MITOCHONDRIAL GENETICS AND EPIGENETICS

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MITOCHONDRIAL GENETICS AND EPIGENETICS

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HYPOTHESIS AND THEORY
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Varied Mechanisms and Models for the Varying Mitochondrial Bottleneck

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Mitochondrial DNA (mtDNA) molecules exist in populations within cells, and may carry mutations. Different cells within an organism, and organisms within a family, may have different proportions of mutant mtDNA in these cellular populations. This diversity is often thought of as arising from a "genetic bottleneck." This article surveys approaches to characterize and model the generation of this genetic diversity, aiming to provide an introduction to the range of concepts involved, and to highlight some recent advances in understanding. In particular, differences between the statistical "genetic bottleneck" (mutant proportion spread) and the physical mtDNA bottleneck and other cellular processes are highlighted. Particular attention is paid to the quantitative analysis of the "genetic bottleneck," estimation of its magnitude from observed data, and inference of its underlying mechanisms. Evidence that the "genetic bottleneck" (mutant proportion spread) varies with age, between individuals and species, and across mtDNA sequences, is described. The interpretation issues that arise from sampling errors, selection, and different quantitative definitions are also discussed.

Keywords: mtDNA, bottleneck, development, inheritance, modeling, uncertainty, heterogeneity

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1. INTRODUCTION

Mitochondria are vital energy-producing compartments in eukaryotic cells. As a result of their evolutionary history, they retain small genomes (mtDNA) which encode important respiratory machinery. In humans and other species, mtDNA molecules are inherited uniparentally, rarely recombine, and can acquire damaging mutations (Wallace and Chalkia, 2013). As hundreds or thousands of mtDNA molecules exist in the same cell, mutations may be present in some but not all molecules: we refer to the fraction of molecules in a cell with a given mutation as the "mutant proportion". MtDNA molecules within the same cell can harbor many different genetic variants at low proportions, a situation called microheteroplasmy (Guo et al., 2013). The mutant proportion associated with each single genetic variant is of scientific and translational interest, particularly as some variants (e.g., point mutations) have pathological consequences above a certain "threshold" proportion (Rossignol et al., 2003; Johnston and Burgstaller, 2019).

If mothers passed an identical mutant proportion onto each offspring, the buildup of mutations would eventually cause extinction (Muller, 1964). As a result, a developmental process has evolved to generate cell-to-cell variability in mutant proportion in animal germlines (Carling et al., 2011; Jokinen and Battersby, 2013; Stewart and Chinnery, 2015; Zhang et al., 2018)[1]. Thus, while some oocytes may receive higher mutant proportions, some will receive lower loads. Rather than all of a mother's oocytes having 50% mutant proportion, for example, they may range from 20 to 80% (**Figure 1A**). Oocytes with lower mutant proportions may then go on to become viable offspring, avoiding the buildup of mutation over generations. This increase in the oocyte-to-oocyte variance

[1]Other mechanisms, outside the scope of this article, exist to mitigate mtDNA mutation in other taxa (Johnston and Burgstaller, 2019).

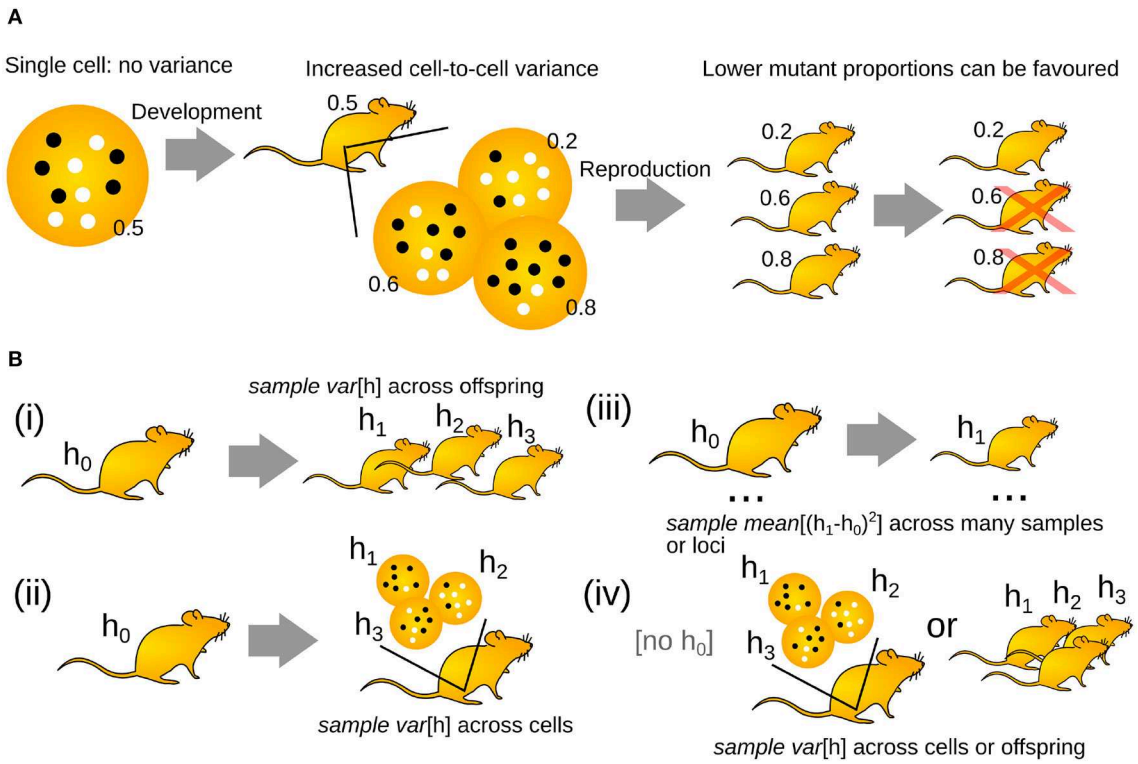

FIGURE 1 | The "genetic bottleneck" increases cell-to-cell mutant proportion spread. **(A)** A mother's life begins as a single cell, with no associated variance in mutant proportion (white circles are wildtype mtDNAs, black circles are mutant mtDNAs; inset numbers give mutant proportion). Development increases cell-to-cell mutant proportion spread in the mother's developing oocytes. In the next generation, oocytes or offspring with lower mutant proportions may be favored. **(B)** Different experimental structures to investigate the generation of mutant proportion spread. (i) Comparing mutant proportion in a mother to her offspring. (ii) Comparing mutant proportion from a reference measurement to a set of individual oocytes. (iii) Comparing mutant proportion differences in a set of mother-child pairs. (iv) Recording mutant proportion differences across oocytes or siblings.

of mutant proportion is typically discussed as resulting from a "genetic bottleneck." Increasing cell-to-cell mtDNA variance has also been reported in somatic tissues, suggesting that the "genetic bottleneck" picture may also apply outside the germline (Sekiguchi et al., 2003; Wilton et al., 2018).

Oocyte-to-oocyte, and offspring-to-offspring, variance in mutant proportion is important in the fundamental biology of inheritance, and in human health and disease. While beneficial from an evolutionary perspective, this variance makes it hard to predict mtDNA inheritance patterns. As diseases result from high mutant proportions (Rossignol et al., 2003; Wallace and Chalkia, 2013), this unpredictability makes clinical planning difficult for families carrying dangerous mtDNA mutations (Poulton et al., 1998; Sallevelt et al., 2013). As such, substantial scientific effort is spent characterizing the processes that give rise to mtDNA variability.

The picture of the "genetic bottleneck" can be useful as a simple comparative statistic. However, experimental technology and mathematical theory has now advanced to the stage where we can ask (and begin to resolve) questions about the detailed physical mechanisms behind this genetic behavior. This article will attempt to compare the effective models and detailed mechanisms used to understand this important process, and discuss how these vary through biology and in the scientific literature.

1.1. Terminology

The "genetic bottleneck" refers to a genetic quantity—an increase in cell-to-cell variability in mutant proportion. In humans and other animals, the genetic bottleneck is achieved in part (though likely not in full) by a "physical bottleneck" (described further below, and recently reviewed in Zhang et al., 2018). This "physical bottleneck" is a physical reduction in the copy number of mtDNA molecules per cell, which occurs during development. Because the word "bottleneck" appears in both terms, it is sometimes tempting to view the genetic and physical bottlenecks as equivalent. This is not generally the case. Unlike the physical bottleneck, the genetic bottleneck does not directly correspond to a observable number of molecules that can be directly measured by some experiment (Birky, 2001; Johnston and Jones, 2016). A genetic bottleneck of size 10, for example, does not mean that the physical copy number of mtDNAs per cell need ever be 10 at any point during development. As such, the term "mutant proportion spread," with less physical and more genetic implication, will be used here as a synonym for

BOX 1 | Calculation and symbols used for mutant proportion statistics.

Given a set of n heteroplasmy measurements $h_1, h_2, \ldots, h_n$, the *sample mean* $[h] = \frac{1}{n}\sum_{i=1}^{n} h_i$. Different ways exist to calculate sample variance. Typically, the "unbiased sample variance" is used, that is $s^2 = \frac{1}{n-1}\sum_{i=1}^{n}(h_i - \textit{sample mean}\,[h])^2$. The "biased sample variance" is the mean squared difference from the mean $s_n^2 = \frac{1}{n}\sum_{i=1}^{n}(h_i - \textit{sample mean}\,[h])^2$. The use of $n-1$ rather than n, known as Bessel's correction, removes bias in the sample variance. As described in the text, studies calculate *sample var* $[h]$ using either s^2 (usually for **Figure 1B**i,ii,iv) or a mean squared difference approach more like s_n^2 (for **Figure 1B**iii).

In the literature, sample variances s^2 may also be found represented by $\mathbb{V}(h)$, $V(h)$, or σ^2 (but the latter is usually used for population variance). Sample means may be written $\bar{h}$, $\mathbb{E}(h)$, $\langle h \rangle$, μ (but the latter is usually used for population mean).

"genetic bottleneck." Note that a smaller "bottleneck" leads to more spread and vice versa. As described below, the "genetic bottleneck" (mutant proportion spread) may vary with species, individual, time, mtDNA sequence and other factors. The term "mutant proportion spread" perhaps captures this fluidity more than the more rigid "bottleneck."

We use "mutant proportion" rather than "heteroplasmy" because a heteroplasmy level over 50% is semantically difficult: the majority mtDNA type should then strictly be considered the reference type, and heteroplasmy redefined with respect to that type.

When taking biological observations and comparing them to models, *population* and *sample* statistics must be considered. Population statistics are summaries of a quantity—like the mean and variance—over the entire population of interest—for example, all oocytes in an organism. Sample measurements of statistics like mean and variance are those derived from a limited number of samples of a larger population. Experimental limitations usually mean that we must consider sample statistics—for example, a set of 20 oocytes from an organism. By contrast, quantitative models typically phrase their predictions in terms of population statistics. Accidents of sampling may lead to differences between sample measurements and population statistics.

When considering these statistics, different studies often use different symbols for the same quantity (**Box 1**). Here, we will attempt to make equations as verbally "readable" as possible. We write *sample var* $\left[h\right]$ for sample variances, *sample mean* $\left[h\right]$ for sample means, *var* $\left[h\right]$ for population variances and *mean* $\left[h\right]$ for population means. The sample quantities are computed as described in **Box 1**.

2. OBSERVATIONS

The fundamental observation that implies the existence of a "genetic bottleneck" (mutant proportion spread) is that offspring have different mutant proportions to their parents (**Figure 1A**). mutant proportions also differ from offspring to offspring. Therefore, at some point(s) between generations, variability in mutant proportion is induced. Parent-to-offspring differences in mtDNA mutant proportion were first reported in cattle (Hauswirth and Laipis, 1982; Ashley et al., 1989; Koehler et al., 1991). Following this, experimental evidence for a "genetic bottleneck" (mutant proportion spread) has been found in animals from flies (Solignac et al., 1984), crickets (Rand and Harrison, 1986), mice (Wai et al., 2008; Burgstaller et al., 2018), salmon (Wolff et al., 2011), and penguins (Millar et al., 2008) to humans (Marchington et al., 1997; Rebolledo-Jaramillo et al., 2014; Li et al., 2016). Some examples of the variety of experimental bottleneck studies are compiled in **Figure 2**.

A mother starts her life as a single fertilized oocyte. As this is a single cell, there is no cell-to-cell variability in mutant proportion; there is only a single value. The oocytes that later develop in that mother, however, may vary substantially in mutant proportion. This suggests that the reason for offspring differences may be the induction of cell-to-cell mtDNA variability in germline development.

To compute the size of the "genetic bottleneck" (mutant proportion spread), we need a set of "before and after" measurements (**Figure 1B**). Often, the "before" measurement is taken from a mother. Different studies have different "after" observation structures. In animal models and some human experiments, sets of "after" observations are obtained: for example, measurements across a set of offspring (**Figure 1B**i), or a set of single-cell oocyte measurements (**Figure 1B**ii). Developmental studies aimed at identifying mechanisms rather than "bottleneck size" may take samples of oocytes or their precursors at different stages of development. In other experiments, particularly in human population genetics, a single "after" observation is taken: for example, a single offspring (**Figure 1B**iii). Many before-after pairs are then used to characterize the population. When a "before" observation is not available, mutant proportion spread may be characterized from "after" measurements and some estimate of the "before" state is constructed (**Figure 1B**iv). This estimate is often the sample mean of the "after" measurements, thus assuming that no selective shift has occurred.

The mutant proportion variability for a system is typically reported as the sampled variance across a set of "after" observations *sample var* $\left[h\right]$ (**Figure 1B**). Most models describing mtDNA statistics (see below) predict that the population variance will follow the form:

$$var\left[h\right] = h_0(1-h_0) \times \ldots, \tag{1}$$

where ... is some expression that may vary according to the model, and h_0 is the mutant proportion in the initial "before" population from which sampling takes place (not the new "after" population). In other words, most models predict cell-to-cell mutant proportion variance to depend on initial mutant proportion h_0, and specifically to be proportional to $h_0(1-h_0)$.

Because most models have the above form, we often work with a quantity which we here call "mutant proportion spread" but which is usually called "normalized heteroplasmy variance":

$$\textit{sample var}'\left[h\right] = \frac{\textit{sample var}\left[h\right]}{h_0(1-h_0)}. \tag{2}$$

Reference	Organism	Intergenerational / developmental?	Tissue	Calculation	sample var' [h] before technical uncertainty
Solignac et al. 1984	Fly	Intergenerational	Oocytes	From V(h)	0.054 to 0.17
Rand et al. 1986	Cricket	Intergenerational	Organism	From V(h)	0.025 to 0.11
Ashley et al. 1989	Cattle	Intergenerational	Brain/liver	From V(h)	0.41
Howell et al. 1992	Human (14560)	Intergenerational	Blood	From V(h)	0.11
Jenuth et al. 1996	Mouse	Intergenerational	Tail	From V(h)	0.033 to 0.18
Bendall et al. 1996	Human	Intergenerational	Blood	Bayesian fit with binomial model	Variable by individual, interpreted as 0.071 to 0.33
Millar et al. 2008	Penguin	Intergenerational	Blood	MSD	0.032
Wolff et al. 2011	Salmon	Intergenerational	Fin	From V(h)	0.0059 to 0.018
Monnot et al. 2011	Human (3243)	Intergenerational	Blood (also PGD samples)	From V(h)	0.27
Guo et al. 2013	Human	Intergenerational	Blood	Correlation approach	Interpreted as 0.005
Rebolledo-Jaramillo et al. 2014	Human	Intergenerational	Blood, cheek	MSD	0.029
Pallotti et al. 2014	Human (3243)	Intergenerational	Several	From V(h)	Interpreted as 0.24 to 0.25
Li et al. 2016	Human	Intergenerational	Blood	MSD	Variable by locus, around 0.11
Wilson et al. 2016	Human	Intergenerational	Blood meta-analysis	Beta	Variable by locus, incl modes around 0.4 for 8993 and 0.1 for 3243
Otten et al. 2018	Human	Intergenerational	PGD samples	Kimura fit	Variable by locus, incl 0.79 for 8993, 0.18 for 3243
Jenuth et al. 1996	Mouse	Developmental	Single oocytes	From V(h)	Variable by stage, 2.4e-7 to 0.0019 PGCs; 0.017 to 0.053 primary oocytes; 0.013 to 0.21 mature oocytes
Marchington et al. 1997 / Poulton et al. 1998	Human	Developmental	Single oocytes	From V(h)	Interpreted as 0.11
Brown et al. 2001	Human	Developmental	Single oocytes	V(h) w/o h0	0.13
Wai et al. 2008	Mouse	Developmental	Single oocytes	V(h) w/o h0	Variable by time, 0.0015 to 0.0022 4 dpc; 0.013 to 0.043 8+ dpc
Wolff et al. 2011	Salmon	Developmental	Single oocytes	From V(h)	0.007 to 0.014
Lee et al. 2012	Monkey	Developmental	Blastomeres	V(h) w/o exact h0	Variable by division, 0.031 2-cell, 0.065 4-cell, 0.095 8-cell
Burgstaller et al. 2018	Mouse	Developmental	Oocytes (also offspring)	From V(h)	Variable by age, around 0.01 for young mice and 0.12 for old mice

FIGURE 2 | Mutant proportion spreads observed in different systems. Some examples of the diverse mutant proportion spread *sample var'* [*h*] observed experimentally. Loci in brackets refer to specific human mtDNA mutations; PGC, primordial germ cell.

The reason for working with *sample var'* $[h]$ is that its normalized value does not typically depend on the specific initial mutant proportion values h_0 from one particular experiment. The results from different experiments, with different values of h_0, can then be more naturally compared.

These variability observations are typically studied from two different perspectives. First, at the "statistical" level: what is the distribution of mutant proportions that will arise from a given mother? This perspective often uses "genetic bottleneck size" as a single number that reflects the observed sample-to-sample mutant proportion spread. Second, at the "mechanistic" level: what physical mechanisms give rise to this distribution of mutant proportions? This perspective attempts to link the coarse-grained outcome of the "genetic bottleneck" to specific, measurable physical rates and properties. In this article, we will first discuss concepts related to this first perspective, before surveying recent progress on the second.

3. THE "GENETIC BOTTLENECK" ABSTRACTED AS SAMPLING EVENTS OR DRIFT

3.1. Abstracting the "Genetic Bottleneck" as a Single Sampling Event

For convenience, studies often describe the "genetic bottleneck" (mutant proportion spread) as the result of a single abrupt event that creates many new individuals, with different mutant

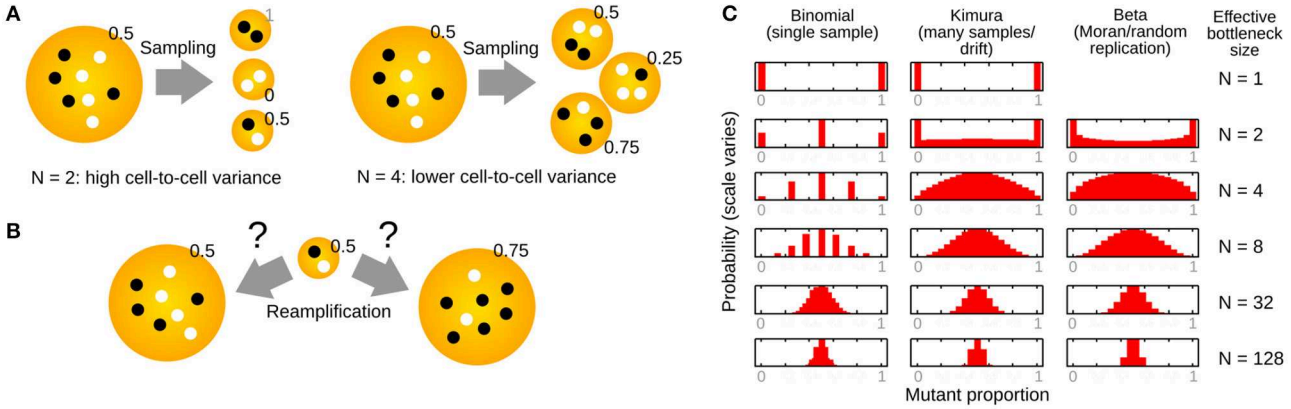

FIGURE 3 | Constructing new populations from random sampling of an initial population. **(A)** Sampling an initial population to construct many new populations of size *N*. Smaller *N* generates more variability between the new populations. **(B)** Reamplification of sampled populations to the original size can be deterministic (left, preserving mutant proportion) or stochastic (right, changing mutant proportion). **(C)** Structures of several distributions related to the study of the "genetic bottleneck" (mutant proportion spread), parameterized by effective "bottleneck size" *N*.

proportions, from an initial individual (**Figure 3A**). In this case, the resulting "bottleneck size" is simply a readout of mutant proportion spread, and does not directly correspond to any physical observable. In particular, it is not generally equal to the minimum copy number of mtDNA molecules (the "physical bottleneck") (Birky, 2001; Jokinen and Battersby, 2013; Johnston and Jones, 2016; Zhang et al., 2018). This is because the "genetic bottleneck" folds together all mechanisms that can influence mutant proportion spread—the physical bottleneck, cell divisions, random mtDNA dynamics, and so on. The specific number associated with "genetic bottleneck size" may therefore be substantially lower than the physical bottleneck during development.

The goal in this perspective is typically to characterize the "genetic bottleneck" (mutant proportion spread) under different conditions. These may involve, for example, different genetic features, different populations, and different species. Knowledge of the value associated with the "genetic bottleneck" (mutant proportion spread) in these cases can inform fundamental biology and clinical planning (Sallevelt et al., 2013).

The concept underlying this approach is a model of "random sampling." Here, we start with an initial population of mtDNA, with mutant proportion h_0. To create one instance of a final population—for example, the population in one oocyte in the next generation—we randomly sample that initial population. Specifically, we pick at random one member of the initial population and put an mtDNA molecule of that type in our final population. If we are sampling "with replacement," we retain the picked member in the original population. The alternative is sampling "without replacement," which involves removing the picked member from the source population so it cannot be picked again.

If we use N picks with replacement to construct one new population, and another N picks with replacement to construct a second new population, and so on, the new populations will likely differ (**Figure 3A**). This is because we are likely to choose different numbers of each mtDNA type when we are constructing the new populations.

Quite how different the new populations will be depends on N, the number of picks. If we just pick $N = 1$ mtDNA from our source population for each new population, different new populations may differ substantially: each will contain only one mtDNA type, so some populations will have a mutant proportion of 0 and some a mutant proportion of 1. By contrast, if N is high, we draw many samples from our initial population, and are likely to end up with new populations that look rather like the initial one (with mutant proportions close to h_0). We can immediately see that our mutant proportion spread (genetic bottleneck) will decrease as N decreases (**Figure 3A**).

This process is called binomial sampling. The actual variance between our new populations is well-known from theory, and is

$$var\left[h\right] = \frac{h_0(1-h_0)}{N}. \tag{3}$$

A common picture of the "genetic bottleneck" is exactly this N. That is, if we observe a certain mutant proportion spread across cells or samples, we work out how large or small N would have to be to generate that amount of spread through this binomial sampling, and call this number the "genetic bottleneck."

How we estimate N depends on the structure of our experiment (**Figure 1B**). First consider the case where we have a single "before" measurement and a set of "after" measurements (for example, a mother mutant proportion and a set of offspring (**Figure 1B**i) or oocyte (**Figure 1B**ii) mutant proportions). Take the sample variance $sample\ var\left[h\right]$ of the "after" measurements. Call the "before" measurement h_0. Then the definition of "bottleneck size" is often taken to be

$$N = \frac{h_0(1-h_0)}{sample\ var\left[h\right]} = \frac{1}{sample\ var'\left[h\right]} \tag{4}$$

based on this binomial sampling picture. If h_0 is not known, as in **Figure 1B**iv, it is sometimes estimated to be equal to

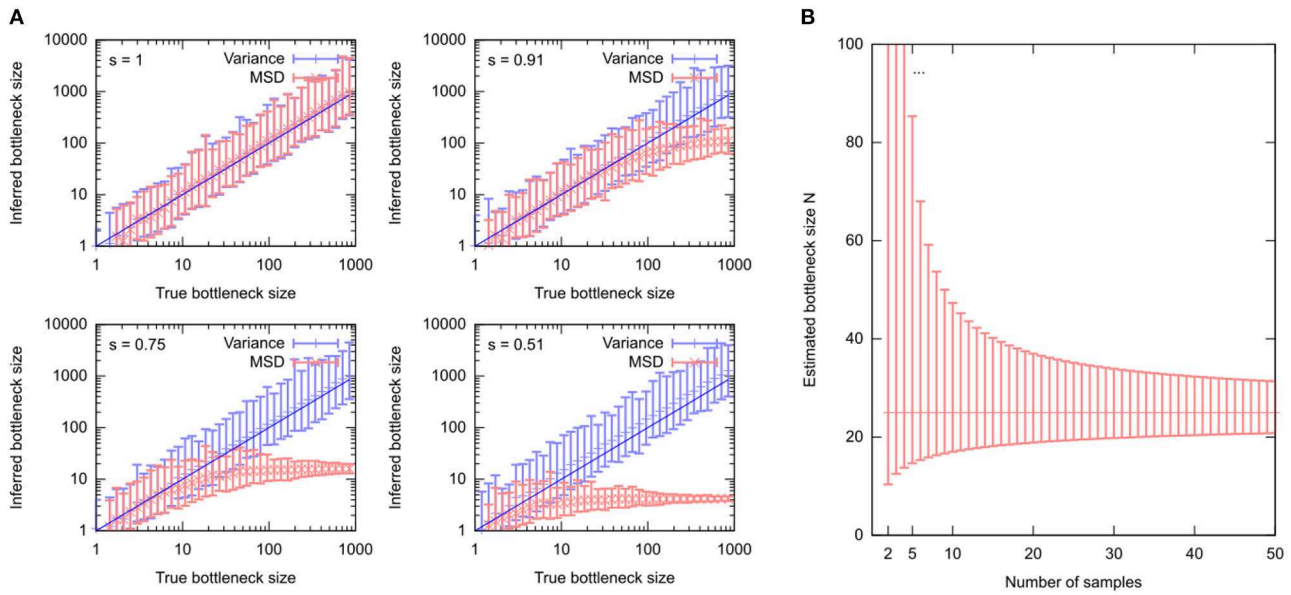

FIGURE 4 | Estimating the "genetic bottleneck" from different data sources. **(A)** Simulated data, comparing estimates of "bottleneck size" N using either 16 "after" measurements ("Variance") or 16 sets of single "after" measurements ("MSD"). Estimates are performed in the presence of different levels of selection ($s = 1$, no selection; decreasing s is increasing selective pressure). **(B)** Uncertainty in a "bottleneck size" estimate using $mean\,[h] = 0.5$, $var\,[h] = 0.01$, and different numbers of samples n. Particularly for $n < 10$, bottleneck size estimates can have large uncertainty.

sample mean $\left[h\right]$. That is, the assumption is made that no shift in mutant proportion has taken place due to selection or accidents of sampling.

The idea here is to convert a less intuitive quantity *sample var'* $\left[h\right]$ into a more intuitive one (an effective number of segregating units). However, this binomial sampling picture has some issues. First, it does not correspond to a plausible biological mechanism. Development does not involve a single, abrupt sampling event. How reamplification of mtDNA back to its original level takes place is rarely considered (**Figure 3B**), although models for reamplification do exist (see below). Second, and related, a binomial sampling regime predicts a binomial distribution for final mutant proportion (**Figure 3C**). For a small value of N, this means that mutant proportion can only take one of a restricted set of values. For example, if N is 4, we would only expect mutant proportions of 0, 25, 50, 75, and 100% after sampling. Other models have been proposed to address these shortcomings (see below).

Next, consider the case where we have a set of paired "before" and "after" observations (for example, the mother-single offspring pairs in **Figure 1B**iii). The prevailing approach to calculate a "bottleneck size" here is via an expression derived in references (Millar et al., 2008; Hendy et al., 2009) based on

$$N = \frac{h_0(1-h_0)}{sample\ mean\left[(h-h_0)^2\right]}. \tag{5}$$

Here, the spread of "after" measurements $sample\ var\left[h\right] = \frac{1}{n-1}\sum_i (h_i - sample\ mean\left[h\right])^2$ has been replaced by the mean square difference between the "before" and "after" measurements $\frac{1}{n}\sum_i (h_i - h_0)^2$. That is, the approach assumes that the average "after" measurement *sample mean* $\left[h\right]$ is equal to the "before" measurement h_0—in other words, that no shifts in mean mutant proportion act between generations. If selection is in fact present, Equations (4) and (5) give different results, and Equation (5) quickly fails to capture the true bottleneck size even in abstracted systems (**Figure 4**). Because Equation (5) deals with squared differences, selective shifts in different directions do not "cancel out" but rather reinforce the resultant discrepancy.

3.2. The "Genetic Bottleneck" Abstracted as Several Sampling Events or Drift

A single binomial sampling event does not represent a real biological mechanism. To improve this picture, some studies consider the "genetic bottleneck" (mutant proportion spread) as arising from a series of sampling events, modeling cell divisions that randomly partition mtDNA molecules between cells. Early work on mtDNA inheritance (Solignac et al., 1984; Rand and Harrison, 1986; Ashley et al., 1989; Howell et al., 1992) drew on a classical result from Sewall Wright (Wright, 1942, 1984) to this end. This result describes the spread of allele frequencies due to "accidents of sampling" in repeated generations, where the individuals in one generation are a random sample from the previous generation. For mtDNA, this "Wright equation" predicts

$$var\left[h\right] = h_0(1-h_0)\left(1-\left(1-\frac{1}{N}\right)^{kn}\right), \tag{6}$$

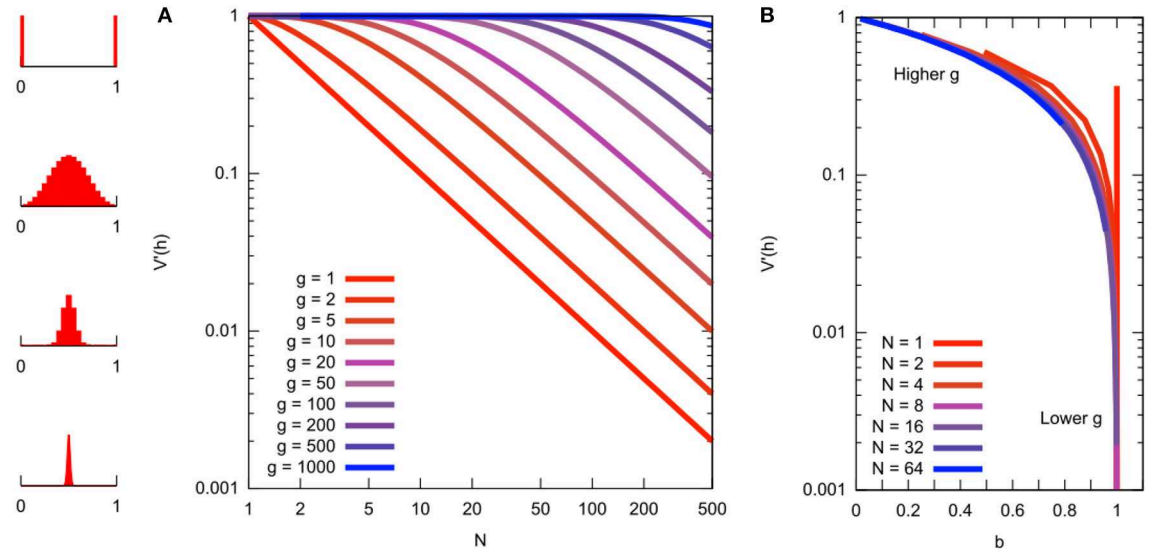

FIGURE 5 | Relationship between different quantities related to mutant proportion spread. **(A)** Mutant proportion spread with different sampling parameters. Horizontal axis gives "bottleneck size" N. Vertical axis gives corresponding mutant proportion spread *sample var'* $[h]$ (from Equation 6 with $g = kn$). Different traces are for different numbers of sampling events g. To convert a *sample var'* $[h]$ value to a "bottleneck size" N, choose the number of sampling events g and read off the corresponding value (distribution sketches at the far left give illustrations of the Kimura distribution for the various *sample var'* $[h]$ values). **(B)** Mutant proportion spread with summary parameter b, from simulated sampling dynamics. Horizontal axes gives "bottleneck parameter" b. Vertical axis gives corresponding mutant proportion spread *sample var'* $[h]$. Behavior at different N and g are now "folded together," collapsing on the same line.

where k is the number of random samplings per generation (for example, the number of cell divisions in germ line formation) and n is the number of generations. The reader will notice that if $kn = 1$, describing a single sampling event as above, Equation (4) is recovered.

For convenience, some studies have since defined new "bottleneck parameters" to simplify this expression. One choice is to set $\alpha = (1 - 1/N)^k$. Another more recent alternative is to define $b = \exp(-g/N)$. Here $g = kn$ represents an amalgamated number of samplings, and the exponential form is used for algebraic convenience because $\exp(-g/N) \simeq (1-1/N)^g$. In these cases, Equation 6 becomes:

$$var\left[h\right] = h_0(1 - h_0)\left(1 - \alpha^n\right) \simeq h_0(1 - h_0)\left(1 - b\right). \qquad (7)$$

The advantage of using these "bottleneck parameters" is that they fold together two unknown quantities: the number of generations and the effective population size. Under Equation (6), readouts of "bottleneck size" (mutant proportion spread) using N are contingent on a particular choice of g, the number of generations for which the bottleneck applies (**Figure 5A**). Readouts using b (and α) absorb this dependency, providing a simple readout of mutant proportion spread that makes no assumptions about the number of sampling events (**Figure 5B**). Given a number of generations, N can be recovered from b via $N = -g/\ln b$.

An approximation of the cell-to-cell distribution of mutant proportion under these repeated-sampling models is the so-called Kimura distribution (Wonnapinij et al., 2008) (**Figure 3C**). Strictly, the assumptions involved in deriving this approximation rely on N being large (Kimura, 1955). However, the Kimura distribution reproduces intuitive behavior for the distribution of mutant proportion under drift, and several studies use a fit to the Kimura distribution to estimate b (Wonnapinij et al., 2008; Otten et al., 2018).

The relationship between these quantities N, b, g, and *sample var'* $\left[h\right]$ is illustrated in **Figure 5**, which may serve as a reference for comparison of reported "genetic bottleneck" (mutant proportion spread) statistics in different studies.

3.3. Drift Manifest Through Random Replication

One issue with a simple sampling picture is that it predicts a set of cellular mtDNA populations consisting of N molecules. In most circumstances, this N value is much lower than the size of typical cellular populations. For example, in animal germline development, the number of mtDNA molecules per cell is amplified several orders of magnitude from a minimum copy number back to a functional level (Cree et al., 2008; Wai et al., 2008; Cotterill et al., 2013; Zhang et al., 2018). If this reamplification happened perfectly deterministically, no further change in $var\left[h\right]$ would occur. However, cell biology is rarely deterministic, and there is good reason to believe that this reamplification process involves a random component (Birky, 1994; Chinnery and Samuels, 1999; Capps et al., 2003; Johnston et al., 2015).

Several recent studies have considered models for this reamplification (Johnston and Jones, 2015; Wilson et al., 2016). Most are based on the idea of random mtDNA replication. That is, an mtDNA molecule is randomly chosen from the current

population, and replicated. Then a new mtDNA molecule is randomly chosen and replicated, and so on until the desired population size is achieved. This is a modified Moran model (Moran, 1958) (the usual Moran model involves removing one molecule per replication, so that overall population size remains constant), also known as a Pólya urn model (Eggenberger and Pólya, 1923; Johnson and Kotz, 1977).

In the limit of infinite reamplification, the model gives rise to a beta distribution for mutant proportion spread (**Figure 3C**). Infinite reamplification may not seem realistic, but actually the structure of this distribution is quickly stabilized after a relatively small number of replications, so the simple infinite limit is similar to more reasonable cases. However, results also exist for intermediate cases, and their exploration may be a fruitful area of future research. The beta distribution takes two parameters, α and β, intuitively corresponding to the number of mutant and wildtype molecules in the cell before any replication. If we $\alpha = h_0 N$ and $\beta = (1 - h_0)N$, the mean of the beta distribution is $mean\left[h\right] = h_0$ as expected, and the variance of the beta distribution is

$$var\left[h\right] = \frac{h_0(1-h_0)}{N+1}. \tag{8}$$

3.4. Uncertainty

The "genetic bottleneck" (mutant proportion spread) is a readout of variance. Revealing trends in cell-to-cell variance is more challenging than revealing trends in average behavior, and requires more data. Wonnapinij et al. (2010) have drawn attention to the challenging nature of obtaining reliable estimates of mutant proportion spread. Uncertainty in estimated mutant proportion spread is often large, challenging precise estimates of the "bottleneck size" and leading to variability in these estimates. Even in the case of no technical error (see below), sampling errors can lead to large variability in estimates of "bottleneck size," particularly if fewer than 10 samples are used (**Figure 4B**).

Uncertainty in readouts of variance can be an unintuitive quantity. We are perhaps more used to thinking about mean values as the quantity of interest, with variance around a mean value corresponding to uncertainty. However, we can—and should—also describe and estimate the uncertainty associated with an observation of variance.

One way to estimate uncertainty in *sample var* $\left[h\right]$ involves assuming that mutant proportion samples are drawn from a normal distribution. This is not generally the case (as seen in **Figure 3C**), but is a simple illustration that may be applied when spread is low. Confusingly, there are two expressions in circulation for the sampling error in this case. Which of these values gets used depends on how the variance was computed. If the variance is calculated using an estimate of the mean taken from the same dataset (employing Bessel's correction, as with many "after" measurements), Wonnapinij et al. (2010) cite:

$$SE\left[sample\ var\left[h\right]\right] = var\left[h\right] \times \sqrt{\frac{2}{n-1}}, \tag{9}$$

for the standard error in *sample var* $\left[h\right]$, where n is the number of samples used to characterize *sample var* $\left[h\right]$. If the mean is estimated from a different source (omitting Bessel's correction, as with mean-squared-difference calculations using a single "after" measurement), an estimate of the variance of the sample variance is $(var\left[h\right])^2(n-1)/n^2$, as quoted in reference (Millar et al., 2008), corresponding to a standard error of $var\left[h\right] \times \sqrt{(n-1)/n^2}$. The standard error associated with a variance measurement can then be estimated by using $var\left[h\right] \simeq sample\ var\left[h\right]$ in these expressions.

However, for wide spreads or means close to 0 or 1, mutant proportion distributions do not have normal structure. In this case (Wonnapinij et al., 2010), cite a more general result:

$$SE\left[sample\ var\left[h\right]\right] = \sqrt{\frac{1}{n}\left(D_4 - (var\left[h\right])^2 \times \left(\frac{n-3}{n-1}\right)\right)} \tag{10}$$

where $D_4 = (n-1)/n^3 \times ((n^2 - 3n + 3)\mu_4 + 3(2n - 3)\mu_2^2)$, and $\mu_2 = 1/n\sum_{i=1}^{n}(h_i - h_0)^2$, $\mu_4 = 1/n\sum_{i=1}^{n}(h_i - h_0)^4$. While more complicated in structure, all these quantities can readily be worked out from the set of observed mutant proportion measurements.

All these expressions have the standard error of *sample var* $\left[h\right]$ scale roughly with the observed value divided by $\sqrt{n}$. Thus, unless a large number n of samples are used to characterize mutant proportion spread, the associated uncertainty in *sample var* $\left[h\right]$ can be rather high. As "bottleneck size" estimates depend on $1/sample\ var\left[h\right]$, the corresponding uncertainty can be enormous for low sample sizes (**Figure 4B**).

These expressions are based on the statistics of sampled variances, and assume that the sample mutant proportion values themselves have no associated uncertainty (in other words, there is no technical error associated with the genetic measurement). Technical error should also be included in the uncertainty associated with these estimates. Several studies include considerations of technical error in their estimates of mtDNA statistics (Bendall et al., 1996; Millar et al., 2008; Li et al., 2016; Wilson et al., 2016). This is typically achieved through simple uncertainty propagation, that is, considering an observed variance to be a combination of natural variance and technical variance. The technical variance may either be quantified through experimental calibration (Millar et al., 2008) or as part of a statistical inference process (Bendall et al., 1996; Li et al., 2016; Wilson et al., 2016).

3.5. Results

Early reports of the size of the genetic bottleneck (mutant proportion spread) varied substantially across organisms. Contributing to this variability was the fact that different studies used different values of kn in Equation (6). These different values reflected, for example, estimates of the number of cell divisions involved in germline development in different species. More recently, it has become more common to set $kn = 1$ and assume a single binomial sampling event, or to use a "bottleneck parameter," usually b, to summarize mutant proportion spread as above. **Figure 2** summarizes the mutant proportion spreads observed in several key experimental studies across species.

The rapid intergenerational shifts observed in cattle (Hauswirth and Laipis, 1982; Koehler et al., 1991) have given rise to the highest mutant proportion spread values so far observed. Insects appear to have lower mutant proportion spreads (Solignac et al., 1984; Rand and Harrison, 1986). In mice, several experiments have observed the increase of mutant proportion spread through germline development (Jenuth et al., 1996; Wai et al., 2008). Fish show similar behavior (Wolff et al., 2011).

Mutant proportion spread in humans was observed some time ago (Bendall et al., 1996; Marchington et al., 1997), but its magnitude remains debated. Variability in the behavior of mutant proportion spread was quickly apparent. Blok et al. found dramatic skew toward extreme mutant proportions in transmission of the 8993 mutation (Blok et al., 1997). Lutz et al. (2000) found evidence for variable mutant proportion spread in a human family; while they did not provide quantitative estimates they noted that the different spreads they observed suggest a varying "bottleneck size" which could be very small. Bendall et al. (1996) used a Bayesian approach to show that it was unlikely that their study families had the same "bottleneck size." More recently, two large-scale population-genetic studies suggest rather different "bottleneck sizes" (Rebolledo-Jaramillo et al., 2014; Li et al., 2016). Pathogenic mutations seem to involve more mutant proportion spread, particularly the 8993 mutation (Blok et al., 1997; Monnot et al., 2011; Wilson et al., 2016; Otten et al., 2018). Ongoing preimplantation genetic diagnoses approaches continue to provide data on mutant proportion spread at different developmental stages (Monnot et al., 2011; Treff et al., 2012; Sallevelt et al., 2013). Pallotti et al. (2014) performed a meta-analysis of 3243 bottlenecks along with their own experiments and found reasonable consistency in mutant proportion spread. Notably, different studies still use different protocols for reporting a "bottleneck size," sometimes setting $g(= kn) = 24$ or $g(= kn) = 1$ in Equation (6).

While not a focus of this article, we note that genetic bottlenecks (increasing mutant proportion spread) (Sekiguchi et al., 2003; Wilton et al., 2018) and physical bottlenecks (Cao et al., 2007; Otten et al., 2016; Floros et al., 2018) have also been reported in somatic tissues.

4. THE "GENETIC BOTTLENECK" AS A SET OF PHYSICAL PROCESSES

In parallel with statistical characterization of the "genetic bottleneck" (mutant proportion spread), related research attempts to understand the physical processes that give rise to an observed "genetic bottleneck" (mutant proportion spread) in a given system. The goal here is typically to identify biological mechanisms and potential targets for intervention.

A plausible physical mechanism for the "genetic bottleneck" (mutant proportion spread) must account for both physical and genetic observations over time during development. The physical observations involve mtDNA copy number per cell and the occurrence of cell divisions; the genetic observations involve cell-to-cell variability in mutant proportion. An example from a meta-analysis of mouse observations is shown in **Figures 6A,B**. The joint prediction of these physical and genetic observations is very important because it constrains the mechanisms that are possible—for example, the size of the physical bottleneck, the timing of cell divisions, and the rate of reamplification all influence the resulting genetic statistics of mtDNA populations.

While not a focus of this article, the specific genetic players behind the physical processes below are increasingly being revealed, and have been reviewed in, for example, references (Carling et al., 2011; Jokinen and Battersby, 2013).

4.1. The Physical Bottleneck During Development

One process that occurs during germline development in animals is a physical reduction in the number of mtDNA molecules per cell (Zhang et al., 2018). This reduction is observed in animals including mice (Cao et al., 2007; Cree et al., 2008; Wai et al., 2008), fish (Wolff et al., 2011; Otten et al., 2016), sheep (Cotterill et al., 2013), and humans (Floros et al., 2018). For some time after fertilization, cell divisions repeatedly halve the cellular mtDNA population, with little compensatory replication. This halving leads to a pronounced drop in mtDNA copy number per cell (**Figure 6A**i). A fertilized oocyte typically contains many mtDNA molecules [hundreds of thousands in mice (Cree et al., 2008; Wai et al., 2008); around a million in humans (Floros et al., 2018)]. The size of the physical bottleneck—that is, the lowest copy number of mtDNA per cell during development—remains debated, but is often orders of magnitude lower; Zhang et al. (2018) have recently provided a survey of mtDNA reduction in different species. In mice, the lowest copy number may lie between 200 and 1,000 (Cao et al., 2007, 2009; Cree et al., 2008; Wai et al., 2008; Johnston et al., 2015) (**Figure 6A**ii). In humans, mean copy numbers around 1400 are observed in progenitor germ cells (Floros et al., 2018). In zebrafish, decreases from tens of millions to hundreds of mtDNAs per cell are observed (Otten et al., 2016). The copy number of mtDNA during development seems to depend on genetic characteristics of the mtDNA (Monnot et al., 2013), potentially making the physical bottleneck sequence-dependent.

Pictured as drawing a random selection of mtDNA molecules from a larger population, copy number reduction provides a way to generate variability between cells. Additionally, the magnitude of variability generated through other random sampling processes is amplified by low copy numbers.

4.2. Random Replication of a Subset of mtDNA Molecules

In this mechanism, at some point(s) in germline development, a random subset of a cell's mtDNA population is allowed to replicate, while all others are eventually subject to degradation or loss (Wai et al., 2008). This subset may be, for example, those mtDNAs within a certain distance of the nucleus (Wallace, 2018). As the random subset chosen will differ in different cells, this process imposes a natural sampling inducing variance between cells. A smaller subset of molecules will lead to more mutant proportion spread.

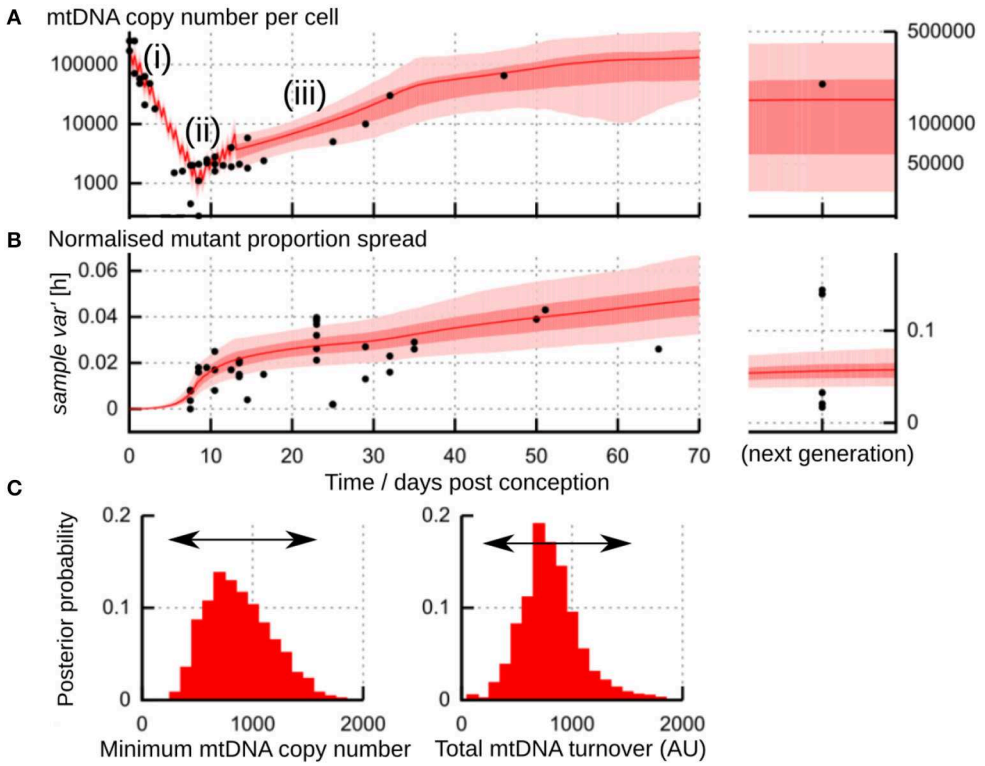

FIGURE 6 | Flexibility in the physical processes underlying the "genetic bottleneck." **(A)** Physical mtDNA dynamics (copy number per cell) in mouse germline development. (i) repeated cell divisions after fertilization with little compensatory mtDNA replication lead to a drop in copy number to a minimum "physical bottleneck" (ii). Copy number is subsequently reamplified (iii) through later development. **(B)** Dynamics of *sample var'* [*h*] in mouse germline development. In **(A,B)**, datapoints (black) are amalgamated from references (Jenuth et al., 1996; Cao et al., 2007; Cree et al., 2008; Wai et al., 2008); shading shows posterior distributions from the most-supported "birth-death-partitioning" model, involving random mtDNA turnover and partitioning at cell divisions (Johnston et al., 2015). **(C)** Observations of physical and genetic dynamics **(A,B)** are best fit by a model that allows a flexible "physical bottleneck" (left, posterior distribution) which can be compensated by a flexible amount of mtDNA turnover (right, posterior distribution). Figure uses results from Johnston et al. (2015).

Wai et al. observed a sharp increase in mutant proportion spread in mice aged between 4 and 8 days (as in **Figure 6B**) (Wai et al., 2008; Samuels et al., 2010). Using microscopy, they showed that only a subset of mtDNA molecules was involved in replication at a given time. They propose this subset replication model as the mechanism by which variability is generated at this development stage (folliculogenesis, **Figure 6A**iii). Johnston et al. (2015) suggest that this observation is also compatible with random mtDNA turnover (see below), where a non-fixed subset of mtDNAs is expected to be involved in replication at any given time.

The random replication model described above, connected to the beta distribution, can describe the dynamics of the subset-replication model. Care must be taken here to ensure that physical copy number dynamics are reproduced: for example, the small amount of replicating mtDNAs must balance the large number of degrading mtDNAs as copy number is amplified (**Figure 6A**iii).

4.3. Random Partitioning of mtDNA Molecules at Cell Divisions

This mechanism is possible during specific times when cells are undergoing divisions. At division, a "parent" cell distributes its population of mtDNA to its two "daughter" cells. The assignment of each mtDNA molecule to one or the other daughter may follow a random process (Birky, 2001; Huh and Paulsson, 2011; Johnston et al., 2012). In this case, each division will increase the cell-to-cell mutant proportion variability between daughter cells. If mtDNA molecules are partitioned in clusters, this increase will be faster (Cao et al., 2007). Larger clusters will lead to more mutant proportion spread.

Whether the "unit of inheritance" of mtDNA is a single molecule or a cluster is a debated question. MtDNA within mitochondria is packaged into complexes called nucleoids. These were thought to contain around 5–10 mtDNA genomes (Jacobs et al., 2000; Cao et al., 2007; Khrapko, 2008), suggesting that clusters of mtDNA may be the natural state. However, evidence from microscopy suggests that nucleoids may only contain around 1 mtDNA genome (Kukat et al., 2011). Model selection for mouse germline development (Johnston et al., 2015) and human transmission (Li et al., 2016) both suggest that single mtDNA molecules are the unit of inheritance.

Observations in rhesus monkeys (Lee et al., 2012) showed a dramatic induction of variance by the 8-cell stage, presumably due to random partitioning of mtDNAs over the first three cell divisions. In this study, mtDNA admixtures in oocytes were

created by fusing two cytoplasm halves from different oocytes. Cell divisions then immediately followed the construction of these admixed oocytes. It is thus not inconceivable that physical heterogeneity in the distribution of mtDNA molecules, remaining from the cytoplasm fusion, may contribute to this high mutant proportion spread. For example, if the fusion process created a "north hemisphere" containing exclusively one mtDNA type and a "south hemisphere" containing exclusively the other, and the first cell division occurred along the "equator," the resultant cells would then immediately have maximum mutant proportion differences. Natural systems may be expected to have more physically mixed mtDNA populations, and so potentially show less extreme mutant proportion spreads in these early stages.

The "repeated sampling" approaches above attempt to model cell divisions during development as a series of random binomial samples. Partitioning dynamics can also be embedded in stochastic models of mtDNA replication and degradation (Johnston and Jones, 2015, 2016). Much of this work assumes binomial partitioning; however, recent work in yeast has suggested that partitioning of mtDNA is tighter than binomial sampling (Jajoo et al., 2016). Mathematical results do exist for more controlled partitioning, or the partitioning of clusters of mtDNA (Johnston and Jones, 2015) but are often complicated, so simulation is often used to make quantitative predictions in these cases (Johnston et al., 2015; Li et al., 2016).

4.4. Random Turnover of mtDNA Molecules

MtDNA replicates and degrades quasi-independently of the cell cycle. The noisy environment of the cell means that these processes have a random component (Birky, 1994; Chinnery and Samuels, 1999; Capps et al., 2003; Johnston et al., 2015). The ongoing action of this random turnover creates cell-to-cell mutant proportion variability. For example, two cells that start with identical mtDNA populations will diverge over time, as different molecules undergo replication and degradation. Faster turnover, or turnover of clusters, will lead to more mutant proportion spread.

To account for the full set of processes that an individual mtDNA molecule may undergo, several stochastic modeling approaches have been developed (reviewed in Hoitzing et al., 2017). These approaches model every individual mtDNA molecule in a cell and subjects them to the physical processes that we may expect to occur during development. Typically, these processes will have a random component, so that if the model is simulated twice, the precise outcomes will differ. These differences can be used to characterize the variability supported by different mechanisms.

A well-known model involves "relaxed replication," that is, replication of mtDNA independent of the cell cycle (Birky, 1994). Models of this process typically involve mtDNA molecules degrading with a fixed rate, and replicating randomly with a rate that depends on population size (Chinnery and Samuels, 1999; Capps et al., 2003). This model generates variability over time because of these random dynamics. Cree et al. propose this mechanism, amplified by the physical bottleneck, to generate mutant proportion spread in mouse development (Cree et al., 2008).

More recently, the different ways that the cell could control this replication rate have recently been explored in detail using "birth-death" models (Johnston et al., 2015; Johnston and Jones, 2016; Hoitzing et al., 2019). Strikingly, this work showed that no matter how the cell controls mtDNA replication, if there is some mutant proportion, the variance of this mutant proportion will increase linearly over time.

Specifically, in a population of N mtDNAs, random turnover of molecules with rate β over time t gives rise to the behavior

$$sample\ var'\left[h\right] = \frac{2f\beta t}{N}, \tag{11}$$

so that, for example, a year of mtDNA turnover, with average rate one degradation event per week, in a cell with 1,000 mtDNA molecules would give a mutant proportion spread of $(2 \times 52)/1,000 = 0.104$. This would be interpreted as a "bottleneck size" around 9.6. In followup theoretical developments (Aryaman et al., 2019), the factor f in Equation (11) has been shown to be the fraction of unfused mitochondria, that is, mitochondria containing mtDNAs subject to mitophagy (Youle and Narendra, 2011; Diot et al., 2016). Mitochondrial quality control, linked to fission-fusion dynamics, contributes to the turnover of mitochondria in the cell (Twig et al., 2008) and provides one way that mitochondrial dynamics may influence both mean and variance dynamics of mtDNA populations (Hoitzing et al., 2015; Johnston, 2018; Latorre-Pellicer et al., 2019). Higher rates of quality control related turnover can result in higher cell-to-cell mutant proportion variance (Johnston et al., 2015) [and, if mitochondria associated with one mtDNA type are preferentially degraded, this selective pressure will also influence mean mutant proportions (Twig et al., 2008; Hoitzing et al., 2015)]. Equation (11) provides a coupling between the physical fission-fusion dynamics of mitochondria and the time behavior of mtDNA mutant proportion spread (Hoitzing et al., 2015; Johnston, 2018; Aryaman et al., 2019).

4.5. Combinations of Mechanisms

Several of these processes are conceptually linked. For example, when a cell divides, it loses around half of its mtDNA content, immediately restricting the subset of mtDNAs that are available for replication. If mtDNA molecules are involved in ongoing random turnover, only a subset of molecules will be replicating at any given time (Johnston et al., 2015).

In each of these cases, a smaller mtDNA population acts to amplify increases in mutant load spread, because the influence of random events is less "smoothed out" in small populations. Therefore, we can end up with the same amount of spread by either (i) generating a smaller amount and amplifying it more through small population size; or (ii) generating a larger amount and amplifying it less. Indeed, analysis of mouse data suggests that the same amount of spread can be achieved with a small physical bottleneck and less mtDNA turnover (less generation, more amplification) or a large physical bottleneck and more mtDNA turnover (more generation, less amplification) (Johnston

et al., 2015) (**Figure 6C**). This flexibility may help reconcile differing reports on the size of the physical bottleneck (Cao et al., 2007, 2009; Cree et al., 2008; Johnston et al., 2015). It is not inconceivable that some mechanism may allow the cell to sense and control this choice, so that, for example, embryos with slightly lower mtDNA turnover have their mtDNA populations depleted more to compensate.

To consider these mechanisms together, the birth-death framework above was coupled to a description of cell divisions to provide a detailed stochastic model of germline development in mice (Johnston and Jones, 2015; Johnston et al., 2015). Compared to other detailed models, this birth-death-partitioning model provided the best fit to a meta-analysis of existing physical and genetic data. The best model for cell-to-cell spread of mutant proportion had two components: a contribution from partitioning at cell divisions and a contribution from ongoing drift due to mtDNA turnover.

The birth-death-partitioning model provides closed-form, though complicated, expressions for full distributional details of mutant proportion at all times through development, which well-predicted independent experimental observations of mutant proportion distributions in oocytes (Johnston et al., 2015). The combined birth-death-partitioning model was also used to provide an update to the Wright equation (Equation 6) to include random mtDNA turnover (Johnston and Jones, 2016), predicting:

$$sample\ var'\left[h\right] = 1 - \left(1 - \frac{1}{N}\right)^{g} + \frac{4t}{3N\tau}, \qquad (12)$$

where N is now a physical mtDNA copy number, g a physical number of cell divisions, t is time and τ is the timescale of mtDNA degradation. Append:

The final term in Equation (12) estimates the ongoing increase in mutant load spread due to mtDNA turnover, increasingly linearly with time t.

5. RECENT TOPICS

5.1. Model Selection and Predictions

We have discussed a range of different proposed mechanisms for the "genetic bottleneck" (mutant proportion spread). A comparatively recent set of studies has attempted to identify the mechanisms that are most supported by data. This has been attempted through the use of model selection (Kirk et al., 2013), a process that compares the statistical support for different mechanisms while guarding against overfitting. Li et al. used likelihood-based model selection with a human dataset to provide support for a "genetic bottleneck" (mutant proportion spread) that varies for different sequences and involves individual mtDNAs (rather than clusters) as segregating units (Li et al., 2016). Johnston et al. used likelihood-free model selection for mouse data to identify the mechanism(s) most supported by data. They found little support for partitioning of clustered mtDNA, and most support for the birth-death-partitioning model above, which was further supported by followup experiments (Johnston et al., 2015). A theoretical comparison of different models for mtDNA control (Johnston and Jones, 2016) revealed the above principles of increasing variance that hold regardless of which specific mechanism is true. More recently, large-scale inter-generational data from mice was used in a statistical framework to identify which processes influence mtDNA statistics during development and aging (Burgstaller et al., 2018).

These detailed mathematical models present the opportunity to refine the prediction of mutant proportion distributions. The birth-death-partitioning model predicted distributional details of oocyte mutant proportion in developing mice (Johnston et al., 2015). Based on the picture of increasing mutant proportion spread in aging oocytes, a simple model involving a variation of a logit-normal distribution for mutant proportion predicted distributional details of mutant proportion in mouse litters (Burgstaller et al., 2018).

5.2. Sequence-Specific Behavior in Mutant Proportion Spread

Substantial recent attention has been focussed on whether the genetic bottleneck (mutant proportion spread) is sequence-specific. Evidence for this hypothesis includes observations from different pathological mtDNA mutations (Monnot et al., 2011; Wilson et al., 2016; Otten et al., 2018). Consideration of different human variants in a population genetic context also suggests that the magnitude of the genetic bottleneck (mutant proportion spread) depends on the specific variant under investigation (Li et al., 2016). A particularly striking difference appears to exist between the 3243 and 8993 mutations (Monnot et al., 2011; Wilson et al., 2016; Otten et al., 2018). The aforementioned population study (Li et al., 2016) also found a variable-size bottleneck to be most statistically supported for non-pathological mutations.

As discussed throughout, sequence-specific proliferative advantages of one mtDNA type over another can confound attempts to analyse the genetic bottleneck (mutant proportion spread). A sequence-specific increase in mutant proportion spread can arise without a proliferative difference between sequences: for example, if one sequence experiences both higher replication and degradation rates, increasing random turnover without an overall selective advantage. Conversely, under some experimental designs, sequence-specific differences in the behavior of mean mutant proportion (i.e., proliferative differences) could be interpreted instead as differences in mutant proportion variance if it is assumed that no proliferative differences exist (as in **Figure 4A**). Further theoretical work unpicking the behavior of mtDNA statistics as mean and variance change together will be useful in interpreting these observations.

5.3. Ongoing Increase of Mutant Proportion Spread During Aging

Recent large-scale intergenerational data in mice has shown an ongoing increase in mutant proportion spread in oocytes over time in adult mice (**Figure 7**). This increasing oocyte-to-oocyte spread of mutant proportion with age has been directly observed in mouse oocytes (Burgstaller et al., 2018), and has been shown to be more statistically supported than a constant-spread model in

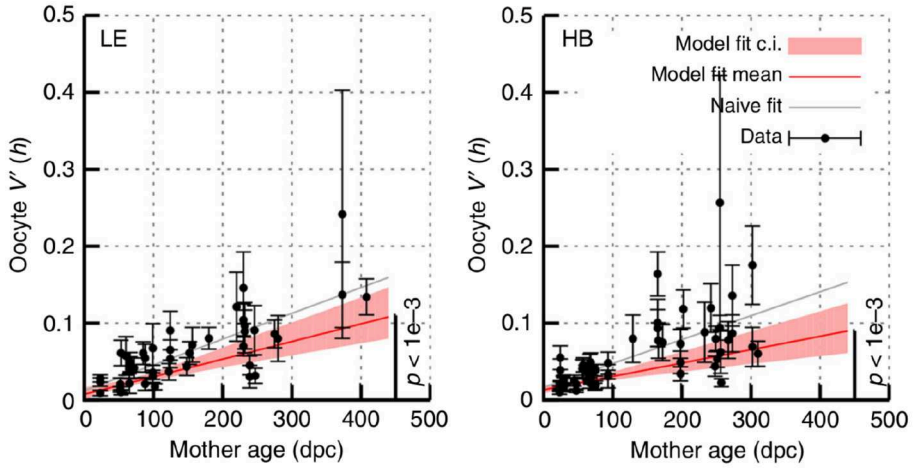

FIGURE 7 | Increasing mutant proportion spread in oocytes with mouse age. Data from Burgstaller et al. (2018), reporting *sample var'* [*h*] in sets of individual oocytes from mice of different ages. HB and LE label two genetic models, involving admixtures of wild-derived haplotypes HB and LE, respectively with haplotype C57Bl/6N. Error bars are derived using Equation (10); "model fit" accounts for this uncertainty and "naive fit" simply fits the bare observations. In all cases a significant linear increase in *sample var'* [*h*] with time, following Equation (11), is observed.

independent observations in flies, mice, and humans (Johnston and Jones, 2016).

The mechanism(s) behind the ongoing shrinking of the genetic bottleneck (increasing mutant proportion spread) remains unclear (Johnston et al., 2015; Zhang et al., 2018). However, random turnover of mtDNA may be a reasonable candidate mechanism (Johnston et al., 2015; Johnston and Jones, 2016; Burgstaller et al., 2018). The cumulative action of stochastic replication (and degradation) is to generate cell-to-cell spread in mitochondrial statistics, including in mutant proportion. Other processes like diversifying selection, physical clustering, and even mutagenesis could all contribute to the observed increase in spread.

These results are from systems involving cellular admixtures of two main haplotypes. Other results suggest a consistent picture, for example, showing an increasing number of heteroplasmic sites in children from older mothers, which the authors suggest is likely attributable to oocyte aging (Rebolledo-Jaramillo et al., 2014). Another study found non-uniform changes in heteroplasmy with age in humans (Sondheimer et al., 2011).

In light of this observation, this article would advocate an additional careful analysis of the contribution of maternal age to observed mutant proportion patterns. As we expect the genetic bottleneck (mutant proportion spread) to decrease with age, any systematic differences in age between these compared variances could confound other relationships. Conversely and more positively, appropriate accounting for age would help increase the statistical power of these comparisons.

6. THE PROBLEM OF SELECTION

Throughout the above, we have alluded to the problems that systematic selection for one or more mtDNA types can cause in these analyses. Theory describing the influence of selection has been established, but is complicated (Johnston et al., 2015). In particular, if approaches that assume the absence of selection are used when it is in fact present, errors can arise in estimates of genetic properties and physical mechanisms. As pointed out above, these issues may lead to dramatic underestimation of "bottleneck size," and cannot be assumed to "cancel out."

Several of the results above are valid only in the absence of selection: when no mtDNA type experiences an advantage over any other. This is known to be false for many mtDNA pairings in many somatic tissues, where selection for one mtDNA type over another is often observed (reviewed in Burgstaller et al., 2014). Selection in the germline has been more debated, but evidence is increasing. In several studies, the transmission of pathological mutations seems to be subject to selective pressure. The maximum level of transmission for the 3243 mutation in humans has appeared to be limited (Monnot et al., 2011; Otten et al., 2018), and selection against severe mtDNA mutations has been observed in mice (Fan et al., 2008). Recent observations in mice (Burgstaller et al., 2018; Latorre-Pellicer et al., 2019) and humans (Wei et al., 2019) have indeed observed selection at different loci. Burgstaller et al. (2018) suggest that selection may act in different directions at different developmental stages (very recently supported by Latorre-Pellicer et al., 2019), and that these directions may either cancel out or provide a net selective shift. Mathematical theory for the behavior of mutant proportion spread when selection is present remains less well-developed and represents an important future theoretical target. The birth-death-partitioning approach in references (Johnston et al., 2015; Johnston and Jones, 2016) can account for selection but are mathematically complicated. Otten et al. (2018) have proposed a truncated Kimura distribution to describe a selective regime where mutant proportions above a certain value are prohibited, and found that it is supported by observations of the 3243 mutation.

Comparison to the Kimura distribution is often used to argue for an absence of selection. However, this approach must be interpreted with caution. Depending on the mechanism of selection, Kimura-distributed samples may be observed even

when selection has occurred. In particular, as approaches using the Kimura distribution sometimes use several "after" but no "before" measurements, it is possible that an early shift in mutant proportion will not be detected.

7. CONCLUSIONS

7.1. The Variable "Genetic Bottleneck"

This article has attempted to review the various models and mechanisms that have been considered for the "genetic bottleneck" (mutant proportion spread). Some diversity in reported mtDNA behavior comes from the choice of analysis protocol: the use of bottleneck parameters, rather than bottleneck sizes that allow a choice of "generation number," can help avoid this. The reporting of *sample var*$'[h]$, the fundamental observation from which these statistics are derived, and its associated uncertainty, will also help interpretability and comparison.

Ongoing research has provided evidence that the "genetic bottleneck" (mutant proportion spread) varies with age, species, individual, and genetic features. Intriguingly, the coupling of physical and genetic behavior of mitochondria (Equation 11; Tam et al., 2013; Aryaman et al., 2019) suggests that heterogeneity in mitochondrial dynamics may induce heterogeneity in mutant proportion.

A diverse range of studies on the mtDNA bottleneck continues to provide a wealth of insight into this important process. However, the very diversity of this research risks confusion arising, particularly around aspects of the prevailing terminology. This article has attempted to clarify some of the concepts involved, to serve as a reference for the increasingly interdisciplinary community working in this field.

Some takehome messages for reference include:

- The "genetic bottleneck" is a readout of mutant proportion spread that is generally not an observable physical quantity, and is measured reported in diverse ways through the literature;
- Observations of mutant proportion spread can have substantial uncertainty both from sampling and technical error, particularly if under 10 samples are used (when the standard error can approach half the observation);
- The physical mechanisms underlying the "genetic bottleneck" (mutant proportion spread) include a combination of copy number reduction (a physical bottleneck), random replication and degradation of mtDNA molecules, and random partitioning at cell divisions;
- The magnitude of the physical bottleneck appears to be flexible, as flexibility in mtDNA turnover can compensate to produce the same effects on mutant proportion spread;
- The presence of mtDNA selection complicates estimates of mutant proportion spread, and different experimental designs report different statistics in this case;
- The "genetic bottleneck" (mutant proportion spread) likely varies by species, individual, age, and mtDNA sequence.

DATA AVAILABILITY STATEMENT

This study did not generate new experimental data. Code for the simulations and visualizations involved is publically available at https://github.com/StochasticBiology/bottleneck-review.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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frontiers in Genetics

REVIEW
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Higher Order Organization of the mtDNA: Beyond Mitochondrial Transcription Factor A

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The higher order organization of eukaryotic and prokaryotic genomes is pivotal in the regulation of gene expression. Specifically, chromatin accessibility in eukaryotes and nucleoid accessibility in bacteria are regulated by a cohort of proteins to alter gene expression in response to diverse physiological conditions. By contrast, prior studies have suggested that the mitochondrial genome (mtDNA) is coated solely by mitochondrial transcription factor A (TFAM), whose increased cellular concentration was proposed to be the major determinant of mtDNA packaging in the mitochondrial nucleoid. Nevertheless, recent analysis of DNase-seq and ATAC-seq experiments from multiple human and mouse samples suggest gradual increase in mtDNA occupancy during the course of embryonic development to generate a conserved footprinting pattern which correlate with sites that have low TFAM occupancy *in vivo* (ChIP-seq) and tend to adopt G-quadruplex structures. These findings, along with recent identification of mtDNA binding by known modulators of chromatin accessibility such as MOF, suggest that mtDNA higher order organization is generated by cross talk with the nuclear regulatory system, may have a role in mtDNA regulation, and is more complex than once thought.

Keywords: ATAC-seq, DNase-seq, G-quadruplex, higher order organization, mtDNA, mitochondrial transcription factor A

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INTRODUCTION

The genome of all organisms undergoes concerted cycles of packaging to reduce its volume and to control access to the regulatory mechanisms of transcription and replication. In the eukaryotic nucleus, DNA is compacted into chromatin, which provides differential accessibility in response to a variety of histone modifications (Zhu and Li, 2016). The bacterial genomes, which lack histones, are folded into nucleoids using a set of dedicated proteins, entitled Nucleoid-Associated Proteins (NAPs), such as HU, Histone-like Nucleoid Structuring protein (H-NS) and Structural Maintenance of Chromosomes proteins (SMC). Alongside their architectural role in DNA packaging, these proteins also play a role in other processes, such as replication and chromosome segregation (Badrinarayanan et al., 2015; Dame and Tark-Dame, 2016). Notably, the most commonly used models for investigation of nucleoid organization are *Escherichia coli*,

Bacillus subtilis and *Caulobacter crescentus* (Dame and Tark-Dame, 2016); the latter is an alphaproteobacterium, which belongs to the same phylogenetic branch from which the mitochondria originated (Wang and Wu, 2015).

The circular genomes of *C. crescentus* are organized in ellipsoidal and helical structures between two opposite poles of the cell, creating two 'arms' that are folded around each other (Le et al., 2013). While analyzing interactions between different regions within the *C. crescentus* genome by genome-wide chromatin conformational capture (Hi-C) (Le et al., 2013), 23 preferential Chromosomal Interaction Domains (CID) were identified. CID boundaries seem to closely associate with transcription and replication units. The boundaries tend to reestablish shortly after, or even during DNA replication, possibly to disentangle the newly formed DNA molecules. Additionally, the CID boundaries can be disrupted by transcription inhibition (Le et al., 2013). Novel CID boundaries can be created by artificially moving loci of highly expressed genes into inherently low expressed regions (Le et al., 2013). These findings, strongly suggest that the bacterial nucleoid, including that of alphaproteobacteria, is a highly regulated structure with great importance to DNA replication and transcription.

In addition to their nuclear genome, all eukaryotic cells contain a much smaller cytoplasmic genome—the mitochondrial DNA (mtDNA). This genome originated ~2.5 billion years ago from an ancient endosymbiosis between a former free-living alphaproteobacterium and the progenitor of all eukaryotic cells (Sagan, 1967; Pittis and Gabaldon, 2016). Although during the course of evolution the ancient bacterium lost most of its inherent genetic material either due to transfer to the nucleus, or due to natural selection, the mitochondria in the vast majority of eukaryotes still harbor their own genomes. Despite its modest size, the mammalian mtDNA encodes 13 critical subunits of the oxidative phosphorylation system (OXPHOS), two ribosomal RNA genes and 22 tRNAs that are required for cellular energy production. Mammalian mtDNA forms a protein-DNA structure that was termed 'nucleoid', to highlight its ancient bacterial heritage (see below). The animal mtDNA is four orders of magnitude smaller than the nuclear genome, and has been long thought to be separately regulated from the nuclear genome (Gustafsson et al., 2016). Accordingly, mitochondrial transcription factor A (TFAM) is believed to be sufficient for mitochondrial nucleoid formation (Kaufman et al., 2007) and the primary driver of mtDNA packaging (Gustafsson et al., 2016; Farge and Falkenberg, 2019). The role of TFAM in mtDNA packaging and higher order organization has been recently thoroughly reviewed, and therefore will be mentioned here only briefly (Farge and Falkenberg, 2019). TFAM is highly conserved across species, and despite the apparently linear mtDNA organization in yeast (Gerhold et al., 2010), the yeast orthologue (Abf2p) of TFAM packs this genome as well (Farge and Falkenberg, 2019). mtDNA condensation positively correlates with the cellular concentration of TFAM so that increased TFAM concentration leads to higher degrees of mtDNA compaction (Kukat et al., 2015).

Thus, our current view of mtDNA regulation suggests that a nuclear-encoded yet mitochondrially restricted set of proteins modulates mtDNA transcription, replication and packaging (Gustafsson et al., 2016). For example, mtDNA genes are transcribed by POLRMT, and not RNA Polymerase II which transcribes nuclear mRNAs, and the mtDNA is replicated by DNA polymerase gamma (POLG), which has no accepted role in replication of the nuclear DNA. However, it would be surprising from an evolutionary point of view if the past 2.5 billion years since mitochondrial endosymbiosis had not led to significant adaptation of the regulation of the mitochondrial and nuclear DNA. Is it plausible that the longtime co-existence of the mitochondrion and its host have been accompanied by adaptation of mtDNA to the host regulatory and packaging systems? Co-adaptation of the nuclear and mitochondrial genomes had been demonstrated in the context of the OXPHOS and in the mitoribosomes, which use nuclear DNA-encoded proteins, and either exclusively mtDNA-encoded proteins (in OXPHOS) or mtDNA-encoded rRNA and tRNA transcripts (in the mitoribosome) (Levin et al., 2014). However, the discovery of transcription factors that directly regulate transcription in both the nucleus and in the mtDNA has suggested that the control of gene expression is coordinated not only by signals, but by dual localization of transcription factors (Barshad et al., 2018). Hence, adaptation of mtDNA regulation to the nuclear regulatory system is plausible.

Mitochondrial DNA is compacted through its interactions with TFAM (Kukat et al., 2015), but there is growing evidence for the involvement of additional nuclear-encoded proteins that also regulate nuclear chromatin. This includes MOF (Chatterjee et al., 2016), members of the AP1 family (c-Jun and JunD) as well as CEBPB (Blumberg et al., 2014) and MEF2D (She et al., 2011). The discovery of mtDNA binding and mitochondrial transcriptional regulation by MOF, a histone lysine acetyltransferase that remodels chromatin, was particularly surprising, as it raises questions about its acetyltransferase target in the mitochondria, and its possible role in mtDNA organization. Secondly, c-Jun and JunD, which were recently shown to bind negatively selected sites in the mtDNA (Blumberg et al., 2014), tend to bind nuclear DNA enhancer regions and affect nuclear DNA gene regulation (Phanstiel et al., 2017). Third, CEBPB, a known chromatin remodeler (Bornstein et al., 2014), not only binds the mtDNA *in vivo*, but also serves as a candidate repressor of human mtDNA gene expression (Barshad et al., 2018). Fourth, DNase-seq and ATAC-seq analysis in multiple human and mouse cells revealed a conserved footprinting pattern, which overlapped known mtDNA regulatory elements, yet correlated with low TFAM occupancy in HeLa cells (Blumberg et al., 2018). This ATAC-seq mtDNA footprinting pattern was gradually formed during the course of embryogenesis in both mouse and humans, as reflected by gradually increasing mtDNA occupancy (Marom et al., 2019). Hence, it is possible that there are mtDNA sites which are consistently occupied, and sites that are consistently under-occupied across the mtDNA, and that the mtDNA is bound not only by TFAM but rather by other additional proteins in an

organized manner. This reflects the existence of an organized protein–DNA structure in the mitochondrial genome, thus providing first clues for the existence of a structured higher order organization of the mitochondrial genome.

We would argue that the investigation of the regulation of the mitochondrial nucleoid in the frame of protein-DNA patterns of interactions and their impact on regulation of mtDNA gene expression and replication is of equivalent importance to our understanding of the organization and compaction of the nuclear chromosome, but that it is markedly less well studied and understood. In this essay we will discuss current knowledge regarding the nature of the higher order organization of the mitochondrial genome (**Figure 1**), and assess its functional potential from an evolutionary perspective.

