Alternative Germ Cell Death Pathway in Drosophila Involves HtrA2/Omi, Lysosomes, and a Caspase-9 Counterpart

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SUMMARY

In both flies and mammals, almost one-third of the newly emerging male germ cells are spontaneously eliminated before entering meiosis. Here, we show that in Drosophila, germ cell death (GCD) involves the initiator caspase Dronc independently of the apoptosome and the main executioner caspases. Electron microscopy of dying germ cells revealed mixed morphologies of apoptosis and necrosis. We further show that the lysosomes and their catabolic enzymes, but not macroautophagy, are involved in the execution of GCD. We then identified, in a screen, the Parkinson’s disease-associated mitochondrial protease, HtrA2/Omi, as an important mediator of GCD, acting mainly through its catalytic activity rather than by antagonizing inhibitor of apoptosis proteins. Concomitantly, other mitochondrial-associated factors were also implicated in GCD, including Pink1 (but not Parkin), the Bcl-2-related proteins, and endonuclease G, which establish the mitochondria as central mediators of GCD. These findings uncover an alternative developmental cell death pathway in metazoans.

INTRODUCTION

Virtually all metazoan cells have the ability to commit suicide by activating a genetic program called programmed cell death (PCD) (Metzstein et al., 1998; Tittel and Steller, 2000). PCD functions to eliminate unwanted or potentially dangerous cells during the development and homeostasis of the organism, and the malfunction of this process is associated with the pathogenesis of a variety of diseases, including cancer and neurodegenerative disorders (White, 2006; Bredesen, 2008; Fuchs and Steller, 2011). Apoptosis, also known as type I cell death, is the most abundant and investigated form of PCD, characterized by a conserved sequence of cytological and morphological events, including cell detachment and shrinkage, nuclear condensation and segmentation, intact organelles, membrane blebbing, and disassembly into apoptotic bodies that are often engulfed by other cells. Biochemically, apoptosis is manifested by the activation of a unique family of cysteine proteases called caspases (Degterev et al., 2003). Caspases play a key role in the signaling and execution of apoptosis, and their activation is tightly controlled by activating and inhibitory proteins (Budihardjo et al., 1999; Salvesen and Dixit, 1999; Bader and Steller, 2009). Activation of caspase-9, the initiator caspase of the intrinsic apoptotic pathway, involves its recruitment to the apoptosome by Apaf-1-Cyt c complex, whereas the apical caspase of the extrinsic apoptotic pathway, caspase-8, is activated within the death-inducing signaling complex (DISC) (Zou et al., 1997; Ashkenazi and Dixit, 1998; Varfolomeev et al., 1998; Rodriguez and Lazebnik, 1999). The full activation of caspases often involves the inactivation of a conserved protein family of endogenous caspase inhibitors called the inhibitor of apoptosis proteins (IAPs) (O’Riordan et al., 2008; Bader and Steller, 2009). Active initiator caspases cleave and activate effector caspases (Rieder and Shi, 2004), which in turn orchestrate cell death through the proteolytic cleavage of hundreds of cellular proteins (Lüthi and Martin, 2007).

Despite their established role in apoptosis, the activation of effector caspases does not always lead to apoptosis, as some cells utilize restricted levels of caspase activity to promote a variety of nonapoptotic vital cellular processes (Feinstein-Rotkopf and Arama, 2009). Furthermore, there is emerging evidence from different experimental systems suggesting that some cells can undergo cell death in the absence of active caspases (Yuan and Kroemer, 2010). In addition to apoptosis, two other types of cell death pathways have been defined based on cytological, morphological, and biochemical/molecular characteristics (Edinger and Thompson, 2004). The type II autophagic cell death is mainly characterized by the accumulation of autophagy-related double-membrane-enclosed vesicles, which function to self-digest the cell by sequestering cellular materials to lysosomes, and many of the key components of the classical macroautophagy pathway are also important in this cell death pathway. Type III cell death, or necrosis, is defined by early plasma membrane rupture, dilation of cytoplasmic organelles (in particular mitochondria), and in some cells, an increase in cytoplasmic calcium, reactive oxygen species (ROS) accumulation, and/or lysosomal membrane permeabilization (LMP), and the concomitant actions of some lysosomal hydrolases in the cytoplasm. In addition, a specific necrotic pathway stimulated by the Fas/TNFR family of death-domain receptors signals the formation of an alternative multiprotein complex, which includes the death domain-containing kinase, RIP1, its interactor kinase, RIP3, and the further downstream kinase, MLKL (Degterev et al., 2008; He et al., 2009; Sun et al., 2012). Cell death pathways
exhibiting mixed morphologies were also reported, and recent analyses of molecular networks may point to some crosstalk between the different pathways (Rubinstein et al., 2011).

Nonapoptotic cell death pathways have received much attention recently because of their potential roles in the pathology of diseases and the promise for possible new therapies (Galuzzi et al., 2011). Despite recent growing interest in unraveling the components and mechanisms underlying alternative death pathways, progress in this area has been relatively slow. One reason for this is that “clean” alternative cell death pathways are often manifested only following nonphysiological conditions/manipulations in certain cell lines, and thus their relevance in normal development and homeostasis and in the pathology of diseases remained largely unknown. Although fascinating, thus far, only very few developmental paradigms of alternative cell death pathways have been described in organisms with conventional apoptotic caspases, such as Caenorhabditis elegans and Drosophila; the connections, if any, between these paradigms and the nonphysiological cell death pathways are still unclear (Berry and Baehrecke, 2007; Denton et al., 2009; Blum et al., 2011).

Here, we show that during normal spermatogenesis in the adult Drosophila testis, between 20%–30% of the spermatogonial cysts undergo spontaneous cell death, dubbed germ cell death (GCD). Immunofluorescence and genetic analyses of core components in the conventional apoptotic machinery reveal that GCD can proceed in the absence of the apoptosome and the main effector caspases, although it involves an apoptosis-independent function of the initiator caspase. Ultrastructural analysis demonstrates distinct morphological features of GCD reminiscent of both apoptosis and necrosis. Examinations of other alternative cell death pathway promoters indicate no role for macroautophagy in GCD, whereas the lysosomes and their catabolic enzymes are active and involved in this cell death pathway, which is also associated with ROS accumulation and cellular acidification. In a screen for mutants that dominantly attenuate GCD, we identified the fly ortholog of the Parkinson’s disease-associated mitochondrial protease, HtrA2/Omi. Functional studies imply that it is the catalytic activity of this protease and not the IAP-antagonizing function that is important for GCD and male fertility. Epistasis analyses place HtrA2/Omi upstream and in parallel to the lysosomal pathway. Finally, the mitochondrial-associated factors, Pink1, the Bcl-2-related proteins, and endonuclease G were all implicated in GCD, revealing a central role of the mitochondria in mediating this alternative cell death pathway.

