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DOI:
10.1073/pnas.2306073120

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Citation for published version (Harvard):

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Download date: 23. Aug. 2023
Milton assembles large mitochondrial clusters, mitoballs, to sustain spermatogenesis

Andy Y. Z. Li, Ying Da, Sumaera Rathore, Ason C.-Y. Chiang, Jan Jezek, and Hansong Ma

Edited by Harmit Malik, Fred Hutchinson Cancer Center, Seattle, WA; received April 25, 2023; accepted July 13, 2023

Mitochondria are dynamic organelles that undergo frequent remodeling to accommodate developmental needs. Here, we describe a striking organization of mitochondria into a large ball-like structure adjacent to the nucleus in premeiotic Drosophila melanogaster spermatocytes, which we term “mitoballs”. Mitoballs are transient structures that colocalize with the endoplasmic reticulum, Golgi bodies, and the fusome. We observed similar premeiotic mitochondrial clusters in a wide range of insect species, including mosquitoes and cockroaches. Through a genetic screen, we identified that Milton, an adaptor protein that links mitochondria to microtubule-based motors, mediates mitoball formation. Flies lacking a 54 amino acid region in the C terminus of Milton completely lacked mitoballs, had swollen mitochondria in their spermatocytes, and showed reduced male fertility. We suggest that the premeiotic mitochondrial clustering is a conserved feature of insect spermatogenesis that supports sperm development.

**Significance**

This work unveils the intriguing phenomenon of large mitochondrial clusters (mitoballs) formed during insect spermatogenesis. It offers unparalleled insights into the dynamic nature of mitochondria and their remarkable ability to adapt to developmental needs. The identification of Milton’s role in mitoball formation and mitochondrial trafficking further exploration of its involvement in human male fertility, given the conserved function of insect and human Milton in mitochondrial trafficking. Moreover, our findings lay the foundation for future investigations connecting subcellular mitochondrial distribution to mitochondrial function and male fertility.

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The authors declare no competing interest.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2306073120/-/DCSupplemental.

**Mitoballs Are Transient Structures That Assemble and Disassemble during the Prophase of Meiosis I.** We constantly observed mitoballs in larval and adult primary spermatocytes (Fig. 1B and C and SI Appendix, Fig. S1 A and C). Like in humans and mice, Drosophila melanogaster spermatogenesis consists of four key steps: 1) differentiation of germline stem cells into spermatogonia cells, 2) mitotic divisions of spermatogonia cells to give rise to primary spermatocytes, 3) meiotic divisions to generate haploid spermatids, and 4) spermatid elongation and maturation (Fig. 1A) (10). In D. melanogaster, it is known that after the last mitotic division, the premeiotic S phase initiates and completes very quickly, and early primary spermatocytes are similar in size to postmitotic spermatogonial cells (10, 11) (with
a nuclear diameter of ~5 μm, Fig. 1D). We found that as primary spermatocytes entered the premeiotic G2 phase (corresponds to prophase I), which is a growth phase that lasts for ~80 h (9), mitoballs began to form (Fig. 1B). The nucleus progressively increased in size, and the three chromatin clumps (SI Appendix, Fig. S1D), due to the separation of paired sister chromatids, became apparent (9, 11). When the nucleus reached ~7.5 μm, round-shaped mitoballs of ~5 μm were clearly visible (Fig. 1D). Mitoballs continued to grow and eventually reached ~11 μm in diameter before mitochondria started to disperse uniformly into the cytoplasm, and the nucleus progressively resumed a central position within the cell (Fig. 1B and D). The entire mitoball stage lasted at least 8 h based on our time-lapse live imaging.

We confirmed the mitochondrial dynamics from the premi- toball to postmitoball stages by electron microscopy imaging (Fig. 1E). Unlike the nebenkern, mitochondria within the mito- ball were not fused. They were highly dynamic rod-shaped indi- viduals, which constantly moved in all directions at an average speed of ~30 nm/s (Fig. 1F and Movie S1). By expressing mito- chondrially targeted Dendra2 (mito-Dendra2), a monomeric fluorescent protein that can be irreversibly photoconverted from an initial green fluorescent form to a red fluorescent form by ultraviolet (UV) light, we converted and tracked a subpopulation of mitochondria within a mitoball. We found that most converted mitochondria (labelled as magenta in Fig. 1G) stayed as discrete populations after 100 min, despite continuously relocating within the mitoball. This result indicates a low level of exchange among the mitochondrial population within the same mitoball.

The gradual increase in mitoball size suggests that mitochon- drial biogenesis occurs during this period. To examine whether mitochondrial DNA (mtDNA) is also being replicated during this stage, we quantified the number of mtDNA foci in primary spermatocytes before, during, and after the mitoball stage by staining testes with anti-dsDNA antibodies (SI Appendix, Fig. S1E). Prior to mitoball formation, the average mtDNA nucleolus copy number was ~65 in each spermatoocyte. The number increased to ~200 when the mitoballs were ~5 μm in diameter. It eventually reached ~360 in the late mitoball stage and remained approximately the same in postmitoball spermatocytes (Fig. 1H).