Mitochondrial Nucleoid and mtDNA Content

The only known structural unit of the mitochondrial genome is the nucleoid, which contains mtDNA and closely interacting proteins (Hensen et al., 2014). The nucleoids are vital for mitochondrial function as they coordinate transcription (Rebelo et al., 2011), translation (He et al., 2012) and interact with enzymatic activities of the mitochondrial inner membrane (Wang and Bogenhagen, 2006). There are a correspondingly large number of proteins that can be pulled down by crosslinking to nucleoids, thus reflecting these diverse activities (Bogenhagen et al., 2008). High-resolution microscopy techniques were used to show that nucleoids are compact and ellipsoidal, suggesting the exclusion of non-nucleoid proteins, and that nucleoids are associated with the mitochondrial inner membrane (Brown et al., 2011; Kukat et al., 2011; Kukat et al., 2015). The number of mtDNA molecules in each nucleoid has been a matter of considerable debate. Logically, the nucleoids must at least transiently contain multiple mitochondrial genomes after the completion of replication. Most studies have observed that the copy number is stably higher with estimates ranging from 1.4 to 7.5 genomes per nucleoid [(reviewed in (Lee and Han, 2017)].

MtDNA Folding and Loops—Current and Future Studies

Higher order organization of both the eukaryotic nuclear genome and the bacterial nucleoid involve regulated steps of protein binding followed by bending and folding to allow the interaction of sequences that are distant in the primary DNA sequence. In a study of the mtDNA binding pattern of the mitochondrial transcription termination factor MTERF1 in mammalian cells, simultaneous binding of MTERF1 was observed at the proximal heavy strand promoter (HSP1) and within the MT-TL1 sequence (Martin et al., 2005). This interaction increased the expression of genes regulated by

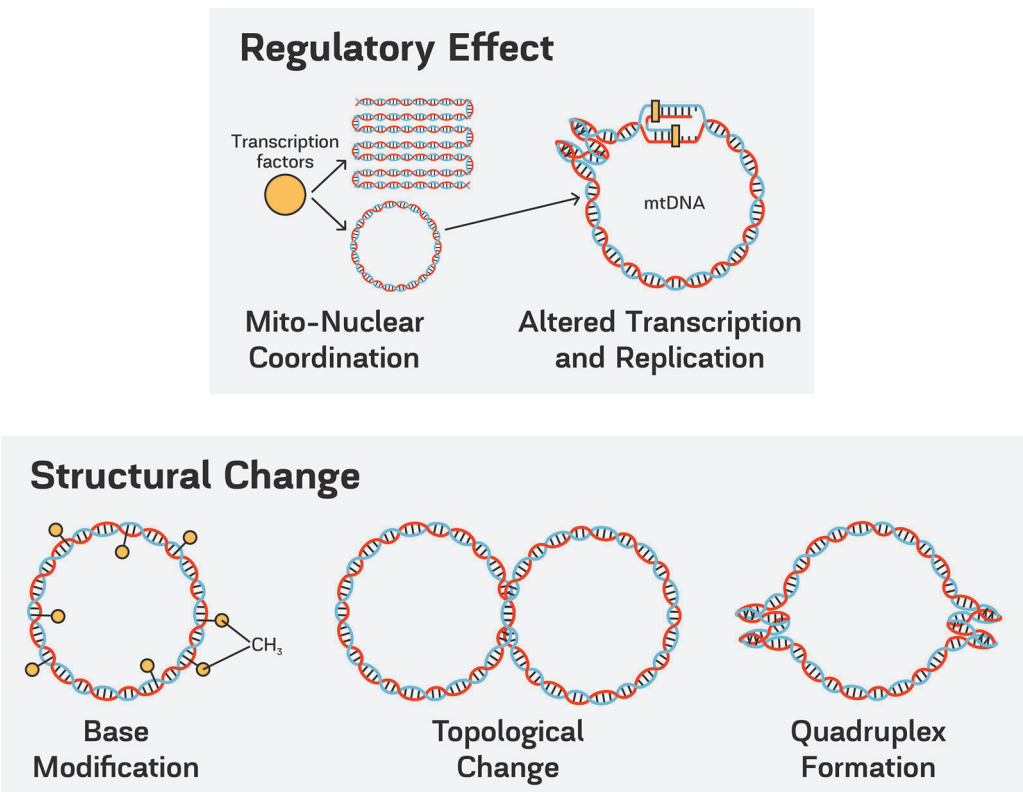

FIGURE 1 | The possible structural changes in the mtDNA and the cross-talk between mtDNA higher order organization and regulation.

HSP1, and a model was proposed that would allow the direct recycling of transcription complexes from the termination point of HSP1 transcription back to its origin. While attractive, the phenotype of an Mterf1 deficient mouse cast doubt upon this elegant concept, as the predicted loss in HSP1 activity was not observed (Terzioglu et al., 2013). Other *cis* interactions along mtDNA are yet to be discovered. Interactions between distant nuclear genomic regions are currently being investigated using sequencing-based techniques such as the Chromosome conformation capture (3C) and subsequent derivative of this methodology (i.e., 4C, 5C and HiC) (Oluwadare et al., 2019), yet all of these techniques are currently designed to identify interactions between regions that are megabases apart, which limit their utility in the study of the human mtDNA. Although a recent study of HiC data claimed to observe direct interactions between the mitochondrial and nuclear genomes (Doynova et al., 2016), no independent study supported such findings. Given the above, there is a need for the development of techniques that will allow mapping of interactions between mtDNA sequences, while taking into account the small size of this genome and its circularity.

Mitochondrial Transcription Responds to Structural Cues Along mtDNA

Mitochondrial transcription uses unique features to allow the differential expression of a very tightly packed genome with a limited number of primary transcripts. Since mitochondrial transcription has recently been reviewed (Gustafsson et al., 2016), here we will only consider the role of the physical structure of mtDNA in the initiation and termination of transcription.

One key challenge for mitochondrial transcription is the use of oppositely oriented promoters that transcribe the same regions in both directions—with strand specific promoters in mammals but bidirectional promoters in birds (L'Abbe et al., 1991; Randi and Lucchini, 1998) and amphibians (Bogenhagen et al., 1986; Bogenhagen and Romanelli, 1988). The human mtDNA harbors a single light-stranded promoter, which is responsible for expression of the OXPHOS complex I subunit *ND6* as well as eight tRNA. The activation of this promoter requires the binding of TFAM, which creates a pronounced U-turn bending in mtDNA, proximal to the site of transcription initiation (Ngo et al., 2011; Rubio-Cosials et al., 2011). The two heavy-strand promoters are closely adjacent to each other, with HSP1 principally driving expression of the two rRNA genes (i.e. the 12S and 16S rRNAs), and HSP2 driving the expression of the remaining twelve protein-coding genes and the distal tRNA genes along the heavy strand (Montoya et al., 1983). Like LSP, TFAM activates HSP1, although studies have come to different conclusions as to the topology of TFAM's interaction at HSP1 (Ngo et al., 2014; Morozov and Temiakov, 2016; Hillen et al., 2017; Uchida et al., 2017).

The balancing of expression of HSP1 and HSP2 has been a matter of some debate. It seems reasonable that some mechanism must exist, since HSP1 is primarily devoted to rRNA and HSP2 to the expression of protein coding genes, which was also shown in living cells (Blumberg et al., 2017). Our group and others have shown that HSP2 is distinct in that TFAM is not only dispensable for activation, but actively inhibits it (Lodeiro et al., 2012; Zollo et al., 2012). We have further shown that the topological state of mtDNA may be important for HSP2 activation. Unique among the promoters, HSP2 is activated by negative supercoiling in a fashion reminiscent of bacterial systems, but no similar effect is seen at LSP and HSP1 (Zollo and Sondheimer, 2017).

The termination of mitochondrial transcription is also regulated by the physical state of mtDNA. Because the molecule is circular and has oppositely oriented promoters, processive transcriptional complexes are at risk of collision. Because of the positioning of genes, the termination of LSP and HSP1 at a point between mt.3229 (the end of the 16S ribosomal RNA as transcribed by HSP1) and mt.4329 (the end of MT-TQ as transcribed by LSP) would allow the simultaneous utilization of LSP and HSP1 without promoter collision. Considerable evidence has been provided for the role of MTERF1 in interaction with mt.3232–3253 (within the coding sequence of MT-TL1), including the crystal structure of the interaction of MTERF1 with its mtDNA target sequences (Yakubovskaya et al., 2010). As noted above, evidence from mouse knockout studies of *Mterf1* agreed only partially with this concept, and suggested that the insulation of the LSP against transcription proceeding back through the promoter might also be important (Terzioglu et al., 2013). It is important to recognize that the regulation of both transcription and mtDNA physical structure in mouse and human may not be identical, but the organization of mtDNA regulatory elements clearly influences interactions with transcription factors to exert control over gene expression. The means of controlling interactions between transcriptional complexes arising from LSP and HSP2 remains undiscovered.

Could mtDNA Packaging and Regulation Be Affected by G-Quadruplex (GQ) Formation?

G-Quadruplexes (GQs) are non-canonical nucleic acid secondary structures that use Hoogsteen hydrogen bonding between guanines on the same strand (Rhodes and Lipps, 2015). The occurrence of GQs within the DNA is not random, and is notably conserved across species, thus supporting selective constraints and hence potential functional importance (Murat and Balasubramanian, 2014). Moreover, whereas transient GQs correlate with binding sites of chromatin remodeling-related transcription factors, genome-wide sites with more stable GQs have been implicated in replication stalling and inhibition of chromatin remodeling (Varizhuk et al., 2019), which support their involvement in regulation of higher order DNA organization. For example, GQ-ChIP-seq experiments revealed that most GQs tend to form within nucleosome-depleted regions with increased transcription activity (Hansel-Hertsch et al., 2016). As GQ structures are mostly resolved by RecQ helicases (Mendoza et al., 2016; Sauer and Paeschke, 2017; Varizhuk et al., 2019), it is noteworthy that one such helicase, RecQ4, is transported into the mitochondria, interacts with DNA POLG

and promotes mtDNA replication (Ding and Liu, 2015). Indeed, due to the asymmetric composition of nucleotides in the heavy (more guanine-rich) and light (more cytosine-rich) strands of mtDNA, the heavy mtDNA strand is prone to GQ formation. Previously, *in silico* analysis suggested the existence of G-quadruplex-forming motifs throughout the human mtDNA (Falabella et al., 2019). Imaging of mtDNA using GQ binding dyes showed that they are widely present (Huang et al., 2015), and the application of compounds that bind to GQ impact mtDNA transcription and replication (Falabella et al., 2019). We have recently demonstrated that GQ formation can even selectively bias the replication of a mixed mtDNA population (heteroplasmy) (Naeem et al., 2019). Hence, similar to the nuclear genome, GQ formation in the human mtDNA affects the regulation of this genome.

Although *in vitro* experiments suggested that TFAM binds to GQ at non-physiological concentrations (Lyonnais et al., 2017), analysis of ChIP-seq TFAM binding experiments in HeLa cells revealed TFAM occupancy throughout the mtDNA (Wang et al., 2013), yet low occupancy of TFAM at GQ-forming regions (Blumberg et al., 2018). Moreover, we showed that G-quadruplex-forming motifs tend to co-localize with conserved DNase-seq footprinting sites in adult cells (Blumberg et al., 2018) and during development (Marom et al., 2019). Other proteins such as the ATP-dependent Lon protease bind GQ sequences *in vitro* (Lu et al., 2003), and *in vivo* (Lu et al., 2007). Thus, it is plausible that investigation of the conformation assumed by such motifs *in vivo* will offer clues for the discovery of novel mtDNA binding proteins that may be involved in the construction and regulation of its higher order organization. Interestingly, nuclear DNA regions that tend to be packed late during the cell cycle, and are prone to breakage, also harbor non-B DNA structures (Dong et al., 2014). Specifically, GQ structures are resolved at the DNA, likely by the Pif1 helicase, to allow maintenance of the mtDNA (Bannwarth et al., 2016). Indeed, double mutant *Pif1* mice exhibit elevated levels of mtDNA damage. As in the nuclear genome, hotspots for chromosomal aberrations and fragile sites tend to correlate with the state of chromatin accessibility (Mishmar et al., 1999). Further investigating the patterns of non-canonical DNA structure may offer additional insights to differential accessibility of sites across the mitochondrial genome.

Structural mtDNA Aberrations in Aging and Disease: Potential Impact on the Higher Order mtDNA Organization

Chromosomal aberrations of various types in the nuclear genome (i.e. inversions, deletions, insertions, duplications and translocations) not only change the location of genes, but also change the location of regulatory elements, thus changing the chromatin structure and regulatory landscape of the modified region. As discussed above, regulatory factors bind the mtDNA not only within the non-coding promoters' region, but rather throughout the mitochondrial genome [reviewed in: (Barshad et al., 2018)].

Therefore, it is logical that mtDNA aberrations such as deletions, duplications, inversions and insertions may not only change the coding content, but will change the location of regulatory elements and hence have the potential impact on mtDNA regulation. Consistent with this hypothesis, and because of the high gene density of mtDNA, structural rearrangements and deletions are poorly tolerated. The association between mitochondrial deletions and pathology is robust. The accumulation of deletions during the process of aging was discovered nearly thirty years ago (Cortopassi and Arnheim, 1990) and at nearly the same time it was recognized that the Kearns-Sayre syndrome was also linked to deletions in mtDNA (Shoffner et al., 1989). The phenotypic impact of mtDNA deletions has been largely interpreted as the result of the loss of genetic material. The effect of such mtDNA aberrations on mtDNA regulation *in vivo* merits further investigation.

Is it possible that structural aberrations are not random, preferentially occurring at positions of special mtDNA organization? Indeed, the 4,977 bp deletion has previously been shown to be flanked by simple repeat sequences with the tendency to form non-B DNA structures (Hou and Wei, 1998). Interestingly, non-B DNA structures tend to co-localize in general with other types of mtDNA deletions that accumulated with aging (Hou and Wei, 1996; Damas et al., 2012). Specifically, as already discussed above, G-quadruplex forming sequences tend to occur at such breakpoints (Dong et al., 2014), and affect mtDNA transcription *in vitro* (Hillen et al., 2017). Hence, it is logical to suggest the existence of mtDNA hotspots for aberrations. In the nuclear genome hot spots for chromosomal aberrations tend to occur in regions with special chromatin organization (Mishmar et al., 1999; Fungtammasan et al., 2012), which calls for assessing such connection in the mtDNA as well.

Structural Differences in mtDNA Across Evolution

mtDNA aberrations do not only associate with human pathologies, but also led to changes in mtDNA gene order and content during the course of evolution. As an example, although the mitochondrial genome remained circular in most studied metazoans, it is linear in *Medusozoa* (Kayal et al., 2012). Secondly, although most vertebrate mtDNAs contain a non-coding region, which harbors most known regulatory elements, the chordate amphioxus nearly lacks a non-coding region (Spruyt et al., 1998; Boore et al., 1999), which prevents identification of the positions of orthologous regulatory elements. Third, fragmentation of the mtDNA into several co-segregating parts that together comprise the full gene content seen in vertebrates has been described in organisms such as lice (Shao et al., 2012) and certain nematodes (Phillips et al., 2016). Do such mtDNA rearrangements affect mtDNA regulation? A recent study of *in vivo* mtDNA transcription using the precision global run-on transcription-sequencing (PRO-seq) revealed, that although the mtDNA gene contents in *Drosophila* and *Caenorhabditis elegans* are nearly identical to that of humans, the gene order and gene content per mtDNA strand profoundly changed (Blumberg et al., 2017). We recently showed that such changes were accompanied by the emergence of a very different mtDNA transcriptional initiation and termination schemes *in vivo* (Blumberg et al., 2017). Specifically, we observed that in

contrast to human mtDNA which harbors two heavy strand and one light strand transcriptional initiation sites, Drosophila had 5-7 initiation sites, and C elegans had a single transcription initiation site, consistent with their mtDNA strand-gene contents. These phenomena exemplify how changes in mtDNA organization, during the course of evolution and in human diseases, likely lead to changes in mtDNA regulation.

As the recently identified mtDNA DNAse-seq and ATAC-seq footprinting patterns appears to be conserved between human and mouse (Blumberg et al., 2018), it would be of interest to study such in organisms with different mtDNA organization, as well as in human cells with pathological mtDNA deletions. Such study will directly assess the impact of mtDNA aberrations on mtDNA higher order organization and while engaging such study with techniques that assess transcriptional pattern *in vivo* (such as PRO-seq) one will be able to assess the connection between such changes with alteration in mtDNA regulation.

The Management of mtDNA Structure—Mitochondrial Topoisomerases as Key Players

The structure of mtDNA and its accessibility is also impacted by topoisomerases, single or double-strand DNA-cleavage proteins that are used to alter the topological state of DNA, keeping it available for transcription and replication and preventing the formation of knots or other unusable structures (Vos et al., 2011). The issues faced by mtDNA that must be resolved by topoisomerase are distinct from those seen in linear chromosomes and include the resolution of concatameric structures formed by mtDNA replication (Kolesar et al., 2013).

There is a single known topoisomerase that is specific for the mitochondrion, TOP1MT (Zhang et al., 2001). This is a type IB topoisomerase, capable of relaxing supercoiling by single-strand cleavage and strand passage. Surprisingly, mice deleted for the homologous *Top1mt*, are viable, although they do show evidence of increased supercoiling of their mtDNA (Zhang et al., 2014). Instead, *Top1mt*$^{-/-}$ animals had increased activity of type IIA topoisomerases, suggesting the capacity for compensation for the loss of Top1mt activity.

The presence of type IIA topoisomerases is probably required in mitochondria, since these proteins fulfill the requirement for the de-catenation of linked molecules of mtDNA. Top2β has dual localization to the mitochondria and nucleus, with a shortened isoform present in the mitochondrion (Low et al., 2003). Top2β is canonically responsible for type IIA activity in non-proliferating cells. Although Top2α was not initially identified in the mitochondrion, recent studies have confirmed that it does locate within the organelle (Zhang et al., 2014).

The topoisomerases collectively appear to play important roles in regulating the supercoiling and also the transcription of mtDNA (Sobek et al., 2013). This provides a striking echo of our growing understanding of the role of topoisomerases in regulating nuclear transcription (McKinnon, 2016). The continuing studies, particularly of the bigenomic type IIA topoisomerases, may increase our understanding of how nuclear and mitochondrial transcription are coordinately regulated using template topology, a mechanism of control that is strikingly conserved from bacteria to man.

MtDNA Methylation and Acetylation of TFAM

Nuclear chromatin is regulated by DNA and protein modifications including the methylation of cytosines and the acetylation of specific lysine residues in histones. Such changes directly correlate with chromatin accessibility and have antagonistic impact on gene expression: whereas H3K27 tri-methylation correlates with gene silencing, K27 acetylation correlates with gene activation (Rada-Iglesias et al., 2011). As histones are not imported into the mitochondria, there is considerable interest in the possibility that equivalent modifications occur in the mtDNA or proteins that bind to it. Recent work reported that acetylation and phosphorylation of TFAM can fine-tune TFAM-DNA binding affinity (King et al., 2018). However, as such results were obtained while testing the binding capacity of TFAM (modified and unmodified) to non-specific DNA, it still remains to be assessed whether such modifications affect TFAM binding to mtDNA in living cells. More intriguing is the discovery of several types of mtDNA methylation in different porcine tissues, which correlated with different patterns of mtDNA transcription and mtDNA copy numbers (Liu et al., 2019). The extent to which mtDNA CpG and GpC methylation affect mitochondrial function in cells and in the entire organism remains still in open discussion (Mposhi et al., 2017), and its very existence has been questioned (Matsuda et al., 2018). Nevertheless, there are reports of association between altered levels of mtDNA methylation and Alzheimer's disease (Stoccoro et al., 2017), suggesting physiological relevance. Taken together, it seems that similar to the nuclear genome, the mtDNA might be 'epigenetically' modified, which correlates with downstream activity. However, the connection between such modifications and mitochondrial higher order organization, and with mitochondrial activities, still remains to be tested.

CONCLUSIONS

The higher order organization of the bacterial nucleoid and the nuclear chromatin are tightly regulated, and the impact of such structures on regulation has been widely studied. In the current essay we discussed current knowledge of the higher order organization of the mitochondrial genome in light of evolution and of the growing usage of functional genomics techniques (**Figure 1**). Recent analysis of DNase-seq and ATAC-seq suggest a conserved mtDNA footprinting pattern between tissues, which does not correlate with the binding sites pattern of the only known mtDNA coating protein—TFAM. As such pattern is conserved between man and mouse, the time is ripe to hypothesize that mtDNA–protein interactions, and hence mtDNA higher order organization, are more complex, and more regulated, than once thought. As functional genomics techniques that determine interactions between genomic regions (such as HiC) grow gradually more sensitive, they

could shed light on the packaging of this small genome, and its impact on regulation.

AUTHOR CONTRIBUTIONS

DM and NS conceived the idea and wrote the manuscript. RL and MN participated in critical reading. RL most contributed to writing the text discussing the bacterial nucleoid and nuclear chromatin. MN participated in writing the text discussing G-quadruplex.

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Molecular Characterization of New FBXL4 Mutations in Patients With mtDNA Depletion Syndrome

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Encephalomyopathic mitochondrial DNA (mtDNA) depletion syndrome 13 (MTDPS13) is a rare genetic disorder caused by defects in F-box leucine-rich repeat protein 4 (FBXL4). Although FBXL4 is essential for the bioenergetic homeostasis of the cell, the precise role of the protein remains unknown. In this study, we report two cases of unrelated patients presenting in the neonatal period with hyperlactacidemia and generalized hypotonia. Severe mtDNA depletion was detected in muscle biopsy in both patients. Genetic analysis showed one patient as having in compound heterozygosis a splice site variant c.858+5G>C and a missense variant c.1510T>C (p.Cys504Arg) in *FBXL4*. The second patient harbored a frameshift novel variant c.851delC (p.Pro284LeufsTer7) in homozygosis. To validate the pathogenicity of these variants, molecular and biochemical analyses were performed using skin-derived fibroblasts. We observed that the mtDNA depletion was less severe in fibroblasts than in muscle. Interestingly, the cells harboring a nonsense variant in homozygosis showed normal mtDNA copy number. Both patient fibroblasts, however, demonstrated reduced mitochondrial transcript quantity leading to diminished steady state levels of respiratory complex subunits, decreased respiratory complex IV (CIV) activity, and finally, low mitochondrial ATP levels. Both patients also revealed citrate synthase deficiency. Genetic complementation assays established that the deficient phenotype was rescued by the canonical version of *FBXL4*, confirming the pathological nature of the variants. Further analysis of fibroblasts allowed to establish that increased mitochondrial mass, mitochondrial fragmentation, and augmented autophagy are associated with FBXL4 deficiency in cells, but are probably secondary to a primary metabolic defect affecting oxidative phosphorylation.

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INTRODUCTION

A dysfunction in the maintenance of the mitochondrial DNA (mtDNA) leads to the reduction of mtDNA copy number and/or the accumulation of defects in mtDNA. mtDNA depletion syndromes (MDSs) are a group of mitochondrial disorders characterized by a severe loss of mtDNA copy number. MDSs are autosomal recessive disorders, genetically heterogeneous, and clinically presented in encephalomyopathic, hepatocerebral or myopathic forms (Suomalainen and Isohanni, 2010; Viscomi and Zeviani, 2017).

The human mtDNA contains genetic coding information for 13 proteins, which are core constituents of the mitochondrial respiratory complexes I, III, and IV (CI, CIII, and CIV) and the F_1F_0-ATPsynthase [complex V (CV)]. The respiratory complexes are embedded in the inner mitochondrial membrane and function together with the tricarboxylic acid (TCA) cycle in the matrix. The TCA cycle, together with the beta oxidation of fatty acids, is pivotal for generation of NADH and $FADH_2$ to be oxidized by the respiratory chain. The electron flux along the chain creates an electrochemical gradient that powers the synthesis of most cellular ATP by CV [oxidative phosphorylation (OxPhos)]. mtDNA depletion therefore causes a combined respiratory chain deficiency and deficiency of oxidative ATP-synthesis.

The study of pathogenic variants in patients with defects in mtDNA maintenance has shown that this process depends on a number of nuclear gene-encoded proteins that function in mtDNA synthesis, either participating in mtDNA replication or in the maintenance of balanced nucleotide pools, which constitute the necessary building blocks (Suomalainen and Isohanni, 2010). Qualitative defects in mtDNA (multiple mtDNA deletions) can in addition be caused by defects in mitochondrial division and fusion processes that influence mtDNA segregation (El-Hattab et al., 2017a; Viscomi and Zeviani, 2017).

Defects in F-box leucine-rich repeat 4 (FBXL4) protein, whose molecular function has yet to be determined, cause an encephalomyopathic type of MDS (MTDPS13; OMIM # 615471). MTDPS13 commonly presents with hypotonia, psychomotor delay, failure to thrive, feeding difficulties, growth failure, and lactic acidosis, among other less common manifestations (El-Hattab et al., 2017a). The age of onset ranges from birth to 2 years (mean 4 months). More than a third of affected children die during childhood and long-term survivors develop severe psychomotor retardation. In skeletal muscle tissue commonly appear cytochrome oxidase (COX)-deficient fibers, decreased activities of all respiratory chain enzymes, particularly CI and CIV, and mtDNA depletion (El-Hattab et al., 2017a). The study of cells derived from affected patients demonstrated that FBXL4 is a mitochondrial protein controlling bioenergetic homeostasis and mtDNA maintenance (Bonnen et al., 2013; Gai et al., 2013). However, the molecular role of FBXL4 in mtDNA maintenance remains unclear.

Here we report the identification of novel *FBXL4* mutations in two independent patients and supported the causal role of those mutations. The study of patient derived fibroblasts provided some clues to understand the molecular function of the protein.

MATERIALS AND METHODS

Cell Culture and Cell Staining With Fluorescent Dyes

S1, S2, and C1 primary skin-derived fibroblasts were obtained from Subject 1, Subject 2, and a 1 month-old control child, respectively. C refers to mix of three primary fibroblasts from 1 month, 3 years and 38 years old controls, respectively. Cells were cultured at 37°C under a 5% CO_2 atmosphere in high-glucose DMEM medium (Gibco-ThermoFisher Scientific) with 10% fetal bovine serum (FBS; Gibco-ThermoFisher Scientific).

Cell staining was performed in six well plates. Logarithmically growing cells were incubated with FBS free DMEM for 30 min at 37°C and then stained for 30 min at 37°C in the dark with 200 nM of either MitoTracker™ Green (Invitrogen) or MitoTracker™ Red CMXRos (Invitrogen) in the culture medium. For flow cytometry, immediately after staining, cells were collected by trypsinization and 10,000 particles were analyzed with a Beckman Coulter CITOMICS FC 500 Flow Cytometer. For fluorescent microscopy, cells grown and stained over cover-slides were fixed following a standard protocol and images were obtained with a ZEISS HAL100 microscope.

Biochemical Analysis

Blood lactate values were determined by automated spectrophotometry. Plasma amino acids and urine organic acids were analyzed by ion exchange chromatography with ninhydrin detection derivatives and gas chromatography/mass spectrometry, respectively.

Genomic Analysis

Nuclear DNA (nDNA) was assessed by next generation sequencing (NGS) using customized gene panels as previously reported (Yubero et al., 2016; Fernandez-Marmiesse et al., 2019), in a NextSeq500 sequencer (Illumina).

Alignment of FBXL4 Reference Sequences

Chordate FBXL4 reference sequences (243) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (accessed July 22nd, 2019), and aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Analysis of *FBXL4* Transcripts and Genetic Complementation

The *FBXL4* cDNA (corresponding to RefSeq NM_012160.4; NP_036292.2) was amplified from retrotranscribed total RNA of control and patient fibroblasts, as in (Emperador et al., 2014), using the specific primers: Fw: GATATCGCCACCATGTC ACCGGTCTTTCC and Rv: GATATCTCACTGAGTAA AGCTC. After cloning with the TOPO™ PCR Cloning system (Invitrogen), six to eight bacterial clones per cell line were isolated and their plasmids sequenced.

For genetic complementation, a sequence checked clone, obtained from control fibroblasts, was transferred to the lentiviral expression vector pWPXLd-ires-Neo^R, that is a modified version of pWPXLd (Tronolab, Addgene #12258). Lentiviral particles were generated as in (Perales-Clemente et al., 2008) and fibroblasts were transduced with lentiviral particles in 100 mm dishes by adding 10 µl of medium with viral particles. Transduced cells were isolated by 10 days selection in the presence of 400 µg/ml geneticin (Invitrogen-ThermoFisher Scientific).

Real Time Quantitative Polymerase Chain Reaction Experiments

mtDNA copy number was quantitated by quantitative polymerase chain reaction (qPCR) as previously described (Andreu et al., 2009), using a StepOne™ Real-Time PCR System (Applied Biosystems™). The mitochondrial probe, labeled with a FAM fluorophore, was targeted to the *MT-RNR1* gene (TGC CAG CCA CCG CG) and the nuclear probe, labeled with a VIC was targeted to the *RNAsa P* gene.

To assess mitochondrial mRNA levels, total RNA was isolated from exponentially growing cells using a NucleoSpin® RNA II kit (Macherey-Nagel). Total RNA (1 µg) was reversed-transcribed (RT) with the Transcriptor First Strand cDNA Synthesis Kit (Roche). The levels of *MT-ND1*, *MT-ND6*, *MT-CYB*, *MT-CO1*, and *MT-ATP6* were determined by RT-qPCR using the One-Step Real-Time system (Applied Biosytems). The expression levels were normalized using the 18S ribosomal RNA. The ΔΔCt method was used to calculate fold expression. StepOne software version 2.0 (Applied Biosystems) was used for data analysis. To quantify *FBXL4* transcripts qPCR was carried out in a LightCycler 2.0 system (Roche), using the specific primers: qFw: TGAGATGTGTCCAAATCTACAGG and qRv: GCTGAGCAGTGCTGTTTGC.

SDS-PAGE and Western Blot Analysis

For Western blotting (WB), 20 µg of either total cellular protein extracted in RIPA buffer (MILLIPORE), or total cell homogenate treated by freeze-thawing (4X) (for LC3B WB) was separated in 12.5% acrylamide/bis-acrylamide SDS/PAGE, electroblotted onto PVDF filter, and sequentially probed with specific antibodies: anti-FBXL4 (Sigma, #SAB2701256), anti-OXPHOS cocktail (Abcam, #ab110411), anti-SDHA (Thermo Fisher Scientific, #459200), anti-Actin (Sigma, #A 2066), anti-CS (Sigma, # SAB2702186), anti-TOMM20 (SantaCruz biotechnology, Inc., #sc-11415), and anti-LC3B (Sigma, #L7543). Luminescence images were acquired using Amersham Imager 600 (GE Healthcare Life Sciences) and quantitative data were obtained with ImageQuant™ TL 8.1 analysis software.

Complex IV Levels and Complex IV and Citrate Synthase Specific Activities

When Microplate Assays were indicated, complex IV (CIV) activity and levels were measured using the CIV Specific Activity Microplate Assay Kit (Mitosciences, Abcam®), according to the manufacturer's instructions, and CS was measured in 96 well plates, using freeze-thawing treated total cell homogenate and a standard protocol (Kirby et al., 2007). Microplate assays were performed in a NovoStar MBG Labtech microplate instrument. Otherwise, CIV and CS activities were measured in an UNICAM UV 500 spectrophotometer using digitonin (Sigma) solubilized cell samples as described previously (Kirby et al., 2007). Activity data were normalized for total protein.

ATP Measurements

ATP levels were measured using the ATP bioluminescence assay kit CellTiter-Glo® Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions. Values were normalized using the CellTiter-Blue® Cell Viability Assay (Promega) according to the manufacturer's instructions. Samples were measured using a NovoStar MBG Labtech microplate instrument.

Statistical Analysis

The statistical package StatView 6.0 was used to perform the statistical analysis. Data are expressed as mean ± SD (standard deviation). The non-parametric Mann-Whitney test was used to evaluate the statistical significance between experimental groups. *P*-values lower than 0.05 were considered statistically significant. All samples were measured at least in biological triplicates.

RESULTS

Subject Description

S1: This female child, born to nonconsanguineous parents of European ancestry, was delivered at 38 weeks gestation with a very low weight for gestational age (2.650 kg, < 1st percentile). Fetal ultrasounds also reported a single umbilical artery and megacisterna magna. In the first days of life, mild hypotonia and nystagmus triggered by Moro reflex were observed. Blood lactate was repeatedly increased (4.8 to 11.3 mmol/L; reference values (RV) < 2.2) along with alanine. Brain magnetic resonance image (MRI) revealed mild cerebellar hypoplasia and probable bilateral simplified temporal and frontal gyration pattern. These results led to the study of mitochondrial disease. At 2 months of age, she had frequent visits to the Emergency Department due to intercurrent respiratory processes. Psychomotor development was delayed with poor eye contact and hypotonia. At 6 months of age, she presented with infantile spasms (West syndrome) that responded to treatment with vigabatrin and prednisolone. Due to

metabolic acidosis, treatment with bicarbonate and L-carnitine was initiated. In addition, she had gastroesophageal reflux with frequent vomiting. She presented with progressive dysphagia with poor control of respiratory secretions, convergent strabismus as well as brain MRI lesions compatible with Leigh syndrome. At 10 months, there was an episode of aspiration with marked deterioration in her general condition, generalized hypotonia and seizures. A worsening of the brainstem lesions was observed on brain MRI. The symptoms were progressive with encephalopathy, metabolic acidosis, and death.

S2: This male child, born to consanguineous parents of Moroccan ancestry, was delivered by caesarean section due to arrest of dilation at 38.4 weeks gestation with a low weight for gestational age (2.650 kg, 8th percentile), short length for gestational age (47 cm, 5th percentile), and normal head circumference (34 cm, 43rd percentile). On the third day of life, he showed an acute neurological and respiratory worsening with cyanosis and hypotonia. The patient was intubated and ventilated. Physical examination showed clinical signs of poor peripheral perfusion and absence of responses to stimuli due to sedoanalgesia. On admission, he presented with metabolic acidosis (pH 7.13, pCO_2 22.2 mmHg on mechanical ventilation, $stHCO_3$ 8.9 mmol/L, EB –20.6 mmol/L), hyperamonemia (224 µmol/L: RV <70), and hyperlactacidemia (25 mmol/L: RV < 2.2). Plasma amino acids exhibited increased alanine (1385 µmol/L: RV 190–337), glycine (658 µmol/L: RV 180–291), glutamine (971 µmol/L: RV 420–750), and lysine (705 µmol/L: RV 67–202). Organics acids revealed a massive accumulation of lactic, 3-hydroxybutyric, acetoacetic, and 2-hydroxybutyric acids, leading to investigate mitochondrial diseases. No analytical signs of infection were detected. Transfontanellar ultrasound showed extensive hyperechogeneity that seemed to correspond to retro cerebellar hemorrhage and enlarged magna cisterna. No brain MRI or lumbar puncture was performed. Echocardiography showed pulmonary hypertension.

Different treatment approaches were initiated to correct the metabolic abnormalities: protein restriction, high energy intake (100–120 cal/kg/day), L-carnitine, arginine, and vitamins (biotin, hydroxycobalamin, pyridoxine, riboflavin, and thiamine). An intravenous insulin and dopamine (maximum dose 5 µg/kg/min) pump and antibiotics (ampicillin and gentamicin) were also started. He received several doses of intravenous bicarbonate and three doses of adrenaline for severe bradycardia. At 24 hours of admission, there was a limitation of the therapeutic effort due to the refractoriness of lactic acidosis. The child died at the age of 4 days.

Although some dysmorphisms have been reported in FBXL4 patients, microcephaly, cataracts, malformed ears, or other dysmorphic facial features were not found in S1 or S2 patients. However, S2 showed distal hypospadias and bilateral cryptorchidism.

Biallelic Mutations in FBXL4 Are Present in the Probands

Exome sequencing analysis of S1 and S2 detected novel mutations in *FBXL4* (**Figure 1A**). S1 was found to be compound heterozygous for the split site variant c.858+5G>C and the missense variant c.1510T>C (p.Cys504Arg). The first variant was detected in the father and the second in the mother. S2 was found to be homozygous for the frameshift variant c.851delC (p.Pro284LeufsTer7), that was detected in both parents in heterozygosis. The variants were confirmed by Sanger sequencing.

Patient RNA-retrotranscription and cDNA cloning revealed two classes of *FBXL4* transcripts in S1 (**Figure 1A**). One transcript harbored the missense variant c.1510T>C (p.Cys504Arg) and a second one lacked exon 4, which can be compatible with a splicing defect. The analysis of S2 detected only one type of transcript harboring the frameshift variant c.851delC (p.P284LfsTer7) (**Figure 1A**).

None of these variants were present in the ExAC browser (accessed July 2019). However, the variant producing an unexpected transcript in S1 has been previously published in heterozygosis in a patient with MTDPS13 (rs1257765682) (Pronicka et al., 2016). The S1 missense variant affects a position conserved in 243 out of 243 reference sequences and is considered pathogenic by several prediction software packages (MutationTaster, PMut and PolyPhen-2). Eighteen months after the death of S1, the parents had a healthy girl that was not a carrier of either of the two mutations. Two years later, the parents had a new twin pregnancy. The amniocentesis of both fetus revealed that they were only carriers of the c.858+5G>C variant inherited from the father. At 2-years of age, both of them are healthy.

Defective OxPhos System Biogenesis Is Associated With the Novel *FBXL4* Mutations

Analysis of mtDNA copy number revealed severe mtDNA depletion in muscle biopsies of the patients (85% in S1 and 93% in S2). In cultured skin fibroblasts, milder mtDNA depletion was detected in S1 (38%) whereas normal levels of mtDNA were observed in S2 (**Figure 1B**). The levels of five mitochondrial transcripts (transcripts of *MT-ND1* and *MT-ND6* subunits from CI; *MT-CYB* subunit from CIII, *MT-CO1* subunit from CIV and *MT-ATP6* subunit from CV) were consistently reduced in S1 and S2 compared with control fibroblasts (**Figure 1C**). Notably, S2, with normal mtDNA copy number, showed the highest reduction of the five transcripts measured.

The steady-state levels of subunits from respiratory chain complexes were also found decreased in fibroblasts from S1, and, to a greater extent, in fibroblasts from S2 (**Figure 1D**). The levels of the nDNA-encoded ATP5A subunit from CV, however, remained unchanged in S1, or were mildly decreased in S2, excluding a global problem in the mitochondrial protein content. The expression of two subunits (SDHA and SDHB) of the nuclear encoded complex II, the citrate synthase (CS) of the TCA cycle, and the translocase of the outer mitochondrial membrane (TOMM20) were partially decreased in S2 (**Figure 1D**). Fully assembled CIV levels were clearly lower in patient fibroblasts relative to controls (**Figure 1E**). Enzymatic measurements provided evidenced of a severe CIV dysfunction (**Figure 1F**). Noteworthy, the CS activity in fibroblasts from S1 and S2 was also significantly diminished (**Figure 1F**).

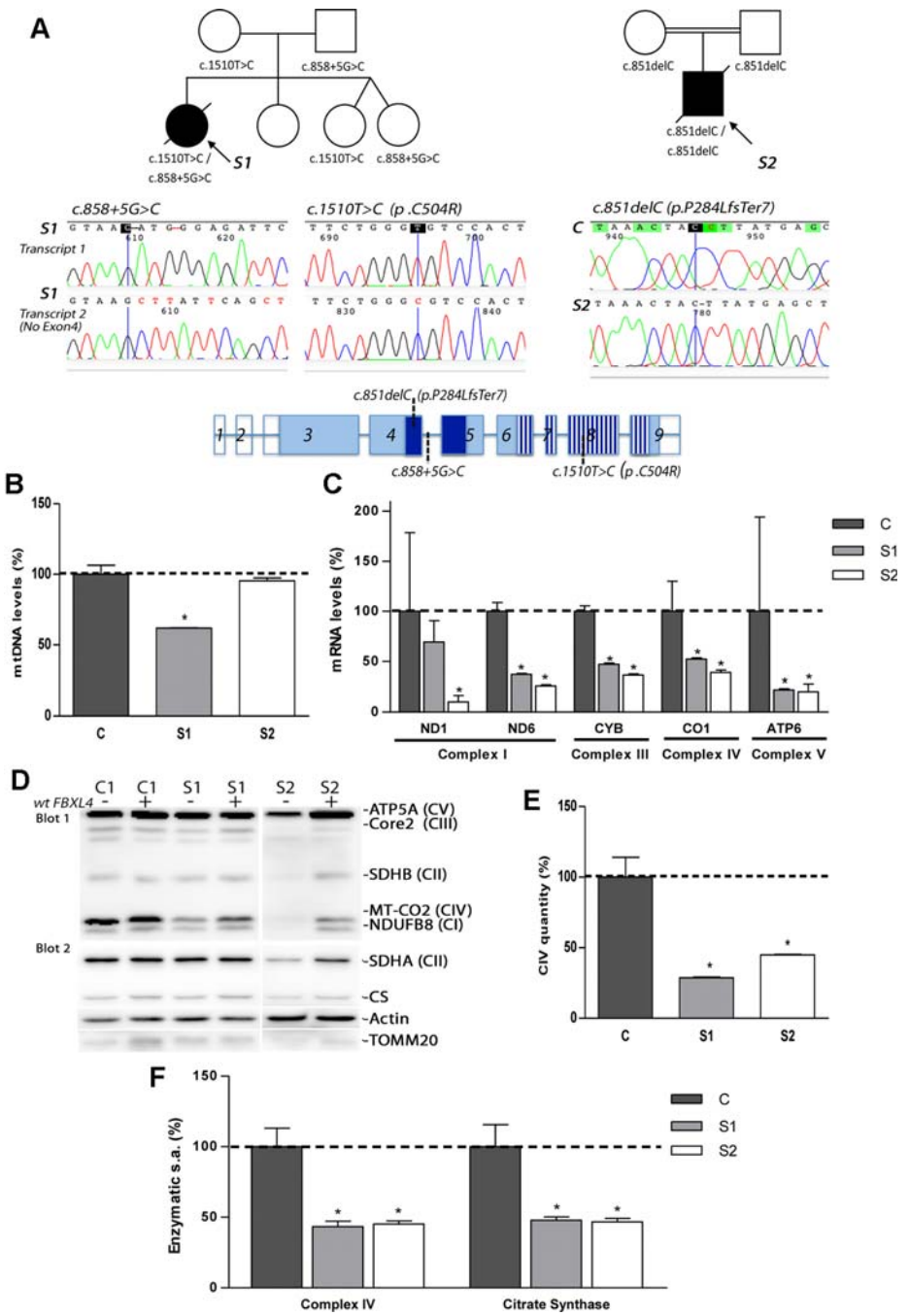

FIGURE 1 | Genetic, molecular, and biochemical characterization of the patients. **(A)** Pedigrees of S1 and S2 with genotypes indicated under each symbol (black symbols designate affected subjects); sequencing electropherograms corresponding to the two different FBXL4-derived transcripts found in S1 and to the unique transcript found in S2; and FBXL4 gene structure (reference sequence NM_012160.4). In gene structure, empty boxes: non-coding exons; dark blue boxes: F-Box domain; striped boxes: leucine-rich repeats domain (LRRs). **(B)** Quantification of mtDNA copy number. The bars represent percentage of mtDNA normalized to nDNA relative to the mean value of controls levels (C, dotted line, 100%). *: Significant mtDNA copy-number reduction, $p < 0.05$, compared with C cells. **(C)** Mitochondrial transcript levels. For each transcript, the bars represents mean values, in percentage, relative to the mean value of control fibroblasts (C, dotted line, 100%). *: significant mtDNA reduction, $p < 0.05$, compared with C cells. **(D)** Steady-state levels of mitochondrial respiratory chain subunits. WB-immunodetection of SDS-PAGE separated total cellular protein isolated from patient S1, S2 and C fibroblasts (–), and those transduced with wt-FBXL4 expressing construct (+). An OXPHOS cocktail of antibodies was used in the upper membrane (blot 1) and the indicated antibodies were sequentially used in the lower membrane (blot 2). **(E)** Complex IV quantity (Microplate Assay). The bars represent the mean value of S1 and S2, in percentage, compared to that of controls fibroblasts (dotted line, 100%). *: $p < 0.05$ (vs. C cells). **(F)** Complex IV (CIV) and CS specific activities (s.a.) (Microplate Assay). The bars represent enzymatic activity of CIV and CS normalized for total cellular protein and compared to the mean value of controls fibroblasts in percentage (dotted line, 100%). *: $p < 0.05$ (vs. C cells).

These results indicated that a decreased expression of mtDNA-encoded genes affects the correct biogenesis and function of the respiratory chain, with the TCA-cycle enzyme CS also affected.

Delivery of Wild-Type *FBXL4* Corrects the OxPhos Dysfunction

In order to confirm the pathogenicity of the FBXL4 mutations, we performed genetic complementation studies. Stably transduced patient cells showed a robust expression of wild-type *FBXL4* (wt-*FBXL4*) at transcript and protein levels (**Figures 2A, B**). The quantification of *FBXL4* transcripts by qRT-PCR revealed decreased steady-state levels of *FBXL4* mRNA in S1 and S2 by approximately 80% when compared with an age matched control cell line, C1. These levels were increased significantly in the over-expressing cell lines (**Figure 2A**). The WB-inmunodetection assay failed to detect the FBXL4 protein in the total protein lysate of non-transduced cells (**Figure 2B**).

Delivery of the wt-*FBXL4* gene increased significantly the amount of mtDNA in both patient cell lines (**Figure 2C**). As a result, the mtDNA copy number deficiency in S1 cells was corrected (compared to C), whereas in S2 cells the mtDNA levels increased up to 200% of the levels of controls. In both S1 and S2 cells over-expressing wt-*FBXL4*, the steady state levels of respiratory complex subunits were fully rescued (**Figure 1D**), the CIV specific activity was increased to control values (**Figure 2D**), and the CS activity and lastly the mitochondrial ATP levels were also increased significantly (**Figures 2E, F**).

These results confirmed the causal role of the mutations identified in FBXL4 in the metabolic dysfunction.

Mitochondrial Mass, Mitochondrial Fragmentation, and Autophagosomes Are Increased in FBXL4 Deficiency

Next, we investigated the mitochondrial mass in cultured patient fibroblasts to determine whether it is reduced as could be suggested by the low CS activity. Assessment of mitochondrial content by staining with the cationic lipophilic dye MitoTracker Green failed to show differences between C1 and wt-*FBXL4* overexpressing C1 cells (**Figure 3A**). However, a small but significant increase in mitochondrial content (15–20%) was detected in S1 and complemented S1 cell lines compared to C1, and a remarkable increase (250% of C1) was detected in S2 fibroblasts and in the S2 cell line overexpressing wt-*FBXL4*.

The mitochondrial network morphology was next examined. As shown in the fluorescence microscopy images (**Figure 3B**), the fluorescent pattern obtained with the mitochondrion-specific dye MitoTracker was mainly tubular in C1 cells. On the contrary, S1 and S2 cell lines showed fragmentation of the mitochondrial network. Genetically complemented S1 cells recovered partially the tubular appearance of the mitochondrial network observed in C1 but interestingly, genetically complemented S2 cells maintained a fragmented mitochondrial network.

The autophagosome marker MAP1 light chain 3B (LC3B) can be found as LC3B-I, mainly cytosolic, or as LC3B-II, which is covalently attached to phosphatidylethanolamine and coats the surface of autophagosomes. Western blotting and immunodetection were used to analyze LC3B-I to LC3B-II conversion and estimate the abundance of autophagy-related structures (Klionsky et al., 2008). The ratio LC3B-II/LC3B-I was found augmented in S1 and S2 cells (**Figure 3C**). Albeit in genetically complemented S1 cells, the ratio was reduced to the control levels; in genetically complemented S2 cells, however, the significantly high levels of autophagy marker LC3-II related to its cytosolic isoform LC3-I persisted.

These results suggested that mitochondrial fragmentation and an increase of autophagosomes are associated with FBXL4 mutations in cultured fibroblasts. Remarkably, the genetically complemented S2 cell line failed to recover normal mitochondrial size or shape and normal autophagy levels, suggesting that these are secondary adaptations to the metabolic dysfunction promoted by FBXL4 deficiency.

DISCUSSION

This work presents two patients with severe encephalopathy and severe mtDNA depletion in muscle associated with novel variants in *FBXL4*; S1 harboring two variants in compound heterozygosis c.[858+5G>C];[1510T>C] and S2 harboring one variant in homozygosis c.[851delC];[851delC]. The pathogenicity of the variants was confirmed by genetic complementation assays in skin-derived fibroblasts. Genetic complementation studies with this gene have only been performed in three subjects previously (Bonnen et al., 2013; Gai et al., 2013).

A review of genotype–phenotype correlation in 87 affected individuals with pathological variants in *FBXL4* indicated that genotypes with missense variants are frequently associated with longer survival (El-Hattab et al., 2017b). Thus, the missense variant encountered in S1 could be associated with preservation of some residual protein function and therefore with a milder phenotype. However, both patients presented with very severe clinical phenotype associated with very early onset and short survival (S1, 10 months, and S2, 4 days), indicating that any possible residual function of the S1 missense variant was not sufficient to maintain *in vivo* protein function.

The finding of severe mtDNA depletion in skeletal muscle biopsies (85% in S1 and 93% in S2) are in line with published data indicating an essential role of FBXL4 in the maintenance of mtDNA. Interestingly, important differences were observed in the mtDNA content of cultured fibroblasts. S1 cells presented with quantitative mtDNA copy number reduction (38%), whereas S2 cells had normal mtDNA copy number. Remarkably, genetic complementation increased the mtDNA levels in at least 50%, still indicating an involvement of FBXL4 in mitochondrial mtDNA copy number maintenance. Despite having normal mtDNA content, S2 derived fibroblasts showed a more severe OxPhos deficient phenotype than S1 cells. The levels of mtDNA-derived transcripts were lower than those in S1, and several subunits of the respiratory chain complexes were absent. Thus, mtDNA levels did not correlate with mitochondrial RNA

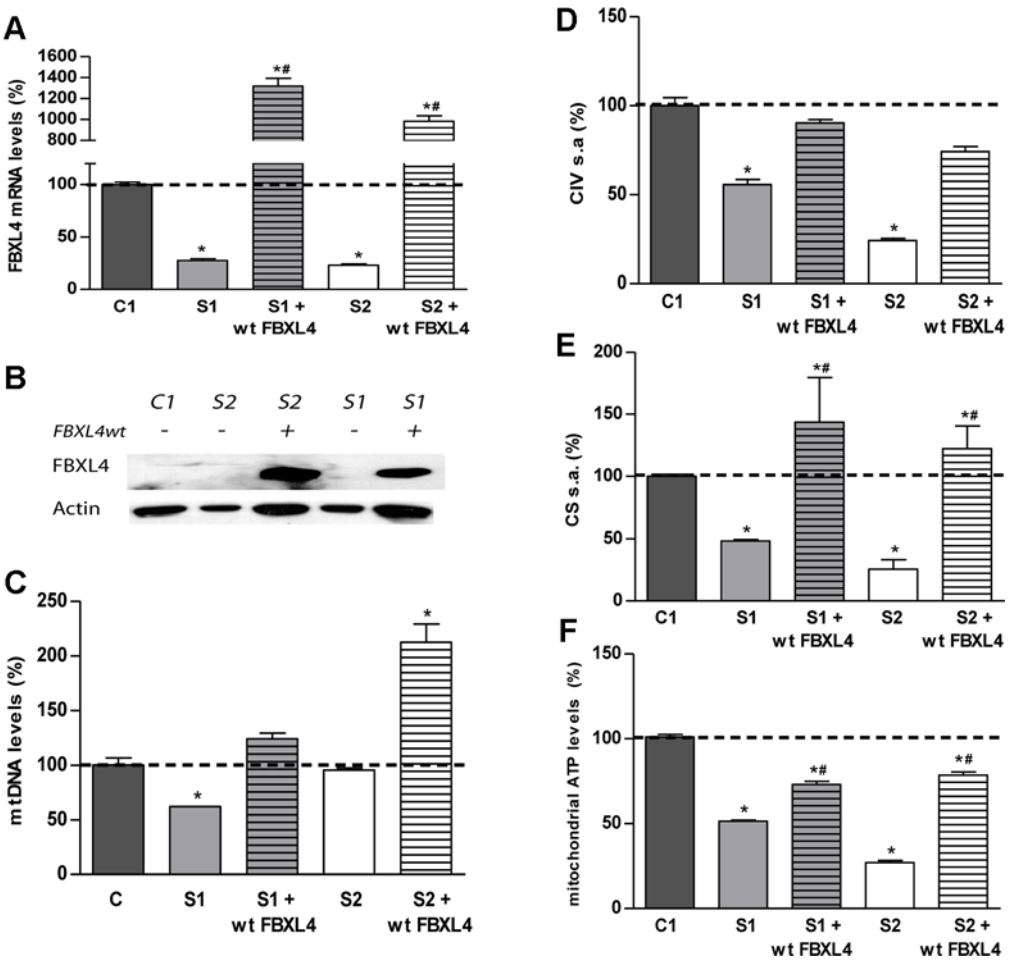

FIGURE 2 | FBXL4 complementation assays. **(A)** *FBXL4* expression levels. Bars represent *FBXL4* mRNA levels of patient fibroblasts compared to the mean value of an age-matched control in percentage (C1 dotted line, 100%). *: $p < 0.05$ (vs. C1 cells); #: $p < 0.05$ (vs. non-transduced cells). **(B)** FBXL4 protein levels. WB-immunodetection with anti-FBXL4 antibody of total cellular proteins isolated from S1, S2, and C1 fibroblasts, and those transduced with wt-*FBXL4* expressing construct. **(C)** Quantification of mtDNA copy number of S1, S2, and C1 fibroblasts and those transduced with the wt-*FBXL4* expressing construct. Bars represent the mean value of mtDNA normalized to nDNA, in percentage, relative to the mean value of controls levels (C, dotted line, 100%). *: $p < 0.05$ (vs. C cells); #: $p < 0.05$ (vs. non-transduced cells). **(D)** Complex IV specific activity of S1, S2, and C1 fibroblasts and of those transduced with the wt-*FBXL4* expressing construct. Bars represent the mean value relative to that of control fibroblasts in percentage (C1, dotted line, 100%). *: $p < 0.05$ (vs. C1 cells). **(E)** CS specific activity of S1, S2, and C fibroblasts and those transduced with the wt-*FBXL4* expressing construct. Bars represent the mean value relative to that of control fibroblasts, in percentage (C1, dotted line, 100%). *: $p < 0.05$ (vs. C1 cells); #: $p < 0.05$ (vs. non-transduced cells). **(F)** Mitochondrial ATP levels. Bars represent the mean values in S1, S2 and C1, S1 and S2 transduced with the wt-*FBXL4* expressing construct, relative to that of control cells in percentage (C1, dotted line, 100%). *: $p < 0.05$ (vs. C1 cells); #: $p < 0.05$ (vs. non-transduced cells).

levels as expected (Gomez-Duran et al., 2012) indicating that FBXL4 deficiency also affects the correct mtDNA expression.

The patient primary fibroblasts showed augmented mitochondrial mass, when assessed by MitoTracker Green fluorescent staining. The increase was particularly high in S2 cells. It can be speculated that FBXL4 deficient fibroblasts might have overcome the defect in mtDNA maintenance by expanding their mitochondrial mass as an adaptation to the life in culture. An attempt to compensate for OxPhos defects increasing mitochondrial mass resembles the massive mitochondrial proliferation observed in muscle of patients with mtDNA-related diseases (resulting in ragged-red fibers) (DiMauro and Schon, 2003). However, in muscle from FBXL4 deficient patients no ragged red fibers have been observed (Gai et al., 2013; Morton et al., 2017), although they are a common feature in newborns and infants with mitochondrial disorders (Jou et al., 2019).

Previous patient reports have described a hyperfragmentation of the mitochondrial network in affected fibroblasts (Bonnen et al., 2013; Gai et al., 2013; Antoun et al., 2016). This has lead to classify FBXL4 as protein participating in mitochondrial dynamics (El-Hattab et al., 2017a). The highly compacted

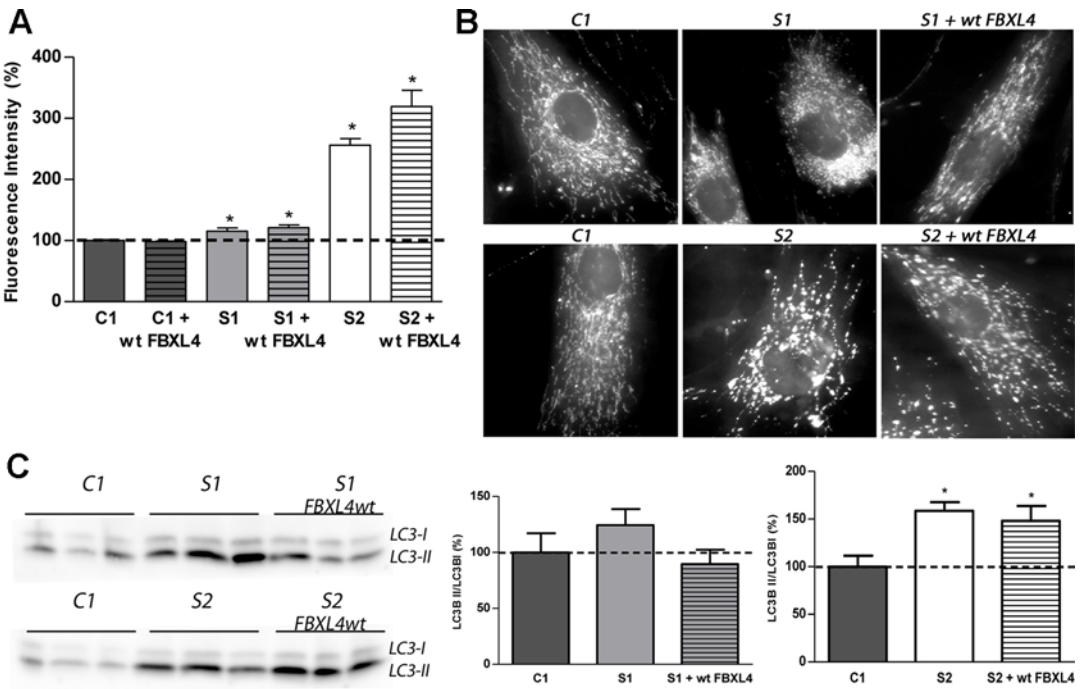

FIGURE 3 | Mitochondrial network shape and size, and autophagy detection. **(A)** Quantification of mitochondrial mass. Bars represent the mean fluorescence values relative to the mean value of age-matched control fibroblasts in percentage (C1, dotted line, 100%). *: $p < 0.05$ (vs. C1 cells). **(B)** Mitochondrial networks of control and patients fibroblasts. Fluorescence microscopy representative images of cells obtained from control and patient fibroblasts and of those transduced with the wt-*FBXL4* expressing construct, as indicated. **(C)** Quantification of autophagy marker LC3-II. WB-immunodetection of the LC3B isoforms (LC3B-I and LC3B-II) of total cell homogenates, and ratio LC3B-II/LC3B-I obtained by quantification of the respective WB-band intensities. The bars represent the mean value, in percentage, compared to that of control fibroblasts (dotted line, 100%). *: $p < 0.05$ (vs. C1 cells).

mtDNA–protein complexes or nucleoids, that usually can be visualized as punctate structures evenly distributed within the mitochondrial network, have also been found altered, enlarged, and clustered (Bonnen et al., 2013). Our patients' fibroblasts also presented a mitochondrial network fragmented into multiple small mitochondria. Interestingly, S1 fibroblasts partially recovered a connected mitochondrial network when functionally complemented with wt-*FBXL4*, whereas mitochondria in S2 complemented fibroblasts remained mostly punctuate. These findings suggest that mitochondrial fragmentation could be secondary to the metabolic defects induced by absence of the FBXL4 protein. Augmented mitochondrial division could be related with attempts to increase mtDNA synthesis because both processes are closely related. In mammalians, tubular ER-mitochondria contacts, by an unknown mechanism, connect the sites of mitochondrial division with the subset of nucleoids engaged in mtDNA synthesis. Thus, following division, nucleoids segregate to both tips of daughter mitochondria (Lewis et al., 2016). This fact, however, could not be confirmed in our studies.

Increased mitochondrial division can also precede mitophagy, because it divides elongated mitochondria into pieces that can be engulfed by autophagosomes to regulate their number and maintain quality control (Youle and Narendra, 2011). Both S1 and S2 fibroblasts demonstrated increase of the autophagic-vesicles coating-protein LC3B-II, suggesting that FBXL4 deficiency promotes autophagic processes. In genetically complemented S1 cells autophagosome formation decreased. It could be speculated that it reflects the need for selective elimination of the organelles lacking a functional OxPhos system and/or the necessity of recycling intracellular components to compensate for the starvation-like situation (Geng and Klionsky, 2008). The reduction of mitochondrial components other than subunits of the respiratory chain clearly observed in S2 can be reflecting its elimination by mitophagy. In genetically complemented S2 cells, the increased autophagy persisted possibly regulating the organelle number since the abnormally high increase in mitochondrial membrane also persisted.

In contrast to patients with typical MDSs due to a disorder of mtDNA replication or nucleoside salvage/synthesis, in patients with FBXL4 deficiency the mtDNA/CS ratio is normal (Huemer et al., 2015). Our patient cells also presented CS deficiency. It can be a secondary effect, because interruptions to the respiratory chain can affect the TCA cycle flux (Vafai and Mootha, 2012). Deficiency in another TCA cycle enzyme, succinyl-CoA synthetase (SCS), has also been associated with an encephalomyopathic form of MDS. However, the involvement of SCS in MDS seems to be related to a role of the enzyme in the mitochondrial nucleoside salvage pathway, facilitating the conversion of dNDPs to dNTPs (Viscomi and Zeviani, 2017).

In summary, this work provides evidence of the pathogenicity of novel variants in FBXL4, demonstrating that FBXL4 is necessary not only for the homeostasis but also for the expression of mtDNA. Since in S2 fibroblasts the rescue of the bioenergetics defects by the *FBXL4* gene can occur independently of the recovery of control mitochondrial mass, mitochondrial network, or autophagic levels, these could be compensatory and subsequent to the bioenergetic defects. Most F-box proteins function as adaptors in phosphorylation dependent ubiquitination-complexes (Craig and Tyers, 1999). A role in post-translational modification of the mitochondrial proteome could reconciled the disparity of effects observed in defective FBXL4, but further work is required to determine the precise molecular function.

DATA AVAILABILITY STATEMENT

The datasets Generated for this study can be found in NCBI https://www.ncbi.nlm.nih.gov/bioproject/PRJNA590845 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA592374.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of the Government of Aragón (CEICA CP- 12/2014). Written informed consent to participate in this study was provided by the participants' legal guardian/ next of kin.

AUTHOR CONTRIBUTIONS

MO, JO-E, AG-C, MI, and MG-C were responsible for the sample collection, analysis of clinical data, and treatment. RA, DY, and AF-M participated in the biochemical and molecular genetic diagnostic studies of the patients. SE, NG-P, JA-G, PG, and JA-S participated in the biochemical and molecular analysis of patient samples. ER-P, JM, and MB-B were responsible of study design, data interpretation, and drafting of the manuscript. All authors have critically reviewed and they approved the final manuscript.

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Mitochondrial Genetics and Epigenetics in Osteoarthritis

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During recent years, the significant influence of mitochondria on osteoarthritis (OA), the most common joint disease, has been consistently demonstrated. Not only mitochondrial dysfunction but also mitochondrial genetic polymorphisms, specifically the mitochondrial DNA haplogroups, have been shown to have an important influence on different OA-related features, including the prevalence, severity, incidence, and progression of the disease. This influence could probably be mediated by the role of mitochondria in the regulation of different processes involved in the pathogenesis of OA, such as energy production, the generation of reactive oxygen and nitrogen species, apoptosis, and inflammation. The regulation of these processes is at least partially controlled by the bi-directional communication between the nucleus and mitochondria, which permits the regulation of adaptation to a wide range of stressors and the maintenance of cellular homeostasis. This bi-directional communication consists of an "anterograde regulation" by which the nucleus regulates mitochondrial biogenesis and activity and a "retrograde regulation" by which both mitochondria and mitochondrial genetic variation exert a regulatory signaling control over the nuclear epigenome, which leads to the modulation of nuclear genes. Throughout this mini review, we will describe the evidence that demonstrates the profound influence of the mitochondrial genetic background in the pathogenesis of OA, as well as its influence on the nuclear DNA methylome of the only cell type present in the articular cartilage, the chondrocyte. This evidence leads to serious consideration of the mitochondrion as an important therapeutic target in OA.

Keywords: mitochondria, genetics, epigenetics, osteoarthritis, methylation

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INTRODUCTION

Osteoarthritis (OA) is the most common chronic progressive disorder that involves movable joints, occurring in 10-20% of the population over 50 years of age; the incidence of OA is estimated to double within the next 30 years (Blanco et al., 2011). The pathogenesis of OA is characterized by extracellular matrix degradation and cell stress initiated by micro- and macro-injuries that lead to the activation of maladaptive repair responses, including proinflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal tissue metabolism) followed by anatomical and/or physiological derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation, and the loss of normal joint function)

that can culminate in illness (Kraus et al., 2015). OA is actually considered a disease of the whole joint as an organ, resulting in organ dysfunction or joint failure (Blanco, 2014). In addition, this disease is one of the most common reasons for visits to primary care physicians and is also the leading cause of permanent work incapacitation. OA has no effective treatment today, and joint replacement is the only choice in cases of total joint dysfunction.

The nature of OA is heterogeneous, because a combination of factors such as occupation, age, gender, body mass index, and genetics have a profound influence on its pathogenesis. As a consequence of this, several different phenotypes characterize this disease, including inflammatory, aging-related, metabolic, pain, and post-traumatic phenotypes (Berenbaum, 2013; Sellam and Berenbaum, 2013; Blanco et al., 2018). In agreement with this heterogeneous nature, it has also been proposed that, on the one hand, mitochondria and mitochondrial DNA (mtDNA) have an important impact on the development of OA (Valdes and Goldring, 2017; Blanco et al., 2018; Li et al., 2019) and, on the other hand, that epigenetics is one of the main actors involved in the phenotypic modulation that articular chondrocytes, the only cell type present in articular cartilage, undergo during the OA process (Roach et al., 2005; Reynard and Loughlin, 2012; Blanco and Rego-Pérez, 2014). In this sense, it has also been consistently shown that specific mtDNA polymorphisms, called mtDNA haplogroups, have been associated not only with different OA-related features such as the incidence or progression of the disease (Fernandez-Moreno et al., 2017a; Fernandez-Moreno et al., 2017b; Koo et al., 2019) but also with the differential methylation status of articular cartilage (Cortes-Pereira et al., 2019). In light of the involvement of mitochondrial DNA in cell behavior and metabolism (Wallace et al., 1999; Martínez-Redondo et al., 2010), in this review, we discuss some of the evidence implicating the epigenetic effect of mtDNA variation in the pathogenesis of OA.

MITOCHONDRIA IN OSTEOARTHRITIS

Because articular chondrocytes are highly glycolytic cells that obtain their energy mainly from anaerobic glucose metabolism, the role of mitochondria in the pathogenesis of OA was not studied in depth until early 2000. However, a large number of studies have demonstrated the profound influence of mitochondria in the pathogenesis of OA.

Terkeltaub and co-workers published a full review describing how mitochondrial impairment of chondrocytes is an important mediator of the establishment of OA (Terkeltaub et al., 2002). Specifically, these and other authors described that mitochondrial dysfunction mediates several specific pathogenic pathways implicated in the OA process, including oxidative stress, chondrocyte apoptosis, cartilage matrix calcification, autophagy, impaired anabolic and growth response of chondrocytes, and increased cytokine-induced inflammation (Terkeltaub et al., 2002; Blanco et al., 2004; Lotz and Loeser, 2012; López de Figueroa et al., 2015). In agreement with these findings, our group also demonstrated that, compared with healthy chondrocytes from patients of the same age, osteoarthritic chondrocytes have reduced mitochondrial activity, mainly in complexes II and III, and increased mitochondrial mass (Maneiro et al., 2003). In addition, the apoptotic mitochondrial pathway is implicated in the apoptosis of osteoarthritic chondrocytes (Hwang and Kim, 2015), and the inhibition of mitochondrial complexes III and V increases the mitochondrial-mediated inflammatory response in OA chondrocytes mediated by an overproduction of reactive oxygen species (ROS) (Vaamonde-García et al., 2012). Mitochondrial dysfunction has also been associated with a significant downregulation of superoxide dismutase 2 (SOD2) (Gavriilidis et al., 2013), one of the major mitochondrial antioxidant proteins, whose levels are also diminished in the superficial layers of end-stage OA cartilage (Ruiz-Romero et al., 2009; Scott et al., 2010).

Mitophagy is a form of autophagy, a process that involves the removal of damaged macromolecules and organelles to regulate cell homeostasis (Choi et al., 2013); specifically, mitophagy consists of the elimination of depolarized and damaged mitochondria. Different studies have demonstrated that the activation of this process protects against mitochondrial dysfunction, prevents ROS production and improves chondrocyte survival under pathological conditions (López de Figueroa et al., 2015; Ansari et al., 2018). It has also been demonstrated that mitochondrial biogenesis is deficient in human OA chondrocytes, leading the chondrocyte to adopt procatabolic responses; however, the activation of the AMP-activated protein kinase (AMPK)-NAD-dependent protein deacetylase sirtuin-1 (SIRT1)-peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α) pathway reverses impaired mitochondrial biogenesis, which is mediated by mitochondrial transcriptional factor A (TFAM) (Wang et al., 2015).

mtDNA HAPLOGROUPS AND OSTEOARTHRITIS

Mitochondria are considered unique organelles because they contain their own maternally inherited DNA, the mtDNA, containing 2 rRNAs, 22 tRNAs, and 13 essential mitochondrial protein-coding genes. The mutation rate of this circular molecule is higher than that of the nuclear DNA, mainly due to i) its proximity to the main source of ROS production, ii) the lack of an efficient repair system, and iii) the higher replication rate of mtDNA (Li et al., 2019). As a consequence of this, mtDNA sequences evolved by sequentially accumulating functional mutations along radiating maternal lineages when humans migrated out of Africa and adapted their energy metabolism to different environments, giving rise to mtDNA haplogroups (Torroni et al., 1996; Wallace, 2016). However, these types of mtDNA variants, though providing an important degree of adaptation as people migrated into new environments, might also have contributed to modern human disorders such as

hypertension, diabetes, obesity, or neurodegenerative diseases (Ruiz-Pesini et al., 2004; Marom et al., 2017).

In this sense, OA is not an exception. During recent years, different studies have shown clinical associations between specific mtDNA haplogroups and different OA-related features, including the prevalence, progression, and incidence of the disease (**Table 1**).

mtDNA Haplogroups and the Prevalence of OA

Different studies have shown significant associations between specific mtDNA haplogroups and the prevalence of OA. Specifically, mtDNA variants belonging to the European cluster JT have been associated with a lower risk of knee and hip OA in a cohort of Spanish patients (Rego-Perez et al., 2008; Rego et al., 2010). mtDNA haplogroup T was also associated with a decreased risk of knee OA in a population from the United Kingdom (Soto-Hermida et al., 2014a); however, a later study in a larger population cohort from the same country failed to replicate these findings (Hudson et al., 2013). In addition, Asian mtDNA haplogroups B and G have been described as protective and risk factors respectively for knee OA in a population of southern China (Fang et al., 2014). The authors have proposed that the mechanisms that could explain that association are related to the alteration of both mitochondrial function and OA-related signaling pathways (Fang et al., 2016). A meta-analysis summarizing most of the studies described above concluded that mtDNA cluster JT is associated with a lower risk of OA prevalence in Spanish populations (Shen et al., 2014).

A plausible cause for the lack of replication among different case-control studies, even when nuclear DNA polymorphisms are analyzed, could be the unavailability of knee (or hip) radiographs for most population-based controls. This is not a minor issue, since up to 50% of people without joint symptoms may develop radiographic changes comparable to OA (Hannan et al., 2000). In this sense, our group has always considered the radiological status, because we believe that both mitochondrial dysfunction and mtDNA variation have a greater impact on the evolution of joint structure than on pain.

mtDNA Haplogroups and Radiographic OA Progression and Incidence

The use of well-characterized prospective cohorts of patients permits rigorous studies to analyze the influence of mtDNA haplogroups on the rate of progression and incidence of OA over time. mtDNA variants within mitochondrial cluster JT were associated with lower rates of radiographic knee OA progression in different world populations, including Spain, the USA, and the Netherlands. Specifically, in a Spanish cohort, mtDNA cluster JT was associated with a lower rate of radiographic progression in terms of Kellgren and Lawrence grade (Kellgren and Lawrence,

TABLE 1 | Published associations of mtDNA variants with specific OA-related features.

Study cohort	Population	Haplogroup	OR (95%CI) p-value/effect on the biomarker	Reference
OA prevalence				
Spanish	457 OA cases, 262 controls	J	OR = 0.460 (0.282-0.748) p = 0.002	(Rego-Perez et al., 2008)
		JT	OR = 0.564 (0.384-0.828) p = 0.005	
Spanish	550 OA cases, 505 controls	J	OR = 0.519 (0.271-0.994) p = 0.048	(Rego et al., 2010)
UK	453 OA cases, 280 controls	T	OR = 0.574 (0.350-0.939) p = 0.027	(Soto-Hermida et al., 2014a)
UK	7846 OA cases, 5402 controls	J	OR = 1.190 (0.720-1.950) ns [&]	(Hudson et al., 2013)
Meta-analysis	2557 OA cases, 1339 controls	J	OR = 0.570 (0.460-0.710) p < 0.0001	(Shen et al., 2014)
	2478 OA cases, 1173 controls	JT	OR = 0.700 (0.580-0.840) p = 0.0002	
Chinese	187 OA cases, 420 controls	G	OR = 3.834 (1.139-12.908) p = 0.003	(Fang et al., 2014)
		B	OR = 0.503 (0.283-0.893) p = 0.019	
OA progression				
OAI	891 knee OA cases	T	HR = 0.499 (0.261-0.819) p < 0.05	(Soto-Hermida et al., 2014b)
Spanish	281 knee OA cases	JT*	HR = 0.584 (0.354-0.964) p = 0.036	(Soto-Hermida et al., 2015)
CHECK	431 knee OA cases	T	HR = 0.645 (0.419-0.978) p < 0.05	(Fernandez-Moreno et al., 2017b)
		JT	HR = 0.707 (0.501-0.965) p < 0.05	
Meta-analysis	1603 knee OA cases	T	HR = 0.612 (0.454-0.824) p = 0.001	(Fernandez-Moreno et al., 2017b)
		JT	HR = 0.765 (0.624-0.938) p = 0.009	
OA incidence				
OAI	2579 subjects	J	HR = 0.680 (0.470-0.968) p < 0.05	(Fernandez-Moreno et al., 2017a)
CHECK	635 subjects	J	HR = 0.728 (0.469-0.998) p < 0.05	(Fernandez-Moreno et al., 2017a)
Meta-analysis	3214 subjects	J	HR = 0.702 (0.541-0.912) p = 0.008	(Fernandez-Moreno et al., 2017a)
Korean	438 subjects	B	RR = 2.389 (1.315-4.342) *p* = 0.004	(Koo et al., 2019)
OA biomarkers				
Spanish	73 knee OA cases, 77 controls	J	Decreased serum levels of catabolic type II collagen biomarkers[&]	(Rego-Perez et al., 2010)
		H	Increased serum levels of catabolic type II collagen biomarkers[&]	
Spanish	73 knee OA cases, 77 controls	J	Decreased serum levels of MMP-13[&]	(Rego-Perez et al., 2011)
		H	Increased serum levels of MMP-13 and MMP-3[&]	
Spanish	79 knee OA cases, 166 controls	J	Lower NO production	(Fernandez-Moreno et al., 2011)
OAI	255 knee OA cases	J	Fewer large tibiofemoral BMLs	(Rego-Perez et al., 2018)

mtDNA, mitochondrial DNA; UK, United Kingdom; OA, Osteoarthritis; OAI, Osteoarthritis Initiative; CHECK, Cohort Hip and Cohort Knee; NO, nitric oxide; BMLs, bone marrow lesions; MMP, metalloproteinase; OR, odds ratio; HR, hazard ratio; RR, risk ratio; ns, non-significant; (*) when compared with mtDNA cluster KU; [(&)] p-value after multiple testing correction.

1957), and even patients with haplogroup H were more prone to requiring total joint replacement (Soto-Hermida et al., 2015). mtDNA haplogroup T was associated with a decreased rate of radiographic knee OA progression, as well as a reduced loss of knee cartilage integrity over time in patients of the Osteoarthritis Initiative (OAI) of the US National Institutes of Health (NIH) (Soto-Hermida et al., 2014b). This association was then replicated in the CHECK cohort (cohort hip and cohort knee), another prospective cohort of OA patients from the Netherlands (Fernandez-Moreno et al., 2017b). Finally, a subsequent meta-analysis including the above-mentioned studies confirmed that mtDNA variants of the JT cluster act as protective factors against the radiographic progression of the disease (Fernandez-Moreno et al., 2017b).

In terms of disease incidence, a meta-analysis including 3217 individuals from the OAI and CHECK cohorts concluded that, compared with the most common Caucasian haplogroup H, subjects with mtDNA haplogroup J show a lower rate of incident knee OA over an eight-year period (Fernandez-Moreno et al., 2017a). This study included the design of a cellular model of transmitochondrial cybrids, consisting of cells with a defined and uniform nuclear background containing mitochondria from different sources, to demonstrate the existence of functional differences between haplogroups H and J; the study concluded that, compared with H cybrids, J cybrids produce less ATP, but this was accompanied with lower amounts of peroxynitrite and mitochondrial superoxide anion together with a lower rate of apoptosis under stress conditions as well as an increased ability to cope with oxidative stress (Fernandez-Moreno et al., 2017a). Another study in Korean populations showed that Asian mtDNA haplogroup B, described as a protective factor against knee OA prevalence in a population from the south of China (Fang et al., 2014), was a risk factor for the incidence of knee OA over an eight-year period (Koo et al., 2019).

mtDNA Haplogroups and Biomarkers in OA

In an effort to detect structural changes in an early stage of the disease, to monitor disease progression, or even to assess therapeutic responses with more sensitivity and reliability, molecular biomarkers have been developed in OA (Garnero et al., 2002; Rousseau and Delmas, 2007; Camacho-Encina et al., 2019). In a set of clinically relevant studies, OA-protective haplogroup J has been significantly associated with lower serum levels of catabolic type II collagen biomarkers and matrix metalloproteinases, in contrast to haplogroup H carriers, which showed significantly higher levels (Rego-Perez et al., 2010; Rego-Perez et al., 2011). Despite not being considered a biomarker of the disease, although higher-than-normal production has been described in OA chondrocytes (Maneiro et al., 2005; Henrotin and Kurz, 2007), the production of nitric oxide (NO) is significantly lower in articular chondrocytes harboring mtDNA haplogroup J than in non-J chondrocytes (Fernandez-Moreno et al., 2011). Based on these findings, haplogroups J and H represent two different OA phenotypes, leading to the consideration of these mtDNA haplogroups as complementary genetic biomarkers of the disease (Fernandez-Moreno et al., 2012).

