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JB Review

Multiple ways to prevent transmission of paternal mitochondrial DNA for maternal inheritance in animals

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Mitochondria contain their own DNA (mtDNA). In most sexually reproducing organisms, mtDNA is inherited maternally (uniparentally); this type of inheritance is thus referred to as 'maternal (uniparental) inheritance'. Recent studies have revealed various mechanisms to prevent the transmission of sperm-derived paternal mtDNA to the offspring, thereby ensuring maternal inheritance of mtDNA. In the nematode *Caenorhabditis elegans*, paternal mitochondria and their mtDNA degenerate almost immediately after fertilization and are selectively degraded by autophagy, which is referred to as 'allophagy' (allogeneic [non-self] organelle autophagy). In the fruit fly *Drosophila melanogaster*, paternal mtDNA is largely eliminated by an endonuclease G-mediated mechanism. Paternal mitochondria are subsequently removed by endocytic and autophagic pathways after fertilization. In many mammals, including humans, paternal mitochondria enter fertilized eggs. However, the fate of paternal mitochondria and their mtDNA in mammals is still a matter of debate. In this review, we will summarize recent knowledge on the molecular mechanisms underlying the prevention of paternal mtDNA transmission, which ensures maternal mtDNA inheritance in animals.

Keywords: autophagy; fertilization; maternal inheritance; mitochondria; mitochondrial DNA.

Abbreviations: EndoG, endonuclease G; FIS1, mitochondrial fission 1; GABARAP, γ-aminobutyric acid receptor associated protein; MO, membranous organelle; mtDNA, mitochondrial DNA; MVB, multivesicular body; VCP, valosin-containing protein.

The mitochondrion is an organelle that produces ATP by oxidative phosphorylation, and exists in most eukaryotic cells. This organelle is thought to have evolved through the symbiosis of aerobic bacteria into anaerobic primitive eukaryotes, about 1.5 billion years ago, and has its own DNA (mitochondrial DNA [mtDNA]) (*1*). In humans, mtDNA consists of a closed 16.5-kbp circular DNA genome encoding thirteen polypeptide subunits required for oxidative phosphorylation, 22 tRNAs, and 2 rRNAs (*2*). Mutations in mtDNA are associated with various diseases including mitochondrial disease and diabetes; these have also been reported be associated with the malignant transformation of some cancers (*3–5*). Interestingly, mtDNA is known to be maternally (uniparentally) inherited in many species, including humans. This phenomenon is referred to as maternal (uniparental) inheritance (*6–9*). Recent studies have revealed that paternal mitochondria and their DNA are actively eliminated before and after fertilization. In this review, we will summarize the current knowledge of the mechanisms underlying maternal inheritance of mtDNA in animals.

Active Degradation of Paternal Mitochondria and Their mtDNA in *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living nematode that generally reproduces as a self-fertilizing hermaphrodite; however, it also produces males (at a low frequency) that can mate with hermaphrodites. In *C. elegans* spermatozoa, there are 50–70 orbicular paternal mitochondria with a 400–600 nm diameter, as well as a similar number of membranous organelles (MOs) which are specialized ER-/Golgi-derived vesicles essential for sperm fertility (Fig. 1) (*10*). In *C. elegans*, paternal mitochondria enter into the fertilized eggs and gradually disappear until the 8- to 16-cell stage (*11*, *12*). We determined, along with others, that the degradation of paternal mitochondria in *C. elegans* embryos is mediated by autophagy. Autophagy delivers various cellular components, including organelles and proteins, to lysosomes for degradation. About 30 min after fertilization, autophagosomes appear around sperm-derived materials and selectively enclose paternal mitochondria and MOs. These autophagosomes are scattered in the cytoplasm by cytoplasmic streaming during the pronuclear fusion stage and ultimately fuse with lysosomes for the degradation of paternal mitochondria and MOs. We named this new type of autophagy for the removal of paternal organelles 'allophagy' (allogeneic [non-self] organelle autophagy) (*13*). If mutations occur in autophagy-related factors such as LGG-1 (an Atg8/LC3 homolog) and UNC-51 (an Atg1/ULK1 homolog), paternal mitochondria are not removed and remain until the larval stage. Paternal mtDNA is not transmitted to the next generation in wild-type animals, but remains even in F1 larvae in autophagy-defective mutants; this clearly indicates that allophagy is essential for the elimination of

