Towards a "druggable" epitranscriptome: Compounds that target RNA modifications in cancer

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Abstract

Epitranscriptomics is an exciting emerging area that studies biochemical modifications of RNA. The field is boosted by the technical efforts of the last decade to characterize and quantify RNA modifications which have led to a map of post-transcripcional RNA marks in normal cell fate and develoment. However, the scientific interest has been fueled by the discovery of aberrant epitranscriptomes associated with human diseases, mainly cancer. The challenge is now to see whether epitrancriptomics offers a tunable mechanims to be targeted by small- molecule intervention. In this review, we will describe the principal RNA modifications (with a focus on mRNA), summarize the latest scientific evidences of their dysregulation in cancer and provide an overview of the state-of-the-art drug discovery to target the epitranscriptome. Finally, we will discuss the principal challenges in the field of chemical biology and drug development to increase the potential of targeted-RNA for clinical benefit.

REVIEW ARTICLE

Themed section on New Avenues in Cancer Prevention and Treatment

Towards a "druggable" epitranscriptome: Compounds that target RNA modifications in cancer

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Running title: Drugs that target RNA modification enzymes in cancer.

ABSTRACT

Epitranscriptomics is an exciting emerging area that studies biochemical modifications of RNA. The field is boosted by the technical efforts of the last decade to characterize and quantify RNA modifications which have led to a map of post-transcripcional RNA marks in normal cell fate and develoment. However, the scientific interest has been fueled by the discovery of aberrant epitranscriptomes associated with human diseases, mainly cancer. The challenge is now to see whether epitrancriptomics offers a tunable mechanims to be targeted by small- molecule intervention. In this review, we will describe the principal RNA modifications (with a focus on mRNA), summarize the latest scientific evidences of their dysregulation in cancer and provide an overview of the state-of-the-art drug discovery to target the epitranscriptome. Finally, we will discuss the principal challenges in the field of chemical biology and drug development to increase the potential of targeted-RNA for clinical benefit.

KEYWORDS

Epitranscriptomics, RNA methylation; pseudouridine, A-to-I-editing, small-molecule inhibitors, therapy, cancer.

ABBREVIATIONS

Ψ, pseudouridine; A-to-I, Adenosine-to-Inosine; AML, Acute Myeloid Leukaemia; AZA, 5-azacytidine; CDS, coding regions; CML, chronic myeloid leukaemia; CTCL, cutaneous T-cell lymphoma; DNMT, DNA methyltransferases; ESCC, oesophageal squamous cell carcinoma; EMT, epithelial-mesenchymal transition; GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; HDAC, histone desacetylases; hm5C, 5-hydroxylmethyl cytidine; HMT, histone methyltransferases; HNSCC, Head and Neck Squamous Carcinoma KMT, lysine protein methyltransferases; m1A, N1-methyladenosine; m5C, 5-methyl cytidine; m6A, N6-methyladenosine; MA, meclofenamic acid; MA2, ethyl ester form of meclofenamic acid;MDS, myelodysplastic syndrome; MM, multiple myeloma; mRNA, messenger RNA; miRNA, microRNA ncRNA, non-coding RNA; NSCLC, non-small cell lung cancer; PDX, patient-derived xenograft; PTCL, peripheral T-cell lymphoma; rRNA, ribosomal RNA; SAM, S-adenosylmethionine; tRNA, transfer RNA; UTR, untranslated regions.

1. INTRODUCTION.

The control of chromatin structure mediated by epigenetic mechanisms has an accepted role in the control of gene expression and other DNA-related biological processes. DNA methylation/demethylation and post-translational modifications of histones set an epigenetic landscape that is stable during cell replication and that could be modulated by specific environmental signals to guide normal development and cell differentiation (Allis and Jenuwein, 2016; Dai et al., 2020). This orchestrated setting is also subject to deviations. Epigenetic alterations are associated with multiple human disorders, including *de novo* epimutations (e.g., cancer, neurological disorders, infectious diseases or cardiovascular pathologies) but also germline-related diseases (e.g. rare disorders associated with genetic mutations affecting epigenetic modifiers) (Berdasco and Esteller, 2018). Our knowledge of epigenetic alterations in disease has improved the discovery and development of small-molecule compounds targeting the catalytic pocket of enzymes with epigenetic activity (Ganesan et al., 2019; Jones et al., 2019). The range of small-molecule inhibitors that target epigenetic proteins include enzymes that add chemical groups into DNA and histones ("*writers* "), proteins that remove these chemical tags ("*erasers*") and specific binding domain proteins that are able to identify and interpret these modifications ("*erasers*") (Ganesan, 2018; Ganesan et al., 2019). DNA methyltransferase (DNMT) inhibitors such as decitabine have been implemented into clinical practice for the treatment of haematological malignancies, such as myelodysplastic syndromes (MDS), acute myeloid leukaemia (AML), and chronic myeloid leukaemia (CML) (Prebet et al., 2014; Diesch et al., 2016). Similarly, histone deacetylase (HDAC) inhibitors have also reached FDA- approval for clinical routine in refractory CML Panobinostat) (Cavenagh and Popat, 2018). New approaches in epidrug development explore the presence of genetic mutations of epigenome-modifying enzymes as a more targeted therapy (Cossío et al., 2020). In this line, the histone methyltransferase (HMT) EZH2 inhibitor tazemotostat reached a Phase II/III clinical trial to treat refractory non-Hodgkin lymphoma with EZH2 amplification (Italiano et al., 2018) or the DOT1L inhibitor pinometostat for the treatment of MLL-fusion leukaemia (Stein et al., 2018). Opportunities have extended

beyond cancer and the potential of epigenetic drugs as therapeutic agents able to revert epigenetic defects is extending to other pathologies, ranging from infectious diseases to brain diseases, cardiovascular and metabolic disorders (Ballestar and Li, 2017; Berdasco and Esteller, 2019; Villanueva et al., 2020). The volume of epigenetic research conducted in academia, R&D sector of pharmaceutical industry and biotech companies have boosted the epigenetic-based market.

Following the epigenetic model, recent discoveries on the role of post-translational modifications at the RNA level (termed "epitranscriptome") have opened new possibilities for the pharmacological targeting of these modifications as an intervention strategy in human diseases with aberrant epitranscriptomes. Over the last 50 years more than 140 posttranslational modifications in RNA molecules have been identified (Boccaletto et al., 2018), most of them affecting the most abundant RNAs: *ribosomal* RNA (rRNA) and*transfer* RNA (tRNA) (Roundtree et al., 2017a). However, it is only during the past decade have we started to construct the first maps of *messenge* r RNA (mRNA) modifications and to envision their impact on gene regulation.

The four RNA bases (A, T, C, U) as well as the ribose sugar can harbour modification sites that range from base isomerization processes to chemical modifications, including inosine (I), 5-methyl cytidine (m5C; also known as 5mC), 5-hydroxylmethyl cytidine (hm5C; also known as 5hmC), pseudo-uridine (Ψ), N6-methyladenosine (m6A) and N1-methyladenosine (m1A). Nowadays, we have identified and characterized mRNA posttranslational modifications that are known to be important for RNA biogenesis, RNA dynamism and RNA function under physiological conditions. In addition, their impact on the onset and progression of human diseases, especially cancer, has been recently examined. Despite all efforts, the field of epitranscriptomics is still in its infancy and we are still far from obtaining a complete landscape of RNA modifications and the molecular and biological pathways in which they are involved. What is clear from the latest evidences, however, is that RNA does not merely act as an effector molecule but it has an active role in the regulation of gene expression. In this review, we will describe the principal RNA modifications (with a focus on mRNA), summarize the latest scientific evidences of their dysregulation in cancer and provide an overview of the state-of-the-art drug discovery efforts. Finally, we will discuss the principal challenges in the field of chemical biology and drug development to increase the potential of targeted-RNA for clinical benefit.

