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# Caspase Activity and a Specific Cytochrome C Are Required for Sperm Differentiation in *Drosophila*

**Eli Arama,[1] Julie Agapite,[2] and Hermann Steller[1,*]**
[1]Howard Hughes Medical Institute
Strang Laboratory of Cancer Research
The Rockefeller University
1230 York Avenue
New York, New York 10021
[2]Department of Biology
Massachusetts Institute of Technology
77 Massachusetts Avenue
Cambridge, Massachusetts 02139

## Summary

**The final stage of spermatid terminal differentiation involves the removal of their bulk cytoplasm in a process known as spermatid individualization. Here we show that apoptotic proteins play an essential role during spermatid individualization in *Drosophila melanogaster*. Several aspects of sperm terminal differentiation, including the activation of caspases, are reminiscent of apoptosis. Notably, caspase inhibitors prevent the removal of bulk cytoplasm in spermatids and block sperm maturation in vivo, causing male sterility. We further identified loss-of-function mutations in one of the two *Drosophila cyt-c* genes, *cyt-c-d*, which block caspase activation and subsequent spermatid terminal differentiation. Finally, a giant ubiquitin-conjugating enzyme, dBruce, is required to protect the sperm nucleus against hypercondensation and degeneration. These observations suggest that an apoptosis-like mechanism is required for spermatid differentiation in *Drosophila*.**

## Introduction

Apoptosis is a morphologically distinct form of cell death that usually serves to remove unwanted and potentially dangerous cells (Meier et al., 2000a; Hengartner, 2000). A key event during apoptosis is the activation of a specific class of cysteine proteases, termed caspases (Thornberry and Lazebnik, 1998; Cryns and Yuan, 1998). Caspases are expressed as inactive zymogens in virtually all animal cells and are specifically activated in cells destined to undergo apoptosis. On the other hand, there are some examples where apoptosis-like events do not lead to the death, but rather the terminal differentiation of certain cell types. For example, lens epithelial cells and mammalian red blood cells lose their nucleus and other subcellular organelles during terminal differentiation but continue to be metabolically active (Jacobson et al., 1997). Likewise, sperm cell terminal differentiation involves complex changes in the cytoarchitecture, some of which are reminiscent of apoptosis. Although mouse mutations in several apoptotic genes produce defects in spermatogenesis, cellular analyses have so far focused on the early stages of sperm development (Knudson et al., 1995; Rodriguez et al., 1997; Print et al., 1998; Honarpour et al., 2000; Furuchi et al., 1996). Therefore, little is known about the exact role of apoptosis during sperm cell terminal differentiation.

Postmeiotic sperm cell differentiation is characterized by a sequence of conserved changes in the morphology and structural organization of the spermatids. In *Drosophila*, these changes include elongation of the flagellum, fusion and subsequent elongation of the mitochondria to the entire length of the spermatid, nuclear condensation, and the expulsion of most of the spermatid cytoplasm (Fuller, 1993). Although spermatid bulk cytoplasm elimination was already described more than three decades ago, the molecular and cellular mechanisms that drive this process are still unknown (Fawcett and Phillips, 1969; Tokuyasu et al., 1972).

We have investigated the role of apoptotic proteins during *Drosophila* spermatogenesis. We find that caspase activity is required for spermatid individualization. More generally, cystic bulges and waste bags, which contain cytoplasm expelled from differentiating spermatids, display many features of apoptotic corpses and contain all the components required for a functional apoptosome. Activation of the effector caspase drICE in spermatids requires one of the two cytochrome c genes of *Drosophila*, *cyt-c-d*. Interestingly, loss-of-function mutants for *cyt-c-d* are male-sterile but viable and have no other apparent defects in caspase activation, apoptosis, cell vitality, and development. Therefore, *cyt-c-d* appears to be specifically required for caspase activation during spermatogenesis. Finally, loss-of-function mutants in dBruce, a giant ubiquitin-conjugating enzyme (Chen et al., 1999; Hauser et al., 1998; Vernooy et al., 2002), have spermatid nuclei that are hypercondensed and degenerate, leading to male sterility. These findings are consistent with the idea that dBruce is required to protect the sperm nucleus from excessive caspase activity and degeneration.

## Results

### Spermatid Individualization

Spermatogenesis in *Drosophila melanogaster* takes place within individual units known as cysts. Each cyst contains 64 spermatids that remain initially connected after meiosis via cytoplasmic bridges and differentiate synchronously. During terminal differentiation, the round-shaped spermatids are transformed into thin, approximately 2 mm long spermatozoa with highly elongated, "needle-shaped" nuclei. In the final stage of spermatogenesis, termed individualization (Figure 1A), the cytoplasmic bridges are disconnected and most of the cytoplasm is expelled, leading to individual sperm. The individualization process involves the assembly of a cytoskeletal-membrane complex, referred to as the "individualization complex" (IC), which contains actin as its major cytoskeletal component. The IC can be detected by staining with phalloidin, which binds to actin (Figures 1B and

*Correspondence: steller@mail.rockefeller.edu

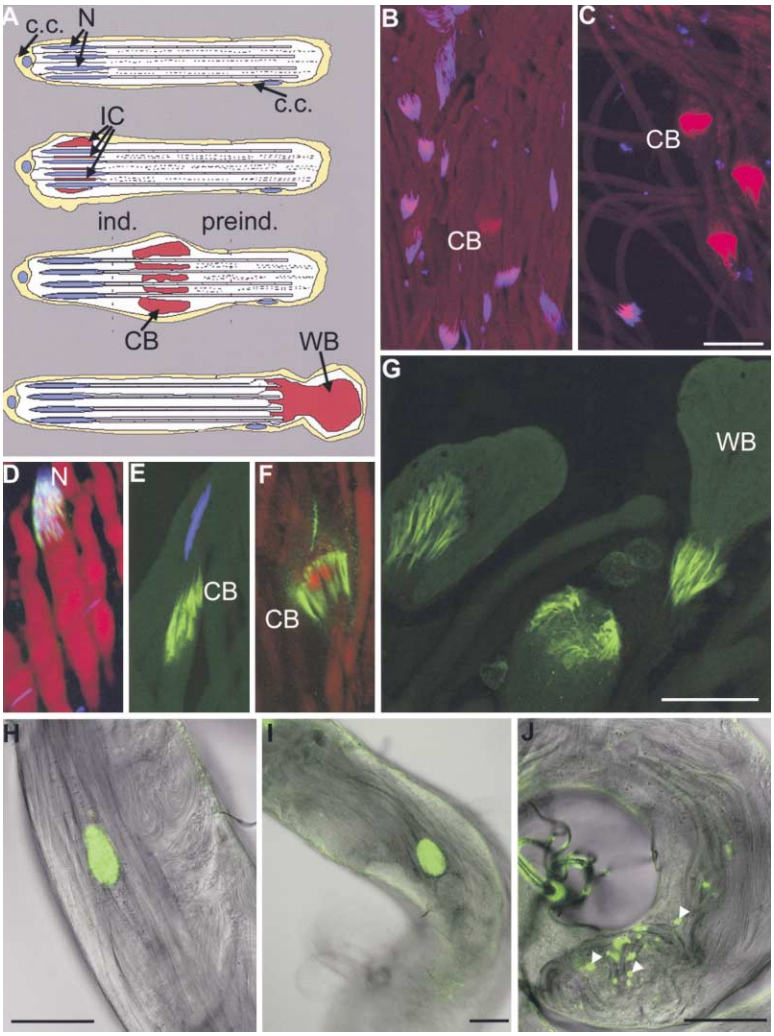


Figure 1. The Removal of Bulk Cytoplasm during Sperm Differentiation Shares Similarities with Apoptosis

