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POINT OF VIEW

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A new layer of rRNA regulation by small interference RNAs and the nuclear RNAi pathway

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ABSTRACT

Ribosome biogenesis drives cell growth and proliferation, but mechanisms that modulate this process remain poorly understood. For a long time, small rRNA sequences have been widely treated as non-specific degradation products and neglected as garbage sequences. Recently, we identified a new class of antisense ribosomal siRNAs (risiRNAs) that downregulate pre-rRNA through the nuclear RNAi pathway in *C. elegans*. risiRNAs exhibit sequence characteristics similar to 22G RNA while complement to 18S and 26S rRNA. risiRNAs elicit the translocation of the nuclear Argonaute protein NRDE-3 from the cytoplasm to nucleus and nucleolus, in which the risiRNA/NRDE complex binds to pre-rRNA and silences rRNA expression. Interestingly, when *C. elegans* is exposed to environmental stimuli, such as cold shock and ultraviolet illumination, risiRNAs accumulate and further turn on the nuclear RNAi-mediated gene silencing pathway. risiRNA may act in a quality control mechanism of rRNA homeostasis. When the exoribonuclease SUSI-1(ceDis3L2) is mutated, risiRNAs are dramatically increased. In this Point of View article, we will summarize our understanding of the small antisense ribosomal siRNAs in a variety of organisms, especially *C. elegans*, and their possible roles in the quality control mechanism of rRNA homeostasis.

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rRNA; risiRNA; NRDE; nucleolus; stress

Ribosomes are ribonucleoprotein machineries that decode the genetic information embedded in mRNAs into proteins. The functional core of ribosomes is rRNA, which promotes the assembly of ribosomes and catalyzes the critical steps of mRNA decoding and amino acid polymerization (reviewed by[1-5]). rRNAs are expressed in almost all cell types, including both prokaryotic and eukaryotic cells. In eukaryotic cells, rRNAs are transcribed into a single 47S polycistronic precursor by RNA polymerase I in the nucleolus, then modified, processed, folded, and matured into 18S, 5.8S, and 28S rRNA. 5S rRNA is independently transcribed by RNA polymerase III in the nucleus. rRNAs and ribosomal proteins associate with each other to form ribosomal subunits in the nucleolus. Then the pre-40S small subunits and pre-60S large subunits are exported into the cytoplasm for final maturation and protein synthesis.

Ribosome biogenesis is a highly orchestrated process involving a variety of molecular components and auxiliary factors. Non-coding RNAs, for example, the small nucleolar RNAs (snoRNAs), are indispensible for the modification, processing, and folding of pre-rRNAs.[1] snoRNAs are an ancient class of small RNA molecules that guide site-specific modifications of other RNAs, including rRNAs, tRNAs (tRNAs) and small nuclear RNAs(snRNAs). snoRNAs associate with protein molecules to form the small nucleolar ribonucleoproteins (snoRNPs). The C/D box snoRNAs direct 2′-O-methylation of certain rRNA nucleotides and the H/ACA snoRNAs guide the site-specific pseudouridylation of rRNAs. These site-specific modifications affect subsequent rRNA folding, maturation and stability, which are essential for ribosome assembly and translation activity. In addition, non-coding RNAs may act in *cis* to modulate the transcription of 5S rRNA in eukaryotic cells.[6] Our recent research of the identification of antisense ribosomal siRNA (risiRNA) further paved the way to investigate the regulation of rRNA by small interfering RNAs (siRNA) and the cellular RNA interference (RNAi) machinery in multicellular organisms.[7]

Small regulatory RNAs direct sequence-specific regulation of gene expression via the mechanism termed RNAi.[8] Small RNAs and the Argonaute proteins play essential roles in RNAi-mediated gene silencing. Small RNAs guide the Argonaute-containing protein complex to bind to complementary nucleic acids and modulate gene expression by several mechanisms, including RNA degradation, translation inhibition, inducing heterochromatin formation, and inhibiting transcription elongation. (reviewed by[9-12])

Small RNAs are produced through a variety of mechanisms. In eukaryotes, microRNA is usually generated by slicing of a stem-loop precursor by the conserved RNase III-like ribonuclease, Dicer.[13] On the other hand, long double-stranded RNA (dsRNA) are cleaved into mature 5′ monophosphorylated siRNAs by Dicer. In *C. elegans*, an additional siRNA amplification step exists, in which secondary siRNAs can be synthesized from targeted RNA by RNA-dependent RNA polymerases (RdRPs). Secondary siRNAs are then loaded into an expanded

