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Title: The role of tRNA-derived small RNAs in aging

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ABSTRACT

Aging is characterized by a gradual decline in biological functions, leading to the increased probability of diseases and deaths in organisms. Previous studies have identified biological factors that modulate aging and lifespan, including non-coding RNAs (ncRNAs). Here, we review the relationship between aging and tRNA-derived small RNAs (tsRNAs), ncRNAs that are generated from the cleavage of tRNAs. We describe age-dependent changes in tsRNA levels and their functions in age-related diseases, such as cancer and neurodegenerative diseases. We also discuss the association of tsRNAs with aging-regulating processes, including mitochondrial respiration and reduced mRNA translation. We cover recent findings regarding the potential roles of tsRNAs in cellular senescence, a major cause of organismal aging. Overall, our review will provide useful information for understanding the roles of tsRNAs in aging and age-associated diseases.

INTRODUCTION

Aging is defined by a progressive decline in the physiological functions of organisms. Mutations in genomic DNA or the impairments of protein homeostasis cause aging at the cellular levels, contributing to organismal aging [1, 2]. RNA, which transmits the information from DNA to proteins during transcription and translation, likely plays a key role in aging as well. We and other research groups have reported that RNA quality control and homeostasis are required for longevity and to delay aging [3-8]. Two DEAD-box RNA helicases, HEL-1 and SACY-1 (suppressor of ACY-4 sterility), which may contribute to RNA homeostasis, are required for longevity conferred by various interventions, including reduced insulin/IGF-1 signaling (IIS) in *Caenorhabditis elegans* [3, 4]. Splicing factor 1 (SFA-1), a key spliceosome component, mediates longevity conferred by dietary restriction [5]. In addition, nonsense-mediated mRNA decay (NMD), which maintains mRNA quality by degrading abnormal

transcripts with premature termination codons, promotes longevity in *C. elegans* [6-8].

Noncoding RNAs (ncRNAs), including microRNAs (miRNAs), circular RNAs (circRNAs), and tRNA-derived small RNAs (tsRNAs), also play important roles in aging regulation.

Many studies have shown that during aging, the levels of diverse ncRNAs are altered, and are implicated in age-related diseases [9-12].

tsRNAs, which are generated by the cleavage of tRNAs [13], are a novel class of ncRNAs.

tsRNAs are categorized by the cleavage sites of tRNAs: tRNA halves, tRNA-derived RNA fragments (tRFs), and internal-tRFs (i-tRFs) (Fig. 1). tRNA halves are also known as tRNA-

derived stress-induced RNAs (tiRNAs), because they are generated under stress conditions, such as starvation, oxidative stress, heat shock, or radiation [14-16]; hereafter, we will refer

to tRNA halves as tiRNAs, and all the other tsRNAs as tRFs. Angiogenin in mammals and

Ribonuclease T2-like 1 (Rny1) in yeast cleave nucleotides in the anticodon loops of tRNAs to generate 30-40 nucleotide-long tiRNAs [16, 17]. tRFs are 14-30 nucleotides in length [13,

18], and were reported to be generated by the cleavage of pseudouridine (T)- or

dihydrouridine (D)-loops of tRNAs by DICER, angiogenin, and RNase T1 [19-21]. Other

enzymes, including angiogenin and RNase T1, also responsible for the generation of tRFs

[21]. Among them 5'- or 3'-tRFs contain the ends of mature tRNAs, whereas i-tRFs contain

only internal regions of mature tRNAs [22, 23]. The length of i-tRFs are more variable than

other types of tsRNAs, 15-36 nucleotides in length [23]. Cleaved 5'-reader and 3'-trailer

sequences of pre-tRNAs are considered as tRFs as well. Additionally, pre-tRNAs can be

cleaved into tRFs, which are approximately 40 nucleotides in length [24]. tsRNAs are not just

byproducts generated from random degradation of tRNAs, because they play active roles in

diverse biological processes that include gene silencing, translation, transposition, apoptosis,

and intergenerational inheritance [13].

