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RNA epigenetics and cardiovascular diseases

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Abstract

Cardiovascular disease (CVD) remains the leading cause of death in the Western world. Despite advances in the prevention and in the management of CVD, the role of RNA epigenetics in the cardiovascular system has been until recently unexplored. The rapidly expanding research field of RNA modifications has introduced a novel layer of gene regulation in mammalian cells. RNA modifications may control all aspects of RNA metabolism, and their study reveals previously unrecognized regulatory pathways that may determine gene expression at a post-transcriptional level. Understanding the role of RNA modifications in CVD may lead towards a better understanding of disease mechanisms and the development of novel biomarkers or therapeutic strategies. In this review, we highlight the most recent and major reports in the field of RNA methylation and adenosine to inosine RNA editing related to the cardiovascular field and we discuss how this breakthrough will advance the field of precision medicine.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in Western society, and places a significant health and economic burden on the US and the world. As instances of obesity, high cholesterol, high blood pressure, and diabetes / metabolic syndrome increase, so too does the risk of CVD [1]. CVD encompasses a wide range of disorders, including coronary heart disease and atherosclerosis, genetic cardiomyopathies, congenital cardiovascular defects, heart rhythm disorders, valvular diseases, and many others. Despite recent advancements in the early diagnosis and management of many of the above disorders, CVD mortality throughout the world remains higher than cancer. Treatment strategies for CVD

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have not often been tailored to the specific needs of the patient in the past, but the landscape for precision medicine in CVD is changing. In recent years, genetic editing and gene-based therapeutics have been applied in the clinic, and remarkable advances are being made in patients with Duchenne muscular dystrophy, spinal muscular atrophy, and CVD [2–8]. The notion that messenger RNA (mRNA) is not a static molecule, but is in fact dynamically regulated by a diverse array of modifications, has broadened the potential for RNA-based therapeutics (antisense oligonucleotides, aptamers, siRNAs, miRNAs, and CRISPR/Cas9) in multiple disease systems including CVD, and many clinical trials for RNA therapies are in progress [9]. Despite the attractiveness of these RNA-based therapeutics for disease management, however, problems with stability, ease-of-delivery, and off-target effects have pushed ongoing research to discover additional, novel mechanisms to regulate RNA and influence disease pathophysiology [10].

RNA contains over 140 distinct chemical modifications, many of which were first identified in tRNAs and other non-coding RNAs in a diverse range of organisms [11]. RNA methylation is the most prevalent chemical addition to RNA nucleotides, and can affect guanosines (7-methylguanosine, m^7G [12]) cytosines (5-methylcytosine, m^5C [13]; 5-hydroxymethylcytosine, hm^5C [14]), adenosines (N^1-methyladenosine, m^1A [15, 16]; N^6-methyladenosine, m^6A [17, 18]; N^6,2'-O-dimethyladenosine, m^6A_m [19]), and ribose (2'-O'methylation, 2'-OMe or N_m [20]). The most prevalent of these, m^6A, was first identified in 1974, and in recent years has captured scientific attention as a conserved and dynamic moiety differentially regulated in many cellular- and state-dependent contexts [21–23]. m^6A occurs in ribosomal RNA (rRNA) [17], transfer RNA (tRNA) [24], small nucleolar RNA (snoRNA) [25], long noncoding RNA (lncRNA) [26], circular RNA (circRNA) [27], microRNA (miRNA) [28], and messenger RNA (mRNA) [22]; it is present on over 7000 (roughly 20%) human mRNAs, distributed throughout the coding and untranslated regions, and enriched in long last exons and upstream of the stop codon [17]. m^6A locations throughout mRNA, and especially in the untranslated regions, hint to the modification's function – a putative connection to RNA processing and translation. Ongoing studies are aimed at defining the precise role of m^6A in cell processes and disease states, as well as investigating the potential of manipulating m^6A to alter these events.

Although m^6A is the most prevalent mRNA modification, it is not the only form of methylation to influence RNA metabolism. m^1A, known to regulate the stability of tRNA and rRNA, can dramatically alter mRNA-protein interactions through electrostatic effects [15, 16]. Its position near translation start sites may upregulate translation, and its deposition is, like m^6A, dynamically regulated during stress [16, 29]. m^6A_m can also have a prominent effect upon translation efficiency by preventing decapping and miRNA-mediated mRNA degradation, thereby stabilizing mRNA [19]. m^5C modifications in tRNA can control cell growth and differentiation, and although also deposited on mRNA and lncRNA by the methyltransferase NSUN2, the precise function of this modification in biological function remains unknown [13, 30]. Finally, like m^1A, 2'-O-Me on ribose can affect RNA secondary structure and accessibility to RNA-binding proteins [31]. Although a great deal of work has focused on identifying the role of methyl modifications during RNA metabolism, additional studies are necessary to elucidate the full range of regulatory mechanisms afforded by RNA methylation.

