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REVIEW ARTICLE

N6-methyladenosine (m6A) modification of ribosomal RNAs (rRNAs): Critical roles in mRNA translation and diseases

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Abstract As key components of the ribosome and the most abundant RNA species, the rRNAs are modified during ribosome formation. N^6-methyladenosine (m^6A) is a conserved RNA modification occurring on different RNA species including rRNAs. Recently, it has been reported that ZCCHC4 and METTL5 are methyltransferases that mediate m^6A modification of human 28S and 18S rRNA, respectively. The newly discovered biological functions of the two methyltransferases include regulation of mRNA translation, cell proliferation, cell differentiation, stress response, and other biological processes. Both of them, especially METTL5, have been proved to be associated with a variety of diseases such as intellectual disability, cancer, congenital dysplasia and have potential clinical application as biomarkers and therapeutic targets.

Introduction

Ribosome, the crucial organelle responsible for protein synthesis, comprises ribosomal RNAs (rRNAs) and ribosomal proteins. rRNA modifications are ubiquitous and can change the local spatial structure of rRNA molecules, thus

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optimizing the protein translation. Meanwhile, rRNA modifications also ensure the heterogeneity of ribosomes to facilitate different functions.[1] With the development of high-resolution ribosomal structure analysis, more and more specific rRNA modification sites have been identified. Recently, ZCCHC4 and METTL5 were identified as methyltransferases that catalyze m^6A modifications on human 28S and 18S rRNAs, respectively. Emerging evidence revealed that rRNA m^6A modification plays critical role in the regulation of ribosome structure and functions, as well as disease pathogenesis. In this review, we first summarize the diversity, characteristics, and significance of rRNA and its modifications. Then, we review the research progresses on the functions, and molecular mechanisms of ZCCHC4 and METTL5 mediated m^6A rRNA modifications in the regulation of mRNA translation and various diseases.

Sequence variation in rRNAs

rRNAs are the key component of the ribosomes where protein synthesis takes place. As the most abundant RNAs in cells, rRNAs account for approximately 80% of the total RNAs. The rRNA types are different between prokaryotes and eukaryotes. There are three kinds of rRNAs in prokaryotes (5S rRNA, 16S rRNA, and 23S rRNA), while in the eukaryotes four kinds of rRNAs are identified (5S rRNA, 5.8S rRNA, 18S rRNA, and 28S rRNA).[1]

The ribosomal DNA (rDNA) is characterized by multiple tandem repeats in Nucleolus Organizing Regions (NORs), locating at the short arms of acrocentric chromosomes (#13, #14, #15, #21, #22 in humans and #13, #14, #15, #21, #22 in mice).[2,3] Due to the instability of the locus, the sequence and copy number of the repeats display extensive inter- and intra-individual variations. Each NOR repeat comprises two parts: the ribosomal part with rDNA sequence and the intergenic sequence (IGS) (Fig. 1).[4] The variable nature of rRNA alleles gives rise to ribosome heterogeneity, but its biological significance is still poorly understood. As the rearrangements of rDNA have been detected in tumor and nutritional stress, the possibility of rDNA variation as a potential pathogenic factor is still being explored.[5,6]

The significance of rRNA modifications

As a multi-component, highly coordinated complex, the eukaryotic ribosome is composed of four kinds of rRNAs and nearly 80 ribosomal proteins.[7] Ribosomal proteins and rRNAs are arranged into two ribosomal subunits of different sizes, known as a small subunit (SSU) and a large subunit (LSU). The SSU and LSU cooperate to translate mRNA into polypeptide chains during protein synthesis. Ribosomal protein assembly begins in the nucleolus and nucleus along with the transcription of rRNA.[8] After the initial assembly of SSU and LSU, the complexes undergo nuclear export and then are further matured in the cytoplasm. The synthetic process involves numerous enzymatic proteins and ribonucleoprotein complexes (RNPs) which mediate the modifications of rRNAs.[9]

