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REVIEW

SUBJECT COLLECTION: MITOCHONDRIA

The role of mitochondrial dynamics in mtDNA maintenance

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ABSTRACT

The dynamic nature of mitochondria, which can fuse, divide and move throughout the cell, allows these critical organelles to adapt their function in response to cellular demands, and is also important for regulating mitochondrial DNA (mtDNA). While it is established that impairments in mitochondrial fusion and fission impact the mitochondrial genome and can lead to mtDNA depletion, abnormal nucleoid organization or accumulation of deletions, it is not entirely clear how or why remodeling mitochondrial network morphology affects mtDNA. Here, we focus on recent advances in our understanding of how mitochondrial dynamics contribute to the regulation of mtDNA and discuss links to human disease.

KEY WORDS: Fission, Fusion, Mitochondria, Mitochondrial dynamics, Mitophagy, mtDNA

Introduction

Mitochondria are dynamic organelles best known for their role in producing energy via oxidative phosphorylation, but which are also important mediators of other cellular functions and processes, such as metabolism, innate immunity and cell death (Nunnari and Suomalainen, 2012). These organelles comprise an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), which define two distinct compartments, the intermembrane space (IMS) and the matrix (Fig. 1, diagram 1). Rather than bean-shaped structures depicted in textbooks, human mitochondria form a constantly changing network as they fuse (Fig. 1, diagrams 2 and 3), divide (Fig. 1, diagrams 4 and 5), and move throughout the cell (Sabouny and Shutt, 2020). Additionally, mitochondria can be degraded by mitochondrial autophagy (mitophagy) (Fig. 1, diagram 6) (Onishi et al., 2021), form vesicles (Sugiura et al., 2014), and interact both transiently and stably with a variety of other organelles (Lackner, 2019). The term 'mitochondrial dynamics' is often used to encompass many of these processes.

A critical aspect of the dynamic mitochondrial network is how it impacts the mitochondrial genome (mtDNA), which encodes proteins that are essential for oxidative phosphorylation (Chapman et al., 2020). Despite being sequenced over 40 years ago (Anderson et al., 1981), there is still much to learn about mtDNA and how it is regulated. For example, even though each cell typically contains ∼100–1000 copies of the mtDNA, we still do not completely understand how mtDNA copy number is determined and maintained, nor how mtDNA is distributed throughout the everchanging mitochondrial network. In this Review, we examine what is known about how mitochondrial fission and fusion impact mtDNA in these respects, as well as cover the implications for mtDNA mutations and human disease. As previous reviews have extensively covered mitochondrial dynamics (Chan, 2019) and mtDNA replication (Gustafsson et al., 2016), here we will only briefly review these key aspects to set the stage for how these topics are related.

The dynamic mitochondrial network

The morphology of the mitochondrial network, which can vary from many small individual puncta to large, reticulated networks, is determined by the balance between fission and fusion events (Fig. 1). Changes in network morphology have important implications for mitochondrial function beyond their impact on the mtDNA. For example, the physical structure of mitochondria can influence biophysical properties (e.g. surface:volume ratio), affecting mitochondrial physiology or interactions with other organelles (Glancy et al., 2020). Meanwhile, fusion and fission events, which are often coordinated (Abrisch et al., 2020; Twig et al., 2008), allow for movement of mitochondria, content mixing and isolation of damaged mitochondria via mitophagy (Chan, 2012). It is also worth noting that defects in fusion and fission perturb many aspects of mitochondrial function, such as oxidative phosphorylation (Chen and Chan, 2010) and apoptosis (Karbowski and Youle, 2003).

Mitochondrial fission is orchestrated by a sequence of events that is initiated at sites where the endoplasmic reticulum (ER) wraps around mitochondrial tubules (Fig. 1, diagram 4) (Friedman et al., 2011). The actomyosin cytoskeleton at these contact sites provides the force to constrict mitochondrial tubules, initiating fission (Moore and Holzbaur, 2018). Next, the dynamin-related GTPase DRP1 (also known as DNM1L) can be recruited from the cytosol by several outer mitochondrial membrane (OMM) adaptor proteins, including MFF, MID49 (also known as MIEF2), MID51 (also known as MIEF1) and FIS1 (Kraus et al., 2021; Loson et al., 2013) (Fig. 1, diagram 5). Once on the OMM, DRP1 assembles into oligomeric rings around mitochondria that mediate scission through GTP hydrolysis and conformational changes.

Mitochondrial fusion begins with tethering and fusion of the OMM, processes mediated by the mitofusin GTPases MFN1 and MFN2 (Fig. 1, diagram 2) (Chen et al., 2003). Next, inner mitochondrial membrane (IMM) fusion is mediated by the GTPase activity of optic atrophy 1 (OPA1) (Fig. 1, diagram 3). Notably, OPA1 interacts with mitochondrial-specific lipid cardiolipin in the IMM to drive inner membrane fusion (Ban et al., 2017, 2018). While the GTPases MFN1, MFN2 and OPA1 constitute the core fusion machinery, recent studies have highlighted additional cytosolic and mitochondrial proteins regulating fusion. Notable among these factors are the cytosolic protein MSTO1

