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Mitochondrial Heterogeneity

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Cell-to-cell heterogeneity drives a range of (patho)physiologically important phenomena, such as cell fate and chemotherapeutic resistance. The role of metabolism, and particularly of mitochondria, is increasingly being recognized as an important explanatory factor in cell-to-cell heterogeneity. Most eukaryotic cells possess a population of mitochondria, in the sense that mitochondrial DNA (mtDNA) is held in multiple copies per cell, where the sequence of each molecule can vary. Hence, intra-cellular mitochondrial heterogeneity is possible, which can induce inter-cellular mitochondrial heterogeneity, and may drive aspects of cellular noise. In this review, we discuss sources of mitochondrial heterogeneity (variations between mitochondria in the same cell, and mitochondrial variations between supposedly identical cells) from both genetic and non-genetic perspectives, and mitochondrial genotype-phenotype links. We discuss the apparent homeostasis of mtDNA copy number, the observation of pervasive intra-cellular mtDNA mutation (which is termed “microheteroplasmy”), and developments in the understanding of inter-cellular mtDNA mutation (“macroheteroplasmy”). We point to the relationship between mitochondrial supercomplexes, cristal structure, pH, and cardiolipin as a potential amplifier of the mitochondrial genotype-phenotype link. We also discuss mitochondrial membrane potential and networks as sources of mitochondrial heterogeneity, and their influence upon the mitochondrial genome. Finally, we revisit the idea of mitochondrial complementation as a means of dampening mitochondrial genotype-phenotype links in light of recent experimental developments. The diverse sources of mitochondrial heterogeneity, as well as their increasingly recognized role in contributing to cellular heterogeneity, highlights the need for future single-cell mitochondrial measurements in the context of cellular noise studies.

Keywords: mitochondria, microheteroplasmy, macroheteroplasmy, complementation, cellular noise, heteroplasmy variance

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1. INTRODUCTION

Cellular heterogeneity plays central functional roles in a variety of biomedically important phenomena, such as development (Vassar et al., 1993; Chang et al., 2008), virus infection (Snijder et al., 2009), chemotherapeutic resistance (Spencer et al., 2009; Márquez-Jurado et al., 2018), and gene expression in aging (Bahar et al., 2006). Inter-cellular heterogeneity may arise from the intrinsically stochastic nature of cellular processes including gene transcription (Elowitz et al., 2002; Swain et al., 2002), but also from “extrinsic” sources such as the cell cycle (Newman et al., 2006) and partitioning noise (Huh and Paulsson, 2011a,b; Johnston and Jones, 2015).

We define mitochondrial heterogeneity as (1) the variation of a mitochondrial feature within the mitochondrial population of a single cell, and (2) the variation of a mitochondrial feature, potentially aggregated at the per-cell level, between supposedly identical cells. Mitochondrial heterogeneity has been found to be an important correlate of extrinsic cellular noise. Das Neves et al. (2010) and Johnston et al. (2012) found that mitochondrial mass, scaled by mitochondrial membrane potential, correlates strongly with global transcription rate at the single-cell level (also accounting for ~50% of the heterogeneity observed in protein levels; Guantes et al., 2015). Since global transcription rate has diverse implications for cellular function (Raj and van Oudenaarden, 2008), this link provides compelling evidence for the importance of mitochondrial heterogeneity as a contributor to cellular noise. Indeed, mathematical modeling predicted that mitochondrial functionality influences stem cell differentiation (Johnston et al., 2012), which has received experimental support (Gaál, 2014; Sukumar et al., 2016; Ansó et al., 2017). Furthermore, mitochondrial heterogeneity has been correlated with cell-to-cell heterogeneity in chemotherapeutic resistance in mammalian cells (Mizutani et al., 2009; Yao et al., 2013; Márquez-Jurado et al., 2018), as well as proliferation rate and drug resistance in yeast (Dhar et al., 2018). While many existing studies on mitochondrial heterogeneity are correlative, rather than showing causal mechanisms, the importance of metabolic noise is becoming more widely recognized, including in prokaryotic systems (Kiviet et al., 2014; Takhaviev and Heinemann, 2018).

In this review, we discuss sources of mitochondrial heterogeneity. Since individual cells consist of a population of mitochondria, intra-cellular heterogeneity may exist and may give rise to inter-cellular heterogeneity. Mitochondria also display a rich physiology, in addition to genetic aspects. We therefore partition our discussion into genetic and non-genetic sources of mitochondrial heterogeneity and indicate their inter-dependence through genotype-phenotype links (**Figure 1**). Our discussion is biomedically focussed, so we will emphasize findings in humans and model organisms used to study human disease such as mouse, fly, worm and yeast (the plant kingdom has several unique aspects of mitochondrial heterogeneity, recently reviewed by Johnston, 2018). We provide a necessarily non-exhaustive discussion of recent developments in these topics, and point out potential areas for future development.

2. GENETIC SOURCES OF MITOCHONDRIAL HETEROGENEITY

Genetic and non-genetic sources of mitochondrial heterogeneity are not independent. Variation in the amount and sequence of mtDNA affects the number and sequence of corresponding transcripts, eventually affecting respiratory output (although these relationships may be non-linear; Rossignol et al., 2003; Rocher et al., 2008; Picard et al., 2014; Aryaman et al., 2017b). Conversely, the non-genetic state of the cell may affect the mitochondrial genetic state, for instance AMP/ATP ratios may alter mitochondrial biogenesis and autophagy (mitophagy) rates (Palikaras and Tavernarakis, 2014). Variation in mtDNA turnover

rates have the potential to affect not only mtDNA copy number but also heteroplasmy (defined as the fraction of a particular variant allele of mtDNA per cell) through neutral genetic drift (Birky et al., 1983; Chinnery and Samuels, 1999; Capps et al., 2003; Wonnapijit et al., 2008; Johnston and Jones, 2016), and selective effects (Larsson et al., 1990; Hart et al., 1996; Bua et al., 2006; Ye et al., 2014; Li et al., 2015; Morris et al., 2017; Floros et al., 2018), as we discuss below.

2.1. Inter-cellular MtDNA Copy Number Appears to Be Under Homeostatic Control

2.1.1. MtDNA Copy Number Appears to Be Under Homeostatic Control

A potential source of mitochondrial genotypic heterogeneity is simply the quantity of mtDNA per cell. A proliferating cell is expected to increase its complement of mtDNAs by a factor of 2 over the cell cycle, and noisily partition those molecules upon division (Johnston and Jones, 2015) (**Figure 1Ai**). However, a number of studies suggest that cytoplasmic mtDNA density is tightly controlled within a variety of replicating cell types (although mtDNA copy number, and density, varies radically between different cell types; Wilson et al., 2001). Work in HeLa cells (Posakony et al., 1977) and budding yeast (Rafelski et al., 2012) have indicated that mitochondrial volume increases approximately in proportion to cytoplasmic volume, although we note that a recent *in vivo* study involving hypertrophic mouse hepatocytes suggested that mtDNA density may reduce with cell size (Miettinen et al., 2014). Further single-cell studies are required to validate this observation. Studies in proliferative human cell lines (Iborra et al., 2004; Tauber et al., 2013), budding yeast (Osman et al., 2015) and fission yeast (Jajoo et al., 2016) have shown that the distribution of inter-nucleoid spacings is significantly perturbed from random, suggesting that mtDNA density is controlled in proliferating cells. Indeed, mathematical modeling using a constant mitochondrial density (Johnston et al., 2012) was able to explain a range of single-cell data for replicating cells (Das Neves et al., 2010).

