Mechanisms of Mitochondrial DNA Deletion Formation

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Abstract

Mitochondrial DNA (mtDNA) encodes a subset of genes which are essential for oxidative phosphorylation. Deletions in the mtDNA can ablate a number of these genes, and result in mitochondrial dysfunction which is associated with bona-fide mitochondrial disorders. Although mtDNA deletions are thought to occur as a result of replication errors or following double-strand breaks, the exact mechanism(s) behind deletion formation have yet to be determined. In this review we discuss the current knowledge about the fate of mtDNA following double-strand breaks, including the molecular players which mediate

the degradation of linear mtDNA fragments, and possible mechanisms of re-circularization. We propose that mtDNA deletions formed from replication errors versus following double-strand breaks can be mediated by separate pathways.

Mitochondrial DNA

The human mitochondrial DNA (mtDNA) is a 16,569 bp circular, double-stranded, supercoiled molecule which was first discovered in 1963 [1]. The mtDNA molecule encodes 37 different genes which are essential for oxidative phosphorylation (OXPHOS) and mitochondrial protein synthesis [2]. Of these, 13 genes encode for subunits of 4 of the 5 multi-subunit enzymatic OXPHOS complexes located on the inner mitochondrial membrane: 7 subunits of Complex I, 1 subunit of Complex III, 3 subunits of Complex IV, and 2 subunits of Complex V. All of the subunits of Complex II are encoded by the nuclear DNA (nDNA), along with the other subunits and assembly factors needed for the biogenesis of the OXPHOS system [3]. Relative to its size, the lack of intron sequences, and the number of encoded genes, there is a high density of genetic information encoded by the mtDNA. To accommodate this density, some of the genes lack complete termination codons or overlap one another [4]. One interesting feature of the mammalian mtDNA molecule is there are two origins of replication, the origin of replication of the heavy (H-) strand (O_H) and the origin of replication of the light (L-) strand (O_L) [3].

Compared to the nuclear genome, where there are 2 copies of each chromosome, there can be 1000's of copies of the mitochondrial genome per cell [5]. Due to its high copy number, wild-type and mutant mtDNA molecules can exist together in a single cell, a concept known as mitochondrial DNA heteroplasmy [6]. MtDNA heteroplasmy levels are dynamic since mtDNA replication is cell-cycle independent, and the mtDNA molecules

selected for replication is random [7]. The type of mutation or deletion and the percentage of heteroplasmy are the major factors which determine the clinical severity of mitochondrial diseases. The percent heteroplasmy must surpass a biochemical threshold for decreased mitochondrial function and a clinical phenotype. While this threshold is dependent on the mutation itself, the affected cell- or tissue-type, heteroplasmy levels can be between 60 – 90% for a detectable phenotype to be present [8].

Mitochondrial DNA deletions are associated with disease

Large deletions in the mtDNA are observed in patients with progressive external ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS) and Pearson's syndrome [9-13]. They are invariably heteroplasmic and can vary in size from 1.8 – 8 kb [14, 15]. They can exist anywhere in the mtDNA, removing many protein coding and RNA genes , though there are some areas of the mtDNA that are much more susceptible to deletion formation than others [11, 16]. The molecular mechanisms underlying the formation of deleted mtDNA molecules remains unknown. It is known that the formation of deletions is usually sporadic, and they are generally thought not to be maternally transmitted [16]. Though a 2004 study analyzing 226 families in which a single mtDNA deletion had been identified, the actual risk of transmission to the offspring was approximately 1 in 24 births [17]. If heteroplasmy is particularly high for a mtDNA deletion in multiple tissues in a young patient, the prevailing theory is that the deletion occurred early in development, shortly after the mtDNA bottleneck and clonally expanded to high heteroplasmy with the potential to affect many organs [17]. Accumulation of mtDNA deletions in post-mitotic tissues is also observed during normal aging [18-22]. It is likely that these deletions are formed by the same mechanisms as disease-associated ones, discussed below.

