Post-transcriptional regulation by cytosine-5 methylation of RNA

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Abstract

The recent advent of high-throughput sequencing technologies coupled with RNA modifications detection methods has allowed the detection of RNA modifications at single nucleotide resolution giving a more comprehensive landscape of post-transcriptional gene regulation pathways. In this review, we focus on the occurrence of 5-methylcytosine (m^5C) in the transcriptome. We summarise the main findings of the molecular role in post-transcriptional regulation that governs m^5C deposition in RNAs. Functionally, m^5C deposition can regulate several cellular and physiological processes including development, differentiation and survival to stress stimuli. Despite many aspects concerning m^5C deposition in RNA, such as position or sequence context, and the fact that many readers and erasers still remain elusive, the overall recent findings indicate that RNA cytosine methylation is a powerful mechanism to post-transcriptionally regulate physiological processes. In addition, mutations in RNA cytosine-5 methyltransferases are associated to pathological processes ranging from neurological syndromes to cancer.

Keywords

5-methylcytosine, 5-hydroxymethylcytosine, RNA methylation, epitranscriptome, post-transcriptional regulation, translation.

1. Introduction

The first modified RNA nucleoside was identified almost 60 years ago and since then around 171 distinct modified nucleotides have been characterized. Most of them are located at abundant non-coding RNAs such as transfer RNAs (tRNAs) from all kingdoms of life [1]. Initial detection studies were based on chromatography techniques and thus were largely limited to detect only more abundant modifications in more stable and abundant RNAs and with little information on their distribution. The presence of chemical modifications on messenger RNA (mRNA) was uncovered in the 70's, when poly-A tail-based purification techniques first allowed for pure mRNA preparations to rule out contamination with other RNA species [2]. These studies identified the existence of internal RNA modifications including N⁶-methyladenosine (m⁶A) and 5-methylcytosine (m⁵C) [2].

However, despite the identification of cytosine-5 methylation in RNA and DNA more than 40 years ago, we are still now beginning to understand its function in RNA and most of our knowledge on cytosine-5 methylation has focused on DNA. Initial reports on the methylation of ribosomal RNA (rRNA) showed that rRNA presented specific patterns with regards to their type of methylation, being the 28S molecules mainly ribose-methylated and 18S rRNA base-methylated [3]. Subsequent studies concluded that methylation of bases was more conserved and present in bacteria and eukaryotes, whereas ribose methylation appeared in eukaryotic cells (extensively reviewed in [4]). Shortly after the discovery of nucleotide methylation in the cap-structure of eukaryotic mRNAs, some studies arose showing the presence of methylated nucleotides in mRNAs in mammalian cells [2] and viral RNAs, including m⁵C [5]. However other studies did not find m⁵C deposition in human mRNAs [6].

While 5-methylcytidine is best known for its central role as an epigenetic mark in DNA, the study of its functional role in RNA is only beginning to emerge. Lately, with the venue of new technological advances in sequencing, special focus has been recovered on detecting RNA modifications. Recent attempts of high throughput detection methods have revealed m⁵C occurrence in coding and non-coding RNAs [7-13]. In addition, the functional roles of m⁵C and other RNA modifications have begun to be elucidated, as well as their writers (enzymes that catalyse their deposition), erasers (enzymes that catalyse their removal) and readers (RNA-binding proteins that specifically bind to RNA modifications) have been identified. It is becoming increasingly clear that the epitranscriptome landscape is highly encoded and it is likely to play many critical functions of RNA metabolism as well as cellular processes including proliferation, differentiation or development.

In this review, we summarise the current knowledge on cytosine-5 RNA methylation distribution in all RNA classes, with special emphasis on the latest advances in m⁵C deposition and function in eukaryotes. We review system-wide available methods for detection of m⁵C sites at nucleotide resolution and discuss their advantages and limitations. We describe the cytosine methyltransferases known so far, the protein readers as well as the recent findings on 5-hydroxymethylcytosine occurrence in RNA. We then examine the molecular role of this modification in RNA metabolism and processing, and emphasise the functional role in expression regulation and protein synthesis. Finally, we discuss the physiological role and the pathological implications of its dysregulation in diseases and analyse the remaining challenges in determining the functions of RNA m⁵C deposition.

2. RNA Cytosine-5 methylation sequencing detection methods

The existence of m⁵C in RNAs has been known since the 1970s [2, 5]. However, its occurrence in low abundance RNAs and its transcriptome-wide distribution have been difficult to determine due to the lack of reliable and sensitive methods to detect m⁵C at nucleoside resolution. The recent advances in next-generation sequencing (NGS) tools have made possible the development of sequencing technologies that have provided a comprehensive view of m⁵C distribution patterns throughout the global transcriptome. These methods have also allowed the definition of the dynamic epitranscriptomic status under different physiological conditions, as well as the characterization of each of the specific substrates of currently known RNA methyltransferases.

Several high throughput m⁵C detection methods have been developed so far. Generally, their methodologies are based on immunoprecipitation techniques, chemical conversion of the modified base or a combination of both. Bisulphite sequencing exploits the chemical reactivity principles of cytosines and it has been widely used [14] (Fig. 1). Other currently used detection techniques are m⁵C RNA immunoprecipitation (m⁵C-RIP) [10], 5-azacytidine-mediated RNA immunoprecipitation (Aza-IP) [15], or methylation-individual nucleotide resolution crosslinking immunoprecipitation (miCLIP) [16]. These three methods are based on immunoprecipitation (IP) protocols and allow for the enrichment of low abundance m⁵C-modified RNAs. These IP-based techniques have overcome some of the limitations of bisulphite sequencing discussed here, including enrichment for low abundance RNAs or RNAs with low m⁵C content. Additionally, Aza-IP and miCLIP enable the identification of enzyme-specific targets. In the following section, we will discuss the methodological approach as well as the advantages and limitations of each of the m⁵C detection sequencing

techniques. Other non-sequencing based approaches that have allowed the detection of m⁵C in RNA since its initial discovery are extensively reviewed elsewhere [17].