(A) Schematic diagram of spermatid individualization. In this diagram, a single cyst with four representative spermatids is depicted at increasingly advanced developmental stages (from top to bottom). The first cyst contains elongated spermatids prior to individualization. In the second cyst, assembly of the individualization complex (IC, red) has begun around the nuclei. The IC progresses caudally from the nuclear region to the end of the tail (in this figure from left to right). The third cyst has a cystic bulge (CB, red), which contains the progressed IC and the cytoplasm of the postindividualized part of the spermatids. At this point, many vesicles and organelles are present in the CB. Finally, most of the spermatids' syncytial cytoplasm is removed into the waste bag (WB, red). The WB is pinched off from the base of the cyst and eventually degrades. Each sperm is now surrounded by its own membrane and is largely devoid of cytoplasm and organelles (with the exception of a single giant mitochondrion). Adapted from Fabrizio et al., 1998. c.c., cyst cell; ind., postindividualized; preind., preindividualized.

(B–G) Both actin and lamin $Dm_0$ are components of the IC. In squashed testes preparations, the IC can be visualized either by TRITC-phalloidin (red) that binds to actin, or by anti-lamin $Dm_0$ (green) that detects the major protein of the nuclear lamina. Bundles of wild-type elongated spermatids (blue) are depicted either before IC assembly (B), associated with an IC ([B and D], IC in pink), or after the IC translocation ([E], IC in green). The CBs' size increases as the spermatid individualization progresses; small CB is detected right after the detachment of the IC from the vicinity of the nuclei ([E], IC in green). As the IC progresses, the CB increases volume ([C], IC in red, and [F], IC in green and red). Finally, the CB reaches the end of the spermatids, becoming a large WB ([G], IC in green).

(H–J) Micrographs of live testes stained with the apoptotic marker acridine orange (AO, green). AO, a specific marker for apoptotic cells in *Drosophila*, specifically stained both CBs (H) and WBs (I).

(J) Scattered AO-positive spots at the base of the testis represent degrading WBs (arrowheads).

The scale bars represent 50 $\mu$m.

1C, red; Fabrizio et al., 1998). In addition, we observed that lamin $Dm_0$ leaves the vicinity of the nucleus and translocates as a component of the IC and thus can also be a useful marker of the IC (Figures 1D–1G, green). The IC is assembled at the nuclear end of the cyst and subsequently translocates caudally along the entire length of the spermatid bundle, expelling most of the cytoplasm in the process. The discarded cytoplasm accumulates in a membrane-enclosed structure, termed the waste bag (WB; Figures 1A, 1G, and 1I; Tokuyasu et al., 1972). The WBs eventually undergo fragmentation and subsequent degradation (Figure 1J).

To investigate the possible occurrence of apoptosis during *Drosophila* sperm differentiation, we stained live wild-type testes with the vital dye acridine orange (AO), which specifically detects apoptotic cells (Abrams et al., 1993). AO staining was observed in intact cystic bulges (CBs) and WBs (Figures 1H and 1I). The staining of the CB and WB with AO in spermatids undergoing individualization suggests that the apoptotic program is activated in the late stages of sperm differentiation, and that CB and WB resemble apoptotic corpses without nuclei.

### Activation of an Effector Caspase at the Onset of Spermatid Individualization

A key feature of apoptosis is the activation of caspases. To investigate whether *Drosophila* effector caspases become activated during the individualization process, testis preparations were stained with the CM1 antibody. CM1 was raised against the active form of mammalian caspase 3 but also crossreacts with the caspase-3-like *Drosophila* effector caspase drICE (Baker and Yu, 2001; Srinivasan et al., 1998; Yu et al., 2002; Ryoo et al., 2002). In addition, we also used an antibody that was raised specifically against the active form of drICE (Dorstyn et

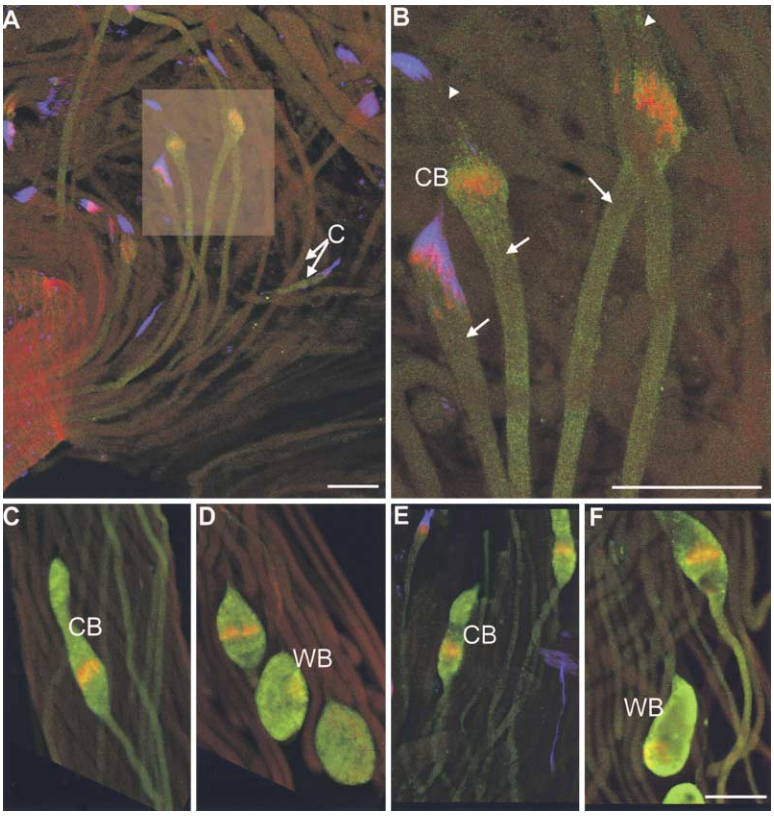


Figure 2. Activation of the Effector Caspase drICE at the Onset of Individualization

(A) and its enlargement in (B). The assembly of the IC (phalloidin, red) at the nuclear end (DAPI, blue) of a cyst, which marks the beginning of the process of spermatid individualization, is followed by a steep gradient of drICE activation (CM1, green). Active drICE accumulates in the preindividualized part of a cyst (arrows) and within the CB. After the caudal translocation of the CB, active drICE is no longer detectable in the postindividualized part of the spermatids (arrowheads). C, elongated spermatid cysts.

(C–F) The activation of drICE during spermatid individualization was detected either by the anti-active mouse caspase 3 (CM1) antibody that crossreacts with drICE ([C and D], green), or by the anti-active drICE antibody ([E and F], green). Both antibodies gave rise to intense staining in the cytoplasmic parts of the spermatids, namely the CB and distal parts of the cell. Once all the cytoplasm is stripped away, active drICE is detectable only in the WB.

The scale bars represent 50 μm.

al., 2002; Yoo et al., 2002). Using either antibody, active drICE became detectable immediately after a mature IC was formed (Figures 2A and 2B). During the caudal translocation of the IC, active drICE became completely depleted from the newly individualized portion of the spermatids. The staining remained abundant, however, in the preindividualized portion of the spermatids, with the highest levels within the CB (Figures 2B, 2C, and 2E). At the end of this process, the newly formed WBs contained high levels of active drICE (Figures 2D and 2F). These observations indicate that caspase activation and apoptosis-like events are intimately associated with the terminal differentiation of spermatids in *Drosophila*.