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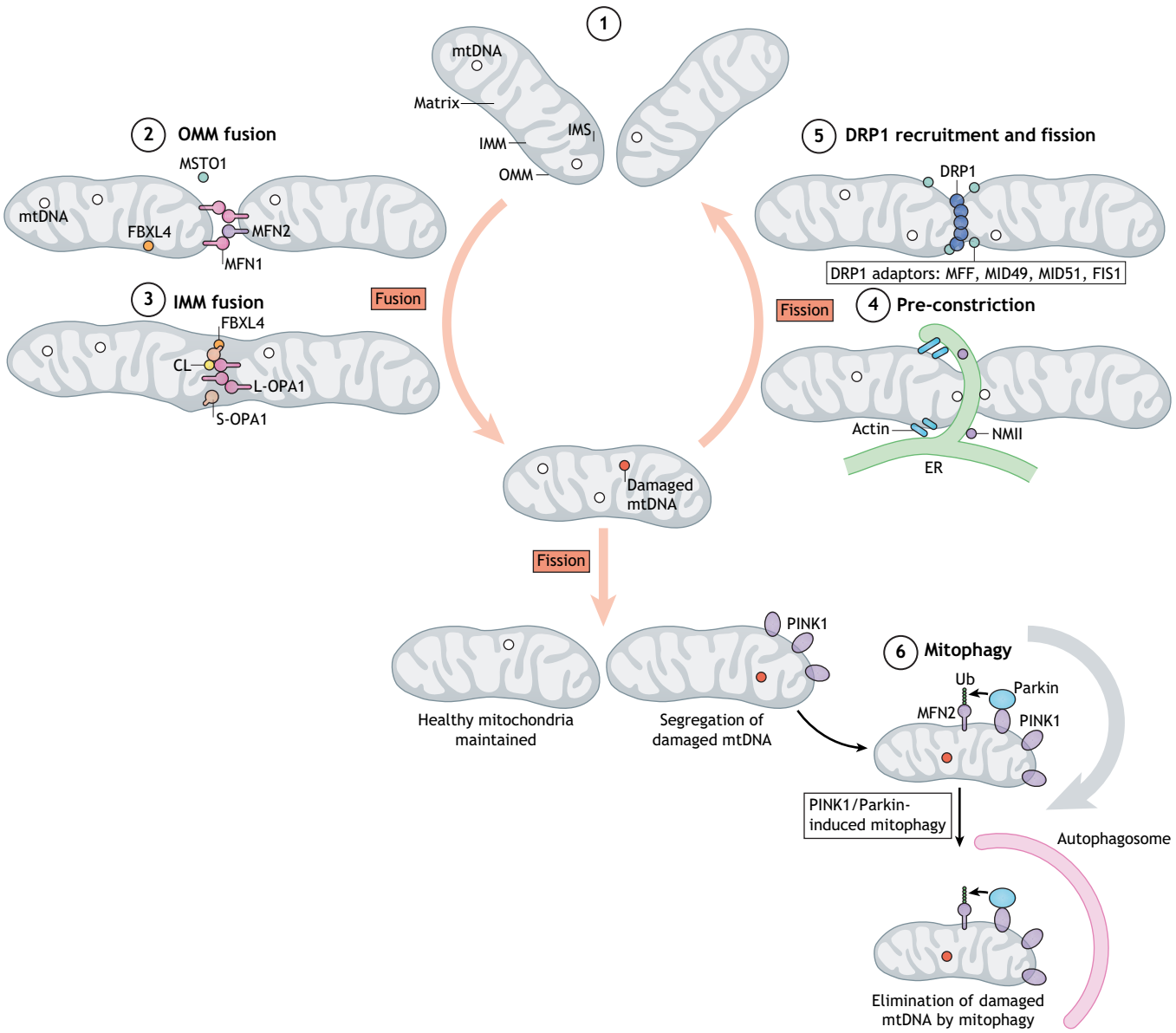

Fig. 1. Schematic representation of dynamic mitochondrial processes. Top, the reciprocal processes of mitochondrial fission and fusion. (1) Diagram showing the outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), intermembrane space (IMS), matrix, and mitochondrial DNA (mtDNA). (2) Mitochondrial fusion begins with tethering and fusion of the OMM, which is mediated by the OMM GTPases mitofusin 1 and 2 (MFN1 and MFN2) that can interact in a homotypic or heterotypic manner. The cytosolic protein MSTO1 mediates fusion through an undefined mechanism. (3) IMM fusion is mediated by the GTPase activity of optic atrophy protein 1 (OPA1), which is present in long and short isoforms (L-OPA1 and S-OPA1, respectively) due to proteolytic processing of multiple splice isoforms. L-OPA1 interacts with the inner membrane lipid cardiolipin (CL) to mediate inner membrane fusion. Notably, the F-box protein FBXL4, proposed to localize to the IMS, also regulates mitochondrial fusion by unknown means. (4) Mitochondrial fission begins with endoplasmic reticulum (ER)-mediated constriction of mitochondria, which is mediated by actin and non-muscle myosins (NMII). (5) The main fission GTPase, dynamin-related protein 1 (DRP1), is recruited to fission sites via the OMM adaptor proteins MFF, MID49, MID51 and FIS1. GTP hydrolysis and conformational change induces final OMM scission and gives rise to mitochondrial fragments. Bottom, following fission events near the ends of mitochondrial tubules, smaller mitochondrial fragments enriched in damaged components can undergo mitophagy. This process allows the eliminate of dysfunctional mitochondria from the cell, which may be due to damaged or mutant mtDNA (depicted in red). (6) Dysfunctional mitochondria accumulate the kinase PINK1 on the OMM, which recruits the E3 ubiquitin ligase Parkin leading to the ubiquitylation (Ub) of OMM proteins, including MFN2, triggering autophagosome formation.

(Donkervoort et al., 2019) and the mitochondrial protein FBXL4 (Sabouny et al., 2019), mutations in which lead to depletion of the mtDNA genome.

Mitophagy, the clearance of damaged, dysfunctional or unwanted mitochondria by a specific form of autophagy, is tightly coordinated with the remodeling of mitochondrial networks (Fig. 1, diagram 6). For example, inhibiting fission results in hyperfused mitochondrial networks that accumulate damage (Parone et al., 2008) and are resistant to mitophagy (Gomes et al., 2011; Rambold et al., 2011). Thus, fission is believed to be required to form the initial mitochondrial fragments that are small enough to be removed by mitophagy. Meanwhile, the mitochondrial fusion protein MFN2 can also play a role in signaling mitophagy (Chen and Dorn, 2013). The molecular mechanisms regulating mitophagy include the well-characterized ubiquitin-dependent pathway mediated by the mitochondrial kinase PINK1 and the cytosolic E3 ubiquitin ligase

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Box 1. Mitochondrial dynamics and disease

The importance of mitochondrial dynamics is evidenced by the fact that pathogenic variants leading to a variety of human disease phenotypes are found in genes encoding several fusion, fission and mitophagy proteins, as well as the fact that mitochondrial dynamics are impaired in many disease models (Chan, 2019). The first indication that mitochondrial fusion is relevant to human disease was the recognition that pathogenic OPA1 variants cause dominant optic atrophy, a neuro-ophthalmic condition characterized by a bilateral degeneration of the optic nerves (Alexander et al., 2000). Shortly thereafter, pathogenic variants in MFN2 were recognized to cause Charcot–Marie–Tooth disease, a progressive motor and sensory neuropathy of the peripheral nervous system (Zuchner et al., 2004). Subsequently, the first report of a pathogenic variant in DRP1 found in a patient with neuronal issues and neonatal lethality also linked fission defects to human disease (Waterham et al., 2007). More recently, additional pathogenic variants have been found in a growing list of proteins involved in both fusion [e.g. MSTO1 (Gal et al., 2017; Nasca et al., 2017) and FBXL4 (Bonnen et al., 2013; Gai et al., 2013)] and fission [e.g. MFF (Koch et al., 2016), MID49 (Bartsakoulia et al., 2018) and NMIIC (Almutawa et al., 2019)]. Similarly, impaired mitophagy is linked to human disease, with the best example being the recognition that pathogenic variants of PINK1 and Parkin cause Parkinson's disease (Onishi et al., 2021). Importantly, as approaches to restore these processes are increasingly recognized to be beneficial in a growing list of human disease models (Whitley et al., 2019), it is crucial to understand how mitochondrial dynamics are regulated, and how their dysfunction leads to disease. Critically, disturbances to mtDNA are a common feature in several diseases arising from impairments in mitochondrial fusion, fission and mitophagy, including accumulation of mtDNA mutations, as well as alterations in mtDNA abundance and nucleoid distribution (Table 1).