Our quantification using dsDNA antibody staining might not reflect the actual increase in mtDNA copy number as individual dots could represent a single mtDNA nucleoid or multiple nucleoids in close proximity. Hence, we further examined mtDNA replication by EdU staining. We observed clear EdU puncta in mitochondria at the mitotic and mitoball stages, but no signal was detected after the mitoball stage even with extended incubation (16 h) with EdU (Fig. 1F and SI Appendix, Fig. S1F). Strong mtDNA EdU staining was only observed again at the nebenkern stage (SI Appendix, Fig. S1F). In line with this observation, the expression level of the endogenous mtDNA polymerase Pogi1 was high in mitotic cells and spermatocytes at the mitoball stage, and much lower in postmito- toball spermatocytes (SI Appendix, Fig. S1G). This suggests that mtDNA replication in early spermatogenesis predominantly occurs before mitoball disassembly. Taken together, we conclude that mito- balls are a collection of dynamic mitochondria that form a spherical cluster on one side of primary spermatocytes in early prophase I and disperse as the cells progress into the later period of this phase. The mitoball stage is also coupled with mitochondrial biogenesis and mtDNA replication.

**Premeiotic Mitoballs and Similar Mitochondrial Clusters Were Found in Many Other Insect Species.** The nebenkern structure is found in many insect species (3). We wondered whether the same applied to mitoballs. By immunostaining, we found mitoball-like structures in all Drosophila species of the melanogaster subgroup, and many distantly related Drosophila species such as Drosophila saltana and Drosophila pseudoobscura (Fig. 2A and B and SI Appendix, Fig. S2A). Mitochondrial clusters were also observed in spermatoocytes of Drosophila serrata, but these clusters were more crescent-shaped rather than spherical (Fig. 2C). Nevertheless, they followed a similar dynamic to mitoballs: they formed during the early premeiotic G2 phase, and the mitochondria dispersed in the cytoplasm as cells progress into a later period of this phase (Fig. 2C). Drosophila bacoqueti, on the other hand, did not seem to have mitoballs, but nebenkerns were observed (SI Appendix, Fig. S2B).

We then examined two mosquito species that are malaria vectors. Like Drosophila, they belong to the order Diptera. Clear mitoballs were observed in Anopheles stephensi (Fig. 2D), but not in the closely related Anopheles coluzzii (SI Appendix, Fig. S2B). Interestingly, neither species seemed to have nebenkerns. We also examined five species in the orders Orthoptera (locusts and crickets) and Blattoidea (cockroaches), which diverged from Diptera about 400 Mya. Mitoball structures were visible in one cockroach (Gromphadorhina portentosa) and one cricket species (Gryllus bimaculatus) (Fig. 2D and SI Appendix, Fig. S2A). In the other cockroach (Blaberus discoidalis) and cricket (Teleogryllus oceanicus) species, mitochondria also formed clusters in the spermatocytes that followed a similar dynamic to mitoballs but, as in Drosophila serrata (Fig. 2C), these clusters were more crescent-shaped (SI Appendix, Fig. S2C). For the locust species, Schistocerca gregaria, no mitoballs were observed while nebenkerns were apparent (SI Appendix, Fig. S2B). These findings show that mitochondrial clustering in spermatocytes is conserved across a wide range of insect species, but the structure and organization can vary even between closely related species.

**Mitoballs Are Packed with Endoplasmic Reticulum (ER), Surrounded by Golgi Bodies and in Direct Contact with the Fusome.** Mitochondrial function and dynamics are modulated through close interactions with other organelles in the cell (15). To visualize whether mitoballs contain other organelles, we imaged D. melanogaster lines expressing fluorescent proteins targeted to either the ER or Golgi. Ubiquitous expression of YFP-KDEL showed that the ER network occupied a larger cytoplasmic region than the mitoballs, but they were heavily condensed in the mitoball area (Fig. 3A). This observation was validated using flies expressing GFP-HDEL in spermatocytes under bamGAL4 (SI Appendix, Fig. S3A). To visualize Golgi bodies, we used an sqh-YFP-Golgi line, which expresses YFP fused with the Golgi targeting sequence of the B4GALT1 gene under a ubiquitously promoter (16), and found that Golgi bodies mainly decorated the periphery of mitoballs (Fig. 3A). Electron microscopic images confirmed that the ER intertwined with mitochondria within mitoballs, while Golgi bodies were mainly located in the peripheral regions (Fig. 3B).

