This is the published version of a paper published in *FEBS Letters*.

Citation for the original published paper (version of record):

Wanrooij, P H., Chabes, A. (2019)
Ribonucleotides in mitochondrial DNA
*FEBS Letters*, 593(13): 1554-1565
https://doi.org/10.1002/1873-3468.13440

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Ribonucleotides in mitochondrial DNA
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The incorporation of ribonucleotides (rNMPs) into DNA during genome replication has gained substantial attention in recent years and has been shown to be a significant source of genomic instability. Studies in yeast and mammals have shown that the two genomes, the nuclear DNA (nDNA) and the mitochondrial DNA (mtDNA), differ with regard to their rNMP content. This is largely due to differences in rNMP repair – whereas rNMPs are efficiently removed from the nuclear genome, mitochondria lack robust mechanisms for removal of single rNMPs incorporated during DNA replication. In this minireview, we describe the processes that determine the frequency of rNMPs in the mitochondrial genome and summarise recent findings regarding the effect of incorporated rNMPs on mtDNA stability and function.

Keywords: dNTP; genome stability; mitochondrial DNA; ribonucleotides

DNA rather than RNA is the preferred medium for long-term storage of genetic information, and this is partly due to the greater stability of DNA. Together with the high fidelity of the replicative DNA polymerases and the plethora of DNA repair mechanisms that the cell possesses, the chemical and structural stability of double-stranded DNA contributes to the faithful maintenance of our genetic information. However, during the past decade the incorporation of ribonucleotides (rNMPs) has become recognised as a major threat to genome stability. Ribonucleotides differ from dNTPs only by the presence of a hydroxyl (−OH) group on carbon-2 of their furanose ring. This seemingly small difference has great implications for genome stability because the reactive 2'-OH group of rNMPs can attack the sugar-phosphate backbone of DNA, and thus incorporation of rNMPs makes the DNA several orders of magnitude more susceptible to strand breaks [1]. The presence of isolated rNMPs in DNA also alters the local structure and elasticity of the DNA [2–4], and this can disrupt the recognition and binding by protein factors. On the other hand, transiently-incorporated rNMPs have been suggested to play positive physiological roles, e.g. as markers of newly-synthesised DNA to guide mismatch repair [5,6] or to relieve torsional stress created during leading strand replication [7]. rNMPs embedded in the genome might thereby influence a variety of fundamental processes including DNA replication, repair, and transcription.

Our two genomes show striking dissimilarity with regard to ribonucleotide content – while rNMPs are virtually absent from nDNA, mature mammalian mtDNA contains an incorporated rNMP approximately every 500–900 nucleotides depending on the source material [8,9]. In vitro, the human mtDNA

Abbreviations
AGS, Aicardi-Goutières syndrome; CSBs, conserved sequence blocks; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; RER, ribonucleotide excision repair; rNMP, ribonucleotide; ssDNA, single-stranded DNA; Top1, topoisomerase 1.
polymerase incorporates an rNMP every 2000–2300 nucleotides, which is comparable or even lower than the rNMP incorporation frequency of nuclear DNA polymerases [8–10], although comparisons are complicated by variations in assays and reaction conditions. Rather than differential rNMP incorporation, the difference in rNMP content between our two genomes is therefore mainly ascribed to the absence of efficient rNMP removal mechanisms in the mitochondria. Numerous excellent reviews have summarised the current understanding of the roles and consequences of rNMPs incorporated into the nuclear genome (e.g. [7,11–13]). Therefore, this mini-review only briefly describes general pathways for ribonucleotide incorporation and removal from nDNA, and then goes on to discuss how rNMPs become embedded in mtDNA. Next, we review the current knowledge on the factors that determine the frequency and identity of rNMPs in mtDNA, and we end by considering the implications of the persistence of rNMPs in mature mtDNA. Although our main focus is on mammalian mtDNA, we include some key findings from the budding yeast system in which many landmark discoveries have been made.