In terms of imaging biomarkers, the identification and quantification of early bone marrow lesions (BMLs) has great relevance for assessing symptomatic progression and radiographic worsening over time in patients with OA (Roemer et al., 2016). In this sense, a longitudinal study including 255 participants from the OAI cohort that developed incident knee OA at 48 months revealed that patients with mtDNA haplogroup J were less likely to develop large BMLs in the tibiofemoral compartment of the knee than those with mtDNA haplogroup H (Rego-Perez et al., 2018). This association could be due to the differential behavior of mtDNA haplogroups H and J in terms of the metabolic activity and inflammation that takes place in BMLs (Kuttapitiya et al., 2017).

In summary, in Caucasian populations, it seems that haplogroups belonging to mitochondrial cluster JT are protective against OA and have even been associated with increased longevity in some European populations (Dato et al., 2004). However, in energy-deficiency diseases, such as LHON (Leber Hereditary Optic Neuropathy) these haplogroups, specifically haplogroup J, are risk factors (Hudson et al., 2007). This controversy is potentially related to the uncoupling nature of the genetic polymorphisms associated with these haplogroups, by which ATP production is reduced but, conversely, lower ROS generation, oxidative damage, and apoptosis are also expected (Ruiz-Pesini et al., 2004).

THE MITOCHONDRIAL GENOME AS AN EPIGENETIC REGULATOR IN ARTICULAR CARTILAGE

It is well known that bi-directional communication exists between the nucleus and mitochondria with the aim of maintaining cellular homeostasis and regulating adaptation to a broad range of stressors (Quirós et al., 2016; Wallace, 2016). This communication implies, on the one hand, that mitochondria are controlled by the nucleus by means of an "anterograde regulation," a mechanism that regulates mitochondrial activity and biogenesis to provide cellular needs; on the other hand, mitochondria and mtDNA variation maintain partial regulatory signaling control over the nucleus through a "retrograde regulation," which leads to the modification of cellular metabolism and function by activating the expression of nuclear genes with the aim of protecting against mitochondrial dysfunction (Jazwinski, 2013; Horan and Cooper, 2014; Matilainen et al., 2017).

Based on the above findings, and taking into account the reported associations between common mtDNA variants and different physiological and pathological phenotypes (Gómez-Durán et al., 2010; Martínez-Redondo et al., 2010; Marom et al., 2017), it can be deduced that interactions between mtDNA sequences, nuclear DNA, and the environment have important effects on mammalian biology (Kenney et al., 2014). In this sense, the effect of specific nuclear polymorphisms classically identified as risk factors for different diseases, such as Parkinson´s disease, cancer, and severe cardiopathy, is also modulated by

mtDNA variation (Maruszak et al., 2008; Strauss et al., 2013; Blein et al., 2015). In the case of OA, as subjects carrying mtDNA haplogroup J are not fully protected from suffering from the disease, it would be interesting to investigate potential interactions between this haplogroup, as well as the biochemically opposite haplogroup H (Wallace et al., 1999; Martínez-Redondo et al., 2010), and the most robust nuclear polymorphisms described in different GWAS performed in OA (Warner and Valdes, 2017; Zengini et al., 2018; Tachmazidou et al., 2019).

In terms of animal models, interesting work in conplastic mice (mice with a constant nuclear background but different mtDNA genomes) showed profound differences in health longevity between conplastic strains. The level of divergence between the two strains, equivalent to that between human African and Eurasian mtDNAs, showed different behavior in terms of mitochondrial proteostasis, reactive oxygen generation, obesity, and insulin signaling as well as in cell-senescence-related parameters such as telomere shortening and mitochondrial dysfunction (Latorre-Pellicer et al., 2016). Most of the altered processes described in the work of Latorre-Pellicer and co-workers are also involved in many common human diseases. In agreement with this, preliminary analyses of OA-related features using the two strains of these animals revealed significant differences between them in terms of the expression of the autophagy-related protein microtubule-associated protein 1 light chain 3 (LC3) and extracellular matrix-degrading protein metalloproteinase-13 (MMP-13) as well as significant differences in the Mankin score, a scoring system for the histopathological classification of the severity of cartilage lesions in OA (Scotece et al., 2019).

In this context of mitochondrial-nuclear interactions, epigenetics emerges as an important mechanism. Specifically, DNA methylation is the best-characterized epigenetic mechanism in OA. DNA methylation consists of the addition of a methyl group (CH_3) by S-adenosyl-methionine (SAM) to a cytosine that lies at 5′ of guanine (CpG site) to give rise to methylated cytosine. When this process occurs in high-density CpG regions of promoters, gene silencing occurs; in contrast, when methylation occurs in gene bodies, it leads to increased gene expression (Hellman and Chess, 2007). In contrast to nuclear DNA, the effects of mtDNA methylation in OA have not been explored so far; however, it is well accepted that mtDNA variation is able to modulate the nuclear methylome. Consistent with this concept, two independent studies using transmitochondrial cybrids showed that global DNA methylation levels are differentially modulated by mtDNA haplogroups J and H (Bellizzi et al., 2012; Atilano et al., 2015); in addition, these two haplogroups also mediate the methylation profile and the expression levels of genes involved in angiogenesis, inflammation, and other signaling pathways (Atilano et al., 2015). Moreover, different haplogroups on a uniform nuclear background of mouse embryonic stem cells were also associated with different methylation profiles and gene expression (Kelly et al., 2013).

In the case of OA, DNA methylation is involved in the phenotypic modulation that articular chondrocytes undergo during the OA process (Roach et al., 2005). After the few first studies, in which the methylation pattern of specific genes involved in OA pathogenesis was explored (Roach et al., 2005; Hashimoto et al., 2013), genome-wide DNA methylation assays were performed. These studies not only showed that knee and hip OA cartilages have different DNA methylation patterns but also identified a subgroup of OA patients with an enrichment of altered genes involved in inflammation and immunity (Fernandez-Tajes et al., 2014; Rushton et al., 2014). The only study that has so far analyzed the effect of mtDNA variation on the methylome of articular cartilage revealed that cartilages harboring haplogroups H and J show a differential methylation pattern, regardless of diagnosis. The study consisted of a genome-wide DNA methylation approach followed by a whole transcriptomic assay and demonstrated that apoptosis is enhanced in haplogroup H cartilage samples, together with an enrichment of overexpressed genes related to cell death; on the contrary, apoptosis appeared more repressed in haplogroup J cartilages. In addition, compared with H cartilages, samples with haplogroup J also showed a significant enrichment of hypomethylated CpGs of genes related to developmental process, including those belonging to the homeobox family of transcription factors, while H cartilages showed an enrichment of genes related to metabolic processes (Cortes-Pereira et al., 2019). These findings reflect that the epigenetic modifications that occur during the pathogenesis of OA and affect different key processes, such as metabolic alterations or apoptosis, vary depending on the mitochondrial genetic background, and this could determine the evolution of the disease (**Figure 1**).

Based on the description above, it is conceivable to consider the mitochondrial genome as an epigenetic regulator of the nuclear genome in articular cartilage. Stimuli such as reactive metabolic intermediates from the mitochondrial metabolism, small RNAs, mitochondria-derived peptides, and/or reactive oxygen species could be considered among the underlying mechanisms by which mitochondrial variation promotes modifications in the nuclear DNA methylome in OA (Schroeder et al., 2013; Horan and Cooper, 2014; Shadel and Horvath, 2015).

CONCLUSIONS AND FUTURE DIRECTIONS

The evidence presented in this review supports the hypothesis that mitochondrial genetics not only influences different features of OA disease but also modulates, in terms of mtDNA haplogroups, the nuclear DNA methylome of the only cell type present in articular cartilage, the chondrocyte. Based on this evidence, a broad field of promising research lies ahead.

Efforts must be made in the use of conplastic mice to investigate the influence of the mitochondrial background on specific OA-related features in animal models that develop spontaneous OA as well as in conplastic animals with induced OA. On the other hand, of special interest would be the design of transmitochondrial cybrids using chondrocytes as stable nuclear donors to subsequently test the influence of mtDNA variants in

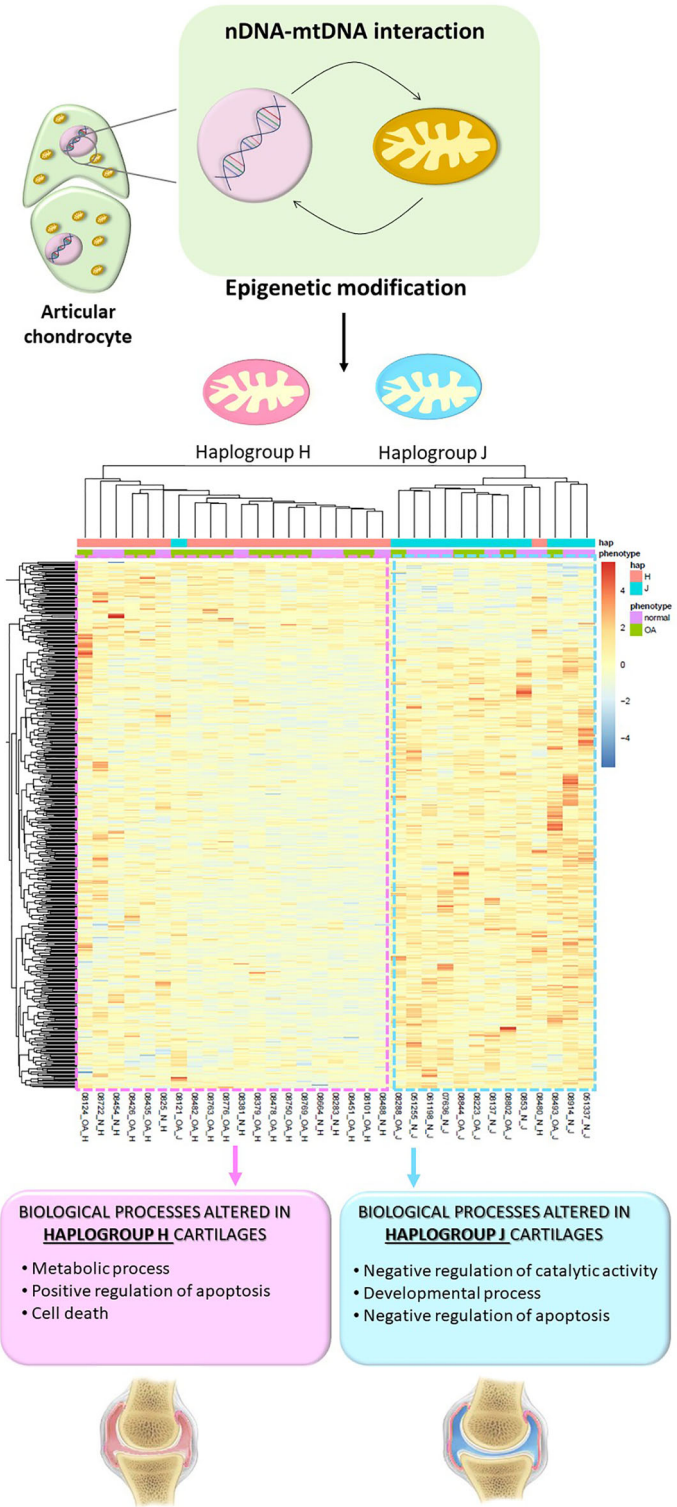

FIGURE 1 | The interactions between the nucleus and mitochondria that take place inside articular chondrocytes give rise to epigenetic modifications that are mediated by mitochondrial DNA haplogroups. As a consequence, different haplogroup-associated methylation patterns condition key processes related to the development of OA. Permission is granted for publication of this figure as a modified version of the figure that appeared on page A17 of the July 2019 issue of Arthritis & Rheumatology (Clinical Connections).

the native cells of articular cartilage. The use of both animal and cellular models, together with the methylation data originating from different genome-wide methylation studies, could contribute to the development of molecular biomarkers aimed at identifying specific OA phenotypes from the design of CpG classifier panels combined with the mitochondrial genetic background.

Given the role of adaptive selection in the origin of mtDNA haplogroups, and recognizing that they could be maladaptive in different environments with new lifestyles (Wallace, 2005), the proposed study of potential interactions between mtDNA variants and different nuclear DNA polymorphisms previously associated with OA susceptibility in various GWAS should be conducted taking into account the specific environment. Because none of the studies described in Table 1 aimed to identify specific or single mitochondrial polymorphisms associated with different OA-related features, the precise identification through next-generation sequencing techniques of these specific mtDNA polymorphisms, characteristic or not of each haplogroup, from both isolated blood and articular cartilage from the same patient, would be a powerful tool for the consideration of mtDNA variation as a potential robust biomarker of OA.

In terms of therapeutic research, the restoration of mitochondrial function in OA chondrocytes would be the ultimate goal. This can be achieved by using different approaches to design different drugs that are capable of: i) suppressing mitochondrial oxidative damage and restoring extracellular matrix homeostasis (Farnaghi et al., 2017), ii) activating the AMPK-SIRT1-PGC1α pathway to induce mitochondrial biogenesis, therefore decreasing the pro-catabolic response of chondrocytes (Wang et al., 2015), iii) activating mitophagy, given its importance in preventing mitochondrial dysfunction (López de Figueroa et al., 2015), or iv) emulating the physiological effects of the OA-protective mtDNA haplogroup J on mitochondrial activity, as well as administering healthy isolated mitochondria into the osteoarthritic joint. On the other hand, given the bi-directional communication between the nucleus and mitochondria, interventions focused on the management of mitochondrial dysfunction by targeting the epigenome, or vice versa (Matilainen et al., 2017), would also be of interest.

AUTHOR CONTRIBUTIONS

FB and IR-P contributed equally to the design and coordination of the study; both conceived the study and participated in its design. AD-S and PR-L contributed to some of the findings described in the manuscript and helped to draft the final version of the manuscript.

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Mitoepigenetics and Its Emerging Roles in Cancer

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In human beings, there is a ∼16,569 bp circular mitochondrial DNA (mtDNA) encoding 22 tRNAs, 12S and 16S rRNAs, 13 polypeptides that constitute the central core of ETC/OxPhos complexes, and some non-coding RNAs. Recently, mtDNA has been shown to have some covalent modifications such as methylation or hydroxylmethylation, which play pivotal epigenetic roles in mtDNA replication and transcription. Post-translational modifications of proteins in mitochondrial nucleoids such as mitochondrial transcription factor A (TFAM) also emerge as essential epigenetic modulations in mtDNA replication and transcription. Post-transcriptional modifications of mitochondrial RNAs (mtRNAs) including mt-rRNAs, mt-tRNAs and mt-mRNAs are important epigenetic modulations. Besides, mtDNA or nuclear DNA (n-DNA)-derived non-coding RNAs also play important roles in the regulation of translation and function of mitochondrial genes. These evidences introduce a novel concept of mitoepigenetics that refers to the study of modulations in the mitochondria that alter heritable phenotype in mitochondria itself without changing the mtDNA sequence. Since mitochondrial dysfunction contributes to carcinogenesis and tumor development, mitoepigenetics is also essential for cancer. Understanding the mode of actions of mitoepigenetics in cancers may shade light on the clinical diagnosis and prevention of these diseases. In this review, we summarize the present study about modifications in mtDNA, mtRNA and nucleoids and modulations of mtDNA/nDNA-derived non-coding RNAs that affect mtDNA translation/function, and overview recent studies of mitoepigenetic alterations in cancer.

Keywords: mtDNA methylation, mitoepigenetics, mtRNA modification, non-coding RNAs, cancer

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INTRODUCTION

Epigenetics is the study of mitotically and/or meiotically heritable phenotype alterations that do not entail a change in DNA sequence (Wu and Morris, 2001). Epigenetics in nuclear genome (nDNA) has been well described and characterized. Generally, epigenetic regulation contains three levels of biological actions, including covalent modifications in DNA bases, histone variants, post-translational modifications of histones, RNA modifications, and non-coding RNA (ncRNA) modulations (Dupont et al., 2009; Peschansky and Wahlestedt, 2014). Epigenetic regulation has been shown to be an important biological process that participates in tumorigenesis and cancer

development, and epigenetic biomarkers or targets can be used in diagnosis, prognosis, and treatment of these diseases (Kondo et al., 2017; Dong and Cui, 2018; Nebbioso et al., 2018; Zhu et al., 2019).

As cellular organelles present in almost all eukaryotic cells, mitochondria are places where ATP is biosynthesized and are essential for various cellular biological processes, including reactive oxygen species (ROS) generation, intracellular Ca^{2+} signaling, heme metabolism, intrinsic apoptosis, mitophagy, metabolism and cell cycle progression (Yien et al., 2014; Picard et al., 2016; van der Bliek et al., 2017; Gómez-Durán et al., 2018). Genetic mutations in mitochondrial DNA (mtDNA) and perturbations in mitochondrial proteins can cause dysfunction of mitochondria that has been shown to be tightly associated with many mitochondrial diseases and cancer (Taylor and Turnbull, 2005; Garone et al., 2012a,b, 2013; Gómez-Durán et al., 2012; Ronchi et al., 2012; Kullar et al., 2017; Chinnery and Gómez-Durán, 2018; Garone and Viscomi, 2018; Andreazza et al., 2019). Therefore, mitochondria are promising targets for the treatment of these diseases (Gómez-Durán et al., 2010; Zong et al., 2016; Dong et al., 2019). Recently, in addition to mitogenetics, epigenetics in mitochondria (mitoepigenetics) also emerges as an important regulatory mode that is related to human physiology and disorders, such as stemness, drug addiction, neurodegenerative diseases, cardiovascular diseases and metabolic diseases (Wallace, 1992; Gómez-Durán et al., 2010; Manev and Dzitoyeva, 2013; Kalani et al., 2014; Sadakierska-Chudy et al., 2014; Ghosh et al., 2015; Gao D. et al., 2017; Stimpfel et al., 2018; Coppede and Stoccoro, 2019). Importantly, this kind of mode of action has also emerged to be associated with cancers (Ferreira et al., 2015; Lozano-Rosas et al., 2018). However, mitoepigenetics has not been well depicted.

Herein, we define the concept of mitoepigenetics as the study of modulations occurring in the mitochondria that induce heritable phenotype alterations in mitochondria without involving a change of mtDNA sequence. Based on current studies, mitoepigenetics comprises of four levels: mtDNA methylation/hydroxylmethylation, mitochondrial nucleoid modifications, mtRNA modifications, and mtDNA-derived or nDNA-derived non-coding RNA modulations during mtDNA-encoded gene translation/function. In this article, we review the current study on mitoepigenetics and its roles in cancers, so as to provide a new sight for the diagnosis and treatment of these disorders.

mtDNA AND ITS MODIFICATIONS

mtDNA

mtDNA exists in the matrix or the inner membrane of mitochondria. Genome signature comparisons reveal that mtDNA is analogous to prokaryotic genome (Campbell et al., 1999). Especially, alpha-proteobacteria seems to be the most likely bacterial ancestor of the mitochondria (Gray, 2012; Muñoz-Gómez et al., 2017; Martijn et al., 2018). mtDNA has a loop structure of guanine-rich heavy chains (H-strand) and cytosine-rich light chains (L-strand) (Chinnery and Hudson, 2013). The length of mtDNA ranges from 15,000 to 17,000 bp in different species (Chinnery and Hudson, 2013). Human mtDNA is a ∼16,569 bp circular DNA that encodes 37 genes (28 on the H-strand and 9 on the L-strand), including 2 rRNAs (12S and 16S rRNAs), 22 tRNAs, and 13 proteins in the electron transport chain (ETC)/oxidative phosphorylation (OxPhos) system (**Table 1** and **Figure 1**) (Andrews et al., 1999; Brandon et al., 2005; Chinnery and Hudson, 2013). Besides, mtDNA also contains some pseudogenes (Woischnik and Moraes, 2002; Gunbin et al., 2017) and encodes non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs) (Rackham et al., 2011) and small non-coding RNAs (sncRNAs), such as microRNAs (Lung et al., 2006; Barrey et al., 2011; Bandiera et al., 2013; Ro et al., 2013; Duarte et al., 2014). Mitochondrial genome has some unique genetic characteristics, including high mutation, heteroplasmy, threshold effect, maternal inheritance and mitotic segregation (Wallace, 2005). The copy number of mtDNA varies between 100 and 10 000 per cell dependent upon cellular energy demand (Srinivasan et al., 2017).

Unlike nDNA, mtDNA contains only one non-coding triple-helical region (16,024-576, ∼1000 bp, ∼7% of all sequence), the displacement loop (D-loop) formed by abortive initiation of replication (Yamamoto, 2001). Both replication and transcription are initiated from D-loop, which contains the H/L-strand promotor (HSP1/LSP1), and the H-strand origin of replication (O_H) (Jemt et al., 2015). Sixty bp upstream of HSP1, there is a HSP2. There are also some specific sites (IT_L, IT_{H1}, IT_{H2}) within the promotors, where mtDNA transcription initiates. Mitochondrial RNAs are firstly transcribed as primary transcripts, which are subsequently cleaved by enzymes and affected by specific nucleotide modifications to yield polycistronic precursors and finally mature RNAs (Van Haute et al., 2015). Besides, like prokaryotic cells, mtDNA lacks intronic regions, and intergenic sequences are either absent or only a few nucleotide bases long. Some genes, such as MT-ATP6/8 and MT-ND4/4L, have overlapping regions.

mtDNA Modifications

Although mitochondrial genome has a diminutive size, mutations in mtDNA occur frequently, because mtDNA lacks the error checking system that nDNA has. Some of mutations are tightly associated with inherited diseases (Taylor and Turnbull, 2005). Apart from mtDNA mutations, mtDNA is also shown to have some modifications, like that in nDNA.

As shown in **Figure 2**, DNA can be methylated by methyltransferases, which can transfer a methyl group from a methyl donor *S*-adenosylmethionine (SAM) onto the C5 position of the cytosine to form 5-methylcytosine (5mC) by the aid of DNA methyltransferases (DNMTs) such as DNMT1, DNMT3A, and DNMT3B (Moore et al., 2013). Methylation leads to changes of the molecular structure in DNA and affects transcription factor, RNA polymerase, topoisomerase, or inhibitory protein binding to DNA, resulting in dysfunction of gene transcription and expression (Moore et al., 2013). DNA 5mC can also be demethylated via either passive demethylation carried out by

TABLE 1 | Protein-coding and RNA genes that encoded by human mtDNA (Pseudogenes and non-coding RNA genes are not included in this table).

Gene symbol	Alternative names	Description	Location in mtDNA (H/L-strand)	Size (nt)
MT-RNR1	12S rRNA	Mitochondrially encoded 12S RNA	648/649–1601, H	954/955
MT-RNR2	16S rRNA	Mitochondrially encoded 16S RNA	1,671–3,229, H	1,559
MT-TL2	mt-tRNA$^{Leu(CUN)}$	Mitochondrially Encoded TRNA-Leu (CUN) 2	12,266–12,336, H	71
MT-TI	mt-tRNA^{Ile}	Mitochondrially Encoded TRNA-Ile (AUU/C)	4,263–4,331, H	69
MT-TQ	mt-tRNA^{Gln}	Mitochondrially Encoded TRNA-Gln (CAA/G)	4,329–4,400, L	72
MT-TW	mt-tRNA^{Trp}	Mitochondrially Encoded TRNA-Trp (UGA/G)	5,512–5,579, H	68
MT-TA	mt-tRNA^{Ala}	Mitochondrially Encoded TRNA-Ala (GCN)	5,587–5,655, L	69
MT-TN	mt-tRNA^{Asn}	Mitochondrially Encoded TRNA-Asn (AAU/C)	5,657–5,729, L	73
MT-TC	mt-tRNA^{Cys}	Mitochondrially Encoded TRNA-Cys (UGU/C)	5,761–5,826, L	66
MT-TY	mt-tRNA^{Tyr}	Mitochondrially Encoded TRNA-Tyr (UAU/C)	5,826–5,891, L	66
MT-TS2	mt-tRNA$^{Ser(AGY)}$	Mitochondrially Encoded TRNA-Ser (AGU/C) 2	12,207–12,265, H	59
MT-TD	mt-tRNA^{Asp}	Mitochondrially Encoded TRNA-Asp (GAU/C)	7,518–7,585, H	68
MT-TK	mt-tRNA^{Lys}	Mitochondrially Encoded TRNA-Lys (AAA/G)	8,295–8,364, H	70
MT-TG	mt-tRNA^{Gly}	Mitochondrially Encoded TRNA-Gly (GGN)	9,991–10,058, H	68
MT-TR	mt-tRNA^{Arg}	Mitochondrially Encoded TRNA-Arg (CGN)	10,405–10,469, H	65
MT-TH	mt-tRNA^{His}	Mitochondrially Encoded TRNA-His (CAU/C)	12,138–12,206, H	69
MT-TS1	mt-tRNA$^{Ser(UCN)}$	Mitochondrially Encoded TRNA-Ser (UCN) 1	7,445–7,516, L	72
MT-TE	mt-tRNA^{Glu}	Mitochondrially Encoded TRNA-Glu (GAA/G)	14,674–14,742, L	69
MT-TP	mt-tRNA^{Pro}	Mitochondrially Encoded TRNA-Pro (CCN)	15,955–16,023, L	69
MT-TT	mt-tRNA^{Thr}	Mitochondrially Encoded TRNA-Thr (ACN)	15,888–15,953, H	66
MT-TF	mt-tRNA^{Phe}	Mitochondrially Encoded TRNA-Phe (UUU/C)	577–647, H	71
MT-TV	mt-tRNA^{Val}	Mitochondrially Encoded TRNA-Val (GUN)	1,602–1,670, H	69
MT-TM	mt-tRNA^{Met}	Mitochondrially Encoded TRNA-Met (AUA/G)	4,402–4,469, H	68
MT-TL1	mt-tRNA$^{Leu(UUR)}$	Mitochondrially Encoded TRNA-Leu (UUA/G) 1	3,230–3,304, H	75
MT-CYB	Cytochrome b	Mitochondrially encoded cytochrome b	14,747–15,887, H	1,141
MT-CO1	COX 1	Mitochondrially Encoded Cytochrome C Oxidase I	5901–7442, H	1,542
MT-CO2	COX 2	Mitochondrially Encoded Cytochrome C Oxidase II	7586–8294, H	709
MT-CO3	COX 3	Mitochondrially Encoded Cytochrome C Oxidase III	9,207–9,990, H	784
MT-ND1	ND 1	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 1	3,305–4,262, H	958
MT-ND2	ND 2	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 2	4,470–5,511, H	1,042
MT-ND3	ND 3	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 3	10,059–10,404, H	346
MT-ND4	ND 4	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 4	10,760–12,137, H	1,378
MT-ND4L	ND 4L	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 4L	10,470–10,766, H	297
MT-ND5	ND 5	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 5	12,337–14,148, H	1,812
MT-ND6	ND 6	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 6	14,149–14,673, L	525
MT-ATP6	ATPase 6	Mitochondrially Encoded ATP Synthase Membrane Subunit 6	8,527–9,206, H	681
MT-ATP8	ATPase 8	Mitochondrially Encoded ATP Synthase Membrane Subunit 8	8,365–8,572, H	207

dilution by replication without *de novo* methylation or active demethylation carried out by oxidation or deamination.

During active demethylation pathway, some of 5mC sites can also be catalyzed and oxidized by 2-oxoglutarate and Fe(II)-dependent oxygenases of the ten-eleven-translocation (TET) proteins, including TET1, TET2, and TET3, to form 5-hydroxymethylcytosine (5hmC), which is considered as a possible intermediate in a replication-independent DNA demethylation pathway (Richa and Sinha, 2014). 5hmC is enriched in active genes that have a strong depletion of 5mC (Mellen et al., 2012). With the aid of TET1/2/3, 5hmC is further catalyzed into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be subsequently excised and replaced via base excision repair (BER). Besides, 5mC and 5hmC can also be deaminated to yield thymine and 5-hydroxymethyluracil (5hmU) by the aid of activation induced cytidine deaminase (AID)/apolipoprotein B mRNA editing enzyme and catalytic polypeptide (APOBEC). This results in a thymine-guanine mismatch that can lead to a DNA repair in which thymine and 5hmU can be replaced by unmethylated cytosine (Jang et al., 2017). However, 5hmC seems to be not only the intermediate of DNA demethylation, but is also a major element in the modulation of chromatin structure and gene expression through binding with methyl-CpG-binding protein 2 (MeCP2) (Mellen et al., 2012).

With the development of technology for detecting methylation, this kind of modification was also found in

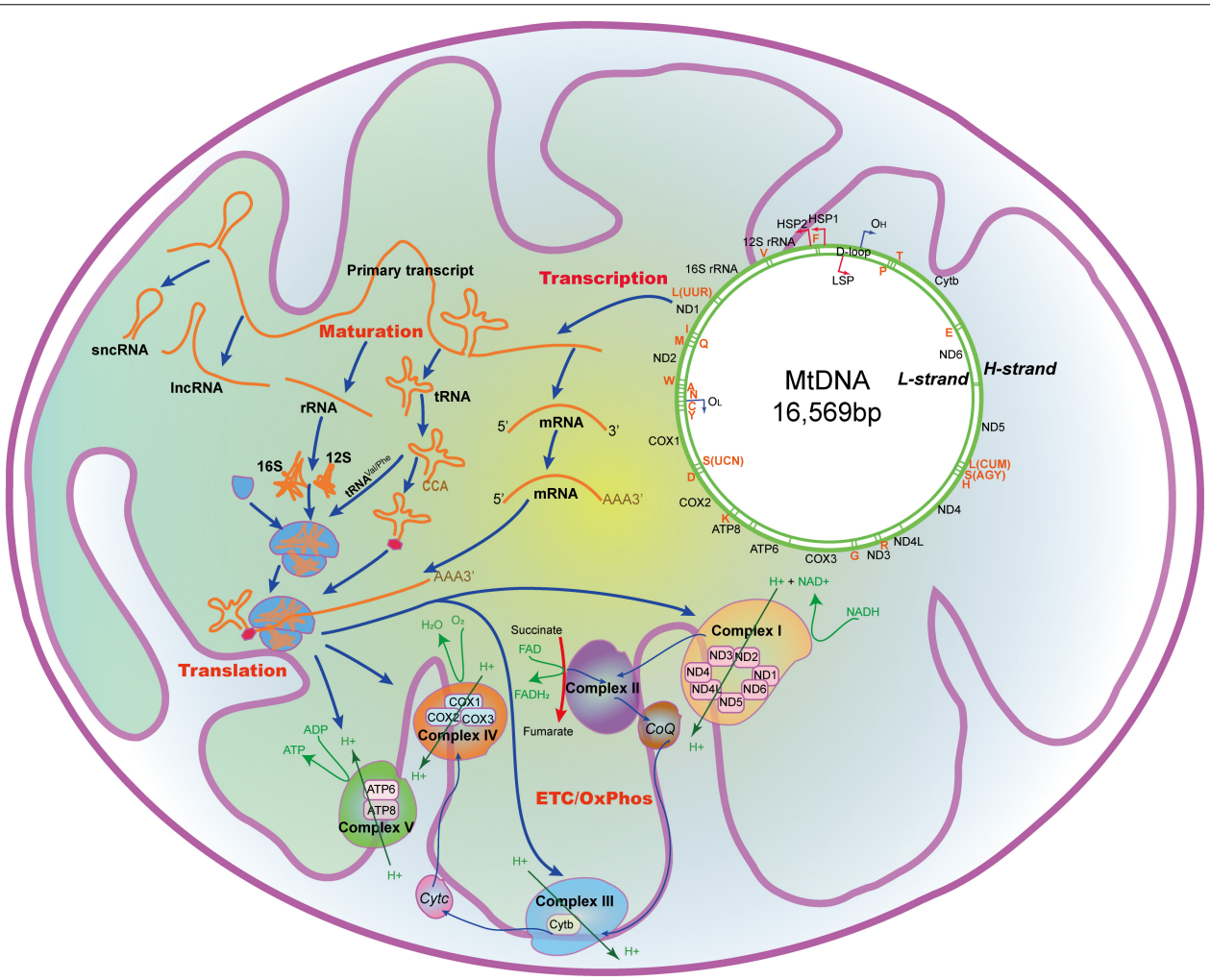

FIGURE 1 | The mtDNA and the processing and function of its encoding genes in the mitochondria. mtDNA with 16,569 nucleotides encodes 22 tRNAs, 2 rRNAs, 13 peptides that constitutes the ETC/OxPhos, and some non-coding RNAs. A, mt-tRNA^{Ala}; C, mt-tRNA^{Cys}; D, mt-tRNA^{Asp}; E, mt-tRNA^{Glu}; F, mt-tRNA^{Phe}; G, mt-tRNA^{Gly}; H, mt-tRNA^{His}; I, mt-tRNA^{Ile}; K, mt-tRNA^{Lys}; L(CUN), mt-tRNA$^{Leu(CUN)}$; L(UUR), mt-tRNA$^{Leu(UUR)}$; M, mt-tRNA^{Met}; N, mt-tRNA^{Asn}; P, mt-tRNA^{Pro}; Q, mt-tRNA^{Gln}; R, mt-tRNA^{Arg}; S(AGY), mt-tRNA$^{Ser(AGY)}$; S(UCN), mt-tRNA$^{Ser(UCN)}$; T, mt-tRNA^{Thr}; V, mt-tRNA^{Val}; W, mt-tRNA^{Trp}; Y, mt-tRNA^{Tyr}; ATP6/8, Mitochondrially encoded ATP synthase membrane subunit 6/8; CoQ, Coenzyme Q; COX1/2/3, Mitochondrially encoded cytochrome C oxidase I/II/III; Cytb, Cytochrome b; Cytc, Cytochrome c; ETC, Electron transport chain; FAD, Flavine adenine dinucleotide; $FADH_2$, Flavine adenine dinucleotide, reduced; HSP1/2, H-strand promotor 1/2; H-strand, Heavy strand; LSP, L-strand promotor; L-strand, Light strand; NAD^+, Nicotinamide adenine dinucleotide; NADH, Nicotinamide adenine dinucleotide, reduced; ND1/2/3/4/4L/5/6, Mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1/2/3/4/4L/5/6; O_L, L-strand origin of replication; O_H, H-strand origin of replication; OxPhos, Oxidative phosphorylation.

mtDNA. Distribution of 5mC seems to be conserved in mitochondrial genomes across all cell and tissue types (Ghosh et al., 2014). mtDNA methylation is usually found within the non-coding D-loop and gene start sites (GSS) (Mposhi et al., 2017), implying that methylation in mtDNA can affect mtDNA replication and transcription. Stimulating mtDNA replication results in increasing methylation (Rebelo et al., 2009), confirming that methylation can also be a feedback regulatory mode that maintains mtDNA copy number.

CpG dinucleotides are the most prominent regions where methylation occurs, however, non-CpG sites, such as CpA, CpT, and CpC also have methylations (Jang et al., 2017). The abundance of CpG sites varies in animal, fungal, protist, and plant mitochondrial genomes. Like nDNA, human mtDNA contains a relatively low frequency of CpG sites (435 in 16 659 nucleotides, 2.61%) (Cardon et al., 1994). Methylation of CpG in the H-strand promoter (HSP1) induces TFAM multimerization to augment cooperativity and enhances its binding affinity to mtDNA, compared to that of the non-methylated DNA. Although TFAM-dependent DNA compaction is not affected by methylation of CpG sites, transcription initiation from the three mitochondrial promoters is significantly impaired by CpG methylation (Dostal and Churchill, 2019). However, a study shows that mtDNA methylation mainly occurs within non-CpG sites of the promoter region of the H-strand,

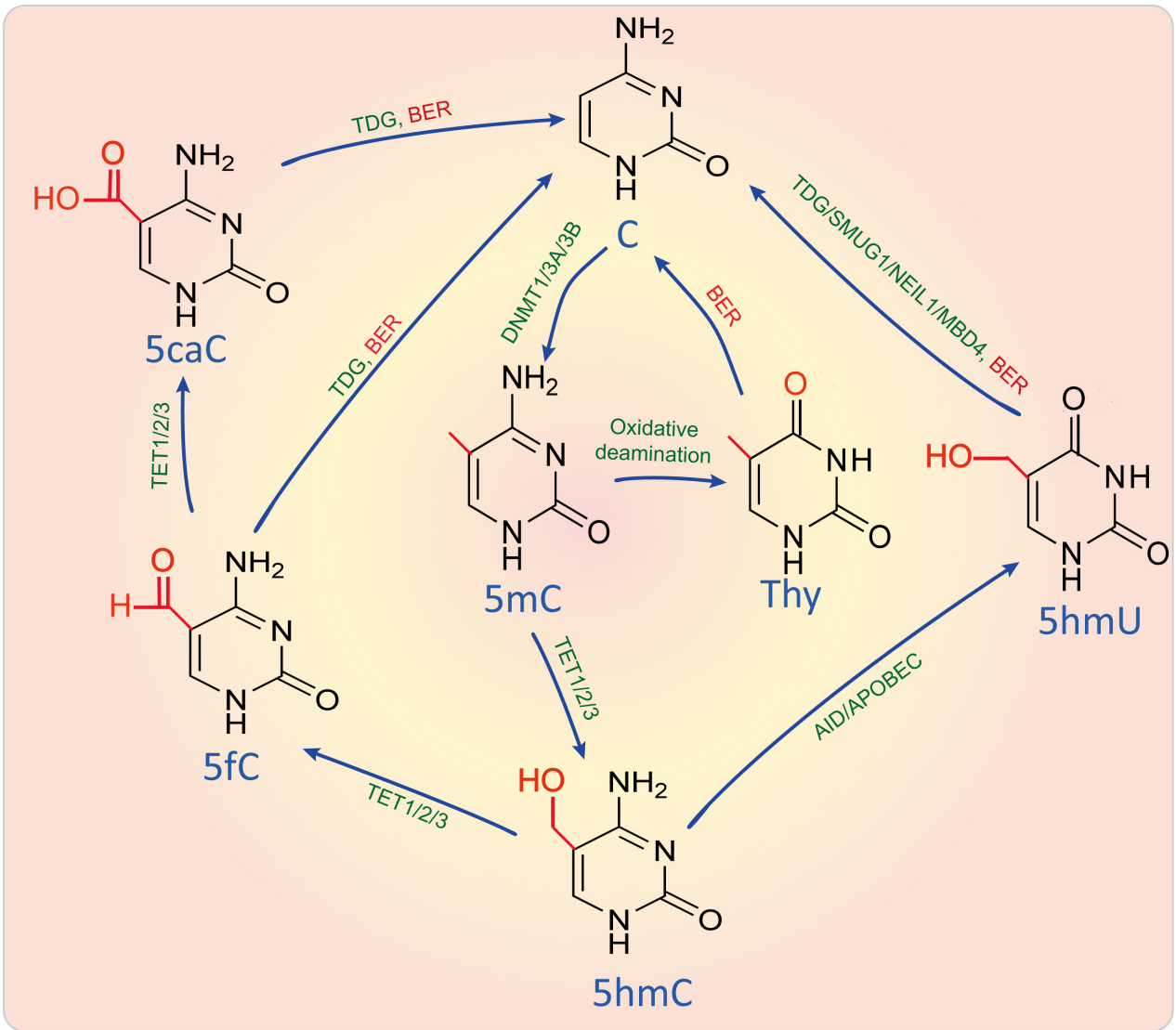

FIGURE 2 | DNA methylation and active demethylation. DNA can be methylated by DNMTs and demethylated by active demethylation through oxidizing, deaminating and base-excision repair. Enzymes were marked in green, metabolites were marked in blue, while biological process like BER was marked in red. 5caC, 5-Carboxylcytosine; 5fC, 5-Formylcytosine; 5hmC, 5-Hydroxymethylcytosine; 5hmU, 5-Hydroxymethyluracil; 5-AID, activation induced cytidine deaminase; APOBEC, Apolipoprotein B mRNA editing enzyme catalytic subunit; BER, Base-excision repair; DNMT1/3A/3B, DNA methyltransferase 1/3A/3B; MBD4, Methyl-CpG binding domain 4, DNA glycosylase; NEIL1, Nei like DNA glycosylase 1; SMUG1, Single-strand-selective monofunctional uracil-DNA glycosylase 1; TET1/2/3, Tet methylcytosine dioxygenase 1/2/3; C, Cytosine; TDG, Thymine DNA glycosylase; Thy, Thymine.

which is essential for mtDNA replication and transcription (Bellizzi et al., 2013).

5mC in mtDNA is catalyzed by mtDNMT1, an isoform of DNMT1. mtDNMT contains a mitochondrial targeting sequence, which can make it translocated into mitochondria (Shock et al., 2011). However, DNA methyltransferases seem to contribute to CpG methylation in the D-loop, while not non-CpG methylation (Bellizzi et al., 2013), because MtDNMT1 binding to the mtDNA is observed to be associated with the density of CpG sites (60).

5hmC is also found in mtDNA and it seems to promote demethylation through impairing mtDNMT1-mediated remethylation during replication (Manev and Dzitoyeva, 2013). However, the detailed process and the enzymes involved are not identified yet.

MITOCHONDRIAL NUCLEOID AND ITS MODIFICATIONS

Mitochondrial Nucleoid

Similarly to nDNA, mtDNA is also packed by proteins to form a protein-DNA structure referred to as a nucleoid. It is located in mitochondrial pseudocompartments, and some of its proteins may play histone-like architectural roles

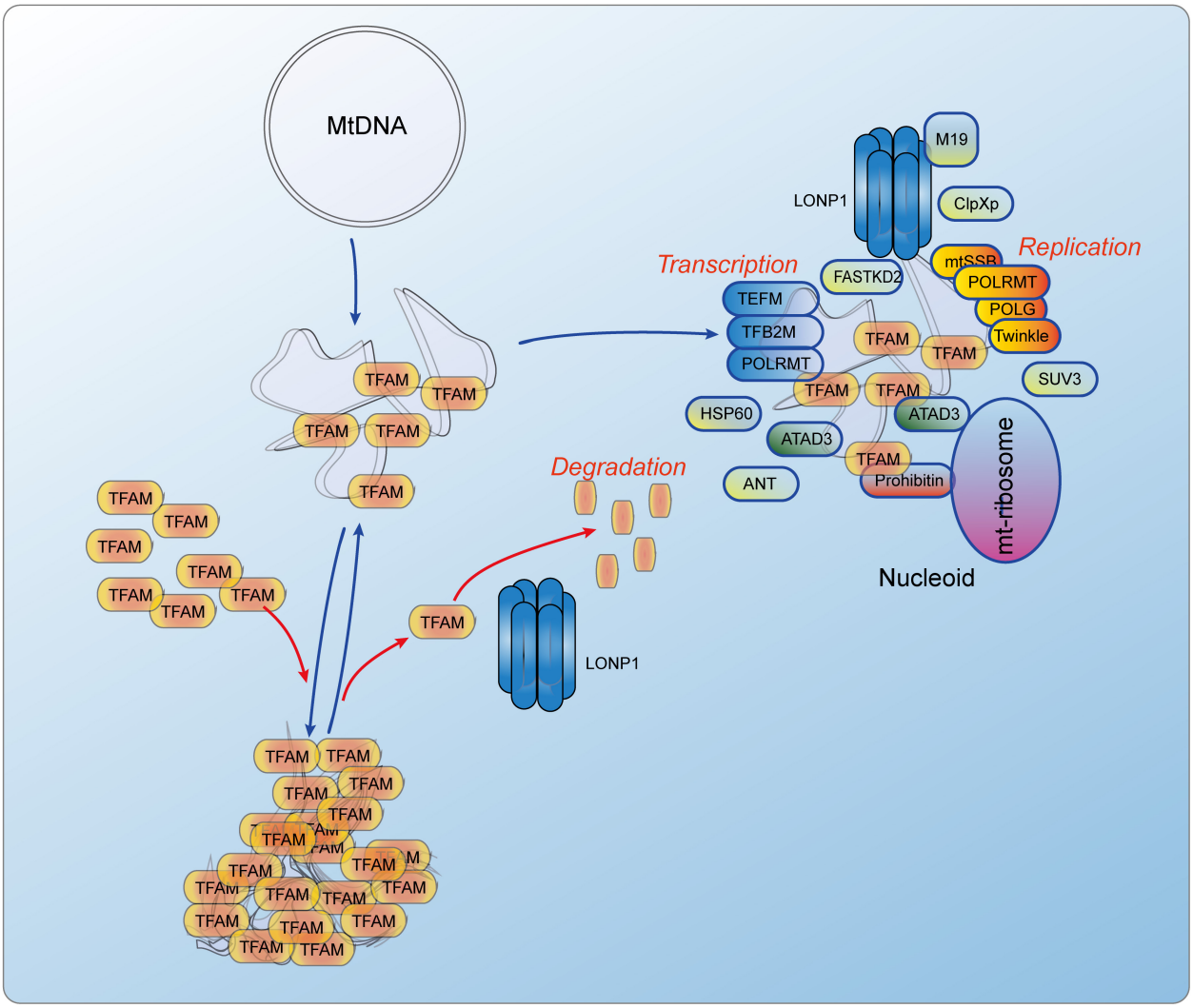

FIGURE 3 | The dynamics of TFAM controlled mitochondrial nucleoid and the constitutions of nucleiod. TFAm can directly bind to mtDNA and functions as a histone-like protein. Its degradation is mediated by LONP1, an AAA + Lon protease. There are more than 50 nucleoid-associated proteins including POLRMT, TFAM, TFB2M and TEFM that initiate the mtDNA transcription, and POLG, Twinkle and mtSSB that initiate the mtDNA replication. There are also some proteins that are related to RNA processing and nucleoid regulation. ANT, Adenine nucleotide translocator; ATAD3, ATPase family AAA domain containing 3; ClpXp, ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial; FASTKD2, FAST kinase domains 2; HSP60, Short heat shock protein 60; LONP1, Lon peptidase 1, mitochondrial; M19, Mitochondrial protein M19; mtSSB, Single-stranded DNA binding protein 1, mitochondrial; POLG, DNA polymerase gamma; POLRMT, RNA polymerase mitochondrial; SUV3, ATP-dependent RNA helicase SUV3, mitochondrial; TEFM, Transcription elongation factor, mitochondrial; TFAM, Transcription factor A, mitochondrial; TFB2M, Transcription factor B2, mitochondrial.

(Kanki et al., 2004). There are more than 50 nucleoid-associated proteins that can either temporarily or permanently associate with mtDNA or other nucleoid-associated proteins to maintain mtDNA and regulate gene expression (**Figure 3**). In nucleoid, mitochondrial transcription factor A (TFAM), mitochondrial polymerase γ (POLG), ATPase family AAA-domain-containing protein 3 (ATAD3), mitochondrial AAA protease (LONP1), and mitochondrial single-stranded DNA-binding protein (mtSSB) possibly directly interact with the D-loop region of mtDNA (Lee and Han, 2017). Mitochondrial RNA polymerase (POLRMT), TFAM, mitochondrial transcription factor B2 (TFB2M) and mitochondrial transcriptional elongation factor (TEFM) are key components of the mitochondrial transcription (Shokolenko and Alexeyev, 2017). POLG, Twinkle, mtSSB are key components of the mitochondrial replication (Young and Copeland, 2016). Besides them, nucleoid-associated proteins also include RNA helicases (e.g., SUV3), RNA-binding proteins (e.g., FASTKD2), quality-control proteases (e.g., lon-like peptidase LONP1 and caseionlytic peptidase CLPXP), as well as mitochondrial RNA processing proteins (Koc and Spremulli, 2003; Szczesny et al., 2013; Popow et al., 2015; Levytskyy et al., 2016; Lee and Han, 2017). These evidences suggest that nucleoid may be a place where mtRNAs are processed and mitoribosomes are assembled. Recently, post-transcriptional mtRNA processing and ribosome

biogenesis including, mtRNA maturation, ribosome assembly, and translation initiation may occur within mitochondrial RNA granules (MRGs), dynamic structures that juxtapose to nucleoids (Jourdain et al., 2013; Antonicka and Shoubridge, 2015; Hensen et al., 2019). MRGs are transiently associated with active nucleoids where they are assembled around the newly synthesized primary transcripts, then MRGs become detached and locate within the inner mitochondrial matrix and subsequent events in mitochondrial gene expression take place (Jourdain et al., 2016).

TFAM is a member of the high-mobility group domain proteins family and can form a U-turn with an overall bend of 180° on unspecific mtDNA sequence, functioning as a transcription and packaging factor (Ngo et al., 2011). In mammalian cells, this protein is very abundant. Per each mtDNA molecule, there are about 1,000 molecules of TFAM protein, which means that there is a TFAM molecule in every 16 bp of mtDNA (Farge and Falkenberg, 2019). Importantly, only with TFAM nucleoid compaction of mtDNA can sufficiently complete (Kaufman et al., 2007). In addition, single TFAM protein can also bridge neighboring mtDNA duplexes to form a cross-strand binding and looping out (Kukat et al., 2015). In the condition of high TFAM/mtDNA ratio, the combination of duplex bending and cross-strand binding result into mtDNA full compaction, which leads to the blockade of mtDNA transcription and replication (Ngo et al., 2014). Besides, TFAM can also act as a homodimer, which promotes looping of the DNA (Ngo et al., 2014). In summary, TFAM is the only nucleoid-associated protein that can stringently fulfill the criteria of a true mtDNA packaging factor (Bonekamp and Larsson, 2018). Unbalanced levels (low or high) of TFAM result in decreasing mtDNA methylation (Rebelo et al., 2009).

Nucleoids have a large size and are dynamically distributed throughout the mitochondrial network. Therefore, nucleoids are unlikely to move freely within mitochondrial matrix (Bonekamp and Larsson, 2018). It may be anchored at the inner membrane of mitochondria and its distribution may depend on mitochondrial fusion and fission (Elgass et al., 2013). There are evidences showing that deficiency of the large GTPase dynamin-related protein 1 (Drp1), a major regulator during mitochondrial fission, leads to remodeling of nucleoid clustering (Ban-Ishihara et al., 2013). Mitochondrial topoisomerase 3a (Top3a) also plays an essential role in genome separation and nucleoid distribution because it can deconcatenate newly replicated mtDNA (Goffart et al., 2019).

Mitochondrial Nucleoid Modifications

The protein scaffold of mtDNA nucleoids can also be epigenetically and post-translationally modified, just like the histones in the nDNA nucleosomes. There are more than 50 nucleoid-related proteins, in which TFAM is the only protein whose behavior is highly similar with that of histones. Therefore, mitochondrial nucleoid modifications refer to TFAM modifications. Based on current studies, TFAM can be acetylated, phosphorylated and ubiquitinated, thereby affecting its function in mtDNA packaging.

TFAM Acetylation

A recent study showed that TFAM is lysine acetylated within its high-mobility-group box 1 (HMGB1), which reduces TFAM to interact with non-specific DNA through distinct kinetic pathways (King et al., 2018). Another study showed that TFAM was acetylated at a single lysine residue and the level of acetylation in rat liver did not change with age (Dinardo et al., 2003). SIRT3 is the deacetylase that mediates the deacetylation of K154 of TFAM (Bagul et al., 2018; Liu et al., 2018).

TFAM Phosphorylation

Serine phosphorylation in HMGB1 domain of TFAM also regulates mtDNA transcription by blocking TFAM to locate at the promoter sites of target genes (King et al., 2018). Extracellular signal-regulated protein kinases (ERK1/2) can mediate phosphorylation of serine 177 in TFAM, thereby downregulating mitochondrial transcription (Wang et al., 2014). The AAA + LONP1, an ssDNA-binding protein, is responsible for the degradation of DNA-free TFAM (Chen et al., 2008). When Lon binds to heavy-strand sequences upstream of light-strand promoter (LSP^{HS}) or dsDNA-TFAM, its protease activity is directly blocked, resulting in TFAM stabilization (Liu et al., 2004). When TFAM is phosphorylated within its HMGB1 domain by cAMP-dependent protein kinase in mitochondria, the ability of TFAM to bind DNA is impaired and gene transcription is activated, then TFAM is degraded by LONP1 (Lu et al., 2013).

TFAM Ubiquitination

In addition to the modifications above, TFAM can also be ubiquitinated and its stability or sub-location may be affected. For instance, in the retina from diabetic rats, high glucose can lead to TFAM ubiquitination, which impedes its transport to the mitochondria, resulting in subnormal mtDNA transcription and mitochondria dysfunction (Santos et al., 2014).

mtRNA MODIFICATIONS

RNA modifications are present in almost all the cellular RNAs in archaeobacteria, bacteria, plants, fungi, and animals. There are almost 150 kinds of modifications that have been found in RNA (Boccaletto et al., 2018). Mitochondrial RNAs (mtRNAs) are processed from single polycistronic precursor RNAs, however, there are also multiple different types of mt-RNAs, including 2 rRNAs, 22 tRNAs, 13 mRNAs and many ncRNAs, present in the mitochondrial matrix. This suggests that the post-transcriptional mechanisms are very important for the translation, maturation, stability and assembly of mtRNAs.

A study on m^6A patterns in the transcriptomes of *Arabidopsis* mitochondria and chloroplast reveals that more than 86% of the transcripts are m^6A methylated. Over 350 m^6A sites were with ∼4.6 to ∼4.9 m^6A sites per transcript are identified in mitochondrial genome. The extent of overall m^6A methylation in mitochondria is much higher than that in the nucleus, but lower than that in the chloroplast. However, the m^6A motif sequences in the transcriptome of mitochondria are similar to those of the nucleus and chloroplast, which means

TABLE 2 | Non-coding RNAs drived from human mtDNA.

Name	Location in mtDNA (H/L-strand)	Size (nt)	Function or relation with disease	References
uc022bqo.2	650–674, H	25	–	Kumarswamy et al., 2014; de Gonzalo-Calvo et al., 2016; Kitow et al., 2016; Thum et al., 2017; Du et al., 2018; Li et al., 2018; Schulte et al., 2019
uc004cor.1	1,603–1,634, H	32	–	
uc022bqp.1	5,543–5,566, L	24	–	
uc022bqq.1	5,585–5,606, L	22	–	
uc022bqr.1	5,690–5,714, L	25	–	
uc022bqv.1	14,674–14,698, L	25	–	
uc004cow.2	12,207–12,264, H	58	–	
uc022bqx.1	15,959–16,024, L	66	–	
uc004coq.4	235–368, L	134	–	
uc004cos.5	1,843–4,264, H	2,421	–	
uc031tga.1	5,904–7,439, H	1,535	–	
uc022bqs.1 (LIPCAR)	15504–15888, L + 7,587–7982, L	781	It predicts survival in patients with type 2 diabetes, heart failure and ST-segment elevation myocardial infarction.	
uc011mfi.2	7,585–9,206, H	1,622	–	
uc022bqt.1	8,367–8472, L + 13450–14,149, L	776	–	
uc022bqu.2	10,060–10,404, H	345	–	
uc022bqu.1	10,059–10,404, H	346	It is upregulated in patients with hypertrophic obstructive cardiomyopathy	
uc004cov.5	10,470–12,138, H	1,669	–	
uc031tgb.1	10,760–14,149, L	3,390	–	
uc004cox.4	12,908–14,149, H	1,242	It provides prognostic information for non-muscle invasive bladder cancer	
uc004cos.4	1,756–4,264, H	2,509	–	
uc022bqw.1	14,857–15,888, H	1,032	–	
uc004coz.1	15,999–16,571, H	5,73	–	
uc004cov.4	10473–12138, H	1,666	It is upregulated in patients with hypertrophic obstructive cardiomyopathy	
uc011mfh.1	5,855–7,427, H	1573	–	
lncND5	12,337–14,148, L	1,812	–	Rackham et al., 2011
lncND6	13,993–14,673, H	681	–	
lncCytb	14,747–15,887, L	1,141	–	
SncmtRNA-1 (GenBank: DQ386868.1)	1,717–2,536, L + 1,672–3,230, H	2,374	It is upregulated in the urine of patients with bladder cancer. It expresses in normal proliferating cells.	Villegas et al., 2007; Burzio et al., 2009; Rivas et al., 2012; Villota et al., 2012; Vidaurre et al., 2014; Bianchessi et al., 2015; Gao Y. et al., 2017
SncmtRNA-2 (GenBank: HM581520.1)	1,775–2,536, L + 1,672–3,230, H	2,311	It is induced by HPV-16/18-encoded in human keratinocytes. It expresses in normal proliferating cells.	
ASncmtRNA-1 (GenBank: EU863789)	2,808–3,124, H + 1,671–3,226, L	1,866	It is downregulated in the urine of patients with bladder cancer. It is downregulated in 17 types of tumor cells. binds to Dicer to recruit to the 3′-UTR of survivin mRNA, resulting in degradation of this mRNA. It is downregulated by HPV-16/18-encoded E2. It inhibits tumor growth and metastasis in the RenCa murine renal adenocarcinoma model.	
ASncmtRNA-2 (GenBank: EU863790)	2,222–2,772, H + 1,671–3,233, L	2,104	It involves in the establishment of replicative senescence by participating in the cell cycle arrest in G2/M phase, possibly through the production of hsa-miR-4485 and hsa-miR-1973. It promotes glomerular fibrosis in diabetic nephropathy via promoting the expression of pro-fibrotic factors. It is downregulated in the urine of patients with bladder cancer. It is downregulated in 17 types of tumor cells. It binds to Dicer to recruit to the 3′-UTR of survivin mRNA, resulting in degradation of this mRNA. It is downregulated by HPV-16/18-encoded E2. It inhibits tumor growth and metastasis in the RenCa murine renal adenocarcinoma model.	

(Continued)

TABLE 2 | Continued

Name	Location in mtDNA (H/L-strand)	Size (nt)	Function or relation with disease	References
$tRNA^{Gln}$AS	4,329–4,400, H	72	–	Gao et al., 2018
$tRNA^{Ala}$AS-$tRNA^{Tyr}$AS	5,587–5,891, H	305	–	
$tRNA^{Ser(UCN)}$AS	7,445–7,516, H	72	–	
ND5/ND6AS/$tRNA^{Glu}$AS	12,337–14,746, H	2,410	–	
$tRNA^{Pro}$AS	15,954–16,023, H	70		
MDL1AS	16,024–407, H	953	–	
MDL1	15,956–576, L	1,192	–	
$tRNA^{Thr}$AS-Cytb	14,747–15,953, L	1,207	–	
ND6-$tRNA^{Asp}$AS	7,518–14,673, L	7,156	–	
COX1AS	5,901–7,442, L	1,542	–	
$tRNA^{Trp}$AS-$tRNA^{Met}$AS	4,402–5,579, L	1,178	–	
$tRNA^{Ile}$AS	4,263–4,331, L	69	–	

that m^6A motif is conserved among them. Besides, the m^6A patterns of rRNAs and tRNAs are also similar. However, the mitochondrial and chloroplastic m^6A patterns in mRNAs are different from those of the nucleus. Methylated transcripts in mitochondria and chloroplast are shown to be associated with rRNA, ribosomal proteins, photosystem reaction proteins, tRNA, NADH dehydrogenase and redox systems. Different organs of the leaves, flowers and roots have differential m^6A methylation, suggesting that m^6A methylation plays an important role during development and differentiation (Wang et al., 2017). Besides which, more than 20 m^1A sites are also identified in mitochondrial genes via using a transcriptome-wide analysis (Zhang and Jia, 2018).

These findings unravel a new concept of mitoepitranscriptome, referring to dynamic regulation of gene expression by the modified mtRNAs. Some mutations in mtDNA or nuclear-encoded mitochondrial modification enzymes can cause RNA modification defects, which are shown to be associated with various mitochondrial diseases. mtRNA modifications in mt-mRNAs, mt-rRNAs and mt-tRNAs are suspected to play essential roles, too. Besides, ncRNAs encoded by mtDNA may also be modified as that reported in the nDNA-derived ncRNAs (Romano et al., 2018). However, reports about mt-ncRNA modifications are absent at present, because mt-ncRNAs are not well identified yet.

Mitochondrial rRNA Modifications

The protein/DNA ratio of mitoribosomes is higher than that of all other ribosomes, indicating that mitochondria need a more stable structure to make sure that mt-rRNA is correctly scaffolded and accurately folded (Greber and Ban, 2016). Therefore, mt-rRNA modifications seem to be important for mitochondria. Currently, there are only eight different types of modifications in 10 nucleotide sites (including m^5U429, m^4C839, m^5C841, $m^6{}_2A936$, $m^6{}_2A937$ in 12S rRNA and m^1A947, Gm1145, Um1369, Gm1370 and ψ1397 in 16S rRNA) identified in mammalian mt-rRNAs (reviewed by Bohnsack and Sloan, 2018), which is lower than that of cytoplasmic and bacterial rRNAs. These sites are clustered at peptidyl transferase center (PTC) in 16S rRNA and decoding (DSC) sites in 12S rRNA, respectively, which are similar with the ribosomal modification features in bacteria and eukaryotic cytoplasm (Amunts et al., 2015).

The study of mitochondrial rRNA modification can be traced since 1970s. A study of methylation in a fungus *Neurospora crassa* shows that the mitochondrial rRNAs have 0.05–0.16 methyl groups per 100 nucleotides (Lambowitz and Luck, 1975). The 25S and 19S rRNAs of this species have methyl contents of approximately 70 and 55%, respectively (Kuriyama and Luck, 1974). Unlike highly modified cytoplasmic rRNA, mitochondrial rRNAs (15S and 21S) of the yeast *Saccharomyces cerevisiae* only contain three modified nucleotides: a pseudouridine (Ψ2918) and two 2′-O-methylated riboses (Gm2270 and Um2791) located at the peptidyl transferase center of 21S rRNA. Mrm2p, a yeast nuclear genome encoding mitochondrial protein, is required for methylating U2791 of 21S rRNA. Mrm2p belongs to a new class of three eukaryotic RNA-modifying enzymes and is the ortholog of *Escherichia coli* FtsJ/RrmJ that can methylate a nucleotide of the peptidyl transferase center of 23S rRNA (Pintard et al., 2002). Nuclear gene-encoded PET56 catalyzes the site-specific formation of 2'-O-methylguanosine on *in vitro* transcripts of both *Saccharomyces cerevisiae* mitochondrial large ribosomal RNA (21S rRNA) and *Escherichia coli* 23S rRNA. This modification is essential for the formation of functional large subunits of the mitoribosome (Sirum-Connolly and Mason, 1993).

In mitochondrial ribosomes of mammals, such as hamster, the large ribosomal subunit RNA (17S rRNA) contains UmpGmpUp, in which Um residue is methylated relatively later than Gm residue (Dubin and Taylor, 1978). The small ribosomal subunit RNA (13S rRNA) contains, on average, approximately one residue of m^4Cp, m^5Cp and m^5Up, and two residues of $m2^6Ap$. m^4Cp in 13 S rRNA is homologous to its ribose-methylated

congener, m^4Cmp of bacterial 16S ribosomal RNA. Neither m^4Cp nor m^4Cmp exists in cell cytoplasmic ribosomal RNA (Dubin et al., 1978).

There are three rRNA 2′-*O*-methyltransferase family members RNMTL1, MRM1, and MRM2 in mammals. MRM1 and MRM2 are bacterial and yeast homologs, whereas RNMTL1 is only found in eukaryotes. They also localize to the mitochondria, especially near mtDNA nucleoids. MRM1, MRM2, and RNMTL1 are responsible for modification of G1145, U1369, and G1370 residues of human 12S rRNA, respectively (Lee and Bogenhagen, 2014). Defective MRM2 can lead to mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS)-like clinical syndrome possibly through reducing of the 2′-*O*-methyl modification at specific uracil position of 12S rRNA (Garone et al., 2017).

However, mitochondrial 16S rRNA can also be methylated (m^1A) by tRNA methyltransferase TRMT61B in all vertebrates (Bar-Yaacov et al., 2016). Pseudouridine synthase RPUSD4 plays a role in the pseudouridylation (ψ) of a single residue in the 16S rRNA, a modification that is essential for its stability and assembly into the mitochondrial ribosome (Antonicka et al., 2017). In addition to mono-methylation, the 3′ end of the rRNA of the 12S rRNA of mouse also contains two dimethylated adenines ($m^6{}_2A$) that are extremely highly conserved. TFB1M is a mammalian mitochondrial dimethyltransferase homologous to bacterial that is responsible for these two dimethylated adenines. Loss of TFB1M is embryonic lethal and deletion of TFB1M in heart results in complete demethylation of these two adenines of the 12S rRNA, thereby impairing mitochondrial ribosome assembly and abolishing mitochondrial translation (Metodiev et al., 2009). Besides, m^5C911 in the mouse 12S rRNA is catalyzed by NSUN4 without forming a complex with MTERF4, which is essential in mitochondrial ribosomal biogenesis (Metodiev et al., 2014).

Modifications in mt-rRNAs are essential for their stability to ensure the normal functions of the mitoribosome. Abnormal modifications in mt-rRNAs can be associated with the dysfunction of the mitoribosome. For instance, mitochondrial mutation 1584A 12S rRNA N6, N6-dimethyladenosine ($m^6{}_2A$) methylation is associated with hearing loss with 1555A > G mutation (O'Sullivan et al., 2014). Lack of the two dimethylated adenines ($m^6{}_2A$) in 12S rRNA is associated with the pathogenesis of type 2 diabetes (Koeck et al., 2011; Sharoyko et al., 2014).

Mitochondrial tRNA Modifications

The genetic code used during mammal mitochondrial gene expression is non-universal. Mitochondria use only 22 mt-tRNAs to decode 60 different codons. Therefore, flexible decoding is needed for mt-tRNAs. Moreover, mt-tRNA modifications are important processes for the biogenesis of the mature tRNA. Based on the current studies, mt-tRNA contains several types of modifications, such as m^1A, m^1G, m^2G, m^5C, m^3C, τm^5U, τm^5s^2U, f^5C, Q, t^6A, i^6A, ms^2i^6A, D, $m^2{}_2G$ and ψ (summarized by Bohnsack and Sloan, 2018), each of which is essential for biogenesis of mature mt-tRNA. These modifications are mediated by different kinds of enzymes such as MRPP1/2, PUS1, GTPBP3, MTO1, MTU1, NSUN2/3, ABH1, TRIT1/5, CDK5RAP1 and TRMT61B (Bohnsack and Sloan, 2018).

For instance, m^1A9 can disfavor the non-functional conformation and shifts the observed equilibrium toward the functional cloverleaf (Voigts-Hoffmann et al., 2007). RNase P, a subcomplex in human mitochondria, is the endonuclease that removes tRNA 5′ extensions and is the methyltransferase responsible for m^1G9 and m^1A9 formation. The ability of the mitochondrial tRNA:m^1R9 (R = G/A) methyltransferase (TRMT10C and SDR5C1) to modify both purines is uncommon among nucleic acid modification enzymes. In this process, PRORP, a short-chain dehydrogenase, is required as a partner protein (Vilardo et al., 2012).

NOP2/Sun RNA methyltransferase family member 2 (NSUN2) is an RNA methyltransferase previously shown to introduce m^5C in tRNAs, mRNAs and microRNAs encoded by nucleic genome. However, NSUN2 can also be imported into mitochondrial matrix and introduces m5C at positions 48, 49, and 50 of several mitochondrial tRNAs, including mt-$tRNA^{Tyr}$, mt-$tRNA^{His}$, mt-$tRNA^{Leu(UUR)}$, mt-$tRNA^{Phe}$, and mt-$tRNA^{Glu}$. However, NSUN2 inactivation does not remarkably affect mitochondrial tRNA stability and OxPhos in differentiated cells (Van Haute et al., 2019).

NSUN3, a RNA methyltransferase that localizes to mitochondria and methylates cytosine 34 (C34) at the wobble position of mt-$tRNA^{Met}$ via specifically recognizing the anticodon stem loop (ASL) of the tRNA. Meanwhile, a dioxygenase ALKBH1/ABH1 can oxidize $^{m5}C34$ mt-$tRNA^{Met}$ to yield an $^{f5}C34$ mt-$tRNA^{Met}$. During translation initiation, mt-$tRNA^{Met}$ recognizes AUG, AUA and AUU codons and mediates incorporation of methionine on these codons, whereas mt-$tRNA^{Met}$ recognizes AUA codons and introduces methionine incorporation during elongation. In fact, mitochondrial translation factors prefer to use $^{m5}C34$ mt-$tRNA^{Met}$ during translation initiation. NSUN3 or ABH1 depletion remarkably affects mitochondrial translation (Haag et al., 2016).

mt-tRNA modifications are essential for their normal functions. Modifications in the anticodon loop can expand the decoding capacity of mt-tRNAs and make sure the fidelity of translation. Core modifications can enable the structural stability of mt-tRNAs, however, this kind of modifications can also affect its recognition by aminoacyl-tRNA synthetases in some cases (Degoul et al., 1998).

Mitochondrial mRNA Modifications

In addition to modifications in rRNAs and tRNAs, the post-transcriptional alterations in mRNA are also important for its maturation and function, as well as regulation. There are 8 H-strand-derived mitochondrial genes (COX3, ND1/2/3/4/4L, Cytb and ATP6/8) that lack translational termination codons, but can be stopped by the addition of a polyadenine (polyA) tail to a terminal U with the help of a mitochondrial PAP poly(A) polymerase (mtPAP) and a polynucleotide phosphorylase (PNPase), thereby generating sequence $(UAA)_{TER}A_n$ (Chang and Tong, 2012; Shokolenko and Alexeyev, 2015). Primary transcript from the H-stand has an approximately 45 nt polyA extension. However, the length of the polyA tail varies in different cell types

and different transcripts (Temperley et al., 2010). PolyA in the 3′-termini can mediate the stability or instability of the transcript possibly depending on additional polyA-binding factors or sequence-specific proteins, which may affect the translation of mt-DNA-encoded genes (Rorbach and Minczuk, 2012).

Recently, a transcriptome-wide analysis revealed that other post-transcriptional modifications also exist in mitochondrial mRNAs (Bohnsack and Sloan, 2018). Intriguingly, m^1A in the coding region of mitochondrial transcripts can block the corresponding protein translation (Zhang and Jia, 2018). In addition to methylation, specific residues in mitochondrial mRNAs can also be pseudouridylated by TRUB2/RPUSD3, thereby affecting mitochondrial protein synthesis and cell viability (Antonicka et al., 2017). These results mean that m1A and ψ in mt-mRNA are essential for the regulation of protein translation.

Mitochondria-Derived Non-coding RNAs

Except for mRNAs, rRNAs and tRNAs, recent reports show that mtDNA can also encode non-coding RNAs (ncRNAs), including lncRNAs and small non-coding RNAs (sncRNAs) (**Table 2** and **Figure 4**), which are termed mtDNA-encoded lncRNAs ($lncRNAs^{mtDNA}$) and mtDNA-encoded sncRNAs ($sncRNAs^{mtDNA}$), respectively. However, circular RNAs (circRNAs) seem to be absent in the mitochondria (Zhang et al., 2019).

$lncRNAs^{mtDNA}$

In rat mitochondrial genome, there are some unidentified RNAs on both the H/L-strand, such as precursors of the ND2 mRNA plus the $tRNA^{Trp}$ and the tRNAs clustered in the Ori L region. Besides them, antisense RNA species in the region of L-strand replication and D-loop region are also observed (Sbisà et al., 1992). A deep RNA sequencing study in human cardiac tissues also shows that there is a high relative abundance (71%) of $lncRNAs^{mtDNA}$ (Yang et al., 2014). Another analysis in data sets from strand-specific deep sequencing shows that there is a significant proportion (15.02%, excluding rRNA and tRNA) of lncRNAs in the transcriptome of mitochondria in cervical cancer cell HeLa. Among them, 3 lncRNAs (lncND5, lncND6, lncCytb) transcripts are highly abundant (**Figure 4B**). These lncRNAs can form intermolecular duplexes and they are expressed in a cell- and tissue-specific manner. Their levels may be regulated by the nuclear-encoded proteins such as ELAC2, MRPP1, MRPP3, PTCD1, and PTCD2 (Rackham et al., 2011).

Recently, the PacBio full-length transcriptome data revealed that there are 6 lncRNAs in the H-strand and 6 lncRNAs in the L-strand of the mtDNA (**Figure 4D**). Among them, 2 novel lncRNAs of MDL1 and MDL1AS from the D-loop region exist ubiquitously in animal mtDNA (Gao et al., 2018).

However, from the UCSC Genome Bioinformatics (2016 version), 24 ncRNAs (including 6 microRNAs) derived from human mtDNA were provided, which are very different from those identified by the PacBio full-length transcriptome (**Figure 4A**). Actually, many researches that focus on the mtDNA-derived lncRNAs used this database to perform their studies. Among them, the mitochondrial lncRNA uc022bqs.1 (long intergenic non-coding RNA predicting cardiac remodeling, LIPCAR) is the best studied. This lncRNA is shown to be downregulated early after myocardial infarction but upregulated during later stages in the circulating blood of the patients (Kumarswamy et al., 2014). In addition, circulating LIPCAR also acts as a biomarker in patients with ST-segment elevation myocardial infarction (Li et al., 2018). Circulating LIPCAR is also inversely associated with diastolic function in patients with type 2 diabetes (de Gonzalo-Calvo et al., 2016). However, LIPCAR does not increase in human cardiac tissue after transcoronary ablation of septal hypertrophy, suggesting that this lncRNA is not originated from the cardiac tissues (Schulte et al., 2019). Besides, another two mtDNA-derived lnRNAs uc004cov.4 and uc022bqu.1 are also upregulated in serum of patients with hypertrophic obstructive cardiomyopathy (HOCM) but not hypertrophic non-obstructive cardiomyopathy (HNCM) (Kitow et al., 2016).

Other researchers also identified 4 $lnRNAs^{mtDNA}$, termed SncmtRNA-1/2 and ASncmtRNA-1/2 (**Figure 4C**). Among them, lncRNA ASncmtRNA-2 is induced in during aging and replicative senescence in endothelial cells, but not in vascular smooth muscle cells (VSMC). Mechanically, ASncmtRNA-2 may be the non-canonical precursor of hsa-miR-4485 and hsa-miR-1973, which are originated at least in part from a mitochondrial transcript. These two microRNAs target p16 and induce a cell cycle arrest at G2M phase, thereby affecting replicative senescence establishment (Bianchessi et al., 2015). Besides, ASncmtRNA-2 is also upregulated in diabetic kidneys and high glucose-treated mesangial cells and can promote glomerular fibrosis via modulating the expression of pro-fibrotic factors in diabetic nephropathy (Gao Y. et al., 2017).

In conclusion, $lncRNAs^{mtDNA}$ are present in the mitochondria, and may function as important epigenetic regulators for the regulation of mitochondrial function. However, a systemic study is needed to clarify the $lncRNAs^{mtDNA}$, and their mechanisms of biogenesis and processing, as well as their functions and mode of actions.

$sncRNAs^{mtDNA}$

Small non-coding RNAs (sncRNAs) are highly structured, less than 200 nt ncRNA fragments that found in bacteria, mitochondria and eukaryotes. According to their functions and characteristics, they can be classified into at least 9 types, such as microRNAs (miRNAs, ∼22 nt), Piwi-interacting RNAs (piRNA), tiny non-coding RNAs (tncRNAs), short interfering RNAs (siRNAs), repeat-associated small interfering RNAs (rasiRNAs), small modulatory RNAs (smRNAs), palindrome small RNAs (psRNAs), guide RNAs (gRNAs) and transcription initial RNAs (tiRNAs). Most of them are encoded by the nucleus genome. However, at present, mtDNA is reported to encode some sncRNAs such as miRNAs, psRNAs, tiRNAs and gRNAs. Among them, only mitochondrial-derived sncRNAs ($sncRNA^{mtDNA}$) are widely reported.

