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Review

Emerging role of dynamic RNA modifications during animal development

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A B S T R A C T

The central dogma of molecular biology statically says that the information flows from DNA to messenger RNA to protein. But the recent advances in mass spectrometry and high throughput technology have helped the scientists to view RNA as little more than a courier of genetic information encoded in the DNA. The dynamics of RNA modifications in coding and non-coding RNAs are just emerging as a carrier of non-genetic information, uncovering a new layer of complexity in the regulation of gene expression and protein translation. In this review, we summarize about the current knowledge of N6-methyladenosine (m6A), N1-methyladenosine (m1A), 5-methylcytosine (m5C) and pseudouridine (Ψ) modifications in RNA, and described how these RNA modifications are implicated in early animal development and in several human diseases.

Several years ago, the idea that the life in the world is dominated by the command of the RNA was proposed (Gilbert, 1986). This idea was supported by the discovery of different kinds of chemical RNA modifications in all kingdoms of life, plus, many of these post-transcriptional modifications are conserved throughout evolution (Kellner et al., 2010; Wachowius and Hobartner, 2010). Time has passed and technologies have advanced, now, the idea of a "RNA world" is more supported since diverse classes of RNA have emerged as key regulators of gene expression, genome stability, defense against foreign genetic elements and recently as heritable element of acquired traits (Gapp et al., 2014; Houri-Zeevi and Rechavi, 2017; Liebers et al., 2014; Wang et al., 2015). In this context, the term "RNA epigenetics" was coined to talk about those RNA modifications that are dynamics trough life and can be heritable (Liu and Pan, 2015; Roundtree and He, 2016b; Zheng et al., 2013b). RNA modifications, which conform the "epitranscriptome", are gaining a great attention and emerging as an attracting field of research, even at comparable degree to what was the epigenomics few years ago. The use of the term "RNA epigenetics" is controversial, and goes beyond the scope of this review, which will outline the recent findings related with the role of dynamic RNA modifications during animal development and disease.

Until now, more than 140 different RNA modifications have been identified in RNA (For more information see: http://modomics.genesilico.pl; http://mods.rna.albany.edu), giving rise to the idea that we are about to reveal a previously ignored code that is outside their primary sequence (Roundtree et al., 2017). Most of these modifications are present in structured RNAs such as tRNA and rRNA, where they play important roles in biogenesis, structural folding and function (Zhang et al., 2016). However, in recent years a diverse set of modifications were also found in transient carriers of information such as mRNAs and ncRNAs to play key roles in gene expression (Soller, 2006).

This review will attempt to summarize recent findings that connect dynamic RNA modification in coding and non-coding RNAs, focusing in underlying their regulatory mechanisms and biological consequences during early animal development and in various human diseases.

1. RNA modification dynamics and early developmental functionalities

The most studied RNA modification is the N6-methyladenosine (m6A), which is found in most of the eukaryotic mRNAs (Dominissini et al., 2012; Meyer et al., 2012; Schwartz et al., 2013). However, several studies have documented other modification including N1-methyladenosine (m1A) (Dominissini et al., 2016; Li et al., 2016b), 5-methylcytosine (m5C) (Westbye et al., 2008) and pseudouridine (Ψ) (Carlile et al., 2014) in coding and non-coding RNAs. Similarly to DNA epigenetics, RNA modifications involve dynamics giving by the "writers" and "erasers" that establish a code which is then decoded by "readers" (Fig. 1). It seems like distinct distribution patterns of specific modifications along RNAs are associated with specific functions, as discussed in the following sections.

1.1. m6A: the most abundant RNA modification

Among all the RNA modifications, methylation of adenosine at position 6 (m6A) is the most abundant in mRNAs and has been shown to