Mitochondrial DNA replication

MtDNA replication occurs independently of the cell cycle because mtDNA levels are generally dependent on the energy status of a cell. While the key proteins which mediate mtDNA replication have been described (including the mitochondrial DNA polymerase gamma (POLG), the mitochondrial DNA helicase (TWINKLE), the mitochondrial single-stranded binding protein (mtSSB), and the mitochondrial RNA polymerase (POLRMT)), the mechanisms of copy number regulation have remained elusive [7, 13, 23, 24]. Though three models for mtDNA replication have been proposed, the strand-displacement model (SDM) [25], the ribonucleotide incorporation throughout the lagging strand model (RITOLS) [26] and the strand-coupled model [27], the SDM model is more widely accepted (Figure 1) [28]. With the SDM mtDNA model replication occurs via continuous synthesis from both O_H and O_L. Replication is initiated at O_H and a new H-strand is produced by POLG as DNA synthesis proceeds. As the mtDNA is unwound by TWINKLE, the displaced original heavy strand is coated with mtSSB, preventing POLRMT-initiated transcription. As POLG approaches O_L, close to $^2/_3$ of the mtDNA molecule has been replicated; when it passes O_L, this single-stranded sequence folds into a stem-loop structure. This structure prevents mtSSB binding, and instead promotes POLRMT binding. POLRMT initiates primer synthesis for approximately 25 nucleotides before it is replaced by POLG to begin L-strand synthesis. From this point on, both H-strand and L-strand synthesis continue out until the entire molecule has been replicated; replication is terminated at either O_H or O_L, dependent on where synthesis was initiated [24, 28-30].

Dual role of mitochondrial DNA replication machinery in degrading damaged mitochondrial DNA

Mutations affecting the genes required for mtDNA replication can lead to the accumulation of mtDNA point mutations or large-scale rearrangements as well as mtDNA depletion [31]. In the clinic, mutations in POLG, TWINKLE, and the mitochondrial genome maintenance exonuclease 1 (MGME1) are associated with mtDNA instability disorders. Mutations in POLG are associated with diseases such as, progressive external ophthalmoplegia (PEO), Alpers-Huttenlocher syndrome, myoclonic epilepsy, myopathy sensory ataxia, and ataxia neuropathy spectrum disorders [24]. Patients with mutations in MGME1 suffer from PEO, skeletal muscle wasting/weakness, emaciation, respiratory distress, microcephalus, mental retardation and gastrointestinal symptoms [32, 33]. Patients with mutations in TWINKLE are also associated with PEO, spinocerebellar ataxia, and mtDNA depletion syndromes [34, 35].

To better understand the roles of these genes in mtDNA instability and mutation/deletion formation, mouse models harboring specific mutations (POLG and TWINKLE) or knockout of the whole protein (MGME1) have been generated. In the mid-2000's two groups generated similar exonuclease-deficient *Polg* mouse models, which are referred to as the "mutator" mouse [36, 37]. The 3' – 5' exonuclease activity of POLG gives the polymerase its proofreading capability and the p.D257A point mutation in the murine POLG abolishes this activity. Mutator mice accumulate mtDNA point mutations and a linear 11 kb fragment, as well as low levels of deletions occurring between O_H and O_L, knowns as major arc deletions, in different tissues. Interestingly, when studying the mtDNA point mutation and deletion load in the mutator mouse, the incidence of deletions

correlates well with the premature aging phenotype [38, 39]. Later studies showed that point mutations in mtDNA-encoded protein coding genes also correlated well with the phenotype [40]. A TWINKLE mouse model was made with p.A360T substitution or an in frame amino acid duplication at position 353-365, which are seen in patients with PEO [41].These mutant Twinkle mice are known as the “deletor” mice as they have multiple mtDNA deletions, along with progressive respiratory dysfunction and late-onset mitochondrial disease. The multiple deletions seen in the deletor mouse have been attributed to replication pausing or stalling, which can also lead to mtDNA depletion, as it is seen in PEO patients [34, 42]. A full body MGME1 knockout mouse was recently generated which also generates mtDNA depletion, duplications, and multiple deletions across many different tissues types commonly seen in patients with PEO [33, 43]. Analysis of the mtDNA point mutation load in the livers of young and aged MGME1 knockouts revealed low levels of mtDNA point mutations, similar to levels observed in wild-type mice. Similar to the mutator mice, the MGME1 knockout mice accumulate a linear 11 kb mtDNA fragment which corresponds to the major arc of the mtDNA (the longer fragment between O_H and O_L). However, unlike the mutator mice, the MGME1 knockouts do not age prematurely, suggesting that increased mtDNA point mutation load, not deletion load, leads to the premature aging phenotype.