## Caspase Activity Is Required for Spermatid Individualization

In order to examine whether caspase activation is necessary for spermatid differentiation, we inhibited caspase activity both in cultured testes and in vivo. Under in vitro culture conditions, each cyst accomplishes individualization within 12 hr (Noguchi and Miller, 2003). To test the effect of caspase inhibition on the caudal movement of the IC in vitro, testes from young adults were isolated. One testis of a pair from the same individual was cultured in the presence of the caspase inhibitor Z-VAD, while the other testis served as a control. Z-VAD was previously shown to block the activity of drICE in vitro and drICE-induced cell death of *Drosophila* S2 cells (Fraser and Evan, 1997). After 26 hr of culture, testes were fixed and analyzed. In testes that were cultured with the caspase inhibitor, the already advanced CBs appeared flat (Figure 3B) as compared to the full, oval shape of CBs in the control (Figure 3A). This suggests that caspase activity is required for the efficient collection of cytoplasm into the CBs. Furthermore, the translocation of the ICs appeared to be defective in the absence of caspase activity. In controls, only a few early ICs were associated with the nuclear complexes because the IC translocates caudally in the process of spermatid differentiation (Figure 3C). In contrast, in the presence of a caspase inhibitor, most of the ICs remained in the vicinity of the nuclei (Figure 3D), indicative of a defect in IC translocation. Therefore, caspase activity appears to be required for proper IC movement and the removal of bulk cytoplasm from differentiating spermatids.

In order to extend these observations to spermatogenesis in vivo, we expressed the baculoviral broad-spectrum caspase inhibitor p35 (Hay et al., 1994) in male gonads using the GAL4-UAS system (Brand and Perrimon, 1993). Transgenic flies were generated that carry the *GAL4* gene fused downstream of the *Drosophila* Hsp83 promoter, which directs strong expression in the germ cells throughout spermatogenesis (Yue et al., 1999). The *Hsp83-Gal4* driver line was crossed to the *UAS-p35* line and male progeny were subjected to a fertility test. Whereas the parental lines that bear two copies of either construct alone were fertile, males with both the driver and the *UAS-p35* construct were sterile. Cytological analyses of these males revealed that the translocation of the IC was severely impaired (Figure 3E). Although the initial assembly of the IC appeared normal (data not shown), there was a failure of cytoplasm

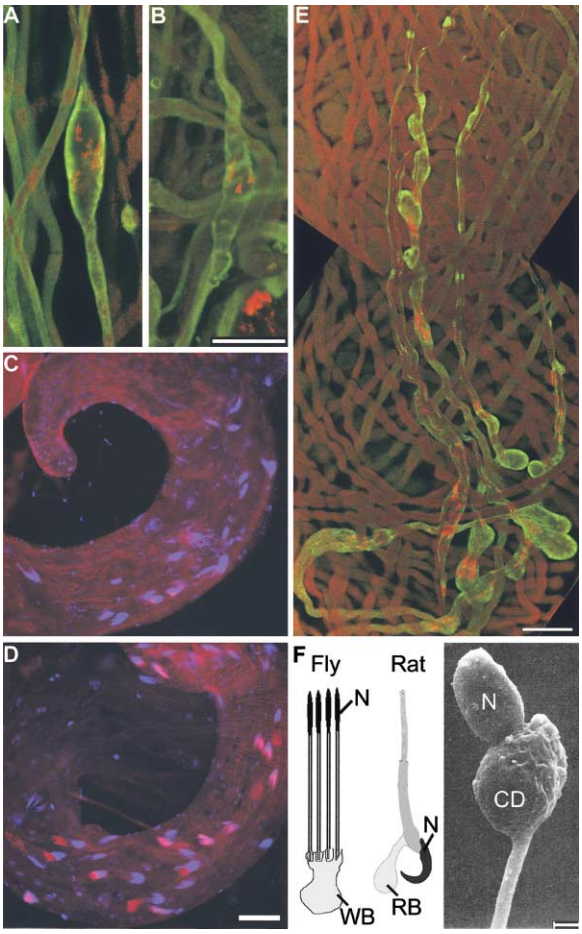


Figure 3. Inhibition of Caspase Activity Blocks Individualization In Vitro and In Vivo

Under in vitro culture conditions, spermatids become individualized within 12 hr. Two testes from the same wild-type individual were cultured for 26 hr under identical conditions, except that the caspase inhibitor Z-VAD was added to one of them. Nuclei were visualized with DAP1 (blue), ICs with phalloidin (red), and active drICE with the CM1 antibody (green). Whereas testes that were cultured without Z-VAD displayed the typical oval appearance of CBs (A), the treated testes displayed flat, irregular CBs, indicating that the bulk cytoplasm was not collected properly in the CBs (B).

(C) After 26 hr of culture, most of the mature cysts underwent individualization in the absence of Z-VAD (note that only a few ICs remain associated with nuclei at the base of the testis).

(D) Testes that were cultured in the presence of Z-VAD displayed many early stages of IC assembly, but no evidence of IC translocation, indicating that caspase activity is necessary for the movement of the IC.

(E) Ectopic expression of the baculoviral broad-spectrum caspase inhibitor p35 blocks individualization. Flies containing two copies of HSP83-Gal4 and at least one copy of UAS-p35 were sterile. Staining of their testes for ICs (red) and active drICE (CM1, green) revealed severe defects in individualization, including scattered IC cones and flat CBs and WBs. Note that inhibition of drICE by p35 does not block CM1 staining, as previously reported (Baker and Yu, 2001; Yu et al., 2002; Ryoo et al., 2002).

(F) Overall similarities of sperm differentiation in *Drosophila* and mammals. On the left is a schematic presentation of the last step of the individualization process in *Drosophila* and mammals (rat). Like in *Drosophila*, mammalian spermatids are also connected by cytoplasmic bridges, which are eliminated together with most of the cytoplasm and organelles during terminal differentiation. In *Drosophila*, the cytoplasm of 64 spermatids accumulates in a structure called WB, whereas in mammals the spermatid cytoplasm ends up in a structure named residual body (RB). On the right is a scanning electron micrograph (SEM) of a human spermatozoon that displays a cytoplasmic droplet (CD) morphological abnormality. This abnormality is characterized by the failure to eliminate spermatid cytoplasm, a phenotype that is very similar to the individualization defects seen in (B), (D), and (E). Reproduced from Hollanders and Carver-Ward, 1996 with permission from Parthenon Publishing, UK. N, nucleus; WB, waste bag; RB, residual body.

The scale bars in (A)–(E) represent 50 μm; in (F), the scale bar represents 1 μm.

to collect into a normal CB (Figure 3E). These observations demonstrate that caspase activity is required for proper individualization, cytoplasmic exclusion, and male fertility.