2.1.2. Interpretation of Apparent mtDNA Copy Number Homeostasis

The conservation of mitochondrial density is somewhat surprising, given that mitochondrial density is a potential axis for cells to control power production in response to differing demands, especially in the context of differing cell volume. Smaller cells have a larger surface area to volume ratio, therefore power demand is not expected to scale linearly with cell volume. Mathematical modeling has suggested that cells may instead modulate their mitochondrial membrane potential, rather than their mtDNA density, to satisfy cellular demands in mammalian cells (Miettinen and Björklund, 2016; Aryaman et al., 2017a), perhaps affording the cell more control since membrane potential may change on a faster timescale than mtDNA biogenesis. The extent to which mtDNA density homeostasis holds in the absence of cell volume variation driven by the cell cycle, i.e., quiescent cells, has yet to be carefully explored (**Figure 1Ai**) despite its relevance for mosaic dysfunction in aging post-mitotic tissues (Kauppila et al., 2017).

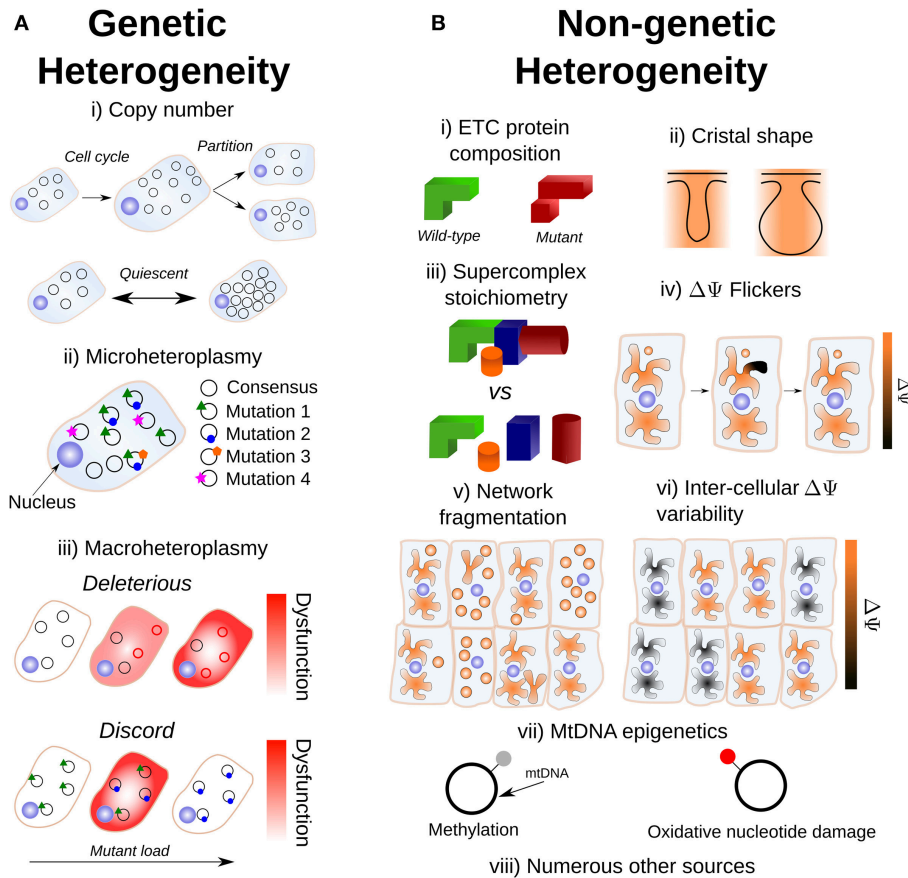


FIGURE 1 | Sources of mitochondrial heterogeneity from genetic and non-genetic mitochondrial sources. **(A)** Potential sources of mitochondrial genetic heterogeneity. (i) A proliferative cell which doubles in volume is expected to replicate its mtDNA complement by approximately $\times 2$ to avoid dilution, which may confound measurements of copy number heterogeneity; noise at partitioning is thought to be somewhat suppressed. The extent of copy number heterogeneity in quiescent cells, and its consequences, are not fully understood. (ii) A cloud of mutations around the consensus sequence is expected given a finite mtDNA mutation rate; we term this “microheteroplasmy.” The actual mutant proportion for most mutations is expected to be very small in reality. (iii) In the more canonical case, “macroheteroplasmy,” a deleterious sequence (such as a deleterious single nucleotide polymorphism or deletion, red circles) induces a pathological phenotype above a threshold heteroplasmy. By contrast, intermediate heteroplasmy has been observed to induce large fitness disadvantages but homoplasmy does not, indicating discord between the two alleles. **(B)** Potential sources of mitochondrial non-genetic heterogeneity. (i) MtDNA sequence variation can induce variation in the structure of corresponding proteins. (ii) Cristal structure is variable and physiologically regulated to control respiratory output. (iii) A landscape of different supercomplex stoichiometries exists. (iv) Spatially-restricted, transient, depolarization/repolarization cycles of the mitochondrial network are observed. (v) The extent of mitochondrial fragmentation influences heteroplasmy dynamics (see Equation 1). (vi) Intercellular heterogeneity in mitochondrial membrane potential has been shown to be an important source of cellular noise. (vii) Potential sources of mitochondrial epigenetic heterogeneity. MtDNA is observed to undergo epigenetic modification by methylation, although the physiological significance of this is uncertain. Also, oxidative damage to mtDNA nucleotides may cause transcriptional errors. (viii) There are numerous other sources of non-genetic heterogeneity: from distinct spatial niches to ER-mitochondrial interactions. For further discussion, see Main Text.

