The RNA modification landscape in human disease

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ABSTRACT

RNA modifications have been historically considered as fine-tuning chemo-structural features of infrastructural RNAs, such as rRNAs, tRNAs, and snoRNAs. This view has changed dramatically in recent years, to a large extent as a result of systematic efforts to map and quantify various RNA modifications in a transcriptome-wide manner, revealing that RNA modifications are reversible, dynamically regulated, far more widespread than originally thought, and involved in major biological processes, including cell differentiation, sex determination, and stress responses. Here we summarize the state of knowledge and provide a catalog of RNA modifications and their links to neurological disorders, cancers, and other diseases. With the advent of direct RNA-sequencing technologies, we expect that this catalog will help prioritize those RNA modifications for transcriptome-wide maps.

Keywords: RNA modification; epitranscriptome; disease; detection methods; direct RNA sequencing

INTRODUCTION

Advances in genomic sequencing technologies have revolutionized our understanding of the mammalian genome and its transcriptional output. It is now evident that most of the genome does not code for protein but is transcribed to produce, in addition to messenger RNAs, a vast pool of intronic, intergenic, and antisense RNAs (Djebali et al. 2012). These non-protein-coding RNAs, most of which have yet to be biologically characterized, are likely to fulfill a wide variety of roles in cell and developmental biology, including the guidance of epigenetic processes (Mattick 2010, 2011; Mercer and Mattick 2013; Morris and Mattick 2014).

While the triplet code of the open reading frame is well understood for mRNAs, the language used by noncoding RNAs to execute their biological functions remains elusive. Dynamic regulation of RNA expression patterns, localization, structure, splicing, stability, and interactions with RNA-binding proteins will intricately dictate this language. In this already complex scenario, RNA modifications, for which more than 100 different types have been described (<http://modomics.genesilico.pl/sequences/>; <http://mods.rna.albany.edu>) (Cantara et al. 2011; Machnicka et al. 2013), overlay the RNA sequence information, expanding its lexicon (Hussain and Bashir 2015).

RNA modifications were first detected in highly abundant “infrastructural” RNAs, such as rRNAs and tRNAs, followed by snoRNAs and snRNAs, and have been generally viewed as irreversible decorations important for RNA structural stability and/or catalytic function (Karijovich and Yu 2010). However, the RNA modification field was greatly stimulated by the discovery that at least some RNA modifications are reversible (Jia et al. 2011; Liu et al. 2016), leading to the birth of the term “epitranscriptome” (He 2010). Comparative analyses of these modification sites across closely related species have shown that these dynamic, reversible modifications are evolutionarily conserved (Schwartz et al. 2013; Batista et al. 2014; Li and Mason 2014; Dominissini et al. 2016).

The functional and evolutionary relevance of the epitranscriptome is yet unknown, but it may represent the crossroads of gene–environment interactions for physiological adaptation and cognition (Mattick 2010; Hussain and Bashir 2015). There is a progressive expansion of enzymes that impart RNA editing and the extent of RNA editing in the brain during cognitive evolution (Mattick and Mehler 2008; Behm and Öhman 2016). A clear example of this expansion is the family of adenosine deaminases acting on RNA (ADARs), responsible for the editing of adenosine to inosine,

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thus increasing gene product diversity, particularly in primates (Paz-Yaacov et al. 2010). Comparative analysis of ADARs suggests that these enzymes evolved from adenosine deaminases acting on tRNAs (ADATs)—present in both Bacteria and Eukarya—after the split of protozoa and metazoa (Grice and Degnan 2015). Thus, A-to-I editing of tRNAs is likely ancestral to editing of other RNAs, achieving its maximal diversity in vertebrates, via the appearance of ADAR3, whose expression is largely restricted to brain (Chen et al. 2000), and the expansion of the ABOBEC family (which catalyze C/meC deamination to U/T) in mammals, with strong positive selection in the primates (Sawyer et al. 2004).

Recent studies have provided detailed maps of the location and abundance of a handful of RNA modifications (Dominissini et al. 2012; Meyer et al. 2012; Khoddami and Cairns 2013; Carlile et al. 2014; Schwartz et al. 2014a; Delatte et al. 2016), mostly obtained by coupling antibody immunoprecipitation or chemical treatments to next-generation sequencing. Through these approaches, N^6 -methyladenosine (m^6A) modification has been identified as an important factor in the determination of mammalian cell fate transition and embryonic stem cell differentiation (Batista et al. 2014; Wang et al. 2014). It is also involved in the regulation of circadian rhythms in hypothalamic mouse brain (Fustin et al. 2013), sex determination in flies (Hausmann et al. 2016; Lence et al. 2016), and maternal mRNA clearance in zebrafish (Zhao et al. 2017). Transcriptome-wide maps have also been obtained for N^1 -methyladenosine (m^1A) (Dominissini et al. 2016), $N^6,2'$ -O-dimethyladenosine (m^6Am) (Linder et al. 2015; Mauer et al. 2016), 5-methylcytosine (m^5C) and pseudouridine (Y), revealing their involvement in biological processes such as stress responses (Carlile et al. 2014), protein synthesis quality control (Tuorto et al. 2012; Hussain et al. 2013; Blanco et al. 2014), and mRNA stability (Mauer et al. 2016), among others. There are several excellent reviews on these few relatively well-characterized RNA modifications (Klungland and Dahl 2014; Li and Mason 2014; Frye et al. 2016; Gilbert et al. 2016; Li et al. 2016; Schwartz 2016; Zhao et al. 2016).

In addition to mapping RNA modifications in a genome-wide fashion, several studies have attempted to characterize the biological function of RNA modifications by comparing wild-type cells to those that lack a specific RNA modification enzyme (Zinshteyn and Gilbert 2013; Nedialkova and Leidel 2015). Although most RNA modifications do not appear to be essential for viability in fungi—but may play important roles in fitness—they have been shown to be critical for maintaining protein homeostasis (Nedialkova and Leidel 2015; Klassen et al. 2016), proper cellular signaling (Zinshteyn and Gilbert 2013), and translation fidelity (Patil et al. 2012; Agris et al. 2017).