### Caspase Activation at the Onset of Spermatid Individualization Is Independent of Terminal Differentiation

To distinguish whether caspase activation is the cause or the consequence of sperm cell differentiation, we examined mutants that disrupt the individualization process. In *purity of essence* (*poe*[1]) spermatids, the ICs are significantly reduced (arrowheads in Figure 4A), and often are not detected at all (arrows in Figure 4A; Fabrizio et al., 1998). However, active caspases are clearly detected in elongated spermatids that lack the IC, suggesting that the activation of caspases at the onset of individualization is independent of IC assembly. In *jaguar* (*jar*[1]) male flies, IC assembly and movement are impaired due to a mutation in a class VI myosin (Hicks et al., 1999). Interestingly, in *jar*[1] flies, active drICE could only be detected in mature cysts, which contain needle-shaped and condensed nuclei (Figure 4B). Therefore, the completion of spermatid morphogenesis rather than the assembly of a functional IC triggers drICE activation. To test whether caspase activation is dependent on the completion of nuclear morphogenesis, we examined testes of *fuzzy onions* (*fzo*) mutant flies. *fzo* flies are defective in the process of mitochondrial fusion during spermatogenesis (Hales and Fuller, 1997), and hence they arrest at mid-spermatid elongation stage. Active drICE accumulated in several immature elongating spermatid cysts containing undifferentiated nuclei (Figure 4C). Thus, we conclude that drICE activation is independent of nuclear maturation.

### Components of the Apoptosome Are Expressed during Spermatogenesis

In mammals, mitochondria play an important role in the activation of the apoptosome, a multiprotein complex that includes caspases, Apaf-1, and cytochrome c (Salvesen and Renatus, 2002). Because orthologs of caspase-9, Apaf-1, and cytochrome c have been identified in *Drosophila* (Meier et al., 2000b; Zhou et al., 1999), we examined whether they are expressed during *Drosophila* spermatogenesis. Using an antibody against full-length Dronc, a caspase-9 homolog (Meier et al., 2000b), revealed that it was expressed along the length of elongated spermatids (arrowheads in Figures 5A and

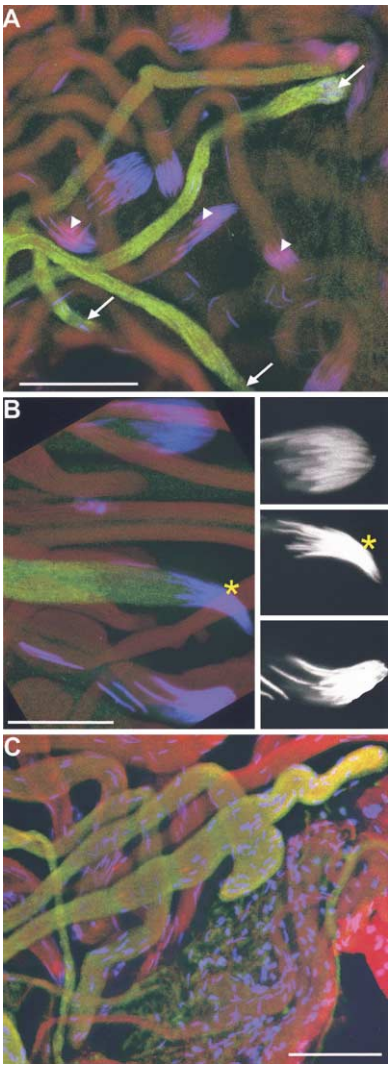


Figure 4. Caspase Activation Is Independent of Nuclear Differentiation or IC Assembly

Mutants that disrupt sperm differentiation retain CM1 staining.

(A) *purity of essence* (*poe*[1]) spermatids contain significantly fewer ICs (arrowheads), and often the ICs cannot be detected at all (arrows). Although caspase activation normally occurs only after IC assembly, CM1 staining (green) can be readily detected in elongated spermatids that lack an IC.

(B) In *jaguar* (*jar*[1]) male flies, IC assembly and movement are impaired. Active drICE (green) accumulates independently of functional IC assembly in *jar*[1] testes, but is correlated with the differentiation stage of nuclei (blue). The black and white panels of nuclear bundles on the right correspond to the blue nuclei on the left; caspase activation was only detected in spermatid bundles with thin, fully differentiated nuclei (see asterisks).

(C) *fzo* spermatids arrest at the mid-spermatid elongation stage and do not complete nuclear condensation. In *fzo*[1]/*fzo*[2] mutants, active drICE (green) accumulates in the more advanced elongating spermatid cysts despite the immature nuclei (blue), demonstrating that drICE activation can occur independently from nuclear differentiation.

The scale bars in (A) and (C) represent 50 μm; in (B), the scale bar represents 25 μm.

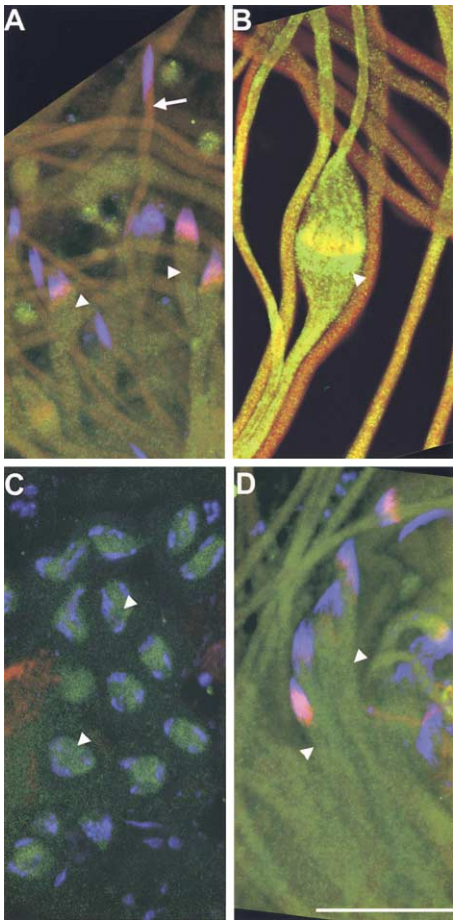


Figure 5. Expression of the *Drosophila* Orthologs of the Apoptosome Complex during Spermatogenesis

Nuclei are stained blue, and the IC is either pink or yellow.

(A) Dronc (green), a caspase-9 ortholog, accumulates along fully elongated spermatid cysts (arrowheads) and is depleted from individualized spermatozoa (arrow).

(B) As the IC progresses caudally, Dronc expression increases within the CB (arrowhead).

(C) The *Drosophila* ortholog of Apaf-1, *hac-1*, is expressed in spermatocytes. Transcription *of hac-1* was visualized using the *l(2)*k11502 line containing a *P*-lacZ insertion in the *hac-1* promoter. In this line, *lacZ* expression (green) mimics the mRNA pattern of *hac-1* (Zhou et al., 1999).

(D) Full-length inactive drICE (green) is expressed uniformly in preindividualizing spermatid cysts (arrowheads), demonstrating that pro-drICE is expressed in early stages of spermatogenesis. However, drICE becomes activated by posttranslation modification only after the assembly of the IC. The scale bar represents 50 μm.

