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Review

Genetic and epigenetic defects of the RNA modification machinery in cancer

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Cancer was initially considered to be an exclusively genetic disease, but an interplay of dysregulated genetic and epigenetic mechanisms is now known to contribute to the cancer phenotype. More recently, chemical modifications of RNA molecules – the so-called epitranscriptome – have been found to regulate various aspects of RNA function and homeostasis. Specific enzymes, known as RNA-modifying proteins (RMPs), are responsible for depositing, removing, and reading chemical modifications in RNA. Intensive investigations in the epitranscriptomic field in recent years, in conjunction with great technological advances, have revealed the critical role of RNA modifications in regulating numerous cellular pathways. Furthermore, growing evidence has revealed that RNA modification machinery is often altered in human cancers, highlighting the enormous potential of RMPs as pharmacological targets or diagnostic markers.

Highlights

In recent years, a growing list of RNA modifiers has been found to be dysregulated in human cancer.

RNA modifications regulate numerous cellular processes, some of which have only recently been identified.

RNA modifiers were identified as new diagnostic and/or prognostic markers in numerous cancers, making them new therapeutic targets.

Several chemical compounds targeting RMPs are currently under development.

Epitranscriptomics as a new layer of gene regulation in cancer

Cancer is a microevolutionary process that was first considered to be driven exclusively by genetic alterations. Further investigation revealed that another important layer of gene regulation, epigenetics – which encompasses the mechanisms of DNA methylation, histone modification, and chromatin organization – also contributes to cancer development. Epigenetic mechanisms are regulators of gene expression, and epigenetic changes in cancer occur mainly through the downregulation of **tumor-suppressor genes** (see Glossary) and the activation of **oncogenes** [1].

Recent research has revealed a third layer of gene regulation, the so-called epitranscriptome, that was also found to be involved in modulating characteristic cancer events collectively known as hallmarks [2]. Epitranscriptomic regulation considers post-transcriptional chemical modifications in coding and noncoding RNA (ncRNA) molecules that affect their function and homeostasis within the cell. Cellular enzymes known as RMPs are engaged within the cell to deposit (writers), remove (erasers), and read (readers) RNA modifications. More than 170 distinct chemical modifications have been identified in RNA molecules in all living organisms [3]. Most of these modifications were initially found in the most abundant RNAs within the cell, such as ribosomal RNA (rRNA) and transport RNA (tRNA). Major technological advances led to the identification of chemical modifications in less abundant types of RNA molecules, including messenger RNA (mRNA), micro RNA (miRNA), and long noncoding RNA (lncRNA) [4]. The importance of RNA modifications in cell behavior is supported by the growing list of RMPs that are dysregulated through genetic or epigenetic mechanisms in various human diseases, including cancer [3,5]. Furthermore, recent evidence has revealed that the aberrant expression of RMPs affects cell survival, proliferation, self-renewal, differentiation, invasion, stress-response, DNA damage response, and resistance to therapy [6]. In this review we focus on the most recent findings about RNA modifications/RMPs that are dysregulated in human cancers, highlighting their impact on tumorigenesis and potential for therapeutic targeting (Figure 1, Table 1).

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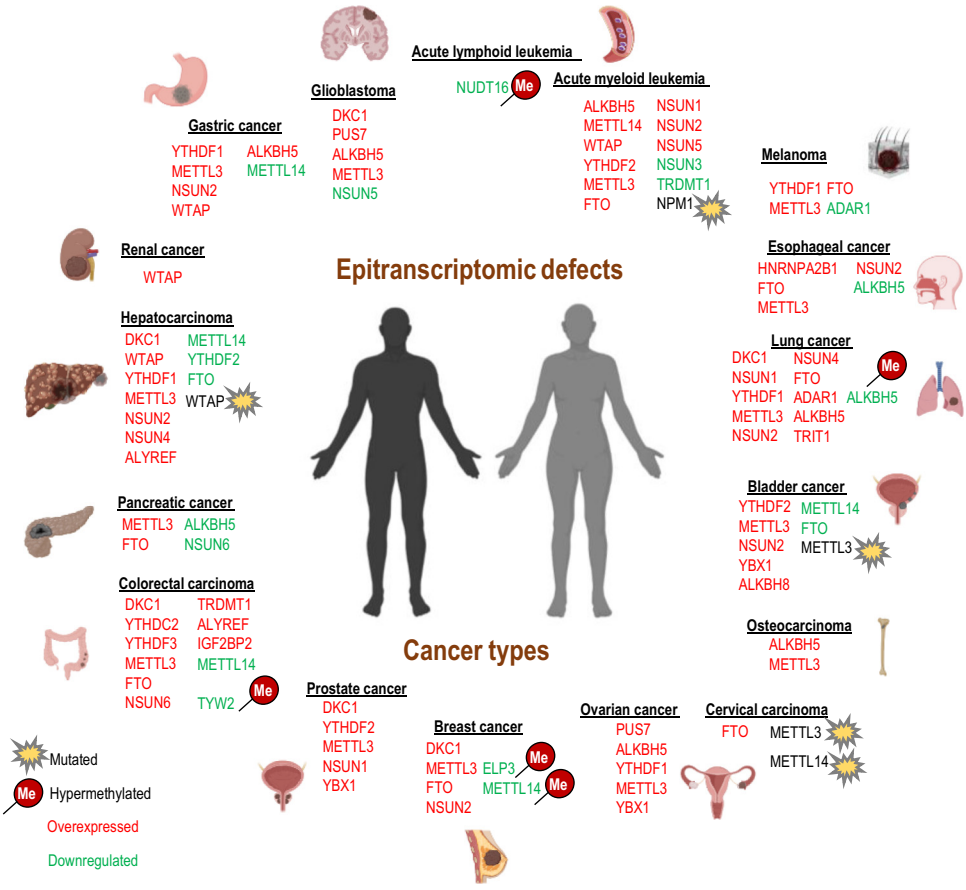

Figure 1. Dysregulated RNA modifiers in human cancers. Changes in the expression of RNA modifiers are illustrated for various cancer types. Sex-specific cancers are shown below the male and female icons. Overexpression and downregulation are indicated in red and green, respectively. RNA modifiers affected by mutations or hypermethylation changes are indicated. The image was created using the BioRender tool (https://biorender.com/).