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group of worm-specific AGOs (WAGOs) to carry out downstream gene silencing.[14-18] tRNAs, apart from their primordial roles in translation, have been suggested to exert regulatory functions through the production of 2 classes of small RNAs derived from their transcripts, tRNA halves of 30-36 nt and tRNA-derived RNA fragments (tRFs) of 18–20 nt.[19,20]

Although small regulatory RNAs have been shown to play several crucial roles in organisms, it remains intriguing whether and how small RNAs and the RNAi machinery can modulate rRNA and ribosome biogenesis. In this review, we will provide an overview of the mechanism of pre-rRNA regulation by a newly identified risiRNA in *C. elegans*, including the biogenesis of risiRNA, pre-rRNA regulation by risiRNA/NRDE complex, and potential physiologic functions of risiRNA.

Small antisense rRNAs are present in several organisms

Small rRNA sequences have long been viewed as degradation products and are usually discarded during high-throughput RNA-seq analyses. Yet several evidence suggests that the antisense rRNAs are widely present in various organisms. For example, in *Schizosaccharomyces pombe (S. pombe)* lacking Cid14, rRNAs become substrates for the RNAi pathway and give rise to siRNAs.[21] Cid14 is a members of the family of non-canonical poly(A) polymerases that play central roles in surveillance mechanism to monitor RNA quality. Cid14 is a subunit of the TRAMP complex and is involved in recognition and targeting of aberrant RNAs for exosome-mediated degradation.[22,23] It is speculated that Cid14 normally promotes the targeting of rRNA precursors and the tRNA-Glu fragment for degradation or processing by exosomes. In the absence of Cid14, such precursors accumulate and become targets for RNA-directed RNA polymerase complex (RDRC), recruit RDRC and Dicr1, and trigger the generation of antisense ribosomal small RNAs (rr-siRNAs).[21]

In the filamentous fungus *Neurospora crassa*, DNA damage induces the expression of the Argonaute protein QDE-2 and a class of small RNAs, qiRNA.[24] qiRNAs interact with QDE-2, are about 20–21 nucleotides long (several nucleotides shorter than normal Neurospora siRNAs), with a strong preference for uridine at the 5′ end, and originate mostly from the rDNA locus correspond to both sense and antisense strands at approximately equal frequency. DNA damage induces the accumulation of aberrant RNAs (aRNAs), which act as precursors for qiRNA production, a process that is dependent on both QDE-1 and QDE-3. QDE-1 is a DNA- and RNA-dependent RNA polymerase that produces aRNAs. The production of qiRNAs also requires the Werner and Bloom RecQ DNA helicase homolog QDE-3 and dicers.

In *Arabidopsis*, the activation of antiviral RNAi induces the production of antisense ribosomal small RNAs as well.[25] These virus-activated siRNAs (vasiRNAs) are predominantly 21 nucleotides long with an approximately equal ratio of sense and antisense strands and require Dicer-like 4 and RNA-dependent RNA polymerase 1 (RDR1) for their generation. These antisense ribosomal siRNAs may aid plants to buffer against virus infection.

We identified risiRNAs and the suppressor of siRNA (SUSI)-1(ceDis3L2) by a chemical-induced genetic screening in *C. elegans*.[7] Here, NRDE-3 is an Argonaute protein that transports siRNAs from the cytoplasm to the nucleus and mediates chromatin modification and co-transcriptional gene silencing.[26-30] NRDE-3 localizes to the nucleus when it binds to siRNAs but accumulates in the cytoplasm in the absence of siRNA ligands. Impairments of the generation of endogenous siRNAs re-localize NRDE-3 from the nucleus to the cytoplasm, while exogenously providing siRNAs re-localizes NRDE-3 to the destined subcellular compartment in a target-specific manner. Using the subcellular localization of GFP::NRDE-3 as a reporter, we performed genetic screenings and identified a series of suppressor of siRNA (*susi*) mutants that are capable of re-distributing NRDE-3 from the cytoplasm to the nucleus.