2. THE EPITRANSCRIPTOME *versus* THE EPIGENOME.

Today, a lot of expectation rests on whether the novel field of epitranscriptomics will follow the exploitation plan reached by epigenetics in drug development. To perform a critical overview, we need to identify common and divergent features between epigenetic and epitranscriptome modifications. Both RNA and DNA modifications share common features, including their reversibility and dynamisms determined by a set of proteins with writer, reader, or eraser function, and these proteins react to changing external conditions (Fu et al., 2014; Dominissini et al., 2016; Roundtree and He, 2016). Regrettably, we still do not have a full picture of the extent of RNA modifications and associated enzymatic machinery, but a general overview could be anticipated. So far, it is known that the number of RNA modifications is high, which involves a considerable number of writers, erasers and readers. These enzymes are potential pharmacological targets guided to modify their catalytic activity or their target binding-sites. In addition, RNA modifications, such as epigenetic modifications, are established in a cell type and time-dependent manner.

Major differences included the following aspects: *(i)* in contrast with the primary role of DNA modifications as regulators of gene transcription, all type of RNA modifications can be associated with wider aspects of gene expression, including splicing, distribution, translation and stability. The function seems to be strongly dependent on the specification of the RNA-specie. The same RNA modification can be recognized by multiple readers in a context- dependent manner resulting in different mechanisms of action and affecting variable biological pathways (Jia et al., 2011; Wang et al., 2014). *(ii)* RNAs demonstrate mobility between cellular compartments, a characteristic that amplifies their effects on multiple biological pathways.*(iii)* The heritability: whilst epigenetic modifications show mitotic inheritance, so far, a transmission of RNA modifications have not been described. When RNA degradation occurs, the epitranscriptomic mark is lost. (*iv*) The structural effect: whilst DNA methylation does not alter the double helix DNA structure, RNA modifications could result in altered charge, base-pairing potential, secondary structure, and protein- RNA interactions

(Liu et al., 2015). This conformational change also influence how the modification works functionally, since changes in RNA modifications could be read directly by their targets but also indirectly though the effect on their structural change.

All together, we can assume that the "RNA word" is tremendously complex and the current level of knowledge is still somewhat limited. In next sections, we will introduce the major research scenarios for making RNA-modifications an actionable target in drug discovery.

3. NON-CANONICAL CODING RNA MODIFICATIONS: DISTRIBUTION, DYNAMISM AND FUNCTION.

3.1. N6-Methyladenosine. N6-methyladenosine (m6A) is the most abundant internal modification detected in mammalian mRNAs (0.2%–0.6% of all adenosines) (Śledź and Jinek, 2016). Its abundance together with the development of robust detection methods led to an intense research interest, and nowadays, m6A is the best characterized RNA modification. It consists of the addition of a methyl group at the nitrogen-6 position of adenosine (**Figure 1**). The methyltransferase-like 3 (METTL3)–METTL14 heterodimer is involved in the methylation process, where METTL3 is the catalytic subunit and METTL14 acts as the RNA-binding scaffold for substrate recognition (Śledź and Jinek, 2016). Another m6A writer protein is METTL16, a U6 snRNA m6A methyltransferase. METTL16 is involved in the regulation of the cellular levels of S-adenosylmethionine (SAM), the methyl donor for methylation, as well as in the mRNA splicing process (Pendleton et al., 2017). Apart from passive m6A demethylation of the transcriptome, this modification is actively removed by the activity of the fat mass and obesity-associated protein (FTO) (Jia et al., 2011) and AlkB homologue 5 (ALKBH5) (Zheng et al., 2013) demethylases. FTO and ALKBH5 proteins are dioxygenases known to demethylate N-methylated nucleic acids. m6A readers have been also identified, included m6A-binding proteins belonging to the YTH family (YTHDF and YTHDC proteins) (Xiao et al., 2016), IGF2BP proteins (Huang et al., 2018), and some heterogeneous nuclear ribonucleoproteins (hnRNP) (Alarcón et al., 2015a).

Generally, m6A deposition on mRNA occurs in a sequence- dependent manner, mainly in the coding regions (CDS) and 3' untranslated regions (UTR) with a significant enrichment just upstream of the stop codon (Dominissini et al., 2012; Meyer et al., 2012). Interestingly, it has been described that trimethylation of histone H3 at Lys36 (H3K36me3) influences m6A deposition into specific genomic sequences by recruiting METTL14 complex (Huang et al., 2019a). Chromatin immunoprecipitation (ChIP)-sequencing studies demonstrated that approximately 70% of m6A peaks overlapped with H3K36me3 sites (Huang et al., 2019a). Altogether, the association between histone H3K36me3 and m6A RNA methylation adds a new layer of complexity in the control of gene expression. An anticipated research scenario focused on the integration of epigenetic and epitranscriptomic signals to explain gene control is expected in the near future.

The wide range of readers could explain why m6A is involved in almost of aspects of postranscriptional gene regulation and mRNA life cycle, including mRNA stability, splicing and translation. For instance, the m6A readers YTHDF1 and YTHDF2 controls \southe mRNA stability during stem cell differentiation and modulates processes such as haematopoietic stem and progenitor cell specification (Zhang et al., 2017a; Li et al., 2018b), neural induction from induced pluripotency stem cells (Heck et al., 2020), mammalian spermatogenesis (Hsu et al., 2017) or circadian regulation of downstream genes involved in lipid metabolism (Zhong et al., 2018). By recognizing m6A on pre-mRNA, YTHDC1, hnRNPC, hnRNPG, and hnRNPA2B1 could also modulate mRNA splicing (Alarcón et al., 2015a; Liu et al., 2015; Xiao et al., 2016). YTHDC1 could also mediate nuclear export of processed RNAs into cytoplasm (Roundtree et al., 2017b). In addition to regulating RNA stability and splicing, m6A reader proteins, including YTHDF1, YTHDF3, IGF2BP1/2/3, YTHDC2, supervise the RNA translation process and RNA decay (Shi et al., 2017; Huang et al., 2018). Strikingly, the deposition of m6A in 3' UTRs suggest that m6A could be incorporated into specific miRNA target sequences to modulate miRNA-binding (Alarcón et al., 2015b). And *vice versa* , it has been recently described that microRNAs regulate m6A modification via a sequence pairing mechanism and influences cell reprogramming in pluripotency (Chen et al., 2015). This finding reinforces the crosstalk between the epigenome and epitranscriptome in the control of gene regulation.

3.2. N1-Methyladenosine. The N1-methyladenosine modification (m1A), or the addition of a methyl group at the nitrogen-1 position of adenosine (**Figure 1**), was described decades ago to primarily affect all classes of RNAs (Barbieri and Kouzarides, 2020). It is predominant in tRNA and rRNA, but it was recently determined that it also exists in mRNA (Boccaletto et al., 2018). Nowadays, there is very little information of its frequency, the key players involved in m1A regulation and its consequences in mRNA. Although its frequency in cytosolic mRNA is controversial, it is accepted that m1A is less abundant than m6A (about ten times) (Dominissini et al., 2016; Safra et al., 2017). The m1A modification maps uniquely to GC-rich, 5'-UTRs positions in coding transcripts (Safra et al., 2017). An aspect of interest is that unlike m6A, m1A occurs in the Watson-Crick interface carrying a positively charged base at this position (Roundtree et al., 2017a). Alterations at protein-RNA interactions and RNA secondary/tertiary structures could be expected. The role of m1A modification is under elucidation, however, some recent works described a function in the initiation of mRNA translation (Dominissini et al., 2016; Li et al., 2016b) by facilitating non-canonical binding of the exon-exon junction complex at 5' UTRs devoid of 5' proximal introns (Cenik et al., 2017). Its role in the control of regulation is supported by its high conservation in mouse and human cells (Cenik et al., 2017).

