REVIEW ARTICLE

Dynamic regulation of mitochondrial genome maintenance in germ cells

Katsumi Kasashima · Yasumitsu Nagao · Hitoshi Endo

Abstract Mitochondria play a crucial role in the development and function of germ cells. Mitochondria contain a maternally inherited genome that should be transmitted to offspring without reactive oxygen species-induced damage during germ line development. Germ cells are also involved in the mitochondrial DNA (mtDNA) bottleneck; thus, the appropriate regulation of mtDNA in these cells is very important for this characteristic transmission. In this review, we focused on unique regulation of the mitochondrial genome in animal germ cells; paternal elimination and the mtDNA bottleneck in females. We also summarized the mitochondrial nucleoid factors involved in various mtDNA regulation pathways. Among them, mitochondrial transcription factor A (TFAM), which has pleiotropic and essential roles in mtDNA maintenance, appears to have putative roles in germ cell regulation.

Keywords Bottleneck · Maternal inheritance · Mitochondrial DNA · Nucleoid · TFAM

Introduction

The mitochondrion, which is referred to as the 'cellular power plant', is an intracellular organelle that produces the majority of cellular ATP through oxidative phosphorylation. Besides this function, mitochondria are also important

K. Kasashima · H. Endo (✉)
Department of Biochemistry, Jichi Medical University,
3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
e-mail: hendo@jichi.ac.jp

Y. Nagao
Center for Experimental Medicine, Jichi Medical University,
Shimotsuke, Tochigi 329-0498, Japan

for the induction of apoptosis, and generate reactive oxygen species (ROS) by respiration. Mitochondria contain their own genomic DNA called mitochondrial DNA (mtDNA) [1]. In animals, mtDNA exists in multiple copies per cell (>1000 copies), and is maternally inherited from egg cells [2]. Human mtDNA is a 16.6 kb circular double-stranded DNA that encodes 13 proteins, which are components of respiratory chain subunits. mtDNA-encoded proteins are translated through mitochondrial ribosomes, and their expression is essential for respiratory function.

Germ cells are essential for the generation of offspring and need to be protected from the accumulation of damaged mtDNA due to ROS. Respiration activity was shown to be suppressed in Xenopus oocytes and the resultant reduced levels of ROS have been suggested to enable accurate mtDNA transmission between generations [3]. It has recently been clarified that mitochondrial genomes and their products (including ribosomal RNA) play important roles in the formation and development of germ cells [4–7], which makes the mitochondrial genome the target for reproductive technology. Mitochondrial transfer (mtDNA replacement) has been attempted as a germline gene therapy for mitochondrial diseases [8] and the efficient development of aged oocytes [9]. However, mitochondrial transfer by replacing the cytoplasm may cause mtDNA carryover and heteroplasmy, in which two or more mtDNA variants co-exist. Since mouse mtDNA heteroplasmy causes reduced physical activity and learning [10], regulating mtDNA segregation, uniparental transmission, and the bottleneck seems to be important for maintaining its homoplasmy.

In this review, we summarized characteristic regulation of the mitochondrial genome in germ cells, such as maternal inheritance and the mtDNA bottleneck (Fig. 1). In addition, we reviewed current understanding of the