Cancer-associated RNA modifications

N6-methyladenosine (m^6A) modification

m^6A is one of the most abundant and most studied RNA modifications identified in coding and ncRNA molecules. The majority of m^6A deposition is carried out by a protein complex composed of catalytic subunit methyltransferase-like 3 (METTL3) and METTL14, together with WTAP, KIAA1429, ZC3H13, HAKAI, RBM15, and RBM15B regulatory partners [7]. Other identified m^6A writers are METTL16, METTL5–TRMT112 complex, and ZCCHC4 involved in deposition of m^6A on U6 small nuclear RNA (snRNA), 18S rRNA, and 28S rRNA, respectively [8–10]. m^6A is dynamic and can be reverted by two erasers belonging to the alkB homolog (ALKBH) family, the fat mass and obesity protein (FTO), and the ALKBH5 protein. m^6A readers ultimately determine the fate of m^6A-modified RNAs and influence various aspects of RNA metabolism (splicing, export, translation, degradation, stability, etc.). The list of m^6A readers is still growing and currently encompasses five members of the YTH domain-containing family of proteins (YTH), YTHDC1–2, and YTHDF1–3, eukaryotic initiation factor 3 (eIF3), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), and three members of insulin growth factor 2 mRNA binding proteins (IGF2BP1/2/3) [7,11].

Glossary

ADAR: adenosine deaminases that act on RNA.
Alu elements: noncoding repetitive transcripts.
BER: base excision repair; cellular mechanism of DNA repair engaged in the removal of small base lesions.
Chromothripsis: mutational process characterized by extensive chromosomal rearrangement in a single event.
DDR: DNA damage response; a cellular signaling pathway that is able to sense and repair damaged DNA or at least to minimize the impact of lesions on cellular homeostasis.
Doxorubicin: DNA damage-inducing cancer therapeutic agent.
EIF3C: component of the eukaryotic translation initiation factor 3 (EIF-3) complex, which is required for several steps in the initiation of protein synthesis.
EZH2: enhancer of zeste homolog 2; lysine methyltransferase involved in lysine 27 histone 3-trimethylation (H3K27me3) and the catalytic subunit of polycomb repressive complex 2 (PRC2), which is involved in gene regulation.
IRES: internal ribosome entry site; specific RNA sequences found in the 5'UTR region of some mRNA molecules required for translation independently of translation initiation factors.
m^1A: methylation of the adenosine base at the N1 position.
m^6A: methylation of adenosine at the N6 position.
m^5C: methylation of cytosine 5.
m^2_2G: N2,N2-dimethylguanosine.
Micronuclei: aberrant nuclear structures formed as a result of chromosome mis-segregation.
NPM1: nucleophosmin, a multifunctional phosphoprotein localized mainly in nucleoli.
2'-O-Me: methylation of the 2' hydroxyl (-OH) group of the ribose moiety of any nucleotide.
Oncogene: a genetically or epigenetically altered gene that has the potential to cause cancer.
PRMT5: protein arginine methyltransferase
Pseudouridylation (Ψ): isomerization of uridine to pseudouridine.
R-loops: structures composed of hybrid DNA–RNA and displaced single-stranded DNA that are a potential source of DNA damage.
RNA editing: a nonreversible RNA modification that involves adenine (A)

m^{6}A in human cancers

Dysregulation of all components involved in m^{6}A modifications have been linked to cancer, although through a dual role (as an oncogene or tumor suppressor) depending on the cancer type and the RNA molecules affected by the modification [12]. METTL3 is especially well investigated, and its overexpression was mostly correlated with oncogenic activities, although in several cancer types its tumor-suppressive or dual role was identified (extensively reviewed elsewhere [13]). More recently, deposition of m^{6}A by METTL3 was shown to regulate genome stability (Box 1) and the recruitment of **TET** proteins on DNA, having an impact on gene expression [14]. In addition, METTL3 was recently identified as a potential therapeutic target (Box 2) and regulates specific processes through the interplay with other RMPs (Box 3). Recently, overexpression of METTL16 was correlated with poor survival of patients with gastric cancer. It has been shown that METTL16 mediates m^{6}A deposition within cyclinD1 mRNA leading to its stabilization and cell cycle progression. By contrast, overexpressed METTL16 was correlated with a good prognosis in patients with liver cancer [15]. Another writer, METTL14, exhibited an antimetastatic effect in clear-cell renal-cell carcinoma. It has been shown that m^{6}A methylation deposited by METTL14 stabilize the lncRNA LSG1 that in turn binds to epithelial specific splicing regulator 2 protein (ESPR2), promoting its degradation and resulting in increased cell migration and invasion. Interestingly, the YTHDC1 reader acts competitively to bind LSG1, uncovering a fine-tuning mechanism between writer and reader to regulate the metastatic process in cancer [16]. Another m^{6}A writer, ZCCHC4, was identified to promote chemoresistance of cells to DNA-damaging agents by binding lncRNA AL133467.2 and preventing its interaction with γH2AX, inhibiting apoptosis induced by DNA damage. The expression of ZCCHC4 was significantly elevated in hepatocellular carcinoma, suggesting a bad prognosis [17]. One example of m^{6}A reader dysregulation is gene amplification and overexpression of YTHDF1 observed in patients with ovarian cancer, where it was associated with poor prognosis affecting the overall survival. Mechanistically, overexpression of YTHDF1 promotes translation of mRNA coding for **EIF3C**, thereby modulating the overall translational rate, thus contributing to tumorigenesis and facilitating the metastatic process [18]. Recently, m^{6}A was also shown to play an important role in telomere maintenance [19]. In addition, two m^{6}A erasers, FTO and ALKBH5, are often dysregulated in human cancers, and are suggested as potential therapeutic targets (Box 2), and ALKBH5 interacts with other RMPs (Box 3).

N1-methyladenosine (m^{1}A) modification

m^{1}A is moderately abundant in humans and decorates tRNAs and rRNAs. m^{1}A writers are the tRNA methyltransferases TRMT6 and TRMT61A, and rRNA processing 8 (RRP8), depending on the type of RNA molecule [20]. In human mitochondrial tRNA (mt-tRNA), TRMT10C and TRMT61B catalyze the m^{1}A in position 9 (m^{1}A9) and 58 (m^{1}A58) (Figure 2) [21]. ALKBH1 and ALKBH3 demethylase are known to erase the m^{1}A marks [22]. Interestingly, m^{1}A modification shares erasers and readers with the m^{6}A modification. These include FTO, which can catalyze the m^{1}A tRNA demethylation, repressing translation [23]. Furthermore, YTHDF1–3 and YTHDC1, described as readers for m^{6}A in the previous section, also bind m^{1}A RNAs and regulate their function. Another member of the AlkB family, ALKBH7, was recently identified as a demethylase required for removing m^{1}A but also **m$^2_2$G** marks within mitochondrial polycistronic RNA, thereby regulating its processing [24]. The initial studies investigating m^{1}A in mRNA molecules presented different results in terms of number of m^{1}A sites in transcripts (473 versus 9) [22,25]. However, these findings were later explained by different experimental conditions between the studies [21]. More recent experiments based on modified HIV-1 reverse transcriptase (RT) enzyme specific to m^{1}A modification identified more than 500 m^{1}A sites in human mRNA transcripts [26]. Even if the exact number of m^{1}A methylation sites within mRNAs is still not clear, some studies highlighted that m^{1}A modification in mRNA regulates its expression. The

and cytosine (C) nucleotides that are converted to inosine (I), and uracil (U).
RNaseH: ribonuclease H; an endonuclease enzyme that catalyzes the cleavage of RNA in an RNA/DNA substrate.
TCGA: The Cancer Genome Atlas; a publicly available database gathering data from a large number of human cancers.
Temozolomide (TMZ): antiproliferative alkylating agent.
TET: 10-11 translocation dioxygenases; enzymes involved in DNA demethylation.
Tumor-suppressor genes: genes encoding proteins that regulate cell growth.
Z-RNA: RNA with a left-handed double-helix structure.

