Paternal Mitochondrial Destruction after Fertilization Is Mediated by a Common Endocytic and Autophagic Pathway in Drosophila

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SUMMARY

Almost all animals contain mitochondria of maternal origin only, but the exact mechanisms underlying this phenomenon are still vague. We investigated the fate of Drosophila paternal mitochondria after fertilization. We demonstrate that the sperm mitochondrial derivative (MD) is rapidly eliminated in a stereotypical process dubbed paternal mitochondrial destruction (PMD). PMD is initiated by a network of vesicles resembling multivesicular bodies and displaying common features of the endocytic and autophagic pathways. These vesicles associate with the sperm tail and mediate the disintegration of its plasma membrane. Subsequently, the MD separates from the axoneme and breaks into smaller fragments, which are then sequestered by autophagosomes for degradation in lysosomes. We further provide evidence for the involvement of the ubiquitin pathway and the autophagy receptor p62 in this process. Finally, we show that the ubiquitin ligase Parkin is not involved in PMD, implying a divergence from the autophagic pathway of damaged mitochondria.

INTRODUCTION

Almost all eukaryotic organisms inherit their mitochondria, the primary cellular power plants, from the maternal parent. Although this renowned phenomenon has been widely accepted among geneticists and developmental biologists, the striking variation among different organisms in the extent and pattern of uniparental inheritance has led to a diverse number of proposed mechanistic models for this phenomenon and thus has been the cause of much uncertainty (Ankel-Simons and Cummings, 1996; Birky, 2001). Until recently, the prevailing mechanistic explanation for maternal mitochondrial inheritance has been a passive model of simple dilution of the paternal mitochondria by an excess copy number of the egg mitochondria (Gyllensten et al., 1991; Birky, 2001). This explanation is mainly based on a study that used unique mitochondrial (mt)DNA nucleotide identifiers to demonstrate that paternally inherited mtDNA molecules can be detected at low frequency in hybrid mice, suggesting that paternal contribution exists but that it may often be overshadowed in mature mice to a point beyond the limits of detection by conventional PCR analyses (Gyllensten et al., 1991). On the other hand, recent studies in Caenorhabditis elegans have reported the active involvement of autophagy in this process. As opposed to organisms with flagellated sperm, in which the mitochondria undergo unique structural remodeling to become part of the flagellum, C. elegans produces nonflagellated amoeboïd sperm with mitochondria of much simpler morphology (Al Rawi et al., 2011; Sato and Sato, 2011). Furthermore, despite recent findings that the autophagy receptor p62 and the ubiquitin-like modifier of autophagy LC3 colocalize to the sperm tail after fertilization in mice (Al Rawi et al., 2011), a more recent report has suggested that sperm mitochondria are not degraded by autophagy in mice but rather that maternal inheritance may be a passive outcome as a result of the elimination of sperm mtDNA before fertilization and the uneven distribution of the paternal mitochondria in the early embryo (Luo et al., 2013). However, the reason and functional consequence of p62 and LC3 localization in the sperm tail soon after fertilization were left unexplained (Luo et al., 2013). Therefore, it remains to be determined whether and what types of active mechanisms may be involved in the elimination of paternal mitochondria in other organisms with flagellated sperm.

The ability of cells to monitor the quality of mitochondria and control their turnover is increasingly recognized as an essential element in maintaining mitochondrial homeostasis (de Castro et al., 2010; Weber and Reichert, 2010). Mitochondrial quality control depends upon a balance between biogenesis, dynamics (fusion and fission), and selective turnover (Twig et al., 2008; Ashrafi and Schwarz, 2013). However, despite its important role in quality control, the molecular mechanisms underlying selective mitochondrial destruction are still not well understood (Wang and Klionsky, 2011). Accumulating data suggest the involvement of the ubiquitin pathway in the selective recognition and autophagy-mediated destruction of cytosolic microbes, protein aggregates, and damaged organelles (Narendra et al., 2008; Kirkin et al., 2009b; Knaevelsrud and Simonsen, 2010; Randow, 2011). However, the function of the autophagic machinery and the ubiquitin pathway in normal mitochondrial turnover (a process also dubbed mitophagy) and their roles...
Figure 1. Visualization of the Paternal MD by Electron and Confocal Microscopy

(A) Schematic representation of Drosophila sperm during three consecutive maturation stages.

(B, C, and F–H) Electron micrographs of cross-sections through (B and C) developing Drosophila sperm cells and (F–H) the anterior regions of fertilized eggs at 10–20 min AEL.

(B and C) Micrographs corresponding to stages II and III in (A), respectively.

(F) Part of the sperm flagellum (Fm) is shown among several maternal mitochondria (mM). Note the presence of the axoneme (AXO) and the MD.

(G and H) Higher magnifications of cross- and transverse sections through the sperm flagellum, respectively.

(B, C, G, and H) The colors indicated were manually added using Adobe Photoshop to correspond to the colored organelles in (A).

(C and G) The asterisks indicate the remnants of the minor MD.

(D, E, and I–N) Confocal images of mature sperm cells from (D and E) seminal vesicles and (I–N) inside fertilized eggs at 15–30 min AEL.

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during development are still vague (Ashrafi and Schwarz, 2013). Whereas in some examples, mammalian sperm mitochondria were shown to be ubiquitinated after fertilization (Sutovsky et al., 1999, 2000; Al Rawi et al., 2011; Luo et al., 2013), other studies in C. elegans and mice have suggested that paternal mitochondria are either not ubiquitinated (Sato and Sato, 2011) or that ubiquitination was not restricted to sperm mitochondria only (Luo et al., 2013), respectively. The establishment of a genetically amiable system to monitor and study selective mitochon-
drial destruction under physiological conditions should therefore help to fill in the large gaps in our understanding of this important process.

Autophagy is a highly regulated membrane-mediated intracel-
lar degradation process, ubiquitous in eukaryotic cells. During autophagy, double-membrane vesicles, called autophago-
somes, engulf cytoplasmic materials, including proteins and organelles, and deliver them to the lysosome for degradation (Nakatogawa et al., 2009; He and Klionsky, 2009; Rubinsztain et al., 2012). The recycled products are subjected to cellular metabolism for production of energy and to build new proteins and membranes (Rabinowitz and White, 2010). In addition to its fundamental role in metabolic adaptation during cell homeostasis, autophagy also functions in cell growth, survival, and cell death and as an intracellular quality control system (Neufeld and Baehrecke, 2008). More recent studies have highlighted the importance of selective autophagy in mediating the turnover of specific unwanted cargo, including damaged organelles (Mizu-
ushima and Komatsu, 2011; Weidberg et al., 2011; Sumpter and Levine, 2011). However, whereas selective clearance of organelles resembles an autophagy-related process at the anatomical level, genetic, molecular, and cellular studies have indicated some divergence from the core autophagic machinery (Xie and Klionsky, 2007; Farré et al., 2009; Zhang et al., 2009; Manjithaya et al., 2010).

Here we uncover a nonclassical, autophagic, and endocytic common pathway involved in the active elimination of the paternal mitochondria in Drosophila (a process also termed paternal mitochondrial destruction; PMD). Our findings suggest that a similar pathway may also mediate PMD in other organisms with flagellated sperm, including humans.