2.1. Bisulphite sequencing

Bisulphite conversion protocols were initially developed to detect m⁵C in DNA [18], and later adapted to detect m⁵C in RNA [19]. By coupling bisulphite conversion protocols to high throughput sequencing techniques, Schaefer and colleagues determined the distribution of m⁵C throughout *Drosophila* tRNAs and rRNAs [14]. Three years later, the protocol was successfully applied at transcriptome-wide level providing the first global view of the m⁵C-methylome in human RNAs at single nucleotide resolution [11]. The technique relies on the differential chemical reactivity of unmethylated and methylated cytosines (C). Unmethylated Cs are converted into uraciles (U) by deamination in the presence of sodium bisulphite. Meanwhile, methylated Cs and double stranded nucleotides are protected from deamination. The extent of modifications at each cytosine residue is then determined by calculating the fraction of reads that do not show the expected C to U change, assuming complete conversion of unmethylated C (Fig. 1). Bisulphite sequencing is the gold standard and it has allowed the identification of m⁵C deposition in RNA at single nucleotide resolution in cells in culture and in vivo and the validation of methylases specific targets [8-13, 20, 21]. The biggest advantages of the technique are its high specificity to detect modified cytosines throughout the transcriptome and its single nucleotide resolution. Yet, it has several limitations: although the chemical conversion is highly specific to cytosines, the sites could include other cytosine modifications that interfere with bisulphite conversion such as 3-methylcytosine and 5-hydroxymethylcytosine among others (reviewed in [22]). Highly structured RNA regions difficult to denature are also protected from the conversion reaction (reviewed in [22]). In addition, its application is limited only for highly abundant RNA molecules, due to the loss of RNA by chemical hydrolysis caused by the harsh conditions (denaturation at high temperature, low pH conditions) during the long bisulphite conversion process. This may explain why the use of bisulphite sequencing has resulted in marked differences in the reported prevalence of m⁵C in low abundance RNAs. For example, from thousand m⁵C sites in mRNA in HeLa cells compared to fewer mRNAs in other cell types or tissues [11, 12]. Therefore, the technique is somehow erratic to detect m⁵C in low abundance RNAs or highly structured RNAs. Increased strand denaturation by using denaturing agents (formamide), random fragmentation of RNA or enrichment of low abundance RNAs can overcome some of the technical limitations [12, 23]. Yet, the technique does not reveal information about substrate specificity and requires genetic manipulation of RNA methylases to reveal methylase-specific positions. In addition, despite

all the advances, analyses of m⁵C deposition in low abundance RNAs have resulted in annotation discrepancies indicating that m⁵C detection by bisulphite sequencing still remains a difficult task [24]. In fact, it requires statistically robust and highly refined bioinformatic tools to allow for a reliable analysis of the RNA methylome.

2.2. m⁵C RNA immunoprecipitation

m⁵C-RIP exploits the use of antibodies that bind to 5-methylcytosine followed by library preparation of enriched m⁵C-modified RNAs and high throughput sequencing. This approach was used to map m⁵C-modified RNAs in bacteria, *Archaea*, yeast and plants transcriptome [9, 10]. m⁵C-RIP overcomes some of the pitfalls derived from bisulphite sequencing such as the hydrolysing conditions during bisulphite conversion protocol, and thus reducing the degradation of low abundance RNAs. Yet, the biggest caveat is the high dependency on the antibody specificity. Furthermore, this protocol relies on RNA-antibody complexes that are non-covalently bound, and thus purification of RNA molecules is performed in low stringency conditions increasing the risk of purifying non-specifically bound RNAs. To overcome these issues and truly define enzyme-specific m⁵C deposition, one can combine this protocol with genetic deletion or knock-down of RNA methylases and RNA-protein cross-linking approaches [25].

2.3. 5-azacytidine-mediated RNA immunoprecipitation

Aza-IP allows the detection of enzyme-specific methylation sites [15]. Aza-IP is based in the use of 5-azacytidine (5-aza-C), a cytidine analogue that forms a stable link between the enzyme and the RNA substrate, preventing the separation of both components [15]. 5-aza-C is fed into cells and randomly incorporated into the nascent RNA transcripts at C positions. The cytidine analogue traps the methylase forming a covalent adduct. Covalently bound RNA-methylase complexes are immunoprecipitated with enzyme-specific or tag antibodies. Next, the RNA is released from the methylase-RNA complex, triggering a hydrolytic opening of the 5-aza-C ring which is read as a guanine during reverse transcription and sequencing [15] (Fig. 1). This approach enables base-resolution mapping of the target sites in RNA. One of its biggest advantages is that m⁵C-modified RNAs are covalently bound to the methylase under study, and the RNA-protein complexes can be purified under high stringency conditions, thus reducing the background of non-specific bound RNAs. Thus, the technique relies on highly specific antibodies against the investigated methylase or the use of genetically engineered cell lines or model organisms that stably expressed a tagged version of the enzyme. Yet, the biggest limitation is the random incorporation of 5-azacytidine and m⁵C sites that are not replaced by 5-azacytidine will be missed. Additionally, the cytidine

analogue is highly toxic, it can be also incorporated into DNA and disrupt the normal transcriptional processes as well as DNA and RNA integrity.

2.4. Methylation-individual nucleotide resolution crosslinking immunoprecipitation

miCLIP follows the same principle as Aza-IP with the difference of its genetic base. This strategy combines individual nucleotide resolution crosslinking and immunoprecipitation (iCLIP) approach to genetic engineering approaches allowing the identification of methylase specific deposition sites with nucleotide resolution [16]. The technique was first used to identify methylation substrates of NSUN2 by exploiting the structural and functional properties of the catalytic domain of NSUN members [16]. A mutation of a conserved cysteine at the catalytic domain (C271A) that releases the RNA from the enzyme led to the establishment of covalently bound RNA-substrates [16, 26]. Methylation positions can be determined because protein crosslinking to the target cytosines lead to termination of reverse transcription during library preparation [16]. This strategy was later used to identify NSUN3 substrates [21]. Despite being a very robust method with high specificity, the technique has limitations too. For instance, cells or organisms expressing the mutant enzymes need to be generated by genetic engineering techniques which increases the time and cost of its performance. In addition, expression of mutant enzymes may produce star activity which could result in the detection of non-bona fide substrates.

In conclusion, all the currently available sequencing techniques show different caveats caused by a variety of reasons ranging from the experimental procedure to the data analysis approaches. This suggests the combinatorial use of different methods and the implementation of different computational strategies to produce a more robust and reliable picture of m⁵C methylomes.

3. Cytosine-5 methylation distribution and cytosine-5 methyltransferases

m⁵C is a conserved and prevalent mark in RNA in all life domains [1]. Yet, its occurrence and prevalence varies among RNA types and organisms. While m⁵C has been found in tRNA and mRNA from eukaryotes and archaea, it is missing in tRNA and mRNA from prokaryotes [1, 2, 7, 9-12, 20, 27, 28]. m⁵C deposition is catalysed by RNA m⁵C methyltransferases (RCMTs) that belong to the superfamily of Rossmann fold-containing enzymes that use S-adenosyl-L-methionine (SAM) as a methyl group donor. To date all confirmed eukaryotic m⁵C-specific RNA methyltransferases belong to either the NOL1/NOP2/sun (Nsun) subgroups of methyltransferases or the DNA methyltransferase member DNMT2 (also known as tRNA Aspartic Acid Methyltransferase 1 or TRDMT1) (Fig.

2) [17, 29]. Below we will examine m⁵C occurrence and distribution in each type of RNAs in eukaryotes, as well as the currently known methyltransferases that specifically target them.