2.1.3. Pathological Consequences of Loss of mtDNA Copy Number Homeostasis

In humans, a variety of nuclear mutations which induce defects in mtDNA maintenance cause mitochondrial depletion syndromes; these are severe disorders and clinically diverse in their physiological impact (El-Hattab and Scaglia, 2013). Conversely, it has been shown that increasing mtDNA copy number can rescue male infertility in mice engineered to accumulate mtDNA mutations, despite unaltered heteroplasmy (Trifunovic et al., 2004; Jiang et al., 2017). It has been hypothesized that failure to maintain homeostasis in the density of functional mtDNAs may underlie the pathology of one of the most common mtDNA

mutations associated with mitochondrial disease (3243A>G tRNA mutation) (Aryaman et al., 2017b). A mathematical model of human cybrid cells with the 3243A>G mutation was consistent with a range of omics data (Picard et al., 2014), made by assuming that cells attempt to maintain mtDNA density homeostasis through cytoplasmic volume reduction, until a minimum cell volume is reached where cells undergo a switch in their metabolic response (Aryaman et al., 2017b). Indeed, assuming constant mitochondrial functionality, the study of Johnston et al. (2012) predicts that a reduction in mtDNA density results in lowered ATP concentrations, which results in lowered transcription rate (Das Neves et al., 2010). These

studies highlight the potential pathophysiological relevance of maintaining mtDNA density homeostasis.

2.2. Intra-cellular Mutations in Mitochondrial DNA Are a Source of Genotypic Heterogeneity

2.2.1. MtDNA Mutation as a Source of Heterogeneity

Mitochondrial DNA is replicated and degraded, even in non-proliferating tissues, which generates opportunities for mtDNA mutations to arise and proliferate. Studies of mtDNA mutation spectra in humans have suggested that point mutations predominantly arise from replication errors (Kennedy et al., 2013; Williams et al., 2013; Stewart and Larsson, 2014) as opposed to oxidative damage (Kauppila and Stewart, 2015; Kauppila J.H. et al., 2018), as is also the case for the “common” 4997 bp deletion (Phillips et al., 2017).

2.2.2. Intra-cellular mtDNA Mutation as a Source of Heterogeneity

Finite mutation rates during replication of mtDNA are expected to give rise to a set of closely-related sequences which do not all necessarily maximize fitness (Eigen and Schuster, 1977; Nowak, 2006). Therefore, at the intra-cellular level, we expect to observe mtDNA sequence diversity (see e.g., Jayaprakash et al., 2015). Recent experimental work in primary cultures of mouse neurons and astrocytes has shown this to be the case (Morris et al., 2017). The authors found 3.9 ± 5.7 single nucleotide variations (SNVs) (\pm standard deviation) per mitochondrion (Morris et al., 2017), with a mitochondrion expected to contain around 5 molecules of mtDNA (Sato and Kuroiwa, 1991). The authors of this study found that the distribution of allele frequencies was skewed toward 0% heteroplasmy (Morris et al., 2017), suggesting the existence of negative selection acting at the intra-cellular level (Birky et al., 1983). However, several moderate/high impact mutations with >90% heteroplasmy were also discovered (Morris et al., 2017), suggesting that certain mutations are able to evade intra-cellular selection and reach high levels of heteroplasmy. Further studies are required to determine whether such high-heteroplasmy variants become established through neutral drift, or whether a positive selection mechanism exists; for example, it is possible that heterogeneity in the intra-cellular environment establishes intra-cellular niches which favor different mtDNA sequences. We use the term “microheteroplasmy” to denote abundant ultra-low heteroplasmic mutations within single cells (Figure 1Aii).

2.2.3. Pathological Consequences of Microheteroplasmy

Whilst the pathological implications of microheteroplasmy remain unclear, comparisons could be drawn to experiments where a naturally occurring, but foreign, mtDNA haplotype is introduced into a cell with which it has not co-evolved. Increasing genetic distance amongst haplotypes has been shown to induce tissue-specific selective pressures in mice (Burgstaller et al., 2014), and heteroplasmy between otherwise healthy haplotypes have been shown to induce fitness disadvantages (Acton et al., 2007; Sharpley et al., 2012), as corroborated by bottom-up

mathematical modeling (Hoitzing et al., 2017a). Recent work has shown that such fitness disadvantages may be mediated through oxidative damage (Bagwan et al., 2018) (Figure 1Aiii).

2.3. Inter-cellular Mutations in Mitochondrial DNA Are a Source of Genotypic Heterogeneity

2.3.1. Cell-to-Cell Heterogeneity in Heteroplasmy Increases Linearly With Time

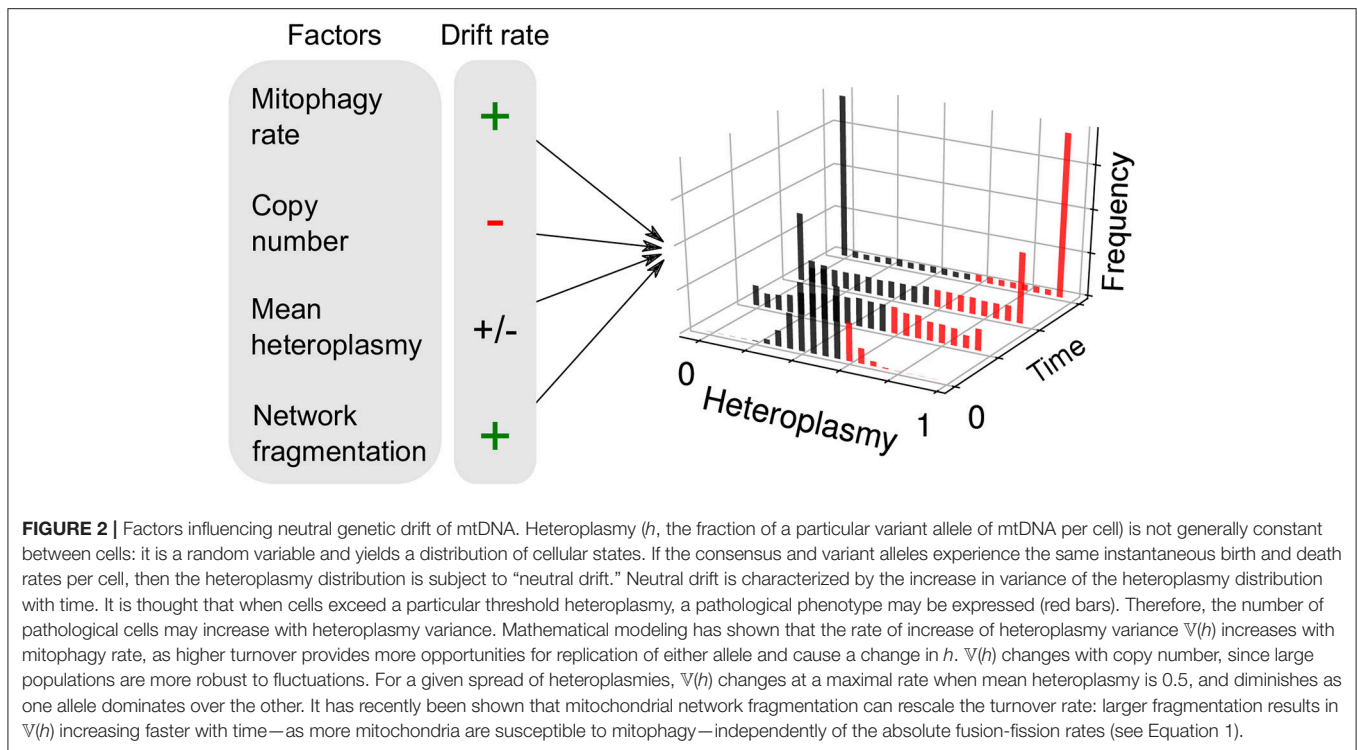
Another consequence of the finite mutation rate of mtDNA, and its stochastic turnover, is that mutations will occasionally drift to very high level heteroplasmies simply by genetic drift (Birky et al., 1983; Ewens, 2004), i.e., “macroheteroplasmy” (Figure 1Aiii). Cell-to-cell heterogeneity in heteroplasmy (heteroplasmy variance) therefore widens through time. Certain deleterious mutations may therefore drift to high levels of heteroplasmy and cause pathology (Rossignol et al., 2003) (see Figure 2). Theoretical work predicts that heteroplasmy variance increases approximately linearly, and increases with the rate of mitophagic turnover of mtDNAs (Poovathingal et al., 2009; Johnston and Jones, 2016), but decreases with total mtDNA copy number with a non-linear dependence upon mean heteroplasmy (Johnston and Jones, 2016). A linear increase in heteroplasmy variance with time has recently been observed in mouse oocytes, and over pup lifetimes (Burgstaller et al., 2018). Mathematical modeling has suggested that increasing heteroplasmy variance might increase the energetic cost of maintaining a tissue (Hoitzing et al., 2017a).