A comprehensive analysis of the human mitochondrial transcriptome across multiple cell lines and tissues shows that sncRNAs exist in the mitochondria (Mercer et al., 2011). Remarkably, mt-$tRNA^{Lys}$ and mt-$tRNA^{Me}$ can be exported to

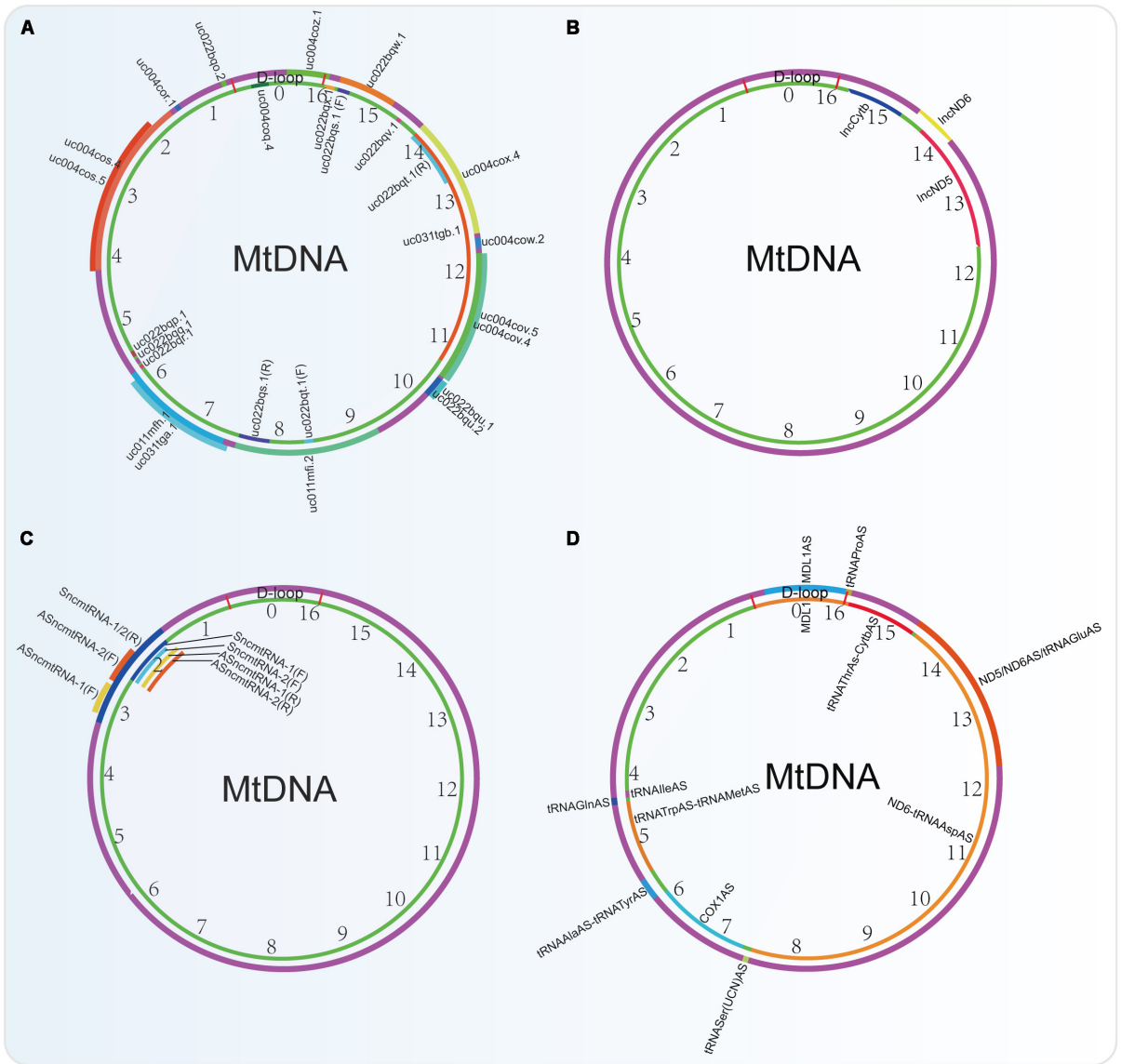

FIGURE 4 | Non-coding RNAs encoded by the mtDNA identified by four different research groups. **(A)** ncRNAs obtained from the UCSC Genome Bioinformatics (2016 version). **(B)** lncRNAs identified by Rackham et al., 2011. **(C)** lncRNAs indented by researchers from Fundación Ciencia para la Vida, Chile. **(D)** ncRNAs identified by using PacBio full-length transcriptome data.

the cytoplasm and bind to argonaute-2 (AGO2), an essential component of the RNA-induced silencing complex (RISC), suggesting that mt-tRNAs may act as miRNA (Maniataki and Mourelatos, 2005; Beitzinger et al., 2007). Mature tRNAs can also be cleaved by stress-activated ribonuclease angiogenin to generate 5′- and 3′-tRNA halves: a novel class of 30–40 nt small non-coding RNAs (Saikia and Hatzoglou, 2015). Besides, miR-1974, miR-1977, and miR-1978 may be encoded by the tRNA and rRNA genes in mtDNA (Bandiera et al., 2011). Some sequences of pre-miR-let7b and pre-miR-302a located in the mitochondria of human muscle can be aligned with the mtDNA, implying that these miRNAs may be derived from mtDNA (Barrey et al., 2011). These evidences suggest that the mtDNA can be a source of microRNAs (Bandiera et al., 2013).

An analysis on the mouse mtDNA identified 6 sncRNAs^{mtDNA}, among which Mt-5 RNA is transcribed in antisense orientation to ND4 and Mt-6 RNA is transcribed in antisense orientation to ND6 (Lung et al., 2006). Further study shows that thousands of sncRNAs are encoded in murine and human mtDNA and most of them derived from the sense transcripts (Ro et al., 2013). The processing of sncRNAs^{mtDNA} is not only dependent on Dicer but also some mitochondrial ribonucleases that are currently unidentified (Ro et al., 2013). Overexpression of mitochondrial-derived sncRNAs can significantly enhance the expression levels of their host genes (Ro et al., 2013). Six miRNAs that are termed as

miR-mit1 to miR-mit6 are identified in mouse mtDNA. Among them, miR-mit3 and miR-mit4 can target MT-RNR2 (16S rRNA) in skeletal muscles (Shinde and Bhadra, 2015). Furthermore, Mt-1 can exhibit variable length due to polyadenylation, in which contains a microRNA-like small RNA, mmu-mir-805, that is encoded in the termination association sequence (TAS) of the mtDNA, and it is upregulated in hippocampus during olfactory discrimination training in the mice (Smalheiser et al., 2011).

Besides that, mtDNA also produces psRNAs, tiRNAs (Gao et al., 2018), and gRNAs (Ochsenreiter and Hajduk, 2006). These RNAs also play essential roles in mitochondria. For instance, precise insertion and deletion of numerous uridines are required to make full-length mitochondrial mRNAs. Guide RNA responsible for COX3 mRNA can be alternatively edited to yield a stable mRNA in the mitochondria of *Trypanosoma brucei brucei* (Ochsenreiter and Hajduk, 2006).

Nucleus-Derived Non-coding RNAs That Target mtDNA Encoded Genes

Apart from the ncRNAs^{mtDNA}, nuclear DNA-derived non-coding RNAs (ncRNAs^{nDNA}) including mainly lncRNAs^{nDNA} and miRNAs^{nDNA} may also mediate mitoepigenetics referring to ncRNAs^{mtDNA} that affect the translation and function of mtDNA-encoded genes. These RNAs encoded by nuclear DNA may be imported into mitochondria. In *Trypanosoma brucei*, RNA import into mitochondria has been studied well. Nuclear encoded tRNA of *T. brucei* can be imported into mitochondria (Schneider et al., 1994). In fact, *T. brucei* imports all mitochondrial tRNAs from the cytosol, and an *in vitro* study showed that there were possible some membrane-bound receptors that can mediate the import of small ribosomal RNAs (srRNAs) and tRNA in protozoon *Leishmania* mitochondria (Mahapatra et al., 1994; Mahapatra and Adhya, 1996). Subsequently, tRNA import into the kinetoplast mitochondrion of the *Leishmania tropica* were shown to be organized into a multiprotein RNA import complex (RIC) that contained 3 mitochondrion- and 8 nuclear−encoded subunits, such as Tim17 and mitochondrial heat-shock protein 70 (mtHSP70) at the inner membrane (Mukherjee et al., 2007; Tschopp et al., 2011).

However, the RNA import systems in different species exhibit some unique features (Schneider and Maréchal-Drouard, 2000). For instance, *Saccharomyces cerevisiae* imports cytoplasmic tRNA^{Gln} into mitochondria without any added protein factors (Rinehart et al., 2005). The RNA import of plant mitochondria is dependent on the voltage-dependent anion channel (Salinas et al., 2006). The study of RNA import into mammalian mitochondria is not so clear. A microarray analysis of highly purified rat liver-derived mitochondria identified 15 miRNAs^{nDNA}, 5 of which were further confirmed by TaqMan 5′ nuclease assays. These miRNAs may be associated with the expression of some genes related to apoptosis, cell proliferation, and differentiation (Kren et al., 2009). Further studies also show that there are possible several ATP-dependent import pathways of nucleus-encoded RNAs to human mitochondria (Duarte et al., 2015). For example, polynucleotide phosphorylase (PNPase) is shown as a contributor to mitochondrial RNases P (MRP), 5S rRNA, tRNAs and miRNAs import into mitochondria (Wang et al., 2010; Shepherd et al., 2017). Mitochondrial enzyme rhodanese was also shown to be responsible for 5 S rRNA import into human mitochondria (Smirnov et al., 2010). Both pre-miRNAs^{nDNA} and mature miRNAs^{nDNA} were shown to be present in the human mitochondria (Barrey et al., 2011; Bandiera et al., 2013), implying that there is possible a miRNA synthesis system in the mitochondria. However, it is also reported that tRNA import into human mitochondria does not take place under normal physiological conditions, but it is possible for mutant human mitochondria to take in nucleus-encoded tRNAs (Kolesnikova et al., 2004; Mahata et al., 2006; Rubio et al., 2008). However, the RNA import to mitochondria needs to be further confirmed and its mechanism investigated.

Selected nucleus-derived ncRNAs are possibly imported into the mitochondria, where they can be involved in multiple mitochondrial biological processes to act as "messengers" between the nucleus and the mitochondria (Vendramin et al., 2017; Jeandard et al., 2019). All the microRNAs present in mitochondria are also called mitomiRNAs (mitomiRs), which is commonly used in some reports (Rippo et al., 2014; Duarte et al., 2015; Giuliani et al., 2018). Interestingly, all the mitomiRs seemed to preferentially target multiple mtDNA sites, other than nuclear-encoded mitochondrial genes, compared with a set of cytosolic miRNAs (Duarte et al., 2015).

lncRNAs^{nDNA} That Target mtDNA Encoded Genes

We summarize the lncRNA^{nDNA} that may affect mtDNA-encoded genes in **Table 3**. The behaviors of lncRNAs are multi-faced and can be implicated in various regulatory levels of gene expression, from transcription to post-translation. For instance, Cerox1 promotes the levels of mitochondrial OxPhos by upregulating mitochondrial complex I subunit transcripts through binding to microRNA-488-3p, thereby leading to a decrease of ROS production (Sirey et al., 2019). Besides, a skeletal muscle- and heart-enriched lncRNA LINC00116 can encode a highly conserved 56-AA mitoregulin (Mtln) that localizes in inner mitochondrial membrane. Mtln can interact with ND5, thereby bolstering protein complex assembly and/or stability. Overexpression of Mtln results in increasing mitochondrial membrane potential and Ca^{2+} retention capacity, decreasing fatty acid oxidation, mitochondrial ROS and matrix-free Ca^{2+}, promoting respiratory complex I activity and oxygen consumption and maintaining lipid composition of the cell (Stein et al., 2018; Chugunova et al., 2019). These evidences show that lncRNAs^{nDNA} act as messengers between nDNA and mitochondria.

miRNAs^{nDNA} That Target mtDNA Encoded Genes

AGO2, a Dicer1-interacting protein that plays an essential role in short interfering RNA-mediated gene silencing, is also found to localize to mitochondria and bind to the mitochondrial transcripts COX3 and tRNA^{Met} (Bandiera et al., 2011), suggesting the activities of microRNA-mediated biological processes in the mitochondria. In addition to the miRNAs^{mtDNA}, mitochondria also contain miRNAs^{nDNA}. **Table 4** summarizes different microRNAs^{nDNA} that can target mtDNA-encoded genes

TABLE 3 | Nucleus-derived long non-coding RNAs that may target mtDNA encoded genes.

Name	Target	Function	Mechanism	References
Cerox1	COX1	Decrease in reactive oxygen species and upregulation in mitochondrial oxidative phosphorylation	It binds with miR-488-3p, which can target COX1	Sirey et al., 2019
LINC00116	ND5	Perturbations in mitochondrial respiratory (super) complex formation and activity, fatty acid oxidation, tricarboxylic acid (TCA) cycle enzymes, and Ca^{2+} retention capacity	It encodes a short peptide named mitoregulin (Mtln) that can interact with ND5	Stein et al., 2018; Chugunova et al., 2019
AK055347	ATP synthase	Inhibition of cell viability of H9C2 cardiomyocytes, dysregulation of mitochondrial energy production	It downregulates ATP synthase	Chen et al., 2016

in multiple species, thereby affecting various biological or pathological processes. For instance, miR-181c was shown to target mt-COX1 in cardiomyocytes of rat (Das et al., 2012). In addition, miR-181c-5p is predicted to have 12 potential targets (12S RNA, 16S RNA, ND1, ND4, ND5, ND6, COX1, COX2, COX3, ATP6, Cytb, $tRNA^{Gly}$) encoded by mtDNA, whereas miR-146a-5p is predicted to have 12 potential targets [16S RNA, ND1, ND2, ND4, ND5, ND6, ATP8, $tRNA^{Ala}$, $tRNA^{Glu}$, $tRNA^{Ser(UCN)}$, $tRNA^{Ser(AGY)}$] on mtRNAs (Dasgupta et al., 2015). Some microRNAs, including miR-1275, miR-1246, miR-328-5p, miR-1908, miR-1972, miR-1977, miR-638, miR-1974, miR-1978 and miR-1201, are also predicted to target mtDNA-derived RNAs (Bandiera et al., 2011). These predictions need to be further validated.

However, functional analysis of miRNAs identified in highly purified rat liver-derived mitochondria showed that they were not targeted to the mitochondrial genome nor nuclear RNAs encoding mitochondrial proteins (Kren et al., 2009). This result implies that there are may be other mechanisms independent of miRNA-mediated mRNA degradation. Interestingly, microRNAs are also shown to directly affect mtRNA translation. For example, miR-1 can promote MT-ND1 and MT-CO1 translation but not their mRNA stability in an AGO2-dependent manner, thereby affecting muscle differentiation in mice (Zhang et al., 2014).

THE ROLES OF MITOEPIGENETICS IN CANCER

Mitochondria are important organelles that are essential for functional eukaryotic cells. In fact, cancer cells also depend much on mitochondria. There are commonly many alterations in mitochondria induced by both extra-mitochondrial or intra-mitochondrial influencing factors that sustain excessive proliferation of cancer cells via providing energy and metabolites (Brandon et al., 2006; Wallace, 2012). For the extra-mitochondrial influencing factors, there are more than 1,000 of nucleus-encoded proteins and thousands of nucleus-encoded non-coding RNAs that can be imported into mitochondria and play a role in function, metabolism, regulation and the production, fission, fusion, trafficking and degradation of the mitochondria. Besides, many extra-mitochondrial signaling pathways such as apoptosis and mitophagy, as well as some factors can directly regulate the function of mitochondria. Intra-mitochondrial influencing factors include mutations in the mtDNA. However, most of these mutations in mtDNA do not inhibit energy metabolism in mitochondria but rather change the mitochondrial bioenergetic and biosynthetic state, which can communicate with the nucleus via modulating signaling pathways, transcriptional circuits and/or chromatin structural remodeling to meet the requirements of the cancer cells (Wallace, 2012). For instance, certain control region mitochondrial single-nucleotide variants (mtSNVs) highly co-occur with MYC oncogene amplification in prostate cancer, and predict a poorer patient survival (Hopkins et al., 2017).

Importantly, besides genetic mutations in mtDNA, mitoepigenetics is emerging as an important regulatory mode. All the mitoepigenetic networks including mtDNA methylation, mitochondrial nucleoid modifications, mtRNA methylation, mtDNA-encoded and nucleus-encoded ncRNAs, have been shown to play essential roles in tumor development and pathogenesis.

mtDNA Methylation in Cancer

Cancer is often related with a low level of total nDNA methylation, hypermethylation of tumor suppressor gene promoters and hypomethylation of oncogene promoters (Kulis and Esteller, 2010). Therefore, it is suspected that methylation in mtDNA should be accurately modulated. The copy number of mtDNA is strictly regulated during cellular differentiation in cancer cells. In breast cancer, mtDNA methylation is maternally inherited in D-loop region, in which 8 aberrant mtDNA methylation sites are tightly dysregulated (Han et al., 2017). mtDNA copy number and ND-2 expression in colorectal cancer tissues are higher than that of the corresponding non-cancerous tissues. Methylation on the D-loop region in colorectal cancer tissues was lower than that of their corresponding non-cancerous tissues. Meanwhile, methylation on the D-loop region in stage III/IV colorectal cancer tissues is also significantly decreased, compared with that in stages I/II colorectal cancer tissues. Furthermore, D-loop region de-methylation is tightly correlated with a high mtDNA copy number and a high ND-2 expression. DNA methylation inhibitor 5-aza-deoxycytidine treatment also increases the mtDNA copy number and ND-2 expression in Caco-2 cells (Gao et al., 2015). Further study reveals that de-methylation of 4th and 6th/7th CpG islands of D-loop promoter can lead to the elevation of mtDNA copy number in colorectal cancer, thereby triggering cell proliferation, cell cycle progression and reducing apoptosis (Tong et al., 2017). These results indicate that mtDNA methylation is negatively correlated with mtDNA number and tumor progression.

TABLE 4 | Nucleus/mtDNA-derived MicroRNAs that have been confirmed to target mtDNA-encoded genes.

Targeted gene	MicroRNA	Species	Organism/Tissues/Cells	Biological/pathological process influenced	References
COX 1	miR-181c	*Rattus norvegicus*	Cardiomyocytes, myoblast	Mitochondrial respiration, reactive oxygen species generation	Das et al., 2012, 2017
	miR-488-3p	*Homo sapiens, Mus musculus*	Mouse Neuro-2a neuroblastoma cells, human embryonic kidney 293HEK cells	Reactive oxygen species, OxPhos	Sirey et al., 2019
	miR-2392	*Homo sapiens*	Multiple types of cancer	OxPhos, glycolysis	Fan et al., 2019
COX 2	miR-26a	*Homo sapiens*	Prostate cancer cells	Cell proliferation, apoptosis	Zhang et al., 2016
Cytb	miR-542-3p	*Homo sapiens*	Human skeletal muscle cell line (LHCN-M2)	Mitochondrial ribosomal stress, muscle wasting	Garros et al., 2017
	miR-151a-5p	*Mus musculus*	Spermatocyte cell line (GC-2)	Asthenozoospermia, mitochondrial respiratory activity	Zhou et al., 2015
	miR-2392	*Homo sapiens*	Multiple types of cancer	OxPhos, glycolysis	Fan et al., 2019
ND 2	miR-24	*Homo sapiens*	Lewis lung carcinoma (LLC) cells	Mitochondrial dysfunction, growth inhibition	Michael et al., 2017
	miR-762	*Homo sapiens*	Cardiomyocytes	Intracellular ATP levels, ROS levels, apoptosis, myocardial infarction	Yan et al., 2019
ND4	miR-2392	*Homo sapiens*	Multiple types of cancer	OxPhos, glycolysis	Fan et al., 2019
ND 4L	miR-214	*Mus musculus*	Kidney	Apoptosis, mitochondrial OxPhos	Bai et al., 2019
ND 6	miR-214	*Mus musculus*	Kidney	Apoptosis, mitochondrial OxPhos	Bai et al., 2019
ATP6	miR-378	*Mus musculus*	Cardiomyocyte cell line (HL-1)	Diabetes mellitus	Jagannathan et al., 2015
16S rRNA	miR-4485	*Homo sapiens*	Breast cancer cells	Mitochondrial complex I activity, the production of ATP, ROS levels, caspase-3/7 activation, and apoptosis	Sripada et al., 2017
	miR-mit3	*Homo sapiens*	skeletal muscles	It targets 16S rRNA	Shinde and Bhadra, 2015
	miR-mit4	*Homo sapiens*	skeletal muscles	It targets 16S rRNA	Shinde and Bhadra, 2015

Similarly, the level of 5mC at several sites of mtDNA is negatively correlated with mtDNA copy number in 143B osteosarcoma cells (Sun et al., 2018a). 5mC in mtDNA D-loop is low during tumor progression and may potentially contribute to the increase in mtDNA copy number observed in these tumor cells, including osteosarcoma and glioblastoma cells (Sun et al., 2018a). 5mC levels of D-loop also negatively correlate with ND5 and ND6 transcription during the tumorigenesis of 143B osteosarcoma cells (Sun et al., 2018a).

However, mtDNA of cancer stem-like cells is also hypermethylated and the mtDNA copy number is low, which makes them to use glycolysis for cell proliferation (Lee and St John, 2015). After sufficient mtDNA is restored in tumors to initiate tumorigenesis, 5mC in D-loop is increased to restrict further mtDNA replication (Sun et al., 2018a). That is the reason why cancer cells have a lower mtDNA copy number to maintain a "pseudo-differentiated" state and why global DNA demethylation can induce cellular differentiation and expansion of mtDNA copy number (Sun et al., 2018a).

However, there are also some studies that do not support the results above. For instance, colorectal adenomas have a low-level methylation of specific sites in mtDNA, but it is not associated with changes of mitochondrial gene transcription (Morris et al., 2018). Maekawa et al. (2004) showed that methylation of mtDNA was a rare event in the CpG sites in cancer cell lines and tissues of gastric and colorectal cancer. Hong et al. (2013) also confirmed that CpG methylation was absent in HCT116 colerectal cancer cell lines. van der Wijst et al. (2017) showed that CpG mtDNA methylation didn't affect mtDNA gene expression, whereas 5mC in the GpC context decreased mtDNA gene transcription.

In conclusion, the relationship between mtDNA methylation and cancer should be studied further. To solve the problems, a more precise method to detect the methylated sites of methylation in mtDNA is needed. A systematic study on mtDNA methylation in both normal tissues and tumor tissues should be performed to show differences between them. Finally, the clinical significance, function and mode of actions of mtDNA methylation should be further elucidated.

In fact, these explorations will not only benefit the study of cancer biology, but also would be useful for studying other mitochondrial diseases. mtDNA methylation is also shown to be connected with various human diseases, such as Down's syndrome and Alzheimer's disease. The levels of mitochondrial SAM is downregulated in Down's syndrome compared to control cells, suggesting that there is a low level of 5mC level in the mtDNA of this disease (Infantino et al., 2011). 5mC in mtDNA D-loop is observed in the blood of patients with late-onset Alzheimer's disease patients (Stoccoro et al., 2017). Besides, 5mC in mitochondrial the transfer RNA phenylalanine (MT-TF), MT-RNR1 gene while not D-loop region is shown to be associated with metal-rich particulate matter (PM1) exposure and mtDNA copy number (Byun et al., 2013). In umbilical cord blood, 5mC in the 12S rRNA (MT-RNR1) or the D-loop control region of mtDNA is positively correlated with the level of free thyroid hormones (FT3 and FT4) and

mitochondrial DNA copy numbers regulated by these two hormones (Janssen et al., 2017).

Mitochondrial Nucleoid Modifications in Cancer

TFAM is the only nucleoid-associated protein that functions as a histone-like factor. Its expression is shown to be positively correlated with the progression of multiple cancers, including melanoma (Araujo et al., 2018), hepatocellular carcinoma (Qiao et al., 2017), non-small cell lung cancer (Xie et al., 2016), colon cancer (Lin et al., 2018), bladder cancer (Mo et al., 2013), epithelial ovarian carcinoma (Gabrielson et al., 2014), glioma (Lee et al., 2017), and breast cancer (Fan et al., 2017). These phenomena may be the results of higher mtDNA copy number that is found in tumors compared to normal tissues (Sun et al., 2018b), which makes tumor cells produce more TFAM for sufficient compaction. Otherwise, mtDNA in cancer cells may be tightly wrapped by more TFAM, which leads to a lower expression of mtDNA encoding ETC/OxPhos-related genes, thereby promoting tumor cells to use aerobic glycolysis.

TFAM can also be regulated by post-translational modifications including acetylation, phosphorylation and ubiquitination. However, there is no direct evidence showing that TFAM modifications are correlated with cancer. Since post-translational modifications of TFAM significantly affect its stability or function, and its modifications may also be tightly associated with tumor progression.

mtRNA Methylation in Cancer

mtDNA is transcribed to produce RNA with continuous polycistrons, implying that post-transcriptional modulations are essential for RNA processing. Recently, mtRNA transcripts were shown to be differently accumulated in tumor tissues (Stewart et al., 2015). Mutation of mtRNA processing enzymes, such as ELAC2, which has RNase Z activity and functions in the maturation of mt-tRNA by removing a 3′-trailer from tRNA precursors to generate 3′ termini of tRNAs, is associated with prostate cancer incidence (Tavtigian et al., 2001). mt-tRNAs are heavily post-transcriptionally modified mtRNAs, mutations within which are also related to cancer (Brandon et al., 2006). A tRNA-dihydrouridine synthases, DUS2, which catalyzes the conversion of uridine residues to dihydrouridine in the D-loop of tRNA, is also commonly upregulated in pulmonary cancer (Kato et al., 2005). These results imply that mtRNA processing is important for cancer.

Recent studies show that mtRNA modifications, which are essential for mtRNA processing, are also major regulatory factors in tumors. In tumor tissues across 12 cancer types, there are remarkable alterations in methylation levels of m^1A and m^1G RNA in mitochondrial tRNAs. In normal tissues, RNA processing pathways are specifically related to mt-tRNAs methylation levels, however, these connections are lost in tumors (Idaghdour and Hodgkinson, 2017). High mt-tRNAs methylation difference predicts a poorer prognosis in a cohort of patients with kidney renal clear cell carcinoma (Idaghdour and Hodgkinson, 2017). The level of m^1A and m^1G methylation in mtRNAs can significantly affect mitochondria-mediated metabolism (Hodgkinson et al., 2014). In conclusions, mt-tRNAs methylation affects their maturation and thus plays emerging roles in tumorigenesis.

ncRNAs^{mtDNA} in Cancer

There are emerging evidences showing that lncRNAs^{mtDNA} may be involved in tumorigenesis by promoting cell proliferation and tumor growth. A lncRNA with an 815 nt inverted repeat (IR) and a stem-loop structure resistant to RNase A is covalently linked to the 5′ end of 16S rRNA in human cells. It is expressed in highly normal proliferating cells while not in resting cells. The expression of this lncRNA can be induced in phytohemagglutinin (PHA)-treated resting lymphocytes, while can be reversibly blocked in aphidicolin-treat DU145 pancreatic cancer cells, in which cell proliferation is also reversibly inhibited (Villegas et al., 2007). Besides which, two antisense mtRNA transcripts that contain stem-loop structures are expressed in normal proliferating cells but significantly downregulated in tumor cells (Burzio et al., 2009). Further study shows that a family of mitochondrial ncRNAs (ncmtRNAs) with stem-loop structures can be divided into sense (SncmtRNAs) and antisense (ASncmtRNAs) members. Both of the SncmtRNAs and ASncmtRNAs are expressed in normal proliferating cells, whereas ASncmtRNAs are downregulated in various types of tumor cells. ASncmtRNAs knockdown induces cell cycle arrest and apoptosis via inhibiting survivin expression in cancer cell lines without impairing cell viability of normal cells. MicroRNAs generated by dicing of the double-stranded stem of the ASncmtRNAs can downregulate survivin. Mechanically, ASncmtRNAs binds to Dicer to recruit to the 3′-UTR of survivin mRNA, resulting in degradation of this mRNA (Vidaurre et al., 2014). Preclinical studies also show that ASncmtRNAs knockdown blocks tumor growth in melanoma and renal cancer models (Olavarria et al., 2018). Immortalization of human keratinocytes with HPV-16/18 downregulates the expression of the ASncmtRNAs and induces the expression of SncmtRNA-2. Furthermore, E6 and E7 are shown to be responsible for SncmtRNA-2 upregulation, whereas E2 oncogene is responsible for ASncmtRNAs downregulation (Villota et al., 2012).

In addition, some specific lncRNAs^{mtDNA} are highly upregulated in tumors or cancer patients' urine and predict poor prognosis of these diseases. For instance, SncmtRNAs are upregulated and ASncmtRNAs are downregulated in the urine of patients with bladder cancer (Rivas et al., 2012). Higher level of lncRNA^{mtDNA} uc004cox.4 in urine is associated with poorer recurrence-free survival (RFS) of non-muscle invasive BC (NMIBC) and act as an independent prognostic factor for RFS of this disease (Du et al., 2018).

In conclusion, ncRNAs^{mtDNA} are important regulators during tumorigenesis and can be promising prognostic markers for cancers. Therefore, it is urgent to identify the mtDNA-encoded ncRNAs to provide a better understanding of this area.

ncRNAs^{nDNA} That Target mtDNA Encoded Genes in Cancer

Emerging evidences also show that ncRNAs derived from nDNA also act as messengers to regulate mitochondrial function in cancer cells. For instance, lncRNA MALAT1 can be transported into mitochondria by RNA-binding protein HuR and mitochondria transmembrane protein mitochondrial carrier 2 (MTCH2) in HepG2 hepatocellular carcinoma cells. Then the 3′-fragment of this lncRNA interacts with multiple mtDNA loci, including D-loop, COX2, ND3, and CYTB. MALAT1 knockdown results in low OxPhos, reduced ATP production, inhibited mitophagy, declined mtDNA copy number, and upregulated intrinsic apoptotic pathway (Zhao et al., 2019).

In addition to lncRNAs^{nDNA}, miRNAs^{nDNA} seem to play roles that are more important in epigenetic regulations of tumor cells. For instance, miR-24 targets ND2 in human Lewis lung carcinoma (LLC) cells, resulting in mitochondrial dysfunction and growth inhibition (Michael et al., 2017). miR-26a targets COX 2 in human prostate cancer cells, inhibiting cell proliferation and inducing apoptosis (Zhang et al., 2016). miR-4485 targets 16S rRNA in human breast cancer cells, leading to the decrease of mitochondrial complex I activity, the production of ATP, and inducing high ROS levels that activates caspase-3/7-dependent apoptosis (Sripada et al., 2017). miR-2392 localizes to mitochondria, silences mtDNA transcription through an AGO2-dependent mechanism, thereby inhibiting ND4, CYTB, and COX1 expression, and promotes cancer cells to chemosensitivity (Fan et al., 2019).

CONCLUSION AND PERSPECTIVES

The puzzle of mitoepigenetics has been uncovered gradually in recent years. New findings also significantly alter the concept of mitoepigenetics. Manev and Dzitoyeva (2013) first proposed the concept of mitoepigenetics in 2013 referring to all epigenetic regulations that are related to mitochondria. Ferreira et al. (2015) also use this concept. According to their definition, mitoepigenetics is comprised of four levels: (i) epigenetic controls of expression of nDNA-encoded mitochondrial genes; (ii) a cell-specific mtDNA content and mitochondrial activity-determined epigenetic alterations in nuclear genes expression; (iii) mtDNA variants-influenced nuclear gene expression patterns and ncDNA methylation levels; (iv) epigenetic modifications in mtDNA like 5mC and 5hmC marks. However, Manev and Dzitoyeva (2013) suggested a restricted usage of mitoepigenetics as the last ones, which also was used by subsequent researchers such as Sadakierska-Chudy et al. (2014). However, van der Wijst and Rots (2015) and Coppede and Stoccoro (2019) used a more restricted definition of mitoepigenetics as 5mC or 5hmC in mtDNA. The concept of mitoepigenetics described by Ghosh et al. (2015) includes 5mC/5hmC in mtDNA, mitochondrial modulation of nuclear DNA methylation and non-coding RNAs regulatory epigenetics in mitochondria.

In this review, we definite the concept of mitoepigenetics as a study of molecular modifications occurring in mitochondria that affect mitochondrial inheritance without involving mtDNA changes. According to this definition, mitoepigenetics refers to mtDNA modifications, mitochondrial nucleoid modifications, mtRNA modifications as well as non-coding RNAs that affect the translation and function of mtDNA-encoded genes (**Figure 5**). This definition is narrower than the concept defined by Manev and Dzitoyeva (2013), but is an extension of the concept used by Ghosh et al. (2015) as it includes mtRNA modifications, mitochondrial nucleoid modifications and a new definition of non-coding RNAs-regulated mitoepigenetics. In fact, according to our definition, all the mitoepigenetic alterations seem to alter the expression and function of mtDNA-encoded proteins, which mainly play essential roles in ETC/OxPhos and participate in mitochondrial cellular metabolism including glucose, lipid and amino acid metabolism. Therefore, mitoepigenetics is tightly related to multiple mitochondria-mediated biological processes, such as intrinsic apoptosis (Dong et al., 2017), mitophagy (Dong and Cui, 2018; Li et al., 2019), ROS generation (Kausar et al., 2018), Ca^{2+} signaling (Bravo-Sagua et al., 2017) and hemoglobin synthesis (Fleming and Hamza, 2012).

Since dysfunctional mitochondria are tightly related to cancer initiation and cancer progression (Wallace, 2012; Vyas et al., 2016), mitoepigenetics can also involve important modulations that occur in the pathological processes of cancer. 5mC in specific sites of mtDNA seems to be decreased during tumorigenesis. This phenomenon suggests that 5mC in these sites may be prognostic markers for cancers. Besides, since recent report shows that 5hmC and 5fC contents are decreased significantly in the very early stage of HCC (Liu et al., 2019), 5hmC found in mtDNA may also make some senses during cancer initiation and progression. Besides, TFAM is also shown to be positively related to malignant progression of multiple cancers, post-translational modifications that found in this protein may also be essential modulations in cancer progression. NcRNAs derived from both the nDNA and mtDNA are also promising prognostic factors that regulate tumorigenesis. Mitoepigenetic alterations may be one of the reasons for carcinogenesis, otherwise they are results of tumorigenesis. These alterations cannot be a main reason for tumorigenesis, because cancer cells without mitochondria (ρ0 cells) still can form tumors *in vivo* (Magda et al., 2008; Sun et al., 2018b). However, epigenetic alterations in mitochondrial indeed affect the development of tumors and mitochondrial Achilles' heel in cancer can also be targeted by mitoepigenetic modulation (Hockenbery, 2002). Anyway, these findings about the connections between cancers and mitoepigenetics may provide some new clues for the prognosis, prevention and even therapeutic strategies for these diseases.

Epigenetic regulation is a kind of reversible mode for gene expression. Until now, there are several epigenetic drugs (epi-drugs), such as 5-azacytosine, decitabine, guadecitabine, belinostat, panobinostat, vorinostat, and romidepsin are approved by FDA in the clinic to treat some diseases including

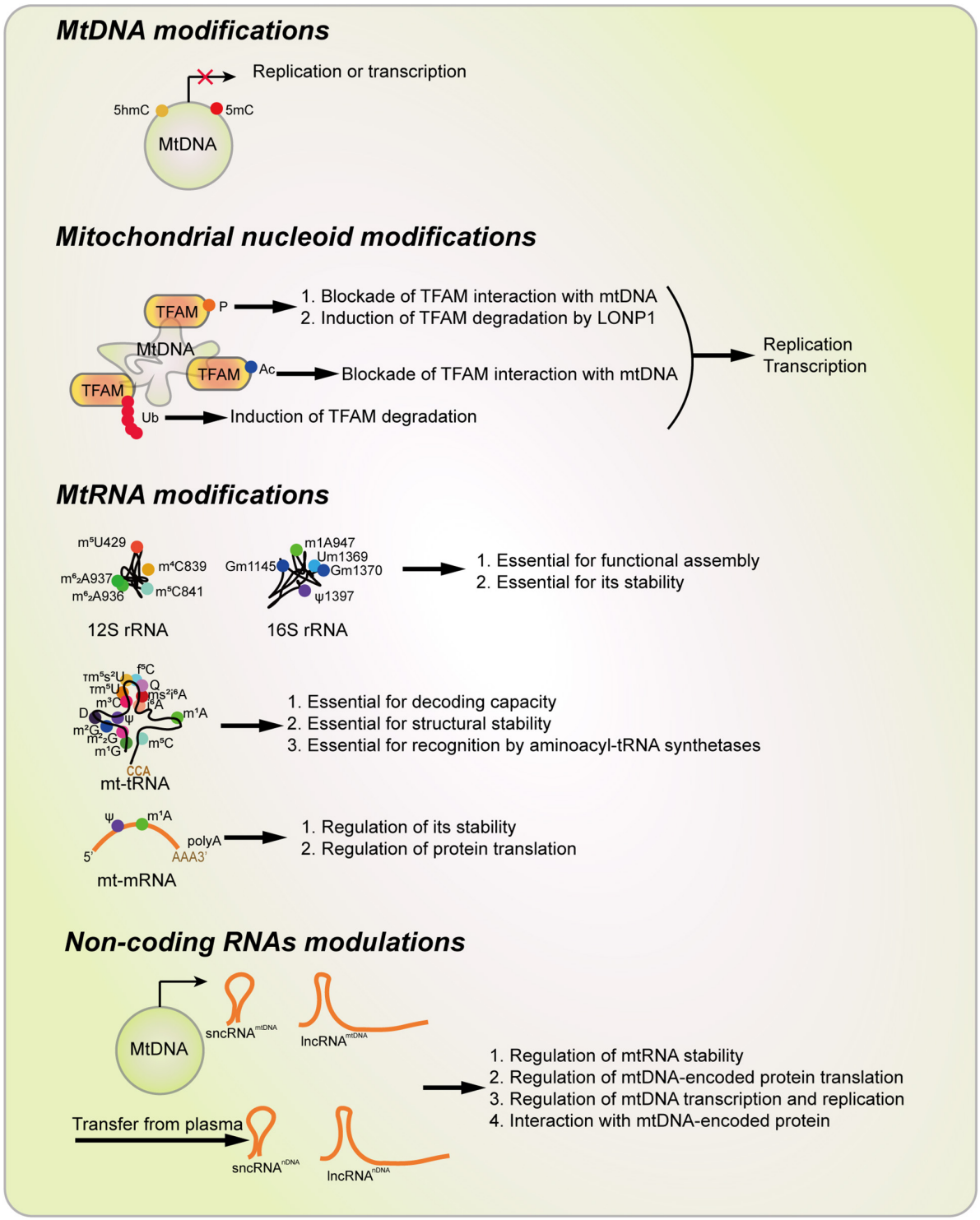

FIGURE 5 | Types of Mitoepigenetics and their functions. Mitoepigenetics constitutes with four different types, including mtDNA modifications, nucleoid modifications, mtRNA modifications, and non-coding RNA modulations. Ac, acetylation; D, Dihydrouridine; f^5C, 5-Formylcytosine; 5hmC 5-Hydroxymethylcytosine; 5mC, 5-methylcytosine; i^6A, *N6*-Isopentenyladenosine; m^1A, 1-Methyladenosine; m^1G, 1-Methylguanosine; m^2G, *N2*-Methylguanosine; $m^2{}_2G$, *N2,N2*-Dimethylguanosine; m^3C, 3-Methylcytosine; m^4C, *N4*-Methylcytosine; m^5C, 5-Methylcytosine; m^5U, 5-Methyluridinep; m^6A, *N6*-Methyladenosine; $m^6{}_2A$, *N6,N6*-Dimethyladenosine; ms^2i^6A, 2-Methylthio-*N6*-isopentenyladenosine; P, phosphorylation; Ψ, Pseudouridine; Q, Queosine; t6A, *N6*-Threonylcarbamoyladenosine; TFAM, Transcription factor A, mitochondrial; τm5U, 5-Taurinomethyluridin; τm5s2U, 5-Taurinomethyl-2-thiouridine, Ub, Ubiquitination.

cancers (Jones et al., 2016; Nebbioso et al., 2018). Besides, other epigenetic drugs such as chidamide, givinostat, quisinostat, GSK2879552 and MAK683 are on clinical trials (Berdasco and Esteller, 2019). These studies have opened a new window for the treatment of cancers. Since mitoepigenetics also plays essential roles in mitochondrial function and processing, it may open a new window for cancer therapy. However, there are some questions need to be further solved. Firstly, mtDNA methylation should be systemically studied with high-resolution methylation sequencing. Secondly, core proteins of the nucleoid should be studied further and the post-translational modifications in TFAM and their connections with cancers should be validated. Thirdly, modifications in mtRNAs including mt-rRNAs, mt-tRNAs, mt-mRNAs and ncRNAs^{mtDNA} should be further explored. Finally, both ncRNAs^{mtDNA} and ncRNAs^{nDNA} should be further characterized, their targets should be systemically identified and their clinical significances should be confirmed.

AUTHOR CONTRIBUTIONS

ZD wrote the manuscript, drew the figures, and made the tables. LP and HC reviewed and revised the manuscript.

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RNase H1 Regulates Mitochondrial Transcription and Translation *via* the Degradation of 7S RNA

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RNase H1 is able to recognize DNA/RNA heteroduplexes and to degrade their RNA component. As a consequence, it has been implicated in different aspects of mtDNA replication such as primer formation, primer removal, and replication termination, and significant differences have been reported between control and mutant *RNASEH1* skin fibroblasts from patients. However, neither mtDNA depletion nor the presence of deletions have been described in skin fibroblasts while still presenting signs of mitochondrial dysfunction (lower mitochondrial membrane potential, reduced oxygen consumption, slow growth in galactose). Here, we show that RNase H1 has an effect on mtDNA transcripts, most likely through the regulation of 7S RNA and other R-loops. The observed effect on both mitochondrial mRNAs and 16S rRNA results in decreased mitochondrial translation and subsequently mitochondrial dysfunction in cells carrying mutations in *RNASEH1*.

Keywords: mitochondria, mtDNA, mitochondrial disease, RNase H1, transcription, translation, 7S DNA, 7S RNA

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INTRODUCTION

Human mitochondrial DNA (mtDNA) encodes 2 rRNAs, 22 tRNAs, and 13 out of 83 proteins that are subunits of the respiratory chain, while the remaining proteins required for mitochondrial function are encoded in the nucleus. Indeed, all proteins responsible for mtDNA maintenance, especially those involved in replication, as well as other proteins necessary for transcription and translation, are encoded in the nucleus (Gustafsson et al., 2016). Human mtDNA replication requires several factors that constitute the replisome and that include DNA polymerase subunits POLG and POLG2, the helicase TWNK, the single-stranded binding protein SSBP1, and DNA topoisomerases TOP1, TOP2A and TOP2B (Gustafsson et al., 2016). The nucleases MGME1, DNA2, FEN1, and RNase H1 have been described in mitochondria, and they have been related to mtDNA replication, especially but not exclusively with regard to primer removal (Kazak et al., 2013; Uhler and Falkenberg, 2015; Al-Behadili et al., 2018; Posse et al., 2019).

The nuclease RNase H1 can be targeted to both the nucleus and mitochondria, and it is able to recognize DNA/RNA heteroduplexes and to degrade their RNA component (Suzuki et al., 2010). The enzyme consists of three domains: a hybrid binding domain and a catalytic domain separated by a connecting domain (Nowotny et al., 2007). The hybrid binding domain is responsible for the recognition of the DNA/RNA hybrids, and it also enhances both the specific activity and the processivity of the enzyme (Nowotny et al., 2008). Despite not being essential, the presence of this

domain results in a protein with greater binding affinity and positional preference for cleavage than the bacterial counterpart (Wu et al., 2001). The catalytic domain is very conserved from bacteria to humans and it contains key residues of the activity (Nowotny et al., 2007). The connecting domain has been less characterized, but it has been described to be required for RNase H activity (Wu et al., 2001).

In the nucleus, RNase H1 activity has been linked to the removal of R-loops (nascent RNA hybridized to template DNA with a single-stranded non-template DNA) in rDNA (Shen et al., 2017) and immunoglobulin sites (Parajuli et al., 2017), Okazaki fragment processing (Lima et al., 2007), DNA repair (Tannous et al., 2015; Amon and Koshland, 2016), telomere elongation (Arora et al., 2014), and hypermutability in the immunoglobulin locus (Maul et al., 2017). In mitochondria, RNase H1 has been implicated in different aspects of mtDNA replication such as replication initiation at origin-specific sites (Posse et al., 2019) and primer removal at both origins of replication (Holmes et al., 2015; Reyes et al., 2015; Al-Behadili et al., 2018), segregation of daughter mtDNA molecules post replication (Akman et al., 2016), R-loop processing (Reyes et al., 2015; Lima et al., 2016; Gonzalez de Cozar et al., 2019), and processing of mitochondrial ribosomal RNA precursor (Wu et al., 2013).

Mutations in genes involved in mitochondrial genome stability result in mtDNA depletion, large-scale multiple deletions, or accumulation of point mutations, which, in turn, can lead to mitochondrial diseases (Almannai et al., 2018; Rusecka et al., 2018). In the past few years, 15 patients with mitochondrial diseases have been found to carry mutations in the *RNASEH1* gene, mainly as compound heterozygous c.424G > A (p.Val142Ile) and c.469C > T (p.Arg157*) (Reyes et al., 2015), c.424G > A (p.Val142Ile) and c.554C > T (p.Ala185Val) (Reyes et al., 2015), c.424G > A (p.Val142Ile) and c.442T > C (p.Cys148Arg) (Bugiardini et al., 2017; Sachdev et al., 2018), and c.487T > C (p.Tyr163His) and c.258_260del (p.Gln86del) (Carreno-Gago et al., 2019) but in some cases as homozygous c.424G > A (p.Val142Ile) (Reyes et al., 2015; Akman et al., 2016). All mutations mapped in the catalytic domain, except c.258_260del (p.Gln86del), which mapped in the connecting domain. Affected individuals presented with adult-onset chronic progressive external ophthalmoplegia (CPEO), ptosis, dysphagia, muscle weakness, ataxia, and respiratory impairment. Mitochondrial DNA depletion and multiple deletions, COX-deficient fibers and low complex I and IV activities are characteristic features of the muscle biopsies from the patients with *RNASEH1* mutations (Reyes et al., 2015; Bugiardini et al., 2017; Sachdev et al., 2018; Carreno-Gago et al., 2019). However, neither significant mtDNA depletion nor the presence of multiple deletions have been observed in skin fibroblasts derived from these patients (Reyes et al., 2015; Akman et al., 2016; Carreno-Gago et al., 2019). Despite this, *RNASEH1* mutant fibroblasts presented lower mitochondrial membrane potential, reduced oxygen consumption, and slower growth than control fibroblasts (Reyes et al., 2015; Reyes et al., 2018). Therefore, RNase H1 may have additional roles not related to mtDNA maintenance that could be held responsible for this phenotype.

In this paper, we show that RNase H1 plays an important role in mtDNA transcription. Mutant *RNASEH1* skin fibroblasts showed a significant decrease in some mitochondrial transcripts, e.g., MT-CO2, MT-ND5, and MT-RNR2 (16S rRNA). Interestingly, the levels of 7S RNA (MT-7S), a small non-coding mitochondrial transcript, were also upregulated in the patient fibroblasts. 7S RNA is involved in the primer synthesis required for mtDNA replication but it has also been suggested to play a role as a negative regulator of mtDNA transcription (Cantatore et al., 1988). Hence, the decrease of transcript levels in the patient fibroblasts could be related to the increase in 7S RNA, as this may not have been efficiently removed by the lower levels and activity of mutant RNase H1 in the patient. In addition, a lack of or slow processing of R-loops in different regions of mtDNA could also affect transcript levels. A decrease in mitochondrial translation due to a decrease in 16S rRNA and possible direct interaction of 7S RNA with 12S rRNA could also explain the mitochondrial dysfunction we detected in these cells.

MATERIALS AND METHODS

Structural Modeling of Mutant RNase H1

The crystal structure of the human RNase H1 catalytic domain in a complex with 18-mer DNA/RNA heteroduplex (PDB ID 2QK9) was downloaded from the Protein Data Bank (PDB) database and loaded onto PyMOL. Conserved residues previously reported to constitute the active site of the protein (Nowotny et al., 2007) were manually colored in yellow and visualized as sticks, while the DNA and RNA components of the heteroduplex were colored in cyan and magenta, respectively. The mutagenesis option available in PyMOL was used to replace Val^{142} with Ile^{142}. These two residues and the neighboring residue Trp^{164} were displayed in different colors and visualized as sticks in order to highlight the possible effect the mutation could have on the structure of the protein.

Cell Culture Conditions

Fibroblasts derived from skin biopsy were obtained from a patient (P) carrying two pathogenic mutations in the *RNASEH1* gene (GenBank: NM_002936.4): c.424G > A (p.Val142Ile) on the paternal allele and a nonsense mutation, c.469C > T (p.Arg157*), on the maternal allele (Reyes et al., 2015). In addition, control fibroblasts were obtained from two healthy controls (C1 and C2). Fibroblast cell lines were maintained in high-glucose medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Primary skin fibroblasts were immortalized by lentiviral transduction of pLOX-Ttag-iresTK (Addgene #12246, Tronolab), as previously described (Reyes et al., 2018). Briefly, human 293T cells were cotransfected with transfer vector (pLOX-Ttag-iresTK), second-generation packaging plasmid (pCMVdR8.74), and envelope plasmid (pMD2.VSVG) (Naldini et al., 1996). Infectious lentiviral particles were collected from the medium

24 h after transfection and used for transduction of all three fibroblast cell lines. Transduced fibroblasts were grown for at least six passages in order to make sure immortalized cells were selected. Changes in cell shape and doubling time were observed as part of the normal process of immortalization. All experiments here reported were carried out on immortalized fibroblasts. When required, high-glucose medium was replaced by glucose-free medium (Gibco) and supplemented with 50 mM galactose (Sigma).

Immunoblot Analysis

Protein gel electrophoresis and blotting analyses were performed on whole cell protein extracts obtained from patient (P) and control (C1 and C2) fibroblasts. Samples containing 30 μg protein were separated by denaturing NuPAGE 4%–12% Bis-Tris gels and transferred to nitrocellulose membrane. Immunodetection was carried out using primary antibodies against target proteins: RNase H1 (ab56560, Abcam), POLG (sc-5931, Santa Cruz), POLG2 (LS-C334882, LSBio), TWNK (gift from M Falkenberg), SSBP1 (ab74710, Abcam), TFAM (gift from RJ Wiesner), POLRMT (ab32954, Abcam), LRPPRC (ab97505, Abcam), SLIRP (ab51523, Abcam), ATAD3 (gift from JE Walker), bL12 (14795-1-AP, Proteintech), uL11 (SAB2701374, Sigma), MDDX28 (ab70821, Abcam), mS35 (16457-1-AP, Proteintech), mS18b (16139-1-AP, Proteintech), NDUFS3 (ab110246, Abcam), NDUFB8 (ab110242, Abcam), SDHA (ab14715, Abcam), SDHB (ab14714, Abcam), UQCRC1 (ab96333, Abcam), UQCRC2 (ab14745, Abcam), MT-CO1 (ab14705, Abcam), MT-CO2 (ab91317, Abcam), COX4l1 (ab14744, Abcam), ATPF1 (ab84625, Abcam), and ATPA1 (ab110273, Abcam), along with GAPDH (ab8245, Abcam), used as loading control. For quantifications, images were digitalized and analyzed with ImageJ software, and data analyses were performed in Microsoft Excel.

DNA Isolation, Gel Electrophoresis, and Hybridization

Total DNA from patient (P) and control (C1 and C2) fibroblasts was extracted using Wizard Genomic Purification Kit (Promega). Total DNA (5 μg) was digested with *Pvu*II (NEB), and the fragments were resolved in 1% agarose gels. After electrophoresis and Southern blot, hybridizations with radiolabelled probes directed against the human mtDNA (nucleotide positions 16,341-151) and nuclear 18S rDNA were carried out overnight at 65°C in 7% SDS and 0.25M sodium phosphate buffer pH 7.4. After washing four times with 1x SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) and twice with 1 x SSC/0.1% SDS, membranes were exposed to Phosphorimager screens for 0.5 to 10 days. ImageQuant software was used for the quantification of the signal.

RNA Isolation and Quantitative PCR (qPCR)

Total RNA from patient (P) and control (C1 and C2) fibroblasts was extracted using Trizol (Invitrogen). RNA was then treated with DNase I (DNA-free kit, Ambion) and reverse transcribed with Omniscript reverse transcription kit (Qiagen). Quantitative polymerase chain reaction (qPCR) analyses were performed with Life Technologies Gene Expression Assays (Applied Biosystems): RNase H1 (Hs00268000_m1, Hs01108220_g1 and Hs01108219_g1 on exons 7-8, 2-3 and 1-2 boundary, respectively), MT-7S (7S RNA, Hs02596861_s1), MT-RNR1 (12S rRNA, Hs02596859_g1), MT-RNR2 (16S rRNA, Hs02596860_s1), MT-CO1 (Hs02596864_g1), MT-CO2 (Hs02596865_g1), MT-CO3 (Hs0259866_g1), MT-ND1 (Hs02596873_s1), MT-ND5 (Hs02596878_g1), MT-ND6 (Hs02596879_g1), MT-CYB (Hs02596867_s1), and MT-ATP6 (Hs02596862_g1) and normalized to levels of GAPDH (Hs02758991_g1).

Mitochondrial Translation

Patient (P) and control (C1 and C2) fibroblast cell lines were subjected to metabolic labeling of mtDNA encoded proteins. [^{35}S]-methionine was added to the medium after treatment with emetine dihydrochloride and labeling was performed for 1 h, as previously described (Chomyn, 1996). Cells were lysed and proteins (30 μg) were loaded onto 12% polyacrylamide gels. Gels were stained with Coomassie blue, dried, and then exposed to Typhoon phosphor screens, with products visualized and quantified with ImageQuant software (GE Healthcare).

Oxygen Consumption

Respiration in patient (P) and control (C1 and C2) fibroblasts, I_{O2} [pmols·s^{-1}·10^{-6} cells], was calculated as the negative time derivate of oxygen concentration as measured by the OROBOROS Oxygraph-2k on one million cell/ml in a 2-ml chamber at 37°C. Basal respiration was measured without substrates, and the proton leak state after the addition of oligomycin (50nM) was also measured. Oxygen consumption coupled to ATP production was calculated as the difference between basal respiration and proton leak. Maximal respiration was measured by stepwise 1.25 μM titration of CCCP and inhibition by 2 μM rotenone and 2.5 μM antimycin A for the final measurement of residual oxygen consumption. Spare capacity was calculated as the difference between maximal respiration and basal respiration.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured in patient (P) and control (C1 and C2) untreated fibroblasts and after treatment with 1 μM FCCP for 5 min at 37°C as the ratio of the red to the green JC-1 signal using a Nucleo Counter NC-3000 Advanced Image Cytometer.

Statistics

Fibroblasts from a single patient with mutations in *RNASEH1* and two non-related healthy individuals were analyzed as controls. All numerical data are expressed as mean ± standard deviation of the mean (SD). Student's unpaired two-tailed t-tests under the assumption of a normal distribution and unequal variance were used for statistical analysis combining the data from both controls against the patient unless specified otherwise.

Control 1 (C1) fibroblasts were randomly chosen as the reference for all experiments, the values obtained in the first biological repeat were arbitrarily assigned as 1 and, subsequently, all other values were corrected accordingly.

RESULTS

Characterization of the Mutations in *RNASEH1*

The two mutations present in the *RNASEH1* gene in the patient were first analyzed *in silico*. The missense mutation, c.424G > A (p.Val142Ile), involved a residue in a conserved position of the β1 strand, close to one of the four key catalytic residues (**Figure 1A**). Modeling of the mutation on the crystal structure of human RNase H1 (PDB ID 2QK9) showed that Ile^{142} is a bulkier residue than Val^{142} and therefore could interfere with another bulky residue nearby, Trp^{164}, causing a change in the orientation of the β1 strand (**Figure 1B**). This could result in a misalignment of the four catalytic residues that constitute the active site and/or the residues involved in the interaction with the DNA/RNA hybrids. The nonsense mutation, c.469C > T (p.Arg157*), affects a residue at the N-terminus of the catalytic domain and, as a consequence, the truncated protein is void of any activity (Reyes et al., 2015). Nonsense-mediated decay is a conserved quality control mechanism that selectively degrades the transcripts harboring premature stop codons (Kurosaki et al., 2019). In order to investigate if the presence of a nonsense mutation was triggering nonsense-mediated decay, we checked *RNASEH1* transcript levels in human control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium with probe Hs00268000_m1, spanning exons 7-8 (**Figure 1C**). *RNASEH1* transcript levels were significantly reduced to at least 50% of controls in both growing conditions. The same results were obtained when different probes upstream of the nonsense mutation were used, Hs01108220_g1 and

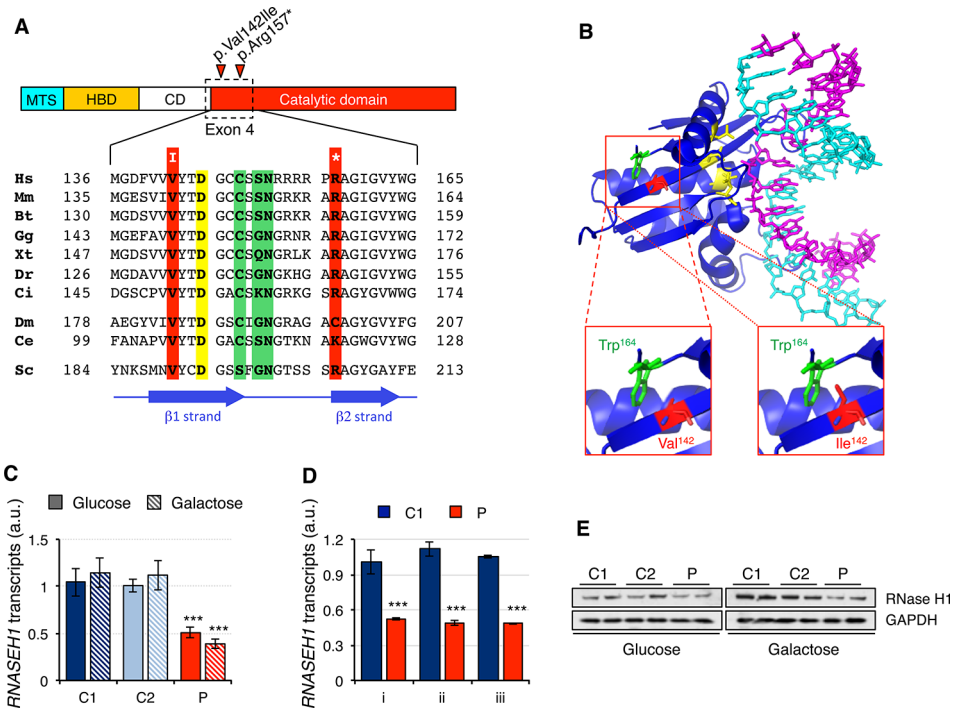

FIGURE 1 | *RNASEH1* mutations, transcript, and protein levels. **(A)** Domains ofhuman RNAse H1 protein (MTS, mitochondrial targeting sequence; HBD, hybrid binding domain; CD, connection domain; catalytic domain). RNase H1 protein sequences from representative species,*H. sapiens* (Hs, NP_002927) *M. musculus* (Mm, NP_035405), *B. taurus* (Bt, NP_001039970), *G. gallus* (Gg, NP_990329),*X. tropicalis* (Xt, NP_001096299),*D. rerio* (Dr, NP_001002659), *C. intestinalis* (Ci, F6QPH0), *D. melanogaster* (Dm, NP_995777), *C. elegans* (Ce, NP_001040786), *S. cerevisiae* (Sc, Q04740), were extracted from the database and aligned using ClustalW2. Conserved residues found mutated in the patient in exon 4 are boxed in red, while residues in the active site and interacting with DNA or RNA are boxed in yellow and green, respectively. Positions of β strands are marked by blue arrows. **(B)** Human RNase H1 crystal structure (PDB ID 2QK9) 18 bp DNA(cyan): RNA (magenta) hybrid is shown respectively. Residues in the active site are colored in yellow. Residues Trp^{164} (green) and Val^{142}, or the mutated variant Ile^{142} (red), are shown as sticks. **(C)** *RNASEH1* transcript levels in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium assessed by qPCR (probe Hs00268000_m1) and normalized to *GAPDH* transcript levels. Data are shown as mean ± SD, n = 4, ***p < 0.001. **(D)** *RNASEH1* transcript levels in control (C1) and patient (P) fibroblasts grown in glucose-containing medium assessed by qPCR with probes Hs00268000_m1 (i), Hs01108220_g1 (ii), and Hs01108219_g1 (iii) and normalized to *GAPDH* transcript levels. Data are shown as mean ± SD, n = 3, ***p < 0.001. **(E)** Western blot analysis of RNase H1 in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. GAPDH was used as loading control.

Hs01108219_g1, spanning exons 2-3 and 1-2, respectively (**Figure 1D**), further supporting nonsense-mediated decay. As a consequence of the decrease in transcript levels, a significant decrease was also observed at protein levels in patient fibroblasts (**Figure 1E**).

Mitochondrial DNA-Related Alterations in Patient Fibroblasts

Since analysis of muscle biopsy from patients carrying mutations in *RNASEH1* has revealed the presence of multiple deletions and depletion in mtDNA (Reyes et al., 2015; Bugiardini et al., 2017; Carreno-Gago et al., 2019), we performed a Southern blot on genomic DNA extracted from control (C1 and C2) and patient (P) fibroblasts grown in either glucose or galactose (**Figure 2A**). No deletions on mtDNA were detected in the patient fibroblasts, and the mtDNA copy number was only marginally reduced to 80% compared to controls when the cells were grown in glucose, with no significant difference observed when cells grew in galactose (**Figures 2A, B**). By contrast, 7S DNA, the third strand of the mtDNA displacement loop, was 10-fold higher in the patient fibroblasts than in control, both in glucose and galactose (**Figures 2A, C**). Furthermore, 7S DNA in controls appears as a net band, as all the molecules have the same length, while in the patient fibroblasts, there is a smear below the main band, indicating that some 7S DNA molecules are shorter than the expected size (**Figure 2A**). This effect is more pronounced in glucose than in galactose.

Then we analyzed the steady-state level of mitochondrial proteins involved in mtDNA maintenance (**Figure 2D**). Other than the overall increase in the steady-state level of all proteins when cells were grown in galactose medium, no significant differences between patient and control fibroblasts were observed. These results are in agreement with the observed minor changes in mtDNA copy number in the patient fibroblasts (**Figure 2A**).

Mitochondrial RNA-Related Alterations in Patient Fibroblasts

Mitochondrial dysfunction has been reported in fibroblasts from patients carrying mutations in *RNASEH1*, but neither mtDNA deletions nor depletion have been observed (Reyes et al., 2015; Bugiardini et al., 2017; Carreno-Gago et al., 2019). Therefore, we first investigated whether there was an effect on mitochondrial transcription. The steady-state levels of 11 transcripts was analyzed by qPCR and included 7S RNA (MT-7S), the two ribosomal RNAs MT-RNR1 (12S rRNA) and MT-RNR2 (16S

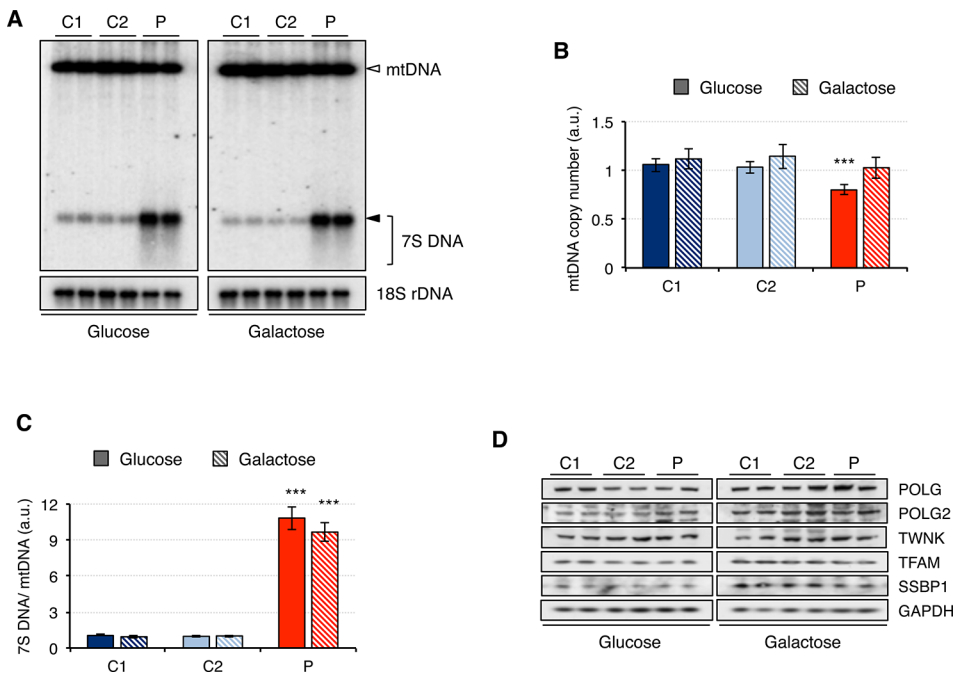

FIGURE 2 | Mitochondrial DNA maintenance in mutant *RNASEH1* fibroblasts. **(A)** Southern blot of total DNA digested with *Pvu*II from control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. A radioactive probe against mtDNA was used to detect both linearized mtDNA (empty arrowhead) and 7S DNA (filled arrowhead and bracket), while a probe against 18S rDNA was used as loading control. **(B)** Relative mitochondrial DNA copy number in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium, calculated as the linearized mtDNA/18S rDNA signal ratio. Data are shown as mean ± SD, n = 4, ***p < 0.001. **(C)** 7S DNA levels in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium calculated as the 7S DNA/linearized mtDNA/18S rDNA signal ratio. Data are shown as mean ± SD, n = 4, Student's unpaired two-tail t-test, ***p < 0.001. **(D)** Western blot analysis of mitochondrial proteins involved in mtDNA maintenance in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. GAPDH was used as loading control.

rRNA), and eight protein mRNAs from all four different oxidative phosphorylation (OxPhos) complexes with mitochondrially-encoded subunits: CI, CIII, CIV, and CV (**Figure 3**). The non-coding 7S RNA is a polyadenylated transcript of about 200 nt whose 5' end maps at the light strand promoter (LSP) and has been implicated in both mtDNA replication and transcription. Transcript levels of 7S RNA in galactose medium were lower than in glucose in all cell lines. Moreover, a two-fold and three-fold increase in 7S RNA was detected in patient fibroblasts compared to controls grown in glucose and galactose, respectively. As a result, patient cells grown in galactose had the same levels of 7S RNA as controls grown in glucose. For all the other transcripts analyzed, the levels in control cells growing in galactose medium were always higher than in glucose medium, suggesting increased mitochondrial biogenesis. In the case of patient fibroblasts, the results varied depending on the transcript. The two ribosomal rRNAs showed a different behavior: a slight decrease in 12S rRNA was observed in patient cells only when they were grown in galactose medium, while a significant decrease in 16S rRNA compared to controls was observed both in glucose and galactose growth (40% and 80% decrease, respectively). The transcripts of both complex IV (MT-CO1, MT-CO2, MT-CO3) and complex I (MT-ND1, MT-ND5, MT-ND6) subunits were moderately decreased in patient cells grown in glucose medium (28-36% and 17-35% decrease for complex IV and I, respectively), and culture in galactose medium did not increase their levels very significantly in most of the cases, which increased the difference with control cell lines (40-43% and 35-50% decrease for complex IV and I, respectively). A completely different trend was observed in transcripts from complex III (MT-CYB) and complex V (MT-ATP6) mitochondrial subunits: transcript levels in glucose growth were higher in the patient fibroblasts than in controls (about 50% increase in both cases), while in galactose growth, they were lower compared to controls (about 50% decrease in both cases).

Next, we analyzed the steady-state levels of proteins involved in RNA metabolism and mitochondrial ribosomal proteins (**Figure 4A**). Overall, protein levels were higher in galactose than in glucose medium, suggesting increased mitochondrial biogenesis. While the mitochondrial RNA polymerase, POLRMT, was not significantly changed in patient fibroblasts, other proteins such as LRPPRC, SLIRP, and ATAD3 were decreased in patient fibroblasts, particularly when grown in glucose medium. Moreover, mitochondrial ribosomal proteins from the large 39S subunit (mt-LSU) but not the small 28S subunit (mt-SSU) were also found to be decreased in patient fibroblasts growing in glucose medium and, to a lesser extent, also in galactose medium (**Figures 4A, B**). As a consequence,

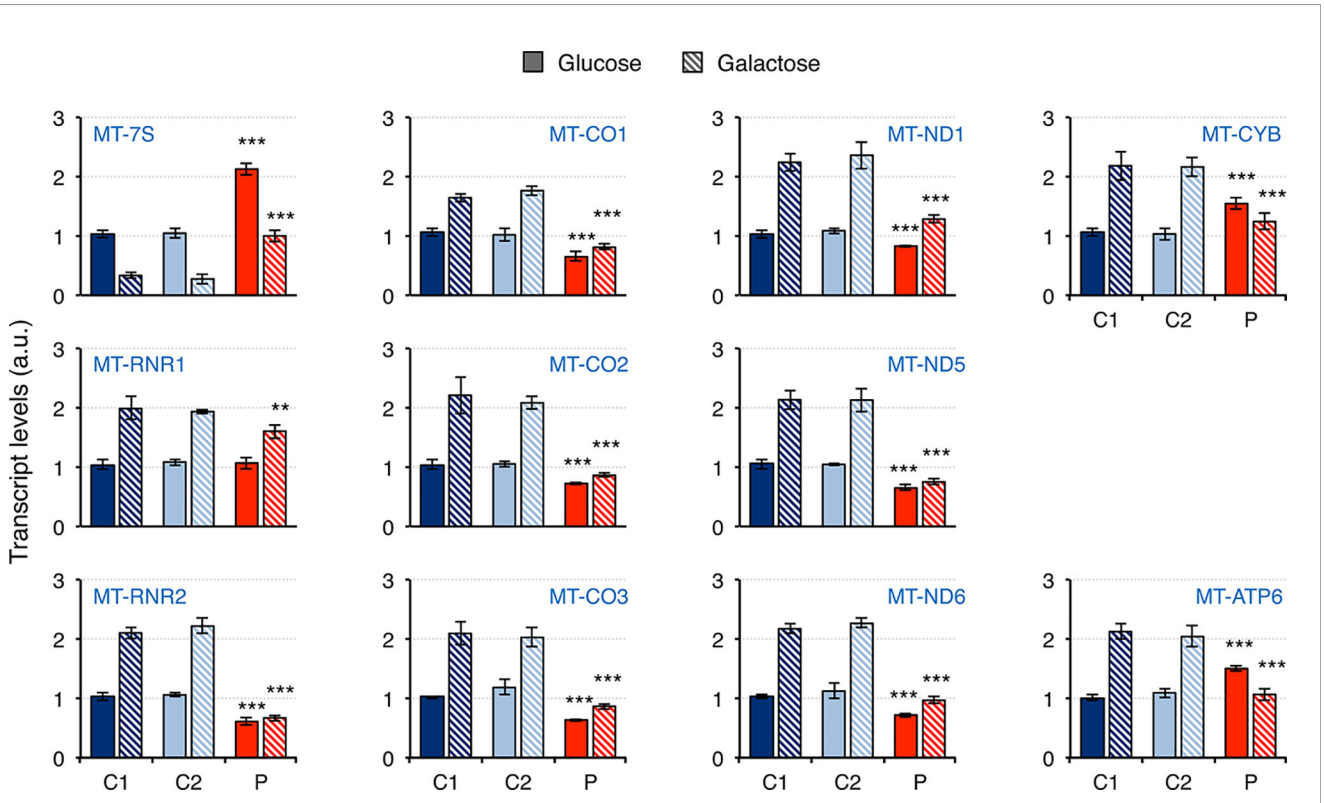

FIGURE 3 | Mitochondrial transcript levels in mutant *RNASEH1* fibroblasts. Mitochondrial transcript levels in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium, assessed by qPCR and normalized to *GAPDH* transcript levels. Analyzed transcripts included the non-coding 7S RNA (MT-7S), the two ribosomal RNAs MT-RNR1 (12S rRNA) and MT-RNR2 (16S rRNA), three complex IV protein mRNAs (MT-CO1, MT-CO2 and MT-CO3), three complex I protein mRNAs (MT-ND1, MT-ND5 and MT-ND6), one complex III protein mRNA (MT-CYB), and one complex V protein mRNA (MT-ATP6). Data are shown as mean ± SD, n = 4, Student's unpaired two-tail t-test, $^{**}p < 0.01$, $^{***}p < 0.001$.

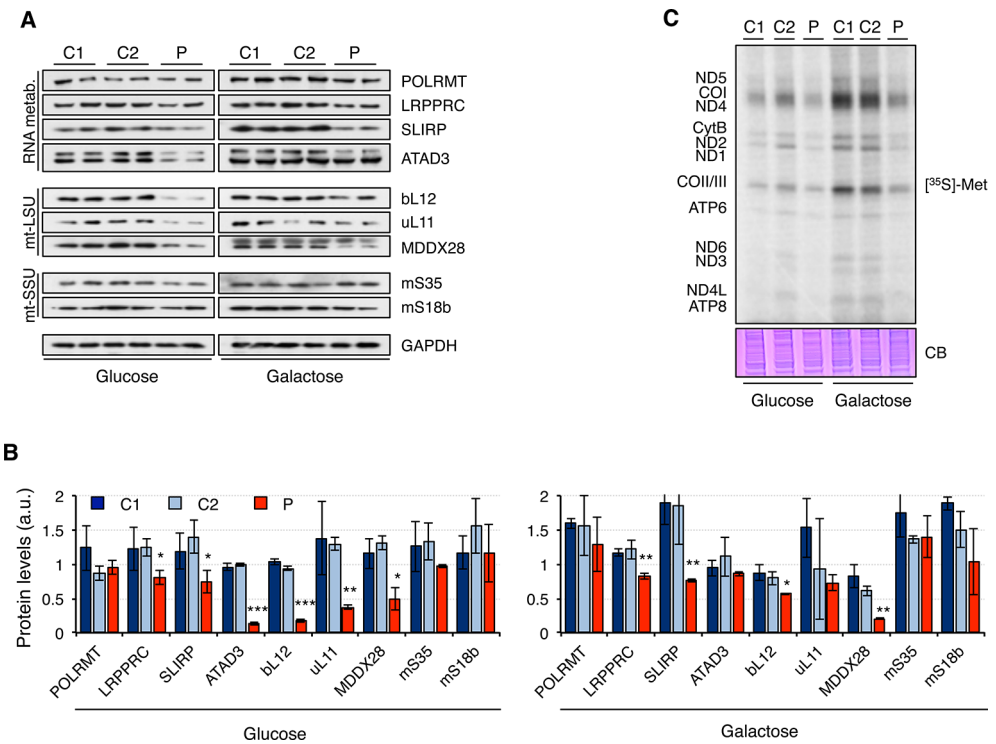

FIGURE 4 | Mitochondrial translation in mutant *RNASEH1* fibroblasts. **(A)** Western blot analysis of mitochondrial proteins involved in mitochondrial RNA metabolism (RNA metab.) and mitochondrial large (mtLSU) and small (mtSSU) ribosomal subunits in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. GAPDH was used as loading control. GAPDH is from the same blot as **Figure1D**. **(B)** Quantification of the Western blots shown in **(A)** normalized to GAPDH levels. Data are shown as mean ± SD, Student's unpaired two-tail t-test, *p < 0.05, **p < 0.01, ***p < 0.001. **(C)** [^{35}S]-methionine *de novo* synthesis of mitochondrially encoded proteins in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. Newly synthesized proteins were visualized after exposure of the dried gel to phosphor screens. The coomassie blue (CB) staining shown below was used as loading control. n = 3.

mitochondrial translation was impaired in patient fibroblasts, with all mitochondrial proteins equally affected (**Figure 4C**).

Mitochondrial Dysfunction in Patient Fibroblasts

Fibroblasts from patients carrying mutations in *RNASEH1* have signals of mitochondrial dysfunction (Reyes et al., 2015; Bugiardini et al., 2017; Carreno-Gago et al., 2019). Indeed, we have shown that patient fibroblasts have alterations in mitochondrial transcription and translation that could lead to mitochondrial dysfunction. Therefore, we first analyzed the steady-state of the OxPhos constituents of all five complexes (**Figure 5A**). Again, overall protein levels were higher in galactose than in glucose medium, supporting increased mitochondrial biogenesis. Patient fibroblasts presented lower steady-state levels of all analyzed subunits of complex I and complex IV, while no difference was detected for complexes III and V. These results are in agreement with the data from mitochondrial transcript levels (**Figure 3**). Complex II subunits were slightly increased in patient fibroblasts compared to controls, most likely as a compensation mechanism. A consequence of the observed decrease in OxPhos protein levels was an alteration in mitochondrial respiration, as measured by oxygen consumption, I_{O2}. Patient fibroblasts showed significant lower basal I_{O2}, ATP-dependent, and maximal I_{O2}, both in glucose and galactose (**Figure 5B**). Mitochondrial membrane potential is usually altered in cases of a dysfunctional electron transport chain and, indeed, we observed a significant decrease in membrane potential in patient fibroblasts both in glucose and galactose (**Figure 5C**). These mitochondrial alterations in the patient fibroblasts have many consequences at a cellular level, and a lower growth rate is one of them (**Figure 5D**).

DISCUSSION

Pathological mutations in *RNASEH1* have been described in patients with mitochondrial depletion and deletion syndromes characterized by CPEO, cerebellar ataxia, and dysphagia (Bugiardini et al., 2017). Mutations in *RNASEH1* are still rare and, to date, only 16 patients have been reported (Reyes et al., 2015; Bugiardini et al., 2017; Sachdev et al., 2018; Carreno-Gago et al., 2019). Mutations involve six different residues and are not randomly distributed: four of them are in exon 4, one in exon 5

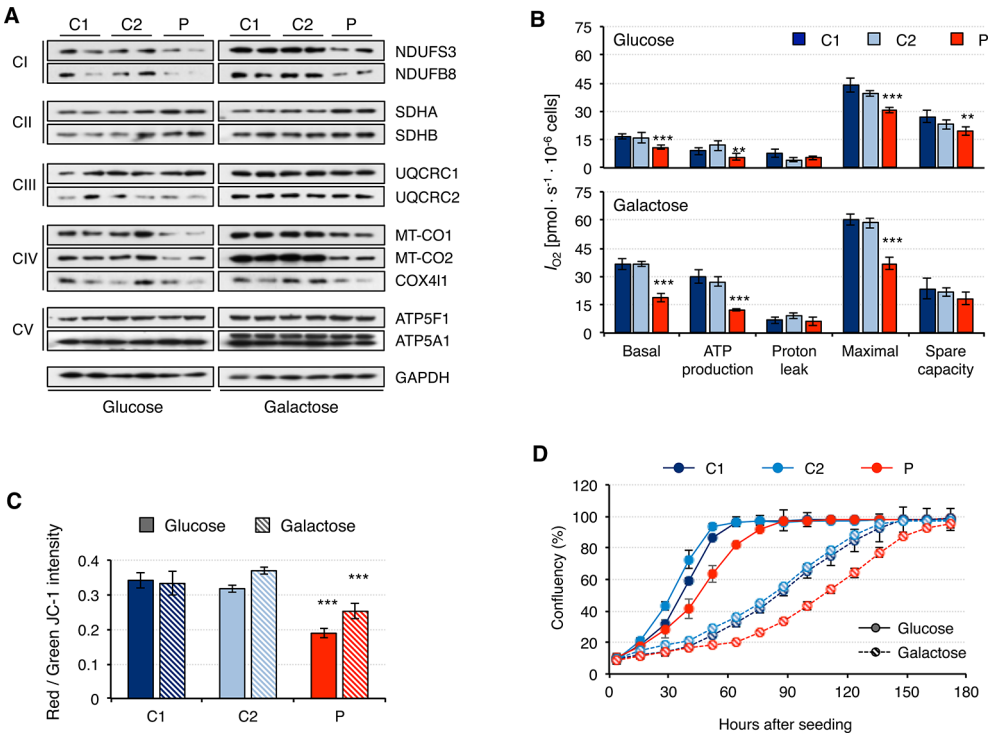

FIGURE 5 | Mitochondrial DNA maintenance in mutant *RNASEH1* fibroblasts. **(A)** Western blot analysis of representative components of the mitochondrial OxPhos complexes I-V in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. GAPDH was used as loading control. **(B)** Oxygen consumption (I_{O2}) measurements in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium. Values of basal and maximal respiration along with ATP production-dependent, proton leak respiration, and spare capacity are presented. Data are shown as mean ± SD, n = 4, Student's unpaired two-tail t-test, $^{**}p < 0.01$, $^{***}p < 0.001$. **(C)** Mitochondrial membrane potential in control (C1 and C2) and patient (P) fibroblasts grown in either glucose- or galactose-containing medium using JC-1 staining. Data are shown as mean ± SD, n = 4, Student's unpaired two-tail t-test, $^{***}p < 0.001$. **(D)** Growth curves of control (C1 and C2) and patient (P) fibroblasts grown in either glucose or galactose. Cell growth was monitored continuously in an Incucyte cell imager (Essen Bioscience). Data correspond to one of the three independent experiments carried out, and they are shown as the mean of three technical replicates ± SD.