RNA modifications have been historically considered to be relatively static fine-tuners of the RNA structure and function. However, in the last few years, it has become evident that the epitranscriptomic layer is not only dynamic and re-

versible (Jia et al. 2011; Zheng et al. 2013; Wang and He 2014; Liu et al. 2016)—catalyzed by RNA modification “erasers” (Meyer and Jaffrey 2017)—but also that the activity of RNA modifications can be regulated by a wide variety of factors, including environmental conditions (Chan et al. 2010; Dedon and Begley 2014; Alings et al. 2015; Han et al. 2015).

The past two decades have witnessed considerable progress in the identification of novel RNA modifications (Grosjean 2015). Unfortunately, the distribution of most remains uncharacterized. Here we provide an overview of current methodologies that have been used to date to map RNA modifications, and consider novel technologies such as direct RNA sequencing, especially useful in the case of RNA modifications for which no other genome-wide method exists. To help decide which RNA modifications to prioritize, we also provide an overview of RNA modifications shown to be linked to human disease, including their distribution and the enzymes involved.

HUMAN RNA MODIFICATIONS AND THEIR IMPLICATIONS IN DISEASE

A comprehensive catalog of RNA modifications and their association to human disease is provided in Table 1 (with the complete list in [Supplemental Table S1](#)). Mutations in approximately half of the currently known RNA modification enzymes have been linked to human diseases, including cancer, cardiovascular diseases, genetic birth defects, metabolic diseases, neurological disorders, and mitochondrial-related defects (Fig. 1). From the more than 100 different associations between mutations in RNA modification enzymes and human disease ([Supplemental Table S1](#)), we find that neurological diseases are largely overrepresented, in agreement with the observed enrichment of several RNA modifications in neuronal tissues (Paul and Bass 1998; Chi and Delgado-Olguin 2013) and in neuronal dysfunction (Najmabadi et al. 2011; Abbasi-Moheb et al. 2012; Davarniya et al. 2015; Lence et al. 2016).

From the battery of known RNA modifications, those present in rRNAs and tRNAs largely dominate the landscape ([Supplemental Table S1](#)). Consequently, previous studies characterizing the associations between RNA modifications and human diseases have been mainly focused on modifications occurring in tRNA molecules (Sarin and Leidel 2014; Torres et al. 2014; Schaffrath and Leidel 2017). However, pseudouridine, originally thought to be exclusive to these infrastructural RNAs, has been detected in several mRNAs (Carlile et al. 2014; Schwartz et al. 2014a), suggesting that additional modifications typically thought to be restricted to “classical” RNA molecules may actually occur more widely.

The mechanisms whereby a lack of modification may lead to disease is a field of active debate. It has been postulated that specific RNA modifications may be essential to tune the proteomic outcome under stress conditions, and that the lack of

TABLE 1. RNA modifications linked to human disease

NT	RNA modification		Associated human disease	Phylogenetic distribution			
	Short nomenclature	Full name		Human enzyme	Yeast enzyme	Bacterial enzyme	Mapping technology
U	m1acp3Y	1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine	Bowen-Conradi syndrome (EMG1)	ACA13 EMG1 NEP1	snR35 EMG1/NEP1		RT Mismatch signature predicted ^a
U	m1Y	1-methyl-pseudouridine	Bowen-Conradi syndrome (EMG1)	EMG1 NEP1	NEP1		
U	s2U	2-thiouridine	Acute infantile liver failure (TRMU) Cancer (TRMU) Deafness (TRMU) MERRF (tRNA/TRMU) MELAS syndrome (tRNA/TRMU) Mitochondrial associated deafness (TRMU) Mitochondrial Infantile Liver Disease (TRMU) Mitochondrial respiratory chain defects (TRMU) MLASA (TRMU)	TRMU	MTU1 NCS2 NCS6	MnmA TusA TusBCD TusE	
U	Um	2'-O-methyluridine	Non-syndromic X-linked mental retardation (FTSJ1)	FTSJ1 FTSJ2 FTSJ3 ALKBH8	TRM7		RiboMethSeq
U	mchm5U	5-(carboxyhydroxymethyl)uridine methyl ester	Bladder cancer (ALKBH8)				
U	ncm5U	5-carbamoyl-methyluridine	Rolandic Epilepsy (ELP4) Breast cancer (ELP3) Dysautonomia (IKBKAP) Familial dysautonomia (IKBKAP) Neuropathy (IKBKAP)	ELP3 ELP4 IKBKAP			
U	cmnm5U	5-carboxymethyl-aminomethyluridine	Hypertrophic cardiomyopathy (MTO1)	MTO1	MSS1 MTO1	MnmE MnmG	
U	mcm5s2U	5-methoxycarbonyl-methyl-2-thiouridine	Bladder cancer (ALKBH8) Cancer (CTU1) Breast cancer (ELP3)[17], (CTU) Amyotrophic lateral sclerosis ALS (ELP3) Rolandic Epilepsy (ELP4) Bronchial asthma (IKBKAP) Dysautonomia (IKBKAP) Familial dysautonomia (IKBKAP) Neuropathy (IKBKAP) Acute infantile liver failure (TRMU) Cancer (TRMU) Deafness (TRMU) MERRF (tRNA/TRMU) MELAS syndrome (tRNA/TRMU) Mitochondrial associated deafness (TRMU) Mitochondrial Infantile Liver Disease (TRMU) Mitochondrial respiratory chain defects (TRMU) MLASA (TRMU)	ALKBH8 CTU1 ELP3 ELP4 IKBKAP TRMU	NCS2 NCS6 TRM9		RT Mismatch signature predicted ^b
U	mcm5U	5-methoxycarbonyl-methyluridine	Bladder cancer (ALKBH8) Breast cancer (ELP3) Amyotrophic lateral sclerosis ALS (ELP3) Rolandic Epilepsy (ELP4) Bronchial asthma (IKBKAP) Dysautonomia (IKBKAP) Familial dysautonomia (IKBKAP) Neuropathy (IKBKAP) Cancer (KIAA1456/TRM9L)	ALKBH8 ELP3 ELP4 IKBKAP KIAA1456/ TRM9L	ELP3 ELP4 TRM9		
U	mnm5se2U	5-methylamino-methyl-2-selenouridine	Lactic acidosis (GTPBP3) Non-syndromic deafness (GTPBP3) Hypertrophic cardiomyopathy (GTPBP3) Mitochondrial respiratory chain defects (GTPBP3) Encephalopathy (GTPBP3)	GTPBP3		MnmCD MnmH	
U	m5U	5-methyluridine	Breast cancer (TRMT2A)	TRMT2A TRMT2B1	TRM2	RlmC RlmCD RlmD RlmFO TrmA TrmFO TrmU54	