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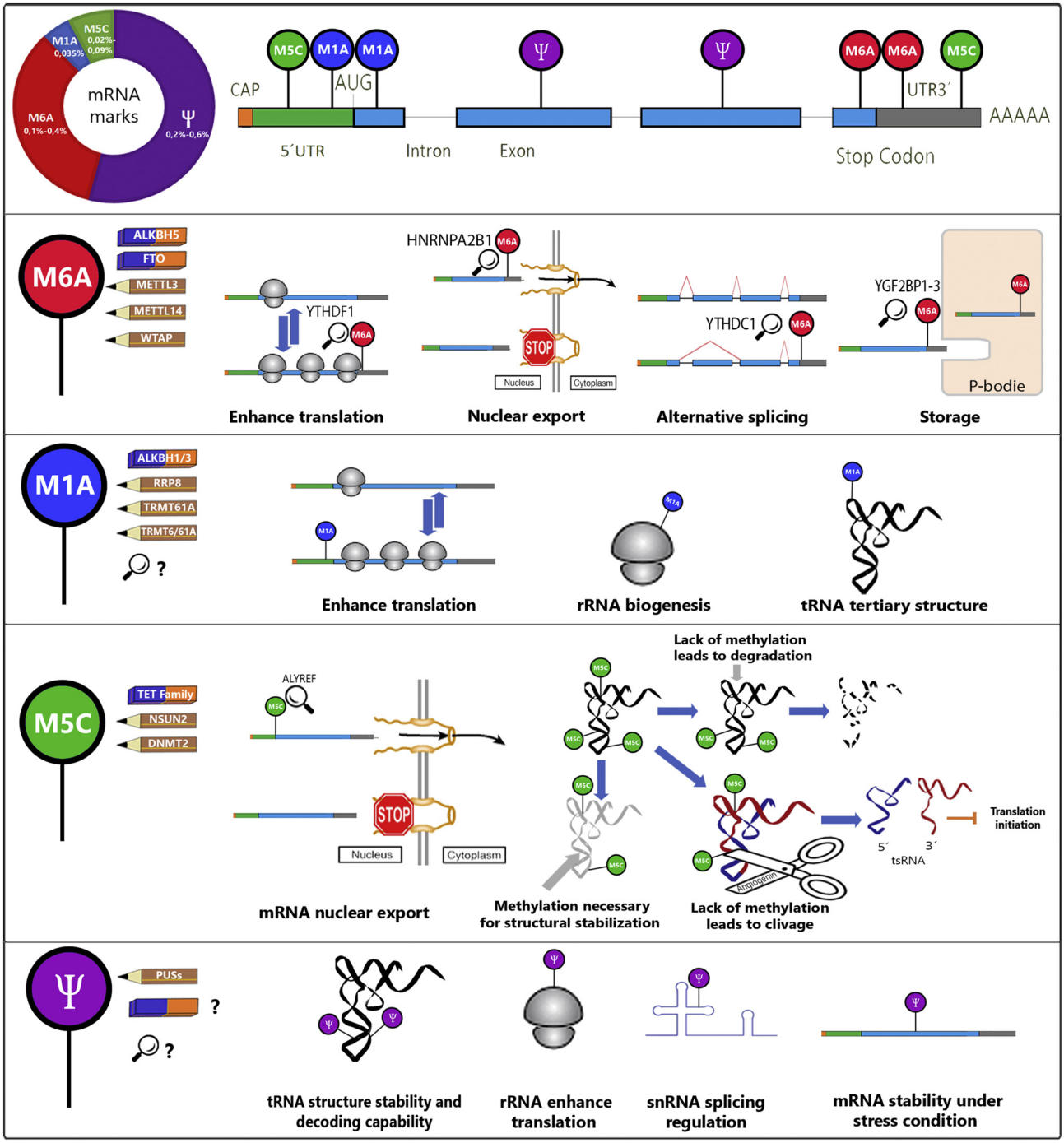

Fig. 1. Occurrence (%), localizations, writers (pens), erasers (rubbers), readers (scopes) and functions of N6-methyladenosine (m6A), N1-methyladenosine (m1A), 5-methylcytosine (m5C) and pseudouridine (Ψ) modifications in eukaryotic RNAs are illustrated.

be 0.1%–0.4% of total adenosine residues (Dubin and Taylor, 1975; Wei et al., 1975), suggesting that this modification may be widespread throughout the transcriptome. Although m6A is found in tRNAs (Saneyoshi et al., 1969), rRNAs (Iwanami and Brown, 1968) and ncRNAs (Pan, 2013), its prevalence in mRNAs has made most of the studies focus their attention on them. In general, the m6A modification can be detected in the mRNAs of over 7000 genes in mammalian cells (Dominissini et al., 2012). Strong enrichment of m6A modification has been found near the stop codons, 3′UTR (Dominissini et al., 2012; Meyer et al., 2012; Schwartz et al., 2013) and long internal exons (Batista et al., 2014). This mark tends to be found within the degenerate consensus sequence RRACH (R = G or A; H = A, C, or U) (Dominissini et al., 2012) and its broad presence in RNAs has led to deeply investigate its functions.

Since its serendipitous discovery in 1974 (Desrosiers et al., 1974), new roles have been associated with the m6A modification. Those functions included: mRNA decay (Y. Wang et al., 2014), circadian clock speed (Fustin et al., 2013), translation efficiency (Wang et al., 2015), RNA nuclear export (Fustin et al., 2013; Zheng et al., 2013a), splicing (Dominissini et al., 2012; Ping et al., 2014), and microRNA processing (Alarcon et al., 2015).

