Regulation of Caspase Activation during *Drosophila* Spermatogenesis

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Apoptosis is a morphologically distinct form of programmed cell death that usually serves to remove unwanted and potentially dangerous cells during development and homeostasis of the animal. Improper control of apoptosis is known to be associated with cancer, AIDS, stroke, myopathies and various neurodegenerative disorders. Remarkable progress has been made in our understanding of the basic mechanism of apoptosis and many of its regulatory pathways. A main feature in all these pathways is the activation of an evolutionarily conserved family of cysteine proteases known as caspases, which are responsible for the execution of apoptosis. Caspases are present in a weak or inactive state in virtually all cells of higher metazoans, and their activity is carefully regulated by both activators and inhibitors. Once activated, caspases cleave a variety of important structural proteins, enzymes and regulatory molecules to ultimately lead to cell death. However, some cells can harness the "death" program and use it to differentiate instead of die. We discovered that during terminal differentiation, *Drosophila* spermatids normally lose their bulk cytoplasm and organelles in a restricted apoptosis-like process which requires active caspases. During spermatid terminal differentiation, in a process known as spermatid individualization, 64 elongated spermatids, connected by cytoplasmic bridges, are separated from each other via the caudal movement of an "individualization complex" (IC), which serves to also eliminate the majority of cytoplasm and cellular organelles from the developing spermatids. These cytoplasmic contents accumulate in cystic bulges (CBs) and are eventually deposited into "waste bags" (WBs; Figure 1a). We demonstrated that apoptotic effector caspases are activated and required for the proper removal of the spermatid's cytoplasm into WBs (Figure 1b), and that acridine orange (AO), a specific marker for apoptotic cells in *Drosophila*, selectively stained both CBs and WBs (Figure 1c), suggesting that CBs and WBs resemble apoptotic corpses (Arama et al., 2003). Moreover, we showed that orthologues of the apoptosome components (cytochrome C, Apaf-1, and caspase-9), which are key regulators of apoptosis, are also required for proper spermatid individualization (Arama et al., 2006). These findings demonstrate that certain cells can use the apoptotic machinery in a restricted manner in order to terminally differentiate. Likewise, there are other examples where apoptosis-like events do not lead to the death, but rather the terminal differentiation of certain cell types. For instance, lens epithelial cells and mammalian red blood cells lose their nucleus and other subcellular organelles during terminal differentiation but continue to be metabolically active. Furthermore, caspases were associated with terminal differentiation of human keratinocytes, differentiation of monocytes into macrophages, formation of platelets from mature megakaryocytes, differentiation of skeletal muscle, and recently, modulation of cell signaling pathways through substrate cleavage in neural precursor development in

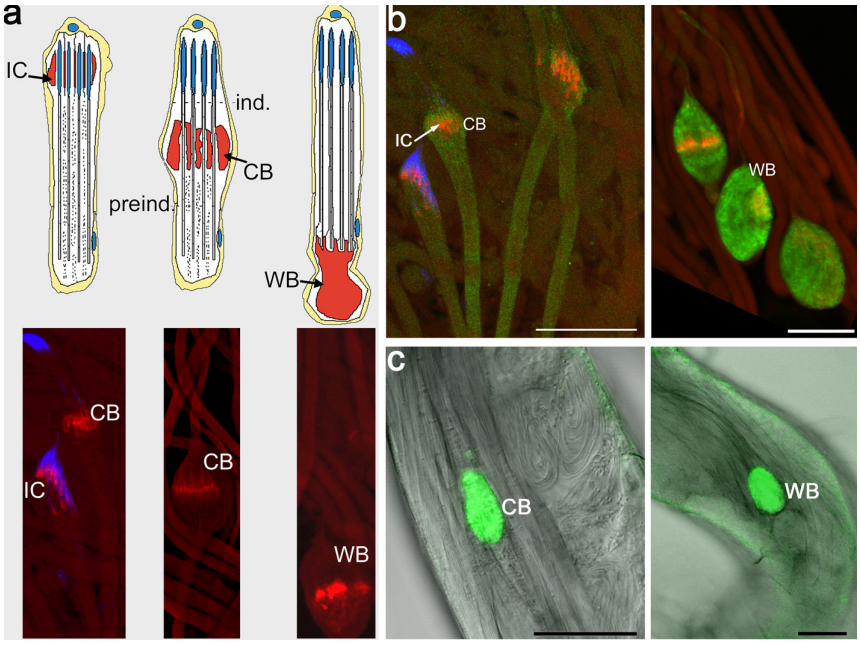

Fig. 1. *Spermatid individualization process is reminiscent of apoptosis.* ***a.*** *Schematic diagram of spermatid individualization (upper panel), and the corresponding developing spermatids (lower panel) stained with DAPI to visualize the nuclei (blue) and with phalloidin to visualize the individualization complex (IC, red). Sixty-four elongated spermatids, connected by cytoplasmic bridges, are separated from each other via the caudal movement of an individualization complex (red), which serves to also eliminate the majority of cytoplasm and cellular organelles from the developing spermatids. These cytoplasmic contents accumulate in cystic bulges (CBs) and are eventually deposited into "waste bags" (WBs).* ***b.*** *A steep cascade of caspase-3 activation (CM1, green) is detected along the entire length of the spermatids once the IC is assembled. Cleaved caspase-3 is detected in the pre-individualized portion of the spermatids, the CB, and eventually in the WB, and is removed from the post-individualized portion of the spermatids (asterisk).* ***c.*** *The apoptotic marker acridine orange (AO, green), which stains apoptotic corpses, also stains the CBs and WBs.*