Damage to the mtDNA can result in either the loss of the damaged mtDNA molecule or the formation of point mutations or rearrangements/deletions. This damage is either from errors in replication or noxious stimuli. Recent data suggests double-strand breask (DSBs) are central to one of the proposed mechanisms for the formation of mtDNA

deletions; another mechanism is through slipped mtDNA replication, which will be discussed later in this review.

DSBs can occur in the mtDNA via exposure to exogenous DNA damaging agents such as ionizing radiation, UV, and chemotherapeutic agents or endogenous agents, such as reactive oxygen species (ROS) [44, 45]. Oxidative damage may be particularly prevalent due to the close proximity of ROS–producing OXPHOS and the mtDNA, which may also promote other types of damage through base modifications and replication errors [44, 45]. The multicopy nature of mtDNA means that when a mtDNA molecule is damaged through DSBs, the damaged molecule is likely to be degraded and healthy molecules will replicate to restore mtDNA copy number rather than expend energy to repair the damaged molecule. Until recently the nucleases which degraded the linear mtDNA fragments were unknown. In addition to POLG and MGME1 there are other nucleases which have roles in the mitochondria including EXOG, APEX2, ENDOG, FEN1, DNA2, MRE11, and RBBP8 [46-48]. It has shown that mtDNA degradation following a DSB is a fast process, as a linear mtDNA intermediate is only seen for approximately 2 hours after DSB induction, but not at later time points implying that mtDNA degradation occurs soon after DSBs [49].

In an *in vitro* model of mitochondrial–targeted restriction endonuclease (mitoRE) *Pst*I (mito*Pst*I) induced DSBs a study tested whether knocking down MGME1, DNA2, EXOG, ENDOG, or FEN1 had an effect on the degradation of linear mtDNA fragments [50]. Using siRNAs to silence each of the nucleases independently and in a pool, the authors did not observe a delay in the degradation of linear mtDNA fragments following mito*Pst*I-induced DSBs at 0 hours, 5 hours, or 1 day [50]. A similar study was carried out

by another group, using a comparable *in vitro* system to express mito*Pst*I after knocking down MGME1, POLG, TWINKLE, EXOG, APEX2, ENDOG, FEN1, DNA2, MRE11, and RBBP8. In contrast to the former study which was unable to attribute any role for MGME1 in degrading linear mtDNA fragments following DSBs, the latter study observed delayed mtDNA degradation (via a linear mtDNA intermediate) 4 hours after the induction of mito*Pst*I in the MGME1-downregulated cell as well as in the POLG and TWINKLE knockdowns, but not the other nucleases [51]. To further characterize the exonuclease activity responsible for this delayed degradation, mito*Eag*I was expressed in HEK293 cells which are either deficient for MGME1 or which harbor the human POLG p.D274A point mutation (abolishing the 3'–5' exonuclease activity). In both cases delayed degradation was seen up to 18 hours after mito*Eag*I expression [51].