Recently, it was found that m6A is a dynamic mark (Zhao et al., 2017a) which is regulated by three groups of proteins: the writer, the erasers and the readers. The writer complex is composed of three core

proteins. First, the methyltransferase-like 3 (METTL3), which is highly conserved in most eukaryotes from yeast to humans, contains the SAM (S-adenosyl methionine) binding domain (Bokar et al., 1997) and catalyzed the m6A methylation (Clancy et al., 2002; Hongay and Orr-Weaver, 2011; Zhong et al., 2008). Second, the METTL14 forms a stable heterodimer with METTL3 and has also been shown to exhibit m6A methyltransferase activity (Liu et al., 2014; Y. Wang et al., 2014). Third, the Wilms' tumor 1-associating protein (WTAP) is required for the subcellular localization and RNA-binding capability of the complex (Ping et al., 2014).

The erasers of m6A in RNAs allow a dynamical control of this modification. Until now, two erasers were found: 1) the fat mass and obesity-associated protein (FTO), which is located in the nucleus and the cytoplasm, and 2) the alkylated DNA repair protein alkB homolog 5 (ALKBH5) found in the nucleus (Jia et al., 2011; Zheng et al., 2013a). FTO and ALKBH5 have different targets based on their localization and tissue distribution. FTO is highly enriched in brain tissue (Hess et al., 2013; Vujovic et al., 2013), while ALKBH5 is predominantly expressed in testes and other tissues (Zheng et al., 2013a). Although FTO was the first enzyme linked to m6A removal, a very recent study has shown that FTO instead demethylates a closely related and highly abundant nucleotide N^6-2-0-dimethyladenosine ($m6A_m$) (Mauer et al., 2017). Thus, making some author to rethink the role of FTO as one of the mayor m6A erasers (Meyer and Jaffrey, 2017).

The previous group of proteins has the function of “mark” or “unmark” the RNAs with m6A in order to allow the reader proteins to specifically recognize the m6A modification and link them with cellular responses. Until now, three different groups of m6A readers were identified. First, the heterogeneous nuclear ribonucleoprotein A2B1 (HNRNPA2B1), which regulated the alternative splicing of exons (Alarcon et al., 2015). Additionally, HNRNPA2B1 binds to m6A marks in a subset of primary microRNA transcripts, interacts with the microRNA microprocessor complex protein DGCR8 and promotes primary microRNA processing (Alarcon et al., 2015). The second group of readers is characterized by the presence of a YTH-RNA binding domain (Dominissini et al., 2012; Xu et al., 2014; Zhu et al., 2014). So far, three YTH-domain-family (YTHDF1–3) and two YTH domain-containing (YTHDC1–2) proteins have been identified. The proteins YTHDF1–3 and YTHDC1 have been validated to bind tightly the RNA when is methylated and are mainly localized in the cytoplasm and nucleus, respectively (N. Wang et al., 2014). Moreover, these m6A readers have been associated with RNA splicing, nuclear export and storage, translation, decay and microRNA processing. In particular, during the splicing process, the reader YTHDC1 recognizes the m6A modification and generates different isoforms through alternative splicing (Roundtree and He, 2016a; Xiao et al., 2016). Specifically, YTHDC1 enhances accessibility to the regulator of splicing HNRNPC (Liu et al., 2015) and facilitates exon inclusion or skipping by recruiting SRSF3 or SRSF10 splicing factors, respectively (Xiao et al., 2016). On the other hand, the reader YTHDF2 binds preferentially to stop codon and coding sequences to accelerate mRNA degradation trough transporting m6A containing-transcripts to decay sites (X. Wang et al., 2014). Contrarily to this notion, YTHDF1 promotes translation efficiency by interacting with the initiation factor 3 (eIF3) and causes slow decay of mRNAs (Wang et al., 2015). The third group of readers belong to the insulin-like growth factor-2 mRNA-biding proteins (IGF2BPs), whose mRNA targets present m6A enrichment in the 3′UTRs and tend to have longer half-lives (Huang et al., 2018; Zhou and Pan, 2018). In agreement with this, IGF2BP co-localize with HuR (RNA stabilizer protein) inside processing bodies (P-bodies) and together stabilize mRNA through MATR3 and PABPC1 proteins in order to protect m6A mRNAs from degradation. This is in agreement with the recent notion that P-bodies are not essentially a decay compartment but are rather involved in mRNA storage and protection where they are translationally repressed, providing a physical reservoir for mRNAs encoding regulatory functions (Brengues et al., 2005; Horvathova et al., 2017; Hubstenberger et al., 2017; Tutucci et al., 2018). Together, YTHDF2 and IGF2BPs seem to control opposite functions, indicating that the recognition of m6A modification by selectively binding proteins may affect the translation status and lifetime of specific mRNAs.