Table 1. Overview of genetic alterations within RNA modifier protein coding genes described in this review[a]

Gene	RNA modification	Type	RNA target	Genomic position	Genomic alteration	Cancer type (frequency %)
HAKAI	m^6A	Writer	mRNA	7q 22.3–31.1	Mutations	UCEC[b] (3.87)
KIAA1429 (VIRMA)	m^6A	Writer	mRNA	8q22.1	Amplification	BLCA (6.33), BRCA (9.41), PRAD (8.1), LIHC (7.26), OV (6.51)
METTL14	m^6A	Writer	mRNA	4q26	Mutations	UCEC (4.16)
METTL16	m^6A	Writer	mRNA, ncRNA	17p13.3	NA	NA
METTL3	m^6A	Writer	mRNA, ncRNA	14q11.2	Mutations	BLCA (4.38)
RBM15	m^6A	Writer	mRNA	1p13.3	Mutations	UCEC (4.91), COAD (3.87)
RBM15B	m^6A	Writer	mRNA	3p21.2	NA	NA
WTAP	m^6A	Writer	mRNA	6q25.3	NA	NA
ZC3H13	m^6A, m^1A	Writer	mRNA	13q14.13	Mutations	UCEC (12.1), STAD (7.73), COAD (5.56), BLCA carcinoma (3.89), SKCM (5.63), LUAD (4.42)
					Deep deletion	PRAD (12.1), BLCA (4.38)
ALKBH5	m^6A	Eraser	mRNA	17p11.2	Amplification	SARC (8.63)
HNRNPA2B1	m^6A	Reader	mRNA, pri-miRNA	7p15.2	NA	NA
IGF2BP2	m^6A	Reader	mRNA	3q27.2	Amplification	LSCC (33.86), OV (17.98), CSCC (13.8), HNSC (13.77)
NPM1	m^6A, 2-O-M	Regulator, writer	mRNA, tRNA, snRNA, rRNA	5q35.1	Amplification	KIRC (6.85)
					Mutations	AML (27)
ALKBH7	m^6A, m^1A	Eraser	mtNApre-tRNA	19p13.3	Amplification	SARC (4.31)
FTO	m^6A, m^1A	Eraser	mRNA, tRNA, snRNA	16q12.2	Mutations	UCEC (3.78)
YTHDC1	m^6A, m^1A	Reader	mRNA, ncRNA	4q13.2	Mutations	UCEC (7.56), SKCM (5.18)
YTHDF1	m^6A, m^1A	Reader	mRNA, ncRNA	20q13.33	Amplification	COAD (6.9), OV (6.34), LUAD (4.06), BRCA (4.06)
YTHDF2	m^6A, m^1A	Reader	mRNA	1p35.3	NA	NA
YTHDF3	m^6A, m^1A	Reader	mRNA, ncRNA	8q12.3	Amplification	LIHC (6.18), BRCA (5.54), PRAD (4.86)
					Mutations	UCEC (4.16)
YTHDC2	m^6A, m^1A	Reader	mRNA	5q22.2	Mutations	UCEC (8.88), COAD (4.71), SKCM (4.95)
KIAA1429 (VIRMA)	m^1A	Writer			Mutations	BLCA (5.11), UCEC (7.94), SKCM (7.88), STAD (4.77), LUAD (4.06), COAD (4.38)
RRP8	m^1A	Writer	rRNA	11p15.4	Mutations	UCEC (5.29)
TRMT10C	m^1A	Writer	mt-tRNA	3q12.3	Amplification	LSCC (6.37), CSCC (4.71)
TRMT6	m^1A	Writer	tRNA, mRNA	20p12.3	NA	NA
TRMT61A	m^1A	Writer	tRNA, mRNA	14q32.32	NA	NA
TRMT61B	m^1A	Writer	mt-tRNA	2p23.2	NA	NA
ALKBH1	m^1A	Eraser	tRNA	14q24.3	NA	NA
ALKBH3	m^1A	Eraser	tRNA, mRNA	11p11.2	NA	NA
NSUN1 (NOP2)	m^5C	Writer	rRNA	12p13.31	Amplification	OV (5.65), BLGG (4.86)
					Mutations	SKCM (5.18)
NSUN2 (TRM4)	m^5C	Writer	mRNA, tRNA	5p15.31	Amplification	LSCC (11.91), LUAD (9.19), BLCA (7.79), OV (6.85), CSCC (4.78)

(continued on next page)

Table 1. (continued)