Increasing evidence revealed that there are multiple post-transcriptional modifications in different kinds of RNAs. It is worth noticing that rRNA is the second most modified RNA type, containing nearly 2% modified nucleotides.[10] The complete set of modification sites in human 80S ribosomes has been identified lately, and there are 228 modification sites with 14 kinds of modifications.[11] Among the 228 nucleotides, except for the most wide-ranging and extensively studied modification—ribose 2′-OH hydroxyl methylations and isomerizations of uridines to pseudo-uridines (ψ), there are other highly conserved modifications such as

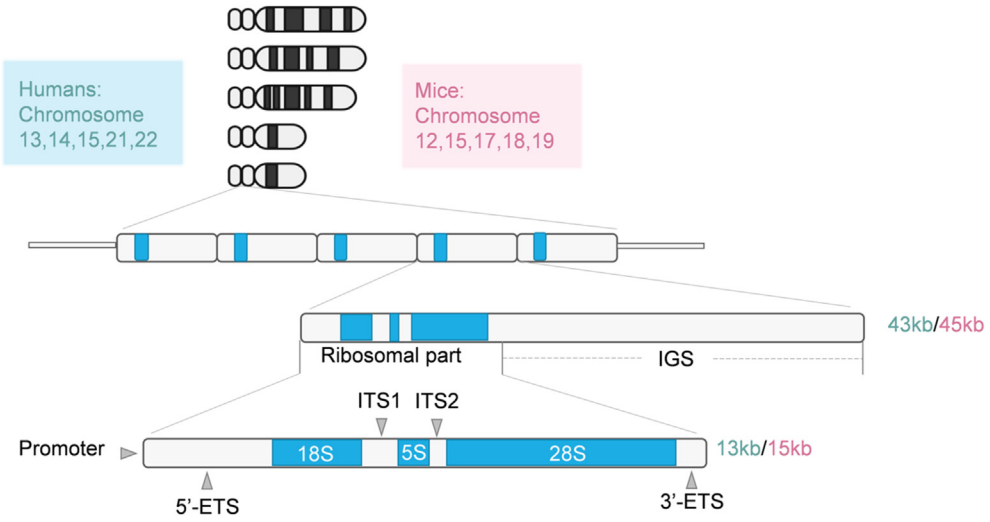

Figure 1 The multi-copy structure of rDNA in humans and mice. Adapted from references 3 and 4. The rRNA genes encoding 18S, 5.8S and 28S exist as tandem repeats on five pairs of chromosomes (the 5S rDNA is on the chromosome 1 in humans and chromosome 8 in mice). A single repeat (43 kb in humans and 45 kb in mice) can be divided into the ribosomal part and the intergenic sequence (IGS). The ribosomal part (13 kb in human and 15 kb in mouse) flanked by external spacers (5′ ETS and 3′ETS) contains three genes separated by internal transcribed spacers (ITS1 and ITS2). The IGS part is a complex with highly repetitive sequences and even more variant than rDNA.

methylations and hypermodifications.[12] Generally, these chemical modifications are installed by enzymatic proteins and ribonucleoprotein complexes (RNPs) to maintain the appropriate structure and function of ribosome.[13–15]

Moreover, rRNA modifications are sources of ribosomal heterogeneity. It was previously recognized that ribosomes are not selective for mRNAs so that they can synthesize all proteins. However, decades of studies have suggested the diversity and heterogeneity of ribosomes.[16] Researchers found that specialized ribosomes are unique to some proteins with certain functions, such as stem cell differentiation and embryonic development.[1,17] Dis-regulation of ribosomal components will lead to a series of rare diseases collectively called ribosomopathies, accompanied by the pathogenesis of congenital hypoplasia, anemia, tumor, and other manifestations.[18,19] Therefore, ribosome modifications are closely related to the assembly and production of ribosome, and have an impact on ribosome heterogeneity and related diseases.