The finding that m6A modification is largely accumulated in 3′UTRs mRNA, where most of the miRNA binding sites are present, is highly suggestive of an association between m6A and miRNA function (Dominissini et al., 2012; Ke et al., 2015; Meyer et al., 2012). Conceivably, the proximity of m6A to a miRNA-binding site could influence the mechanism of miRNA-mediated transcript inhibition by promoting either transcript degradation or translational repression. Inversely, it is possible that miRNA binding influences m6A levels within 3′UTRs. Indeed, the authors found that abundant miRNAs within the brain are significantly enriched in m6A peaks, raising the possibility that miRNAs regulate methylation status (Dominissini et al., 2012; Ke et al., 2015; Meyer et al., 2012). Recent studies put light into these speculations and discovered that m6A modification is regulated by miRNAs *via* sequence pairing mechanism by recruiting METTL3 (Chen et al., 2015), and m6A sites typically precede, but do not overlap, the miRNA sites in the 3′UTRs (Huisman et al., 2017).

1.1.1. m6A during development

Different studies, either using *in vitro* cell culture or *in vivo* models, determined the importance of m6A modification during the processes of cellular self-renewal, pluripotency and differentiation. All of these processes are key events occurring during embryo development that lead to the generation of different tissues and organs. Interestingly, it has been demonstrated that pluripotency regulators (*Nanog*, *Sox2*, *Oct4*, *Myc* and *Klf4*) exhibit m6A methylation inversely correlating with mRNA stability and gene expression (Batista et al., 2014). Inactivation or depletion of methyltransferases *Mettl3* and *Mettl14* prolonged pluripotency regulators expression upon differentiation, and impaired embryonic stem cells (ESC) exit from self-renewal towards differentiation into several lineages *in vitro* and *in vivo* (Y. Wang et al., 2014). Recent studies identified a chromatin-associated zinc finger protein 217 (ZFP217) which modulates m6A deposition on pluripotency transcripts by sequestering the METTL3. Consistently, *Zfp217* depletion compromises ESC self-renewal and somatic cell reprogramming (Aguilo et al., 2015). Moreover, reduction in m6A methylation decreases Ago2-binding (key factor involved in microRNA mediated silencing) and increases HuR binding (Y. Wang et al., 2014). Collectively, these findings shed light on how m6A modification can tightly couple mRNA stability and translation to ensure ESC identity.

In zebrafish, *Mettl3* and *Wtap* are both expressed since four cells stage until 36 h post-fertilization (hpf), where is mostly detected in the brain. Their knockdown leads to reduced head and eyes, causes small cerebral ventricles and induces a general apoptosis (Ping et al., 2014). M6A is present in mRNA at low levels throughout mouse brain embryogenesis but increases dramatically in adulthood (Meyer et al., 2012). In addition, their transcriptional-wide analysis revealed that m6A peaks mapped to many genes linked to neurodevelopmental and neurological disorders. Interestingly, recent reports demonstrated that *Mettl14* conditional knockout in embryonic mouse brains prolongs the cell cycle of radial glia cells and extends cortical neurogenesis into postnatal stages (Yoon et al., 2017). In Drosophila, *Ime4* (*Mettl3*) knock out displays severe locomotion defects in orientation, walking speed and general activity due to the impaired neuronal function (Lence et al., 2016). Moreover, Ime4-null mutants show a sex bias towards maleness. This is because m6A is required for female-specific alternative splicing of Sxl, which determines female physiognomy, but also translationally represses male-specific lethal 2 (msl-2) to prevent dosage compensation in females (Haussmann et al., 2016).

A recent study has implicated m6A modification with the maternal-to-zygotic transition *via* YTHDF2-mediated maternal mRNA clearance in Zebrafish (Zhao et al., 2017b). Taking into account that during early developmental transitions many transcripts need to be translated in a

very narrow period of time, Zhao and collaborators proposed that m6A may function as a mark for fast mRNA processing, translation and decay (Zhao et al., 2017a).

It was found that ALKBH5 eraser is expressed in mouse testes and its knockout produces an increase of apoptosis in this organ impairing male fertility (Zheng et al., 2013a); while FTO eraser deficiency generates a decrease of body mass and early mortality (Fischer et al., 2009; Gerken et al., 2007). However, recent studies showed that allelic variants within introns of FTO, associated with increased risk for obesity, are functionally connected at megabase distances with the homeobox gene IRX3 (Smemo et al., 2014). Irx3-deficient mice exhibit a body weight reduction of 25 to 30% through the loss of fat mass (Smemo et al., 2014). Based on this, obesity-associated single nucleotide polymorphisms are associated with the expression of IRX3, but not FTO or its effect on m6A modification.