Gene	RNA modification	Type	RNA target	Genomic position	Genomic alteration	Cancer type (frequency %)
					Mutations	UCEC (6.62)
NSUN3	m^5C	Writer	mt-tRNA	3q11.2	Amplification	LSCC (8.21), CSCC (4.71), HNSC (3.82)
NSUN4	m^5C	Writer	mt-rRNA	1p33	Amplification	OV (5.14)
NSUN5	m^5C	Writer	rRNA	7q11.23	NA	NA
NSUN6	m^5C	Writer	tRNA, mRNA	10p12.31	Mutations	UCEC (4.73)
TRDMT1 (dnmt2)	m^5C	Writer	tRNA	10p13	Mutations	UCEC (3.97)
ALYREF	m^5C	Reader	mRNA	17q25.3	Amplification	LIHC (4.3)
YBX1	m^5C	Reader	mRNA	1p34.2	Amplification	OV (6.34), BRCA (3.89)
ADAR1	A-I	Writer	mRNA, tRNA, miRNA	1q21.3	Amplification	LIHC (10.48), LUAD (8.66), BRCA (8.21), BLCA (4.14), OVC (3.94)
					Mutations	SKCM (4.05), UCEC (5.86)
DKC1	Ψ	Writer	mRNA	Xq28	Mutations	UCEC (4.73)
NOP10	Ψ	Writer	rRNA	15q14	NA	NA
PUS7	Ψ	Writer	tRNA, mRNA	7q22.3	Mutations	UCEC (4.54)
FBL	2-O-M	Writer	rRNA	19q13.3	Amplification	OV (8.73), LSCC (5.75)
CMTR1	2-O-M	Writer	mRNA, snoRNA	6p21.2	Amplification	OV (4.97)
					Mutations	SKCM (4.05), UCEC (5.29)
CMTR2	2-O-M	Writer	mRNA, snRNA	16q22.2	Mutations	UCEC (6.05), LUAD (5.48), SKCM (5.63)
SNU13	2-O-M	Writer	rRNA	22q13.2	NA	NA
ELP3	cm5U, ncm5U mcm5U, mcm5s2U	Writer	tRNA	8p21.1	Deep deletion	LIHC (6.18), PRAD (6.07), OV (6.16), BLCA (5.35), COAD (4.38), LUAD (4.77), LSCC (4.31), BRCA (4.06)
					Mutations	UCEC (4.73)
TRIT1	i6A	Writer	tRNA	1p34.2	Amplification	OV (8.22), BLCA (6.33)
NUDT16	$m^7Gpp(pN)$	Eraser	mRNA	3q22.1	Amplification	LSCC (6.37), CSCC (5.72)
ALKBH8	mchm5U	Writer	tRNA	11q22.3	mutations	UCEC (3.78)
TRMT12 (TYW2)	o2yW	Writer	tRNA	8q24.13	Amplification	OV (25.68), BRCA (12.36), LIHC (10.75), STAD (7.73), UCEC (4.16), BLCA (5.6), PRAD (7.49), HNSC (7.27), LUAD (5.48)

[a]cBioPortal for Cancer Genomics based on TCGA PanCancer Atlas Studies (10 967 samples). Cancer type and frequencies are listed in tumors with more than 200 samples available and with the frequency higher than 3.75%.
[b]Abbreviations: AML, acute myeloid leukemia; BLCA, bladder urothelial carcinoma; BLGG, brain lower grade glioma; BRCA, breast invasive carcinoma; CSCC, cervical squamous-cell carcinoma; COAD, colorectal adenocarcinoma; HNSC, head and neck squamous-cell carcinoma; KIRC, kidney renal clear-cell carcinoma; LIHC, liver hepatocellular carcinoma; LSCC, lung squamous-cell carcinoma; LUAD, lung adenocarcinoma; NA, not altered; OV, ovarian serous cystadenocarcinoma; PRAD, prostate adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; UCEC, uterine corpus endometrial carcinoma.

methylation on adenine71 in the first exon of mRNA coding for ATP5D inhibits its translation elongation by increasing the binding of YTHDF1/eRF1 complex, thereby affecting glycolysis in cancer cells [27]. Another study showed that demethylation of m^1A by ALKBH3 on Aurora A transcript enhances its stability, leading to a promotion of ciliogenesis in vertebrates (human RPE-1 cells and zebrafish) [28]. However, further studies are necessary to completely elucidate the effect of m^1A within mRNA molecules [29].

Box 1. RNA modifications and genome stability

Double-strand breaks (DSBs) are one of the most important sources of genomic instability within the cell. A recent investigation revealed that RNA molecules are indirectly involved in DNA DSB repair through the regulation of the expression of other factors necessary for the repair, through ncRNAs or the regulation of DNA–RNA hybrids, the so-called **R-loops** [94].

m^6A has an important function in regulating R-loop accumulation. Deposition of the m^6A mark within R-loops contributes to their removal, and cells depleted for METTL3 accumulate R-loops due to the absence of m^6A modifications (Figure 2). Although the exact mechanism for R-loop removal is not fully determined, the same study highlighted the important role of YTHDF2 in this process, as the depletion of YTHDF2 leads to increased levels of DNA DSBs [95]. Another study indicated that m^6A is selectively deposited on RNA related to DSBs through the involvement of METTL3 in the **DDR** [96]. Another recent study highlighted the opposite effect on the accumulation of R-loops [97]. There is a possibility that contradictory results are due to the different cellular contexts of R-loop formation, although this speculation requires additional studies.

Recent research identified the key role of ADAR1 p110 isoform in regulating the extent of R-loops in telomeric repeats. It has been shown that ADAR1 p110-mediated editing within R-loops facilitates their resolution by **RNaseH** [98]. This function of ADAR1 p110 is crucial, since the ineffective resolution of R-loops may cause telomeric instability. ADAR2 protein also acts on DDR, but this time through the regulation of DNA–RNA hybrids that are formed at DSB sites as one of the initial processes in DNA repair [99]. Cells depleted in ADAR2 are more sensitive to genotoxic agents, identifying ADAR2 as a potential target in synthetic lethal cancer treatments. As described previously, editing events contribute to the resolution of R-loops, thereby lowering the potential source of DNA damage within the cell. However, the enhanced editing by the ADAR1 p150 isoform was also described as a source of DNA damage under some specific circumstances. During **chromothripsis**, enhanced editing of R-loops within **micronuclei** in cells with pathological **BER** results in fragmentation of micronuclei chromosomes [100]. We may speculate that the mutational signature of some specific cancers is due to the amplification of the ADAR1 gene and its enhanced catalytic activity.

m^1A as a diagnostic and prognostic marker

In the last 2 years, tremendous research effort has been expended to determine the relevance of genes coding for m^1A modifiers in tumorigenesis and their potential value as diagnostic or prognostic markers. Based on the **TCGA** data analysis, m^1A modifiers exhibited distinct levels of expression between paired cancer and normal tissue and were overexpressed in major cases. Specifically, the global expression profile linked to the m^1A modifiers in the liver hepatocellular