RESULTS

Ultrastructural Analysis of the Paternal Mitochondrial Destruction Process in Drosophila

In the fruit fly Drosophila melanogaster, mitochondrial remodeling initiates immediately after meiosis in the haploid round spermatid with the fusion of all the mitochondria into a giant sphere called the Nebenkern. The Nebenkern is then resolved into two mitochondrial derivatives (MDs), which unfold and elongate alongside and in close association with the growing axoneme to the length of the sperm flagellum (nearly 2 mm long; Figure 1A, I and II; Figure 1B). During advanced spermatid elongation stages, one MD (“minor”) constantly loses volume and shrivels, whereas the other MD (“major”) accumulates dark amorphous material of an unknown nature termed paracrystalline material (Figure 1A, III; Figure 1C) (Lindsey and Tokuyasu, 1980; Fuller, 1993).

Upon fertilization, a single sperm cell completely penetrates the egg through a specialized structure, the micropyle, a single hole in the chorion located at the anterior end of the egg (Karr, 1991). To uncover the fate of the paternal MD after fertilization, we first used transmission electron microscopy (EM) of ultrathin sections prepared from the anterior part of early fertilized eggs. At 15–30 min after egg laying (AEL), the intact sperm flagellum could be readily distinguished from the egg’s organelles by virtue of its unique organellar contents (i.e., the axoneme and the attached MD; Figures 1F–1H). The still largely intact flagellum is approached by globular clusters of microvesicles (also referred to as multivesicular clusters; MCVs; Figures 2A and A’), which then associate with the sperm plasma membrane (Figures 2A’–2B’; also note the microvesicles, which are attached to the plasma membrane). The MCVs are also abundant in unfertil-
ized eggs, suggesting that the developing egg may already be prepared for sperm penetration (Figures S2A and S2A’ available online). In addition to the MCVs, other large vesicular organelles reminiscent of either multivesicular bodies (MVBs) or multilamel-
lar bodies (MLBs), and which are completely wrapped around some regions of the flagellum, were also detected (Figures 2C and 2C’, respectively). For convenience, we will hereafter refer to the MCVs, MVBs, and MLBs as MVB-like vesicles. At a later stage, the mitochondrial derivative separates from the axoneme (Figures 2D and 2D’), a process also readily visualized by fluores-
cence microscopy (Figures 1L–1N; Figure S1). Subsequently, still within the preblastoderm (mitotic cycles 1–9), the MD breaks into oval fragments (MFs) with diameters between 0.1 and 0.4 μm, which are then enveloped by autophagosome-like double membranes (Figures 2E and 2E’). The MFs are eventually sequestered to lysosomes, where they are completely degraded and assimilated into the egg cytoplasm (Figures 2F and 2F’; see also Figures S2C–S2E for autolysosomes that are in the process of digesting the MFs), and they are no longer detectable by the cellular blastoderm stage (130–180 min AEL). We conclude that immediately after fertilization, the paternal MD is degraded by a network of vesicles originating in the egg and reminiscent of elements of the endocytic (MVB-like vesicles) and autophagic pathways. Because this is a stereotypical active process, we hereafter refer to this process as paternal mitochondrial destruction.

The Kinetics of PMD

In order to investigate the mechanisms underlying PMD, we first established a fluorescence-based imaging system to monitor the sperm MD within early fertilized eggs. We generated transgenic flies carrying mitochondrial targeted red (MTS-DsRed) and yellow (MTS-Venus) fluorescent proteins under the
Figure 2. Ultrastructural Characterization of the PMD Process
Electron micrographs of cross-sections through fertilized eggs at 10–60 min AEL.
(A and A') Soon after fertilization, multivesicular clusters approach and associate with the flagellum (detected in six sections of three embryos). Note that the sperm plasma membrane is readily visualized (light green arrow), mv, microvesicles.
(B and B') The microvesicles specifically attach to the sperm membrane, but not to the maternal mitochondria (detected in 14 different sections of four embryos).
(C and C') Multivesicular bodies (C) and multilamellar bodies (C'), which completely enwrap some flagellar regions, are also detected (detected in two different sections of one embryo). Note that the MD and the axoneme are still enveloped by the sperm plasma membrane, as indicated by the double membrane of the MD and the surrounding plasma membrane within the MVB (light green arrow), and that the highly ordered 9 + 2 microtubule structure of the axoneme is disrupted (11 white dots).

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regulatory regions of the sperm-specific gene don juan (dj). The red and yellow (but not green) fluorescent transgenes were preferentially selected because of the large amount of masking green autofluorescence coming from the egg yolk. By crossing these flies to transgenic flies expressing the sperm-specific mitochondrial protein marker DJ-GFP (Santel et al., 1998), we validated that these transgenes specifically label the sperm MD (Figures 1D and 1I–1K). Furthermore, staining the DJ-GFP-expressing sperm with the anti-polyglycylated tubulin antibody (AXO 49), which specifically detects the mature axoneme (Bré et al., 1998; Arama et al., 2007), revealed a clear distinction between these two organelles despite their close alignment throughout the sperm flagellum (Figures 1E and 1L–1N). Transgenic male flies carrying MTS-DsRed, MTS-Venus, and DJ-GFP were fertile and gave rise to normal progeny (Figures S3C and S3E and data not shown), and can thus serve for further analyses of the PMD process.

To determine the kinetics of the PMD process, we monitored the elimination rate of the sperm MD by live imaging of eggs fertilized by the MTS-DsRed sperm. Beginning at 10 min AEL, time-lapse sequences of z projection images with a 4 min interval were captured for about 2 hr. These analyses revealed that the MD almost completely disappears at 80–90 min AEL (i.e., at the onset of the syncytial blastoderm stage; see Movie S1 and selected still images from this movie at the top of Figure 5A and the quantification of the fluorescence intensities in Figure 5B). It is noteworthy that the disappearance of the fluorescent signal does not occur in a uniform fashion throughout the sperm flagellum, as some regions fade away before others, suggesting that PMD is initiated at different points along the MD. This is also consistent with our observation that the detachment of the MD from the axoneme is also initiated at different points along the flagellum (Figure S1).

**Autophagy-Related Vesicles Associate with the Sperm MD Soon after Fertilization**

To further characterize the molecular nature of the MVB-like vesicles and the autophagosome-like vesicles, we used fluorescent protein markers. Males expressing the MTS-Venus sperm were first crossed to females maternally expressing a genetic reporter of autophagy, eGFP-mCherry-Atg8a. This double-labeled reporter can reveal autophagosomes and autolysosomes by virtue of the Atg8 ability to localize to phagophore-assembly sites and thus fluorescently label autophagosomes in green and red (i.e., yellow) and autolysosomes in red only because of the quenching of the eGFP signal in the acidic environment (Nezis et al., 2010). Significantly, as opposed to classical autophagosomes, which are usually smaller than 0.5–0.7 μm in diameter, this reporter labeled much larger vesicles in the range of 0.5–1.8 μm in diameter, which were specifically associated with the MD (an embryo at 40–60 min AEL is shown in Figure 3A). These vesicles were all detected in both the green and red fluorescent channels, suggesting that they were not fused with lysosomes. In addition, the MD was still detected even when the embryo reached the cellular blastoderm stage (2 hr AEL; data not shown), suggesting that this reporter may abrogate the function of the vesicles and thus attenuate the PMD process.

