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Review Article

The molecular machinery for maturation of primary mtDNA transcripts

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Abstract

Human mitochondria harbour a circular, polyploid genome (mtDNA) encoding 11 messenger RNAs (mRNAs), two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs). Mitochondrial transcription produces long, polycistronic transcripts that span almost the entire length of the genome, and hence contain all three types of RNAs. The primary transcripts then undergo a number of processing and maturation steps, which constitute key regulatory points of mitochondrial gene expression. The first step of mitochondrial RNA processing consists of the separation of primary transcripts into individual, functional RNA molecules and can occur by two distinct pathways. Both are carried out by dedicated molecular machineries that substantially differ from RNA processing enzymes found elsewhere. As a result, the underlying molecular mechanisms remain poorly understood. Over the last years, genetic, biochemical and structural studies have identified key players involved in both RNA processing pathways and provided the first insights into the underlying mechanisms. Here, we review our current understanding of RNA processing in mammalian mitochondria and provide an outlook on open questions in the field.

Keywords: Mitochondria; RNA; RNA Processing; Mitochondriopathy

Introduction

The human mitochondrial genome (mtDNA) is a circular, double-stranded DNA molecule of approximately 16.5 kilobase pairs (kb). It encodes for 13 essential subunits of the oxidative phosphorylation machinery (OXPHOS) on 11 messenger RNAs (mRNAs, two are bicistronic), as well as 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs) (12S and 16S rRNA) [1–4]. Mammalian mtDNA can be separated into a "heavy" (H) and "light" (L) strand due to their different nucleotide composition. Both strands are tightly packed with intron-less genes that contain no or only short untranslated regions (UTRs). Some open reading frames overlap and require polyadenylation to complete their stop codons [1, 5]. Transcription of mtDNA is carried out by a dedicated single-subunit RNA polymerase (POLRMT), which initiates from three promoters located in a non-coding region of the genome [5–9]. Two promoters on the light (LSP1 and LSP2) and one on the heavy strand (HSP) [5–9] give rise to long, polycistronic transcripts that cover almost the entire length of the genome [2]. The primary transcripts then undergo numerous processing and maturation steps, which include cleavage, modification, and/or polyadenylation to become mature and functional tRNAs, rRNAs, or mRNAs [10]. These steps are critical for correct gene expression and mitochondrial function, and defects in this process are associated with severe mitochondriopathies and other diseases [11–14].

The very first step of RNA processing consists of cleavage of the primary transcripts, which separates mRNAs, tRNAs, and rRNAs [10]. This is carried out by dedicated molecular machineries that must recognise the individual gene junctions and catalyse their endonucleolytic cleavage. The mammalian mtDNA encodes for two types of gene junctions that are recognised by two fundamentally different RNA processing mechanisms (Fig. 1) [10, 14]. In a seminal paper, Attardi and colleagues recognized that the rRNAs and most mRNAs are interspersed by tRNAs in the human mitochondrial genome, and proposed a 'tRNA punctuation model' in which the tRNAs act as excision sites to release the individual RNAs [2]. Since then, the core molecular machinery for this 'canonical' processing mode has been identified and studied extensively. However, four gene junctions are not flanked by tRNAs, and their processing occurs through distinct mechanisms. The players and mechanisms underlying these "non-canonical" RNA processing pathways remain largely unknown.

Despite its crucial role in mitochondrial gene expression and relevance in disease, the molecular mechanisms of mitochondrial RNA processing remain poorly understood. While structural