3.1. Cytosine-5 methylation in tRNAs

m⁵C deposition in tRNAs is structure- and enzyme-specific. It is mostly found at conserved positions at cytosines at the junction of the variable loop and the T stem spanning positions 48-50 of majority of tRNAs. It is also found at the anti-codon loop at positions 34 and 38 and at the acceptor arm at position 72 of fewer tRNAs [10, 11, 13, 16, 28, 30, 31] (Fig. 2). DNMT2, NSUN2, NSUN3 and NSUN6 all methylate tRNAs, yet with different specificity [8, 13, 21, 31-36]. The best characterized RNA cytosine-5 methyltransferases in eukaryotes are DNMT2 and NSUN2 [8, 13, 16, 32, 33, 37-48]. NSUN2 methylates the majority of the cytoplasmic tRNAs at the variable loop at position 48-50, and leucine at position C34 at the anti-codon loop in mammals [8, 15, 16]. Likewise, NSUN2 yeast homologue Trm4 and *Arabidopsis* homologue TRM4B methylate similar positions indicating a highly conserved functional role [9, 20, 28, 30, 31]. NSUN6 targets C72 at the acceptor stem of cysteine and threonine cytosolic tRNAs [35]. Meanwhile, mammalian NSUN3 catalyses the formation of m⁵C at position C34 of mitochondrial methionine tRNAs [21, 34, 36]. DNMT2-mediated methylation is restricted to three cytoplasmic tRNAs (Asp, Gly and Val) at position C38 at the anti-codon loop in mammals and *Drosophila* [13, 32, 37]. In comparison, fission yeast homologue Pmt1, *Dictyostelium discoideum* homologue DnmA and plant TRDMT1 all methylate tRNAs at the anti-codon loop [20, 49, 50].

3.2. Cytosine-5 methylation in rRNAs

Cytosine-5 methylation in rRNA is the most conserved across all forms of life and thus reflecting its biological significance (Fig. 2) [1]. The nuclear large subunit 25S rRNA in yeast and 28S rRNA in high eukaryotes contains two m⁵C sites that cluster around conserved structural and functional ribosome regions, suggesting functional relevance [51, 52]. NOP2 and the yeast homologue Nop2 catalyses the transfer of a methyl group onto C2870, which resides close to the peptidyltransferase center of the 28S rRNA (25S rRNA in yeast) of the large ribosomal subunit [27, 31, 52]. NSUN5, and the yeast homologue Rcm1, methylate position C2278 of 28S rRNA (and 25S rRNA in yeast), located at the interface between large and small subunit [27, 31, 52]. NSUN4 targets C911 on the small subunit 12S of mitochondrial rRNA [53].

3.3. Cytosine-5 methylation in mRNAs

The existence of m⁵C in eukaryotic mRNA has been difficult to prove due to its low prevalence and the lack of effective detection techniques. By using mass spectrometry, early studies in the 1970's, detected m⁵C in mammalian mRNAs [2], although some other studies failed to do so [6, 54]. In recent years, advanced and more sensitive high performance liquid chromatography coupled to mass spectrometry (LC-MS) methods allowed the detection of m⁵C in mRNAs and thus ratifying that indeed mRNA is methylated at cytosines [55-57]. In addition, high throughput detection methods coupled to RNA sequencing have allowed the detection of m⁵C at single nucleotide resolution [7, 9, 11, 12, 20]. For instance, following bisulphite sequencing of total RNA from human HeLa cells, Squires and co-workers found 10275 methylated positions in the transcriptome and about 8000 sites were located in mRNA sequences [11]. Likewise, by using bisulphite sequencing of poly-A-enriched RNAs of the same cell line, 2000 mRNAs were found to be methylated [12]. In addition, bisulphite sequencing of poly-A-enriched RNAs from mouse tissues including brain, and from mouse embryonic stem cells (ESC) revealed few thousand mRNAs being methylated [7]. Regarding its m⁵C sites distribution, several studies have identified m⁵C deposition in different mRNA positions including 3' and 5' UTRs regions, at CDS (coding sequence) [7, 9, 11, 12, 16, 20]. In contrast, by using more stringent quality controls, tailored filters for sequencing and alignment artefacts and statistical modelling to eliminate non-conversion artefacts, Lyko and co-workers could not identify any clear m⁵C site in mouse mRNAs [24].

Attempts to detect m⁵C in mRNA from other species have identified methylated mRNAs in various plant species [9, 10, 20]. By using bisulphite sequencing, m⁵C-RIP-seq and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), m⁵C residues were detected in mRNA from different plant organs [9, 20]. One study found 100 sites and enrichment at 3'UTR [20] and the other reported more than 6000 m⁵C sites and enriched in the CDS [9]. Combining bisulphite sequencing and m⁵C-RIP-seq approaches on archaeobacteria, bacteria and budding yeast mRNAs, Sorek and colleagues identified m⁵C residues only in archaeal mRNAs, although at very low prevalence [10]. m⁵C residues have not been detected in *Drosophila* mRNAs [24].

Attempts to determine the specific writers of m⁵C in mRNA have indicated that human NSUN2 and its homologue in plants TRM4B are currently the only cytosine-C5 methylases with capacity to modify mRNAs (Fig. 2) [9, 11, 12, 16, 20]. Whether other cytosine-5 methyltransferases target mRNA remains unknown.

Overall, multiple lines of evidence show that mRNA is methylated [7, 10-12, 55-57], yet its prevalence and site distribution in mRNA still remain elusive.

3.4. Cytosine-5 methylation in other non-coding RNAs

m⁵C has also been detected in small non-coding RNAs and long non-coding RNAs (lncRNA) [9, 11, 15, 16, 20, 58]. Bisulphite sequencing on polyA-RNAs from several human cell lines identified m⁵C sites within or near functionally important regions of lncRNAs HOTAIR and XIST [58]. Using bisulphite sequencing, miCLIP and Aza-IP for human NSUN2, m⁵C sites were identified in the three vault RNAs (vtRNA) vtRNA1.1, vtRNA1.2 and vtRNA1.3 [15, 16]. In addition, NSUN2-mediated methylation sites were identified in Ribonuclease P RNA component H1 (RPPH1), 5S rRNA, the C/D box small nucleolar (snoRNA) small cajal body specific RNA 2 (SCARNA2), RNY1, signal recognition particle RNA (7SL RNA) and the small nuclear RNA 7SK [11, 15, 16]. Although the number of methylated ncRNAs that have been described so far is still small, most are methylated by NSUN2. These results indicate that mammalian NSUN2 is currently the cytosine-C5 methylase with a broader substrate specificity as it methylates ncRNAs, tRNAs and mRNAs [8, 11, 12, 15, 16]. Similar to human NSUN2, plant TRM4B methylates a wide range of non-coding RNAs including small nuclear RNAs, snoRNAs and lncRNAs [20].

Another NSUN family member with affinity for ncRNAs is NSUN7. NSUN7 was described to interact with the transcriptional coregulator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) and with promoter-derived enhancer RNAs (eRNAs) of several PGC-1α target genes in mouse hepatocytes (Fig. 2) [59]. Bisulphite conversion and Aza-IP experiments followed by qPCR of eRNAs from cells depleted of *Nsun7* showed a decrease of the presence of m⁵C sites, suggesting that eRNAs selectively undergo cytosine methylation [59]. The report further showed that *NSun7* expression and m⁵C content on RNA increased after fasting, suggesting that both events were regulated upon metabolic stress [59].