(in the N-terminal portion of the catalytic domain), and one in exon 3 (in the connecting domain). The fibroblasts from the patient presented here carry two mutations in exon 4: a missense (c.424G > A, p.Val142Ile) and a nonsense mutation (c.469C > T, p.Arg157*). Nonsense mutations are often associated with a decrease in protein level due to nonsense mediated decay (Kurosaki et al., 2019), and this has been reported not only in fibroblasts carrying *RNASEH1* mutations (Reyes et al., 2015) but also in fibroblasts with nonsense mutations in other genes such as *PYCR2* (Zaki et al., 2016), *TIMM50* (Reyes et al., 2018), and *TAOK1* (Dulovic-Mahlow et al., 2019). Similarly, albeit to a lesser degree, two missense mutations in *RNASEH1* (p.Val142Ile and p.Gln86del) have also been described to have an effect on protein stability and therefore to result in a decreased steady-state level of the protein in cultured fibroblasts (Reyes et al., 2015; Akman et al., 2016; Carreno-Gago et al., 2019). In addition, p.Val142Ile mutant RNase H1 activity is only 36-40% of that of wild-type protein based on *in vitro* assays (Reyes et al., 2015; Al-Behadili et al., 2018). With our current knowledge, it is not possible to ascertain whether it is the amino acid substitution itself, the decrease/lack of activity, or a combination of both that is responsible for the observed protein instability.

Notwithstanding the mtDNA depletion and deletion observed in muscle biopsies from patients with mutations in *RNASEH1*, skin fibroblasts derived from the same patients display normal to slightly decreased mtDNA content (Reyes et al., 2015; Akman et al., 2016; Carreno-Gago et al., 2019). Thus, it not surprising that the levels of proteins involved in mtDNA maintenance are not markedly affected either. This could be achieved, on the one hand, by other proteins with similar or complementary functions, such as MGME1, FEN1, and DNA2, helping to maintain the minimum requirements for mtDNA replication, and on the other hand, by changes in the cellular processes like a slow down of cellular growth that could compensate for the slower or less active replication in cells with mutant RNase H1 (Reyes et al., 2015).

Despite the lack of effect on mtDNA, *RNASEH1* mutations have a marked impact on several mitochondrial transcripts in the patient fibroblasts. Significant decreases in mitochondrially encoded complex IV (MT-CO1, MT-CO2, MT-CO3) and

complex I (MT-ND1, MT-ND5, MT-ND6) transcripts have been observed in *RNASEH1* patient fibroblasts. However, no such decrease was observed in transcripts from complex III (MT-CYB) and complex V (MT-ATP6) mitochondrial subunits in glucose medium. Although this is the first report of transcript levels in patient fibroblasts, our data are in agreement with recent reports in a *Rnaseh1* liver-specific knockout (KO) mouse model (Lima et al., 2016) and in *Drosophila* S2 cell *rnh1* knockdown (KD) (Gonzalez de Cozar et al., 2019). In the *Rnaseh1* KO mouse, a decline was observed in all mitochondrial transcripts over time from six to 14 weeks of *Rnaseh1* ablation (Lima et al., 2016). In *Drosophila rnh1* KD, transcript levels of Cox3 and ND5 were decreased, while cyt b and ATP8 remained unaltered (Gonzalez de Cozar et al., 2019). The main difference from our patient fibroblasts is that in those cases, mtDNA depletion was also present, making it more difficult to segregate the direct effect of RNAse H1 on transcription from its secondary effect due to partial mtDNA depletion. The non-coding 7S RNA is the only transcript that was increased in the patient fibroblasts in both glucose and galactose medium. This transcript has been described to be involved in the synthesis of the 7S DNA (Gustafsson et al., 2016), and therefore it is not surprising that 7S DNA levels were also increased in the patient fibroblasts, albeit to a much higher level. Much less is known about its role in transcription, despite the fact that early studies suggested that 7S RNA could regulate mitochondrial transcription by preventing the formation of new transcription initiation events (Cantatore et al., 1988). More recently, it has been demonstrated for the first time that RNase H1 is required for the effective removal of 7S RNA, as the *Rnaseh1* KO mouse presents higher levels of 7S RNA, which results in failure to transcribe mtDNA (Lima et al., 2016). In our *RNASEH1* patient fibroblasts, we detected a concomitant increase in 7S RNA and a decrease in seven out of 10 mitochondrial transcripts, supporting the idea that 7S RNA plays a role in their transcription levels. Not only 7S RNA but also other transcripts are able to form R-loops throughout the mitochondrial genome (Brown et al., 2008), and, subsequently, inefficient removal of these structures could block ongoing transcription anywhere along the genome. In spite of this, the mitochondrial degradosome, composed by SUV3 and PNPase, has also been described to be involved in preventing the accumulation of pathological R-loops in mtDNA (Silva et al., 2018), providing a salvage pathway in cells carrying mutations in *RNASEH1*. However, two of the mitochondrial protein transcripts, MT-CYB and MT-ATP6, did not seem to be affected in the patient fibroblasts. This could be explained by a differential transcript half-life, as MT-ATP8/6 transcript is among the longest half-life mitochondrial transcripts in HeLa cells (Nagao et al., 2008). In certain situations, the stabilization of some transcripts could be modified by the up- or downregulation of certain proteins. It has been reported that upon decrease in the steady-state levels of LRPPRC/SLIRP complexes, some transcript levels, including MT-CYB, are less prone to degradation (Chujo et al., 2012). Both LRPPRC and SLIRP were downregulated in patient fibroblasts and therefore could have an effect on MT-CYB transcript stability.

Mitochondrial rRNAs are essential components of the mitochondrial ribosomes, and alterations in their levels often result in mitochondrial translation defects (Boczonadi et al., 2018). The *RNASEH1* patient fibroblasts displayed lower levels of 16S rRNA (MT-RNR2) than controls and, in agreement with these results, lower levels of mitochondrial ribosomal proteins associated with the mt-LSU were observed. This is not the case for 12S rRNA (MT-RNR1) and associated ribosomal proteins, mt-SSU. As discussed above for mitochondrial mRNAs, the steady-state levels of mitochondrial rRNAs can also be modulated by the levels of 7S RNA since this molecule could impede transcription initiation not only at the light but also at the heavy strand promoter (LSP and HSP, respectively). However, this would result in lower levels of both 12S and 16S rRNAs, and we have only detected a decrease in the levels of 16S rRNA. RNase H1, along with P32, has been shown to be involved in the processing of guanosine-cytosine rich mitochondrial ribosomal RNA precursor (12S/16S rRNA precursor) (Wu et al., 2013). Downregulation of RNase H1 increases the levels of the 12S/16S rRNA precursor with one and two species containing 12S and 16S rRNA, respectively (Wu et al., 2013). This suggests that processing of the pre-rRNA by RNase H1 is sequential, originating the mature 12S rRNA in the first step and after further processing, the mature 16S rRNA. A delay in this second processing step could result in the degradation of the partly processed rRNA containing 16S rRNA we observed in the patient fibroblasts. Lower levels of 16S rRNA would result in a decrease of mt-LSU ribosomal proteins, leading to decreased mitochondrial translation. In addition, mitochondrial translation could also be directly modulated by 7S RNA, since this molecule contains a region complementary to the 3' end of 12S rRNA (Cantatore et al., 1988), and therefore it could alter the structure of the ribosomal subunit, preventing the formation of the full ribosome. A decrease in mitochondrial translation has also been observed in *RNASEH1* patient fibroblasts carrying the p.Val142Ile mutation in homozygosity; however, transcript levels were not analyzed in that case (Akman et al., 2016). The mitochondrial topoisomerase IB (TOP1MT) has also been reported to have a role beyond the resolution of replication and transcription stress, as it has been found to regulate mitochondrial translation through protein–protein interaction with at least one mtSSU ribosomal protein, uS22 (Baechler et al., 2019).

As a result of the alterations in mitochondrial transcription and translation, patient fibroblasts showed OxPhos deficiency with lower oxygen consumption that was not related to mtDNA depletion and slower growth compared to controls. Previous studies also reported lower oxygen consumption in *RNASEH1* patient fibroblasts carrying the p.Val142Ile mutation (Reyes et al., 2015; Akman et al., 2016) and a slower cell growth rate (Reyes et al., 2015; Reyes et al., 2018). However, neither *RNASEH1* patient fibroblasts carrying p.Tyr163His and p.Gln86del mutations (Carreno-Gago et al., 2019) nor *Drosophila rnh1* KD (Gonzalez de Cozar et al., 2019) showed any defect on cell growth. This highlights the fact that *RNASEH1* mutations are rare and subsequently, the number of patients with

mutations in this gene is still very low. More comprehensive analyses, including more patient fibroblasts and different mutations, will be needed in order to better establish the role of RNase H1 in mitochondrial transcription and translation and, in particular, the contribution of 7S RNA to these processes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Informed consent for participation in this study was obtained from all investigated subjects in agreement with the Declaration of Helsinki, and the study was approved by the ethical committees of the centers where biological samples were obtained and the Ethical Committee of the Fondazione IRCCS Istituto Neurologico 'Carlo Besta,' Milan, Italy.

AUTHOR CONTRIBUTIONS

AR conceived the study and designed the experiments, immortalized the skin fibroblasts, performed most of the experiments, interpreted the results, and wrote the manuscript. JR performed some of the experiments. KT critically reviewed the manuscript. MZ provided the funding for the study and critically reviewed the manuscript. All authors read and approved the submitted version of the manuscript.

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BRIEF RESEARCH REPORT
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Novel *MT-ND* Gene Variants Causing Adult-Onset Mitochondrial Disease and Isolated Complex I Deficiency

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Mitochondrial complex I deficiency is associated with a diverse range of clinical phenotypes and can arise due to either mitochondrial DNA (mtDNA) or nuclear gene defects. We investigated two adult patients who exhibited non-syndromic neurological features and evidence of isolated mitochondrial complex I deficiency in skeletal muscle biopsies. The first presented with indolent myopathy, progressive since age 17, while the second developed deafness around age 20 and other relapsing-remitting neurological symptoms since. A novel, likely *de novo,* frameshift variant in *MT-ND6* (m.14512_14513del) and a novel maternally-inherited transversion mutation in *MT-ND1* were identified, respectively. Skewed tissue segregation of mutant heteroplasmy level was observed; the mutant heteroplasmy levels of both variants were greater than 70% in muscle homogenate, however, in blood the *MT-ND6* variant was undetectable while the mutant heteroplasmy level of the *MT-ND1* variant was low (12%). Assessment of complex I assembly by Blue-Native PAGE demonstrated a decrease in fully assembled complex I in the muscle of both cases. SDS-PAGE and immunoblotting showed decreased levels of mtDNA-encoded ND1 and several nuclear encoded complex I subunits in both cases, consistent with functional pathogenic consequences of the identified variants. Pathogenicity of the m.14512_14513del was further corroborated by single-fiber segregation studies.

Keywords: mitochondrial DNA, muscle biopsy, myopathy, deafness, tissue segregation

INTRODUCTION

Mitochondrial NADH:ubiquinone oxidoreductase (Complex I) is the first and largest (~1 MDa) complex of the mitochondrial respiratory chain involved in the oxidative phosphorylation (OXPHOS) pathway and generation of ATP. It comprises 45 structural subunits of which seven are encoded by mitochondrial DNA (mtDNA), the remaining subunits being encoded by the

nuclear genome as are the ~20 ancillary proteins required for assembly and biogenesis (Formosa et al., 2018). As such, genetic defects in both mitochondrial and nuclear DNA can result in isolated complex I deficiency.

Complex I deficiency is the most common biochemical defect associated with mitochondrial disease (Alston et al., 2017). Identical biochemical defects are associated with phenotypic heterogeneity, (Kirby et al., 1999; Janssen et al., 2006) ranging from a tissue specific manifestations such as Leber hereditary optic neuropathy (LHON), (Man et al., 2002) to devastating, severe phenotypes including Leigh syndrome, (Distelmaier et al., 2009) mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome, multi-system disease, (Alston et al., 2010) and hypetrophic cardiomyopathy and severe lactic acidosis (Distelmaier et al., 2009). Pathogenic variants have been identified in all seven mtDNA-encoded subunits of complex I; however, there is no clear genotype-phenotype correlation (Distelmaier et al., 2009; Hoefs et al., 2012). While incomplete penetrance is frequently observed in the homoplasmic variants associated with LHON, (Man et al., 2002) the heteroplasmy levels in other pathogenic variants such as m.13513G > A and m.13094T > C in *MT-ND5,* both reported in Leigh Syndrome and MELAS syndrome, show good correlation with the severity of disease burden (Ng et al., 2018). Conversely, some *de novo* pathogenic variants in the *MT-ND* (Mitochondrially-encoded NADH:ubiquinone oxidoreductase core subunit) genes cause slowly progressive, non-syndromic presentations such as myopathy and exercise intolerance (Gorman et al., 2015).

In this report, we present two adult patients with complex I deficiency manifesting with different clinical pictures, one developing an insidious-onset myopathy while the other presents with deafness in her 20s and subsequent neurological symptoms that follow a relapsing-remitting pattern. Novel variants in the mtDNA-encoded MT-ND6 and MT-ND1 proteins were identified, respectively, and characterized fully to demonstrate causality.

MATERIAL AND METHODS

Case Reports

Patient 1

A 27-year-old man was referred to a neurology service with a 10-year history of exercise intolerance and mild muscle weakness. In addition, the patient also complained of intermittent drooping of his eyelids and double vision. There was no history of myoglobinuria, deafness, optic atrophy, or retinitis pigmentosa. There was no family history of neuromuscular disorder. Clinical examination revealed very mild proximal lower limb weakness with MRC grade 4+/5. The upper limb muscle bulk was reduced, and subtle scapular winging, and an excessive lumbar lordosis were apparent. The rest of the neurological examination was normal. Routine laboratory investigations were normal except for an elevated serum creatine kinase (CK) (1,212 IU/L). He underwent electromyography (EMG) study which showed polyphasic myopathic units in most muscles sampled. No myotonia or abnormal decrement was evident. He was found to have dipstick proteinuria, and a 24-h urine collection confirmed the presence of microalbuminuria. The following investigations were either negative or normal: serum lactate level, anti-acetylcholine receptor and anti-muscle specific kinase autoantibodies, forearm ischaemic lactate test, serum alpha-glucosidase levels, cardiac investigations (including ambulatory electrocardiogram (ECG) and echocardiogram), renal ultrasound scan, magnetic resonance imaging (MRI) of the brain, and MRI of the upper and lower limb muscles. He had a muscle biopsy at the age of 28 years.

Patient 2

This patient presented with painless, sequential visual loss over four months during pregnancy at the age of 35 years. Her visual acuity at the nadir was documented to be 6/60 bilaterally with the presence of relative afferent pupillary defect in one eye. Her medical history included endometriosis, gestational diabetes and hearing impairment since her late 20s. Both her mother and maternal grandmother developed hearing impairment in their 40s. Retrobulbar optic neuritis was initially suspected, however, her MRI head (including angiography) did not identify any acute structural changes and the visual evoked potentials (VEP) were normal. Her vision gradually improved over several months. Three years later, she developed a gradual-onset, severe headache. MRI head showed several subcortical T2 hyperintensities. The possibility of raised intracranial pressure was excluded with normal cerebrospinal fluid (CSF) opening pressure. CSF constituents were normal, and CSF-restricted oligoclonal bands were not detected. Her headache settled a week later. At age 44 years, she presented with left arm weakness. A CT head scan was normal and the weakness improved spontaneously a week later. Three months later, she re-presented with vertigo, poor balance, sensory disturbances on the left hand, and fatigue. A repeat MRI head showed an increase in subcortical and periventricular white matter lesions with sparing of the corpus callosum. However, repeat CSF studies and VEP remained unremarkable. Her resting serum lactate was 1.2 mmol/L (normal < 2.2 mmol/L). At this point, mitochondrial disease was considered, and a muscle biopsy was performed. In the last clinical review at the age of 46, she developed diabetes mellitus and complained of unsteadiness and fatigue. She had a mild dysarthric speech and reduced muscle strength in the hip flexion (MRC grade 4+/5). Her recent cardiac investigations were normal.

Histochemical and Quadruple Immunohistochemistry (IHC) Studies of Diagnostic Muscle Biopsies

Standard histological (modified Gomori trichrome) and histochemical (individual cytochrome c oxidase (COX), succinate dehydrogenase (SDH), and sequential COX-SDH) analyses of skeletal muscle biopsies were performed on fresh-frozen skeletal muscle sections (10 μm) as previously described (Old and Johnson, 1989). Quadruple OXPHOS immunofluorescence

was undertaken on transversely-orientated frozen muscle sections (10 μm) according to a previously validated protocol to establish evidence of complex I or complex IV deficiency (Rocha et al., 2015).

Molecular Genetic Analyses

Total DNA was extracted from available tissues including sketelal muscle, blood, buccal epithelia, and urinary sediments. In both patients, muscle mtDNA rearrangements were investigated using several long-range PCR strategies prior to sequencing of the entire mitochondrial genome as described elsewhere (Krishnan et al., 2007; Zierz et al., 2019). Analytical sensitivity for single nucleotide variants present at ≥5% heteroplasmy is ≥95% (95% confidence intervals).

Assessment of mtDNA Mutation Load by Quantitative Pyrosequencing

Mutation loads of m.14512_14513del *MT-ND6* and m.3761C > A *MT-ND1* variants were determined in homogenate tissue by quantitative pyrosequencing; quantification of the heteroplasmy level of each variant was achieved using Pyromark Q24 software (Grady et al., 2018). For Patient 1 (m.14512_14513del mutation), we also determined the mutation loads in individual, laser-microdissected muscle fibers for two groups: COX-positive reacting fibers and COX-positive, ragged-red fibers showing marked subsarcolemmal mitochondrial accumulation.

BN–PAGE and Western Blot Analysis of Patient Muscle

Blue-Native Polyacrylamide Gel Electrophoresis (BN–PAGE) was performed using mitochondrial proteins isolated from skeletal muscle samples (25 mg of tissue) as described previously (Thompson et al., 2016) using antibodies against COXI (abcam ab14705), SDHA (abcam ab14715), VDAC1 (abcam ab14734), UQCRC2 (abcam ab14745), NDUFB8 (abcam ab110242), and ATP5A (abcam ab14748); all primary antibodies were used at a dilution of 1 in 1,000. Total protein extraction from human muscle for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting was carried out as described (Olahova et al., 2015a) using the following commercially available antibodies: NDUFB8 (abcam ab110242), NDUFV1 (Proteintech 11238-1-AP), NDUFS3 (abcam ab110246), SDHA (abcam ab14715), UQCRC2 (abcam ab14745), COXI (abcam ab14705), ATP5A (abcam ab14748), and VDAC1 (abcam ab14734), which served as a loading control. The antibody against ND1 was a kind gift from Dr Anne Lombès.

RESULTS

Histochemical and Quadruple Immunohistochemistry (IHC) Studies of Muscle Biopsy

In Patient 1, the oxidative enzyme reactions (SDH and COX) revealed numerous fibers with increased activity at the fiber periphery, confirmed by modified Gomori trichrome staining, which showed subsarcolemmal accumulations typical of "ragged-red" changes affecting >30% of all fibers (**Figure 1A**). Quadruple OXPHOS IHC assay detected >75% of fibers showing a complete loss of NDUFB8 immunoreactivity, again associated with preserved COX-I immunoreactivity (**Figure 1B**). Many of these fibers showed high porin levels, reflecting enhanced mitochondrial numbers fibers showing subsarcolemmal mitochondrial accumulation. A histopathological assessment of the muscle biopsy from Patient 2 failed to detect significant mitochondrial changes; a single COX-deficient fiber was noted following sequential COX-SDH histochemistry, likely as a result of somatic mtDNA mutation (**Figure 1D**). However, the IHC mitochondrial respiratory chain profile shows a loss of NDUFB8 immunoreactivity, associated with preserved COX-I immunoreactivity, for >60% of all fibers and consistent with isolated complex I deficiency (**Figure 1E**).

Identification of Novel Pathogenic *MT-ND6* and *MT-ND1* Mutations

Long-range PCR assays were used to exclude mtDNA rearrangements in the muscle from both patients, prompting the sequencing of the entire mitochondrial genome which identified candidate pathogenic variants in genes encoding structural subunits of mitochondrial complex I. We determined the mtDNA sequence in muscle from both patients identifying novel, candidate pathogenic *MTND* mutations. Patient 1 was shown to harbor a novel m.14512_14513del, p.(Met54Serfs*7) variant, also predicting the premature truncation of the relevant complex I protein subunit (ND6). Quantitative pyrosequencing showed that the m.14512_14513del variant was present at high levels of heteroplasmy in skeletal muscle (76%); at low levels (10%) in a urinary sediment-derived DNA sample but undetectable in blood and buccal epithelial-derived DNA samples. Concurrent studies in his mother's blood, urine and buccal epithelial DNA samples failed to detect the m.14512_14513del variant, strongly implicating a *de novo* mutation event.

Patient 2 harbored a novel m.3761C > A transversion (predicting p.(Ser152*) and the premature truncation of the ND1 protein) which was present at high levels of heteroplasmy in skeletal muscle (80%), and lower levels in other tissues including urinary sediment (46%), buccal epithelia (35%), and blood (12%). Testing of the samples from the patients clinically-unaffected mother confirmed maternal transmission of the m.3761C > A variant, with lower levels of mtDNA heteroplasmy detected in urinary sediments (38%) and blood (5%).

Neither the m.14512_14513del nor m.3761C > A variants were reported within online databases of mtDNA variation, nor did we detect these within our own in-house database of >1,950 human mtDNA sequences. Using quantitative pyrosequencing, we detected significantly higher levels of the m.14512_14513del variant in COX-positive ragged-red fibers [90.9 ± 0.74% ($n = 21$)] than in COX-positive non-ragged-red fibers [31.7 ± 9.6% ($n = 17$)] ($p < 0.0001$, two-tailed Student's t test), confirming segregation of the m.14512_14513del genotype with a histopathological abnormality in Patient 1 (**Figure 1C**).

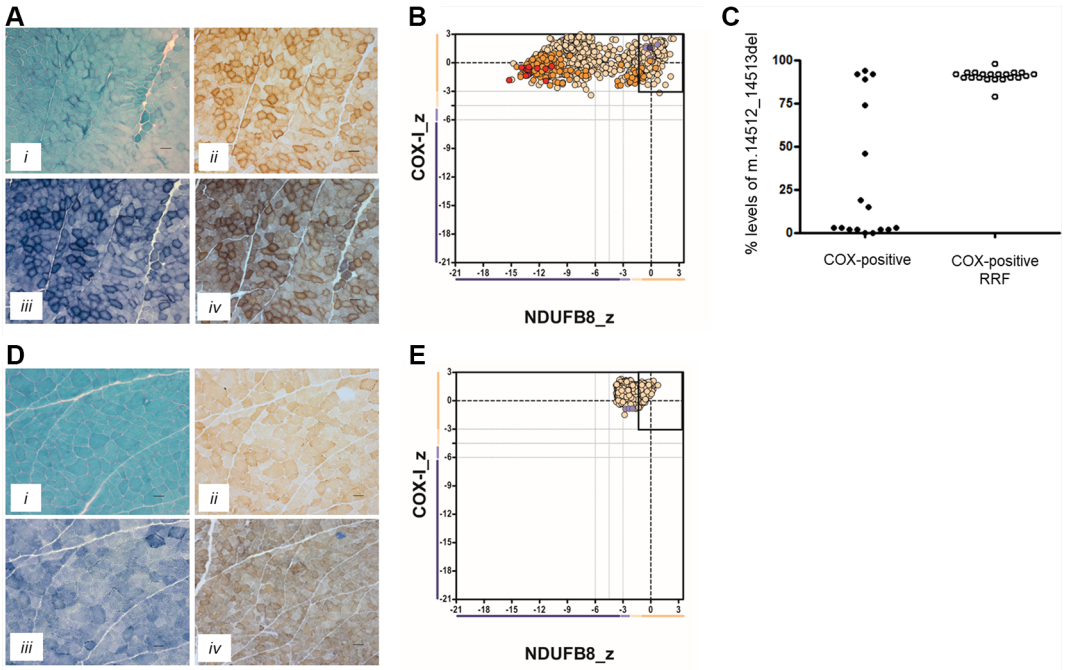

FIGURE 1 | Muscle biopsy findings in two patients with isolated complex I deficiency. **(A)** Histopathological analysis of skeletal muscle sections from Patient 1 showing modified Gomori trichrome staining (i), cytochrome *c* oxidase (COX) histochemistry (ii), succinate dehydrogenase (SDH) histochemistry (iii), and sequential COX-SDH histochemistry (iv), highlighting the presence of COX-positive ragged-red fibers (RRF) showing mitochondrial accumulation. Scale bars = 100 μm. **(B)** Respiratory chain profile following quadruple oxidative phosphorylation immunofluorescence analysis of cryosectioned muscle from Patient 1, confirming the presence of numerous fibers lacking complex I (NDUFB8) protein. Each dot represents the measurement from an individual muscle fiber, color coded according to its mitochondrial mass (blue-low, normal-beige, high-orange, very high-red). Gray dashed lines indicate SD limits for the classification of fibers. Lines next to x- and y-axes represent the levels (SDs from the average of control fibers after normalization to porin/VDAC1 levels; _z = Z-score, see Methods section of Rocha et al. (2015) for full description of statistics (Rocha et al., 2015) of NDUFB8 and COX1, respectively (beige = normal (>−3), light beige = intermediate positive (−3 to −4.5), light purple = intermediate negative (−4.5 to −6), purple = deficient (<−6). Bold dotted lines indicate the mean expression level observed in respiratory normal fibers. **(C)** Single fiber PCR analysis shows significant segregation of higher m.14512_14513del, p.(Met54Serfs*7) *MTND6* mutation load within COX-positive RRF than COX-positive fibers not showing obvious subsarcolemmal mitochondrial accumulation. **(D)** Histopathological analysis of skeletal muscle sections from Patient 2 showing modified Gomori trichrome staining (i), COX histochemistry (ii), SDH histochemistry (iii), and sequential COX-SDH histochemistry (iv). COX-SDH histochemistry identified a single, COX-deficient fiber which is likely the result of somatic (age-related) mtDNA mutation. **(E)** Respiratory chain profile following quadruple oxidative phosphorylation immunofluorescence analysis of cryosectioned muscle from Patient 2, again confirming the presence of fibers lacking complex I (NDUFB8) protein.

Novel *MTND* Gene Mutations are Associated With Impaired Complex I Assembly and Loss of Immunoreactive Complex I Subunits

To assess the ability of complex I to assemble in the inner mitochondrial membrane, a one-dimensional BN-PAGE was performed with muscle samples isolated from both patients and two age-matched healthy controls. A band representing fully assembled complex I (980 kDa) was detectable in both controls, but Patient 2 showed very weak signal and no signal was detected in Patient 1 (**Figure 2A**). However, the assembly of all other OXPHOS complexes were unchanged between patients and controls, confirming an isolated complex I defect in skeletal muscle from both patients. SDS-PAGE and immunoblotting was performed in skeletal muscle samples from each patient and showed a decrease in the steady-state protein levels of all complex I subunits tested (ND1, NDUFV1, NDUFS3, and NDUFB8) (**Figure 2B**), whereas subunits of complexes II-V (SDHA, UQCRC2, COXI, and ATP5A, respectively) were unchanged between patients and controls.

DISCUSSION

Mitochondrial disease presentations that do not exhibit classical syndromic clinical phenotypes can be difficult to diagnose. Both patients described in this report have undergone multiple investigations over several years, with the eventual diagnosis being underpinned by clear evidence of mitochondrial complex I deficiency in a diagnostic muscle biopsy.

A heteroplasmic m.14512_14513del *MT-ND6* variant was identified in Patient 1 who presented with exercise intolerance, mild myopathy, and hyperCKaemia. This novel mtDNA variant has likely arisen *de novo* as it is not detectable in several mitotic tissues of his clinically-unaffected mother although we

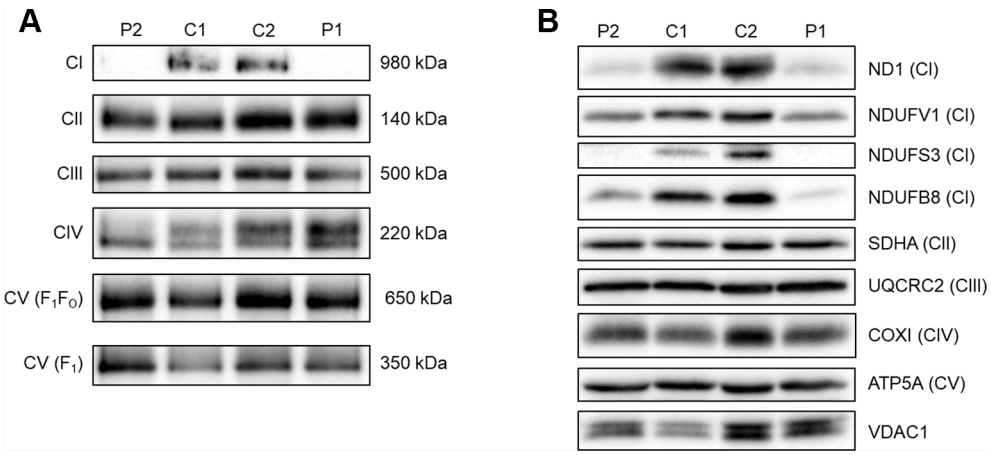

FIGURE 2 | Assessing OXPHOS complex assembly and protein levels in patient muscle. **(A)** BN-PAGE of muscle samples from two age-matched controls (C1 and C2) and Patients (P1 and P2). Antibodies used were anti-NDUFB8 for complex I (CI), anti-SDHA for complex II (CII), anti-UQCRC2 for complex III (CIII), anti-COX1 for complex IV (CIV), and anti-ATP5A for complex V (CV). Complex II was used as a loading control. Blots are representative of two technical repeats. **(B)** SDS-PAGE and immunoblotting analysis of muscle samples from two age-matched controls (C1 and C2) and Patients (P1 and P2). Antibodies against ND1, NDUFV1, NDUFS3, NDUFB8 were used as markers of complex I; SDHA for complex II; UQCRC2 for complex III; COXI for complex IV; ATP5A for complex V and VDAC1 as a mitochondrial mass marker. Blots are representative of two independent experiments.

demonstrate skewed tissue segregation of this variant in the patient. A novel, m.3761C > A; p.(Ser152*) *MT-ND1* variant is the likely cause of Patient 2's personal and maternal history of deafness and her relapsing-remitting neurological presentations. Both mtDNA variants clearly result in the isolated complex I deficiency, as identified with the IHC findings of decreased expression of complex I subunit (NDUFB8), BN-PAGE showing perturbed assembly of the complex I holoenzyme and immunobloting showing a decrease in the steady-state protein of complex I subunits. Moreover, the pathogenicity of the m.14512_14513del variant is further supported by the single-fiber segregation analysis confirming higher levels of the variant are present in ragged-red fibers.

The maternal inheritance of the m.3761C > A in *MT-ND1* variant and the observed decrease in ND1 protein levels in skeletal muscle samples from Patient 2 strongly indicate pathogenicity of this variant. Skeletal muscle from Patient 1, harboring the m.14512_14513del variant in *MT-ND6,* also had decreased ND1 protein levels as well as decreased levels of several nuclear encoded complex I subunits (NDUFV1, NDUFS3, and NDUFB8). This is consistent with decreased ND6 levels leading to a complex I assembly defect and subsequent degradation of many complex I subunits and is similar to what is seen in Patient 2 due to the loss of ND1. ND6 could not be directly assessed by immunoblotting due to the lack of availability of an antibody to ND6. In both patients, NDUFV1 is the least affected subunit. This is likely due to NDUFV1 being part of the N module of complex I which is assembled separately to the Q/ND1 and ND2 modules that ND1 and ND6 are part of respectively (Mimaki et al., 2012; Formosa et al., 2018).

Progressive exercise intolerance and myopathy identified in Patient 1 are infrequent clinical findings associated with pathogenic *MT-ND* variants (Musumeci et al., 2000; Gorman et al., 2015). The putative link between the mitochondrial complex I defect and glomerular dysfunction is highly conceivable given no other cause has been identified, and renal involvement is increasingly recognized as part of the multisystem manifestation in mitochondrial diseases (O'Toole, 2014).

A retrospective review of the history of bilateral visual impairment in Patient 2 raised the suspicion of LHON. However, the details of initial retinal examination were not available and it is not known whether characteristic acute findings of LHON such as disc hyperemia, oedema of the peripapillary retinal nerve fiber layer andretinal telangiectasia were evident . The relapsing-remitting nature of subsequent neurological presentations mimicked multiple sclerosis but the radiological, VEP and CSF findings were not supportive of the diagnosis. While there are some uncertainties on establishing the causal link between visual disturbance, white matter changes and the novel *MT-ND1* variant, the presence of sensorineural hearing loss, the development of diabetes mellitus, myopathy and maternal history of deafness are typical findings in primary mtDNA disease.

Next generation sequencing (NGS) technology has been increasingly integrated in the diagnostic pathway of a wide range of genetic disorders including mitochondrial disease (Thompson et al., 2019). One of the proposed advantages is that NGS could mitigate the need and the risk of invasive, diagnostic muscle biopsies, especially in the paediatric population. However, primary mtDNA mutations account for two-third of the diagnosis of adult cases, (Gorman et al., 2015) and the skewed segregation of some mtDNA mutations between non-invasive tissues (e.g., blood) and post-mitotic tissues (e.g., muscle) could pose a significant challenge on the interpretation of any variant of unknown significance detected at low heteroplasmy levels in blood-derived DNA. Moreover, the expression of some mtDNA mutations is tissue specific and testing the blood-derived DNA alone could yield a false negative finding, such as in Patient 1 and other reported cases

(Andreu et al., 1999; Musumeci et al., 2000). Given these diagnostic caveats listed above, muscle biopsy would retain its crucial role in establishing the diagnosis of primary mtDNA disease, (Hardy et al., 2016; Zierz et al., 2019) especially in cases without apparent maternal history and *de novo* variants.

In conclusion, isolated complex I deficiency is associated with an increasingly diverse phenotypic expression of mitochondrial disease. We highlight two novel mutations causing isolated complex I deficiency and diverse clinical features. Our findings also serve to highlight the importance of diagnostic muscle biopsy in proving the pathogenicity of novel mtDNA variants, particularly in cases with non-syndromic presentations.

DATA AVAILABILITY STATEMENT

Both novel mtDNA variants have been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), with the following Accession Numbers: m.14512_14513del (SCV001132040); m.3761C>A (SCV001132041).

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RT has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: YN, KT, and RT. Acquisition, analysis, or interpretation of data: all authors. Drafting of the manuscript: YN, KT, JL and RT. Critical revision of the manuscript for important intellectual content: all authors.

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REVIEW
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ncRNAs: New Players in Mitochondrial Health and Disease?

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The regulation of mitochondrial proteome is unique in that its components have origins in both mitochondria and nucleus. With the development of OMICS technologies, emerging evidence indicates an interaction between mitochondria and nucleus based not only on the proteins but also on the non-coding RNAs (ncRNAs). It is now accepted that large parts of the non-coding genome are transcribed into various ncRNA species. Although their characterization has been a hot topic in recent years, the function of the majority remains unknown. Recently, ncRNA species microRNA (miRNA) and long-non coding RNAs (lncRNA) have been gaining attention as direct or indirect modulators of the mitochondrial proteome homeostasis. These ncRNA can impact mitochondria indirectly by affecting transcripts encoding for mitochondrial proteins in the cytoplasm. Furthermore, reports of mitochondria-localized miRNAs, termed mitomiRs, and lncRNAs directly regulating mitochondrial gene expression suggest the import of RNA to mitochondria, but also transcription from the mitochondrial genome. Interestingly, ncRNAs have been also shown to hide small open reading frames (sORFs) encoding for small functional peptides termed micropeptides, with several examples reported with a role in mitochondria. In this review, we provide a literature overview on ncRNAs and micropeptides found to be associated with mitochondrial biology in the context of both health and disease. Although reported, small study overlap and rare replications by other groups make the presence, transport, and role of ncRNA in mitochondria an attractive, but still challenging subject. Finally, we touch the topic of their potential as prognosis markers and therapeutic targets.

Keywords: mitochondria, ncRNA, lncRNA, miRNA, mtDNA, micropeptide

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BACKGROUND

Molecular biology has historically described RNA as an intermediate between genetic information stored in DNA and protein synthesis. The estimated number of protein-coding genes is around 20,000 (Pertea et al., 2018). Classical approaches to classify RNAs with protein-coding potential—the messenger RNAs (*mRNAs*)—were typically based on the existence of open reading frame (ORF) longer than 300 nucleotides (nt), conservation, and/or functional domains (Dinger et al., 2008). Nevertheless, as protein-coding regions encompass only ∼2% of the human genome, the rest has been considered as "dark matter". Detected RNAs not translated into proteins were named non-

coding RNAs (*ncRNA*) and initially regarded as a transcriptional noise or the byproducts of genetic information flow from DNA to protein. Nevertheless, since the discovery of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), the number and understanding of new and putative functional ncRNAs have expanded. Moreover, the boundaries between the coding and non-coding RNAs have become more blurry. Evidence is emerging that some RNAs, initially classified as non-coding, hide small ORFs (sORFs, < 300 nt) encoding for small functional peptides- micropeptides. Currently, we know dozen of different ncRNAs, which can be can be classified as housekeeping or regulatory ncRNS, according to Szymanski et al. (2003).

Housekeeping ncRNAs are constitutively expressed and mostly well functionally characterized classes of rRNAs, tRNAs, small nuclear RNAs (snRNAs), small-nucleolar RNAs (snoRNAs), Ribonuclease P RNA (*RNase P*), Ribonuclease MRP RNA (*MRP RNase*, *RNRP*), and Telomerase RNA component (*TERC*). *rRNAs* are the most abundant class of RNAs in most cells, composing around 80% of cellular transcriptome. They serve as the essential binding site for ribosomal proteins within the assembled ribosome and contribute to the binding of extra-ribosomal factors and ribosome-associated proteins, resulting in the protein translation machinery (Noller et al., 2017; Simsek et al., 2017). *tRNAs* provide the interface between nucleic acids and proteins during translation by carrying an amino acid on its 3′ end and reading the mRNA by base-pairing induced by the ribosome, which uniquely determines the position of amino acids in proteins (Schimmel, 2018). *snRNAs* participate in the assembly and function of canonical spliceosomes (Wang and Burge, 2008). *snoRNAs* are localized to the nucleolus and guide the methylation and pseudouridylation of rRNAs, tRNAs, and snRNAs (Maxwell and Fournier, 1995). *RNase P* has a role in precursor-tRNA cleavage, *RMRP* in precursor-rRNA cleavage, and *TERC* in telomere synthesis (discussed later).

Regulatory ncRNAs are mostly produced in a cell- or tissue-specific fashion during certain stages of cell differentiation or organism development, or as a response to changes in the environment. They are still poorly understood and a very heterogeneous group that can act in different ways, from gene expression regulation to modulation of protein and RNA distribution within cells (Szymanski et al., 2003). They are divided based on their length into short (<200 nt) and long (>200 nt, lncRNAs) RNAs. *Short ncRNAs* consist of microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-associated RNAs (piRNAs). *miRNAs* are endogenous, single-stranded, 19-23 nt in length RNAs that can bind to a target mRNA with a complementary sequence to induce its cleavage, degradation, or interfere with translation. Similar in size, *siRNAs* are exogenous RNAs that undergo processing and function in post-transcriptional gene silencing (Carthew and Sontheimer, 2009). *piRNAs* are single stranded, 26-31 nucleotides long RNAs that form complexes with the piwi family of proteins. These complexes have a role in RNA and epigenetic silencing of transposons (Siomi et al., 2011). Longer than 200 nt, *lncRNAs* represent the most abundant, yet least understood class of RNAs, with an average length ~ 1000 nt (Ulitsky and Bartel, 2013). They share some features typical for mRNAs, such as transcription by the RNA-polymerase II (Pol II), 5′end cap, 3′end polyadenylation and presence of alternative splicing isoforms (Kopp and Mendell, 2018). However, compared to the mRNAs, they exhibit lower expression levels, more tissue-specific expression, and poor sequence conservation (Derrien et al., 2012; Djebali et al., 2012; Kopp and Mendell, 2018; Fazal et al., 2019). Although often considered as nucleus-enriched, lncRNAs exhibit variety of subcellular localization, which often helps to determine their biological function (Carlevaro-Fita and Johnson, 2019). Finally, *circular RNAs (circRNAs)* are a special class of RNAs with the 3′ and 5′ ends covalently linked, generally formed by alternative splicing of pre-mRNA (Salzman et al., 2012). They have been proposed to act as miRNAs sponges or even as templates for protein synthesis (Ragan et al., 2019).

Interest in the ncRNAs has been stimulated by the development of high-throughput OMICS technologies. Genome-, transcriptome-, translatome- and proteome-wide measurements by the whole genome sequencing (WGS), RNA-sequencing (RNA-seq), ribosome profiling (Ribo-seq) and mass spectrometry (MS), respectively. In combination, these methods offer the possibility of a systematic analysis of different stages of gene expression (Ori et al., 2015; Wang et al., 2019). RNA-seq data have shown that up to 85% of the genome is transcribed and identified, among others, novel transcript isoforms, transcripts arising from intergenic regions, overlapping transcripts, and transcribed pseudogenes (Consortium, 2012; Djebali et al., 2012; Hangauer et al., 2013). Ribo-seq has shown widespread and pervasive translation on cytosolic RNAs, with surprisingly ~40% lncRNAs being engaged with the ribosome (Ingolia et al., 2009; Kearse and Wilusz, 2017). Reported ribosomal occupancy of RNAs indicated on the one side presence of different protein isoforms and regulatory upstream open reading frames ORFs (uORFs) from the mRNAs, and on the other, more exciting side, new ways of translational regulation and possible micropeptide production from lncRNAs (Morris and Geballe, 2000; Andrews and Rothnagel, 2014). It must be taken into account that the ribosomal occupancy of transcripts need not automatically lead to the production of stable, functional polypeptides, and that further evidence is needed in order to reclassify transcripts as indeed protein-coding (Guttman et al., 2013). MS has proven as a useful tool to inspect the postulated translational event, with developing proteogenomics approaches confirming the presence of some peptides encoded by previously non-coding regions (Slavoff et al., 2013; Fields et al., 2015; Wang et al., 2019). However, in order to omit the possibility of false-positive findings from MS, further functional studies on revealed peptides are needed, and these studies remain sparse.

The complexity of gene expression has in most cases been published on the levels of detection and its functional relevance remains elusive. Still, it has revealed that the distinguishment between mRNAs and ncRNAs is more challenging than initially assumed and that automatic gene annotation systems, although straightforward across large datasets, can sometimes be misleading. Traditional arbitrary ORF cutoff can lead to misclassification of some ncRNAs as mRNAs as they can by

chance contain putative ORFs. This is especially true for lncRNAs, such as functionally characterized *H19*, *Xist*, *Mirg*, *Gtl2*, and *KcnqOT1* (Prasanth and Spector, 2007). Some ncRNAs have evolved from the protein-coding genes, and so will keep certain features and homologies to mRNAs (Duret et al., 2006). For example, *Xist* has evolved into the ncRNA through the process of pseudogenization, during which proto-*Xist* had lost its protein-coding function and its flanking genes had turned into pseudogenes (Duret et al., 2006). On the contrary, micropeptide-encoding regions may be incorrectly classified as non-coding due to their size (Yeasmin et al., 2018). Next, the absence of ORF conservation does not guarantee an absence of protein-coding potential. Indeed, the majority of micropeptide-encoding regions are not conserved (Ji et al., 2015), suggesting their role in encoding evolutionary young proteins (Ruiz-Orera et al., 2014). Finally, some genes are bifunctional, and its products function independently both as RNAs and proteins. The first report of such a gene was the human Steroid Receptor Activator (SRA) (Lanz et al., 1999; Chooniedass-Kothari et al., 2004). SRA was initially characterized as ncRNA which co-activates steroid hormone receptors (Lanz et al., 1999) and later was revealed to also encode a functional protein (SRAP), which seems to modulate *SRA* activity (Chooniedass-Kothari et al., 2004).

Emerging discoveries in the ncRNA field have also raised the possibility that some ncRNAs affect mitochondrial biology. Mitochondria are crucial organelles for the integration of several key metabolic processes and the primary powerhouses in the cell (Spinelli and Haigis, 2018). The control of mitochondrial protein homeostasis is unique in that its components have origins in both mitochondria and nucleus (**Figure 1**). Mitochondria contain their own circular genome (mtDNA). In humans, it is 16,569 bp in length and contains 37 genes- encoding for 2 rRNAs, 22 tRNAs, and 13 proteins of the oxidative phosphorylation (OXPHOS) system (Anderson et al., 1981) (**Figure 2**). The rRNA coding sequences and all but one protein-coding sequences are separated by tRNAs and deprived of introns. The mtDNA is transcribed entirely from both strands, named heavy (H) or light (L). Transcription is initiated from the two H-strand (HSP1/2) and one L-strand promoter, located in the major non-coding region named "control region", resulting in long polycistronic transcripts.

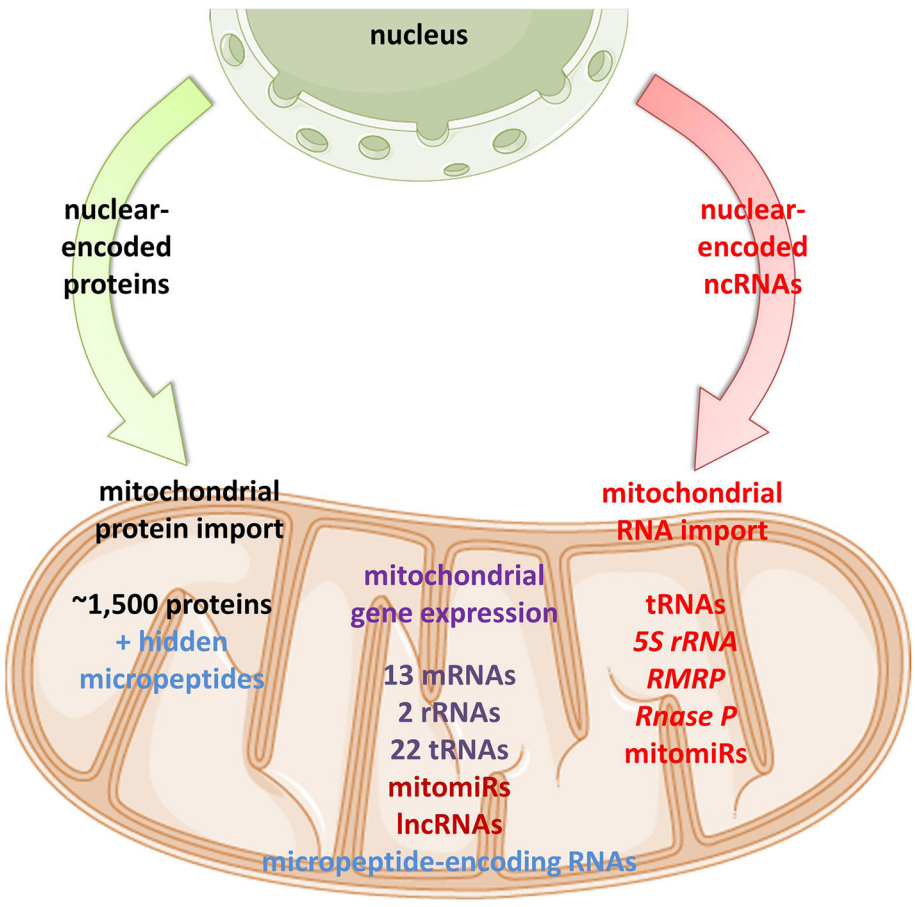

FIGURE 1 | Proposed mitochondrial proteome and transcriptome. Mitochondrial homeostasis is depending on its own gene expression, but also on the import of nuclear-encoded proteins from the cytoplasm. In recent years, emerging evidence suggests import, but also mtDNA-transcription of different classes of ncRNAs in mitochondria.

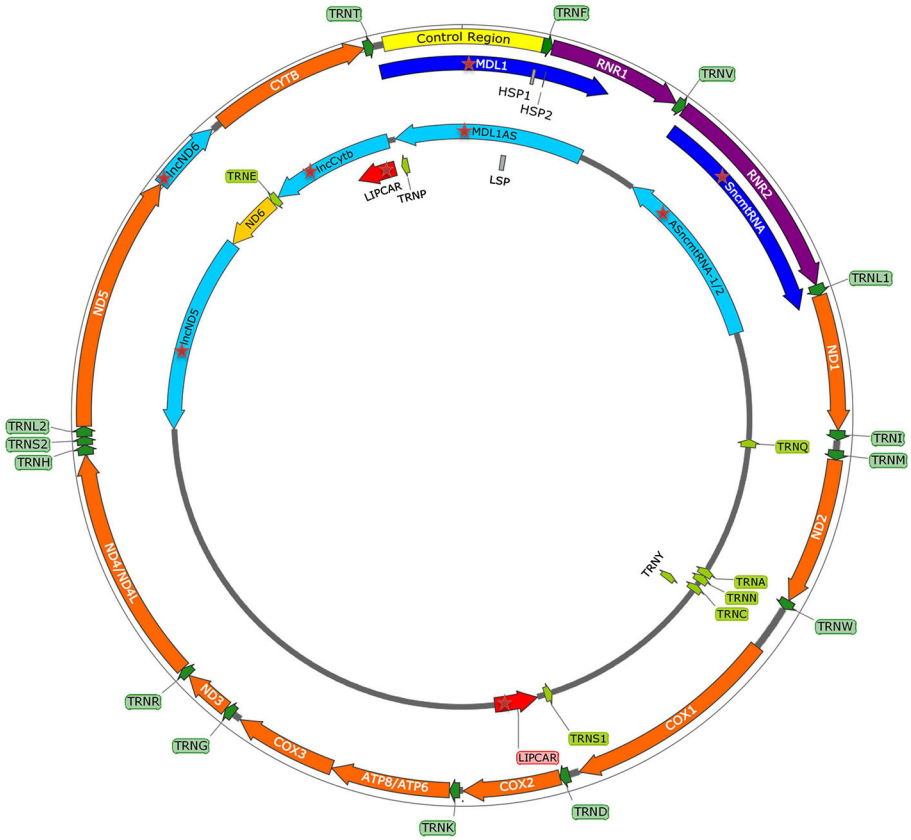

FIGURE 2 | mtDNA map showing heavy (outside circle) and light (inside circle) strand and within them the control region with promoters (HSP1, HSP2, LSP), and genes encoding for 13 mitochondrial proteins, 2 rRNAs, 22 tRNAs, and recently discovered mitochondria-encoded lncRNAs (mtlncRNAs) (highlighted with red star).

LSP controls the transcription of eight tRNAs and the ND6 gene. HSP1 transcription produces a transcript containing $tRNA^{Phe}$, $tRNA^{Val}$, and the rRNAs, while transcription from HSP2 generates a transcript that spans almost the entire genome (Montoya et al., 1983; Chang and Clayton, 1984). The main proteins controlling the process are the RNA polymerase (POLRMT), two transcription factors (TFAM and TF2BM), transcription elongation factor (TEFM), and transcription termination factor (mTERF1) (Barshad et al., 2018). The "tRNA punctuation" model (Ojala et al., 1981) proposes that individual mRNA, rRNAs, and tRNAs are released from the polycistronic transcripts by the cleavage of tRNAs, which is in humans performed by endonucleases RNase P complex and ELAC2 (Holzmann et al., 2008; Brzezniak et al., 2011). After release, the rRNAs undergo chemical nucleotide modifications before becoming part of mitoribosome, the tRNAs undergo chemical nucleotide modifications, CCA addition at the 3′-end, deadenylation and finally aminoacylation, and the mRNAs get 3′ end polyadenylated (D'Souza and Minczuk, 2018). The half-life of mitochondrial transcripts and the decay of RNA intermediates are mediated by a complex of polynucleotide phosphorylase (PNPase) and SUV3 (Borowski et al., 2013). Finally, the mature mRNAs, tRNAs, and the assembled mitoribosome come together in the translation apparatus, for the synthesis of 13 subunits of OXPHOS system.

As mtDNA's coding capacity is very limited, mitochondria are heavily dependent on the import of about 1,500 nuclear-encoded proteins. Besides, there have been indications that mitochondrial homeostasis is maintained not just through proteins, but also ncRNAs (**Figure 1**). The presence of housekeeping mitochondrial nuclear-encoded ncRNAs has been postulated for decades. These ncRNAs include tRNAs ($tRNA^{Leu}_{UAA}$, $tRNA^{Gln}_{UUG}$, $tRNA^{Gln}_{CUG}$, $tRNA^{Lys}_{CUU}$), 5S rRNA, *RMRP*, and *RNase P* (Chang and Clayton, 1987a; Chang and Clayton, 1987b; Kiss et al., 1992; Yoshionari et al., 1994; Magalhaes et al., 1998; Puranam and Attardi, 2001; Holzmann et al., 2008). A systematic analysis of mitochondrial transcriptome further strengthened these claims. RNA-seq from 143B cells mitochondria and mitoplasts revealed the presence of several nuclear- and mitochondrial-encoded small RNAs and antisense transcripts (Mercer et al., 2011). Soon afterward, Rackham et al. (2011) observed by RNA-seq on HeLa cells that ncRNAs, excluding rRNAs and tRNAs, make up 15% of the human mitochondrial transcriptome, and identified three lncRNAs transcribed from the mtDNA. Follow-up studies have

also reported the presence of ncRNAs encoded by the nuclear DNA, especially miRNAs and lncRNAs, within mitochondria across various cell types and tissues, suggesting that these ncRNAs may play important roles in the mitochondrial homeostasis (Kim et al., 2017b; Jeandard et al., 2019). The summary of the proposed nuclear-encoded ncRNAs is given in **Table 1**.

Although detection of ncRNAs in mitochondria paved the way to more extensive research in this field with several examples of ncRNAs functionally described as directly impacting mitochondrial biology, these transcripts are far from being well characterized. It is important to mention that there are (still) many controversies and debates ongoing about the sole existence of ncRNA in mitochondria. The main obstacle presents the technical challenge of truly separating isolated and uncontaminated mitochondria from other membrane vesicles (endoplasmic reticulum (ER), the Golgi apparatus, the endosomes) they are tightly associated within the cell (Vendramin et al., 2017). Therefore, to assess the purity of mitochondria or mitoplasts, ER or other membrane vesicles should be used instead of cytosol or nucleus, which was not always the case. Mitoplasts—rather than mitochondria—should be subjected to RNase treatment before lysis in order to minimize the risk of contamination. Unfortunately, these control steps have not always been performed systematically, so the published data is to date a complicated topic of many debates (Vendramin et al., 2017). Moreover, implementation of high sensitive NGS techniques such as deep sequencing is likely to detect small amounts of contaminants, leading to data misinterpretation. Finally, as this field is still very fresh, many studies miss independent replicates and functional studies are published by one research group.

Despite these controversies, an increasing body of evidence has connected ncRNAs and their machinery with mitochondrial biology. In this review, we focus on classes of ncRNAs described to be functionally related with and/or localized in mitochondria: the housekeeping ncRNAs, miRNAs, and lncRNAs. We also take up the topic of mitochondrial micropeptides, recently discovered to be encoded within regions initially annotated as non-coding. Overall, we summarize knowledge on ncRNAs in mitochondrial

TABLE 1 | Nuclear-encoded ncRNAs discovered in mitochondria.

RNA	Function in cytosol/nucleus	Proposed function in mitochondria	Evidence for mitochondrial localization	Reference
tRNAs ($tRNA^{Leu}_{UAA}$, $tRNA^{Gln}_{UUG}$, $tRNA^{Gln}_{CUG}$, $tRNA^{Lys}_{CUU}$)	Translation	Translation?	RNA-seq RT-qPCR Enrichment in mitoplasts compared to crude mitochondria	Rubio et al., 2008 Mercer et al., 2011 Gowher et al., 2013
5S rRNA	Component of the cytosolic ribosome	Translation?	RT-qPCR and Northern blot Enrichment in mitoplasts compared to crude mitochondria Import into isolated mitochondria RNA-seq Fluorescence microscopy FISH	Yoshionari et al., 1994 Magalhaes et al., 1998 Entelis et al., 2001 Mercer et al., 2011 Autour et al., 2018 Zelenka et al., 2012
RMRP	5.8S rRNA processing	RNA metabolism?	Enrichment in mitoplasts compared to crude mitochondria RT-qPCR RNA-seq Import into isolated mitochondria, Electron microscopy	Chang and Clayton, 1987a Wang et al., 2010 Mercer et al., 2011 Noh et al., 2016
RNASE P	Component of RNase P	Pre-tRNA processing?	RT-qPCR Enrichment in mitoplasts in comparison to crude mitochondria Import into isolated mitochondria RNA-seq	Bartkiewicz et al., 1989 Puranam and Attardi, 2001 Wang et al., 2010 Mercer et al., 2011
hTERC	Component of telomerase	Processed and transported to cytosol?	RT-qPCR	Cheng et al., 2018
miRNAs and pre-miRNAs	mRNA degradation/ repression of mRNA translation	Repression or activation of translation, repression of transcription	RNA-seq miRNA-microarray Northern blot Enrichment in mitoplasts in comparison to crude mitochondria FISH Immunostaining	Summarized in **Table 3**
SAMMSON	Facilitates p32 targeting to the mitochondria in melanoma cells	?	RT-qPCR FISH	Leucci et al., 2016 Vendramin et al., 2018
SRA	Co-activates steroid hormone receptors	?	Computational screen	Baughman et al., 2009
MALAT1	Transcriptional regulator	Mitochondrial metabolism?	FISH	Zhao et al., 2019

biology and discuss their discovery, biosynthesis, import, and function in the context of both health and disease. Finally, we touch their potential as prognosis markers and therapeutic targets.

HOUSEKEEPING NCRNAS LOCALIZED IN MITOCHONDRIA

Several tRNAs, *5S rRNA*, *RMRP,* and *RNase P* present housekeeping ncRNAs whose mitochondrial localization, transport, and function have been discussed for years. For some of them, their interacting RNA-binding proteins (RBPs) have been proposed and associated with mitochondrial import and function (**Figure 3**, **Table 1**). However, the exact import mechanism across mitochondrial membranes and the function of these ncRNAs remain unclear. It is important to note that reports of these ncRNAs have been sparse and therefore questionable, so more evidence is needed to confirm/deny their presence and role in mitochondria.

Nuclear-encoded *tRNAs* have been observed in mitochondria across many species, as most eukaryotes lack some of the essential tRNAs in their mtDNA and must import them (Tarassov et al., 2007; Schneider, 2011). Even though human mtDNA encodes all the necessary tRNAs, published data indicate that they are able to import some of the cytosolic tRNAs through conserved protein machinery. *In vitro* experiments have shown that the synthetic transcripts of yeast tRNAs could be internalized by the isolated human mitochondria (Kolesnikova et al., 2000; Entelis et al.,

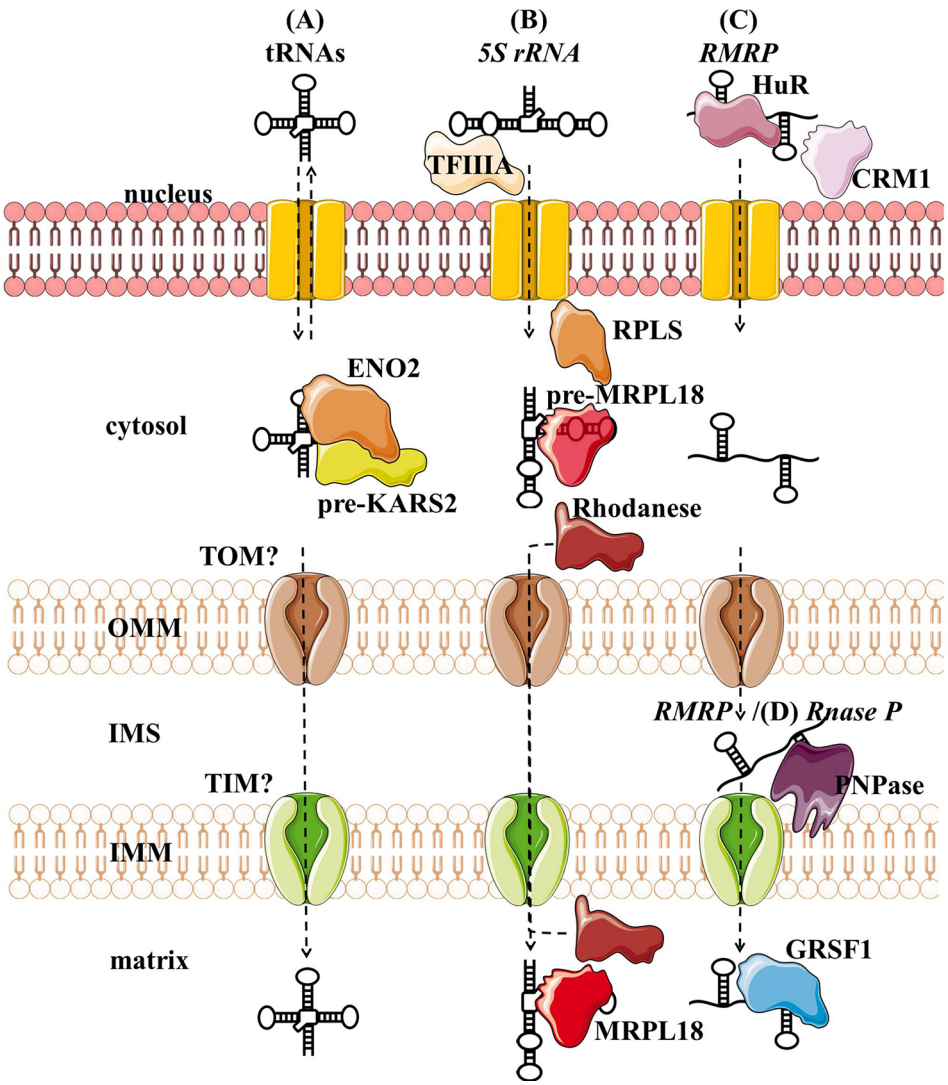

FIGURE 3 | Proposed import mechanisms of tRNAs **(A)**, 5s rRNA **(B)**, and *RMRP* **(C)** into human mitochondria. ncRNAs could be targeted by various nuclear-encoded proteins localized in the nucleus and close or inside the organelle. The mechanism behind translocation across mitochondrial membranes is still unknown, but *RMRP* and *Rnase P* seem to require the PNPase **(D)**.OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane.

2001). Later, nuclear-encoded tRNAs have been detected in mitochondria (Rubio et al., 2008; Mercer et al., 2011), namely $tRNA^{Leu}_{UAA}$, $tRNA^{Gln}_{UUG}$, and $tRNA^{Gln}_{CUG}$. Gowher et al. (2013) successfully targeted yeast $tRNA^{Lys}_{CUU}$ into human mitochondria *in vivo*, suggesting similarities in the tRNA import between the two species (**Figure 3A**). The current proposal by Gowher et al. (2013) is that tRNAs are recruited from the cytosol to the mitochondria with the precursor pre-KARS2 (mitochondrial lysyl-tRNA synthetase), helped by ENO2 (glyolitic enlolase). It is still unclear how the tRNA-pre-KARS2 complex then gets internalized into the mitochondrial matrix (Gowher et al., 2013; Kim et al., 2017b). Possible protein import pathway could consist of the translocase of the outer (TOM) and inner (TIM) mitochondrial membrane, as in yeast (Tarassov and Martin, 1996). Although the import of tRNA is yet to be fully understood, it could present a novel concept for therapy for disorders caused by defects in mtDNA-encoded tRNAs. Successful import of tRNA compensating the mutated mtDNA could rescue defects in mitochondrial translation. Rescue of mtDNA mutations by the import of designed tRNAs to mitochondria has been reported *in vitro* and *in vivo* (Salinas et al., 2008; Wang et al., 2012a), but more recent reports are missing.

Several studies have suggested that *5S rRNA* is imported to the mammalian mitochondria (Yoshionari et al., 1994; Magalhaes et al., 1998). Entelis et al. (2001) suggested that mitochondrial 5S rRNA might substitute for its lost counterpart and be part of mitoribosome large subunit. Smirnov et al. (2008) proposed a model of mitochondrial 5S rRNA import (**Figure 3B**), starting with the recognition and transport of 5S rRNA from the nucleus to the cytoplasm by TFIIIA (Ciganda and Williams, 2011). In the cytosol, 5S rRNA was proposed to interact with pre-MRPL18 (precursor of mitochondrial ribosomal protein L18). This interaction might induce a conformational change in 5S rRNA that makes it recognized and bound by the mitochondrial enzyme Rhodanese, which helps it possibly translocate into mitochondria through a yet unknown mechanism. In the matrix 5S rRNA was proposed to associate with the mature MRPL18 and with mitoribosomes, affecting mitochondrial translation efficiency (Smirnov et al., 2010; Smirnov et al., 2011). However, as cryo-electron microscopy did not detect 5S rRNA within the mammalian mitoribosome 5S rRNA (Greber et al., 2015), its possible function in mitochondria remains enigmatic.

RMRP is a part of the RNase MRP, a ribonucleoprotein complex whose function has been discussed for decades. In the nucleus, it is involved in the pre-rRNA processing (Schmitt and Clayton, 1993; Chu et al., 1994; Goldfarb and Cech, 2017). In mitochondria, it was postulated to cleave RNA complementary to the light chain near the D-loop sites that mark the transition from RNA to DNA synthesis (Chang and Clayton, 1987b; Lee and Clayton, 1997). Three RNA-binding proteins (RBPs- HuR, PNPase, and GRSF1) have been implicated in the *RMRP* transport and role in mitochondria (**Figure 3C**). In the nucleus, *RMRP* is bound to HuR, which promotes its export to the cytosol in a CRM1-dependent manner (Noh et al., 2016). The exported *RMRP* might be then targeted into the mitochondrial intermembrane space through yet unknown mechanisms where PNPase was suggested to enable its import into the matrix (Wang et al., 2012b), after which its abundance in the matrix was reported to be increased through the interaction with GRSF1 (Noh et al., 2016). However, recent studies cast a shadow on the role of RMRP complex in mitochondria. Agaronyan et al. (2015) have shown that the RNA primer formation is a result of a premature arrest of the mitochondrial RNA polymerase after a G-quadruplex. Moreover, only the 3′ half (~130 nt) of *RMRP* could be found in mitochondria, indicating a processing that would result in a loss of catalytic activity (Esakova and Krasilnikov, 2010). These reports indicate that *RMRP* unlikely acts as an endonuclease in mitochondria. However, its interaction with GRSF1, an important component of the RNA granules (Antonicka et al., 2013; Jourdain et al., 2013), might still make it involved in the RNA metabolism.

RNase P processes the 5′ leader of precursor tRNA, which is a critical step of processing mitochondrial polycistronic transcripts (Ojala et al., 1981; Rackham et al., 2016). Two types of RNase P are known: ribonucleoproteins RNases P containing *RNase P* and protein-only RNases P (PRORP) (Lechner et al., 2015; Klemm et al., 2016). In the majority of species, including humans, it is assumed that the ribonucleoprotein RNase P acts in the nucleus and PRORP in mitochondria (Holzmann et al., 2008; Lechner et al., 2015). Strengthening this assumption, studies have reported that mammalian mitochondrial RNAse P does not require the catalytic RNA component for catalysis (Rossmanith et al., 1995; Holzmann et al., 2008). Nevertheless, *RNase P* was partially purified from HeLa cells mitochondria. Detected "*mtRNase P*", together with the observed sensitivity of RNAse P to the nuclease treatment, suggested that RNAse P acts as a ribonucleoprotein also in mitochondria (Doersen et al., 1985). In addition, several groups indicated that *mtRNase P* is imported into the mitochondrial matrix through interaction with PNPase (Wang et al., 2010; Mercer et al., 2011; Noh et al., 2016) (**Figure 3D**). However, as so far functional RNase P ribonucleoprotein has not been reported in mitochondria, the existence of *mtRNase P* remains controversial (Jeandard et al., 2019).

hTERC is the RNA component of the human telomerase, where it serves as a sequence template for the telomere replication (Gall, 1990). As its sequence contains a region similar to an *RMRP* and *RNase P* short stem-loop that was proposed to enable their entry into mitochondria (Wang et al., 2010), *hTERC* was also proposed to be mitochondria-localized (Cheng et al., 2018). It was detected by the RT-PCR in purified mitoplasts, but as as a shorter, 195 nt-long transcript, which was termed TERC-53. Zheng et al. (2019) demonstrated that TERC-53 is mostly localized in the cytosol, where it regulates cellular senescence and is involved in cognition decline in mice hippocampus without affecting telomerase activity or mitochondrial functions. Having this in mind, the authors hypothesized that TERC-53 is exported from the mitochondria back to the cytosol (Cheng et al., 2018; Zheng et al., 2019). However, this hypothesis indicates *hTERC* processing occurring within the mitochondria, which has so far not been reported.

MICRORNAS

Vertebrate genomes contain thousands of miRNAs: according to MiRBase catalog, with the human genome containing 2,654 mature sequences (Kozomara et al., 2019). The biogenesis and biological functions of miRNAs have been widely studied in eukaryotic cells (Bartel, 2009) (**Figure 4**). In short, miRNAs are transcribed from the intergenic regions or in antisense orientation to coding regions as the primary miRNA transcript (pri-miRNA). pri-miRNA is processed in the nucleus by Drosha and/or DiGeorge syndrome chromosomal region 8 (DGCR8). This results in premature miRNA (pre-miRNA) which is then bound by exportin 5 (XPO5). XPO5, along with RanGTP, enables the export of the pre-miRNA through the nuclear pore into the cytosol. There RNase Dicer (DICER1 in humans) cleaves it, producing mature double-stranded miRNA. From two strands, the "passenger strand" undergoes RNA degradation while the remaining "guide strand" associates with argonaute 2 (AGO2) and becomes part of a multiprotein RNA-induced silencing complex (RISC) (Han et al., 2006). The main function of miRNA within RISC is post-transcriptional gene regulation by promoting mRNA degradation or translational repression by sequence-specific binding to the target mRNA. mRNA degradation is achieved *via* AGO2 (Carthew and Sontheimer, 2009; Chekulaeva and Filipowicz, 2009). Translational control is mediated by GW182 (Czech and Hannon, 2011; Iwakawa and Tomari, 2015). Moreover, miRNAs have also been implicated in some non-canonical functions, such as direct transcription and chromatin state regulation in the nucleus, and even translational promotion (Vasudevan, 2012; Yao et al., 2019). Each miRNA can target multiple genes, enabling them to regulate the expression of over 60% of the human genes and therefore moderate any part of cellular biology (Bartel, 2009; Friedman et al., 2009). Focusing on mitochondria, based on their localization and genetic origin, three different classes of mitochondria-related miRNAs can be distinguished **(1)** cytoplasmic, nuclear-encoded miRNAs targeting mitochondria-related transcripts; **(2)** mitochondrial, nuclear-encoded miRNAs; and **(3)** mitochondrial, mtDNA-encoded miRNAs (Bandiera et al., 2013) (**Figure 4**). The two latter classes, termed mitomiRs, are yet to be functionally deciphered.

Cytoplasmic miRNAs With Impact on Mitochondria

As about 1,500 nuclear-encoded proteins are imported into mitochondria and involved in diverse mitochondrial functions, many miRNAs have been described as directly targeting their mRNAs in the cytoplasm. By downregulating transcripts encoding for proteins involved in a variety of mitochondrial processes, reported miRNAs can indirectly influence mitochondrial biology and homeostasis. A summary of miRNAs reported to target nuclear-encoded mitochondrial transcripts is given in **Table 2**.

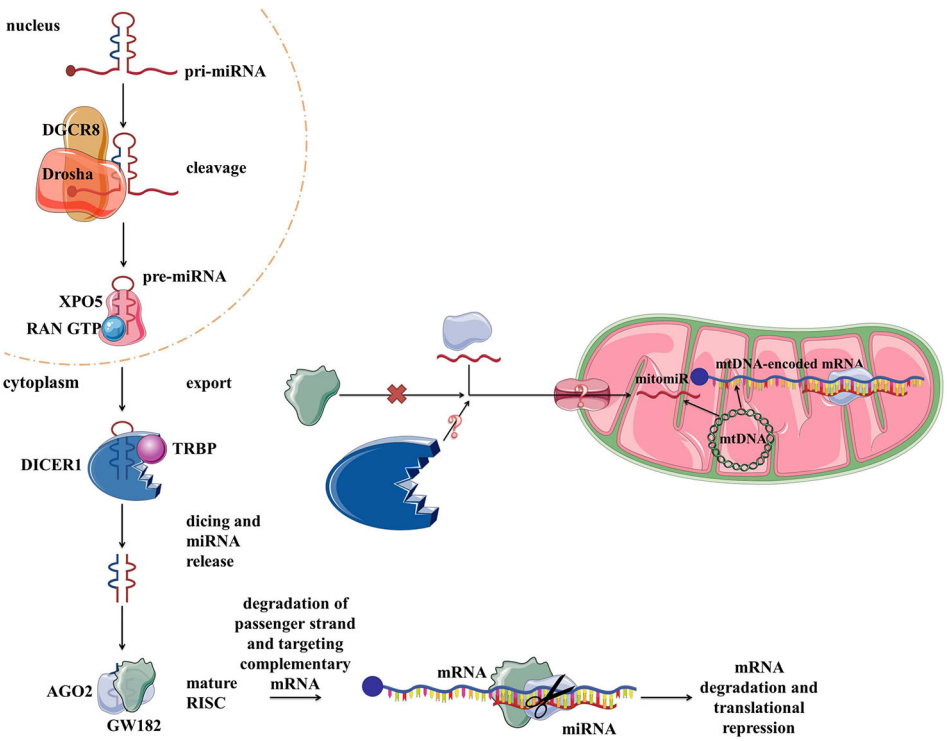

FIGURE 4 | miRNA biogenesis, function in the cytoplasm within RISC, and proposed transport/presence in mitochondria. RISC, RNA-induced silencing complex.