Parkin (PRKN) (Pickles et al., 2018). While additional mitophagy pathways exist (Onishi et al., 2021), there is still much work required to understand exactly how these pathways work together or independently to maintain mitochondrial quality control and abundance under different contexts.

The importance of these dynamic processes is highlighted by the growing list of diseases caused by their impairment (Box 1), while the impact of fusion and fission on mtDNA are further highlighted by loss-of-function studies (Table 1). Although the link between maintenance of the mitochondrial genome and mitochondrial dynamics is an intriguing facet of fusion and fission dynamics, there remain many unanswered questions. However, before we examine recent advances in our understanding on how mitochondrial dynamics influence mtDNA, we will first review some basics of the mitochondrial genome.

mtDNA maintenance and organization

The circular 16,569 bp human mitochondrial genome is replicated independently of the nuclear genome, and encodes 37 genes, including 13 proteins that are essential for oxidative phosphorylation. Replication, maintenance and expression of the multicopy mtDNA depends on nuclear-encoded proteins that are imported into the mitochondrial matrix. The core mtDNA replication machinery comprises the mitochondrial polymerase γ (POLG), the mitochondrial DNA helicase (Twinkle; TWNK) and the mitochondrial single-stranded DNA-binding protein (mtSSB; also known as SSBP1) (Gustafsson et al., 2016), which work in concert with several additional proteins. Importantly, components of the mitochondrial replication machinery need to be balanced stoichiometrically, as imbalance can lead to mtDNA depletion and deletions (Phillips et al., 2017). Replication of mitochondrial genomes also requires a balanced pool of intra-mitochondrial deoxyribonucleotide triphosphates (dNTPs) (Gorman et al., 2016).

The mtDNA copy number varies in different cell types, probably to help mitochondria meet differing energetic demands. However, the processes that regulate copy number are not completely understood. Nevertheless, the importance of maintaining sufficient copies of the mtDNA genome is evidenced by a class of mitochondrial diseases termed mitochondrial DNA depletion syndromes (MTDPS), which are characterized by a significant reduction of mtDNA levels in affected tissues (Viscomi and Zeviani, 2017). Notably, MTDPS arise from mutations in nuclear genes encoding factors important for mtDNA replication, maintenance of mtDNA nucleotide pools or mitochondrial dynamics (Table 1). Although it is not surprising that defects in mtDNA replication or imbalances in nucleotides would lead to mtDNA loss, exactly how fusion–fission dynamics affect mtDNA levels is not fully understood.

Another way through which mtDNA impairment can lead to dysfunction is through the acquisition of point mutations, as well as both small- and large-scale deletions or duplications. Critically, mtDNA mutations can and do accumulate to a point where they negatively impact mitochondrial function, and lead to diseases such as mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonus epilepsy with ragged-red fibers (MERFF) and Leigh syndrome (Stewart and Chinnery, 2015). A mixture of wild-type and mutant mtDNA is referred to as heteroplasmy, while the term homoplasmy refers to a situation where all mtDNA copies in the cell are identical (either wild-type or mutant). As part of the mitochondrial genome, deleterious mtDNA variants are inherited maternally. Additionally, mtDNA mutations arise *de novo* in somatic cells, and their accumulation has been proposed to contribute to both neurodegenerative conditions (Keogh and Chinnery, 2015) and cancers (Yuan et al., 2020). The level of heteroplasmy required to meet a pathogenic threshold varies depending on the specific mutation, the affected tissue and the genetic background of an individual. Generally, increased levels of mutant mtDNA correspond to worse disease phenotypes. Whereas heteroplasmy levels can vary significantly between cells in the same individual, the factors and mechanisms leading to selective expansion of mutant mtDNA molecules are not entirely clear, although, as discussed later, mitochondrial dynamics have been implicated. Nevertheless, as heteroplasmy levels can change, approaches such as using targeted nucleases (Zekonyte et al., 2020) or editing enzymes (Mok et al., 2020) to reduce the abundance of mtDNA mutations offer promise for mtDNA disease patients, although they are still not yet applicable clinically (Al Khatib and Shutt, 2019).

The mtDNA is organized into nucleoprotein structures known as nucleoids, which are localized in the mitochondrial matrix (Gustafsson et al., 2016). Nucleoids are tethered to the IMM, which facilitates their movement and distribution within the mitochondrial network (Nicholls and Gustafsson, 2018). Although early work estimated that nucleoids contained approximately six to ten mtDNA copies (Iborra et al., 2004), more recent high-resolution microscopy studies show that nucleoid are ∼100 nm in diameter and estimated to contain 1.4 copies of mtDNA on average, reflecting the ongoing replication of mitochondrial genomes (Kukat et al., 2011). The fact that individual nucleoids are comprised of a single mtDNA genome supports earlier work showing that nucleoids do not exchange genomes (Gilkerson et al., 2008). However, despite this fact, cells with a mixture of distinct mtDNA mutations can have normal mitochondrial function owing to fusion-dependent content mixing and trans-complementation (Schon and Gilkerson, 2010). Although nucleoids are typically distributed evenly throughout the

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Table 1. Summary of reports linking impairment of mitochondrial fusion and fission proteins to mtDNA abnormalities and patient phenotypes.