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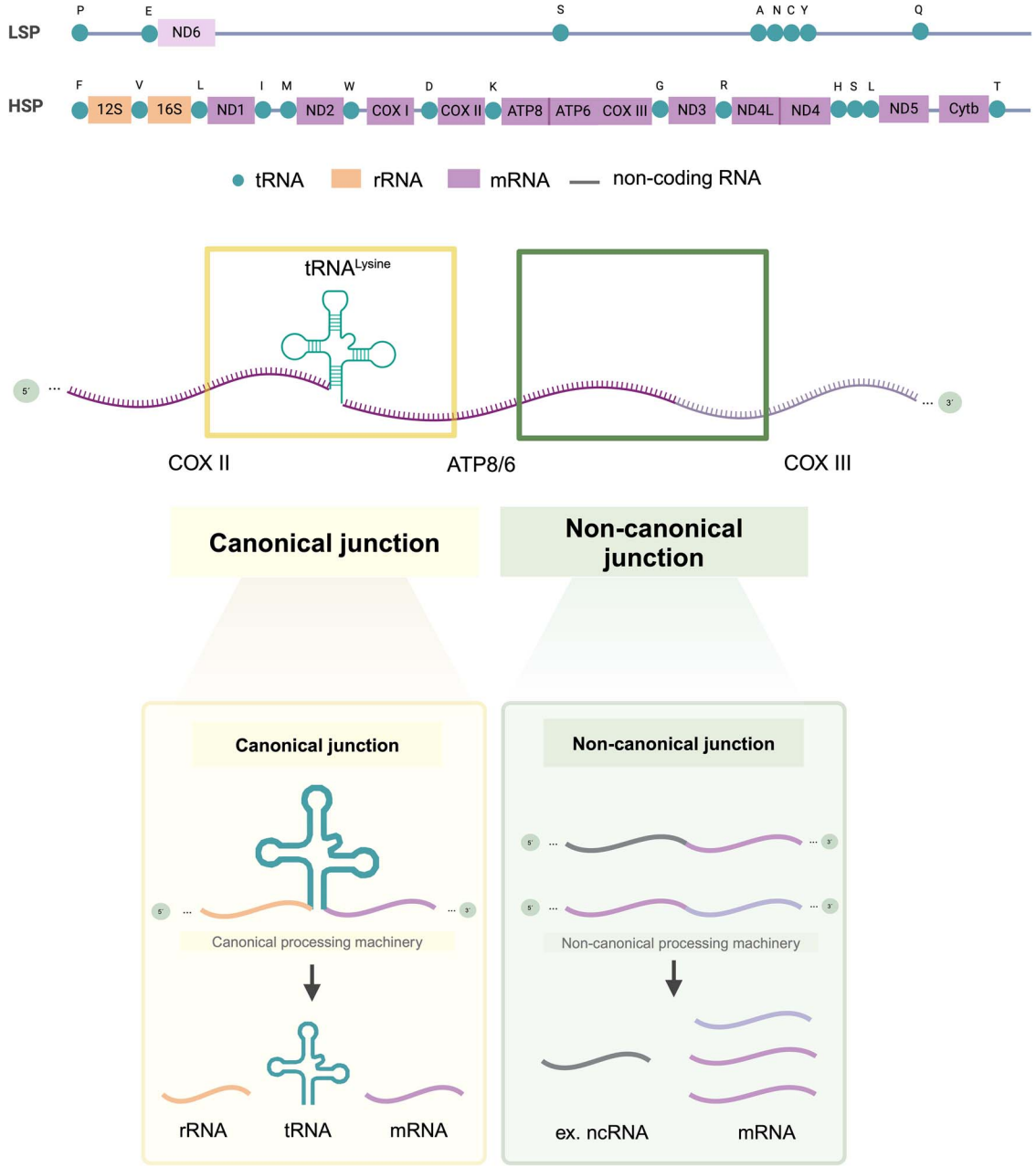

Figure 1. Overview of primary mitochondrial transcript processing. The primary transcripts from light- and heavy strand are shown schematically at the top, with the different RNA regions indicated. Two types of gene junctions exist, canonical and non-canonical, which are processed by distinct molecular machineries. Created with BioRender.com.

and biochemical studies have provided a molecular picture of transcription in human and yeast mitochondria [15–20], we lack a comparable understanding of the first steps of RNA processing. Recently, the first mechanistic insights into the molecular machinery of canonical mitochondrial RNA processing have been obtained, and several factors have been identified to be involved in non-canonical processing. This review will summarise recent advancements in our understanding of mitochondrial RNA processing and provide an outlook on open questions in the field.

Canonical RNA processing

At most gene junctions in mitochondrial RNAs, mRNAs or rRNAs are flanked by tRNAs. These junctions are processed by sequential action of two nuclease complexes, which recognize and excise the tRNA segments. The 5′ end of the tRNAs is cleaved by the mitochondrial ribonuclease P complex (mtRNase P) and the 3′ end is cleaved by the mitochondrial ribonuclease Z complex (mtRNase Z or ELAC2), thus leading to the release of the intervening segment (Fig. 2, left panel) [21–23].