Fig. 1 Characteristic mtDNA regulation in germ cells. Schematic representation of mtDNA regulation in germ cells, maternal inheritance, and the mtDNA bottleneck. In females, the rapid segregation of mtDNA is enabled by the mtDNA bottleneck during PGCs and mature oocytes. There are three possible mechanisms in the mtDNA bottleneck, all of which are based on the low segregation unit number. In males, a decrease in mitochondria occurs during spermatogenesis, which includes a reduction in the mtDNA copy number and trimming of mitochondria. After fertilization, selective degradation of paternal mitochondria by autophagy or proteasomes further enhances the maternal inheritance of mtDNA
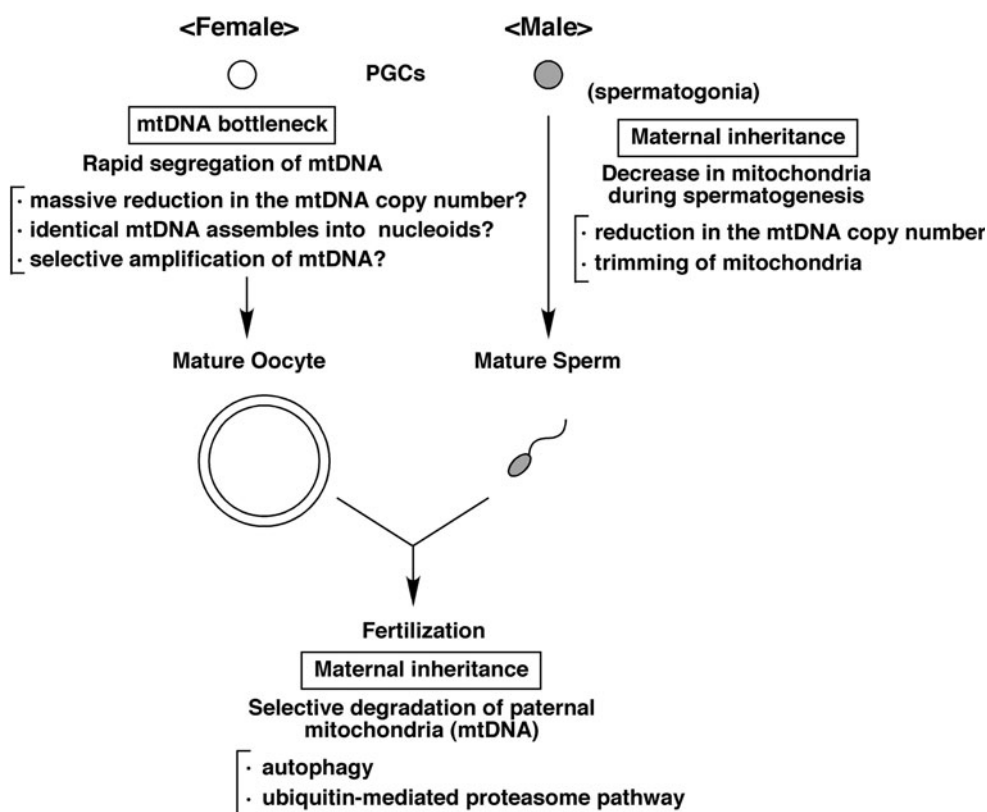
major mitochondrial nucleoid factor TFAM, which has pleiotropic functions and may regulate the mtDNA copy number in germ cells.

Maternal inheritance of the mitochondrial genome

In most organisms, mtDNA is maternally inherited and transmitted to offspring, although paternal mitochondria enter into the egg cell after fertilization [11]. Maternal inheritance has been explained by differences in the size of the gamete; the paternal gamete (sperm) is much smaller than the maternal gamete (egg). The mtDNA copy number in germ cells is also very different; in animals, the egg cell contains 10^{5-8} copies of mtDNA [2], whereas mature sperm contain only 100 [12, 13]. A dilution of sperm mtDNA in the ooplasm has been considered as a simple model for explaining maternal inheritance. However, chloroplast DNA is inherited from the maternal gamete in *Chlamydomonas reinhardtii*, in which the size of the two gametes is similar [14], and this is caused by the active digestion of paternal DNA [15]. Thus, some active eliminating mechanisms have been suggested to work in the paternal mitochondrial genome.

Recent studies have shown that the mtDNA copy number was decreased during spermatogenesis in various organisms. In humans, the amount of mtDNA decreased