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Another class of modifications are the substitutional RNA modifications. In RNA editing, RNA molecules are enzymatically modified on specific nucleic acids following transcription. RNA editing was first identified more than 30 years ago as an unwinding activity of transfected RNA duplexes in *Xenopus laevis* embryos [32, 33]. This was the result of covalent modification of RNA and was specific to double-stranded RNAs [34]. Editing is an important mechanism regulating gene expression at the RNA level, but only recently have methodological advances enabled a thorough investigation and highlighted an essential role for editing in cell physiology [35]. RNA editing is a ubiquitous and crucial posttranscriptional modification of the genome-encoded RNA sequence that modifies primary RNA transcripts and provides an additional layer of gene regulation to expand both the protein-coding potential of the transcriptome and the range of RNA transcript functions.

The most prominent form of RNA editing is adenosine to inosine (A-to-I) deamination, catalysed by the adenosine deaminases acting on RNA-1 and −2 (ADAR-1/−2) in mammals [36]. Another less prevalent form of RNA editing is the hydrolytic deamination of cytidine to uracil (C-to-U), catalysed by the cytosine deaminases of the apolipoprotein B mRNA editing catalytic polypeptide family APOBEC1 and APOBEC3A [37]. RNA editing occurs only on double-stranded RNAs (dsRNAs), with the majority of editing sites present in repetitive sequences such as *Alu* and long interspersed elements (LINEs) located in introns and 5' and 3' untranslated regions (UTR). Different editing enzymes demonstrate specific editing preferences: for example, ADAR1 appears to preferentially edit the *Alu* elements in non-coding regions while ADAR2 targets more non-repetitive sequences in exons or primary/precursor microRNAs [38]. Recently, millions of adenosines (mostly within *Alu* repeats) in the human transcriptome have been mapped as potential sites of ADAR-mediated A-to-I editing, demonstrating the likely wide-ranging impacts of this modification [39].

This review will discuss in more detail the role of m^{6}A and A-I editing, as these two RNA modifications have been recently implicated in crucial regulation of cardiovascular homeostasis and stress responses [40–43]. Specifically, we will discuss the impact of m^{6}A upon RNA regulation, including the m^{6}A 'life cycle' (deposition, recognition, and removal), m^{6}A alterations during development and various diseases, including recent studies investigating m^{6}A in cardiac disease, and finally the potential of m^{6}A modulation in understanding CVD pathogenesis and therapeutic design. Key aspects related to m^{6}A are also summarized in figure 1. We will also present a brief overview of A-to-I RNA editing and its impact on cardiovascular RNA epigenetics (visualized in figure 2). Recent reviews are also available for a deeper understanding of the molecular mechanisms and the pathophysiological process triggered by RNA editing [36, 44, 45].

2. m^{6}A and A-to-I RNA modifications control mRNA fate

2.1 m^{6}A dictates mRNA metabolism

As the most abundant internal mRNA modification, N^6-adenosine methylation can affect almost every aspect of RNA metabolism, from splicing and processing in the nucleus to translation and degradation in the cytoplasm (see Figure 1). Typically, m^{6}A is deposited onto mRNA at a conserved consensus sequence, RRm^{6}ACH ([G/A/U][G/A]m^{6}AC[U/A/C]), at particular locations on mRNA that can dictate the modification's function [23, 46]. The

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process of m^6A deposition and removal is highly regulated, and differs significantly based on cell type, differentiation state, presence or absence of stress, and countless other factors. Furthermore, m^6A recognition ('reading') is equally (if not more) complex, and recent studies have focused on identifying m^6A reader proteins and investigating how similar reader enzymes can have separate and unique impacts on RNA fate [47–49]. In addition to the direct effect of m^6A and its recognition by specific proteins, m^6A also acts indirectly on RNA molecules by weakening local RNA duplexes, which opens up the sequence to additional RNA-binding proteins (termed the 'm^6A switch') (see Figure 1, path B) [50–52].

2.2 The m^6A lifecycle: writers, readers, and erasers

The components of the m^6A methyltransferase complex were discovered starting in 1994 with the work of Bokar, Rottman, and colleagues, who cloned methyltransferase-like 3 (METTL3, also known as MTA-70, MTA, or IME4) [53]. This was followed closely by the discovery of the METTL3 adaptor protein Wilms tumor 1-associated protein (WTAP) and later, METTL14 and KIAA1429 (also called VIRMA) [54–57]. METTL3 is the canonical catalytic enzyme responsible for m^6A addition to mRNA, although all parts of the methyltransferase complex are required for efficient m^6A deposition. Despite METTL14's significant (approximately 43%) sequence homology with METTL3 and the presence of similar motifs required for catalytic activity, several studies have determined that METTL14 is in fact not catalytically active and instead acts as a scaffold for RNA binding to promote METTL3 activity; therefore METTL14 is dubbed a 'pseudomethyltransferase,' albeit an indispensable component of the methyltransferase complex [58–60]. WTAP mediates METTL3-METTL14 localization to nuclear speckles and is a similarly necessary component of the complex, as is KIAA1429/VIRMA (although the function of this component is not yet understood) [54]. Finally, recent work has identified RBM15 and RBM15B as additional components of the methyltransferase complex, which confer targeting specificity to certain adenosines [26, 61, 62].