RESULTS

Premeiotic Male Germ Cells Undergo Spontaneous Cell Death in Drosophila

In the course of our studies of late spermatogenesis in the adult Drosophila testis, we noticed that in nearly all adult testes, germ cells in some premeiotic spermatogonial cysts spontaneously and synchronously assume disintegrated morphology suggestive of cell death (the apical tip of a Drosophila testis is illustrated in Figure 1A). To start characterizing this phenomenon, testes were first stained with DAPI, to visualize the nuclei, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), which labels fragmented DNA in dying cells. Indeed, dying spermatogonial cysts could be identified by virtue of their rounded and packed nuclei and abundant TUNEL labeling (Figure 1B, insets). In more advanced stages of the cellular demotion, the DAPI signal disappeared, while the TUNEL signal further increased (Figure 1C, insets). Subsequent analyses of numerous testes from 1- to 7-day-old flies indicate an average of 1.65 ± 0.05 (mean ± SE, n = 600) dying spermatogonial cysts per testis. Most of this germ cell death (GCD) occurs at the eight-cell stage, albeit a low percentage of dying 4- and 16-cell spermatogonial cysts were also recorded (Figures 1B and 1C; data not shown). Finally, because the adult testis normally contains 5–8 spermatogonial cysts at each developmental stage (Lindsley and Tokuyasu, 1980), we estimate that between 20%–30% of the newly emerging spermatogonial cysts undergo spontaneous cell death in the adult testis.

GCD Is Independent of Effector Caspases

A key feature of apoptotic cell death is the activation of caspases, a process commonly visualized in situ using the anti-cleaved caspase-3 antibody, which specifically detects the cleaved active forms of the main effector caspases in Drosophila, Drice and Dcp-1 (Fan and Bergmann, 2010; Florentin and Arama, 2012). However, in contrast to the pronounced activation of caspases in terminally differentiating (individualizing) spermatids, no caspase activation was detected at earlier stages and, in particular, during GCD (Figure 1D). Conversely, marked expression of activated caspases was detected in ectopically dying spermatogonial cells following targeted knockdown of the major fly IAP, Diap1 (Figure 1E; Figure S1A available online). Collectively, these findings indicate no association of spontaneous GCD with detectable levels of activated caspases.

To directly test this hypothesis, we first examined the effect of complete loss-of-function of Drice (drice<sup>de</sup>) and Dcp-1 (dcp-1<sup>de</sup>) on GCD levels. Significantly, not only were the levels of GCD not reduced in these mutants, a mild increase was recorded in both mutants (Figures 2A–2C). We then tested for a possible additive effect by specifically expressing the pan-effector caspase inhibitor protein from baculovirus, p35, and the Drosophila caspase inhibitor, Diap1, in spermatogonial cells. Indeed, a marked, 50%–80%, increase in GCD levels was detected when effector caspase activity was globally inhibited (Figures 2D–2G and S1B). Therefore, GCD can proceed in the absence of effector caspase activity, which in turn acts to inhibit this cell death pathway.

The Initiator Caspase Dr onc Promotes GCD in an Apoptosome-Independent Manner

The main activator of the effector caspases during Drosophila apoptosis is the initiator caspase-9 ortholog, Dr onc (Chew et al., 2004; Xu et al., 2005). As in vertebrates, apoptosome assembly and Dr onc activation is usually triggered by binding of pro-Dr onc to the Apaf-1-like adaptor protein, Ark, and in accordance, inactivation of these proteins lead to almost identical phenotypes (Xu et al., 2005; Srivastava et al., 2007). To test the effect of the apoptosome on GCD, we examined several independent loss-of-function alleles of d r onc and ark. Unexpectedly, however, whereas a marked increase in GCD
levels was recorded in the ark mutants, the dronc mutants displayed a significant 40%–60% decrease in GCD levels (Figures 2H–2J). There was no effect on GCD levels in mutants of the two other initiator-like caspases, Strica and Dredd (Figure 2J; data not shown). Altogether, these results suggest that Dronc promotes GCD in an apoptosome-independent manner.

**GCD Displays Mixed Morphologies**

Apoptosis has been originally characterized by virtue of its unique cellular morphologies (Wyllie et al., 1980). To explore the morphological traits of spontaneous GCD, we used transmission electron microscopy (TEM) to look at ultrathin sections of the apical tips of testes. These traits were subsequently contrasted with morphological features of apoptotic spermatagonia triggered in the diap1 RNAi-expressing flies (Figure 1E). Spontaneously dying spermatogonial cysts could be distinguished from their healthy counterparts based on their unusually dense and disintegrated morphologies (red and white arrows, Figures 3A, 3D, and 3G). In addition to significant cellular shrinkage, these cells also exhibited chromatin condensation and chromosomal DNA fragmentation, both features that are reminiscent of apoptosis (a purple asterisk in Figure 3B and green arrowheads in Figure 3C, respectively), and which were also associated with apoptotic spermatagonia (Figure S2C). Unlike apoptosis, however, the nuclei in spontaneously dying spermatagonia remained largely intact and were enclosed by membranes even during late degenerative stages (Figures 3B, 3C, and 3E). Of note, the crenellated morphology of the nuclei is not unique to GCD, as healthy spermatogonial cells normally contain unusually crenellated nuclei at these stages. Nonetheless, apoptotic spermatagonia exhibited increased nuclear crenellation and were sometimes also mildly fragmented.

**Figure 1. Spontaneous GCD Is Associated with DNA Fragmentation but Not Effector Caspase Activation**

(A) Schematic representation of the Drosophila testis apex. At the very tip of the testis are the nondividing somatic hub cells (orange), which constitute a niche for the germline stem cells (GSC; blue). Asymmetric division of a GSC yields a stem cell and a gonialblast (dark purple). The latter then undergoes four mitotic divisions with incomplete cytokinesis producing interconnected 2-, 4-, 8-, and 16-cell spermatogonial bundles called cysts (light purple), which, after an extensive growth phase, enter meiosis as primary spermatocytes. Germ cell cysts remain enveloped by a pair of somatic “cyst cells” (light blue), originating from cyst progenitor cells (CPC; green), throughout spermatogenesis. Note the illustrated dying eight-cell spermatogonial cyst (red).

(B and C) Apical tips of wild-type testes stained to visualize the nuclei (DAPI; blue) and fragmented DNA (TUNEL; red). The insets present channel separations and enlargements of the squared areas.

(D) A wild-type testis labeled as in (B) and additionally stained to visualize activated effector caspases (cCasp.3; green). IS, individualizing spermatids; WBs, waste bags.

(E) A testis expressing a diap1 RNAi transgene in spermatogonial cells by the bam-Gal4 driver line was labeled as in (D). Dying cysts are either active caspase-positive but TUNEL-negative (early cell death stage; arrows) or TUNEL-positive with a faint active caspase signal (late stage; white arrowheads). A spontaneously dying spermatogonial cyst in a region excluded from the bam-Gal4 expression domain is indicated by a yellow arrowhead.