		mtDNA abnormalities				
Gene (Protein)	Model used	mtDNA deletions	mtDNA depletion	Altered nucleoid distribution	Patient Phenotype	References
Fusion Proteins						
MFN1 (MFN1)	Knockout MEFs	NR	Y	Y	–	Chen et al., 2007
MFN2 (MFN2)	p.Q74R, p.V226_S229del, p.M376V, p.R707P	Y	Y	NR	CMT2A	Vielhaber et al., 2013
	p.D210V	Y	N	NR	Optic atrophy 'plus' (axonal neuropathy, mitochondrial myopathy)	Rouzier et al., 2012
	p.D210Y	N	Y	NR	Hypotonia, ataxia, axonal neuropathy, optic atrophy, hearing loss	Renaldo et al., 2012
	Knockout MEFs	NR	Y	Y	–	Chen et al., 2007
MFN1 and *MFN2* (MFN1 and MFN2)	Knockout MEFs	NR	Y	Y	–	Chen et al., 2007; Silva Ramos et al., 2019
	Conditional double knockout mice	Y (skeletal muscle) N (cardiac muscle)	Y	NR	–	Chen et al., 2010; Silva Ramos et al., 2019
OPA1 (OPA1)	p.G439V, p.V910D, p.R455H, p.S545R, p.A357T	Y	N	NR	DOA 'plus' (sensorineural deafness, ataxia, myopathy, CPEO)	Amati-Bonneau et al., 2008; Hudson et al., 2008
	p.L534R	N	Y	NR	Encephalomyopathy, optic atrophy, hypertrophic cardiomyopathy	Spiegel et al., 2016
	Knockout MEFs	NR	Y	Y	–	Chen et al., 2007
MSTO1 (MSTO1)	Most variants examined to date	NR	Y	Y	Myopathy, ataxia, muscular dystrophy	Donkervoort et al., 2019; Gal et al., 2017; Iwama et al., 2018; Nasca et al., 2017
FBXL4 (FBXL4)	Most variants examined to date	N	Y	Y	Encephalomyopathy, lactic acidemia, cardiac hypertrophy	Antoun et al., 2015; Ballout et al., 2019; Baroy et al., 2016; Bonnen et al., 2013; Dai et al., 2017; Ebrahimi-Fakhari et al., 2015; El-Hattab et al., 2017; Gai et al., 2013; Huemer et al., 2015; Morton et al., 2017; Sabouny et al., 2019
Fission proteins						
DNM1L (DRP1)	Multiple pathogenic variants	NR	NR	NR	Infantile lethality, encephalopathy, epilepsy, optic atrophy, microcephaly, lactic acidosis	Fahrner et al., 2016; Nasca et al., 2016; Vanstone et al., 2016
	siRNA knockdown	NR	N	Y	–	Ban-Ishihara et al., 2013
	shRNA knockdown	NR	Y	Y	–	Parone et al., 2008
MFF (MFF)	p.R298*	N	NR	NR	Epileptic encephalopathy, optic atrophy, peripheral neuropathy, microcephaly	Koch et al., 2016; Nasca et al., 2018
	siRNA knockdown	NR	N	Y	–	Ban-Ishihara et al., 2013
MIEF2 (MID49)	p.Q92*	N	N	NR	Myopathy	Bartsakoulia et al., 2018
MYH10 (NMIIB)	Knockout MEFs	NR	N	Y	–	Reyes et al., 2011
	siRNA knockdown	NR	N	N	–	Reyes et al., 2011
MYH14 (NMIIC)	p.R941L	N	N	Y	Peripheral neuropathy hearing loss, hoarseness	Almutawa et al., 2019; Choi et al., 2011

The list of pathogenic mutations is certainly not exhaustive; only pathogenic mutations with a clear link to mtDNA perturbations are outlined. Y, yes (denoting detection of mtDNA deletions, mtDNA loss or altered nucleoid distribution); N, No (denoting absence of mtDNA perturbation phenotype); NR, not reported. CMT2A, Charcot–Marie–Tooth disease type 2A; DOA, dominant optic atrophy; het, heterozygous; cPEO, chronic progressive external ophthalmoplegia; MEFs, mouse embryonic fibroblasts. Underlined mutations are homozygous, all other reported mutations were heterozygous.

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Box 2. mtDNA nucleoids

A number of different approaches have been developed to visualize mtDNA nucleoids (Prole et al., 2020), revealing a typical organization whereby uniformly sized nucleoids are distributed evenly throughout mitochondrial networks, regardless of whether they are elongated or fragmented (Chen et al., 2007). However, altered nucleoid distribution with enlarged nucleoids is reported in response to a variety of mitochondrial stresses. Notably, super-resolution microscopy has confirmed that in some cases these enlarged nucleoids, sometimes called 'mito-bulbs', are clusters of individual nucleoids (Ban-Ishihara et al., 2013; Nasonovs et al., 2021). Here, we will use the generic term enlarged nucleoid, as most studies to date only report such enlarged nucleoids under normal microscopy where it has not been confirmed that these are indeed clusters. Various mitochondrial stresses linked to enlarged nucleoids include those induced by drugs that intercalate into mtDNA (Alán et al., 2016; Ashley and Poulton, 2009), inhibit oxidative phosphorylation (Tauber et al., 2013) and uncouple mitochondrial membrane potential (Tauber et al., 2013), as well as upon genetic modifications that target several mitochondrial proteins (Dalla Rosa et al., 2014). Notably, these targets include the mtDNA-binding protein TFAM (transcription factor A, mitochondrial) (Kasashima et al., 2011), which induces a U-turn in the DNA structure and mediates compaction of mtDNA into nucleoids (Kukat and Larsson, 2013). As an aside, TFAM can spontaneously phase separate *in vitro*, which contributes to the recently described phase separation properties of mtDNA nucleoids (Feric et al., 2021). Highlighting their relevance to human heath, enlarged nucleoids are also reported in response to viral infection (West et al., 2015) and in several disease models, including mtDNA mutations (Newell et al., 2018), fibrotic lung disease (Ryu et al., 2017) and Hutchinson–Guilford progeria (Feric et al., 2021). Although it is not clear why enlarged nucleoids occur in the cases listed above, as discussed in the main text, mitochondrial fusion and fission are recognized to be important mediators of nucleoid size and distribution (Fig. 2).

mitochondrial network, this distribution can be disturbed by several perturbations, including impaired mitochondrial dynamics (Box 2) (Fig. 2).

Integrating mitochondrial dynamics and mtDNA

Nucleoid distribution

A link between mitochondrial dynamics and nucleoid distribution was first recognized during early studies of mitochondrial dynamics, with the observation that nucleoids were often located near fission and fusion sites (Iborra et al., 2004; Margineantu et al., 2002). Subsequently, abnormally enlarged nucleoid structures were observed upon disruption of the mitochondrial fission machinery (e.g. through perturbing DRP1 or MFF) (Ashley and Poulton, 2009; Ban-Ishihara et al., 2013; Parone et al., 2008). In the context of diseases with impaired mitochondrial fission, larger nucleoids are reported in fibroblasts with reduced fission due to pathogenic variants in *MYH14* and *DNM1L* (Almutawa et al., 2019; Ilamathi et al., 2021 preprint). Similarly, the abundance of nucleoids within extremely hyperfused regions of cells from patients with pathogenic variants in the fission protein DRP1 and non-muscle myosin IIC (NMIIC; heavy chain encoded by *MYH14*) are reduced (Almutawa et al., 2019; Ilamathi et al., 2021 preprint).