2.3.2. Heteroplasmy Variance and Selective Thresholds May Counter the Progressive Increase in Mutant Load During Development

Increases in heteroplasmy variance can also purge mitochondrial mutations when combined with selection, as observed between generations in the human mitochondrial bottleneck (Johnston et al., 2015; Burr et al., 2018; Floros et al., 2018). During development, through a reduction of cellular mtDNA copy number, heteroplasmy variance is increased. When combined with a selective apoptotic threshold, whereby cells above a heteroplasmic threshold undergo cell death, mean heteroplasmy can be reduced. The mechanisms and timings of the mitochondrial bottleneck are debated (Jenuth et al., 1996; Cao et al., 2007; Cree et al., 2008; Wai et al., 2008); however, stochastic modeling has shown that several proposals are compatible with the induction of heteroplasmy variance through a combination of random mtDNA partitioning at division and passive turnover of mtDNA (Johnston et al., 2015; Johnston and Jones, 2016; Hoitzing et al., 2017b).

2.3.3. Clonal Expansions of Mitochondrial Mutations With Age Have Pathological Consequences

Mitochondrial mutations which survive the bottleneck can result in severe congenital diseases (Schon et al., 2012), and accelerate aging in the case of severe mutant loads in mice (Ross et al., 2013). Under physiological conditions, mtDNA mutations are also observed to accumulate with age across tissues in humans (Khrapko et al., 1999; Taylor et al., 2003; Bender et al., 2006;



Kraysberg et al., 2006; Li et al., 2015; Kauppila et al., 2017). Certain cases of mutant accumulation (or “clonal expansion”) may be explained by neutral drift, for instance partitioning noise in highly proliferative colonic crypt cells can result in highly mutated crypts (Taylor et al., 2003). However, positive selection through replicative advantage (Samuels et al., 2013) and tissue-specific niches (Avital et al., 2012; Li et al., 2015; Ahier et al., 2018) can also influence heteroplasmy dynamics. The extent to which mtDNA mutations present at birth, as opposed to somatic mutation post-development, contribute to healthy aging remains open. Bulk heteroplasmy measurements in monozygotic twins have shown that inherited mutations can contribute mitochondrial mutations, including heteroplasmic mutations with a low mutant load (Avital et al., 2012). The vast amount of DNA replication during development itself is also likely to seed and expand a number of potentially pathological mtDNA mutations (Hahn and Zuryn, 2018), as is thought to be the case in the nuclear genome (Keogh et al., 2018). Mitochondrial mutations which arise and focally expand post-development in skeletal muscle contribute to pathological age-related loss in muscle mass (Khrapko et al., 1999; Aiken et al., 2002; Bua et al., 2006; Vincent et al., 2018). In the case of human muscle fibers of healthy individuals, the number of fibers exhibiting electron transport chain (ETC) abnormalities increases from approximately 6% at age 49–31% at age 92 (Bua et al., 2006). Serial sections through laser-captured single skeletal muscle fibers showed that heteroplasmy in mtDNA deletions may exceed 90% (Bua et al., 2006). Deletion mutations have been associated with local fiber atrophy and breakage in mice (Wanagat et al., 2001; Khrapko and Vijg, 2009) and rhesus

monkeys (Aiken et al., 2002) (although the causative role of mtDNA mutations in aging for shorter-lived animals is contested; Vermulst et al., 2007; Kauppila T.E. et al., 2018; Lakshmanan et al., 2018). It is noteworthy that mitochondrial dysfunction in a particular tissue is able to induce stress responses in distal tissues through the mitochondrial unfolded protein response (Zhao et al., 2002; Durieux et al., 2011; Zhang et al., 2018) and other hormonal signaling pathways (Tyynismaa et al., 2010; Khan et al., 2017), suggesting potential organism-wide consequences of focal mitochondrial mutations. The development of novel, quantitative, dyes for electron transport chain (ETC) deficiency may allow more refined measurements of the causes of deletion proliferation in somatic tissues in the future (Simard et al., 2018; Vincent and Picard, 2018).

2.3.4. Inter-cellular Mitochondrial Exchange as a Physiological Means to Slow Heteroplasmy Variance

Mitochondria have been observed to be transferred between cells (Spees et al., 2006; Torralba et al., 2016), and the intercellular transfer of other organelles has also been observed (Rustom et al., 2004). Several mechanisms by which mitochondrial transfer is achieved are known, including tunneling nanotubes (Koyanagi et al., 2005) and microvesicles (Phinney et al., 2015), amongst others (Torralba et al., 2016). Intercellular mitochondrial exchange has largely been understood through *in vitro* experiments (Spees et al., 2006; Jayaprakash et al., 2015; Berridge et al., 2016a); however, recent developments have shown the effect to also be relevant *in vivo* (e.g., Islam et al., 2012; Ahmad et al., 2014; Berridge et al., 2016a). For instance, Islam et al. (2012) have shown that mouse mesenchymal

stem cells (MSCs) are able to release microvesicles containing mitochondria, and protect against sepsis-induced acute lung injury in mice. Recent evidence from xenograft mouse models have also shown that mtDNA can be transferred between cells via exosomes, mediating an escape from dormancy in therapy-resistant breast cancer cells (Sansone et al., 2017). Furthermore, astrocytes have been found to donate mitochondria to neurons after stroke (Hayakawa et al., 2016), although interpretations of these data exist which do not require mitochondrial exchange (Berridge et al., 2016b). In terms of mitochondrial genetic dynamics, intercellular exchange of mtDNA appears to slow down heteroplasmic drift (Jayaprakash et al., 2015) and thereby reduce cell-to-cell mitochondrial genetic heterogeneity. It is possible that inter-cellular exchange of mitochondria, and mitochondrial DNA, is an evolved mechanism to ameliorate heteroplasmy variance and therefore the build-up of cells with pathological levels of heteroplasmy. Although the existence of inter-cellular mitochondrial exchange has now been established, the extent and dynamics of mtDNA transfer remain incompletely understood.