N6-methyladenosine (m^6A) in human rRNAs

N^6-Methyladenosine (m^6A) is one of the most common post-transcriptional modifications on RNAs, affecting multiple RNA metabolism processes, including RNA alternative splicing, translation efficiency, degradation.[20] Moreover, it is widely involved in the regulation of cell proliferation, differentiation, embryonic development, and tumorigenesis.[21–24] The m^6A modification is reversible, mediated by methyltransferases, demethylases, and downstream reading proteins.[25,26] It has been proved that m^6A exists in most RNAs, including mRNA, tRNA, rRNA, microRNA, snoRNA, etc. Nevertheless, most of the previous studies of m^6A focused on mRNA, while the function and mechanism of m^6A on rRNA remain poorly understood.

Early in 1986 and 1988, Maden B.E. et al discovered two m^6A methylation sites on 18S rRNA and 28S rRNA in human and *Xenopus laevis*, which were 18S rRNA m^6A1832 and 28S rRNA m^6A4220.[27,28] However, the methyltransferases responsible for the two rRNA m^6A sites were identified as ZCCHC4 and METTL5 until 2019 (Fig. 2).[29,30] Below we summarize in detail about their structures, functions, and roles in diseases.

The characteristics of ZCCHC4

The structure of ZCCHC4

The ZCCHC4 protein consists of three flanking zinc finger domains and a central methyltransferase (MTase) domain with β-sheets intertwined by α-helices. The flanking domains, including an N-terminal GRF (Gly-Arg-Phe)-type zinc finger domain followed by an adjoining C2H2 zinc finger domain and a C-terminal CCHC zinc finger domain, are required for RNA binding affinity (Fig. 2).[31,32] ZCCHC4 is characterized by an autoinhibitory conformation: a junction sequence, namely the regulatory ring, blocks the catalytic center and therefore bridges the MTase domain and the C-terminal CCHC domain, thus forming a self-inhibitory conformation. In addition, the methyl donor s-adenosine-L-methionine (SAM) binding pocket adopts a completely closed conformation, indicating the binding of the cofactor is in the wake of RNA substrate recognition. Moreover, specific substrate recognition lies in the stem-loop structure of the RNA substrate rather than the overhang sequence.[32]