So far, only one epidemiological study found evidence that ALKBH5 confers genetic risk for major depression disorder (Du et al., 2015). Similarly, two studies identified an association between FTO mutations and growth retardation and developmental delay (Boissel et al., 2009; Daoud et al., 2016). In conclusion, the relevance of m6A modification is being re-discovered, but still, much remains to be known about transcript, site and tissue selectivity to coordinates gene expression during animal development.

1.2. m1A: the mark for RNA structure and translation

N1-methyladenosine (m1A) is another type of methylation in adenosine that was found to mark thousands of different transcripts in eukaryotic cells, from yeast to mammals (Dominissini et al., 2016; Iyer et al., 2016). m1A was first discovered at the position 58 in tRNAs (Schevitz et al., 1979), where has an important role in stabilizing the tertiary structure (Li et al., 2016b; Schevitz et al., 1979). Similarly, in human 28S rRNA, m1A was also detected and this methylation is necessary for proper rRNA biogenesis (Peifer et al., 2013). Very recent works have also demonstrated the existence of this modification in mammalian mRNA, whose m1A/A ratios are ranging from 0.015–0.054% in cell lines and up to 0.16% in different tissues (Dominissini et al., 2016; Li et al., 2016b). Most of the methylated transcripts harbor a single m1A that is located around the start codon upstream of the first splice site, in a region with high GC-content. This localization is highly conserved and could markedly alter RNA structure and protein-RNA interactions (Liu and Pan, 2016). The importance of mRNA structure in translation initiation is well known (Dominissini et al., 2016), and different authors speculated that positively charged m1A mark may alter the mRNA structure around this site, thus facilitating translation initiation (Dominissini et al., 2016; Iyer et al., 2016; Li et al., 2016b; Zhao et al., 2017a).

SAM-dependent methyltransferases that install m1A on tRNA and rRNA are known in eukaryotes (Guy and Phizicky, 2014; Oerum et al., 2017). Particularly, TRMT6 and TRMT61A complexes catalyzed the methylation of cytoplasmic tRNA; and TRMT61B the mitochondrial tRNA (Guy and Phizicky, 2014). On the other side, 28S rRNA methylation is catalyzed by RRP8 (also known as NML) (Peifer et al., 2013).

On the other side, the demethylases ALKBH1 and ALKBH3, whose belongs to AlkB family, are involved in the removal of m1A in tRNA and mRNA (Li et al., 2016b; Liu et al., 2016). However, specific readers and writers for m1A in mRNAs are largely unknown. Therefore, the finding of potential readers that specifically recognize m1A and/or the RNA structure on methylated transcripts are highly desirable as aids to a full understanding of the biological roles of this modification.

1.2.1. m1A during development

Since the early discovery of functional m1A as transcriptional regulator, a few studies exist on its role during early development. The m1A level in mouse tissues showed significant variability, with kidney and brain possessing the highest m1A levels (Dominissini et al., 2016). In the same study, a comparison between wild-type and obese mice revealed that brains of the former contain 75% more m1A. Moreover, other authors have demonstrated in human cells that m1A methylation in mRNAs responds dynamically to stimuli, such as serum starvation conditions and H_2O_2 treatment, and identified hundreds of stress-induced m1A sites (Li et al., 2016b). This observation highlights that m1A in mRNA is a dynamic modification in response to certain stresses and/or physiological conditions. Collectively, the recent discovery of m1A in mRNAs, its association with protein translation, its highly conserved localization and the dynamic capacity to respond to certain stresses, make this modification a potentially important player during embryonic development.

1.3. m5C: not only a DNA mark

The function of m5C as an epigenetic mark in DNA have been intensively studied, but the role of this modification in RNA, even when it was discovered more than 40 years ago, took the attention just recently (Desrosiers et al., 1974; Dubin and Taylor, 1975; Edelheit et al., 2013; Marbaniang and Vogel, 2016). Initially, m5C was frequently detected in various non-coding RNAs such as tRNAs, rRNAs and sncRNAs (Motorin et al., 2010). Recent advances in transcriptome-wide analysis have led to the identification of m5C modification in mRNAs (Squires et al., 2012; Yang et al., 2017). Specifically, m5C/C ratio in mRNAs is ~0.02–0.09 suggesting to be present at very specific target sites (Squires et al., 2012). Even when m5C is under-represented in mRNA, their distribution is biased towards untranslated regions and relatively depleted within the coding sequence (Schaefer, 2015). Although a consensus motif (AUCGANGU) has been established in mRNAs from archaea (Edelheit et al., 2013), a consensus has not been defined in animals or plants so far (David et al., 2017; Hussain et al., 2013a; Squires et al., 2012).