during spermatogenesis and was associated with a reduction in TFAM, which plays an essential role in maintaining the mtDNA copy number (reviewed below) [16]. A reduction in the amount of mtDNA during spermatogenesis has also been observed in rodents [12, 17], Japanese medaka (*Oryzias latipes*) [18], and drosophila [19], which suggests a conserved mechanism for the active digestion of paternal mtDNA in spermatogenesis. Furthermore, paternal mtDNA in mice was selectively degraded in the fertilized egg through an unknown mechanism [20]. This elimination occurred specifically in mtDNA derived from spermatids and not in mtDNA from liver cells, indicating that spermatid mitochondria display specific factors that are recognized as eliminating signals [21]. In addition, although paternal mtDNA remained in the fertilized eggs of F1 hybrids by interspecific mouse crosses, it was eliminated by a subsequent backcross, which suggested that species-specific elimination of paternal mtDNA is particularly stringent [22]. These elimination systems enable strict maternal inheritance of mtDNA in mice. The rapid digestion of mtDNA was observed just after fertilization in *O. latipes*, in addition to the decreased amount of mtDNA during spermatogenesis [18]; however, the molecular mechanism is still unknown. Two barriers to paternal mtDNA transmission, which act before fertilization, were identified in drosophila. First, endonuclease G was shown to be involved in the degradation of sperm mtDNA during

spermatogenesis [19]. This was the first evidence to show that the molecular mechanism involved the elimination system. Second, the cellular remodeling process that trims and shapes the tail of sperm cells was shown to eliminate sperm mitochondria independently of endonuclease G activity [19]. The cytoplasm of developing spermatids, which includes mitochondria, was shown to be similarly trimmed in mammals [12, 23], suggesting that the trimming of mitochondria is also a conserved mechanism for reducing the amount of mtDNA in sperm (Fig. 1). Taken together, multistep regulation seems to be required for strict uniparental transmission.

The involvement of the ubiquitin–proteasome system has been suggested for the molecular mechanism underlying the digestion of sperm mtDNA in fertilized eggs [24]. Mitochondria and some mitochondrial nucleoid proteins (reviewed below) are known to be ubiquitinated in sperm [25, 26]; thus, this modification may be involved in this regulation. More recently, paternal mitochondria in *Caenorhabditis elegans* were shown to be degraded after fertilization by autophagy [27–29], which degrades cytoplasmic proteins and organelles by lysosomes. In autophagy-defective zygotes, parental mitochondria including mtDNA were shown to remain in the developmental stage, indicating the essential role of autophagy in paternal elimination. Because paternal mitochondria seem to be heavily damaged by ROS, autophagy is a plausible system for regulation.

mtDNA bottleneck and rapid segregation

Another genetic characteristic of mtDNA in germ cells is the 'mtDNA bottleneck' [30]. In mammals, individuals typically have a single mtDNA variant, called homoplasmy. mtDNA variants rapidly shift between generations and revert to homoplasmy during a few generations [30–34]. The mtDNA bottleneck, with a very low mtDNA copy number, has been considered as an explanation for this genetic characteristic [30]. Mature oocytes in humans contain an mtDNA copy number of over 200000 per cell, whereas primordial germ cells (PGCs), produced by cell division after fertilization, contain only about 200 copies per cell [2]. Similar decreases in the mtDNA copy number have been observed in mouse PGCs [35, 36], and this marked reduction was considered to be the cause of the rapid segregation of mtDNA variants [35]. However, the Yonekawa group proposed that the mtDNA bottleneck occurred without a severe reduction in the mtDNA copy number in mice [37, 38]. They showed that PGCs contained consistent, moderate mtDNA copy numbers during oocyte maturation by quantitative PCR using a single germ line cell [37]. In addition, they reported that a severe

reduction in mtDNA content did not occur in early PGCs through an improved PGC-isolation method [38]. Therefore, they concluded that a reduction in mtDNA content was not required for the mtDNA bottleneck. A bottleneck through nucleoid formation or the selective amplification of mtDNA has been considered as alternative models for the selection of transmitted units. In the nucleoid formation model, identical mtDNA molecules assemble into and form nucleoids, which act as the segregation unit. A low amount of the mtDNA variant is thought to be transmitted through unequal partitioning of the decreased segregation unit. Wai et al. also demonstrated that genotypic variance (heteroplasmy) was increased during postnatal oocyte maturation, and not during embryonic oogenesis, which indicated that the mtDNA genetic bottleneck does not occur in the early period with a very low mtDNA copy number. Therefore, they suggested that the rapid segregation of the genotype was due to the selective replication of a subgroup of mtDNA within postnatal oocyte maturation (the selective amplification model), rather than a reduction in mtDNA content [36]. Regarding the timing of the bottleneck, the Chinnery group recently showed that mammalian mtDNA heteroplasmy was determined prenatally during the developing female germline [39]. Thus, it appears that there are still three proposed models for bottleneck regulation (Fig. 1). Confirming the precise timing when the mtDNA bottleneck occurs will be required to clarify the real bottleneck mechanism from these models [40].