We considered the possibility that the double-labeled Atg8 reporter may be somewhat harmful to the function of the vesicles, and hence we instead used a single-labeled reporter, mCherry-Atg8a. Indeed, although the single-labeled reporter still caused a mild attenuation in the PMD process (data not shown), we could clearly and reproducibly follow the dynamics of the Atg8-positive vesicles, concomitant with the destruction of the MD (Figures 3B–3E). At 15–20 min AEL, only small scattered mCherry-Atg8-positive vesicles were detected in the anterior region of the egg, without any specific association with the MD (Figure 3B). Similar distribution of Atg8-positive vesicles was also detected in unfertilized eggs (Figure S2B). However, at about 30 min AEL onward, mCherry-Atg8 started accumulating in the vicinity of and became associated with the MD, which in turn displayed gradually increasing fragmented morphology (Figures 3C–3E). Interestingly, these vesicles gradually increased in size, becoming unusually large, reminiscent of the size range of the vesicles detected with the double-labeled reporter as well as the sizes of the MVB-like vesicles detected by the EM (Figures 3D and 3E). Furthermore, similar to the MVBs and MLBs, which entirely encapsulated some flagellar regions/pieces, large mCherry-Atg8-positive vesicles encapsulating fragments of the MD and which were already detached from the main MD piece were also detected (Figure 3E). Taken together, these observations suggest an unconventional role of the autophagy pathway during PMD.

**PMD Is Mediated by a Network of Vesicles Displaying Markers Common to the Autophagic and Endocytic Pathways**

Existing evidence suggests that under certain conditions, the endocytic and autophagic pathways may con vene, giving rise to hybrid vesicular organelles termed amphiosomes (Berg et al., 1998; Filimonenko et al., 2007; Fader et al., 2008; Lamb et al., 2013). Amphiosomes are believed to constitute prelysosomal intermediate vesicles involved in autophagosomal maturation. The facts that Atg8 was localized to unusually large vesicles during PMD and that MVBs and MLBs, but not autophagosomes, are the only vesicles of a similar size range associated with the sperm during this process (the EM data), raised the hypothesis that the Atg8-positive vesicles may also constitute hybrid organelles of the endocytic and autophagic pathways. To test this idea, we used available markers of the endosomal pathway. First, we stained early fertilized eggs with the anti-Hrs antibody, which detects the endosome-associated, ubiquitin-binding protein Hrs/Vps27, a member of the endosomal sorting complexes required for transport 0 (ESCRT-0) (Lloyd et al., 2002). Whereas...
this antibody faintly stained large vesicles (1.3–1.9 μm) found in the vicinity of the sperm flagellum (data not shown), vesicles that were already wrapped around flagellar material displayed a stronger signal, suggesting that the MVBs may be highly active during and/or after the encapsulation process (white arrow in the inset of Figure 4B). To further test this possibility, we used an antibody that specifically recognizes the nonclassical K63-linked ubiquitin side chains (Newton et al., 2008; see also the legend for Figure 6). Large ubiquitin-positive vesicles (1.4–3.5 μm) were detected in the vicinity of the flagellum, some of which completely enwrapped flagellar material (arrows in Figure 4C, inset). Interestingly, ubiquitin-positive vesicles that were still wrapped around some regions of the main flagellar piece could also be detected, suggesting that the encapsulation process may promote subsequent clipping of these flagellar regions (upper arrow in the inset of Figure 4C). Taken together, these results are consistent with the idea that at least some of the large vesicles associated with the sperm flagellum are related to the endocytic (MVB) pathway.

Rab GTPases function as master regulators of endocytic trafficking, and thus can serve as protein markers for specific steps in the endocytic pathway (Figure 4A) (Pfeffer, 2013). To directly test whether the Atg8-positive vesicles may also contain features of the endocytic pathway, we examined early fertilized eggs expressing the mCherry-Atg8 reporter and either Rab5, -7, or -11 fused to yellow fluorescent protein (YFP). Indeed, YFP-Rab7, a protein marker for mature endosomes/MVBs, colocalized with the large Atg8-positive vesicles associated with the sperm flagellum (Figures 4D and 4E). In contrast, the respective markers for early and recycling endosomes, YFP-Rab5 and YFP-Rab11, marked unrelated vesicles in the egg (data not shown). Therefore, the large vesicles associated with the sperm flagellum display features common to the endocytic and autophagic pathways.

Inactivating Mutations in the Autophagic and Endocytic Pathways Attenuate PMD

To examine the functional involvement/requirement of the autophagic and endocytic pathways in PMD, we next inactivated key
Figure 4. Vesicular Elements of the Endocytic and Autophagic Pathways Overlap during PMD

(A) A simplified diagram depicting the endocytic pathway and the relative vesicular locations of the proteins examined in this study.
(B and C) Early fertilized eggs stained to visualize the axoneme (green) and (B) the Hrs protein (red) or (C) ubiquitinated proteins with K63-polyUb (red). Anterior is to the left. The diameters of selected vesicles are indicated by arrows in the insets. The scale bar represents 20 μm.
(D and E) Live confocal images of early fertilized eggs maternally expressing the autophagy reporter, mCherry-Atg8 (red), and the late endosome marker, YFP-Rab7 (green).

All insets are enlargements of the respective regions in the small white squares.
components in the two pathways in the eggs and monitored the effects on the kinetics of PMD using a live imaging assay. To compromise the autophagic pathway, we first analyzed eggs from females carrying two \textit{atg7} null alleles in trans to one another (atg7\textsuperscript{21d/kd77}; see also complex 3a in Figure S3A). These mutant flies complete metamorphosis and survive to adulthood, despite showing some severe defects in autophagy (Juhász et al., 2007; McPhee and Baehrecke, 2009). Furthermore, \textit{atg7} maternal mutant embryos develop with normal kinetics at least until the end of the cellularization stage and beginning of gastrulation, a stage when the MD is already completely eliminated in wild-type counterparts (Figures S3B–S3E). Critically, \textit{atg7} mutant eggs displayed a significant delay in the clearance of the MD, showing about 60% reduction in the fluorescence intensity of the MD at 45 min AEL as compared with 85% reduction in the wild-type counterparts (compare upper panels with lower panels in Figure 5A and the quantifications in Figure 5B). This finding that there is a significant but still partial effect is consistent with the idea that autophagy does not play an orthodox role during the initial stages of PMD. On the other hand, the final stages of PMD, namely the clearance of the MD fragments by classical autophagosomes, also appeared not to be completely blocked in this mutant. The latter, however, is mainly attributed to a technical limitation of our system in quantifying the fluorescence of the small MD fragments, as they are dispersed with time throughout the egg until they become too dispersed to be distinguished from the background autofluorescence.

Another possibility for the observed incomplete blockage of the clearance of the MD fragments in the \textit{atg7} mutant eggs may be the involvement of an \textit{Atg7}-independent mechanism of autophagy (Zhang et al., 2009; Chang et al., 2013). To test this idea, we thus explored the involvement of other key components in the autophagic pathway using available transgenic RNAi lines, which were designed to induce efficient knockdown of autophagy (Kirkin et al., 2009b). In these paradigms, the targets involved in the target specificity of cargo cleared by selective autophagy (Kirkin et al., 2009b). In these paradigms, the targets are largely devoid of microvesicles (Figures S4C–S4E). Finally, monitoring PMD in eggs from mutant females for \textit{atg13} (atg13\textsuperscript{213GLC}), as well as maternal knockdown of \textit{atg7} and \textit{fip200} (\textit{atg1\textsuperscript{shR}} and \textit{fip200\textsuperscript{shR}; Figure S3G}), all autophagy pathway components in the initiation complex (complex 1 in Figure S3A), revealed no (atg13\textsuperscript{213GLC}) or mild (atg1\textsuperscript{shR} and \textit{fip200\textsuperscript{shR}}) effects on the clearance of the MD (Figure S3F), suggesting that the initiation signal of the autophagic/endocytic pathway in PMD is distinct from that of classical autophagy. Interestingly, another noncanonical autophagy form, called LC3-associated phagocytosis (LAP), also proceeds in the absence of the initiation complex, suggesting some possible similarities between PMD and LAP (Sanjuan et al., 2007; Florey et al., 2011). Finally, the fact that a mild attenuation was detected in the \textit{atg1\textsuperscript{shR}} and \textit{fip200\textsuperscript{shR}} eggs may suggest that the initiation complex may still be involved in the subsequent step of the clearance of the MFS by classical autophagy and that at 70 min AEL the MFS are too dispersed to be detected.