2.3.5. Gene Therapy as a Means to Therapeutically Control Heteroplasmy Distributions

Gene-editing technologies targeted at the mitochondrial genome are under development (Bacman et al., 2013, 2018; Gammage et al., 2014, 2018; Reddy et al., 2015; Pereira et al., 2018). Such technologies sequence-specifically bind and cleave mitochondrial DNA, which is subsequently degraded rapidly (Peeva et al., 2018). Mathematical modeling of mitochondrially-targeted gene therapies predicts that tissues with high mean heteroplasmy and large heteroplasmy variance are generally more difficult to treat (Hoitzing et al., 2017a); there is therefore a close link between these promising therapeutic technologies and inter-cellular heterogeneity in heteroplasmy.

3. NON-GENETIC SOURCES OF MITOCHONDRIAL HETEROGENEITY

Apart from the mitochondrial genome, there are many non-genetic properties of mitochondria which can vary. One such property which is particularly clear is ETC protein structure, which is influenced by sequence heterogeneity in mtDNA (Figure 1Bi)—this being a potentially important source of both intra- and inter-cellular variability. Other non-genetic differences in mitochondria can include mitochondrial membrane composition/structure, ion content, membrane potential and network structure. Intra-cellular spatial heterogeneity through the existence of sub-cellular mitochondrial niches (Palmer et al., 1977; McKenna et al., 2000; Benador et al., 2018), e.g., perinuclear vs. peripheral locations, has been highlighted as an important axis of mitochondrial heterogeneity by previous authors (see Wikstrom et al., 2009). Below, we discuss several other aspects in which non-genetic attributes of mitochondria may vary, and the potential pathological consequences of such variation. We also draw attention to how non-genetic heterogeneity may be driven by, or drive, genetic heterogeneity through genotype-phenotype links.

3.1. Stoichiometric and Structural Heterogeneity in the Inner Mitochondrial Membrane as a Potential Amplifier of the Genotype-Phenotype Link

3.1.1. MtDNA Genotype as a Driver of IMM Phenotype

The inner mitochondrial membrane (IMM) is heterogeneous in both its composition and its topology. The connection between the IMM and the mitochondrial genotype is particularly relevant since mitochondrial DNA is situated in the mitochondrial matrix and in close proximity to the IMM (Brown et al., 2011). Consequently, one might expect mtDNAs to affect their local respiratory units more than distal mtDNAs (Busch et al., 2014), thus allowing greater control of respiration at a local level (Allen, 1993, 2003; Lane, 2011). Below we discuss aspects of how IMM physiology may generate mitochondrial heterogeneity, and how cardiolipin may act as a sensor of the mitochondrial genotype. We discuss how cardiolipin reacts to differences in reactive oxygen species (ROS) production and pH, and may amplify the mitochondrial phenotype through supercomplex formation and cristal structure.

3.1.2. Mitochondrial Supercomplex Stoichiometry as an Axis of IMM Heterogeneity

The stoichiometry of respiratory units within the IMM can vary, since respiratory units organize into supramolecular structures termed supercomplexes (Schägger and Pfeiffer, 2000; Enríquez, 2016) (Figure 1Biii). A variety of supercomplex stoichiometries exist in mammalian cells (Schägger and Pfeiffer, 2000; Schäfer et al., 2006) as well as free respiratory subunits (Schägger and Pfeiffer, 2000). The “plasticity model” has therefore been suggested (Acín-Pérez et al., 2008), whereby a landscape of combinations between respiratory complexes coexist. Dysfunction in the assembly of mitochondrial supercomplexes in mice results in decreased muscle activity and heat production in the cold due to reduced CIV activity (Ikeda et al., 2013), showing that supercomplex assembly is required for fully-functional respiration. Consequently, dysfunction in supercomplex assembly could affect mitochondrial quality control pathways and influence the mitochondrial genotype.

3.1.3. Cristal Structure as an Axis of IMM Heterogeneity

The topologies of mitochondrial membranes are heterogeneous (Mannella, 2006; Enríquez, 2016), and this heterogeneity may potentially be driven by mitochondrial genetic heterogeneity (see above), as we will discuss at the end of this subsection. ATP-synthase forms dimers in the IMM which often arrange into micron-scale rows, which are associated with high local curvature to form mitochondrial cristae (Strauss et al., 2008; Davies et al., 2012). Recent work has suggested that the mitochondrial fusion protein OPA1 stabilizes ATP synthase oligomers by modulating cristal shape (Quintana-Cabrera et al., 2018). Crista membranes show an enrichment of respiratory complexes relative to the inner boundary membrane (Gilkerson et al., 2003; Vogel et al., 2006), and it has been suggested that cristae exist to increase the packing density of respiratory

units (Rieger et al., 2014). Individual cristae are morphologically heterogeneous in mammalian cells (Frey and Mannella, 2000), and can be modulated in response to altered metabolic demands (Eisner et al., 2018): cristae become narrower in mammalian cells in response to starvation (Patten et al., 2014), suggesting that cristal shape influences respiratory efficiency, perhaps by modulating local substrate concentrations (Mannella et al., 2001) (**Figure 1Bii**). Remodeling of mitochondrial morphology occurs during cell death (Scorrano et al., 2002; Yamaguchi et al., 2008), whereby supercomplexes and dimers of ATP synthase disassemble and cristal structure becomes disorganized (Cogliati et al., 2016), allowing the release of cytochrome *c* (an electron carrier of the ETC) to trigger the intrinsic cell death pathway (Taylor et al., 2008) (although the importance of crista remodeling for cytochrome *c* release has been questioned; Tam et al., 2010). The intimate connection between mitochondrial physiology and cell death provides insight into the recent observation that mitochondrial heterogeneity can partially explain variability in chemotherapeutic resistance in HeLa cells (Márquez-Jurado et al., 2018).