Scale bars, 20 μm. See also Figure S1.
In addition, in both cell death types, only mild nuclear condensation was recorded, which may be secondary to the global condensation of these dying cells (the circumference of a dying cell nucleus and the circumference of its healthy neighboring counterpart are indicated in red and white, respectively, Figure 3E; Figure S2C). As opposed to apoptosis, where organelles remain largely intact, spontaneously dying spermatogonia, but not apoptotic spermatogonia, exhibited deformed mitochondria with abnormally swollen cristae (yellow asterisks in Figures 3 F and 3I; compare with Figures S2 A, S2B, and S2D). Also, degradative foci accompanied by clear vesicles and/or disorganized membranes and other materials, but not autophagosome-like double-membrane vesicles, were often detected during GCD, indicating extensive catabolic activity (blue and yellow arrowheads, respectively, Figures 3B and 3H).

On the other hand, other typical apoptotic features, including significant cell detachment, membrane blebbing, and disassembly into apoptotic bodies were all readily detected in the apoptotic spermatogonia, but not in spontaneously dying spermatogonia (Figures S2 D, S2D, and S2D′, receptively). We conclude that GCD is manifested by coexistence of mixed morphologies, some of which are reminiscent of apoptosis and necrosis, but not autophagic cell death.

We occasionally detected spermatogonial cysts at advanced GCD degradative stages that were surrounded by common cellular material, implying that as opposed to the piecemeal clearance of apoptotic bodies, dying spermatogonial cysts are engulfed as single entities by other cells (Figure S2E).
Furthermore, TUNEL labeling of testes expressing GFP under the control of the phagocyte-specific driver, croquemort (crq)-Gal4 (Franc et al., 1996), revealed some unusually large and often amorphic GFP-expressing cells, which contain several separated TUNEL-positive cellular entities (arrows in Figures S2F and S2G). Therefore, phagocytes residing in the testis appear to clear spontaneously dying spermatogonial cysts.

GCD Is Associated with High Lysosomal Activity, ROS Accumulation, and Cellular Acidification, but Not Macroautophagy

Macroautophagy has been suggested to play a role in developmental cell death of certain Drosophila cells (Ryoo and Baehrbecke, 2010). To investigate whether the autophagic machinery may also be involved in GCD, we first examined flies with loss-of-function mutations in two major autophagy-related genes, atg8 and atg7. Furthermore, we overexpressed a dominant-negative form of yet another important autophagy protein, Atg1, in early spermatogonial cells. However, no significant effects on the levels of GCD have been recorded in any of these mutant backgrounds (Figure S3A). Consistently, expression of the common LC3(Atg8)-GFP autophagy reporter in these cells revealed no typical puncta of lipidated Atg8 accumulating on autophagosomal vesicles, but rather more homogenous cytoplasmic GFP distribution in living cells and a much dimmer signal in dying cells (Figure S3B). Taken together, our ultrastructural, genetic, and cytological analyses do not support a role for macroautophagy in GCD.

One of these Atg8-based autophagy reporters was fused in tandem to eGFP and mCherry, thus labeling autophagosomes in both green and red fluorescence, whereas autolysosomes are in red only because of the quenching of the eGFP in the acidic environment of the lysosomes (Nezis et al., 2010). Interestingly, despite the dimming of the eGFP signal, the mCherry signal was still pronounced in dying spermatogonial cysts, suggesting that the eGFP protein is not degraded but rather quenched because of global acidification of the cellular environment during GCD (Figure S3C).

The necrosis-like morphological features and the evidence of cellular acidification prompted us to further explore other possible mechanistic similarities, such as ROS production or lysosomal activity. Staining testes with the oxidant-sensitive dye, dihydroethidium (DHE), revealed specific accumulation of ROS at the time when dying spermatogonial cysts became round and compact and the DNA was still visible (mid-GCD stage; Figure 4A). Furthermore, using the lysosomal probe, LysoTracker, which selectively accumulates in cellular compartments with low internal pH, revealed a profound signal in what appeared to be dying spermatogonial cysts during mid- and late-GCD stages based on nuclear morphology (Figure 4B). Indeed, when testes were costained with LysoTracker and TUNEL, the dying cysts were positive for both (Figure 4C).

Next, to directly investigate the involvement of lysosomes in GCD, we examined the effects of mutations in lysosomal catabolic or biogenesis genes. Significantly, mutant flies homozygous for either a null allele of the abundant aspartyl protease, cathepsin D (cathD1), a hypomorphic allele of the acid Deoxyribonuclease (DNase) II (dnaseIIlo), or two hypomorphic alleles of the genetically linked lysosomal biogenesis proteins, Deep-orange and Carnation (dor4 and car1, respectively), all displayed a marked, 40%–60%, decrease in the levels of GCD (Figures 4D–4F). Of note, in addition to the reduction in GCD levels, TUNEL labeling often appeared diffused and/or incomplete in the dnaseII mutants, further demonstrating the role of this nuclease in DNA fragmentation during GCD (Figures S3D and S3E). Collectively, these findings...
Figure 4. ROS Accumulation, High Lysosomal Activity, and Some Lysosomal Hydrolases Are Involved in GCD

(A–C) Apical tips of wild-type testes stained with (A and B) Hochest or (C) DAPI to reveal the nuclei (blue) and either (A) DHE to detect ROS (red) or (B and C) LysoTracker to detect lysosomal activity (red). (C) To reveal the dying cysts, testes were also labeled with TUNEL (green). (B) A yellow circle outlines a dying cyst with visible packed nuclei (an early-mid GCD stage), whereas a white circle outlines a dying cyst with faint Hochest signal of the nuclei (an advanced GCD stage). (C) Arrowheads indicate two dying cysts at either early-mid GCD stage (the lower cyst) or advanced stage (the upper cyst).

(D and E) Representative testes from the indicated genotypes were stained as in Figure 1B.

(F) Quantification of GCD levels in testes from the indicated genotypes. All calculations were performed as in Figure 2C.

Scale bars, 20 μm (A–E). See also Figure S3.
A Genetic Screen Identifies the Mitochondrial Serine Protease HtrA2/Omi as an Important Mediator of GCD

To gain more insight into the regulation of GCD, we conducted a deficiency screen for chromosomal deletions that dominantly suppress GCD, as visualized by TUNEL labeling. After narrowing down one of the suppressive regions to 35 genes, we used a candidate approach to prioritize genes based on high expression in the tests (according to the FlyAtlas database) and known functions. Importantly, one of these genes encodes the *Drosophila* ortholog of the mitochondrial serine protease, HtrA2/Omi, which has been previously implicated in both cell death and the pathology of Parkinson’s disease and is thus a strong candidate for the recorded effect on GCD. To explore this possibility, we first generated two partially overlapping small deficiency lines, omi<sup>PT1</sup> and omi<sup>PT2</sup>, covering the entire *htrA2/omi* gene as well as several adjacent genes (Figure 5A). In parallel, using the fly-TILL service, we identified two mutant alleles of *htrA2/omi*, omi<sup>RD</sup> (containing a 123 bp in-frame deletion), which lacks 41 amino acids (aa) in the protease domain, including the catalytic serine, and omi<sup>V110E</sup> (contains a T-to-A transversion), where a conserved valine was replaced with glutamic acid within the homotrimerization domain (Figure 5B). Finally, in the course of this study, an *htrA2/omi* null allele, omi<sup>A1</sup>, was also generated (Figure 5A) (Tain et al., 2009).