The Paternal MD Is Ubiquitinated through Lysine 63-Linked Polyubiquitin Side Chains Soon after Fertilization

Recent studies suggest that the ubiquitin system may be involved in the target specificity of cargo cleared by selective autophagy (Kirkin et al., 2009b). In these paradigms, the targets are surface ubiquitinated through nonclassical ubiquitin side chains, such as lysine 63-linked polyubiquitin (K63-polyUb) chains, which may preferentially bind to autophagy receptors, thus linking the ubiquitin pathway with the autophagy pathway. To explore the possibility that the paternal MD may also be ubiquitinated, fertilized eggs were stained with a linkage-specific antibody that recognizes polyubiquitin chains joined through K63 of ubiquitin (Newton et al., 2008) and with AXO 49 antisemur, which detects the axoneme. Interestingly, already at 10–20 min AEL, one of the two elongated sperm tail organelles was entirely decorated by K63-polyUb (Figure 6A; Figure S4A). Although for technical reasons we could not detect both the K63-polyUb staining and the fluorescent MD sperm at the same time (due to the insufficient fluorescence of the MD sperm after fixation), the ubiquitinated organelle clearly corresponds to the MD, as evident in flagellar regions where the ubiquitinated organelle (i.e., the MD) starts separating from the axoneme (Figure 6A, inset; see also Figures S2, S4B, and S4C).

These findings raised the question of whether the ubiquitination occurs before or after egg penetration. Although staining of the testes revealed a strong K63-polyUb signal in elongating spermatids, this signal completely disappeared during a later stage when the spermatids removed their bulk cytoplasmic contents (i.e., the “individualization” stage; Figure S4D). Consistently, no staining was detected in mature sperm from the male storage organ (i.e., the seminal vesicle) and the female storage organs (i.e., seminal receptacle and the paired spermathecae) (Figures S4E and S4F). We therefore...
Figure 5. The Clearance Kinetics of the MD Is Attenuated upon Inactivation of Components in the Autophagic and Endocytic Pathways

(A) Selected frames from two 2 hr movies of paternal mitochondrial elimination after fertilization in eggs from a wild-type female (top) and an atg7 mutant female (bottom) fertilized by males with mito-DsRed sperm. The windows of time AEL are indicated at the top of the panels. Anterior is to the left.

(B) Quantification of fluorescence intensity in 4 min intervals in live fertilized eggs mutant for the indicated genes/proteins. Error bars represent standard errors. The number (N) of examined embryos from each genotype is indicated in parentheses. p values of the differences in the fluorescence intensities between the mutants and the wild-type were calculated for each time point (TP) as follows: p < 0.005 for atg7d14/d77 and stamshR (second TP onward), atg7shR, atg6shR, uvragshR, and Rab7-DN (fourth TP onward). For the other TPs, p < 0.05.

(C–E) Electron micrographs of cross-sections through fertilized eggs at 10–40 min AEL showing (C) a wild-type MVC and (D and E) biogenesis-deformed MVCs (dMVCs) in atg7 mutant and uvrag knockdown eggs, respectively. The scale bars represent 200 nm (C) and 500 nm (D and E).

See also Figure S3 and Movie S1.
Figure 6. The MD Is Associated with Lysine 63-Linked Polyubiquitin Chains and p62 Soon after Fertilization

(A and B) Fertilized wild-type eggs at 10–30 min AEL were double stained to visualize K63-polyUb (red) and (A) the axoneme (green) or (B) p62 (green). Anterior is to the left. Insets are enlargements of the boxed areas. Arrows point at (A) a region where the MD was separated from the axoneme and (B) an MD fragment soon after its detachment from the main MD, whereas arrowheads point at MD fragments distant from the main MD. The scale bars represent 50 μm.

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conclude that ubiquitination of the MD occurs after egg penetration.

The Ub-Binding Autophagy Receptor p62 Is Recruited to the Paternal MD
 Autophagy receptor/adaptor proteins, such as p62/SQSTM1 and its structurally related protein, NBR1, can interact with both polyubiquitin-tagged proteins and Atg8/LC3 (Pankiv et al., 2007; Kirkin et al., 2009a). In Drosophila, the single p62 ortholog, called refractory to Sigma P (ref(2)P; CG10360) (Nezis et al., 2008), was recently shown to mediate the clearance by autophagy of mitochondria with enhanced levels of misfolded proteins (de Castro et al., 2012). To examine whether p62 may be recruited to the paternal MD, early fertilized eggs (10–30 min AEL) were double labeled with anti-Ub-K63 and anti-Drosophila p62 antibodies. Importantly, p62 was specifically associated with the ubiquitinated MD, as well as with some of the MD fragments that were detached from the main derivative, suggesting a direct binding between the ubiquitinated targets on the surface of the MD and p62 (Figure 6B).

The Ubiquitin/p62 Pathway Is Involved in PMD
 Next, we wanted to examine the consequence of this ubiquitination on the PMD process. For this, we maternally expressed the yeast deubiquitinating protease (DUB) UBP2, which has been previously shown to antagonize some ubiquitination pathways in Drosophila neurons (DiAntonio et al., 2001). Interestingly, DUB-expressing eggs display a moderate yet significant delay in the clearance of the MD compared to wild-type eggs, suggesting that ubiquitination may indeed be involved in the clearance of the MD (Figure 6C). Furthermore, monitoring PMD in eggs from p62 mutant mothers (Nezis et al., 2008) also revealed only a moderate delay in the clearance of the MD (Figure 6C), as compared with the autophagy and endocytic mutants (compare with Figure 5B). Although we used a viable transheterozygous combination of two mutant p62 alleles, ref(2)P<sup>od2</sup> and ref(2)P<sup>od3</sup>, they were shown to be strong alleles of this gene, producing stable truncated p62 proteins (Nezis et al., 2008). Therefore, the relatively weak effect may be attributed to the fact that other adaptor proteins have been recently identified that may also be involved in this process (Schweers et al., 2007; Kirkin et al., 2009a; Narendra et al., 2010a; Wild et al., 2011). In addition, other types of ubiquitination of MD surface proteins (i.e., through other lysines) may also occur, and may be less efficiently antagonized by UBP2. Collectively, these findings indicate that the ubiquitin/p62 pathway is involved in the selective clearance of the paternal MD after fertilization, and that additional components in this pathway should also be important for this process.