In this review, we discuss the roles of tsRNAs in organismal aging and cellular senescence. We describe studies that showed changes in tsRNA levels during aging. We discuss the roles of tsRNAs in age-related diseases, including cancer and neurodegenerative diseases. We also review studies suggesting potential aging-regulating roles of tsRNAs. Because several papers on tsRNAs employed inappropriate methods for performing gain-of- or loss-of-function experiments, here we only discussed papers that used proper methods [25]. Understanding the roles of tsRNAs in the regulation of aging will provide key information about how RNAs affect organismal aging and cellular senescence, similar to what DNA and protein homeostasis does.

tsRNAs as potential biomarkers of aging

Studies have revealed that in multiple species including *C. elegans*, *Drosophila melanogaster*, mice, and rats, the levels of various tsRNAs are altered in an age-dependent manner, raising the possibility that tsRNA levels may be used as biomarkers of aging [26]. In *C. elegans*, overall levels of tiRNAs increase during aging. Bioinformatic analysis shows that tiRNAs from the 5'-end of tRNAs (5'-tiRNAs) are generally upregulated during aging, while those from the 3'-end of tRNAs (3'-tiRNAs) exhibit mixed patterns of expression changes [27]. In the *Drosophila*, CCA-containing tRFs loaded onto Argonaute (Ago), a key component of RNA-induced silencing complex (RISC), accumulate during aging. Thus, *Drosophila* tRFs may contribute to age-related changes through Ago-containing RISC [28]. The levels of tsRNAs change during aging in mammals as well. In the brains of rats, 3'-tRFs are upregulated during aging [29]. In addition, many tsRNAs are differentially expressed between the brains of senescence-accelerated mouse prone 8 (SAMP8) progeria mice, and those of control mice [30]. Therefore, age-dependent changes in tsRNA levels appear to be conserved among different species, ranging from the nematode to mammals.

The roles of tsRNAs in age-related diseases

tsRNAs are associated with age-related diseases that include cancer and neurodegenerative disorders [13]. Specific tsRNAs are differentially expressed, and play important roles in several types of human cancer. Sequencing analysis identified tsRNAs that are differentially expressed in nasopharyngeal carcinoma and their potential targets that contribute to cancer [31]. tsRNAs appear to directly regulate cancer progression, because overexpression of, or treatment with specific tsRNAs whose levels are increased in cancer cells, can further enhance cancer cell proliferation [32, 33]. tsRNAs influence cancer metastasis, as exemplified by a study with a tRF derived from the 5'-end of tRNA^{Cys} (5'-tRF^{Cys}) [34]. 5'-tRF^{Cys} stabilizes the mRNAs of platelet activating factor acetylhydrolase 1b regulatory subunit (*Pafah1b1*) and methylenetetrahydrofolate dehydrogenase 1 like (*Mthfd1l*), by increasing the formation of mRNA-stabilizing nucleolin oligomers. This leads to enhanced breast cancer metastasis, because *Pafah1b1* and *Mthfd1l* are metastasis-promoting metabolic transcripts. Interestingly, other tsRNAs contribute to the inhibition of cancer. For example, 5'-tRNA^{Gly} is downregulated in laryngeal carcinoma patients and suppresses the growth of the cancer cells by silencing a phosphoinositide 3-kinase catalytic subunit (*PIK3CD*) [35]. Overall, these studies indicate that tsRNAs play both positive and negative roles in the pathophysiology of various types of cancer.