Continued

TABLE 1. Continued

NT	RNA modification		Associated human disease	Phylogenetic distribution			
	Short nomenclature	Full name		Human enzyme	Yeast enzyme	Bacterial enzyme	Mapping technology
U	tm5U	5-taurinomethyl-uridine	MELAS (tRNA)	GTPB3			
U	Y	pseudouridine	Dyskeratosis congenital (DKC1) Pituitary tumorigenesis (DKC1) Prostate cancer (DKC1) Lactic acidosis (PUS1) Mitochondrial myopathy (PUS1) MLASA (PUS1) Sideroblastic Anemia (PUS1)	PUS1 PUS3 RPUSD2 PUS7	Pus1 Pus2 Pus3 Pus4 Pus6 Pus7 Pus8		Pseudo-seq
C	Cm	2'-O-methylcytidine	Non-syndromic X-linked mental retardation (FTSJ1)	FTSJ1 FTSJ2 FTSJ3 CCDC76	NOP1 TRM13 TRM7	RlmM RsmI TrmJ TrmL aTrm56	RiboMethSeq
C	m3C	3-methylcytidine	Asthma (METTL2B) Breast cancer (METTL6) Lung cancer (METTL6)	METTL2B METTL2A METTL8 METTL6	TRM140		RT Mismatch signature predicted ^{a,b}
C	f5C	5-formylcytidine	Hypotonia / Floppy baby syndrome (NSUN3) Lactic acidosis (NSUN3) Leber hereditary optic neuropathy (NSUN3)	NSUN3			
C	m5C	5-methylcytidine	Autosomal recessive intellectual disability (NSUN2) Autistic features (NSUN2) Breast cancer (NSUN2) Cancer (NSUN2) Dubowitz syndrome (NSUN2) Intellectual disability syndromes (NSUN2) Noonan-like syndrome (NSUN2) Metabolism (NSUN2) Hypotonia / Floppy baby syndrome (NSUN3) Lactic acidosis (NSUN3) Leber hereditary optic neuropathy (NSUN3) Cri du chat syndrome (NOP2) Breast cancer (TRDMT1) Spina bifida (TRDMT1) Metabolism (TRDMT1) Williams-Beuren syndrome (WBSCR20/NSUN5)	NSUN2 DNMT2 NSUN1 NSUN3 NSUN4 NSUN5 NSUN6 WBSCR20 hNOP2 NOL1 p120 TRDMT1	TRM4 RCM1/BMT3 NOP2/BMT4		Bisulfite sequencing AZA-IP
C	ac4C	N4-acetylcytidine	Laminopathy (NAT10) Hutchinson-Gilford progeria syndrome (NAT10) Malignant disease (NAT10)	NAT10 U13	KRE33 RRA1 TAN1		RT Mismatch signature predicted ^c
C	m1G	1-methylguanosine	Diabetes II (TRMT10A) Intellectual disability (TRMT10A) X-linked intractable epilepsy (TRMT10C) Neurodevelopmental regression (TRMT10C) Multiple Respiratory Chain Deficiencies (TRMT10C) Mitochondrial respiratory chain defects (TRMT10C) Head and neck cancer (TRMT5) Multiple Respiratory Chain Deficiencies (TRMT5) Colorectal cancer (RG9MTD2)	TRMT5 TRMT10A TRMT10B TRMT10C RG9MTD2 RG9MTD1 RG9MTD3 SDR5C1	TRM5 TRM10	AciRa RlmA(I) RlmA(II) Taw22 Trm5b TrmD	RT Mismatch signature predicted ^{a,b}
C	Gm	2'-O-methylguanosine	Non-syndromic X-linked mental retardation (FTSJ1) Liver cancer (TARBP1) Cancer (RNMTL1)	TARBP1 FTSJ1 MRM1 RNMTL1	MRM1 NOP1 SPB1 TRM3 TRM7	RlmB TemH	RiboMethSeq
G	m7G	7-methyl-guanosine	Intellectual disability (WDR4) Primordial dwarfism (WDR4) Cancer (WBSCR22/TRMT112) Inflammation (WBSCR22/TRMT112) Neoplastic human lung pathology (WBSCR22/TRMT112) Williams-Beuren (WBSCR22/TRMT112)	WBSCR22/ TRMT112 WDR4 METTL1	BUD23- TRM112 TRM8/TRM82	ArmA RlmKL RmtB RsmG Sgm TrmB	
G	m2,2G	N2,N2-dimethyl-guanosine	Intellectual disability (TRMT1)	TRMT1 TRMT1L	TRM1		RT Mismatch signature predicted ^{a,b}
G	m2G	N2-methylguanosine	Prostate cancer (TRMT11)	TRMT11	TRM11	TRMT1	RT Mismatch signature predicted ^b