susi-1 encodes a 3′-5′ exonuclease that is homologous to DIS3L2. Human DIS3L2 has been shown to involve in Perlman syndrome, Wilm's tumor development, early embryogenesis, and stem cell proliferation.[31,32] DIS3L2 preferentially degrades polyuridylated RNA transcripts, which might serve as a quality control mechanism to eliminate aberrant RNAs.[33] In *susi-1* mutant animals, the risiRNAs are enriched and NRDE-3 is accumulated in the nucleoli (Fig. 1-III). Interestingly, when *C. elegans* is exposed to environmental stimuli, such as cold shock and UV illumination, risiRNAs is enriched and trigger the nucleolar accumulation of NRDE-3 as well. risiRNAs exhibit characteristics of 22G RNA, both biochemically and genetically, in *C. elegans*. They are 22 nt in length and have a triphosphorylated guanidine at their 5′-end. The generation of risiRNA depends on RdRPs. Interestingly, unlike the normal 22G RNAs binding to NRDE-3, the biogenesis of risiRNA is independent of the ERI/Dicer complex.

Uridylation of targeted mRNA is a signal for recruiting RdRPs to produce 22G RNA (Fig. 1-II).[34] The endoribonuclease, RDE-8, is recruited to targeted mRNA by RDE-1, cleaves mRNA, recruits RDE-3, and enables 3′ uridylation of the 5′ mRNA fragments. These processes are required to promote RdRP activity and ensure the amplification of 22G RNA. Similar to other classes of 22G RNA, the generation of risiRNAs requires the presence of 3′-end polyuridylated rRNA template as well (Fig. 1-III). By using a 3′ tail-seq method, we found that the oligouridylated 26S rRNA is modestly enriched in *susi-1(ceDis3L2)* mutant and in cold temperature-treated animals.[7] The injection of 3′ end oligouridylated 26S rRNA fragments is sufficient to trigger the nuclear localization of GFP::NRDE-3 in *C. elegans*, which further supports that the generation of risiRNAs can be induced by oligouridylated rRNAs.

Different small RNA pathways have been shown to compete for limiting factors that are required for RNAi. The ERI/Dicer complex is essential for the production of a subset of endogenous 22G RNA (Fig. 1-I).[35] The loss of function of ERI/Dicer complex may disrupt the normal biogenesis machinery of endogenous 22G RNA and re-distribute RdRPs for the generation of risiRNA. Both ERI/Dicer-independent risiRNA and ERI/Dicer-dependent 22G RNA engage the Nrde pathway to silence their respective genomic loci. Further experiments should be performed to examine how these two classes of siRNA compete and/or coordinate with each other to regulate cell homeostasis.

