Our views of dynamic N^6-methyladenosine RNA methylation

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INTRODUCTION

We thank Darnell and coworkers in their Divergent Views article for noting our contributions to recent progress in the field of RNA modifications (Darnell et al. 2018). We agree with many of the viewpoints expressed: that a majority of messenger RNA N^6-methyladenosine (m^6A) methylation occurs cotranscriptionally, that one of the main functions of m^6A methylation on mRNA is to mark sets of transcripts for expedited turnover, and that this methylation may not dramatically affect splicing in HeLa cells. However, although the impact of m^6A methylation on splicing appears to be modest in many cell lines, we suggest caution because m^6A methylation is enriched in long exons and overrepresented in transcripts with alternative splicing variants (Dominissini et al. 2012). Several recent examples have revealed methylation-dependent changes in splicing: One demonstrated m^6A-modulated sex determination in *Drosophila melanogaster* (Haussmann et al. 2016; Lence et al. 2016), another found enhanced SAM synthetase expression mediated by a specific m^6A site installed by METTL16 (Pendleton et al. 2017), and recent reports uncovered extensive m^6A-dependent splicing changes mediated by ALKBH5 in male germ lines (Tang et al. 2017), as well as FTO-involved pre-mRNA splicing changes (Bartosovic et al. 2017). The potential effects of RNA methylation on constitutive and alternative splicing in additional physiological contexts need to be further evaluated.

DISCUSSION

There are three points with which we respectfully have different views: (i) the occurrence and functional relevance of RNA demethylation; (ii) the implication that "regulatory" and "dynamic" events should reside primarily in the cytoplasm; (iii) the general notion of "dynamic" RNA methylation.

First, the occurrence and functional relevance of RNA demethylation: Darnell and coworkers investigated HeLa cells, in which the m^6A demethylases FTO and ALKBH5 are primarily localized in the nucleus, and thus noticeable cytoplasmic demethylation would not be expected (Ke et al. 2017; Darnell et al. 2018). In addition, changes of modification stoichiometry were ignored in the argument. In situations where FTO resides in the nucleus, we have repeatedly observed measurable increases in nuclear mRNA m^6A levels upon FTO knockdown using quantitative LC–MS/MS measurements. The second demethylase, ALKBH5, has clearly defined demethylation functions during spermatogenesis (Zheng et al. 2013a) and in cancer progression (Zhang et al. 2016, 2017). We have also identified and characterized the first tRNA demethylase that mediates tRNA demethylation in the cytoplasm (Liu et al. 2016).

While the role(s) of RNA demethylation in the regulation of gene expression has yet to be fully understood and is currently being investigated in our and other laboratories, we will summarize recently published work. In collaboration with Jianjun Chen's laboratory, we recently reported that FTO is highly expressed in certain subtypes of human acute myeloid leukemia (AML), and that this elevated FTO expression promotes leukemogenesis and affects drug response (Li et al. 2017). In an analysis of m^6A-seq data from human AML cells with or without FTO knockdown (GSE84944; the data were reported in Li et al. 2017), we identified 7795 peaks with significant changes ($P < 0.01$) between the two samples. The vast majority of them (6991, 89.7%) display a notable increase in m^6A abundance in shFTO cells compared with control cells, and 95% of these peaks are located internally in mRNAs, demonstrating active cellular m^6A demethylation by FTO. In particular, we showed that *ASB2* and *RARA* are two functionally important mRNA targets of FTO in AML and that internal m^6A sites on these transcripts are required for FTO-mediated post-transcriptional regulation of their stability and expression (Li et al. 2017). Forced expression of wild-type FTO (but not a catalytically dead mutant) decreases the stability of *ASB2* and *RARA* transcripts by reducing m^6A levels, thereby down-regulating their expression (Li et al. 2017). Thus, this study demonstrates both the presence and the importance of FTO-mediated m^6A mRNA

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Article is online at http://www.rnajournal.org/cgi/doi/10.1261/rna.064295.117.

demethylation. It also suggests that alternative m^{6}A reading processes, other than the YTHDF2-dependent process we have described (Wang et al. 2014, 2015), may exist, which recognize m^{6}A sites in FTO target transcripts to promote their stability rather than reduce the stability. FTO activity on N^6-2′O-methyladenosine (m^{6}A$_m$) in the cap of mRNA has been reported (Mauer et al. 2017); however, the total cap m^{6}A$_m$ methylation level is less than 1/20 compared with internal m^{6}A, and close to 95% of the observed m^{6}A increases occur on internal sites when knocking down FTO in this AML system. These FTO-mediated demethylation events work on internal m^{6}A and could occur in the cytoplasm, as FTO localizes to both nucleus and cytoplasm in these cells.