Another independent study observed a similar role for the exonuclease activity of the mouse POLG in the degradation of linear mtDNA fragments both *in vitro* and *in vivo* using adenoviral mediated expression of mito*Sca*I (Ad-mito*Sca*I) in the mutator model [52]. Ad-mito*Sca*I had a peak of expression in lung-derived fibroblasts from mutator and wild-type mice one to two days after infection, but by days five and 10, expression was lost. Linear mtDNA fragments were observed in a southern blot at Day 1, but only the full length mtDNA molecule was observed at Days 5 and 10 in the mutator fibroblasts, implying that the exonuclease activity of POLG plays an early role in degrading linear mtDNA fragments, but it is not the only nuclease which can degrade these fragments. When Ad-mito*Sca*I was injected retro-orbitally in wild-type and mutator mice, a similar phenomenon was observed in the liver. Along with a general depletion of mtDNA, linear mtDNA fragments were also observed four days after Ad-mito*Sca*I infection in DNA

extracted from the liver of the mutator mouse, compared to the liver of the Ad-mito*Sca*I injected wild-type mouse [52].

In addition to its function in degrading linear mtDNA fragments following DSBs, POLG appears to have evolved to have many roles in regulating mtDNA stability. Recent work done has shown that MIP1, the yeast homolog of POLG, controls mtDNA copy number by regulating mtDNA synthesis and degradation in starving yeast cells which were deficient in Atg7 (∆Atg7) [53]. Atg7 is a conserved component of the ubiquitin-like system required for autophagy in yeast [54]. Additionally, they found that the 3' – 5'exonuclease activity of MIP1 regulated mtDNA copy number in wild-type yeast, as well as mediated the degradation of mtDNA in ∆Atg7 yeast during starvation-induced nucleotide insufficiency [53]. MtDNA deletion levels were analyzed in yeast MIP1 models of disease associated POLG mutations and an increase in mtDNA deletions was only seen in exonuclease-deficient MIP1 compared to wild-type or other pathogenic mutations [55].

Formation of mitochondrial DNA deletions following double-strand breaks

In a mouse model expressing the mitochondrially-targeted restriction endonuclease, mito*Pst*I (which has two recognition sites in the mouse mtDNA), in forebrain neurons showed the depletion of the full length mtDNA molecule [56]. However, recombination events were identified that occurred either between 1) the free ends generated by *Pst*I cleaving the mtDNA at both sites (resulting in an approximately 12 kb mtDNA molecule) or 2) one of the free ends generated by *Pst*I and a region close the D-loop (resulting in an approximately 8 kb mtDNA molecule) [56-58]. The possibility of "repair" occurring between the two free ends could be explained by the recombinogenic

features of these DNA ends. However, the break-points detected for a *Pst*I end and the D-loop have been proposed to occur due to the relaxed three-stand confirmation of the D-loop, allowing for single strand annealing and recombination to occur [59].

In the recent studies described above that showed roles for POLG, MGME1, and TWINKLE in degrading linear mtDNA following DSBs, both showed that a consequence of the persisting linear mtDNA fragments is the increased formation of mtDNA rearrangements. PCR analysis across the mito*Pst*I breakpoint showed an increase in the presence of a recombined molecule in the POLG, MGME1, and TWINKLE knockdown samples compared to controls at 2 and 4 hours after mito*Pst*I induction [51]. Another study analyzed the formation of three different putative mtDNA rearrangements which could have formed following Ad-mito*Sca*I, one which contained only O_H, one which contained only O_L, and one which contained both O_H and O_L [52]. Fibroblasts derived from the mutator mouse showed higher levels of all three putative recombined molecules at Days 1 and 2 after Ad-mito*Sca*I infection, however the levels of the molecules which contained only O_H or only O_L were reduced by Days 5 and 10. On the other hand, recombined molecules which contained both O_H and O_L were able to replicate and levels continued to increase through Day 10. These breakpoints were also analyzed in the livers of the mice retro-orbitally injected with Ad-mito*Sca*I and the levels of the O_H and O_L containing rearrangements were higher than those containing only a single origin of replication.

