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CITATION:

Sahayasheela, Vinodh J. ...[et al]. Mitochondria and G-quadruplex evolution: an intertwined relationship. Trends in Genetics 2023, 39(1): 15-30

ISSUE DATE:

2023-01

URL:

http://hdl.handle.net/2433/281774

Trends in Genetics

CellPress OPEN ACCESS

Opinion

Mitochondria and G-quadruplex evolution: an intertwined relationship

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G-quadruplexes (G4s) are non-canonical structures formed in guanine (G)-rich sequences through stacked G tetrads by Hoogsteen hydrogen bonding. Several studies have demonstrated the existence of G4s in the genome of various organisms, including humans, and have proposed that G4s have a regulatory role in various cellular functions. However, little is known regarding the dissemination of G4s in mitochondria. In this review, we report the observation that the number of potential G4-forming sequences in the mitochondrial genome increases with the evolutionary complexity of different species, suggesting that G4s have a beneficial role in higher-order organisms. We also discuss the possible function of G4s in mitochondrial (mt)DNA and long noncoding (lnc)RNA and their role in various biological processes.

Highlights

G-quadruplexes (G4s) are nucleic acid secondary structures comprising stacked planar guanine (G)-tetrads. Their ability to influence biological processes, such as replication, transcription, and genome instability, has been observed in mitochondria.

The mitochondrial G4-forming sequence has increased in higher organisms through evolution, showing its key role in mitochondrial regulation.

We discuss the possible function of DNA G4 in mitochondria in various functions, such as transcription and genome stability, and their regulation.

We report potential G4-forming sequences in the long noncoding RNA produced in mitochondria and their possible role in RNA granules and heme scavenging.

G-quadruplex and its biological role

Nucleic acids are known to form structures other than the Watson–Crick canonical double-helical structure. A **G4** (see Glossary) is a stable secondary structure of nucleic acid that can arise from single-stranded G-rich DNA and RNA sequences (Figure 1) [1]. The formation of G4 tetrameric structures was first reported in 1962 [2], after the original observation of the self-assembly of guanylic acid in 1910 [3], decades before the structure of DNA was proposed by Watson and Crick. G4s are formed by **Hoogsteen hydrogen bonding** between four Gs to form a planar G-tetrad and exhibit extremely high stability under physiological conditions in the presence of monovalent metal cations (such as K^+, Na^+, and Li^+) and resistance to degradation by nucleases. G4s can fold into various topologies based on the orientation of the G-tract and the interconnecting loops, and these polymorphic structures have an important influence on G4-related biological functions [4]. Computational tools are available to identify potential G4-forming sequences, based on algorithms [5]; and G4-specific probes, antibodies [6], and G4 sequencing (G4-seq) [7] have been developed to study G4s in cells.

The ability of genomic DNA to form G4s was first reported for G-rich sequences from the immunoglobulin switch region [8] and, subsequently, the crystal structure of G4 in the human telomeric DNA sequence was elucidated [9]. The formation of G4s in the G-rich telomere repeat has a role in genome stability and inhibits telomerase function. Since then, numerous studies have reported G4 formation, mostly in the promoter region of various genes, such as *cMYC* [10], *VEGF* [11], and *HIF1a* [12]. G4-forming sequences in the promoter region were proposed to function as transcription repressors by stabilizing the G4 structure using ligands such as TMPyP4, BRACO19, and pyridostatin (PDS) [13]. Using computational approaches, several G4-forming sites were also found to be enriched in the promoter region of various oncogenes in the genome [14,15]. These findings raised the possibility of developing ligands to target G4s for various therapeutic interventions. However, G4 formation in promoters resulting in transcription suppression within the chromatin is not fully understood and a study using genome-wide G4 mapping identified that the

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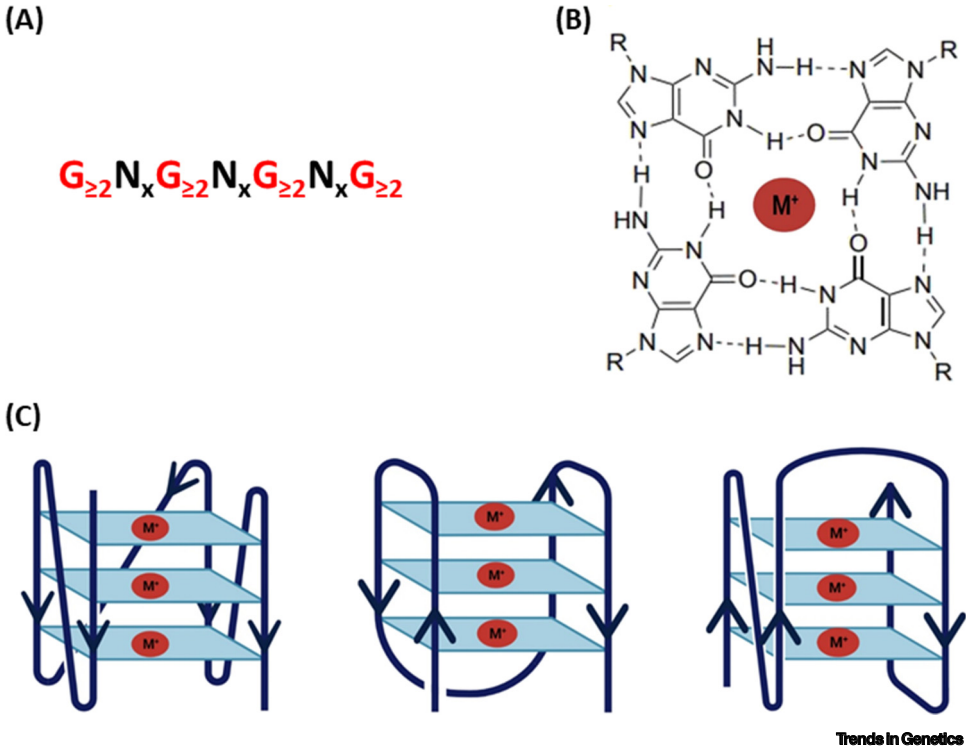

Figure 1. G-quadruplex (G4) structure and topologies. (A) The nucleotide sequence of the G4 motif, where N denotes the loop sequences. (B) A planar guanine tetrad formed by Hoogsteen bonds and stabilized by the metal cation M^+. (C) Schematic of topologies of G4s.

Glossary

Circos plot: Circos visualizes data in a circular layout ideal for exploring relationships between objects or positions. It greatly enhances the visualization of scientific results, especially in the genomics field.
G-quadruplex (G4): secondary structures formed in nucleic acids sequences that are rich in G, where four G bases can associate through Hoogsteen hydrogen bonding to form a square planar structure called a G tetrad.
GC skew: the relative excess of G nucleotides over C nucleotides on one strand compared with other; calculated by GC skew = (G – C)/(G + C).
Hoogsteen hydrogen bonding: variation of base-pairing in nucleic acids where two nucleobases, one on each strand, can be held together by hydrogen bonds in the major groove.
Mitochondrial heavy (H) and light (L) strand: mtDNA comprises 'light' and 'heavy' strands, with the H strand containing a higher proportion of G and adenine nucleotides and the light strand containing a higher proposition of cytosine and thymine.
R-loop: during transcription, the nascent RNA strand can base pair with its template DNA, displacing the nontemplate strand as single-stranded DNA and forming a structure called an R-loop.