3.1.4. Cardiolipin Is Necessary for Supercomplex and Crista Stabilization

Cardiolipin is a phospholipid found in the IMM, and stabilizes both supercomplexes and cristal structure. When exposed to a pH gradient, cardiolipin-containing lipid vesicles spontaneously form crista-like membrane invaginations (Khalifat et al., 2008, 2011), thus providing a potential connection between respiratory activity, which is influenced by mtDNA genotype, and cristal shape. Furthermore, flies with deficient cardiolipin levels show reduced ATP synthase abundance in high-curvature regions of cristae, resulting in disorganized cristae, cardiac insufficiency, motor weakness and early death (Acehan et al., 2011). In yeast, cardiolipin has been shown to be necessary for supercomplex stabilization (Zhang et al., 2002; Pfeiffer et al., 2003). Indeed, patients with Barth syndrome who are deficient in cardiolipin due to a mutation in the tafazzin gene show both aberrant crista formation (Acehan et al., 2011) and reduced supercomplex formation (McKenzie et al., 2006).

3.1.5. Cardiolipin as a Potential Amplifier of mtDNA Heterogeneity Through Alterations in pH and ROS Generation

Cardiolipin is particularly susceptible to damage by ROS. In isolated bovine mitochondria, ROS exposure resulted in loss of CI activity, but exogenously added cardiolipin could restore CI activity (Paradies et al., 2002). It has recently been shown that a mitochondrially-targeted antioxidant (MitoQ) is able to increase cardiolipin expression and content in liver mitochondria of rats fed on a high-fat diet, resulting in increased mitochondrial functionality and ATP synthase activity (Fouret et al., 2015). Together, this suggests that mitochondrial dysfunction, which alters pH gradients across the IMM and ROS production, can cause cristae to become disorganized and affect supercomplex assembly via cardiolipin, potentially resulting in further loss of mitochondrial efficiency and ROS production. It is possible that heterogeneity in pH and ROS production, for instance through

mtDNA mutation heterogeneity (Lane, 2011), could be amplified through such mechanisms and thus strengthen the genotype-phenotype link between mtDNA and their local respiratory complexes.

3.2. Mitochondrial Membrane Potential Heterogeneity, Mitochondrial Networks, and Mitochondrial Genotype

3.2.1. Inter-cellular Mitochondrial Membrane Potential Heterogeneity as a Predictor of Cell-Physiological Heterogeneity

The inner membrane potential ($\Delta\Psi$) is an indicator of mitochondrial functionality, generated by the ETC, which drives the synthesis of ATP by ATP-synthase. Mitochondrial output is highly sensitive to $\Delta\Psi$: a 14 mV change in $\Delta\Psi$ corresponds to a 10-fold change in the maximum ATP/ADP ratio (Nicholls, 2004), where $\Delta\Psi$ typically ranges between 150 and 180 mV (Perry et al., 2011). Quantification of the absolute value of $\Delta\Psi$ in millivolts at a single-cell level through fluorescence probes (Perry et al., 2011) is possible but technically challenging, requiring deconvolution from other confounding factors such as fluctuations in plasma membrane potential, the matrix: cell volume ratio, dye activity and binding affinity in the matrix/cytosol, and spectral changes resulting from binding (Gerencser et al., 2012, 2016). Many studies involving $\Delta\Psi$ measurements through Nernstian dyes neglect these possible confounding variables and assume differences in fluorescence are always directly attributable to differences in $\Delta\Psi$, so some caution is required. With these caveats in mind, measurements of mitochondrial mass scaled by $\Delta\Psi$ has been shown to explain much of the variation in transcript elongation rate (Das Neves et al., 2010; Johnston et al., 2012) and protein noise (Guantes et al., 2015), as well as predicting phenomena such as cell cycle duration (Johnston et al., 2012) and chemotherapeutic resistance (Márquez-Jurado et al., 2018) in mammalian cells (**Figure 1Bvi**).

3.2.2. Calcium and pH Transients as Determinants of Intra-cellular $\Delta\Psi$ Fluctuations

In addition to inter-cellular $\Delta\Psi$ heterogeneity, individual mitochondria have been shown to undergo transient depolarization/repolarization cycles, termed “flickers” in animals (Duchen et al., 1998; O’Reilly et al., 2003) or “pulses” in plants (Schwarzländer et al., 2012) (**Figure 1Biv**). In freshly dissociated smooth muscle cells, flickers range from <10 mV to >100 mV, typically lasting on the order of seconds (O’Reilly et al., 2003). It has been proposed that mitochondrial flickers are regulated by various mechanisms, including mitochondrial inner membrane fusion (Santo-Domingo et al., 2013), Ca^{2+} influx (Duchen et al., 1998; Jacobson and Duchen, 2002), and transient opening of the mitochondrial permeability transition pore (mPTP) (Hüser and Blatter, 1999; Jacobson and Duchen, 2002). The mPTP is thought to be a non-selective mitochondrial channel that induces cell death when open for prolonged periods (Bernardi et al., 2006). In rat myocytes, opening of the mPTP through pharmacological intervention has been found to correlate with the frequency of transients in circularly permuted yellow fluorescent protein

(cpYFP) fluorescence (Wang et al., 2008). cpYFP has been shown to be sensitive to pH (Schwarzländer et al., 2011, 2014; Santo-Domingo et al., 2013), therefore transient cpYFP fluorescence corresponds to transient alkalinization of the mitochondrial matrix (i.e., an increase in pH gradient across the IMM). Since one would expect a reduction in pH gradient if the mPTP were behaving in a non-selective mode, transient alkalinization potentially implies an ion-selective mode of the mPTP. The coincidence of an increase in pH gradient, and loss of membrane potential, indicates a redistribution of the proton motive force, which has pH and electrical contributions (Nicholls, 2004). This redistribution may be mediated by selective ion movement. Flickers have been shown to exist *in vivo* in mouse astrocytes, and are enhanced by both neuronal activity and oxidative shifts (Agarwal et al., 2017), which is consistent with previous *in vivo* observations in mice (Breckwoldt et al., 2014, 2016). Although no clear consensus exists on the mechanisms of transient mitochondrial depolarization/alkalinization cycles, such cycles appear to be a likely means of regulating metabolic rate, and perhaps ROS production, at the single mitochondrion level.

3.2.3. Mitochondrial Membrane Potential Influences Mitochondrial Genotype Through Quality Control

The canonical means by which mitochondrial membrane potential feeds back into the genetic state is through mitophagy and mitochondrial network dynamics (Twig et al., 2008). Mitochondria are not static organelles but undergo dynamic fusion and fission, the purpose of which is incompletely understood (Hoitzing et al., 2015). In rat pancreas cells, it has been shown that fission often results in a daughter mitochondrion which has a lower $\Delta\Psi$ than its sister (Twig et al., 2008). These depolarized mitochondria have a lowered propensity for fusion, and are more likely to be degraded (Twig et al., 2008). Selective fusion, when combined with non-selective mitophagy, is sufficient to preferentially degrade depolarized/damaged mitochondria (Hoitzing et al., 2015; Aryaman et al., 2018), although the selective strength of mitophagy itself is not yet fully understood. The extent to which mitochondria which are degraded via selective/non-selective forms of mitophagy possess mitochondrial genomes which are perturbed from the consensus sequence also remains incompletely understood (Lemasters, 2014). However, if depolarized mitochondria are more likely to be perturbed from the consensus mitochondrial sequence, mitochondrial quality control mechanisms would exert a negative selective pressure against variant alleles. Indeed, negative selection pressures have been observed in human tissues (Li et al., 2010, 2015; Avital et al., 2012; Ye et al., 2014) and at the intracellular level in mice (Morris et al., 2017), which may be due to a combination of mitochondrial networks and mitophagy.

