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Review

Emerging functions of mitochondria-encoded noncoding RNAs

Bingbing Ren,[1,2,3] Min-Xin Guan,[4,5] Tianhua Zhou,[2,6,7] Xiujun Cai,[2,8,9,10,11] and Ge Shan[1,2,3,12,*]

Mitochondria, organelles that harbor their own circular genomes, are critical for energy production and homeostasis maintenance in eukaryotic cells. Recent studies discovered hundreds of mitochondria-encoded noncoding RNAs (mt-ncRNAs), including novel subtypes of mitochondria-encoded circular RNAs (mecciRNAs) and mitochondria-encoded double-stranded RNAs (mt-dsRNAs). Here, we discuss the emerging field of mt-ncRNAs by reviewing their expression patterns, biogenesis, metabolism, regulatory roles, and functional mechanisms. Many mt-ncRNAs have regulatory roles in cellular physiology, and some are associated with, or even act as, causal factors in human diseases. We also highlight developments in technologies and methodologies and further insights into future perspectives and challenges in studying these noncoding RNAs, as well as their potential biomedical applications.

Highlights

The mitochondrial genome encodes hundreds of noncoding RNAs (mt-ncRNAs). Mt-rRNAs, mt-tRNAs, mt-lncRNAs, mitosRNAs, along with recently identified mecciRNAs and mt-dsRNAs, are major species of mt-ncRNAs.

A variety of mt-ncRNAs have been shown to have critical roles in physiology and pathology, through diverse regulatory mechanisms. Mutations in mt-tRNAs are known genetic causes of several human hereditary diseases. Members of other mt-ncRNA species also participate in key regulatory events and disorders, such as metabolic diseases and cancers.

Major advancements have been made in the toolbox for studying mitochondrial biology. Technologies such as those that allow mitochondrial genome editing will continue to drive further understandings of mt-ncRNAs in terms of their biogenesis, metabolism, functions, and functional mechanisms.

Mitochondria transcription and transcript processing

Mitochondria are at the center of energy production and cellular homeostasis maintenance in eukaryotic cells. These organelles have retained their own genome (mitochondrial DNA, mtDNA) throughout evolution. Human mtDNA was the first genome to be completely sequenced and is a 16.5-kb circular dsDNA [1]. Different from the nuclear genome, the human mitochondrial genome is highly condensed, with a total of 13 proteins, 22 tRNAs, and two rRNAs as classic products of mitochondrial gene expression. The only noncoding region in human mtDNA is 1.1 kb in length, and contains a distinct DNA loop structure known as the displacement loop (D-loop) [2] (Figure 1).

The two DNA strands of mtDNA are known as the heavy (H) and light (L) strands, based on their buoyancy in CsCl density gradients. Both strands are transcribed into long **polycistronic transcripts** (see Glossary) by POLRMT, a DNA-dependent RNA polymerase, which shows structural similarity to RNA polymerases in T7 bacteriophages [2,3]. Two heavy-strand promoters (HSP1 and HSP2) and a light-strand promoter (LSP) are all located in the D-loop. HSP1 and HSP2 drive transcription that gives rise to 12 mRNAs, two rRNAs, and 22 tRNAs [4]. By contrast, LSP1, which controls the expression of ND6 protein and eight tRNAs, promotes mtDNA transcription in the opposite direction [5] (Figure 1).

For their evolution into mature mRNAs, tRNAs, and rRNAs, the long primary transcripts need to undergo endonucleolytic excision by **RNase P** and **RNase Z** [6,7]. According to the commonly acknowledged 'tRNA Punctuation Model', mitochondrial rRNAs (mt-rRNAs) and the majority of the protein-coding sequences (mt-mRNAs) are separated by mitochondrial tRNAs (mt-tRNAs); therefore, excision of mt-tRNAs from the long primary transcripts also releases rRNAs and mRNAs [8]. However, not all mt-mRNAs are flanked by tRNAs. The discovery of various types of mt-ncRNA over the past few years also suggests that the tRNA Punctuation Model cannot fully explain the cleavage events on mitochondrial primary transcripts or the biogenesis of all mitochondria-encoded RNAs.

[1]Department of Pulmonary and Critical Care Medicine, Regional Medical Center for National Institute of Respiratory Disease, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
[2]Cancer Center, Zhejiang University, Hangzhou 310058, China
[3]Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
[4]Division of Medical Genetics and Genomics, The Children's Hospital, School of Medicine, Zhejiang University, Hangzhou 310052, China
[5]Zhejiang Provincial Key Lab of Genetic and Developmental Disorder, Institute of Genetics, School of Medicine, Zhejiang University, Hangzhou 310058, China

With the help of RNA-sequencing (RNA-seq) technologies and bioinformatics methods, novel mt-ncRNAs are constantly being discovered, ranging from long noncoding RNAs (lncRNAs) and small noncoding RNAs (sncRNAs) to recently identified circular RNAs (circRNAs) and dsRNAs [9–12]. Increasing evidence suggests that these mt-ncRNAs have important roles in human cells, and perturbations in their expression are associated with mitochondrial dysfunction and a series of diseases, such as mitochondrial myopathies, cardiovascular diseases, and cancers [13–16].

Mitochondria-encoded lncRNAs

lncRNAs are defined as nonprotein-coding transcripts that are >200 nucleotides in length. According to the recent GENCODE release 41, in total 19 095 lncRNAs have been annotated in human cells [17]. Many of these lncRNAs have indispensable roles in development, metabolism, aging, and reproduction, among others. For instance, lncRNA Xist is probably the most well-established trigger and master regulator of X-chromosome inactivation, which is crucial for female embryonic development [18]. hTERC, the RNA component of human telomerase, is essential for maintaining telomere length and controlling cell aging [19]. 5S-OT, a lncRNA relatively conserved in eukaryotes, has evolved a novel function to modulate alternative splicing due to the insertion of a primate-specific mobile element in humans [20]. More information about the physiological functions and molecular mechanisms of these nuclear-encoded lncRNAs can be found in recent reviews [21,22].

The first mitochondria-encoded lncRNA (mt-lncRNA) in human cells, named sense noncoding mitochondrial RNA (SncmtRNA), was discovered in 2007 [23]. It comprises an 815-nuleotide inverted repeat (IR) contiguous to the 16S mt-rRNA (Figure 1). SncmtRNA and two antisense transcripts identified later (ASncmtRNA-1 and ASncmtRNA-2) are widely expressed in proliferating cells and ubiquitously localized in the nucleus [14]. In accordance with the observation that ASncmtRNAs are universally downregulated in 17 types of cancer cells examined, knocking down ASncmtRNAs with Andes-1537, a chemically modified **antisense oligonucleotide (ASO)**, specifically induced apoptotic cell death in a series of human cancer cell lines without affecting healthy cells [24]. This effect was mediated by the activation of procaspase-3 and downregulation of the key cell cycle control proteins cyclin B1, cyclin D1, CDK1, CDK4, and survivin [24]. A Phase 1a clinical trial on Andes-1537 (ClinicalTrials.gov Identifier: NCT02508441), completed in 2018, tested a dosage range of 100–800 mg in 22 patients with advanced unresectable solid tumors, determining a maximum tolerated biweekly subcutaneously injected dose of 600 mg. For one patient with pancreatic cancer and one with cholangiocarcinoma, Andes-1537 showed an efficacy signal even at a dosage of 200 mg and stabilized the disease beyond 6 months [25]. A follow-up and ongoing clinical trial (Clinical Trials.gov Identifier: NCT03985072) was launched in 2019 to test Andes-1537 in patients with one of the five specific types of advanced solid tumor (gall bladder cancer, cervical cancer, gastric cancer, pancreatic cancer, and colorectal cancer).