Features of mitochondrial DNA deletion breakpoints are potential indicators of the mechanism of deletion formation

As previously discussed, mammalian mtDNA molecules containing DSBs are usually rapidly eliminated, with the components of the replication machinery, including POLG, MGME1, and TWINKLE being involved in this process. However, evidence exist that in some instances DSB can be re-ligated resulting in a rearranged molecule [56]. While it was previously thought that DSB repair mechanisms do not exist in the mitochondria, the presence of rearranged molecules upon induction of DSB suggests that an end-joining activity actually operates within the mammalian mitochondria. This repair mechanism, however, is likely to be different from the precise mechanisms operating in the nucleus, because of the absence of specific bona-fide DSB repair factors, such as Rad51, Nbs1, members of the MRN complex [60-63]. Nonetheless, the observation that the free ends come together indicates a rudimentary DSB repair mechanism, resulting in the formation of partially-deleted mtDNA.

Whereas mtDNA deletions that appear to arise from slipped replication are usually flanked by direct repeats, analysis of the mtDNA repair breakpoints after a DSB did not reveal the same features (Figure 2B). Instead there is evidence suggesting repair is mediated mostly by non-homologous end joining (NHEJ), or micro-homolog mediated end joining (MMEJ) [48, 56, 58, 64-66].

In *Saccharomyces cerevisiae* mtDNA DSBs are repaired by homologous recombination (HR) and the proteins which facilitate this repair in yeast have been previously identified [67, 68]. However, a number of factors of yeast mtDNA HR that do not have identified counterparts in the mammalian system, including the Rad52-like protein or a resolvase, while the other factors have known homologs (reviewed in [60]).

There is a report that one of the factors of nDNA HR, Ku80, has an alternate form, XRCC1, which is found in mammalian mitochondria [62].

The observation that deletion breakpoints seen in the human mtDNA across different mitochondrial diseases can have large, micro or no homologies, is consistent with some mechanism of NHEJ existing in the mitochondria [3]. These breakpoints would be formed purely through ligation of the free ends, and not show any sequence homology on either side of the break. This has been observed in the analysis of breakpoints between either two free mtDNA ends introduced by mitoRE or a free, mitoRE-specific mtDNA end and the displacement loop (D-loop), showing that these breakpoints are not flanked by any homologous sequences [56, 58].

A more recent study found that classical NHEJ is unlikely to occur in the mitochondria following DSBs. Using an *in vitro* system, the authors incubated either cell free extracts or mitochondrial extracts isolated from different tissues (brain, testes, spleen, and kidney) from rats with [γ-^{32}P]ATP labeled double-stranded oligomeric DNA [48]. These oligonucleotides had either compatible 5' overhangs, non-compatible 5'-5' overhangs, non-compatible 3'-3' overhangs or blunt ends which mimicked endogenous DSBs. While total cell extracts from all tissues were able to catalyze the formation of dimers, trimers, and other multimers with all types of overhangs by NHEJ, there was only evidence of the compatible 5' overhangs ligating in the mitochondrial extracts from testes [48]. This type of joining does not require any type of processing before ligation, so while it does not rule out the possibility, it suggests that classical NHEJ does not occur in mitochondria.

MMEJ is an alternative NHEJ pathways that was has also been explored. MMEJ was first thought to have a central role in mtDNA maintenance and repair following DSBs in 2008, when it was found that approximately 90% of mtDNA deletions in human cells were flanked by short, direct or imperfect repeat sequences [64, 69]. Using a similar *in vitro* system, double-stranded DNA oligos harboring 13 nucleotide direct repeats were incubated independently with mitochondrial or cell free extracts, PCR amplified, post-digested, and resolved by polyacrylamide gel electrophoresis. The authors found the presence of a larger fragment which would be expected from the MMEJ Sequence analyses of the breakpoints showed 68% of perfect microhomologies, while 32% showed imperfect microhomologies with up to 2 mismatches [48]. In summary, the mechanism by which DSB lead to mtDNA deletion formation is unclear and could be as simple as ligase 3-mediated ligation.