3.2.4. If Quality Control Is Weak, Mitochondrial Network Fragmentation Slows Heteroplasmy Variance Through a Rescaling of Time

The existence of mitochondrial diseases (Schon et al., 2012), the ubiquity of heteroplasmy (Payne et al., 2012; Morris et al., 2017), and the accumulation of heteroplasmy with age (Li et al., 2015), suggest that mitochondrial quality control may be weak

for certain sequences. Building on insights from previous work (Mouli et al., 2009; Tam et al., 2013, 2015; Johnston and Jones, 2016), recent mathematical modeling (Aryaman et al., 2018) suggests that, if quality control is weak, heteroplasmy variance $\mathbb{V}(h)$ follows the equation

$$\mathbb{V}(h) \approx f_s \frac{2\mu t}{n} h_0(1 - h_0) \quad (1)$$

where t is time, f_s is the fraction of unfused mitochondria, μ is the mitophagy rate, n is copy number and h_0 is the initial heteroplasmy, which is equivalent to mean heteroplasmy under neutral drift (see **Figure 2**). This equation arises through the assumption that larger mitochondrial fragments are at a reduced susceptibility to degradation, as is observed empirically (Twig et al., 2008). As a consequence, total mitochondrial turnover is modulated by the fraction of unfused mitochondria, independently of the absolute magnitude of fusion-fission rates (Aryaman et al., 2018). Since heteroplasmy variance is proportional to mitochondrial turnover through mitophagy (Johnston and Jones, 2016), mitochondrial fragmentation may therefore modulate the rate of accumulation of pathologically mutated cells in a tissue, independently of selective effects (Aryaman et al., 2018) (**Figure 1Bv**). Mitochondrial network fragmentation may also slow *de novo* mutation (Aryaman et al., 2018) through a rescaling of mitochondrial turnover (which is known to modulate the *de novo* mutation rate; Poovathingal et al., 2009). As a consequence, promoting mitochondrial fusion earlier in life, when mean heteroplasmy is low, may delay the rate of accumulation of cells with pathological levels of mutated mtDNA (Aryaman et al., 2018), which may have implications for healthy aging.

3.3. Mitochondrial Complementation May Dampen the Genotype-Phenotype Link but Its Extent Is Incompletely Understood

3.3.1. Mitochondrial Complementation Is Thought to Partially Buffer Genotype-Phenotype Links via the Threshold Effect

Mitochondrial “complementation” consists of mitochondria sharing their contents through fusion-fission events, and potentially complementing each others’ genetic defects (Hayashi et al., 1994; Yoneda et al., 1994; Enriquez et al., 2000; Ono et al., 2001). This is supported by experiments involving photoactivatable fluorescent proteins which show that intra-mitochondrial contents mix over time (Twig et al., 2008; Wilkens et al., 2013). Inhibition of fusion and fission have also been shown to induce heterogeneity in the distribution of aged mitochondrial proteins (Ferree et al., 2013). Beyond depending on fission and fusion rates, and the degree of mitochondrial mobility, the strength of complementation is closely linked to the diffusivity of the mitochondrial matrix: if the matrix is a high-diffusivity environment then gene products are expected to be promiscuous within the matrix and not remain local to their parental mtDNA. Therefore, in cells heteroplasmic between wild-type and a pathological mutant, healthy versions of any particular transcript would be found in the matrix. The idea of complementation is also closely linked to the mitochondrial

threshold effect (Rossignol et al., 2003; Stewart and Chinnery, 2015; Aryaman et al., 2017b), whereby complementation effects allow cells to withstand high levels of mutant load (60–90%, Chomyn et al., 1992; Miyabayashi et al., 1992; Rossignol et al., 2003) before displaying a respiratory defect.

3.3.2. Mitochondrial Complementation Remains Incompletely Understood

Much of the progress in understanding mitochondrial complementation derives from cell fusion studies where cells harboring different mtDNA mutations are fused, and mitochondrial functionality recovers through sharing of transcription products (Ono et al., 2001; Gilkerson et al., 2008; Yang et al., 2015). Such experiments highlighted physiological subtleties which remain incompletely understood. For instance, the experiment of Ono et al. (2001) between cells harboring two different mt-tRNA mutations required an adaptive period of 10–14 days before respiratory activity was restored—the reason for this was not fully understood. Furthermore, cell fusion studies between cells harboring partially-functional (3271T>C) and completely dysfunctional (3243A>G) mitochondrial tRNA-Leu mutations resulted in a sigmoidal relationship between heteroplasmy and COX-activity, whereas a linear relationship was observed for simple co-culture between the two cell types (Ono et al., 2004), which again lacks a complete explanation. In contrast to the experiment of Ono et al. (2001), an experiment by Gilkerson et al. (2008) between two non-overlapping mitochondrial deletions showed recovery of MTCO2 protein production on a relatively fast timescale (4 days). Furthermore, Gilkerson et al. (2008) found that fused cells cycled between heteroplasmic and homoplasmic states during long-term culture, despite being on a medium which mildly selected for mitochondrial function (but still allowed ATP production through glycolysis). Yet, a straightforward interpretation of complementation would suggest that heteroplasmic states would show maximal fitness. This effect was originally explained as a tension between a neutral genetic drift effect, deriving from the assumption that nucleoids possess several mtDNAs (Jacobs et al., 2000), and selective pressure for heteroplasmy (Gilkerson et al., 2008). However super-resolution microscopy studies have revealed that nucleoids harbor only 1–2 mtDNAs per nucleoid (Kukat et al., 2011, 2015). It is possible that large drift rates could be explained by the passive partitioning of very large clusters of genetically homogeneous mtDNA at cell division. Furthermore, active asymmetric apportioning of damaged or aged mitochondria has been observed in yeast (McFaline-Figueroa et al., 2011) and stem cells (Katajisto et al., 2015) (which has been shown to influence cellular fate decisions in immune cells; Adams et al., 2016); therefore, active mechanisms of asymmetric apportioning of mitochondrial genomes may also exist. Cycles between heteroplasmy and homoplasmy highlight an area for future experimental and theoretical investigation.