In maternal germ cells, the copy number of mtDNA was shown to be markedly increased during oocyte maturation, from PGCs to mature egg cells, through resumed replication. Partitioning mtDNA into dividing cells may play an important role in the transmission of the mtDNA genotype in processes that go through cell division [41].

As described above, the two characteristic processes that regulate mtDNA in germ cells appear to be related to the copy number regulation of mtDNA [40], which suggests the importance of mitochondrial nucleoid proteins in this regulation.

Mitochondrial nucleoids

Several studies have shown that mtDNA exists not as naked DNA, but as a highly organized structure associated with multiple proteins (the nucleoprotein complex), which is different from the nuclear chromatin structure. These complex structures, called mitochondrial nucleoids, associate with the inner membrane, and are considered to regulate the stability, replication, transcription, and segregation of mtDNA [42]. Two major proteins have been associated with mtDNA: TFAM and mitochondrial single-stranded DNA-binding protein (mtSSB).

Table 1 Mitochondrial nucleoid proteins with known functions

Protein	Functions in mtDNA metabolism	References
TFAM	Transcription, replication, packaging, copy number regulation, segregation	[60, 66]
mtSSB	Replication	[89]
Polg/Polg2	Replication, repair	[90]
Polrmt	Transcription, replication	[91]
TFB2M	Transcription	[92]
TFB1M	Transcription, translation	[54, 93]
mTERF	Transcription, replication	[94]
Twinkle	Replication	[95]
Supv3L1	RNA stability	[96]
Lon	Quality control of mtDNA, TFAM degradation	[71, 97]
ClpX	Distribution of mtDNA, quality control of TFAM	[77]
Top1mt	Replication	[98]
LRP130	Transcription, RNA stability	[99, 100]
PHB1/PHB2	Organization of nucleoids, copy number regulation, translation	[44, 74]
ATAD3A	Organization of nucleoids, segregation	[45, 101]

The Bogenhagen and Holt groups have comprehensively identified human mitochondrial nucleoid proteins using affinity-purification with TFAM, mtSSB, or HU, a bacterial histone-like DNA-binding protein [43–46]. Several proteins have been redundantly found in these reports and their fundamental roles in mtDNA metabolism have been suggested. Some proteins play key roles in the maintenance of mtDNA; including the transcription, replication, repair, translation, copy number regulation, distribution, and organization of the nucleoids. Thus, we summarized the major nucleoid proteins mainly included in the complex and their identified roles in mtDNA metabolism (Table 1). Beside these factors, other mitochondrial ribosome proteins and translation factors including elongation factor Tu (EFTu) have also been identified as interacting partners with nucleoids [43, 44, 46], which suggests the close interplay between mtDNA maintenance and its translation. The Holt group also reported that cytoskeletal proteins, such as the non-muscle type myosin heavy chain and β-actin, were included in nucleoids, and regulated their organization and distribution [47]. Because mtDNA segregation in budding yeast was previously shown to be closely related to actin [48], this cytoskeletal interaction may also be important for active mtDNA segregation in higher organisms. Furthermore, several repair proteins that mainly act in the nucleus have been shown to interact with mitochondrial nucleoids and perform DNA repair in mitochondria [49].

Among these proteins, TFAM appears to be an essential protein that plays a fundamental role in the regulation of mtDNA in germ cells. We then focused on the pleiotropic function of TFAM (Fig. 2) and its regulation (Fig. 3).