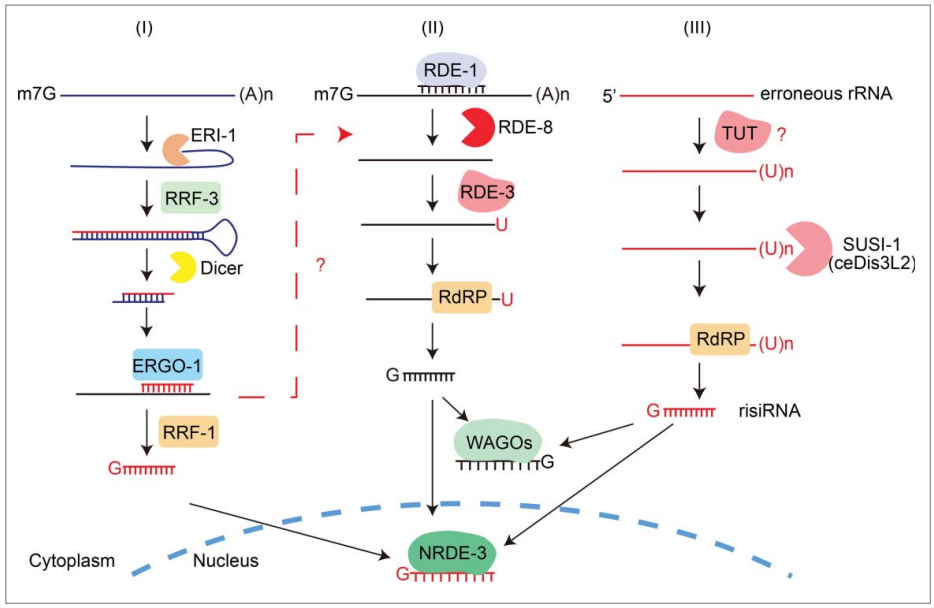

Figure 1. The biogenesis pathways of WAGO-associated 22G RNA in *C. elegans*. (I) The ERI/Dicer complex-dependent 22G RNA generation pathway. ERI-1 is an exonuclease that recognizes a distinct subset of mRNAs in the cell and recruits the RNA-dependent RNA polymerase RRF-3 and the ribonuclease Dicer to generate 26G RNA, which further associates with the Argonaute proteins ERGO-1 and triggers the generation of 22G RNA by recruiting another RNA-dependent-RNA polymerase RRF-1. These 22G RNAs associate with WAGO class Argonaute proteins to silence their targets through nuclear and cytoplasmic silencing pathways. (II) RDE-8-dependent 22G RNA biogenesis pathway. The targeted mRNA is recognized by the primary Argonaute RDE-1, cleaved by the ribonuclease RDE-8, oligouridylated by terminal nucleotidyltransferase RDE-3, and followed by the recruitment of RNA-dependent RNA polymerases to elicit the production of 22G RNA. (III) The biogenesis pathway of risiRNAs. Misprocessed or stress-induced rRNAs are recognized as errors and polyuridylated by unknown terminal uridylyl transferases (TUTs), which are further degraded by the exoribonuclease SUSI-1(ceDis3L2). The oligouridylated rRNAs can also serve as templates to recruit RNA-dependent RNA polymerases and generate risiRNAs. risiRNAs can guide nuclear and cytoplasmic Argonaute proteins to suppress the accumulation of the erroneous rRNA.

risiRNAs inhibit the expression of rRNA via multiple RNAi machineries in *C. elegans*

Small regulatory RNAs mainly function via binding to distinct Argonaute proteins and eliciting different gene silencing mechanisms to negatively regulate their respective targets. In *S. pombe*, the newly derived rr-siRNAs associates with Ago1 in Cid14 mutant.[21] In *Neurospora crassa*, DNA damage induces the expression of the Argonaute protein QDE-2 and rRNA-derived qiRNAs simultaneously, while qiRNAs bind to QDE-2.[24] In *Arabidopsis*, the activation of antiviral RNAi induces the accumulation of rRNA-derived vasiRNAs, which are loaded into both AGO1 and AGO2 proteins.[25]

In *C. elegans*, risiRNA may engage multiple WAGO-mediated gene silencing machineries, including but not limiting to the nuclear RNAi pathway. The *C. elegans* genome encodes 27 Argonaute proteins, among which there are 12 worm specific Argonaute proteins that are termed as WAGOs.[15,18] Secondary siRNAs mainly function via binding to distinct WAGOs and eliciting respective gene silencing mechanisms. We detected the association of risiRNAs to multiple WAGO proteins,[7] which suggests that risiRNA may utilize a variety of mechanisms to silence rRNA expression. This flexibility may aid *C. elegans* to quickly and efficiently respond to environmental alterations and facilitate the survival under stress conditions. Further investigating the association of risiRNAs to these Argonaute proteins under various stress conditions or in several distinct *susi* mutants is desired to pinpoint the functional significance and mechanisms of risiRNA-engaged gene silencing.

risiRNAs direct the nucleolar localization of the Argonaute protein NRDE-3 and the association with pre-rRNA.[7] Previously, we showed that siRNAs guide NRDE-3 to the nucleus, bind to targeted nascent pre-mRNA transcripts, induce histone modifications, pause RNA polymerase II, and inhibit transcription elongation.[26-29,36] It is intriguing to examine whether risiRNA/NRDE complex uses a similar mechanism to induce histone modifications at the rDNA loci and silence RNA polymerase I-mediated transcription (Fig. 2).

The physiologic functions of risiRNA

Noncoding RNAs, such as snoRNAs, play essential roles in rRNA processing and maturation. Other nucleolar noncoding RNAs have also been identified to act in pre-rRNA processing.[37,38] The microRNA, *let-7*, regulates nucleolar size by targeting the 3′ UTR of ncl-1 and inhibits its expression, which further restores the translation of FIB-1/fibrillarin.[39] Recently, two groups have reported that RNAi-induced rDNA silencing helps cells to enter into the quiescence state in *S. pombe*.[40,41] The loss of RNAi factors results in the loss of ability to maintain the long-term quiescence. In *Leishmanis*, drug-induced apoptosis-like programmed cell death triggers the fragmentation of antisense rRNA (asrRNA) complementary to the large subunit gamma (LSU-γ) rRNA.[42] Heat and oxidative stress also induce the fragmentation of asrRNA. Extensive asrRNA cleavage correlates with rRNA breakdown and translation inhibition.