4. Functional role of cytosine-5 methylation

Early studies in single cell organisms shed light on the molecular and biological functions of m⁵C methylases indicating multiple roles stabilizing tRNA molecules and in mRNA decoding [60-62]. In this section, we will discuss the biological roles associated to known methyltransferases, focusing mainly on eukaryotic organisms, as well as the molecular role of m⁵C in each RNA type.

4.1. Molecular and physiological role of NSUN2

Cytosine-5 methylation of tRNAs by NSUN2 has been linked to survival, differentiation and development in eukaryotes [8, 9, 20, 30, 31, 43, 46-48, 63]. In humans and mice, comparative results of gene expression data with global cytosine-5 RNA methylome from *Nsun2*-deficient cells and tissues elucidated that loss of NSUN2-mediated tRNA methylation increased angiogenin-mediated endonucleolytic cleavage of tRNAs [8]. Consequently, *Nsun2*-deficient cells aberrantly accumulated 5' tRNA-derived small RNA fragments (5' tRFs) [8]. Molecularly, accumulation of 5' tRFs resulted in global repression of protein translation (Fig. 3A). Ribosome profiling studies further showed a favoured increased translation of mRNAs encoding for stress response genes [47]. The study, similar to previous works in yeast [30], linked NSUN2-mediated RNA methylation to stress-specific reprogramming of translation and demonstrated that translational control through RNA methylation is a conserved stress survival mechanism. Consequently, *Nsun2*-deficient cells were hypersensitive to stress [8, 47]. Likewise, *Trm4B* loss in plants (*Nsun2* homologue in plants) resulted in increased sensitivity to oxidative stress [9, 20], and double null mutants *trm4B trdmt1* were hypersensitive to the antibiotic hygromycin B [31]. Altogether, demonstrating a function of RNA methylation in regulating stress responses and survival.

Studies in mice also revealed that lack of NSUN2-mediated RNA methylation repressed the translation of transcripts encoding for differentiation genes in mammals [47]. Consequently, loss of *Nsun2* in mouse led to differentiation failure, causing developmental delayed and tissue differentiation defects in mice [8, 43, 46, 48]. For instance, deletion of *Nsun2* in mice decreased differentiation of hair follicle stem cells and germ cells leading to hair growth defects and infertility in male mice [43, 46]. Likewise, loss of *Nsun2* in mice caused accumulation of intermediate progenitors and a decrease in differentiated upper-layer neurons in the cortex due to decreased differentiation and migration of neural progenitors [48]. Together these indicate that NSUN2-mediated RNA methylation regulates developmental and differentiation processes. Not surprisingly, NSUN2 was initially described as a downstream target gene of Myc in a population of progenitor and differentiating cells in human and mouse epidermis [64]. NSUN2 expression was also shown to be highest during embryo development in ectodermal tissues and in the compartment of differentiating cells in adult tissues [43, 46, 48, 65].

NSUN2 also targets other non-coding RNAs such as vault RNAs. Molecularly, loss of *NSUN2* in human cells induces cleavage of vault RNAs into small RNAs that interfere with

the silencing machinery (Fig. 3B) [16]. Nevertheless, the physiological significance of NSUN2-mediated methylation of vault RNAs or other non-coding RNAs remains elusive.

4.2. Molecular role of the RNA methyltransferase NSUN3

NSUN3-mediated methylation of mitochondrial methionine tRNA at position C34 has been shown to be further oxidized by the alphaketoglutarate and Fe(II)-dependent dioxygenase ALKBH1/ABH1 to generate 5-formylcytidine (f^5C) at this position [21, 34, 66]. Functional studies in mice and humans showed that loss of *NSUN3* and lack of formylated methionine tRNA resulted in reduced mitochondrial translation (Fig. 3D) [21, 34, 36, 67]. In addition, it was shown that catalytic inactivation of *Nsun3* in mouse ESCs further caused impaired differentiation into the neuroectodermal lineages [67]. Nonetheless, the mechanistic link between the loss of mitochondrial methionine tRNA formylation and the impairment of differentiation remains unknown.

4.3. Molecular role of the RNA methyltransferase NSUN6

NSUN6 methylates two cytosolic tRNAs at C72 at the acceptor stem of only mature tRNAs [35]. The molecular and functional role of NSUN6-mediated methylation at tRNAs is unknown. However, its proximity to the amino acid charging position suggests a role in accurate discrimination of amino acids by the aminoacyl-tRNA synthetases and ultimately accurate protein synthesis. Whether NSUN6 has a broader substrate specificity and can target other RNA types remains unknown.

4.4. Functional role of the rRNA methyltransferases NOP2, NSUN4, NSUN5

Several studies have indicated that cytosine-5 methyltransferases that target rRNA play a role in rRNA biogenesis and ribosome assembly. For instance, functional studies in mouse showed that the mitochondrial family member NSUN4 was required for the methylation of 12S rRNA of the mitochondrial small ribosomal subunit and also to interact with MTERF4 facilitating the assembly of the large subunit (Fig. 3D) [53]. Likewise, functional studies in eukaryotic cells showed that lack of the rRNA methyltransferase targeting these positions, NOP2 and NSUN5, led to defects in ribosome biogenesis and assembly [27, 52, 68]. Studies in yeast further showed that Nop2p interaction, regardless of its methylation activity, was required for pre-rRNA processing [27, 69]. In addition, polysome profiling of *Rcm1* null mutant in yeasts showed an increased in translation of transcripts encoding stress response genes (Fig. 3C) [70]. All together, these studies show that NSUN members with rRNA specificity possess two related functions in rRNA processing: an essential factor for pre-

rRNA processing and methylation activity. The first supports the notion of quality control during ribosome synthesis by such enzymes [27, 53, 68]. The second suggests that the occurrence of m⁵C could directly influence ribosomal activity to favour translational programmes in response to environmental changes [70]. Nonetheless, fluctuations of the fraction of methylated cytosines in rRNA have not been described yet. However, profiling of other modifications in human rRNA (ribose 2'-O-methylation) has revealed a subset of sites showing variable levels of modification [71], suggesting a role of rRNA modifications in the specialisation of the translating machinery. Mechanistically, whether the level of modified residues can dictate the recruitment or displacement of the ribosome onto specific mRNAs is still elusive. However, studies on the mechanism of 80S complex formation on cellular internal ribosome entry sites (IRES) elements have shown that the 2'-O-methylation status of rRNA impaired the assembly of the ribosome on IRES, indicating a role for rRNA modifications on cellular IRES-mediated translation regulation [72]. All these findings suggest that RNA modifications may play a role in mRNA-specific translation regulation by ribosome specialisation.