Another important organelle for germ cells is the fusome, which branches through the ring canals and connects the germ cells in each cyst (Fig. 3C). The fusome contains several cytoskeletal proteins including Adducin (coded by bts in D. melanogaster) and α-spectrin (17–20). During early oogenesis, mitochondria in cyst cells migrate along the fusome, and that facilitates the delivery of mitochondria from the nurse cells to the future oocyte (21). Our live imaging did not detect mito- chondrial movement between spermatocytes via the fusome.
Fig. 1. Mitoballs are transient structures that assemble and disassemble during prophase I of Drosophila spermatogenesis. (A) Illustration of D. melanogaster spermatogenesis. The germline stem cells (GSCs) are contained at the apical tip of the testis. Each GSC undergoes numerous asymmetric cell divisions and each time, it self-renews and produces a gonialblast. The gonialblast then goes through four mitotic divisions to form 16 spermatogonia cells interconnected by the fusome (known as a cyst) due to incomplete cytokinesis. The premeiotic S phase occurs quickly after the last mitotic division, and the bulk of primary spermatocyte development occurs in a long premeiotic G2 phase of the cell cycle, which increases the cell size by ~25 times (9). This is followed by two meiotic divisions to generate 64 spermatids, sperm elongation, and maturation (10). Mitoballs form in the primary spermatocytes and nebenkerns form in early spermatids, prior to sperm elongation (SI Appendix, Fig. S1A and B). Mitochondria and nuclei are labeled in green and blue, respectively. Drawings are not to scale. (B) The apical tip of a D. melanogaster testis showing germ cells in mitotic and prophase I stages. The testis was stained with DAPI (blue), anti-ATP5A antibodies (green) and phalloidin (magenta) to visualize DNA, mitochondria, and cell/cyst boundaries, respectively. (Scale bar: 20 μm.) (C) A zoomed-in view of a mitoball in sqh-mito-YFP flies. (Scale bar: 5 μm.) (D) The diameters of nuclei and mitoballs of spermatocytes at different stages (n = 28). Error bars: SDs. (E) Electron microscopic images of spermatocytes at premitoball, mitoball and postmitoball stages. (Scale bars: 5 μm.) (F) The speed of mitochondrial motion in mitoballs (n = 48) (Movie S1). (G) Time-lapse images illustrating the dynamics of mitochondria within mitoballs. The expression of mito-Dendra2 in early germ cells was driven by bamGAL4. A subpopulation of mito-Dendra2 was converted from the green to red fluorescent form and followed for 100 min. (Scale bar: 5 μm.) (H) The total mtDNA copy number in spermatocytes of different stages (n = 4). Antibodies against dsDNA were used to stain mtDNA nucleoids and count the total mtDNA copy number per cell (SI Appendix, Fig. S1E). Error bars: SDs, one-way ANOVA and Tukey’s post hoc, ns P > 0.05, **P < 0.01, ***P < 0.005. (I) EdU staining reveals active mtDNA replication during the mitoball stage (SI Appendix, Fig. S1F). (Scale bars: 5 μm (1 μm for the zoomed-in view).) Testes were dissected from sqh-mito-YFP (green), incubated with EdU (magenta) for 4 h, fixed, and stained with DAPI (blue).
However, the fusome was in direct contact with the mitoball in each spermatocyte, with the nucleus localizing to the other side (see Movie S2 and SI Appendix, Fig. S3B for the relative positions of the fusome, mitoballs, nuclei and ring canals in a cyst). In fact, when only mitochondria were visualized, the fusome could be identified within the mitoball by what resembles a cavity, devoid of any mitochondria, under certain focal planes (SI Appendix, Fig. S3C). Close contact with the fusome was observed for mitoball-like structures in many other species we examined, such as A. stephensi (SI Appendix, Fig. S3D). In summary, mitoballs are densely packed with ER, surrounded by Golgi bodies, and connected by the fusome within a cyst.

Milton Is Required for Mitoball Formation. Given the close contact of the fusome and mitoballs, we wondered whether the fusome was required for mitoball formation. To test this, we examined mitoballs in mutants of two genes required for fusome formation: hts and α-spectrin. Despite the absence of the fusome (SI Appendix, Fig. S3E), the mitoball appeared normal in the hts/ Df(2R)BSC135 mutant (Fig. 3D) and α-spectrin knockout (KO) cysts (Fig. 3E). Hence, the fusome is dispensable for mitoball formation.

To identify factors required for mitoball formation, we screened mutants and/or RNAi knockdowns (KD) of 115 candidate genes in early germline cells using nosGAL4 and BamGAL4 (Fig. 4A and SI Appendix, Tables S1 and S2). These genes encode proteins...
highly expressed in primary spermatocytes (22) or involved in relevant processes, such as mitochondrial dynamics, mtDNA replication, or cell polarity. Through our screen, we found that knocking down/out genes known to regulate mitochondrial morphology including marf, opa1, MICU3 and rho-7 caused defects in mitochondrial morphologies and/or testis development; however, mitoballs were still formed in many of these KD/KO lines (SI Appendix, Tables S1 and S2 and Fig. S4A). Knocking down milton, on the other hand, completely abolished mitoballs without causing any other obvious abnormalities in mitochondrial or testis morphology. The milton RNAi flies had mitochondria distributed evenly in the cytoplasm of the primary spermatocytes where the mitoball normally presents (Fig. 4B and Movie S3). To confirm the role of Milton in mitoball formation, we isolated two null mutants that carry premature stop codons near the N terminus by CRISPR/Cas9-based editing (miltKO1 and miltKO2, SI Appendix, Fig. S4B). miltKO mutants were homozygous lethal, so we generated KO cyst clones in heterozygous flies. We found that milt KO cysts completely lacked mitoballs, whereas neighboring wild-type cysts at a similar developmental stage had clear mitoballs (Fig. 4C and SI Appendix, Fig. S4C). Hence, Milton is required for mitoball formation.