Parkin Does Not Play a Main Role in the Clearance of the Paternal MD after Fertilization
 Genetic studies in Drosophila have placed the two fly orthologs of the Parkinson’s disease-associated genes pink1 and parkin in a similar pathway; dispensable for the fly’s viability but required for normal mitochondrial remodeling during flight muscle and spermatid differentiation (Greene et al., 2003; Clark et al., 2006; Park et al., 2008). In addition, more recent studies in mammalian cells have uncovered a role of the Pink1/Parkin pathway in the turnover of damaged mitochondria (Narendra et al., 2008, 2010b). According to this model, Pink1 is stabilized on the surface of damaged mitochondria, which promotes the recruitment of Parkin to the site and the subsequent clearance of these organelles by autophagy. Finally, it has been reported that Parkin mainly induces K63-linked chain ubiquitination (Lim et al., 2005; Oizmann et al., 2007; Geisler et al., 2010). Therefore, we wanted to test the hypothesis that Parkin may be involved in the process of PMD (e.g., through ubiquitination of the paternal MD). Because the source of Parkin can be either maternal or paternal, parkin mutant females (using a null allelic combination; Greene et al., 2003; park<sup>25/dj</sup>; see also lane 3 in Figure S5B) were first crossed with males producing MTS-DsRed sperm, and the clearance of the MD was monitored using a live imaging assay. However, no significant effect on the clearance of the paternal MD was detected in these mutant eggs (Figure S5C), suggesting that if Parkin is involved in this process, it may originate in the sperm.

We next set up to test for a possible effect on the kinetics of PMD when Parkin is inactivated in males. As opposed to fertile females, parkin mutant males are completely sterile, displaying severe spermatid differentiation defects, which hinder the accumulation of mature sperm in the seminal vesicle (this study and Greene et al., 2003). To overcome this technical obstacle, we devised a complex genetic scheme to allow inducible inactivation of Parkin at a late spermatid developmental stage, thus still enabling normal spermatid differentiation and sperm production (Figure S5A). For this, we modified a genomic clone containing the parkin gene, such that the engineered gene contains TEV protease cleavage sites within its open reading frame, and generated transgenic flies carrying this construct. The ability of this modified transgene (t-parkin) to restore functional spermatid production and fertility in parkin mutant males was then validated (data not shown). Subsequently, another transgene, consisting of the TEV protease gene under the regulatory regions of the dj gene, was brought into the parkin rescue background, and the cleavage of t-Parkin was demonstrated by western blotting of testis and seminal vesicle extracts (Figure S5B). Importantly, efficient inactivation/elimination of t-Parkin could be detected in the seminal vesicles, which contain the mature sperm (Figure S5B, lane 5). The MTS-DsRed transgene was also crossed to these flies to mark the sperm MD, and these males (i.e., with the following genotype: dj-Tev/t-parkin; park<sup>25/dj</sup>; dj- MTS-DsRed) were shown to be fertile, giving rise to viable progeny (data not shown). Finally, monitoring PMD in eggs that were fertilized by the Parkin-devoid sperm revealed no significant effect on the clearance of the MD (Figure S5C). Taken together, we conclude that Parkin does not play any major role in PMD.
DISCUSSION

Active Mechanisms Originating in the Egg Function to Eliminate the Paternal Mitochondria after Fertilization in Drosophila

In this work, we demonstrate that the paternal MD in D. melanogaster is rapidly eliminated after fertilization by active mechanisms. Our study supports a multistep model for this process, which we also term PMD (illustrated in Figure 7). After sperm penetration of the egg (Figure 7, I), a network of MVB-like vesicles originating in the egg and displaying molecular features of both the endocytic and autophagic pathways associates with the sperm tail (yellow arrows in Figure 7, II). These vesicles are involved in the initial recognition and disintegration of the sperm plasma membrane after fertilization, and may also mediate clipping of sperm flagellum at different regions along the tail, as vesicles that completely encapsulate flagellar regions (containing the sperm plasma membrane, axoneme, and MD) are detected by both electron and confocal microscopy (white arrow in Figure 7, II). MVCs making direct contacts with sperm plasma membrane were also often detected by EM and may also contribute to membrane disintegration and subsequent MD separation from the axoneme, as they were only detected prior to the separation of these two organelles (arrowhead in Figure 7, II). Although the exact mode of action of these vesicles is unclear, it is attractive to suggest that their attachment to the sperm plasma membrane and release of microvesicles into the sperm are reminiscent of conventional MVB attachment to the cell plasma membrane and release of their internal vesicles/exosomes into the extracellular region. Indeed, similar to the observed PMD attenuation caused by expression of the Atg8-based autophagy reporters, a reporter was also found to inhibit amphisome release of exosomes into the extracellular medium in an erythroleukemic cell line (Fader et al., 2008). Moreover, the strong PMD attenuation in atg7 mutant eggs is likely attributed to the observed deformed MVCs, which are largely devoid of microvesicles.

The Significance of Paternal Mitochondrial Ubiquitination and Association with p62 and LC3/Atg8

Decoration of the paternal MD by K63-linked polyubiquitin chains and the subsequent association with p62 and Atg8 occur early during the PMD process at a stage when the MD is still much too large to be enwrapped by autophagosomes, suggesting that these events may serve another role, different from marking the mitochondria for selective autophagy. Consistent with this idea, our findings support an unconventional role of a common autophagic and endocytic vesicular pathway in promoting the initial disintegration of the sperm tail and separation between the MD and the axoneme, both of which occur at a stage when the MD is already ubiquitinated. Furthermore, we demonstrate that Parkin, which has been shown to target damaged mitochondria for selective autophagy in both mammalian systems and Drosophila (Narendra et al., 2008; de Castro et al., 2012), does not play a role during PMD, implying some divergence from the classical process of damaged mitochondrial clearance by selective autophagy.

Interestingly, a similar unconventional pathway may also operate in mammals, as ubiquitination of sperm mitochondria via K63-linked polyubiquitin chains and the subsequent association with p62 and LC3 association were also reported to occur soon after fertilization in several mammalian systems (Sutovsky et al., 1999; Al Rawi et al., 2011; Luo et al., 2013). This is an important point, because a recent report suggested a passive model of mitochondrial dispersal to explain paternal mitochondrial elimination after fertilization in mice, even though an early process of sperm tail disintegration that facilitates the subsequent dispersal of the mitochondria was also noted (Luo et al., 2013). Indeed, this may also be in agreement with the authors’ finding that atg5 knockout mice displayed no effect on the kinetics of the sperm mitochondrial dispersal, implying a nonorthodox role of some...
elements of the autophagy system but not others during this process in mammals (Luo et al., 2013). Furthermore, in organisms that produce nonflagellated sperm with already dispersed mitochondria, such as C. elegans, this pathway does not exist, and the mitochondria are directly targeted for clearance by autophagy (Al Rawi et al., 2011; Sato and Sato, 2011). Future studies in mice aimed to uncover a similar unconventional autophagic and endocytic common pathway, as well as further characterization of the MVCs and their contents in *Drosophila*, will shed light on the generality and exact mode of action of the ubiquitin/p62/Atg8 pathway in the elimination of the paternal mitochondria in organisms with flagellated sperm.

**Why Is the Paternal Mitochondria Eliminated after Fertilization?**

Several hypotheses have been proposed to address this question, but the exact answer still remains a mystery. One idea is that the egg may regard the paternal mitochondria as a potentially dangerous trespasser, similar to the way somatic cells react against intrusive bacteria. Indeed, the mitochondria are believed to originate from a bacterial endosymbiont (Andersson et al., 1998). Furthermore, microbes that invade cells during infection are selectively disposed by a specialized form of autophagy called xenophagy (Levine and Deretic, 2007; Schmid and Münn, 2007). However, the fact that maternal mitochondrial inheritance has been conserved throughout eukaryotic evolution strongly suggests that this phenomenon is not merely a preserved remnant of a primitive defense mechanism but rather reflects a crucial need to prevent the delivery of certain paternal mitochondrial factors to the developing embryo. Along these lines, it is widely believed that elimination of paternal mitochondria may be important to prevent mtDNA heteroplasmy, which in extreme cases may cause mitochondrial disorders (Kraytsberg et al., 2004; Montoya et al., 2009). Furthermore, inducing mtDNA heteroplasmy in mice was recently reported to result in significant physiological, cognitive, and behavioral complications (Sharpley et al., 2012).