Multiple studies have provided evidence for the key roles of tsRNAs in neurodegenerative diseases. Abnormal tRNA metabolism by mutations in cleavage and polyadenylation factor I subunit 1 (*CLPI*), which regulates tRNA splicing, contributes to neurodegenerative disorders in zebrafish, mice, and humans [24, 36, 37]. Interestingly, mutations in *CLPI* lead to the accumulation of several tsRNAs, including the pre-tRNA^{Tyr}-derived tRF, and these tsRNAs sensitize cells to the tumor suppressor p53-dependent cell death [24]. Angiogenin, which

cleaves tRNAs into tiRNAs, promotes the survival of neuron-like SH-SY5Y cells [38], suggesting the protective roles of tiRNAs against neuronal loss. In contrast, another study using *D. melanogaster* and cultured rat neurons indicates that tsRNAs can cause cell swelling and necrosis by reducing translation through participation in ribosomal stalling [39]. In the brains of the progeric SAMP8 mice, many tsRNAs are differentially expressed, compared with those in control mice, and their potential targets are implicated in neurodegenerative disorders [30]. In the brains of Alzheimer's disease patients, the levels of tiRNA^{Tyr} and tiRNA^{Arg} are decreased, although the functional significance remains unknown [40]. Overall, these findings raise the possibility that tsRNAs may modulate neurodegenerative disorders.

tsRNAs may affect aging by modulating gene expression

Previous studies have established that protein synthesis greatly affects aging rates in animals [41]. Overall protein synthesis is reduced in long-lived mutants, such as animals with reduced insulin/IGF-1 signaling or TOR signaling [42, 43]. Additionally, the inhibition of mRNA translation contributes to longevity in *C. elegans* [41]. Because tsRNAs play roles in the regulation of global translation, tsRNAs likely affect aging through modulating protein synthesis. For example, 5'-tiRNAs inhibit global protein translation by displacing eukaryotic initiation factor 4F (eIF4F) complexes that contain eIF4G from capped mRNAs [44] (Fig. 2A). In addition, pseudouridylated 5'-tRFs generated by pseudouridine synthase 7 (PUS7) globally reduce protein synthesis [45]. In contrast, tRF derived from the 3'-end of tRNA^{Leu} (3'-tRF^{Leu}) can enhance overall protein synthesis by increasing the translation of ribosomal proteins S28 (*RPS28*) and S15 (*RPS15*) mRNAs [46, 47]. As tsRNAs can increase or decrease translation, specific tsRNAs may affect longevity in opposite directions, which need to be dissected in future research. tsRNAs also downregulate specific genes by acting through mRNA-silencing pathways [48-51]. However, whether target mRNAs that bind tsRNAs

regulate organismal aging or cellular senescence remains elusive. Overall, these studies raise the possibility that tsRNAs regulate aging by modulating gene expression.

tsRNAs generated from pre-tRNAs may influence organismal longevity

Although the direct roles of tsRNAs in cellular senescence or organismal aging remain poorly understood, emerging evidence indicates their modulatory roles in aging. A study using multiple model organisms, including *Saccharomyces cerevisiae*, *C. elegans*, and *D. melanogaster*, demonstrated that downregulation of RNA polymerase III, which transcribes pre-tRNA, enhances longevity [52]. Because subsets of tsRNAs are generated from pre-tRNAs [24], their depletion by downregulation of RNA polymerase III may reduce some fractions of tsRNAs (Fig. 2B). tRNA-processing enzyme ELAC2 affects the generation of tsRNAs, and is a key factor that is required for the functions of mitochondria in mammals and in *C. elegans* [18, 53-56] (Fig. 2B). Because lifespan extension by mild inhibition of mitochondria or depletion of RNA polymerase III is conserved among species [52, 57, 58], tsRNAs generated from pre-tRNA processing may affect aging and longevity in diverse organisms, including *C. elegans*.

tsRNAs that affect the inheritance of metabolic diseases may contribute to aging regulation

Several studies revealed that the composition of sperm tiRNAs are altered by diet or during sperm maturation, and sperm tiRNAs contribute to epigenetic inheritance [59-61]. High-fat diets, which can cause metabolic diseases, alter tiRNA fractions in mouse sperm, and the offspring of males fed with high-fat diets suffer from glucose intolerance [60]. Inflammation-induced metabolic diseases in adult male mice also alter the composition of sperm tiRNAs, leading to increased body weight and glucose intolerance in their offspring [62] (Fig. 2C).