The only known m1A writer of cytosolic mRNA is the TRM6-TRM61 complex, however, its activity does also cover m1A in the mitochondrial-encoded transcripts (Li et al., 2017a; Safra et al., 2017). m1A modification can be removed from mRNA by ALKBH3, a m1A demethylase both in mRNA and tRNA (Dominissini et al., 2016; Li et al., 2016b; Esteve-Puig et al., 2020). The YTH protein family of m6A readers could also interpret m1A signal. Specifically, YTHDF1-3 and YTHDC1 were shown to bind directly to m1A in mRNA in human cancer cells (Dai et al., 2018). New insights into the functions of m1A in RNA biology are needed; so far, only a role in the response to various types of cellular stress has been proposed (Dominissini et al., 2016; Li et al., 2016b).

3.3. 5-Methylcytosine . Like DNA, all types of RNA molecules can be methylated at carbon 5 of cytosine giving rise to 5-methylcytosine (m5C) (**Figure 1**) covering diverse functions depending on the RNA specie (Trixl and Lusser, 2019). The abundance of m5C in mRNA is under strong debate and discrepancies come from the technical difficulties to establish the transcriptome-mapping of m5C, mainly due to incomplete conversion of cytidine and m5C during bisulfite treatment. It is estimated that about 62-70% of \soutthe cytosine sites had low methylation levels (<20% methylation), while 8-10% of the sites were moderately or highly methylated (>40% methylation) (Huang et al., 2019b). The location of m5C modifications primarily maps to CDS, although an enrichment has been also observed in the 5'-UTR and the 3'-UTR regions (Huang et al., 2019b).

The writers of RNA m5C modifications in mammals include seven members of the NOL1/NOP2/SUN domain family member (NSUN) family (NSUN1-7), and DNA methyltransferase-like 2 (DNMT2). However, so far only NSUN2 has been proved to methylate mRNA (Yang et al., 2017b). In this regard, only overexpression/suppression of NSUN2 but not of any other NSUN enzyme, affected overall m5C levels in mRNA from HeLa cells (Yang et al., 2017b). Regrettably, enzymes that remove 5mC from RNA species have not yet been identified.

As we are only beginning to uncover the biology of m5C in mRNA, not much is known about the potential functional consequences. A role for m5C in the regulation of nuclear export has been discovered (Yang et al., 2017b). Specifically, the activity of the nuclear export factor ALYREF/THOC4 is strongly affected by the m5C level of its target mRNAs (Yang et al., 2017b). The 5mC deposition is not a random event since 5mC accumulates at translational start codon and in a CG sequence context. In addition, m5C can act as a modulator of protein translation. Examples include the m5C accumulation at 5'UTR of cyclin-dependent kinase inhibitor ^{p27}KIP1 during replicative senescence (Tang et al., 2015), or m5C deposition in the 3' UTRs of the cell cycle regulators CDK1 and p21 during the cell division cycle (Xing et al., 2015).

Physiologically, NSUN2 is enrolled in multiple biological pathways. It has been identified as a direct target gene of the transcription factor Myc and its activation is relevant for the differentiation of primary human keratinocytes (Frye and Watt, 2006). Mice models consisting of*Nsun2* knockdown exhibit additional develop-

ment defects, such as impaired cerebral cortex organization, immature skeleton, among others (Tuorto et al., 2012) . Nsun2 was also implicated in testis differentiation (Hussain et al., 2013). The molecular mechanisms connecting NSUN2 deficiencies and impaired cell differentiation were not identified.

3.4. Pseudouridine. Pseudouridylation is the isomerization of the uridine base via breakage of the glycosidic bond, 180° base-rotation, and bond reformation (Hamma and Ferré-D'Amaré, 2006) (**Figure 1**). It is the most frequent modification in total human RNA; however, the mapping of pseudouridine (ψ) in mRNAs was recently addressed (Penzo et al., 2017). Methodological limitations introduce serious controversy on the distribution and abundance of ψ, but the general consensus is that ψ sites in mRNA are much less abundant than m6A (Schwartz et al., 2014). Besides mRNAs, non-coding RNAs (ncRNAs) have emerged as highly interesting targets with ψ sites (Rintala-Dempsey and Kothe, 2017). The enzymology associated with pseudouridylation is very complex. In eukaryotes, uridine is transformed into ψ by a class of enzymes known as pseudouridylases. Pseudouridylases are represented in humans by pseudouridine synthases (PUS) encoded by 13 genes. Human PUS enzymes are far less studied than their counterparts in other organisms but recent discoveries allow a better identification of PUS enzymes, including those acting on mRNA (PUS1, PUS3, PUS4, PUS6, PUS7 and PUS9) (Penzo et al., 2017). Their mode of action or potential redundancy in their functions has not yet been completely resolved (Carlile et al., 2014a; Penzo et al., 2017). Currently, any specific eraser or reader associated with ψ modifications have been identified (Barbieri and Kouzarides, 2020).

It is well known that ψ enhances the function of tRNA and rRNA by stabilizing the RNA structure as well as regulating the splicing process by modifying specific snRNAs (Carlile et al., 2014b; Barbieri and Kouzarides, 2020). The physiological relevance of ψ in mRNA is more unclear with only a few evidences of its role. Mutations in genes encoding human PUS enzymes cause inherited diseases affecting muscle and brain function which reinforced their emerging role as regulators of gene expression (Shaheen et al., 2019). Notably, ψ content in 3'UTR mRNA is regulated in response to environmental signals, such as serum starvation in human cells, suggesting a function in the flexible adaptation of the genetic code through inducible mRNA modifications (Carlile et al., 2014b). A role in mRNA translation throughout the control of ribosome pausing and RNA localization has been also suggested (Carlile et al., 2014b; Schwartz et al., 2014).

3.5. Adenosine-to-inosine editing. Another RNA modification in mammals is the irreversible deamination of adenosine to inosine, a process also known as A-to-I editing (**Figure 1**). A-to-I editing occurs in multiple genomic sequences, ranging from coding regions of mRNAs to non-coding regions (e.g., Alu repeats, pre-miRNAs or pri-miRNAs) (Nishikura, 2016a). Inosine is interpreted at cellular level like a guanine and, consequently, A-to I editing could alter the biogenesis and/or function of miRNAs or mRNAs as well as proteins (Nishikura, 2016b). However, a comparative study among animal A-to-I modifications revealed that non-coding parts of the genome were the main targets for the editing process. A role in protecting against activation of innate immunity by self-transcripts have been proposed (Eisenberg and Levanon, 2018). A second type of A-to-I editing is hyper-editing, which could be understood as \soutan editing enriched regions (Porath et al., 2014). A large proportion of adenosines in close proximity to each other within the same transcript is a requisite for hyper-editing. In mammals, this class of editing is mostly associated with regions of repetitive sequences, intronic regions and 3' UTRs (Porath et al., 2017).

A-to-I edition is catalysed by \soutthe adenosine deaminase acting on dsRNA family of proteins, ADAR. ADAR1 and ADAR2 are the catalytically active proteins, whereas ADAR3 lacks editing activity and may act as a negative regulator of ADAR1 and ADAR2 activity (Nishikura, 2016b). Both ADAR1 and ADAR2 proteins have essential roles in cellular differentiation. In mammals, ADAR1 is widely expressed, especially in the myeloid component of the blood system, and plays a prominent role in promiscuous editing of long dsRNA (Zipeto et al., 2016). Additional studies indicate that ADAR1 forms a complex with Dicer to promote miRNA processing (Ota et al., 2013). ADAR2 has a higher expression in brain and is primarily required for site-specific editing of key transcripts for central nervous system development (Behm et al., 2017). A role for ADAR2 in the control of the circadian clock has been revealed (Terajima et al., 2017).