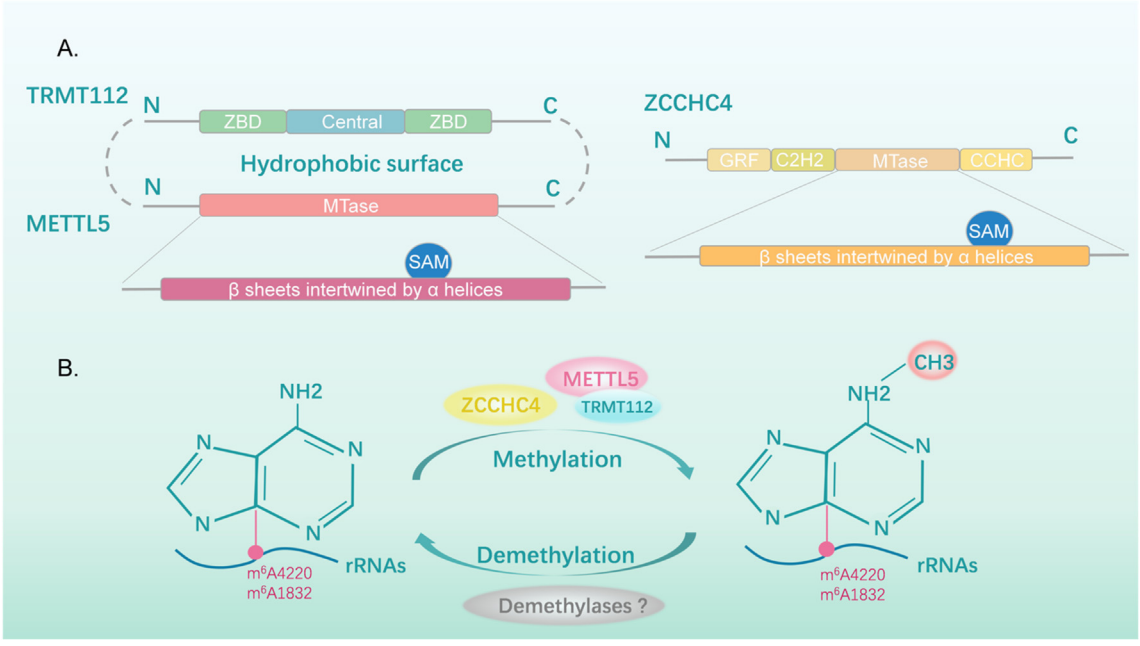

Figure 2 The functional domains and enzymic activity of ZCCHC4 and METTL5. **(A)** Schematic representation of functional domains of ZCCHC4 and TRMT112-METTL5 complex. **(B)** ZCCHC4 and METTL5 mediated the methylation of 28S rRNA m^6A4220 and 18S rRNA m^6A1832, respectively, by adding a methyl group to the sixth nitrogen atom of adenine. Previous studies have shown that m^6A is a reversible modification process, but the demethylase responsible for rRNA demethylation has not been identified.

The role of ZCCHC4 in biological processes

Since ZCCHC4 is the enzyme that mediates the modification of 28S ribosomal RNA, it is intriguing to determine whether its defects will affect the ribosome production. Then 28S subunits can be produced normally in the absence of ZCCHC4 while the overall translation level decreases in ZCCHC4 deficient cells.[30] Compared to WT cells, ZCCHC4 KO cells display up to 25% reduction of translation efficiency, which can be restored by transfection of WT flag-ZCCHC4 plasmid but not catalytically mutant flag-ZCCHC4 plasmid.[30] Striking difference is observed when viewing the codon occupancy of WT and ZCCHC4 KO cells. The ribosomal profiling data reveals that genes representing RNA metabolism and nucleosome are up-regulated while genes related to signal transduction and neural development are down-regulated.[31] Furthermore, ribosome translation is closely related to cell growth. Several researches reported differential conclusions on the effect of ZCCHC4 deletion on cell proliferation. This indefinite conclusion may be due to the usage of different cell lines.[29,30]

The role of ZCCHC4 in tumors

ZCCHC4 is highly expressed in many tumor tissues, revealing the relationship between m^6A of human ribosomal RNA and tumorigenesis. By measuring ZCCHC4 in hepatocellular carcinoma tissues and adjacent tissues, the expression of ZCCHC4 protein is significantly up-regulated in cancerous tissues, and 28S rRNA in cancerous tissues has a higher level of m^6A modification. In the nude mice tumor model, the tumor volume and weight of mice inoculated with ZCCHC4 KO cells are significantly diminished than those of the control group.[30] At present, the significances of ZCCHC4 in other tumors and the tumor-promoting mechanism are still poorly understood. And the report on the phenotype of ZCCHC4 deficient animals and the relationship between ZCCHC4 and diseases other than tumor remains to be characterized.

The characteristics of METTL5

The structure of METTL5

The special feature of METTL5 is that it needs TRMT112 to form a stable complex for its function. And the methylation activity of the complex can be weakened upon increased ionic strength.[33] METTL5 is comprised of a single catalytic domain containing seven-stranded β-sheet with a SAM binding motif flanked by α-helices. TRMT112 has a central domain flanked by zinc binding domain (ZBD) formed by residues of both N-terminal and C-terminal.[29] METTL5 and TRMT112 proteins interact with each other through a large surface formed by 29 METTL5 and 28 TRMT112 residues. When the complex is integrated, METTL5 is covered by TRMT112 to form a large central hydrophobic core, which ensures the metabolic stability of METTL5.[29] Similar to ZCCHC4, METTL5 also has a special recognition effect on the substrate, but the specific mechanism such as the recognition of RNA sequence or RNA length has not been elucidated.[34]

The role of METTL5 in biological processes

Like ZCCHC4, 18S ribosomal subunit can be generated in METTL5 knockout cell lines, but the overall translation efficiency is also reduced.[35] Rong et al further confirmed that in the absence of METTL5, the binding of translation initiation factors to mature ribosomes is attenuated. The phosphorylation level of ribosomal protein S6 kinase 1 (S6K) related to translation initiation is decreased upon METTL5 depletion.[35] Accordingly, these results suggest that the two human rRNA m^6A sites are unlikely to perturb ribosome assembly and biogenesis but may alter the overall translation activity and preference for certain mRNAs.