Continued

TABLE 1. Continued

NT	RNA modification		Associated human disease	Phylogenetic distribution			
	Short nomenclature	Full name		Human enzyme	Yeast enzyme	Bacterial enzyme	Mapping technology
G	yW	wybutosine	Breast cancer (TRMT12) Leukemia (TRMT12)	TRMT12	TYW1 TRM12 (TYW2) TYW3 TYW4		RT Mismatch signature predicted ^c
G	m1A	1-methyladenosine	Mitochondrial respiratory chain defects (TRMT10C) Multiple Respiratory Chain Deficiencies (TRMT10C) Neurodevelopmental regression (TRMT10C) X-linked intractable epilepsy (TRMT10C) Obesity (NML/Nucleomethyin)	TRM6 TRMT10C TRM61A TRM61B NML hRRP8 SDR5C1 ALKBH1 CDK5RAP1	TRM6 (GCD10) TRM61 (GCD14) RRP8/BMT1 BMT2		RIP-Seq RT mismatch signature ^{a,b}
G	ms2i6A	2-methylthio-N6-isopentenyl-adenosine	Alzheimer's disease (CDK5RAP1) Breast cancer (CDK5RAP1)	CDK5RAP1		MiaB	RT Mismatch signature predicted ^b
G	ms2t6A	2-methylthio-N6-threonylcarbamoyl-adenosine	Diabetes II (CDKAL1)	CDKAL1		MtaB	
A	Am	2'-O-methyl-adenosine	Non-syndromic X-linked mental retardation (FTSJ1)	FTSJ1 FTSJ2 FTSJ3	NOP1 NSR TRM13 TSR		RiboMethSeq
A	i6A	N6-formyladenosine	Breast cancer (FTO) Cancer (FTO) Coronary heart disease (FTO) Diabetes II (FTO) Developmental delay (FTO) Intellectual disability/Mental retardation (FTO) Leukemia (FTO) Prostate cancer (FTO) Obesity (FTO) Zika virus (FTO)	FTO			
A	i6A	N6-isopentenyl-adenosine	Breast cancer (TRIT1) Mitochondrial respiratory chain defects (TRIT1)	MOD5 TRIT1	MOD5	MiaA Trit1	RT Mismatch signature predicted ^b
A	m6A	N6-methyl-adenosine	Acute myelogenous leukemia (WTAP) Hypospadias (WTAP) Breast cancer (FTO) Cancer (FTO) Coronary heart disease (FTO) Diabetes II (FTO) Developmental delay (FTO) Intellectual disability/Mental retardation (FTO) Leukemia (FTO) Prostate cancer (FTO) Obesity (FTO) Zika virus (FTO) Infertility (ALKBH5) Major depressive disorder (ALKBH5)	ALKBH5 FTO Mettl14 Mettl3	IME4 RSMA	ErmAM ErmBC ErmC RlmF RlmJ RsmA TrmM	RIP-Seq (anti-m6A)
A	I	inosine	Intellectual disability (ADAT3) Strabismus (ADAT3) Aicardi-Goutieres syndrome (ADAR1) Chronic myeloid leukemia (ADAR1) Deliberate self harm (ADAR1) Esophageal squamous cell carcinoma (ADAR1) Human hepatocellular carcinoma (ADAR1) Metastatic melanoma (ADAR1) ALS (ADAR2) Alzheimer's disease (ADAR2) Glioblastoma multiforme (ADAR2)	ADAT2-ADAT3 ADAT1 ADAR2 ADAR1	TAD2-TAD3		RNA-Seq (A-to-G conversion) ice-Seq

^aRT mismatch/block predicted from Motorin et al. (2007).^bRT mismatch/block predicted from Rykvin et al. (2013).^cAdditional RT mismatch/block predicted based on disturbance of W-C base pairing.

FIGURE 1. RNA modifications and their links to human disease. The set of known RNA modifications classified by their reference nucleotide, highlighting those that have been associated to human diseases (red), as well as those for which a transcriptome-wide detection method has been established (circled in green).

regulation may affect the ability of the cell to responsively tune its proteome (Patil et al. 2012; Deng et al. 2015). On the other hand, the lack of specific RNA modifications may affect global and/or local translation rates, and consequently cause increased protein aggregation (Nedialkova and Leidel 2015). Finally, it has also been proposed that RNA modifications transduce information that connect the cell's metabolic state to its translational output, and therefore, that their dysregulation may cause an imbalance between metabolic rates and protein synthesis (Helm and Alfonzo 2014). Future work will be needed to disentangle the causal relationship between RNA modification dysregulation and human disease.

Neurological diseases

Defects in RNA metabolism, including RNA synthesis, processing, function, and degradation, have been found to be associated with motor neuron disorders (Lemmens et al. 2010). In this regard, several RNA methyltransferase-encoding genes have been linked to intellectual disability, supporting the relevance of RNA modifications in the development of cognitive functions (Bednářová et al. 2017). These include FTSJ1, identified in X-linked nonsyndromic intellectual disability in which mental retardation is the sole clinical feature (Freude

et al. 2004); TRMT1, which has been identified as the cause of autosomal-recessive intellectual disability (ARID) (Najmabadi et al. 2011; Davarniya et al. 2015); and the m⁵C methyltransferase NSUN2, which has been associated with defects in memory and learning in *Drosophila* and NSUN2-deficient mouse models (Abbasi-Moheb et al. 2012; Blanco et al. 2014). Mechanistically, it has been shown that lack of NSUN2 in mice leads to fragmentation of tRNAs, which may trigger apoptosis in the brain (Blanco et al. 2014). However, it is still unclear to which degree this mechanism may actually contribute to the intellectual disability phenotypes observed in human.

Defects in demethylation of RNAs have been also linked to neurological defects. Deletion of the FTO gene in mice, which is one of the two enzymes responsible for reversing or “erasing” m⁶A modifications (Zhao et al. 2016), results in an impairment of dopamine receptor control of neuronal activity and behavioral responses (Hess et al. 2013). ALKBH5, also responsible for m⁶A demethylation, has been linked to major depressive disorders (Du et al. 2015), suggesting that m⁶A may be playing an important regulatory role in the function of the mammalian brain.

A-to-I editing defects have also been associated with neurological diseases (Hideyama and Kwak 2011; Hideyama et al. 2012; Gaisler-Salomon et al. 2014; Tomaselli et al. 2015), such as amyotrophic lateral sclerosis (ALS), the most common adult-onset motor neuron disease (Hideyama and Kwak 2011; Hideyama et al. 2012). More specifically, the glutamate receptor 2 (GluA2) mRNA, which is constitutively edited in some of its nucleotides, has been found to be unedited in motor neurons in individuals with sporadic ALS (Hideyama and Kwak 2011). On the other hand, ADAR2 expression levels were found to be down-regulated in ALS individuals, further supporting the importance of editing in proper motor neuronal functioning (Hideyama et al. 2012). In addition, ADAR2 knockout mice show increased cell death rates in their motor neurons (Sasaki et al. 2015), in agreement with the results observed in ALS individuals, indicating a pivotal role of A-to-I editing in proper neuronal functioning and brain development in mammals.