The features of deletion breakpoints after DSBs showed a paucity of homology in the regions involved in the breakpoint, occurring at or close to the cleavage site, suggesting limited degradation of the free ends [52]. These features contrast with observations in most patients with mtDNA deletions, where nearly 60% of deletions are flanked by short, directly repeated sequences between 4 – 13 bp, known as Class I deletions [64, 70-78]. Thirty percent of patient-derived deletions are flanked by imperfect repeat sequences, known as Class II deletions, and the remaining deletions, are flanked by non-repetitive sequences, known as Class III deletions [69]. Most clinically observed deletions are located between the two origins of replication when the H-strand is displaced, suggesting replication intermediates mediate deletion formation. This is consistent with the SDM of mtDNA replication. Over the years, different models for the

formation of these mtDNA deletions have been proposed. One such model, the slipped-strand replication model, has been proposed to explain the formation of the common deletion, a 4,977 bp deletion found in the major arc that is one of the most common mtDNA mutations [11, 76]. One of these studies suggested that during the displacement of the nascent H-strand, a repeat sequence on the H-strand anneals with a homologous sequence on the L-strand, creating a wild-type mtDNA molecule and a deleted mtDNA molecule [11]. Later work suggested that the activity required for mtDNA strand annealing are mediated by members of the minimal mitochondrial replisome and is coupled to mtDNA replication [79]. Furthermore, in a more recent model, named “copy-choice recombination”, experimental data suggested that mtDNA deletions can be formed via slipped replication during the synthesis of the nascent L-strand. According to this model, the 3′ end of the nascent L-strand is displaced from the template H-strand soon after passing one of the sequences of a tandem repeat and can reanneal to the second repeated sequence on the template H-strand. This 3′-end slippage would lead to a mtDNA deletion upon a subsequent replication round (Figure 2A) [80].

Concluding remarks and future perspectives

A hallmark feature of mtDNA is the ability to quickly recover from a rapid loss of mtDNA molecules following DSBs both *in vitro* and *in vivo*. Up until recently the nucleases which degrade linear mtDNA fragments following DSBs had not been determined, but now we know that members of the mtDNA replication machinery play dual roles in synthesizing and degrading mtDNA, and these roles are likely independent of one another. If linear mtDNA molecules are not rapidly degraded following DSBs there is the increased likelihood of the formation of mtDNA deletions, either between the free ends

generated by the DSB or between a free end and a region close to the D-loop. Therefore, DSB is at least one of the mechanisms by which mtDNA deletions can be formed. It is likely that mtDNA deletions without homologies in the breakpoint may be formed after DSBs. On the other hand, replication errors are also likely to generate mtDNA deletions, and these would be mediated by some homology at the breakpoint region (Figure 2). Presently, gene therapy tools, such as mitoTALENs and mtZFNs, have been utilized to specifically create DSBs to cleave mtDNA to effectively shift mtDNA heteroplasmy both *in vitro* and *in vivo* [81-84]. The off-target effects of these DSBs, including the formation of mtDNA deletions, is a concern with moving these techniques into the clinical setting. However, recent work has shown that functionally relevant levels of mtDNA deletions form only when there are mutations in the mtDNA replication machinery, opening the door to move these tools forward.

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Figure Legend

Figure 1. Strand-displacement model of mitochondrial DNA replication.

Schematic representation of mitochondrial DNA replication through the strand displacement model. Mitochondrial DNA replication is initiated at O_H and proceeds unidirectionally to produce the nascent H-strand. When replication of the H-strand passes O_L, replication of the nascent L-strand is initiated. Continuous synthesis of the nascent H- and L-strands proceeds until two full-length, double-stranded mtDNA molecules have been formed.

Figure 2. Mitochondrial DNA deletions have two origins of formation.

Deletions in mtDNA can form by copy-choice recombination mtDNA replication or after a double-strand break. (A) During copy-choice recombination, which assumes the strand-displacement model of replication, mtDNA replication begins at O_H, and proceeds to O_L where synthesis of the nascent L-strand begins. As the repeat closest to O_L, marked as the 3' repeat, is displaced from the template H-strand, it can reanneal to the repeat closest to O_H, marked as the 5' repeat, on the template H-strand. After the first round of replication, there would be a complete wild-type mtDNA molecule, and a mtDNA molecule harboring both a wild-type mtDNA strand and a deletion strand. A subsequent round of replication leads to a wild-type mtDNA molecule and a deleted mtDNA (∆mtDNA) molecule, where the breakpoint is flanked by direct or imperfect repeats. (B) Following a double strand break, the mtDNA is either rapidly degraded by members of the mtDNA replication machinery (including as POLG, MGME1, and TWINKLE) or only partially degraded, where the free ends are able to ligate and a ∆mtDNA molecule can form. This ∆mtDNA molecule breakpoint is typically not flanked by direct or imperfect repeats.