The effect of METTL5 on cell proliferation has recently been reported in several studies. After knocking down METTL5 in several breast cancer cell lines, the cell growth is significantly inhibited in the METTL5 knockdown group, accompanied by increased apoptosis and G2/M phase arrest.[35] However, the growth of some cells, including HeLa cells, mouse embryonic stem cells (mESCs), and HCT116 cells reported previously, is not affected by METTL5 deficiency, which can be interpreted as the existence of METTL5-independent bypass mechanism to maintain normal cell growth.[35–37]

In mESCs, the absence of METTL5 resulted in significant inhibition of differentiation, and the multipotential stem cells could maintain pluripotency for a longer time. Mechanistically, METTL5 deficiency decreases the level of FBXW7 and results in the accumulation of c-Myc, which delays the differentiation of mESCs.[38] Therefore, METTL5 plays an essential role in the pluripotency and differentiation of pluripotent stem cells.[36] However, METTL5-deficient mice have not completely lost the ability of differentiation and can normally complete embryonic development. It can be necessary to continuously monitor the markers of pluripotency and differentiation during embryonic development to find some compensatory mechanism.

Some studies indicated that METTL5 affects the translation of specific mRNAs under stress.[35,39,40] In B16 melanoma cells of METTL5 knockout mice, the translation efficiencies of multiple stress response related mRNAs including Activating Transcription Factor 4 (ATF4) are impaired. ATF4 is a key transcription factor that mediates the integrated stress response (ISR), including endoplasmic reticulum (ER) stress.[41] During ER stress, the transcription of ISR effector genes in METTL5 KO cells decreases continuously, representing a new mechanism to modulate cellular stress responses.

METL-5 in *C. elegans*, the ortholog of human METTL5, increases the selective translation of CYP-29A3, leading to more oxidation of omega-3 polyunsaturated fatty acid eicosapentaenoic acid to eicosanoids, increasing the sensitivity of multiple internal and external stresses. METL-5 mutant strains of *C. elegans* show no difference in natality and gross morphology but are more resistant to various stresses such as heat shock, cold stress, and ultraviolet (UV) irradiation.[39] Similarly, another study reported that *C. elegans* with METL-5-knockout show a longer

lifespan and stronger stress resistance.[35] When METL-5 is knocked down, the expression of p-S6K related to translation initiation and cell proliferation is also down-regulated. The extended lifespan and enhanced stress resistance of METL-5 knockout worms are consistent with the phenotype of S6K/rsks-1 mutant worms, and this phenotype could be rescued by inhibiting AMP-activated protein kinase (AMPK) signaling pathway.[35] S6K is a crucial effector protein downstream of mammalian target of rapamycin (mTOR) signaling pathway, which can sense the signal changes of extracellular nutrition, energy level and growth factors.[42] When the extracellular environment changes, mTOR regulates cell growth by activating downstream effector protein S6K to promote cell proliferation and protein synthesis. As an important kinase regulating energy homeostasis, AMPK can phosphorylate TSC2 and Raptor, resulting in the down-regulation of mTOR complex 1 (mTORC1).[43] Although more evidence is needed, METTL5 is likely to be mediated by AMPK/mTOR to participate in longevity, nutrition, and stress resistance.