Cancer

Dysregulation and mutations in several RNA modification enzymes have been associated with various types of cancers, including breast cancer, bladder cancer, and leukemia,

among others (Supplemental Table S1). For example, the enzymes responsible for mcm⁵s² modification, ELP3 and CTU1/2, have been found to be up-regulated in breast cancer and to sustain metastasis (Delaunay et al. 2016). Similarly, the methyltransferase NSUN2 has been found to be overexpressed in breast cancer and its expression levels have been shown to correlate with cancer development and progression (Yi et al. 2016). In contrast, the tRNA methyltransferase TRM9L/KIAA1456 has been found to be down-regulated in breast cancer cells, as well as in other forms of cancer (Begley et al. 2013). Lastly, TRMT12 was found to be overexpressed in 87% of breast tumors (Rodriguez et al. 2007). Taken together, these point to a role of RNA modifications in cancer, although it is yet to be shown whether these enzymes could be used as possible targets for cancer treatment or as biomarkers of disease prognosis.

Genetic defects

Most serious genetic birth defects are caused by mutations in protein-coding genes, but the regulation of gene expression may also cause deviation from normal development. Numerous genetic birth defects have been associated with mutations in RNA modification enzymes, such as the *Cri du chat* syndrome (NOP2/NOL1/p120/NSUN1) (Wu et al. 2005), the Dubowitz syndrome (NSUN2) (Martinez et al. 2012), the Noonan-like syndrome (NSUN2) (Fahiminiya et al. 2014), or the William–Beuren syndrome (WBSCR20/WBSCR22/NSUN5) (Doll and Grzeschik 2001). Furthermore, mutations in RNA modification enzymes have also been shown to cause developmental defects, such as Hutchinson–Gilford progeria syndrome (NAT10) (Larrieu et al. 2014), and primordial dwarfism (WDR4) (Shaheen et al. 2015). In addition, mutations in RNA modification enzymes can cause the spinal cord to expose outside the body like spina bifida (TRDMT1) (Franke et al. 2009) and infant death (EMG1) (Armistead et al. 2009).

Note of caution

Here we provide an updated comprehensive catalog of RNA modifications and their associations with human disease, which we expect will provide useful starting points to prioritize the study of additional RNA modifications. However, caution must be taken when interpreting the available data. For example, the fat mass and obesity-associated (*FTO*) gene obtained its name in 2007 after a strong association between a single-nucleotide polymorphism (SNP) in the *FTO* locus and obesity had been identified in multiple populations. Ever since, *FTO* has been widely cited as an example of how an RNA modification dysregulation can be linked to human disease. However, this SNP was recently shown to be unrelated to *FTO* function (Claussnitzer et al. 2015). Instead, this intronic SNP disrupts a conserved motif for the ARID5B repressor, which in turn leads to a de-repression of a potent

pre-adipocyte enhancer, causing increased expression of two nearby genes, *IRX3* and *IRX5*, involved in early adipocyte differentiation (Claussnitzer et al. 2015).

In a similar fashion, a variant of *CDKAL1* was found to be associated to type 2 diabetes in various ethnic groups (Benrahma et al. 2014; Lasram et al. 2015), but a different study on Caucasian UK residents found evidence against a role of deregulated expression of *CDKAL1-v1* in susceptibility to type II diabetes (Locke et al. 2015), emphasizing the fact that caution should be taken in inferring causality from an association between disease-risk genotypes and expression levels. These examples highlight the importance of understanding the underlying mechanisms driving the association between RNA modification enzyme mutations and human diseases, as well as the need to validate these associations.

DETECTING RNA MODIFICATIONS: PAST, PRESENT, AND FUTURE EFFORTS

Classical approaches to detect modified ribonucleosides have relied on thin-layer chromatography, capillary electrophoresis, or related techniques. In all cases, the sample is reduced to nucleotides, which are then separated based on their physicochemical properties. To increase sensitivity, ³²P-radioactive labeling can be combined with two-dimensional TLC separation on cellulose, enabling the detection of femtomole quantities of modified nucleotides (Keith 1995). However, these methods are labor-intensive, require the use of radioactive labeling, and are semiquantitative at best (Reddy et al. 1981; Zhao and Yu 2004; Hengesbach et al. 2008).

More recent approaches have used liquid chromatography–tandem mass spectrometry (LC–MS/MS) methodologies, which can provide accurate quantitation of multiple RNA modifications across conditions and cell types (Chan et al. 2010; Addepalli and Limbach 2011; Yan et al. 2013; Su et al. 2014) and have been successfully applied to a wide variety of species (Chan et al. 2010; Patil et al. 2012; Begley et al. 2013). Selective enzymatic digestions of individual RNAs with a battery of RNases, coupled to LC–MS/MS techniques, has been shown to be extremely useful for comparative RNA modification analysis across species and conditions (Li and Limbach 2012). In addition, the use of isotopically-labeled compounds (¹³C, ¹⁵N, ¹⁸O) has lowered the detection methods to the femtomole scale, allowing the detection of small differences of RNA modifications between RNA samples (Nikcevic et al. 2011; Li and Limbach 2012).

A key requirement to obtain meaningful results from LC–MS/MS approaches, however, is the isolation of specific RNA species, free from contamination with other RNAs. Thus, LC–MS/MS analyses of RNA modifications have been mostly focused on rRNA or tRNA molecules, due to their high abundance of modifications and simplicity of isolation (Chan et al. 2010; Su et al. 2014). Among the few attempts to identify RNA modifications beyond tRNAs and rRNAs (Yan et al. 2013), several were detected in other RNA pools,

suggesting that some may have broader distribution. Unfortunately, a major limitation of LC–MS/MS approaches is that the information of both the transcript that carried the modified nucleosides, as well as their location within the sequence, remains unknown.