While enlarged nucleoids can be associated with mitochondrial dysfunction, they may not lead to dysfunction per se. For example, A549 human lung carcinoma cells contain enlarged nucleoid clusters that are functional with respect to transcription and replication competence (Nasonovs et al., 2021). Notably, the number of enlarged nucleoids in A549 cells increases in high-glucose conditions and decreases with TGFβ treatment,

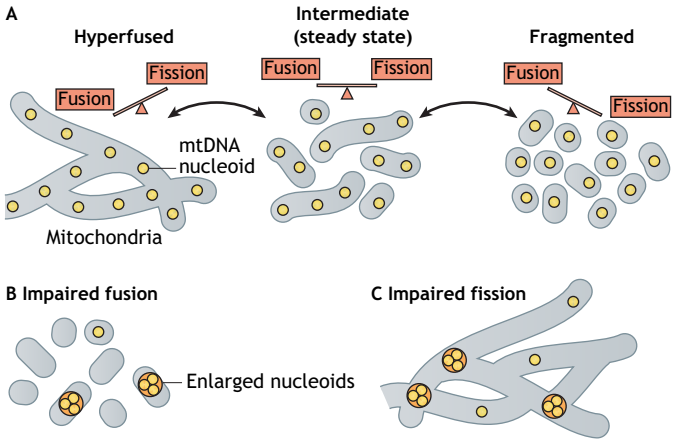

Fig. 2. Mitochondrial network remodeling and coordinated mtDNA nucleoid distribution following fusion and fission. (A) Mitochondrial morphology is determined by the balance between fusion and fission events (i.e. hyperfused, intermediate or fragmented). Mitochondria become hyperfused when there is more fusion than fission, or become fragmented when there is excess fission. Regardless of the mitochondrial network morphology, in healthy cells, mtDNA nucleoids are typically evenly distributed and copy number is maintained even upon normal shifting of mitochondrial morphology, which can be modulated in response to various physiological stimuli. (B) When mitochondrial fusion is impaired, this can lead to reduced mtDNA copy number, and can gives rise to small mitochondrial fragments that are either completely devoid of mtDNA nucleoids, or contain enlarged nucleoids that consist of clusters. (C) When mitochondrial fission is impaired, enlarged nucleoids can accumulate in hyperfused networks.

demonstrating that dynamic changes in nucleoid arrangement can occur in response to physiological cues (Nasonovs et al., 2021). Nevertheless, the question of why enlarged nucleoids form remains elusive. In this regard, recent work found that mtDNA replication is coordinated with fission events, as fission sites occur in close proximity to replicating nucleoids (Lewis et al., 2016). As such, fission has been proposed to be important for the segregation of nascent mtDNA molecules after replication (Fig. 3), which may explain the nucleoid clustering observed when fission is inhibited.

Impairments to mitochondrial fusion also impact the distribution of nucleoids throughout the mitochondrial network in two important ways. First, whereas mitochondria in normal cells typically each contain at least a single nucleoid, even when fragmented into smaller mitochondria, cells with impaired fusion can have mitochondrial fragments that do not contain nucleoids. Such an uneven distribution is reported in mouse embryonic fibroblast (MEF) cells lacking any one of the fusion proteins MFN1, MFN2 or OPA1 (Chen et al., 2007), as well as in fibroblasts from patients with pathogenic mutations in FBXL4 (Sabouny et al., 2019) and MSTO1 (Donkervoort et al., 2019). Second, upon impaired fusion, nucleoids appear larger, likely due to clustering of multiple genomes. Enlarged nucleoids are reported upon double knockout of MFN1 and MFN2 (Silva Ramos et al., 2019), as well as in cells with pathogenic variants of MSTO1 (Donkervoort et al., 2019) and FBXL4 (Sabouny et al., 2019). Intriguingly, there are conflicting reports about the role of OPA1 in enlarged nucleoids. Whereas siRNA-mediated knockdown of an alternatively spliced isoform of OPA1 (OPA1-exon4b) that is reported to bind mtDNA leads to enlarged nucleoids (Elachouri et al., 2011; Vidoni et al., 2013; Yang et al., 2020), OPA1-knockout MEFs lacking all isoforms have normal nucleoids (Ban-Ishihara et al., 2013; Silva Ramos et al., 2019). These contradictory findings suggest that an imbalance of the different OPA1 isoforms may be more detrimental

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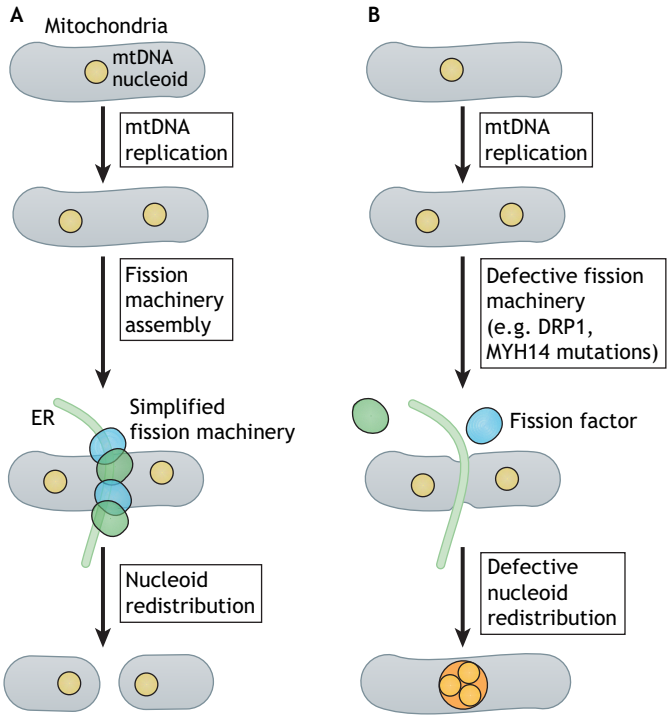

Fig. 3. Role of mitochondrial fission in the appropriate distribution of newly replicated mtDNA nucleoids. (A) Following mtDNA replication, mitochondrial fission factors assemble and divide mitochondria, segregating nucleoids into separate daughter mitochondria. (B) In cells with a defective fission machinery, mitochondria do not undergo fission after mtDNA replication, impairing the distribution of mtDNA nucleoids and giving rise to enlarged clusters of nucleoids. However, the exact underlying mechanisms are not well understood.

than their complete absence, or the possibility of cell-type-specific responses to loss of OPA1. However, it should be noted that we do not have a complete understanding of the physiological role played by the OPA1-exon4b isoform, whose proposed topology (Elachouri et al., 2011) does not agree with the majority of OPA1 research suggesting the N-terminus resides within the matrix.

Another potential link between fusion and nucleoid remodeling is the lipid cardiolipin, which can bind nucleoids (Luévano-Martínez et al., 2015), and has a direct role in mediating IMM fusion (Ban et al., 2017). Importantly, manipulation of enzymes regulating cardiolipin impacts mitochondrial morphology and mtDNA nucleoids (Ban et al., 2018; Huang et al., 2020; Li et al., 2012). One interesting observation is that knockdown of the Barth syndrome protein Tafazzin (Hauff and Hatch, 2006), an enzyme that regulates remodeling of cardiolipin, causes accumulation of large nucleoids and mtDNA depletion, but does not impair mitochondrial fusion (Ban et al., 2018). This finding suggests that subtle changes in cardiolipin might impact nucleoids independently of its effects on mitochondrial fusion.