Initial analysis of these *htrA2/omi* mutants revealed that all are viable and female fertile but male sterile, either as homozygotes or in trans to the small deficiencies or to one another. This is in agreement with previous studies reporting that inactivation of *htrA2/omi* caused male sterility in flies (Yun et al., 2008; Tain et al., 2009). We then examined whether GCD levels may be attenuated in the *htrA2/omi* mutants. Critically, in accordance to the dominant effect on GCD recorded for the original deficiency from the screen, flies heterozygous for either *omi<sup>PT1</sup>*, *omi<sup>PT2</sup>*, or *omi<sup>RD</sup>* displayed 50%–60% reduction in the levels of GCD, uncovering a dosage-sensitive effect (haploinsufficiency) of the *htrA2/omi* gene (Figure 5C). This effect may be attributed to the fact that HtrA2/Omi is functional in cell death as a homotrimer rather than a monomer (Li et al., 2002). Consistent with this idea, suppression of GCD was not significantly enhanced in homozygotes or transheterozygous allelic combinations of the *htrA2/omi* mutants, including the null *omi<sup>A1</sup>* allele (Figures 5C, 5D, and 5G). Of note, flies heterozygous for the H99 deficiency, which deletes the four primary proapoptotic genes, reaper, grim, hid, and sickle, displayed no significant effect on GCD levels (Figure 5C).

To further validate that the loss of *htrA2/omi* is the cause of the attenuation in GCD, we generated two “rescue” transgenes inserted at a defined site of the *Drosophila* genome. The first transgene, omi<sub>IR-1</sub>BAC, is a large (bacterial artificial chromosome; BAC) genomic clone encompassing *htrA2/omi* and three additional flanking genes, whereas the second transgene, omi<sub>IR-2</sub>, is a smaller genomic segment containing the *htrA2/omi* locus only (red bars in Figure 5A). A third “rescue” transgene, omi<sub>IR-1</sub>, which moderately expands beyond the 5’ and 3’ sequences of omi<sub>IR-2</sub>, but is still confined to only the *htrA2/omi* locus, was obtained from another group (Figure 5A) (Tain et al., 2009). All the “rescue” transgenes were then crossed to the *omi<sup>PT1/IFD</sup>* transheterozygous mutants and analyzed for their ability to restore normal levels of GCD. Two copies of each of the three transgenes restored GCD levels to about 80% of the levels in wild-type testes, confirming the important role of HtrA2/Omi in promoting GCD (Figures 5E and 5F; Figures 6B and 6C). It is noteworthy that when tested for its ability to restore normal levels of GCD in a yet another mutant background, (i.e., the *omi<sup>A1</sup>* homozygotes), the omi<sub>IR-1</sub> transgene elevated the levels of GCD to 120% of that in wild-type, indicating some alterations in the “rescue” efficiency of the transgenes in the different mutant backgrounds (Figure 5G).

The Catalytic Activity of HtrA2/Omi, and Not the IAP-Dependent Function, Is Important for Both GCD and Male Fertility

After being processed in the mitochondria, the mature form of HtrA2/Omi exposes a N-terminal IAP-binding motif (IBM), related to the tetrapeptide motifs found in IAP antagonists of the Reaper-family proteins in *Drosophila* and Smac/DIABLO in mammals (Suzuki et al., 2001). Various apoptotic insults in cell culture were reported to trigger translocation of the mature HtrA2/Omi to the cytosol, where it can promote either caspase-dependent or -independent cell death by binding to IAPs or through its self-protease activity, respectively (Vande Walle et al., 2008). To gain more insight into the function of HtrA2/Omi in promoting GCD, we performed functional studies in vivo by mutating either the catalytic serine or the three suggested IBMs of HtrA2/Omi within the *omi<sub>R-2</sub>* “rescue” transgene and testing whether they can still rescue GCD levels in the *omi<sup>PT1/IFD</sup>* transheterozygous mutants (Figures 6A and 6E). Importantly, the elimination of the catalytic serine, not the IBMs, significantly (albeit not completely) deteriorated the ability of the *omi<sub>R-2</sub>* transgene to induce GCD in the mutant (Figures 6B and 6C). Therefore, it is the catalytic activity of HtrA2/Omi, and not the IAP-dependent function, that may be important for its function in GCD.

Fertility tests revealed that *omi<sup>PT1/IFD</sup>* mutant males produce only 2% of the number of progeny generated by wild-type (Figure 6D). Whereas the cause of this sterility is unclear as these mutants still produce mature and motile sperm cells (data not shown), it may be, at least in part, unrelated to the role of HtrA2/Omi in GCD, because normally the majority of spermatogonial cysts (70%–80%) are not destined to die. Nevertheless, the function of HtrA2/Omi in male fertility appears to depend on its catalytic activity rather than its ability to interact with IAPs, because expression of the IBM-less form of HtrA2/Omi, but not the catalytic mutant, rescued the sterility of *omi<sup>PT1/IFD</sup>* mutant males to similar levels as those obtained with the intact, *omi<sub>R-2</sub>* transgene (about 75% of wild-type levels; Figure 6D). Interestingly, although the mutation in the catalytic serine strongly deteriorated the ability of the *omi<sub>R-2</sub>* transgene to rescue GCD and sterility, it may still preserve some residual activity because it was sufficient to cause a 25% increase in GCD levels, as well as a 3-fold increase in the number of progeny produced by mutant males (Figures 6C and 6D).
Figure 5. The Mitochondrial Serine Protease HtrA2/Omi Is an Important Mediator of GCD

(A) An in-scale diagram of the genomic organization of htrA2/omi (red arrow trapezoid) and other flanking genes (black arrow trapezoids) is highlighted in pink. The directions of the arrows correspond to the relative 5'–3' directions of the genes. Chromosomal bands (gray) are indicated above. Green bars denote the deleted areas in the small deficiencies and the omiD1 mutant, whereas dashed gray lines depict their distal and proximal breakpoints. The genomic segments used in the “rescue” constructs are indicated by red bars.

(B) A schematic structure of the Drosophila HtrA2/Omi protein. The relative locations of the different domains and the fly-TILL mutants are indicated. MTS, mitochondrial targeting sequence.