**General pathways for ribonucleotide incorporation and removal from nuclear DNA**

**Ribonucleotide incorporation into nDNA**

A major process contributing to the incorporation of rNMPs in the genome is the priming of DNA replication. In the nucleus, the Pol α-primase complex synthesises a 20–30 nucleotide primer that initiates DNA replication at origins of replication and at each Okazaki fragment on the lagging strand [14]. The first 7–10 nucleotides of the replication primer are rNMPs, and even the subsequent DNA extension of the primer might be interspersed with occasional rNMPs because of the relatively high propensity of Pol α to incorporate rNMPs [15]. Given that eukaryotic Okazaki fragments are only about 200 nucleotides in length, priming is frequent on the lagging strand, making it by far the largest source of rNMP incorporation. Although replication primers have been thought to be efficiently removed during Okazaki fragment maturation, this view has been questioned in light of recent findings suggesting that rapid re-association of DNA-binding proteins post-replication might prevent the full removal of primers synthesised by Pol α-primase [16]. Therefore, some rNMPs inserted by Pol α-primase might escape the Okazaki fragment maturation machinery and thus remain in mature nDNA.

Another source of rNMPs in nDNA are the main nuclear replicative DNA polymerases Pol ε and Pol δ that occasionally incorporate rNMPs during genome replication. Both polymerases exhibit high selectivity for dNTPs over rNTPs, being at least 100-fold more likely to insert a dNTP than an rNTP [15,17,18]. This high selectivity for dNTPs is often achieved by use of a bulky steric gate residue that clashes with the 2'-OH group of rNTPs and thus obstructs the entry of the rNTP into the polymerase active site [19,20]. The insertion frequency of rNMPs is also influenced by the ratios of free rNTPs to dNTPs. Free rNTPs are present in great excess over dNTPs [15,21], so even the highly selective replicative polymerases will occasionally insert rNMPs into their product. Finally, DNA polymerases show poor, if any, ability to remove inserted rNMPs with their 3’–5’ exonuclease activity [18,22,23]. Therefore, the majority of inserted rNMPs will become incorporated into the growing DNA chain as polymerisation continues. The average frequency of rNMP incorporation by *Saccharomyces cerevisiae* Pol ε *in vitro* is 1 rNMP per 640–1250 nucleotides inserted, depending on the assay used. Pol δ incorporates fewer rNMPs, with frequencies of 1 rNMP every 720–5000 nucleotides [10,15]. Given that Pol ε and Pol δ replicate the vast majority of the eukaryotic genetic material, they are estimated to introduce over 10 000 rNMPs per round of replication of the *S. cerevisiae* nuclear genome [15]. For the mammalian genome, estimates exceed 3 million rNMPs inserted per round of replication [15,18,22].

Additional processes that might contribute to insertion of rNMPs into the genome include DNA repair and translesion synthesis. Repair and translesion polymerases such as Pols μ, λ, β, τ, and ζ can all use rNMPs during polymerisation and generally exhibit poorer selectivity for dNTPs over rNTPs than replicative polymerases (reviewed in [7]). Furthermore, the novel human archaeal-type primase/polymerase PrimPol that can re-prime stalled replication forks downstream of DNA damage or hard-to-replicate sites prefers to initiate primer synthesis with a ribonucleotide and, although it favours dNTPs during elongation of the primer, can also insert rNMPs when elongating [24,25].

**Removal of rNMPs from nDNA**

Ribonucleotides are removed from the nuclear genome primarily through the action of a dedicated repair pathway known as ribonucleotide excision repair (RER). Removal is initiated by cleavage on the 3' side of the rNMP by the endonuclease RNase H2 [26,27],
Next, strand displacement synthesis by Pol δ creates a displaced rNMP-containing flap that is subsequently removed by Fen1 and/or Dna2, and the repair is completed by ligation of the nick by DNA ligase 1 [10]. RER has high capacity – it removes virtually all rNMPs present in the nuclear genome of budding yeast even in the presence of dNTP pool imbalances that result in the increased incorporation of rNMPs [28]. RNase H2 is critical for initiation of RER at single embedded rNMPs, but is additionally involved in processing longer stretches of rNMPs or R-loops, which are RNA/DNA hybrids that are the outcome of an RNA transcript hybridising to DNA [29]. R-loops and stretches of four or more consecutive rNMPs are also substrates of the RNase H1 enzyme [30], which differs from RNase H2 in that it is unable to cleave at single rNMPs.