Specifically, the injection of sperm tRNAs from mice with metabolic disorders into normal mouse zygote is sufficient to cause metabolic impairments in developed animals [60, 62]. These studies suggest that tsRNAs play direct roles in the transmission of paternal metabolic diseases to offspring. Obesity is accompanied by physiological changes that are similar to those observed during aging, as exemplified by the accumulation of senescent cells and DNA instability resulting from oxidative stress [63]. In addition, metabolic diseases are considered as age-associated diseases [64]. Therefore, these studies raise the possibility that tsRNAs inherited from sperm may accelerate aging, accompanied by metabolic defects, including obesity and insulin resistance.

tsRNAs may regulate cellular senescence

The functions of tsRNAs are also associated with cellular senescence, a major hallmark of organismal aging. Cellular damage drives cells into several fates, including cellular senescence and apoptosis [65]. Noticeably tRNAs are generated by external stress, including oxidative stress, heat shock, and radiation, which can accelerate cellular senescence. tRNAs inhibit apoptosis by binding to cytochrome c, which elicits apoptosis in the cytosol [66] (Fig. 3A). Therefore, tRNAs upregulated by sublethal cellular damage enhance the survival of damaged cells, possibly leading to the accumulation of senescent cells.

Transposable elements, also known as transposons, are the regions of DNA that can jump within the genome. Because transposons are inserted into genome during transposition, dysregulated transposons can negatively affect DNA integrity by increasing insertion mutations and DNA damage [67]. The activation of transposons, including long-interspersed element-1 (LINE-1), increases during aging, and can cause cellular senescence [68]. Therefore, proper suppression of transposons is important to prevent premature aging. A study revealed that 3' terminal CCA-containing 3'-tRFs inhibit the retrotransposition of long-

terminal repeat (LTR) retrotransposons [69] (Fig. 3B). Several 18 nucleotide-long 3'-tRFs compete with mature tRNAs for binding transcripts generated from retrotransposons, thereby inhibiting the jumping of LTR retrotransposons. In addition, 22 nucleotide-long 3'-tRFs contribute to the suppression of transposons by inhibiting the expression of transposon-encoded reverse transcriptase. Thus, tRFs may prevent cellular senescence caused by transposable elements by contributing to the maintenance of genomic stability.

Conclusions

In this review, we have discussed the roles of tsRNAs in aging and age-related diseases. Many studies have reported the identities and the functions of various kinds of tsRNAs, thanks to advances in whole transcriptome sequencing and bioinformatic technologies. Although tsRNAs were initially thought of as byproducts of RNA metabolism, recent studies indicate that tsRNAs modulate multiple biological processes, including mRNA silencing, translation, transposition, and apoptosis. Subsequent reports have identified tsRNAs whose levels are altered during aging in a wide range of species, including *C. elegans* and mammals. These findings suggest that tsRNAs are putative biomarkers of aging and/or aging-regulating factors. Furthermore, tsRNAs display altered levels in multiple age-related diseases, including cancers and neurodegenerative disorders, and play direct roles in those diseases. The functions of tsRNAs are also associated with various factors that regulate aging and cellular senescence, such as the components of mitochondrial electron transport chain and mRNA translation machinery. Thus, tsRNAs appear to be a new class of aging-regulating small RNAs.

As the biology of tsRNAs is still at an early stage, numerous questions remain to be addressed for future research. The mechanisms by which tsRNA levels are altered during aging remain elusive. For example, tsRNA levels are increased during aging in *C. elegans*,

but its genome does not contain a homolog of mammalian angiogenin or yeast Rny1, tRNA-generating enzymes. Identification of such enzymes can lead to insights into the mechanisms and functions of age-dependent increases in tRNA levels with respect to animal physiology. The causal effects of tsRNAs on aging also remain obscure, although multiple studies that we covered in this review have suggested putative roles of tsRNAs in organismal aging and cellular senescence. For example, whether the modulation of protein synthesis by tsRNAs or the inhibition of transposons directly contributes to longevity is elusive. Furthermore, functional conservation of tsRNAs among species is very rare, despite the evolutionally conserved sequences of tRNAs among eukaryotes. Additionally, the functions of tsRNAs are characterized mainly by using cultured mammalian cells. Therefore, it will be important to determine whether the aging-regulating roles of tsRNAs are conserved in various species at the organismal level. Overall, further studies from multiple research directions will provide critical information for understanding the role of tsRNAs in aging.