Mammalian mtRNase P is unique both in its architecture and functionality. RNase P enzymes are present in all domains of life and are typically either ribozymes [24, 25], single-subunit monomeric [26, 27] or homo-multimeric enzymes [28]. In contrast, mtRNase P is a multi-subunit protein-only complex that consists of the three subunits MRPP (for mitochondrial ribonuclease protein) 1-3 [29]. First identified and reconstituted by Walter Rossmanith and colleagues [22], this complex was subsequently

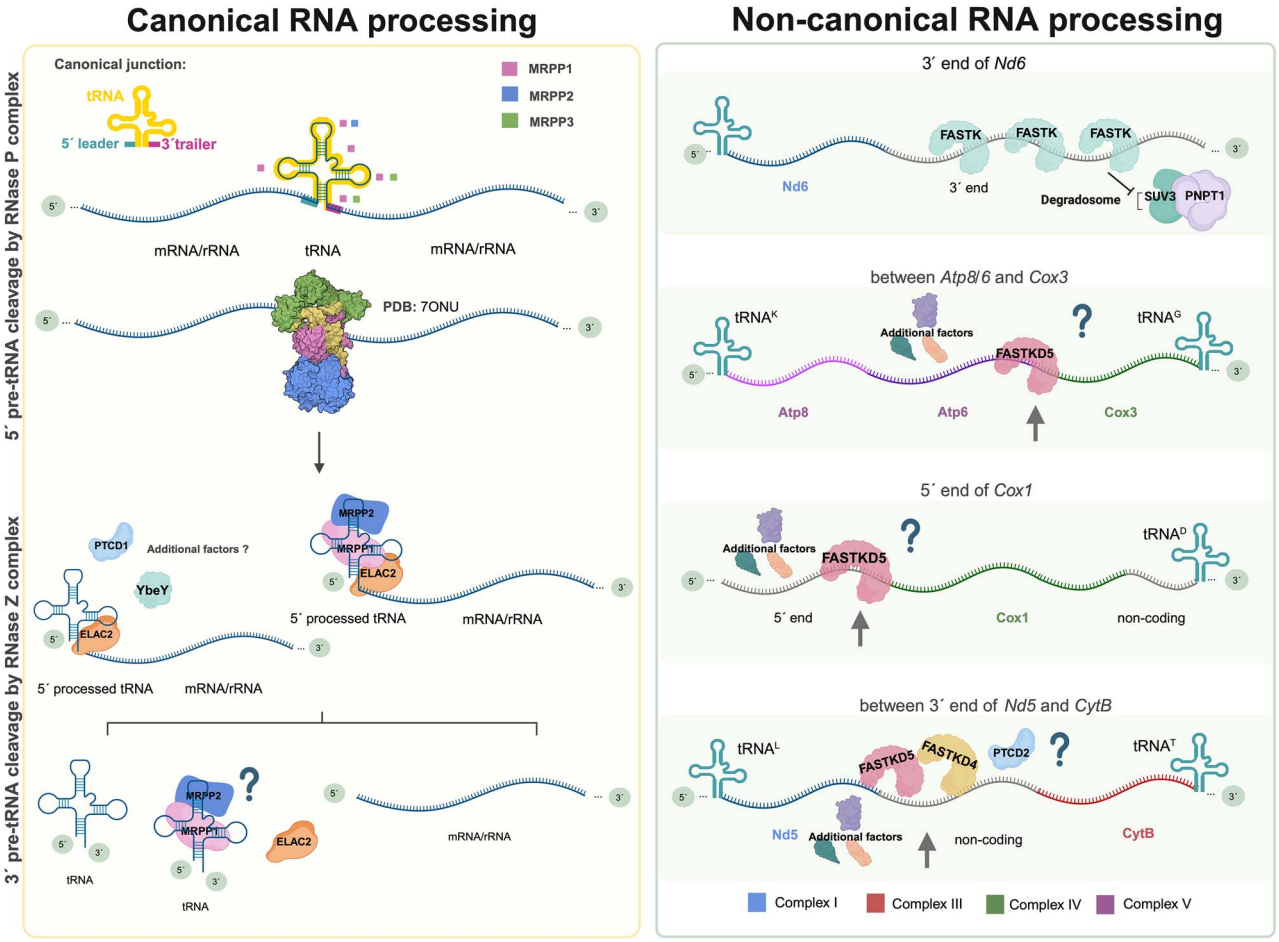

Figure 2. Details of canonical and non-canonical RNA processing in mitochondria. (left) schematic depiction of the sequential cleavage at canonical RNA junctions by mtRNase P and mtRNase Z. The structure of mtRNase P (PDB: 7ONU [33]) is depicted as surface view, while the other players, for which structural data is lacking, are shown schematically. (right) schematic depiction of the four non-canonical RNA processing sites in human mitochondria and potential players involved. Putative cleavage sites are indicated with arrows. Created with BioRender.com.