There are also examples of functional demethylation in several other types of cancers. Recent work in breast cancer stem cells (BCSC) reported that hypoxia stimulates the expression of ALKBH5, which demethylates a 3′UTR m^{6}A site in *NANOG* mRNA and increases its expression, causing enhanced proliferation and enrichment of BCSC in tumors (Zhang et al. 2016). This effect can be diminished by the knockdown of ALKBH5. Our laboratory has also contributed to two recent studies on glioblastoma, demonstrating the critical functions of m^{6}A demethylases in the regulation of glioblastoma stem cell (GSC) self-renewal and tumorigenesis. In the first study, inhibition of FTO increases m^{6}A levels in GSC and decreases the expression of several oncogenes (*ADAM19*, *EPHA3*, and *KLF4*), which in turn suppresses GSC growth and prevents tumor progression (Cui et al. 2017). In a second study, it was revealed that ALKBH5 is highly expressed in GSC and specifically removes a 3′UTR m^{6}A on nascent transcripts of the oncogene *FOXM1*, sustaining its expression and leading to enhanced GSC proliferation and tumorigenesis (Zhang et al. 2017). These are recent examples of nuclear demethylation that have significant functional outcomes.

Dynamic nuclear demethylation has also been reported to play critical roles during stress response processes. Using the same heat shock response example mentioned in Darnell et al. (2018), new m^{6}A sites were found on the 5′UTR of newly transcribed mRNA during the heat shock response, facilitating selective cap-independent translation initiation (Zhou et al. 2015). These internal 5′UTR m^{6}A sites are bound by the m^{6}A reader YTHDF2, limiting the ability of FTO to demethylate them. In another recent study to probe nuclear factors facilitating the DNA damage response, it was discovered that ultraviolet irradiation-induced DNA damage recruits METTL3 and then FTO sequentially to damaged DNA sites, triggering a rapid and transient surge (~2–10 minutes after irradiation) of m^{6}A levels in adjacent RNA transcripts (Xiang et al. 2017). This transient increase of methylated RNA is required to recruit Pol κ to DNA damage sites for subsequent efficient DNA repair. Both examples demonstrate that nuclear methylation can be followed by demethylation, sometimes in quick succession, resulting in dynamic regulation of m^{6}A patterns to mediate responses to stress. These examples illustrate that the fates of m^{6}A-modified mRNA transcripts can be determined by nuclear demethylation in transcript- and sometimes site-specific manners. Recent work also points to non-nuclear regulatory roles of FTO-mediated m^{6}A demethylation in the context of localized translation (Yu et al. 2017).

Second, nuclear versus cytoplasmic regulation: The Divergent Views by Darnell et al. (2018) states: "Even if it is true that these pre-mRNA nascent molecules undergo partial demethylation in the nucleus before completion of processing, this nuclear demethylation is hardly evidence of a 'dynamic' methylation/demethylation in the cell at large …. Rather, such likely nuclear demethylation as described above in HeLa cells may represent a possibly necessary removal of a methylation for the correct nuclear processing and/or function of that particular mRNA when it reaches the cytoplasm. The possible function of this set of nuclear methylations and apparent demethylations surely requires additional investigation. But the present results are hardly the foundation for a regulatory 'RNA epigenetics' that is 'dynamic'."

We fully agree that "The possible function of this set of nuclear methylations and apparent demethylations surely require additional investigation" as RNA demethylases are known to mostly localize in the nucleus in HeLa cells. We further suggest that nuclear methylation and demethylation represent a dynamic process with regulatory effects in both the nucleus and cytoplasm, with methylation and demethylation in the nucleus playing primary regulatory roles. Methylation status determined within the nucleus may affect the fate of transcripts through effects on pre-mRNA processing, and ultimately tune cytoplasmic functions (Lin et al. 2017).