In a recently published study, researchers started with the weight loss of METTL5 knockout mice and found that METTL5 deficiency would lead to metabolic defects. In gross anatomy, a decrease in body fat was also observed in the knockout mice. The difference of gene expression in brain and liver reveals the metabolic changes caused by METTL5 knockout. The metabolic pathways of lipid, lipoprotein, and fatty acid in knockout mice were disordered, accompanied by the down-regulation of Thyroid Hormone Responsive Protein (Thrsp) and accumulation of T3.[44] Overall, these researches show that METTL5 is related to nutrition and metabolic homeostasis and may be used as a therapeutic target for metabolic diseases.

The role of METTL5 in diseases

METTL5 is up-regulated in a variety of tumors, especially in solid tumors.[35] Besides, the expression of its cofactor TRMT112 is also increased in tumor tissues.[40] TCGA analysis reveals that METTL5 is overexpressed in breast cancer, lung adenocarcinoma, and other cancers. The negative correlation between the expression level of METTL5 and survival rate is also proved in the overall survival analysis.[35,45] Together with the other four genes (*RAC1*, *C11of24*, *RCCD1*, and *SLC7A5*), the METTL5-associated prognostic score (MAPS) was constructed to certify the predictive value of METTL5 in lung adenocarcinoma. The validity of MAPS was verified in the GEO database, and MAPS was related to some immune factors and pathways.[45] The reason for the high expression of METTL5 in tumor tissues may be that 18S rRNA methylation can maintain the high expression level of ATF4 in response to the stress response in the tumor microenvironments, such as hypoxia, low pH, and nutrient deficiency, which also raises the possibility that METTL5 can act as a biomarker and a therapeutic target for tumor.[40,46]

METTL5 whole-body knockout mice are viable but exhibit some developmental and behavioral phenotypes, including **(1)** short stature and lightweight; **(2)** low birth rate (reported as $<$12.5%); **(3)** craniofacial dysplasia (reported as nasal bone distortion and incomplete fusion of the frontal bone suture); **(4)** increased hearing thresholds; **(5)** anomalous retrolental tissue (reported as asymmetric with prevalence in the right eye); **(6)** sterility with degenerative lesions in male reproductive system; **(7)** lack of activity and exploratory behavior; **(8)** impaired learning and memory capacities.[36,37] Furthermore, METTL5 is widely expressed in the drosophila central nervous system during embryonic development and is highly expressed in the early development of the human brain with a considerable expression in various substructures until adulthood.[47,48] The intelligence development and learning ability of METTL5 knockout mice are also affected. RNA-seq of brain tissue showed that METTL5 regulates the nervous system development of mice by regulating the myelination process of neurons.[37]

Interestingly, the phenotypes of METTL5 knockout mice share a lot in common with the characteristics of METTL5 deficiency in human diseases. Recently, exon sequencing of the patients revealed that mutations of METTL5 are related to neurodevelopmental disorders.[49,50] Richard et al identified two bi-allelic frameshift variants: c.344_345delGA and c.571_572delAA, which are associated with damaged stability and conformation of METTL5. The truncating variants in the patient family pedigree cause autosomal recessive intellectual disability (ID) and microcephaly. The patients suffer from cognitive abilities and impaired adaptive behaviors, usually accompanied by craniofacial abnormalities, such as overhanging nasal tip, wide nasal base, and abnormal dental morphology. Besides, knocking down METTL5 in embryos of zebrafish results in microcephaly, resembling the human phenotype.[48] The effect of METTL5 on the development of the nervous system has also been verified in drosophila, and the METTL5 knockout drosophila shows disordered movement trajectory and impaired sense of direction.[47] Generally, the above studies elucidated the regulatory role of METTL5 in the development of the nervous system of various model organisms and provided a new molecular mechanism for ID.