The regulatory functions of m5C modification in RNA are still not fully understood. In tRNAs, m5C can influence their secondary structure (Chen et al., 1993; Motorin and Helm, 2010), protect them from degradation (Schaefer et al., 2010; Tuorto et al., 2012) and induce a site-specific cleavage (Alexandrov et al., 2006; Chernyakov et al., 2008). Specifically, loss of m5C methylation increases endonuclease angiogenin-mediated cleavage of tRNAs, generating tRNA-derived small RNAs (5′-tsRNAs and 3′-tsRNAs) (Blanco et al., 2016; Blanco et al., 2014). Interestingly, the accumulation of tsRNAs has been associated with reduced protein translation rates (Ivanov et al., 2011). On the other side, in rRNA and mRNA, m5C is thought to play a role in nuclear-cytoplasmic shuttling, stability and translation (Squires et al., 2012; Squires and Preiss, 2010; Yang et al., 2017).

Up to now, two m5C writers have been identified. The first to be discovered was the tRNA-specific methyltransferase 4 (*Trm4*) present in yeast (Blanco et al., 2011). Later the orthologue protein was identified in animals and named as NOP2/Sun domain protein 2 (*NSun2*) (Moon and Redman, 2014; Tuorto et al., 2012). Initially, this protein was exclusively related to the methylation of a single tRNA target. However, recent studies revealed that NSUN2 target several tRNAs at multiple sites, mRNAs, and noncoding RNAs (Moon and Redman, 2014). The second writer identified was the DNA methyltransferase 2 (*Dnmt2*, also known as *Trdmt1*). Initially, the search for a *Dnmt2* substrate was exclusively focused on DNA until the discovery of its methylation activity in position 38 of different tRNAs (Goll et al., 2006). Interestingly, the RNA methyltransferase activity of this protein is still controversial and only two mRNA targets were identified in a human cell line (Khoddami and Cairns, 2013).

Same as in DNA, the erasers of m5C in RNAs are the Tet-family enzymes that oxidize m5C to hm5C, f5C, and ca5C (Basanta-Sanchez et al., 2017; Fu et al., 2014; Xu et al., 2016). It is remarkable to mention that TET-null ES cells show detectable levels of hm5C in RNA. Similarly, hm5C modification is also detectable in RNAs from *C. elegans* and *A. thaliana*, species that lack orthologs of TET in their genomes (Fu

et al., 2014). Thus, these evidences reveal the possible existence of a Tet-independent mechanism that can oxidize m5C in RNAs. While the final oxidation and eventual decarboxylation of ca5C in RNA provides a possible route to methylation reversibility, evidence of these key steps have yet to be reported. Interestingly, multiple m5C modification have been found in vault non-coding RNAs (vault ncRNAs), which are part of the vault ribonucleoprotein complex that are thought to play a role in intracellular and nucleocytoplasmic transport (Mossink et al., 2003). Finally, the recent discovery of the first m5C reader, the mRNA export adaptor protein ALYREF (Yang et al., 2017), suggesting an essential role in nuclear export of m5C-containing transcripts. Therefore, other potential reader proteins that selectively recognize m5C are also highly desirable as aids to a full understanding of the biological roles of this modification.

1.3.1. m5C during development

Deletion of the m5C methyltransferases *NSun2*, alone or in combination with the *Dnmt2* can impair cellular differentiation pathways in skin, testes, and brain (Blanco et al., 2011; Hussain et al., 2013b; Rai et al., 2007; Tuorto et al., 2012). Besides, loss of m5C methyltransferases caused microcephaly and other neurological abnormalities in mice (Blanco et al., 2014), flies (Schaefer et al., 2010), and zebrafish (Khoddami and Cairns, 2013). So far, no human disease has been associated with mutations in *Dnmt2*. But, mutations in the *NSun2* gene causes a syndromic form of intellectual disability and neurological abnormalities, such as microcephaly, behavioral deficits, speech delay, abnormal gait, as well as morphological features including growth retardation, unusual facies and cutaneous abnormalities (Abbasi-Moheb et al., 2012; Martinez et al., 2012). However, whether and how loss of RNA methylation can cause those anomalies are largely unknown. Interestingly, using patients cells lacking the NSUN2 protein, Hussain et al. (2013b) show that loss of m5C in vault RNAs causes aberrant processing of these RNAs into Argonaute-associated small RNA fragments, that can function as microRNAs. Thus, impaired processing of vault ncRNA may contribute to the etiology of *NSun2*-deficiency human disorders. Similar to *NSun2*, mutations in the endonuclease angiogenin gene, involved in tRNA cleavage, are also linked to neurological disorders (van Es et al., 2011). Furthermore, inhibition of tRNA cleavage in Nsun2-deficient mice revert brain phenotype to normal (Blanco et al., 2014). Taking together these studies suggest that lack or aberrant m5C modification on RNA may be a novel and important contributor to human diseases and tsRNAs may be the mediators.