It is still not fully understood why mtDNA heteroplasmy can cause such deleterious effects to the organism. It has been suggested that sperm mtDNA has a higher mutation rate than egg mtDNA, and that spermatozoa even from normal men may contain significant levels of mtDNA deletions (Cummins, 2000). However, the possibility of increased accumulation of mutations in sperm mtDNA may not be the only reason for the deleterious effects of heteroplasmy, as even under conditions in which the two mixed mtDNA were normal, induction of heteroplasmy was shown to be genetically unstable and to produce adverse physiological effects (Sharpley et al., 2012).

Paternal mitochondrial factors other than mtDNA may also be detrimental to the developing embryo. Indeed, the estimated number of maternal mtDNA copies in the animal egg far exceeds that of sperm by a factor of $10^3$ to $10^6$, indicating that most of the sperm mtDNA is already excluded during sperm differentiation (Birky, 2001). Furthermore, elimination of sperm mtDNA in the Japanese medaka fish has been reported to happen in two steps before the destruction of the vacuolar mitochondria (Nishimura et al., 2006). Moreover, recent work in *Drosophila* suggested that essentially all of the sperm mtDNA is already degraded during late spermatogenesis (DeLuca and O’Farrell, 2012). Finally, in the majority of mouse sperm cells, most of the mtDNA is also eliminated before fertilization (Luo et al., 2013). Therefore, our findings that active mechanisms still operate to eliminate the paternal MD in the *Drosophila* egg, despite the fact that the MD is already devoid of mtDNA, suggest the intriguing hypotheses that either other detrimental factors or, alternatively, some beneficial factors may also exist, which may provide evolutionary pressure for eggs to clear and recycle the paternal vacuolar mitochondria.

In conclusion, our studies have uncovered an autophagic/endocytic pathway, which might have evolved in organisms with flagellated sperm to efficiently eliminate paternal mitochondria. Understanding the mechanisms that govern PMD is not only of academic interest, as sophisticated human in vitro fertilization (IVF) technologies are being widely used in the clinic and the risk attributed to paternal factors has been the subject of ongoing debate (Verpoest and Tournaye, 2006). An understanding of the paternally and maternally derived pathways and factors that mediate PMD may be critical to determining possible risks associated with new IVF and cloning technologies.

**EXPERIMENTAL PROCEDURES**

**Fly Strains and Expression Vectors**

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

**Preparation of Samples for Ultrastructural Studies**

Fertilized eggs were collected for 10 min and either immediately dechorionated or aged at 25°C for 20 min and then dechorionated by hand peeling on double-sided adhesive tape. See further details of sample preparations and analyses in Supplemental Experimental Procedures.

**Live Imaging Studies**

For kinetics experiments, fertilized eggs were collected 0–5 min AEL and hand dechorionated as described above. Dechorionated eggs were transferred to a glass slide coated with a thin adhesive layer (extracted by heptane from double-sided adhesive tape, dispersed on a slide, dried, and bordered by hydrophobic pen), mounted in a drop of PBS, and visualized under the confocal microscope (Zeiss LSM 510). For each embryo, 12 z stacks of 4 μm each were taken every 4 min for 25 rounds. Frame series were converted into movies using Imaris software (Bitplane).

For live imaging of eggs expressing mCherry-Atg8a and YFP-Rab7, adjusted z stacks of 2 μm each were taken every 20 min for consecutive time points.

Twenty-five projections (one for each time point) from 12 z stack images per each time point of live dechorionated fertilized eggs (beginning at 5–10 min AEL and lasting for about 2 hr) were analyzed by a dedicated MATLAB script (MathWorks) designed to quantify the fluorescence intensity of the MD (with the help of the Department of Veterinary Resources at the Weizmann Institute of Science). The first time point of each embryo was set as 1, and the values of the ensuing time points were expressed as the percentage of the first time point. Fluorescence intensity levels are represented as the mean ± SEM of all the examined embryos at each time point. Statistical analysis was performed using the two-tailed unpaired Student’s t test. Values of p < 0.05 were accepted as statistically significant.

**Immunostaining**

Fertilized eggs were collected for 10 min, aged at 25°C for 10, 30, or 50 min, and processed for whole-mount antibody staining using standard techniques (Asburner, 1989). Antibodies used in this study are described in Supplemental Experimental Procedures. Eggs were mounted in Fluoromount-G (SouthernBiotech) and observed by confocal microscopy (Zeiss LSM 510 and LSM 710).
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.04.005.

AUTHOR CONTRIBUTIONS

Y.P., L.G., and E.A. conceived and designed the experiments, analyzed the data, and wrote the paper; Y.P. and L.G. performed the experiments, except for Figure S5, which were designed and performed by L.R. and E.A.; Y.K. was involved in the initial stages of the project, helping to establish the electron and confocal microscopy assays; and Z.E. provided advice and insight during different stages of the project.

ACKNOWLEDGMENTS


REFERENCES


Paternal Mitochondrial Destruction after Fertilization Is Mediated by a Common Endocytic and Autophagic Pathway in *Drosophila*

Yoav Politi, Liron Gal, Yossi Kalifa, Liat Ravid, Zvulun Elazar, and Eli Arama
Figure S1. The MD separates from the axoneme at different regions along the flagellum, related to Figure 1

(A-C) Confocal images showing the detachment of the MD (marked by DJ-GFP; green) from the axoneme (red; AXO; using the AXO 49 antiserum) in 3 different fertilized eggs at different times AEL (indicated on the left). Anterior is to the left. (A) At 10-20 minutes AEL, the axoneme and the MD are largely aligned throughout their entire length. (B, C) At 20-40 and 40-60 minutes AEL, the two organelles progressively separate from one another. Scale bar, 20 μm.
Figure S2. The unfertilized egg already contains numerous MVCs, related to Figure 2

(A and the enlargement in A’) Electron micrographs of a cross section through an unfertilized egg soon AEL. The area confined by a black square was magnified and presented in a separated micrograph (A’). (B) Live confocal imaging of an early unfertilized egg. The inset is enlargement of the respective region in the small white square. The egg maternally expressed the singly-labeled autophagy reporter, mCherry-Atg8 (red). Atg8-positive vacuoles are dispersed throughout the egg. (C-E) Electron micrographs of cross sections through fertilized eggs at 10-60 minutes AEL. Note the presence of autolysosomes which still contain MD fragments (MF) together with degraded material.