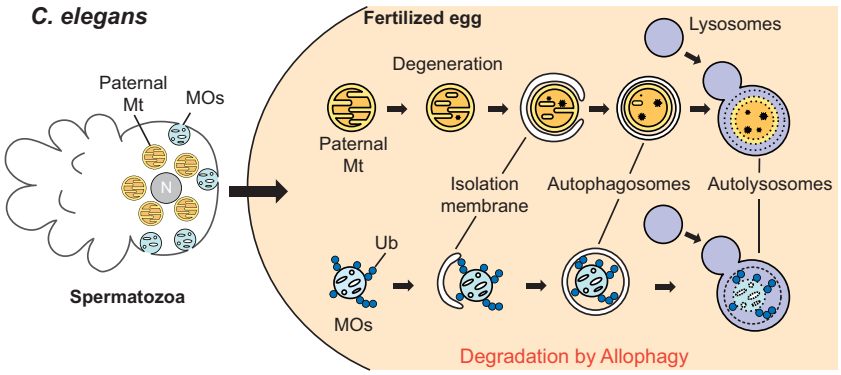

Fig. 1 Maternal inheritance of mtDNA by selective degradation of paternal mitochondria and mtDNA in *C. elegans*. In *C. elegans*, paternal mitochondria start to lose their integrity and are degenerated by a CPS-6 (endonuclease G)-mediated mechanism shortly after fertilization (*17*). Paternal mitochondria are then engulfed by autophagosomes and delivered to lysosomes for degradation (*11*, *12*). Ubiquitination of MOs occurs after fertilization; they are then selectively degraded via autophagy. Mt, mitochondria; MOs, membranous organelles; Ub, ubiquitin.

paternal mtDNA and maternal inheritance of mtDNA in *C. elegans*. It was also reported that the degradation of paternal mtDNA is delayed by knockdown of proteasome subunit genes (*14*). In addition, the localization of the 19 S regulatory subunit of proteasomes around MOs is observed in sperm and fertilized embryos (*12*). These findings suggest that the ubiquitin-proteasome system is also directly or indirectly involved in the degradation of paternal mtDNA in *C. elegans*.

Autophagy was originally discovered as a system for recycling cellular components during starvation (*15*). However, it has been revealed that certain substrates, such as pathogens and damaged mitochondria, are removed by selective autophagic mechanisms (*16*). In selective pathways, such as mitophagy, ubiquitination of the substrate is known to be one of the signals for autophagy. Notably, MOs are highly ubiquitinated in embryos after fertilization (*11*, *12*). Although the ubiquitin signal is still difficult to detect in *C. elegans* paternal mitochondria, ubiquitination of paternal organelles at low levels may be involved in allophagy. Sperm-derived mitochondria and MOs could be incorporated into autophagosomes separately, although they are engulfed together in some autophagosomes. This observation suggests that there may be mechanisms to recognize paternal mitochondria and MOs independently.

Recently, it has been reported that the membrane structure of paternal mitochondria begins to degenerate immediately after fertilization (*17*). In fertilized eggs, paternal mitochondria form multiple dark aggregates in the matrix and their cristae gradually disappear. In addition, these paternal mitochondria show loss of membrane potential. Interestingly, this degeneration is dependent on CPS-6, a homolog of mitochondrial endonuclease G (EndoG). CPS-6 seems to localize close to the mitochondrial membrane, but moves to the matrix after fertilization, where it participates in paternal mtDNA degradation. In *cps-6* mutant embryos, degeneration of the paternal mitochondria, as well as autophagosome formation around them, is partially delayed. These findings suggest that CPS-6-mediated degeneration of the paternal mitochondrial membrane in embryos leads to allophagy. Since the effect of CPS-6 seems to depend on its endonuclease activity, paternal mtDNA is likely to be digested by CPS-6, at least in part. In contrast, paternal mtDNA persists in larvae of autophagy-defective mutants, even if CPS-6 is intact. These observations suggest that allophagy is required for the complete elimination of paternal mtDNA in embryos (*11*, *12*).