Milton is an adaptor protein that links mitochondria to microtubules, which exhibit a dense network in spermatocytes at the...
Fig. 4. A candidate screen revealed that Milton is required for mitoball formation to sustain mitochondrial function and male fertility. (A) A list of 115 genes knocked down (RNAi) or knocked out (mutants) to examine their effect on mitoball formation. (B) No mitoballs were found in testes expressing Milton-RNAi driven by nosGAL4 and bamGAL4. Mitochondria (green) were visualized by anti-ATP5A antibody staining. (Scale bar: 50 μm.) (C) Germline cysts that lack Milton had no mitoballs. Control cysts (outlined by dotted white lines in the zoomed-in view) at a similar stage carry mitoballs. Milton KO cells were marked by the absence of RFP (magenta) in their nuclei. (Scale bars: 20 μm.) (D) The function of Milton protein regions based on previous studies: the AA138–450 region is highly conserved and binds to Khc (23); the AA450–750 region binds to, and is a substrate for the cytosolic glycosylating enzyme O-GlcNAc transferase (OGT) (24); ROI: region of interest identified in this study to be essential for mitoball formation. (E) A schematic illustration of the Milton–Miro–Khc complex in transporting mitochondria along microtubules. The protein names for the human orthologs are in brackets. (F) Knocking out Miro compromised mitoball formation. Miro KO cysts had either no mitoballs (39%) or partial mitochondrial clustering (61%). (Left) A single wild-type cyst (outlined by dotted white lines) is surrounded by multiple KO cysts with no mitoballs. (Right) A KO cyst (outlined by solid yellow lines), next to a control cyst (outlined by dotted white lines), shows partial mitochondrial aggregation. (Scale bars: 20 μm.) (G) Mitoballs were found in Khc KO cysts, but not in Khc KO cysts. Mutant and control cyst clones are outlined by solid yellow and dotted white lines, respectively. (Scale bars: 20 μm.) (H) Mitoballs were only observed in flies containing the AA850–903 region of Milton. (Scale bars: 20 μm.) (I) Homozygous Milton mutants with no mitoball showed reduced male fertility (n = 20). The numbers of progeny produced at 25 °C by homozygous mutants were reduced compared to wild-type controls (p < 0.05). (J) Electron micrographs illustrating a Milton mutant spermatocyte at the mitoball stage with no mitoballs, but abnormally swollen mitochondria surrounded by a layer of membrane reminiscent of ER. The ER-like membrane is indicated by pink arrowheads for some mitochondria in the zoomed-in view. (Scale bar: 5 μm.) (K) Representative images of mito-QC expression in a Milton mutant testis. Some cysts with more mCherry-only foci are outlined. (Scale bars: 20 μm.)
mitoball stage (SI Appendix, Fig. S4D). It was first identified in D. melanogaster for its role in recruiting kinesin heavy chain (Khc) to mitochondria and mediating their anterograde movement in axons (23, 25). Milton contains a Khc-binding domain near its N terminus (136 to 450) (Fig. 4D) (23). Its association with mitochondria is mediated by Miro, which is a Rho-like GTPase that locates in the outer membrane of mitochondria (Fig. 4E) (23, 26). Miro binds to the N terminus of Milton (1 to 750), and a dominant negative Miro that lacks its mitochondrial transmembrane domain can displace Milton from mitochondria (23). In humans, the Milton ortholog proteins TRAK1 and TRAK2 also form a complex with Miro (RHOST1/2) to mediate microtubule-based mitochondrial trafficking in neurons and other cell types (24, 27).

To test whether Miro is also required for mitoball formation, we isolated two CRISPR mutants with premature stop codons near the N terminus (SI Appendix, Fig. S4B) and generated KO cyst clones in heterozygous mutants. Mitoballs were not formed in 39% of miro KO cysts (Fig. 4F). The remaining 61% of KO cysts showed partial clustering, with mitochondria aggregated more on one side of the nucleus (Fig. 4F). These data show that mitoball formation requires Milton and Miro, but knocking out Milton abolishes mitochondrial clustering more thoroughly. This suggests that Milton could accomplish some mitochondrial trafficking independently of Miro during spermatogenesis.

Besides kinesin-based mitochondrial trafficking toward the plus end of microtubules, Milton and Miro have also been shown to influence mitochondrial mobility toward the minus end of microtubules by dynein in D. melanogaster and human cells, although the interactions between Milton, Miro, and the dynein complex are less well characterized (28–30). To identify the motor protein required for mitoball formation, we generated KO cysts for khc, kinesin light chain (klc), and dynein heavy chain (dhc64C), respectively. Mitoball formation was abolished in khc KO clones, although partial mitochondrial aggregations assembling those found in the miro KO cysts were observed in some clones (Fig. 4G). On the other hand, knocking out the klc, which is dispensable for Milton-Miro-Khc-mediated mitochondrial trafficking in axons (23), had little impact on mitoball formation (Fig. 4G). Similarly, in dhc64C KO clones, mitoball-like clusters still formed next to the fusome, although the spermatoocytes were organized more in a rosette in some cysts, with all the 16 mitoballs located in the center (SI Appendix, Fig. S4F). Based on these data, we conclude that Milton-mediated mitoball formation relies primarily on Khc.