5B). To investigate whether the *Drosophila* Apaf-1 homolog *hac-1/dark/dapaf* is expressed during spermatogenesis, we examined the β-galactosidase distribution in testes of the enhancer-trap line *l(2)*k11502, which mimics the mRNA pattern of expression of *hac-1* (Zhou et al., 1999). As shown in Figure 5C, *hac-1* accumulated in late primary spermatocytes. Finally, antibody staining for full-length drICE revealed that pro-drICE accumulated in primary spermatocytes and was uniformly distributed in preindividualized elongated spermatids (data not shown and Figure 5D). Interestingly, a significant proportion of cellular Dronc and drICE appears to localize near the mitochondria (Dorstyn et al., 2002). These observations suggest that *Drosophila* apoptosomes may form at the surface of mitochondria. However, the

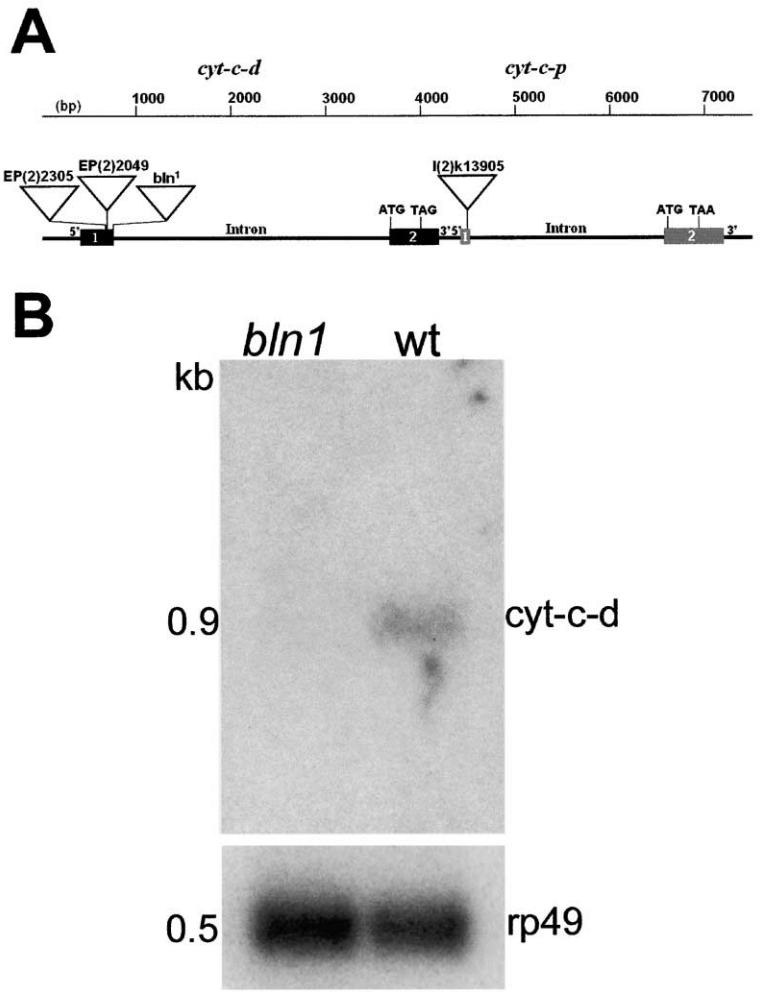


Figure 6. Molecular Genetic Analysis of the *Drosophila* Cytochrome C Genes

(A) Genomic organization of the two cytochrome c genes and transposon insertions. The map illustrates the *cyt-c-d* and *cyt-c-p* exons (thick lines in black and gray), introns (thin lines), and insertion points of the *P* element transposons in strains *EP*(2)*2305*, *EP*(2)*2049*, *bln*$^1$, and *l*(2)*k13905* (triangles). Three independent insertions were identified in the 5′ UTR of *cyt-c-d* (exon-1). The *p*[Z] element of the *bln*$^1$ allele is inserted 2 bp upstream of the last nucleotide of exon-1, while the *EP*$^{2305}$ and *EP*$^{2049}$ insertions are 83 bp and 77 bp upstream of the last nucleotide of exon-1. The predicted ORF for *cyt-c-d* (ATG-TAG within exon-2) encodes a protein 105 amino acids long. The *cyt-c-p* gene is located 241 bp downstream of *cyt-c-d*. The predicted ORF for *cyt-c-p* (ATG-TAA within exon-2) encodes a protein of 108 amino acids. The *l*(2)*k13905* allele is a *P-lacW* insertion in the first nucleotide of the *cyt-c-p* intron.

(B) Transcript analysis of the *cyt-c-d* gene. Northern blot analysis of adult male poly(A+) RNA of wild-type and *bln*$^1$ mutant. A probe unique for the 3′ UTR region of *cyt-c-d* detected a single band of the predicted size for *cyt-c-d* (0.87 kb) in wild-type (wt, right lane), but not in the *bln*$^1$ mutant (left lane). The *Drosophila rp49* gene was used as a loading control marker.

role of cytochrome c for caspase activation in *Drosophila* has remained unclear, as no release from mitochondria was seen in previous studies, and RNAi experiments in SL-2 cells produced negative results as well (Dorstyn et al., 2002; Varkey et al., 1999; Wang, 2001; Zimmermann et al., 2002).

### Caspase Activation Requires a Specific Cytochrome C

To critically examine the requirement of cytochrome c for caspase activation and sperm cell differentiation in *Drosophila*, we examined the phenotype of loss-of-function mutants. *Drosophila* contains two closely linked but distinct cytochrome c genes, termed *cyt-c-d* and *cyt-c-p* (Limbach and Wu, 1985). The *cyt-c-p* gene encodes the major form of cytochrome c and is expressed at much higher levels than *cyt-c-d*. Searching FlyBase for possible mutations, we identified three independent *P* element insertions located in *cyt-c-d* (*bln*$^1$, *EP*$^{2305}$, and *EP*$^{2049}$), and one insertion in *cyt-c-p* (Figure 6A). The *P* insertion in *cyt-c-p* was homozygous lethal, and all three *P* insertions into the *cyt-c-d* gene were homozygous viable but male-sterile. All three *cyt-c-d* alleles produced male sterility when placed in *trans* with each other, and over the *DF*(2L)H20 deletion. Complementation analysis revealed that the *bln*$^1$ allele complemented the lethality of the *l*(2)*k13905* allele, and that the latter complemented the sterility of *bln*$^1$. *l*(2)*k13905/DF*(2L)H20 trans-heterozygotes are also recessive embryonic lethal, demonstrating that this phenotype is due to the loss of *cyt-c-p* function. Significantly, one of the *cyt-c-d* alleles, *blanks* (*bln*$^1$), was previously reported as a male-sterile mutant (Castrillon et al., 1993). Detailed phenotypic analyses (see below) revealed that these various insertions and trans-heterozygous combinations can be arranged into an allelic series, with *bln*$^1$ behaving as a genetic null. Furthermore, in order to verify that the mutant phenotypes are caused by the transposon insertions, we mobilized the *P* elements in all three lines and generated revertants (see Experimental Procedures). Finally, Northern blot analysis with a probe directed against the unique 3′ UTR of *cyt-c-d* identified one band of the predicted size in wild-type, but no transcript was detected in *bln*$^1$ mutants (Figure 6B). Therefore, *bln*$^1$ appears to be a complete loss-of-function allele for *cyt-c-d*.