(C) Quantification of GCD levels in testes from the indicated genotypes. All calculations were performed as in Figure 2C.

(D and E) Representative testes from the indicated genotypes were stained as in Figure 1B. Scale bar, 20 μm.

(F and G) Quantification of GCD levels in testes from the indicated genotypes. The presence of a specific “rescue” transgene is depicted in red letters. All calculations were performed as in Figure 2C.

See also Figure S4.
HtrA2/Omi and the Lysosomes Act in Both Common and Parallel Pathways during GCD

Lysosomes have been implicated in both the initiation and execution of cell death (Kroemer and Jaätelä, 2005; Golstein and Kroemer, 2007). To explore the relationship between the mitochondria and lysosomes in GCD, we performed epistatic experiments by first analyzing double mutants for htrA2/omi and cathD. Significantly, GCD levels in the double mutant were further decreased by half, from 40% of the wild-type levels in the single mutants to about 20% in the double mutant, suggesting the existence of some independent functions of these two pathways in GCD (Figure S4A). However, when measuring the levels of LysoTracker-positive spermatogonia in htrA2/omi mutant testes, we recorded a 50% decrease as compared to wild-type levels, indicating that lysosomes act both downstream and in parallel to HtrA2/Omi to promote GCD (Figure S4B).

The Parkinson’s Disease-Associated Gene pink1, Not parkin, Is Involved in GCD

Previous genetic studies in mouse and Drosophila have suggested that HtrA2/Omi may physically and genetically interact with the Parkinson’s disease (PD)-associated mitochondrial kinase, Pink1 (Plun-Favreau et al., 2007; Tain et al., 2009). Pink1 has been demonstrated to act upstream of another important PD-associated gene, parkin, to promote a mitochondrial homeostasis pathway (Vande Walle et al., 2008). We therefore tested whether the pink1-parkin pathway may also be involved in GCD. Whereas a significant 40% decrease in GCD levels was recorded in loss-of-function mutants for pink1 (pink1B9), no significant effect was detected in a null allelic combination of parkin (park25/Df) (Figure S5A). Previous reports in Drosophila have similarly demonstrated common phenotypes for pink1 and htrA2/omi mutants that are distinct from the phenotypes shared by pink1 and htrA2/omi mutants (Yun et al., 2008; Tain et al., 2009). Furthermore, although mutations in all three genes cause male sterility, phenotypic analyses imply distinct roles of Parkin and HtrA2/Omi during late spermatogenesis (data not shown; Yun et al., 2008). We therefore conclude that the pink1-htrA2/omi pathway is involved in GCD independently of Parkin and that both pathways act at different stages to promote proper sperm production.

The Bcl-2 Family Proteins Debcl and Buffy and the Mitochondrial Nuclease EndoG Are Associated with GCD

Similar to Cyt-c, HtrA2/Omi is also released from the mitochondria into the cytosol during apoptosis in mammals (Vande Walle et al., 2008), a process mediated by the proapoptotic members of the Bcl-2 family (Youle and Strasser, 2008). Two members of this protein family exist in Drosophila, Debcl and Buffy, and...
their involvement in apoptosis is marginal, if any (Sevrioukov et al., 2007; Galindo et al., 2009). We examined the levels of GCD in strong debcl (debcl€25€) and buffy (buffyH37) mutants, using homozygous, hemizygous (in trans to deficiencies), and double-mutant allelic combinations (Sevrioukov et al., 2007). All these mutant allelic combinations displayed a 60%–80% reduction in GCD levels, which was not further enhanced in the double mutants, suggesting that both proteins may act in a linear pathway to promote GCD (Figure S5B). These findings suggest an important role of the Drosophila Bcl-2 proteins in developmental cell death.

Endonuclease G is another prodeath enzyme, which resides in the mitochondrial intermembrane space and is released to the cytosol during apoptosis (Widlak and Garrard, 2005). A loss-of-function allele of the ubiquitously expressed archetype endoGMB07150, has been recently described (DeLuca and O’Farrell, 2012). Using this mutant, we detected a 60% decrease in GCD levels (Figure S5C). Altogether, these findings establish the mitochondria as central regulators of GCD.

DISCUSSION

In the present study, we describe an alternative cell death pathway in Drosophila, distinct from the conventional apoptotic pathway, which operates to execute some of the early developing male germ cells in the adult testis (Figure 7).

Caspases and GCD

Our findings that GCD levels increase when caspase activity is blocked is reminiscent of a phenomenon previously noted in certain mammalian cells lacking key apoptotic components, yet induced to undergo apoptosis; instead of maintaining cellular viability these cells may shift the apoptotic pathway to another cell death pathway (Vercammen et al., 1998). Although the mechanisms and physiological relevance of this phenomenon are still vague, it has been hypothesized that some cells may maintain baseline levels of caspase activity in order to prevent, possibly through cleavage and inactivation of proapoptotic factors, a default induction of necrosis (He et al., 2009). It is important to note that in addition to this phenomenon, GCD shares other similarities with certain necrotic pathways, including some morphological traits. In particular, Jäättelä and colleagues have described an alternative cell death pathway induced by tumor necrosis factor (TNF) in murine embryonic fibroblasts (MEFs), which largely proceeds in the absence of effector caspase activity but requires the lysosomal pathway. Strikingly, similar to GCD, which involves Dronc but not Ark, it is caspase-9 and not Apaf-1 that is required for this TNF-induced cell death pathway. Furthermore, caspase-9 activity in these cells was shown to trigger LMP and the concomitant release of cathepsins into the cytosol (Gyrd-Hansen et al., 2006). However, the signals that trigger the lysosomal pathway in MEFs may be distinct from GCD, as mutations in the sole fly TNF ortholog, Eiger, did not attenuate GCD (data not shown). Furthermore, whereas in MEFs, the apical caspase-8 is required for the apoptosome-independent activation of caspase-9, loss-of-function mutants for the two other initiator-like caspases in Drosophila, Dredd and Strica, did not affect GCD levels.

The Lysosomal Pathway in GCD and the Crosstalk with the Mitochondrial Pathway

The notion that under certain conditions the lysosomes can serve as “suicide bags” is not a new one (Kirkegaard and Jäättelä, 2009). Lysosomes contain over 50 different hydrolases, including proteases that are highly important for not only nonspecific but also selective protein degradation (Turk et al., 2002). Two major mechanisms for the action of the lysosomes in cellular degradation during alternative cell death pathways have been proposed: sequestration of cellular contents to lysosomes by the autophagic machinery or spillage of lysosomal catabolic enzymes to the cytosol following LMP induction. Interestingly, whereas most of these enzymes function optimally in the acidic environment of the lysosomal lumen, some of them, including cathepsins B and D, are also functional in the cytosol (Golstein and Kroemer, 2007; Kirkegaard and Jäättelä, 2009). Our findings that GCD is not dependent on the autophagy pathway, while it is still associated with cytosolic acidification and lysosomal activity, including cathepsins D, favors an LMP-related mode of action in GCD. This idea is also supported by the finding that dying spermatogonia accumulate ROS, which has been proposed to be an important inducer of LMP (Kroemer and Jäättelä, 2005).