**Mitoball-Free Mutants Show Reduced Male Fertility and Carry Swollen Mitochondria in Their Spermatocytes.** Since completely knocking out milton is lethal at the organismal level, we designed a number of guide RNAs to generate hypomorphic alleles that are homozygous viable, as they allow us to examine the impact of Milton and mitoball formation on sperm development and male fertility. There are four protein isoforms of Milton in D. melanogaster, which differ at their N terminus but are identical from the Khc-binding region onwards. One of the large isoforms (Milton-A) is 1,166 amino acids (AA) long (Fig. 4D). By designing guide RNAs targeting different regions of milton, we found that terminating Milton upstream of the AA840 was lethal (e.g., milton and milton, SI Appendix, Fig. S4B), while mutants that carried premature stop codons or in-frame deletions after that were also homozygous viable. We examined mitoball formation in three such mutants: milton, milton, and milton, which carried a point mutation at AA850, which transforms the Glu to a premature stop codon (SI Appendix, Fig. S4B). They completely lacked mitoballs (Fig. 4H), just like the milton KD or KOs (Fig. 4B and C and SI Appendix, Fig. S4C). Their nebenkerns, however, appeared normal based on confocal images (SI Appendix, Fig. S4E).

We also generated milton and milton transheterozygotes, which were viable, and confirmed that mitoballs were absent while nebenkerns were present in these flies (SI Appendix, Fig. S4G). In contrast, milton, which carried a nucleotide deletion at AA946 that generates a premature stop codon at AA948, had mitoballs (SI Appendix, Fig. S4B and H). Similarly, milton, which had an in-frame deletion that removes the AA904 to 945 region without affecting the rest of the C terminus, also had mitoballs (SI Appendix, Fig. S4B and H). Therefore, the AA850-903 region of Milton is essential for mitoball assembly.

Leveraging the mitoball-free milton mutant, we examined the impact of losing mitoballs on male fertility. At 25 °C, the number of progeny produced by milton and milton homozygous males, which have mitoballs, was similar to the respective heterozygous controls (Fig. 4I). milton homozygous males were also fertile and gave rise to viable adults, but they produced ~23% fewer progeny than the heterozygous controls (Fig. 4I). On the other hand, the female fertility of milton homozygotes was comparable to controls (SI Appendix, Fig. S4H). Thus, the loss of mitoballs is associated with reduced male fertility.

Since mtDNA replication is a prominent feature of the mitoball stage, we tested whether the reduced male fertility is due to compromised mtDNA replication in flies lacking mitoballs. We stained wild-type testes with anti-dsDNA antibodies and counted the mtDNA nucleoids in spermatocytes from premitoball to postmitoball stages based on the nuclear sizes (Fig. 1D). We found that milton spermatocytes had similar mtDNA copy numbers to the wild-type cells at the corresponding stages (Fig. 1H and SI Appendix, Fig. S4I). Hence, mitoball formation is not necessary for mtDNA replication in the premeiotic growth phase.

We next examined whether the mitoball functions to eliminate dysfunctional mitochondria and thus improve mitochondrial quality for the later stages of sperm development. To this end, we expressed mCherry-GFP-Fis1 in wild-type flies to probe mitophagy (31, 32), a process that removes damaged mitochondria through autophagy. The mitochondria of the mito-QC line fluoresce both red and green under the steady state. Upon mitophagy, as mitochondria are delivered to lysosomes, mCherry fluorescence remains stable whereas GFP fluorescence becomes quenched by the acidic environment. This results in the appearance of mCherry-only foci that can be quantified as an index of cellular mitophagy. We found that the numbers of mCherry-only foci remained low and comparable from premitoball to postmitoball stages (SI Appendix, Fig. S4J), indicating no increased mitochondrial degradation at the mitoball stage.

The subcellular distribution of mitochondria could affect energy production and metabolic status (33, 34). We thus measured the adenosine triphosphate (ATP) level of testes by a quantitative bioluminescence assay and found it was significantly lower in milton and milton mutants compared to the controls (Fig. 4J). This finding raised the question of whether mitochondrial function was compromised in spermatocytes with no mitoballs. As there are no efficient methods nor good markers to assess mitochondrial respiratory status at a single-cell resolution in tissues, we examined mitochondrial morphology by electron microscopy imaging. Interestingly, we found that ~33% of spermatocyte cysts in milton mutants had strikingly swollen mitochondria, which were not observed in wild-type testes (Fig. 4K). The width of these swollen mitochondria was ~463 ± 93 nm, whereas wild-type mitochondria at a similar stage had a width of 304 ± 46 nm (SI Appendix, Fig. S4K). In addition, the individual swollen mitochondria were surrounded by a single layer of ER-like membrane and the overall ER network was more truncated (Fig. 4K) than the wild-type...
controls (Figs. 1E and 3B). Furthermore, these cysts showed an increase in mCherry-only foci when mito-QC is expressed, suggesting that the swollen mitochondria were either being degraded by mitophagy, or the cytoplasmic pH of these cysts was lower than that in controls, so more GFP fluorescence was quenched (Fig. 4L and SI Appendix, Fig. S4L).

It is surprising that disrupting mitochondrial trafficking could have such a profound effect on the mitochondrial volume and its interactions with other organelles. Mitochondrial swelling often indicates the opening of the mitochondrial permeability transition pore and is a hallmark of mitochondrial dysfunction. The increase in matrix volume could compromise K⁺ and Ca²⁺ fluxes, and lead to cristae remodeling and ultimately cell death (35, 36). We only observed cysts with swollen mitochondria in promeiotic stages, so it is possible that these cysts did not progress to the later stages of spermatogenesis, and the spermatids with nebenkerns we observed in mito-QC flies (SI Appendix, Fig. S4 E and G) were derived from healthy spermatocytes with normal mitochondrial networks. It could also be that only spermatocytes in certain developmental points are particularly sensitive to mitochondrial-trafficking disruption and will show the mitochondrial swelling phenotype. Further studies are required to gain a better understanding of how mitoballs and Milton modulate mitochondrial morphology, function, and turnover to support sperm development.