G4 ligand PDS elicited DNA damage and caused gene downregulation [16]. In addition, contrary to the finding that G4s have a vital role in transcription suppression in the promoter region, other researchers have reported a role for G4s as enhancers of various genes [17], by mechanisms such as G oxidation [18] and transcription factor binding [19]. Further accumulating scientific data suggest that G4s have the potential to alter gene expression at many different levels, perturb the chromatin architecture, and act as regulatory elements in epigenetics (reviewed in [20]). G4s further can affect the stability of the genome and manipulate the DNA damage response (DDR) as a valuable anticancer strategy (reviewed in [21]).

In addition to DNA, G4s can also form in RNA and have been implicated in a range of biological processes. Compared with their DNA counterparts, RNA G4s are relatively stable, unconstrained, and can readily form secondary structures. Although studies of G4s are mainly focused on DNA, many studies also report the potential of RNA G4s and their biological significance (reviewed in [22]). Computational analysis identified nearly 3000 putative RNA G4-forming sites in the 5′-untranslated region (UTR) of mRNA in the human genome, revealing their potential role in cellular function [23]. Recent studies used a G4-specific probe to perform BioTASQ G4-RNA-specific precipitation (G4RP)-seq and identified a large number of G4-forming RNAs, demonstrating the existence of RNA G4 in *in vivo* conditions [24]. The formation of G4s in the 5′-UTR of mRNA inhibited the translation of several genes, such as *NRAS* [23] and *BCL2* [25]; in cap-independent translation, G4s conferred internal ribosome entry site (IRES)-mediated translation initiation in *hVEGF* [26] and *FGF2* [27]. Furthermore, RNA G4s have multiple roles in cells, such as splicing regulation, mRNA localization, and miRNA maturation, and G4-binding

proteins (G4BPs) were found to have an essential role in cellular processes. Hence, G4 formation and its functional roles have gained considerable attention, with efforts to explore its role in biological processes beyond a simple roadblock of the transcription process.

Many studies of G4s focus solely on the nuclear genome, but little is known about G4s and their functional role in mitochondria, despite an abundance of G and a favorable G4-forming environment. A review by Falabella and colleagues [28] elaborates on the possible regulatory function of G4 in mitochondria, but mainly focused on DNA G4 and its role in transcription, translation, and genome instability. Thus, in this review, we highlight mitochondrial G4 (mitoG4) from an evolutionary perspective, reporting an increase in potential G4-forming sequences from lower- to higher-order organisms, reflecting its gradual evolution. Furthermore, we discuss RNA G4s and their abundance in mitochondrial lncRNA and their potential roles in translation, granules, and reactive oxygen species (ROS) scavenging. Finally, we discuss the experimental tools available to study mitoG4s and provide insight into their future perspectives and challenges.

Mitochondria

Mitochondria are known as the powerhouse of the cell because of their ability to generate ATP, which is essential for normal cellular function. They also play a vital part in various cellular functions, including calcium homeostasis, apoptosis, stem cell generation, and heme synthesis [29]. Most eukaryotic mtDNAs are double-stranded, circular molecules that are typically present at several hundred to thousands of copies per cell. mtDNA lacks histones but is usually packaged into slightly elongated DNA–protein structures known as mitochondrial nucleoids, comprising one or two genome copies per nucleoid [30]. In most eukaryotes, nearly 90% of mtDNA comprises coding regions, unlike nuclear DNA, and its genetic code differs slightly from that of nuclear DNA [31]. Most mammalian mtDNAs encode 37 genes, including 13 genes that form the essential subunits of the mitochondrial respiratory chain complexes; however, the remaining ~77 respiratory chain subunits of the organism are encoded by the nuclear genome [30].

mtDNA comprises two strands that can be distinguished by their nucleotide composition and are termed **mitochondrial heavy (H) and light (L) strands** (Figure 2). Typically, mtDNA strands are separated on the basis of density using the classical biochemical technique of ultracentrifugation, producing the H strand, which has a high G + thymidine (T) content, and the L strand, which has a low G + T content [32]. Mitochondria are proposed to have evolved from endosymbiotic bacteria, and phylogenetic analysis confirmed that the lineage of mtDNA is closely related to that of Alphaproteobacteria [33,34]. This has raised several intriguing questions for the fields of mitochondrial and evolutionary research, such as how mitochondria integrate and adapt within the host, and whether mitochondria had an important role in the transition from prokaryote to eukaryote.

Genomic DNA G-quadruplex evolution

Computational analyses [5] and *in vitro* [35–37] and *in vivo* [38,39] techniques have been developed to identify and validate G4 sequences across the genomes of various organisms, but the role of secondary DNA structures and their contribution to the evolution of each species is not well explored. However, a study using G4-seq mapped the DNA G4 formation in 12 species widely used as model organisms (i.e., multiple species of bacteria, plants, and eukaryotes, including human, mouse, and drosophila) [40], and found the experimentally observed G4s to be diverse in sequence composition and genomic location across different organisms. G4s were strongly depleted in the genome of bacteria and yeast but, interestingly, were found to be enriched in gene promoters and transcription start sites (TSS) of mammals, which suggests a specific role of G4 in transcription. Ding and colleagues [41], who analyzed the quadruplex-forming sequences (PQSs) of microbial genomes, found that enrichment of PQSs was