By analyzing the strand-specific deep sequencing data sets from HeLa cells, three more mt-lncRNAs (lncCytb, lncND5, and lncND6) have been identified [9]. These lncRNAs are expressed in comparable abundance to their complementary mRNAs and are specifically enriched in mitochondria [9,10]. The processing of their 5′ and 3′ ends requires the participation of mitochondrial RNase P protein 1 (MRPP1) [9]. It is proposed that these lncRNAs form intermolecular duplexes with their complementary mRNAs and regulate mRNA expression, although only one *in vitro* RNase I^f resistance assay, in which the endoribonuclease RNase I^f cleaves single-stranded RNAs but not dsRNAs, has been used to support the potential existence of certain lncRNA–mRNA duplexes in mitochondria [9] (Table 1).

[6]Department of Cell Biology and Department of Gastroenterology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
[7]Institute of Gastroenterology, Zhejiang University, Hangzhou 310016, China
[8]Zhejiang Provincial Key Laboratory of Laparoscopic Technology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
[9]Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
[10]Zhejiang Minimal Invasive Diagnosis and Treatment Technology Research Center of Severe Hepatobiliary Disease, Zhejiang University, Hangzhou 310016, China
[11]Zhejiang Research and Development Engineering Laboratory of Minimally Invasive Technology and Equipment, Zhejiang University, Hangzhou 310016, China
[12]Department of Clinical Laboratory, The First Affiliated Hospital of USTC, the CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Basic Medical Sciences, Division of Life Science and Medicine, University of Science and Technology of China, Hefei 230027, China

*Correspondence:
shange@zju.edu.cn (G. Shan).

Cell- and tissue-specificity expression patterns of mt-lncRNAs imply that they are subject to tight regulation, and might also conduct corresponding functions in particular cells (Table 1). A striking example is that more than 70% of lncRNAs in human left ventricle are mt-lncRNAs, suggesting their importance in the heart [26]. Among them, long intergenic noncoding RNA predicting cardiac remodeling (LIPCAR) is a powerful biomarker for predicting cardiac remodeling and heart failure outcome [15,27] (Figure 2 and Table 1). LIPCAR is highly expressed and readily detectable in plasma samples, and thus, has potential value in clinical diagnostics [15,27]. However, its physiological functions are poorly understood. A recent study reported that the LIPCAR expression level in atrial fibrillation was closely associated with TGF-β1 and Smad2/3 phosphorylation, suggesting that it regulates atrial fibrosis via the TGF-β/Smad pathway [28].

New sequencing technologies and analysis methods have allowed the identification of more mt-lncRNAs [29,30]. Mitochondrial D-loop 1 (MDL1) and mitochondrial D-loop 1 antisense (MDL1AS), two transcripts that cover the full length of the D-loop and $tRNA^{Pro}$ coding region, were identified by PacBio full-length transcriptome sequencing and confirmed by PAN RNA-seq analysis (Box 1) [29,30]. Similar lncRNAs originating from D-loop regions are being ubiquitously identified in rats, mice, and insects [30,31]. The expression level of MDL1AS is much lower than that of MDL1 [30]. MDL1 and MDL1AS are proposed as long transcription initial RNAs (ltiRNAs), which give rise to mitochondria transcription initiation RNAs (tiRNAs), which are discussed further below. Other than being considered as precursors of tiRNAs, so far no experimental evidence has been offered to show a direct role of ltiRNAs in regulating mitochondrial transcription [30].

By contrast, 7S RNA, another lncRNA that originated from the D-loop region, recently came into focus for modulating mitochondrial transcription [32]. It is transcribed from an ~180-bp region between LSP and a conserved sequence element named conserved sequence block I (CSBI) (Figure 1) [33]. Distinct from other transcripts derived from the D-loop region, 7S RNA is polyadenylated at its 3′ end [34]. Since its initial identification during the 1970s, 7S RNA has been detected in humans and other vertebrates [33,35]. Although having been known for decades, the physiological function of 7S RNA was not uncovered until recently [32]. Using a range of experiments, including cryo-electron microscopy (cryo-EM) imaging of reconstructed complexes, Zhu *et al.* demonstrated that 7S RNA directly targets POLRMT to induce homodimerization and block the promoter recognition and catalytic domains of this protein from transcription initiation, thereby inhibiting mitochondrial transcription [32]. In isolated mitochondria, the 7S RNA expression level is increased by high pH or high ATP concentration in the incubation buffer [36]. 7S RNA levels are also upregulated in skin fibroblasts from patients with chronic progressive external ophthalmoplegia (CPEO) with disease-causing RNase H1 mutations [37]. Thus, it appears that 7S RNA is responsive to cellular metabolic changes and pathological conditions. Further functional studies of 7S RNA may provide new insights for understanding how mitochondrial transcription is fine-tuned to meet cellular metabolic needs [34].

Mitochondria-encoded sncRNAs

sncRNAs are RNAs shorter than 200 nucleotides in length. Distinct types of sncRNA have been identified based on their size, biogenesis, and functional mechanism. Similar to its nuclear counterpart, the mitochondrial genome encodes diverse kinds of sncRNA (mitosRNAs), including PIWI-interacting RNAs (piRNAs), miRNAs, and other types of sncRNAs derived from tRNAs, rRNAs, or lncRNAs. For example, tiRNAs, likely generated from ltiRNAs, and mitochondrial tRNA fragments (mt-tRFs), are cleavage products of mt-tRNAs [30,38]. In 2013, a systematical sequencing study of mitochondria from a variety of human and murine tissues revealed a total of 1499 and 2540 mitosRNAs in mouse and human, respectively [39]. Of these, 1285 murine

