Results

*Mutations that affect the Nebenkern, a unique mitochondrial formation, block caspase activation and spermatid individualization*

The requirement of cytochrome C-d for caspase activation during spermatid individualization provides a link between this apoptosis-like process and mitochondrial function. Because sperm in insects, such as *Drosophila*, carry a unique mitochondrial formation, known as the Nebenkern, we were interested in further exploring this connection. Immediately after completion of meiosis, the mitochondria of the spermatid assemble on one side of the haploid pronucleus and fuse together into two giant aggregates (Fuller, 1993). These aggregated mitochondria then wrap around one another to produce the spherical Nebenkern (yellow arrows in Supplementary Figure 1C). When viewed in cross-section under the transmission electron microscope, the Nebenkern resembles a sliced onion, and hence this early stage of spermatogenesis is called the “onion stage”. When the flagellum elongates, the two mitochondrial regions of the Nebenkern unfold and elongate down the side of the 1.8 mm long axoneme (Supplementary Figure 1E-G and K-N; Fuller, 1993). However, while the use of electron microscopy facilitated a detailed and insightful morphological description of this extensive mitochondrial organization (Tokuyasu, 1974; Tokuyasu, 1975), the role of the Nebenkern for the development of the spermatid is still unknown. Interestingly, in addition to a block in caspase activation, almost a third of the mutants identified in the genetic screen (described in the “Results” section) also displayed severe defects in Nebenkern organization (Supplementary Figure 1 and data not shown). In contrast, we previously reported that the *fuzzy onions* mutant, which blocks mitochondrial fusion but not elongation (Hales and Fuller, 1997) still displayed caspase activation (Arama *et al*, 2003). Thus, to shed light on the specific Nebenkern organization property required for caspase activation, we extensively analyzed the morphological defect of the mitochondria of one of these mutants, which we called *pln* (because the defective mitochondrial derivatives in these spermatids are aggregated in a “neck”-like region immediately adjacent to the nucleus as seen in Supplementary Figure 1J, which is reminiscent of the
Pa Dong Long Neck women who live along the Thai and Burmese border; this mutant corresponds to Zuker stock #Z2-0516). Using phase-contrast microscopy, we found that while no gross mitochondrial defects were observed during early stages of spermatocyte development (data not shown), a pronounced defect of grainy and highly vacuolated Nebenkerns was observed in pln$^{Z2-0516}$ round spermatids (compare Supplementary Figure 1C and D). Staining testes with DAPI, which binds to both nuclear and mitochondrial DNA also reveals the smooth versus grainy Nebenkerns in wild-type and pln$^{Z2-0516}$ round spermatids, respectively (yellow arrows in Supplementary Figure 1E and H). Furthermore, in pln$^{Z2-0516}$ mutants, the Nebenkerns fail to elongate, displaying non-homogeneous agglomerations of mitochondrial DNA adjacent to the nuclear pole (compare Supplementary Figure 1F and G with I and J). To obtain a more accurate characterization of the mitochondrial defect in pln mutants and to confirm the mitochondrial elongation defect observed, we used transmission electron microscopy. In wild-type, the elongating Nebenkern splits into the major and the minor mitochondrial derivatives (red and blue, respectively in Supplementary Figure 1K-N), which are associated with the axoneme (green) throughout the entire period of flagellar elongation. As the spermatids mature, the volume of the minor derivative is reduced until after individualization when it becomes a tiny wedge (blue in Supplementary Figure 1N). Meanwhile, as the major derivative matures, it accumulates an amorphic material known as the “paracrystalline” material (dark mitochondrial density in Supplementary Figure 1L-N). In pln$^{Z2-0516}$ spermatids however, most of the axonemes (green in Supplementary Figure 1O-R) are not associated at all or only sometimes associated with only one mitochondrial derivative with no “paracrystalline” material inside (yellow in Supplementary Figure 1O-Q). In addition, we frequently observed immature mitochondrial derivatives, which fail to unfurl properly resembling the early onion-like shaped Nebenkern in round spermatids (purple in Supplementary Figure 1P and R). These results demonstrate that pln is required for Nebenkern elongation and that disruption of this process causes a block in caspase activation. Hence, we have established a link between mitochondrial reorganization and caspase activation in Drosophila spermatids.
Mutations which affect mitochondrial organization block caspase activation and spermatid individualization. (A) While CM1 staining is clearly visualized (green) in testes of a representative mutant (Z2-0706) that displays a strong block in mid spermatid elongation stage, (B) no CM1 staining was detected in the pln mutants. The nuclei (blue) are stained with DAPI. (C) Round spermatids from wild-type flies visualized by phase microscopy. The mitochondria fuse into a giant mitochondrion, known as the Nebenkern (yellow arrows) which is about the same diameter as the nucleus (red arrows). Note the smooth oval shape of the Nebenkern. (D) In contrast, the Nebenkern in round spermatids of pln mutants are grainy and highly vacuolated (yellow arrows). Using DAPI to stain nuclei and mitochondrial DNA also indicates that in wild-type the mitochondrial DNA in Nebenkerns (E, weak blue, yellow arrows) and in the elongating mitochondrial derivatives (F, G, yellow arrows) appear smooth and homogenous, while in plnZ2-0516-/- round spermatids’ Nebenkerns the DNA is grainy (H, yellow arrows) and later fails to elongate properly, displaying non-homogeneous agglomerations of mitochondrial DNA (I, J, yellow arrows). (K-R) Electron micrographs of cross sections through elongating spermatids. The colors indicated were manually added using Adobe Photoshop program to emphasize the described organelles. (K-N) In wild-type, as the Nebenkern elongates, it splits into two parts; the major (red) and the minor (blue) mitochondrial derivatives. Note that the minor derivative is reduced as the spermatids mature until it becomes a tiny wedge after individualization (blue in N). The two mitochondrial derivatives assume a characteristic angular relationship with the axoneme (green) throughout the period of flagellar elongation. (O-R) In plnZ2-0516-/- elongating spermatids, no or only one out of the two mitochondrial derivatives (yellow) is visualized in association with the axoneme (green). Furthermore, immature mitochondrial derivatives, which fail to unfurl properly, is frequently visualized (purple). The magnification of the electron micrographs is 50,000.
References