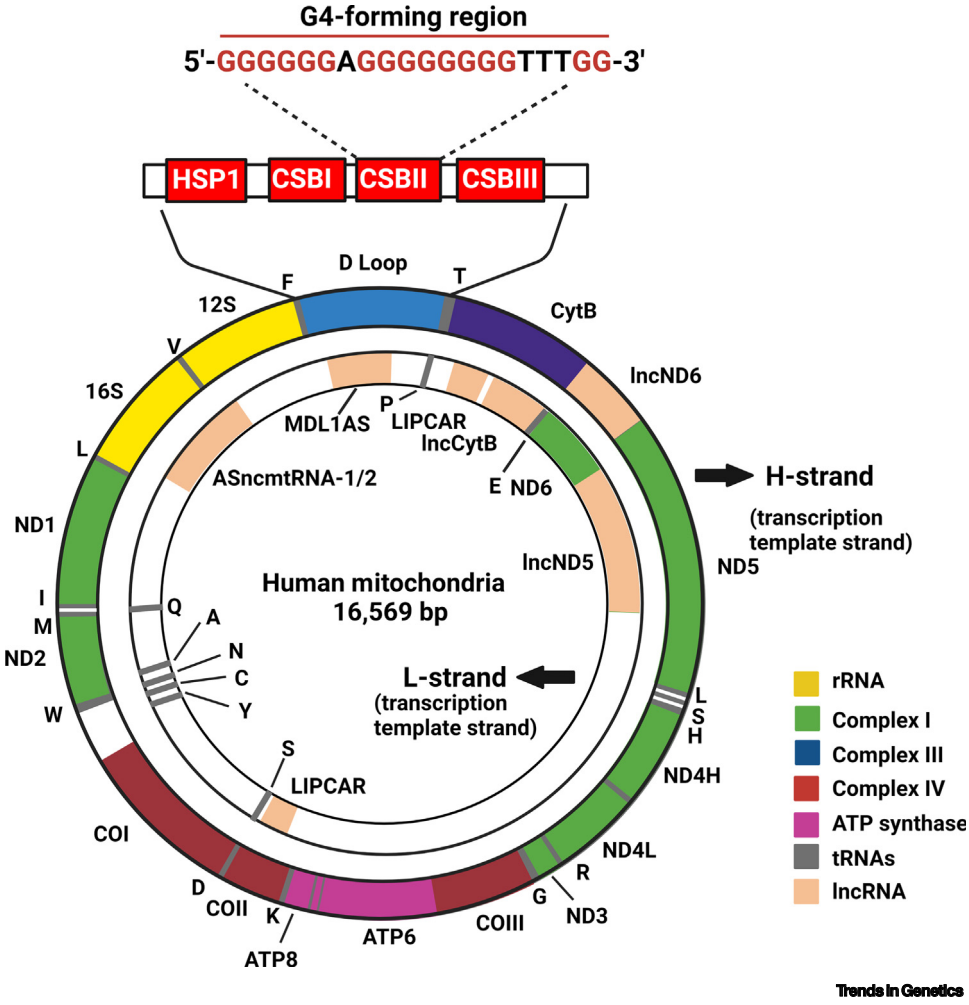

Figure 2. Map of human mitochondrial DNA. The highlighted region in the D-loop indicates the conserved block sequence II (CSBII), the G4-forming sequence, which is highly conserved in most vertebrates.

randomly distributed in thermophilic organisms, while, in the order *Deinococcales*, the PQS was enriched and biased in the TSS of genes. This led to the hypothesis that the different bacteria evolved G4s for different beneficial functions, such as gene regulation or thermal stability at high temperatures. Another study using comparative bioinformatics analysis of seven species of *Saccharomyces* revealed that G4 structures are relatively more conserved than expected by chance throughout evolution [42]. The conserved G4 motifs maintained a strong association with promoters and rDNA, but not with double-strand break sites (DSBs), supporting the theory that G4 has *in vivo* functions that are evolutionarily constrained. A recent study comparing the genomes of 37 evolutionary-representative species found that G4 number, length, and density generally increased with evolution [43]. This study also found G4-bearing genes particularly enriched at the TSS in higher organisms and identified an antagonistic relationship between G4s and DNA methylation sites. It was hypothesized that the increase in G4 structures might facilitate the development of new gene regulatory mechanisms to achieve increasingly complex cellular, physiological, and behavioral activities.

Mitochondrial G-quadruplex evolution

mtDNA can form G4 secondary structures more easily compared with genomic DNA due to more favorable conditions. Most importantly, mtDNA is known to have an excessive number of Gs in the H strand compared with the L strand, violating the second parity rule, allowing more free G to be available for secondary-structure formation. This asymmetry is quantified in terms of AT skew (A – T)/(A + T) and **GC skew** (G – C)/(G + C), which studies suggest are associated with replication [44]. The replication of mtDNA is highly asymmetric at the origin of replication at the H strand (Ori_H) in a unidirectional manner, remaining single-stranded until the synthesis of L strand replication starts 11 kb downstream [45]. Mitochondrial replication is very slow, taking ~2 h; during this time, the single-stranded G-rich H strand has a very high opportunity to form G4 structures [46]. In addition, mitochondria maintain a K^+ concentration of 150–180 mM, which is favorable for forming G4 structures [47].

To understand mitochondria G4 from an evolutionary perspective, the mtDNA of 16 different biological model organisms belonging to various families was studied and the G4-forming sequences were mapped using QGRS mapper [48]. The parameters for the G4-forming sequences were set to a maximum length of 30 nucleotides and a minimum of two G4 groups. Most mitochondrial genomes range between 14 and 17 kb in size, although *Plasmodium* mtDNA was a relatively small exception at 6 kb. Interestingly, there was an increase in G4-forming motifs in higher-order vertebrates compared with primitive eukaryotes, indicating a gradual evolution of G4s. Figure 3 illustrates the phylogeny tree of the mitochondrial genomes studied with their respective size, GC content, and the number of G4-forming sequences in the H and L strands. The analyses revealed that, as species evolved, the G4 motif density increased, although the mitochondrial genome size remained largely the same, suggesting no association with genome size [127]. The **Circos plot** in Figure 4A illustrates the evolutionarily increasing GC content and high GC skew of mtDNA, demonstrating a correlation between these characteristics and higher-order organisms. The density of G4 motifs in these species indicated an even distribution within the mitochondrial genome, but the motifs were clustered densely in the higher taxonomic species with a stepwise reduction to lower organisms. To test the influence of GC content on G4 formation, the GC content of each species was plotted relative to their respective G4 motifs (Figure 4C), which showed a high correlation between the PQS and GC content (R = 0.8557 by Pearson and S = 0.8939 by Spearman's rank correlation coefficients).

Furthermore, examination of the frequency of PQS sites relative to the genome length of 140 organisms of different taxonomies (Supplementary S1 in the supplemental information online) revealed that it was associated with evolutionary distance. Closely related vertebrates, such as birds, reptiles, mammals, amphibians, and fish, had the highest PQS frequencies, while lower taxonomy groups, such as fungi, nematodes, and insects, had the lowest frequency (Figure 4D). Among the different orders, there was a strikingly high G4 frequency in avian species. The mtDNA organization among the vertebrates was very similar, with noncoding sequences grouped together in the control region (CR), which controls mtDNA replication and transcription [66]. Interestingly, birds have an additional CR termed the pseudo CR (YCR), which is unique to avian species [49–51]. This YCR was found to have a strong association with an increased lifespan, despite birds having characteristics that generally have a negative effect on longevity by increasing ROS, including higher metabolic rates, high blood glucose levels, and high body temperature [52]. One of the well-studied G4s in mtDNA lies in the CSBII (GCGGGGGAGGGGGG GTTTG) of the CR and is a highly conserved sequence among vertebrates [53]. More surprisingly, the same G4-forming sequence was also found in the YCR region of birds, showing its important role and evolutionary conservation in mtDNA [54]. A potential role of the conserved G4 in CSBII is the formation of an **R-loop** hybrid G4 three-stranded nucleic acid structure that comprises a DNA:

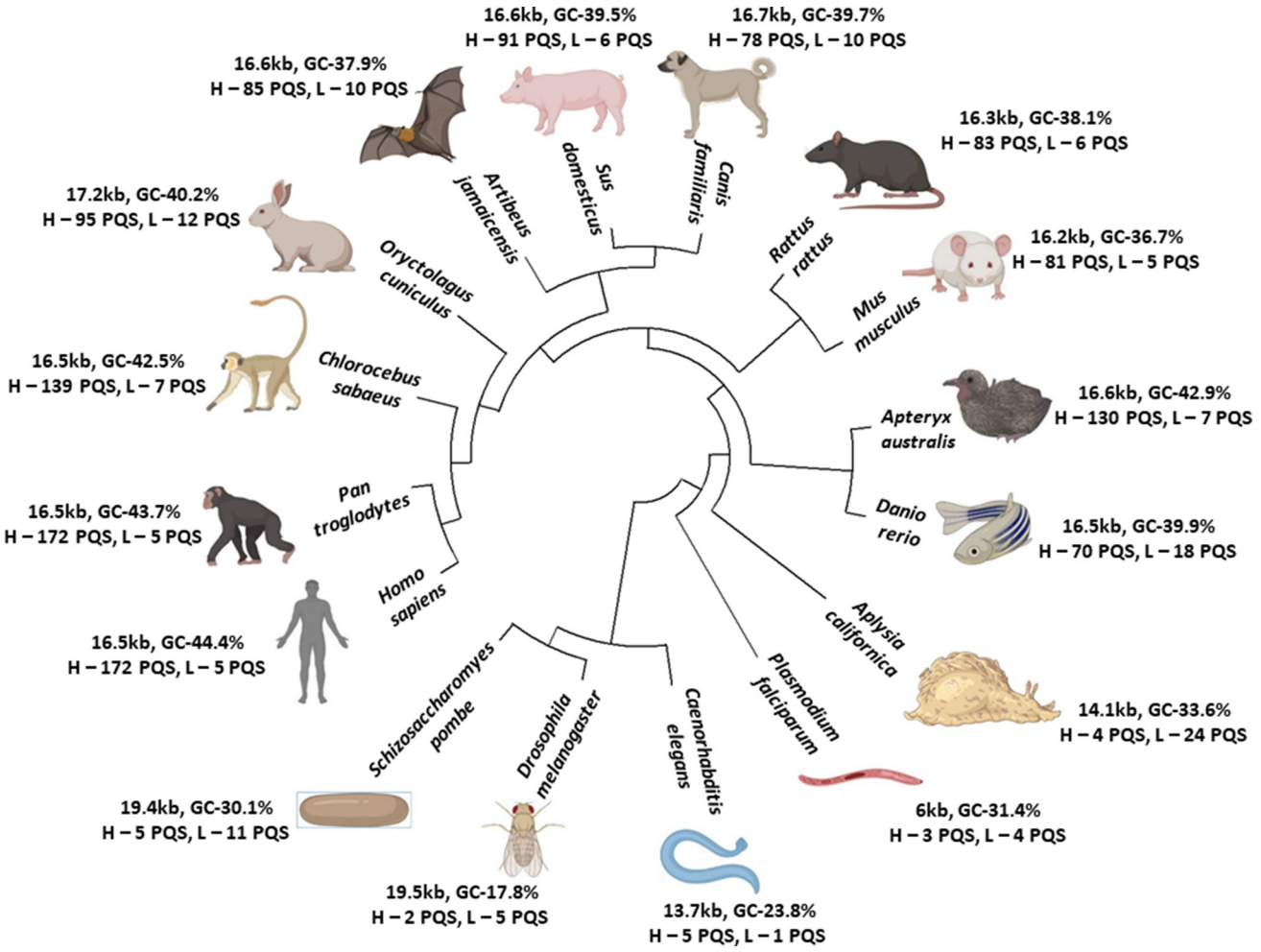

Figure 3. The phylogeny tree of 16 species together with their mitochondrial genome size. The guanine–cytosine (GC) content and the number of potential G-quadruplex (G4)-forming sequences (PQS) in the heavy (H) and light (L) strands are denoted.

RNA hybrid and a displaced strand of DNA [55], which has an important role in mitochondrial replication and transcription [56]. Furthermore, the G4 stabilized R-loop is reported to increase transcription by a mechanism involving successive rounds of R-loop formation [57]. Hence, the duplication of the CR might provide an advantage for efficient replication and transcription, generating more mitochondria, which can lead to more effective energy production for flight. However, the reason why G4 is conserved in the YCR and how it contributes to the longevity of birds requires experimental validation. Recently, Yang and colleagues identified the co-formation of G4s and R-loops spatially linked into unique structures called G-loops at telomere regions [58]. It would be interesting to evaluate if such pronounced structures are also formed in mitochondria.

Control and regulation of G-quadruplexes in mitochondria

G4 formation depends on the sequence composition within the G4 motif and its flanking sequences, while its stability depends on the number of G-tetrads, the loop length, and its topology. G4 formation is also highly dependent on monovalent cations, such as $K^+ > Na^+ > NH_4^+ > Li^+$, and on the presence of K^+ and Na^+ in the cell environment [59]. Furthermore, the presence of