Figure 1

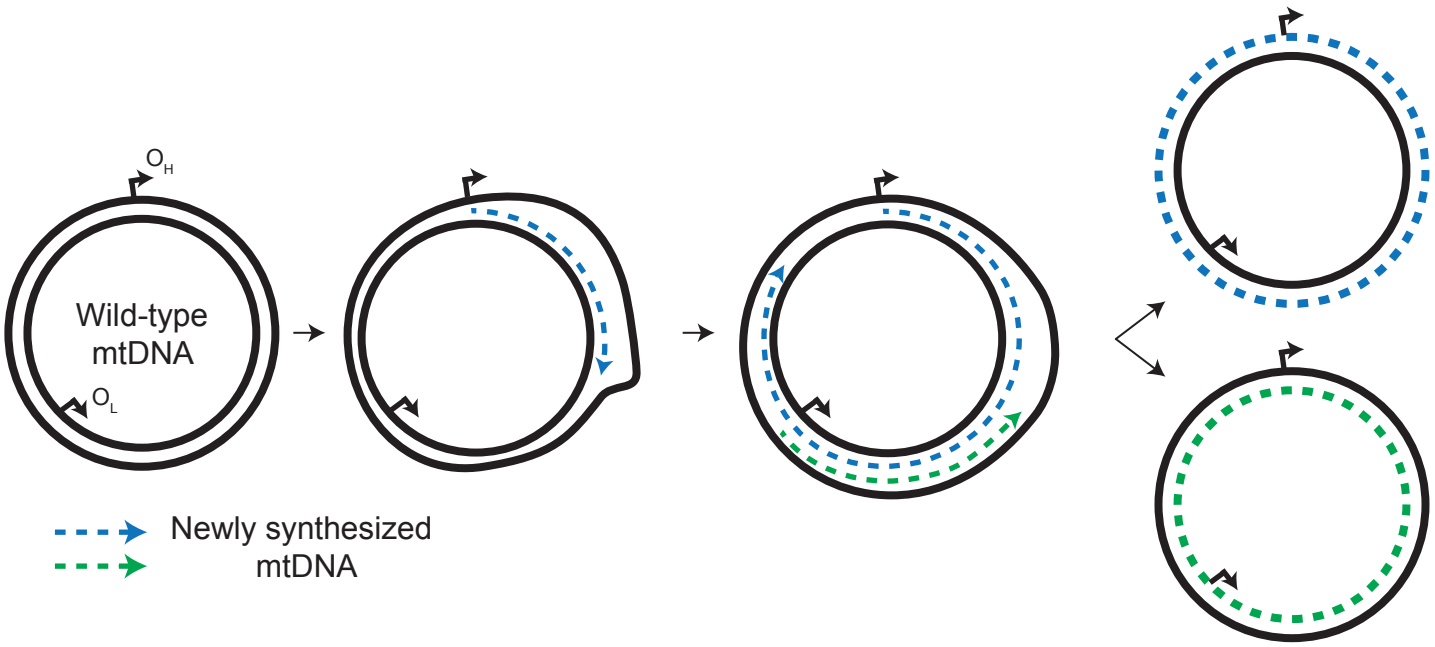
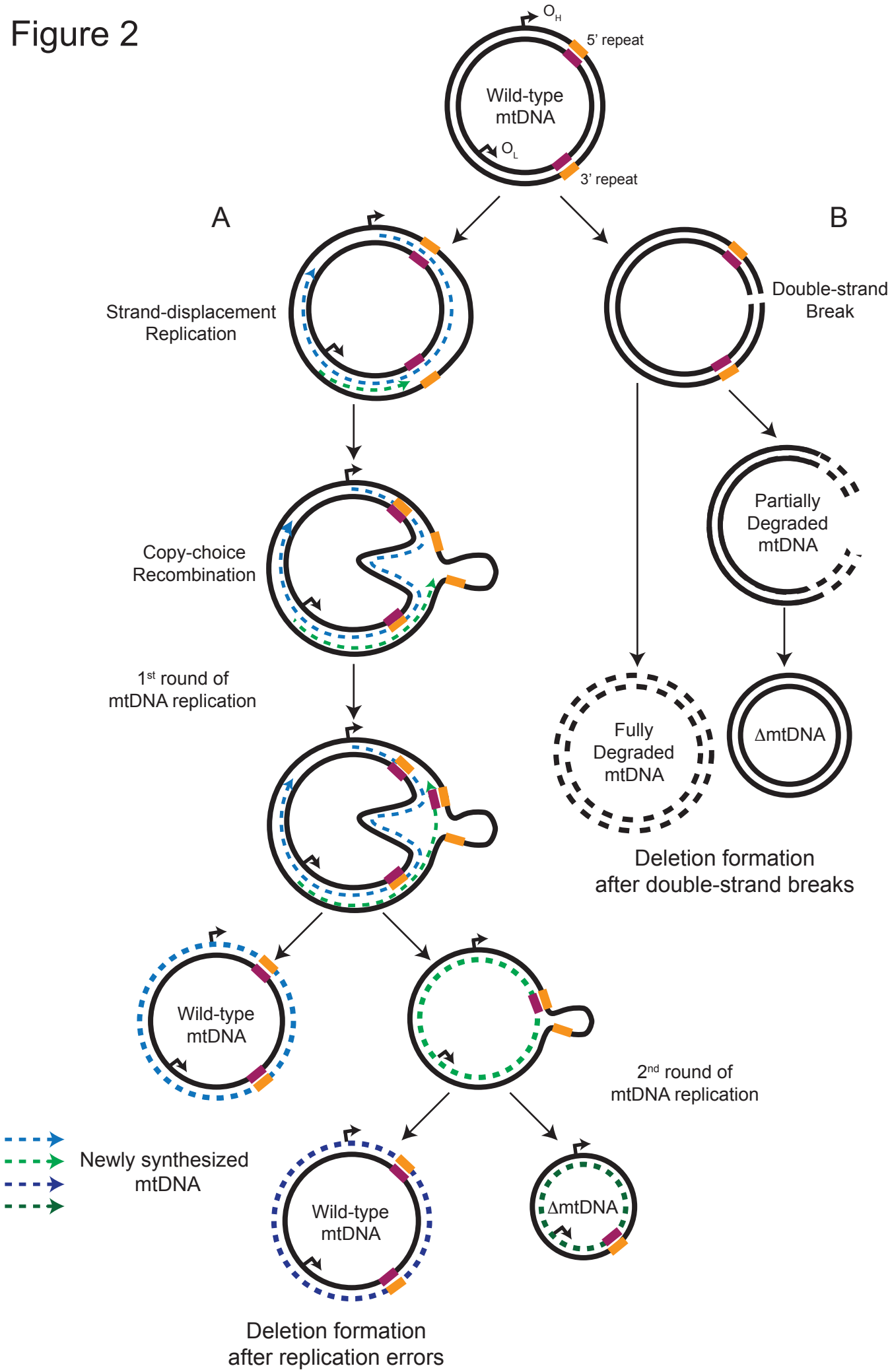


Figure 2


O_H
5' repeat
Wild-type mtDNA
O_L
3' repeat
A
B
Strand-displacement Replication
Double-strand Break
Copy-choice Recombination
Partially Degraded mtDNA
1st round of mtDNA replication
Fully Degraded mtDNA
ΔmtDNA
Deletion formation after double-strand breaks
Wild-type mtDNA
2nd round of mtDNA replication
Newly synthesized mtDNA
Wild-type mtDNA
ΔmtDNA
Deletion formation after replication errors