Another study mentioned that METTL5 is associated with inflammation-mediated alveolar bone loss (iABL), which is one of the characterizations of periodontitis. iABL is a polygenic trait and relates to eleven genes on chromosome 2 including METTL5. The authors suggested that these genes are related to innate immunity and bone metabolism, especially macrophage and osteoblast function.[51]

Connection among ribosomopathies, neurocristopathies and METTL5

The disorders in ribosome components may cause ribosome stress or nucleolar stress and finally lead to ribosomopathies.[52] Interestingly, ribosomopathies impede embryonic development, but this effect is tissue and organ specific, usually manifested as bone marrow dysfunction, tumor, and craniofacial dysplasia.[19,53] At present, the recognized ribosomal diseases include Diamond-Blackfan anemia (DBA), dyskeratosis congenita (DKC), cartilage hair

hypoplasia (CHH), and Treacher Collins syndrome (TCS), etc.[18] Under normal conditions, p53 and MDM2 form a negative feedback loop, in which MDM2 can degrade p53 through ubiquitin proteasome pathway.[54] However, in the case of ribosome stress, free ribosomal proteins (RPs) such as RPL5, RPL11, RPL23 will bind to MDM2, making MDM2 unable to degrade p53 normally, leading to p53 accumulation and apoptosis.[55,56] Neural crest cells, a kind of pluripotent and migratory progenitor cells, produce most of the cartilage, bone, and connective tissue in the craniofacial region during embryonic development.[57,58] When ribosomal stress occurs in neural crest cells, neural crest cells undergo apoptosis and cannot migrate normally, resulting in craniofacial dysplasia, namely neurocristopathies.[59] Ribosomopathies and neurocristopathies are the general names of a large class of diseases with similar origins. A widely studied disease that belongs to the above two categories is the Treacher Collins syndrome (TCS).[60] TCS is a congenital craniofacial malformation with autosomal dominant inheritance.[61] Its main features include blepharoplasty, eyelid defect, hypoplasia of facial bone, especially for the mandible and zygomatic complex, and deformity of external and middle ear.[62] So far, four genes related to ribosome are known to cause TCS including treacle ribosome biogenesis factor 1 (TCOF1), RNA polymerase I subunit C (POLR1C), RNA pol I subunit D (POLR1D), and RNA polymerase I subunit B (POLR1B).[62–64] Therefore, we can infer that the normal structure and function of ribosomes play an important role in craniofacial development (Fig. 3).

Previous studies demonstrated that epigenetic regulation of DNA methylation and histone modifications can activate the expression of specific genes in neural crest cells and affect the migration of neural crest cells. When epigenetic factors change, it may lead to neural crest development defects.[65–67] The craniofacial dysplasia such as intellectual disability and microcephaly caused by METTL5 mutations in humans and model animals showed common characteristics with ribosomopathies and neurocristopathies. Therefore, dysregulation of the rRNA m^6A modification by METTL5 could be a novel mechanism in craniomaxillofacial development, providing a new approach for the diagnosis and treatment of developmental diseases.

Perspective

Because tumor cells need to synthesize more energy to meet their overgrowth needs, the protein translation is usually accelerated. Therefore, the expression of ribosome-related components including ribosomal proteins and rRNAs are elevated in cancers.[68–70] As mentioned earlier, ZCCHC4 and METTL5 are related to the proliferation of tumor cells. Their expressions are up-regulated in a variety of tumors and associated with poor prognosis of patients. Moreover, TRMT112, the interacting factor of METTL5, is also up-regulated in cancers. Therefore, ZCCHC4 and METTL5 can be used as biomarkers for the diagnosis and prognosis of malignant tumors.

Overall, human rRNA m^6A methyltransferases ZCCHC4 and METTL5 affect the overall translation efficiency in cells and participate in biological processes like cell proliferation and differentiation (Table 1). In addition, they are also involved in the occurrence and progress of a variety of diseases (Table 1), and therefore could serve as biomarkers and therapeutic targets. Although recent studies have provided us with a preliminary understanding of ZCCHC4 and METTL5, more in-depth research should be carried out to uncover the specific molecular mechanisms underlying their functions in regulation of development and disease pathogenesis.