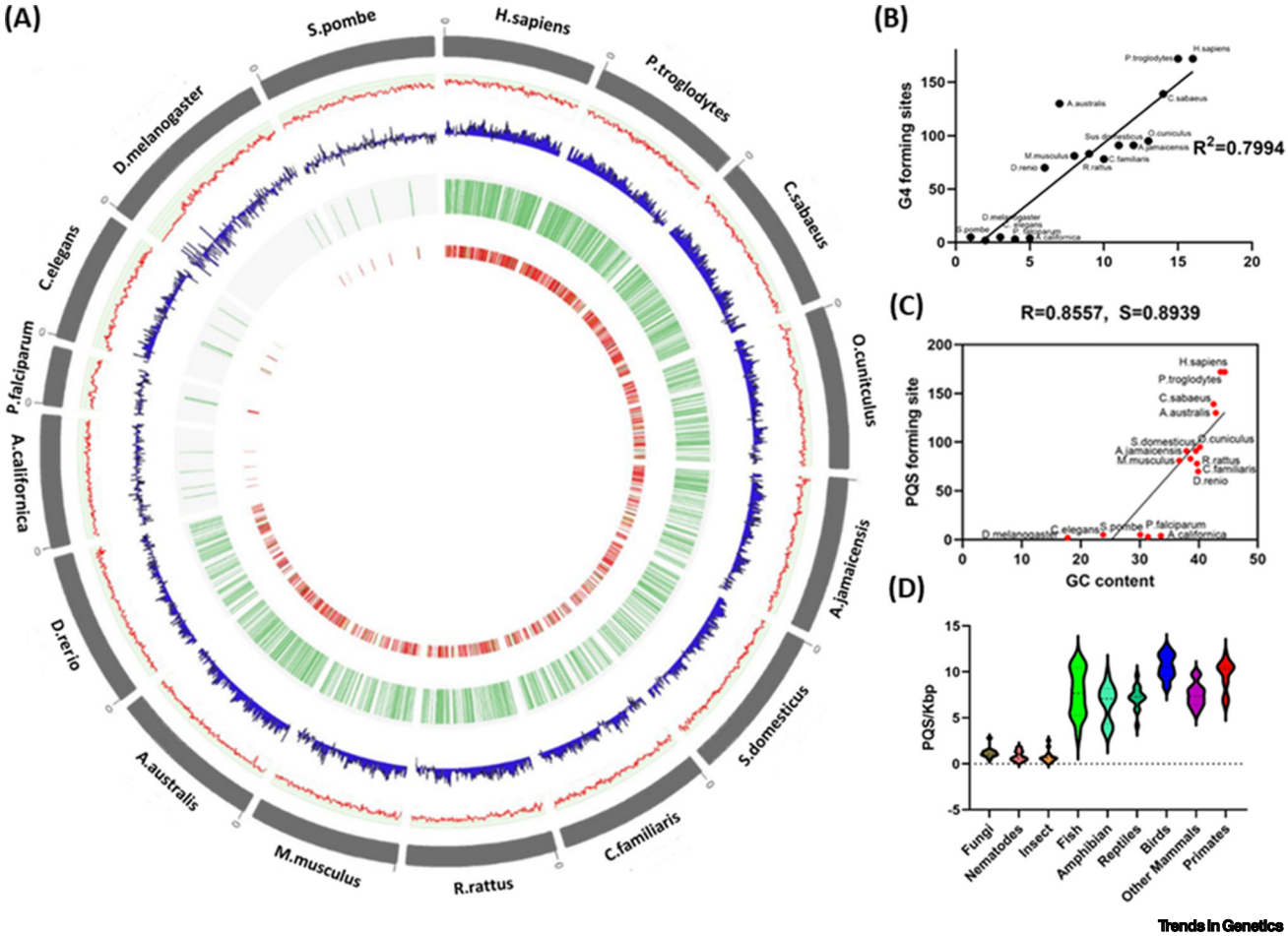

Figure 4. Mitochondrial genomic landscape of G-quadruplex (G4) motifs in selected species. (A) Circos plot of representative mitochondrial genomes. The outer circle represents the organism and its mitochondrial DNA (mtDNA) in gray followed by the guanine–cytosine (GC) content in the form of a line plot in red, further followed by GC skew in blue. The next circle indicates the localization of the G4 plotted in the form of tiles in green, and the inner circle represents the score of the G4-forming regions plotted in the form of a heatmap. (B) The GC content of the mitochondrial genomes of the 16 species and potential G4-forming motifs in the mitochondrial heavy strand of the 16 species. The *x*-axis represents the ranking of organisms based on their complexity by phylogeny analysis, from lower- to higher-order organisms. (C) Scatter plot indicating the G4-forming sequence (PQS) for all species on the *y*-axis with respect to GC content on the *x*-axis. The R and S values at the top denote the Pearson correlation coefficient and the Spearman correlation coefficient, respectively. (D) The frequency of PQS-forming sites, relative to genome length, expressed as PQS/kbp in the different taxonomic subgroups.

molecular crowding and the induction of negative DNA super helicity during transcription can influence G4 formation [60]. In general, RNA G4s are more thermally stable compared with their DNA counterparts, but are limited to a parallel confirmation only, in contrast to different topologies adapted by DNA G4s.

G4s within a cell are highly regulated by G4BPs, which can stabilize or destabilize G4 formation, influencing biological processes, such as transcription, translation, and genomic stabilization [61]. Various techniques using G4 bait and mass spectrometry [62,63] have been developed to profile G4-interacting proteins in cells. Most G4BPs belong to the helicase family, such as DEAH-box helicase (DHX36) [64], RecQ class [Bloom protein (BLM)] [65], and Werner's syndrome protein (WRN) [66]. Some G4BPs can also recognize specific sequences and bind to selective G4 topology. For example, POT1 selectively binds to the telomeric antiparallel G4 and promotes its unfolding and refolding with the TPP1 complex [63,64]. Many G4BPs that bind to DNA G4s

can also bind to RNA G4 because of their structural similarity. RNA G4BPs include DHX36, nucleolin, heterogeneous nuclear ribonucleoproteins (hnRNPs), serine/arginine-rich splicing factors (SRSFs), fragile X mental retardation 2 (FMR2), and ribosomal proteins [61]. However, there are still other G4BPs to be discovered and a full understanding of their effect on biological functions requires further study.

While many studies have focused on G4BPs in the nucleus and cytoplasm, there are reports of G4BPs in mitochondria, which regulate mitoG4s. It is reported that TWINKLE can unwind the mitoG4 structures inefficiently close to the DNA deletion breakpoint, assisting mitochondrial replication machinery and preventing G4s from causing instability [67]. A knockdown of TWINKLE caused severe mtDNA depletion and affected the respiratory chain, demonstrating that TWINKLE is essential for replication [68]. Another example is Pif1, a DNA helicase that displays 5′–3′ helicase activity on both DNA/DNA and DNA/RNA hybrids. It can unwind G4 structures, and is located in both the nucleus and mitochondria [69,70]. PIF1 helicase inactivation was demonstrated to cause mitochondrial myopathy in mice, suggesting that this enzyme has a role in the regulation of G4 [71]. Similar to PIF1, DNA2 nuclease/helicase has also been reported to unwind G4, and its mutation has been shown to impair mitochondrial function, suggesting that DNA2 also has an important role in the regulation of G4 [72,73]. While other helicases with G4-unwinding function, such as RECQL4, are reported to localize in mitochondria, their role in mtDNA G4 has not yet been identified [74]. The mitochondrial transcription factor A (TFAM), a high-mobility group (HMG)-box protein, is the major binding protein of human mtDNA and has a critical role in its expression and maintenance. It is reported to bind to mtDNA G4 with an affinity similar to that of double-stranded DNA, suggesting functional recognition of G4 in mitochondria [75].

The transcription of mtDNA starts in the D-loop and continues throughout the entire genome; as a result, a large number of noncoding (nc)RNA transcripts are released [76]. These ncRNAs are relatively enriched in G, leading to a high G4-forming capability. A recent study identified that the protein G-rich RNA sequence binding factor 1 (GRSF1) was able to localize in mitochondria [77] and melt G4 structures in mtRNAs, which facilitated their degradation with the Suv3-PNPase complex [78]. Interestingly, this protein is present exclusively in vertebrates that have a high number of G4 motifs. We hypothesize that GRSF1 evolved to regulate and control G4 formation in mitochondria as it transitioned from G4-poor to G4-rich sequences. GRSF1 together with the SUV3 helicase aids the regulation of G4-rich lncRNA, and has an important role in maintaining mitochondrial homeostasis [79]. The RNA-binding protein, SLIRP, was reported to be localized in mitochondria and to regulate mitochondrial protein synthesis; it was also identified as a G4-interacting protein at low nanomolar concentrations [80,81]. G4BPs influence biological processes by either resolving or binding G4, but these effects do not always equate with the abundance of G4 motifs in the mtDNA. These reports support the likelihood of discovering novel proteins with roles in mitochondrial maintenance, stability, and evolution.