Overview of the mitochondrial function of TFAM

The best-characterized and most abundant nucleoid protein is TFAM. TFAM was initially identified as a mitochondrial transcription activator [50]. It contains two high mobility group (HMG)-box domains and directly binds to mtDNA through these domains. In addition, TFAM possesses a C-terminal tail abundant in basic amino acid residues. The C-terminal tail was originally identified as the domain required for the activation of transcription [51] and interacts with mitochondrial transcription factor B1/B2 (TFBM) [52]. The tail is also important for efficient binding of TFAM to mtDNA [53].

TFAM exhibits some specific DNA-binding properties within light and heavy strand promoters (LSP and HSP1) and stimulates the initiation of mitochondrial transcription with mitochondrial RNA polymerase (Polrmt) and TFBM [54]. Recent X-ray crystallographic analyses demonstrated that TFAM forced LSP DNA to undergo a U-turn structure, which allowed the C-terminal tail to approach the transcription initiation site [55, 56]. Since a short RNA molecule transcribed from the LSP (RNA primer) is required for genome replication, transcription activation by TFAM appears to initiate replication and increase the mtDNA copy number. Thus, the specific DNA-binding of TFAM around the promoter regions regulates mitochondrial transcription and replication (Fig. 2).

TFAM also exhibits non-specific DNA binding activity and completely wraps mtDNA [57]. This non-specific DNA binding allows mtDNA packaging and compaction [58]. Single TFAM proteins were recently shown to diffuse extensively on DNA and the compaction of DNA was caused by the local denaturation of DNA [59]. The TFAM-
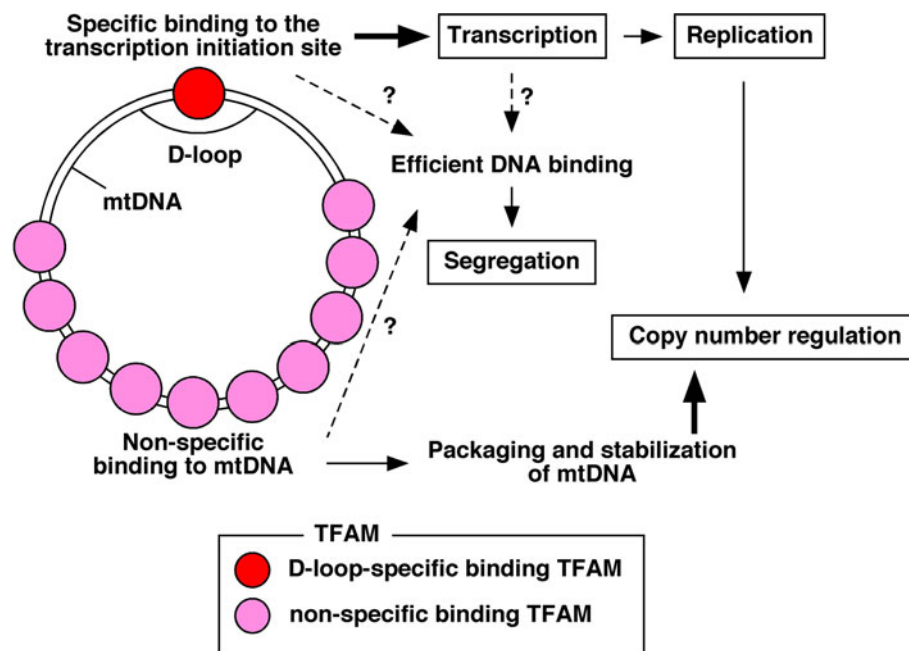
Fig. 2 Pleiotropic mtDNA regulation by TFAM. The functions of TFAM are summarized from the point of view of its DNA-binding characteristic. Specific DNA-binding activity of TFAM (*red circle*) around the transcription initiation site within the D-loop is involved in the transcription of mtDNA (*double open circles*) and resultant activation of replication. Non-specific DNA-binding activity of TFAM (*pink circle*) enables mtDNA packaging and stabilization and regulates the copy number. Although it is unknown which DNA-binding property is essential for this regulation, efficient DNA-binding through the C-terminal tail is required for the segregation and distribution of mtDNA by TFAM
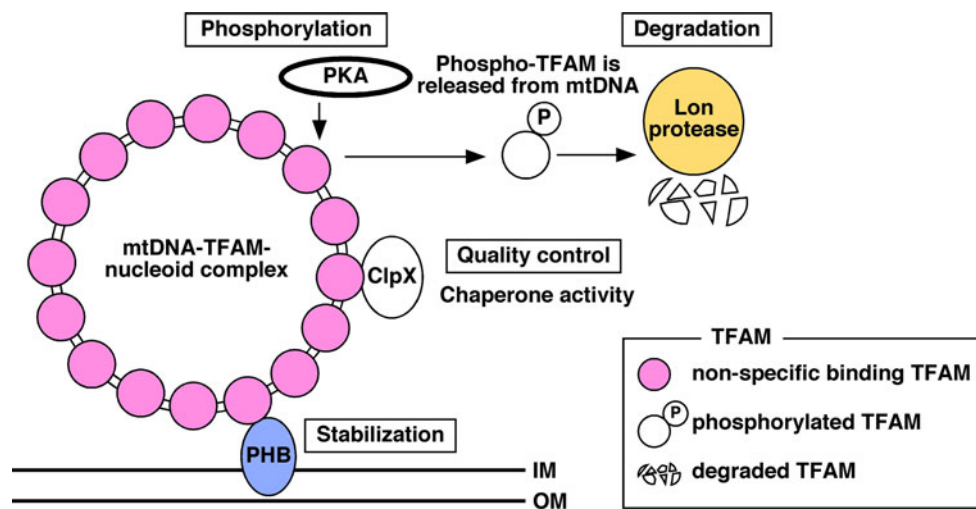
Fig. 3 Post-translational regulation of TFAM. The amount and quality of the TFAM protein are regulated by various factors. Possible regulation pathways are shown. TFAM and mtDNA are represented as a *pink circle* and *double open circle*, respectively, as described in the legend of Fig. 2. After PKA phosphorylates the TFAM bound to mtDNA, it is released from mtDNA, and the free TFAM is specifically degraded by Lon protease. Prohibitin (PHB) proteins maintain the stability of TFAM and anchor the nucleoids to the inner membrane (IM). Outer membrane; OM. ClpX regulates the DNA-binding activity of TFAM in vitro, suggesting the quality control of TFAM by the chaperone activity