TABLE 2 | miRNAs and their target genes across mitochondrial functions.

miRNA	Target	Reference
(A) TCA cycle		
miR-148a	*CS*	Tibiche and Wang, 2008
miR-148b	*CS*	Tibiche and Wang, 2008
miR-299-5p	*CS*	Tibiche and Wang, 2008
miR-19a-3p	*CS*	Tibiche and Wang, 2008
miR-19b-3p	*CS*	Tibiche and Wang, 2008
miR-122a	*CS*	Tibiche and Wang, 2008
miR-421	*CS*	Tibiche and Wang, 2008
miR-494	*CS*	Tibiche and Wang, 2008
miR-183	*IDH2*	Vohwinkel et al., 2011
miR-743a	*MDH2*	Shi and Gibson, 2011
miRNA-26a	*PDHX*	Chen et al., 2014
miR-210	*SDHD*	Puissegur et al., 2011
miR-147b	*SDHD*	Zhang et al., 2019
miR-124	*SUCLG2*	Wang and Wang, 2006
(B) OXPHOS		
miR-101-3p	*ATP5B*	Zheng et al., 2011
miR-127-5p	*ATP5B*	Willers et al., 2012
miR-338-5p	*ATP5G1*	Aschrafi et al., 2012
mitomiR-378	*ATP6*	Jagannathan et al., 2015
miR-181c	*COX1*	Das et al., 2014
miR-338	*COX4*	Aschrafi et al., 2008
miR-34a	*CYC*	Bukeirat et al., 2016
miR-210-5p	*ISCU*, *COX10*	Chan et al., 2009; Chen et al., 2010
miR-210	*SDHD*	Puissegur et al., 2011
miR-147b	*SDHD*	Zhang et al., 2019
miR-663	*UQCC2*	Carden et al., 2017
(C) Fatty acid metabolism		
miR-204-5p	*ACACB*	Civelek et al., 2013
miR-224-5p	*ACSL4*	Peng et al., 2013
miR-122	*Aldoa*	Esau et al., 2006
miR-212	*CACT*	Soni et al., 2014
miR-132	*CACT*	Soni et al., 2014
miR-370	*CPT1A*	Iliopoulos et al., 2010
miR-33b	*CPT1A*	Rottiers and Naar, 2012
*miR-378, miR-378**	*CRAT*	Carrer et al., 2012
miR-33a	*CROT*	Gerin et al., 2010
miR-107	*PANK*	Wilfred et al., 2007
miR-103	*PANK*	Wilfred et al., 2007
miR-29a-3p	*PPARδ*	Kurtz et al., 2014
miR-199a-5b	*PPARδ*	el Azzouzi et al., 2013
(D) Aminoacid metabolism		
miR-29b	*DBT*	Mersey et al., 2005
miR-23a-3p	*GLS*	Gao et al., 2009
miR-23b-3p	*GLS*	Gao et al., 2009
miR-193b	*SHMT2*	Leivonen et al., 2011
(E) Nucleotide metabolism		
miR-502	*DHODH*	Zhai et al., 2013
miR-940	*MTHFD2*	Xu et al., 2019
miR-149	*MTHFR*	Wu C. et al., 2013
miR-125	*MTHFR*	Stone et al., 2011
miR-22	*MTHFR*	Stone et al., 2011
(F) Mitochondrial transport		
miR-15b	*Arl2*	Nishi et al., 2010
miR-16	*Arl2*	Nishi et al., 2010
miR-195	*Arl2*	Nishi et al., 2010
miR-424	*Arl2*	Nishi et al., 2010
miR-25	Mitochondrial calcium uniporter	Marchi et al., 2013
miR-155	*SLC25A19*	Kim et al., 2015
miR-132	*SLC25A20*	Soni et al., 2014
miR-212	*SLC25A20*	Soni et al., 2014
miR-184	*Slc25a22*	Morita et al., 2013
miR-141	*Slc25a3*	Baseler et al., 2012
(G) Mitochondrial dynamics		
miR-30a-5p	*DRP1*	Li et al., 2010
miR-483-5p	*Fis1*	Fan et al., 2015
miR-484	*Fis1*	Wang K. et al., 2012
miR-499	*Fnip1, Calcinurin*	van Rooij et al., 2009; Wang et al., 2011; Liu L. et al., 2016
*miR-9/9**	*GTPBP3, MTO1, TRMU*	Meseguer et al., 2015
miR-27	*MFF*	Tak et al., 2014
miR-761	*MFF*	Long et al., 2013
miR-593	*MFF*	Fan et al., 2015
miR-200a-3p	*MFF*	Lee et al., 2017
miR-140	*MFN1*	Guan et al., 2016
miR-19b	*MFN1*	Li X. et al., 2014; Joshi et al., 2016
miR-382-5p	*MFN1, MFN2, OPA, SIRT1, PGC1-α*	Dahlmans et al., 2019
miR-214	*MFN2*	Bucha et al., 2015
miR-106a	*MFN2*	Zhang et al., 2016
miR-195	*MFN2*	Zhou et al., 2016
miR-30 family	*P53*	Li et al., 2010
miR-149	*PARP-2*	Mohamed et al., 2014
miR-23a	*PGC1-α*	Russell et al., 2013
miR-696	*PGC1-α*	Aoi et al., 2010
miR-27	*PHB*	Kang et al., 2013
miR-494	*TFAM*	Yamamoto et al., 2012
miR-23b-5p	*TFAM*	Jiang et al., 2013
miR-590-3p	*TFAM*	Wu et al., 2016
miR-155-5p	*TFAM*	Quinones-Lombrana and Blanco, 2015
miR-200a	*TFAM*	Yao et al., 2014
miR-26	*UCP1*	Karbiener et al., 2014
miR-15a	*UCP2*	Sun et al., 2011
miR-133a	*UCP2*	Chen et al., 2009
miR-7	*VDAC1*	Chaudhuri et al., 2016
(H) Autophagy, mitophagy and ROS		
miR-146a	*Bcl-2*	Rippo et al., 2014
miR-181a	*Bcl-2*	Rippo et al., 2014
miR-195	*Bcl-2*	Singh and Saini, 2012
miR-24-2	*Bcl-2*	Singh and Saini, 2012
miR-365-2	*Bcl-2*	Singh and Saini, 2012
miR-497	*Bcl-2*	Yadav et al., 2011
miR-146	*Bcl-2*	Zhang et al., 2017
miR-15a	*Bcl-2* and *Mcl-1*	Cimmino et al., 2005
miR-16	*Bcl-2* and *Mcl-1*	Cimmino et al., 2005
miR-9	*BCL2L11*	Li Y. et al., 2014
miR-30a	*Becn-1*	Zhu et al., 2009
miR-17-92	*Bim*	Molitoris et al., 2011
miR-92a	*Bim*	Tsuchida et al., 2011
miR-145	*BNIP3*	Du et al., 2017
miR-101	*Mcl-1*	Frankel et al., 2011
miR-29	*Mcl-1*	Mott et al., 2007
miR-181	*Mcl-1, Bcl-2*	Ouyang et al., 2012
miR-137	*NIX, FUNDC1*	Li W. et al., 2014
miR-504	*P53*	Hu et al., 2010
miR-125b	*P53, Bak*	Le et al., 2009; Sun et al., 2013
miR-21	*PTEN*	Meng et al., 2007; Zhang et al., 2010
miR-128	*SIRT1*	Adlakha et al., 2013

(Continued)

TABLE 2 | Continued

miRNA	Target	Reference
miR-335	*SOD2, TXNRD2*	Bai et al., 2011;
miR-34a	*SOD2, TXNRD2, Bcl-2, SIRT1*	Yamakuchi et al., 2008; Bai et al., 2011; Rippo et al., 2014
*miR-17**	*SOD2, TXNRD2, GPX2*	Xu et al., 2010

TCA Cycle

The tricarboxylic acid (TCA) cycle is a central pathway in the metabolism of sugars, lipids, and amino acids. Several miRNAs have been described to directly target transcripts of enzymes involved in its chemical reactions (**Figure 5**, **Table 2A**). For example, *miR-26a* targets subunit X of pyruvate dehydrogenase (PDH). As PDH catalyzes a crucial reaction before acetyl-coA enters the TCA cycle, its repression is leading to the decreased levels of acetyl-coA and the accumulation of pyruvate (Chen et al., 2014). In cancer research, miRNAs have been discovered to have a role in developing drug tolerance. Altered *miR-147b* initiates a reversible state of tolerance to osimertinib in lung cancer cells by binding *SDHD* (Zhang et al., 2019). Pretreatment with a *miR-147b* inhibitor delayed osimertinib-associated drug tolerance, providing a promising target for preventing tumor relapse (Zhang et al., 2019).

Oxidative Phosphorylation System (OXPHOS)

OXPHOS system is composed of five protein complexes in the inner mitochondrial membrane that through oxidoreductase reactions generate a proton gradient, ultimately driving ATP synthesis. Several miRNAs have been described as directly targeting the OXPHOS subunits or assembly factors (**Figure 6**, **Table 2B**). It was shown that *miR-663* positively regulates OXPHOS subunit and assembly factor protein levels by direct stabilization of complex III assembly factor *UQCC2* (Carden et al., 2017). In breast cancer cell lines, mitochondrial dysfunction downregulates *miR-663* through hypermethylation of its promoter, which leads to decreasing OXPHOS proteins levels and enzymatic activity and stability of supercomplexes, which promotes tumorigenesis (Carden et al., 2017).

Fatty Acid Metabolism

Fatty acid metabolism includes catabolic and anabolic processes that involve triglycerides, phospholipids, steroid hormones, and ketone bodies. Several miRNAs have been described as regulators of these processes (**Table 2C**). As fatty acid oxidation defects have been linked to the obesity and the development of insulin resistance (Kusunoki et al., 2006), these miRNAs could serve as potential therapeutic targets. As an example, *PPARGC1B* encodes for PGC-1β, a transcriptional coactivator that promotes mitochondrial biogenesis. Interestingly, this locus can also encode for *miR-378* and *miR-378**, which counterbalance the effect of PGC1-β by targeting carnitine-O-acetyltransferase (*CRAT*) (Carrer et al., 2012). miR-378/378* knockout (KO) mice showed significantly greater mitochondrial function and oxidative capacity.

Amino Acid Metabolism

The main steps of breakdown and synthesis of amino acids occur in mitochondria. Several miRNAs have been connected to amino

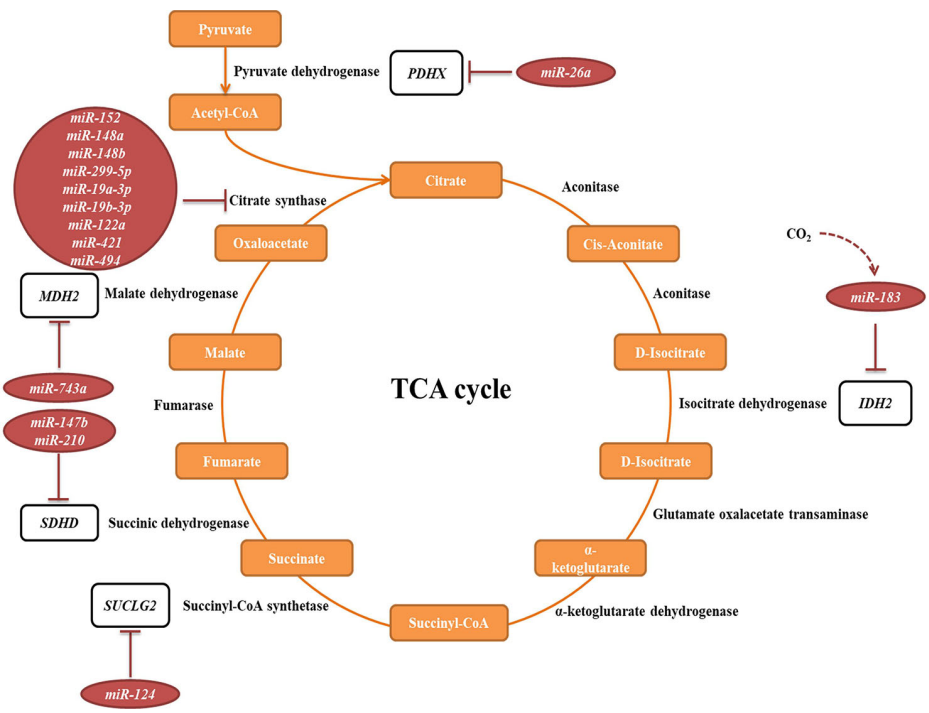

FIGURE 5 | miRNAs targeting transcripts encoding proteins involved in the TCA cycle. Red arrows present the repressing effect of miRNA on its target mRNA.

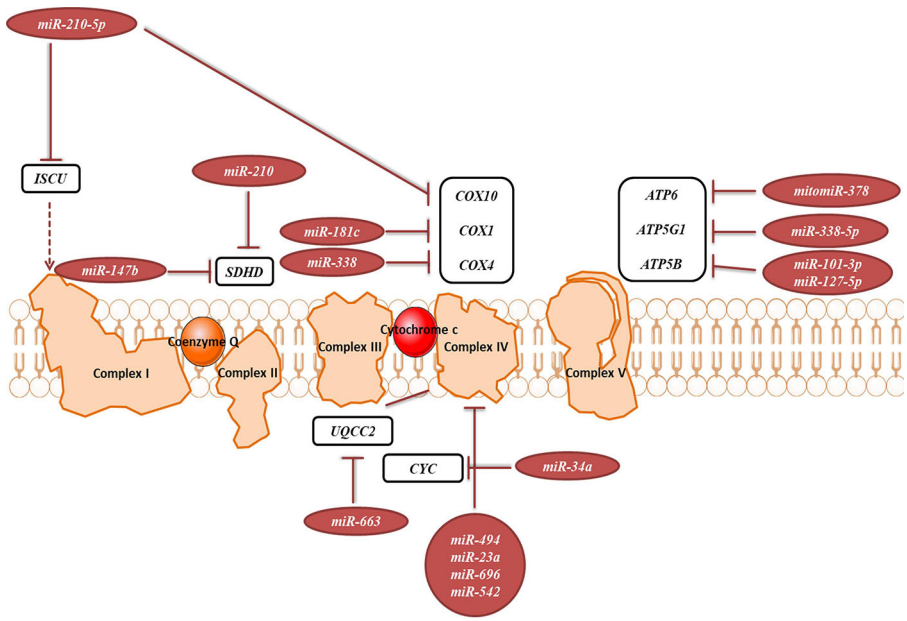

FIGURE 6 | miRNAs targeting transcripts encoding proteins involved in the OXPHOS. Red arrows present the repressing effect of miRNA on its target mRNA.

acid metabolism (**Table 2D**). Most of the published work is focused on the regulation of glutaminase (GLS), which catalyzes the conversion of glutamine to glutamate. *miR-23a* and *miR-23b* participate in targeting glutaminase and thereby contribute to the mitochondrial amino acid metabolism (Gao et al., 2009).

Nucleotide Metabolism

Parts of the nucleotide and one-carbon metabolism are occurring in mitochondria. Various miRNAs can influence these processes (Desler et al., 2010) (**Table 2E**). For example, *miR-149*, *miR-125,* and *miR-22* have been found to target *MTHFR* (Stone et al., 2011; Wu C. et al., 2013).

Mitochondrial Transport

Many mitochondrial transporter and carrier proteins enable the import and export of molecules across the mitochondrial membranes. By targeting the transcripts encoding for these proteins, miRNAs are able to influence mitochondrial biology (**Table 2F**). It has been shown that the *miR-15/16* cluster, composed of *miR-15b*, *miR-16*, *miR-195*, and *miR-424*, target *Arl2* (Nishi et al., 2010).

Mitochondrial Dynamics

Mitochondria are constantly changing their size, shape, and number to maximize the capacity for OXPHOS and answer the cell needs. This is achieved through the coordinated processes of biogenesis, fission, and fusion (Tilokani et al., 2018). Several miRNAs have been shown to be involved in the regulation of mitochondrial dynamics by directly or indirectly targeting these key factors (**Figure 7**, **Table 2G**). *miR-149* indirectly promotes mitochondrial biogenesis by inhibiting *PARP-2*, which increases the NAD+ levels and SIRT-1 activity, finally leading to the increased activity of PGC-1α, the master regulator of mitochondrial biogenesis. Skeletal muscles from a high fat diet-fed obese mice have low levels of *miR-149* and present with mitochondrial dysfunction, which might be due to *miR-149*-induced SIRT-1/PGC-1α pathway dysregulation. Noteworthy, miRNAs have been implicated in the mitochondria-mediated transition of skeletal muscle fiber types. *miR-499* directly targets *Fnip1*, a negative regulator of AMPK, a known activator of PGC-1 α, and thereby triggers a muscle mitochondrial oxidative metabolism program (Liu L. et al., 2016). The *miR-30* family, highly expressed in heart, was reported to regulate mitochondria fission and apoptosis by directly targeting *p53*, a transcriptional activator of Drp1 (Li et al., 2010). In addition, Drp1 is indirectly regulated by *miR-499*, which targets Drp1 activator dephosphatase calcinurin (van Rooij et al., 2009; Wang et al., 2011). Finally, *miR-499* transcription is regulated by p53 on the transcript level (Wang et al., 2011).

MELAS syndrome is caused by mutations in mtDNA affecting $tRNA^{Leu}_{UUR}$. One of the phenotypes of MELAS patients is the increased oxidative stress. In addition, mutant $tRNAs^{Leu}_{UUR}$ have reduced levels of the taurine-containing chemical modification at the wobble uridine (U34). Meseguer et al. (2015) reported that elevated oxidative stress in mutant cells leads to induction of *miRNA-9/9**, which then act as post-transcriptional repressors of the tRNA-modification enzymes GTPBP3, MTO1, and TRMU. Downregulation of these enzymes disrupts the chemical modification at U34 of non-mutant tRNAs and contributes to mitochondrial dysfunction (Meseguer et al., 2015).

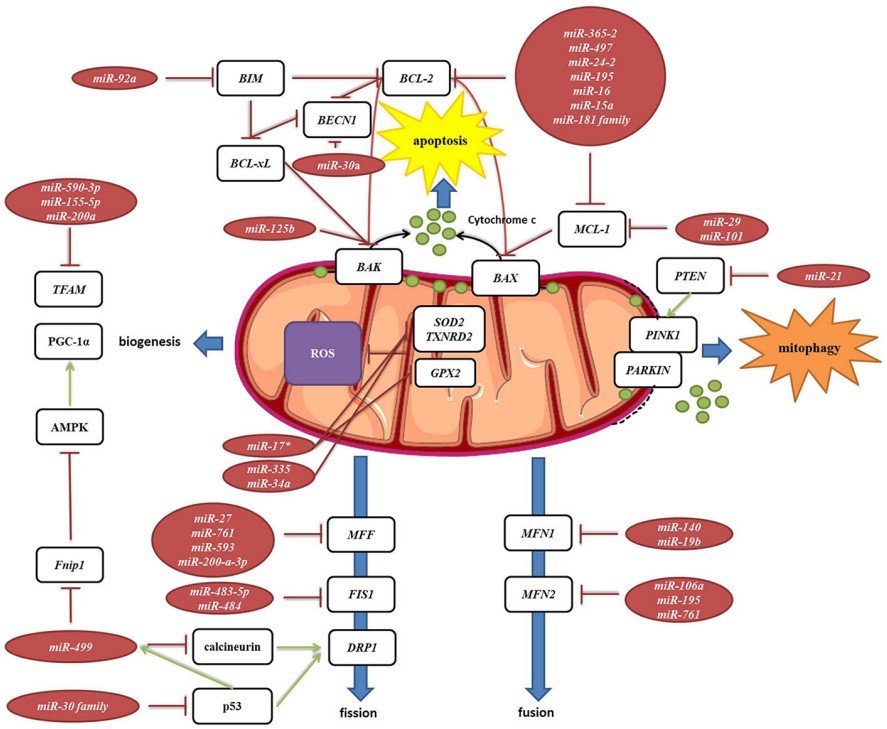

FIGURE 7 | miRNA targeting transcripts encoding proteins involved in the mitochondrial dynamics, autophagy, mitophagy and ROS production. Red arrows present inhibitory effect of miRNA on its target mRNA or repressive effect of protein on its interaction partners, and green arrows present the activating effect of protein on its interaction partner.

Autophagy, Mitophagy, and Reactive Oxygen Species (ROS) Production

Autophagy is a catabolic process which prevents cell damage and promotes the cell survival by degrading and/or recycling dysfunctional components during cellular stress (Dikic and Elazar, 2018). Mitophagy is a form of autophagy that removes faulty or superfluous mitochondria, regulating their number to match the cellular needs (Pickles et al., 2018). miRNAs are also involved in the mitochondria-mediated apoptosis (**Figure 7**, **Table 2H**). Moreover, they are frequently dysregulated in human cancers, where they may function as potent oncogenes or tumor suppressors (Peng and Croce, 2016). Since mitochondrial dysfunction is one of the hallmarks of cancer (Wallace, 2012), miRNAs targeting apoptosis-related transcripts could be important in the development of cancer therapies. *miR-101* (Frankel et al., 2011), *miR-30a* (Zhu et al., 2009), *miR-15a,* and *miR-16* (Cimmino et al., 2005) have been reported to target oncogenic *Bcl-2* and *Mcl-1*, and are frequently deleted or decreased in chronic lymphocytic leukemia. *miR-21* levels have been shown to be significantly increased, leading to reduced expression of PTEN in human lung and hepatocellular carcinomas (Meng et al., 2007; Zhang et al., 2010).

mitomiRs

MitomiRs are defined as miRNAs with mitochondrial localization (Bandiera et al., 2011). The majority of mitomiRs were suggested to originate from the nuclear genome, but also there were reports of mtDNA-encoded miRNAs. Different experimental approaches across mammalian tissues and cell lines indicated the mitochondrial presence of miRNAs, but also proteins involved in miRNAs biogenesis and function, suggesting miRNAs import, transcription, and/or processing and function within mitochondria themselves. Intriguingly, mitomiRs have some unique features which distinguish them from conventional cytosolic miRNAs (Bandiera et al., 2011; Barrey et al., 2011). Most of the nuclear-encoded mitomiRs loci are located within mitochondrial gene clusters or close to mitochondrial genes, and their transcriptions are often coregulated (Baskerville and Bartel, 2005; Bandiera et al., 2011). Their size slightly differs (between 17 and 25 nt instead of the average 22 nt), and they contain short 3′ overhangs, stem-loop secondary structures, and unique thermodynamic features (Vendramin et al., 2017). They lack 5′ cap and most were predicted *in silico* to target multiple mtDNA sites. It has thus been speculated that at least some of these features could present a signal for entry into mitochondria (Bandiera et al., 2011; Barrey et al., 2011).

mitomiRs have been found *via* different approaches (from miRNA microarray and RT-qPCR to deep sRNA-sequencing) and across various tissues and organisms. To begin with, sequence analysis of cDNA libraries from mice mitochondrial RNA identified clones mapping to four nuclear-encoded miRNAs and three regions within the D-loop (Lung et al.,

2006). Other reports on miRNAs localized in mammalian mitochondria have expanded in the past decade (Kren et al., 2009; Bandiera et al., 2011; Barrey et al., 2011; Mercer et al., 2011; Sripada et al., 2012; Jagannathan et al., 2015), as summarized in **Table 3**. For example, Kren et al. (2009) reported by miRNA microarray 15 nuclear-encoded miRNAs from highly purified rat liver mitochondria and further strengthened their findings with Northern blot and stem-loop RT-qPCR analyses. Barrey et al. (2011) *in silico* predicted 33 pre-miRNAs and 25 miRNAs targeting mtDNA and experimentally confirmed localization of *pre-mir302a*, *let-7b,* and *mir-365* to isolated mitochondria from the human myotubes. Mercer et al. (2011) detected 31 mitochondria-encoded small RNAs in human 143B mitoplasts by sRNA-seq, the majority (84%) derived from mt-tRNA genes.

The presence of miRNA-associated proteins in the mitochondria was only recently recognized (summarized in **Table 4**). Wang et al. (2015a) and Vargas et al. (2016) reported Dicer in the rat brain, but it was reported as absent in the mitochondria isolated from the heart (Chen et al., 2010; Das et al., 2012; Jagannathan et al., 2015). So far, only one colocalization of *pre-miR-338* and Dicer in rat brain mitochondria has been published (Vargas et al., 2016). If indeed true, the presence of Dicer could indicate that mature miRNA are formed from the precursors in mitochondria, from where they could directly affect the mitochondrial transcripts or even be exported to act in the cytosol (Bienertova-Vasku et al., 2013). However, mitochondrial localization of Dicer, Drosha, and DGCR8 has not yet been validated by other groups. Several studies have documented the presence of RNA-interference components, most notably AGO2, in the mitochondria, implying the functional importance of mitomiRs. As an example, Ago2 immunoprecipitated with miRNA from mitochondria in rat cardiac myocytes (Das et al., 2012). In addition, FXR1, a postulated RISC subunit, has been found together with Ago2 in the mitochondrial matrix of mouse cardiomyocytes (Jagannathan et al., 2015). However, an important factor for miRNA-mediated translational repression-GW182 has not been detected in any studies (Ro et al., 2013; Zhang et al., 2014). Finally, the presence of Dicer and AGO2 in mitochondria need not necessarily imply processing and function of mitomiRs, as these enzymes are involved also in other, miRNA-independent, processes (Janowski et al., 2006; Song and Rossi, 2017).

Although protein transport across mitochondrial membranes is well described, the translocases for RNA transport across mitochondrial membranes remain speculative. Several

TABLE 3 | miRNAs detected in mitochondria, mitomiRs.

mitomiR	Tissue	Method of detection	Reference
Mt-1; Mt-2; Mt-3; Mt-4; let7f-;, let-7g; 122a; 101b	Mouse liver and kidney	cDNA library	Lung et al., 2006
130a; 130b; 140; 290; 320; 494; 671; 202; 705; 709; 721; 761; 763; 198; 765	Rat liver	miRNA microarray, Northern blot, RT-qPCR	Kren et al., 2009
690; 122; 451; 720; let-7f; let-7b; let-7g; 29a; 26a; 192; 101; 22; 805; 29c; 7a; 98; 26b; 30b; 7c; 709	Mouse liver	miRNA microarray, RT-qPCR	Bian et al., 2010
1973; 1275; 494; 513a-5p; 1246; 328; 1908; 1972; 1974;638; 1977;1978;1201	HeLa cells	miRNA microarray, RT-qPCR	Bandiera et al., 2011
pre-mir302a; pre-let-7b; 365; 720; 133b; 1974; 24; 133a; 125a-5p; 1979; 103; 125b; 103; 221; 23a; let-7b; 423-3p; 106a; 23b; 92a; 193b; 93; 532-3p; 20a; 149; 181a; 503; 210; 107; 574-3p; 34a; let-7g; miRPlus-D1033; 19b; 197; 324-3p; 127-3p; 324-5p; 484; 151-5p; 486-5p; 542-5p; 199a-5p; 501-3p; 675; 134; 490-3p; 598*	Human myotubes	FISH, RT-qPCR	Barrey et al., 2011
103-3p; 146a-5p; 16-5p	143B cells	sRNA-seq	Mercer et al., 2011
181c-5p	Rat cardiac myocytes	miRNA microarray, immunostaining, RT-qPCR	Das et al., 2012
107; 181a-5p; 221-5p; 320a; let-7b; let-7g	HEK293 and HeLa cells	sRNA-seq, RT-qPCR	Sripada et al., 2012
1	C2C12 cells	CLIP-seq, miRACE, RT-qPCR	Zhang et al., 2014
143-3p; 378a-3p; 146a-5p; 181c-5p; 501-3	143B and 206 ρ° cells	sRNA-seq, RT-qPCR	Dasgupta et al., 2015
let-7d-5p; let-7b-5p; let-7c-5p; let-7f-5p; mghv-M1-7-3p; 1187; 1224-5p; 125a-3p; 125b-5p; 126-3p; 130a-5p; 133a-3p; 133a-5p; 133b; 135a-1-3p; 139-3p; 1-3p;144-3p; 149-3p; 149-5p; 188-5p; 1894-3p; 1895; 1897-5p; 1904; 1934-3p; 1982-5p; 211-3p; 2137; 21a-5p; 22-3p; 23a-3p; 23b-3p; 24-3p; 26a-5p; 27a-3p; 27b-3p; 2861; 29a-3p; 29b-3p; 29c-3p; 3072-3p; 3081-5p; 3082-5p; 3085-3p; 3092-3p; 3095-3p; 3098-5p; 30a-5p; 30c-1-3p; 30d-5p; 30e-5p; 3102-5p; 3102-5p.2-5p; 3470a; 378a-5p; 451a; 466b-3p; 466i-5p; 483-5p; 486b; 494-3p; 497-5p; 574-5p; 652-5p; 671-5p; 680; 705; 709; 712-5p; 721; 877-3p; 99a-5p	Mouse heart, HL-1 cells	Microarray, RT-qPCR, CLIP-seq, sRNA-seq	Jagannathan et al., 2015
142-5p; 142-3p; 146; 150a	Rat hippocampus, rat astrocytes	RT-qPCR	Wang et al., 2015a
Has-mit-miR-1; Has-mit-miR-2; Has-mit-miR-3; Has-mit-miR-4; Has-mit-miR-5; Has-mit-miR-6	Human skeletal muscle myoblasts	Northern blot, RT-qPCR	Shinde and Bhadra, 2015
pre-miR-338	Rat SCG neurons	qRT-PCR, co-localisation	Vargas et al., 2016
371a-5p; 1246; 664b-3p; 513b; 4271; 2392; 4462; 1290; 4449; 3934-5p1268a	TSCCs	miRNA microarray, RT-qPCR	Fan et al., 2019

TABLE 4 | miRNA biogenesis and RISC proteins detected in mitochondria.

Protein	Tissue	Method of detection	Reference
DICER	Rat hippocampus	Western blot, immunoprecipitation	Wang et al., 2015a
	Rat total brain, SCG neurons	Western blot, immunostaining	Vargas et al., 2016
AGO2	Mouse liver	Western blot	Bian et al., 2010
	HeLa cells	Western blot, immunostaining, immunoprecipitation	Bandiera et al., 2011
	Rat cardiac myocytes	Immunoprecipitation	Das et al., 2012
	HeLa cells	Immunostaining	Sripada et al., 2012
	C2C12 cells	Western blot, immunoprecipitation	Zhang et al., 2014
	143B and 206 ρ° cells	Western blot	Dasgupta et al., 2015
	Mouse cardiomyocytes, HL-1 cells	Western blot, immunoprecipitation	Jagannathan et al., 2015
	Rat hippocampus	Western blot, immunoprecipitation	Wang et al., 2015a
	TSCC	Western blot	Fan et al., 2019
AGO3	HEK293 cells	Immunostaining	Sripada et al., 2012
FXR1	Mouse cardiomyocytes	Western blot, immunoprecipitation	Jagannathan et al., 2015

mechanisms of miRNAs transport into the mitochondria have been proposed. As shown in **Figure 8**, the potential players are AGO2, processing bodies (P-bodies), polynucleotide phosphorylase (PNPase) and voltage-gated ion channels (VDAC). AGO2 has been proposed as an important factor in the subcellular localization of miRNAs. Zhang et al. (2014) have shown an association of *miR-1* with Ago2 in mitochondria and proposed their mechanism of action. At the baseline, *miR-1* is found in the cytoplasm within RISC with 3′UTR of *HDAC4*. However, during myogenesis, GW182 detaches and *HDAC4* loses 5′cap and poly(A) tail, suggesting that loss of GW182 alone or in combination with changes in *HDAC4* facilitates the transport of Ago2:*miR-1* into mitochondria (**Figure 8A**). Still, it remains unclear if AGO2 and miRNA translocate together as a complex (**Figure 8B**) or separately (**Figure 8C**) into the mitochondria and by which mechanism. Another hypothesis involves P-bodies, as they interact with mitochondria and can regulate mRNA decay, mRNA storage, and possibly miRNA import into different cellular compartments (Huang et al., 2011; Bandiera et al., 2013; Luo et al., 2018). Activation of several pathways and phosphorylation at the Ago2 Ser387 site has been shown to separate the Ago2/miRNA complex from the RISC and activate its intake into the P-body (Huang et al., 2011; McKenzie et al., 2016) (**Figure 8D**). As GW182 is also a P-body subunit (Liu et al., 2005), it might still have significance for the Ago2-miRNA import. PNPase is another candidate, as it has already been postulated to recognize specific structures of the housekeeping ncRNAs and help RNA fold properly to migrate through the mitochondrial membranes and return to its original conformation when they arrive in the mitochondrial matrix (Wang et al., 2010; Wang et al., 2012a) (**Figure 8E**). Several pre-miRNAs share the specific stem-loop structure that PNPase could recognize and enable import (Wang et al., 2010; Barrey et al., 2011; Lin et al., 2012). PNPase levels were reported to affect *mitomiR-378* mitochondrial localization and co-immunoprecipitation showed Ago2 association with PNPase, suggesting that PNPase can bind to the miRNA within the complex with Ago2 (Shepherd et al., 2017). Transport across mitochondrial membranes could occur *via* TOM/TIM complexes (**Figure 8F**). Still, additional studies are needed to prove whether and how Ago2 can go through such small pores, even if facilitated by PNPase. Finally, it has been demonstrated that VDAC, the most abundant outer mitochondrial membrane protein in plants, could help transport of tRNAs across the outer mitochondrial membrane in plant cells (Salinas et al., 2006) (**Figure 8G**). This mechanism is yet to be tested in the animal systems.

Although many have been detected, very few mitomiRs were functionally described to impact mitochondria (Baradan et al., 2017). Das et al. (2012) found *miR-181c*, Ago2, and *COX1* in mitochondrial co-immunoprecipitate, suggesting that mature *miR-181c* could translocate to mitochondria and together with Ago2 repress the translation of this mitochondrial transcript. Overexpression of *miR-181c* seems to lead to a loss of COX1 and an increase COX2 and COX3, resulting in complex IV remodeling. *miR-378* has been proposed to bind *ATP6* in mitochondria in the presence of Ago2 and FXR1, leading to a decrease of ATP6 in mouse type 1 diabetic heart (Jagannathan et al., 2015). *miR-1*, specifically induced during myogenesis, is able to promote translation of COX1 and ND1 within Ago2-miRNA complex in mitochondria, while, on the contrary, suppressing its target transcripts in the cytosol (Zhang et al., 2014). However, the binding of *miR-1* to mitochondrial transcripts has been suggested only by Ago2 CLIP experiments, and to date, *miR-1* is the only example of this non-canonical mitomiR function. Nevertheless, as many mitochondrial diseases are caused by defects in mitochondrial translation (Pearce et al., 2013), the upregulation of mitochondrial translation *via* miRNAs may be a new therapeutic route for these diseases which currently have no cure and few treatment options. Finally, a recent report reveals the role of mitomiRs in mitochondrial transcriptional regulation. *mitomiR-2392*, together with Ago2, was reported to recognize target sequences in the H-strand and partially inhibit polycistronic mtDNA transcription in a tongue squamous cell carcinoma (TSCC) cells, leading to downregulation of oxidative phosphorylation and upregulation of glycolysis (Fan et al., 2019).

To summarize, the identification of a miRNA inside mitochondria has, without a doubt, raised the interest in studying mitomiRs. However, mitomiRs are far from being well recognized. It is initially crucial to prevent any contamination during mitochondrial/mitoplast isolation to certain their mitochondrial localization. Furthermore, the mechanisms of their import, including interaction factors and

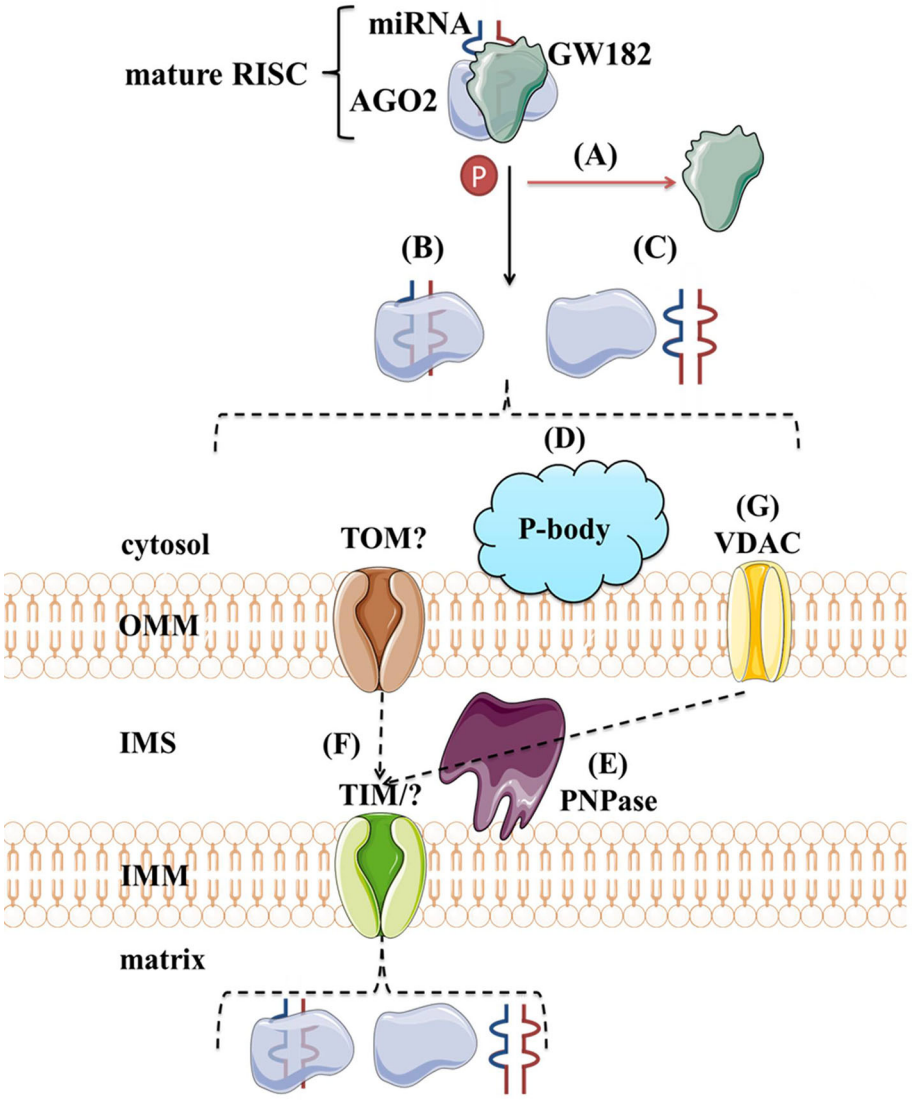

FIGURE 8 | Proposed import mechanisms of miRNAs to mammalian mitochondria. A detachment of AGO2 and miRNA from RISC or just GW182 due to AGO2 phosphorylation or some other signal activation **(A)** could promote their translocation together **(B)** or separately **(C)** into the mitochondria. This process could be stimulated by P-bodies **(D)**. Translocation across mitochondrial membranes is unknown but suggested to be promoted by PNPase **(E)** and occur within TOM/TIM complexes **(F)**. Alternatively, miRNAs could rely on VDAC **(G)** at the OMM, as proposed for tRNAs in plants. OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane; TOM/TIM, translocases of OMM/IMM; VDAC, voltage-gated ion channels.

important sequence features, and functions in mitochondria are yet to be elucidated. One should be aware that mitomiRs reported across various cell types and species show a very poor overlap. This could reflect species and cell type-specific expression of mitomiRs (Geiger and Dalgaard, 2017). On the other hand, such low reproducibility raises urgent questions regarding the techniques used in the published studies (Vendramin et al., 2017). Although several hypotheses concerning miRNA import into mitochondria have been proposed, it remains without convincing experimental validation. Finally, mitomiRs mode of action in mitochondria is largely enigmatic. On the one hand, only AGO2 from RISC has been proposed to reside in the mitochondria and on the other hand, mitochondrial mRNAs contain no or very small 3′ UTRs, questioning if they can function as canonical miRNAs.

LONG NON-CODING RNAS

The number of lncRNA genes in mammals varies broadly between different sources, from less than 20,000 to more than 100,000 in humans (Zhao et al., 2016; Kopp and Mendell, 2018). According to noncode.org, they are encompassing ∼144 000 loci in humans (Zhao et al., 2016). Intriguingly, although nucleus-enriched,

lncRNAs have been observed in different cell compartments, including mitochondria (Dong et al., 2017). Their biological activities are highly influenced by their localization in the cell (Mercer and Mattick, 2013; Fatica and Bozzoni, 2014). lncRNAs have been suggested to regulate cellular biology *via* transcriptional regulation, organization of nuclear domains, and bindings to proteins or other RNAs (Ulitsky and Bartel, 2013; Kopp and Mendell, 2018). It is therefore not surprising that their disruption has been associated with different diseases (Briggs et al., 2015; Huarte, 2015; Uchida and Dimmeler, 2015).

lncRNAs can be functionally classified into those that act *in cis*, and those that act *in trans* (Kopp and Mendell, 2018). ***In cis***, the lncRNA locus can regulate chromatin or gene expression of nearbye genes in at least three potential mechanisms: **(1)** DNA elements within the lncRNA promoter or locus carry the regulatory function, which is not related to the lncRNA or its production; **(2)** the act of transcription and/or splicing of the lncRNA affects nearby genes, irrespective of the transcribed lncRNA sequence; and **(3)** the lncRNA transcript alone affects the nearby genes, most commonly leading to the establishment of repressive or activating chromatin states. Some lncRNAs function *in trans* throughout the cell in, again, at least three potential mechanisms**: (1)** lncRNAs affect chromatin states and gene expression of distant genetic regions, **(2)** lncRNAs take part in the nuclear structure and organization (for example, as parts of speckles and paraspeckles), and **(3)** lncRNAs interact with proteins and/or other RNA molecules and modulate their expression and function (Lee, 2012; Rinn and Chang, 2012). Moreover, some transcripts initially annotated as lncRNAs are not non-coding, but actually encoding for biologically active *micropeptides* (Anderson et al., 2015; Matsumoto et al., 2017; Kopp and Mendell, 2018).

Over twenty lncRNAs have been described so far to affect the mitochondrial biology directly or indirectly. Some act in the cytosol, by regulating mitochondria-associated genes, often in interaction with miRNA, thus creating a complex mRNA-ncRNA regulation network. Other nuclear-encoded lncRNAs have been described to localize and act in mitochondria. As their transport mechanism into mitochondria is unknown their presence remains questionable. Finally, several lncRNAs have been discovered to be transcribed from mtDNA. These two latter mitochondria-localized, but origin-different lncRNAs could be refered to as nuclear-transported mitochondria-associated lncRNAs (ntmtlncRNAs) and mitochondria-encoded lncRNAs (mtlncRNAs) (Zhao et al., 2018).

Cytoplasmic lncRNAs With Impact on Mitochondria

Several lncRNAs, some previously well described in the non-mitochondrial function, have been associated with mitochondrial metabolism. As in the case of miRNAs, these lncRNAs were proposed to impact a variety of mitochondrial functions by directly targeting or indirectly influencing mitochondrial-related genes/transcripts/proteins. It should be noted that most of these studies report an indirect effect of lncRNAs perturbations on mitochondria function. Besides, most of these lncRNAs were reported in the context of complex systems such as cancer. Nevertheless, they could present possible treatment strategies (De Paepe et al., 2018). A summary of these findings is given in **Table 5**, with several examples given below.

Cerox1 (cytoplasmic endogenous regulator of oxidative phosphorylation 1) has been described as the first direct lncRNA modulator of OXPHOS. It has been reported to positively regulate the levels of at least 12 complex I transcripts in miRNA-dependent fashion, by binding *miR-488-3p* and blocking its post-transcriptional repression of these transcripts and enabling translation. *Cerox1* knockdown was shown to decrease the enzymatic activities of complex I and IV. Accordingly, its overexpression was shown to increase their enzymatic activities and halve the cellular oxidative stress (Sirey et al., 2019).

Long et al. (2016) have described *Tug1* as a regulator of *PGC-1α* transcription in diabetic nephropathy (DN). *Tug1*-binding site was identified upstream of the Ppargc1a promoter region. *Tug1* interaction with this region recruited PGC-1α to promote its own gene transcription. *Tug1* expression was significantly repressed in the podocytes of diabetic mice and its overexpression lead to improved mitochondrial bioenergetics (Long et al., 2016).

Li et al. (2017) proposed the pro-oncogenic role of lncRNA *UCA1* in bladder tumors. *UCA1* is supposed to regulate mitochondrial function through upregulating *ARL2*, a direct target of *miR-195*. In this way, it inhibits the *miR-195* signaling pathway, leading to a tumor growth (Li et al., 2017).

Nuclear-Transported Mitochondria-Associated lncRNAs (ntmtlncRNAs)

Several nuclear-encoded lncRNAs have been reported in mitochondria and proposed to regulate their biology

TABLE 5 | Nuclear-encoded lncRNAs affecting mitochondria-related genes.

lncRNA	Target	Reference
AK055347	*Cyp450, ATP synthase, MSS51*	Chen G. Y. et al., 2016
ANRIL	*PARP, Bcl-2*	Zhu et al., 2015; Liu B. et al., 2016
CARL	*PHB2*	Wang et al., 2014
BATE1	hnRNPU	Alvarez-Dominguez et al., 2015
CCAT2	*GLS*	Redis et al., 2016
Cerox	*miR-488-3p*	Sirey et al., 2019
ENSMUST00000136025	*BIM*	Chen X. et al., 2016
FAL1	*DRP1*	Liu et al., 2019
GAS5	*BAX, BAK*	Gao et al., 2015
HOTAIR	MICU1, UQCRB	Kong et al., 2015; Zheng et al., 2015
H19	VDAC1	Li et al., 2016
HOTTIP	*GLS*	Ge et al., 2015
MEG3	*Bcl-2*	Wang et al., 2015b; Liu B. et al., 2016
MPRL	*miR-483-5p*	Tian et al., 2019
Pvt1	*c-Myc, Lipe, Cpt1a*	Alessio et al., 2019
Tug1	*PGC1-α*	Long et al., 2016
UCA1	*ARL2, miR-16, GLS*	Li et al., 2015; Li et al., 2017
UIHTC	*PGC1-α*	Zhang et al., 2018

(Vendramin et al., 2017; Zhao et al., 2018). However, due to a very limited number of publications and unresolved import mechanism, the presence and role of these lncRNAs are yet to be confirmed.

SAMMSON is predominantly expressed in aggressive melanomas, where it was described as a promoter of cell growth (Leucci et al., 2016; Vendramin et al., 2018). It has been proposed to bind to CARF and promote its binding to p32 in the cytosol (Vendramin et al., 2018). p32 is a mitochondrial and cytosolic protein that is required for the maturation of mitochondrial rRNAs (Wu H. et al., 2013), but also described as an important player in tumor metabolism (Fogal et al., 2010). Its interaction with CARF *via SAMMSON* promotes its mitochondrial targeting, where it increases protein synthesis, leading to an increased tumor cell growth (Vendramin et al., 2018). Knockdown of *SAMMSON* was shown to impair the p32 targeting to the mitochondria, resulting in mitochondrial protein synthesis defects and increased apoptosis, which could be of therapeutical potential (Leucci et al., 2016). As a fraction of *SAMMSON* was found to co-localize and co-purify with mitochondria, Leucci et al. (2016) proposed that it is accompanying p32 to the mitochondria.

The steroid receptor RNA activator (*SRA)* is an important coactivator of nuclear hormone receptors and a target for several RBPs, namely SHARP and SLIRP (Colley et al., 2008). By interaction with SRA, SHARP represses *SRA*-augmented estrogen-induced transactivation (Shi et al., 2001). SLIRP binds to the complex of *SRA* and SHARP and interferes with the repressing activity of SHARP. However, SLIRP is predominantly localized to the mitochondria (Colley et al., 2008; Pagliarini et al., 2008), where it regulates the expression, processing, and stability of mRNAs (Baughman et al., 2009; Dong et al., 2017). *SRA* and SLIRP were found in mitochondria, but their import and roles are yet to be explained (Dong et al., 2017).

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is one of the most-studied lncRNAs, mostly associated with cancer and metastasis (Wu et al., 2015; Sun and Li, 2019). Recently, Zhao et al. (2019) discovered that *MALAT1*, although normally enriched in the nucleus, to be also enriched in the mitochondria collected from HepG2 cells. *MALAT1*-deficient HepG2 cells produced less ATP and had impaired cell invasion, suggesting a role of this lncRNA in the mitochondrial metabolism (Zhao et al., 2019).

Mitochondria-Encoded lncRNAs (mtlncRNAs)

Sets of lncRNAs have been reported to be transcribed from the mtDNA (**Figure 2**). Surprisingly, it has been noted that some of these lncRNAs seem to operate in the nucleus. However, their trafficking raises questions far beyond the current knowledge (Dietrich et al., 2015; Vendramin et al., 2017). Up to this date, the existence and functional relevance of these lncRNAs are still debatable. Mitochondria-encoded lncRNAs are divided into three categories:

1. Simple antisense mitochondrial DNA-encoded lncRNAs

Antisense transcripts arising from the ND4 and ND6 loci were initially detected in cDNA libraries of mice mitochondria, but Northern blot failed to confirm their presence (Lung et al., 2006). Later, strand-specific RNA-seq of purified mitochondria identified *lncND5*, *lncND6*, and *lncCytb* as antisense transcripts (Mercer et al., 2011). Rackham et al. (2011) confirmed existence of these transcripts by RNA-seq and RT-qPCR, additionally revealing that they are 58%, 34% and 14% as abundant as their mRNA counterparts, respectively. These antisense RNAs create RNA-RNA duplexes with their complementary mRNAs, suggesting their role in mRNAs expression and stability (Rackham et al., 2011). Interestingly, Zhao et al. (2019) discovered that *lncCytB* is aberrantly transported to the nucleus in hepatoma HepG2 cells as compared with normal hepatic HL7702 cells, suggesting a new function of this lncRNA as a mitochondria-nuclear communicator in cancer. Furthermore, Gao et al. (2018) discovered within the PacBio full-length transcriptome dataset the lncRNA *MDL1*, which covers the $tRNA^{Pro}$ antisense gene and the entire D-loop region, and its antisense transcript *MDL1AS*.

2. Chimeric mitochondrial DNA-encoded lncRNAs

The first member of this class was discovered in mouse cells, comprised of the 16S rRNA linked to a 121 nucleotide 5′-leader sequence deriving from its complementary strand (Villegas et al., 2000). Afterward, similar transcript, called sense mitochondrial ncRNA (*SncmtRNA*), was identified in humans, and in this case, the mitochondrial 16S rRNA is linked to an 815 nucleotide 5′-leader sequence from its complementary strand (Villegas et al., 2007). *SncmtRNA* forms an 820 bp, double-stranded structure with a 40 nucleotide loop (Dietrich et al., 2015). Interestingly, *SncmtRNA* was only detected in the proliferating tumor but not in resting cells, suggesting that it might serve as a marker of cell proliferation (Villegas et al., 2007). Later, two antisense lncRNAs, called *ASncmtRNA-1* and *ASncmtRNA-2* were discovered. Here, the antisense mitochondrial 16S rRNA is linked to a 310 or 545 nucleotide 5′-leader sequence deriving from the complementary sense strand (Burzio et al., 2009). These two transcripts also form distinct double-stranded structures with a nucleotide loop. In contrast to *SncmtRNA*, they were detected mainly in normal cells and were much less expressed in proliferating tumor cells, suggesting their role as tumor suppressors (Burzio et al., 2009). Later, they were reported to be present in the nucleus associated with heterochromatin (Landerer et al., 2011). However, more data is needed to support this claim. It has been postulated that *ASncmtRNA-2* gets transported into the nucleus, where it presents a precursor of two miRNAs (*hsamiR-4485* and *hsa-miR-1973*), which could potentially regulate survivin, an inhibitor of apoptosis (Vidaurre et al., 2014; Bianchessi et al., 2015). Indeed, knockdown of *ASncmtRNAs* promoted apoptotic cell death due to the survivin downregulation at the translational level (Vidaurre et al., 2014).

3. Putative mitochondrial DNA-encoded lncRNAs

These lncRNAs have been identified in the heart disease studies (Kumarswamy et al., 2014; Yang et al., 2014; Dietrich

et al., 2015). RNA-seq revealed a high relative abundance (over 70%) of these transcripts in the total lncRNA population from patients with a severe heart failure (Yang et al., 2014). The most significant lncRNA has been named long intergenic noncoding RNA predicting CARdiac remodeling (*LIPCAR)*. Aligning the *LIPCAR* sequence to the human mtDNA revealed that the 5′ half aligns to the *lncCytb*, while the 3′ half aligns to the antisense region of *COX2* (Dorn, 2014). As its circulating levels were increased in the late stages of left ventricular remodeling and patients with chronic heart failure, *LIPCAR* could be used as a prognostic biomarker (Kumarswamy et al., 2014; Dietrich et al., 2015).

To conclude, lncRNAs are slowly but surely drawing attention with their complex mechanisms behind gene regulation. However, the physiological relevance of lncRNAs in mitochondria is still enigmatic. The crucial issue is the investigation of transport of the nuclear- or mtDNA-encoded lncRNAs to mitochondria and even to the nucleus. Unfortunately, there is no published data on the topic so far. Finally, the questions of specific lncRNAs mechanisms of gene regulation remain to be solved.

LNCRNA-ENCODED MICROPEPTIDES

Micropeptides are a class of small peptides encoded by a sORFs, without N-terminal signaling sequence and as such are released into cytoplasm immediately after translation. Due to their sORF that escapes automatic gene annotation, they tend to be overlooked and therefore misannotated as non-coding. Indeed, lncRNAs and TUFs (transcripts of unknown function) represent the greatest source for sORFs (Yeasmin et al., 2018). Although numerous ribosome profiling studies have reported substantial ribosome occupancy of the lncRNA transcripts, the MS and the proteogenomic approaches have confirmed only a small portion of them, numbers ranging from less than 100 to up to 1600 (van Heesch et al., 2019). With a lack of consensus in the datasets, the true coding potential of lncRNAs currently remains open to speculation. Several in-depth investigations have characterized lncRNA-derived micropeptides with important roles in the ion channel modulation (Anderson et al., 2015), cell signaling (Matsumoto et al., 2017) and RNA regulation (D'Lima et al., 2017). It is important to state that the mammalian mitochondrial proteome is surprisingly enriched in micropeptides, accounting for 5% of its proteins (Calvo et al., 2016). In recent years, several micropeptides within lncRNA were discovered and characterized with a role in mitochondria, some even encoded by the mtDNA (Kim et al., 2017a). Termed mitochondrial-derived peptides (MDPs) (Kim et al., 2017a), these mtDNA-encoded peptides-humanin, MOTS-c, and SHLPs were described as potential mitochondrial bioenergetics and metabolism regulators.

Mitoregulin (MOXI, MPM) has been discovered by four different groups recently as a muscle- and heart-enriched 56-amino acids inner mitochondrial membrane micropeptide encoded within *LINC00116*. It has a role in mitochondrial respiratory chain supercomplexes support, fatty acids oxidation, and Ca^{2+} dynamics (Makarewich et al., 2018; Stein et al., 2018; Chugunova et al., 2019; Lin et al., 2019). Lin et al. (2019) highlighted its importance in the muscle tissue, finding it upregulated during myogenic differentiation and knockout mice exhibiting smaller skeletal muscle fibers, worse muscle performance, and slower regeneration.

Humanin is a 24-amino acids micropeptide whose sORF is embeded within the 16S rRNA of mtDNA (Yen et al., 2013). It was initially discovered in the surviving cells of Alzheimer's disease brain (Hashimoto et al., 2001), suggesting its neuroprotective and cytoprotective role that has later been investigated and acknowledged across various diseases (Hashimoto et al., 2001; Muzumdar et al., 2009; Bachar et al., 2010; Oh et al., 2011; Gong et al., 2014; Kim et al., 2018). It was shown to block apoptosis, improve insulin sensitivity, decrease inflammation, and reduce oxidative stress during aging (Guo et al., 2003; Muzumdar et al., 2009; Zhao et al., 2013; Sreekumar et al., 2016). Its effects are yet to be assessed for therapeutic purposes, especially in the treatments of diabetes and neurodegenerative disorders.

MOTS-c (mitochondrial open reading frame of the 12S rRNA type-c) is a 16-amino acids micropeptide with an sORF within the 12S rRNA mtDNA and reported to act in the cytoplasm (Lee et al., 2015). The micropeptide was found to target the methionine-folate cycle and *de novo* purine biosynthesis pathway, increase AICAR levels, and activate AMPK, by which it increases glucose utilization, fatty acid oxidation, and changes nucleotide metabolism. MOTS-c has been proposed as a biomarker for metabolic function, as it correlates with markers of insulin resistance and obesity (Du et al., 2018). In high fat diet-induced obese mice, it prevented obesity, fat accumulation, and hyperinsulinemia, making it a possible therapeutic target (Lee et al., 2015).

SHLPs (small humanin-like peptides) are a group of 6 peptides discovered by an *in silico* approach to be encoded in the 16S rRNA region of mtDNA in mice (Cobb et al., 2016). Each peptide is 20-38 amino acids long, and their names were given due to similar biological effects as Humanin. Each SHLP showed a unique expression pattern across different tissues. Incubation of each synthetic SHLP with cells affected cell viability, proliferation, and apoptosis differentially, suggesting a specific role of each. Moreover, SHLP2 and SHLP3 induced oxygen consumption rate (OCR) and increased cellular ATP levels, which indicated them as mitochondrial modulators (Cobb et al., 2016). Indeed, the administration of SHLP2 to a cellular model of macular degeneration rescued its defects in the OXPHOS and the mtDNA copy number, and induced anti-apoptotic effects, indicating its therapeutic potential (Nashine et al., 2018). In addition, an intracerebral infusion of SHLP2 increased glucose uptake and suppressed hepatic glucose production (Cobb et al., 2016). Further supporting their role as insulin sensitizers, both SHLPs promoted pre-adipocyte differentiation (Cobb et al., 2016). Similarly to humanin, the circulating levels of MOTS-c and SHLP2 declined with age, indicating that they are potential regulators of aging (Lee et al., Lee et al., 2015; Cobb et al., 2016).

CONCLUDING REMARKS

Development of high-throughput OMICS techniques, especially the next-generation sequencing, has shed new light on the non-coding fraction of the genome. Transcription of the majority of the eukaryotic genome generates not only mRNAs but a much bigger fraction of different ncRNA species that show complex structure, patterns of expression and regulation. It is now becoming apparent that RNAs are not important for cell only in the context of mRNAs as intermediates between DNA and protein, but also as powerful players themselves by affecting basically any stage of gene expression. The now expanding RNA field highlights the importance of bioinformatics analysis in order to predict and examine existence, evolution, structure, and function of non-coding regions and transcripts. Focusing on mitochondria, dozens of ncRNAs acting in the cytosol have been described to indirectly influence mitochondrial biology, usually by targeting mitochondria-related, nuclear-encoded transcripts. More surprisingly, recent research indicated that the mitochondrial transcriptome could represent a mixture of the intrinsic transcriptome and complemented by some extrinsic RNA, implying RNA import (**Figure 1**). Although dozens of papers reported ncRNAs in mitochondria, their existence is still under a question mark. Further research will need to identify their interacting partners and elucidate the molecular mechanisms behind their synthesis, transport, and function. Housekeeping ncRNAs have been proposed to have a mitochondrial localization even for decades, however, recent deeper insights into the mitochondrial biology have cast a shadow on their hypothesized role. It is clear that the re-evaluation of their presence and especially function in mitochondria is needed. Focusing on miRNA, they are well-described fine-modulators of gene regulation in the cytosol. It is not surprising that they can impact mitochondria by targeting its transcripts in the cytosol. Additionally, recent discoveries of mitomiRs suggest an attractive, even closer interplay of miRNAs and mitochondria occurring in mitochondria themselves. Yet, these findings are still a topic of many debates and therefore should be handled with caution. On the one side, the discovery of mitomiRs across different tissues and cell types by different techniques promises they are more than a false-positive finding. However, on the other side is the poor overlap between datasets that raises doubts concerning methods used. Focusing on lncRNAs, although they are among the least well-understood of these transcript species, they are slowly but surely emerging as important components of gene regulatory networks. Although the field of lncRNAs has just started to expand, published reports indicate that they influence mitochondria in different ways. Moreover, mtDNA seems to encode some lncRNAs itself. However, this field is still very fresh and further confirmation is needed, especially in the case of mitochondria-imported lncRNAs. Of clinical relevance, ncRNAs dysregulation has been noted in various mitochondria-related diseases, mostly cancer. Their association with tumorigenesis has been increasingly demonstrated. As ncRNAs often exhibit cancer-type-specific expression patterns (Iyer et al., 2015), targeting them could prove as a very selective and specific approach. Notably, they can be targeted by the antagomiRs or antisense oligonucleotides (ASOs) (reviewed by Matsui and Corey, 2017). Indeed, several pre-clinical studies have already demonstrated the therapeutic benefits of ncRNA inhibition. For example, inhibition of *SAMMSON* in melanoma xenografts suppressed the tumor growth (Leucci et al., 2016). ASOs targeting *ASncmtRNA* reduced the progression of renal adenocarcinoma and melanoma metastases in mice (Lobos-Gonzalez et al., 2016; Borgna et al., 2017). Finally, ncRNA-derived micropeptides, although biologically active as peptides, are especially interesting in terms of their discovery. As many ribosomal-profiling studies report significant ribosomal occupancy of non-coding transcripts, it is evident that further confirmation of these findings by mass spectrometry is needed in order to recognize the importance of these reported translational activities. Discoveries of mitochondrial-derived peptides and enrichment of the mammalian mitochondrial proteome in micropeptides suggest the organelle as an evolutionary playground for small proteins, either due to still unknown localization signals or import system or simply driven by the size or amino acid (positive charge) composition (van Heesch et al., 2019). This also promises that there could be many micropeptides hidden in the non-coding region, awaiting discovery and characterization. Of clinical interest, discovered mitochondria-derived micropeptides have exhibited a variety of cyto- and neuroprotective effects, and promising results of both *in vitro* and *in vivo* studies further strengthen their therapeutic potential. Overall, ncRNAs in mitochondria present a thought-provoking, but unfortunately still neglected field of study. It raises many interesting, but also challenging questions whose answers might be of clinical importance. It may reveal some enigmatic biological mechanisms (such as the RNA import in mitochondria) and eventually lead to the development of new therapeutic strategies for mitochondria-related diseases. However, before the field of ncRNA truly expands, there are still a lot of experimental approaches to be optimized and biological mechanisms to be deciphered to conclude their importance for mitochondria.

AUTHOR CONTRIBUTIONS

MG: conceived the topic for the review, wrote the manuscript and created the tables and figures. HP: helped shape the review, supervised the writing process, provided the critical feedback, contributed to the final version of the manuscript.

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CORRECTION
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Corrigendum: ncRNAs: New Players in Mitochondrial Health and Disease?

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In the original article, there were mistakes in **Figure 5** and **Figure 6** as published. Figures are stating miR-167b instead of the correct miR-147b. The corrected **Figure 5** and **Figure 6** appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

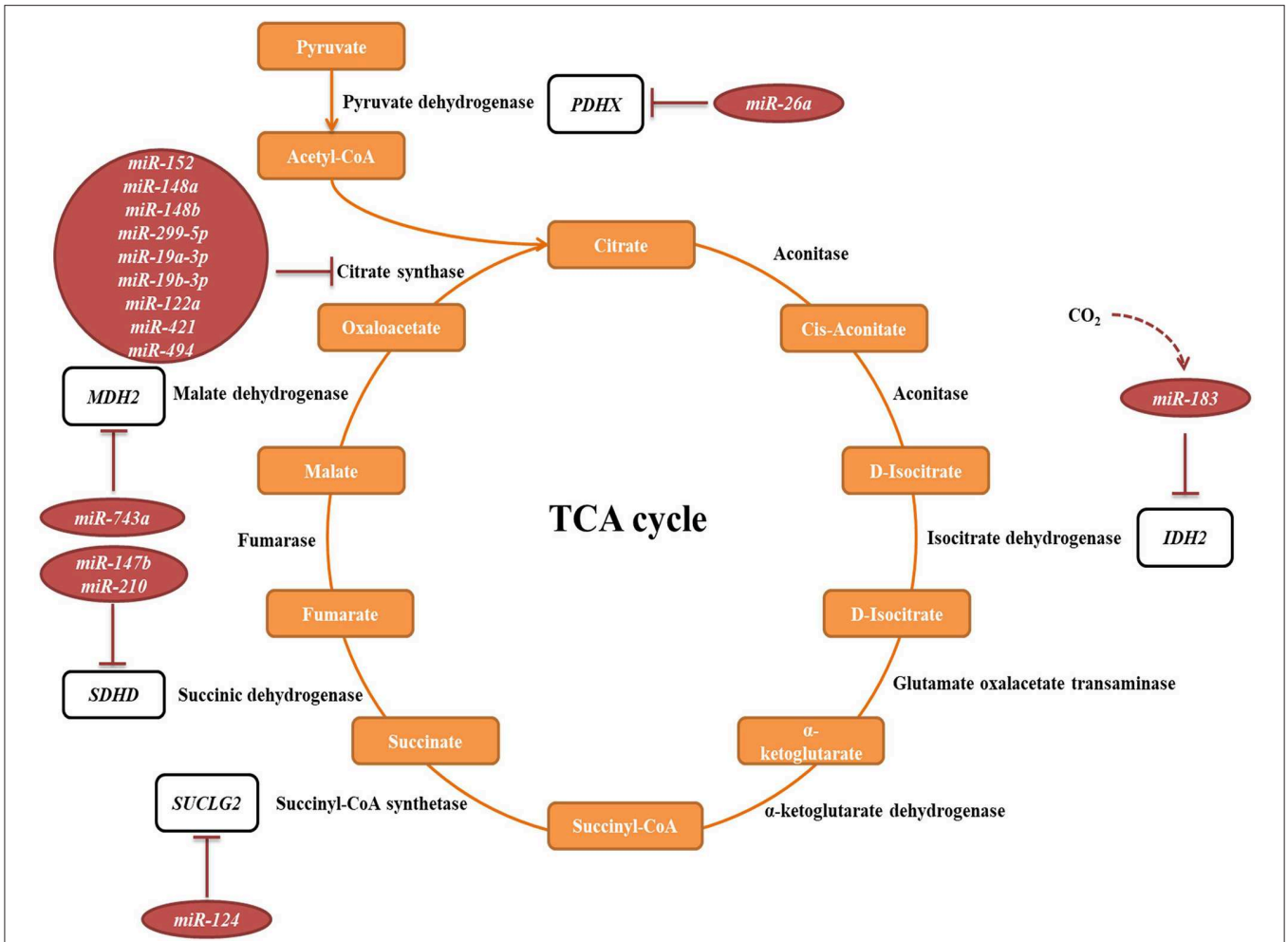

FIGURE 5 | miRNAs targeting transcripts encoding proteins involved in the TCA cycle. Red arrows present the repressing effect of miRNA on its target mRNA.

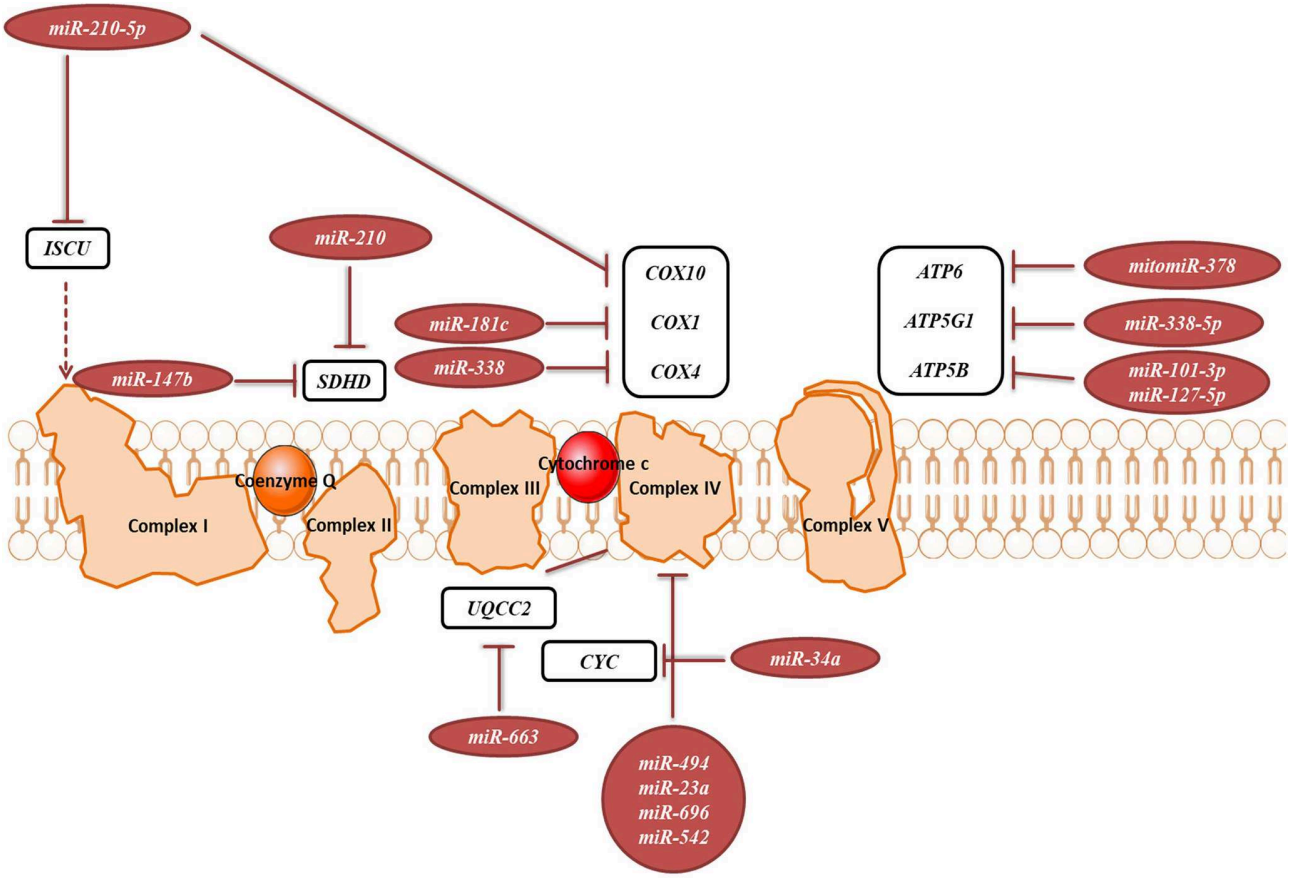

FIGURE 6 | miRNAs targeting transcripts encoding proteins involved in the OXPHOS. Red arrows present the repressing effect of miRNA on its target mRNA.

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BRIEF RESEARCH REPORT
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No Evidence of Persistence or Inheritance of Mitochondrial DNA Copy Number in Holocaust Survivors and Their Descendants

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Mitochondrial DNA copy number has been previously shown to be elevated with severe and chronic stress, as well as stress-related pathology like Major Depressive Disorder (MDD) and post-traumatic stress disorder (PTSD). While experimental data point to likely recovery of mtDNA copy number changes after the stressful event, time needed for full recovery and whether it can be achieved are still unknown. Further, while it has been shown that stress-related mtDNA elevation affects multiple tissues, its specific consequences for oogenesis and maternal inheritance of mtDNA has never been explored. In this study, we used qPCR to quantify mtDNA copy number in 15 Holocaust survivors and 102 of their second- and third-generation descendants from the Czech Republic, many of whom suffer from PTSD, and compared them to controls in the respective generations. We found no significant difference in mtDNA copy number in the Holocaust survivors compared to controls, whether they have PTSD or not, and no significant elevation in descendants of female Holocaust survivors as compared to descendants of male survivors or controls. Our results showed no evidence of persistence or inheritance of mtDNA changes in Holocaust survivors, though that does not rule out effects in other tissues or mitigating mechanism for such changes.

Keywords: mitochondrial DNA, posttraumatic stress disorder, copy number variation, quantitative PCR, Holocaust-psychic trauma

INTRODUCTION

Mitochondrial DNA (mtDNA) occurs in hundreds to thousands of copies in each cell. The levels of mtDNA is tissue-specific (Robin and Wong, 1988; Falkenberg et al., 2007; Kelly et al., 2012), and dependent on genetic factors (Scarpulla, 2008; Cai et al., 2015a; Kukat et al., 2015) and environmental stimuli. Increase in mtDNA copy number has been found to be associated with a wide range of psychological stress, including childhood parental loss, maltreatment (Tyrka et al.,

2016; Ridout et al., 2019), sexual abuse (Cai et al., 2015b) and a wide range of stressful events over one's lifetime (Cai et al., 2015b). As such, alterations to mitochondrial function are increasingly investigated as a key mechanism underlying stress-related conditions (Zhang et al., 2006; Manoli et al., 2007; Su et al., 2008). An increase in mtDNA levels obtained from blood samples has been reported in recurrent Major Depressive Disorder (MDD) (Cai et al., 2015b; Edwards et al., 2016) and a decreased in mtDNA levels has been shown in moderately severe post-traumatic stress disorder (PTSD) (Bersani et al., 2016). Other reports showing conflicting results. The former has been shown to be more pronounced in those experiencing an episode of MDD than those with a history of it, and are reversible upon cessation of the stressful stimuli in animal models (Cai et al., 2015b), demonstrating both disease state-dependence and potential for recovery. Among individuals with a history of severe recurrent MDD, chronicity of disease state was positively associated with mtDNA levels (Edwards et al., 2016), suggesting complete recovery may not be achieved or requires a long time.

Stress-related changes in mtDNA may be mediated by an alteration of hypothalamic–pituitary–adrenal (HPA) axis reactivity (Cai et al., 2015b), likely partly accounting for mtDNA changes seen in both MDD (Holsboer, 2000; Kloet et al., 2005) and PTSD (van Zuiden et al., 2012). Other biological processes have been proposed as mechanisms for stress-related changes in mtDNA, including mitochondrial biogenesis mediated by stress-induced increase in reactive oxygen species (ROS) (St-Pierre et al., 2006; Scarpulla et al., 2012), and mtDNA damage induced apoptosis and release of mtDNA (Lindqvist et al., 2018). A range of broad, converging or co-existing pathways may explain inconsistencies between studies primarily capturing effects of different pathways (Kageyama et al., 2018; Tymofiyeva et al., 2018; Verhoeven et al., 2018; Wang et al., 2018; Tsujii et al., 2019; Czarny et al., 2019), an increasing number of conditions associated with changes in mtDNA levels (Carew et al., 2004; Lan et al., 2008; Malik et al., 2009), and tissue-specificity of mtDNA changes (Cai et al., 2015b).

The mechanism of intergenerational transmission of trauma is not understood. One possibility is that the transmission is based on social mechanisms, another is it is mediated by genetic or epigenetic changes. While animal models have shown that stress-related mtDNA changes likely affect multiple tissues including the ovary (Cai et al., 2015b), it is unknown if stress has an effect on mtDNA in mature oocytes in females, and if such changes may be inherited. It is widely recognized that there is an mtDNA bottleneck during the development of primordial germ cells in female human embryos (Giles et al., 1980), followed by a gradual thousand-fold expansion (Cotterill et al., 2013) of mtDNA during oogenesis (Jenuth et al., 1996; Reynier et al., 2001; Barritt et al., 2002; Cree et al., 2008; Wai et al., 2008). However, little is known of whether maternal stress in the prenatal or gestational period would affect the mtDNA bottleneck or mtDNA expansions during oogenesis. Though placental mtDNA levels at birth has been found to be inversely correlated with maternal prenatal negative events, PTSD, depressive symptoms and lifetime stress (Brunst et al., 2017), it remains unclear whether maternal stress has any lasting effect on mtDNA levels in descendants and their health and fertility (Reynier et al., 2001; Santos et al., 2006).

In this study, we investigate whether mtDNA increases during a stressful life event may persist throughout one's lifetime and affect the mtDNA levels of one's descendants, and whether that is dependent on the development of a persistent stress-related pathology. We assess the relative levels of mtDNA obtained from peripheral blood mononuclear cells (PBMC) of Holocaust survivors in Czech Republic, most of them from the Jewish communities of Brno and Prague, and of two generations of their descendants, many of whom suffer from PTSD, with age-matched individuals from the same generations who were not exposed to this extreme circumstance and its consequences (Konečná et al., 2019). The Holocaust survivors represent a group of people who have gone through extreme physical and psychological trauma early in life, and have lived to a relatively old age. We took the unique opportunity to examine this specific group of people in this study.

RESULTS

Holocaust Survivors and Their Descendants

We obtained DNA samples from peripheral blood mononuclear cells (PBMCs, see *Methods*) from 235 individuals recruited for this study, 196 of which passed DNA quality control and were successfully analysed with quantitative polymerase chain reaction (qPCR) for mitochondrial DNA (mtDNA) copy number (*Methods*) and used for all analysis in this manuscript. Seventy seven of these individuals were men and 119 women. One hundred seventeen of them were first generation (G1) Holocaust survivors or their second (G2) and third generation (G3) descendants (n = 15, 60 and 42 for G1–3 respectively) and 79 (n = 22, 37, 20 for G1–3 respectively) were controls from all three generations (**Figure 1A**). Using this sample, we have 0.8 statistical power to find effect sizes of Holocaust survivor status on mtDNA copy number that are larger than 0.48, 0.29 and 0.32 in G1 to G3 using linear regression respectively. We will not have adequate power to detect effects smaller than this.

Holocaust survivors and their descendants are not significantly different in terms of age from the controls as a whole cohort (t-test P = 0.148, **Figure 1B**), in individual generations (t-test P = 0.048, 0.982 and 0.105 respectively for G1–3), or in each sex (t-test P = 0.095 and 0.855 in males and females respectively, **Figure 1C**). While there are different numbers of men and women in each generation, there is no significant difference by sex between the number of controls and Holocaust survivors and their descendants across generations (Fisher's exact test P = 0.768) or within individual generations (G1 Fisher's exact test P = 1; G2 Fisher's exact test P = 0.27; G3 Fisher's exact test P = 0.59, **Figure 1D**).

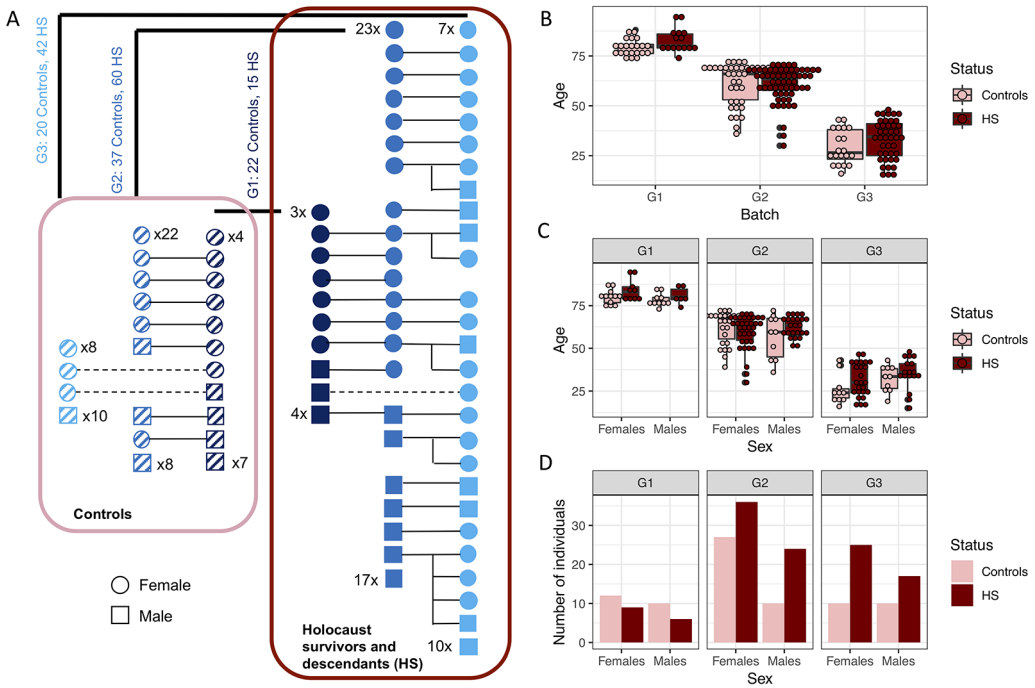

FIGURE 1 | Study participants. **(A)** Schematic diagram showing relationships between generation 1 (G1) Holocaust survivors and controls with generations 2 and 3 (G2 and G3) descendants. Males are depicted as squares and females as circles; Holocaust survivors and their descendants are depicted colored solid navy, blue and cyan squares and circles for G1, G2 and G3 respectively, and controls of each generation are depicted as squares and circles filled with slanted dashes. Numbers of those individuals without first degree relationships with other individuals in this study are written for each generation. All Holocaust survivors and their descendants are encircled by a dark red rectangle, while all controls are encircled by a pink rectangle. Numbers of Holocaust survivors or their descendants and controls are written for each generation. **(B)** Boxplot of the age of Holocaust survivors and their descendants (in dark red), and controls (in pink), for each generation. **(C)** Boxplot of age of female and male Holocaust survivors and their descendants (in dark red) and controls (in pink) for each generation. **(D)** This figure shows the number of female and male Holocaust survivors and their descendants (in dark red) and controls (in pink) participating in this study with mtDNA successfully quantified through qPCR.

mtDNA Copy Number Is Associated With Age and Sex

mtDNA copy number was quantified using qPCR on 196 individuals across three batches and 17 plates, each with a different threshold quantification cycle (CT threshold). As individuals were randomized across the plates, there was no significant difference between the plates on sex (Chi-squared test P value = 0.856), holocaust experience (Chi-squared test P value = 0.396), or PTSD status (Chi-squared test P value = 1). In addition to normalizing the raw mtDNA copy number measured in each plate using measures from a reference DNA sample of constant concentration (resulting in ΔΔCT values representing raw measures of mtDNA copy number, *Methods*), we further corrected the raw mtDNA copy number measure for the following: CT threshold across plates (ANOVA P value $< 10^{-16}$, variance explained = 0.45, **Figure 2A**), PCR batch (ANOVA P value = 1.26×10^{-4}, variance explained = 0.05, **Figure 2B**), and concentration of DNA extracted from PBMC before dilution for qPCR (P value = 0.01, variance explained = 0.01, **Figure 2C**). These capture plate effects, batch effects due to difference in reagent batches, and experimental error in dilution respectively. We then quantile normalized the residuals across all individuals to obtain our final measure of mtDNA copy number for analysis.