It remains unclear how impaired fusion results in enlarged nucleoids. One possibility, given that fission and fusion events are often coordinated (Abrisch et al., 2020; Twig et al., 2008), is that cells with reduced rates of fusion also have reduced rates of fission, and that it is a secondary reduction in fission accounting for nucleoid clustering. Alternatively, it could be that the balance between fission and fusion events is critical for the proper distribution of nucleoids. With respect to this latter possibility, it is worth noting that enlarged nucleoids observed in DRP1-knockdown cells were suppressed when either OPA1 or both of MFN1 and MFN2 were also depleted, whereas MFN1 overexpression induced nucleoid enlargement (Ban-Ishihara et al., 2013), suggesting that balanced fission and fusion is important. Finally, whereas enlarged nucleoids and mtDNA depletion can occur in cells with both impaired fission or fusion, they do not always occur together (e.g. *OPA1* and *MYH14*). Another example of this separation of phenotypes is the fact that whereas treatment with the fission inhibitor M-divi1 rescues mtDNA copy number in cells of patients with pathogenic FBXL4 variants, some nucleoids remain enlarged (Sabouny et al., 2019). Thus, it does not appear that mtDNA depletion and enlarged nucleoids are necessarily caused by the same underlying mechanisms.

Fission and mtDNA replication

Although fission is clearly important for segregating mtDNA genomes to avoid enlarged nucleoids, it has also been proposed to play a role in initiating mtDNA replication. One idea that remains to be tested is that ER-mediated mitochondrial constriction initiates mtDNA replication (Lewis et al., 2016). However, it is not clear how the fission machinery, which is located outside mitochondria, would influence mtDNA in the matrix. Although a two-membrane spanning structure linked to replicating nucleoids has been described in yeast (Meeusen and Nunnari, 2003), similar machinery in mammalian cells is yet to be identified. In this regard, several factors worth discussing in the context of mtDNA replication and segregation include the mitochondrial contact site and cristae organizing system (MICOS) complex, which may provide a physical tether from across the OMM and IMM, IMM lipids, which physically interact with mtDNA, and the mitochondrial transport machinery, which is also implicated in nucleoid segregation.

The MICOS complex is involved in many functions, including lipid metabolism, protein import and maintaining mitochondrial architecture (Khosravi and Harner, 2020). Notably, this large multi-subunit structure links the IMM and the OMM, and is suggested to be a tether that could connect nucleoids to the exterior of the mitochondria (van der Laan et al., 2012). Disruption of the MICOS subunit Mic60 (also known as mitofilin, encoded by *IMMT*) leads to enlarged nucleoids, which appears to be partially independent of DRP1 (Li et al., 2016). Also supporting the notion of a role for the MICOS complex in mtDNA maintenance is the identification of pathogenic variants in the MICOS subunit MICOS13 (also known as QIl1) that lead to ∼90% mtDNA depletion (Kishita et al., 2020). Furthermore, pathogenic variants in CHCHD10, which regulates MICOS but is not a core subunit, lead to fewer but larger nucleoids (Genin et al., 2016), as well as mtDNA deletions (Bannwarth et al., 2014). Finally, loss of SAMM50, an OMM protein that interacts with MICOS, leads to an accumulation of large nucleoids (Jian et al., 2018). Thus, several lines of evidence demonstrate that the proper function of the MICOS complex is important for mtDNA maintenance.

The fact that IMM lipids are relevant to mtDNA maintenance should come as no surprise given that nucleoids interact with the IMM (Nicholls and Gustafsson, 2018). Two lipids in particular, cholesterol and cardiolipin, potentially link fission and mtDNA. Cardiolipin, which interacts physically with mtDNA and the MICOS complex (Friedman et al., 2015), can also localize to the OMM, where it interacts with DRP1 to promote mitochondrial fission (Mahajan et al., 2021). Meanwhile, despite its low mitochondrial abundance, cholesterol has also been implicated in mediating nucleoid interactions with the IMM (Gerhold et al., 2015). A role for cholesterol in mtDNA maintenance is supported

by the fact that pharmacological disruption of cholesterol homeostasis leads to the accumulation of large nucleoids (Desai et al., 2017). Furthermore, defects in the intracellular cholesterol transporter NPC1, which cause Niemann–Pick disease type C, result in enlarged nucleoids (Desai et al., 2017). Similarly, ATAD3A, a regulator of mitochondrial cholesterol import, is also important for mtDNA maintenance, as its deletion results in mtDNA depletion and accumulation of mtDNA deletions (Peralta et al., 2018). Moreover, pathogenic variants in ATAD3A lead to enlarged nucleoids (Desai et al., 2017). However, there are several ways in which ATAD3A could impact mtDNA in addition to affecting cholesterol, as ATAD3A is proposed to be a nucleoid-associated protein (Bogenhagen et al., 2008; He et al., 2007), and its loss also destabilizes the MICOS complex (Peralta et al., 2018). Finally, as the import of cholesterol into mitochondria is mediated by interactions between ER and mitochondria (Martin et al., 2016), it is tempting to speculate that this process is coordinated with fission initiation to promote cholesterol import. Such a coordination could favor a localized cholesterol accumulation in the IMM, perhaps creating a platform for replication that signals the initiation of mtDNA replication.

Another consideration for nucleoid segregation is the dedicated transport machinery that moves mitochondria about the cell via the cytoskeleton (Kruppa and Buss, 2021), components of which are associated with nucleoids. While early work showed that nucleoids localize near the KIF5B kinesin motor, which moves mitochondria along microtubules (Iborra et al., 2004), recent work also showed an enrichment of MIRO1, a mitochondrial OMM adaptor protein involved in transport, with the MICOS subunit MIC60 and nucleoids at mitochondria–ER contacts (Qin et al., 2020). Thus, it is possible that the mitochondrial transport machinery is tethered to mtDNA nucleoids via the MICOS complex, which might help to distribute mitochondria with newly synthesized nucleoids within the mitochondrial network following fission by physically separation.

Further arguing for a role of fission in initiating mtDNA replication, impaired fission can also cause reduced mtDNA copy number. For example, blocking fission leads to mtDNA depletion in several experimental models (e.g. genetic ablation of DRP1, MFF and MYH10) (Parone et al., 2008; Reyes et al., 2011). However, it is notable that a transient reduction in fission (e.g. siRNA-mediated knockdown of DRP1 or MFF) does not lead to mtDNA depletion, despite leading to nucleoid clustering (Ban-Ishihara et al., 2013). These discrepancies could be explained if a more severe inhibition of fission is required to induce loss of mtDNA, or if nucleoid clustering precedes mtDNA depletion. Nevertheless, it seems that nucleoid clustering and mtDNA depletion are not necessarily linked.