mtDNA ratio is important for TFAM function; low levels of TFAM allow replication, while high levels are associated with repressed transcription [60]. Thus, TFAM appears to dynamically regulate mtDNA organization. TFAM has been shown to be essential for maintaining the mtDNA copy number in knockdown and knockout studies [61–64]. The exogenous expression of TFAM also increased the mtDNA copy number, indicating that TFAM is a dose-limiting factor for mtDNA. The C-terminal deletion of TFAM, which has reduced transcription activity

in vitro, can maintain the mtDNA copy number [62], which suggests that transcription and copy number maintenance are independently regulated. These results support the finding that the non-specific DNA binding activity of TFAM mainly regulates the mtDNA copy number (Fig. 2). mtDNA titration by TFAM has been suggested as a regulation model, in which TFAM and mtDNA stabilize each other through binding and formation of the nucleoid structure [65].

Besides the above functions, we recently demonstrated that human TFAM was required for the equal distribution and symmetric segregation of mtDNA in cultured cells [66]. Enlarged mtDNA nucleoids have been observed in TFAM-knockdown HeLa cells, and these cells showed the asymmetric segregation of mtDNA nucleoids between dividing cells. The overexpression of TFAM in mice resulted in enlarged mtDNA nucleoids in some tissues, suggesting its role in the segregation of mtDNA [67]. Down-regulation of the functions of TFAM may be the cause of this phenotype. Although the molecular mechanism underlying this regulation is largely unknown, our complementation assay demonstrated that the C-terminal domain was required for the appropriate distribution of mtDNA. The requirement of the C-terminal tail suggests that mtDNA segregation is coupled with transcription, but not with copy number regulation. However, it remains unclear whether the specific binding of TFAM to the promoter region is essential for the regulation of segregation. The C-terminal tail enhances general DNA-binding activity [53] and also mediates the cooperative formation of the TFAM dimer on DNA [68]. Thus, the efficient and cooperative DNA binding of TFAM may be important for mtDNA segregation (Fig. 2).