Role of G-quadruplexes in mitochondrial replication and transcription

In mammals, mtDNA replicates in a distinct manner compared with nuclear DNA. Given the circular nature of mtDNA, the transcription and replication machinery may collide, which can have a detrimental effect on mtDNA gene expression [82,83]. The mitochondrial RNA polymerase (mitoRNAP) transcribes the mtDNA and generates primers for replication, while the mitochondrial transcription elongation factor, TEFM, has a key role in replication–transcription [82]. Transcription of human mtDNA is directed by two promoters, the light-strand promoter (LSP) and the heavy-strand promoter (HSP), located in opposing mtDNA strands, which results in polycistronic transcripts that undergo extensive processing, terminated at the CSBII (Figure 5). Transcription termination is a result of the formation of R-loops and is more efficient when the CSBII has

more G tracts in G_6AG_8, rather than the rare variant G_5AG_7, which suggests an effect of G4 on transcription [82]. The presence of G4 structures causes genome instability, and has an important role in various cancers and genetic diseases [84,85]. The distribution of G4 in the genome is not random and is often localized with chromosomal breakage points, further highlighting the importance of G4 in the genome [84]. Given the higher number of potential G4 sequences in mitochondria, they may have an important role in mtDNA instability. mtDNA deletions are prominent in cancer and genetic disorders and also have a role in aging [86,87]. The stalling of mitochondrial replication machinery by G4 sequences during DNA synthesis is a prominent source of mitochondrial genome instability [67]. G4 sequences have accumulated significantly in higher-order organisms, suggesting that these sequences have important functional roles, despite their deleterious effect on mtDNA stability.

In recent years, it has been reported that phase separation has a key role in many critical nuclear functions, such as transcription [88], chromatin higher-order structure organization [89], membrane-less organelles [90], and histone modification [91,92]. Phase separation is driven by weak multivalent interactions between nucleic acid and nucleic acid-binding proteins that have intrinsically disordered regions (IDRs), which are sensitive to the NaCl concentration [93]. While studies of phase separation have traditionally focused on proteins, recent studies revealed that nucleic acid secondary structure can also trigger phase separation even in the absence of proteins. G4s can undergo liquid–liquid phase separation (LLPS) by connecting multiple nucleic acid strands in an intermolecular configuration, or by π-stacking between G-tetrads of different G4s. Such interactions form droplets by combining G4 and histone H1, which mediate dynamic chromatic condemnation in the nucleus [94]. In the mitochondrial context, it was recently discovered that TFAM undergoes phase separation with mtDNA to drive nucleoid self-assembly, which regulates mitochondrial transcription [95]. Therefore, we propose that the G-dense mtDNA might also be key in promoting LLPS.

ROS, produced mostly by mitochondria, contribute significantly to mitochondrial damage, but also have a prominent role in redox signaling to other parts of the cell [96]. Among the four bases, G has the lowest redox potential, being oxidized by ROS generation into 8-oxo-7,8-dihydroguanine (8OG) [97]. Therefore, G4s are an easy target for G oxidation, potentially affecting their stability. An interesting role of G4s is the epigenetic control of genes based on their 8OG modification. DNA damage inflicted on the promoter G4 region can up- or downregulate gene expression [98]. Furthermore, G4-rich DNA was found to accumulate in the cytoplasm and to participate in stress granule assembly upon oxidative stress, thereby also regulating gene expression [99]. Given the abundance of ROS in mitochondria, we wonder whether this can regulate mitochondrial gene expression and DDRs, similar to nuclear DNA, although this has yet to be evaluated in detail.

Mitochondrial RNA G-quadruplexes

The human mitochondrial genome is densely packed with genetic information that encodes two rRNAs, 22 tRNAs, and 13 mRNAs for the oxidative phosphorylation (OXPHOS) system. The entire mitochondrial genome is transcribed from both H and L strands as long polycistronic transcripts that undergo multiple processing before becoming functional. While most mRNAs are transcribed from the template of the G-rich H strand under the control of the HSP, the complementary L strand serves as the template for ND6 mitochondrial mRNA from the LSP. The transcribed polycistronic RNA undergoes processing at ribonucleoprotein structures called mitochondrial RNA granules (MRGs) before protein synthesis [100,101]. Several proteins associated with the processing and maturation of primary transcripts have been identified in MRGs, suggesting their role in the regulation of mitochondrial translation [102].

Genomes generate numerous lncRNAs, sequences longer than 200 nucleotides that do not translate into protein. Via their interaction with DNA, RNA, and proteins, lncRNAs are linked to gene regulation, epigenetic control of chromatin structure, and membrane-less bodies, respectively [103]. The importance of lncRNAs in evolution is reflected by their increasing presence in higher-order organisms, contributing to development and differentiation processes [104]. The formation of G4s is reported in lncRNAs, such as TERRA [105] and MALAT [106], with interest growing in identifying their functional role.

In mitochondria, while the L strand was known to be a template for ND6 and several tRNAs, it was also reported to generate several lncRNAs, which contain numerous potential G4-forming motifs [76,107]. The regions of the mitochondrial genome complementary to the genes that encode ND5, ND6, and Cytb mRNAs were found to have high levels of lncRNAs [76]. Using QGRS mapper, 21 G4-forming motifs were identified in G-rich lncND5 and 17 in lncCYTB. In a different study, the mitochondrial lncRNA, LIPCAR, was found to be a biomarker associated with chronic heart failure, although the actual mechanism remains elusive. Another heart failure study using deep sequencing of RNA revealed an abundance of mRNA (37%) and lncRNA (71%) of mitochondrial origin [108]. Given its abundance and G4 enrichment, it is possible that lncRNA generated from mitochondria may have a biological significance that has yet to be identified.

Newly transcribed RNA and RNA-processing protein form MRGs in mitochondria, although the exact mechanism of their formation is not fully understood [109]. The presence of G4 structures forms droplets by LLPS without the presence of protein in short-hair root RNA [110]. and ALS/FTD-associated C9ORF72 repeat RNA [111]. Given that this lncRNA with the presence of G4 may form droplets, it may constitute mitochondrial granules (Figure 6), but whether the G4-enriched lncRNA alone or other molecular determinants, such as protein, promotes the formation of granules has yet to be elucidated. Apart from producing ATP for cells, mitochondria also generate heme, an essential cofactor for many enzymes, by inserting ferrous iron into protoporphyrin IX using ferrochelatase. However, free heme is toxic even at very low levels, catalyzing the formation of ROS and inducing oxidative stress. This risk is mitigated by G4 because it can sequester heme in live cells, thus preventing oxidative DNA damage [112,113]. Earlier studies showed that G4s in complex with porphyrins, such as heme, have peroxidase and peroxygenase enzyme-like activity capable of reducing hydrogen peroxidase and other hydroperoxidases [114]. Furthermore, the DNA and RNA sequences that form parallel-stranded G4 were found to be optimal for heme binding and peroxidase activity [115]. It has been reported that, in Alzheimer's disease, amyloid-β-peptide sequesters heme, leading to heme deficiency and strong peroxidase activity at the intracellular level [116,117]. Given this evidence, we hypothesize that mtDNA and lncRNA can sequester free heme and protect cells from ROS arising from G4/heme complexes (Figure 6).