Glossary

Antisense oligonucleotide (ASO): short, synthetic, chemically modified nucleotides that target transcripts of interest; usually single-stranded and complementary to the target sequence.
Bax–Bak pore: Bax and Bak are both Bcl-2 protein family members. They can form hetero- and homo-oligomeric pores in the outer mitochondrial membrane to initiate irreversible mitochondrial outer membrane permeabilization (MOMP). Proapoptotic factors, such as cytochrome c, transfer to the cytoplasm through the Bax–Bak pore.
Genetic heterogeneity: phenomenon whereby a single phenotype or disorder is caused by multiple alleles or non-allele mutations.
Mitochondrial permeability transition pore (mPTP): nonspecific channel across the inner mitochondrial and outer mitochondrial membranes that allows the passage of molecules <1.5 kD, such as protons and ions.
Nuclear mitochondrial DNA segments (NUMTs): nuclear DNA sequences that are homologous to the mitochondrial genome. They were proposed to have migrated from the mitochondrial to the nuclear genome.
Pleiotropy: phenomenon whereby a single gene influences two or more distinct phenotypic traits.
Polycistronic transcript: single RNA transcripts that encode two or more proteins or peptides.
Polynucleotide phosphorylase (PNPase): 3′-to-5′ exoribonuclease that also has 3′-polyA polymerase activity. Canonically, PNPase is known to regulate the import of nuclear-encoded RNAs into mitochondrial matrix.
RNase P and RNase Z: endonucleases responsible for processing the 5′ and 3′ ends of precursor tRNAs to generate mature tRNAs.
TOM40: main component of the outer mitochondrial membrane translocase holocomplex. It is embedded into mitochondrial outer membranes and is conventionally required for mitochondrial protein import.

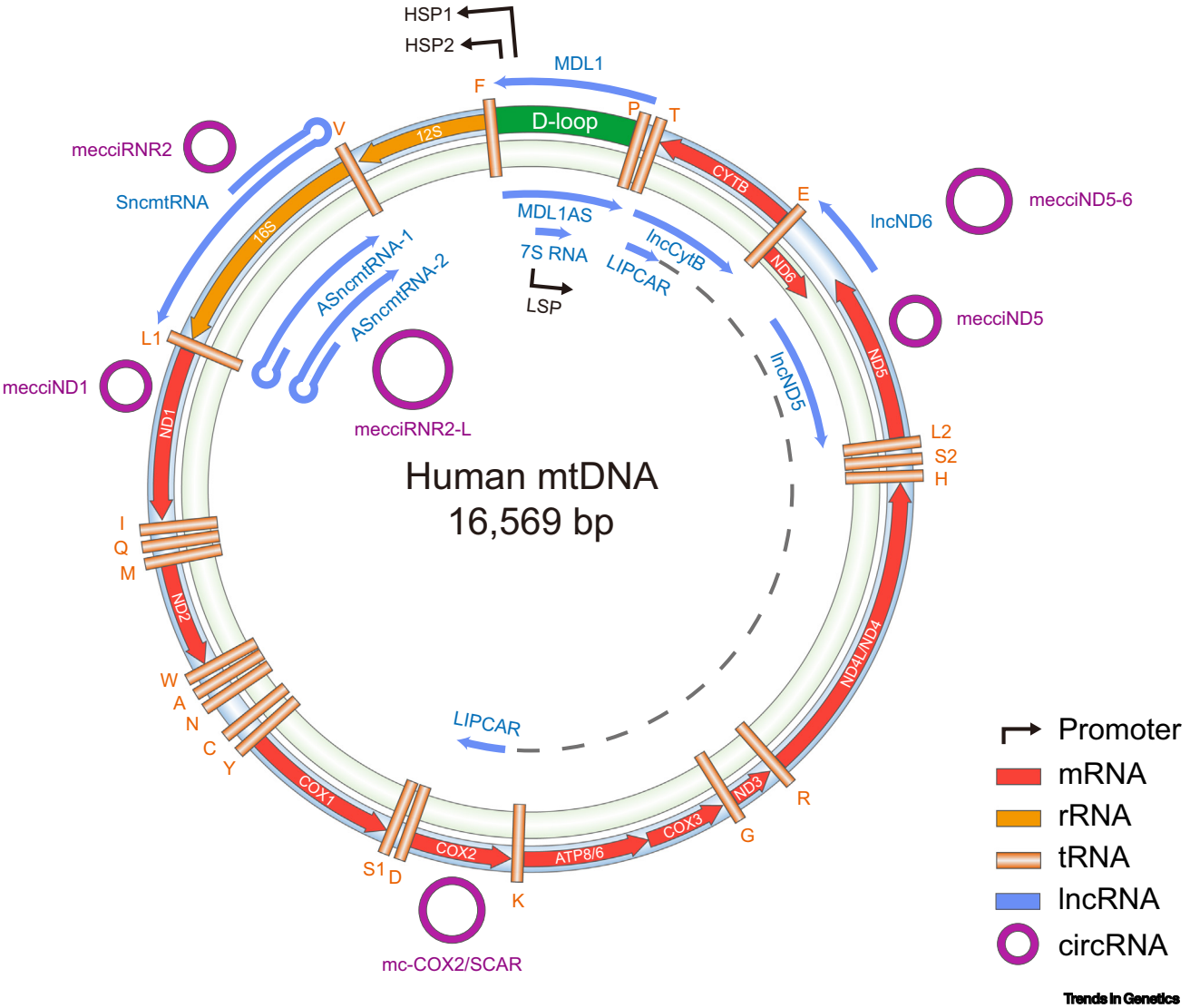

Figure 1. Schematic of the human mitochondrial genome and the mt-ncRNAs. The outer and inner tracks represent heavy (H) and light (L) strands, respectively. Abbreviations: HSP, heavy-strand promoter; LSP, light-strand promoter.

mitosRNAs and 2110 human mitosRNAs were derived from H-strand transcripts, and the remaining mitosRNAs were derived from L-strand transcripts [39]. The major sizes of mitosRNAs range from 30 to 39 nucleotides in mice and from 20 to 29 nucleotides in humans; mitosRNAs also display distinct tissue-specific expression patterns and sequence features in both humans and mice [39].

The nuclear tiRNAs, with lengths of 18–25 nucleotides, are derived from regions adjacent to transcription start sites, and are intrinsically linked to transcription initiation [40,41]. In mitochondria, it is suspected that MDL1AS and MDL1 serve as ltiRNAs to give rise to tiRNAs [30]. The expression level of the tiRNA hsa-tir-MDL1AS associates with hepatocellular carcinoma (HCC), although details of the biogenesis and functions of tiRNAs are not yet understood [30].