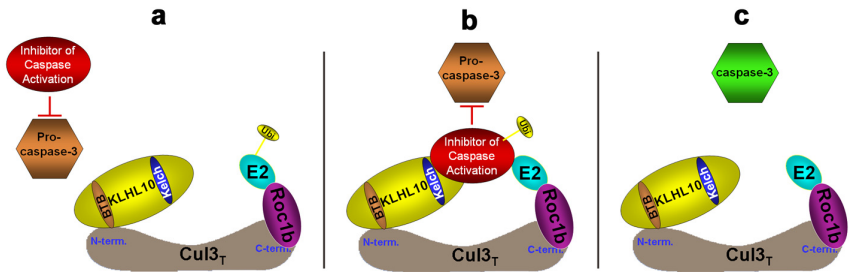

Fig. 2. *A working model for the function of Cullin-3-containing ubiquitin ligase complex in the testis. The diagram represents the assembly of the testis Cullin-3-containing ubiquitin ligase (E3) and its possible function in caspase activation during spermatid individualization.* ***a.*** *The model suggests that once an active Klhl10-Cul3Testis-Roc1b E3 ubiquitin ligase complex is assembled, it recruits an "Inhibitor of caspase activation" protein (red) via the Kelch domain of the substrate recruitment protein Klhl10 (yellow).* ***b.*** *Then, the ubiquitin conjugating enzyme (E2, blue), which is recruited by the RING finger protein Roc1b (purple), ubiquitinates the "Inhibitor of caspase activation" protein.* ***c.*** *Subsequent degradation of this inhibitor allows the activation of caspases at the onset of spermatid individualization process.*

Drosophila and the removal of sensory neuron dendrites during pruning in *Drosophila*. Collectively, these striking observations counter the dogma that cells expressing active caspases, the core of the evolutionarily-conserved apoptotic cell death machinery, are doomed to die.

In the course of exploring the role of apoptotic factors in spermatid differentiation, we addressed another major controversy in the field: the role of cytochrome C for caspase activation in *Drosophila*. Cytochrome C has two very different functions within the cell. As traditionally appreciated, it acts in the respiratory chain for energy metabolism, but it also serves as a regulator of the 'intrinsic' apoptotic pathway. While the role of mitochondria and cytochrome C in the assembly of the apoptosome and caspase activation has been established for mammalian cells, the existence of a comparable function for cytochrome C in invertebrate cells has been highly controversial. We found that one of the two *Drosophila* cytochrome c genes, *cyt-c-d*, is required for caspase activation and spermatid individualization, whereas the second gene, *cyt-c-p*, is required for respiration in the soma. We demonstrated that both cytochrome C proteins can function interchangeably in respiration and caspase activation upon ectopic expression, while the difference in their genetic requirements *in vivo* can be attributed to differential expression in the soma and the testes (Arama *et al.*, 2006). Importantly, the findings which were obtained from the study of spermatid individualization can also be extended to "normal" apoptosis. For example, in collaboration with Bertrand Mollereau, we demonstrated that cytochrome C-d also regulates "conventional" developmental apoptosis in the *Drosophila* eye (Mendes *et al.*, 2006).

Research in my laboratory

is concerned with the molecular mechanisms of programmed cell death or apoptosis and its role in *Drosophila* development. One main focus in the lab is to study the molecular, cellular, and anatomical mechanisms that underlie late spermatid differentiation in *Drosophila*. We aim to uncover the signaling pathways that regulate caspase activation during cellular remodeling of the sperm and investigate how caspases facilitate cell terminal differentiation. We also aim to identify the caspase substrates required for individualization, and how they differ from those cleaved during apoptosis. Answering questions such as these will also advance the understanding of how spermatids avoid death in the presence of active caspases that would normally induce apoptosis in other cells.

The Role of the Ubiquitin/ Proteasome Pathway for Caspase Activation in Spermatids