Box 2. RMPs as therapeutic targets

FTO demethylase is one of the most promising targets in cancer therapy, as the overexpression of FTO promotes tumorigenesis, progression, and resistance to chemotherapy, radiotherapy, and immunotherapy in several types of human cancers [101]. Polymorphisms within the gene coding for FTO are also known to contribute to the development of several human disorders, with increased cancer susceptibility [102]. Two recently identified small-molecule inhibitors (CS1 and CS2) were shown to target FTO and to reduce m^6A demethylation *in vitro* in AML, breast cancer, glioblastoma, and pancreatic cell lines, with confirmed *in vivo* efficacy in AML and breast cancer models. Moreover, the inhibition of FTO was shown to suppress immune evasion and hamper leukemia stem/initiating cell (LSC/LIC) self-renewal [103]. Another study revealed the potential for synthetic lethal interactions between the inhibition of FTO and VHL tumor-suppressor in renal clear-cell carcinoma [104]. The ethyl ester form of meclofenamic acid (MA2), another FTO-targeting compound, was shown to enhance the effect of the chemotherapeutic drug **temozolomide** by suppressing the proliferation of glioblastoma cell lines [105]. However, targeting FTO can also be unfavorable, as the downregulation of FTO seems to play a dual role depending of the cancer type; it was recently shown that a hypoxic tumor environment induces FTO ubiquitin-mediated protein degradation, promoting metastasis of colorectal cancer cells *in vitro* and *in vivo* [106]. Besides FTO, another m^6A demethylase, ALKBH5, was also considered to have a dual tumorigenic effect, depending on the tumor type. Overexpression of ALKBH5 was shown to be an oncogene in several tumor types, and two recently identified ALKBH5 inhibitors were found to reduce proliferation in AML cell lines [107]. An additional study confirmed that the deletion or inhibition of ALKBH5, using small-molecule inhibitors in melanoma and colon cancer mouse models, enhances the response to immunotherapy [108]. In addition, new studies have identified novel compounds for investigating the biological functions of ALKBH5 inhibition in the context of cancer [109]. Although most attempts to target RMPs involve demethylase inhibitors, the drug-targeting catalytic activity of METTL3, namely STM2457, has recently been discovered, and its efficiency has been tested in AML cell lines and xenograft mouse models. It has been shown that the application of STM2457 increases differentiation and apoptosis, thereby reducing AML growth. The *in vivo* application of the latter exhibited impaired engraftment and prolonged survival in various mouse models of AML [110]. The same compound was used in a study where the m^6A mark proved to be crucial for the conversion of B cells to macrophages; the inhibition of METTL3 or shRNA targeting METTL3 reduced the m^6A content, with a subsequent negative effect on cellular transdifferentiation [111].

Box 3. Interplay between RNA modifications/RMP

Many recent and current epitranscriptomic studies have highlighted the role of individual RNA modifications and their role in cancer and other human diseases. However, interest in investigating the interplay between different types of modifications is growing substantially. A recent study investigated the role of RNA modifications in the chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells (BMSCs). Simultaneous Nsun4-mediated m^5C and Mettl3-mediated m^6A modification in the 3′UTR of mRNA coding for Sox9 transcription factor enhance its translation and chondrogenic differentiation. Furthermore, it has been confirmed that this regulation is mediated by the formation of a complex between Nsun4, Mettl3, Ythdf2, and eEF1α-1 [112]. Another interesting example is the interplay between m^6A and A–I, which has an important role in regulating innate immune cell response. Mechanistically, modifications within the conserved m^6A site in mRNA coding for interferon-inducible ADAR1p150 are recognized by YTHDF1, which mediates ADAR1 translation, leading to the induction of ADAR1-mediated A-I editing and preventing excessive activation of interferon (IFN) responses [113]. In glioblastoma, ADAR1 was identified as a new target of METTL3, exhibiting a pro-oncogenic role by an editing-independent mechanism. In short, upregulated METTL3 methylates ADAR1 mRNA and increases its protein level through YTHDF1 binding. ADAR1 in turn stabilizes CDK2, promoting cell proliferation [114]. Besides the interplay between different types of modifications, it has been shown that direct interaction between RMPs involved in distinct modifications may affect sensitivity of cells on chemotherapeutic drugs. As described in Box1, m^6A modification regulates DDR. Furthermore, **doxorubicin** was shown to elevate cellular m^6A mark. In breast cancer, upon doxorubicin treatment **PRMT5** methylates cytoplasmic ALKBH7 leading to its stabilization. ALKBH7 in turn binds to ALKBH5 promoting its translocation to the nucleus where it removes m^6A from BRCA1 coding mRNA thereby enhancing its stabilization and expression. In this way wild-type BRCA1 cancers overexpressing PRMT5 may confer sensitivity to DNA-damaging agents that can be reverted with simultaneous application of PRMT5 inhibitor and doxorubicin [115].

carcinoma was significantly higher [30]. Amplification and overexpression of m^1A writers (TRMT6, TRMT61A, TRMT10C), erasers (ALKBH1, ALKBH3), and readers (YTHDF1–3, YTHDC1) was identified in gastrointestinal cancers, where this event was related to the clinical stage of cancer and was found to affect PI3K/AKT/mTOR and ErbB pathways. The effect on these pathways was confirmed by RNA sequencing (RNA-seq) data obtained from HEK293T cells upon ALKBH3 knockdown [31]. Moreover, promoter DNA-methylation silencing of ALKBH3 was identified in Hodgkin lymphoma, leading to an increase of m^1A in two collagen transcripts (COL1A1 and COL1A2) and conferring poor clinical outcomes [32]. In pancreatic cancer, a low level of expression of ALKBH1 is related to a poor prognosis, and it has been suggested that ALKBH1 also activates or regulates the mTOR and ErbB signaling pathways [33].

Ten m^1A-related regulatory genes were also identified as a part of the gene signature in the TCGA dataset of hepatocellular carcinoma – taking into account RNA expression, copy number variants (CNVs), mutations, and clinical characteristics – where mutations within these genes were identified in 6.33% of patients. The high level of expression of four of these regulators (TRMT6, TRMT61A, TRMT10C, and YTHDF1) was associated with a bad prognosis and had a negative impact on the overall survival of patients with hepatocellular carcinoma (HCC) [34]. Furthermore, by an unsupervised clustering approach, it was possible to identify three specific expression patterns of m^1A regulators with different tumor microenvironment immune cell infiltration profiles in ovarian cancer [35]. As a new tool, this kind of m^1A score, which is correlated with the immune microenvironment, can also be used to predict the outcome in HCC and oral squamous-cell carcinoma (OSCC) [36]. A recent study in colon cancer highlighted different distributions of m^1A within lncRNAs between tumor and healthy tissue. However, a more detailed analysis of this event is needed to fully elucidate its impact on the tumorigenic process [37].

N5-methylcytosine (m^5C) modification

m^5C occurs in all types of RNA molecules. It is catalyzed by seven enzymes belonging to the NOL1/NOP2/SUN family (NSUN1–7) and the DNA methyltransferase homolog DNMT2 [20]. As found in DNA, m^5C within RNA is dynamic and can be erased by the TET family of proteins and ALKBH1, and the readers for this modification are ALYREF and YBX1.