Table 1. Mitochondria-encoded lncRNAs and circRNAs[a]

lncRNA/circRNA	Distribution	Tissue specificity	Function	Clinical potential	Refs
SncmtRNA	Ubiquitous	Highly expressed in proliferating cells, but not in resting cells	Cell cycle regulation		[15–17,70]
ASncmtRNA	Ubiquitous	Downregulated in 17 types of cancer cells		Andes-1537 in clinical trials for gallbladder cancer, cervical cancer, gastric cancer, etc.	
lncCytb	Mitochondria	Abundant in ovary	Stabilize mRNAs by forming RNA–RNA duplexes		[9]
lncND5	Mitochondria	Abundant in testes, ovary, and skeletal muscle			
lncND6	Mitochondria	Abundant in spleen, testes, and thymus			
LIPCAR	Plasma	Highly expressed in heart	Regulate atrial fibrosis via TGF-β/Smad pathway	Potential diagnostic biomarker for cardiac remodeling	[19]
MDL1	Mitochondria		Suspected precursors of tiRNAs		[22]
MDL1AS	Mitochondria				
7S RNA	Mitochondria		Transcription initiation		[32]
mecciND1	Mitochondria, cytoplasm		Mediates mitochondrial entry of proteins	Upregulated in HCC cells	[11]
mecciND5	Mitochondria, cytoplasm		Mediates mitochondrial entry of proteins		
mc-COX2/SCAR	Mitochondria, exosome		Inhibit ROS output	Highly expressed in plasma exosomes of patients with CLL Downregulated in NASH fibroblasts	[42,43]

[a]Abbreviations: CLL, chronic lymphocytic leukemia; HCC, hepatocellular carcinoma; NASH, nonalcoholic steatohepatitis; ROS, reactive oxygen species.

tRFs are sncRNAs derived from tRNA precursors or mature tRNAs, and depending on the incision positions in the parental tRNAs, can be further classified into five subtypes: 5′-tRF, 3′-tRF, i-tRF, 5′-tRH (tRNA half), and 3′-tRH [42]. Nuclear-encoded tRFs have been reported to have regulatory roles, such as regulating gene expression through miRNA-like mechanisms, binding to RNA-binding proteins, and promoting ribosome biogenesis or interfering with translation initiation [42,43]. However, although the existence of mt-tRFs was recently described, their exact biogenesis steps and physiological roles remain largely unexplored [16,44].

piRNAs are sncRNAs that are processed and bound to PIWI proteins, which are usually 26–31 nucleotides long [45]. Using small RNA-seq data sets, Kwon *et al.* identified 29 piRNAs (mito-piRNAs) that are perfectly aligned with human mtDNA, although the identification of these sncRNAs as piRNAs was based on their size only, rather than on their PIWI-interacting or functional roles [46]. Of those, 12 mapped to tRNA-coding regions, 14 mapped to rRNA-coding regions, and the other three mapped to the coding sequences of COX2, ND4L, and ND5 [46]. Noticeably, some of these piRNAs are present not only in mitochondria, but also in the nucleus and cytosol [45]. A later *in silico* study showed that mito-piRNAs are the most abundant mitosRNAs in mouse male germ cells, gametes, and zygotes [47]. However, the biogenesis, functions, and corresponding regulatory mechanisms of mito-piRNAs are yet to be revealed.

Historically, the term ‘mitochondrial miRNA’ (mitomiRs) referred to miRNAs located inside mitochondria, regardless of their genome origins [48]. The existence of **nuclear mitochondrial DNA segments (NUMTs)** has cast difficulties in determining the nuclear or mitochondrial genomic origin of mitomiRs [49]. Currently, the mainstream view is that most mitomiRs originate from

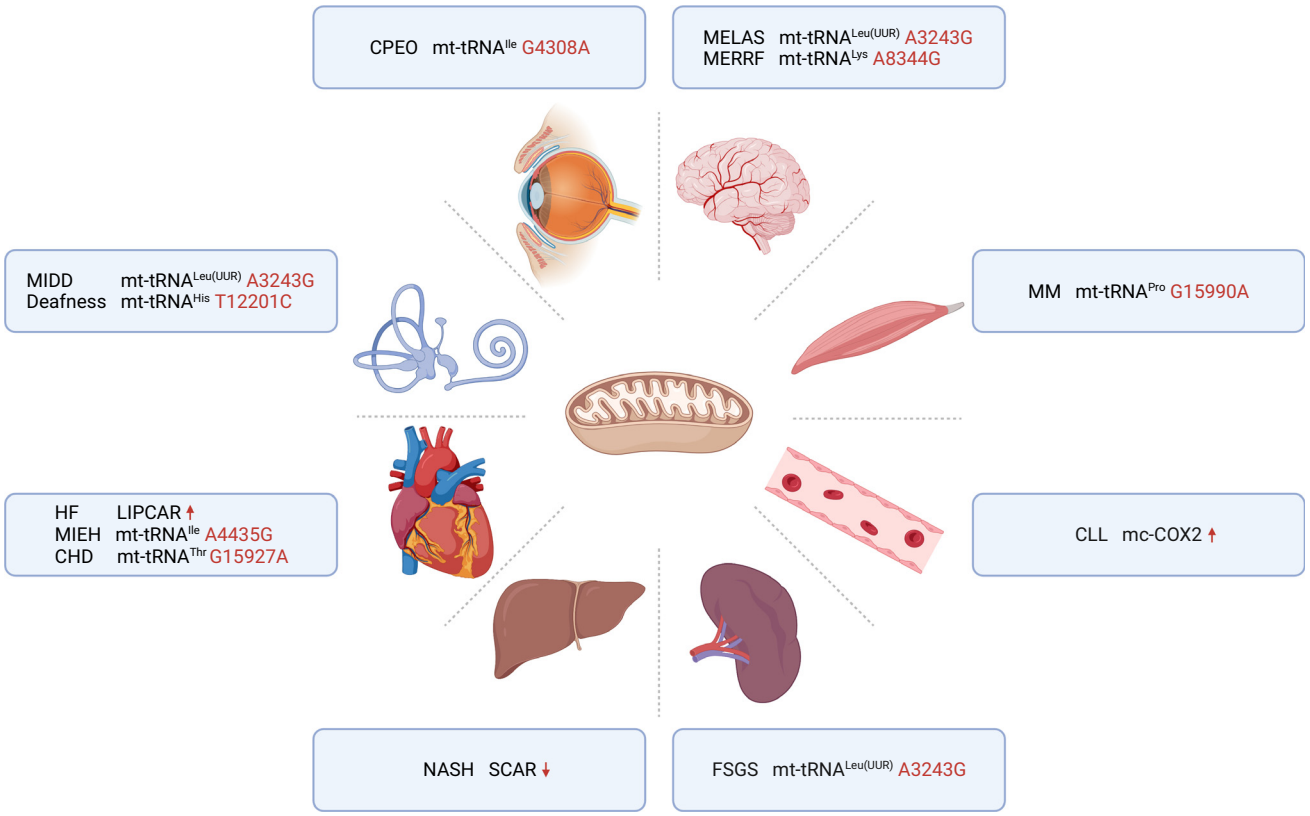

Figure 2. Mitochondria-encoded noncoding RNAs (mt-ncRNAs) with strong disease associations. Upregulation, downregulation, and mutation of representative pathogenic mt-ncRNAs are highlighted in red. Abbreviations; CHD, coronary heart disease; CLL, chronic lymphocytic leukemia; CPEO, chronic progressive external ophthalmoplegia; FSGS, focal and segmental glomerulosclerosis; HF, heart failure; MELAS, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibers; MIDD, maternally inherited diabetes and deafness; MIEH, maternally inherited essential hypertension; MM, mitochondrial myopathy; NASH, nonalcoholic steatohepatitis.

the nucleus, while only a small portion map exclusively to the mtDNA, and thus can be named as 'mitochondria-encoded miRNAs' (mt-miRs) [48]. For example, nucleus-derived miR-1 efficiently enters the mitochondria [50], whereas hsa-miR-4461 and has-miR-4463 are aligned to mitochondrial *ND4* and *ND5* genes, respectively [48]. Further studies are required to reveal the biology related to mt-miRs, such as hsa-miR-4461 and hsa-miR-4463.