Active Degradation of Paternal Mitochondria and Their mtDNA in *Drosophila*

In the fruit fly *Drosophila melanogaster*, paternal mtDNA is mostly eliminated during spermatogenesis (Fig. 2). During spermatogenesis, paternal mitochondria fuse with each other, elongate to a length of ~1.8 mm, and finally form two very long mitochondria. During this process, when the mitochondria have extended to lengths of 1.7–1.8 mm, mitochondrial nucleoids containing mtDNA gradually disappear, starting at the basal (nuclear) ends and moving to the apical ends (*18*). In the final stages of spermatogenesis, a unique actin-containing structure called the 'investment cone' moves from the base of the flagellum to the tip and sequesters all unnecessary cytoplasmic components inside the flagella to a waste bag. EndoG, which is present in the mitochondria, is involved in the degradation of mtDNA during the mitochondrial elongation process (*18*). In EndoG mutants, the elimination of nucleoids containing mtDNA is significantly delayed. However, all remaining paternal mtDNA is ultimately removed by the movement of the actin-containing investment cone and discarded to the waste bag with other cytoplasmic components (*18*). Therefore, mtDNA is hardly detected in the mature sperm (Fig. 2).

In *Drosophila*, paternal mitochondria also enter the fertilized egg and are eventually degraded by endocytic and autophagic degradation systems (*19*). Upon fertilization, multivesicular body (MVB)-like vesicles surround the sperm-derived flagellum, which is largely

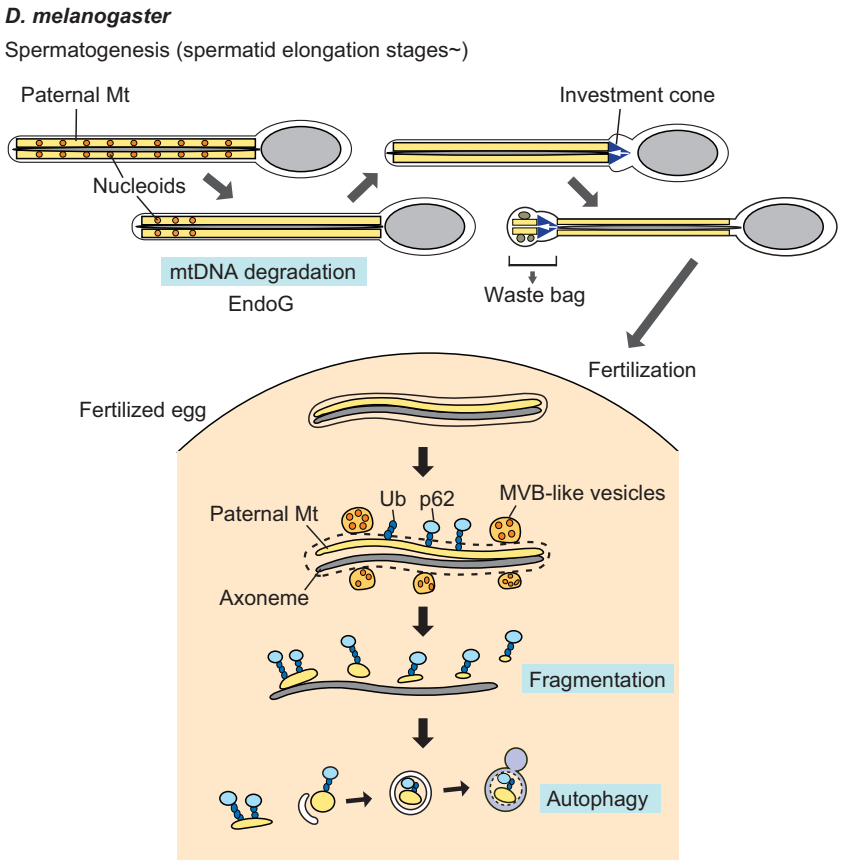

Fig. 2 Maternal inheritance of mtDNA by selective degradation of paternal mtDNA in *D. melanogaster*. In *Drosophila*, paternal mtDNA is mostly degraded by an endonuclease G (EndoG)-mediated mechanism during the process of spermatogenesis (*18*). Any remaining paternal mtDNA is removed by an actin-based structure called the investment cone, which move from the base to the distal region of the flagellum to deliver unnecessary cytoplasmic components to the waste bag (*18*). After fertilization, the paternal mitochondria and axoneme are surrounded by MVB-like vesicles (*19*). After being released from the axoneme, long paternal mitochondria are fragmented and finally degraded by autophagy. K63-linked polyubiquitin signals and p62/SQSTM1 have been observed on paternal mitochondria in fertilized eggs (*19*).