Small RNA therapeutics are emerging as promising means for targeting various diseases, including cancer [70]. The majority of small RNA therapeutics utilize miRNA mimetics or anti-miRNA drugs [71]. tsRNAs can potentially serve as valuable tools for small RNA therapeutics, based on their aforementioned roles in cancer and potential functions in aging. Furthermore, tsRNA-targeting drugs may have systemic effects, because of their abundance in circulating blood systems [72]. Therefore, understanding the molecular actions and physiological functions of tsRNAs will help develop novel small RNA therapeutics for treating aging-related diseases or degenerative processes associated with aging.

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251 **CONFLICTS OF INTEREST**

252 The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Biogenesis of tRNA-derived small RNAs (tsRNAs). Intron (yellow), 5'-reader (green), and 3'-trailer sequences (brown) are cleaved from pre-tRNAs to generate mature tRNAs. Angiogenin (ANG) in mammals and Ribonuclease-T2 like 1 (Rny1) in yeast cleave nucleotides in the anticodon loops (red) of tRNAs to generate 30-40 nucleotide-long tRNA-derived stress-induced RNAs (tiRNAs), also known as tRNA halves. Despite controversy, DICER, ANG, and RNase T1 were shown to cleave nucleotides in the pseudouridine (T) - or dihydrouridine (D) -loops (blue) of tRNAs to generate 5'- and 3'-tRNA-derived RNA fragments (tRFs), 14-30 nucleotides in length. Inter-tRFs (i-tRFs) are generated by multiple cleavages of tRNAs, and contain internal regions of tRNAs, which are 15-36 nucleotides in length.

Figure 2. Association of tsRNAs with aging-regulating pathways. (A) A group of tiRNAs reduce overall translation by displacing eukaryotic initiation factor 4F (eIF4F) complexes from the 5'-cap of mRNAs. In contrast, a specific tRF can increase overall translation rates by enhancing the mRNA translation of ribosomal protein S (*RPS*) gene. Modulation of protein synthesis by tsRNAs may affect longevity in multiple species, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and mouse. (B) tsRNAs may mediate the effects of abnormal tRNA metabolism on aging. Depletion of RNA polymerase III (RNA pol III) may reduce tsRNA generation, possibly contributing to longevity conferred by depletion of RNA pol III in *C. elegans* and *D. melanogaster*. Abnormal tRNA processing causes the accumulation of tsRNAs, likely leading to mitochondrial dysfunction. Mitochondrial impairments enhance or suppress longevity in a context-dependent manner in multiple

species, including *C. elegans*. (C) High-fat diet (HFD) feeding or lipopolysaccharide (LPS) injection alters the composition of sperm tRNAs and causes metabolic disorders in mice. The tRNAs in mouse sperm contribute to the inheritance of metabolic diseases to the offspring, and the inherited metabolic diseases may cause premature aging.

Figure 3. Association of tsRNAs with cellular senescence. (A) Cellular damages cause the release of cytochrome c from mitochondria, eliciting apoptosis. tRNAs inhibit apoptosis by binding cytochrome c. The inhibition of apoptosis in damaged cells may lead to cellular senescence. (B) Activation of transposable elements can cause cellular senescence. tRFs inhibit the translation of reverse transcriptase (RTase)-encoding long-terminal repeat (LTR) retrotransposons. tRFs also inhibit the reverse transcription of LTR retrotransposons, and may prevent cellular senescence resulting from activated transposons.