Small rRNA sequences have been usually viewed as rRNA degradation products for decades. Our work demonstrated that risiRNAs act to maintain the homeostasis of pre-rRNA and

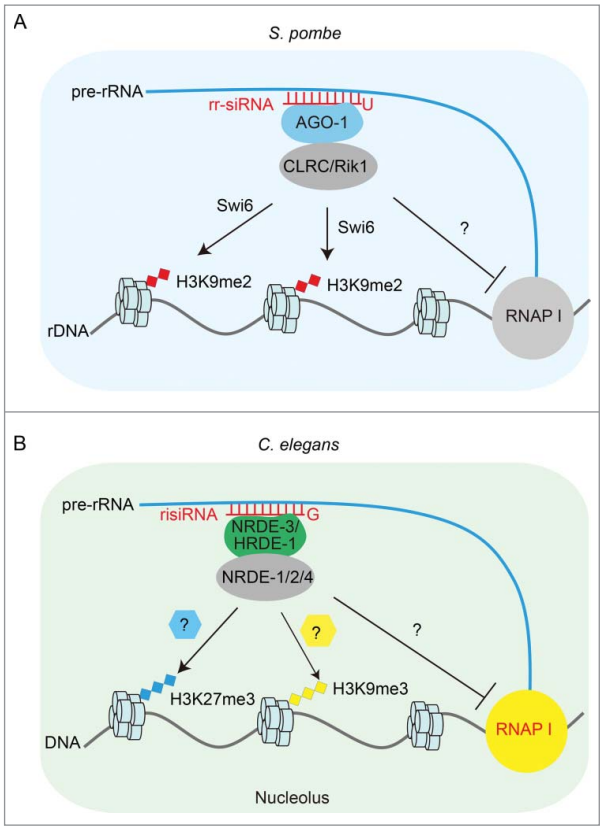

Figure 2. The working model of risiRNA-induced rRNA silencing in the nucleolus. (A) In *S. pombe*, antisense ribosomal siRNAs (rr-siRNAs) are loaded to AGO1, bind to pre-rRNA, recruit CLRC/Rik1 and the histone methyltransferase Swi6, which leads to the accumulation of H3K9me2 marks and the silencing at the rDNA loci. (B) In *C. elegans*, risiRNAs direct the nuclear Argonaute protein NRDE-3 (HRDE-1 in germline) to nascent pre-rRNA transcripts, recruit other NRDE factors, and inhibit RNA polymerase I-engaged rRNA transcription. Meanwhile, the rDNA loci may be modified by unknown histone methyltransferases to elicit H3K9me3 and H3K27me3 modifications.

may have important biologic roles in anti-stress response or developmental regulations. Temperature has profound effects on the biogenesis and processing of rRNA, the assembly of ribonucleoprotein particles, and the genome integrity of rDNA.[43-46] We showed that lower temperature could trigger risiRNA expression and downregulate pre-rRNA via the Nrde pathway. We further showed that lower temperature can impair 3′-end maturation of 26S rRNA. It is unclear whether the enzymatic activity of SUSI-1(ceDis3L2) and/or other RNA processing and surveillance factors are altered by this lower temperature treatment.

UV exposure has complex effects on macromolecules in the cell, including but not limiting to inducing intra- and inter- molecular crosslinking, which might further be recognized by the cellular surveillance system as errors and subjected to repair and/or degradation. We observed that UV irradiation also increased risiRNA expression and elicited the cytoplasm to nucleus translocation of NRDE-3. This result suggests that the UV-induced erroneous nucleic acids, including rRNAs, could recruit RdRPs to trigger the generation of 22G RNA.