shown to not only cleave the 5′ end of mt-tRNAs, but also catalyse the conserved formation of N(1)-methylguanine (m^1G) and N(1)-methyladenine (m^1A) at position 9 in 19 out of 22 mitochondrial tRNAs [30]. This methylation reaction is carried out by MRPP1 (also known as tRNA methyltransferase 10 homolog C, TRMT10C), a S-adenosylmethionine (SAM)-dependent methyltransferase. MRPP2 (also known as SDR5C1 or hydroxysteroid 17-β dehydrogenase 10, HSD17B10), is a member of the short-chain dehydrogenases superfamily. Biochemical studies suggested that MRPP2 has a moonlighting function in RNA processing, where it acts as a non-catalytic subunit that forms a stable subcomplex with MRPP1 [30–32]. MRPP3 (also known as protein-only RNase P catalytic subunit, PRORP) is the catalytic subunit responsible for 5′ tRNA processing [29].

A recent cryo-electron microscopy (cryo-EM) structure of human mtRNase P bound to a pre-tRNA [33] revealed the architecture and mechanism of substrate recognition, methylation, and cleavage by this complex [33]. In this structure, a homotetramer of MRPP2 forms a base to which MRPP1 binds to form a stable subcomplex. This complex acts as a surface- and charge-complementary binding platform for the pre-tRNA. MRPP1 and MRPP2 interact with the pre-tRNA via the anticodon loop, which may facilitate the processing of the structurally highly variable mitochondrial tRNAs. This is consistent with biochemical studies that suggested that MRPP1 and MRPP2 form a processing platform on which several subsequent RNA processing and maturation steps can take place and explains why the methyltransferase activity of MRPP1 is dependent on MRPP2 [30, 32]. The interaction of MRPP3 with the MRPP1/MRPP2/pre-tRNA complex allows for the correct positioning and precise 5′ cleavage by MRPP3. From these studies, a detailed molecular picture of the first step of canonical RNA processing in mammalian mitochondria has emerged.

In contrast, the second step of canonical RNA processing, 3′ cleavage of the pre-tRNAs by RNase Z, is much less characterized at the molecular level. Similar to RNase P, RNase Z enzymes are ubiquitous throughout all domains of life. In humans, ELAC2 is the RNase Z responsible for tRNA 3′ processing in the nucleus and in mitochondria [34, 35]. Silencing of *ELAC2* results in the accumulation of various 3′-unprocessed mitochondrial tRNA precursors [23, 34], and pathological variants in *ELAC2* cause mitochondrial respiratory chain deficiencies, hypertrophic cardiomyopathy, lactic acidosis, and have even been proposed to contribute to the pathogenesis of prostate cancer [36–38].

Canonical processing events occur in a hierarchical fashion, where 5′ cleavage precedes 3′ cleavage both *in vivo* and in reconstituted *in vitro* systems [32, 39, 40]. Intriguingly, ELAC2 requires the presence of MRPP1 and MRPP2 for efficient cleavage of some but not all tRNAs under physiological salt conditions *in vitro* [32]. This is consistent with the suggestion that the MRPP1/MRPP2

complex serves as a processing platform [32], and suggests that mitochondrial RNase Z is likely a multi-subunit complex. No structures of ELAC2 have been reported to date, and the molecular mechanisms underlying the hierarchical 3′ processing of primary transcripts remain elusive. However, structural and biochemical studies on bacterial homologs of ELAC2 demonstrate that these enzymes recognise their substrate through interactions with the acceptor stem and the so-called elbow region of the tRNA [41]. Whether this mode of interaction is conserved in ELAC2 and what role the MRPP1/MRPP2 subcomplex might play remains to be determined.