Factors regulating the amount and activity of TFAM

As described above, TFAM is a key factor for mtDNA maintenance. Thus, it is important to understand how the amount and function of TFAM are regulated. TFAM expression at transcription levels is regulated by transcription factors involved in mitochondrial biogenesis, such as PGC-1α and NRF-1 [69]. Some of the factors regulating the amount and function of TFAM at the posttranslational level have been identified in recent studies.

Matsushima et al. [70] initially reported that Lon protease, which is also included in nucleoids, degraded TFAM in drosophila cells. Lon knockdown increased TFAM protein levels and the amount of mtDNA, whereas Lon overexpression had the opposite effect [70]. Although the trigger for Lon-mediated TFAM degradation was unknown, the Suzuki group very recently reported that PKA-mediated phosphorylation of the N-terminal HMG domain of TFAM induced Lon-mediated degradation in human cells [71]. PKA phosphorylates TFAM in mitochondria, leading to the dissociation of TFAM from DNA and resultant degradation by Lon. Thus, Lon selectively degrades phosphorylated TFAM and regulates the amount of TFAM in accordance with cellular signaling (Fig. 3).

Prohibitin proteins (PHB1 and PHB2) have been shown to regulate pleiotropic functions in mitochondria including apoptosis and mitochondrial morphology through OPA1 stabilization, a mitochondrial fusion factor [72, 73]. We also reported that PHB1 was required for the organization and stability of mitochondrial nucleoids [74]. PHB1 knockdown resulted in a reduction in TFAM protein levels and the amount of mtDNA in cultured cells [74]. Because PHBs are membrane-anchored molecular chaperons and protein stabilizers [75], TFAM may be stabilized and anchored to the inner membrane by PHBs (Fig. 3). In support of mtDNA copy number regulation by PHBs, the conditional knockout of PHB2 reduced the mtDNA copy number in vivo [76]. Interestingly, PHB1 is known to be ubiquitinated in sperm cells [26]. The modification of PHB1 may be involved in the down-regulation of TFAM and mtDNA copy number during spermatogenesis.

We recently found that mitochondrial AAA + protein ClpX was required for the equal distribution of mtDNA nucleoids in cultured cells [77]. Enlarged mtDNA nucleoids, which are very similar and related to those in TFAM-knockdown cells, have been observed in ClpX-knockdown cells. ClpX knockdown did not significantly alter TFAM protein levels or the mtDNA copy number, indicating that ClpX has small effect on TFAM levels. However, TFAM overexpression suppressed the enlarged mtDNA nucleoids caused by ClpX knockdown, suggesting that TFAM and ClpX regulate the distribution of mtDNA in the same pathway. In support of this regulation, TFAM and ClpX were shown to closely interact in mitochondria. We also found that ClpX enhanced the DNA-binding activity of TFAM in vitro [77], suggesting that ClpX regulates TFAM activity in vivo. Although the molecular mechanism is still largely unknown, ClpX appears to regulate the distribution of mtDNA through quality control of TFAM as a chaperone (Fig. 3).