4. DEREGULATED EPITRANSCRIPTOME IN CANCER.

Given the importance of RNA modifications in regulating RNA life cycle and their role in cell fate and normal human development (Esteller and Pandolfi, 2017; Roundtree et al., 2017a; Morena et al., 2018), it is therefore not surprising that abnormal expression of the epitranscriptome lead to human diseases. In the last years, a number of studies have revealed that deregulated epitranscriptomes are associated with human pathologies, mainly cancer (but not limited to) (**Table 1**) through the deregulation of main tumourogenic pathways, including stem cell differentiation, cell invasion, immune responses, tissue renewal, viral infection or angiogenesis, among others. In this section, we will summarize the major evidences of altered epitranscriptome associated with cancer. This is a growth area with major interest, so it is foreseeable that it will open up a wide range of possibilities for oncology research.

4.1. Alterations of N6-Methyladenosine contents in cancer. The study of m6A dysregulation has been the main focus in oncology. The expression of m6A methyltransferases is frequently altered in cancer, and the functional consequences could be compatible with oncogenic but also tumour suppressor properties depending on the tumour type (Barbieri and Kouzarides, 2020; Rosselló-Tortella et al., 2020). Although the supporting evidences are still limited, this dual role in cancer seems to be determined by the cancer-specific downstream targets of m6A-related enzymes (Zheng et al., 2019).

An oncogenic role for METTL3 has been proposed in AML (Barbieri et al., 2017), clear cell renal cell carcinoma (Wang et al., 2020), gastric cancer (Yang et al., 2020) or pancreatic cancer (Taketo et al., 2017), among others. The role of METTL3-METTL14 complex in the AML model has been widely reported (Barbieri et al., 2017; Vu et al., 2017). m6A methylation is essential for the maintenance of crucial mRNAs involved in the self-renewal of haematopoietic stem/progenitor cells, so that METTL3 overexpression contributes to the maintenance of undifferentiated leukemic cells (Barbieri et al., 2017; Vu et al., 2017). Mechanistically, overexpression of METTL3 results in increased translation of oncogenic transcripts such as MYC, BCL2 or PTEN, as firstly demonstrated in *in vitro* studies performed in the MOLM-13 cell line (Vu et al., 2017). *In vivo* assays performed in METTL3- knockdown immunodeficient mice result in increased differentiation of leukemic cells and decreased anti-tumoural effect (Barbieri et al., 2017), confirming the oncogenic role of METTL3. A MYC-dependent oncogenic role of METTL14 overexpression has been also described in AML (Weng et al., 2018). Epithelial-mesenchymal transition (EMT), a crucial process for cancer metastasis, has been also associated with METTL3 dysregulation. m6A in *Snail* CDS causes polysome-mediated translation of Snail mRNA in liver cancer cells (Lin et al., 2019). Moreover, the upregulation of METTL3 and its reader YTHDF1 could be used as a prognosis factor for adverse overall survival of liver cancer patients (Lin et al., 2019). Overexpression of the EMT effectors metallopeptidase 2 (MMP2) and N-cadherin has been also observed in melanoma cells together with increased METTL3 expression (Dahal et al., 2019). Interestingly, the METTL3 mode of action also includes an effect on miRNA processing (Alarcón et al., 2015b). METTL3 promotes the maturation of miRNAs by interacting with the microprocessor protein DGCR8 (Alarcón et al., 2015b). In this model, METTL3 is able to dually modulate oncogenes or tumour suppressor genes by regulating the maturation of multiple miRNAs with pro- or anti-tumoural activity. For example, METTL3 overexpression promotes the maturation of pri-miR221/222, resulting in decreased expression of the tumour suppressor gene PTEN, and leading to the proliferation of bladder cancer (Han et al., 2019b). On the contrary, METTL14- DGCR8 interaction positively modulates the primary miRNA126 process in an m6 A-dependent manner leading to decreased metastatic potential in hepatocellular carcinoma (Ma et al., 2017). Tumour suppressor functions on METTL3- METTL14 complex have been identified (Cui et al., 2017; Liu et al., 2018; Deng et al., 2019). Human endometrial cancer carrying hotspot mutations in METTL14, and consequently reductions in m6A methylation, showed increased proliferation and tumorigenicity. Reductions in m^6A methylation lead to decreased expression of the negative AKT regulator PHLPP2 and increased expression of the positive AKT regulator mTORC2 (Liu et al., 2018). METTL3 also acts as tumour-suppressor in colorectal cancer through p38/ERK pathways (Deng et al., 2019)

In the case of altered m6A RNA demethylation in cancer, the first studies provide an oncogenic role for FTO in melanoma (Iles et al., 2013; Yang et al., 2019). FTO decreases m6A methylation and increases the stability of pro-tumorigenic melanoma genes such as PD-1 (PDCD1), CXCR4, and SOX10, in a mechanism dependent on the m6A reader YTHDF2. A role for FTO in the promotion of resistance to immunothera-

py (i.e., anti-PD-1) in melanoma therapy has been also demonstrated in mice models (Yang et al., 2019). FTO also promotes tumour progression in AML with t(11q23)/MLL rearrangements, t(15;17)/PML-RARA, FLT3-ITD, and/or NPM1 mutations. FTO demethylase m6A levels in ASB2 and RARA mRNA transcripts leading to inhibition of all-trans-retinoic acid (ATRA)-induced AML cell differentiation and promotion of leukemogenesis (Li et al., 2017b). In contrast, it could have a suppressive effect in IDH1/2-mutant AML tumours. FTO is an α-ketoglutarate dependent dioxygenase that is competitively inhibited by the structurally related oncometabolite R-2-hydroxyglutarate, which aberrantly accumulates in IDH1/2-mutant AML tumours. This FOT inhibition results in an increase of m6A content at specific targets that contribute to leukaemia suppression (Elkashef et al., 2017). In sum, the FTO effect on tumorigenesis strongly depends on the genomic context and the down-stream pathways that are involved.

The role of the m6A demethylase ALKBH5 has been well characterized in a glioblastoma model (Zhang et al., 2017c). ALKBH5 is highly expressed in glioblastoma stem-like cells. ALKBH5 target in glioblastoma is the transcription factor FOXM1, which is crucial for the maintenance of glioblastoma stem-cells properties and self-renewal. As a result of loss of m6A in FOXM1 mRNA transcript, its stability is increased, the FOXM1 expression is enhanced and cell differentiation diminished. Interestingly, ALKBH5 expression is correlated with poor prognosis in glioblastoma (Zhang et al., 2017c). Similarly, ALKBH5 overexpression (stimulated by hypoxia-inducible factor (HIF)-1α- and HIF-2α) in breast cancer demethylated the mRNA transcript of the pluripotency factor Nanog. The gain of Nanog stability favours a breast cancer stem cell phenotype (Zhang et al., 2016).