Targeting mitochondrial G-quadruplexes

In living cells, the presence of G4s in genomic DNA has been demonstrated with various imaging and sequencing techniques. G4 ligands cannot easily target mtDNA due to the highly dense, impermeable mitochondrial inner membrane [118]. The first direct evidence of mitoG4 was reported by developing a fluorescent G4 ligand, 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC), which localizes within mitochondria [119]. It was found that a sufficient quantity of BMVC suppressed mtDNA gene expression, eventually inducing cell death. Using live-cell imaging, another G4 ligand, RHPS4, was found to be localized primarily in mitochondria, even at low doses. Treatment with RHPS4 also induced acute inhibition of mitochondrial transcript elongation, leading to respiratory complex depletion [120]. Furthermore,

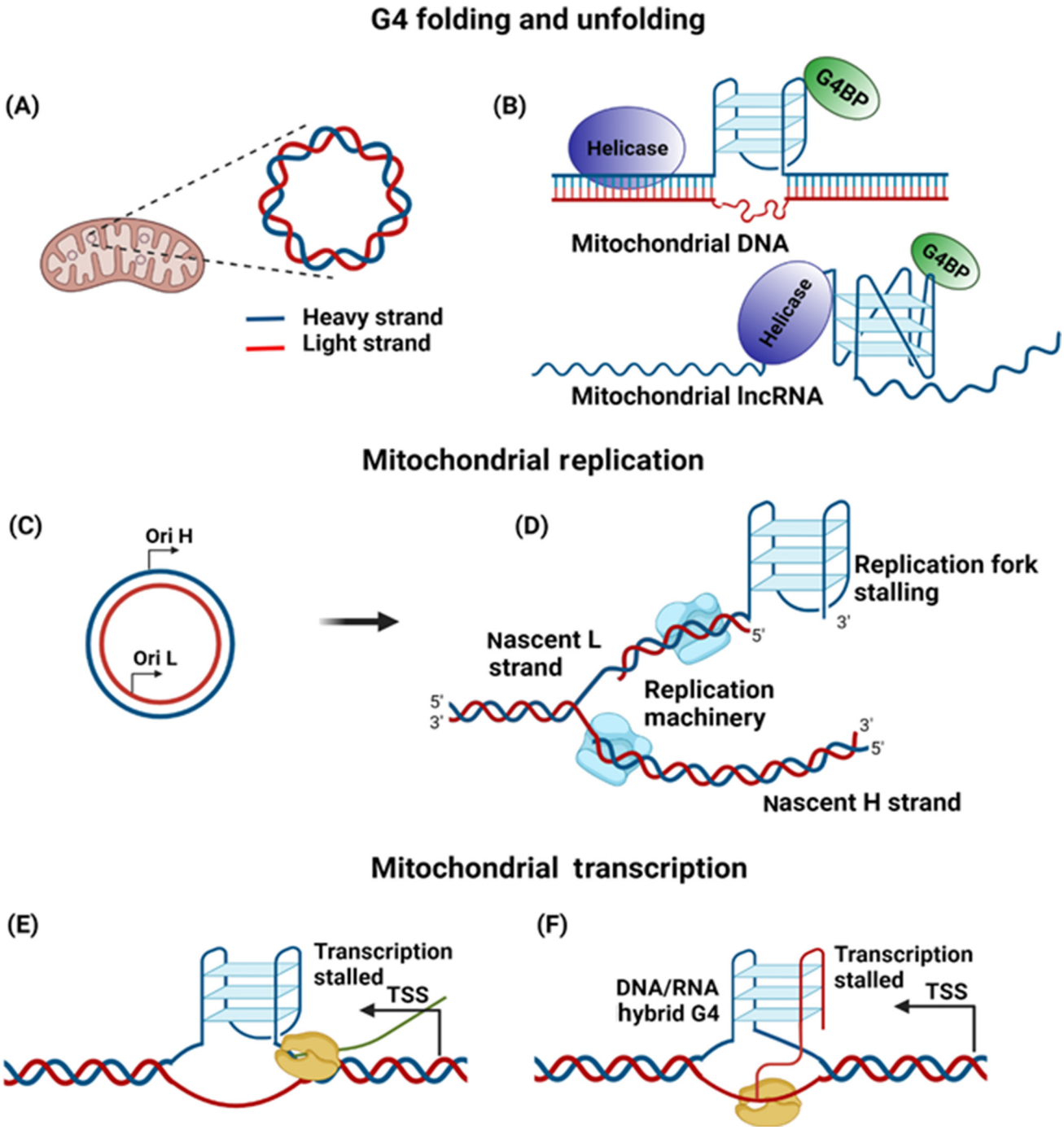

Figure 5. Role of G-quadruplexes (G4s) in mitochondrial DNA (mtDNA). (A) mtDNA is represented as a heavy (H) strand and a light (L) strand, based on guanine (G) content. (B) The presence of proteins that can bind and stabilize G4 (G4 binding protein; G4BP), as well as helicases that can unwind G4, can influence the G4 in mitochondria. (C) The mitochondrial replication origin in the H and L strand (O_H and O_L) initiates DNA replication in the respective strands. (D) The formation of G4 can influence mtDNA replication by stalling the process. (E) The presence of G4 in the transcription start site (TSS) can block the progression of mtRNA polymerase, resulting in altered transcription. (F) Formation of hybrid DNA:RNA G4 in the conserved block sequence II (CSBII) region terminates the transcription.