Arguably the event that re-kindled scientific excitement towards m^6A was the discovery that the fat mass and obesity-associated (FTO) protein, which regulates metabolism and energy utilization and is implicated in obesity, is an m^6A demethylase or 'eraser', thus implicating m^6A in human physiological processes [63]. FTO's ability to erase m^6A marks suggested the dynamic, regulatory, and potentially targetable nature of mRNA methylation (Fig. 1, path A). Despite initial enthusiasm for this protein as both a demethylase and a culprit in obesity, several studies have recently demonstrated that obesity-associated mutations in *FTO* do not affect the FTO protein, but rather the neighboring genes *Irx3* and *Rpgrip1l* [64, 65]. In addition, several groups have hinted that m^6A may not be the only FTO substrate [19]. Antibodies to m^6A do not discriminate between m^6A and the similar m^6A_m in m^6A-RNA Immunoprecipitation followed by RNA sequencing (MeRIP-seq) experiments, and Mauer et al. demonstrated that FTO knockdown significantly affects m^6A_m, but does not appreciably increase m^6A levels *in vitro* [19]. Despite this, there remains significant controversy about FTO substrate specificity, as Wei *et al* have demonstrated that FTO preferentially targets nuclear m^6A in various RNAs and both m^6A and m^6A_m in cytoplasmic mRNA [66]. They have addressed controversial FTO-m^6A_m specificity by demonstrating that indeed FTO demethylates m^6A_m with higher affinity *in vitro*, but in various cell lines FTO may act on

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both substrates, a system which depends on cell state and even RNA species [67]. A common theme surrounding methylation deposition and removal appears to be extreme complexity in regulation, which is not fully understood. In the future, this intricate system may be exploited to target particular mRNAs, or even specific m^{6}A moieties, to regulate physiological processes.

In addition to FTO, alkB homolog 5 RNA demethylase (ALKBH5) is an m^{6}A demethylase, but it does not act on m^{6}A$_m$. ALKBH5 is localized in the nucleus, hinting that demethylation can rapidly occur after methylation, before the mature mRNA is shuttled to the cytoplasm [68]. However, due to its nuclear location ALKBH5 is able to demethylate other m^{6}A-containing noncoding RNAs, such as the lncRNA *MALAT1* and some small nuclear RNAs (snRNAs) and snoRNAs [25]. Murine global ALKBH5 knockout confers a relatively mild phenotype - knockout mice have defective spermatogenesis but are otherwise normal, suggesting that ALKBH5-mediated m^{6}A demethylation plays a subtle role in signaling that may be compensated by other pathways (Figure 1, path A) [68].

The final class of proteins directly involved in RNA metabolism are the m^{6}A readers or YTH proteins (YT521-B homology), which fall into three major classes: the DF family (YTHDF1, 2, and 3), YTHDC1, and YTHDC2. The YTH domain of these 5 proteins is the conserved site of selective m^{6}A binding [69]. The DF family members are highly similar to each other, reside in the cytoplasm, and have a large low-complexity domain enriched in Q, N, and P residues [69]. DC1 appears to be the major nuclear m^{6}A reader and can mediate splicing m^{6}A-regulated splicing events (see Figure 1, path C), whereas DC2 binds noncoding RNAs and intronic and intergenic regions, but its function is poorly understood [26, 70, 71]. As the DF readers are a major determinant of the impact of a particular m^{6}A modification on RNA metabolism, they will be discussed in more detail below.

2.3 m^{6}A-protein interactions specify RNA fate

The YTHDF proteins were originally thought to have separate, discrete functions on m^{6}A-modified mRNAs. He and colleagues first studied the function of YTHDF2, demonstrating that YTHDF2 knockdown induces half-life increases in several thousand transcripts, concluding that YTHDF2-m^{6}A binding induces mRNA instability [66]. Despite this, ribosome profiling experiments showed a negligible effect of YTHDF2-m^{6}A binding on translation efficiency, suggesting that this reader may be influencing mRNA transcript turnover more than translation efficiency [66]. More recently, YTHDF2 has been shown to recruit the CCR4-NOT deadenylase complex to m^{6}A-containing mRNAs, thereby directing them to cytoplasmic P bodies and promoting their decay (Figure 1, path D) [72].