Besides ELAC2, several additional factors have been implicated in tRNA 3′ processing. For instance, the pentatricopeptide repeat-containing protein 1 (PTCD1) has been proposed to negatively regulate mitochondrial tRNA leucine levels and affect 3′ tRNA processing [21]. Moreover, the metalloendoribonuclease YbeY was implicated in mitochondrial ribosome assembly and tRNA processing [42, 43]. However, besides their circumstantial association, how these factors are involved in canonical processing is unclear, although interactions with ELAC2 for specific tRNAs have been proposed [21, 43]. Finally, knockdown or loss-of-function of the Suv3-like RNA helicase SUPV3L1 results in the loss of mature tRNAs and the accumulation of unprocessed RNA intermediates in yeast [44–46], flies [47], human cell lines [48], and patients with mutations in SUPV3L [49]. SUPV3L and the polynucleotide phosphorylase (PNPase) form the mitochondrial RNA degradosome [50], but how SUPV3L might aid processing remains unclear. Nevertheless, data from yeast suggest that the SUPV3L ortholog SUV3 forms a stable supercomplex with yeast RNase P and Z, supporting such a role [51]. In summary, while it is clear that the second step of canonical RNA processing is mediated by ELAC2, its precise mechanism is still unknown.

Non-canonical RNA processing

In mammalian mtDNA, four gene junctions do not include a tRNA (Fig. 2, right panel): 1. between the bicistronic transcript of *Atp8/6* and *Cox3*; 2. between the 3′ untranslated region (UTR) of *Nd5* and *CytB*; 3. at the 5′ end of *Cox1*; and 4. at the 3′ end of *Nd6* [1, 52, 53]. These junctions are not defined by any obvious sequence or structural motifs and, as in the case of *Atp8/6-Cox3*, do not even involve UTRs [23, 53]. To date, neither the exact players nor the molecular mechanisms underlying the processing of these non-canonical junctions are known.

Over the past years, genetic studies have identified several proteins that may be involved in non-canonical RNA processing. Surprisingly, knockdown of the mtRNase P subunits MRPP1 or MRPP3 leads to the accumulation of unprocessed COXI, while the other three non-canonical junctions are unaffected [21, 23]. In humans, *Cox1* is preceded by a cluster of four tRNAs encoded on the opposing strand and a 12-14 nucleotide gap. It was therefore suggested that the antisense tRNAs might act as “mirror” tRNAs [52, 54, 55] that are recognised by mtRNase P [21, 23]. However, whether this is indeed the case is not clear, as the cleavage site would be further upstream than the short 3-nt 5’ UTR reported for mature *Cox1* transcripts [52, 53]. Depletion of the pentatricopeptide repeat domain-containing protein, PTCD2, results in the accumulation of unprocessed *Nd5-CytB* precursors and a marked reduction in ubiquinol–cytochrome c reductase activity [56]. However, whether PTCD2 is directly involved in non-canonical processing remains unclear, as recent data suggested that PTCD2 rather interacts with the mitochondrial ribosome to regulate COXIII translation [57].

More recently, members of the Fas-activated serine/threonine kinase (FASTK) family of proteins have been suggested to play key roles in non-canonical RNA processing [58]. Humans encode six FASTK family members (FASTK and FASTKD1-FASTKD5), which all localise to mitochondria. Although their functions are not fully elucidated, they have been implicated in maintaining rRNA and mRNA stability, mRNA processing, and regulation of mitochondrial translation [58–65]. Specifically, FASTK, FASTKD4, and FASTKD5 have been suggested to be important for non-canonical processing. FASTK has two isoforms, with the shorter isoform localising to mitochondria to protect ND6 mRNA from degradation [61]. Silencing or deletion of FASTKD5 results in the accumulation of three unprocessed transcripts (5′ UTR-Cox1, Atp8/6-Cox3, and Nd5-CytB), and the loss of FASTKD4 leads to an accumulation of unprocessed Nd5-CytB precursor transcript, heavily implicating involvement of FASTKD4 and 5 in non- canonical processing [60, 63, 65, 66]. Interaction and double-knockout studies further suggest that both FASTKDs interact *in vivo*, where FASTKD4 acts at the *Nd5-CytB* junction, and FASTKD5 taking a more general involvement at all three junctions [63]. In addition to their proposed roles in processing non-canonical junctions, FASTK proteins have also been implicated in the clearance of non-coding mitochondrial RNAs, and it has been suggested that this may be functionally coupled to processing [63]. Nevertheless, despite their clear importance in non-canonical processing, their exact roles or structures are unclear.