To gain more insight into the caspase regulation in this system, we initiated a systematic mutant screen for genes controlling caspase activation during spermatid terminal differentiation. In collaboration with colleagues in Hermann Steller's lab, we screened a large collection of 1000 male-sterile flies for mutants that block effector caspase activation at the onset of spermatid individualization, and we identified 33 mutant alleles representing 22 different complementation groups. Among them are one of the two *Drosophila* cytochrome *c* genes, *cyt c-d*, eight different genes that affect mitochondrial structural organization, and two components of an E3 ubiquitin ligase complex, Cullin-3 and the BTB-Kelch domains protein Klhl10. Cullins are major components of E3-ubiquitin ligases, serving as the scaffolding for two functional modules: a catalytic module comprised of a small RING-domain protein that recruits the E2 ubiquitin-conjugating enzyme, and a substrate recognition module that binds to the substrate and brings it within proximity to the catalytic module. From the genetic screen, we identified several *cullin-3* and *klhl10* mutant alleles, and showed that these two proteins, together with the small RING-domain protein Roc1b, can physically interact to form an E3 ubiquitin ligase complex that activates effector caspase at the onset of spermatid individualization (Arama *et al.*, 2007). Our results suggest a simple working model in which the Cullin-3-Roc1b-Klhl10 complex promotes caspase activation via ubiquitination and degradation of a caspase inhibitor (Figure 2). Identifying this inhibitor protein is, therefore, an important step towards elucidating the Cullin-3 pathway in spermatids. Using different approaches, we have identified already two candidate substrates for the E3 complex, which are currently further characterized.

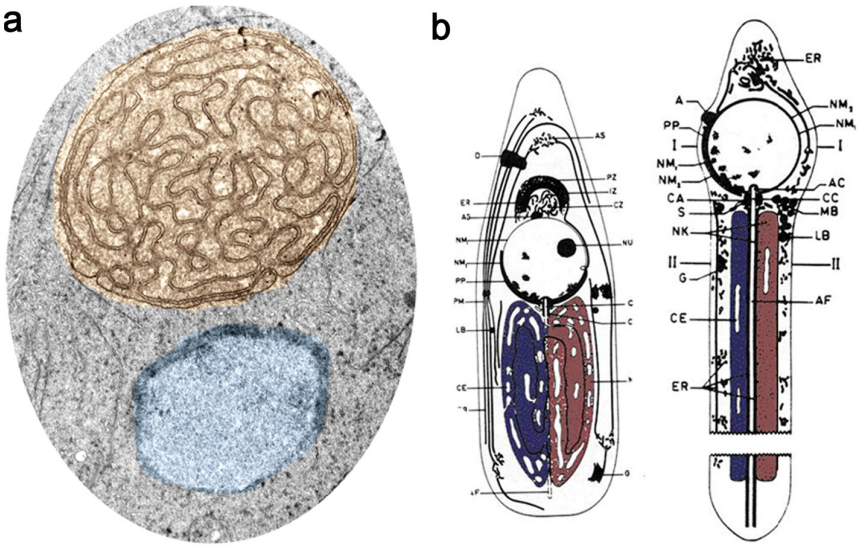

Fig. 3. *Mitochondrial dynamics during sperm maturation in Drosophila.* ***a.*** *Electron micrograph of section through a Drosophila melanogaster early spermatid at the clew stage. By this stage, the mitochondria have fused and formed a spherical mass of about 6.7 mM in diameter known as the Nebenkern (manually highlighted in beige), located to one side of the nucleus (manually highlighted in blue).* ***b.*** *After completion of this striking transformation, the mitochondrial derivative unfolds, splits into two parts, and elongates together with the growing axoneme along the length of the sperm tail (1.8 mm). The major mitochondrial derivative in the scheme of the elongating spermatids is in red and the minor derivative is marked blue. The nuclei are the "oval" structures on top, whereas the axoneme is represented by a thin white line in between the two mitochondrial derivatives. The image in a was acquired with the help of Janet Rollins at St. John's University in NY. The image in b was modified from Fuller MT (1993) Spermatogenesis in Drosophila. In: Bate M, Arias AM, editors. The development of Drosophila melanogaster. CSH (New York): CSHL Press.*

The Role of Mitochondrial Dynamics for Caspase Activation in Spermatids

In a second line of research in the lab, we investigate the role of mitochondrial dynamics for caspase activation in spermatids. Being at the core of the intrinsic apoptosis pathway, mitochondria have emerged as the central regulators of the apoptotic program in mammalian cells, providing a reservoir of protein factors that induce caspase activation. One of these mitochondrial factors, cytochrome C, is largely sequestered within the intracristae compartment, and must migrate into the boundary intermembrane space in order to allow passage across the outer membrane to the cytosol. Recent evidence argues that inner mitochondrial membrane dynamics may regulate this process; however the mechanisms behind these processes and their roles for caspase activation are largely unclear.

The *Drosophila* sperm system can serve as an excellent model to study the significance of the mitochondrial pathway and dynamics for caspase activation. In *Drosophila*, spermatids are the only known cells that absolutely require the mitochondrial pathway (cytochrome C) for caspase activation. During the development of the spermatid, the mitochondria undergo dramatic morphological changes and structural organizations. In the haploid spermatid, the mitochondria fuse together into two giant aggregates, which wrap around one another to produce a spherical, giant mitochondrion known as the Nebenkern. When the flagellum elongates, the two mitochondrial regions of the Nebenkern unfold and elongate down the spermatid tail (Figure 3). We isolated eight different mutants in our screen that displayed defects in these mitochondrial organization processes.

We are now mapping the responsible genes and characterizing the specific mitochondrial organization defects in these mutants. Results from this study will further our understanding of the underlying molecular mechanisms of mitochondrial dynamics and their relevance to caspase activation in a model organism.

Selected publications

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