The role of the lysosomes in promoting GCD, as well as the crosstalk between lysosomes and the mitochondria may be...
complex. The current study suggests that the lysosomes may act both downstream and in parallel to the mitochondrial pathway and fulfill at least some of the execution functions of GCD. Both the mitochondria and the lytic vacuole (the functional equivalent of the lysosome) have been recently implicated in a developmental program of nuclear destruction during yeast sporogenesis under starvation, reminiscent of some aspects of GCD, which raises the hypothesis that GCD might have evolved before the emergence of the caspases and apoptosis (Eastwood et al., 2012).

**HtrA2/Omi and the Mitochondrial Pathway in GCD**

One exciting implication of the current study concerns the discovery of a role for HtrA2/Omi in developmental cell death. A large body of circumstantial evidence indicates two distinct mechanisms of action for HtrA2/Omi in mammalian cell death. HtrA2/Omi can induce caspase activation and apoptosis by binding to IAPs and relieving their inhibition of caspases, but it can also trigger caspase-independent cell death through its own serine protease activity (Suzuki et al., 2001; Li et al., 2002). Interestingly, several lines of evidence from the current work and other studies suggest that it is the catalytic activity of HtrA2/Omi, and not its IAP-dependent function, that is important during physiological cell death. In accordance, although these IBDs were shown to interact with the baculovirus IAP repeat (BIR) domains of Diap1, cell death induced by ectopic expression of the different mature (cleaved) forms of HtrA2/Omi in S2 cells and the *Drosophila* eye could not be blocked by coexpression of the baculoviral effector caspase inhibitor protein, p35, indicating that at least in *Drosophila*, HtrA2/Omi mainly induces caspase-independent cell death (Igaki et al., 2007; Challa et al., 2007; Khan et al., 2008). Indeed, as opposed to the compelling evidence for the importance of the Reaper-family proteins in the induction of most if not all developmental and stress-induced cell death in *Drosophila* (Fuchs and Steller, 2011), HtrA2/Omi has been shown to be dispensable for apoptosis in flies (Yun et al., 2008; Tain et al., 2009). Likewise, htrA2/omi knockout/mutant mice displayed no apoptosis-related phenotypes (Jones et al., 2003). It is also interesting to note that the IBM of HtrA2/Omi is not conserved in all mammalian homologs (Li et al., 2002). Therefore, disruption of the IAP-caspase interaction may not be the major cell death function of HtrA2/Omi in at least some mammals.

Very little is known about how HtrA2/Omi protease activity can trigger caspase-independent cell death. Overexpression of an extramitochondrial form of HtrA2/Omi in mammalian cells induced caspase-independent cell death with atypical morphology, largely reminiscent of the morphological features of GCD, including some apoptosis-like morphological changes (i.e., cell rounding and shrinkage) and the lack of other apoptotic events, implying that HtrA2/Omi may either directly or indirectly trigger the cleavage of at least some caspase substrates (Suzuki et al., 2001). Along this line, several htrA2/Omi cytosolic substrates were identified in a proteomic study, most of which are also known targets of caspases (Vande Walle et al., 2007).

In conclusion, whereas it is still unclear why relatively high numbers of spermatogonia undergo cell death during normal testis development, cell death of premeiotic male germ cells has also been reported in many mammalian species (Allan et al., 1987). Two phases of developmental germ cell death have been reported in rodents: an early coordinated wave of cell death, which is estimated to eliminate approximately 80% of the newly generated spermatocytes during the first round of spermatogenesis and constant spontaneous cell death in the adult testis (Allan et al., 1992; Rodriguez et al., 1997). In striking similarity to *Drosophila*, however, this spontaneous cell death is mainly restricted to spermatogonia and is estimated to eliminate about 30% of these newly generated germ cells. Moreover, whereas the early wave of cell death was blocked in mice overexpressing the apoptosis inhibitory proteins, Bcl2 and BclXL, or in mice deficient for the proapoptotic protein, Bax, the spontaneous cell death in the adult testis was not affected in these animals, suggesting a possible divergence from the conventional apoptotic program (Knudson et al., 1995; Rodriguez et al., 1997).

**EXPERIMENTAL PROCEDURES**

**Fly Strains**

All fly strains and the generation of all the constructs and transgenes mentioned in this study are described in detail in the Supp. Experimental Procedures.

**The Deficiency Screen**

We used the “Bloomington deficiency kit,” a collection of several hundred chromosomal deletion lines, which collectively cover 98% of the genome (Cook et al., 2012). We initially screened through the third chromosome subset, encompassing about one-third of the total collection, and identified three deficiency lines with nonoverlapping chromosomal deletions that significantly suppressed GCD as heterozygotes. We then screened deficiency lines with smaller deletions in the respective suppressive regions, which limited one of these regions to a relatively small chromosomal segment, bands 88B2–88C9, containing only 35 genes, including htrA2/omi.

**TUNEL, LysoTracker, and DHE Labeling**

TUNEL labeling of the testis was carried out using the ApopTag kit (Millipore, Billerica, MA, USA) as described in Arama and Steller (2006). For TUNEL and cleaved caspase-3 double staining, the rabbit anti-cleaved caspase-3 antibody (1:100; Asp175; Cell Signaling Technology, Danvers, MA, USA) was added to the anti-digoxigenin solution. The secondary antibody was added after the washes of the following day.
For LysoTracker and Hoechst double staining, testes were dissected in PBS, incubated in staining solution (100 μM LysoTracker Red DND-99 [Molecular Probes, Carlsbad, CA, USA] and 16.2 μM Hoechst 33342 [Molecular Probes]) for 10 min, rinsed in PBS, mounted in a drop of PBS, and immediately photographed. For DHE and Hoechst double staining, we performed a similar procedure using instead a staining solution with 20 μM DHE (Molecular Probes) and incubating for 30 min. Images were taken on a confocal microscope (LSM510 Meta Inverted Axio Observer; Carl Zeiss) and captured using the LSM510 operating software.

Ultrastructural Studies

Testes were prepared for TEM analysis as described in Arama et al. (2006). Ultra-thin sections were prepared with ultramicrotome Leica UCT (Leica), analyzed under 120kV transmission electron microscope Tecnai 12, and digitized with EAGLE CCD camera using TIA software (FEI, Eindhoven). The electron microscopy studies were conducted at the Irving and Cherna Moskowitz Center for Nano and Bio-Nano Imaging at the WIS.

Fertility Test

Young adult males were placed individually with three wild-type virgin females in separate vials at 25 C, and vials were scored for offspring pupa after 12 days.