In the absence of RNase H2, numerous rNMPs remain in the nuclear genome and result in embryonic lethality in mice and in milder signs of replication stress in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* [17,31–33]. The number of rNMPs remaining in the nuclear genome when RER is defective has been estimated at 1500 and 1.3 million in *S. cerevisiae* and mice, respectively [16,32]. In the absence of RNase H2, some of these rNMPs can be removed by an alternative pathway dependent on topoisomerase 1 (Top1) that resolves DNA supercoiling by transiently nicking one DNA strand. Top1 incision at an rNMP creates a 2′-3′ cyclic phosphate end that must be processed further to allow re-ligation [34,35]. When present at repetitive sequences, processing of these ‘dirty’ DNA ends can result in 2–5 bp deletions that in turn trigger checkpoint activation, replication stress, and genome instability [34,36,37]. The use of this alternative rNMP-removal pathway therefore comes at a cost.

**Defects in rNMP removal have detrimental consequences**

As mentioned above, RNase H2 is essential in mammals, and knockout of one of its three subunits leads to embryonic lethality after embryonic day 9. In accordance with the adverse effects of excess rNMPs on genome stability, RNase H2-deficient cells exhibit increased single-stranded DNA (ssDNA) breaks and an activated p53-dependent DNA damage response [32,33]. These defects have been attributed to the presence of single or at most double rNMPs rather than longer stretches of rNMPs in the genome because rNMPs present in the nuclear genome of RNase H2-deficient MEFs is not sensitive to recombinant prokaryotic RNase H1 that digests at sites with multiple consecutive rNMPs [32]. Furthermore, separation-of-function mutations in mouse RNase H2 support the interpretation that the embryonic lethality observed in RNase H2 knockouts is caused by single un-repaired rNMPs rather than longer RNA/DNA hybrids [38].

In humans, mutations that lead to partial loss of RNase H2 activity cause Aicardi-Goutières syndrome (AGS), a neuro-inflammatory autoimmune condition that involves activation of the type I interferon system [39,40]. It appears that while the high levels of un-repaired rNMPs found in RNase H2-deficient mice lead to an activated DNA damage response and death, the somewhat lower rNMP load observed in AGS mutant strains or cell lines instead triggers an innate immune response that is dependent on the cGAS/STING pathway [38,41].

In contrast to RNase H2, loss of RNase H1 activity does not adversely affect nDNA. This likely reflects the fact that the majority of the RNA/DNA hybrid-processing activity observed in the cell is actually derived from RNase H2 [32], whereby loss of RNase H1 can be compensated for by RNase H2 in the nucleus. However, RNase H1 is a dual-localised enzyme with two in-frame start codons. Translation from the first start codon produces a mitochondrially-targeted RNase H1, while initiation from the second produces the nuclear enzyme [42]. Given that RNase H2 is absent in the mitochondrial compartment, it cannot compensate for the loss of mitochondrial RNase H1 activity and hence knockout of RNase H1 results in a severe mitochondrial phenotype [42,43] that will be discussed in the following section on mtDNA rNMPs.

**Ribonucleotides are incorporated during mtDNA replication and repair**

**Features of mtDNA and its replication**

Mammalian mtDNA is a circular multicopy genome of 16.6 kb that encodes for 13 proteins, 22 rRNAs, and 2 rRNAs. Its copy number ranges from a few hundred to tens of thousands of mtDNA molecules per cell, depending on the cell type. The 13 polypeptides encoded by mtDNA are core components of the mitochondrial electron transport chain that is essential for efficient energy production. The integrity and sufficient copy number of mtDNA is therefore of critical importance for the cell, as illustrated by the myriad of disease symptoms associated with mtDNA mutations or depletion [44,45].