Accurate transcriptome-wide mapping of modified nucleosides is now possible due to advances in next-generation sequencing technologies (Saletore et al. 2012; Helm and Motorin 2017; Novoa et al. 2017). These fall into three different categories: (i) immunoprecipitation of fragmented RNAs using modification-specific antibodies, followed by sequencing of the enriched RNA fragments (RIP-seq), which has been used for mapping m⁶A (Dominissini et al. 2012; Meyer et al. 2012), hm⁵C (Delatte et al. 2016) and m¹A (Hauenschild et al. 2015; Dominissini et al. 2016); (ii) chemical treatment of RNA prior to sequencing, which exploits the differential reactivity of modified bases, such as using sodium bisulfite for detection of m⁵C (Squires et al. 2012) or CMC [*N*-cyclohexyl-*N*⁹-(2-morpholinoethyl)-carbodiimidemetho-*p*-toluenesulphonate] for detection of pseudouridine (Carlile et al. 2014; Schwartz et al. 2014a) (Chem-seq); and (iii) nonrandom mismatch signatures in RNA sequencing data produced during conversion of RNA to cDNA by reverse transcriptase during library preparation (Fig. 2; Hauenschild et al. 2015). To date, these technologies

and sequencing adaptations have produced transcriptome-wide maps for six RNA modifications including 5-methylcytidine (m⁵C), *N*⁶-methyladenosine (m⁶A), *N*⁶-2'-*O*-dimethyladenosine (m⁶Am), pseudouridine (Ψ), 1-methyladenosine (m¹A), 5-hydroxymethylcytidine (hm⁵C), in addition to adenosine to inosine (A-to-I) editing, which can be detected using traditional RNA-seq protocols (Ramaswami et al. 2013; Shafik et al. 2016).

RIP-seq

Building on the principles of chromatin immunoprecipitation-sequencing (ChIP-seq), antibody-based detection methods have been successfully applied to detect RNA modifications in a transcriptome-wide fashion, where read densities of immunoprecipitated modified RNA are compared to an untreated input (Fig. 2A; Dominissini et al. 2012; Batista et al. 2014). These methods have proven to be highly sensitive, but they are limited by the available repertoire of commercial antibodies (i.e., at present only those against m⁶A, m¹A, m⁵C, and hm⁵C) (Table 2). They have had the disadvantage of lacking single-nucleotide resolution, although this limitation can be overcome by slight modification of the protocol (Linder et al. 2015).

FIGURE 2. Current genome-wide detection methods used to identify RNA modifications. (A) In the *left* panel, antibody-based methods (RIP-seq) show how RNA-modification enriched fragments are selected using pool-down, and compared to a total fragmented sample (input), which is used for normalization, obtaining genome-wide maps with peak resolution. (B) In the *middle* panel, RNA samples are pretreated with chemical reagents (Chem-seq), which inhibit the reverse transcription reaction beyond the chemically modified position. (C) In the *right* panel, mismatch signature-based methods, which are based on the increased mismatch rates that occur upon reverse transcription at certain RNA-modified positions, are depicted.

TABLE 2. Comparison of current detection methods to map RNA modifications transcriptome-wide

	Chemical-based detection	Antibody-based detection	Nonrandom mismatch signature detection	Oxford Nanopore Technologies (direct sequencing)
Advantages	Single-nucleotide resolution	High selectivity Low false-positive rate	Single-nucleotide resolution	Single-nucleotide resolution Quantitative Virtually all RNA modifications can be detected
Limitations	Limited capacity to expand this method to additional modifications High false-positive rate due to reverse-transcription drop-off and untemplated nucleotide additions	Limited capacity to expand to additional modifications Peak resolution (not single nucleotide)	Limited to modifications that produce mismatch signatures Requires high coverage, strongly limiting the detection to highly expressed RNAs (or whichever RNA pool selected)	Limited to RNA modifications for which you can perform a “training” (e.g., available NTPs containing the modification, to be incorporated in synthetic sequences)
RNA modifications detected	Pseudouridine (Y) Inosine (I) 5-methylcytosine (m ⁵ C) 2'-O-methylation (Gm, Um, Cm, Am)	N ⁶ -methyladenosine (m ⁶ A) 5-hydroxymethylcytosine (hm ⁵ C)	N ¹ -methyladenosine (m ¹ A), Inosine (I)	
Resolution	Single nucleotide	Peak-based	Single nucleotide	Single nucleotide
Isoform identification	In general, no	In general, no	In general, no	Yes
Quantitative measurement	Difficult, requires the use of modified RNA spike-ins	Difficult, requires the use of modified RNA spike-ins	Difficult, requires the use of modified RNA spike-ins	Yes
Length of reads	Typically under 100 bp due to selection of truncated RT reads	Typically under 200 bp	Typically under 200 bp	Extremely long, record >500 kb
PCR in library preparation	Required	Required	Required	Not required
Library preparation cost	Medium (>\$US 100/sample)	High (> \$US 200/sample)	Medium (>\$US 100/sample)	Medium (>\$US 100/sample)
Need to sequence input	Yes	Yes	No	No
Library sequencing cost	Medium (~\$US 2000/lane)	Medium (~\$US 2000/lane)	Medium (~\$US 2000/lane)	Low (~\$US 500-900/flowcell)

Chem-seq

Chemical-based detection methods rely on the use of chemical reagents that selectively react with specific modified RNA nucleotides (Fig. 2B). Upon reverse transcription (RT), chemically modified positions induce RT drop-off, leading to the accumulation of reads ending at the same position. These chemically modified positions can be then precisely located through the identification of increased reverse transcription termination sites (RTTS). Successful application of this technique is exemplified by Pseudo-seq (Carlile et al. 2014; Schwartz et al. 2014a), where CMC-modified pseudouridines block reverse transcription. Although chemical-based detection methods have the strength of producing single-nucleotide resolution RNA modification maps, RT drop-off can be caused by many factors, such as increased RNA secondary structures (Aviran

and Pachter 2014), the presence of binding of proteins (Konig et al. 2010), or other RNA modifications (Table 2; Motorin et al. 2007; Ryvkin et al. 2013). In addition, RT enzymes have the undesirable ability of adding nontemplated nucleotides, thus generating an additional source of false positives (Chen and Patton 2001).