In support of a role in RNA processing, bioinformatic and genetic studies suggest that FASTK proteins may harbor RNA cleavage activity. All FASTK proteins contain an N-terminal mitochondrial targeting signal (MTS), two FAST domains (FAST_1, FAST_2), and a RAP (RNA-binding domain abundant in *Apicomplexas*) domain [58, 67, 68]. To date, no experimental structural data are available for any member of the FASTK protein family, but sequence comparisons suggest that the RAP domain of FASTK proteins shows homology to the PD-(D/E)XK endonuclease superfamily and may thus harbor endonuclease activity [68]. Proteins containing this fold play an important role in nucleic acid maintenance and are found to be present in all domains of life [69]. FASTK proteins show the highest similarity to the bacterial Very Short Patch Repair (VSR) endonuclease, which contains a catalytic aspartate in its active site [60, 70]. Strikingly, expression of a mutant human FASTKD4 protein that was modified at the corresponding aspartate (D529) failed to complement FASTKD4 knockout cells [60, 66], suggesting that the RAP domain is essential for its function in non-canonical RNA processing. In addition, the RAP domain of FASTK was shown to be critical for the binding and maturation of the ND6 transcript [61]. Thus, circumstantial evidence suggests that FASTK family members are responsible for cleavage during non-canonical processing, but direct biochemical evidence has not yet been formally demonstrated.

An additional piece of the puzzle was recently presented when it was proposed that cleavage of non-canonical junctions results in the formation of 3′- or 2′,3′-cyclic phosphate (2′,3′-cP) residues [71]. Several ribonucleases are known to cleave the phosphodiester bond downstream of the bridging phosphorus atom, resulting in unconventional free 3′ phosphates or 2′,3′-cP, and 5′ hydroxyl groups [72]. Using fly and mouse knockout models of the carbon catabolite repressor 4 domain-containing family member CCR4D, also known as ANGEL2, it was demonstrated that *Atp6/8* and *Nd5* accumulate 3′ terminal phosphates, preventing their polyadenylation [71]. Furthermore, the *Drosophila* ortholog *DmAngel* (cg12273) co-precipitated with the *Drosophila* FASTK family member CG13850. The fly has only two FASTK homologs,

and CG13850 most closely aligns with human FASTKD4 and 5, further indicating that these factors may be directly responsible for cleavage during non-canonical processing. Thus, ANGEL2 appears to be responsible for the dephosphorylation of the 3′ ends of non-canonically processed mitochondrial transcripts.

Taken together, genetic, and biochemical studies have identified a number of players that may be involved in non-canonical RNA processing. In contrast to the situation in canonical RNA processing, where the same machinery is responsible for processing most tRNA-flanked junctions, the mechanisms of processing appear to differ at each non-canonical junction. Recent studies strongly suggest that FASTK proteins play a pivotal role in non-canonical processing and may cooperate with various other players at different junctions. However, the precise molecular mechanisms underlying non-canonical RNA processing remain to be unravelled.

Open questions

Since its proposition 40 years ago, detailed biochemical and structural insights into the mechanism of the first step of the mitochondrial tRNA punctuation model have been obtained. However, it remains unknown how RNase P activity is regulated or whether different structures are adopted for the different, structurally divergent mt-tRNAs. Furthermore, the mechanism of the second step catalyzed by RNase Z remains unknown. In particular, it is not known (i) how is ELAC2 recruited to the 3′ processing sites, (ii) why the MRPP1/MRPP2 subcomplex is required for efficient processing of a subset of mitochondrial tRNAs, and (iii) how the strict hierarchy of RNAse P and ELAC2 activity is ensured. Thus, our molecular understanding of canonical RNA processing in mammalian mitochondria remains incomplete.

In comparison, the molecular mechanisms of non-canonical RNA processing are even less well understood. From recent studies, the FASTK protein family has emerged as a central player in this process. However, it remains open (i) whether the FASTK proteins are indeed endonucleases that carry out cleavage at non-canonical junctions, (ii) how FASTKs interact with other players to recognise the substrate junctions and how specificity may be achieved, and (iii) what molecular mechanism these non-canonical machineries employ. Combining genetic, biochemical, and structural studies bears an exciting potential to answer these open questions in the future.

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