As both sex and age have previously been shown to contribute to variations in mtDNA copy number, we first asked if we can observe previously found trends among the controls. While mtDNA copy number is not significantly associated with age in the whole cohort (linear regression P = 0.58, beta = −0.002, se = 0.004), it significantly decreases with age in G2, the single generation with the largest age range (30–73, P = 0.05, beta = −0.02, se = 0.01, **Figure 2D**) where no individuals had personally experienced holocaust. This is consistent with previous reports demonstrating decrease of mtDNA levels with age (Mengel-From et al., 2014). We also observed significantly higher levels of mtDNA in males in the whole cohort (linear regression P = 0.01, beta = 0.37, se = 0.14, **Figure 2E**), as well as in the youngest generation G3 (age range 15–48, 35 males/27 females, linear regression P = 0.02, beta = 0.51, se = 0.21). We found no interactions between age and sex effects on mtDNA copy number in the whole cohort (interaction P value = 0.67, **Figure 2F**) or any individual generations (interaction P values = 0.985, 0.258 and 0.448 for G1–3 respectively).

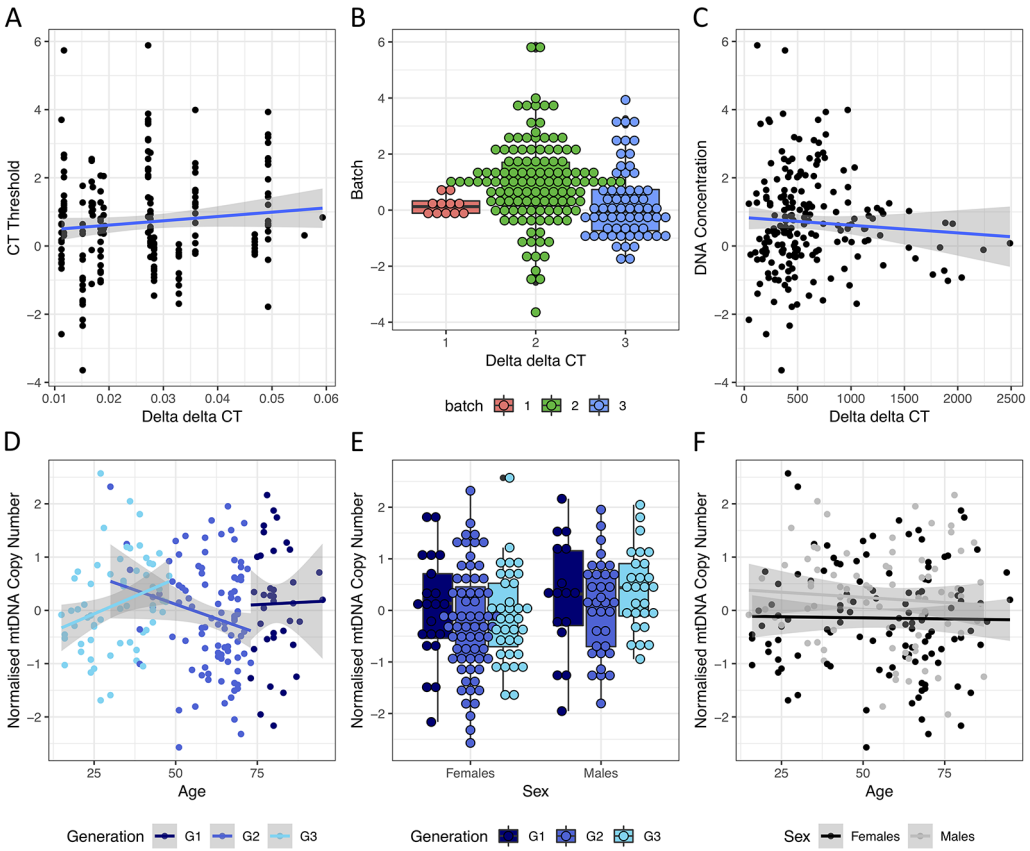

FIGURE 2 | Effects of technical and biological covariates on mtDNA copy number measurements. **(A)** Relationships between ΔΔCT values, representing raw mtDNA copy number measures from qPCR, and the threshold CT values for each qPCR run. **(B)** Boxplot of ΔΔCT values from each qPCR batch. **(C)** Relationship between ΔΔCT values and starting DNA concentration (before dilution) of each sample. **(D)** Relationship between normalized mtDNA copy number measure (after correcting for threshold CT values, qPCR batch and starting DNA concentration) and age in individuals from G1, G2 and G3 in navy, blue and cyan respectively. **(E)** Boxplot of normalized mtDNA copy number in female and male individuals from G1, G3 and G3 in navy, blue and cyan respectively. **(F)** Relationship between normalized mtDNA copy number and age in females (in black) and males (in grey) respectively.

Low Variation in mtDNA Copy Number Among Haplogroups

As all Jewish individuals in Czech Republic were persecuted and experienced the Holocaust during World War II, one of the confounding factors in this study is the controls are not of the same origin as the Holocaust survivors, and may not have the same mtDNA Haplogroups as them. If mtDNA haplogroups have an effect on mtDNA copy number, systematic differences in mtDNA haplogroups between Holocaust survivors and controls can lead to spurious findings. We therefore investigated whether mtDNA copy number differ between Haplogroups in an independent sequencing dataset, in order to assess how likely a mismatch in mtDNA Haplogroups can lead to spurious findings. We obtained mtDNA copy numbers (*Methods*) from whole-genome sequencing of lymphoblastic cell lines (LCLs) of individuals in Phase 3 of the 1000 Genomes Project (Consortium and 1000 G. P. and The 1000 Genomes Project Consortium, 2015) (1000G) for this investigation (**Figure 3A**). To obtain the Haplogroups of each individual in 1000G, we called homoplasmic variants from the mtDNA using HaplogroupCaller in GATK v4 (*Methods*), and called Haplogroups using these variants with Haplogrep v2 (Consortium and 1000 G. P. and The 1000 Genomes Project Consortium, 2015; Weissensteiner et al., 2016) (**Figure 3B**, *Methods*).

Figure 3C shows the relative mtDNA copy number in different Haplogroups represented in 1000G. Testing each haplogroups against all others for association with mtDNA copy number (**Table 1**), we found that mtDNA copy number is significantly higher in Haplogroup L (beta = 0.38, se = 0.05, P = 9.43×10^{-15}), and lower in Haplogroups A (beta = −0.50, se = 0.10, P = 3.14×10^{-7}), B (beta = −0.54, se = 0.09, P = 6.32×10^{-10}), C (beta = −0.53, se = 0.15, P = 3.16×10^{-4}), D (beta = −0.38, se = 0.0.10, P = 9.31×10^{-5}), and F (beta = −0.49, se = 0.12, P = 2.11×10^{-5}), none of which occur at high frequencies in Europe (Torroni et al., 2000; Simoni et al., 2000). As such, it is unlikely that Haplogroup differences between Holocaust survivors and their descendants and controls, if any, would lead to spurious associations with mtDNA copy number.

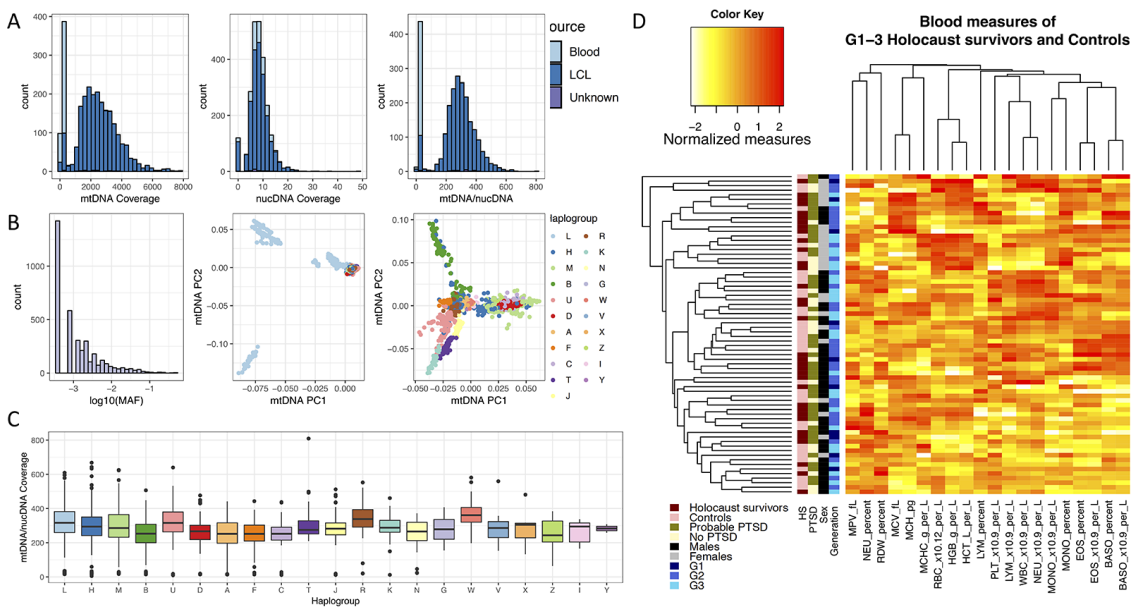

FIGURE 3 | Effects of mtDNA haplogroup and blood cell count on mtDNA copy number measurements. **(A)** Left and middle panel show the sequencing coverage over mtDNA and nuclear DNA in Blood (light blue) and lymphoblastic cell lines (LCL, blue) in the 1000 Genomes Phase 3 project, and the right panel shows the ratio between them. **(B)** The left panel shows the number of SNP variants called per minor allele frequency (MAF) on the log10 scale from sequencing of mtDNA in LCL samples of 1000 Genomes Phase 3 individuals; the middle panel shows the distribution of all individuals by their first two principle components computed from mtDNA SNPs (mtDNA PC1, mtDNA PC2), colored by their mitochondrial haplogroups. Individuals of Haplogroup L (mostly of African origin) contribute the greatest mtDNA diversity as shown in the middle panel, and removing these individuals from the principle component analysis gives greater resolution for visualization of the mtDNA diversity among all other Haplogroups, as shown in the right panel. **(C)** Boxplot of effect of mtDNA Haplogroups on mtDNA copy number, estimated using the ratio of mtDNA and nuclear DNA sequencing coverage. All Haplogroups with significant effects on mtDNA copy number (L, A, B, C, D, F) are rare in European populations. **(D)** Heatmap and clustering of normalized blood cell count measures obtained from 70 individuals in this study.

TABLE 1 | Effect of mtDNA Haplogroups on mtDNA copy number.

Haplogroup	Beta	SE	P value
W	0.735	0.242	2.46e–03
R	0.444	0.159	5.25e–03
L	0.381	0.049	9.43e–15*
U	0.263	0.089	3.29e–03
T	0.110	0.152	4.70e–01
H	0.072	0.056	2.03e–01
M	0.001	0.080	9.87e–01
K	–0.064	0.187	7.32e–01
J	–0.104	0.156	5.03e–01
V	–0.104	0.277	7.07e–01
Y	–0.112	0.705	8.74e–01
X	–0.144	0.333	6.65e–01
G	–0.194	0.243	4.24e–01
I	–0.304	0.408	4.55e–01
N	–0.368	0.224	1.01e–01
Z	–0.368	0.377	3.29e–01
D	–0.375	0.096	9.31e–05*
F	–0.495	0.116	2.11e–05*
A	–0.505	0.098	3.14e–07*
C	–0.535	0.148	3.16e–04*
B	–0.549	0.088	6.33e–10*

This table shows the effect (Beta) of each mtDNA Haplogroup in 1000 Genomes Phase 3 on mtDNA copy number quantified through whole-genome sequencing, ordered by their effects. The ones with significant effect after multiple testing correction for 21 Haplogroups (P value threshold = 0.0024) on mtDNA copy number are marked with asterisk (*).

Platelet Levels in Whole Blood Sample Affect mtDNA Copy Number in Extracted PBMC

A subset of 70 individuals had their whole blood counts assessed on their blood sample prior to extraction of PBMCs and DNA (*Methods*). While there is variation among individuals in all blood measures, we found no significant effect of Holocaust, probable PTSD diagnosis, or age on individual quantile-normalized blood cell counts after multiple-testing correction (P threshold = 0.05/80 = 2.63×10^{-4}). We did find significant effects of sex (being male) on red blood cell count (RBC, P = 1.47×10^{-4}, beta = 0.86, se = 0.21), haemoglobin levels (HGB, P = 5.57×10^{-7}, beta = 1.09, se = 0.20), haematocrit levels (HCT, P = 6.16×10^{-7}, beta = 1.07, se = 0.19) and monocyte percentage (MONO_Percent, P = 2.32×10^{-6}, beta = 0.95, se = 0.21). All results are summarized in **Table 2**. We performed hierarchical clustering on all independent quantile-normalized blood measures and found no clustering by experience of Holocaust, probable PTSD diagnosis, sex and generation (**Figure 3D**).

We tested for effects of all blood cell count measures on mtDNA copy number jointly, after controlling for age and sex. Only platelet levels showed significant effects on mtDNA copy number (PLT, P = 0.043, beta = -5.45×10^{-3}, se = 2.61×10^{-3}). Of note, we have specifically chosen to use DNA extracted from

TABLE 2 | Effect of covariates on blood cell counts.

Blood cell count	Age			Sex			Holocaust survivor status			Probable PTSD		
	Beta	SE	P value	Beta	SE	P value	Beta	SE	P value	Beta	SE	P value
WBC_x10.9_per_L	−0.001	0.006	8.98E−01	−0.302	0.234	2.01E−01	0.571	0.218	1.08E−02	0.351	0.233	1.37E−01
RBC_x10.12_per_L	−0.009	0.006	1.30E−01	**0.857**	**0.213**	**1.47E−04**	0.273	0.226	2.31E−01	0.424	0.231	7.11E−02
HGB_g_per_L	0.000	0.006	9.66E−01	**1.087**	**0.197**	**5.58E−07**	−0.008	0.228	9.71E−01	0.184	0.235	4.37E−01
HCT_L_per_L	0.002	0.006	6.90E−01	**1.067**	**0.194**	**6.17E−07**	−0.205	0.223	3.62E−01	0.281	0.230	2.27E−01
MCV_fL	0.018	0.005	1.18E−03	0.087	0.237	7.13E−01	−0.751	0.210	6.35E−04	−0.371	0.233	1.16E−01
PLT_x10.9_per_L	−0.001	0.006	9.13E−01	−0.820	0.215	3.02E−04	0.004	0.228	9.87E−01	−0.056	0.237	8.15E−01
MCH_pg	0.014	0.006	1.41E−02	0.228	0.235	3.35E−01	−0.506	0.220	2.45E−02	−0.473	0.230	4.33E−02
MCHC_g_per_L	−0.007	0.006	2.52E−01	0.269	0.234	2.53E−01	0.524	0.219	1.92E−02	−0.102	0.236	6.67E−01
RDW_percent	0.004	0.006	4.69E−01	−0.409	0.232	8.20E−02	0.137	0.228	5.48E−01	−0.130	0.236	5.84E-−01
MPV_fL	−0.006	0.006	2.71E−01	0.142	0.236	5.50E−01	−0.077	0.228	7.37E−01	0.128	0.236	5.90E−01
NEU_percent	0.001	0.006	8.56E−01	−0.241	0.238	3.16E−01	0.027	0.230	9.05E−01	0.194	0.237	4.15E−01
LYM_percent	−0.002	0.006	7.64E−01	−0.105	0.240	6.62E−01	0.147	0.229	5.25E−01	−0.037	0.238	8.78E−01
MONO_percent	−0.004	0.006	4.86E−01	**0.955**	**0.210**	**2.32E−05**	−0.339	0.226	1.39E−01	−0.351	0.234	1.39E−01
EOS_percent	0.007	0.006	2.23E−01	0.636	0.227	6.63E−03	0.028	0.230	9.04E−01	−0.435	0.232	6.45E−02
BASO_percent	0.008	0.006	1.71E−01	0.033	0.238	8.89E−01	−0.216	0.227	3.43E−01	−0.196	0.235	4.08E−01
NEU_x10.9_per_L	0.000	0.006	9.79E−01	−0.286	0.237	2.33E−01	0.441	0.224	5.30E−02	0.345	0.234	1.45E−01
LYM_x10.9_per_L	0.000	0.006	9.70E−01	−0.311	0.237	1.94E−01	0.519	0.221	2.20E−02	0.228	0.236	3.39E−01
MONO_x10.9_per_L	−0.004	0.006	4.41E−01	0.549	0.230	2.00E−02	0.227	0.228	3.23E−01	0.094	0.237	6.93E−01
EOS_x10.9_per_L	0.005	0.006	3.50E−01	0.482	0.232	4.16E−02	0.254	0.227	2.67E−01	−0.231	0.236	3.31E−01
BASO_x10.9_per_L	0.006	0.006	2.52E−01	−0.211	0.232	3.66E−01	−0.103	0.224	6.48E−01	−0.154	0.231	5.08E−01

This table shows the effect (Beta) of each covariate (age, sex, holocaust survivor status, and probable PTSD diagnosis) on each blood cell type assessed through linear regression, its standard error (SE) and its P value. The significant effects after multiple testing correction are highlighted in bold.

PBMCs instead of whole blood for this study to remove DNA contribution from platelets—platelets contain only mtDNA and no nuclear DNA, as variation in platelet levels will confound measurements of mtDNA copy number relative to nuclear DNA copy number (Hurtado-Roca et al., 2016).

mtDNA Copy Number Does Not Index Previous Holocaust Experience or Potential PTSD Diagnosis

Having controlled for technical and biological confounding factors, we asked if mtDNA copy number was significantly different between controls and Holocaust survivors and their descendants. We first examined the generation who has personally experienced Holocaust. In G1, we do not have adequate sample size to detect significantly higher mtDNA copy number (beta > 0.48) in Holocaust survivors than controls, after controlling for age and sex as covariates in a linear regression model at our sample size (*Methods*, P = 0.60, beta = 0.07, se = 0.14, **Figure 4A**). The same is true if the analysis was conducted separately among males and females, controlling for age as a covariate (females: P = 0.48, beta = 0.13, se = 0.19; males: P = 0.97, beta = −0.009, se = 0.22). While this is inconsistent with our hypothesis that there will be an increase in mtDNA copy number due to chronic stress during the Holocaust in Holocaust survivors, it is consistent with previous reports of the dynamic nature of mtDNA copy number increase in response to chronic stress and their reversing to normal levels with time.

As it was previously shown that persistent mtDNA copy number increase was dependent on the depressive state (Cai et al., 2015a), we asked if this is also true for PTSD. All but 10 individuals were screened for markers of PTSD were evaluated using the 17-item civilian version of the PCL questionnaire (PCL-C, *Methods*), where individual items are scored 0 to 5, and total scores range from 0 to 85. A cutoff of 30 was used to indicate potential diagnosis of PTSD. Among the 15 G1 Holocaust survivors, seven has a potential diagnosis of PTSD. We found no significant differences in mtDNA copy number in Holocaust survivors with potential diagnosis of PTSD when compared to those without a potential diagnosis of PTSD after correcting for age and sex (P = 0.67, beta = −0.08, se = 0.17) or controls (P = 0.80, beta = −0.07, se = 0.26).

We found significantly more Holocaust survivors and their descendants with PTSD (Fisher's exact test $P = 8.71 \times 10^{-5}$, OR = 3.56, 95% CI = 1.80–7.31) than controls in the whole cohort without accounting for the different generations, in both females (Fisher's exact test P = 0.021, OR = 2.57, 95% CI = 1.09–6.38) and males (Fisher's exact test $P = 8.39 \times 10^{-4}$, OR = 6.04, 95% CI = 1.85–23.78). Interestingly, this trend is observed in both G1 (Fisher's exact test P = 0.017, OR = 8.18, 95% CI = 1.21–97.32) and G2 (Fisher's exact test P = 0.010, OR = 3.35, 95% CI = 1.23–9.92), but not in G3 (Fisher's exact test P = 0.278, OR = 2.02, 95% CI = 0.60–7.27, **Figure 4B**). We do not find significant associations between mtDNA copy number with potential diagnosis of PTSD in G1 (P = 0.54, beta = −0.30, se = 0.50), G2 (P = 0.69, beta = 0.09, se = 0.22) or G3 (P = 0.74, beta = 0.06, se = 0.19, **Figure 4C**).

No Evidence of Inheritance of Elevated PBMC mtDNA Levels in Descendants of Holocaust Survivors

Finally, we tested if potential previous elevation of mtDNA copy number in Holocaust survivors could be inherited by their children. Eight G2 and six G3 participants are identified as

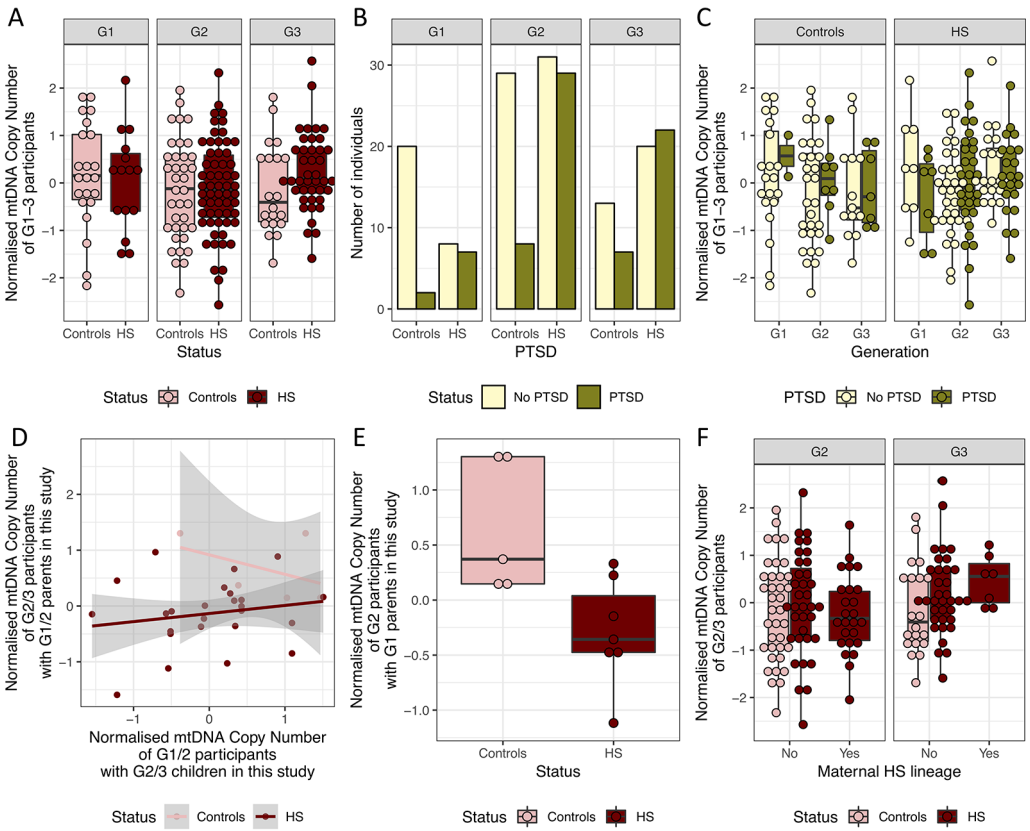

FIGURE 4 | mtDNA copy number is not significantly different between HS and controls. **(A)** Boxplot of the normalized mtDNA copy number in holocaust survivors and their descendants (HS, in dark red) and controls (in pink) in G1–3. **(B)** Number of HS and controls with and without potential PTSD diagnosis from PCL-C in G1–3. **(C)** Boxplot of normalized mtDNA copy number in HS and controls who have a probable PTSD diagnosis and those who do not in each generation. **(D)** Relationship between normalized mtDNA copy number in G1/2 parents and that in their G2/3 children who are also participants in the study. **(E)** Boxplot of normalized mtDNA copy number in G2 HS and controls who are sons or daughters of G1 HS and controls in this study. Controls have significantly higher normalized mtDNA copy number. **(F)** Boxplot of normalized mtDNA copy number in all G2 and G3 HS and controls with and without maternal HS lineage, including those who are not children or grandchildren of G1 and G2 HS and controls.

children and grandchildren of G1 Holocaust survivor participants in the study, and a further 17 G3 participants are children of 12 G2 Holocaust survivor descendant participants. Similarly, six G2 and one G3 participants are identified as children and grandchildren of G1 control participants in the study. This is summarized in **Figure 1A**.

Using all known parent–child relationships in the dataset, we found that parents' mtDNA copy number does not significantly correlate with children's mtDNA copy number, after controlling for age and sex of both parents and children (P = 0.30, beta = 0.47, se = 0.41). This is true when performed just in Holocaust survivors and their descendants (P = 0.07, beta = 0.32, se = 0.17, **Figure 4D**), and we do not have enough data points to perform this analysis in controls. However, we found that G1 Holocaust experience does have a significant impact on their G2 descendants' mtDNA copy number after controlling for age and sex of both parents and children (P = 0.01, beta = −1.18, se = 0.32, **Figure 4E**). In other words, we found lower mtDNA copy number in children of G1 Holocaust survivors than in children of G1 participants who did not experience Holocaust. We get a similar result performing this analysis in only female G1 participants and their G2 descendants (P = 0.06, −1.17, se = 0.44), though the effect of parental Holocaust experience is no longer significant. We are unable to perform this analysis on their G3 participants' mtDNA copy number, as none of them are children or grandchildren of G2 and G1 controls in the study.

Using all participants, including those without records of relationships with one or more other participants in the study, we found that G2 and G3 descendants of Holocaust survivors do not differ significantly in their mtDNA copy number from controls in their generations after controlling for age and sex (G2: P = 0.97, beta = 0.007, se = 0.21, G3: P = 0.15, beta = 0.33, se = 0.23). Further, in both G2 and G3, mtDNA copy number is not significantly different in descendants of female G1 Holocaust survivors than other individuals in their generations, after controlling for age and sex in a linear regression model (G2: P = 0.45, beta = −0.18, se = 0.23, G2: P = 0.22, beta = 0.40, se = 0.33, **Figures 4F**).

DISCUSSION

Holocaust was one of the most horrific periods of the twentieth century. The Holocaust survivors experienced varied forms of persecution during WWII. For all of them, it was six years lasting humiliation, deprivation of basic human rights, deportation, imprisonment in the concentration or death camps or in hiding, under false identities or in mountains or combating in partisans groups. All of them were under threat to be assassinated. Those who survived this psychological and physical ordeal continued to experience trauma due to the murdering of their families, friends and community after the war.

In this study mtDNA copy number in PBMCs obtained in HS were compared to controls without Holocaust experience. While it was impossible to determine levels of mtDNA immediately following the Holocaust experience, our study assumed that they would have been increased in Holocaust survivors, consistent with previous reports of elevated mtDNA after severe and chronic stress. Under this assumption, we asked whether their mtDNA copy number remained elevated decades after their experience, and whether it could be inherited by their descendants. After accounting for variation in mtDNA copy number that may be due to technical between qPCR runs and biological differences between individuals, we investigated the mtDNA copy number differences between G1 Holocaust survivors, their G2 and G3 descendants, and controls from G1 to G3. Bearing in mind we are limited in statistical power by the small sample size of our cohort, and we would not be able to account for effect sizes smaller than beta = 0.29, we found no significant difference in mtDNA copy number or their age-related dynamic in Holocaust survivors as compared to controls in any generation. This is consistent with findings of no significance difference in telomere length and their age-related dynamics in Holocaust survivors from the same cohort (Konečná et al., 2019), as mtDNA copy number was previously shown to be negatively correlated with telomere length in chronic stress (Cai et al., 2015b; Edwards et al., 2016).

There are several explanations for the lack of difference in mtDNA copy number in G1 Holocaust survivors as compared to controls. First, any elevated mtDNA copy number due to the experience may have reversed with time (Cai et al., 2015b). In particular, if release of mtDNA from apoptotic cells rather than intra-cellular mtDNA increase can explain the increase in mtDNA copy number observed in chronic stress and MDD (Lindqvist et al., 2018), discontinuation of apoptotic reaction upon removal of stressful stimuli may lead to complete recovery of elevation in mtDNA copy number, and explain why it cannot be observed decades later. Second, persistent mtDNA copy number changes may be dependent on MDD or other disease states (Cai et al., 2015b). While we were able to perform this test using mtDNA copy number in G1 Holocaust survivors with PTSD only, it is possible PTSD does not have the same molecular signature as MDD, or we do not have enough statistical power to identify it at current sample sizes. Third, Holocaust survivors may consist of highly resilient individuals who were able to survive both prolonged physical and psychological trauma. It was previously shown that Holocaust survivors have higher life-expectancy as compared to those who did not go through the same experience, potentially due to selection during the Holocaust (Sagi-Schwartz et al., 2013), and a genetic basis to this resilience was proposed (Lindqvist et al., 2018; Konečná et al., 2019). Genetic factors contributing to this resilience may contribute to recovery of elevated mtDNA copy number and maintaining telomere length (Konečná et al., 2019), though it is unclear if the same factors confer protection against PTSD and other disorders. Fourth, we may not be observing the right tissue for lasting molecular changes in Holocaust survivors. While an increase of mtDNA due to chronic stress was shown in multiple tissues including saliva, blood and liver in animal models (Cai et al., 2015b), a post-mortem study on suicide completers showed opposite changes in mtDNA levels in blood and dorso-lateral prefrontal cortex (Otsuka et al., 2017). As such, a lack mtDNA copy number difference between Holocaust survivors and controls in blood cells with rapid turnovers does not exclude lasting changes in mtDNA copy number in other tissues that may have important consequences on health and disease. Finally, other factors may influence and confound differences in mtDNA copy number between groups in our sample, including disease, epigenetic factors and ageing. While we were able to account for some of them using blood cell counts as proxy, an exhaustive assessment of these other factors are needed to fully account for their effects on mtDNA copy number.

Interestingly, we found that Holocaust experience of G1 Holocaust survivors was associated with lower mtDNA copy number in their children who also participated in this study. This cannot be completely accounted for by current mtDNA copy number of the G1 Holocaust survivors, and may be mediated through other biological or environmental factors that may be specific to descendants of Holocaust survivors. However, we found no replication of the effect of parental Holocaust experience on children's mtDNA copy number in our whole cohort, where many participants did not indicate direct familial relationships with other participants. Whether this can conclusively dismiss the effect of parental Holocaust experience (or other traumatic experiences) in one's mtDNA levels needs to be further investigated, ideally using a study design where all members of the family participate. This may no longer be possible with Holocaust survivors, many of whom have already passed away.

Finally, we observed higher rates of PTSD in G2 Holocaust survivor descendants than controls, consistent with previous findings (Yehuda et al., 1998), but not in G3, suggesting single generational inheritance of certain risk factors for PTSD, though we have no evidence mtDNA copy number elevation is one of them. While our results do not show evidence of maternal stress or PTSD effects on mtDNA copy number in their descendants, they do not rule out changes in mtDNA replication dynamics in oocyte development due to prenatal and gestational stress, and only targeted analysis on the relevant tissues may answer this question.

In summary, we did not find conclusive evidence of persistent mtDNA copy number changes or differences in age-related dynamics and inheritance of mtDNA in Holocaust survivors as compared to controls, and mtDNA copy number cannot be used

as a marker for PTSD in Holocaust survivors or explain the inheritance of risk for PTSD in their descendants. This study is, to the best of our knowledge, the first comprehensive study of effect of stress and PTSD on mtDNA copy number dynamics and inheritance among Holocaust survivors and their descendants.

METHODS

Participants in This Study

The study was conducted at Central European Institute of Technology (CEITEC), Masaryk University in Brno, Czech Republic. A part of DNA samples was obtained with the cooperation of the National Institute of Mental Health, Klecany, Czech Republic. All of the volunteers were Czechs or Slovaks (people with a similar geopolitical background). Participants had no brain trauma injury history or cognitive or mental impairment. While all Holocaust survivors and their descendants were fully or partially of Jewish origin, none of the controls were due to lack of Jewish persons without the Holocaust history in Czech Republic.

Informed Consent and Ethical Approval

All participants were recruited through voluntarily responding positively to a public appeal presented by Masaryk University and Czech national media (Konečná et al., 2019), with the cooperation of the Jewish community of Brno and Prague. Written informed consent was obtained from all participants, except for in the cases of participants below the age of 16, where written informed consent was obtained from the next of kin/legal guardian. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Masaryk University, Brno, Czech Republic (project NV18-04-00559).

Experience of Chronic Trauma in the Participants

G1 Holocaust survivors suffered from the extreme and chronic stress during the World War II, inclusive and often a combination of long-term humiliation, deprivation of basic human rights, life threating internment in prisons, death camps, hiding, false identities, fighting as partisans and assassination of family members. For participants in G2 and G3 generations, we obtained their relationships to G1 Holocaust survivors. For the participants in the control group, none were exposed to similar extreme and chronic stress.

Evaluation of Post-Traumatic Stress Disorder (PTSD)

Markers of PTSD were evaluated using the 17-item civilian version of the PCL questionnaire (PCL-C), a general civilian version that is not linked to a specific event but to "a stressful experience from the past", rather than PCL-M which is used in the case of military experiences and PCL-S for specific stressful events. Each item is rated 1 to 5 indicating the degree to which a participant has been affected by the item in the past month; a cut-off score of 30 was used to indicate a diagnosis of probable PTSD. The same questionnaire was administered to all participants by trained professionals.

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and DNA

A blood sample were collected from every participant in the years 2016 to 2019. Full blood count was performed on 136 participants using this blood sample. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples using ficoll (Histopaque, Sigma) density gradient centrifugation. Genomic DNA was purified from PBMC samples by proteinase K (Roth) treatment, chloroform extraction, and isopropanol precipitation, as previously described. Quality and concentration of DNA were analyzed by agarose electrophoresis and spectrophotometrically using Thermo Fisher Scientific Nanodrop 2000.

Quantification of mtDNA Copy Number Using Quantitative Polymerase Chain Reaction (qPCR)

qPCR was carried out using the TaqMan® Universal PCR Master Mix, No AmpErase® UNG. A nuclear genomic fragment was amplified from the RNase P gene using the TaqMan® RNase P Detection Reagents Kit, and a fragment of the mitochondrial genome (positions 14747-15887) was amplified from the Cytochrome B (CYB) gene using TaqMan® Gene Expression Assays—Hs02596867_s1—MT-CYB. qPCR was performed under the following conditions: incubation at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. qPCRs of were carried out on 96-well plates; all samples were randomized for the plate they were analyzed on and their positions on the plates, a reference DNA sample was analyzed in triplicates across all plates, and all samples were run in duplicates. Threshold quantitation cycle (CT) was obtained of each sample including the reference DNA samples (REF); we obtain the difference between the mean CT (ΔCT) at for both genes between each sample and REF to correct for plate effects, before obtaining the difference between the CT of the two genes ($\Delta\Delta$CT) as an estimate of mtDNA copy number. PCR runs were discarded if they failed to meet the following criteria: no template control (NTC) with a quantitation cycle (CT) > 38 cycles, sample with a CT <30 cycles.

Quantification of mtDNA Copy Number From Whole-Genome Sequencing in 1000G Samples

We extracted reads mapping to the rCRS mitochondrial reference genome (NC_012920) from WGS in 2,558 individuals in Phase 3 of the 1000 Genomes Project (1000G). Reads mapping to chr20 and mtDNA with the following SAM flags are removed with –F 3852 using samtools (Li et al., 2009) to ensure unique and high quality mapping to chr20 and mtDNA reference genomes respectively: read unmapped (0 × 4), mate unmapped (0 × 8), not primary alignment (0 × 100), read fails platform/vendor quality checks (0 × 200), read is PCG or optical duplicate (0 × 400), and supplementary alignment (0 × 800). We

quantified total read depth across all positions across chr20 and mtDNA and obtained mean coverage for each, and obtained a mtDNA copy number by correcting mean coverage over mtDNA with mean coverage over chr20. We then quantile normalized the measure to obtain a normalized mtDNA copy number.

Haplogroup Calling Using mtDNA Variants Obtained From WGS of 1000G Individuals

We called mtDNA variants from WGS in 2,558 individuals in Phase 3 of the 1000 Genomes Project (1000G) using GATK v4 (McKenna et al., 2010; DePristo et al., 2011), obtaining 3,779 high quality biallelic SNPs. We use all 3,779 SNPs for assigning Haplogroups to each individual in 1000G using Haplogrep v2 (Weissensteiner et al., 2016). We built a genetic related matrix of all 1000G samples using all 3,779 mtDNA SNPs using LDAK (Speed et al., 2017) with options –ignore-weights YES –power -1 –hwe-stand NO, such that each mtDNA SNP contribute the same to the genetic covariance between individuals regardless of their minor allele frequencies, and are not standardized according to Hardy–Weinberg Equilibrium as if they were diploid. We then performed principal component analysis (PCA). As 626 SNPs are private to the 657 individuals with Haplogroup L, and within-Haplogroup diversity in Haplogroup L is greater than diversity across all other Haplogroups combined, we performed PCA on 3,153 mtDNA SNPs in the remaining 1,903 individuals to obtain PCs that show clustering of individuals by their mtDNA Haplogroups.

DATA AVAILABILITY STATEMENT

Datasets from 1000 Genomes Project Phase 3 (ENA Study Accession: PRJNA262923) were analyzed in this study. All other data is available on request to the authors.

ETHICS STATEMENT

Written informed consent was obtained from all participants, except for in the cases of participants below the age of 16, where written informed consent was obtained from the next of kin/legal guardian. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Masaryk University, Brno, Czech Republic (project NV18-04-00559).

AUTHOR CONTRIBUTIONS

NC, MiF, JF, and IR designed the study. NC, SW, KK, and EC performed the experiments. NC performed the analysis. IR obtained funding from the Czech Health Research Council. MoF, KK, MiF, JF, MP, and IR and collected the data through public appeal presented by Masaryk University and Czech national media. NC interpreted the results and wrote the manuscript with the help of MiF, JF, NS, and IR with approval of all authors.

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Mitochondrial Toxicogenomics for Antiretroviral Management: HIV Post-exposure Prophylaxis in Uninfected Patients

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Background: Mitochondrial genome has been used across multiple fields in research, diagnosis, and toxicogenomics. Several compounds damage mitochondrial DNA (mtDNA), including biological and therapeutic agents like the human immunodeficiency virus (HIV) but also its antiretroviral treatment, leading to adverse clinical manifestations. HIV-infected and treated patients may show impaired mitochondrial and metabolic profile, but specific contribution of viral or treatment toxicity remains elusive. The evaluation of HIV consequences without treatment interference has been performed in naïve (non-treated) patients, but assessment of treatment toxicity without viral interference is usually restricted to *in vitro* assays.

Objective: The objective of the present study is to determine whether antiretroviral treatment without HIV interference can lead to mtDNA disturbances. We studied clinical, mitochondrial, and metabolic toxicity in non-infected healthy patients who received HIV post-exposure prophylaxis (PEP) to prevent further infection. We assessed two different PEP regimens according to their composition to ascertain if they were the cause of tolerability issues and derived toxicity.

Methods: We analyzed reasons for PEP discontinuation and main secondary effects of treatment withdrawal, mtDNA content from peripheral blood mononuclear cells and metabolic profile, before and after 28 days of PEP, in 23 patients classified depending on PEP composition: one protease inhibitor (PI) plus Zidovudine/Lamivudine (PI plus AZT + 3TC; $n = 9$) or PI plus Tenofovir/Emtricitabine (PI plus TDF + FTC; $n = 14$).

Results: Zidovudine-containing-regimens showed an increased risk for drug discontinuation (RR = 9.33; 95% CI = 1.34–65.23) due to adverse effects of medication related to gastrointestinal complications. In the absence of metabolic disturbances, 4-week PEP containing PI plus AZT + 3TC led to higher mitochondrial

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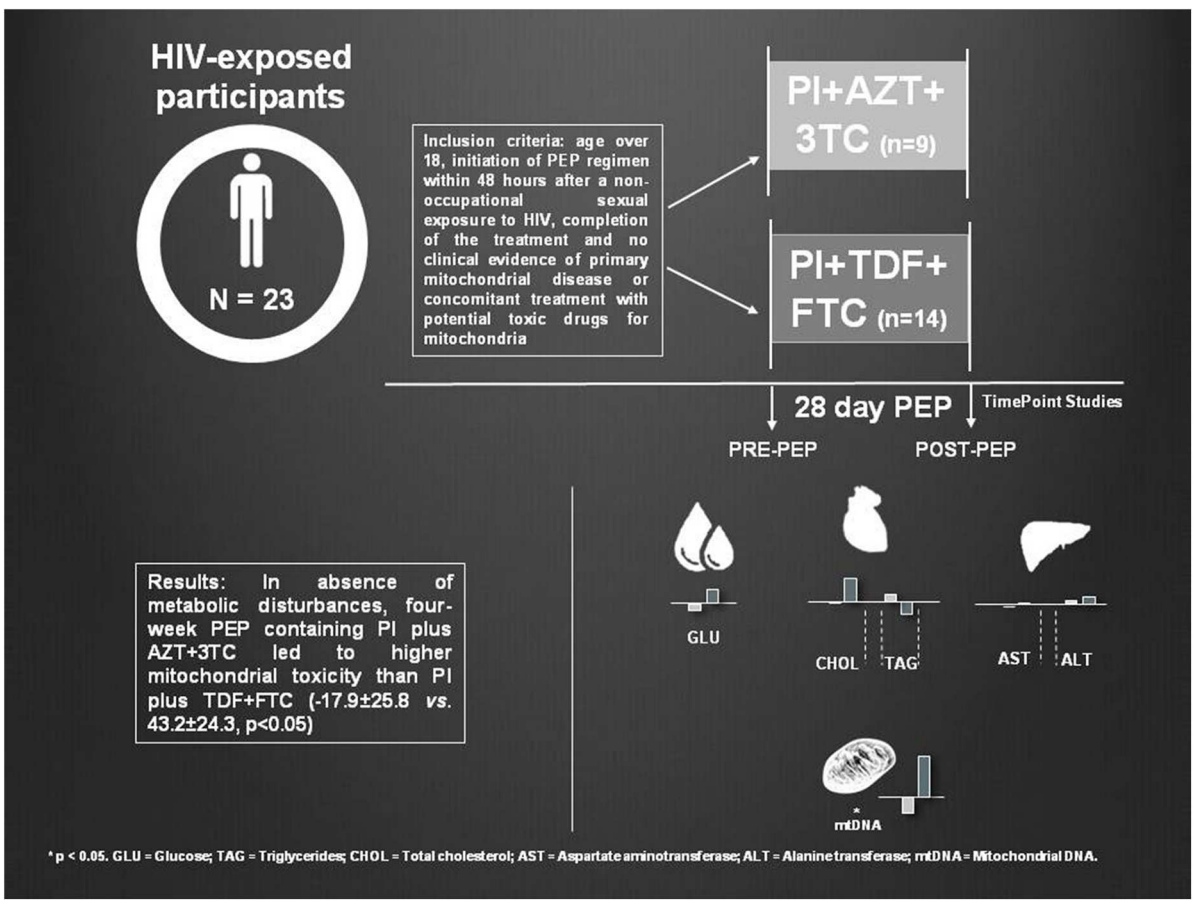
toxicity (−17.9 ± 25.8 decrease in mtDNA/nDNA levels) than PI plus TDF + FTC (which increased by 43.2 ± 24.3 units mtDNA/nDNA; $p < 0.05$ between groups). MtDNA changes showed a significant and negative correlation with baseline alanine transaminase levels ($p < 0.05$), suggesting that a proper hepatic function may protect from antiretroviral toxicity.

Conclusions: In absence of HIV infection, preventive short antiretroviral treatment can cause secondary effects responsible for treatment discontinuation and subclinical mitochondrial damage, especially pyrimidine analogs such as AZT, which still rank as the alternative option and first choice in certain cohorts for PEP. Forthcoming efforts should be focused on launching new strategies with safer clinical and mitotoxic profile.

Keywords: ART, HIV, mitochondria, mtDNA, PEP

HIGHLIGHTS

- PEP regimens are metabolically safe.
- PEP antiretrovirals, in absence of HIV infection, is able to induce mitochondrial toxicity. Currently recommended PEP regimens show less mitochondrial toxicity than the old ones containing pyrimidine analogs such as AZT and 3TC. However, AZT is still used in certain clinical and geographical settings.
- AZT-containing regimens showed a higher risk of drug discontinuation.
- Reduction of mitochondrial toxicity of PEP regimens may improve tolerability and toxicity issues.



GRAPHICAL ABSTRACT| Post-exposure prophylaxis (PEP) myotoxicity.

- Current and forthcoming efforts to elaborate global policy guidelines should consider mitochondrial toxicity of PEP as an important issue for compliance and patient care.
- PEP-treated patients convey an outstanding opportunity to assess antiretrovirals toxicity *in vivo*.
- mtDNA is confirmed as the gold standard for mitochondrial toxicogenomics in antiretroviral management.

INTRODUCTION

Mitochondria are the energy and heat power plants of the cell (Nunnari and Suomalainen, 2012). These organelles harbor their own enzymatic machinery and all the structures required for the transcription and translation of their own genome, the mitochondrial DNA (mtDNA) (Anderson et al., 1981). Any disbalance in mitohormesis can lead to disease (Boczonadi and Horvath, 2014; Suomalainen and Battersby, 2017; Eisner et al., 2018). Thus, genetic but also epigenetic modifications in the mitochondria can be associated with a variety of metabolic modifications described in a multitude of adverse conditions, including cancer and neurodegenerative diseases as well as biological processes as aging (Moosavi and Motevalizadeh Ardekani, 2016; Weinhouse, 2017; Raimundo and Krisko, 2019). Moreover, the study of mitochondrial genome has been used in fields as population genetics, forensic science, clinical diagnosis, and toxicogenomics (Castro Antönia and Ramon, 1998; Budowle et al., 2003; Chinnery and Hudson, 2013).

A multitude of evidence demonstrates that any toxic agent interfering at genetic or epigenetic level with mtDNA can potentially disrupt mitochondrial function and induce metabolic disturbances and their associated clinical consequences (Alston et al., 2017; Matilainen et al., 2017).

Historically, several compounds have been found to damage mtDNA, including biological and therapeutic agents. This is the case with both the human immunodeficiency virus (HIV) and its antiretroviral treatment (ART) (Miro et al., 2005; Margolis et al., 2014; Smith et al., 2017). HIV induces mitochondrial-driven apoptosis, indirectly reducing mtDNA content (Mbita et al., 2014). Moreover, ART—especially nucleoside reverse transcriptase inhibitors analogs (NRTIs)—interferes with the replication of the viral genome, but secondarily by off-targeting the replication of the mtDNA through the inhibition of mtDNA-polymerase-γ (Brinkman et al., 1999; Kakuda, 2000; Nolan and Mallal, 2004; Feeney et al., 2010; Zhang et al., 2014). This process subsequently triggers mtDNA depletion and derived mitochondrial and cell dysfunction, which has been postulated as the basis for associated clinical toxicity (Carr and Cooper, 2000; Lim and Copeland, 2001).

Zidovudine (AZT), the prototype NRTI class drug, is a pyrimidine analog linked to long-term secondary effects. Included in this group, and combined with AZT is Lamivudine (3TC), with lesser harmful effects (World Health Organization, 2018). Both of these drugs in long-term usage result in different secondary effects such as myelosuppression or myopathy, among others (Kinloch-de Loës et al., 1995; Quercia et al., 2018).

To avoid these adverse effects, other NRTIs such as Tenofovir (TDF) emerged (Scherzer et al., 2012; Margolis et al., 2014; Yap et al., 2019). TDF in combination with Emtricitabine (FTC), another NRTI, constitutes the main 2xNRTI combination included in the ART proposed by the main institutions (Centers for Disease Control Prevention, 2016; Battegary et al., 2018; World Health Organization, 2018). FTC is a dideoxycytidine analog with a structure similar to 3TC, being considered as bioequivalent drugs even from the toxic point of view (Birkus et al., 2002; Margolis et al., 2014).

In vitro studies have ranked the potencies of these four NRTIs to inhibit mtDNA synthesis as follows: Zidovudine > Lamivudine = Emtricitabine = Tenofovir (Kakuda, 2000; Birkus et al., 2002). Therefore, mtDNA quantification has been established as the hallmark of antiretroviral toxicity and the gold standard for assessing mitochondrial toxicity even in new ART regimens (Margolis et al., 2014).

Current guidelines associate two different NRTIs with other antiretroviral families such as integrase inhibitors or, alternatively, with protease inhibitors (PI), which have also been associated with metabolic alterations (Mallon et al., 2005; Domingo et al., 2010; Hammond et al., 2010). To control these subclinical events, a glucose, lipid, and hepatic profile is usually monitored in clinical settings to manage chronic HIV-infected and treated patients aiming to avoid further clinical manifestations (AIDSinfo, 2018).

Although ART has dramatically reduced acquired immune deficiency syndrome (AIDS) development, major concerns have been ascribed to its mitochondrial and metabolic toxicity, especially primary ART (Martinez et al., 2001; Garrabou et al., 2009; Hargreaves et al., 2016). Despite current available drugs and regimens are almost free from toxicity, some of these primary antiretrovirals, including AZT, are still used in certain geographic or clinical settings (World Health Organization, 2018). Both mitochondrial and metabolic disturbances caused by the virus and its ART were postulated as one of the bigger etiological bases of adverse events including hyperlactatemia, hepatic failure, decreased bone mineral density, neuropathy, myopathy, lipodystrophy, and metabolic syndrome (Brinkman et al., 1999; Carr and Cooper, 2000; Pfeffer et al., 2009; Caron-Debarle et al., 2010; Hammond et al., 2010; Güerri-Fernández et al., 2018). However, the contribution of each one of these entities (the virus or its treatment) to associated adverse clinical manifestations is difficult to elucidate in HIV-infected and treated patients. While viral consequences without therapeutic interference have been historically evaluated in naïve patients (Miró et al., 2004), assessment of isolated ART toxicity without viral interference usually requires *in vitro* assays (Kakuda, 2000). Therefore, the *in vivo* consequences for ART for mitochondrial and metabolic toxicity in an HIV-free environment requires novel experimental approaches and cohorts of patients that have been scarcely evaluated to date.

Despite the main goal of ART being the treatment of HIV infection, these drugs may also be used to prevent vertical mother-to-child transmission or can also be administered as pre-exposure or post-exposure prophylaxis (PrEP or PEP, respectively, Yap et al., 2019). PEP involves counseling,

assessment of risk of exposure to the infection, HIV testing, and the prescription of a 1-month course of antiretroviral drugs with appropriate support and follow-up (Katz and Gerberding, 1997; Chauveau et al., 2019). While the necessity of PEP is undeniable, it is still limited by a low-adherence, non-negligible secondary effect and some tolerability issues of unknown etiology (Beymer et al., 2017; Chauveau et al., 2019), showing worse tolerability than the ART prescribed for long-term HIV-infected patients under chronic treatment (Rabaud et al., 2005). Such diversity of secondary effects and differential level of mitotoxicity has been attributed to different PEP regimens depending on their composition, but there is little molecular data supporting such differential safety/toxic profile (Groener et al., 2011).

This toxicity has prompted clinical organizations to gradually change the composition of PEP regimens. Between 2008 (Ibarguren et al., 2008) and 2014 (Azkune et al., 2011; World Health Organization, 2013), the PEP regimen consisting of PI plus AZT + 3TC was replaced by a new regimen containing PI plus TDF + FTC. This change in PEP policies offered the perfect occasion to compare these two regimens, which still rank as first-choice treatments in certain patients' cohorts or countries (**Supplementary Table 1**).

Due to HIV prevalence, the use of PEP is highly advisable when an acknowledged risk of HIV transmission is detected, and there is the need for understanding the secondary or toxic effects of this treatment. PEP-treated patients offer an outstanding opportunity to determine the short-term mitochondrial and metabolic effects of PEP *in vivo*, without viral interference. Hence, we designed the present study to assess whether the 28-day PEP regimens can cause clinical, mitochondrial, or metabolic toxicity and whether there are any variances between the different PEP regimens, thus confirming the usefulness of mitochondrial toxicogenomics for antiretroviral management.

MATERIALS AND METHODS

Design, Criteria, and Participants

We performed a multicentric observational study in HIV-1-exposed and uninfected patients to evaluate mitochondrial and metabolic disturbances before and after a 28-day PEP treatment comparing two different regimens: PI plus AZT + 3TC ($n = 9$) or PI plus TDF + FTC ($n = 14$).

Patients were recruited in two hospitals: the Hospital Clinic of Barcelona (Barcelona, Spain) and the Hospital of Granollers (Granollers, Spain).

All participants initiated their PEP regimen within 48 h after a non-occupational sexual exposure to HIV and provided informed consent to be enrolled in the study, which was approved by the Ethical Committee of our institutions.

The inclusion criteria were adults over 18 years old with no clinical evidence of primary mitochondrial disease, or concomitant treatment with potential toxic drugs for mitochondria (antipsychotics, statins or antibiotics, among others) and the full completion of the 28-day treatment (*per-protocol* analysis).

Although the initial sample of the study included a total of 30 participants, 7 of them were lost or excluded from the study. These excluded participants requiered changes of their PEP regimen due to the manifestation of intolerability recorded during the clinical interview.

Epidemiological, virological, and therapeutic characteristics of the HIV-exposed participants were equivalent in both PEP arms. There were no statistically significant differences between both groups with respect to gender and age distribution. These treatment groups were composed by men exclusively, with mean age ranging from 33 to 34 years. The duration of treatment was consistent in both groups, as all patients received full-length PEP regimen and, once concluded, all participants were negative for HIV antibody testing.

Epidemiological, Clinical, and Metabolic Data

As aforementioned, epidemiological, virological, and therapeutic parameters including age, gender, HIV antibody (ELISA), PEP regimen, and treatment intervention were gathered during the study. Similarly, data regarding tolerability, adherence, and reasons for PEP discontinuation were collected in the follow-up on account of clinical interviews.

Glucose, lipid, and hepatic profile data included information about blood glucose (measured using the glucose-oxidase method), triglycerides, and total cholesterol (by enzymatic approaches), as well as aspartate and alanine aminotransferase hepatic enzymes (AST and ALT), which were quantified by atomic absorption spectrophotometry (Siemens Diagnostics®, New York).

Collection of Blood Samples

Fasting samples of 20 ml of venous blood were collected in Vacutainer™ EDTA tubes. For each subject of the study (and for both groups), two sets of samples were obtained, one just after HIV exposure and before PEP, and another after a 28-day course of treatment. Blood was first centrifuged at room temperature for 15 min at 1,500g to reduce platelet contamination through plasma removal. Peripheral blood mononuclear cells (PBMCs) were immediately isolated by means of Ficoll density gradient centrifugation procedure (Histopaque®-1077, Sigma Diagnostics, St. Louis, MO) (Cossarizza, 2003; Mallone et al., 2011). After isolation, PBMCs were resuspended in phosphate-buffered saline and stored frozen at −80°C until analysis.

Nucleic Acid Isolation From PBMC and Quantification of mtDNA

An aliquot of PBMC was used for extracting total DNA using a standard phenol-chloroform procedure. For mtDNA quantification, a fragment of the mitochondrial conserved gene mt12SrRNA and the nuclear constitutive gene nRNAseP were amplified simultaneously and in duplicate by multiplex quantitative Real-Time PCR. We used Applied Biosystems technology (CA, USA) in a 96-well plate and results were expressed in relative units as the ratio between mtDNA to nuclear DNA (mt12SrRNA/nRNaseP), as previously validated (Côté et al., 2011) and reported by our group (Moren et al., 2015; Catalán-García et al., 2016; Barroso et al., 2019) and other groups

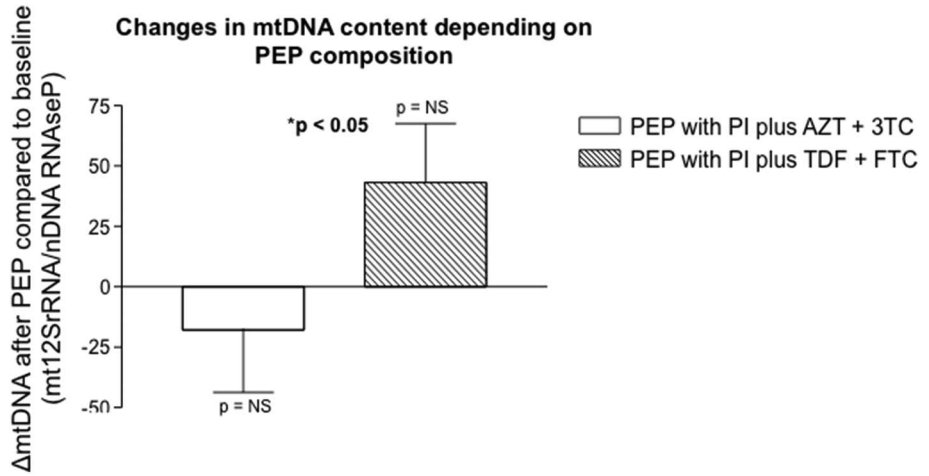


FIGURE 1 | Non significant differences in mtDNA content were observed within each therapeutic group intervention (before and after each treatment), but significant differences were found between the different PEP regimens (PI plus AZT + 3TC vs. PI plus TDF + FTC). Results were expressed as the ratio of mitochondrial 12SrRNA gene with respect to the constitutive nuclear RNAseP gene.

(Villarroya et al., 2011; Navarro-Sastre et al., 2012; Carreño-Gago et al., 2019).

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM) or in percentage of change ± SEM with respect to the baseline measurement. Longitudinal differences between both study time points (in each treatment arm) and cross-sectional differences between treatment intervention groups (PI plus AZT + 3TC vs. PI plus TDF + FTC) were determined with the non-parametric Kolmogorov–Smirnov test for paired and independent measures, respectively. Correlation analysis between all quantitative parameters was determined using the non-parametric Spearman test. Statistical analysis was performed using the Statistical Package for Social Sciences version 23.0 (SPSS, Chicago, Illinois, USA). Statistical significance was set at a $p < 0.05$.

RESULTS

As previously stated, from the initial 30 participants of the study, 7 discontinued PEP before 4 weeks due to gastrointestinal secondary effects including bloating, diarrhea, nausea, and/or vomiting. Consequently, longitudinal mitochondrial and metabolic toxicity profile could not be assessed in these 7 patients due to lack of follow-up. From these patients, 6 were treated with PI plus either AZT + 3TC and 1 with a PI plus TDF + FTC (relative risk or RR for PI plus AZT + 3TC vs. PI plus TDF + FTC discontinuation = 9.33; 95% CI = 1.34–65.23).

After 6 months of HIV exposure, all subjects that continued the study ($n = 23$) remained uninfected and blood analysis for HIV antibodies were all confirmed as negative.

There were no statistically significant intragroup differences between initial and final mtDNA levels within each PEP regimen: baseline 133.5 ± 19.8 mtDNA/nDNA copies vs. final 115.7 ± 22.4 levels for PI plus AZT + 3TC regimen and initial 136.5 ± 20.9 vs. final 177.3 ± 22.8 copies for PI plus TDF + FTC regimen. However, when comparing differences between groups, mtDNA content was significantly reduced in the PI plus AZT + 3TC regimen vs. the PI plus TDF + FTC group: −17.9 ± 25.8% vs. 43.2 ± 24.3%, respectively, $p < 0.05$ (**Figure 1**).

There were no statistically significant differences before and after treatment in glucose, lipid, or hepatic metabolic profiles in both groups, either concerning glucose, triglycerides, total cholesterol, AST, or ALT levels, regardless of the PEP regimen followed, as summarized in **Table 1** and **Supplementary Figures 1, 2**.

Some metabolic parameters were correlated, showing their strong dependence to maintain physiologic homeostasis (**Supplementary Table 2**). In addition, mtDNA levels after treatment were negatively correlated to initial ALT levels ($R^2 = 0.090$ and $p < 0.05$) regardless of the PEP regimen (**Figure 2**).

DISCUSSION

HIV infection and ART toxicity (especially of NRTIs) have been postulated as the etiopathological basis of several side effects in HIV-infected and chronically treated patients (Carr and Cooper, 2000; Kohler and Lewis, 2007). Both have been demonstrated to induce mtDNA depletion and derived mitochondrial and metabolic dysfunction (Garrabou et al., 2009; Margolis et al., 2014) even after short periods of treatment (Carr, 2000; Pilon et al., 2002). However, the differential contribution of each agent (HIV or ART) to the observed mitochondrial toxicogenomic profile that is present in HIV-infected patients under ART is difficult to elucidate. Isolated HIV-induced mitochondrial damage has been studied in HIV-infected and untreated individuals (naïve), but ART-related mitochondrial toxicity has

TABLE 1 | Glucose, lipid, and hepatic profile of all participants before and after PEP treatment.

	HIV-exposed patients				
	PEP regimen with PI plus AZT + 3TC (n = 9)		PEP regimen with PI plus TDF + FTC (n = 14)		
	Before	After	Before	After	*P*-value
Glucose (mg/dl)	93.4 ± 5.4	86.5 ± 3.8	79.5 ± 3.2	94.6 ± 10.0	NS
Triglycerides (mg/dl)	98.5 ± 24.5	106.5 ± 20.8	135.9 ± 19.0	122.6 ± 22.0	NS
Total cholesterol (mg/dl)	181.5 ± 15.9	180.3 ± 17.3	155.3 ± 6.7	181.3 ± 16.4	NS
AST (U/L)	31.4 ± 3.1	30.9 ± 3.6	20.7 ± 1.4	21.6 ± 2.3	NS
ALT (U/L)	29.4 ± 4.8	32.0 ± 5.4	19.7 ± 1.1	27.9 ± 4.7	NS

No differences were observed in metabolic parameters after the therapeutic intervention or between regimens. All values are expressed as mean ± SEM. ALT, alanine transaminase; AST, aspartate transaminase; AZT, Zidovudine; FTC, Emtricitabine; HIV, human immunodeficiency virus; NS, non-significant; PEP, post-exposure prophylaxis; PI, protease inhibitor; SEM, standard error mean; TDF, Tenofovir; 3TC, Lamivudine.

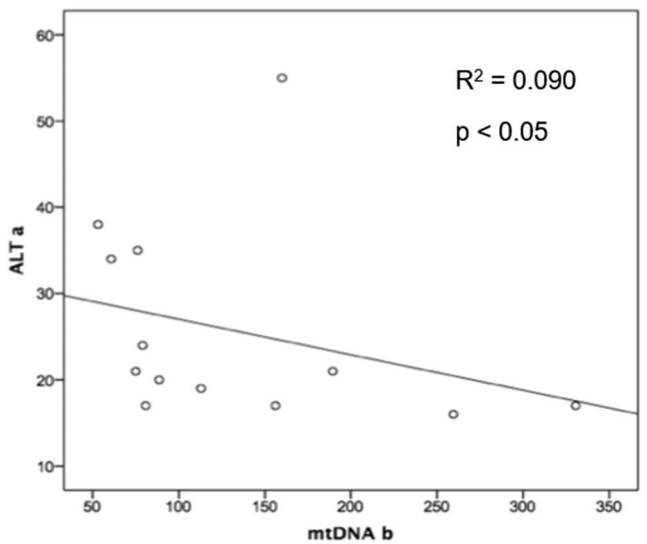


FIGURE 2 | Spearman Rho coefficient was significant and showed a negative correlation for basal levels of ALT and mitochondrial DNA after treatment intervention in both PEP groups (PI plus AZT + 3TC or PI plus TDF + FTC), suggesting that proper basal hepatic function protects from further drug toxicity (p-value = 0.015). ALT a, Alanine transaminase baseline levels; AZT, Zidovudine; mtDNA b, mitochondrial DNA after treatment; PEP, post-exposure prophylaxis.

been poorly explored in uninfected subjects on account of ethical concerns.

HIV-exposed patients subjected to PEP prophylaxis convey a unique opportunity to test ART toxicity without HIV interference. Additionally, we took advantage of the use of different PEP regimens to compare different clinical, metabolic, and mitochondrial ART toxicity profiles.

Regarding PEP efficacy, all tested alternative treatments showed identical immunotherapeutic efficacy in preventing HIV infection, both in the present study and in the literature (Sultan et al., 2014).

Regarding clinical manifestations and despite its short length (28 days according to up-to-date guidelines), serious complications were raised: the low compliance, the appearance of several secondary or toxic effects, and the little commitment of some patients led to further discontinuation of AZT-containing regimens, herein demonstrated. As previous reported, the main secondary effects for both of these regimens that led to discontinuation were gastrointestinal symptoms (Chowta et al., 2018). These clinical side effects make PEP prone to become a difficult treatment to be fully completed. However, few toxicological studies have been done to assess molecular causes of differential safety/toxic profile of PEP regimens or antiretroviral toxicity in human subjects without HIV interference.

With respect to mitochondrial toxicity, a previous study performed in 18 individuals reported a decrease in the mitochondrial transmembrane potential over a 4 weeks of HIV-PEP, suggesting that PEP toxicity may be confirmed in larger cohorts (Groener et al., 2011). We herein tested the mitochondrial target of nucleoside analog toxicity, considered the gold standard for monitorization of antiretroviral toxicity, that is mtDNA content.

According to our findings, when comparing PEP regimens including PI plus AZT + 3TC with respect PI plus TDF + FTC, subclinical mtDNA depletion was higher in those receiving AZT + 3TC. This confirms previous reported higher mitochondrial toxicity for these older drugs derived from *in vitro* (Kakuda, 2000) or *ex vivo* studies in HIV-infected and long-term treated individuals (Gardner et al., 2013; Sun et al., 2014).

Despite that the use of pyrimidine analogs in PEP regimens, and particularly AZT, is being reduced in developed countries, it still ranks as the alternative option in the CDC, WHO, and EACS guidelines for certain patients (Centers for Disease Control Prevention, 2016; World Health Organization, 2018). Specifically, (i) it is the alternative treatment in subjects over 13 years old with renal dysfunction (creatinine clearance ≤59 ml/min); (ii) it is the alternative treatment for children aged 2–12 years; or (iii) it is the preferred treatment for children aging 4 weeks to 2 years old; and (iv) it is the alternative choice of treatment in adults (Battegary et al., 2018). In these cases, AZT is chosen with 3TC. Furthermore, in numerous developing countries, AZT administration in PEP regimens is still the treatment of choice.

These results, among others (Morén et al., 2012; Margolis et al., 2014), give light to the capacity for antiretrovirals to target

and disrupt mtDNA expression even after short treatments. Translating all these findings into emerging fields such as epigenetics opens new gates in research to elucidate whether these changes into gene expression can cause drug resistance, metabolic disturbances, and different secondary effects that can lead to drug discontinuance and its subsequent treatment failure (Nyce et al., 1993; Lucarelli et al., 1996; Bozzi et al., 2008; Koczor et al., 2015). It has been shown that some miRNAs that participate in the regulation of mitochondrial translation are mitochondrial-genome-encoded miRNAs (Stimpfel et al., 2018). Consequently, mtDNA depletion produced by NRTIs, as AZT by itself, may reduce miRNA content, thus having effects in mitoepigenetics (Koczor et al., 2015). Additionally, some studies propose a possible surrogate effect in neonates under AZT-containing regimens, as they show an altered nuclear heterochromatin organization that persisted after the treatment was terminated (up to 9 years of age) (Senda et al., 2007; Zuena et al., 2013; García-Otero et al., 2019). Whether all these levels of regulation of mtDNA expression are additionally influencing the toxicity of tested PEP regimens in our work should be addressed in further studies.

Finally, the metabolic profile of PEP-treated patients did not show any differences either in basal or endpoint levels between groups, indicating that in a 28-day interval, there are no visible effects on glucose, lipid, or hepatic enzyme levels regardless of PEP composition. Interestingly, lower initial ALT levels have been associated with higher content in mtDNA after PEP in both groups. While all patients had standard liver enzyme levels, these results point out the association between mitochondrial toxicity and hepatic function, probably because proper basal liver function protects from further drug toxicity by promoting hepatic drug detoxification.

Noticeably, this study has several constraints. The most relevant limitation may be its small sample size. Because of the singularity of these individuals, the lack of compliance, and the need for fast sample processing (to immediately isolate fresh PBMC), it was difficult to gather all the participants for the study in a short period of time. In fact, we needed to perform a multicenter study to include the minimum sample size required to reach our aim. However, we cannot discard a type II error due to the small sample size of the cohorts herein tested, which may be bypassed in further studies with bigger sample sizes and controlled designs. Additionally, the fact that male patients exclusively composed our sample may be considered as the second limitation of the study. However, in current clinical settings, this characteristic may reflect the differences in prevalence of HIV infection according to gender in general population and eradicates potential gender interference in observed results. Regarding the source of sample, we acknowledge that mitochondrial parameters may be exacerbated in more energy-dependent tissues than PBMCs. Likewise, we are aware that assessing specific PBMC composition would be of interest to assess potential interference of cell populations in observed findings, as well as preventing platelet contamination (Tin et al., 2016; Sun et al., 2018). However, we should take into consideration that PBMCs have been demonstrated to be a reliable and non-invasive model to perform mitochondrial studies and that is the present gold standard for mitochondrial toxicity evaluation (Garrabou et al., 2009; Moren et al., 2015; Barroso et al., 2019). Additionally, the potential follow-up of patients for an extended period of time over PEP administration and additional measures for evaluation of mitochondrial toxicity or specific cell toxicity profiling may be of interest for further approaches.

CONCLUSIONS

The results herein presented indicate that, first, short-term ART in the absence of HIV infection can induce mitochondrial toxicity and, second, in the context of HIV-PEP, new antiretrovirals regimens including PI plus TDF + FTC show less mtDNA depletion and therefore are less harmful to mitochondria than the old ones with PI plus AZT + 3TC. The latter regimen also showed a higher risk of drug discontinuation due to a lack of tolerance, while capable of maintaining identical therapeutic activity. Whether mitochondrial toxicity relies at the base of adverse PEP effects has to be further demonstrated. However, considering the reported association between mitochondrial toxicity and clinical adverse effects in chronic antiretrovirals-treated HIV individuals, these results should be considered to elaborate guidelines to potentially reduce tolerability and toxicity issues of PEP.

Fortunately, efforts are being raised to elaborate global policy makers and coordinate program managers, researchers, and activists around the world at a moment of a paradigm shift of the global response to HIV (24), where toxicity of PEP regimens should be considered and AZT should be discouraged.

PEP-treated patients convey an outstanding opportunity to assess antiretroviral toxicity *in vivo* and mtDNA is confirmed as the gold standard for mitochondrial toxicogenomics in antiretroviral management.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Hospital Clinic of Barcelona. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

We also acknowledge the contribution of each author: GG, along with EM, FC, EP, and ÒM, who conceived the study and supervised the data collection and analysis. JR and AL participated in patient inclusion and supervised the clinical aspects of this study, in collaboration with JM and EM. Each author participated in the recruitment of epidemiological, clinical, and metabolic data of patients. GG, MB, MG-M, and IG-C managed the collection of samples and isolation of cells. EL, JC-S, DJ, CM, and MB were responsible for experimental

analysis of mitochondrial DNA content, under the supervision of GG. ET provided technical assistance for reagent preparation. A database was also created by MB and SB to collect all the clinical and experimental parameters to perform the statistical analysis of the data, under the supervision of GG. All authors participated in drafting and critical revision of the manuscript, especially MB, SB, EM, and GG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00497/full#supplementary-material

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Evidence of Selection Against Damaged Mitochondria During Early Embryogenesis in the Mouse

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There is evidence of a purifying filter acting in the female germline to prevent the expansion of deleterious mutations in the mitochondrial DNA (mtDNA). Given our poor understanding of this filter, here we investigate the competence of the mouse embryo to eliminate dysfunctional mitochondria. Toward that, mitochondria were damaged by photoirradiation of NZB/BINJ zygotes loaded with chloromethyl-X-rosamine (CMXRos). The resultant cytoplasm was then injected into C57BL/6J zygotes to track the levels of NZB/BINJ mtDNA during the preimplantation development. About 30% of NZB/BINJ mtDNA was present after injection, regardless of using photoirradiated or non-photoirradiated cytoplasmic donors. Moreover, injection of photoirradiated-derived cytoplasm did not impact development into blastocysts. However, lower levels of NZB/BINJ mtDNA were present in blastocysts when comparing injection of photoirradiated (24.7% ± 1.43) versus non-photoirradiated (31.4% ± 1.43) cytoplasm. Given that total mtDNA content remained stable between stages (zygotes vs. blastocysts) and treatments (photoirradiated vs. non-photoirradiated), these results indicate that the photoirradiated-derived mtDNA was replaced by recipient mtDNA in blastocysts. Unexpectedly, treatment with rapamycin prevented the drop in NZB/BINJ mtDNA levels associated with injection of photoirradiated cytoplasm. Additionally, analysis of mitochondria-autophagosome colocalization provided no evidence that photoirradiated mitochondria were eliminated by autophagy. In conclusion, our findings give evidence that the mouse embryo is competent to mitigate the levels of damaged mitochondria, which might have implications to the transmission of mtDNA-encoded disease.

Keywords: mitochondria, embryo, mitochondrial DNA, mtDNA, mouse, cytoplasmic transfer, NZB, photosensitization

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INTRODUCTION

Mitochondria play a central role in cellular energy production (i.e., ATP), besides being involved in several other functions including Ca^{2+} buffering, innate immunity, biogenesis of iron-sulfur clusters and apoptosis (Wallace and Chalkia, 2013). Most proteins needed for mitochondrial function are encoded in the nucleus and imported by the organelle (Wallace and Chalkia, 2013). Yet, the mitochondrion also relies on 37 genes (13 mRNAs, 22 tRNAs, and two rRNAs) encoded by its own genome, the mitochondrial DNA (mtDNA). The importance of these genes is revealed

by mutations in mtDNA, which can result in mitochondrial dysfunction and severe pathologies in humans (Stewart and Chinnery, 2015). Manifestation of these pathologies is difficult to predict though as it depends on the level of mutant mtDNA. Multiple copies of mtDNA are present in each cell and mutations commonly coexist with wild-type molecules, a condition termed heteroplasmy (Schon et al., 2012; Burr et al., 2018). Given that most mtDNA mutations are recessive, wild-type molecules can complement the mutation defect. A threshold level of mutant mtDNA is needed to impair mitochondrial function (i.e., 60–80%), but this threshold level varies for different mutations and tissues (Schon et al., 2012; Burr et al., 2018).

Due to the lack of efficient methods to treat mitochondrial disease, much attention has been given to prevent its transmission to the next generation. Yet, the non-Mendelian pattern of mtDNA inheritance makes difficult to predict transmission of such disease (Stewart and Chinnery, 2015). Despite few exceptions (Luo et al., 2018), autophagic elimination of paternal mitochondria shortly after fertilization assures mtDNA to be exclusively inherited from the mother (Rojansky et al., 2016; Wei et al., 2020). In addition, in case of heteroplasmy, several mechanisms take place during germline development toward reestablishing homoplasmy (i.e., existence of a single mtDNA genotype, regardless of mutant or wild-type). For instance, the mitochondrial genetic bottleneck allows for quick changes in mtDNA genotype frequency (Jenuth et al., 1996; Cree et al., 2008; Wai et al., 2008; Floros et al., 2018; Latorre-Pellicer et al., 2019). Also, there is increasing evidence in support of purifying selection acting in the female germline to prevent deleterious mutations (i.e., non-synonymous) in mtDNA from accumulating in the population (Burr et al., 2018).

One of the most consistent evidence of purifying selection first came from the work by Stewart et al. (2008). Using a mouse model with a burden of randomly generated mtDNA mutations, the authors found that synonymous mutations in protein-coding genes are preferentially transmitted to offspring than non-synonymous mutations. In addition, mutations in tRNA and rRNA genes were more often present in offspring than mutations in protein-coding genes (Stewart et al., 2008). Similar findings have been reported in mice and humans (Sato et al., 2007; Fan et al., 2008; Freyer et al., 2012; Sharpley et al., 2012; Li et al., 2016; Floros et al., 2018; Latorre-Pellicer et al., 2019; Wei et al., 2019). However, the mechanism underpinning purifying selection is currently unclear (Burr et al., 2018). Here we provide evidence that early embryos mitigate the levels of photoirradiated mitochondria introduced by cytoplasmic transfer (CT), which suggests they are virtually competent to tackle dysfunctional mitochondria harboring deleterious mtDNA mutations.

MATERIALS AND METHODS

All chemical and reagents were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, United States), unless otherwise stated. All experiments were performed in compliance with the regulations and policies of the National Council for Control of Animal Experimentation (CONCEA, Brazil) and were approved by the Animal Care and Use Committee at Universidade de São Paulo (USP—protocol number 13.1.1832.74.8).

Source of Mouse and Embryos

Mice containing mtDNA of NZB/BINJ (NZB) origin were obtained by backcrossing NZB females to C57BL/6J (B6) females for five generations. Thereafter, females with NZB mtDNA in a ∼100% B6 background were maintained by brother–sister mating (Machado et al., 2015). Mice containing mtDNA of B6 origin were obtained from F1 females from a cross of B6 females with males of CBA origin. Mice with mtDNA of NZB or B6 origin are hereafter termed NZB and B6, respectively.