m^{6}A RNA methylation mediated by METTL3–METTL14 is essential in mammals. This seems puzzling given that mRNAs can be turned over in a matter of hours, but may suggest that m^{6}A RNA methylation represents a mechanism of gene regulation that is distinct from other known pathways. We noticed that m^{6}A mRNA methylation was found on transcripts with shorter half-lives back in 2014 (Fig. 4b in Fu et al. 2014). Since then we have focused our studies primarily on the "reader" proteins, and our investigations of YTHDF2 and various animal models have led us to a proposed mechanism of coordinated transcriptome turnover (Fu et al. 2014; Wang et al. 2014, 2015; Ivanova et al. 2017; Roundtree et al. 2017; Zhao et al. 2017a,b). Biochemically, it has already been shown that YTHDF2 recruits CCR4–NOT to accelerate deadenylation of target mRNAs (Du et al. 2016). In order for methylation to coordinate groups of transcripts during differentiation, development, or in response to cellular or environmental signaling, the proper establishment of the methylation landscape within the nucleus must be critical. Most of this methylation occurs cotranscriptionally, as demonstrated by Darnell and coworkers (Ke et al. 2017), and transcriptional regulation likely has a major influence on which sets of transcripts are methylated, properly processed, and subsequently metabolized in the cytoplasm.

Besides grouping sets of transcripts for coordinated turnover, we believe that there are additional essential functions of m^{6}A that we do not currently understand. The knockout mouse models for *Mettl3* and *Mettl14* exhibit much more severe phenotypes (early embryonic lethality) than the *Ythdf2* knockout mouse (Ivanova et al. 2017). In most developmental processes, the *Ythdf2*-dependent phenotypes account for only a portion of the phenotypes observed in the *Mettl3* or *Mettl14* knockout mice. During mouse germ cell development, YTHDF2 is reported to only be required for oocyte maturation (Ivanova et al. 2017). Another m^{6}A reader, YTHDC2, is essential for fertility in both male and female mice (Hsu et al. 2017; Soh et al. 2017; Wojtas et al. 2017), while both METTL3 and METTL14 are required at various stages of spermatogenesis (Lin et al. 2017; Xu et al. 2017). It is not clear what factors determine the distinct specificity of m^{6}A regulators toward different gametogenesis processes. Collectively, in early embryo development, stem cell differentiation, and tissue development, the impacts of m^{6}A are quite complex and broad. How the methyltransferase complex achieves both transcript- and methylation-site selectivity remain major mysteries in the field. The phenotypes of knockout mice of both demethylase genes (*Alkbh5* knockout results in spermatogenesis defects and a portion of *Fto* knockout mouse embryos do not last to birth) indicate critical functions and are likely based on their nuclear functions. We propose that there is a new layer of nuclear regulation involving RNA methylation and demethylation that will explain these complex knockout phenotypes, and we think this should be a main research focus for the community in coming years.

Third, dynamic RNA demethylation: Darnell et al. (2018) referred to two of our early commentary papers with regard to using the term "RNA epigenetics" (He 2010; Zheng et al. 2013b). We first noticed that FTO works on single-stranded nucleic acids and suggested that it might work on RNA in 2008 (Jia et al. 2008). Our 2010 commentary paper was published to propose to the community for the first time the idea of reversible RNA modifications with regulatory roles (He 2010), preceding our own discovery of the RNA demethylase in 2011 (Jia et al. 2011) and m^{6}A mapping in 2012 by others (Dominissini et al. 2012; Meyer et al. 2012). Later discoveries of mRNA and tRNA demethylases confirm the presence of RNA demethylation in the nucleus and cytoplasm. For the lack of an appropriate word to describe this notion, and to draw comparisons to the well-known DNA and histone methylations, we used "RNA epigenetics." We have restrained from using this phrase in recent years, and others invented the terms "epitranscriptome" and "epitranscriptomics" (Saletore et al. 2013) as a way to describe dynamic and regulatory RNA modifications. However, subsequent studies of RNA methylation have revealed many similarities between RNA and DNA/histone methylations: (i) chemical reversibility; (ii) the presence of dedicated "writer," "reader," and "eraser" proteins that regulate methylation levels and their functional consequences; (iii) that methylation patterns can change in response to cellular signaling and environmental cues; (iv) that because one methyl group provides limited binding affinity, methylation tends to cluster in DNA and RNA and the density of the methylation influences the biological outcome. In addition, while 2%–6% of total mammalian DNA cytosines can be 5-methylcytosine (5mC) methylated, only a portion undergoes TET-mediated demethylation in specific cells or biological processes (Yu et al. 2012; Hon et al. 2014; Wu and Zhang 2017), which is likely the case for RNA methylation and demethylation as well. There are of course fundamental differences between DNA, histones, and RNA. The primary roles of most RNA species, in particular mRNA, prevent them from storing long-term information. RNAs are turned over and they are less likely to directly carry heritable information. RNA methylation patterns could be maintained through generations, but such mechanisms would likely be mediated through crosstalk with transcriptional regulation and based on changes in DNA methylation and chromatin states.