Similarly, the reader YTHDF1 is also directly involved mRNA stability and translation. The He group found that, in contrast to YTHDF2, YTHDF1 acts to promote translation by directly interacting with eiF3 and other translation initiation factors, promoting cap-independent translation of m^{6}A-containing mRNAs [49]. Although first thought to have Different mRNA targets than YTHDF2, recent work has shown that YTHDF1 in fact shares the same pool of mRNA targets as its family members YTHDF2 and YTHDF3 (Figure 1, path D) [26].

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The final DF reader, YTHDF3, does not seem to have as clearly defined a function as YTHDF1 or YTHDF2. In fact, YTHDF3 interacts cooperatively with YTHDF2 to enhance mRNA decay, but can also act in concert with YTHDF1 to promote translation of targets [47, 73]. He's group suggested a dynamic model by which m^{6}A-methyated mRNAs are shuttled out of the cytoplasm and recognized by YTHDF3, which then acts as a "buffering agent" to mediate interactions with YTHDF1 (for translation) or to YTHDF2 (for decay) (Figure 1, path D) [73]. This system would allow for precise control of protein production during states in which cells require rapid protein expression turnover, such as cell differentiation and circadian rhythm. For more detailed reviews on m^{6}A-binding proteins and impacts on RNA metabolism, see Refs. [61, 69, 74, 75].

2.4 A-to-I RNA editing in RNA metabolism

Similar to m^{6}A modifications, RNA editing can affect every aspect of RNA metabolism, from transcription to RNA degradation. ADAR1 and ADAR2 exert a similar catalytic activity that modifies adenosine to inosine. RNA editing by ADARs may control RNA metabolism through Differential regulation of the binding of RNA-binding proteins to their targets [76]. This is dependent on the precise location of editing on the RNA segment. Edited transcripts have varied fates, which may be either physiological or pathological, depending on context (Figure 2). RNA editing may participate in genome recoding events at the RNA level which can in turn influence protein function [76]. Since RNA editing occurs cotranscriptionally, it can regulate alternative splicing, [77] RNA silencing, [78] trapping of the RNA in the nucleus, [79] or induce RNA degradation [80]. The many Different fates of edited RNA transcripts suggest that ADAR-mediated editing acts to regulate RNA metabolism in various ways depending on cell state (Figure 2).

3. m^{6}A and A-to-I RNA editing regulate cellular processes: implications for CVD

3.1 m^{6}A in development

During development, cells respond to an array of signals dictating how they must Differentiate spatially and functionally, and this requires precise coordination of transcription and translation to guarantee that the necessary genes are expressed at appropriate times. As previously described, m^{6}A deposition, recognition, and removal can dramatically impact the stability and translation of certain mRNAs, and therefore m^{6}A was hypothesized to play a role in stem cell differentiation. METTL3 knockout in mouse embryonic stem cells prevented differentiation and instead maintained cells in a naïve pluripotent state [81, 82]. In addition, several transcripts essential for maintaining pluripotency in humans and mice (such as *Nanog*) are normally m^{6}A methylated and thereby targeted for degradation [81–83]. The impact of m^{6}A upon differentiation is so significant that global knockout of METTL3 in mice is lethal during early development [82]. Interestingly, as previously discussed, global knockout of the demethylase ALKBH5 produces mice with a relatively mild phenotype, namely impairments in testes development and spermatogenesis, and these defects have been hypothesized to be caused by aberrant gene expression regulation [68]. Noticeably similar phenotypes (i.e. developmental

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impairment) are obtained between global METTL3 knockout, global ALKBH5 knockout, and germ-cell-specific METTL3 knockout (which also causes depletion of spermatogonial stem cells and sterility) [84]. This reinforces the notion that proper m^6A deposition, recognition, and removal is necessary for coordinated expression of pluripotency and lineage commitment markers and proper, timely cellular differentiation.

3.2 ADAR1 and ADAR2 in development

RNA modifications catalysed by ADAR1 or ADAR2 are an essential component of life, and therefore homeostatic and regulated levels of these enzymes are critical. Genetic ablation of either ADAR1 [85] or ADAR2 [86] in mice led to either prenatal or early postnatal lethality, highlighting the importance of RNA editing in normal physiology. ADAR1 seems to play an essential role in haematopoiesis, organ homeostasis, and suppression of innate immune system activation [87]. However, the exact mechanisms at the cellular level are poorly understood [88]. In contrast, ADAR2–/– mice are prone to seizures and die prematurely before P20, caused by neuronal death after excess influx of Ca^{2+} through the unedited glutamate receptor [86]. In this case, RNA editing of the glutamate receptor pre-mRNA is essential for the physiologic function of this gene. Although ADAR1 and ADAR2 possess the same deaminase domain, the site of RNA editing and the RNA molecules being targeted are subtly Different and may explain the Different phenotypes observed.