Visualization of mitochondrial genome/nucleoids in germ cells

Visualizing mtDNA/nucleoids in germ cells is needed to detect and understand mtDNA dynamics in these cells. To date, several mtDNA labeling methods have been reported as follows. Immunolabeling using an anti-DNA antibody [78] or fluorescent in situ hybridization (FISH) [79] is commonly selected in fixed cells. In addition, several
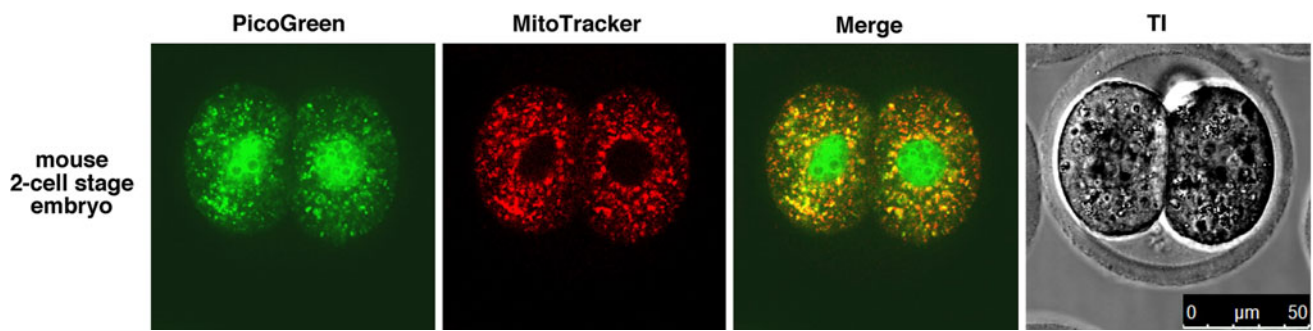
Fig. 4 Visualization of mtDNA nucleoids in mouse embryo. mtDNA nucleoids in a 2-cell stage mouse embryo were visualized by staining with PicoGreen. The embryo was incubated in the M2 medium supplemented with PicoGreen at 1:200 dilutions for 1 h. The embryo was also stained with MitoTracker (500 nM). A merged image (Merge) and transmission image (TI) are also shown. *Scale bar* 50 μm

fluorescent DNA intercalators, such as 4′,6′-diamidino-2-phenylindole (DAPI) [80], SYTO13 [81], ethidium bromide [78, 82], SYBR Green I [83, 84], PicoGreen [85], and ditercalinium chloride [86], have been shown to label mtDNA nucleoids in living cultured cells or organisms. These fluorescent dyes are useful in a multistaining procedure because they have different excitation and emission spectrums. PicoGreen and SYBR Green I enabled the visualization of mtDNA nucleoids in mitochondria stained with MitoTracker in drosophila [19] and *O. latipes* spermatids [18], respectively. Because fluorescent intensity correlates well with the actual amount of mtDNA, it is possible to chase the amount of mtDNA and its dynamics in living cells using these fluorescent dyes. We succeeded in labeling mtDNA in mouse fertilized eggs with Pico-Green staining (Fig. 4). This shows that mtDNA nucleoids are almost equally segregated into dividing egg cells, and indicates that the method is useful for investigating mtDNA dynamics such as segregation during embryogenesis.

An alternative method to label mtDNA nucleoids uses fluorescent proteins such as GFP. A core nucleoid factor Twinkle was previously fused to GFP, and the fusion protein was shown to closely co-localize with mtDNA [87]. Using time-lapse observations of the protein, this study showed that mtDNA nucleoids were not static, but mobile, which was also observed in ethidium labeling of nucleoids [78]. We recently identified the *C. elegans* TFAM ortholog HMG5, and showed that it was included in mitochondrial nucleoids and bound mtDNA in human cells [88]. We also reported that the mtSSB-GFP fusion protein showed close co-localization with mtDNA in human cells (our unpublished data). Thus, these nucleoid proteins may be useful for labeling mtDNA nucleoids in living cells. Taken together, these mtDNA-binding proteins are potential tools for visualizing mtDNA nucleoids in vivo.

Conclusion

In germ cells, the content and quality of mtDNA are appropriately regulated by characteristic mechanisms, paternal mtDNA degradation, and the selection of transmitted units by the mtDNA bottleneck. As described above, mitochondrial nucleoid proteins play an essential role in mtDNA metabolism and may be involved in this regulation. TFAM, in particular, regulates the copy number and segregation of mtDNA, making it a key factor in these processes. To further understand these molecular mechanisms, germ-line specific TFAM knockdown or knockout studies in combination with improved mtDNA visualization techniques will be important and are required.

Conflict of interest The authors declare no conflict of interest.

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