Since siRNAs share similar processing and functioning machinery with miRNAs, it is tempting to utilize RNA interference (RNAi) for studying gene and ncRNA functions inside mitochondria. Recently, Gao *et al.* successfully transfected exogenous siRNAs into the mitochondrial matrix and specifically silenced the targeted mitochondrial transcripts in both mouse and human cells (mitoRNAi; Box 1) [51]. Interestingly, the same researchers previously demonstrated that miR-1, when entering mitochondria, forms a complex with Ago2 to enhance rather than repress mitochondrial translation [50]. A possibility is that, in mitochondria, perfect base-paring between siRNAs and their targets directs them to mRNA degradation, whereas imperfect base-pairing between miRNAs and their targets promotes translation [51]. Perfect base-paring between siRNAs and their targets would trigger splicing activity of Ago2 [51]. GW182, a canonical functional partner of AGO2, is required for the inhibitory effect of miRNA in the cytosol [52]. However, GW182 is

Box 1. Toolbox for studying mitochondria-encoded noncoding RNAs

Identification of mt-ncRNAs

Since the production and maturation of mt-ncRNAs are not yet fully understood, short reads from RNA-seq are incompetent for the *de novo* assembly of full-length mt-ncRNAs except for mitosRNAs. Therefore, the single-molecule real-time sequencing (SMRT-seq) technology has been used to identify mt-ncRNAs. Currently, PacBio and Oxford Nanopore are the two main platforms for SMRT-seq, both of which can obtain full-length transcripts [117,118]. The PacBio platform performs better on error rates (99.8% accuracy with an average read length of 13.5 kb), whereas the Oxford Nanopore platform can produce longer reads [119].

Manipulation of mitochondrial transcript levels

MitoRNAi is an approach that enables perturbation of mtDNA-encoded transcripts using siRNAs and short hairpin RNAs (shRNAs) [51]. Although mechanisms underlying the mitochondrial entrance of siRNA are unknown, this approach has been shown to effectively knockdown specific mitochondrial transcripts [51].

Mito-NP is a nanoparticle-based delivery system for mitochondria-specific gene delivery [56]. An amphiphilic peptide, TACP, is responsible for the mitochondrial targeting of this system [56].

Mitochondrial genome editing

MtZFN is an engineered zinc finger nuclease [68]. It contains a Cys2His2 zinc finger protein (ZFP) domain, which can be engineered to bind specific DNA sequences, and a C-terminal catalytic domain of FokI, which is a dimeric restriction enzyme [68]. Two modules of specifically designed ZFPs can align two FokIs into a specific site of the mtDNA, where dimerization is required for their DNA cleavage activity [68]. Usually, a nuclear export signal peptide and a mitochondrial targeting sequence (MTS) are also needed to ensure mitochondrial targeting of the enzymes [68,70].

Similar to mtZFN, MtTALEN is another engineered nuclease that selectively cleaves mtDNAs [69]. Its major difference from mtZFN is that, instead of ZFP, it uses a DNA-binding domain from transcription activator-like effector (TALE) to convey sequence specificity [69]. TALE is a protein originating from *Xanthomonas*, which contains tandem repeat modules in its DNA-binding domain [120]. Each repeat can recognize one DNA base pair; therefore, ideally mtTALEN can be designed to target any sequence [120].

Both the DdCBE and TALED editing systems are also based on TALE [74,75]. In DdCBE, FoKI is replaced by a split-DddA. DddA is a dsDNA cytidine deaminase, which can catalyze the C-to-T conversion without generating double-strand breaks (DSBs) on DNA [74]. By contrast, in TALED, an adenosine deaminase, TadA8e, is used to enable A-to-G conversion. Since TadA8e only works on single-stranded DNA, a catalytically impaired DddA is still kept in TALED to temporarily unwind dsDNA for TadA8e to work [75].

In summary, mtZFN and mtTALEN can be designed to cleave mtDNA and generate DSBs at specific sites. Due to the lack of a DNA damage repair system for DSB in mitochondria, the whole mtDNA would then be removed. By contrast, DdCBE and TALED enable precise editing of mtDNA, allowing single-nucleotide C-to-T and A-to-G conversions, respectively.

not present in mitochondria, which may be the reason for the translation-promoting effect of miR-1 in this organelle [50]. The development of mitoRNAi provides a promising tool for manipulating RNA expression inside mitochondria, although clearer mechanisms of mitoRNAi and successful applications in more scenarios are required for its further application.

Mitochondria-encoded circRNAs

CircRNAs are defined as covalently closed single-stranded ncRNAs, with a wide range of lengths, from less than 50 nucleotides to more than several kilonucleotides [53]. They are pervasively present in eukaryotes and act as indispensable regulators of various cellular events [53]. Although mitochondria of plants and lower eukaryotes have been reported to produce circular transcripts from splicing in mitochondria, circRNAs originated from animal mitochondrial genome were only discovered very recently [11]. In 2020, Liu *et al.* reported the identification of hundreds of circRNAs originating from human and murine mitochondria, naming them mecciRNAs [11]. This finding has since been validated in mouse brain cells by other researchers through nanopore sequencing (Box 1) [54].

MecciRNAs not only reside in mitochondria, but are also distributed in the cytosol and even extracellularly [11,55]. Several pioneer studies demonstrated important physiological functions of this novel type of circRNAs and their roles in pathogenesis (Figure 3). MecciND1 and mecciND5 are two mecciRNAs derived from the H-strand transcript, and map to the *ND1* and *ND5* coding regions, respectively (Figure 1). MecciND1 and mecciND5 have been proposed as molecular chaperones that selectively facilitate the mitochondrial entry of specific proteins [11]. The expression level of mecciND1 increases under environmental stressors, such as UV and H_2O_2 exposure, and is positively associated with mtDNA copy number; by contrast, mecciND5 binds to hnRNPA proteins and promotes their mitochondrial importation (Figure 3). *In vitro* assays have shown that mecciND1 and mecciND5, but not linear RNAs with the same sequences, can be imported into, and exported from, isolated mitochondria [11]. Moreover, both mecciRNAs show physical interactions with **TOM40** and **PNPase**, which are key mediators for mitochondrial protein and RNA importation [11]. It is proposed that both mecciRNAs facilitate mitochondrial localization of newly synthesized polypeptides [11].

