Wild-type levels of staining are also present in males lacking *dcp-1* (data not shown), hid, fadd or dredd (Huh et al., 2004b). Therefore, although we cannot exclude the possibility that spermatid anti-active Ice staining reflects the activity of an uncharacterized

caspase, at this point a relationship between this staining and caspase activity remains to be demonstrated. It will be interesting to determine if the cleavage-specific caspase 3 immunostaining observed in mammalian spermatids has a similar unexpected origin (cf. Kissel et al., 2005). That said, the epitope recognized by these sera serves as an excellent marker for the process of individualization, becoming apparent throughout spermatids just as the process initiates. Characterization of loci such as *Iceless*, mutation of which result in elimination of this staining (Huh et al., 2004b), may provide insight into the identity of the target recognized and its role in individualization.

Financial support was provided by NIH grant GM057422 to B.A.H., by NIH grant GM59136 to E.H.B., and by NIH grants NS042580 and NS048396 to M.G.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/17/3305/DC1

References

Abbott, M. K. and Lengyel, J. (1991). Embryonic head involution and rotation of male terminalia require the *Drosophila* locus *head involution defective*. *Genetics* **129**, 783-789.

Adam, G., Perrimon, N. and Noselli, S. (2003). The retinoic-like juvenile hormone controls the looping of left-right asymmetric organs in *Drosophila*. *Development* **130**, 2397-2406.

Akdemir, F., Farkas, R., Chen, P., Juhasz, G., Medved'ova, L., Sass, M., Wang, L., Wang, X., Chittaranjan, S., Gorski, S. M. et al. (2006). Autophagy occurs upstream or parallel to the apoptosome during histolytic cell death. *Development* **133**, 1457-1465.

Arama, E., Agapite, J. and Steller, H. (2003). Caspase activity and a specific cytochrome c are required for sperm differentiation in *Drosophila*. *Dev. Cell* **4**, 687-697.

Arama, E., Bader, M., Srivastava, M., Bergmann, A. and Steller, H. (2005). The two Drosophila cytochrome C proteins can function in both respiration and caspase activation. *EMBO J.* **25**, 232-243.

Arbeitman, M. N., Furlong, E. E., Imam, F., Johnson, E., Null, B. H., Baker, B. S., Krasnow, M. A., Scott, M. P., Davis, R. W. and White, K. P. (2002). Gene expression during the lifecycle of *Drosophila melanogaster*. *Science* **297**, 2270-2275.

Baehrecke, E. H. (2002). How death shapes life during development. *Nat. Rev. Mol. Cell Biol.* **3**, 779-787.

Baehrecke, E. H. (2003). Autophagic programmed cell death in *Drosophila*. *Cell Death Differ.* **10**, 940-945.

Baker Brachmann, C. and Cagan, R. L. (2003). Patterning the fly eye: the role of apoptosis. *Trends Genet.* **19**, 91-96.

Benedict, C. A., Norris, P. S. and Ware, C. F. (2002). To kill or be killed: viral evasion of apoptosis. *Nat. Immunol.* **3**, 1013-1018.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Cagan, R. L. and Ready, D. F. (1989). The emergence of order in the *Drosophila* pupal retina. *Dev. Biol.* **136**, 346-362.

Chen, P., Nordstrom, W., Gish, B. and Abrams, J. M. (1996). *grim*, a novel cell death gene in *Drosophila*. *Genes Dev.* **10**, 1773-1782.

Chen, P., Rodriguez, A., Erskine, R., Thach, T. and Abrams, J. M. (1998). *Dredd*, a novel effector of the apoptosis activators *reaper*, *grim*, and *hid* in *Drosophila*. *Dev. Biol.* **201**, 202-216.

Chew, S. K., Akdemir, F., Chen, P., Lu, W.-J., Mills, K., Daish, T., Kumar, S., Rodriguez, A. and Abrams, J. M. (2004). The apical caspase *dronc* governs programmed and unprogrammed cell death in *Drosophila*. *Dev. Cell* **7**, 897-907.

Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S. I. and Abrams, J. M. (2002). The damage-responsive Drosophila gene *sickle* encodes a novel IAP binding protein similar to but distinct from *reaper*, *grim*, and *hid*. *Curr. Biol.* **12**, 137-140.