Finally, as previously mentioned, the m6A signal is interpreted by a set of reader proteins that exert their function in multiple biological pathways. Although the dysregulation of m6A readers should not result in changes in m6A patterns, alterations in the expression levels of these effector proteins could result in changes in the molecular function of the RNA modification. Furthermore, it still needs to be elucidated whether these readers have a crucial role in cancer independently of the m6A-signal. The reader YTHDF1 has a suppression effect of the antigen presentation in dendritic cells facilitating stable neoantigen-specific immunity (Han et al., 2019a). *In vivo* studies using mice models demonstrated that a loss of YTHDF1 in classical dendritic cells enhanced the cross-presentation of tumour antigens and the cross-priming of $CD8^{+}$T cells through \souta mechanisms involving the control of mRNA translation of lysosomal cathepsins. Most important, the therapeutic efficacy of PD-L1 checkpoint blockade is enhanced after YTHDF1 abolition, highlighting the potential therapeutic application of YTHDF1 expression in immunotherapy (Han et al., 2019a). YTHDF2 reader is overexpressed in metastatic colorectal cancer, leading to gain of expression of the metastasis-related gene HIF-1α, of tumour cells both*in vitro* and *in vivo* . A potential biomarker role for predicting metastasis has been proposed (Tanabe et al., 2016). Although based on preliminary results, the prediction potential is extended to HNRNPC reader (Liu et al., 2019). YTHDF2 is overexpressed in several subtypes of AML and is required for disease initiation as well as propagation in mouse and human AML (Paris et al., 2019). YTHDF2 decreases the half-life of tumour necrosis factor receptor Tnfrsf2 transcript avoiding apoptosis in leukaemia stem cells promoting their expansion.

4.2. Alterations of N1-Methyladenosine contents in cancer. Most of the efforts for the elucidation of the role of m1A dysregulation in cancer mainly refer to tRNA demethylation. Recently, it was described that loss of m1A contents mediated by ALKBH3 increased the mRNA transcript abundance of colony-stimulating factor (CSF1) promoting cell invasion without affecting cell proliferation or migration in ovarian and breast cancer cells (Woo and Chambers, 2019). This study anticipates a pathological role of m1A dysregulation in mRNA species.

4.3. Alteration of 5-Methylcytosine content in cancer. Consistent with its role in cellular differentiation, alterations in NSUN2 expression has been associated with human cancer progression. Gain of protein expression of NSUN2 was quantified in multiple cancer types, including oesophageal squamous cell carcinoma (ESCC), stomach, liver, pancreas, uterine cervix, prostate, kidney, bladder, thyroid, and breast cancer (Chellamuthu and Gray, 2020). In some cases, NSUN2 expression has potential biomarker applications. Ovarian cancer patients with high NSUN2 expression and low IGF-II expression exhibit higher overall and

disease progression-free survival (Yang et al., 2017a). In contrast, NSUN2 upregulation in Head and Neck Squamous Carcinoma (HNSCC) was associated with shorter overall survival and a higher mortality risk (Lu et al., 2018). Interestingly, NSUN2 expression have been proposed as a biomarker for the prediction of response to immunotherapy in HNSCC. The effect could be mediated by an association of NSUN2 expression and T-cell activation (Lu et al., 2020). In a similar manner to m6A or m1A RNA modifications, we are still far away from the understanding of the molecular pathways governing tumorigenesis. An elegant work aimed at identifying 5mC mRNA modifications at single-nucleotide resolution in human bladder carcinoma showed that hypermethylation of m5C mRNAs is highly enriched in well-known cancer-related pathways, including PI3K–AKT35 and ERK–MAPK36, and the oncogene heparin binding growth factor (HDGF), resulting in enhanced mRNAs stabilization (Chen et al., 2019). In addition, authors found a 5mC reader, named Y-box binding protein 1 (YBX1), whose expression is aberrantly increased in bladder cancer, providing new molecular clues on the 5mC dynamism (Chen et al., 2019). A separate study described a role of NSUN2-dependent methylation in the stabilization of the oncogenic lncRNA NMR (LINC01672) in ESCC. As a result of its increased stability, NMR transcript could directly bind to chromatin regulator BPTF, and potentially promote the expression of the metalloproteinase MMP3 and MMP10 by ERK1/2 activation (Li et al., 2018a). Conversely, other NSUN familiy members, such as NSUN5, exert tumor suppressor roles, being epigenetically inactivated in human brain tumors (Janin et al., 2019).

4.4. Pseudouridyne alterations associated with cancer .

Despite pseudouridylation biogenesis not being well understood, it is likely ψ plays a role in various physiological and pathological contexts. Unfortunately, its implication in disease and mode of action has been only partially explored up to now. Furthermore, most of the defects of Ψ modification linked to cancer are mediated by its control of non-mRNA species, mainly rRNA or tRNA.

Altered dyskerin pseudouridine synthase (DKC1) activity has been recognized as a potential trigger for cancer onset in hereditary syndrome-associated tumours and sporadic cancers. DKC1 expression levels have been correlated with tumour progression and poor overall survival in breast cancer, hepatocellular carcinomas, lung, and prostate cancers (Montanaro et al., 2006; Sieron et al., 2009; Penzo et al., 2017). Interestingly, the molecular consequences of dyskerin overexpression in cancer has been linked to the stabilization of the telomerase RNA component (TERC) (Penzo et al., 2015). Additionally, impairment of DKC1 function has been associated with aberrant p53 mRNA translation and p53 inactivation in human breast cancer cells (Montanaro et al., 2010).

The pseudouridine synthase PUS1 has been associated with melanoma and breast cancer through the pseudouridylation activity on its target ncRNA, the steroid receptor RNA activator 1 (SRA1). SRA1-associated PUS1 then binds the nuclear receptor domains of target genes (e.g., retinoic acid receptor-γ) to help establish the transcriptional pre-initiation complex (Zhao et al., 2004). Nevertheless, the molecular mechanisms underlying this phenomenon have not yet been clarified.

On the other hand, a mechanism involving the pseudouridine synthase PUS10 in TRAIL-induced apoptosis was discovered (Jana et al., 2017). PUS10 is exported from the nucleus to the mitochondria in the early stages of TRAIL-induced apoptosis. A feedback loop between PUS10 and caspase 3 is involved: active caspase-3 is required for PUS10 export whilst the movement of PUS10 reciprocally amplifies caspase-3 activity (Jana et al., 2017). Whether the pseudouridine synthase is involved it is still uncertain. Recently, it has been described that PUS10 binds to pre-miRNAs and interacts with the microprocessor DROSHA-DGCR8 complex to promote miRNA biogenesis in multiple cell types. Mechanistically, this process is also independent of the catalytic activity of PUS10 (Song et al., 2020).

4.5. Adenosine- to- inosine editing in cancer .

The overall biological significance of ADARs by affecting both the base pairing properties of mRNAs transcripts and ncRNAs but also altering codons after translation (and proteins) explains why their dysregulation result in multiple human diseases, including cancer. Alterations in ADAR activity has been described for multiple cancers, including lung cancer, hepatocellular carcinoma, chronic myelogenous leukaemia, glioblas-

toma and melanoma, among others (Chen et al., 2013; Jiang et al., 2013a; Chan et al., 2014; Qin et al., 2014).