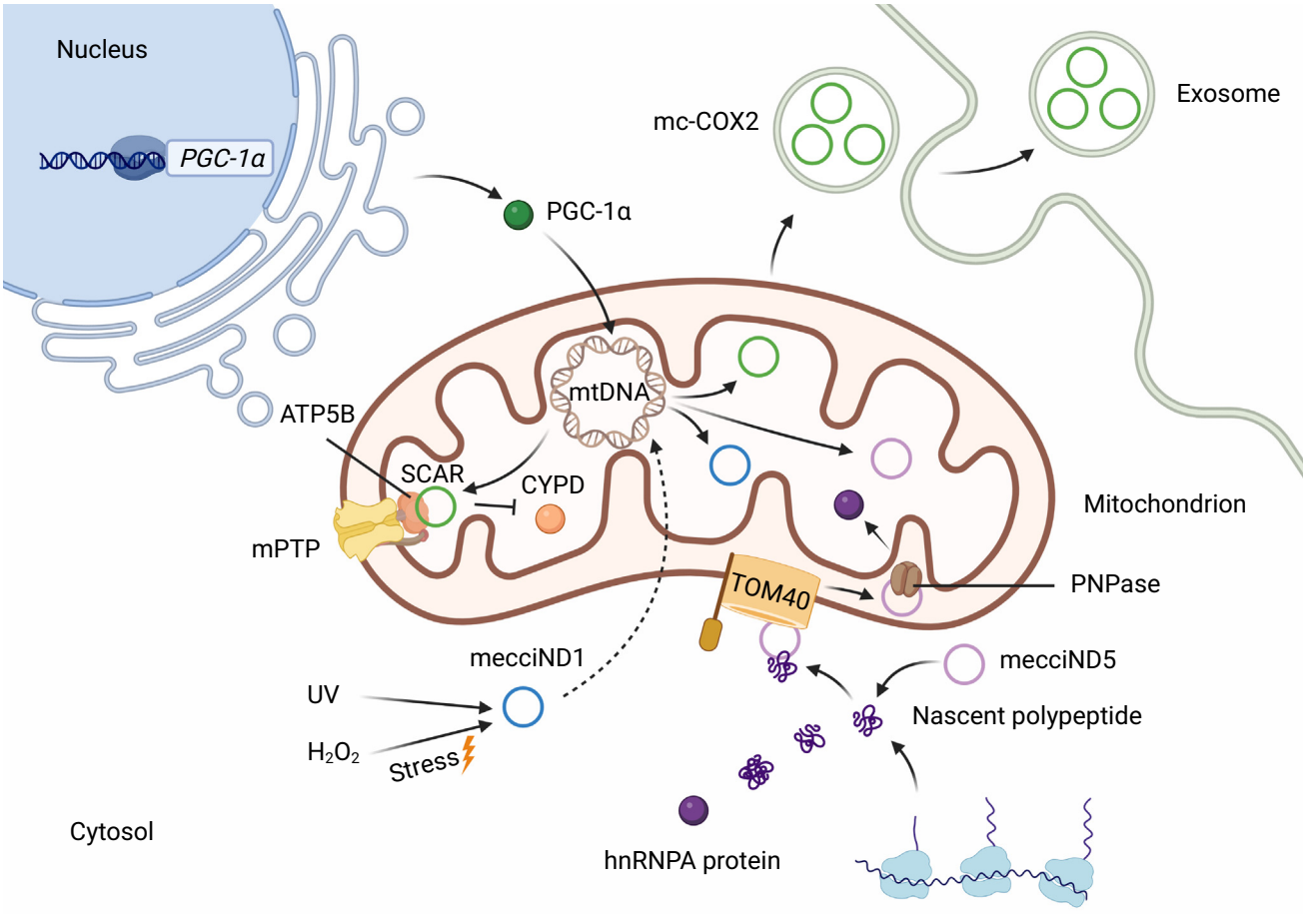

Figure 3. Proposed functional mechanisms of mecciRNAs. MecciND1 expression increases under the stimulus of UV or H_2O_2, affecting mtDNA copy number. MecciND5 interacts with nascent polypeptides and facilitates mitochondrial importation of hnRNPA proteins through the TOM40 complex. SCAR, the expression of which is mediated by PGC-1α, binds to ATP5B and shuts down mPTP by blocking the CypD–mPTP interaction. In CLL cells, mc-COX2 is delivered to the plasma through exosomes.

Another mecciRNA, mc-COX2, also known as steatohepatitis-associated circRNA ATP5B regulator (SCAR), was found to affect the progression of chronic lymphocytic leukemia (CLL) and nonalcoholic steatohepatitis (NASH) [55,56]. SCAR/mc-COX2 is produced from the *COX2* locus on the L-strand (Figure 1). This mecciRNA occurs at high levels in the plasma exosomes of patients with CLL, and its expression level is highly associated with the progression and prognosis of this disease (Figure 2) [55]. SCAR is one of several mecciRNAs significantly downregulated in primary fibroblasts isolated from patients with NASH [56]. It directly binds to ATP5B to shut down the **mitochondrial permeability transition pore (mPTP)** in mitochondria and then inhibits the output of reactive oxygen species (ROS) (Figure 3). The expression levels of SCAR strongly correlate with steatosis-to-NASH progression in patients (Figure 2). By mitochondrial-specific delivery of a SCAR-expression plasmid using the mito-NP nanoparticle (Box 1), alleviation of metaflammation was observed in a mouse model of NASH, suggesting the potential therapeutic value of SCAR [56]. The mito-NP nanoparticle, if vigorously verified for efficient mitochondria-specific delivery in future studies, could provide a unique tool for basic research or even biomedical applications.

MecciRNAs may have other, as-yet unknown, functions, and we cannot rule out the possibility that some mecciRNAs are in fact coding rather than noncoding. In mitochondrial gene expression, the generation of linear transcripts and mecciRNAs has to be finely controlled, and little is known about the corresponding mechanism. Details of the biogenesis, turnover, and regulation of mecciRNAs remain elusive. The lack of introns and linear splicing events in the mitochondria of higher eukaryotes suggests that mecciRNAs are synthesized through an alternative mechanism independent of splicing [10,11]. In fact, some mecciRNAs are derived from two neighboring mitochondrial coding genes [11,54] (Figure 1). Besides their biogenesis mechanism, factors that regulate the expression and transportation of mecciRNAs are also pending elucidation. MecciRNAs function not only inside the mitochondria, but also in the cytosol (Figure 3). By contrast, a recent study showed that circPUM1, a nuclear-encoded circRNA, enters and functions inside mitochondria, although the mitochondrial importation mechanism of this circRNA is unknown [57]. Thus, further studies are needed to conclusively dissect the mitochondrial export and import of circRNAs.