3.3 m^6A in stress responses

Environmental perturbations and cellular stress have profound effects on gene expression and translation, and given the dynamic nature of m^6A modifications and their ability to influence mRNA metabolism, several studies have focused on the role of m^6A in cell stress. Zhou et al. discovered that following heat shock, increased m^6A methylation in the 5’UTR promotes translation initiation in a cap-independent manner [89]. This response is directly linked with increased expression of YTHDF2, which the authors demonstrated directly competed with FTO in the nucleus to bind m^6A on select heat-shock-response mRNAs and promote their translation [89]. Similarly, Xiang et al. showed that METTL3 activity, and therefore presence of m^6A, was required for efficient repair of UV-induced DNA damage in a human osteosarcoma cell line [90]. In this scenario, m^6A is necessary for timely localization of the DNA repair enzyme DNA Polymerase κ (Polκ) to the site of DNA damage [90]. Finally, a recent study has examined the roles of both m^6A and m^6A_m (m^6A/m) in stress response regulation in a mouse model of fear behavior and human Major Depressive Disorder (MDD); m^6A/m changes alter transcriptome regulation following acute stress in mice, and the authors propose that m^6A/m regulation in the peripheral blood of MDD patients may approximate the brain’s response to the same modifications [91]. A clear understanding of which transcripts are methylated during stress, and furthermore their larger effects on the cell, will likely be complicated by the diverse stress-response mechanisms employed by Different cell types.

m^6A is crucial for stem cell fate and regulation of the stress response, and therefore seems to have a natural connection to cancer biology. Aberrant *FTO* expression has been described in certain subtypes of acute myeloid leukemia (AML), where forced *FTO* expression enhances AML cell survival and proliferation by decreasing levels of *ASB2* and *RARA*, suggesting

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FTO as an 'oncogenic m^{6}A demethylase' [92]. Notably, this is Different from the observation that many transcripts are stabilized by demethylation, e.g. by overexpression of FTO. Similarly, the demethylase ALKBH5 has been shown to enhance self-renewal of glioblastoma stem cells via demethylation and subsequent upregulation of *FOXM1*, and to promote proliferation of breast cancer stem cells via *NANOG* in a similar manner [93, 94]. As the major components of the methyltransferase complex, METTL3 and METTL14 has also recently been implicated in cancer pathogenesis. Lin and Choe *et al* first reported that METTL3 interacts with cap-dependent translation machinery, specifically eiF3, to promote the translation of oncogenes such as *EGFR* and *TAZ*, and furthermore that METTL3 is required for the proliferation of lung adenocarcinoma cells [95]. Subsequently, the methyltransferase writer complex was confirmed to be crucial for AML progression in three independent studies [96–98]. These studies, which have been reviewed extensively elsewhere, ([69, 99]) demonstrate the importance and specificity of m^{6}A 'writing' and 'erasing' in Different cancers, especially leukemias, and the potential therapeutic benefit m^{6}A modulation may provide. Despite this, future work is necessary to fully understand and exploit methylation-dependent transcriptome changes during stress and cancer, and these will likely vary depending on the type of cancer and the cellular environment which it creates.

3.4 A-to-I RNA editing in stress responses

Accumulating evidence suggests that cellular stress responses are critically regulated by RNA editing. The first work on this area showed that disruption of the *dADAR* gene in *Drosophilia* melanogaster leads to heat shock vulnerability and necessitates increased adaptation periods after oxygen deprivation [100]. Following this, it was suggested that A-to-I RNA editing may be one of the mechanisms cells use to regulate changes in gene expression after hypoxia, as RNA editing patterns are altered after hypoxia in mammalian cell lines. In addition to hypoxia, Differential regulation of ADAR1 has been demonstrated following inflammation [101] and increased oxidative stress [102]. Environmental factors such as energy and nutrient deprivation have been also shown to influence the expression or activity of ADARs [103, 104]. All these stress responses are firmly associated with cardiovascular disease, however there is little known at transcript level regarding the specific role of A-to-I RNA editing in these pathologies.

3.5 mRNA methylation and demethylation in cardiac disease

Given the explosion of studies investigating the role of m^{6}A in development, stress responses, and cancer, it is not surprising that there has recently been interest in elucidating the role of m^{6}A in cardiovascular health and disease. The importance of m^{6}A for proper timing and regulation of the circadian rhythm hinted to its connection with cardiovascular disease, as misalignment of the circadian clock is also shown to increase CVD risk factors (hypertension, inflammatory markers, etc.) in humans [105]. In addition, METTL3 and appropriate m^{6}A deposition is necessary for directed differentiation of mouse embryonic stem cells into cardiomyocytes and for the maintenance of *MyoD* in proliferative skeletal muscle myoblasts, underscoring the importance of this modification in muscle physiology [81, 106].