intact. The autophagy-related factor Atg8a and the late endosomal protein Rab7 localize to these MVB-like vesicles around the paternal mitochondria, indicating that these vesicles may be hybrid organelles between the endocytic and autophagic pathways, similar to amphisomes. At a later stage, the long paternal mitochondria are separated from the axoneme and fragmented into oval shapes (0.1–0.4 μm in diameter). Fragmented paternal mitochondria are ultimately engulfed by autophagosomes and degraded in lysosomes. The degradation of paternal mitochondria is partially inhibited in autophagy-defective mutants, such as the *atg7* null mutant and a strain expressing a Rab7-dominant negative mutant; this suggests that the autophagic and endocytic pathways are required for paternal mitochondria degradation. Because some autophagy-defective mutants also exhibit defects in the formation of MVB-like vesicles around the paternal mitochondria and the fragmentation of paternal mitochondria, autophagy-related factors may play roles in such processes. K63-linked polyubiquitin chains and a homolog of ubiquitin-binding autophagy receptor p62/SQSTM1 have also been detected on paternal mitochondria in fertilized eggs. In addition, the overexpression of a deubiquitinating protease causes a delay in paternal mitochondria degradation. These observations suggest that the ubiquitin-p62 pathway is also involved in paternal mitochondria elimination.

Multiple Ways to Prevent Transmission of Paternal mtDNA in Vertebrates

In the Japanese rice fish (*Oryzias latipes*), the number of nucleoids containing paternal mtDNA is gradually reduced over the course of spermatogenesis. After fertilization, paternal mtDNA disappears in penetrated paternal mitochondria, which still maintain their shape, suggesting a two-step digestion of paternal mtDNA inside the paternal mitochondria, both before and after fertilization (*20*).

Textbooks used to describe that mtDNA is maternally inherited in mammals, since the paternal mitochondria do not enter the egg. This is true in the case of the Chinese hamster (*Cricetulus griseus*). Since the spermatozoa of the Chinese hamster are very large, the male nucleus in the head of the sperm enters the fertilized egg, but the sperm tail—including the mitochondria—does not enter the oocyte (*21*, *22*). However, this turned out to be an exceptional case, rather than the norm, because the paternal mitochondria of many

animals—including human and mouse—have been shown to enter the fertilized egg (*6*). Recently, the fate of paternal mitochondria and their mtDNA in embryos has been a hot topic of debate (Fig. 3). Many groups have reported that paternal mitochondria and their mtDNA in fertilized eggs gradually disappear during early embryogenesis (*23–28*), suggesting the degradation of paternal mitochondria in embryos. In the case of cattle and rhesus monkeys, it has been shown that paternal mitochondria are ubiquitinated and degraded during early embryogenesis (*28*). Since ubiquitin signals are already detected in sperm produced in the male reproductive organs, ubiquitin may be added in advance to paternal mitochondria as a marker for selective degradation after fertilization. One candidate for ubiquitinated proteins in sperms is the mitochondrial inner membrane protein, prohibitin (*29*, *30*). Interestingly, this protein was recently identified as a mitophagy receptor for the elimination of damaged mitochondria (*31*). It may be possible that prohibitin is also involved in paternal mitochondria elimination in mammalian embryos.