Mitochondria-encoded tRNAs

Mt-tRNAs are probably the most-studied mt-ncRNAs. They are the foundation of the mitochondria-specific decoding system. Notably, mt-tRNAs can undergo various post-transcriptional nucleotide modifications, four types of which [2-methylthio-N^6-isopentenyl adenosine (ms^2i^6A), 5-formylcytosine (f^5C), 5-taurinomethyluridine (τm^5U), and taurinomethyl-2-thiouridine (τm^5s^2U)] are mitochondria specific [58]. Accumulating evidence suggests that these mt-tRNA modifications are crucial for efficient mitochondrial translation, and deficiency in some modifications would lead to mitochondrial dysfunction. For instance, τm^5U modifications on mt-$tRNA^{Leu(UUR)}$ and mt-$tRNA^{Lys}$ are associated with mitochondrial diseases [59]. Loss of τm^5U modifications on mt-$tRNA^{Leu(UUR)}$ and mt-$tRNA^{Lys}$ impaired mitochondrial protein translation, and in patients with mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged red fibers (MERRF), the U34 position of these mt-tRNAs is hypomodified [59]. 3-Methyl-cytidine (m^3C) methylation at position C32 of the mt-$tRNA^{Ser(UCN)}$ and mt-$tRNA^{Thr}$ is catalyzed by METTL8 [60]. Balanced m^3C32 methylation is important for mitochondrial function, and a lack of METTL8 triggers ribosome stalling on mt-$tRNA^{Ser(UCN)}$- and mt-$tRNA^{Thr}$-dependent codons [60]. By contrast, a high expression level of METTL8 in pancreatic cancer cells enhances respiratory chain activity and correlates with lower patient survival [60]. A newly released study reported that 5-methylcytosine (m^5C) at position 34 in mt-$tRNA^{Met}$ is catalyzed by NSUN3, and hypomethylation of mt-$tRNA^{Met}$ downregulates mitochondrial encoded proteins and changes

mitochondria morphology [61]. Importantly, in a subpopulation of oral carcinoma cells (CD36-dependent metastasis-initiating cells), m^5C on mt-$tRNA^{Met}$ is required to activate cancer cell metastasis [61].

Hundreds of pathological mutations have been identified on mt-tRNAs, associated with a variety of human diseases (Table 2 and Figure 2). Novel pathogenic mutations are constantly being identified and updated on MITOMAP[i] and MitoMiner[ii] [62,63]. Considering the importance of mitochondrial activities in energy production, it is not surprising that more disease phenotypes are observed in high energy-consuming tissues and organs, such as nervous and muscular systems, compared with low energy-consuming tissues. For instance, MELAS is a maternally inherited neurometabolic disorder, characterized by seizure, headache, and multiple neurological symptoms. It is reported that ~80% of patients with MELAS carry the A3243G mutation on mt-$tRNA^{Leu(UUR)}$ [64]. Patients with another mitochondria disorder, MERRF, clinically present with myoclonus, generalized epilepsy, cerebellar ataxia, and dementia; MERRF is mainly caused by A8344G mt-$tRNA^{Lys}$ (Figure 2 and Table 2) [65,66].

mtDNA heteroplasmy has impeded studies of pathogenic mt-tRNA mutants and the underlying mechanisms. The coexistence of **genetic heterogeneity** and **pleiotropy** makes it challenging

Table 2. Disease-associated mt-tRNA mutations[a]

mt-tRNA	Mutation hotspot	Structural location	Associated disease	Refs
$tRNA^{Phe}$	G611A	AC loop	MERRF	[89]
$tRNA^{Leu(UUR)}$	A3243G	D loop	Deafness, diabetes, FSGS, MELAS, MIDD	[64,90–93]
	T3250C	D loop	MM	[94]
	T3291C	T loop	MELAS, MM	[94,95]
$tRNA^{Ile}$	A4263G	ACC stem	MIEH	[96]
	C4277T	D loop	MCH	[97]
	A4295G	AC loop	Deafness, MIEH	[98,99]
	G4308A	T stem	CPEO	[100]
	A4317G	T loop	Deafness	[101]
$tRNA^{Met}$	T4409C	ACC stem	EXIT	[102]
	A4435G	AC stem	Diabetes, LHON, MIEH	[103–105]
$tRNA^{Tyr}$	T5885del	T loop	CPEO	[106]
$tRNA^{Ser(UCN)}$	T7511C	ACC stem	Deafness	[107]
$tRNA^{Asp}$	A7551G	AC loop	Deafness	[108]
$tRNA^{Lys}$	A8344G	T loop	CD, MERRF	[65,109]
	T8356C	T stem	MERRF	[110]
$tRNA^{His}$	T12201C	ACC stem	Deafness	[111]
$tRNA^{Ser(AGY)}$	C12258A	ACC stem	Deafness, PR	[112]
$tRNA^{Leu(CUN)}$	A12308G	Viable loop	Stroke	[113]
$tRNA^{Thr}$	G15927A	AC stem	CHD, LHON	[114,115]
$tRNA^{Pro}$	G15990A	AC loop	MM	[116]

[a]Abbreviations: CHD, coronary heart disease; CPEO, chronic progressive external ophthalmoplegia; EXIT, exercise intolerance; FSGS, focal and segmental glomerulosclerosis; LHON, Leber's hereditary optic neuropathy; MIDD, maternally inherited diabetes and deafness; MIEH, maternally inherited essential hypertension; MM, mitochondrial myopathy; PR, pigmentary retinopathy.

to determine the genotype–phenotype relationship. For instance, despite its original identification in MELAS, the most common disease induced by A3243G mutant is maternally inherited diabetes and deafness (MIDD) [67]. In addition, focal and segmental glomerulosclerosis (FSGS), maternally inherited deafness, and diabetes were also found to be associated with this mutation (Table 2). By contrast, although $tRNA^{Lys}$ A8344G remains the major pathogenic mutant of MERRF, T8356C on the same mt-tRNA and several mutations on other mt-tRNAs were also shown to be highly associated with MERRF (Table 2).

To clarify the complicated pathogenic mechanisms of mt-tRNAs mutations and other mutations on mtDNA, genetic tools that permit the editing of mtDNA are needed. Previously, two engineered mitochondria-targeted nucleases, mitochondrial-targeted zinc finger nucleases (mtZFNs) and mitochondrial-targeted transcription activator-like effector nuclease (mtTALEN), have been used to cleave specific sites on mtDNA (Box 1) [68,69]. Despite the difference in their DNA sequence recognition modules, the dimeric restriction enzyme FokI is used to catalyze DNA cleavage in both systems. Without a DNA damage repair system inside mitochondria, the DNA double-strand breaks (DSBs) generated by FokI cannot be fixed on mtDNAs, leading to mtDNA elimination [70,71]. Using mtZFNs and mtTALENs, researchers have been able to reduce mutant mtDNA load in cellular models of MELAS and MERRF and to revert disease-related phenotypes in corresponding mouse models [70–73]. Inspiringly, the cutting-edge development of novel mtDNA-editing technologies, DddA-derived cytosine base editors (DdCBE) and transcription-activator-like effector-liked deaminases (TALEDs) now enable base editing in mtDNA (Box 1) [74,75]. These editing systems make use of the cytidine deaminase DddA and the adenosine deaminase TadA8e to mediate single-nucleotide C-to-T and A-to-G conversions, respectively, on mtDNA, without creating DSBs. However, several groups recently raised serious concerns about the substantial nuclear off-target effects induced by current mitochondrial base-editing methods [76,77]. Therefore, thorough evaluations of the off-target effects and improvements in these base-editing tools are required for their applications in basic research and perhaps eventually in the clinic.