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Two studies have been recently published investigating the role of m^{6}A, through modulation of either FTO or METTL3, in cardiac remodeling and function following stress [40, 41]. The first of these studies, by the Sahoo group, examined the functional effect of FTO on cardiac contractile function and contractile transcripts during both cardiac homeostasis and myocardial ischemia-induced heart failure. They found that m^{6}A is increased in failing human, pig, and mouse hearts, hinting at the modification's clinical relevance in cardiac disease. MeRIP-seq experiments identified hypermethylation following myocardial infarction in mice in transcripts associated with cardiac hypertrophy, contraction, and sarcomere dynamics (*NPPA*, *SERCA2a*, *MYH7*, etc.) [40]. In addition, the authors show a corresponding decrease in FTO expression levels during heart failure, and reasoned that increased FTO expression could attenuate maladaptive cardiac remodeling following myocardial ischemia [40]. FTO knockdown induced arrhythmic events in primary isolated cardiomyocytes as well as proarrhythmic remodeling and altered ventricular repolarization in mice lacking FTO. Conversely, FTO overexpression attenuated hypoxia-induced cardiomyocyte dysfunction and restored calcium handling and sarcomere dynamics [40]. The authors of this study concluded that, due to m^{6}A-mediated downregulation of several calcium-handling and sarcomere contractile transcripts and corresponding decreased protein expression, forced expression of FTO attenuated ischemia-induced cardiac remodeling and may therefore represent a potential therapeutic target [40]. Overall, this study presents a novel role for the demethylase FTO in the regulation of cardiac contractility and remodeling following ischemic injury.

Recently, our group has published a similar study examining the effects of m^{6}A and its writer, METTL3, on cardiac homeostasis and hypertrophy. We have found that m^{6}A is a dynamic modification increased in specific transcripts (including members of the Mitogen-Activated Protein Kinase (Mapk) family) in isolated primary cardiomyocytes stimulated to hypertrophy, suggesting that METTL3 and m^{6}A act to regulate the hypertrophic response [41]. Cardiomyocyte-specific METTL3 overexpression both *in vitro* and *in vivo* stimulates physiological cardiomyocyte hypertrophy, and interestingly does not induce cardiac dysfunction in METTL3-overexpressing mice following long term pressure-overload stress [41]. In contrast, cardiomyocyte-specific METTL3 knockout prevents hypertrophy *in vitro* and negatively impacts cardiac homeostasis *in vivo*; METTL3-knockout hearts develop maladaptive eccentric remodeling and cardiac functional defects with aging and rapid, progressive dysfunction following acute pressure-overload stress [41]. Interestingly, the defects in hypertrophy seen in METTL3 knockout mice seem to affect cardiomyocyte morphology prior to the development of functional defects, suggesting that m^{6}A content is crucial for regulating cardiomyocyte geometry and structural adaptation to stress prior to symptom onset [41]. In fact, it appears that m^{6}A is both necessary and sufficient for cardiac hypertrophy to occur, and therefore targeting the m^{6}A-METTL3 pathway is a potential, novel therapeutic avenue for patients in which cardiac hypertrophy is aberrant and homeostasis is perturbed.

3.6 A-to-I RNA editing in CVD

The relevance and mechanistic role of adenosine to inosine RNA editing in the cardiovascular system and disease is largely unknown, with the exception of only few

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reports documenting a critical role of RNA editing in cardiovascular biology. A pilot study towards this aim has previously reported that children with cyanotic congenital heart disease manifested significantly higher rates of A-to-I RNA editing in the MED13 RNA than acyanotic, suggesting that RNA modification may influence the cellular and metabolic pathways in congenital cardiac defects [42]. We have recently shown that ADAR1 plays a critical role in the regulation of proinflammatory endothelial cells. Specifically we described that ADAR-1 induced A-to-I RNA editing controls the mRNA stability of the extracellular matrix degradation enzyme cathepsin S, and that the increase of ADAR1 expression in atherosclerotic inflammatory diseases results in increased expression of cathepsin S [38]. Interesting other findings have also contributed to establishing a link between RNA editing and CVD. Fei *et al* unraveled a new mechanism of contractile protein repression in smooth muscle cell (SMC) dedifferentiation through ADAR1-mediated RNA editing [43]. As mentioned previously, miRNA can also be affected by RNA editing. The editing of certain miRNAs such as miR487b, which is increased in murine muscle tissue during postischemic neovascularization, results in a new proangiogenic RNA with Different target specificity [107]. More recently, Jain *et al* demonstrated that Filamin A pre-mRNA editing by ADAR2 triggers a Q-to-R codon exchange at the end of exon 42 which regulates the activity of key smooth muscle contraction regulators such as PLC and ROCK machinery [108]. Lack of editing in Filamin A pre-mRNA produces a Filamin A isoform that only encodes a glutamine residue (Q) leading to mislocalization of p190RhoGAP, misregulation of PLC and ROCK signaling, increased MLC phosphorylation, aortic hypercontraction, thickening of the smooth muscle layer, and increased perivascular collagen deposition [108]. Consequently, loss of Filamin A editing leads to persistently elevated diastolic blood pressure resulting in left ventricular hypertrophy in mice [108].