Degradation of paternal mitochondria in mammalian embryos was reported to be affected by lysosome inhibitors and proteasome inhibitors (*32*). In mice, autophagy is transiently induced in one-cell stage embryos shortly after fertilization (*33*). In addition, the accumulation of autophagosomal marker proteins was observed in the vicinity of paternal mitochondria in fertilized eggs (*13*). These observations support the possibility that paternal mitochondria and their mtDNA in mammalian embryos are degraded via autophagy. Recently, Chan's group reported that mouse paternal mitochondria experience a loss in membrane potential by 48 h after fertilization, and are progressively degraded 60–84 h after fertilization (*34*). They also showed that the degradation of paternal mitochondria is delayed by the loss of Parkin, an E3 ubiquitin ligase required for mitophagy, and strongly inhibited by the simultaneous loss of Parkin and the mitochondrial E3 ubiquitin ligase MUL1 (MULAN/MAPL). They also reported that the knockdown of PINK1 kinase, autophagy receptor p62/SQSTM1, mitochondrial fission 1 (FIS1), and FIS1-interacting protein Tbc1d15 partially inhibits the degradation of paternal mitochondria. Since these factors are involved in Parkin-dependent mitophagy, a similar mechanism may function in the elimination of paternal mitochondria in mouse embryos. In the case of the pig and rhesus monkey, p62/SQSTM1 and valosin-containing protein (VCP), ubiquitin-binding protein dislocase, localize to paternal mitochondria in zygotes (*35*). γ-aminobutyric acid receptor associated protein (GABARAP) also localizes around paternal mitochondria in porcine zygotes. The study by Song *et al.* claimed that simultaneous inhibition of p62/SQSTM1, GABARAP and VCP prevents the degradation of paternal mitochondria in embryos, implying that the autophagy and ubiquitin-proteasome systems function cooperatively in the degradation of paternal mitochondria in these species.

In contrast, Luo *et al.* (*36*) reported a different aspect of maternal inheritance of mtDNA in mice (Fig. 3). This study argued that mouse paternal mtDNA is largely eliminated during spermatogenesis and detectable only in parts of the embryo. They also

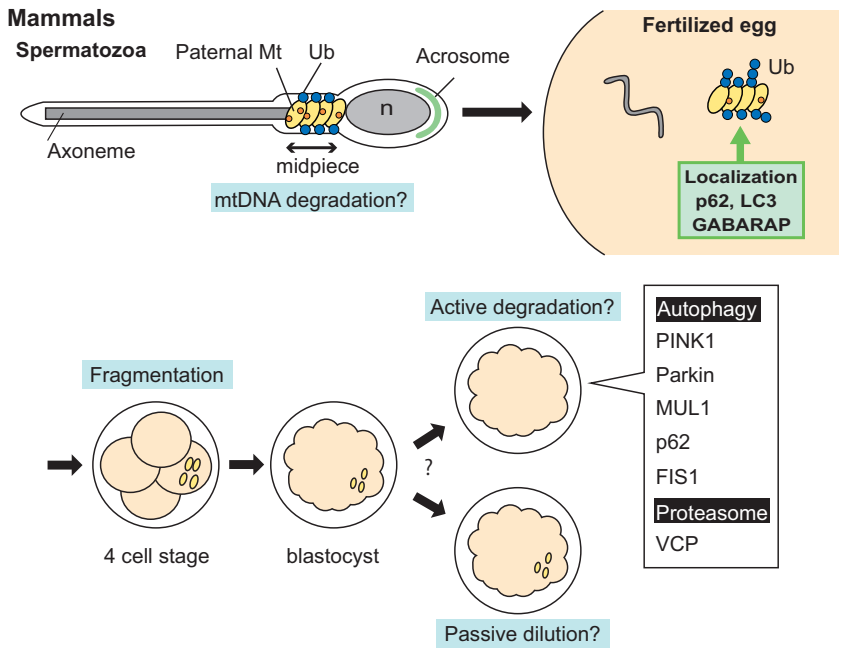

Fig. 3 The fate of paternal mitochondria in mammals. Paternal mitochondria enter into fertilized eggs in many mammals (*23–28*). Polyubiquitin signals have been observed on paternal mitochondria during spermatogenesis (*28*). After fertilization, autophagy-related factors localize around the paternal mitochondria (*12*). It remains unclear whether paternal mitochondria are eliminated in embryos. Several groups have reported that paternal mitochondria are degraded by mitophagy and/or ubiquitin-proteasome systems (*34*,*35*), whereas other groups have claimed that paternal mitochondria are not eliminated; rather, they are distributed unevenly to blastomeres during embryogenesis (*36*). Mt, mitochondria; Ub, ubiquitin.

reported that paternal mitochondria, including remnants of mtDNA, are not actively removed and persist in embryos at least until the morula stage. Instead, paternal mitochondria are unevenly distributed to one of the blastomeres until the four-cell stage; they are then disaggregated and scattered to the cytosol after the morula stage. Certainly, autophagosomal proteins such as LC3 are detected near paternal mitochondria at the one-cell stage, but these are dissociated from paternal mitochondria at the four-cell stage. In addition, the behaviour of paternal mitochondria was unchanged even in mouse autophagy-defective embryos, implying that autophagy is not involved in the degradation of paternal mitochondria in embryos. Finally, this study reported that paternal mtDNA is detectable even in newborn mice, albeit at a very low rate, and is distributed unevenly in parts of tissues after implantation and birth. From these observations, the authors proposed that the elimination of mtDNA during spermatogenesis and the uneven distribution of paternal mitochondria during embryogenesis ensure maternal inheritance of mtDNA in mice. Whether paternal mitochondria are eliminated in mammalian embryos should be further verified.