In hepatocellular carcinoma (HCC), ADAR1 is fundamental in the earlier stages of tumorigenesis. A-to-I editing of antizyme inhibitor 1 (AZIN1) transcripts lead to a substitution of serine to glycine at residue 367, facilitating the tumorigenic phenotype and increased invasion properties (Chen et al., 2013). This effect is mediated by the prevention of the degradation of the ornithine decarboxylase ODC and cyclin D1 oncoproteins. Additionally A-to-I editing targets for ADAR1 in HCC has been proposed, including FLNB and COPA (Chan et al., 2014). AZIN1- dependent editing, together with FLNB A-to-I edition, is also involved in the pathogenesis of ESCC (Qin et al., 2014). Interestingly, type I interferon, and its associated JAK/STAT pathway, upregulates ADAR1 expression resulting in aberrant A-to-I editing process in ESCC (Zhang et al., 2017b). In contrast, a tumour suppressor role for ADAR2 has been described and downregulation of ADAR2 enzyme has been linked to ESCC progression. In this work, authors demonstrated that ADAR2 catalytic activity is necessary for the edition and stabilization of insulin-like growth factor binding protein 7 (IGFBP7) leading to cell apoptosis and inhibition of tumour growth (Chen et al., 2017). The mechanism by which ADAR1 expression is associated with CML involved the inflammatory pathway. In CML carrying BCR-ABL fusion gene, the expression of IFN-γ pathway genes promotes ADAR1 expression and editing activity (Jiang et al., 2013a). Through *in vitro* genetic assays, authors demonstrated that over-expression of ADAR1 positively correlated with expression of PU.1 (myeloid transcription factor) inducing a malignant reprogramming of embryonic stem cells (Jiang et al., 2013b). In lung cancer, ADAR1 gene amplification and overexpression has been observed in HNSCC cell lines and primary tumours, and it has been proposed as a biomarker for prediction of poor outcome (Anadón et al., 2016a). Mechanistically, ADAR1 overexpression enhances the editing frequencies of target transcripts such as NEIL1 (a DNA repair gene) and the oncogenic miR-381 (Anadón et al., 2016b). ADAR1- mediated editing of miRNAs (e.g., miR-455-5p) has been also described for metastatic melanoma by a mechanism involving the inhibition of the tumour suppressor gene CPEB1 (Shoshan et al., 2015). In gastric cancer, ADAR1 expression influences the phosphorylation level of crucial players of mTOR signalling pathway (i.e., mTOR, p70S kinase, S6 ribosomal protein) enhancing oncogenesis (Dou et al., 2016), while ADAR2 exerts a tumour suppressor role through the A-to-I editing of the PODXL (podocalyxin-like) transcript (Chan et al., 2016).

Indeed, ADAR2 seems to be more associated with tumour-suppressor properties than ADAR1 in several types of cancers as it was widely described in highly aggressive brain tumours. In high-grade astrocytoma or glioblastoma multiforme (GBM), ADAR2 regulates key cell cycle proteins, such as Skp2, p21 and p27, which control the G1/M phase and inhibits cellular growth (Galeano et al., 2013). A role of ADAR2 in the editing of oncogenic and tumour suppressors (e.g., miRNAs miR221, miR222 and miR-21) in GMB has been revealed (Tomaselli et al., 2015). The contribution of ADAR3 in glioblastoma revealed a potential binding competition between ADAR2 and ADAR3 to target specific transcripts and subsequently regulate their editing activity (Oakes et al., 2017a). Overexpression of ADAR3 in astrocyte and astrocytoma cell lines inhibits RNA editing at the Q/R site of the transcript *GRIA2*. Most importantly, the relation between ADAR2 and ADAR3 expression contributes to the relative level of *GRIA2* editing in primary tumour samples taken from glioblastoma patients (Oakes et al., 2017b).

5. PHARMACOLOGICAL STRATEGIES AIM TO TARGET THE EPITRANSCRIPTOME.

Taking into consideration the aforementioned dysregulation of the epitranscriptome in cancer, the design and development of small molecules that potentially revert defects in the epitranscriptome opens new and exciting opportunities for drug discovery in oncology (Table 2). Inevitabily, the successful introduction of epigenetic-based drugs into the clinic delineates a model for chemical biology and drug discovery research also on RNA-epitranscriptomics. Although the field of epitranscriptome-targeting is still in its beginnings, preclinical evidences of the benefits of RNA-modifying therapy are found in specific experimental models. Most important, several biotech companies are addressing the therapeutic potential of this promising field. Below, we will highlight the emerging results and challenges for the use of RNA- modifications as actionable targets for cancer drug discovery.

5.1. RNA methyltransferase inhibitors. There are several parallelisms between DNA and RNA methylation that support their exploitation within the framework of pharmacological inhibition. The most similar chemical modification is the addition of a methyl group at position 5 of cytidine resulting in 5-methylcytosine both at DNA or RNA molecule. It is however not unreasonable to assume that current DNA methyltransferases influence the dynamism of m5C RNA. As mentioned before, drugs inhibiting DNA methylation (e.g., 5-Azacytidine (AZA) or decitabine) have been FDA- approved and included in a clinical setting for the treatment of haematological tumours (Berdasco and Esteller, 2018). However, it is known that the vast majority (˜90%) of AZA is incorporated into the RNA molecule and that DNA methylation status does not correlate with the clinical response to hypomethylating treatment. Whether the antiproliferative effect is mediated by RNA or DNA methylation is still under debate and further investigation is needed. Recently, a mechanism involving members of the RNA methyltransferases NSUN (NSUN1 and NSUN3) together with DNMT2 has been proposed as a mediator of AZA response in a AML and myelodysplasic syndrome model (Cheng et al., 2018). Specifically, authors proposed a mode of action that involves the formation of two chromatin complexes including distinct RNA modifiers to explain positive response or resistance to AZA treatments. In AZA sensitive cells, the reader hnRNPK directly recognized NSUN3, DNMT2 and CDK9/P-TEFb to recruit RNA-pol-II \soutand resulting in an active conformation of chromatin in sensitive AML cells. In AZA-resistant cells, the interaction of NSUN1 with the chromatin remodelling factor BRD4 (but not hnRNPK) and RNA pol-II results in an active chromatin structure that is resistant to AZA. However, these AZA-resistant cells are sensitive to the BRD4 inhibitor JQ1 and NSUN1 interference by siRNA (Cheng et al., 2018). Supporting evidences on the impact of NSUN2 in AZA response previously showed that NSUN2 and METTL1 abrogation (by genetic knockdown) results in increased hypomethylating drug sensitivity in HeLa cells (Okamoto et al., 2014). Since acquired resistance to chemotherapy is a major bottleneck in cancer treatments, unravelling the multiple molecular mechanisms that guides therapy response is a mandatory concern to improve precision medicine in cancer.

Histone lysine methyltransferases (KMT) also contain SAM-binding pocket and a substrate-binding domain that have been successfully targeted (Ganesan et al., 2019). The first attempt to inhibit KMT activity was based on the discovery that the natural product sinefungin reversibly competes with SAM for its binding site (Kaniskan et al., 2015a). Thereafter, several potent SAM-mimetics that are selective by taking advantage of differences in the cofactor binding pocket have been developed as KMT inhibitors. The DOT1L inhibitor, Pimenostat, was the first KMT inhibitor to enter clinical trials for leukaemia therapy, followed by EZH2 inhibitors (GSK2816126 and tazemetostat) approved for B-Cell lymphoma treatments (Berdasco and Esteller, 2018). Although the protein structure of specific RNA methyltransferases has specific and unique features, like DOT1L, m5C and m6A RNA methyltransferases belong to the Rossmann fold family of methyltransferases. This similarity portends that KMT inhibitors could be used as a starting point for the chemical design and drug development of RNA methyltransferases; however, no strong preclinical data has been given to support this observation.

Considering that m6A is the most universal RNA modification together with the well-defined aberrant m6A patterns associated with cancers, the research community has already drawn attention to the importance of strengthening the pharmacological manipulation of m6A methyltransferase activity. METTL3-METTL4 is upregulated in cancers, and it has been previously showed that genetic manipulation by CRISPR-Cas9 technology guided to downregulate METTL3 enzyme prevent cell proliferation and invasion in AML *in vitro* and *in vivo* models (Barbieri et al., 2017). Consequently, drug developers from biotech companies have started the race for early drug discovery targeting RNA methyltransferases, mainly METTL3. Three companies, STORM Therapeutics (Cambridge, UK), Accent Therapeutics (MA, USA) and Gotham Therapeutics (NYC, USA), have announced to have METTL3 inhibitors in preclinical phases ready for phase I clinical trials (Cully, 2019). To date, the most advanced results have been achieved by STORM Therapeutics. In October 2020, STORM \southas announced that its first-in-class drug candidate targeting METTL3, named STC-15, has been selected to enter human Phase I clinical trial as a therapy for refractory AML. Preclinical studies on a mouse model of AML, showed that oral administration of SCT-15 reduced both splenomegaly and the number of circulating monocytes. Similarly, tumour growth was reduced in patient-derived-xenografts

(PDX)-AML models after treatment with the METTL3 inhibitor (Cully, 2019). The company is now studying the application on solid tumours. Accent Therapeutics has started its drug discovery program with an initial investment of $40M to optimize the selection of RNA-modifier inhibitors. They have announced that the company has already found around 20 targets, with METTL3/METTL14 inhibitors for AML treatment at the front of their research (Boriack-Sjodin et al., 2019; Cully, 2019). Similarly, Gotham Therapeutics launched in October 2018 with a $54 million program is the third company with a METTL3 inhibitor in preclinical development for AML therapy (Cully, 2019).