Next, we stained mutant testes from all *cyt-c-d* alleles as homozygotes and all the different trans-heterozygous combinations for active drICE using the CM1 antibody. Strikingly, there was no CM1 immunoreactivity in *bln*$^1$ homozygotes and *bln*$^1$/*DF*(2L)H20 flies (Figures 7B, 7D,

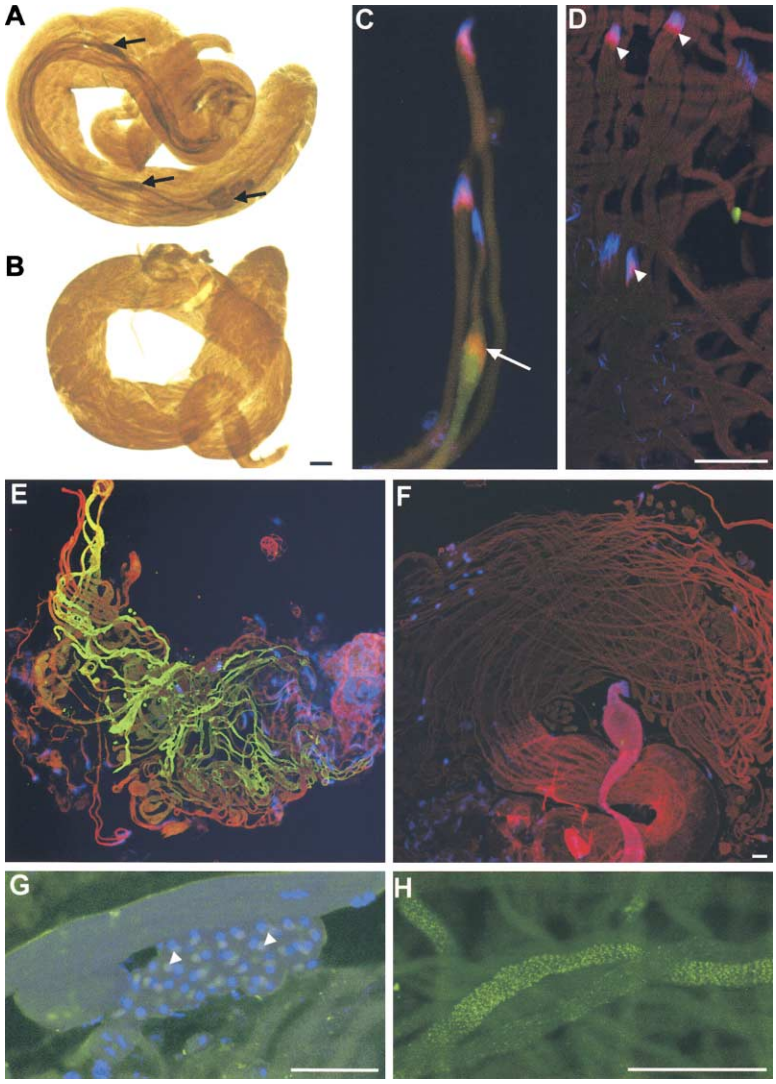


Figure 7. Mutations in *cyt-c-d* Block Caspase Activation at the Onset of Spermatid Individualization

(A and B) Visualization of active drICE with CM1 in wild-type (A) and *bln*[1] testes (B).

(A) Whereas CM1-positive cysts at different individualization stages can be readily seen in wild-type testes (black arrows pointing at CBs and WBs), no CM1-positive cysts were detected in testes of flies homozygous for the *bln*[1] allele (B).

(C–F) Nuclei, blue; IC, red; active drICE, green.

(C) CM1-positive spermatids were easily detected in wild-type individualizing cysts (green, white arrow). However, no CM1 staining was detected in *bln*[1] mutant testis (D) and (F), although *bln*[1] spermatids showed no obvious morphological defects. Note that the spermatids appeared elongated (F), that the nuclei differentiated and acquired a normal needle-like shape, and that the IC was assembled normally ([D], arrowheads).

(E) Spermatids of the *hephaestus* (*heph*[2]) mutant showed strong immunoreactivity with CM1 despite other severe defects in the individualization process. Similar results were obtained for ten additional different mutants that show defects in spermatid individualization process (data not shown).

(G) mAB 2G8, which detects cytochrome c only in apoptotic cells, stains the mitochondria of round spermatids (arrowheads) and then acquires a punctate expression pattern in elongated spermatids typical of mitochondrial protein expression at this stage (H). Note that the giant mitochondrion of round spermatids can be easily visualized by DAPI due to staining of the mitochondrial DNA.

The scale bars represent 50 μm.

and 7F), even though *bln*[1] testes contained elongated spermatids with an intact IC and with the characteristic needle-shaped nuclei that are typical for advanced stages of spermatid differentiation (arrowheads in Figure 7D). Furthermore, whereas in wild-type the IC translocates caudally (arrows in Figures 7A and 7C), in *bln*[1] mutants the IC does not separate from the nuclei (Figure 7D). Very similar observations were made for the other allelic combinations. *EP*[2305] and *EP*[2049] homozygotes showed some occasional CM1 staining and more progressed ICs, but all allelic combinations were male-sterile, although we observed some occasional escapers in *EP*[2049] −/− and *EP*[2305]/*EP*[2049] flies (data not shown). All *P* element revertants that we generated were fertile and had normal CM1 staining and individualization, demonstrating that the phenotypes reported here are caused by the transposon insertions into the *cyt-c-d* locus.

Finally, we stained testes with the mAB2G8 anti-cytochrome c antibody (Varkey et al., 1999). As previously reported for apoptotic cells, we saw increased mAB2G8 immunoreactivity in elongated spermatids of both wild-type and *bln*[1] mutants (Figures 7G and 7H). We attribute this to the expression of the major cytochrome c, *cyt-c-p*. Because *cyt-c-p* has no apparent function in caspase activation/apoptosis but is expressed at much higher levels than *cyt-c-d*, this may explain the previous difficulties in detecting cytochrome c release during apoptosis in *Drosophila*. Alternatively, it is possible that cytochrome c is only required for caspase activation during spermatogenesis.

We also examined 11 other mutants that displayed severe defects in individualization, and all of them had intense CM1 staining (see, for example, the *hephaestus* mutant in Figure 7E). These results demonstrate that the lack of CM1 staining is not simply the consequence of defective individualization, but rather reflects a specific requirement of the "minor" cytochrome c protein in *Drosophila*. Therefore, we conclude that *cyt-c-d* is required for caspase activation and for the subsequent IC translocation during spermatid individualization.

**Role of *dBruce* during Sperm Terminal Differentiation**

Effector caspases, such as drICE, normally cleave a variety of nuclear targets and thereby destroy the nucleus and chromosomal DNA (Hengartner, 2000). Therefore, somehow the sperm nucleus must be protected

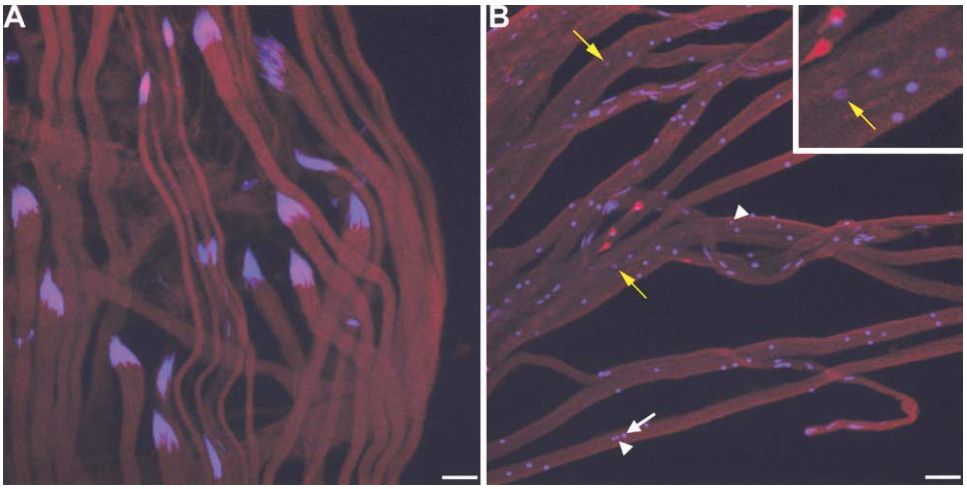


Figure 8. Spermatids in *dbruce* −/− Mutants Have Hypercondensed Nuclei and Degenerate

Nuclei are stained in blue (DAPI), and ICs are stained in red (phalloidin).