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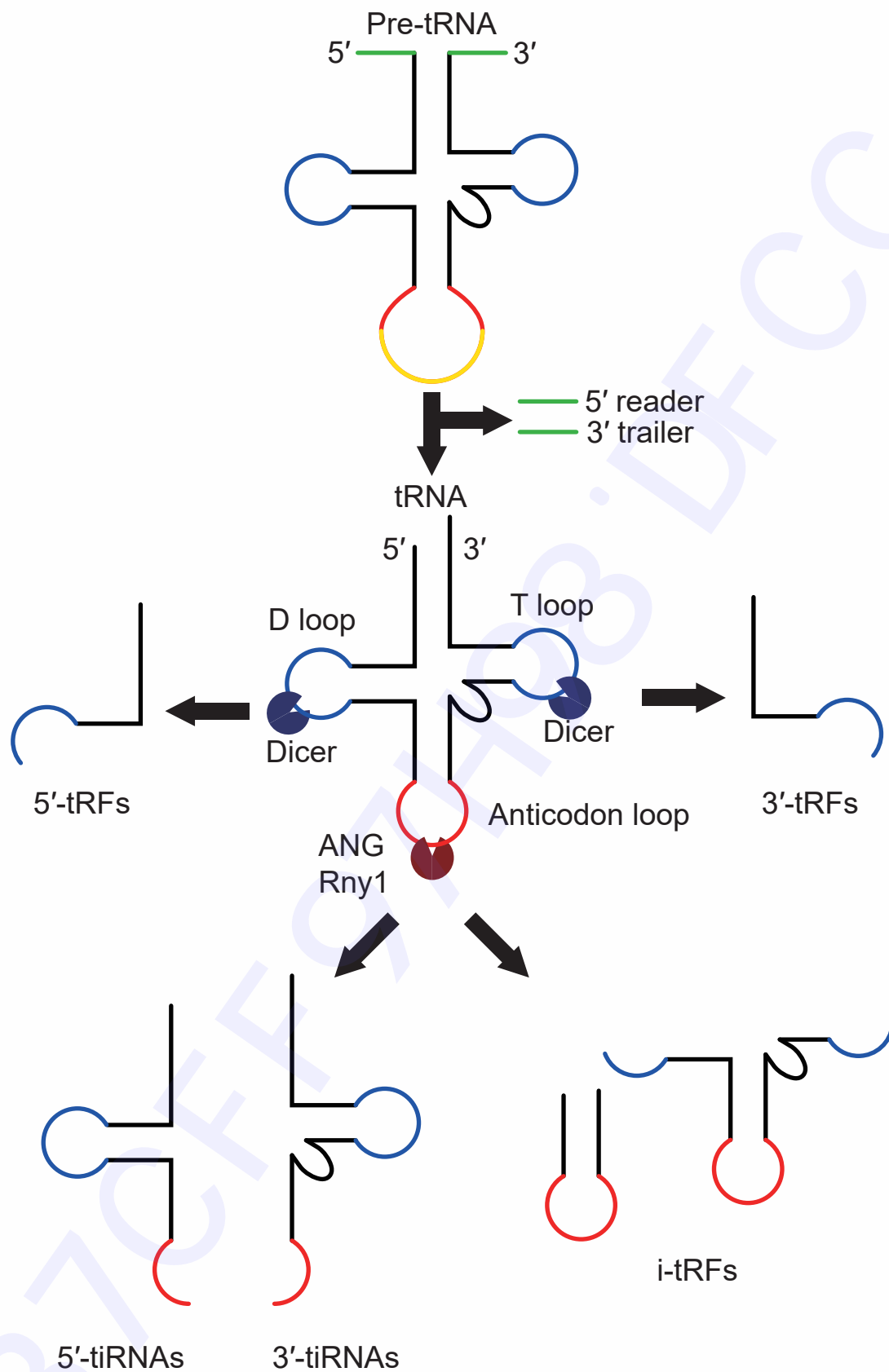
Figure 1