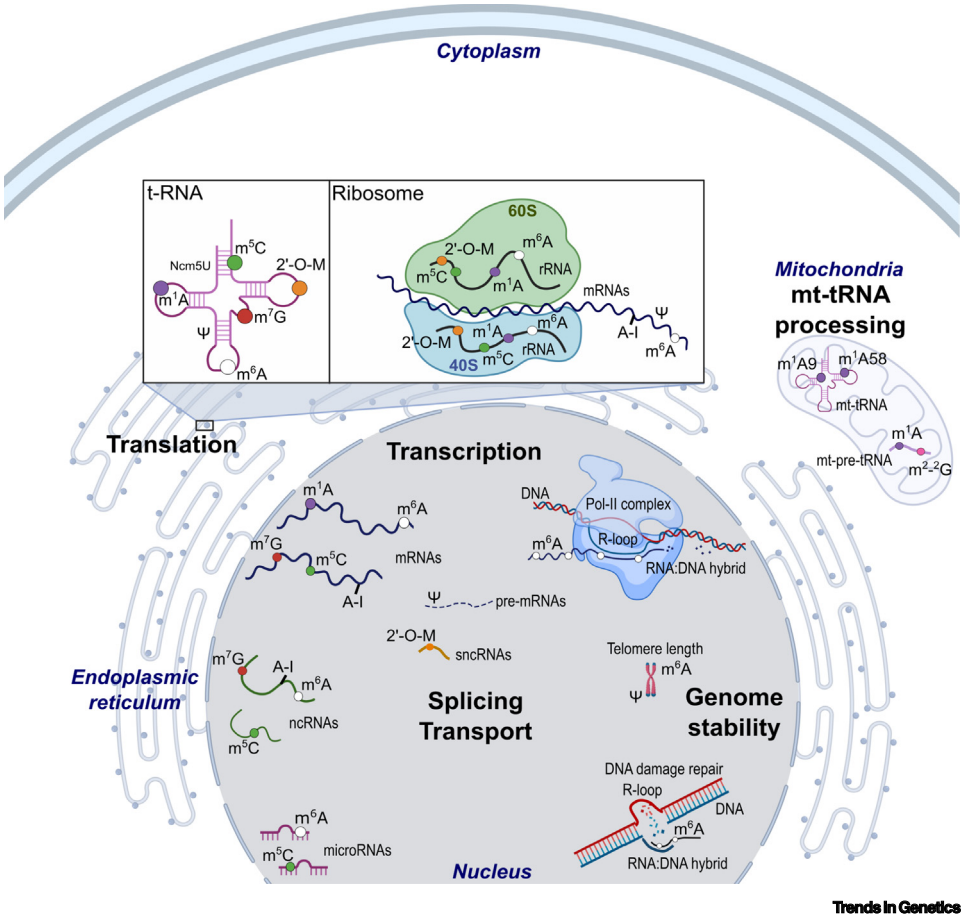

Figure 2. Subcellular localization of the described RNA modifications in the cell and their involvement in specific cellular processes. Dysregulation of RNA modifications affects translation, genome stability, and mitochondrial gene expression regulation. Specific modifications on tRNA and rRNA involved in the regulation of translation are highlighted in squares. Other RNA modifications are highlighted according to the cellular compartment in which the regulated process occurs. The image was created using the BioRender tool (https://biorender.com/).

m^{5}C in the context of cancer

YBX1 was recently shown to recognize m^{5}C marks within lncRNAs, causing their stabilization and affecting their oncogenic or tumor-suppressive role in different ways according to the cancer type [38,39]. In bladder cancer, m^{5}C modification deposited by NSUN2 and recognized by YBX1 in the 3′ untranslated region (UTR) of oncogenic heparin-binding growth factor (HDGF) mRNA causes its stabilization, thereby driving the tumorigenic process. Furthermore, the elevated level of coexpression of NSUN2, YBX1, and HDGF was associated with poor patient survival [40]. m^{5}C writers and readers were shown to be upregulated and mutated in gastrointestinal cancer, affecting the ErbB and PI3K–Akt signaling pathways and revealing GSK3B to be a downstream target of m^{5}C regulators [41]. m^{5}C profiles of mRNA that were distinct from those in the adjacent normal tissue were also identified in HCC [42]. Most recently, NSUN2 has been the focus of many studies that have found alterations involving overexpression to be common events in breast, colon, and lung carcinoma [43]. Furthermore, NSUN2-mediated methylation was identified as a potential biomarker, since aberrant modification of H19 lncRNA is associated with poor differentiation in HCC [44]. It was recently shown that NSUN3 can directly methylate mt-tRNA at the C34

position on a mt-RNAmet, and that mechanism is necessary to allow metastasis formation derived from oral carcinoma cell lines. The inhibition of NSUN3 leads to an extinction of mitochondrial energy production critical for the initiation of the metastasis. Furthermore, the authors identified a NSUN3 gene signature that is prognostic for lymph-node metastasis and for higher pathological stage in patients with HNSCC [45]. In glioma, epigenetic inactivation of NSUN5 by promoter DNA methylation was observed to lead to hypomethylation of 28S rRNA and to drive the specific translational program that enables cell survival under stress conditions [46]. In glioblastoma, loss of NSUN6 was correlated with **temozolomide (TMZ)** resistance due to a global loss of m^5C within RNAs. This change induced an accumulation of negative elongation factor B (NELFB) and ribosomal protein S6 kinase B2 (RPS6KB2) and preinitiation of growth transcription factors leading to transcription pausing [47]. By contrast, the expression of NSUN6 in pancreatic cancer was shown to inhibit cell proliferation and tumor growth *in vitro* and *in vivo* with a possible prediction of tumor recurrence and patient survival [48]. Finally, recent studies have highlighted that variable expression of enzymes involved in m^5C modification can modulate the immune microenvironment in several types of cancers – such as colorectal cancer, triple-negative breast cancer (TNBC), and lung adenocarcinoma – and can be used as a prognostic tool [49,50].

Pseudouridylation (Ψ)

Pseudouridylation (Ψ) is the most abundant RNA modification found in human cells, and it is present in multiple RNA molecules [51]. Thirteen RMPs have been identified in humans as writers for this nonreversible modification, but the erasers and readers are still unknown [52]. Ψ can occur by two mechanisms: an RNA-independent mechanism that requires one of the seven pseudouridine synthase (PUS) enzymes, and an RNA-dependent mechanism that requires a complex between RNA and protein, known as H/ACA RNP. The protein component of H/ACA RNP consists of nonhistone protein 2 (NHP2), nucleolar protein 10 (NOP10), glycine–arginine-rich protein 1 (Gar1), and dyskerin (DKC1) that has a catalytic function [53]. Depending on the type of RNA molecule decorated, Ψ regulates different processes within the cell, such as ribosome biogenesis (RB) (pre-rRNA ψ), translation (tRNA and rRNA ψ) and splicing (pre-mRNA and U1–U6 snRNAs ψ) [54,55]. Furthermore, investigation on yeast and human cellular models suggested Ψ as a highly dynamic process that is reshaped in response to cellular stressors. It is interesting to note that human rRNA may exhibit 14 types of modification at 228 sites, more than 100 of which are pseudouridines, additionally highlighting its importance in RB and translation (Figure 2) [56].