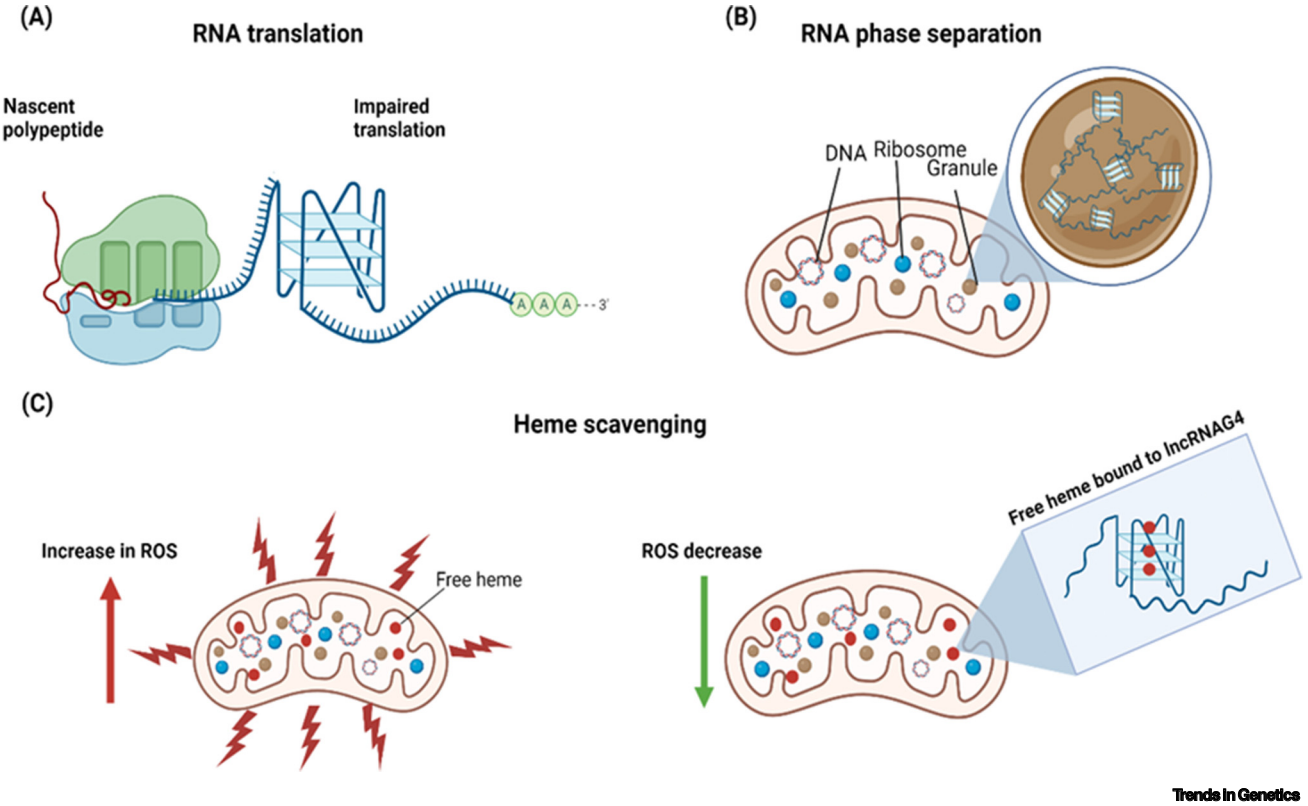

Figure 6. Role of G-quadruplexes (G4s) in RNA. (A) Formation of RNA G4s can impair the translation of mRNA rich in guanine (G). (B) Long noncoding (lnc)RNA with G4 structures can trigger phase separation and contribute to RNA granules. (C) The lncRNA can scavenge free heme in the mitochondria and reduce reactive oxygen species (ROS), thereby reducing oxidative stress.

mitochondria-specific probes targeting G4s for live-cell imaging were recently developed [121]. The probe showed selectivity almost 1000-fold higher than that of mitochondrial double-stranded DNA and sensitivity toward mitoG4, making it a suitable candidate for monitoring the dynamic process of mitochondria. More recently, a mtDNA G4-targeting probe integrating an active photosensitizer and mitochondria- targeting functional group was developed for mtDNA G4 sensing [122]. Furthermore, Hu developed near-IR fluorescent ligand for tracking mtDNA G4 [123]. Another study developed a set of chemical probes to thoroughly investigate mtDNA G4s: MitoISCH, a mtDNA G4-specific fluorescent probe, and MitoPDS, a mtDNA-targeted, G4-stabilizing agent [124]. MitoPDS caused glycolysis-related gene activation in cancer cells, revealing a connection of mtDNA G4s to glycolysis. Many types of cancer display increased glycolysis even in the presence of oxygen and competent mitochondrial function [125], and mtDNA instability contributes to the enhancement of glycolysis in cancer cells [126]. Given the increase in mtDNA instability caused by G4 stabilization, it is hoped that unraveling the associated mechanism will provide insights that will facilitate the development of cancer treatments.

Concluding remarks and future perspectives

Evolutionary erosion of GC-rich isochores in the nuclear genome is a common trend in most organisms, but mitochondria were found to have transformed from GC poor to GC rich as evolution progressed. The enrichment of G4 in birds and primates is particularly interesting, and suggests a need for further study of how these adaptations facilitate advantages in

higher organisms (related to aging, meeting the needs of complex cellular mechanisms, and physiological activities, etc.). While the role of mtDNA G4 has been explored, we believe it is worth investigating G-rich lncRNA produced by mtDNA, and its biological role inside mitochondria and the cytoplasm. Current knowledge of mitoG4s and their functional relevance is limited because of a lack of tools, unlike nuclear G4s, for which many probes, ligands, antibodies, and sequencing techniques have been developed. We believe that the potential role of G4 structures in mitochondria can be unraveled by developing probes capable of infiltrating these respiratory organelles. With many questions still remaining (see Outstanding questions), we believe that the field of mitoG4 research will continue to grow and provide a better understanding of secondary structures and their role in the evolution of higher organisms and their cellular processes.

Outstanding questions

G4 and mitochondria evolution: Why does G increase in H strands despite its deleterious effects? How do mtDNA G4s contribute to the evolution of higher organisms? Do they evolve to meet the physiological demands of higher organisms? Does mtDNA G4s have a role in the lifespan of an organism?

Does mitoG4 evolve in birds to meet their energy needs? How does the YCR contribute to bird lifespan? Why is the G4 sequence in the CR also conserved in YCR? Does it contribute to enhanced mitochondrial replication and transcription?

What is the functional role of G-rich lncRNA produced by mitochondria? Does it have an important role in mitochondrial function? Do the lncRNAs from mitochondria shuttle to the cytoplasm or nucleus as a molecular signal? Why do the levels of lncRNA from mitochondria vary in cardiac diseases?

lncRNA G4s and granules: Does G4 in mitochondrial lncRNA contribute to RNA granules? If so, are there any other molecular determinants that govern their formation? Do they have an important role in heme scavenging?

Interplay between mtDNA G4s and glycolysis: How does G4 in mtDNA contribute to enhanced glycolysis in cancer cells? How do they activate glycolysis-related genes? Do mtDNA G4s control mitochondrial OXPHOS? Do mtDNA G4s have a key role in the Warburg effect?

Author contributions

V.J.S. conceptualized, performed the analysis, wrote the manuscript, and drew the figures. Z.Y. conceptualized and supported manuscript editing. T.H. gave critical suggestions and supported manuscript editing. H.S. conceptualized, supervised, and edited the review process as well as funding acquisition.

Acknowledgments

We express sincere thanks for a Grant-in-Aid Priority Research (KAKENHI 21H04705 to H.S.) and 20H05936 from JSPS. This work was also supported by AMED [JP20am0101101 to H.S. (Platform Project for Supporting Drug Discovery and Life Science Research) (BINDS)] and NIH award R01CA236350 to H.S. The authors sincerely acknowledge Jeffrey Friedl for help in proofreading the manuscript. We also acknowledge Shinjiro Suzuki, Manendra Lankadasari, Vipin Mohan Dan, and Vinay Rajput for their input. Figures 2, 5, 6 were created using BioRender (BioRender.com).

Declaration of interests

The authors declare no conflicts of interest.

Supplemental information

Supplemental information to this article can be found online at https://doi.org/10.1016/j.tig.2022.10.006.

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