Mitochondria-encoded dsRNAs

In 2018, Dhir *et al.* discovered mitochondria-encoded dsRNAs (mt-dsRNAs) in HeLa cells using J2 antibody, which specifically recognizes dsRNAs [12]. RNA-seq profiles showed that sequences of these mt-dsRNAs covered all parts of the H and L strands. Mt-dsRNAs are highly unstable and under the strict surveillance of the mitochondria degradosome, the components of which include SUV3 and PNPase [12,78]. Loss of either SUV3 or PNPase leads to massive accumulation of mt-dsRNAs. *Drosophila* with orthologs of SUV3 or PNPase deletions also show mt-dsRNA accumulation [79].

Notably, mt-dsRNA is able to escape into the cytoplasm in a PNPase-dependent manner, triggering a type I interferon response [12]. The release of mt-dsRNAs into cytoplasm depends on **Bax–Bak pores**, which resembles the reported release of mtDNA into the cytosol during apoptosis or mtDNA breaks [80–82]. p53 mutant mouse embryonic fibroblasts contain immune-stimulating endogenous mt-dsRNAs, which can be cleaved by RNase L [83]. Mt-dsRNAs have been postulated as a key agonist of the innate immune response, and the especially immunogenic nature of mt-dsRNAs also leads to the suspicion that their accumulation might be associated with autoimmune disorders [83].

Concluding remarks

The discovery of numerous mt-ncRNAs over the past few years has expanded our knowledge of the mitochondria transcriptome. In contrast to its small size and limited protein-coding capacity, the mitochondrial genome encodes an ncRNA profile of remarkable complexity. Although only a

Outstanding questions

How are mt-ncRNAs synthesized and what RNA metabolism pathways participate in the regulation of the biogenesis and degradation of distinct types of mt-ncRNA?

What are the factors that regulate the expression and turnover of mt-ncRNAs? Which factors determine the tissue-specific expression of certain mt-ncRNAs? How can they make active responses to intracellular and intercellular stimuli?

How do RNA molecules selectively enter or exit mitochondria, and how is this regulated? What functions do mt-ncRNAs conduct inside and outside the mitochondria, and how are these functions regulated and orchestrated?

To what extent do mt-ncRNAs participate in the pathogenesis of human diseases? What more can be done to develop mt-ncRNA-based diagnosis and treatments?

small portion has been characterized in detail, mt-ncRNAs have demonstrated diverse functions, including, but not limited to, regulating mitochondrial gene expression and metabolism. Some mt-ncRNAs (e.g., SncmtRNA and ASncmtRNAs) can act as retrograde signals to regulate nuclear gene expression, whereas others can shuttle between mitochondria and the cytoplasm as molecular chaperones (e.g., mecciND5). Several mt-ncRNAs can be detected extracellularly (e.g., LIPCAR and mc-COX2). Numerous mt-ncRNAs have been reported to associate with human diseases (Figure 2). Their diverse roles in pathogenesis make mt-ncRNAs promising biomarkers, therapeutic targets, or even therapeutic RNA drugs, although the biomedical application of these mt-ncRNAs is currently in its infancy, with specific ones, such as ASncmtRNAs, in clinical trials. Chemical modifications of mt-tRNAs and supposedly unappreciated modifications in other mt-ncRNAs require more research but could provide additional opportunities for biomedical applications. Thus, there is no doubt that the growth in our understanding of mt-ncRNAs over the past few years has resulted in a new field in ncRNA research.

Some unsolved questions are challenging the basic research and clinical application of mt-ncRNAs (see Outstanding questions). Due to the existence of NUMTs, it is difficult to determine the genome origin of some mt-ncRNAs, especially mitosRNAs. Accordingly, the biogenesis mechanisms are not clear for many mt-ncRNAs. For instance, Dicer is compulsory for the biogenesis of nuclear-encoded miRNAs. However, this protein is not found inside mitochondria [84]. In fact, it was shown that knocking out Dicer had a limited impact on some mitomiRs [10,39]. A similar conundrum also applies to mecciRNAs, in that canonical splicing machinery, which is absent in mammalian mitochondria, may not be responsible for their biogenesis.

Regarding their fundamental role in energy production, mitochondria are sensitive to environmental changes and physiological challenges. In accordance, many mt-ncRNAs have been reported to change drastically in response to cellular stresses or pathological conditions. For instance, mecciRNA SCAR is significantly downregulated in NASH fibroblasts. The change in SCAR level is mediated by PGC-1α in response to lipid-induced endoplasmic reticulum stress [56]. However, for most mt-ncRNAs, it is not yet known how they respond dynamically. Exploring the factors that regulate the expression and mediate the turnover of mt-ncRNA would improve our understanding of the physiological and pathological roles of mt-ncRNAs.

Noticeably, some mt-ncRNAs not only reside in mitochondria, but are also present in the cytosol, nucleus, or extracellular vesicles. By contrast, nuclear-encoded lncRNAs (e.g., SAMMON and RMRP) [85,86], miRNAs (e.g., miR-1) [50], circRNAs (e.g., circPUM1) [57], as well as previously discovered nuclear-encoded tRNAs, were also found to have critical roles in mitochondria [87]. In fact, a recent study on the subcellular distribution of ncRNAs has identified lots of nuclear-encoded ncRNAs inside mitochondria [88]. Despite some advances in our understanding of how RNAs enter and exit mitochondria, further research is required.

Features of mitochondria, such as being enclosed by outer and inner membranes and having their own genomes and gene expression apparatuses, have impeded studies and manipulations of mt-ncRNAs. The heteroplasmic nature of mitochondria creates an additional level of complexity. Fortunately, recent biotechnology developments have allowed the identification of previously hidden mt-ncRNAs by novel sequencing methods, such as PacBio SMRT-seq, and tools, such as DdCBE and TALED, have made it possible to edit mtDNA. Methods, such as mitoRNAi and mito-NP nanoparticles for mitochondrial delivery of plasmids, are available to manipulate levels of mitochondrial transcripts. These methods are ready for application, and could be improved further. Hopefully, such brisk growth of this mitochondrial toolbox will herald the dawn of a new era in basic and clinical research on mt-ncRNAs.

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Declaration of interests

The authors declare no conflict of interest.

Resources

[i]www.mitomap.org

[ii]https://mitominer.mrc-mbu.cam.ac.uk/

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