Several successful chemical-detection methods do not induce RT drop-off, but instead change the pairing ability of the modified position. In the case of RNA editing, treatment with glyoxal protects guanosines but not inosines (the product of adenosine deamination, which behaves like guanosine) from RNase T1 activity prior to reverse transcription (Cattenoz et al. 2013). Similarly, in the case of bisulphite sequencing, unmethylated cytosine is sulfonated by sodium bisulphite and is subsequently deaminated to uridine, while methylated cytosine is refractory, remaining as cytosine (Schaefer et al. 2009). Upon conversion to cDNA,

unmethylated cytosine will be read by the sequencer as thymine. Unfortunately, due to the incomplete conversion of C-to-U (in the case of bisulfite sequencing) or glyoxal protection of guanosines, these methods suffer from high false-positive rates. Thus, to correct for false positives, these methods require deep sequencing of RNAs both from a wild-type in combination with the knockout/knockdown of the enzyme of interest.

A hybrid method combining chemical modification and immunoprecipitation is 5-azacytidine-mediated RNA immunoprecipitation (Aza-IP), a mechanism-based technique that exploits the covalent bond formed between an RNA methyltransferase and the cytidine analog 5-azacytidine to selectively recover m⁵C modified RNA targets by immunoprecipitation (Khoddami and Cairns 2013).

Mismatch signature analysis

Lastly, mismatch-signature-based analyses have been used to produce transcriptome-wide maps of RNA modifications with single-nucleotide resolution (Hauenschild et al. 2015). These methods rely on the interference of certain RNA modifications in Watson–Crick (W–C) base-pairing, generating nonrandom mismatch patterns at modified positions during enzymatic RT readthrough (Fig. 2C). Unfortunately, multiple RNA modifications disturb W–C base-pairing and generate increased mismatch rates, hindering the proper identification of the specific underlying modification. To deconvolute these signatures and identify the underlying modifications, previous efforts have used bioinformatic approaches to classify the nonrandom mismatch signatures, using mismatch patterns observed at known tRNA modification sites to train the algorithm (Rykin et al. 2013). In our hands, however, tRNA modification signatures are not representative of mismatch patterns observed in other RNA classes and locations, perhaps due to the rich modification environment of tRNA molecules.

Overall, current transcriptome-wide mapping methods have provided highly valuable information to broaden our understanding of the epitranscriptome, but are constrained by the limited repertoire of commercial antibodies (e.g., those against m⁶A and hm⁵C) and the lack of selective chemical reactivities of uncharacterized RNA modifications (Table 2).

Future approaches: direct RNA sequencing

A major limitation of current genome-wide sequencing methods is that they are based on sequence-by-synthesis (SBS) technologies, and consequently, are blind to DNA and RNA modifications (with the exception of A-to-I editing, which causes an A-to-G mismatch). In the case of DNA modifications, this information is lost in the amplification step, e.g., m⁵C will be read as a C, and a G will be placed in this position. In the case of RNA modifications, the loss occurs

during reverse transcription, whereby RNA is converted back to cDNA in order to be sequenced. These processes strip all edited bases and epigenetic information from the molecules, and occasionally introduce substantial artifacts (Chen and Patton 2001). In addition, standard RT enzymes are sensitive to RNA length and may terminate early when encountering stable RNA structures during extension (Aviran and Pachter 2014).

Third generation sequencing (TGS) appeared only a few years ago, and has emerged as a promising alternative to genome-wide map RNA modifications. TGS technologies distance themselves from second generation sequencing (SGS) technologies for their capability of generating very long reads (>100 kb) (Laver et al. 2015; Rhoads and Au 2015). First single-molecule sequencing attempts were performed by the now defunct Helicos Biosciences, which used a single-molecule SBS strategy. Its ability to identify modified and non-standard RNA bases is unknown, but would likely be subject to similar constraints as cDNA-based technologies, given that a polymerase is nonetheless required for sequencing.

The first alternative SBS approach for detecting RNA modifications was offered by Pacific Bioscience's single-molecule real-time sequencing (SMRT) platform, which was proven successful in detecting the differences between m⁶A-modified and unmodified synthetic sequences of RNA, but was too labor-intensive and expensive for subsequent development (Saletore et al. 2012). Another promising TGS technology has been developed by Oxford Nanopore Technologies (ONT). This platform is based on direct measurement of disruptions in the current as the DNA or RNA molecules pass through a porous bacterial transmembrane protein (Loman and Watson 2015). These changes in current intensity can then be used to identify the transiting nucleotides, including modified RNA and DNA nucleotides (Fig. 3A). Initially limited to DNA sequencing, ONT published their first results of direct RNA sequencing at the end of 2016 (Garalde et al. 2016). Several months later, a direct RNA-sequencing (DRS) kit finally became available to the general public in April 2017 (Fig. 3B). In the first step of the DRS library preparation protocol, a double-stranded DNA adapter with a poly(T) or sequence-specific complementary single-stranded overhang is ligated to the 3' end of template RNA molecules. Next, an optional reverse-transcription step can be performed to linearize and stabilize the template RNAs. Finally, a proprietary sequencing adapter is ligated to the double-stranded DNA adapter before being loaded into a flow cell for sequencing. It is important to note that only the RNA strand will be sequenced (in 3'–5' orientation). Remarkably, the low sample manipulation required for the library preparation (Fig. 3B) vastly diminishes the biases typically introduced during SBS library preparation, such as those introduced by fragmentation, PCR amplification, or immunoprecipitation.

The simplest way to convert ionic current traces into base-called nucleotides is to run local "live" base-calling, where samples are base-called *on-the-fly* as the molecules exit

FIGURE 3. Direct RNA sequencing library preparation steps using Oxford Nanopore Technologies. (A) Schematic representation of a nanopore embedded in the membrane of the flowcell. (B) Overview of the main library preparation steps in ONT direct RNA sequencing.

individual pores using ONT's proprietary algorithm Albacore (which combines a recurrent neural network and a hidden Markov model). Base-calling can also be performed a posteriori with Albacore on a personal computer, a high performance computing server, or ONT's cloud-based analysis service known as Metrichor (<https://metrichor.com>). A third option is to use one of the multiple open-source base-calling algorithms, which use various machine- or deep-learning algorithms, including hidden Markov models (David et al. 2017; Simpson et al. 2017) and recurrent neural networks (Boža et al. 2017; Stoiber and Brown 2017). Unfortunately, these algorithms are typically trained to predict exclusively four bases (A, C, G, T), and thus cannot directly identify DNA- or RNA-modified nucleotides. There have nonetheless been recent reports describing computational models capable of detecting modified DNA bases, by training models from biological control data and by observing conspicuous alterations of ionic current at specific positions (Stoiber et al. 2016; McIntyre et al. 2017; Rand et al. 2017; Simpson et al. 2017). With respect to DRS, these strategies have recently been applied to characterize the epitranscriptome, namely the identification of m⁶A (Garalde et al. 2016) and conserved 16S ribosomal RNA base modifications and a 7-methylguanosine modification associated with antibiotic resistance

(Smith et al. 2017). It is likely that, following the release of the direct RNA-sequencing kit, additional algorithms to detect and predict RNA modifications from DRS data will become available.