Fusion and mtDNA replication

One of the earliest connections between mitochondrial network remodeling and mtDNA copy number dynamics was noted in yeast, where disruption of the OPA1 and MFN homologs, Mgm1 and Fzo1, respectively, led to a complete loss of mtDNA (Jones and Fangman, 1992; Rapaport et al., 1998). This observation has since been extended to mammalian models of impaired mitochondrial fusion, as MEFs lacking either OPA1 or both MFN1 and MFN2 have a lower mtDNA copy number (Chen et al., 2007; Silva Ramos et al., 2019). Likewise, mice in which these mitochondrial fusion proteins are conditionally knocked out also exhibit reduced mtDNA copy number in targeted tissues (Chen et al., 2010; Silva Ramos et al., 2019).

Insights from mitochondrial diseases has strengthened the connection between fusion and mtDNA. For example, some (but not all) pathogenic variants of both MFN2 and OPA1 can cause mtDNA depletion (Elachouri et al., 2011; Spiegel et al., 2016; Vielhaber et al., 2013). An explanation for this discrepancy could be that not all pathogenic variants impair mitochondrial fusion to the same degree. Alternatively, it is worth noting that MFN2 and OPA1 also have additional cellular functions that are independent of fusion (e.g. mitochondria–ER tethering and stabilizing cristae junctions, respectively), which could cause mitochondrial dysfunction if they are impaired but not affect mtDNA. In addition, pathogenic variants of the fusion regulators FBXL4 and MSTO1 also cause mtDNA depletion (Donkervoort et al., 2019; Sabouny et al., 2019). Of particular interest, restoring the mitochondrial network morphology by treatment with the fission inhibitor m-Divi1 was sufficient to restore mtDNA copy number in cells harboring pathogenic variants in FBXL4 (Sabouny et al., 2019). Similarly, mtDNA copy number rescue was observed when crossing mice harboring a fission defect (MFF deletion) with mice that have a fusion defect (MFN1 deletion) (Chen et al., 2015). Thus, there is hope that therapeutic strategies to restore mitochondrial morphology by reducing fission or increasing fusion could provide clinical benefits for patients with mtDNA depletion owing to impaired mitochondrial fission or fusion.

Despite the evidence that impaired fusion leads to mtDNA depletion, the underlying mechanism is not completely understood. There are two, non-mutually exclusive, explanations for how impaired mitochondrial fusion leads to mtDNA depletion – reduced replication and/or increased mtDNA turnover. Supporting the reduced mtDNA replication hypothesis, the relative levels of mitochondrial replisome enzymes have been shown to be significantly disrupted in models of impaired fusion (Silva Ramos et al., 2019). Whereas the protein levels of mtSSB and POLG are reduced in MEFs and conditional MFN-knockout mice, the helicase Twinkle was upregulated in these animals (Silva Ramos et al., 2019). Thus, the authors posit that mitochondrial fusion and content mixing are important for maintaining the stoichiometric balance in mitochondrial replisome components (Silva Ramos et al., 2019). In support of the increased turnover notion, loss of the fusion regulator FBXL4 increases mitochondrial removal via mitophagy (Alsina et al., 2020). In this case, it is tempting to speculate that reduced fusion leads to smaller mitochondria that are simply more likely to be degraded by mitophagy. Whether this increase in mitophagy leads to increased turnover of mtDNA and reduced copy number is unknown.

Another intriguing connection between mtDNA and mitochondrial fusion is the recent finding that the anti-inflammation drug leflunomide increases hyperfusion of the mitochondrial network by increasing the expression of both MFN1 and MFN2 (Miret-Casals et al., 2018). Interestingly, leflunomide inhibits dihydroorotate dehydrogenoase (DHODH), a mitochondrial enzyme required for the synthesis of pyrimidine nucleotides, suggesting that cells may increase expression of MFN1 and MFN2 in response to reduced levels of pyrimidines. Whether this previously unappreciated connection between mitochondrial fusion and dNTPs is also relevant to mtDNA depletion disorders remains unknown. Although loss of mitochondrial fusion does not appear to affect cellular dNTP levels (Silva Ramos et al., 2019), the effects on mitochondrial dNTP pools have not been investigated. Given the role of fusion in content mixing to distribute the mtDNA replication machinery, it is easy to envision fusion playing a similar role in delivering dNTPs for mtDNA replication. Regardless, as reduced

DHODH activity also leads to reduced mtDNA copy number (Fang et al., 2013), leflunomide may not be an appropriate choice as a therapeutic for mtDNA depletion syndromes due to impaired mitochondrial fusion.

Mitochondrial dynamics and mtDNA heteroplasmy

Another link between impaired mitochondrial dynamics and the mitochondrial genome is the accumulation of mtDNA mutations. For example, blocking fission in cultured cells leads to increased levels of mutant mtDNA (Malena et al., 2009). Another interesting study supporting a role for fission in mediating heteroplasmy shifting involved crossing a mouse model of mtDNA heteroplasmy comprising a mix of two non-pathogenic mtDNA haplotypes (NZB and BALB) with the Python mouse harboring a pathogenic mutation in DRP1 (Jokinen et al., 2016). This work showed selection for mtDNA haplotypes, but only in liver, kidney and hematopoietic tissues, emphasizing the importance of tissue specificity. With respect to fusion, disruption of MFN1 and MFN2 in mouse skeletal muscle leads to elevated levels of mtDNA point mutations and deletions (Chen et al., 2010). However, these effects are likely tissue specific, as no differences in mtDNA deletions or point mutations were observed in a cardiac-specific double MFN1 and MFN2 knockout mice (Silva Ramos et al., 2019). Relevant to disease, accumulation of mtDNA deletions has been reported in patient cells harboring certain pathogenic variants in MFN2 (Vielhaber et al., 2013) and OPA1 (Amati-Bonneau et al., 2008; Hudson et al., 2008). As fusion-mediated content mixing is important for the distribution of enzymes that regulate mtDNA, the accumulation of point mutations when fusion is impaired may be due to a reduced capacity to repair mtDNA. Supporting this notion, fibroblasts from patients with pathogenic MFN2 variants showed reduced mtDNA repair (Rouzier et al., 2012). Additionally, given that human mtDNA can be degraded by components of the mtDNA replication machinery (Nissanka et al., 2018; Peeva et al., 2018; Wiehe et al., 2018), reduced content mixing of enzymes that can degrade mtDNA could also lead to reduced turnover of mutant mtDNA. With respect to mtDNA deletions, which cannot be repaired by the limited mtDNA repair machinery, their accumulation must be solely due to increased mutational rates. Given that stalled mtDNA replication complexes are believed to be a contributing factor to the production of mtDNA deletions (Nissanka et al., 2019), it is possible that reduced content mixing and uneven distribution of the mtDNA replication machinery causes stalling, promoting the generation of mtDNA deletions. Supporting the importance of fusion-dependent content mixing to protect against mutant mtDNA accumulation is the finding that crossing MFN1$^{-/-}$ mice with the POLGA mutator mice, which accumulate mutations due to a proofreading deficiency in the polymerase, is embryonic lethal, despite the individual lines being viable (Chen et al., 2010). Nevertheless, it is curious that mtDNA deletions are only linked to a few pathogenic variants in MFN2 or OPA1, and are not observed in cells harboring variants in FBXL4 (Sabouny et al., 2019). These observations suggests that a threshold of impaired fusion is required for deletions, that deletions occur in a tissue-specific manner or that other factors may also be involved in eliminating mtDNA mutations.