3.3.3. Restricted Diffusion in the IMM May Inhibit Complementation

Experiments by Wilkens et al. (2013) fused HeLa cells with mitochondrially-targeted fluorescent proteins of differing colors

to investigate the extent of mitochondrial diffusivity. The authors found that, through cycles of mitochondrial fusion and fission, IMM proteins appear to experience slow diffusion relative to the outer mitochondrial membrane, and retain cristalline structure (Wilkens et al., 2013). Consequently, complementation may be diminished since gene products may remain local to their parental mtDNA (Busch et al., 2014), allowing local control of respiration (Allen, 1993, 2003; Lane, 2011). This is supported by the observation that mitochondrial transcripts are particularly concentrated around mtDNA (Ozawa et al., 2007). Mathematical modeling of progressive increases in heteroplasmy of the pathological 3243A>G mutation is compatible with the interpretation that tRNAs are enriched in the vicinity of their local mtDNA, and that mutated mtDNAs experience a local depletion of ATP resulting in a transcriptional defect (Picard et al., 2014; Aryaman et al., 2017b). Hence, restricted complementation and local phenotype-genotype links appear to be explanatory.

4. DISCUSSION

Mitochondrial heterogeneity can occur at various scales. In this review, we have focussed on heterogeneity in the mitochondrial population within a cell (e.g., microheteroplasmy Morris et al., 2017) and inter-cellular heterogeneity of aggregate per-mitochondrion observables (e.g., variation in inter-cellular membrane potential; Das Neves et al., 2010; Johnston et al., 2012). Heterogeneity also exists at larger scales, for instance between organs of a particular individual (e.g., tissue-specific genetic selective pressures upon mtDNA; Burgstaller et al., 2014; Li et al., 2015; Ahier et al., 2018), and heterogeneity between individuals (e.g., variation in the consensus sequence between individuals of different mitochondrial haplotypes; Wallace and Chalkia, 2013). We have pointed out the genetic and non-genetic sources of such intra- and inter-cellular mitochondrial heterogeneity, as well as its pathological significance.

We note that mitochondrial epigenetic modification is also a potential layer of non-genetic mitochondrial heterogeneity (Figure 1Bvii). Mitochondrial DNA can undergo methylation (van der Wijst and Rots, 2015); although its physiological impact is still being unraveled, it has been suggested that methylation may regulate mtDNA gene expression (van der Wijst et al., 2017). Furthermore, whilst oxidative damage to mtDNA nucleotides have classically been known for their potential role in mutagenesis, it has been proposed that the formation of 8-OHdG, which is a ROS-modified version of guanine, induces mitochondrial mutations at the transcriptional level (Nakanishi et al., 2012) and may be responsible for premature aging phenotypes in mice bred to rapidly accumulate mtDNA mutations (Trifunovic et al., 2004; Safdar et al., 2011, 2016). Post-transcriptional modifications of mitochondrial transcripts (Bar-Yaacov et al., 2013), potentially modulated by heterogeneity in the sequence of nuclear DNA (Hodgkinson et al., 2014), are also possible sources of mitochondrial heterogeneity, which potentially constitute a rich and fascinating avenue for future research. Further downstream sources of mitochondrial heterogeneity, such as translation errors, have also been shown

to have physiological consequences in budding yeast (Suhm et al., 2018). The age-related functional decline of mitochondria in shorter-lived animals (Itsara et al., 2014; Brandt et al., 2017) might not be explained directly by mtDNA mutations (Vermulst et al., 2007; Lakshmanan et al., 2018) (see also recent, contrasting, results in *Drosophila*; Kauppila T.E. et al., 2018; Samstag et al., 2018). However, the mechanisms described above which are downstream of mtDNA mutation, but are nevertheless constrained by mtDNA, may still be able to explain this functional decline.

We have also discussed how genotype-phenotype links provide feedback between genetic and non-genetic states. The mitochondrial genotype may drive the phenotype through e.g., the genotype influencing ROS formation and pH gradients. This may, in turn, affect cardiolipin, supercomplex formation and cristal structure, which ultimately affect respiratory capacity. On the other hand, the mitochondrial phenotype may influence the genotype through e.g., the mitochondrial network and mitophagy. This may, in turn, modulate the mean and variance of the heteroplasmy distribution.

The interdependence between genetic and non-genetic sources of mitochondrial heterogeneity means that it is difficult to state which sources of mitochondrial heterogeneity are the most important in general. However, one may argue that genetic variability occurs on a slower timescale and may drive slowly-varying aspects of non-genetic mitochondrial heterogeneity. Slowly-varying aspects of mitochondrial heterogeneity may be especially important in explaining heterogeneous health outcomes during healthy aging (Lowsky et al., 2013; Sun et al., 2016; Kauppila et al., 2017). Therefore, understanding the dynamics of mitochondrial genetics through time has the potential to be particularly explanatory for age-related mitochondrial dysfunction. There exist various competing theoretical models which have the potential to describe mitochondrial genetic dynamics (Chinnery and Samuels, 1999; Wonnapijit et al., 2008; Poovathingal et al., 2009; Johnston and Jones, 2016; Aryaman et al., 2018); future interdisciplinary studies are required to constrain which of these models are statistically best able to describe experimental data (see e.g., Johnston et al., 2015 for an example of how statistical inference can constrain different theoretical explanations of the mitochondrial bottleneck).

In order to deepen our understanding of mitochondrial genetics, it is important for experimental studies to shift away from heteroplasmy measurement in bulk cellular samples, and toward single-cell studies. In bulk measurements, there is no way to determine whether individual cells are homoplasmic or heteroplasmic: this has important consequences for development and mitochondrial inheritance

(Johnston and Jones, 2015; Johnston et al., 2015). Furthermore, in the context of variations in mtDNA copy number between experimental conditions, inferences about differences in heteroplasmy in tissue homogenate can be erroneous and apparently display selective effects where there are in fact none (Hoitzing et al., 2017a). Such difficulties may be circumvented when heteroplasmy is measured at the single-cell level.

We have highlighted several other important outstanding questions in the field of mitochondrial heterogeneity:

- What explains the apparent ubiquity of mtDNA copy number density homeostasis, despite mtDNA density being a potential axis of energetic control?
- What is the extent, and physiological importance, of microheteroplasmy (**Figure 1Aii**)?
- To what extent do inherited mtDNA mutations, vs. somatic mtDNA mutations, contribute to healthy aging?
- To what extent does mitochondrial exchange affect heteroplasmy dynamics?
- To what extent can non-genetic mitochondrial heterogeneity in factors such as pH, ROS production, and cristae structure be explained by genetic mitochondrial heterogeneity?
- Under what circumstances, and to what extent, is mitophagy selective under physiological conditions?
- To what extent are mtDNAs asymmetrically partitioned amongst proliferating cells *in vivo*?
- To what extent may mtDNAs complement each others genetic defects through sharing of gene products?

The richness in sources of mitochondrial heterogeneity, as well as the growing appreciation of its pathophysiological importance, will likely provide future insight into the determinants of cellular heterogeneity and its associated pathologies.

AUTHOR CONTRIBUTIONS

NJ conceived the project. JA performed the literature review and wrote the manuscript with input from IJ and NJ.

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