(A) Wild-type cysts. Normally, the elongated spermatid nucleus acquires a highly elongated, needle shape morphology; each cyst contains a bundle of 64 needle-shaped nuclei located at one end.

(B) *dbruce*$^{E81}$ −/− cysts. *dbruce* −/− mutants are homozygous viable but male-sterile. Mutant spermatid nuclei are significantly more condensed (arrowheads) and rounded (arrows and arrowheads) and are scattered throughout the cyst. In addition, there are fewer nuclei, and many nuclei are stained faintly (yellow arrows and enlargement in the inset), presumably because they undergo degeneration. Although an IC forms, it is highly reduced and scattered (red).

The scale bars represent 20 μm.

against this lethal activity of drICE. One candidate for such a protective function is dBruce, the *Drosophila* ortholog of mammalian Bruce/Apollon (Chen et al., 1999; Hauser et al., 1998; Vernooy et al., 2002). Bruce/Apollon proteins are giant E2 ubiquitin-conjugating enzymes that are thought to inhibit apoptosis. The presence of the BIR domain, a domain also found in IAPs, suggests that these proteins may bind to caspases (Salvesen and Duckett, 2002). We identified many mutations in *dBruce* from a screen for genetic modifiers of Reaper-induced apoptosis (J.A., K. McCall, and H.S., unpublished results). These alleles are homozygous viable but male-sterile, and they behave genetically like loss-of-function alleles. In all *dBruce* −/− mutants, we saw evidence for nuclear hypercondensation and degeneration, indicative of excessive caspase activity (Figure 8). Whereas nuclei in wild-type acquire a highly elongated, needle shape morphology, nuclei in *dBruce* −/− mutants appeared much more condensed and rounded, and were scattered throughout the cyst. In addition, many nuclei stained very faintly, and eventually they degenerated. These observations are consistent with a role of dBruce in protecting spermatids against excessive caspase activity and death.

## Discussion

### Removal of the Bulk Cytoplasm during Sperm Differentiation Resembles Apoptosis

Generation of functional sperm in all metazoan animals requires the elimination of most of the cytoplasm to generate a highly condensed, compact cell. The molecular and cellular mechanisms that drive this process are poorly understood. In this study, we provide evidence that the elimination of the cytoplasm during terminal differentiation of elongated spermatids involves an apoptosis-like process. However, unlike in "regular" apoptosis, this process is restricted to the cytoplasmic compartment. An apoptotic marker, acridine orange (AO), specifically stains the CBs and WBs, indicating that these structures resemble apoptotic corpses. Furthermore, we find that effector caspases, such as drICE, are activated during *Drosophila* spermatogenesis and are necessary for the removal of cytoplasm and the generation of functional sperm. Other key proapoptotic proteins are also expressed and become upregulated during *Drosophila* spermatogenesis. Our observations suggest that an apoptosome-like complex is assembled prior to individualization and is important for the removal of bulk cytoplasm from spermatids.

### A Specific Cytochrome C Is Required for Caspase Activation

In mammals, mitochondria are an important organelle for the induction of apoptosis, and it has been shown that they can release several proapoptotic proteins into the cytosol in response to apoptotic stimuli (Green and Reed, 1998; Meier et al., 2000a; van Loo et al., 2002). The best-studied case is the release of cytochrome c, which binds to and activates Apaf-1, which in turn leads to the activation of caspase-9 (Wang, 2001). However, no comparable role of mitochondrial factors for caspase activation has yet been established in invertebrates. Here, we present evidence that a specific form of cytochrome c, encoded by the *cyt-c-d* gene, is required for the activation of the effector caspase drICE at the onset of spermatid individualization. Loss-of-function mutants for *cyt-c-d* are homozygous viable but male-sterile. Significantly, these mutants were defective in drICE activation and failed to exclude the bulk cytoplasm, producing phenotypes virtually identical to the ones resulting from

the application/expression of caspase inhibitors. This provides compelling evidence for a role of the *cyt-c-d* gene for caspase activation during spermatogenesis in *Drosophila*. Interestingly, it was previously suggested that only *cyt-c-p*, but not *cyt-c-d*, functions in respiration (Inoue et al., 1986). Consistent with an essential role of *cyt-c-p* in respiration, a *P* element insertion into this locus resulted in recessive lethality (data not shown). Likewise, targeted gene inactivation of the murine cytochrome c gene causes very early embryonic lethality, and this has precluded functional studies on the role of cytochrome c for caspase activation during normal development in mammals (Li et al., 2000). We propose that the two cytochrome c genes in *Drosophila* fulfill distinct functions in respiration (*cyt-c-p*) and caspase activation/apoptosis (*cyt-c-d*). Previous arguments against a role of cytochrome c for caspase activation in *Drosophila* were largely based on the failure to detect release of cytochrome c from mitochondria. However, because *cyt-c-d* is expressed at much lower levels than *cyt-c-p* (Figure 6B; Limbach and Wu, 1985), it would be virtually impossible to detect the release of the relevant protein in the absence of highly specific antibodies. Furthermore, because *cyt-c-d* null flies are viable and, apart from male sterility, have no obvious anatomical defects, it is unlikely that this gene is broadly required for the activation of apoptosis. A complete block of apoptosis in *Drosophila* interferes with normal embryogenesis, and mutants with significantly reduced apoptosis can be viable but are phenotypically abnormal (White et al., 1994; Grether et al., 1995; Peterson et al., 2002). Therefore, although we cannot rule out that the loss of *cyt-c-d* function may affect and/or delay apoptosis in somatic tissues, the main function of this gene appears to be in caspase activation during spermatid differentiation.

### Protection of the Sperm Nucleus

Effector caspases, such as drICE, Dcp-1, and caspase-3, normally can cleave a variety of nuclear targets, including lamins, I-CAD, and PARP (Hengartner, 2000). Therefore, the sperm nucleus must be protected against this potentially lethal activity of drICE. Our data indicate that dBruce, which encodes a giant E2 ubiquitin-conjugating enzyme, may exercise this function (Chen et al., 1999; Hauser et al., 1998; Vernooy et al., 2002). Loss of dBruce function results in nuclear hypercondensation, degeneration, and male sterility, consistent with a role of dBruce to restrain or limit caspase activity. Interestingly, dBruce contains a BIR domain, a motif also found in IAPs. This suggests that dBruce may bind to either caspases or Reaper/Hid/Grim-like (RHG) proteins (Salvesen and Duckett, 2002). Previous work has argued against RHG proteins as direct targets for dBruce (Vernooy et al., 2002). Therefore, it is attractive to speculate that dBruce functions by directly binding to and degrading caspases. Obviously, this proposed function would have to be spatially restricted during spermatogenesis, for example, by localizing dBruce to protected compartments, or by spatially limiting its E2 activity. An additional possibility is that drICE activation occurs only locally, in the affected compartment. Consistent with this idea, strong CM1 staining was only observed distal to the nuclei, in the cytoplasmic compartment that will be eliminated. One plausible mechanism for locally restricting drICE activation may be the local release of the "minor" cytochrome c from mitochondria, which are known to undergo dramatic morphological changes only in the post-individualized portion of the cyst (Fuller, 1993).