Clem, R. J. (2001). Baculoviruses and apoptosis: the good, the bad, and the ugly. *Cell Death Differ.* **8**, 137-143.

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.

Cullen, K. and McCall, K. (2004). Role of programmed cell death in patterning the *Drosophila* antennal arista. *Dev. Biol.* **275**, 82-92.

Daish, T. J., 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.

Degterev, A., Boyce, M. and Yuan, J. (2003). A decade of caspases. *Oncogene* **22**, 8543-8567.

Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999a). DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA* **96**, 4307-4312.

Dorstyn, L., Read, S. H., Quinn, L. M., Richardson, H. and Kumar, S. (1999b). DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. *J. Biol. Chem.* **274**, 30778-30783.

Doumanis, J., Quinn, L., Richardson, H. and Kumar, S. (2001). STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. *Cell Death Differ.* **8**, 387-394.

Eschalier, G. (1997). *Drosophila Cells in Culture*. New York: Academic Press.

Fraser, A. G. and Evan, G. I. (1997). Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. *EMBO J.* **16**, 2805-2813.

Fraser, A. G., McCarthy, N. J. and Evan, G. I. (1997). drICE is an essential caspase required for apoptotic activity in *Drosophila* cells. *EMBO J.* **16**, 6192-6199.

Gleichauf, R. (1936). Anatomie und variabilitat des geschlechtapparates von *Drosophila melanogaster* (meigen). *Z. Wiss. Zool.* **148**, 1-66.

Goyal, L., McCall, K., Agapite, J., Hartwieg, E. and Steller, H. (2000). Induction of apoptosis by *Drosophila reaper*, *hid* and *grim* through inhibition of IAP function. *EMBO J.* **19**, 589-597.

Green, D. R. and Evan, G. I. (2002). A matter of life and death. *Cancer Cell* **1**, 19-30.

Grether, M. E., Abrams, J. M., Agapite, J., White, K. and Steller, H. (1995). The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev.* **9**, 1694-1708.

Harvey, N. L., Daish, T., Mills, K., Dorstyn, L., Quinn, L. M., Read, S. H., Richardson, H. and Kumar, S. (2001). Characterization of the *Drosophila* caspase, DAMM. *J. Biol. Chem.* **276**, 25342-25350.

Hawkins, C. J., Wang, S. L. and Hay, B. A. (1999). A cloning method to identify caspases and their regulators in yeast: identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. *Proc. Natl. Acad. Sci. USA* **96**, 2885-2890.

Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y. and Hay, B. A. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem.* **275**, 27084-27093.

Hay, B. A. (2000). Understanding IAP function and regulation: a view from *Drosophila*. *Cell Death Differ.* **7**, 1045-1056.

Hay, B. A. and Guo, M. (2006). Caspase-dependent cell death in *Drosophila*. *Annu. Rev. Dev. Cell Biol.* **22**, 623-650.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**, 2121-2129.

Hay, B. A., Wassarman, D. A. and Rubin, G. M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**, 1253-1262.

Hay, B. A., Huh, J. R. and Guo, M. (2004). The genetics of cell death: approaches, insights and opportunities in *Drosophila*. *Nat. Rev. Genet.* **5**, 911-922.

He, B. and Adler, P. N. (2001). Cellular mechanisms in the development of the *Drosophila* arista. *Mech. Dev.* **104**, 69-78.

Holley, C. L., Olson, M. R., Colon-Ramos, D. A. and Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nat. Cell Biol.* **4**, 439-444.

Huh, J. R. and Hay, B. A. (2002). Sculptures of a fly's head. *Nature* **418**, 926-927.

Huh, J. R., Guo, M. and Hay, B. A. (2004a). Compensatory proliferation induced by cell death in the *Drosophila* wing disc requires activity of the apical caspase Dronc in a nonapoptotic role. *Curr. Biol.* **14**, 1262-1266.

Huh, J. R., Vernooy, S. Y., Yu, H., Yan, N., Shi, Y., Guo, M. and Hay, B. A. (2004b). Multiple apoptotic caspase cascades are required in nonapoptotic roles for *Drosophila* spermatid individualization. *PLoS Biol.* **2**, 43-53.

Igaki, T., Yamamoto-Goto, Y., Tokushige, N., Kanda, H. and Miura, M. (2002). Down-regulation of DIAP1 triggers a novel *Drosophila* cell death pathway mediated by Dark and DRONC. *J. Biol. Chem.* **277**, 23103-23106.