MVC, multivesicular clusters; Fm, flagellum; mM, maternal mitochondria. Scale bars: A, 1 μm; A’, 500 nm; B, 20 μm; C-E, 2 μm.
Figure S3

A

1. Atg1 kinase/initiation complex
   - FIP200
   - Atg1, Atg18

2. Ptdins kinase/nucleation complexes
   - UVrag
   - p150
   - p14
   - Atg16

3a. Autophagosomal expansion
   - Alg8
   - Alg9
   - Alg10
   - Alg11
   - Alg7

3b. Elongation complexes
   - Alg1
   - Alg12
   - Alg15

B. Cephalic furrow
   - wt x wt
   - wt x MTS-DsRed
   - atg7^{145077} x MTS-DsRed

C. Image of cephalic furrow

D. Image of cephalic furrow

E. Graph showing fluorescence intensity over time AEL (min)

F. Graph showing fluorescence intensity over time AEL (min)
   - wt (N=18)
   - alg13^{1017} (N=15)
   - alg1^{1017} (N=18)
   - fip200^{1017} (N=17)

G. Genotype:
   - wt
   - atg6^{1017}
   - atg7^{1017}
   - uvrag^{1017}
   - "-

   Transcript:
   - un
   - alg6
   - alg7
   - uvrag
   - "-
Figure S3. Compromised autophagy does not affect fertilized egg progression through the early embryonic stages, related to Figure 5

(A) Schematic illustration of the four main autophagic machinery units and complexes. The proteins which make each unit are depicted as rectangles or circles. The bold (proteins) names correspond to the genes examined in this study. (B-D) Dissecting scope images of embryos from the indicated genotypes and crosses (top). Note the presence of the cephalic furrow (yellow arrows) in all embryos, which marks the gastrulation stage. Anterior is to the left. (E) Quantification of the results described in (B-D). Note that although the atg7 mutant females lay fewer eggs than wild-type females, fertilized atg7 mutant eggs develop normally at least until the gastrulation stage, at which time the MD is normally already eliminated. (F) Quantification of fluorescence intensity in 4-minute intervals in live fertilized eggs mutant for the indicated genes/proteins. Error bars represent standard errors. The number (N) of examined embryos from each genotype is indicated in parenthesis. (G) Validation of the potency of the different shRNA transgenes to efficiently knockdown the indicated genes in the egg. Reverse transcriptase (RT-PCR) analyses on RNA from unfertilized eggs of the indicated genotypes are presented. The tubulin (tub) gene was also amplified from each RNA sample to control for the presence and levels of RNAs.
Figure S4. Ubiquitination of the MD occurs soon after fertilization and declines with time during the PMD process, related to Figure 6

(A-C) Confocal images of three wild-type eggs at the indicated times AEL (indicated on the left) stained to visualize the axoneme (green; AXO; the AXO 49 antiserum) and lysine 63-linked polyubiquitin chains (anti-Ub-K63; red), which decorate the MD. Anterior is to the left. Arrows are pointing at regions where the MD is completely detached from the axoneme. Note the gradual decrease in the level of ubiquitination as PMD progresses. (D-F) Ubiquitination of the MD does not occur on late differentiating spermatids or mature sperm before fertilization. (D) Terminally differentiating spermatids in the testis were double stained to visualize lysine 63-linked polyubiquitin chains (anti-Ub-K63; red) and the individualization complex (IC) found in the waste bags (WB) and marks the late stage of spermatid individualization (F-actin; green). Note the massive K63-polyUb staining before the individualization stage and its elimination from individualized spermatid regions (arrows pointing at post-individualized regions). (E) Mature sperm from the male seminal vesicle (SV) expressing the MD marker, DJ-GFP (green), do not display K63-polyUb staining (red). (F) Mature sperm in the female storage organs (spermathecae and seminal receptacle) expressing the MD marker, MTS-DsRed (red), do not display K63-polyUb staining (green). Scale bars: A-C, 100 µm; D-E, 50 µm.
Figure S5

A

parkin (BACPAC)

Targeting (PCR) Fragment

Recombinant parkin (BACPAC)

t-parkin (gen.)

t-parkin (mRNA)

Translation (in flies)

Splicing (in flies)

Floxing (in bacteria) & generation of transgenic flies

Recombination (in bacteria)

200 bp

Inactivated t-Parkin

B

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Legend:

- wt (N=18)
- park mutant female (N=15)
- park mutant male (N=13)
Figure S5. Maternal and paternal Parkin are not involved in PMD, related to Figure 6

(A) Schematic view of the recombineering-based approach used to inactivate Parkin during late spermatogenesis. The relative region of the inserted 3 x TEV-protease recognition sites is indicated in gray. *parkin* DNA and RNA coding exon sequences are shown in light green bars, UTR regions in light blue bars, and introns in solid dark lines. The numbers of exons appear inside the bars. The targeting (PCR) fragment which also includes the *kanamycin* resistant gene (Kan^R^; red bar) flanked by *LoxP* sites (light blue rectangles) is indicated without the flanking homology sequences. A light blue rectangle (a *LoxP* site) inside a yellow bar indicates the remaining sequences after floxing. Parkin protein sequences are indicated by dark green bars and the relative locations of its different domains are indicated in purple. See also the main text and the Experimental Procedures for more details about the procedure. (B) Cleavage of the t-Parkin protein was assessed by Western blotting of protein extracts from dissected testes (denoted by blue text) or seminal vesicles (denoted by red text) of the indicated genotypes using the anti-Parkin antibody (top panels). Note that the t-Parkin (lane 4) is larger than the endogenous Parkin (endo; lanes 1 and 2) because of the inserted TEV-protease recognition sites in t-Parkin. Also note that no endogenous Parkin is detected in the *park25/Df* background (lane 3), and that after expression of the TEV-protease, t-Parkin is cleaved (cParkin; middle panel; lane 4) and both t-Parkin and cParkin are almost completely eliminated from mature sperm (lane 5). It is also noteworthy that despite the cleavage of t-Parkin, the uncleaved form was still readily detected in the testis; this is due to the fact that in addition to individualizing spermatids, the testis also contains germ cells of various (earlier) stages, which express t-Parkin but still do not express the TEV protease, as the latter is not expressed until the onset of the spermatid individualization stage (lane 4). The same membrane was reblotted with the anti–β-tub antibody for loading control (bottom panels). wt, wild-type. Molecular mass is indicated in kilodaltons. (C) Quantification of fluorescence intensity in live fertilized eggs mutants for maternal and paternal *parkin*. The number (N) of examined embryos from each genotype is indicated in parenthesis. All
calculations were performed as in Figure 5B. The PMD kinetics of the wild-type (wt) and mutant eggs were not statistically significant.

**Movie S1. PMD in wild-type egg, related to Figure 5**

Shown is a time-lapse movie of a wild-type egg fertilized by males producing the MTS-DsRed sperm for 2 hours AEL. Elapsed time is shown as hh:mm:ss:msmsms. The scale bar represents 30 μm. See also in the supplementary experimental Procedures
Supplementary Experimental Procedures

Fly strains and expression vectors

All strains were grown at 25°C. yw flies were used as wild-type controls. Fly mutant and transgenic alleles used in this study are as follows: \{atg7^{d14}\} and \{atg7^{d77}\} (Juhasz et al., 2007); \{FRT82B, Atg13^{Δ81}\}, \{ref(2)P^{od2}\}, \{ref(2)P^{od3}\} and \{UASp-eGFP-mCherry-DrAtg8a\} (Nezis et al., 2010); \{park^{21}\} and \{park^{Z1-472}\} (Greene et al., 2003); \{UASt-UBP2\} (DiAntonio et al., 2001); \{UASp-mCherry-Atg8a\} and \{UASp-eGFP-Atg8a\} (Rusten et al., 2007); \{DJ-GFP\} (Bazinet and Rollins, 2003).