The specifics of mtDNA replication have recently been reviewed by others (e.g. [46–48]), and only the
aspects that are critical for the discussion of mtDNA rNMP incorporation will be summarised here. Mammalian mtDNA replication relies on a core machinery consisting of the replicative mtDNA polymerase Pol γ, the helicase TWINKLE, and the mitochondrial ssDNA-binding protein mtSSB [49–51]. No dedicated replicative primase has been identified in mammalian mitochondria; instead, the replication primers are synthesised by the mitochondrial RNA polymerase POLRMT [52,53]. In addition to these factors, other proteins are required, e.g. for ligation of the completed replication products and for relaxing supercoiling [46].

Not only the machinery, but also the mechanism of mtDNA replication differs from that of nDNA. According to the strand-displacement model [54,55], replication of the two mtDNA strands occurs continuously on both strands and is asymmetric, with considerable delay in replication of the so-called light (L) strand relative to the heavy (H) strand. Replication of the H-strand initiates in the H-strand origin (OH) region and proceeds three quarters of the way around the genome until it exposes the L-strand origin of replication (OL). Once in single-stranded form, OL folds into a stem-loop structure that enables POLRMT to synthesise the L-strand primer that is extended by Pol γ [52,56]. The synthesis of both strands then proceeds full-circle, producing two genome-length mtDNA molecules.

During the first phase of replication before H-strand synthesis reaches OL, the parental H-strand is displaced as ssDNA. According to the strand-displacement model, the displaced H-strand is coated and stabilized by mtSSB. This model of mtDNA replication has been challenged by the RITOLS model where the displaced H-strand is instead covered by long rNMPs. No dedicated replication origin in the RITOLS model is extended by Pol γ [52,53]. The synthesis of both strands then proceeds full-circle, producing two genome-length mtDNA molecules.

Conceptually, the rNMPs in mtDNA might derive from various processes such as priming, replication, and/or repair. As previously mentioned, POLRMT synthesises the primer for mtDNA replication. In the OH region, transcription from the L-strand promoter that is located a few hundred base pairs upstream of the designated replication origin creates the RNA primer for replication of the H-strand [53]. In vitro studies suggest that a prematurely terminated transcript with a 3’ end at CSBII, one of the three conserved sequence blocks (CSBs), forms a G-quadruplex-stabilised RNA/DNA hybrid with the non-template DNA strand [66–69]. This stable RNA/DNA hybrid, termed the mitochondrial R-loop, acts as the replication primer for H-strand synthesis. In support of this model of priming, RNA-to-DNA transition sites have been mapped to the CSB region in vivo [67,70–73]. The majority of the free 5’ ends in the OH region do not contain covalently-attached RNA at their 5’ terminus unless RNase H1 is defective, indicating that RNase H1 is essential for primer removal and that it normally functions in an efficient manner [74]. It should also be noted here that most free 5’ ends in this region are located about 100 base pairs downstream of CSBII in the vicinity of nucleotide position 191, which is formally defined as OH [67,70,72,73]. The gap between the observed RNA-to-DNA transitions near CSBII and the free 5’ ends can be explained by further processing of the 5’ termini of newly-replicated H-strand.

illustrated by the susceptibility of purified mtDNA to cleavage by RNase H2 but not RNase H1 [32]. The rNMPs found in mature mtDNA are therefore not related to the lengthy RNA species that temporarily coat the displaced H-strand in the RITOLS model nor are they longer RNA primers left unremoved after replication initiation. The estimated number of rNMPs per double-stranded mtDNA molecule ranges from 36 and 54 rNMPs in cultured HeLa and primary fibroblast cell lines, respectively [9], to 65 rNMPs in mouse liver [8]. In accordance with these numbers, Moss et al. [65] reported a higher frequency of rNMPs in solid tissues than in cultured cell lines. The location of mtDNA rNMPs has been mapped using next-generation sequencing approaches, and they appear to be randomly distributed across the mammalian mitochondrial genome [9,65]. There is no obvious strand bias in rNMP frequency between the two strands of mtDNA isolated from mouse tissues or from cultured human cells [8,9].
molecules by the mitochondrial exonuclease MGME1 [75–77]. A current model for primer removal at the O_h region therefore involves sequential activity of RNase H1, which removes the RNA primer ranging from the L-strand promoter to CSBII, and MGME1, which further digests the 5’ end of the H-strand replication product from CSBII to O_h [75].