### Similarities between *Drosophila* and Mammalian Sperm Differentiation

Terminal differentiation of sperm shares many morphological and biochemical features with apoptosis. However, rather than causing the death of the entire cell, in this case apoptotic proteins are used to specifically eliminate cytoplasmic components, thereby producing a highly specialized living cell. Interestingly, a similar phenomenon is observed in mammals. As in *Drosophila*, intracellular bridges between spermatids and the bulk of the spermatid cytoplasm need to be eliminated during mammalian spermatogenesis. In mammals, the cytoplasm collects in the residual body (RB), which is functionally homologous to the WB in *Drosophila* (Figure 3F). Consistent with this idea, mammalian RBs display several features of apoptosis (Blanco-Rodriguez and Martinez-Garcia, 1999). Although a role of caspases for the removal of bulk cytoplasm during mammalian spermatogenesis remains to be established, we have preliminary data showing that active caspase-3 is present in RBs in the testes of mice (H. Kissel, E.A., and H.S., unpublished results). Consequently, there are both anatomical and biochemical similarities between insects and mammals that warrant more detailed studies. This is not only of academic interest, as various types of caspase inhibitors are being considered as drugs for therapeutic purposes, and effects on human fertility have not been studied. Furthermore, the abnormal spermatozoa with residual cytoplasm resulting from caspase inhibition in *Drosophila* (Figure 3E) bear a striking resemblance to one of the most commonly seen abnormalities of human spermatozoa, known as cytoplasmic droplet sperm (Figure 3F, right panel; Hollanders and Carver-Ward, 1996). Therefore, it is possible that defects in proper caspase activation may be responsible for this pathology, and further studies of apoptotic proteins may shed light on the etiology of some forms of human male infertility.

## Experimental Procedures

### Fly Strains

Canton S and *yw* were used as wild-type controls. *fuzzy onions* (*fzo*) mutant alleles were obtained from M.T. Fuller (Stanford University), *jaguar* (*jar*$^1$), *purity of essence* (*poe*$^1$), *hephaestus* (*heph*$^2$), *blanks* (*bln*$^1$), *Df*(2L)H20, *l*(2)*k13905*, and Δ2-3 "jumpstart" lines from the Bloomington Stock Center, and *EP*(2)*2305* and *EP*(2)*2049* lines from Exelixis and Szeged *Drosophila* Stock Centre, respectively. *dbruce* alleles were isolated in our lab (J.A., K. McCall, and H.S., unpublished results). *HSP83-Gal4* lines were generated by using CasperR-HSP83 (Horabin and Schedl, 1993; Hicks et al., 1999) to drive GAL4 (Brand and Perrimon, 1993).

### Antibodies

Primary antibodies used in this study were anti-lamin $Dm_0$ (mAb-ADL84; P. Fisher; 1:100), rabbit CM1 antiserum (IDUN Pharmaceuticals; 1:1000), rabbit anti-FL-DRICE and rabbit anti-active DRICE (B. Hay; 1:500 to 1:1000; Dorstyn et al., 2002), anti-cytochrome c (mAb2G8; R.J. Jemmerson; 22 μg/ml; Varkey et al., 1999), guinea

pig anti-Dronc (H.D. Ryoo and H.S., unpublished, 1:2000), and rabbit anti-β-galactosidase (Cappell; 1:1000). All secondary antibodies were from Jackson Laboratories (1:500).

### Antibody Staining

At least 20 testes were examined for each experiment. Testes of young adults were dissected in testis buffer (TB; 10 mM Tris-HCl [pH 6.8], 183 mM KCl, 47 mM NaCl, 1 mM EDTA, and 1 mM PMSF), transferred to a 2.5 μl drop of TB on a siliconized coverslip (GOLD SEAL), opened using thin forceps, and sandwiched with a poly-L-lysine-coated slide. The sandwich was frozen in liquid nitrogen, the coverslip was removed with a razor blade, and the slide was placed in ice-cold absolute ethanol. The slides were drained and a hydrophobic ring surrounding the opaque tissue was drawn using a PAP PEN (Zymed Laboratories). The tissue was fixed in 4% formaldehyde in PBS for 20 min, rinsed twice with PBS for 5 min, incubated in PBT (PBS + 0.1% Triton X-100) for 30 min, and rinsed twice again. The fixed testes were then blocked with PBS/BSA (1% BSA in PBS) for 45 min, incubated with primary antibody (diluted in PBS/BSA) within the hydrophobic ring overnight at 4°C inside a humid chamber, and rinsed twice for 5 min in PBS. Testes were incubated with the secondary antibody (diluted in PBS) for 1 hr at room temperature, rinsed twice in PBS for 5 min, incubated with 1 ng/μl TRITC-phalloidin (Sigma) in PBTw (PBS + 0.1% Tween 20) for 1.5 hr in 37°C in a humid chamber, rinsed once for 15 min at room temperature, and mounted in Vectashield mounting medium with DAPI (Vector Laboratories). For AO staining, testes were dissected in TB, incubated for 5 min in fresh $1.6 \times 10^{-6}$ M AO (Sigma) in TB, rinsed briefly in TB, and mounted in TB. All pictures were taken with a Zeiss confocal microscope.

The immunocytochemistry procedure was essentially as described in Gonczy et al. (1992).

### RNA Isolation and Northern Blotting

Total RNA was extracted from 100 adult male flies by using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen). Poly(A) RNA was isolated (PolyATract III; Promega), and 2.5 μg poly(A) RNA was loaded per lane. Northern blots were probed with a 200 bp fragment from the 3′ UTR of *cyt-c-d* (BDGP's EST clone LP05614 cleaved with BseRI and XhoI).

### Testes Cultures

Testes were dissected in a drop of culture media (10% fetal calf serum, modified Shields and Sang M3 medium [−bicarbonate; Sigma], with 1× penicillin/streptomycin [GIBCO-BRL]). Each isolated testis was incubated at 25°C for 24–26 hr with or without 30 μM caspase inhibitor Z-VAD(OMe)-FMK (Enzyme Systems Products).

### 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 after 1 week.

### *P* Element Excisions and Isolation of Fertile Revertants

To generate revertants from either the $bln^1$ or the *EP* strains, the *p*[Z] and *EP* transposons were excised using the transposase-producing Δ2-3 jumpstart strain (Robertson et al., 1988). For the $bln^1$ strain, we used the following excision scheme: $bln^1/bln^1$ females were crossed to *Sco/CyO*; Δ2-3, *ki* males, and progeny of the $bln^1$/CyO; Δ2-3, *ki* genotype were crossed to the original $bln^1$/CyO strain. Groups of five males, which contain a potential excised allele over the $bln^1$ allele, were allowed to copulate with wild-type females for 4 days for a fertility test. Then, testes of fertile groups were dissected and analyzed for the presence of active drICE and proper individualization structures.

### Isolation and Characterization of *dbruce* Alleles

In a genetic screen for cell death genes, we isolated 13 alleles of the *Drosophila* IAP-related gene, *dbruce*. Most of our *dBruce* alleles dominantly enhance cell death induced by Reaper and Grim, but have no effect on Hid-induced apoptosis. $dbruce^{E81}$ allele bears a deletion of 534 bp in-frame, which predicts to encode a truncated protein form that lacks the BIR domain (amino acids 234–411).

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