Many authors have proposed that m5C serves as a general strategy to regulate the function of ncRNAs (Motorin and Helm, 2010; Squires and Preiss, 2010). This assumption was supported with a study where they found in fasting mice deposition of m5C in enhancers RNAs (eRNAs) (Aguilo et al., 2016). In this experiment, the author demonstrated that the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), a transcriptional co-activator, need to bind with eRNAs containing m5C to target specific genes. This study shows for the first time a complex epigenetic circuit between an epigenetic protein and modified ncRNAs to regulate gene expression. The authors concluded that enrichment of m5C within eRNAs species is highly correlated with metabolic stress of fasting, remarking that probably this is a new level to fine-tune rapid changes during energy metabolism.

In the last years, several works highlighted the importance of tsRNAs, mainly 5′-tsRNAs, as they are highly enriched in mature sperm and their expression profile is altered under different environmental exposures in mammals. Furthermore, zygotic injection of a combination of tsRNAs or a single tsRNA could change early embryonic gene expression (K. Chen et al., 2016). These evidence indicated that sperm tsRNAs may play a pivotal role in the epitranscriptomic transmission of paternally acquired experiences by affecting early embryonic development. Thus, very recent and elegant works demonstrated their functional role as an epitranscriptomic mediator by injecting tsRNA-enriched RNA fragments from the sperm of mice fed with high-fat diet into wild zygotes (Q. Chen et al., 2016). Interestingly, the injected tsRNA-enriched RNA fragments presented a significant increase in the level of m5C modification after paternal high-fat diet consumption and induced metabolic disorders in offspring. However, the functional mechanism by which sperm tsRNAs affects early embryonic development and mimics paternally acquired traits remains unclear and warrants future investigations.

1.4. Pseudouridine, the fifth ribonucleotide

Pseudouridine (Ψ) modification comes from the isomerization of the uridine base and shines for being the first discovered and globally most abundant RNA modification (Ge and Yu, 2013). It was discovered 60 years ago in rRNAs (Cohn, 1951; Cohn, 1960), but later identified in other ncRNAs such as tRNAs, snRNAs, snoRNAs and telomerase RNA (Ge and Yu, 2013; Machnicka et al., 2013). Ψ presence in mRNA was recently established using high-throughput protocols (Carlile et al., 2014; Li et al., 2015; Schwartz et al., 2014). These studies revealed that the Ψ/U ratio in mammalian mRNA is ~0.2–0.6%, which is comparable in content to m6A. The identification of Ψ in more than 2000 sites, mostly detected within the coding sequences and 3′UTRs, but underrepresented in the 5′ UTR, demonstrated that this modification can be more important than was previously consider (Li et al., 2015; Schwartz et al., 2014).

Ψ is dynamically regulated across species, tissues and stress stimulus, suggesting plasticity and a regulatory purpose for this mark (Li et al., 2016a). Moreover, the presence of this mark in non-coding RNAs can influence diverse cellular processes, including transcription, translation efficiency and accuracy, splicing and telomere length (Li et al., 2015; Schwartz et al., 2014). On the other side, pseudouridylation is found in a great number of mRNAs and showed to be dynamically regulated upon stress conditions (Li et al., 2016a). However, the exact biological consequences of normal or inducible pseudouridylation in mRNAs remain to be investigated.

Pseudouridylation is catalyzed by pseudouridine synthases (PUSs) which may act in two ways. First, the RNA-independent pseudouridylation, where a single PUS carries out both, substrate recognition and catalysis in tRNAs and rRNAs (Hamma and Ferre-D'Amare, 2006). Second, the RNA-dependent manner, which required a RNA-protein complex, known as box H/ACA small RNPs, formed by a box H/ACA ncRNA and a PUS-containing complex (Karijolich and Yu, 2011). The PUS-containing complex is formed by four core proteins including centromere-binding factor 5 (CBF5, or dyskerin in mammals), nonhistone protein 2 (NHP2), nucleolar protein 10 (NOP10) and glycine-arginine-rich protein 1 (GAR1). Altogether, the box H/ACA small RNPs complex is responsible for substrate recognition, through complementary base-pairing interactions with the substrate RNA, where dyskerin (DKC1) catalyzed the pseudouridylation (Hamma and Ferre-D'Amare, 2006; Karijolich and Yu, 2011). In human, 23 genes with a PUS domain exist and most of the Ψ modifications are introduced by PUS1, PUS4 and PUS7 (Schwartz et al., 2014).