Ψ in the context of cancer

DKC1 is one of the best characterized writers of Ψ acting within H/ACA RNP and regulating mainly rRNA Ψ and telomere maintenance [51]. The most studied model of *DKC1* mutations is X-linked dyskeratosis congenita (X-DC), an inherited bone-marrow failure syndrome associated with increased cancer susceptibility. Despite the established role of DKC1 in telomere maintenance, DKC1 mouse models suggest that changes in rRNA Ψ are responsible for the disease phenotype. This occurs through the changes in internal ribosome entry site (**IRES**)-dependent translation of key mRNAs coding for tumor-suppressor genes and oncogenes [4]. Dysregulated expression of DKC1 has been observed in many human cancers, having contrasting roles depending on the cancer type. However, elevated expression of DKC1 was mostly correlated with a worse prognosis [54]. Recently, the oncogenic role of DKC1 was mechanistically explored in colon cancer. It has been shown that elevated levels of DKC1 and its consequent Ψ activity promotes stabilization of mRNAs coding for ribosomal proteins (RPs) L10A, L22L1, L34, and S3, leading to their overexpression and cancer progression. These RPs were also found to suppress Ras/Raf/Mek/Erk signaling pathway through their interaction with H-Ras, pointing to the possibility of combinational therapy by inhibiting DKC1 and Mek1/2 in colorectal cancer treatment

[57]. Similarly to DKC1, mutations in genes coding for NHP2 and NOP10 were identified in autosomal dyskeratosis congenita, and dysregulated expression of these genes and small nucleolar RNA (snoRNA) components of H/ACA RNP were identified in several cancers [54,58]. In non-small-cell lung cancer (NSCLC), increased expression of NOP10 is related to poor prognosis, and its deletion negatively affects tumorigenesis through dysregulation of specific snoRNAs and reduced Ψ [59]. In addition, increased expression of NOP10 and NHP2 was correlated with poor survival in patients with breast cancer (especially those receiving chemotherapy who had higher risk of developing metastasis) and colon cancer (especially in aged patients), respectively [60,61]. In glioblastoma, overexpression of the stand-alone enzyme PUS7 was correlated with poor survival. Specifically, it was found that overexpressed PUS7 regulates translation of factors essential for growth of glioblastoma stem cells (GSCs) by tRNA Ψ [62].

2′-O-methylation (2′-O-Me)

2′-O-Me is deposited on rRNA, tRNA, mRNA, and small ncRNAs, affecting stability, secondary structure, and the interaction of modified RNAs with other RNAs or proteins [63]. rRNA is highly decorated with 2′-O-Me, with more than 100 sites being affected within the mature ribosome [64]. 2′-O-Me is deposited by the RNP complex composed of the catalytic subunit fibrillarin (FBL), box C/D snoRNAs that serve as sequence-specific guides, NOP56/58 heterodimer, and SNU13 protein [65].

2′-O-Me in the context of cancer

Changes in the expression of all components of the 2′-O-Me machinery have been the subject of many studies over the past 10 years [66]. The importance of maintaining the right level of FBL is emphasized by the fact that it is directly regulated by the p53 tumor-suppressor, which is mutated in almost 50% of human cancers [67]. A high level of expression of FBL has been associated with poor prognosis in several types of human cancers [68]. It has been shown that upregulation of C/D box snoRNAs caused by common leukemia oncogenes leads to an increase in 2′-O-Me of rRNA, which is critical for self-renewal of leukemic cells [69]. More recent work identified snoRNA SNORD42A – which directs 2′-O-Me at uridine 116 of 18S rRNA – as being overexpressed in patients with acute myeloid leukemia (AML). Detailed investigation of SNORD42A revealed its importance for survival and proliferation of AML cells, as a decrease in 2′-O-Me at uridine 116 caused changes in the translation of distinct sets of genes. Cells with deleted SNORD42A exhibited decreased translation of RPs, indicating that methylation of this residue regulates RB and the abundance of translational machinery within the cell. Knowing that elevated RB and protein translation is essential for cancer cells, snoRNAs might be considered potential targets in cancer treatment [70]. Recent analysis of the 2′-O-Me landscape in human breast cancer samples have revealed inter- and intra-tumor heterogeneity of 2′-O-Me in rRNA, implying the existence of specialized ribosomes and giving rise to a plethora of new possibilities for predicting clinical outcome and biological characteristics of tumors [71]. lncRNAs were also identified as regulators of 2′-O-Me. For example, lncRNA ZFAS1 interacts with NOP58, promotes its recruitment, and accelerates the assembly of SNORD12C and SNORD78 snoRNPs that guide 2′-O-Me at specific sites within 28S rRNA. In colon cancer, ZFAS1 is upregulated and exhibits its oncogenic role through the regulation of 2′-O-Me [72]. In recent years, some other proteins were found to be involved in regulating 2′-O-Me. For example, **EZH2** interacts with FBL and alters rRNA methylation, thereby demonstrating its function in regulating rRNA 2′-O-Me and IRES-dependent translation independent of its methyltransferase activity [73]. **NPM1** is also involved in regulating 2′-O-Me at five specific sites within 28S rRNA through its interaction with C/D box snoRNAs and FBL, by which process it regulates IRES translation [64]. It is interesting to note that NPM1 mutations were identified in patients with X-DC who presented dysregulation of another epitranscriptomic mark (besides Ψ) as a cause of this disease. Besides its involvement

in X-DC, NPM1 is frequently genetically altered in various hematological diseases, and a mutant cytoplasmic form of NPM1 that is incapable of regulating 2′-O-Me was identified in 30% of AML cases. Furthermore, NPM1 mutations were found in other cancer types in the TCGA dataset, highlighting aberrant 2′-O-Me as a cancer driver event [64]. Hypoxia is the major feature of solid tumors that enhances tumor progression and increases treatment resistance. Hypoxia has recently been found to cause changes in the 2′-O-Me pattern of rRNA, creating specialized ribosomes that in turn preferentially translate vascular endothelial growth factor C (VEGF-C), thereby promoting vascularization [74].

A–I RNA editing

In humans, the most common type of **RNA editing** is deamination of adenine (A) to inosine (I) [75]. This conversion is carried out by the three enzymes of the **ADAR** family: catalytically active ADAR1 and ADAR2, and catalytically inactive ADAR3. ADAR1 has two isoforms generated from distinct promoters and through different splicing events. The larger p150 isoform is involved in immune response regulation and is inducible by interferon signaling, while the nuclear p110 isoform is constitutively expressed [75]. ADAR1 and ADAR2 are ubiquitously expressed in all human tissue types, with ADAR2 being extremely important for editing events in the brain [76]. ADAR3 was suggested as a negative regulator of A–I editing via its competitive binding to target RNAs. As the expression of ADAR3 is mostly restricted to the brain, it can be assumed that it mostly competes with ADAR2 RNA targets [77].