Discussion

This study characterized premeiotic mitochondrial clusters formed in insect spermatogenesis. We showed that mitoball-like structures were transient organizations formed in a wide range of insect species. In D. melanogaster, mitoballs assembled and dispersed in prophase I, and they contained other organelles including ER, Golgi bodies and the fusome. Microtubule-dependent mitochondrial trafficking was essential for mitoball formation as knocking out the adapter proteins Milton and Miro that link mitochondria to motor proteins abolished mitoball formation. Loss of mitoball was associated with reduced male fertility, suggesting its role in sperm development and potency. The mitoball of the male germ line resembles the Balbiani body of the female germ line. The Balbiani body is a cytoplasmic aggregation enriched with mitochondria. It is found in the oocytes of many animal species including insects and vertebrates (21, 37). Like the mitoball, the Balbiani body is a transient structure formed in early meiosis, and it contains other organelles such as ER and Golgi bodies (37–40). Furthermore, the Balbiani body in D. melanogaster also depends on Milton-mediated mitochondrial trafficking (28). However, the development of the Drosophila Balbiani body in oocytes relies on the transportation of mitochondria via the fusome from the rest of cyst cells and is blocked by mutations in hts, whereas mitoball formation does not rely on hts or an intact fusome (Fig. 3D). It is also unclear whether the mitoball is rich in proteins and RNAs, and organized by functional amyloid into a dense matrix that sequesters mitochondria and other cellular components like the Xenopus Balbiani body (37, 41).

Nevertheless, the similarities between the mitoball and the Balbiani body suggest that they may serve common functions in germ cell development. Since the discovery of the Balbiani body in the 19th century, it has been suggested to play roles in a number of processes including the localization of organelles and macromolecules to the germplasm, lipogenesis, and the selection/elimination of dysfunctional mitochondria from female germline cells, although evidence for these suggested roles is sparse (37, 42). Our study shows that the mitoball in D. melanogaster does not operate to eliminate dysfunctional mitochondria in early spermatogenesis as mitophagy-mediated mitochondrial degradation was not up-regulated at the mitoball stage (SI Appendix, Fig. S4J). Instead, we found mitoball-free testses had reduced ATP levels, and they carried cysts with abnormally swollen mitochondria that might be undergoing mitophagy (Fig. 4 J, K, and L). This suggests that mitochondrial trafficking and distribution could play a role in shaping mitochondrial morphology, function, and turnover, which allows efficient energy and metabolite production during the spermatocyte growth phase to support subsequent development. Mitochondrial movement could modulate mitochondrial function directly, or indirectly by affecting the distributions of other components as the mitoball might act as a scaffolding hub that assembles other organelles and germplasm components to facilitate certain biological processes that support mitochondrial function and sperm development. It is worth exploring whether mitochondrial swelling is a male germ-cell-specific defect associated with the milt850T mutation or also occurs in other tissues.

We showed that Milton and Miro mediated mitoball formation. We further deduced that a 54 AA region in the Milton C terminus was essential for this process. Milton and Miro are part of a conserved protein complex and function together to regulate mitochondrial transport in various species, including humans. However, polarized mitochondrial clustering was more clearly abolished when we knocked out milt than miro, suggesting that Milton can regulate mitochondrial trafficking independently of Miro during spermatogenesis. Similarly, a recent study showed that Milton and Miro affected mitochondrial transport in D. melanogaster bristle cells differently, with Milton being required for antegrade transport and Miro being needed for retrograde mobility (29). Recently, the human Milton proteins have also been found to form functional anterograde mitochondrial transport complexes in the absence of Miro (43). How Milton operates independently from Miro is unclear. Milton has neither a mitochondrial import sequence nor a transmembrane domain. Its interaction with mitochondria is via binding to Miro, which is a mitochondrial outer membrane protein. However, in a previous study, the C terminus of D. melanogaster Milton (AA847-1116), which does not bind to Miro, was shown to localize to mitochondria when expressed in COS7 cells. Hence, it has been proposed that the C-terminal region could tether Milton to mitochondria, likely through interacting with another mitochondria-anchoring protein (23). The 54 AA region essential for mitoball formation (AA850-903) lies in the C terminus of Milton, suggesting that it could be the domain that mediates the Milton-independent interactions between Milton and mitochondria and thus allows Milton to regulate mitochondrial transport in the absence of Miro.