Quantification of GCD

The number of fixed TUNEL or LysoTracker-positive spermatogonial cysts in each testis was manually counted under the confocal microscope. The mean score of the wild-type control group was set as 100%, and the values of the other genotype groups were expressed as percentage of the wild-type group. GCD levels are represented as the mean ± SEM of pooled results obtained from the indicated number of samples. Statistical analysis was performed by Fisher’s protected least significant difference posttest for multiple comparisons using the StatView Program (Abacus Concepts, Berkeley, CA, USA). Significance level was considered as p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.02.002.

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REFERENCES


Omi and Lysosomes Mediate Alternative Cell Death


Supplemental Information

Alternative Germ Cell Death Pathway in *Drosophila* Involves HtrA2/Omi, Lysosomes, and a Caspase-9 Counterpart

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Inventory of Supplementary Materials:

Figure S1, related to Figure 1. *bag of marbles (bam)* and *nanos (nos)* expression domains in the *Drosophila* adult testis

Figure S2, related to Figure 3. TEM analysis of apoptotic spermatogonial cysts

Figure S3, related to Figure 4. Global cellular acidification, and not macroautophagy, is associated with GCD

Figure S4, related to Figure 5. Epistatic relationships between the mitochondrial and lysosomal pathways during GCD

Figure S5, related to Figure 7. The mitochondrial-associated components, Pink1, the Bcl-2 family proteins, Debcl and Buffy, and EndoG are all involved in GCD

Supplemental Experimental Procedures

Supplemental References
Figure S1. *bag of marbles* (*bam*) and *nanos* (*nos*) expression domains in the *Drosophila* adult testis, related to Figure 1

(A) *bam-Gal4* and (B) *nos-Gal4* driver lines were each crossed to a fly line carrying a *UAS-GFP* transgene to reveal their respective patterns of expression in the adult testis. Note that *nos* expression is confined to spermatogonial cysts at the 2-8-cell stages, whereas the *bam* is expressed in 8-16-cell cysts. Testes were counterstained with DAPI (blue). Scale bar 20 μm.
Figure S2. TEM analysis of apoptotic spermatogonial cysts, related to Figure 3

(A-D’’) Electron micrographs of cross sections through the apical tip of adult testes from (A and A’) wild-type and (B-D’’) flies expressing the diap1 RNAi transgene in spermatogonial cells by the bam-Gal4 driver line. Areas confined by yellowish squares and marked by yellow letters were magnified and presented in separated micrographs with corresponding letters.

(A and A’) A normal wild-type spermatogonium containing mitochondria of similar width but with different lengths (yellow asterisks mark some of the elongated mitochondria). Note the fine and almost invisible cristae.

(B, B’ and D’) Apoptotic spermatogonia display regular mitochondrial morphology (yellow asterisks mark some of the mitochondria).

(B) An apoptotic spermatogonia exhibiting highly crenellated nuclei with some fragmentation (magenta asterisks).

(C and C’) Apoptotically dying spermatogonial cyst with significantly shrunken cells (a magenta arrow; note the dark color of these cells), displaying condensed and fragmented chromatin (green arrowheads).

(D) The detachment of the apoptotic spermatogonia from their surrounding cells is highly pronounced (blue asterisks mark empty spaces).

(D’) Membrane blebbing is often detected in circumference of the dying cells (light blue arrowheads).

(D’’) Apoptotically dying spermatogonia are eventually disintegrated to apoptotic bodies (red arrows; the blue arrow points at a newly generated apoptotic body confined within a membrane.)
(E) Spontaneously dying spermatogonial cysts are engulfed as single entities by phagocytes. Electron micrograph of cross section through the apical tip of wild-type adult testis. At least two dying spermatogonial cysts at advanced GCD stages (red and yellow asterisks) are engulfed by the same macrophage.

(F and G) TUNEL labeling (red) of testes expressing GFP (green) under the control of the phagocyte-specific driver, *croquemort (crq)*-Gal4. The nuclei are in blue (DAPI). Professional phagocytes are visualized, which engulfed several dying TUNEL-positive spermatogonia found at advanced disintegration states (white arrows).

Scale bars in A, 2 μm; B and C’, 1 μm; C, D and E, 5 μm; A’, B’, D’ and D’’, 0.5 μm; F and G, 20 μm.
Figure S3. Global cellular acidification, and not macroautophagy, is associated with GCD, related to Figure 4

(A) Quantification of GCD levels in a mutant of atg8 (atg8\textsuperscript{KG07569}) and a null allelic mutant combination of atg7 (atg7\textsuperscript{d14/d77}), and in a separate experiment, flies expressing GFP (serving as control) or a dominant negative form of Atg1 (Atg1\textsuperscript{KQ}) in spermatogonial cells using the nos-Gal4 driver line. All calculations were performed as in Figure 2C.

(B and C) Apical tips of testes expressing either (B) human or (C) Drosophila Atg8-based autophagy reporters in spermatogonial cells using the nos-Gal4 driver line. Testes were stained with DAPI (blue) to reveal the nuclei, (B) GFP or (C) eGFP (green), which mark the LC3/Atg8 subcellular localization, and either (B) TUNEL or (C) mCherry (red). The positions of dying cysts are outlines by white circles. Note the quenching of eGFP in contrast to the persistence of the mCherry throughout the dying cyst, suggesting global acidification of the cellular compartment. Arrowheads point at two living 8-cell spermatogonial cysts which display some puncta. However, no puncta was detected in dying cysts.

(D and E) Nonuniform/incomplete DNA fragmentation during GCD in dnaseII mutants. Apical tips of testes from dnaseII mutants stained to visualize DNA fragmentation (TUNEL, red) and the nuclei (DAPI, blue). White arrowheads points to dying spermatogonial cysts during early-mid GCD stages (nuclei are still readily visible) with only partially fragmented DNA. Yellow arrowheads points to dying spermatogonial cysts during mid-late GCD stages (note that the DAPI signal is fading out) displaying diffused TUNEL labeling. Scale bars 20 µm.
Figure S4. Epistatic relationships between the mitochondrial and lysosomal pathways during GCD, related to Figure 5

(A) Quantification of GCD levels in testes from the indicated genotypes. All calculations were performed as in Figure 2C.