Although A–I editing can emerge in coding and ncRNA molecules, most of the editing sites occur in **Alu elements**, with an as yet unknown impact in the context of disease [76]. Editing within coding RNAs occurs mostly in the brain, resulting in the synthesis of proteins that differ from those encoded in the genome, as inosines are recognized by the translational machinery as guanosines [76]. Besides its impact on protein recoding, A–I editing also affects RNA splicing, stability, translation, localization, and biogenesis of ncRNAs [75]. Although ADAR enzymes were found to bind and edit double-stranded RNA (dsRNA), *in vitro* experiments have shown that ADARs can also edit DNA within DNA–RNA hybrids, although the catalytic activity of this event is relatively low [78]. ADARs regulate genome stability, as described in Box 1, and exhibit an interplay with m^6A modification/modifiers (Box 3). Furthermore, the employment of natural characteristics of ADARs was recognized as a potential tool for correcting disease-associated mutations at the RNA level, without affecting the genome [75]. This strategy is appealing as epitranscriptomic editing has been shown to have many advantages over the genome-editing tools [79].

Changes in A–I RNA-editing machinery in human diseases

ADAR coding genes are genetically and epigenetically altered in several human diseases, including cancer. Mutations of ADAR1 were identified as causative in hereditary autoimmune disorder Aicardi–Goutières syndrome. It has been revealed that mutations within the ADAR1p150-Zα domain prevent its binding with **Z-RNA**, resulting in activation of pathogenic interferon signaling [80,81]. Gene amplification of ADAR1 (p110 isoform) was identified as causative in NSCLC development, in *in vitro* models, and in primary tumors. Mechanistically, it was confirmed that elevated levels of ADAR1 p110 affect the editing of the coding transcript involved in DNA repair (NEIL1) and the ncRNA (miR-381) transcript implicated in stemness and chemoresistance, among other cancer-related pathways [82]. Amplification and high expression levels of ADAR1 were also identified in advanced stages of gastric cancer, revealing its importance to the metastatic process [83]. Overexpression of ADAR1 was also implicated in the development of TNBC, highlighting ADAR1 as a potential therapeutic target [84]. Recently, it was shown that ADAR1 loss elevates sensitivity of tumors to immunotherapy by enhancing tumor inflammation and elevating its sensitivity to interferon [85].

Other cancer-associated tRNA modifications

A plethora of modifications has been identified in the components of the translational apparatus. For tRNA alone, 100 distinct modifications have been identified, most of which affect the anticodon loop and thereby shape the translational process [6]. Of these, methylation on N7 (m^7G) is catalyzed by the METTL1–WDR4 protein complex. METTL1 is frequently amplified and overexpressed in human cancers, leading to a greater abundance of Arg-TCT-4-1 tRNA that in turn favors translation of mRNAs encoding cell-cycle regulators, and leads to malignant transformation [86]. Increased m^7G was also identified as relevant for intrahepatic cholangiocarcinoma, hepatocarcinoma, and lung cancer progression [87]. In bladder cancer, the m^7G modification of tRNAs promotes the translation of mRNAs coding for epidermal growth factor receptor (EGFR)/EGF-containing fibulin extracellular matrix protein 1 (EFEMP1), thereby promoting cellular proliferation, migration, and invasion [88]. Epigenetic silencing by promoter hypermethylation of the tRNA modifier *TYW2* responsible for correct hypermodification of guanine 37 on phenylalanine-tRNA was observed in several cancer types. It has been shown *in vitro* that hypomodification of this position can affect translation, leading to increased ribosomal frameshifting and downregulated translation of specific RNA molecules (Figure 2) [89]. After transcription, RNA molecules can undergo m^7G capping at the 5′ end by NUDT16, which was shown to be epigenetically silenced in T-cell acute lymphoblastic leukemia (T-ALL), leading to c-Myc oncogene activation [90]. Gene amplification and overexpression of another tRNA modifier, TRIT1, was observed in small-cell lung cancer, leading to increased translation of selenoproteins, which in turn promotes tumorigenesis [91]. 5-Carbamoylmethylation of uridine (Ncm5U) is a tRNA modification catalyzed by the elongator complex, which is composed of six different subunits (Elp1-6). It has been shown recently that silencing of Elp3 in hematopoietic stem cells (HSCs) activates p53-dependent apoptosis, resulting in bone-marrow failure [92].

The list of RNA modifications, especially those in tRNAs, is still growing. The most recent study identified 2′-phosphouridine modification (U^p) at position 47 of tRNAs in thermophilic archaea, with ArkI and KptA being the writer/eraser for this modification [93]. It is the first internal RNA phosphorylation found to maintain the stability of tRNA under extremely high temperatures. This finding raises the possibility that similar modifications might be found in human RNAs.

Concluding remarks

Studying the impact of the epitranscriptome on the pathogenesis of cancer and other human diseases has been the focus of the work of many research groups in recent years. The identification of 170 modifications in eukaryotes and around 100 in humans heralds the emergence of this important area of knowledge. In the last decade, RNA modifications have been found to play an important role in regulating cellular pathways that are important for preserving cellular homeostasis. Thus, it is not surprising that genetic and epigenetic defects of the RNA modification machinery are often dysregulated, boosting the pathological processes. Exploiting this knowledge has tremendous potential for the development of diagnostic, prognostic, and therapeutic tools and targets. To date, several RMPs have already been identified as targetable, although their application has to be considered with caution as many RMPs exert dualistic roles depending on the cancer type. Moreover, the interplay between different RNA modifications and modifiers is just beginning to be elucidated, adding another layer of complexity. In addition, some specific readers/erasers are shared by different modifications, and the list of readers is still growing and their novel roles are being uncovered. By keeping in mind that the list of RNA modifications and RMPs is still not definitive, epitranscriptomics will undoubtedly continue to be an attractive research field in the coming years (see Outstanding questions).

Outstanding questions

How many RNA modifications still await discovery, and what novel functions might these modifications regulate?

What would be the benefit of future developments of novel techniques for studying RNA modifications at a higher level of resolution?

To what extent is there interplay between different RNA modifications?

To what extent could RNA modification and RNA modifier profiling be exploited as diagnostic, prognostic, and therapeutic tools in cancer treatment?

What is the future of targeting RNA modifiers in cancer treatment? Some molecules improve the response to immunotherapy treatment, indicating new possibilities for combinational therapy, but what other possibilities exist to exploit synthetic lethal interactions?

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Declaration of interests

M.E. is a consultant of Ferrer International, Incyte and Quimatryx. No interests are declared by the other authors.

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