4. Conclusions and Future Perspectives

The above studies contribute to a field of cardiovascular epitranscriptomics that is still in its infancy, and more work will be necessary to determine how m^{6}A writers, erasers, and readers, as well as ADAR-associated events, are able to influence cardiac function and pathophysiology. Research will no doubt require focus on unraveling the complexities associated with this field, such as the importance of m^{6}A in cardiomyocytes versus other cell types in the heart, Different forms of cardiac injury (ischemia +/− reperfusion, pressure overload, response to neurohumoral stimulation, etc.), and the interplay between the cardiovascular system and other organ systems frequently perturbed during CVD. As it appears that a proper amount of m^{6}A is necessary on very specific targets is necessary for proper cardiomyocyte function, both at baseline and with injury, a ‘simple solution’ of inhibiting or activating m^{6}A writers or erasers in a particular cell type or organ would likely not provide a lasting solution for CVD *in vivo*. Further specificity may be achieved by manipulating the levels of particular m^{6}A readers in a cell-type or state-dependent manner, however these types of studies are only beginning to be done. Besides m^{6}A mRNA methylation, it is also unclear how other RNA modifications (methylations of other nucleotides or even small and noncoding RNAs, pseudouridine formation, etc.) contribute to transcript regulation in CVD, or whether these modifications will have entirely distinct functions on RNA metabolism. Also, the use of the most recent technological advances in

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multiplex genome-editing tools such as CRISPR/Cas [109, 110] coupled with the power of A-to-I RNA substitutional editing, may provide a new therapeutic option. Overall, current advances in sequencing and mapping technologies for RNA modifications will undoubtedly lead to the discovery of even more novel mechanisms of gene expression regulation for CVD in the future.

Glossary

m^6A	N^6-methyladenosine
mRNA	messenger RNA
METTL3	Methyltransferase-like 3
METTL14	Methyltransferase-like 14
WTAP	Wilms tumor-1 associated protein
KIAA1429	vir like m^6A methyltransferase associated
FTO	fat mass and obesity-associated
ALKBH5	AlkB homolog 5, RNA demethylase
RNA-BP	RNA binding protein
YTHDC1	YTH domain-containing protein 1
YTHDF	YTH domain-containing family protein
P body	processing body
CCR4-NOT	CCR4-NOT deadenylase complex
eiF3	elongation initiation factor 3
40S	40S ribosomal subunit

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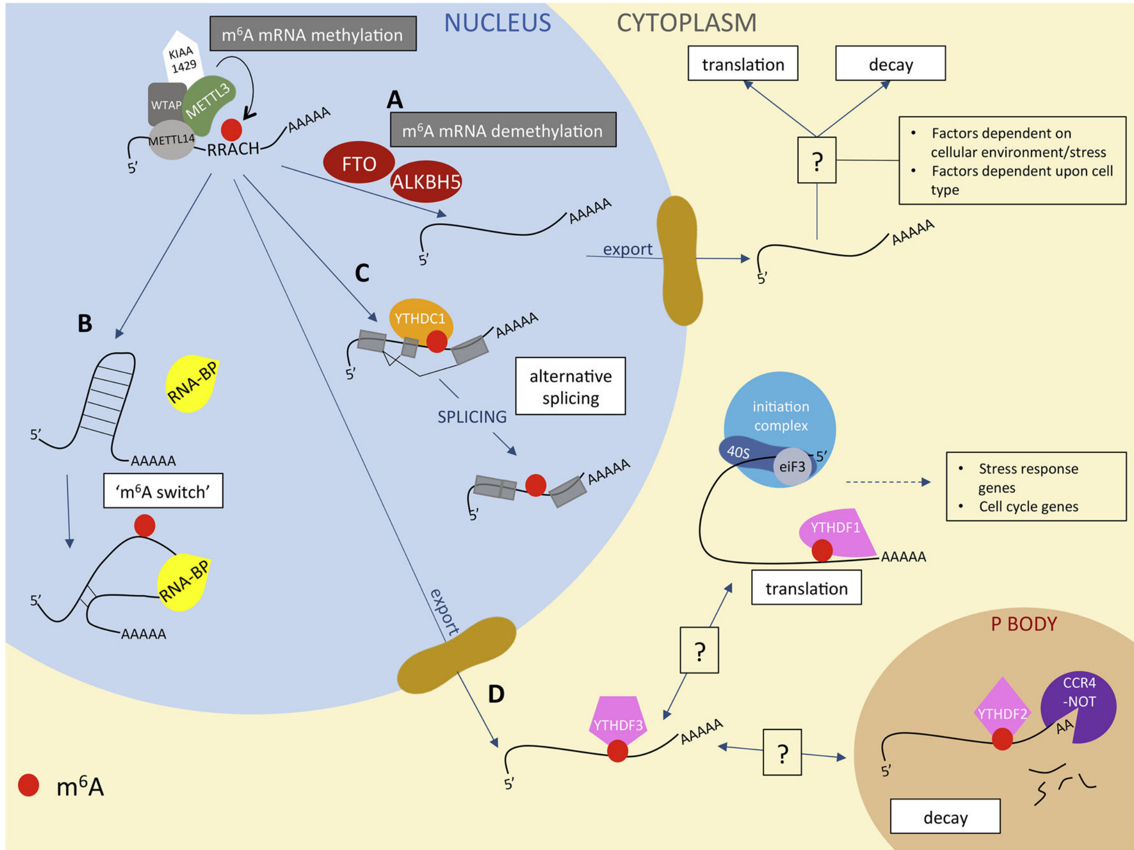