James, E. R. and Green, D. R. (2004). Manipulation of apoptosis in the host-parasite interaction. *Trends Parasitol.* **20**, 280-287.

Jiang, C., Baehrecke, E. H. and Thummel, C. S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* **124**, 4673-4683.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H. and Miura, M. (1999). Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4- related caspase activator. *Mol. Cell* **4**, 757-769.

Kimura, K.-I., Kodama, A., Hayasaka, Y. and Ohta, T. (2004). Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila*. *Development* **131**, 1597-1606.

Kissel, H., Georgescu, M.-M., Larisch, S., Manova, K., Hunnicut, G. R. and Steller, H. (2005). The *Sept4* septin locus is required for sperm terminal differentiation in mice. *Dev. Cell* **8**, 353-364.

Klambt, C., Jacobs, J. R. and Goodman, C. S. (1991). The midline glia 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.
Kurada, P. and White, K. (1998). Ras promotes cell survival in *Drosophila* by downregulating *hid* expression. *Cell* **95**, 319-329.
Lakhani, S. A., Masud, A., Kuida, K., Porter, G. A., Jr, Booth, C. J., Mehal, W. Z., Inayat, I. and Flavell, R. A. (2006). Caspases 3 and 7, key mediators of mitochondrial events of apoptosis. *Science* **311**, 847-851.
Laundrie, B., Peterson, J. S., Baum, J. S., Chang, J. C., Fileppo, D., Thompson, S. R. 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.
Lee, C.-Y. and Baehrecke, E. H. (2001). Steroid regulation of autophagic programmed cell death during development. *Development* **128**, 1443-1455.
Lee, C.-Y., Simon, C. R., Woodward, C. T. and Baehrecke, E. H. (2002). Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Dev. Biol*. **252**, 138-148.
Lindsley, D. I. and Tokuyasu, K. T. (1980). Spermatogenesis. In *Genetics and Biology of Drosophila* (ed. M. Ashburner and T. R. Wright), pp. 225-294. New York: Academic Press.
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.
Macias, A., Romero, N. M., Martin, F., Suarez, L., Rosa, A. L. and Morata, G. (2004). PVF1/PVR signaling and apoptosis promotes the rotation and dorsal closure of the *Drosophila* male terminalia. *Int. J. Dev. Biol*. **48**, 1087-1094.
Madhavan, M. K. and Madhavan, K. (1980). Morphogenesis of the epidermis of adult abdomen of *Drosophila*. *J. Embryol. Exp. Morphol*. **60**, 1-31.
Martin, D. N. and Baehrecke, E. H. (2004). Caspases function in autophagic programmed cell death in *Drosophila*. *Development* **131**, 275-284.
Meier, P., Silke, J., Leevers, S. J. and Evan, G. I. (2000). The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J*. **19**, 598-611.
Mills, K., Daish, T., Harvey, K. F., Pfleger, C. M., Hariharan, I. K. and Kumar, S. (2006). The Drosophila melanogaster Apaf-1 homologue Ark is required for most, but not all, programmed cell death. *J. Cell Biol.* **172**, 809-815.
Muro, I., Hay, B. A. and Clem, R. J. (2002). The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J. Biol. Chem*. **277**, 49644-49650.
Muro, I., Monser, K. and Clem, R. J. (2004). Mechanism of Dronc activation in *Drosophila* cells. *J. Cell Sci.* **117**, 5035-5041.
Opferman, J. T. and Korsmeyer, S. J. (2003). Apoptosis in the development and maintenance of the immune system. *Nat. Immunol.* **4**, 410-415.
Perez-Garijo, A., Martin, F. A. and Morata, G. (2004). Caspase inhibition during apoptosis causes abnormal signaling and developmental aberrations in *Drosophila*. *Development* **131**, 5591-5598.
Quinn, L. M., Dorstyn, L., Mills, K., Colussi, P. A., 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.
Robertson, C. W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. *J. Morphol*. **59**, 351-399.
Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X. and Abrams, J. M. (1999). Dark, a *Drosophila* homologue of Apaf-1/CED-4, functions in an evolutionarily conserved death pathway. *Nat. Cell Biol*. **1**, 272-279.