For maternal ectopic expression we used the driver line \textit{P\{mat\α4-GAL-VP16\}V37} (Bloomington Stock #7063). Other Bloomington stocks used in this study are as follows: \{FRT82B, ovoD^{1-18}\} (#2149); \{Df(3L)Pc-MK\} (#3068); \{UASp-YFP.Rab7\} (#23270); \{UASp-YFP.Rab7DN\} (#9778); \{UASp-YFP.Rab5\} (#24616); \{UASp-YFP.Rab11\} (#9790).

For the knockdown experiments, the triple maternal driver MTD (Bloomington #31777) was crossed to the following shRNA lines from the TRiP collection at Harvard Medical School: \{atg7^{shR}\} (#34369), \{atg6^{shR}\} (#35741), \{uvrag^{shR}\} (#34368), \{atg1^{shR}\} (#35177), \{fip200^{shR}\} (#36918) and \{stam^{shR}\} (#35016).

To generate the MTS-DsRed and MTS-Venus flies, a pDsRed2 or a Venus gene were fused to an N-terminal mitochondrial targeting signal (MTS) from the subunit VIII of human cytochrome c oxidase (mito-DsRed; Clontech), PCR amplified and cloned into a pCaSpeR4 vector downstream of a \textit{don juan} promoter and upstream of the \textit{cyp-c-d} 3’UTR (Santel et al., 1998; Blumer et al., 2002; Bader et al., 2011). Transgenic flies were generated by micro-injection into embryos using standard procedures (performed by Genetic Services Inc, Sudbury, MA).

Embryos were staged by developmental hours at 25°C in a humid incubator, in hours after egg laying (AEL). Females can sometimes retain embryos for a longer period before they lay them due to being
fed on limited diet or to being too old (more than 7 days old). Therefore, we used young females which were fed on a reach yeast diet. Under these conditions, embryogenesis is synchronized and lasts 21 ± 1 hour at 25°C.

**Inactivation of paternal Parkin**

To inactivate *parkin* during late spermatogenesis (see also the scheme in Figure S5A), we first cloned a genomic fragment of *parkin* (*g-parkin*) encompassing the 2nd intron and the 3rd exon of the gene (isoform RB of the CG10523 gene) into the pL452 vector (a gift from Stephen P. Creekmore, NCI-Frederick) using the *Bam*HI and *Sac*II restriction sites. This genomic fragment was amplified from the 23 kb BAC PAC genomic clone CH322-72M15 (BACPAC resources), which includes *parkin* and 4 additional flanking genes, using the following forward primer: CGGCGGATCCGAAGTTAACTTTTACTGTCTTTC and reverse primer: AGTCCCGGTATTCAGACGTCCCTGGAAATAAAGATTTTCGAGCTCTCCTTGGAAAGTAAA GGTTCAGAAGCACCTGAAAAATACAAATTCCTCTCGCTTCCAGCTGCA. The reverse primer contained 3 tandem arrays of the TEV-recognition sequence ENLYFQG. Recombineering of the “targeting (PCR) fragment” (which contains the *kanamycin* resistant gene flanked by two *LoxP* sites and followed by the *g-parkin* fragment) with the *parkin* BAC PAC (which is cloned in attB-P[acman]-CmR-BW) was performed as previously described (Venken et al., 2006). The following primers were used to amplify the “targeting (PCR) fragment” from the pL452 plasmid, which also added two flanking *parkin* homology sequences 50 bp and 70 bp, respectively, for the subsequent recombineering process: forward primer: CGATGCCACGACAATAGAGGTAAGTTTTCTTATATGTATCCGCTACATGCAGCCCAAT TCCGATCATATTTTC; reverse primer: GGGTTTCGTAAAAAAAAAAACCAAACTAATTGTTGCCCTACCTCATCGGTAT TATTCAGACGTCCCTGGAAA. Recombineering followed by floxing (in a Cre producing bacteria)
resulted in the insertion of the Tev recognition sequences into parkin 3rd exon, while leaving one LoxP site inside the 2nd intron of parkin. This t-parkin construct was then injected into embryos (containing the attP-VK2 landing site; BestGene Inc) to generate transgenic t-parkin flies. The single LoxP site in the t-parkin transgene is then removed by conventional splicing, thus ultimately promoting the generation of t-parkin mRNA which contains the Tev recognition sites within its ORF.

The dj-Tev transgene was generated by PCR amplification of the TEV protease sequence from a plasmid (a gift from Kim Nasmyth, University of Oxford) and its subsequent cloning into the pCaSpeR4 vector downstream of a don juan promoter and upstream of the cyt-c-d 3’UTR (see above), using the BamHI and NotI restriction sites. Transgenic flies were generated using standard procedures as indicated above.

**TEM analysis**

Eggs were fixed for 20 minutes in 1:1 volume of heptane and 2.5% glutaraldehyde (diluted in 0.1M Cacodylate buffer - CaCo, pH=7.4). Fixed eggs were hand-devitellinized on a metal lattice, re-fixed in 2.5% glutaraldehyde, and kept at 4°C. Preserved eggs were washed with 0.1M CaCo buffer, post fixed in 1% osmium-tetroxide (diluted in 0.1M CaCo buffer) for 2 hours without shaking, and washed with 2% uranylacetate in DDW. Samples were dehydrated in graded ethanols, embedded in Epon 812, and hardened at 60°C for 2 days. Testes were prepared for TEM analysis as described in (Arama et al., 2006). Ultrathin sections (70-90 nm thickness) of eggs and testes were prepared with ultramicrotome Leica UCT (Leica). Samples were analyzed under 120kV Transmission Electron Microscope (TEM) 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.
**Immunostaining (antibodies)**

The primary antibodies used in this study are human monoclonal anti-Ub-K63 antibody (anti-ubiquitin lys63-specific clone Apu3; 1:100; Genetech) (Newton et al., 2008), mouse monoclonal anti-polyglycylated tubulin antibody (AXO 49; 1:5000) (Bre et al., 1996), rabbit polyclonal anti-*Drosophila* p62 antibody (anti-ref(2)P; 1:100) (Wyers et al., 1995), and guinea-pig polyclonal anti-Hrs antibody (1:100) (Lloyd et al., 2002). All secondary antibodies were used in a dilution of 1:100 (Jackson ImmunoResearch).

To preserve the endogenous fluorescence of the DJ-GFP transgene after fixation, we performed the hand devitellinization of embryo procedure, which is methanol free, as described in (Rothwell and Sullivan, 2000). Fixation was performed in 4% paraformaldehyde. This procedure, however, preserved the immunoreactivity of the AXO 49 antibody, but not that of the anti-Ub-K63 and anti-p62 antibodies.

**Western blot**

Proteins were extracted from testes and seminal vesicles from 1 and 5 days old male flies, respectively. Samples were run in SDS-PAGE and transferred to nitroglycerin membrane, which was reacted with rabbit anti-Parkin antibody (1:5000) (Greene et al., 2005) and mouse anti-β-tubulin (clone E7, Hybridoma Bank; 1:1000).

**Gastrulation assay**

Fertilized eggs were collected for 30 minutes, allowed to develop for 3 hours at 25°C, fixed, and mounted. Embryos were then monitored for gastrulation using phase contrast or digital interference contrast (DIC or Numarski) microscopy.
RNA isolation and RT-PCR

Total RNA was extracted from 10-20 unfertilized eggs using the PureLink™ Total RNA Purification System (Invitrogen) according to the manufacturer’s recommendations. Purified RNA was reversed transcribed using Qiagen OneStep RT–PCR kit (Qiagen). cDNA was then PCR amplified for 25-30 cycles using specific primers.
**Supplementary References**