Functional studies have shown that cytosine-5 rRNA methyltransferases play roles in development, proliferation and cell survival processes. For example, NSUN4 is essential for embryonic development in the mouse [53]. Studies in yeasts showed that *Nop2* (NOP2 homologue in yeast) and *Rcm1* (NSUN5 homologue in yeast) null mutants were not lethal, however, *rcm1* mutants were hypersensitive to anisomycin and oxidative stress [52, 70]. Human NOP2 functional role has been associated to cell cycle [73]. Whether the enzymatic activity of NOP2 is directly required to mediate these cellular processes remains unclear. In *C. elegans* and flies, deletion of *Nsun5* increased the lifespan of both organisms, indicating a role of rRNA cytosine-5 methylation in aging through protein synthesis control [70]. The functional role of NSUN5 in mammals has yet to be determined. These findings indicate that rRNA cytosine-5 methyltransferases control stress responses and developmental programmes.

4.5. Functional role of NSUN7

Little is known on the functional role of this NSUN member. During embryogenesis NSUN7 is highly expressed in different mouse tissues, indicating a role in development [65]. *Nsun7* mutations in adult male mice have shown to lead to infertility and impaired sperm motility and in male mice [74], however, the underlying molecular mechanism remains unknown. In a recent report, NSUN7 was found to interact with the transcriptional coactivator PGC-1 α and promoter-derived eRNAs of PGC-1 α target genes (Fig. 3E) [59]. Furthermore, *NSUN7*

knock-down in human cells reduced eRNAs levels as well as the mRNA levels of PGC-1 α target genes, indicating a role of NSUN7 in gene expression regulation mechanisms [59].

4.6. Functional role of DNMT2

Although initial studies showed that Dnmt2-deficient mice, flies and plants were viable, fertile and morphologically indistinguishable from wild-type organisms [32]. Studies in the past decade have shown that methylation of C38 by DNMT2 plays a role in several processes including tRNA processing, translation accuracy, survival and differentiation [13, 33, 37-40, 75]. Studies in flies and mice have shown that molecularly, loss of *Dnmt2* causes increased tRNA cleavage and decreased tRNA stability leading to erratic discrimination of near-cognate codons for Asp, Gly and Val codons (Fig. 3F) [13, 33]. In addition, studies in mice also showed that DNMT2-mediated methylation in Asp tRNA, stimulated amino acid charging of the tRNA *in vitro* and *in vivo*, facilitating translation of poly-Asp containing proteins [41]. Phenotypic studies demonstrated that the absence of the methyltransferase resulted in endochondral ossification, cardiac hypertrophy and hematopoietic system differentiation defects in mice [33, 76]. Morpholino knock-down of *Dnmt2* in zebrafish embryos caused retina, liver and brain differentiation defects due to a failure to conduct late differentiation [40].

In addition to the role that DNMT2 plays in differentiation, several studies in *Drosophila* have described the significance of DNMT2 in other physiological processes including survival to stress, immunity, regulation of silencing pathways as well as regulation of silencing of mobile genetic elements [13, 38, 39, 75]. Null mutations of *Dnmt2* in adult flies resulted in increased stress-induced tRNA cleavage and reduction of viability of mutant flies under heat and oxidative stress conditions [13]. In addition, Dnmt2 mediated tRNA methylation contributed to the control of siRNA pathway components during the heat shock response [39]. This study showed that increased of tRNA-derived double stranded RNA fragments induced upon *Dnmt2* loss, promoted their binding to Dicer-2. Consequently Dicer-2 complex activity was inhibited and resulted in misregulation of siRNA pathway-controlled gene expression (Fig. 4A) [39]. Interestingly, tRNA fragments can affect the efficiency of siRNA silencing pathways in human cells raising the possibility that tRNA-derived RNA fragments may play a role in gene silencing pathways in human cells too [77]. Because the activity of Dicer-2 is required for the production of virus-derived small-interfering RNAs, it is not surprising that Dnmt2 is also required for efficient innate immune responses in *Drosophila* [38]. In fact, infectious studies showed that lack of Dnmt2 resulted in increased levels of Drosophila C virus in infected mutant flies. The study further showed that Dnmt2 efficiently regulated viral

replication by binding to viral RNA (Fig. 4B) [38]. Another recently described phenotype in *Dnmt2* and *Nsun2* null mutant flies was the characteristic silencing defects of transposable elements and impaired chromatin formation (Fig. 4C) [75]. Schaefer and colleagues further showed reduced tRNA stability influenced the expression or metabolism of TE-derived sequences [75]. Whether *Dnmt2* and *Nsun2* can methylate transposable elements remains unknown.

In summary, although we are only now beginning to understand the physiological relevance of cytosine-5 methylation in RNA, it is becoming clear that it represents a new layer of control for several cellular processes.

5. Role of m⁵C in mRNAs

The molecular role and physiological relevance of m⁵C occurrence in mRNA remains still elusive. Despite many recent advances, little is known on m⁵C deposition dynamics, site distribution or protein readers in mRNA. While some system-wide studies have analysed m⁵C site distribution on mRNA to gain insights on its molecular role, yet no defined consensus on the site distribution has been found [7, 9, 11, 12, 16, 20]. Other studies have attempted to gain information about m⁵C physiological function by performing gene ontology (GO) term enrichment analyses of m⁵C-modified mRNAs, yet any common GO term arose from those studies [7, 11, 12]. Likewise, a few studies have also investigated the contribution of m⁵C occurrence in mRNAs stability [7, 9, 12]. Generally, there was no correlation between mRNA expression levels and m⁵C occurrence. In addition, few studies have analysed the correlation of m⁵C occurrence in mRNA with translation efficiency or accuracy. *In vitro* studies employing a bacterial translation system showed that, while single m⁵C modifications did not strongly inhibit protein synthesis independent of their localization within a codon, m⁵C led to a 4% mis-recoding of proline as leucine [78]. In eukaryotes, some reports have investigated the effect of m⁵C on translation of few single transcripts [79-81]. Yet nobody has reproduced these data requiring further validation. Thus, the effect on translation efficiency of methylated mRNAs still remains uncertain in eukaryotes.

Recently, it has been evidenced Aly/REF export factor as a mRNA-binding protein that recognises and binds m⁵C-containing mRNAs [12]. The study further reported that Aly/REF knock-down resulted in increased nuclear retention of m⁵C-modified mRNAs, an effect that was suppressed by overexpression of wild-type but not m⁵C-binding defective Aly/REF. Thus, the study suggested a role for m⁵C occurrence in mRNA export (Fig. 5) [12]. It is unknown whether Aly/REF is the only m⁵C reader protein specific for mRNA and if the only

molecular function of m⁵C in mRNAs is to favour their export. In addition, whether increased nuclear export of m⁵C-containing mRNAs favours their translation or impacts their degradation in the cytoplasm remains uncertain and needs further evaluation.

Overall, while some studies have attempted to investigate the regulatory role of m⁵C in mRNA metabolism or fate, our knowledge is still very limited.