1.4.1. Pseudouridylation during development

As we mentioned before, it has been described that both RNA-dependent and RNA-independent mechanisms exist for the isomerization of uridines into Ψ. Those mechanisms can influence important RNA processes including translation, gene regulation and telomere maintenance (Zhao and He, 2015). Based on this, it is not surprising that pseudouridylation may play a pivotal role during early development. Indeed, mutation in *DKC1*, from the Ψ RNA-dependent PUS-containing protein complex, was associated with the multisystem disorder X-linked dyskeratosis congenita (Heiss et al., 1998), Hoyeraal-Hreidarsson syndrome (Chhabra, 2015; Knight et al., 1999) and cancer predisposition (Bellodi et al., 2010; Sieron et al., 2009). In mice, deletion of *Dkc1* causes early embryonic lethality (He et al., 2002). This is in agreement

with the observation that DKC1 is highly expressed in pluripotent ESC and regulated the expression of pluripotency factors OCT4 and SOX2, raising the possibility that *DKC1* might control pluripotency (Agarwal et al., 2010; Fong et al., 2014). In this context, high-throughput studies have found in patients with dyskeratosis congenital that the telomerase RNA component (TERC) has lower Ψ and this leads to poor maintenance of telomeres (Schwartz et al., 2014).

On the other side, a missense mutation in the PUS1 gene, which belongs to the RNA-independent writers, causes impaired mitochondrial and cytoplasmic tRNA pseudouridylation leading to various conditions such as myopathy, lactic acidosis and sideroblastic anemia (Patton et al., 2005). However, little is known about the molecular mechanism implicated in these diseases, opening questions about the role of this dynamic modification during early development.

2. Conclusion and perspectives

During several years we thought that the term epigenetics exclusive concern to chromatin (epigenome), and RNA was just a bridge between DNA and proteins. However, this idea is certainly changing since RNA modifications (epitranscriptome) are gaining a tremendous interest in the scientific community. Thanks to the high-throughput technologies, a great progress was achieved in the novel world of dynamic RNA modifications. In agreement with this, very recent findings have suggested that RNA modifications are an additional central mode of transcriptome malleability that has the potential to determine the biological sophistication and the evolutionary outcomes (Hussain, 2017). This idea is supported by the fact that m6A occurrence has generally steadily increased during primate evolution (Ma et al., 2017).

The dynamism of m6A is well known and stands out because of the following major points: 1) it is the most abundant in mRNA; 2) it is important during RNA metabolism as well as gene expression; 3) it is present in mRNA of transcription factors that regulate differentiation and pluripotency, and 4) loss of m6A methyltransferases and demethylases are lethal during embryo development. However, we must consider that multiple RNA modification may contribute together to regulate the expression of specific transcripts (Song and Yi, 2017).

Comparing with the well-studied dynamism of m6A modification in RNA, the m1A is until now a puzzle. Even though m1A is less abundant than m6A, its distribution is highly conserved in mammals, drawing attention for its tentative functions. Tacking together, m6A and m1A are both involved in regulating gene expression and translation, thus they would be handling a fine control of protein expression during stages where fast and precise transcriptomics changes are needed, such us early development.

The dynamism of m5C has been well established and several functions were associated with this mark related with structural, stability, localization and translation. One of the most recent and interesting aspects of this modification was the fact that modified tsRNAs mediated inheritance of paternally acquired traits. However, the epitranscriptomic mechanism through which specific acquired experience become encoded in RNA and how this impact on the offspring are fundamental questions that require further investigation and have obvious medical and social implications.

Ψ is one of the most abundant RNA modification. Although a very dynamic distribution is observed under stress conditions, little is known about the enzymes implicated in this dynamism. Up to now, studies of loss-of-function for Ψ writers cause early embryonic lethality and several human diseases were associated with mutation on them. Nevertheless, little is known about which are the molecular mechanism involved and certainly, further investigations are needed to understand the role of this abundant RNA modification during animal development.

As we have described, the central role of RNA modifications is reflected by the fact that aberrant modifications have deleterious effects on animal development, cellular differentiation, cancer occurrence, inflammation, obesity, infertility and several neurological disorders (Dezi et al., 2016; Lence et al., 2017; Li et al., 2016c; Licht and Jantsch, 2016; Meyer and Jaffrey, 2017; Song and Yi, 2017). Thus, further emphasize the importance of these emerging post-transcriptional modifications that will ultimate impact on the cellular proteome.

We are confident that in the coming years the alphabet of RNA epigenetics will be more understood and will make epitranscriptome more complex and diverse. Finally, it was proposed that in the near future new technologies will contribute to alter RNA modifications artificially by engineering RNA-modifying enzymes with altered substrate specificity or activity (Frye et al., 2016). This approach will help to develop new therapies for human diseases caused by RNA modification deficiency. However, we are aware that there are still remaining questions to be answered about the dynamics of RNA modifications and how these changes lead different regulatory processes in development and disease.

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