We speculate that risiRNA may act to suppress the accumulation of erroneous rRNA in *C. elegans*.[7] The cellular erroneous transcripts are usually scrutinized and suppressed via several mechanisms. In mammalian cells, TUT-4 and TUT-7, known as the RNA terminal uridyltransferases (TUTs), selectively recognize and add untemplated U to the 3′ end of targeted RNAs.[47-51] The cytoplasmic exoribonuclease Dis3L2-involved surveillance system further directs the degradation of oligouridylated RNA.[52,53] The environment stimuli or gene mutation can lead to the generation of erroneous rRNA, which can be modified by oligouridylation and degraded by exoribonucleases. The disruption of exoribonucleases will result in the accumulation of oligouridylated erroneous rRNA, which recruits RdRP to synthesize risiRNA and silence rRNA expression to limit the accumulation of erroneous rRNA.[7] The risiRNA/RNAi-directed feedback loop therefore compensate for the dysfunction of exoribonuclease-engaged degradation of erroneous transcripts. However, due to the sequence characteristics of RNAi, it is unlikely that risiRNA themselves can distinguish between damaged and functional rRNA molecules in the cell. Otherwise, risiRNAs are capable of silencing pre-rRNA via the nuclear RNAi pathway in the nucleoli.

Perspectives

Ribosome biogenesis is a very sophisticated multi-step process, in which mistakes can take place at any step. Due to the crucial roles of ribosomes, cells have to carefully surveil every step of the pre-rRNA processing and the assembly of ribosomal subunits. Misprocessed rRNAs are usually surveyed and degraded by multiple surveillance machineries, including the exosome complex and TRAMP complex, etc.[1,4,54] The exoribonuclease SUSI-1(ceDis3L2) degrades 3′-uridylated noncoding RNA.[55] However, it is unclear which terminal uridyltransferase contributes to the untemplated addition of the 3′end uracil. The *C. elegans* genome encodes 3 polyuridylation polymerases, in which PUP-2/3 are the homologues of TUT4/7 in mammals. Identifying which terminal uridyltransferase is involved in the 3′-oligouridylation of rRNA fragments will further aid to clarify the scrutinization system of cellular nucleic acids.

risiRNAs bound multiple Argonaute proteins, including WAGO-1. WAGO-1 localized to the peri-nuclear P-granule, suggesting that risiRNAs may have functions in the cytoplasm. Whether and how risiRNAs direct different cytoplasmic Argonaute proteins to regulate the rRNA surveillance, ribosome turnover, and translation in the cytoplasm is unclear. In the cytoplasm, risiRNAs may be able to both induce the cleavage of its targeted RNAs and/or inhibit translation. risiRNAs may modulate translation through several mechanisms, for example, affecting the assembly of ribosomes and/or blocking protein synthesis. It will be very interested to test whether and how risiRNA regulate translation in the cytoplasm besides silencing rRNA transcription in nucleolus.

risiRNAs directs the translocation of the nuclear Argonaute protein NRDE-3 into the nucleus and nucleolus and the association with pre-rRNA. It is intriguing whether risiRNA/NRDE complex uses the classic nuclear RNAi mechanism to bind to targeted nascent pre-rRNA transcripts, induce H3K9 and H3K27 trimethylation, pause RNA polymerase I, and inhibit rDNA transcription (Fig. 2).

How environmental stimuli elicit risiRNA expression is unclear. Systematic analyzing the enzymatic activities of various

rRNA processing and maturation factors under stress conditions will aid to elucidate the mechanism and regulation of the generation of erroneous rRNAs and risiRNAs. Since risiRNA is strongly suppressed in wild type animals growing at laboratory culturing conditions, it is intriguing to investigate whether risiRNAs may have normal physiologic roles besides stress conditions. The genome-wide single-cell mapping technology will help to illustrate the roles of risiRNA in embryonic development and cell fate determination.[56,57] Our current knowledge of risiRNAs is very limited. Investigating the regulation of risiRNAs in different cell types and at different developmental stages will further our understanding of the rRNA regulation.

SUSI-1(ceDis3L2) is a 3′ to 5′ exoribonuclease from the RNase II/RNB family that acts preferentially on oligo(U)-tailed transcripts, which is engaged in the regulation of a variety of RNA species in mammals.[52,55,58] Human Dis3L2 has been linked to Perlman syndrome, Wilm's tumor development, early embryogenesis, and stem cell proliferation.[32,59-61] The accumulation of oligouridylated rRNA and risiRNA in *susi-1* (ceDis3L2) mutant suggests that the deficiency of rRNA biogenesis and risiRNA may contribute to the development of diseases. Whether and how risiRNA and these erroneous rRNAs act in human disease need further investigation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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