6. Deposition and role of cytosine-5-hydroxymethylation and cytosine-5-formylation

Similar to DNA, recent findings have shown that existing 5-methylcytosine in RNA can suffer subsequent modifications. For instance, m⁵C can be oxidized to generate cytosine-5-hydroxymethylation (hm⁵C) *in vitro* and *in vivo* by enzymes of the ten-eleven translocator family (TET) (Fig. 6) [55, 82]. Mass spectrometry studies revealed low but significant levels of hm⁵C in mRNA, which is in consistence with the low abundance of the m⁵C modification overall detected in mRNA [55, 57, 83]. Regarding to hm⁵C, the highest *in vivo* levels were found in mouse and human brain relative to other tissues [55, 57, 84]. These results indicate a specificity that is reminiscent of hm⁵C amounts in DNA [85], suggesting that the cellular environment or activity of TET enzymes may cause hyper 5-cytosine hydroxymethylation in both DNA and RNA.

Studies in humans have contributed to shed some light of the broader regulatory mechanisms of RNA hm⁵C, in particular, the role that hm⁵C has on pathogen infection and endogenous retroviral control [82, 86]. A recent study demonstrated that TET2-mediated oxidation of methylated cytosines in mRNAs, increased the binding of the editing enzyme ADAR-1 to hydroxymethylated mRNAs [82]. In particular, it was shown that ADAR-1 binding to SOCS3 mRNA increased its editing and degradation, resulting in increased myelopoiesis [82]. These findings indicated that immune responses are regulated through a very sophisticated mechanism combining modifications and editing of mRNAs. hm⁵C deposition in human cells was also found in transcripts derived from endogenous retroviruses integrated in the genome that are transcriptionally and epigenetically controlled via DNA and RNA hm⁵C deposition [86].

Thus, those studies have revealed a significant role of hm⁵C in mRNAs besides its low prevalence. Not surprisingly, hm⁵C occurs also in other species of RNA such as tRNAs, which show higher prevalence of m⁵C modification compared to mRNAs, and thus might be more likely to show higher prevalence of hm⁵C too. In tRNAs, *in vitro* and *in vivo* studies showed that Alkylation repair homologues family member ALKBH1 can first hydroxylate m⁵C

to form hm⁵C, and subsequently oxidize hm⁵C to form 5-formylcytosine (f⁵C) in leucine and methionine tRNA [34, 66]. In particular, the m⁵C-oxidation derivatives found in tRNAs are hm⁵C and f⁵C at position 34 in cytoplasmic leucine tRNA, and 5-formylcytosine at position 34 in mitochondrial methionine tRNA (f⁵C34 mt-tRNA), being this change driven by ALKBH1 [34, 66], instead of by the TET family members.

Finally, *in vitro* studies showed that TET1 enzyme can catalyse the formation of 5-carboxylcytosine (ca⁵C) from existing f⁵C groups, however the RNA type was not specified (Fig. 6) [87].

Overall, the existence of m⁵C-oxidized derivatives in RNA are just beginning to be appreciated and have been postulated to be equally dynamic as the reversible epigenetic marks previously reported on DNA. All these recent studies have demonstrated that while there is a broad repertoire of RNA and DNA cytosine-5 methylating enzymes, synthesis of hm⁵C is mediated by TET and ALKBH enzymes. This indicates that the very same TET enzymes that modify DNA can promiscuously target RNA, and therefore it is harder to discriminate DNA- or RNA-related biological functions.

7. Pathologies associated to alterations in m⁵C deposition

In the last few years, our understanding about RNA cytosine-5 methylation pathways has significantly increased, indicating to be essential for a wide range of biological processes (Table 1). However, in some diseases, the precise molecular mechanisms and cellular processes regulated by these modifications need to be further studied to have a deeper knowledge and a better prognosis.

7.1. NOP2

NOP2 (also known as NSUN1 or p120) is a nucleolar protein and was initially found to be overexpressed in human cancer cell lines [88]. Its overexpression was shown to increase proliferation of mouse fibroblasts [73], and has been considered a prognostic marker for cancer cells with a high proliferative potential [89].

7.2. NSUN2

NSUN2 expression alterations have been also linked to several cancer types [64, 90, 91]. In fact, NSUN2 was initially described as a novel downstream target of Myc oncogene [64]. In

addition, it is overexpressed in various solid tumours including breast, skin and colon [44, 64]. Initial studies showed that NSUN2 protein, regardless of its methyltransferase activity, was required for mitotic spindle stability, and its overexpression in cancer cells led to aberrant mitosis [45]. In mouse skin tumours, its expression was lower in tumour initiating or stem cell populations compare to cell populations with high differentiation potential and low self-renewal potential [47]. Consequently, *Nsun2* deletion in mouse skin tumours led to increased tumour growth due to an increase on tumour initiating cells with high self-renewal capacity [47]. Thus, NSUN2 expression levels do not always correlate with good or poor overall survival [90-92]. The most intriguing was its role in cancer cell survival. *Nsun2* deletion and reduced tRNA cytosine-5 methylation in mouse tumours were shown to increase the sensitivity of tumour cells to anti-cancer drugs [47]. This suggested the combination of conventional chemotherapeutic agents together with RNA methyltransferases inhibitors as most effective therapeutic strategies to reduce tumour growth.

With the venue of the NGS technologies, mutations in some NSUN member genes have been associated to other human diseases. Inactivating mutations in *NSUN2* have been found associated to autosomal recessive intellectual disability syndromes in humans characterized by microcephaly and mental and growth retardation [93-97]. In most cases, *NSUN2* mutations lead to protein loss or mis-localization, and thereby a loss of site-specific 5-cytosine methylation [93, 94]. For instance, a homozygous variant in an intronic region of the gene *NSUN2* (chr. 5 base position 6,622,214 C>G; hg19), located one base pair upstream of the start of exon 6, which abolishes the canonical splice acceptor site, led to a subsequent mRNA and protein loss [94]. The mutation was associated to Dubowitz-like syndrome, an autosomal recessive syndrome defined by microcephaly, intellectual disability, skin disorders like eczema, growth and peculiar facies [94]. Mechanistically, the neurological abnormalities were linked to hypomethylated tRNAs, increased angiogenin-induced tRNA cleavage and increased sensitive to stress of cells during development [8, 48, 94]. Indeed, treatment of *Nsun2*^{-/-} mouse embryos during development with an angiogenin inhibitor rescued the brain development abnormalities [8, 48].

In addition, homozygous 1-bp deletion (c.915del) in *NSUN2* gene has been associated to an autosomal syndrome defined by bilateral ptosis, hypotonia and facial features suggestive of Noonan syndrome [98]. The deletion resulted in a truncated *NSUN2* transcript presumably degraded via the nonsense-mediated decay pathway [98].

7.3. NSUN3

Loss-of-function mutations in *NSUN3* have been identified in patients with muscular weakness, combined developmental disability, microcephaly, failure to thrive, recurrent increased lactate levels in plasma, proximal accentuated, external ophthalmoplegia and convergence nystagmus [21]. Analysis of patient-derived fibroblasts exhibited severe defects in mitochondrial translation due to lack of mitochondrial tRNA 5-cytosine methylation, suggestive of mitochondrial respiratory chain complex deficiency and OXPHOS deficiency in skeletal muscle [21]. In addition, pathogenic mutations in methionine mt-tRNA that are associated with mitochondrial diseases were showed to significantly impaired m⁵C34-forming activity by NSUN3, strongly suggesting that pathological symptoms in patients harboring these mutations were due to decreased levels of f⁵C34 [36].