Out of these few examples, discovery of METTL3-METTL14 inhibitors are also explored in academia. Adenosine, one of the two moieties of SAM, is a SAM-competitive inhibitor of METTL3 activity. Recently, starting from a library of 4000 analogues and derivatives of the adenosine moiety of SAM and using high-throughput docking into METTL3 and protein X-ray crystallography, an adenosine derivative showed low μM potency and good ligand efficiency (Bedi et al., 2020). Interestingly, authors showed that the ribose of adenosine can be replaced by other ring systems, opening new opportunities for additional modifications (Bedi et al., 2020). Further development in preclinical models is still needed to explore the biological effect and mode of action of these adenosine derivatives.

5.2. RNA demethylases inhibitors. RNA demethylases also exhibit structural similarities with the protein lysine demethylases from the Jumonji C (JMJC) family. These are part of the 2-oxoglutarate and iron (II)-dependent dioxygenase family. This similarity is very interesting given that current inhibitors of JMJC proteins are available (Hauser et al., 2018) and that the mechanistic similarity between JMJC and RNA demethylases could facilitate the drug discovery for the inhibition of RNA modifications.

Interestingly, RNA demethylases have been targeted by specific small-molecule inhibitors. As m6A dysregulation impact in normal development and disease, its inhibition has been in the spotlight in the last years (**Table 2**). A pioneer study of small-molecule inhibitors of the human FTO demethylase was achieved by an chemical optimization of the natural product rhein (Chen et al., 2012). Rhein competitively binds to the FTO active site *in vitro* and globally increases cellular m6A on mRNA in the BE-2(C) cell line (Chen et al., 2012). Rhein also binds to ALKBH2 and ALKBH3 m1A and m3C demethylase, respectively; however, different binding sites are involved for ALKB or FTO inhibition (Li et al., 2016a).

A selective inhibition of m6A demethylase FTO rather than ALKBH5 was reported (Huang et al., 2015). The work was based on the identification of the differences in the displacement of m^6A-containing ssDNA binding to FTO and ALKBH5. This screening provides meclofenamic acid (MA), previously identified as an anti-inflammatory, as the best match to specifically inhibit FTO over ALKBH5 (Huang et al., 2015). *In vitro* studies demonstrated that treatment of HeLa cancer cells with the ethyl ester form of MA (MA2) increased m6A mRNA levels (Huang et al., 2015). Furthermore, the antiproliferative effect of MA2 treatment has been tested in *in vivo* models of glioblastoma (Cui et al., 2017). MA2 increased mRNA m6A levels in glioblastoma-stem cells (GSC) leading to suppression of the GSC-initiated brain tumour development and prolonged the lifespan of GSC-engrafted mice (Cui et al., 2017). This result suggests that targeting m6A methylation could be a promising strategy for the treatment of glioblastoma. Using leukaemia *in vitro* and *in vivo* models, a role for the pharmacological inhibition of FTO to prevent resistance to tyrosine kinase inhibitor (TKI) therapy has been demonstrated (Yan et al., 2018). Mechanistically, exposure to rhein or MA increases m6A and mRNA stability of survival and proliferation genes (e.g. BCL-2 or MERTK) improving the sensitivity of the tumour to the TKI nilotinib and PKC412 (Yan et al., 2018).

The knowledge gained on the basis for MA selectivity for FTO over ALKBH5 has facilitated the design of additional FTO inhibitors. In a later step, a study based on a screening of many fluorescent molecules with structures similar to MA revealed that fluorescein (and some of its derivatives) selectively inhibited FTO demethylation as well as directly labelled FTO protein (Wang et al., 2015). Two fluorescein derivatives with improved cell permeability, FL6 and FL8, could efficiently inhibit FTO demethylation and modulate the level of m6A in the mRNA of living cells (Wang et al., 2015).

Research aimed at developing selective and cell-active small molecule inhibitors of AlkB subfamilies of

demethylases have also explored the nucleotide-binding site instead of the 2OG-binding site (Toh et al., 2015). Compound 12 exhibits 30-fold to 130-fold selectivity for FTO over other AlkB subfamilies, and what is probably more interesting, the compound also discriminates against other human 2OG oxygenases, as PHD2 and JMJD2A protein demethylases. Treatment with an ethyl ester derivative of compound 12 increases m6A in HeLa cells (Toh et al., 2015).

Using structure-based rational design, which maintains the benzyl carboxylic acid to keep MA selectivity for FTO but extends the dichloride-substituted benzene to a deeper pocket that could be fully occupied by a bulky ligand, two FTO inhibitors were developed (FB23 and FB23-2) (Huang et al., 2019c). Based on the discovery that FB23 showed increased m6A levels due to FTO inhibition in *in vitro* AML cell lines, researchers have optimized the physicochemical property of FB23 and produced FB23-2 compound with a significantly improved antitumoural ability in *in vitro* but also *in vivo* conditions. FB23-2 treatments reduces the proliferation of a panel of AML cell lines, but most importantly, FB23-2 also inhibits primary leukaemia stem cells in PDX –AML mice models. Mechanistically, gain of m6A levels after FB23-2 treatment modulates mRNA transcripts associated with proliferation (e.g., MYC, CEBPA, RARA, and ASB2) (Huang et al., 2019c).

Additional FTO inhibitors were discovered using an elegant high-throughput screen using the fluorogenic methylated Broccoli substrate HTS assay (Svensen and Jaffrey, 2016). These Broccoli assays are based on the construction of a fluorescent RNA-dye complex that appear non-fluorescent when it contains m6A but becomes fluorescent after demethylation. The study identified several selective compounds for FTO inhibition which increase m6A levels at FTO target mRNAs (bone morphogenetic protein 6 (BMP6) and ubiquitin C (UBC) in HEK293C cells (Svensen and Jaffrey, 2016).

The possibilities extend beyond rational drug design as we learn more of the mode of action of the m6A-FTO axis. For example, the oncometabolite R–2-hydroxyglutarate (R-2HG) is produced at high levels in mutant isocitrate dehydrogenase 1/2 (IDH1/2) leukaemia cells (Su et al., 2018). However, R-2HG also has an antitumoral effect through the inhibition of FTO activity. FTO inhibition results in a gain of m6A levels and the stabilization of the mRNA transcripts MYC/CEBPA, leading to the suppression of relevant proliferation pathways (Su et al., 2018).

Undoubtedly, the primary focus of attention is now in FTO inhibition. However, additional RNA demethylases could be "druggable" targets. For example, the small compound 1-(5-methyl-1H-benzimidazol-2-yl)-4-benzyl-3- methylpyrazol-5-ol (HUHS015) was able to inhibit the prostate cancer antigen-1 (PCA-1/ALKBH3) axis in prostate cancer cell lines and murine xenograft models for prostate cancer (Nakao et al., 2014).