If the primary criticism stems from the impression that we propose that most RNA methylation and demethylation occurs repeatedly, we have never meant to imply this and we accept the criticism. Our views have always been that mRNA regulation through methylation occurs at multiple levels and has to couple with other cellular processes. The first level is the methylation step, during which the writers determine transcript selectivity and site selectivity, which is most likely coupled with transcriptional regulation. A curious question that has interested us in the past few years is why nature evolves demethylases then. These demethylases are clearly present and functional. We suspect that the second level of regulation involves demethylation of subsets, though certainly not all, transcripts by demethylases in response to signaling or stress. The heat shock and UV damage studies are two examples, but we also believe that during developmental events such as spermatogenesis and early embryo development, the involvement of demethylation to erase m^{6}A on sets of transcripts offers a better option than directly tuning the methyltransferase complex, which is much bigger, more complex, and already engaged with transcription. It is perhaps more economical or efficient to swiftly change RNA methylation profiles using targeted demethylation of selected transcripts during rapid differentiation processes. The third level is binding by reader proteins to mediate the functional outcome of RNA methylation. All of these processes are subjected to regulation by cellular signaling and protein post-translational modifications. In this mechanistic scheme, the majority of methylated transcripts are methylated once. A portion of them could be demethylated at the second layer. Once transcripts are methylated, reader proteins can still mediate additional functions by binding or not binding the substrate under different conditions. This hypothesis correlates with the fundamental property of mRNA, which carries genetic information flowing from nuclear DNA to cytoplasmic translation sites to accomplish protein synthesis. However, we

cannot exclude that demethylation of mRNAs could occur in the cytoplasm, and that certain nuclear RNAs, mRNAs, and tRNAs could undergo multiple rounds of methylation and demethylation. We propose that repeated methylation and demethylation could occur in certain nuclear RNA species, as hinted by recent findings (Xiang et al. 2017). Lastly, cell lines grown under normal growth conditions may not require dynamic RNA methylation in gene expression regulation. RNA methylation has been shown to be critical to cell differentiation and development (Geula et al. 2015; Hsu et al. 2017; Ivanova et al. 2017; Lin et al. 2017; Zhao et al. 2017b), and plays important roles in stress responses (Zhou et al. 2015; Xiang et al. 2017), and thus RNA methylation and demethylation could significantly impact gene expression regulation in response to signaling and/or stresses.

CONCLUSION

In summary, we appreciate the opportunity for an exchange of views that can advance our understanding. Both mRNA and tRNA demethylation in the nucleus and/or cytoplasm have been discovered in recent years, supporting what we proposed back in 2010 (He 2010). Recent examples of RNA demethylation have started to show substantial functional relevance. We suggest that nuclear regulation of m^{6}A RNA methylation is critical and warrants careful future studies. We have focused much of our past investigations on reader proteins and physiological functions of mRNA methylation, but our own laboratory has come back to demethylase research. We anticipate that additional examples of critical roles of RNA demethylation will be reported by our laboratory and others in the near future. We think RNA methylation can be dynamic and hypothesize that the landscape of methylation throughout biological contexts varies significantly, and thus is a dynamic, regulatory feature of cell biology.

The field is still in its infancy. We still have no idea how the selectivity, both at the transcript level and at specific modification sites, is achieved, and how this is coupled with transcriptional events. We still do not know how effector proteins are tuned. Importantly, beyond simple cell lines, we really need to dive into complex in vivo biological systems and further reveal functional relevance and significance, as well as the potential implications for human diseases. Our understanding of this field, like in many other emerging fields, is continuously evolving. New discoveries, mechanistic investigations, and in-depth discussions and exchange of ideas will continue to further broaden our views.

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