POLRMT also synthesises the primer at O_l. The structural elements of the O_l sequence that are critical for priming have been defined and the process has been reconstituted in vitro [52,56,78], as has the process for removing the RNA primer through RNase H1 and Fen1 or a Fen1-like activity [79]. The key role of RNase H1 in primer removal at both mtDNA origins is highlighted by the fact that RNase H1 knockout mice exhibit progressive mtDNA depletion resulting in embryonic lethality after embryonic day 8.5 [42]. Accordingly, mutations in human RNase H1 cause progressive external ophthalmoplegia characterised by depletion of mtDNA levels and the accumulation of deletions [43]. Together with the finding that RNA primers are retained at both the O_h region and O_l in RNase H1-deficient cells [74], these observations imply that RNase H1 is the factor responsible for the normally efficient removal of mtDNA RNA primers.

**Incorporation of single rNMPs during mtDNA replication and repair**

As with nDNA duplication, single rNMPs are also incorporated during replication of mtDNA by Pol γ. Biochemical studies indicate that yeast Pol γ (Mip1) has an rNMP incorporation rate that is comparable to its nuclear counterparts (1 rNMP per 640 nucleotides in a long-template assay where Pol ε and Pol δ insert 1 rNMP per 640 and 720 nucleotides, respectively) [10,28]. Human Pol γ exhibits high discrimination against rNTPs and inserts only one rNMP per 2300 nucleotides in a similar assay [8,80]. The persistent rNMPs found in mtDNA are therefore not due to unusually poor discrimination against rNTPs by the mtDNA polymerase. As has been found for many other DNA polymerases, the exonuclease activity of Pol γ does not contribute to rNMP removal [8,9].

Although Pol γ is the only replicative DNA polymerase identified in the mitochondria, there is at least one other DNA polymerase that is required for mtDNA maintenance under certain conditions. The PrimPol primase-polymerase is partially localised to mitochondria [24] and is required for repriming of mtDNA replication after DNA damage [81]. Repriming and/or translesion synthesis by PrimPol might therefore contribute to the rNMP load of mtDNA, although due to its low processivity and presumably only occasional involvement in mtDNA synthesis, its contribution can be expected to be minor. Furthermore, rNMP insertion by some of the repair or translesion polymerases that have been suggested to be present in mitochondria, such as Pol β, Pol ζ, Pol η, and Pol θ, cannot be excluded until the mitochondrial role of these polymerases is studied in more detail (reviewed in [82]).

**Mitochondria lack efficient repair mechanisms for single embedded rNMPs**

The frequency and identity of mtDNA rNMPs is partly defined by the dNTP pool

As reviewed above, mtDNA contains single (or at most double) rNMPs that are broadly scattered over both strands (Fig. 1). These rNMPs persist in mtDNA over time and are thus best explained by the absence of repair mechanisms rather than by exceptionally frequent incorporation during mtDNA replication. Indeed, it was found that elimination of RER by deletion of RNase H2 did not increase mtDNA rNMP frequency in *S. cerevisiae* [28,83]. Furthermore, analysis of a panel of yeast strains with permanent dNTP pool imbalances revealed that the size and balance of the cellular dNTP pool determines the frequency of individual rNMPs in mtDNA, i.e. an insufficient supply of one dNTP results in increased incorporation of the corresponding rNMP in mtDNA [28]. In contrast, rNMP levels in nDNA were unaffected by dNTP pool alterations unless RER was eliminated. These results demonstrate that yeast mitochondria lack efficient mechanisms for replacing incorporated rNMPs with dNMPs. Furthermore, given that the base identity of embedded rNMPs was determined by the rNTP/dNTP ratios present in the cell, the majority of mtDNA rNMPs are likely to derive from replication by Pol γ rather than being remnants of replication primers.