Conclusion

Various mechanisms for preventing the transmission of paternal mtDNA to offspring in animals have been revealed, as follows: (i) degradation of paternal mtDNA before or after fertilization, (ii) blocking paternal mitochondria from entering the oocyte, (iii) elimination of paternal mitochondria by autophagy and/or ubiquitin-proteasome systems and (iv) uneven distribution of paternal mitochondria with remaining paternal mtDNA during embryogenesis. In some cases, a combination of several mechanisms is adopted to prevent paternal mtDNA transmission. However, many questions remain to be addressed. It is not understood how the timing of paternal mtDNA digestion is controlled. In mice and fish, the corresponding enzymes have not been identified. It is still unclear how paternal mitochondria are recognized for selective degradation. In the case of *C. elegans*, sperm-derived MOs are highly ubiquitinated in embryos and eliminated by allophagy. Thus, low levels of ubiquitin signal on paternal mitochondria may be a signal for allophagy, although ubiquitin signals are still difficult to detect on paternal mitochondria in *C. elegans*. Recently, it has been reported that prohibitin 2 functions as an inner mitochondrial membrane autophagy receptor for Parkin-dependent mitophagy, and the knockdown of its *C. elegans* homolog *phb-2* also inhibits allophagy (*31*). It is unknown whether this also works for the elimination of paternal mitochondria in mammalian embryos. As described above, paternal mitochondria elimination in mouse embryos seems to be mediated by Parkin- and MUL1-dependent mechanisms. In contrast, in *Drosophila*, the loss of Parkin does not affect the elimination of paternal mitochondria in embryos, suggesting the existence of a Parkin-independent mitophagy mechanism. This inconsistency may be due to species-specific mechanisms for paternal mitochondria elimination in embryos. It is also possible that unidentified ubiquitin ligases act redundantly with Parkin to eliminate paternal mitochondria in *Drosophila* embryos. Further studies will reveal how sperm-derived organelles, such as paternal mitochondria, are ubiquitinated and recognized by the autophagy and/or ubiquitin-proteasome systems.

An important question is why mtDNA has to be maternally and uniparentally inherited. Although there is diversity in the mechanisms of mtDNA elimination, maternal (uniparental) inheritance of mtDNA has been observed in many sexual organisms. Thus, there should be some evolutionary or physiological advantage to the phenomenon of maternal inheritance of mtDNA. In this regard, it is necessary to consider another feature of mtDNA inheritance. It is thought that mtDNA accumulates mutations at a higher frequency than nuclear DNA (*37*). Since mtDNA is present in multiple copies, it is expected to tend to be in a state of heteroplasmy, i.e. a mixture of mtDNA with different sequences. However, heteroplasmy of mtDNA is genetically unstable and rarely inherited. Even if heteroplasmy of mtDNA occurs, it rapidly changes to a homoplasmic state over several generations via rapid segregation (*38–40*). Recently, an interesting study was reported where mice containing mtDNA from two wild-type strains (129S6 and NZB) in a heteroplasmic state were generated with a congenic C57BL/6 J nuclear background (*41*). The inheritance of two variants of mtDNA was analyzed over 14 years. There is only a 91-bp difference between 129S6 mtDNA and NZB mtDNA. Nevertheless, mice that maintained heteroplasmy in the mtDNA exhibited several changes in behaviour and metabolism, such as reduced food intake, activity, energy consumption and cognitive function, compared with mice with homoplasmic mtDNA of either variant. Although a mechanism to cause such differences has not been identified, this result suggests that a homoplasmic state of mtDNA is preferable within an organism. Mechanisms for maternal inheritance and rapid segregation may have evolved to maintain the homoplasmic state of mtDNA.

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Conflict of Interest

None declared.

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