7.4. NSUN4

Loss-of-function mutations in *NSUN4* have not been identified in patients, however its deletion in the heart in mice causes mitochondrial cardiomyopathy due to mitochondrial impaired translation and mitochondrial dysfunction [53].

7.5. NSUN5

Although there are no mutations associated to *NSUN5* gene, it is located in a genomic region deleted in patients with Williams-Beuren syndrome (WBS) [99]. WBS is associated to a contiguous gene deletion of approximately 1.5 Mb at 7q11.23. It is a complex developmental disorder characterized by intellectual disability and developmental delay, as well as craniofacial and cardiovascular abnormalities [100]. Whether deletion of *NSUN5* directly contributes to these symptoms is unknown.

7.6. NSUN7

A deletion of adenine in location 11337 in exon 4 and a T26248G-transversion mutation in exon7 of the *NSUN7* gene were found in infertile men with reduced sperm motility [101, 102]. The A11337-deletion resulted in a TAG-early stop codon, and reduced, leading to a shorter protein product and reduced binding capacity to the S-adenosyl-L-methionine cofactor [101]. Whether defects on NSUN7 methylation activity can cause infertility is unknown.

7.7. DNMT2

DNMT2 has also been found consistently up-regulated in hundreds of tumour samples listed in the COSMIC database and 60 somatic mutations in *DNMT2* have been identified in tumours originated from various tissues [103]. To investigate whether the mutations found in cancer tissues had a functional effect in the activity of the enzyme, 13 mutations were selected and the mutant proteins were generated. One of the mutations (E63K) caused a twofold increase in activity, two of them led to a strong decrease in activity (G155S and L257V), and two additional mutant proteins were almost inactive (R371H and G155V). The strong effect of some of the somatic cancer mutations on DNMT2 activity suggests that these mutations have a functional role in tumorigenesis although further investigation is needed.

In summary, although the precise molecular and biological functions of RNA m⁵C methyltransferases are still poorly understood, there is a high number of alterations in NSUN-proteins associated with human disease syndromes. The diseased phenotypes associated to the alterations in the cytosine-5 methylation machinery are diverse ranging from growth retardation, neurological deficits, muscular weakness, mitochondrial diseases, sterility or tumorigenesis, indicating that cytosine-5 methylation is physiologically highly relevant in human health. Mutations or protein expression alterations of NSUN4 and NSUN6 have been associated to human diseases.

8. Conclusion and future directions

While modifications have long been known to be present in RNA, over the last six years there has been a renewed focus on the study of RNA modifications. Yet despite all of the latest advances, our knowledge about m⁵C in RNA is still in its infancy and many questions remain open. For instance, m⁵C site distributions and prevalence in low abundance RNAs such as mRNAs are still vague. The variable results on m⁵C distribution sites may reflect a lack of consistency of the technical approaches and poor computational methods. This requires a critical next phase to first determine m⁵C sites whose detection is robust to technical variation and computational analysis. Second, to increase the number of system-wide m⁵C detection studies before urging any conclusion on the specificity and regulation of the precise dynamics of this modification *in vivo*.

Novel approaches are still needed to overcome some of these issues. For instance, the combination of NGS techniques with well-defined and widely used techniques such as mass spectrometry could bring a broad new vision of the whole epitranscriptome. In this regard,

advances have been made based on NAIL-MS (nucleic acid isotope labelling coupled mass spectrometry), which relies on the quantification of RNA modifications using stable isotope-labelled internal standards (SILIS) [104]. The adaptation of the nascent single-molecule, real-time (SMRT) sequencing technology for the detection of RNA sequences [105], may contribute to resolve site distribution maps in low abundance RNAs. Moreover, development of robust data analysis tools will be required to improve calling of m⁵C sites and elimination of stochastic artefacts.

While we are beginning to understand the molecular function of m⁵C on non-coding RNAs, the molecular role of m⁵C occurrence in mRNA metabolism and the biological relevance of such a low prevalence mark still remains elusive. A unifying theme among most of the cytosine-5 methyltransferase's roles is that they are linked to stress responses, development and cell differentiation. Yet, the functional roles of several RNA cytosine-5 methyltransferases remain to be established, and novel genetically modified cells and organism models are needed. Furthermore, the level of protein redundancy in RNA methylation pathways is unknown and depletion of individual RNA methyltransferases may not be sufficient to comprehensively understand their roles.

Expression alterations or mutations of m⁵C methyltransferases have been linked to several diseases, indicating an essential role in human physiology. Further identification of m⁵C and hm⁵C dynamic changes as well as their context-specific writers, erasers and protein readers are now the priority, that will shed light into the wide range of cellular processes being regulated by this epitranscriptomic marks. However, the main effort should be made in the characterization of the role of m⁵C in disease, leading to the search of molecules to rewire m⁵C deposition. Recent studies have indicated an association between alterations on m⁵C deposition and cancer, suggesting the targeting of RNA methyltransferases activity as a promising strategy to treat cancer [47]. The recent findings that TET and ALKBH1 have also the ability to oxidize m⁵C in RNA raise the question as to whether defects of m⁵C oxidative derivatives on RNA can lead to human diseases. Mutations in *TET2* are associated to leukaemia and myeloid malignancies [106]. In addition, *ALKBH1* loci is susceptible to B-cell acute lymphoblastic leukaemia in children [107]. Mechanistically, the cause has been always associated to DNA demethylation defects. However, RNA hydroxymethylation defects could be also linked to TET and ALKBH1 associated diseases. Future findings of the potential of m⁵C-modified RNA as diagnostic tool or RNA-modifying proteins as therapeutic targets to treat complex diseases including cancer and neurological diseases will be extremely interesting.