For many years, a major limitation of ONT technologies has been its relatively low base-calling accuracy. However, in the last three years, its base-calling accuracy has increased from 70%–88% (using R7 pore technologies and HMM-based algorithms) to 90%–98%, due to a more efficient protein nanopore (currently R9.5), homopolymer-aware RNN base-calling, and a paired-end consensus read strategy. Nonetheless, base-calling errors can be corrected a posteriori either by determining a consensus sequence, probabilistic refinement (Jain et al. 2015), or via a process known as “polishing” (Loman et al. 2015; Sarkozy et al. 2017), where reads are aligned to a reference genome or transcriptome to guide error correction by revisiting the raw signal. However, initial base-calling attempts using ONT to sequence 16S rRNA from *E. coli* only using DRS only yielded an accuracy of 87% (Smith et al. 2017). The authors found that these errors were mainly due to deletion errors occurring in G-rich regions, which are abundant in noncoding infrastructural RNAs such as 16S rRNA. It is likely that the highly modified nature of rRNA molecules may be in fact a confounder for proper RNA base-calling. Newer algorithms, previously trained with known modified RNA nucleotides, will likely produce higher base-calling identities in RNA molecules.

A second major limitation of direct RNA-sequencing technologies is the yield of each individual sequencing run. Although the throughput of ONT sequencing has greatly increased in the last years for (c)DNA sequences, achieving yields of 3–15 billion bases (Gb) per run in a standard R9.4 MinION FLO-MIN106 flow cell (Lu et al. 2016; Jain et al. 2017), the yields obtained from direct RNA sequencing are still far from these values. More specifically, the expected number of cDNA reads using a high-quality FLO-MIN106 flow cell ranges between 6–10 million reads, whereas the expected number of reads from direct RNA sequencing is only 1 million (<https://nanoporetech.com/rna>).

Despite these limitations, the possibility of detecting RNA modifications in each individual RNA molecule opens new avenues to explore the cross talk and dependencies that may exist between multiple RNA modifications within the same RNA molecule. Current indirect SBS-based methods are unable to decipher whether two RNA modifications present in a given mRNA sequence actually coexist in the same RNA molecule, or if instead, they are exclusively present in different molecules. Furthermore, compared with SBS-based methods, ONT offers the possibility to identify in which RNA transcript isoform the modification is found, and thus may be able to provide quantitative stoichiometric measurements of modified RNA nucleotides at each position in an isoform-specific manner.

DISCUSSION

During the past few decades we have learned how vital epigenetic processes are for learning and memory formation (Day et al. 2013). RNA modifications form an additional language, much less characterized, which is capable of overwriting and redefining the hard-wired transcriptome, extending and diversifying the function of transcripts, thus adding an uncharted layer of regulation affecting genome function.

One of the major surprises in the last decades was the observation that developmental complexity is not correlated with the number of protein-coding genes. One of the answers to this enigma came with the discovery of long noncoding RNAs, whose number increases with developmental complexity (Mattick 2011). Thus, the appearance of multitudes of noncoding RNAs created additional layers of gene expression and genetic information, which provides the regulatory power and plasticity required for the developmental and cognitive capacity of mammals and, in particular, primates (Qureshi et al. 2010; Mattick 2011).

In a similar fashion, RNA modifications may not be simple fine-tuners of RNA function, such as mRNA half-lives (Batista et al. 2014; Schwartz et al. 2014b) or translation efficiency (Wang et al. 2015), but also provide additional capacity for increasing developmental and cognitive complexity. RNAs undergo an enormous amount of editing, especially in primates and especially in the brain (Paul and Bass 1998). A large variety of studies have shown that the activity ADARs (adenosine deaminases acting on RNAs), responsible for A-to-I editing, are highly expressed in nervous systems (Picardi et al. 2015), and markedly increased during primate evolution (Paz-Yaacov et al. 2010). These modifications do not only affect protein-coding genes, but also noncoding transcripts, and thus may be central to learning and plasticity in brain function (Lence et al. 2016; Nainar et al. 2016).

Moreover, there is now evidence that transgenerational inheritance can be mediated by RNAs (Chen et al. 2016; Sharma et al. 2016), thus raising the possibility that RNA is not just the underlying engine of cell biology, developmental biology, and cognition, but perhaps also of evolution itself. Interestingly, the identified molecules involved in transgenerational inheritance were tRNA-derived fragments, which are likely highly modified. Whether these RNAs display different RNA modifications to execute or regulate their function is still an open question, hopefully to be answered in the following years. Once we are capable of systematically mapping RNA modifications in a genome-wide fashion, we may be able to uncover the potential roles of RNA modifications in human development, as well as the effects of their dysregulation in disease.

In recent years, TGS technologies have made possible the sequencing of very long reads from single RNA/DNA molecules. Despite being in their infancy, they have already demonstrated to be powerful technologies capable of overcoming challenges that could not be solved by SGS, such as providing

quick in situ diagnoses of virus outbreaks (Quick et al. 2016, 2017), or obtaining genome-wide structural variant information from patient genomes (Cretu Stancu et al. 2017). While the use of TGS to detect RNA modifications is still not fully benchmarked, we expect that in the near future TGS will provide us with single-molecule genome-wide maps of RNA modifications, allowing us to investigate the dependencies between different modified sites, as well as those between different RNA modification types (e.g., m⁶A and pseudouridine), in a genome-wide fashion.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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