To obtain pronuclear zygotes, females were intraperitoneally injected with 5 I.U. of equine chorionic gonadotropin (eCG; Folligon, MSD Animal Health, Summit, United States) and 5 I.U. of human chorionic gonadotropin (hCG; Chorulon, MSD Animal Health), given 46–47 h apart. Immediately after the hCG injection, females were paired with B6 males and inspected for the presence of vaginal plug in the next morning. Pronuclear zygotes were collected from the oviduct (ampulla) of plugged females ∼18 h after the hCG injection using HEPES-buffered KSOM medium (Erbach et al., 1994; Nagy et al., 2003; Machado et al., 2015). Viable zygotes were denuded of cumulus cells by vigorous pipetting in the presence of 0.3% hyaluronidase in HEPES-buffered KSOM. Groups of 20 zygotes were cultured *in vitro* under mineral oil in a 40 μl drop of KSOM. After 96 h of culture in an incubator (set at 37°C, maximum humidity, and 5% CO_2 in air), embryos were assessed as for the blastocyst rate (Nagy et al., 2003; Machado et al., 2015).

Induction of Mitochondrial Damage

Chloromethyl-X-rosamine (CMXRos; MitoTracker Red; ThermoFisher Scientific, Waltham, MA, United States) is a mitochondrion-selective fluorescent probe with a strong photosensitizing action (Minamikawa et al., 1999; Lum et al., 2002; Palermo et al., 2002; Lum and Nagley, 2003; Takeuchi et al., 2005). Under photoirradiation, CMXRos absorbs light, leading to excitation of the outer shell electrons and generation of reactive species such as hydroxyl radicals and singlet oxygen within the mitochondrion. These reactive species may damage mitochondrial structures, with evidence of organelle swelling and membrane depolarization (Minamikawa et al., 1999; Lum et al., 2002; Palermo et al., 2002; Lum and Nagley, 2003; Takeuchi et al., 2005).

To induce mitochondrial damage, NZB zygotes at the pronuclear stage were incubated for 30 min with 500 nM CMXRos in HEPES-buffered KSOM at 37°C. Next, based on a previous report (Takeuchi et al., 2005), zygotes were rinsed three times in HEPES-buffered KSOM and photoirradiated for either 0, 2.5, 5, 10, 20, or 60 s. Photoirradiation was performed in groups of 20 zygotes using an inverted microscope (Eclipse TS 100, Nikon Instruments Inc., Tokyo, Japan) equipped with an epifluorescence attachment (50-W mercury burner) with a Texas Red filter (excitation wavelength, 540–580 nm; emission wavelength, 600–660 nm) at 200x magnification (Minamikawa et al., 1999; Lum et al., 2002; Palermo et al., 2002; Takeuchi et al., 2005). Control zygotes were photoirradiated for either 0 or 60 s without prior loading with CMXRos.

Cytoplasmic Transfer

Five experimental groups were considered during CT experiments: control B6 embryos not subjected to either CMXRos loading or photoirradiation—termed "B6-control"; NZB embryos subjected to CMXRos loading and photoirradiation (P) for either 0 or 20 s—termed "NZB-P0" and "NZB-P20," respectively; and, B6 embryos subjected to CT using cytoplasm from either NZB-P0 or NZB-P20—termed "CT-P0" and "CT-P20," respectively.

Micromanipulation was performed using an inverted microscope (Leica DMI RB, Leica, Wetzlar, Germany) equipped with micromanipulators and microinjectors (Narishige, Tokyo, Japan), as previously reported (Machado et al., 2015). Briefly, pronuclear zygotes were incubated for 15 min in HEPES-buffered KSOM medium containing 5 μg/ml cytochalasin and 5 μg/ml nocodazole. Next, ∼30% of cytoplasm was removed from B6 zygotes (calculated as previously reported; Chiaratti et al., 2010), followed by injection in the perivitelline space of a similar amount of cytoplasm derived from NZB zygotes. Pronuclei were always visualized during the micromanipulation procedure to prevent their unintended removal. After micromanipulation, zygotes were placed in an electrofusion solution (0.28 M mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES, and 0.05% BSA) and subjected to a single electrical pulse of 1 kV/cm (DC) for 45 μs (Multiporator, Eppendorf, Hamburg, Germany) to induce fusion of the NZB cytoplast with the B6 recipient zygote. Successfully fused zygotes were cultured *in vitro* as described above. When applicable, embryos were cultured in the presence of 250 nM rapamycin (Lee et al., 2011; Gilkerson et al., 2012). After 96 h of *in vitro* culture, the blastocyst rate was assessed.

Evaluation of NZB Levels and Mitochondrial DNA Copy Number

Embryos used for molecular evaluation were sampled immediately before (at the pronuclear stage) or after (at the blastocyst stage) *in vitro* culture. These were rinsed three times in phosphate buffer solution (PBS) containing 0.1% polyvinyl pyrrolidone (PVP) and stored individually in 1 μl PBS plus 0.1% PVP in 0.2 ml tubes at −20°C. Embryos were lyzed for 3 h at 55°C in 50 mM KCl, 10 mM Trix-Cl (pH 8.3), 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Igepal CA-630, 0.45% Tween 20, and 125 μg/ml proteinase K (ThermoFisher Scientific). Following, lysates were incubated at 95°C for 10 min for proteinase K inactivation, diluted with 45 μl ultrapure H_2O, and centrifuged at 10,000 × *g* for 5 min. The supernatant was finally used for analysis of NZB levels and mtDNA copy number (Machado et al., 2015).

The levels of NZB mtDNA in zygotes and blastocysts were assessed by quantitative PCR (qPCR) as previously reported by Machado et al. (2015). Briefly, two set of primers were used to amplify either a 118-bp fragment of NZB mtDNA or a 146-bp fragment of B6 mtDNA. Reactions consisted of a final volume of 15 μl containing 5 μl of sample lysate, 200 nM of each primer, and 1x Power SYBR Green Master Mix (ThermoFisher Scientific). Amplifications were performed using the 7500 Fast Real-Time PCR System (ThermoFisher Scientific) and the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 62°C for 1 min. SYBR Green fluorescence was read at the end of each extension step. The percentage of NZB mtDNA was calculated in relation to the sum of NZB and B6 mtDNA, as reported by Machado et al. (2015).

Total mtDNA copy number (sum of NZB and B6 mtDNA) in zygotes and blastocysts was assessed as reported by Machado et al. (2015). Toward that aim, a 736-bp fragment of B6 mtDNA was cloned into a plasmid vector (pCR2.1-TopTA; ThermoFisher Scientific). Part of this construct (at concentration of 10^7, 10^6, 10^5, 10^4, and 10^3 copies/reaction) was amplified by qPCR in parallel with zygote and blastocyst samples. Conditions of qPCR were the same described above, except for the use of non-discriminative primers that amplify a common fragment (148 bp) from both NZB and B6 mtDNA. The number of mtDNA copies was calculated as reported by Machado et al. (2015).

Analysis of Mitochondria-Autophagosome Colocalization

Embryos at the two-cell stage (21 h of culture) were fixed in 3.7% paraformaldehyde in PBS with 0.5% Triton X-100 and 0.1% PVP for 15 min at room temperature. Next, embryos were rinsed three times in PBS with 0.1% PVP, and incubated for 1 h at room temperature with a primary antibody (anti-MAP1LC3B raised in rabbit; Cat# L7543, Sigma–Aldrich). Afterward, embryos were rinsed in PBS with 0.1% PVP, and incubated for 1 h at room temperature with an Alexa Fluor 488-tagged secondary antibody raised against rabbit (Cat# A11008, ThermoFisher Scientific). Both antibodies were diluted 1:200 in PBS with 0.1% PVP. Finally, embryos were thoroughly washed in PBS with 0.1% PVP, and mounted on slides with coverslips using Prolong Gold (ThermoFisher Scientific). Embryos were evaluated by confocal microscopy (LSM 780, Zeiss, Oberkochen, Germany) at 1000x magnification. Autophagosomes were visualized at 495 and 519 nm, respectively, for excitation and emission. NZB mitochondria (previously stained with CMXRos for the photosensitization treatment) were visualized at 543 and 580–650 nm, respectively. Images were analyzed using the ZEN lite (Zeiss).

Statistical Analyses

Statistical analyses were performed using SAS v.9.3 (SAS/STAT, SAS Institute Inc., Cary, NC, United States). When necessary, data were transformed to fit a normal distribution. Data were analyzed by one-way or two-way ANOVA followed by Tukey's *post hoc* test. Values are reported as mean ± standard error of the mean (SEM).

RESULTS

Photoirradiation of CMXRos-Loaded Zygotes Prevents Development Into Blastocysts

Aiming to set up photosensitization conditions, zygotes were loaded with CMXRos and photoirradiated for either 0, 2.5, 5,

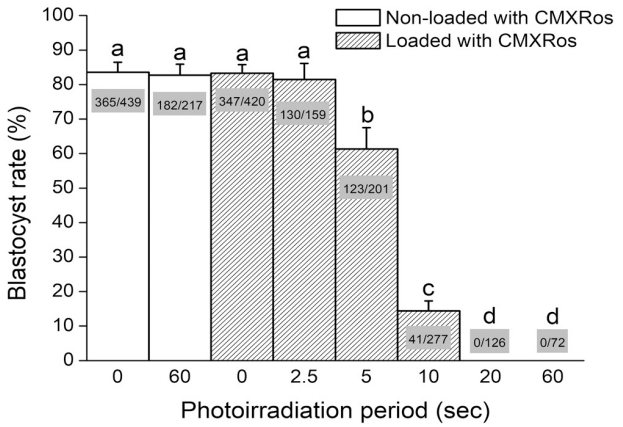

FIGURE 1 | Photoirradiation of CMXRos-loaded zygotes prevents development into blastocysts. Percentage of NZB zygotes that developed into blastocysts after 96 h of *in vitro* culture. Zygotes were either loaded or not with CMXRos prior to photoirradiation. Bar insets represent the number of blastocysts in relation to total number of cultured zygotes. Different letters over bars depict statistical difference ($P < 0.05$).

10, 20, or 60 s before *in vitro* culture and analysis of blastocyst development. Zygotes photoirradiated for either 0 or 60 s, without prior incubation with CMXRos, were used as controls. As a result, photoirradiation of non-loaded zygotes for 60 s did not impact blastocyst rate in comparison with zygotes that were neither incubated with CMXRos nor photoirradiated (**Figure 1**). Likewise, no effect was seen when CMXRos-loaded zygotes were photoirradiated for 0 or 2.5 s (**Figure 1**). Yet, photoirradiation for 5 s or more progressively impacted on blastocyst rate ($P < 0.05$); a photoirradiation period of 20 or 60 s was sufficient to completely prevent blastocyst formation (**Figure 1**). In summary, these findings show a linear impact of photoirradiation time on blastocyst rate, which relied on the prior loading with CMXRos.

Photoirradiated Mitochondria Injected Into Zygotes Are Selected Against During Early Embryogenesis

To investigate whether damaged mitochondria are selectively eliminated during early embryogenesis, donor zygotes (containing NZB mtDNA) were loaded with CMXRos and photoirradiated for either 0 or 20 s. We chose a 20-s exposure time given this was the shortest period that completely precluded development into blastocysts (**Figure 1**). To validate our system, we first assessed in recipient zygotes (CT-P0 and CT-P20) the levels of NZB mtDNA following CT. As a result, comparable levels ($P > 0.05$) of NZB mtDNA were present in CT-P0 (30.8 ± 1.73) and CT-P20 (30.6 ± 1.73) zygotes (**Figure 2A**). Similarly, mtDNA copy number (sum of B6 and NZB mtDNA) was not different ($P > 0.05$) between CT-P0 (365,022 ± 33,062) and CT-P20 (365,704 ± 33,314) zygotes (**Figure 2B**). These zygotes also presented similar mtDNA copy number ($P > 0.05$) compared with zygotes not subjected to CT: B6-control (348,850 ± 23,696), NZB-P0 (375,461 ± 33,388), and NZB-P20 (359,852 ± 23,132). In summary, neither photoirradiation nor CT altered the levels of NZB and total mtDNA in pronuclear zygotes.

We next sought to assess the levels of NZB mtDNA after development of CT-derived zygotes into blastocysts. Toward this, CT-P0 and CT-P20 embryos were cultured *in vitro* for 96 h, reaching the blastocyst stage with similar rates (CT-P0 = 92.3% vs. CT-P20 = 83.8%) when compared to that of B6-control (84.5%) and NZB-P0 (77.9%) embryos. In comparison, only 5.8% of NZB-P20 zygotes developed into blastocysts ($P < 0.05$). In regard of the levels of CT-derived mitochondria, similar ($P > 0.05$) levels of NZB mtDNA were found between blastocysts (31.4% ± 1.43) and zygotes (30.8% ± 1.73) of the CT-P0 group (**Figure 2A**). Conversely, the levels of NZB mtDNA in CT-P20 embryos dropped ($P = 0.008$) from 30.6% ± 1.73 in zygotes to 24.7% ± 1.43 in blastocysts (**Figure 2A**). The levels of NZB mtDNA also proved to be lower ($P < 0.05$) in CT-P20 than CT-P0 blastocysts (**Figure 2A**). On the other hand, mtDNA copy number remained stable (**Figure 2B**) between groups at the blastocyst stage (CT-P0 = 336,497 ± 14,551 vs. CT-P20 = 371,063 ± 20,054). This was also true when compared with NZB-P0 (352,179 ± 15,704) and B6-control (366,065 ± 11,322) blastocysts. Therefore, these findings provide evidence that photoirradiated mitochondria introduced into zygotes were eliminated during development into blastocysts.

Rapamycin Treatment Precludes Elimination of Photoirradiated Mitochondria During Early Embryogenesis

To investigate whether autophagy was linked with the drop in the levels of photoirradiated mitochondria in blastocysts, zygotes subjected to CT were cultured in the presence of rapamycin—an autophagy agonist (Lee et al., 2011; Gilkerson et al., 2012; Dai et al., 2014). As a result, the rapamycin treatment precluded elimination of photoirradiated-derived mtDNA, resulting in similar ($P > 0.05$) levels of NZB and total mtDNA between CT-P0 and CT-P20 blastocysts (**Figures 3A,B**). Additionally, analysis of CT-derived embryos (at the two-cell stage) provided no evidence of increased mitochondria-autophagosome colocalization, regardless of the rapamycin treatment (**Figure 4**). Taken together, these results do not support a link between autophagy and elimination of photoirradiated-derived mitochondria during early embryogenesis.

DISCUSSION

Our present findings provide new evidence that damaged mitochondria are eliminated during early embryogenesis through an autophagy-independent mechanism.

After absorption of light, certain biocompatible photosensitizers are capable of generating reactive species (i.e., hydroxyl radicals and singlet oxygen), which may damage neighboring biomolecules such as membrane unsaturated lipids, proteins, and DNAs (Foote, 1968). Given that some photosensitizers accumulate in specific

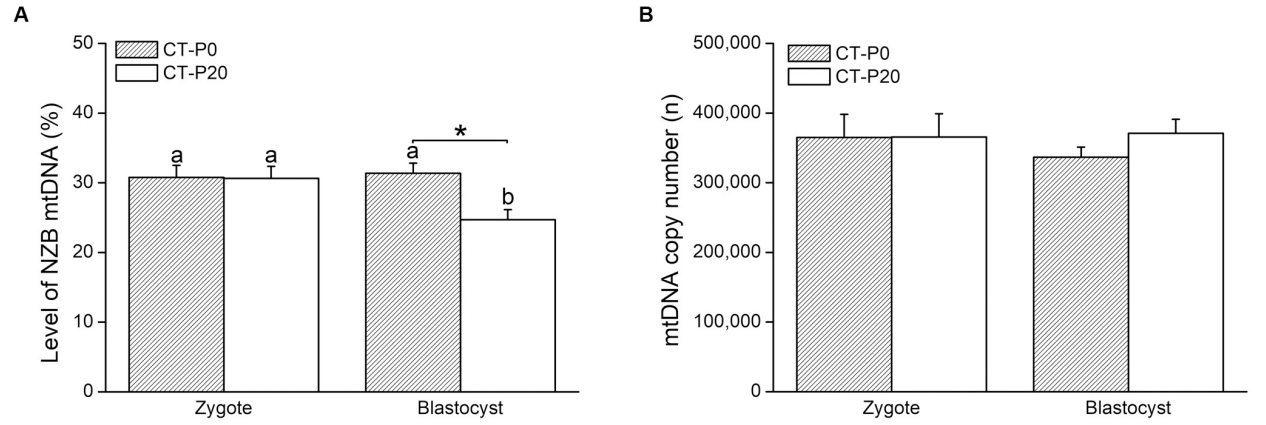

FIGURE 2 | Photoirradiated mitochondria injected into zygotes are selected against during early embryogenesis. CMXRos-loaded zygotes containing mtDNA of NZB origin were photoirradiated for either 0 (P0) or 20 (P20) s to induce mitochondrial damage. Mitochondria from these zygotes were injected by cytoplasmic transfer (CT) into B6 zygotes, resulting in CT-P0 and CT-P20 groups, respectively. CT-derived embryos were assessed at zygote and blastocyst stage as for the levels of NZB **(A)** and total **(B)** mtDNA. Different letters over bars depict statistical difference within group ($P < 0.05$). *Statistical difference within stage ($P = 0.008$).

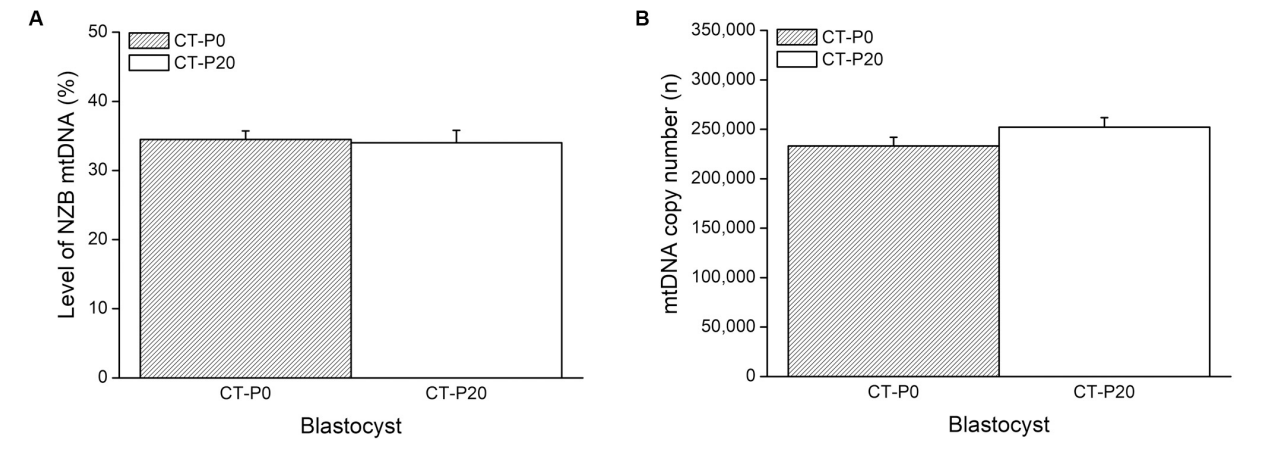

FIGURE 3 | Rapamycin treatment precludes elimination of photoirradiated mitochondria during early embryogenesis. CMXRos-loaded zygotes containing mtDNA of NZB origin were photoirradiated for either 0 (P0) or 20 (P20) s to induce mitochondrial damage. Mitochondria from these zygotes were injected by cytoplasmic transfer (CT) into B6 zygotes, resulting in CT-P0 and CT-P20 groups, respectively. CT-derived blastocysts were assessed as for the levels of NZB **(A)** and total **(B)** mtDNA. No statistical difference ($P > 0.05$).

subcellular compartments, damage can be efficiently targeted to mitochondria (Gabrielli et al., 2004; Oliveira et al., 2011; Hammerer et al., 2018; Taba et al., 2018). In this respect, CMXRos has been shown, both in cultured cells and mouse oocytes, to be a potent mitochondrial photosensitizer (Minamikawa et al., 1999; Lum et al., 2002; Palermo et al., 2002; Lum and Nagley, 2003; Takeuchi et al., 2005). Hence, we have used CMXRos and photoirradiation to specifically induce mitochondrial damage in mouse zygotes. As a result, we found that either CMXRos or photoirradiation alone have no effect on development into blastocysts. Yet, photoirradiation of CMXRos-loaded zygotes for 5 s or more led to a linear decline (up to 20 s) on blastocyst rate. These results corroborate previous findings that photosensitization of oocytes leads to mitochondrial dysfunction and developmental arrest after fertilization (Palermo et al., 2002; Thouas et al., 2004, 2006; Takeuchi et al., 2005).

Given the time-depend effect of photoirradiation, we decided to photoirradiate NZB zygotes, loaded with CMXRos, for either 0 or 20 s. Next, their cytoplasm was transferred into B6 zygotes aiming to track injected mitochondria in blastocysts. Injected and recipient mitochondria were distinguished based on mtDNA origin, respectively, NZB and B6. Importantly, ∼30% of NZB mtDNA were present in zygotes, regardless of the photoirradiation treatment. However, the levels of NZB mtDNA dropped in blastocysts only when photoirradiated-zygotes were used as cytoplasmic donors. Once zygotes injected with either photoirradiated or non-photoirradiated cytoplasm developed into blastocysts with similar rates, this drop cannot be attributed to an impact of CT on embryogenesis. Moreover, mtDNA copy number remained stable between stages (zygotes vs. blastocysts) and treatments (photoirradiated vs. non-photoirradiated), indicating

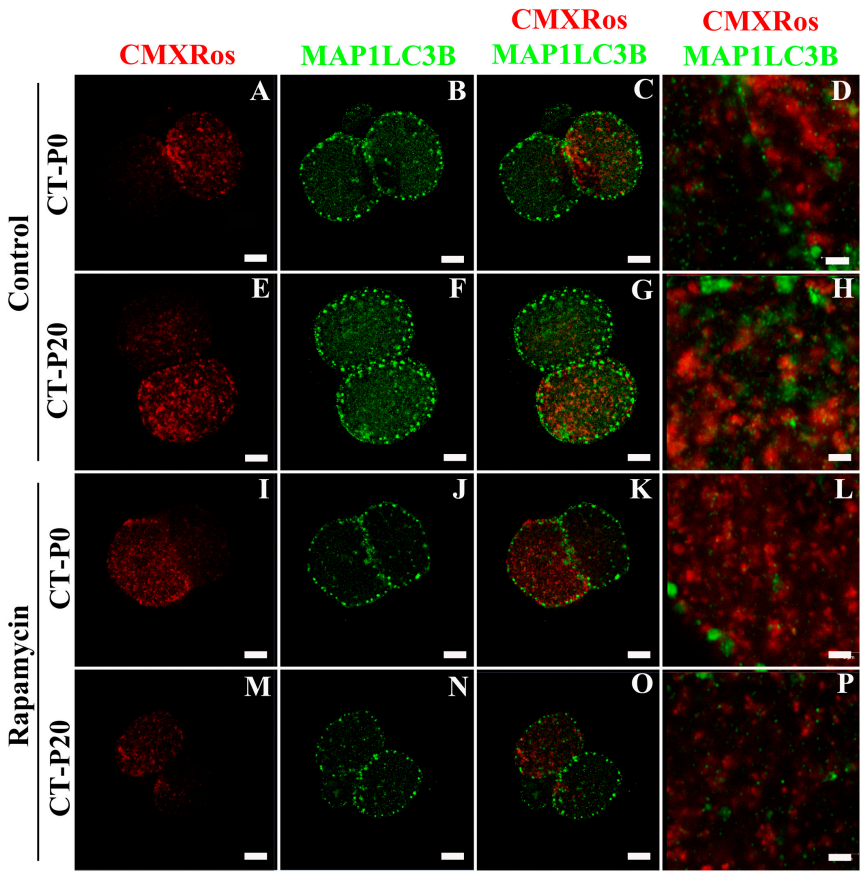

FIGURE 4 | Autophagy is not linked with elimination of photoirradiated mitochondria in early embryos. CMXRos-loaded zygotes were photoirradiated for either 0 (P0) or 20 (P20) s to induce mitochondrial damage. Mitochondria from these zygotes were injected by cytoplasmic transfer (CT) into recipient zygotes, resulting in CT-P0 (A-D and I-L) and CT-P20 (E-H and M-P) groups, respectively. CT-derived zygotes were cultured *in vitro* for 24 h in either absence **(A–H)** or presence **(I–P)** of rapamycin. Autophagosomes in two-cell embryos were detected by immunofluorescence using a primary antibody against MAP1LC3B (green; **B,F,J,N**). Injected mitochondria were visualized based on CMXRos fluorescence (red; **A,E,I,M**). Pictures were merged to assess mitochondria-autophagosome colocalization **(C,G,K,O,D,H,L,P)**. Bars in **(A–C,E–G,I–K,M–O),** and **M–O** correspond to 10 μm, while in **(D,H,L,P),** they correspond to 2 μm.

that photoirradiated-derived mtDNA was replaced by recipient mtDNA in blastocysts.

There is mounting evidence in support of purifying selection acting in the female germline to prevent the accumulation of deleterious mtDNA mutations (Sato et al., 2007; Fan et al., 2008; Stewart et al., 2008; Freyer et al., 2012; Sharpley et al., 2012; Li et al., 2016; Floros et al., 2018; Lieber et al., 2019; Wei et al., 2019). Among other stages of germline development, purifying selection may take place during early embryogenesis as reported by Lee et al. (2012) and Latorre-Pellicer et al. (2019). In agreement with these reports, our present data indicate that the levels of NZB mtDNA dropped in blastocysts only when it derived from photoirradiated cytoplasm. Given that photoirradiation leads to mitochondrial damage (Minamikawa et al., 1999; Lum et al., 2002; Palermo et al., 2002; Lum and Nagley, 2003; Takeuchi et al., 2005), including on mtDNA (Battogtokh et al., 2018), we argue that photoirradiated mitochondria were targeted for destruction in preimplantation embryos (Wang et al., 2012). This hypothesis is in keeping with autophagic elimination of paternal mitochondria following fertilization (Rojansky et al., 2016), suggesting that a similar mechanism might be involved with elimination of dysfunctional mitochondria inherited from the oocyte.

To address the hypothesis that photoirradiated mitochondria were destroyed by autophagy, injected embryos were cultured in the presence of rapamycin. We expected with this treatment to enhance the drop in the levels of NZB mtDNA as rapamycin is a canonical inducer of macroautophagy; by inhibiting mTORC1, rapamycin induces autophagosome formation and degradation of cellular components such as dysfunctional mitochondria (Kim et al., 2002; Narendra et al., 2008; Twig et al., 2008; Suen et al., 2010; Gilkerson et al., 2012; Dai et al., 2014). In opposite to our prediction, rapamycin prevented the drop in NZB mtDNA associated with injection of photoirradiated cytoplasm. Although difficult to explain, we propose that rapamycin mitigated mitochondrial damage induced by photoirradiation. This hypothesis is supported by a previous report showing that rapamycin upregulates DNA repair enzyme OGG1 (Habib et al., 2010). Thus, rapamycin might have countered mitochondrial damage by enhancing mtDNA repair on photoirradiated-derived

mitochondria. In addition, embryos were assessed as for colocation between injected mitochondria and autophagosomes. Two-cell embryos were used as an autophagic wave takes place at this stage (Tsukamoto et al., 2008), coinciding with destruction of paternal mitochondria in mice (Rojansky et al., 2016). However, no skewed colocalization of photoirradiated mitochondria and autophagosomes was seen, even when considering the rapamycin treatment. Together, these data do not implicate autophagy in the elimination of photoirradiated-derived mitochondria.

Our current findings support the hypothesis that damaged mitochondria are destroyed during early embryogenesis, suggesting that the same mechanism might take place to counter expansion of deleterious mtDNA mutations. Such mechanism is in accordance with the "Muller's ratchet" theory, which proposes that uniparental inheritance of mtDNA in the absence of recombination would lead to accumulation and fixation of deleterious mutations (Muller, 1964). In fact, few highly deleterious mutations in mtDNA have become fixed in the human population, lending further support to purifying selection (Rand and Kann, 1996; Elson et al., 2004; Rand, 2008; Wei et al., 2019). Considering that deleterious mtDNA mutations may impact mitochondrial function, mutations might be selected against at the organelle level (Burr et al., 2018). In support of this notion, previous reports have provided evidence that autophagy acts to eliminate dysfunctional mitochondria with deleterious mtDNA mutations (Narendra et al., 2008; Twig et al., 2008; Suen et al., 2010; Gilkerson et al., 2012; Dai et al., 2014). Although our findings do not support a link between autophagy and the lower levels of photoirradiated-derived mitochondria in blastocysts, this requires further investigation as it might be a rapamycin-independent mechanism (Yamamoto et al., 2014) or take place at a different embryonic stage (Tsukamoto et al., 2008).

CONCLUSION

The preimplantation embryo is competent to mitigate the levels of damaged mitochondria. This finding is of relevance for the transmission of mitochondrial disease as a similar mechanism might take place during early embryogenesis to counter expansion of deleterious mtDNA mutations. Limitation of the study: lack of mtDNA sequencing data. Further studies are needed to show whether the lower levels of damaged mitochondria in blastocysts are linked with elimination of potentially deleterious mtDNA mutations derived from photoirradiation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee at Universidade de São Paulo (USP—protocol number 13.1.1832.74.8).

AUTHOR CONTRIBUTIONS

MRC designed the experiments and wrote the manuscript. TM, CM, MDC, and MRC carried out the experiments, data organization, and statistical analyses. FG and FM contributed new reagents and analytical tools. All authors read and approved the final manuscript.

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Methylation of Ribosomal RNA: A Mitochondrial Perspective

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Ribosomal RNA (rRNA) from all organisms undergoes post-transcriptional modifications that increase the diversity of its composition and activity. In mitochondria, specialized mitochondrial ribosomes (mitoribosomes) are responsible for the synthesis of 13 oxidative phosphorylation proteins encoded by the mitochondrial genome. Mitoribosomal RNA is also modified, with 10 modifications thus far identified and all corresponding modifying enzymes described. This form of epigenetic regulation of mitochondrial gene expression affects mitoribosome biogenesis and function. Here, we provide an overview on rRNA methylation and highlight critical work that is beginning to elucidate its role in mitochondrial gene expression. Given the similarities between bacterial and mitochondrial ribosomes, we focus on studies involving *Escherichia coli* and human models. Furthermore, we highlight the use of state-of-the-art technologies, such as cryoEM in the study of rRNA methylation and its biological relevance. Understanding the mechanisms and functional relevance of this process represents an exciting frontier in the RNA biology and mitochondrial fields.

Keywords: mitochondria, RNA, ribosome, methylation, methyltransferases, epigenetics

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EPIGENETIC MODIFICATIONS OF rRNA

RNA modifications are present in all living organisms and play important roles in RNA metabolism. The number of experimentally identified RNA modifications is growing, and to date, more than 170 RNA modifications have been reported (Boccaletto et al., 2018). RNA modifications are predominantly found in transfer RNA (tRNA), with modifications identified in up to 20% of nucleotides (Jackman and Alfonzo, 2013). Although not as common as in tRNA, human cytosolic ribosomal RNA (rRNA) contains 14 distinct types of post-transcriptional modifications in 228 sites (Taoka et al., 2018), while *Escherichia coli* rRNAs contain 36 modified nucleotides (Sergiev et al., 2011). Among the different types of rRNA modifications, 2′-O-methylation of the ribose followed by pseudouridylation is the most common (for a review on this abundant RNA modification see Charette and Gray, 2000).

In bacteria, most of the methylated nucleotides in the small subunit (SSU) of the ribosome are located on the surface and are introduced during the late stages of ribosome assembly, while nucleotide modifications in the large subunit (LSU) occur during early stages of assembly (Siibak and Remme, 2010). In eukaryotes, the introduction of rRNA modifications is closely linked to rRNA processing events and coupled to various stages of ribosome assembly (Armistead et al., 2009; Bourgeois et al., 2015; Zorbas et al., 2015).

In most cases, the precise role of rRNA modifications remains unclear. Some rRNA modifications are located on highly conserved nucleotides and cluster in functionally important areas of the ribosome, including the peptidyl transferase center and decoding site (Decatur and Fournier, 2002; Polikanov et al., 2015). This suggests that they may play important roles, including alteration of ribosomal active sites and stabilization of the rRNA scaffold (Demirci et al., 2010b; Polikanov et al., 2015). Furthermore, the presence of partial modifications, including 2′-O-methylation in a subset of rRNAs, indicates that nucleotide modifications may play additional roles under different physiological conditions (Krogh et al., 2016). Alterations in rRNA modification patterns have also been described during development (Blanco and Frye, 2014), in response to environmental changes (Schwartz et al., 2014) and in disease (Armistead et al., 2009). While it remains unclear how rRNA modifications affect overall cellular function, it is becoming evident that rRNA modifications are dynamic factors in the regulation of gene expression and may contribute to the fine-tuning of translation regulation.

METHYLATION OF rRNA IN BACTERIA AND THE EUKARYOTIC CYTOSOL

Universally Conserved rRNA Methylation Sites

Methylation of rRNAs is a ubiquitous feature in all living organisms, and the presence of methylated rRNA residues at corresponding sites in prokaryotes and eukaryotes indicates that it is evolutionarily conserved. There are two universally conserved methylated residues in the SSU, N^6-dimethylated adenines $m^6{}_2$A1518 and $m^6{}_2$A1519 (*E. coli* 16S rRNA numbering) located in helix 45 (Poldermans et al., 1979). Methylation at these sites facilitates contact between helices 44 and 45 near the decoding center of the ribosome through the formation of a hydrogen-bonding network that stabilizes this contact site (Wimberly et al., 2000; Demirci et al., 2010b). Absence of this dimethylation results in the rearrangement of the ribosomal decoding center and decreases fidelity of translation initiation and elongation (Demirci et al., 2010b). Methylation at these sites is introduced by KsgA, a highly conserved methyltransferase present in almost all living organisms (Poldermans et al., 1979; Formenoy et al., 1994). DIMT1L, the mammalian homolog of KsgA, is responsible for dimethylation in helix 45 and also plays a role in the assembly of the small ribosomal subunit through its independent function in pre-rRNA processing (Zorbas et al., 2015). In mitochondria, corresponding modifications are introduced by TFB1M, described in detail in the "mt-SSU Methyltransferases" section below.

The LSU contains two universally conserved modified nucleotides, Gm2251 and Um2552 (*E. coli* 23S rRNA numbering), located in the P-loop and A-loop (helices 80 and 92, respectively; Kiss-László et al., 1996; Lövgren and Wikström, 2001). Structural analyses indicate that the Um2552 methylation intercalates between the adjacent bases G2553 and U2554, thus preserving the active conformation of the G2553 base, which is directly involved in accommodating the aminoacyl-tRNA (Polikanov et al., 2015). Similarly, the 2′-O-methyl group of Gm2251 forms hydrophobic contacts with C2065 and U2449 that maintain the active conformation of the nucleotides involved in base pairing with P- and A-site tRNAs (Polikanov et al., 2015). While the absence of Gm2251 in *E. coli* has no phenotypic effects (Lövgren and Wikström, 2001), the lack of Um2552 results in a significant accumulation of assembly intermediates of the LSU (Tan et al., 2002). In bacteria, Gm2251 and Um2552 are introduced by the methyltransferases RlmB and RlmE, respectively (Caldas et al., 2000; Lövgren and Wikström, 2001), while the human cytoplasmic equivalents, Gm4196 and Um4498, are catalyzed *via* a small nucleolar RNA (snoRNA)-guided mechanism (Kiss-László et al., 1996) and an unknown mechanism, respectively. In mitochondria, modifications corresponding to Gm2251 and Um2552 are introduced by mitochondrial rRNA methyltransferase 1 (MRM1) and MRM2 respectively (described below in "mt-LSU Methyltransferases" section).

Enzymes Responsible for Methylation of rRNA

Methylation of rRNAs takes place during ribosomal biogenesis either by enzymes guided by an antisense snoRNA or by conventional protein enzymes. All enzymes responsible for known rRNA methylation sites in *E. coli* and the yeast, *Saccharomyces cerevisiae*, have now been identified (Sharma and Lafontaine, 2015). However, the enzymes responsible for the modification of nucleotides Um4498, Gm4499, and m^3U4530 in humans remain to be identified.

In eukaryotes, the most common rRNA modifications, 2′-O methylation and pseudouridylation, are catalyzed by small nucleolar ribonucleoprotein (snoRNP) particles that consist of snoRNA and proteins and occur simultaneously with the processing of rRNA precursors (Phipps et al., 2011). snoRNAs act as guides for snoRNPs *via* sequence complementarity with their respective rRNA target sequence (Reichow et al., 2007). Most snoRNPs fall into two large categories, C/D and H/ACA snoRNPs; C/D snoRNPs mediate 2′-O methylation, while H/ACA snoRNPs are responsible for pseudouridylation modifications (for a review see Watkins and Bohnsack, 2012). Other modifications are catalyzed by methyltransferases that modify specific rRNA nucleotides and do not require the participation of snoRNPs. To date, 57 RNA methyltransferases have been identified in humans (Schapira, 2016). With rare exceptions (Lesnyak et al., 2006; Kimura et al., 2012), each methyltransferase is responsible for the methylation of one rRNA nucleotide only.

In addition to methylation, several rRNA methyltransferases are involved in other aspects of ribosomal biogenesis, including pre-rRNA processing (Armistead et al., 2009). Interestingly, it has been shown that the role of some methyltransferases in pre-rRNA processing may be more critical to cellular function than their role in modifying rRNA. This is likely explained by the fact that eukaryotic 5.8S, 18S, and 28S rRNAs are encoded by a single, long polycistronic transcript that requires extensive processing by multiple assembly factors, including RNA-modifier enzymes, to release mature rRNAs (Kressler et al., 1999a). The existence of pre-rRNA processing enzymes that also function

as methyltransferases may thus reflect a quality control mechanism, whereby methylation of certain rRNA nucleotides is dependent upon the generation of mature rRNAs.

BIOLOGICAL SIGNIFICANCE OF rRNA METHYLATION

Numerous studies have shown that methylation of rRNA may have important implications for human health. This is primarily due to its role in antibiotic resistance, a potential role in cancer development, and because of genetic diseases caused by mutations in the rRNA methylation machinery components.

Antibiotic Resistance

Most ribosome-targeting antibiotics interact exclusively with bacterial rRNA. Bacteria have evolved several mechanisms of resistance to antibiotics, including through the methylation of specific rRNA nucleotides that prevents the binding of protein synthesis inhibitors to their target sites on the bacterial ribosome. For instance, N^1 methylation of A1408 in the bacterial 16S rRNA confers resistance against aminoglycosides (Kanazawa et al., 2017). Loss of methylation can also decrease antibiotic sensitivity. A classic example of this is the lack of methylation at A1518 and A1519 in 16S rRNA by KsgA, which confers resistance to kasugamycin (Poldermans et al., 1979). Similarly, the loss of m^2A2503 in 23S rRNA, catalyzed by RlmN, confers resistance to antibiotics that target the peptidyl transferase center of the ribosome (Stojković et al., 2016). These examples highlight the important role of methylation in regulating the response to antibiotics.

Cancer

There is increasing evidence linking messenger RNA (mRNA) or tRNA methylation and cancer. For instance, the m^6A modification in mRNAs is associated with tumor proliferation in endometrial cancer (Liu et al., 2018), while m^5C methylation of tRNAs by NSUN2 in skin cancer cells has been associated to tumorigenesis (Blanco et al., 2016). Similarly, altered ribosome biogenesis has been associated with the development of various cancers (Truitt and Ruggero, 2016). Evidence linking rRNA methylation and cancer comes from the inactivation of the tumor-suppressor gene *p53*, which resulted in an altered rRNA methylation pattern (Marcel et al., 2013). Future studies may elucidate the role of individual rRNA modifications in cancer. This is of particular interest given that modulation of ribosome biogenesis may also provide an alternative mechanism to arrest cell proliferation and delay tumor formation (Brighenti et al., 2015).

Pathogenic Mutations in Methyltransferases

There is a growing list of human genetic disorders named ribosomopathies that are caused by mutations in genes encoding ribosomal proteins or ribosome biogenesis cofactors, including those involved in the rRNA methylation machinery. For instance, a point mutation in the EMG1 methyltransferase causes Bowen-Conradi syndrome, a ribosomopathy characterized by severe developmental delay and growth failure that often leads to early infant death (Armistead et al., 2009). Prader-Willi syndrome, a neurological disease characterized by intellectual disability, obesity, and muscle hypotonia is caused by deletions in the locus 15q11–q13, which contains a cluster of snoRNAs involved in RNA 2′-O-methylation (Sahoo et al., 2008). Similarly, mutations in the family of NOL1/NOP2/sun (Nsun) domain-containing genes encoding RNA methyltransferases in humans are associated with neurodevelopmental disorders (Blanco and Frye, 2014). However, due to the dual role of some methyl transferases in pre-rRNA processing, the exact contribution of impaired rRNA methylation to the pathology of these disorders requires further investigation.

MITOCHONDRIAL RNA EXPRESSION

Evolutionarily originated from α-proteobacteria that were engulfed by a primitive cell (Roger et al., 2017), mitochondria retain their own circular double-stranded DNA along with their own protein translational machinery. Mammalian mitochondrial DNA (mtDNA) is 16,569 bp and is maternally inherited (Kaneda et al., 1995). It encodes a total of 37 genes, including 2 rRNAs, 22 tRNAs, and 13 polypeptides of the oxidative phosphorylation (OxPhos) system (Anderson et al., 1981). mtDNA exists as compactly-packed nucleoid structures of ~100 nm with mitochondrial transcription factor A (TFAM) being the core packaging factor (Brown et al., 2011; Kukat et al., 2011).

Unlike their cytosolic counterparts, mitochondrial RNAs (mt-RNAs) are transcribed as long polycistronic transcripts and require endonucleolytic cleavage for individual transcripts to be released. Processing of mitochondrial transcripts flanked by mitochondrial tRNAs (mt-tRNAs) involves cleavage by Ribonuclease P (RNaseP) complex and ElaC Ribonuclease Z 2 (ELAC2; Holzmann et al., 2008; Brzezniak et al., 2011; Sanchez et al., 2011), while mitochondrial transcripts that are not flanked by mt-tRNAs require additional protein factors for processing, including FASTKD4, FASTKD5, and GRSF1 (Jourdain et al., 2013, 2017). Similar to their cytosolic counterparts, mt-RNAs also are polyadenylated; however, the poly(A) tails are shorter, with an average length of 45–55 nucleotides, while the ND6 transcript is not polyadenylated at all (Temperley et al., 2010). Polyadenylation of the 3′ end of mt-RNAs is essential for the completion of stop codons of several mitochondrial transcripts and, therefore, for correct translation of their open-reading frames. Mutations in poly(A) polymerase (mtPAP) have been linked to neurodegenerative disease (Crosby et al., 2010).

Mitochondria maintain their own ribosomes (mitoribosomes) and translation system. The mammalian mitoribosome consists of RNA and proteins, with 16S mitoribosomal rRNA (mt-rRNA) and a mt-tRNA belonging to the mitoribosome large subunit (mt-LSU), and 12S mt-rRNA belonging to the mitoribosome small subunit (mt-SSU). There are 82 mitoribosomal proteins (**Table 1**), 36 of which are mitochondria-specific, while many proteins with homologs in bacteria have mitochondria-specific extensions.

Although mitoribosomes are similar to their bacterial counterparts, there are some key differences. For instance, while

TABLE 1 | Composition of eukaryotic and prokaryotic small and large ribosomal subunits.

Ribosome	Monosome sedimentation rate	Small subunit	Large subunit
Eukaryotic	80S	40S: 18S rRNA and 33 ribosomal proteins (mammals)	60S: 5S, 5.8S, and 28S (mammals) rRNAs and 47 ribosomal proteins (mammals)
Prokaryotic	70S	30S: 16S rRNA and 22 ribosomal proteins	50S: 5S and 23S rRNAs and 34 ribosomal proteins
Mitochondrial	55S	28S: 12S rRNA and 30 ribosomal proteins	39S: mitochondrially-encoded tRNA, 16S rRNA, and 52 ribosomal subunits

the RNA:protein ratio in bacterial ribosomes is 2:1, it is 1:2 in mitoribosomes, due to the large rRNA reductions and recruitment of new proteins stabilizing the mitoribosomal structure (Mears et al., 2006). Structural studies of the mammalian mitoribosome revealed that 5S rRNA is absent from the central protuberance of the mt-LSU. Instead, a mitochondrially-encoded $tRNA^{Val}$ was detected in human and $tRNA^{Phe}$ in porcine mitoribosomes (Amunts et al., 2015; Greber et al., 2015). Another significant adaptation of mitoribosomes is the presence of mitochondria-specific proteins with highly hydrophobic amino acid residues facing the ribosomal exit tunnel, due to the hydrophobic nature of the mtDNA-encoded OxPhos subunits (Amunts et al., 2015; Greber et al., 2015).

RNA MODIFICATIONS IN MITOCHONDRIA

Numerous nuclear-encoded enzymes have been shown to introduce a wide range of modifications on mt-tRNAs. To date, 15 different types of modifications have been detected at 118 positions in mt-tRNAs, some of which occur within the anti-codon loop and are important for tRNA decoding, while others are important for the stabilization of tRNA structures and their recognition by aminoacyl-tRNA synthetases (reviewed in Suzuki and Suzuki, 2014). In contrast, while recent findings reported the presence of multiple pseudouridine and m^1A sites in mt-mRNAs (Carlile et al., 2014; Antonicka et al., 2017; Li et al., 2017; Safra et al., 2017), their importance in the regulation of mitochondrial gene expression still needs to be elucidated.

The total number of modifications mapped to mammalian mt-rRNAs is significantly lower than that for bacterial and cytoplasmic rRNAs. There are 10 modifications identified to date in mt-rRNAs, including three 2′-O-ribose methylations, six base methylations, and one pseudouridylation (**Figure 1** and **Table 2**). The majority of these modifications were identified around 40 years ago by Dubin and colleague in hamster cells (Dubin, 1974; Dubin and Taylor, 1978; Baer and Dubin, 1980, 1981). Since then, new modifications have been uncovered, and the enzymes responsible for all thus far identified modifications have been described. The roles of mitochondrial methyltransferases and their mt-rRNA targets are discussed below.

MITOCHONDRIAL rRNA METHYLTRANSFERASES

mt-SSU Methyltransferases

TFB1M (m^6_2A936/m^6_2A937)

TFB1M is a homolog of the universally conserved methyltransferase KsgA (also known as RsmA). Structural analyses of bacterial KsgA in complex with the 30S subunit indicated that the enzyme binds to the inactive conformation of the SSU with helix 44 in a displaced conformation (O'Farrell et al., 2004; Boehringer et al., 2012). This binding blocks the interaction between helices 44 and 45, which form the decoding center in the mature ribosomal subunit. Once the 30S platform and helix 45 reach a near mature conformation, KsgA methylates helix 45, which leads to its dissociation. The release of KsgA is required for helix 44 to assume its native position in the 30S subunit and for 17S rRNA processing (Boehringer et al., 2012). KsgA's role in the formation of the translationally active 30S subunit conformation may explain its conservation across all domains of life.

Initially, mitochondrial TFB1M was considered to function as a transcription factor alongside TFB2M. However, it was instead shown to be a dimethyltransferase responsible for m^6_2A modification of the 12S rRNA (Cotney and Shadel, 2006; Liu et al., 2019). This modification occurs within two adjacent adenines, A936 and A937 (human mtDNA position: m.1583A and m.1584A, respectively), located in the tetraloop "GGAA" of helix 45 at the 3′-end of the mt-12S rRNA, which is extremely conserved in both sequence and structure (McCulloch et al., 2002). TFB1M binds S-adenosylmethionine (SAM), the methyl-donating substrate of methyltransferase enzymes, and can functionally complement KsgA ablation by restoring the dimethylation of the conserved stem-loop (McCulloch et al., 2002; Seidel-Rogol et al., 2003).

Studies in the fly *Drosophila melanogaster* (Dm) demonstrated that the Dm-TFB1M ortholog is mainly involved in translation regulation, while Dm-TFB2M is involved in transcription (Matsushima et al., 2004, 2005). This is consistent with studies showing that human TFB1M has a greater rRNA methyltransferase activity compared to TFB2M (Cotney and Shadel, 2006). The importance of the m^6_2A modification has been subsequently highlighted in an *in vivo* study, showing that mouse *Tfb1m* knock-out is embryonic lethal, while heart conditional knock-out causes loss of mt-12S rRNA dimethylation, affecting the stability of the mt-SSU and leading to altered mitoribosome assembly and mitochondrial translation (Metodiev et al., 2009).

Recently, the crystal structure of TFB1M in complex with helix 45 and SAM has revealed its unique properties compared to its paralogue TFB2M (Liu et al., 2019). Notably, TFB1M has a clear acid-active pocket, which accommodates SAM, while the same region in TFB2M is highly positively charged, thus facilitating interaction with DNA molecules. Furthermore, A937 has been recognized as the first adenine to be methylated and G934 is necessary for this methylation, since it brings A937 into the active center where SAM is sheltered (Liu et al., 2019).

Interestingly, two mt-SSU assembly factors, the human ribosome-binding factor A (RBFA) and Era-like 12S mitochondrial rRNA chaperone 1 (ERAL1), have been identified to interact with the hairpin at the 3′-terminus of mt-12S rRNA, where dimethylation

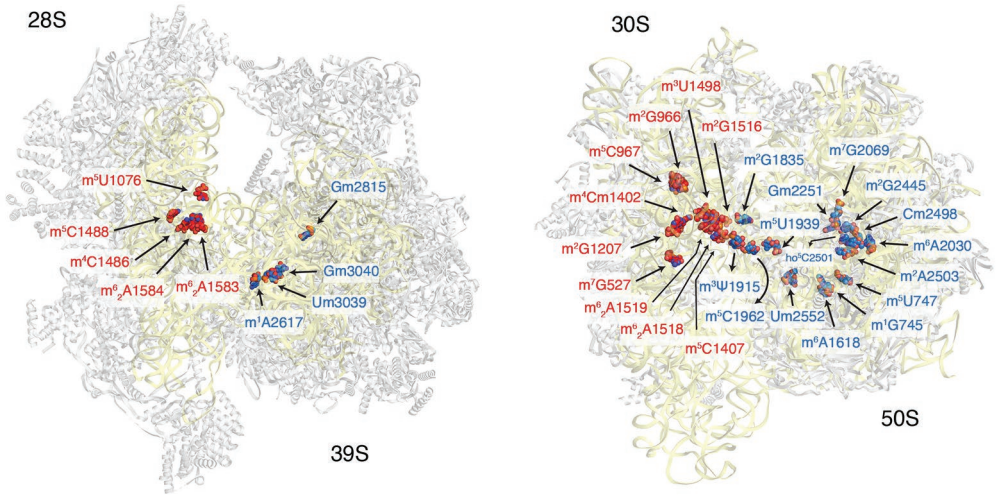

FIGURE 1 | Distribution of ribosomal RNA (rRNA) methylation sites on human mitoribosome and bacterial ribosomes. The location of rRNA-methylation sites on ribosomal small subunit (red) and large subunit (blue) are displayed on the structure of human mitoribosome (**left**, PDB: 3J9M) and *Escherichia coli* (**right**, PBD: 4YBB). The ribosomal proteins are colored gray and the rRNA is in yellow.

TABLE 2 | Mitochondrial rRNA methyltransferases, corresponding bacterial homologs, and modified RNA residues.

Homo sapiens **Enzyme**	***H. sapiens*** **rRNA**	**Reference**	***E. coli*** **Enzyme**	***E. coli*** **rRNA**	**Reference**
TFB1M	m^6_2A936 m^6_2A937	(Cotney and Shadel, 2006; Liu et al., 2019)	KsgA/RsmA	m^6_2A1518 m^6_2 A1519 16S rRNA	(Helser et al., 1972; Poldermans et al., 1979; Formenoy et al., 1994)
NSUN4	m^5C841	(Metodiev et al., 2014)	RsmF	m^5C1407 16S rRNA	(Demirci et al., 2010a)
TRMT2B	m^5U429	(Laptev et al., 2019)	RlmD	m^5U1939 23S rRNA	(Madsen et al., 2003; Auxilien et al., 2011)
METTL15	m^4C839	(Haute et al., 2019)	RsmH	m^4Cm1402 23S rRNA	(Kimura and Suzuki, 2010)
MRM1	Gm1145	(Lee et al., 2013; Lee and Bogenhagen, 2014)	RlmB	Gm2251 23S rRNA	(Lövgren and Wikström, 2001)
MRM2	Um1364	(Lee et al., 2013; Lee and Bogenhagen, 2014; Rorbach et al., 2014)	RlmE	Um2552 23S rRNA	(Caldas et al., 2000)
MRM3	Gm1370	(Lee et al., 2013; Lee and Bogenhagen, 2014; Rorbach et al., 2014)	No homolog		
TRMT61B	m^1A947	(Bar-Yaacov et al., 2016)	TrmI	m^1A58 tRNA	(Droogmans et al., 2003)

by TFB1M occurs (Dennerlein et al., 2010; Uchiumi et al., 2010; Rozanska et al., 2017). RBFA was observed to bind directly the dimethylation site of mt-12S rRNA and *RBFA* knock-down resulted in a reduced level of modification, suggesting that RBFA helps to expose adenines for subsequent methylation by TFB1M (Rozanska et al., 2017). Future studies are necessary to describe the molecular details of RBFA involvement in this process.

TRMT2B (m5U429)

Although methyl-5-uridine (m^5U) is one of the most abundant RNA modifications (Boccaletto et al., 2018), it remains poorly characterized. Bacterial RlmD, which modifies m^5U1939 of the 23S rRNA in *E. coli*, is considered to be the ancestral m^5U RNA methyltransferase (Auxilien et al., 2011). Due to gene duplication and specialization, RlmC and TrmA enzymes have evolved in addition to RlmD. RlmC modifies m^5U747 of the 23S rRNA (Madsen et al., 2003), and TrmA introduces m^5U at position 54 in the T-loop of several tRNAs (Ny and Björk, 1980). In *S. cerevisiae*, Trm2 catalyzes this tRNA modification (Nordlund et al., 2000). Sequence homology analysis identified two mammalian proteins, TRMT2A and TRMT2B, as m^5U methyltransferase candidates (Carter et al., 2019). TRMT2A was identified as the enzyme responsible for m^5U54 in the cytosol (Carter et al., 2019; Powell and Minczuk, 2020), while TRMT2B was suggested to methylate tRNAs in mitochondria (de Crécy-Lagard et al., 2019).

A recent study by Laptev et al. showed that *Trmt2b* knock-out in mouse cells leads to a lack of m^5U425 methylation (mouse numbering, equivalent to human m^5U429, mtDNA position: m.1076T) in mt-12S rRNA as well as of U54 in certain mitochondrial tRNAs, indicating that TRMT2B might act as a dual tRNA/rRNA methyltransferase (Laptev et al., 2019). At the same time, Powell and Minczuk showed that TRMT2B is located in human mitochondria and plays an essential role in methylation of both tRNAs and 12S rRNA (Powell and Minczuk, 2020). Similar to yeast Trm2 (Nordlund et al., 2000), no apparent impairment of mitochondrial tRNA stability, mitoribosome integrity, or mitochondrial protein synthesis was detected upon TRMT2B loss in human cells (Powell and Minczuk, 2020). Interestingly, while the rate of protein synthesis was also not affected in mouse *Trmt2b* knock-out model, a small, but statistically significant, decrease in the activity of OxPhos complexes I, III, and IV was detected, which may be explained by a reduction of protein synthesis fidelity (Laptev et al., 2019).

The mild phenotype upon TRMT2B loss is in contrast to a detrimental effect on mitochondrial translation observed upon the loss of other mt-rRNA modifying enzymes, including for example TFB1M (Metodiev et al., 2009) or NSUN4 (Metodiev et al., 2014). The exact role of the modification introduced by TRMT2B and its contribution to mitochondrial function in different environmental conditions and/or specific tissues requires further investigation.

NSUN4 (m^5C841)

NSUN4 is a mitochondrial rRNA methyltransferase that belongs to the m^5C methyltransferase family and introduces m^5C911 modification of mt-12S rRNA in mice (human m^5C841, mtDNA position: m.1488C; Metodiev et al., 2014).

In *Thermus thermophilus*, the corresponding residue (position C1404) is modified by the methyltransferase RsmF, which also modifies C1400 and C1407. All three m^5C residues modified by RsmF in *T. thermophilus* 16S rRNA are clustered around the decoding center, close to sites of contact with tRNA, mRNA, and elongation factor G. The *T. thermophilus* RsmF null mutants were shown to be thermosensitive. *In vitro*, RsmF methylates C1404 to around 35% with naked 16S rRNA as a substrate and to 100% in the context of 30S subunit, suggesting that this modification is likely to be introduced at the later stages of SSU biogenesis (Demirci et al., 2010a)

In mice, knock-out of *Nsun4* results in defective embryonic development, while heart conditional knock-out causes an OxPhos impairment, leading to severe cardiomyopathy (Metodiev et al., 2014). Sucrose gradient centrifugation analysis revealed that NSUN4 ablation leads to the accumulation of free mt-SSU and mt-LSU, preventing monosome formation (Metodiev et al., 2014).

Interestingly, NSUN4 has been shown to form a stable heterodimeric complex with MTERF4 that is targeted to the mt-LSU and plays an essential role in mt-LSU assembly, independent of the methylation activity of NSUN4 (Cámara et al., 2011). NSUN4 lacks RNA binding domains; instead, structural studies revealed that a positively charged surface forms an RNA binding path from MTERF4, along NSUN4, all the way into its active site, suggesting that both proteins contribute to RNA recognition (Spahr et al., 2012). *In vitro* methylation experiments showed that MTERF4 strongly stimulates the specificity of NSUN4; however, the monomeric NSUN4 is still able to methylate the substrate albeit with lower specificity (Yakubovskaya et al., 2012).

METTL15 (m^4C839)

Recently, METTL15, from the methyltransferase-like (METTL) family, was reported to be involved in m^4C839 (human mtDNA position: m.1486C) modification of human 12S rRNA (Haute et al., 2019; Chen et al., 2020). An equivalent position in bacteria (C1402) has two modifications, N^4 and 2′-O-methylations (m^4Cm), introduced by RsmH and RsmI, respectively (Kimura and Suzuki, 2010), while in the human mitoribosome 2′-O-methylation at C839 is not conserved. *In vitro*, recombinant *E. coli* RsmH and RsmI reconstitute $m^4Cm1402$ on the 30S subunit, but not on the naked 16S rRNA, suggesting that these modifications are formed at a late step during 30S assembly. Moreover, RsmH prefers 2′-O-methyl cytosine as a substrate and, therefore, m^4C in bacteria likely occurs subsequent to the 2′-O-methylation. Modified $m^4Cm1402$ interacts directly with the P-site codon of the mRNA and the lack of N^4 methylation increases the efficiency of non-AUG initiation and decreases the rate of UGA read-through, implying that $m^4Cm1402$ plays a role in fine-tuning the ribosomal decoding center, thus increasing decoding fidelity (Kimura and Suzuki, 2010).

In human cells, METTL15 localizes to mitochondria, and the lack of this enzyme leads to mitochondrial dysfunction. METTL15 was shown to interact with the mt-SSU, and knock-out of *METTL15* results in significantly decreased m^4C839 levels in 12S rRNA (Haute et al., 2019; Chen et al., 2020). Loss of m^4C839 modification leads to aberrant assembly of the mt-SSU and accumulation of late-stage assembly intermediates, suggesting an important role of this modification in the 12S rRNA folding and, consequently, interaction with the mitoribosomal proteins. Importantly, both published reports detected reduction in the m^5C841 modification catalyzed by NSUN4 (Metodiev et al., 2014) concomitant to decreased m^4C839 modification, revealing a potential crosstalk between modifications of these two nearby residues.

Interestingly, Shi et al. have recently identified another member of the METTL family, METTL17, to modulate m^4C839 modification (Shi et al., 2019). METTL17 localizes to mitochondria and associates with the mt-SSU. Loss of METTL17 leads to around 70% reduction of m^4C840 and 50% reduction of m^5C842 of 12S mt-rRNA, severely compromising integrity of the mt-SSU and mitochondrial protein translation (Shi et al., 2019). Collectively, these data suggest an important role for METTL17 in mitochondrial function, although further work is needed to assess potential interdependence of METTL15 and METTL17 in m^4C839 methylation.

mt-LSU Methyltransferases

MRM1 (Gm1145)

Human mitochondrial 16S rRNA contains three 2′-O-ribose methylation sites: Gm1145, Um1364, and Gm1370 (human mtDNA positions: m.2815G, m.3039T, m.3040G, respectively). These methylations reside in highly conserved sites found within the peptidyl transferase center (Decatur and Fournier, 2002).

The peptidyl transferase region of 16S rRNA involved in the binding of tRNA in the P-site (referred to as peptidyl-transferase loop, P-loop) undergoes 2′-O-ribose methylation

at G1145 by MRM1. This modification is highly conserved across ribosomes of different species and seems to play a direct role in peptidyl-tRNA recognition (Sergiev et al., 2018). In yeast, the equivalent modification, Gm2270, on mitochondrial 21S rRNA is catalyzed by Pet56p/MRM1 (Sirum-Connolly and Mason, 1993). The loss of Pet56p in *S. cerevisiae* leads to a defect in the maturation of the mt-LSU with an accumulation of slower sedimenting particles by sucrose gradient (Sirum-Connolly and Mason, 1993). Interestingly, a variant of Pet56p with an amino acid substitution in the SAM pocket that abolishes its methyltransferase activity does not alter the formation of fully functional mitoribosomes (Lövgren and Wikström, 2001). This suggests that the role of Pet56p in ribosome assembly is independent of its methyltransferase activity.

In bacteria, Gm2251 of 23S rRNA is catalyzed by RlmB (Lövgren and Wikström, 2001). The crystal structure of RlmB revealed the presence of an N-terminal domain connected *via* a linker to a catalytic C-terminal domain, responsible for the dimerization of RlmB in solution (Michel et al., 2002). A strong similarity between the N-terminal domain and the ribosomal proteins L7 and L30 was observed; in particular, the presence of conserved residues that are essential for binding of L30 to RNA suggested that the N-terminal domain might be important for RlmB interaction with the 23S rRNA (Michel et al., 2002). Interestingly, in contrast to Pet56p, no effect on growth rate or ribosome assembly was observed upon RlmB depletion (Lövgren and Wikström, 2001).

Human methyltransferase MRM1 was shown to localize in mitochondria in close proximity to mtDNA nucleoids (Lee et al., 2013) and was found to co-sediment with the mt-LSU through gradient sedimentation experiments. Primer extension and DNAzyme-mediated RNA cleavage assays were used to assign the 2′-O-ribose methylation of Gm1145 to MRM1 (Lee et al., 2013; Lee and Bogenhagen, 2014). Further studies are essential to understand the role of MRM1 and Gm1145 modification in mitoribosome biogenesis and function.

MRM2 (Um1369)

MRM2 is a uridine 2′-O-methyltransferase that modifies U1369 position of the mitochondrial 16S rRNA. This highly conserved modification is located in the peptidyl transferase center and is implicated in the interaction of the ribosome with an aminoacyl(A)-site tRNA. Human MRM2 is closely related to yeast MRM2p and bacterial FtsJ/RlmE (Lee et al., 2013). Both MRM2p and FtsJ/RlmE have been extensively studied and their ablation has been shown to lead to severe growth defects and thermosensitive phenotypes (Caldas et al., 2000; Pintard et al., 2002b).

In *S. cerevisiae* mitochondria, Mrm2p was shown to co-sediment with 21S rRNA by sucrose gradient centrifugation analysis and to methylate, both *in vitro* and *in vivo*, U2791 of 21S rRNA in the context of the LSU, but not naked rRNA (Pintard et al., 2002a). Alignment analysis with its putative bacterial ortholog FtsJ/RlmE showed high similarities between the two proteins. The *ftsJ* gene in *E. coli* was originally identified as a heat-inducible gene (Richmond et al., 1999), and subsequently FtsJ was shown to be a SAM-dependent methyltransferase responsible for 2′-O-methylation of U2552 in 23S rRNA (Caldas et al., 2000).

RlmE depletion was shown to cause striking defects in the ribosome assembly process, leading to an accumulation of intermediates of the 30S and 45S particles, and a decrease of the 70S particles and polysomes (Bügl et al., 2000; Caldas et al., 2000; Arai et al., 2015). Initially, RlmE was thought to methylate the 23S rRNA in the context of the 50S subunit rather than the 45S intermediates that accumulate upon its depletion (Bügl et al., 2000). However, it was later shown that 45S, the precursor of the 50S subunit, is the real substrate of RlmE and that methylation of U2552 triggers the formation of the 50S subunit (Arai et al., 2015). Intriguingly, expression of two GTPases, EngA and ObgE, restored the defective phenotypes caused by RlmE ablation despite the absence of the U2552 modification, suggesting an interesting link between GTPase activity and RNA methylation (Tan et al., 2002).

As for MRM1, primer extension assay and DNAzyme-mediated RNA cleavage analysis allowed to identify MRM2 to be responsible for modification of Um1369 (Lee et al., 2013; Lee and Bogenhagen, 2014; Rorbach et al., 2014). Silencing of *MRM2* in cultured human cells led to decreased mitochondrial translation and OxPhos impairment, while immunoprecipitations and sucrose gradient centrifugation analyses revealed an interaction between MRM2 and the mt-LSU (Rorbach et al., 2014). Upon *MRM2* silencing, the steady-state levels of mt-LSU were found to be decreased, without affecting the mt-SSU levels, confirming that Um1369 is important for the biogenesis of the mt-LSU. Furthermore, *MRM2* downregulation resulted in a partial decrease in Gm1370 modification, alongside Um1369, with the former modification being introduced by MRM3. This suggests an interdependence between methylation of U1369 and G1370 and implies that MRM2 may act at an earlier stage of mitoribosome biogenesis than MRM3.

MRM3 (Gm1370)

MRM3 is responsible for methylation of G1370, adjacent to Um1369 in the A-loop of the mt-LSU. The equivalent residue in *E. coli* 23S, G2553, pairs with C75 of the aminoacyl tRNA in the bacterial ribosomal A-site (Kim and Green, 1999). In *E. coli*, G2553 is not modified, and neither is the yeast equivalent in mitochondrial 21S rRNA (G2792). Interestingly, yeast cytosolic LSU 25S rRNA has 2′-O-methylguanosine modifications in the analogous site (Gm2922) introduced by nucleolar protein Spb1p (Kressler et al., 1999b). Modification of G2922 is a late event occurring on the 27S ribosome intermediate and is essential for ribosome biogenesis (Lapeyre and Purushothaman, 2004). In human cytoplasmic 28S rRNA, the corresponding site is G4499 and it is 2′-O-methylated *via* a snoRNA-guided mechanism (Sergiev et al., 2018).

Human MRM3 associates with the mt-LSU, as revealed by co-immunoprecipitation and sucrose gradient sedimentation analyses (Lee et al., 2013). *MRM3* silencing reduced Gm1370 methylation and, consequently, mitochondrial translation and OxPhos function. Moreover, downregulation of MRM3 expression resulted in the accumulation of species consistent with mt-LSU pre-ribosomal particles, suggesting that methylation of G1370 likely occurs during the late-stage of mitoribosome assembly (Rorbach et al., 2014).

TRMT61B (m^1A947)

TRMT61B is a dual function methyltransferase that modifies both mt-tRNA (Chujo and Suzuki, 2012) and mt-rRNA (Bar-Yaacov et al., 2016). The conserved residues of its bacterial homolog,

TrmI, are well characterized for their catalytic function (Barraud et al., 2008) and contribution to binding of SAM (Dégut et al., 2016). TrmI is responsible for SAM-dependent N^1-methylation of adenosine 58 in the T-loop of many tRNAs and its inactivation in the hyperthermophilic bacterium *T. thermophilus* results in a thermosensitive phenotype (Droogmans et al., 2003).

Initially, TRMT61B was found to act as a mitochondrial tRNA methyltransferase responsible for m^1A58 of $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$, and $tRNA^{Ser(UCN)}$ (Chujo and Suzuki, 2012). However, a more recent study identified TRMT61B as the enzyme responsible for the m^1A modification at position 947 (mtDNA: m.2617A) of the mt-16S rRNA (Bar-Yaacov et al., 2016). This was supported by siRNA experiments coupled with primer extension assay and RNA sequencing analyses showing a hypomethylation of m^1A947 upon TRMT61B depletion. *In vitro* methylation assays further confirmed the ability of TRMT61B to modify naked mt-16S rRNA, suggesting a possible role for TRMT61B in the early stages of mt-LSU maturation (Bar-Yaacov et al., 2016).

The m^1A947 modification of the 16S rRNA occurs in most vertebrates and is enriched in the mature mammalian mitoribosome (Bar-Yaacov et al., 2016). Mapping of m^1A947 into the 55S monosome structure revealed that the modification is located in helix 71 of the mt-LSU, in proximity to the intersubunit bridge B3, where interaction with the mt-SSU occurs (**Figure 1**). Phylogenetic studies show that this region is structurally conserved in bacterial and cytoplasmic ribosomes, where the same position is evolutionarily occupied by an unmodified guanine and an unmodified uracil, respectively (Yusupova and Yusupov, 2014; Greber et al., 2015; Noeske et al., 2015).

In mitoribosomes, helix 71 seems to form an interdomain interaction with helix 92 and to stabilize a tertiary interaction with helix 64 *via* an electrostatic bond. Notably, *in vivo* substitution of the unmodified guanine in bacterial ribosomes with an unmodified adenine led to an alteration of protein synthesis and slower growth rates, while no effect was detected in the presence of an unmodified uracil (Bar-Yaacov et al., 2016). This data corroborates the hypothesis that methylation of A947 is essential for the maintenance and stabilization of the mitoribosome structure, as the unmodified adenine lacks the positive charge needed to bind the negatively charged backbone of helix 64. In contrast, in bacteria, the unmodified guanine can interact with the 23S rRNA *via* a hydrogen bond, while in the cytoplasm the unmodified uracil can interact *via* a water molecular bridge with the rRNA. It is intriguing to notice how vertebrate mitochondrial ribosomes diverged from their bacterial ancestors by replacing an unmodified nucleotide with an rRNA modification that requires the recruitment of a nuclear-encoded rRNA methyltransferase. Although the exact function of m^1A947 still needs to be elucidated, it is clear that this modification is important for the stabilization of the mitoribosome structure.

Mitochondrial rRNA Methyltransferases and Disease

Our current knowledge of the pathological role of mitochondrial rRNA-modifying enzymes is limited. A patient manifesting symptoms of mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome was found to carry a mutation in *MRM2* (Garone et al., 2017). While the patient fibroblasts did not exhibit the same phenotypes ascertained in *MRM2* knock-down experiments (Rorbach et al., 2014), complementation of the *MRM2* knock-out yeast model with the patient MRM2 variant could not rescue the respiration defect detected, thus supporting the pathogenicity of *MRM2* mutation in MELAS syndrome (Garone et al., 2017). To date, MRM2 is the only rRNA modifying enzyme in mitochondria with a pathogenic mutation directly linked to a primary mitochondrial disorder.

TFB1M was initially linked to aminoglycoside antibiotic-induced deafness because studies using TFB1M transgenic mice showed activation of pro-apoptotic factor E2F1 caused by TFB1M-hypermethylation of mt-12S rRNA (Raimundo et al., 2012). However, patients carrying the mt-DNA mutation m.A1555G, previously identified as a cause of deafness and located in proximity to the two adenines methylated by TFB1M, did not manifest changes in mt-12S rRNA methylation levels compared to controls, thus putting into question the role of TFB1M in the pathogenesis of this disorder (O'Sullivan et al., 2015). Interestingly, another study found a common variant of *TFB1M* to be associated with reduced insulin secretion and increased risk of type 2 diabetes in Tfb1m-deficient mice (Koeck et al., 2011). Similar observations were documented for a mouse model with beta cell-specific knock-out of *Tfb1m* that resulted in lower insulin secretion, mitochondrial dysfunction, and eventual development of type 2 diabetes (Sharoyko et al., 2014).

TRMT61B transcript expression was altered in total RNA extracted from astrocytes of Alzheimer's disease patients compared to controls (Sekar et al., 2015). In a separate study, functional and expression quantitative trait loci analyses linked TRMT61B to estrogen receptor-negative breast cancer (Couch et al., 2016). Further research is needed to clarify the potential role of TFB1M or TRMT61B, as well as other rRNA modifying enzymes, in human disease.

FUTURE PROSPECTS: EMERGING TECHNOLOGIES TO INVESTIGATE RRNA METHYLATION

Although there has been significant progress in the detection of mt-RNA modifications and corresponding enzymes, the complete landscape of mt-rRNA methylations and the specific roles of these modifications remain to be fully elucidated. Several cytosolic rRNA modifications exist at very low levels and only recent technical advances have enabled their detection (Taoka et al., 2018). Furthermore, there is increasing evidence that ribosomal modifications are dynamic, and their levels can be regulated under different physiological conditions (Erales et al., 2017). Further studies are needed to assess if the same is true for mt-rRNA modifications.

Due to the numerous types of RNA modifications, there is no universal technique to identify all of them simultaneously. Some recent approaches include nuclease protection assays and reversed-phase high-performance liquid chromatography (Yang et al., 2016) or sequencing profiling to measure reverse

transcriptase drop-off rates coupled to mass spectrometry (Enroth et al., 2019). Transcriptome-wide next-generation sequencing and mass spectrometry methods have also been used to estimate the abundance of individual RNA modifications (Zhang et al., 2019). Alternative approaches use immunoprecipitation and next-generation sequencing in pooled samples to gain insights into the stoichiometry of modified nucleotides while preserving sequence information (Dominissini et al., 2013). Additionally, emerging technologies aim to detect RNA modifications at the single-cell level (Ranasinghe et al., 2018).

Among the 172 RNA modifications reported to date, more than 40% involve the methyl-group (Boccaletto et al., 2018). Recent reviews have highlighted various approaches, including immunochemical, methylation-sensitive enzymes, hybridization, and high-throughput sequencing technologies to identify specific RNA methylations (Li et al., 2016; Ovcharenko and Rentmeister, 2018), while biochemical approaches have enabled mapping of modifications that do not interfere with Watson–Crick base pairing, including m^6A (Hartstock et al., 2018). Furthermore, a transcriptome-wide, single-base resolution method based on the modification of RNA bisulfite sequencing was reported to simultaneously detect m^5C, pseudouridylations, and m^1A modifications (Khoddami et al., 2019).

Despite these advancements, the aforementioned techniques often do not cover the mt-RNA modifications efficiently, partially due to the lower abundance of mt-RNA in comparison to the cytosolic RNA pool. Nevertheless, the suitability of these methodologies for mitochondrial studies is promising, and the enrichment of mitochondria by standard isolation methods from cultured cells or tissues (for example, Mercer et al., 2011) can be introduced in the adapted protocols to yield explicit and deeper coverage on mt-RNA modifications.

Advances in the field of cryoEM have significantly contributed to the characterization of ribosomes, providing structural insights at atomic resolution. Progress in the cryoEM field has enabled the detection of rRNA modifications by detecting the positions of extra densities in electron density maps (Liu et al., 2017). Recently, a high-resolution cryoEM 3D structure of the human 80S ribosome identified 136 rRNA modification sites, including 60 2′-O methylations, 25 pseudouridylation sites, and 51 other base modifications, all located in or close to functionally important sites within the ribosome (Natchiar et al., 2017). Some discrepancies between the data obtained by cryoEM studies (Natchiar et al., 2017) and other quantitative techniques detecting modifications (Taoka et al., 2018) illustrate the need for complementary techniques to elucidate the entire epitranscriptome map of rRNA modifications.

Our preliminary analysis of the already available high-resolution maps of mt-SSU (Khawaja et al., 2020) allowed us to identify densities corresponding to all five methylations of the 12S rRNA (**Figure 2**), proving that cryoEM is indeed a great tool to investigate mt-rRNA modifications. There is no doubt that the same will be achieved soon for 16S rRNA and can be expanded to mitoribosomes isolated from different tissues, thanks to the continuous improvements in cryoEM methodology.

As mitochondria are a central organelle critical for a variety of cellular processes, an in-depth understanding of RNA

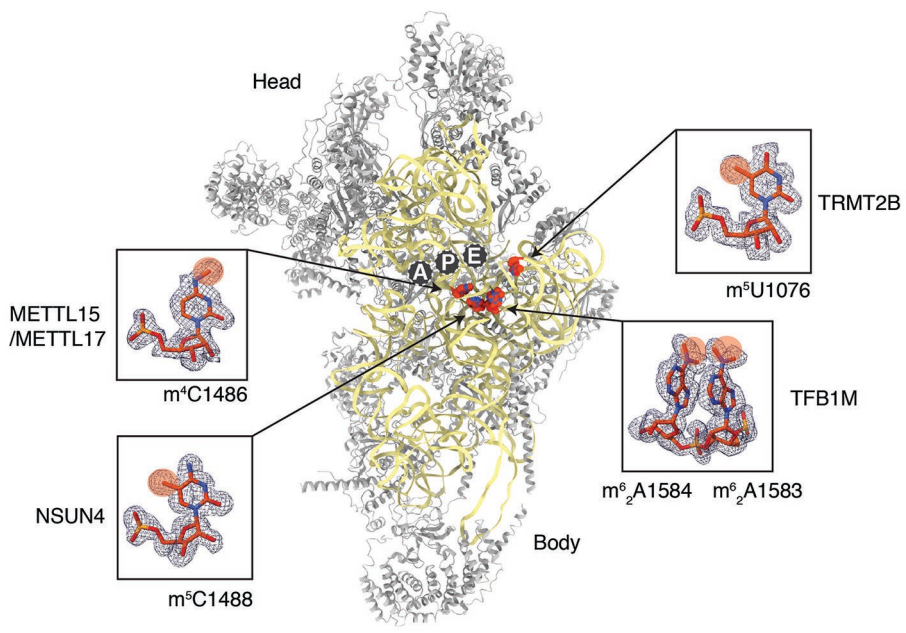

FIGURE 2 | Methylated 12S mitoribosomal rRNA (mt-rRNA) residues in the human mitoribosomal small subunit as revealed by cryoEM. The structure of human 28S (PDB: 6RW4) reveals the distribution of 12S rRNA methylation sites (red). The proteins of the 28S are colored gray and the 12S mt-rRNA is in yellow. The zoom-in panel displays the methylated rRNA residues with corresponding density maps. Chemical groups that are added enzymatically through the action of specific enzymes are highlighted in orange.

modifications, including methylation within mitochondria, will improve our understanding of mitochondrial gene expression regulation and its link to human pathophysiology.

AUTHOR CONTRIBUTIONS

ML and JR designed and coordinated the writing of the manuscript. AK prepared the figures. ML, JR, MC, and SG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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