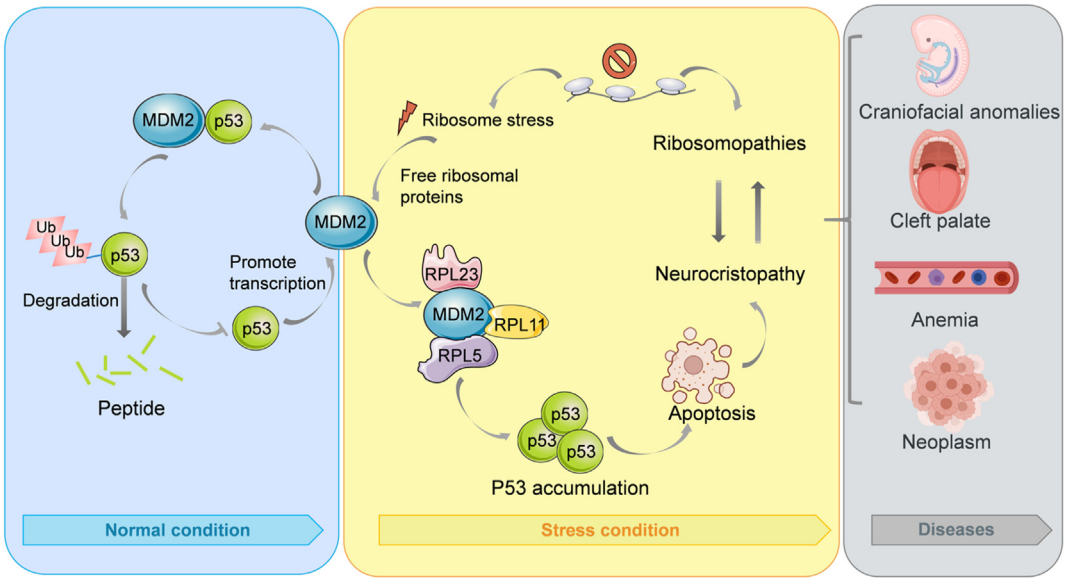

Figure 3 Ribosome stress leads to the disorder of p53-MDM2 negative feedback loop and a series of diseases. p53 and MDM2 form a negative feedback loop under normal circumstances. When abnormal ribosome synthesis leads to ribosome stress, ribosome associated proteins will bind to MDM2, bringing to p53 accumulation and cell apoptosis, which leads to a series of diseases classified into ribosomopathies or neurocristopathies.

Table 1 The comparisons of ZCCHC4 and METTL5.

	ZCCHC4	METTL5
Modification site	➢ 28S rRNA m^6A4220[29,30]	➢ 18S rRNA m^6A1832[29]
Subcellular localization	➢ Nucleus[29]	➢ Nucleus[29] ➢ Cytoplasm[50]
Structural feature	➢ Formed by three flanking zinc finger domains and the central methyltransferase domain[31] ➢ Autoinhibitory conformation[31] ➢ Specific substrate recognition[32]	➢ Interact with cofactor TRMT112[29] ➢ Act like DNA methyltransferase[29] ➢ Specific substrate recognition[34]
Biological function	➢ Translation efficiency[30] ➢ Tumor cell proliferation[30]	➢ Translation efficiency[29,35] ➢ Tumor cell proliferation[35] ➢ Cellular pluripotency and differentiation[36,38] ➢ Stress response[35,39,40]
Animal phenotype	➢ Promotion of hepatocellular carcinoma growth in nude mice[30]	➢ Stronger stress resistance in METTL5 deficient C. elegans[35,39] ➢ Nervous system development in drosophila[47] and mice[37] ➢ Craniofacial dysplasia[36] ➢ Microcephaly in zebrafish[48] ➢ Metabolism disorder in mice[44]
Related diseases	➢ Tumor[30]	➢ Tumor[35] ➢ Intellectual disability[37,49,50,51] ➢ Congenital hypoplasia[48] ➢ Periodontitis[51]

Author contributions

KL wrote the manuscript and designed the figures; SL edited the manuscript; QY provided guidance and revised this manuscript. All authors approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

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