Rodriguez, A., Chen, P., Oliver, H. and Abrams, J. M. (2002). Unrestrained caspase-dependent cell death caused by loss of Diap1 function requires the *Drosophila* Apaf-1 homolog, Dark. *EMBO J*. **21**, 2189-2197.
Ryoo, H. D., Gorenc, T. and Steller, H. (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and wingless signaling pathways. *Dev. Cell* **7**, 491-501.
Song, Z., McCall, K. and Steller, H. (1997). DCP-1, a *Drosophila* cell death protease essential for development. *Science* **275**, 536-540.
Sonnenfeld, M. J. and Jacobs, J. R. (1995). Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development* **121**, 569-578.
Srinivasula, S. M., Datta, P., Kobayashi, M., Wu, J. W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y. et al. (2002). *sickle*, a Novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-Inhibitory protein. *Curr. Biol*. **12**, 125-130.
Srivastava, M., Scherr, H., Lackey, M., Xu, D., Chen, Z., Lu, J. and Bergmann, A. (2006). ARK, Apaf-1 related killer in Drosophila, requires diverse domains for its apoptotic activity. *Cell Death Differ.* (in press).
Tenev, T., Zachariou, A., Wilson, R., Paul, A. and Meier, P. (2002). Jafrac2 is an IAP antagonist that promotes cell death by liberating Dronc from DIAP1. *EMBO J*. **21**, 5118-5129.
Thummel, C. S. and Pirotta, V. (1992). New pCaSpeR P element vectors. *Drosoph. Inf. Serv*. **71**, 150.
Vaux, D. L. and Silke, J. (2005). IAPs, RINGs and ubiquitylation. *Nat. Rev. Mol. Cell Biol*. **6**, 287-297.
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.
Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H. A. and Hay, B. A. (1999). The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**, 453-463.
Wharton, K. A. and Crews, S. T. (1993). CNS midline enhancers of the *Drosophila* slit and Toll genes. *Mech. Dev*. **40**, 453-463.
White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. *Science* **264**, 677-683.
White, K., Tahaoglu, E. and Steller, H. (1996). Cell killing by the *Drosophila* gene *reaper*. *Science* **271**, 805-807.
Wing, J. P., Karres, J. S., Ogdahl, J. L., Zhou, L., Schwartz, L. M. and Nambu, J. R. (2002). *Drosophila sickle* Is a novel *grim-reaper* cell death activator. *Curr. Biol*. **12**, 131-135.
Wolff, T. and Ready, D. F. (1991). Cell death in normal and rough eye mutants of *Drosophila*. *Development* **113**, 825-839.
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.
Xu, D., Wang, Y., Willecke, R., Chen, Z., Ding, T., Bergmann, A. (2006). The effector caspases *drICE* and *dcp-1* have partially overlapping functions in the apoptotic pathway in *Drosophila*. *Cell Death Differ.* doi: 10.1038/sj.cdd.4401920.
Yan, N. and Shi, Y. (2005). Mechanisms of apoptosis through structural biology. *Annu. Rev. Cell Dev. Biol*. **21**, 35-56.
Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M. R., Clem, R. J., Muller, H.-A. J. and Hay, B. A. (2002). Apoptosis inducers Hid, Rpr and Grim negatively regulate levels of the caspase inhibitor DIAP1 by distinct mechanisms. *Nat. Cell Biol*. **4**, 416-424.
Yu, S.-Y., Yoo, S. J., Yang, L., Zapata, C., Srinivasan, A. and Hay, B. A. (2002). A pathway of signals regulating effector and initiator caspases in the developing *Drosophila* eye. *Development* **129**, 3269-3278.
Zhou, L., Hashimi, H., Schwartz, L. M. and Nambu, J. R. (1995). Programmed cell death in the *Drosophila* central nervous system midline. *Curr. Biol*. **5**, 784-790.
Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L. M., Steller, H. and Nambu, J. R. (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. USA* **94**, 5131-5136.
Zhou, L., Song, Z., Tittel, J. and Steller, H. (1999). HAC-1, a *Drosophila* homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. *Mol. Cell* **4**, 745-755.
Zimmermann, K. C., Ricci, J. E., Droin, N. M. and Green, D. R. (2002). The role of ARK in stress-induced apoptosis in *Drosophila* cells. *J. Cell Biol*. **156**, 1077-1087.