Figure 1: m^{6}A dictates mRNA metabolism.

m^{6}A is deposited onto mRNAs in the nucleus by the methyltransferase complex, comprised of METTL3, METTL14, WTAP, and KIAA1429. It can then be then demethylated by FTO and ALKBH5 (path **A**), after which it exits the nucleus and, depending on signals from the host cell, can either be translated or marked for decay (Unknown, additional signals from the cell are represented with "?"). Alternatively, m^{6}A remains on mRNAs, allowing for additional regulation of mRNA metabolism. m^{6}A can disrupt RNA secondary structure, exposing binding sites for RNA binding proteins, termed the 'm^{6}A switch' (path **B**). The m^{6}A reader YTHDC1 can bind m^{6}A on mRNAs and dictate alternative splicing (path **C**). m^{6}A-modified mRNAs can exit the nucleus and be bound by the m^{6}A 'readers' YTHDF1, YTHDF2, and YTHDF3 in the cytoplasm (path **D**). YTHDF1 generally promotes translation of the methylated mRNA, whereas YTHDF2 promotes decay by recruiting the mRNA to P bodies and binding the CCR4-NOT deadenylase complex. YTHDF3 can promote either translation or decay, depending upon as-yet-undefined signals from the host cell.

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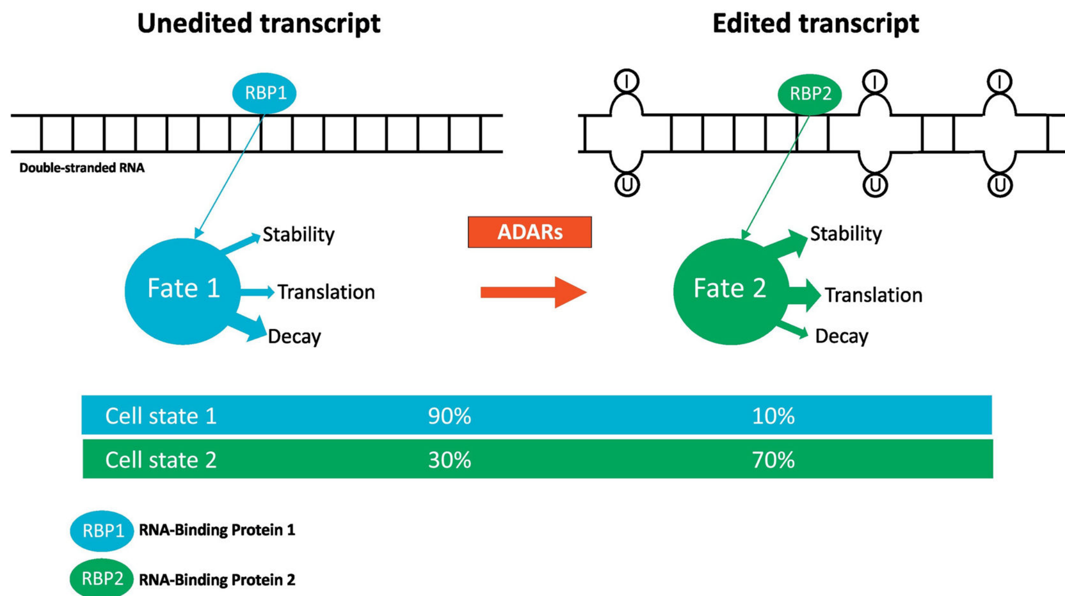

Figure2: RNA editing controls RNA-protein interaction and cellular function.

Cartoon illustrates the effect of adenosine to inosine RNA editing on double-stranded RNAs (dsRNAs) by the Adenosine Deaminases Acting on RNA (ADARs) enzymes. ADARs deaminate the adenosine residues into inosines, which no longer pair with uracil. This induces a change in RNA secondary structure, which may affect the binding of proteins to RNA molecules and thus may control several aspects of RNA metabolism and cellular function.