Humans have two milt orthologs TRAK1 and TRAK2, which mediate microtubule-based mitochondrial trafficking in a similar manner. The function of TRAK1 and TRAK2 have been characterized in neurons and some other cell types (24, 27), but not in spermatogenesis. In D. melanogaster spermatogenesis, besides playing a role in mitoball assembly, Milton is also known to mediate mitochondrial elongation and microtubule sliding during spermatid development (44, 45). In mutant clones completely lacking Milton or Miro, spermatid elongation was slowed and arrested prematurely (44, 45). As a hypomorphic mutant, milt850T flies had nebenkerns and did not show obvious abnormalities in mitochondrial elongation or premature arrest of spermatid elongation (SI Appendix, Fig. S4M). However, due to difficulties in measuring the speed of mitochondrial and spermatid elongation across different testes within and between different genotypes, we cannot rule out the possibility that the reduced male fertility of milt850T flies is a combined effect of the lack of mitoballs and minor defects in spermatid development. Further investigation is needed to examine the impact of
the mitoball as an evolutionary strategy. It is worth noting that alternative mechanisms for spermatogenesis do not involve the mitoball as an evolutionary strategy. It is worth considering alternative mechanisms for spermatogenesis which do not involve the mitoball as an evolutionary strategy. It is worth examining alternative mechanisms for spermatogenesis which do not involve the mitoball as an evolutionary strategy.

In summary, this study provides unique insights into the dynamic nature of the mitochondrial network during insect spermatogenesis. It serves as a foundation for future characterizations of premeiotic mitochondrial clustering and how it impacts mitochondrial function and sperm development. This work also encourages investigations of Milton's role in human mitochondrial function and male fertility, given its conserved role in mitochondrial trafficking.

Materials and Methods

Drosophila Husbandry and Stocks. All D. melanogaster stocks were raised on standard media at 25 °C unless otherwise stated. D. melanogaster stocks and other Drosophila and insect species used in this study are listed in SI Appendix, Tables S1–S3. For the milt850 and miroKO2 mutants, flies carrying both the mutant allele and an FRT site on the same arm of the chromosome were generated by recombinant DNA technology.

Immunoﬂuorescence imaging by Confocal. Dissected testes were fixed in phosphate buffered saline (PBS) with 4% paraformaldehyde (pH 7.4) for 30 min, washed three times in PBS, and permeabilized with PBS containing 0.1% TritonX-100 (PBS) for 1 h at room temperature. The samples were then blocked in PBS supplement with 3% bovine serum albumin for 1 h. If subsequent immunostaining was required, tests were incubated with primary antibodies overnight at 4 °C, followed by another overnight incubation with secondary antibodies. Some samples were incubated with phalloloid Alexa Fluor 647 (1:200, ThermoFisher Scientific, A22878) at the secondary antibody staining step. Samples were then mounted in VECTASHIELD with DAPI (Vector Laboratories, H-1200) and imaged as described above. Immunostaining samples were sputter coated with 30- nm carbon using a Quorum Q150 T E carbon coater. Samples were imaged in a Verios 460 scanning electron microscope (FEI/ThermoFisher Scientific) at 4 keV accelerating voltage and 0.2 nA probe current in backscatter mode using the concentric backscatter detector in immersion mode at 29 °C to allow early and strong expression of RNAi. Testes were dissected from 1 to 2-d-old bam-GAL4 > UAS-mito-Dendra2 males at room temperature and immediately transferred with a drop of Schneider’s media to a glass slide. The coverslip was gently placed onto the top of the testes to allow a small volume of Schneider’s media to be retained during imaging. The edges of the glass slide were then sealed with nail polish, and samples were imaged on the Leica SP8 confocal microscope. If photo conversion was required, an area of interest was selected and illuminated with a UV laser (405 nm) for 10 s until all the Dendra2 had been converted from the green to the red form. The samples were then imaged with 488-nm and 647-nm lasers to detect green and red fluorescence signals, respectively.

Electron Microscopy Imaging. Samples were fixed in the fixative media (2% glutaraldehyde/2% formaldehyde in 0.05 M sodium cacodylate buffer pH 7.4 containing 2 mM CaCl2) overnight at 4 °C. After washing five times with 0.05 M sodium cacodylate buffer (pH 7.4), samples were osmicated (1% osmium tetroxide, 1.5% potassium ferricyanide, 0.05 M sodium cacodylate buffer pH 7.4) for 3 d at 4 °C. After washing five times in distilled H2O (dH2O), samples were treated with 0.1% (w/v) thio-carbohydrazide for 20 min at room temperature in the dark. After washing five times in dH2O, samples were osmicated a second time for 1 h at room temperature in 2% osmium tetroxide. Samples were washed five times in dH2O, and stained with maleate buffer (2% uranyl acetate in 0.05 M maleate buffer, pH 5.5) for 3 d at room temperature. Samples were washed again five times in dH2O and then dehydrated in a graded series of ethanol (50/70/95/100%/100% dry), 100% dry acetone and 100% dry acetonitrile, three times in each for at least 5 min. Samples were infiltrated with a 50/50 mixture of 100% dry acetonitrile/Quetol resin [without benzyl dimethylamine (BDMA)] overnight, followed by 3 d in 100% Quetol resin (without BDMA). Samples were then infiltrated for 5 d in 100% Quetol resin with BDMA, exchanging the resin each day. The Quetol resin mixture contains 12 g Quetol 651, 15.7 g nonenyl succinic anhydride, 5.7 g n-butyl naphthyl anhydride and 0.5 g BDMA. Samples were placed in embedding molds and cured at 60 °C for 3 d. Ultrathin sections (~70 nm) were cut using a Leica Ultratome microtome and mounted on mica in plastic coverslips. The coverslips were mounted on aluminum EM stubs using conductive carbon tabs and the edges of the slides were painted with conductive silver paint. Then, samples were sputter coated with 30-nm carbon using a Quorum Q150 T E carbon coater. Samples were imaged in a Jeol 4506 scanning electron microscope (FEI/ThermoFisher Scientific) at 4 keV accelerating voltage and 0.2 nA probe current in backscatter mode using the concentric backscatter detector in immersion mode at a working distance of 3.5 to 4 mm, 1,536 × 1,024 pixel resolution, 3 us dwell time, 4 line integrations.