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Table 1. Human Diseases associated to m^5C deposition alterations

Factor	Role	Associated pathologies	Disease Area	References
NOP2	Writer	Increased expression in several cancer types	Cancer	J.W. Freeman, P. McGrath, 1991; S.M. Jhiang, M. Yaneva, 1990; T. Bocker, A. Bittinger, 1995; T. Ueki, Y. Nakayama, 1997; Kuwano, M. Tsuneyoshi, 1997.
NSUN2	Writer	Dubowitz-like syndrome and intellectual disability	Neurological	M.A. Khan, M.A. Rafiq, 2012; F.J. Martinez, J.H. Lee, 2012; L. Abbasi-Moheb, S. Mertel, 2012; M. Komara, A.M. Al-Shamsi, 2015; S. Ghadami, H.M. Mohammadi, 2015.
NSUN2	Writer	Noonan-like syndrome	Cardiovascular	S. Fahiminiya, M. Almuriekh, 2014.
NSUN2	Writer	Altered expression of NSUN2 in several cancer types	Cancer	M. Frye, F.M. Watt, 2006; J.C. Yang, E. Risch, 2017; Y. Li, J. Li, M. Luo, 2018, L. Lu, G. Zhu, 2018.
NSUN3	Writer	Mitochondrial respiratory chain complex deficiency	Mitochondrial	L. Van Haute, S. Dietmann, 2016; S. Nakano, T. Suzuki, 2016.
NSUN4	Writer	Loci susceptibility in breast, ovarian and prostate cancer	Cancer	S.P. Kar, J. Beesley, 2016.
NSUN5	Writer	Williams-Beuren syndrome Neurodevelopmental disorder	Neurological	A. Doll, K.H. Grzeschik, 2001; G. Merla, C. Ucla, 2002; M.A. Martens, S.J. Wilson, 2008.
NSUN7	Writer	Impaired sperm motility	Infertility	N. Khosronezhad, A.H. Colagar, 2015; N. Khosronezhad, A. Hosseinzadeh Colagar, 2015; H.Y. Ren, R. Zhong, 2015; S.A. Forbes, D. Beare, 2015.
DNMT2	Writer	Altered expression and mutations in several cancer types	Cancer	W. Elhardt, R. Shanmugam, 2015; S.A. Forbes, D. Beare, 2015.
TET1	Eraser	Leukaemia	Cancer	R.B. Lorsch, J. Moore, 2003.
TET2	Eraser	Myelodysplastic syndrome, Leukaemia	Cancer	S. Weissmann, T. Alpermann, 2012; W.C. Chou, S.C. Chou, 2011; B. Euba, J.L. Vizmanos, 2012; M. Ko, Y. Huang, 2010.
ALYREF	Reader	Altered expression in a variety of tumours	Cancer	Y. Saito, A. Kasamatsu, 2013; M.S. Dominguez-Sanchez, C. Saez, 2011.
Vault RNA	Substrate	Play a role in resistance development of cancer cells to chemotherapeutic drugs	Cancer	J. Chen, H. OuYang, 2018; S.C. Gopinath, R. Wadhwa, 2010.
ALKBH1	Eraser	Susceptibility to childhood B-cell acute lymphoblastic leukaemia	Cancer	N.P. Archer, V. Perez-Andreu, 2016.

Figure legends

Figure 1. Schematic overview of RNA cytosine methylation detection techniques followed by next generation sequencing. Bisulphite sequencing (BS-Seq) relies on the differential reaction of cytosines to sodium bisulphite. m⁵C-RNA immunoprecipitation (m⁵C-RIP), 5-azacytidine mediated RNA-IP and methylation-individual nucleotide resolution crosslinking-IP (mi-CLIP) are based on the use of antibodies that bind specifically to m⁵C marks or to the complex methyltransferase-RNA. Aza-IP and mi-CLIP provide information of methyltransferase particular substrates. In mi-CLIP, RNA-enzyme adducts are formed by using methyltransferase inactive mutants that bind covalently to the substrate. IP: immunoprecipitation.

Figure 2. m⁵C deposition landscape and cytosine-5 methyltransferases. Coding (mRNA) and non-coding RNAs are methylated at cytosine-C5 by the cytosine-5-methylases of the NOP2/Sun domain RNA methyltransferase family (NOP2, NSUN2-7 and TRM4B in higher eukaryotes, Trm4, Nop2 and Rcm1 in yeasts) and DNA methyltransferase family (DNMT2/TRDMT1 in higher eukaryotes, Pmt1 in yeast and DnmA in *Dictyostelium*). Methylated positions are indicated by coloured balloons, as well as the responsible enzymes. Star indicates protein interaction. Arrows indicate nuclear to cytosol export of methylated RNAs. tRNA: transfer RNA; pre-mRNA: pre-messenger RNA; eRNA: enhancer long non-coding RNA; rRNA: ribosomal RNA. ? indicates m⁵C positions catalysed by an unknown RNA methylase.

Figure 3. Post-transcriptional expression regulation by cytosine-5 methylation of RNAs. **A)** NSUN2 methylation protects tRNAs from Angiogenin (ANG)-mediated cleavage into tRNA fragments (tRFs) that repressed translation. **B)** NSUN2 methylation protects Vault RNAs from cleavage into small RNAs that interact with the silencing machinery. **C)** Rcm1-mediated methylation of 25S rRNA at the large ribosomal subunit re-programmes the translation machinery. **D)** NSUN3-mediated methylation of mitochondrial tRNAs and NSUN4-mediated methylation of mitochondrial rRNAs enhances protein translation at the mitochondria. RISC: RNA-induced silencing complex. **E)** NSUN7 interacts with the transcriptional co-activator PGC-1 α (Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha) and promoter-derived eRNAs (enhancer RNAs) and reinforces transcription of PGC-1 α -transcriptionally dependent genes. **F)** The lack of C38 tRNA methylation reduces the capacity of tRNAs to properly discriminate between Asp and Glu codons.

Figure 4. Functional role of tRNA methyltransferases in *Drosophila*. **A)** Lack of Dnmt2 leads to increased tRNA fragmentation in stress conditions. The accumulation of tRFs inhibits Dicer-2 (DCR-2) complex activity, thereby decreasing the production of siRNA (small-interfering RNAs) and miRNAs (microRNAs) leading to inefficient silencing pathway activity. **B)** Upon infection of *Drosophila* C virus (DCV), Dnmt2, in wild-type (wt) flies, binds to viral RNAs (vRNA) leading to the neutralisation of the virus and anti-viral defence. Without Dnmt2 (*Dnmt2-null* flies), virus detection is inefficient leading to the virus to propagate. **C)** Lack of Dnmt2- and Nsun2-mediated methylation leads to silencing defects of transposable elements (TEs) in a heat-shock dependent and independent manner, respectively. tRFs: tRNA fragments; dsRNA: double-stranded RNA; ARGO: Argonaute.

Figure 5. m⁵C regulates mRNA export to the cytoplasm. NSUN2-methylated mRNAs are selectively transferred to the cytoplasm by the export factor AlyREF. SAM: S-Adenosyl-Methionine; SAH: S-Adenosyl-Homocysteine.

Figure 6. Enzymatic oxidation of 5-methylcytosine. 5-methylcytosine formation in RNA is catalysed by the NSUN family and DNMT2. 5-methylcytosine is oxidized to 5-hydroxymethylcytosine by Ten-eleven Translocation (TET) enzymes in mRNA and by the Alkylation repair homologues family member ALKBH1 in tRNAs. 5-formylcytosine formation is catalysed ALKBH1. TET1 converts 5-formylcytosine to 5-carboxycytosines. ? indicates that it is yet unknown whether m⁵C can be completely removed.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Cytosine

***NSUN
DNMT2***

5-methyl-
cytosine

TET (*mRNA*)
ALKBH1 (*tRNA*)

5-hydroxy
methyl-cytosine

↑ ?

TET

5-carboxy-cytosine

5-formyl-cytosine

ALKBH1 (*tRNA*)