The dNTP pools also affect the type and frequency of rNMPs embedded in the mtDNA in mammalian cells and tissues. For instance, fibroblasts from patients with defects in mitochondrial dNTP metabolism show altered frequencies of the individual rNMPs [9]. Furthermore, mice defective in the mitochondrial inner membrane protein MPV17 that is implicated in mitochondrial disease [84–86] exhibit decreased dGTP and dTTP pools in the liver [87] and increased frequency of rGMP embedded in mtDNA [65]. However, the correlation between rNTP/dNTP ratios and mtDNA rNMP frequency in mammals might not be as clear-cut as in budding yeast. For example,
although dNTP pools were only found to be altered in the liver of MPV17 knockout animals, additional tissues (the heart and brain) showed increased mtDNA rGMP incorporation that could not be explained by an increased rGTP pool [65]. One possible explanation for why rNTP/dNTP ratios might not directly predict mtDNA rNMP incorporation is that the nucleotide levels measured from cellular or mitochondrial extracts might not accurately reflect the levels available to the mitochondrial replisome during mtDNA synthesis, for example, due to spatial and temporal variations in dNTP levels.

The dNTP levels fluctuate greatly over the cell cycle, with the highest concentrations found during S-phase when nDNA replicates [88,89]. However, mtDNA replication is not strictly limited to S-phase or to cycling cells [90], and it thus also occurs when cellular dNTP levels are low and rNTP/dNTP ratios are high. The rNTP/dNTP ratios from dividing and quiescent mouse Balb/3T3 fibroblasts have been shown to be up to 10-fold higher in quiescent cells ([21]; Fig. 2). Elevated rNTP/dNTP ratios can therefore explain the higher frequency of rNMPs observed in the mtDNA of non-dividing vs. cycling cells [65]. Furthermore, the ratios of each dNTP-rNTP pair are a major determinant of the relative frequency of the individual rNMPs in mtDNA. In line with the high cellular concentration of rATP (a 188-fold and 169-fold excess of rATP over dATP in logarithmically growing \( S. \) cerevisiae and cultured mouse cells, respectively) [15,21], two independent next-generation sequencing approaches found rAMP to be the most frequent rNMP in mammalian mtDNA, followed in declining frequency by rGMP, rCMP, and rUMP [9,65]. However, the discrimination ability of the replicative DNA polymerase also influences the relative frequency of rNMPs in the mitochondrial genome. Human Pol \( \gamma \) discriminates most efficiently against rUTP, showing a 77 000-fold preference for dTTP over rUTP, while the discrimination factors for the other rNTPs range from 9300 to 1100 in the order rATP > rCTP > rGTP [80]. When considered together, the discriminatory capacity of Pol \( \gamma \) and the rNTP/dNTP ratios for each dNTP-rNTP pair appear to largely explain the observed relative frequencies of the individual rNMPs in mtDNA. Still, further research is called for in order to obtain a complete understanding of the factors that determine mtDNA rNMP levels, especially in different cell types and during different stages of development.

**The implications of mtDNA rNMPs**

As discussed in the first section of this mini-review, the presence of rNMPs in nDNA has detrimental consequences for genome stability [32,33,38]. An additional point to consider is that the mitochondrial matrix is thought to be more basic (pH 8.0) than the nucleus (pH 7.2) [91], which is expected to render mtDNA more prone to alkaline hydrolysis at sites of rNMP incorporation than nDNA. Given the undisputedly negative effects of persistent rNMPs in nDNA, why are rNMPs in mtDNA so well tolerated? One central reason might be the reverse transcriptase activity of Pol \( \gamma \) [92,93] that allows it to efficiently replicate DNA templates containing incorporated rNMPs. Accordingly, in vitro analyses have shown that Pol \( \gamma \) bypasses...
single rNMPs embedded in a DNA template without any major negative effects on the processivity or fidelity of replication [8, 80]. In contrast, multiple consecutive rNMPs greatly reduce the efficiency of rNMP bypass by Pol γ. However, the polymerase is unlikely to encounter persistent stretches of more than two or three rNMPs in vivo because of the presence of RNase H1 in the mitochondria. Additional factors that have been suggested to contribute to the tolerance of rNMPs in mtDNA include its slower replication rate [94], its small genome size, and the partial redundancy conferred by the presence of multiple mtDNA copies per mitochondrion. In support of the latter explanation, super-resolution microscopy studies have provided evidence suggesting that only a subset of the mtDNA molecules in cultured fibroblasts are undergoing active replication [95, 96], while the other mtDNA molecules might simply be 'storage' of mtDNA. In principle, DNA breaks caused by hydrolysis at rNMPs should only be problematic if they occur in the molecule that is actively being replicated.