The Candidate RNAi/KO Screen. For the RNAi screen, males carrying the UAS-RNAi constructs were crossed to females carrying both nosGAL4 and bamGAL4 at 29 °C to allow early and strong expression of RNAi. Testes were dissected from 1 to 2-d-old male progeny and stained with anti-AIPSA antibodies and DAPI for immunofluorescence imaging as described above.

For KO mutants, 10 to 15 testes were dissected from males if the mutant was homozygous viable and prepared for immunostaining. If the mutant was not homozygous viable, Germline KO clones/cysts were generated in heterozygous mutants. To generate germline KO clones, flies carrying both the mutant allele and an FRT site on the same arm of the chromosome were generated by recombination through genetic crosses. The FRT-mutant flies were then crossed to flies with a heat-shock inducible or germline-driven flipase (hs-FLP or nosGAL4 > UAS-FLP), the same FRT site, and a nuclear targeted fluorescent protein marker, which was present in heterozygous mutant clones and wild-type clones, but not
in homozygous mutant clones. Genetic crosses were performed at 25°C in vials. If the hs FLP was used, the vials were shifted to 37°C (heat shock treatment) for 2 h each time for 3 consecutive days once the progeny reached the wandering 3rd instar larval stage. Testes were dissected from 1 to 2-d-old male progeny and prepared for immunofluorescence staining as described above. The FRT/FLP lines used in this study are listed in SI Appendix, Table S3.

Image Analyses and Quantifications. The nuclear and mitochondria diameters were measured using the Leica LAS X software. In total, 28 spermatocytes from each genotype were measured using the Leica "draw scale bar" tool. The total distance moved by each mitochondrion was divided by the total time between the frames to obtain the average speed in μm/s

To quantify mtQC, the number of red (mCherry signal) and green (GFP signal) puncta of 0.2 to 0.5 μm in diameters were counted in cells at the respective developmental stage. Where all the cells at a given developmental stage show the number of dsDNA puncta per cell for four cells at each 100 μm

**ATP Assay.** ATP levels were measured using the ATP determination kit (ThermoFisher Scientific, A22066). For each genotype, 10 testes were dissected from 1 to 2-d-old males and homogenized in 100 μL of 6 M guanidic HCl (ThermoFisher Scientific, 24115) and 0.01 M Tris-HCl (pH 7.3), and frozen in liquid nitrogen for 5 min. The sample was then incubated at 95°C for 5 min and centrifuged at 12,000 × g for 10 min at 4°C. 10 μL of the sample was used for each 100 μL reaction as instructed in the kit manual. ATP levels for each sample were normalized to protein concentration measured using the Pierce BCA protein assay kit (ThermoFisher Scientific, 23225). For each genotype, six replicates were performed.

**Statistical Analyses.** Statistical analyses were performed using R (version R 4.0.5) and graphs were plotted using Prism 9 (GraphPad). All comparisons of means for the mitochondrial genome quantification, normalized proportion of mCherry-only foc and female fertility were performed using one-way (ANOVA) with Tukey’s honestly significant difference post hoc test. For the male fertility and mitochondrial width data, the Mann–Whitney U test was used as the data were not normally distributed. Student’s t test was used to compare the means for the ATP assay. For all statistical tests, key assumptions were formally tested in R, and the datasets were deemed suitable. Statistical significance was defined by ns (not significant) P > 0.05, *P < 0.05, **P < 0.01, and ***P < 0.005.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information. All fly stocks will be made available upon request.

**ACKNOWLEDGMENTS.** We thank Prof Daniel St Johnston (Gurdon Institute, University of Cambridge) for sharing fly stocks related to cell polarity and cytokinesis regulation, Prof Alex Whitworth (MRC Mitochondrial Biology Unit, University of Cambridge) for fly stocks related to mitochondrial calcium transporters, Prof Jake Baum (Imperial College London and University of New South Wales) and Dr Andrew Blagborough (Department of Pathology, University of Cambridge) for providing the two mosquito species, and Dr Steve Rogers (Department of Zoology, University of Cambridge) for supplying various other insect species used in this study. We thank Dr Richard Butler (Gurdon Institute, University of Cambridge) for providing an in-house FIJI plugin for the mito-QC image analysis and quantification. We also thank Dr Karin Müller and Filomena Gallo at Cambridge Advanced Imaging Centre for their help in acquiring electron microscopic images, and the Gurdon Institute Core Facilities for their support. This work is funded by a Biotechnology and Biological Sciences Research Council Studentship to Sumeraa Rathore, Wellcome Trust Sir Henry Dale Fellowship 202269/Z/16/B, ERC Starting Grant 803852 and an Isaac Newton Trust/Wellcome ISSF/University of Cambridge Joint Grant to Dr Hansong Ma. The Gurdon Institute Core Facility is funded by Wellcome Trust grant 203144 and Cancer Research UK grant C6946/A24843.