5.3. Targeting other RNA modifications. Epigenetic modifications of DNA molecules are interpreted by a set of reader proteins with essential functions. Most importantly, these readers for epigenetic marks are altered in human diseases, leading to the discovery of small compound inhibitors of their activity (Ganesan et al., 2019). Potent drug inhibitors have been identified for the H3K27me3 reader Polycomb protein EED from the Polycomb repressive complex 2 (PRC2) family (He et al., 2017). Like The YTF family of RNA methyl readers, Polycomb protein EED contains an aromatic cage crucial for the recognition of the methyl group. Whether the success of drug discovery associated with methyl-lysine readers could be translated to the methyl-RNA-reader field is still uncertain and unexplored. One barrier to the development of YTF- inhibitors is that YTF members from the same family of proteins exhibit high structural homology. It still needs to be determined whether the application of Pan-YTF inhibitors could have a tissue-specific effect and contribute to increased specificity (Cully, 2019). Efforts to inhibit the m7G reader eukaryotic translation initiation factor 4E (eIF4E) as an actionable target have been proposed (Soukarieh et al., 2016). Through its role in the regulation of mRNA translation of oncogenic pathways, eIF4E is implicated in cell transformation, tumorigenesis, and angiogenesis. Guanine-based inhibitors of eIF4E were evaluated in *in vitro* cell-based assays and provides a set of compounds with inhibitory activity at physiological doses (Soukarieh et al., 2016).

There are unique RNA modifications that could not be compared with DNA or histone modifications. The

RNA modifications involved in pseudouridylation or A-to-I editing have no precedents in drug discovery. However, chemical biology and drug discovery in this area requires a better characterization of the modes of action and pathological implications in a context-specific manner. The 3' terminal uridylyl transferase Zcchc11 (TUT4) is recruited to precursor let-7 RNA to selectively block let-7 miRNA biogenesis, a miRNA with tumour suppressor properties. Downregulation of Let7 miRNA has been described in cancer. It is therefore of great application to develop an inhibitor for the uridylation of precursor let-7 resulting in restored Let-7 expression in cancer (Lin and Gregory, 2015). Using an automated high-throughput screen of 15,000 chemicals, some small compounds have been selected as putative TUTase inhibitors (Lin and Gregory, 2015). The understanding of the TUT4-let-7 mediated inhibition is not addressed, so the consequences in preclinical models need to be determined.

In terms of pharmacological inhibition of the A-to-I edition, targeting ADAR family of proteins could be a promising strategy for cancer therapy. As mentioned before, ADAR1 is involved in multiple cancer-related pathways, and loss of function of ADAR1 in tumour cells profoundly sensitizes tumours to anti-PD1 immunotherapy (Ishizuka et al., 2019). Consequently, a strategy to repress ADAR1 expression is particularly challenging. Nowadays, there is not any public compound targeting ADAR1; but biotech companies such as Accent Therapeutics have declared to be working on this target for NSCLC therapy (Cully, 2019). In addition, by studying analogues of a naturally adenosine analogue, the 8-azaadenosine compound showed *in vitro* inhibition activity of ADAR2 (Véliz et al., 2003).

6. CONCLUSION

Rapidly accumulating evidences of the role of RNA modifications in cell differentiation and development and their dysregulation in cancer have emerged in the last years (Barbieri and Kouzarides, 2020; Rosselló-Tortella et al., 2020). Although still in its infancy, the potential of pharmacological targeting of RNA modifiers for reverting aberrant epitranscriptomes strongly energized the research of chemical biology and drug discovery in this field (Boriack-Sjodin et al., 2018). Below, we highlight some of the key limitations and challenges that will need to be addressed in the near future to improve the drug discovery in the RNA scenario (**Figure 2**).

It should be noticed that the field does not start from the zero \soutpoint, since the structures of key enzymes (writers, erasers) and reader proteins are currently well-characterized. This is a good starting point for guided and precise design of small compounds targeting these structures based on computational methods. To date a sizeable number of epitranscriptomic inhibitors have been reported, but in actuality not all small molecule inhibitors have demonstrated acceptable target potency or enzyme selectivity. Thus, there is an unmet need to develop selective and more effective small-molecule inhibitors of RNA modifiers for therapeutic applications. Apart from rational chemical design approaches, we still need to learn more about the biochemistry of RNA modifiers, their ligand binding pockets and the downstream pathways.

A very important matter to consider is our current limited vision of the mode of action of RNA modifiers. In other words, we are establishing associations between two observations (i.e., an altered m6A level and a tumour-suppressive effect) but, to date, we cannot determine causality between both observations. Most of the studies trying to establish phenotypic connections applied approaches that involve genetic manipulation of the full RNA-related gene. These approaches do not allow to solve whether the resulting phenotype is due to "druggable" biochemical mechanisms (e.g., enzyme catalysis or ligand binding) or other non-easy actionable targets linked to protein interactions (e.g., scaffolding, protein–protein interactions or chaperone mechanisms) (Boriack-Sjodin et al., 2018). Or even less, whether the RNA modifications result in changes in secondary structures or protein-protein interactions is still uncertain.

More sophisticated biological approaches apart from cell-based assays are essential to identify the functional effects of targeting the epitranscriptome. On the basis of what we have learned from epigenetic- based therapy, it is expected that RNA modifications work in multimeric protein complexes. Consequently, the translation of the results obtained in *in vitro* cell based assays do not replicate the physiological conditions from *in vivo* models. Animal models carrying RNA modification defects (e.g., inducible knockout or mutant

mice for RNA-modifying enzymes) are a move in the right direction to unravel the physiological function of RNA modifications. Furthermore, as previously described (Cheng et al., 2018; Huang et al., 2019a), most of the actual RNA modifying enzymes shared catalytic sites and cofactors with DNA and histone epigenetic machinery, and thus, drug-based intervention on RNA modifications can induce unforeseen effects on other regulation systems including epigenetics.

Although we are seeing improvements, the high number of possible RNA modifications and the complexity of molecular pathways involved are still an impediment to evaluate the biological consequences of small molecules intervention targeting the epitranscriptome (Jia et al., 2011; Wang et al., 2014). An aspect of intense debate is whether the ubiquitous nature of RNA modifications could increase the toxicity of associated drugs as it would be extremely difficult to prevent a pleiotropic effect. This knowledge, undoubtedly, also draws from the technical side. RNA modification detection methods have shown a tremendous improvement, particularly since the development of valid methods for studying the epitranscriptome at wide scale using NGS (Linder et al., 2015). Improvements to avoid methods dependent on antibody recognition or site-specific cleavage linked to radiolabelling, like MAZTER-seq (Garcia-Campos et al., 2019) or m6A-REF-seq (Zhang et al., 2019), held promises to allow a better quantification and precision of the epitranscriptome in specific contexts. Technological advances for the identification and quantification of low-abundance RNA modifications, establishment of internal standards as controls or development of bioinformatic tools to generate, analyse, and standardize protocols should be also a priority to ensure the reliability of the data (Morena et al., 2018).

In overcoming these (and other) chemical, biological and technical barriers, we will have a better and clearer view of the epitranscriptome map, its contribution to signalling pathways and its role in human health and disease. With this comprehensive overview of "epitranscriptome Science", the development of innovative therapeutic intervention of RNA modifications will be an exciting reality.

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FIGURE LEGENDS

Figure 1 . Schematic diagram of the predominant mRNA covalent modifications. Reactions for methylation at nucleosides, pseudouridylation and adenosine-to-inosine edition are illustrated. The known writers (W), erasers (E) and readers (R) for each RNA modification are listed together with the modification. Ψ, pseudouridine; I, Adenosine-to-Inosine edition; m1A, N1-methyladenosine; m5C, 5-methyl cytidine; m6A, N6-methyladenosine.

Figure 2. Challenges and difficulties for drug discovery targeting RNA-modifiers.

AUTHOR CONTRIBUTIONS

All authors contributed to all aspects of the manuscript.

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CONFLICT OF INTEREST STATEMENT

MB discloses no conflicts of interest. M.E. is consultant of Ferrer and Quimatryx.

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