What, if any, is the consequence of an increased mtDNA rNMP frequency? Yeast strains with a decreased mtDNA rNMP load showed improved mtDNA stability [28]. One possible interpretation of this finding is that rNMPs jeopardise mtDNA stability, although alternative explanations also exist given that the same strains have increased mtDNA copy number. Moss et al. [65] reported the formation of late-onset mtDNA deletions in the brains of MPV17 knockout mice that also show increased rGMP incorporation. However, because the overall frequency of mtDNA rNMPs does not appear to be affected (assayed from the liver of the same mice), it is at present unclear whether the deletions are caused by increased rNMP-dependent instability or by some other, yet-undescribed mechanism. In summary, the effects of rNMPs on mtDNA stability and on mtDNA-related processes are not yet well-defined. One must also consider the possibility that the rNMPs in mtDNA play a positive physiological role, as has been suggested for the transient rNMPs in nDNA (discussed above). If this were to be the case, then a drastic reduction in mtDNA rNMPs could have some form of negative consequences. Because even single embedded rNMPs alter the properties of DNA, they can be envisioned to affect the interaction of DNA-binding proteins with the mtDNA molecule and thus influence processes such as mtDNA replication, repair, gene expression, packaging, and segregation.

**Conclusions and perspectives**

Although the presence of rNMPs in mtDNA has been acknowledged for over four decades, we still lack a complete understanding of how these atypical DNA
building blocks influence the stability of the mitochondrial genome and the many mtDNA-related processes that are essential for mitochondrial function. With increased interest in the role of rNMPs in the nuclear genome and the introduction of new methods for mapping them, mitochondrial rNMPs have experienced a boost in interest and have been the subject of several recent studies. The potential connection of mtDNA rNMPs to mitochondrial disease via defects in enzymes involved in mitochondrial nucleotide metabolism makes them even more interesting to study.

Many questions currently remain unanswered. What is the consequence of increased rNMPs, and would a higher frequency of mtDNA rNMPs be tolerated in vivo? Are rNMP levels really relevant to mitochondrial disease, or are they more of a proxy for mitochondrial dNTP pools? A number of studies have demonstrated that increasing dNTP pools elevates mtDNA copy number [97–99]. While elevated dNTP pools might act directly by increasing mtDNA replication efficiency and thus mtDNA copy number, it is interesting to consider the option that some of the positive effects of increased dNTP pools on mtDNA copy number might in fact be explained by a decreased frequency of mtDNA rNMPs. The presence of fewer rNMPs in the mtDNA should increase its stability and consequently mtDNA copy number. Because the effect of dNTP pools cannot be unlinked from mtDNA rNMP frequency, the individual contributions of these factors on mtDNA copy number have not been determined. Finally, there is a discrepancy between the frequency of rNMPs found in mtDNA in vivo (1 rNMP per 500–900 nucleotides) and the frequency of rNMP incorporation in vitro (1 rNMP per 2000–2300 nucleotides) [8,9]. Is this discrepancy due to differences in rNTP/dNTP ratios or has the contribution of factors other than Pol γ been underestimated as a source of rNMPs in vivo? We hope for many interesting developments and new insights to address these and other pressing questions within the coming years.

References
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ribonuclease H2 subunits cause Aicardi-Goutières syndrome and mimic congenital viral brain infection. 


*Am J Hum Genet.*