Figure 2

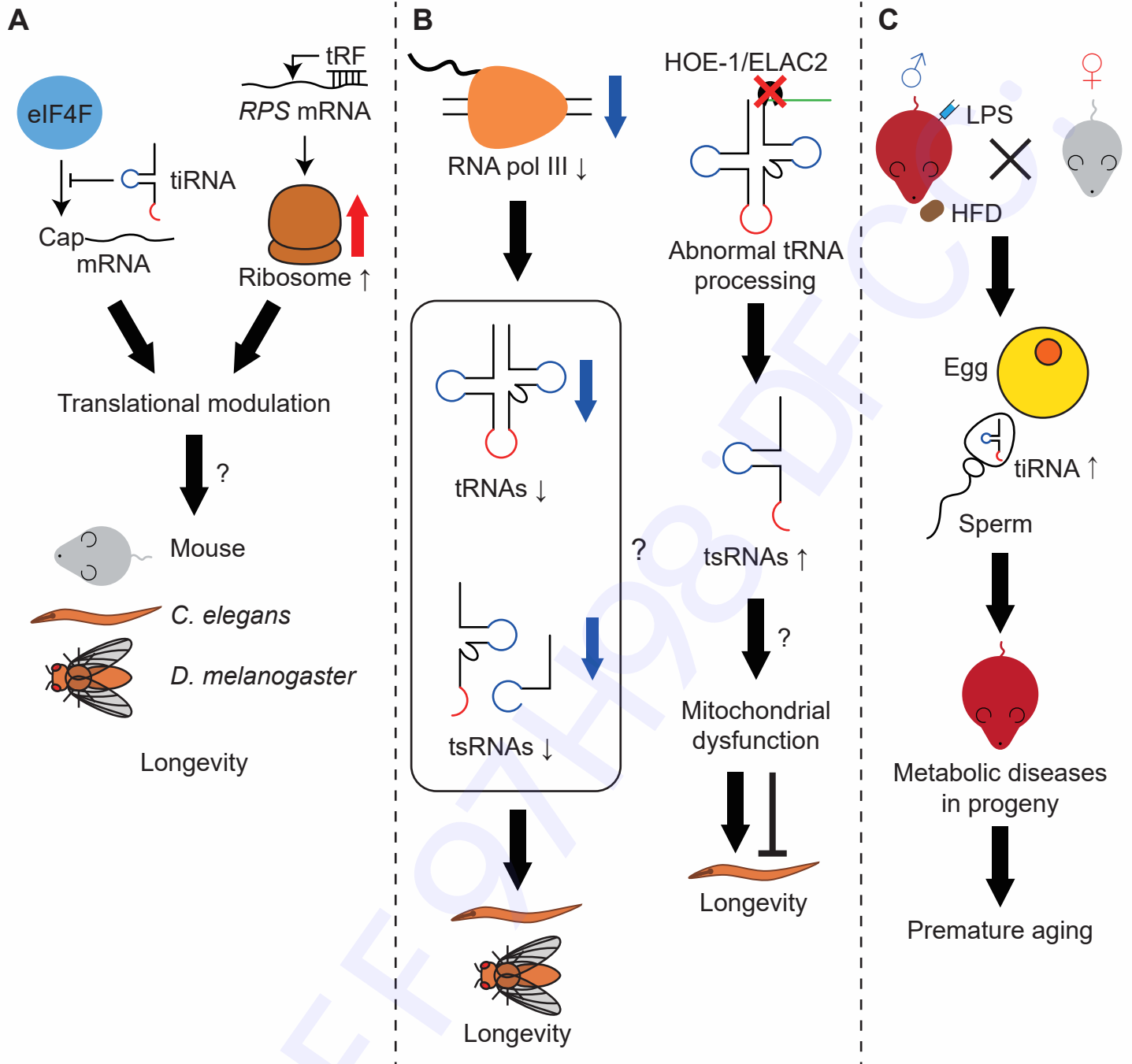


Figure 3