Mitophagy and mtDNA

Mitophagy is another dynamic process relevant to mtDNA regulation in the context of quality control and potential mtDNA elimination. Exciting recent work highlights the fate of mitochondria following fission and describes a distinct type of fission event that occurs near the ends of mitochondrial tubules, with smaller mitochondrial fragments that contain damaged cargo recruiting Parkin and subsequently undergoing mitophagy (Kleele et al., 2021). Given that mitophagy requires mitochondrial fission, initial observations that blocking fission increased mutant mtDNA levels (Malena et al., 2009), implicated mitophagy as a process important for the removal of mutant mtDNA. Similarly, mitophagy is required for the degradation of damaged mtDNA (Bess et al., 2013; Shu et al., 2021). Moreover, the induction of DNA damage leads to increased prevalence of dysfunctional mtDNA in mitochondrial fragments destined for mitophagy (Kleele et al., 2021). These findings suggest a mechanism by which mitochondria harboring mutant or damaged mtDNA genomes are preferentially recognized for mitophagy. In this regard, cells harboring mutant mtDNA genomes are reported to have reduced IMM fusion (Mishra et al., 2014), which may lead to smaller mitochondrial fragments that are more susceptible to mitophagy.

Several lines of evidence demonstrate that mitophagy selects against deleterious mtDNA mutations (Hahn and Zuryn, 2019). For example, loss of the mitophagy protein Parkin leads to an increase in the predicted pathogenicity of mtDNA mutations in the striatum of POLGA mutant mice (Pickrell et al., 2015). Similarly, Parkin overexpression reduces heteroplasmy levels of mutant mtDNA genomes in both cultured cells (Suen et al., 2010) and in *Drosophila* (Kandul et al., 2016). Conversely, mutations in PINK1 and Parkin that reduce mitophagy favor the accumulation of mutant mtDNA in several *C. elegans* models (Valenci et al., 2015). Beyond the well-studied PINK1–Parkin-mediated form of mitophagy, Parkin-independent mitophagy mediated by ATAD3B also promotes the removal of pathogenic mtDNA variants (Shu et al., 2021). However, despite evidence that mitophagy can eliminate mutant mtDNA in certain circumstances, the fact that mtDNA mutations can and do accumulate to pathogenic levels suggests that normal rates of mitophagy are insufficient to completely prevent mtDNA disease (de Vries et al., 2012). In this respect, it is notable that Parkin expression is downregulated in response to certain mtDNA mutations (Gilkerson et al., 2012). Such a response may be a compensatory mechanism to prevent mitophagy from eliminating too many mitochondria, which would be detrimental to the cell. Nevertheless, approaches to upregulate mitophagy offer a potential approach to reduce mtDNA heteroplasmy, and thus may have therapeutic benefits (Diot et al., 2015). In this regard, it is notable that promoting general autophagy by rapamycin treatment reduces mtDNA heteroplasmy in cultured cells (Dai et al., 2014).

Another emerging connection between mitophagy and mtDNA relates to the release of mtDNA into the cytosol (McArthur et al., 2018; Riley et al., 2018), where it can activate innate immune pathways such as the cGAS-STING axis (West et al., 2015). While it remains to be seen how mtDNA release is influenced by fusion or fission, and whether it can also contribute to mtDNA depletion, two recent reports link the PINK1–Parkin mitophagy pathway to mtDNA release and activation of the cGAS-STING pathway. The first study showed that following exhaustive exercise, mice lacking either PINK1 or Parkin had a strong inflammatory response dependent on STING (Sliter et al., 2018). A second study has directly linked PINK1 deficiency to mtDNA release into the cytosol (Bueno et al., 2019). Intriguingly, activation of the cGAS-STING pathway, which can be mediated by release of mtDNA into the cytosol, induces autophagy (Gui et al., 2019). Although this increase in autophagy has not yet been linked to mitophagy specifically, it is tempting to speculate that cytosolic mtDNA acts as

a signal of mitochondrial distress that can induce mitophagy to target damaged mitochondria.

Conclusions

Mitochondrial dynamics are important for regulating many aspects of mitochondrial function, including mtDNA maintenance. As highlighted above, both fission and fusion of the mitochondrial network are important for mediating the distribution of nucleoids throughout the mitochondrial network, maintaining mtDNA copy number, and regulating mtDNA heteroplasmy. In addition, mitophagy, which is linked to mitochondrial fission and fusion, is also important for mtDNA turnover, which could impact both the copy number and heteroplasmy levels of mutant mtDNA. Nonetheless, many critical questions remain to be answered about how exactly mitochondrial dynamics regulate mtDNA. The fact that impairment of the opposing forces of fusion and fission has similar outcomes with respect to mtDNA is somewhat surprising as our current understanding suggests that the underlying mechanisms for how both fusion and fission affect mtDNA may not be the same. For example, fission appears to be directly involved in initiating the replication of mtDNA, whereas fusion appears to be required for an even distribution of the mtDNA replication machinery. Future work will need to address whether the mechanisms leading to loss of mtDNA are indeed distinct in different models of impaired fission and fusion. The fact that different pathogenic variants in both fusion and fission proteins can affect mtDNA differently suggests that the underlying mechanisms are more complicated than currently appreciated. Another question that will need to be answered is how fission events, which are mediated by cytosolic and OMM proteins, are coordinated with the replication of the mtDNA in the matrix. With respect to the distribution of nucleoids throughout the mitochondrial network, the functional consequences of their clustering remain to be elucidated. There are also still questions with regard to whether nucleoid distribution is linked to copy number maintenance, although it does not appear that this is always the case. Moreover, although fission appears to be required to segregate nucleoids, exactly why nucleoids cluster upon inhibition of fusion remains unknown. Overall, although there is certainly more to learn mechanistically about how fusion, fission and mitophagy impact mtDNA, the lessons gleaned from both basic mechanistic models and human diseases clearly highlight the importance of this link. A better understanding of how and why mitochondrial dynamics impact mtDNA will be important in developing novel therapeutic approaches to treat diseases of mitochondrial dysfunction by restoring mtDNA copy number or eliminating mutant mtDNA.

Competing interests

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