(B) Quantification of LysoTracker-positive spermatogonial cysts in testes from the indicated genotypes. All calculations were performed as in Figure 2C.
Figure S5. The mitochondrial-associated components, Pink1, the Bcl-2 family proteins, Debcl and Buffy, and EndoG are all involved in GCD, related to Figure 7 (A-C) Quantification of GCD levels in testes from the indicated genotypes. All calculations were performed as in Figure 2C. (A) Note that GCD levels are not significantly reduced in the parkin hemizygous mutant (park^{25/Df}). (B) The fact that the debcl homozygous mutants (debcl^{E26}) display a stronger effect than the hemizygous mutants (debcl^{E26/Df}) may suggest either some background effect or an unforeseen dominant effect of this mutant.
Supplemental Experimental Procedures

Fly Strains

yw flies were used as wild-type controls. Fly mutant alleles used in this study are *dronc*¹²⁴, *dronc*²⁹ and *dronc*³² (Xu et al., 2005), *ark*¹⁰⁸ and *ark*¹⁴⁶ (Srivastava et al., 2007), *ark*²⁹ (Akdemir et al., 2006), *dcp-1*prev (Laundrie et al., 2003), *strica*⁴ (Baum et al., 2007), *drice*¹ (Muro et al., 2006), *cathD*¹ (Myllykangas et al., 2005), *dor*⁴ and *car*¹ (Sriram et al., 2003), *dnaseII*lo (Mukae et al., 2002), and *omi*¹ and *omiR-1* (Tain et al., 2009). The UAS-*diap1-IR* line was obtained from P. Meier (ICR, London), *nos-Gal4* and *bam-Gal4* from L. Gilboa (WIS, Israel), UAS-*myc-diap1* from H.D. Ryoo (NYU, NY), and UAS-*p35* and *Df(3L)H99* from H. Steller (Rockefeller Univ., NY), *atg⁷d¹⁴* and *atg⁷d⁷⁷* (Juhasz et al., 2007), UAS-*atg-1*K³⁸Q and *atg8*K⁰⁷⁵⁶⁹ (Scott et al., 2007), UAS-*eGFP-mCherry-DrAtg8a* (Nezis et al., 2010), UAS-*eGFP-huLC3* (Rusten et al., 2004), *pink*B⁹ (Park et al., 2006), *park*²⁵ (Greene et al., 2003), *crq-Gal4*, UAS-*GFP* (Franc et al., 1996), *endoG*MB⁰⁷⁵⁰ (Deluca and O'Farrell, 2012), *debc*²⁶ and *buffy*H³⁷ (Sevrioukov et al., 2007), and the *Df(2R)ED1552*, *Df(2R)BSC259*, *Df(2R)BSC699* and *Df(3L)Pc-MK* from the Bloomington Stock Center.

Expression Constructs

For generation of the *omiR-2* rescue construct, a 1.97 kb genomic fragment spanning the *htrA2/omi* locus was amplified by PCR from purified yw genomic DNA using the PFU polymerase (Stratagene), the forward primer, 5’-GGCGAATTCGTGGTGGTGGAGAGAAGAAGA-3’, and reverse primer, 5’-
GCGGTACCAGGTTGTCAGGATTTC-3’, and cloned into the PattB vector using the EcoRI and Acc65I restriction sites.

To generate the \(omi_{R.2(S266A)}\) mutant form where the conserved serine residue in position 266 was replaced by an alanine residue, we performed standard site-directed mutagenesis using the \(omi_{R.2}\) construct as a template and the forward primer, 5’-CGTGTGCAAGCCGCGGCCGGTCC-3’, and its complementary reverse primer.

To generate the \(omi_{R.2(-IBM)}\) mutant form, the three putative IBM tetrapeptides in positions 74, 79 and 92 were simultaneously mutated by replacement of the first two amino acids in each tetrapeptide, including the invariable first alanine, by two glycine residues. This was done in two steps using the \(omi_{R.2}\) construct as a template. The tetrapeptide in position 92 was first mutated as before using the forward primer, 5’-CGGCAACGATCGCCTGGCGAATAATGACCAGTCG-3’ and its complementary reverse primer, and subsequently this construct was further mutated simultaneously in positions 74 and 79 using the forward primer, 5’-GCCCTTCTCCCTGGGCGGTCGAGTGCCGAAAAATGCAGTGGGCGGGAAGAC-3’, and its complementary reverse primer.

To generate the \(omi_{R.BAC}\) rescue construct, a P[acman] BAC genomic clone (from a genomic DNA library inserted in the pattB vector), spanning approximately 18.2 kb of the genomic region encompassing the \(htrA2/omi\) locus (BAC# CH322-129B23), was obtained from the BACPAC Resource Center [BPRC; Children's Hospital Oakland Research Institute, Oakland, CA; (Venken et al., 2009)], and the DNA was purified from the DH10B bacteria using the alkaline lysis miniprep method (according to
The clone was validated by restriction enzyme digest and transformed into EPI300 bacteria (for the high copy number production step).

To generate transgenic flies, these (pattB) rescue constructs were all injected to embryos bearing the defined attP40 landing site on chromosome 2. omiR-2, omiR-2(S266A), and omiR-2(IBM) were injected by Genetic Services Inc. (Cambridge, MA), whereas omiR-BAC was injected by BestGene Inc. (Chino Hills, CA).

**RNA isolation and RT-PCR**

Total RNA was extracted from twenty young adult male testes using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer’s recommendations. Purified RNA was reversed transcribed using Qiagen OneStep RT–PCR kit (Qiagen). cDNA for HtrA2/Omi was amplified using forward primer: 5’-GTGAGGCTATCCGATGGCAG-3’ and a reverse primer: 5’-TGAGTTCTTGATCTCCTTCTTG-3’. The primers were designed to amplify a 659 bp-long cDNA fragment from wild-type (wt) and the omi transgenes (omi\textsuperscript{trans}), a 536 bp-long truncated omi cDNA fragment from the omi\textsuperscript{Df1/IFD} mutant (omi\textsuperscript{IFD}), and a larger, 812 bp-long fragment of omi genomic DNA, which includes two small introns (omi\textsuperscript{gen}).
Supplemental References

upstream or parallel to the apoptosome during histolytic cell death. Development 133,
1457-1465.

and Dronc function redundantly in programmed cell death during oogenesis. Cell Death.
Differ. 14, 1508-1517.

Deluca,S.Z. and O'Farrell,P.H. (2012). Barriers to male transmission of mitochondrial

Croquemort, a novel Drosophila hemocyte/macrophage receptor that recognizes
apoptotic cells. Immunity. 4, 431-443.

(2003). Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin

promotes neuronal health, stress tolerance, and longevity but is dispensable for
metamorphosis in Drosophila. Genes Dev. 21, 3061-3066.

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

of the innate immunity in Drosophila by endogenous chromosomal DNA that escaped
apoptotic degradation. Genes Dev. 16, 2662-2671.

Muro,I., Berry,D.L., Huh,J.R., Chen,C.H., Huang,H., Yoo,S.J., Guo,M., Baehrecke,E.H.,
and Hay,B.A. (2006). The Drosophila caspase Ice is important for many apoptotic cell
deaths and for spermatid individualization, a nonapoptotic process. Development 133,
3305-3315.

(2005). Cathepsin D-deficient Drosophila recapitulate the key features of neuronal ceroid

Nezis,I.P., Shravage,B.V., Sagona,A.P., Lamark,T., Bjorkoy,G., Johansen,T.,
of dBruce controls DNA fragmentation in nurse cells during late Drosophila


