The epitranscriptome in stem cell biology and neural development

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Abstract

The blossoming field of epitranscriptomics has recently garnered attention across many fields by findings that chemical modifications on RNA have immense biological consequences. Methylation of nucleotides in RNA, including N6-methyladenosine (m6A), 2'-O-methyladenosine (m2’A), N1-methyladenosine (m1A), 5-methylcytosine (m5C), and isomerization of uracil to pseudouridine (Ψ), have the potential to alter RNA processing and regulatory systems that drive these dynamics? The first step of the process, DNA to RNA, unfolds into many fields, including chromatin regulation, epigenetics, and transcription. For example, reversible chemical modifications are dynamically added to DNA and histones to alter gene expression and RNA levels (Akichika et al. 2019; Guo et al. 2011; Kohli and Zhang 2013; Strahl and Allis 2000). The next step, making protein from RNA, has largely been focused on the regulation of translation. However, the recent exploration of post-transcriptional modifications on RNA has shown that RNA is much more than a middleman between DNA and protein (Wang and Yi 2019). In fact, over 100 “epitranscriptomic” modifications have been identified, though their biological consequences are largely unknown (Cantara et al. 2011; Machnicka et al. 2013). A particular methylation at the N6 position of adenosine, termed m6A, has garnered the most attention for its powerful role in regulating mRNA processing (Roundtree et al. 2017).

While the existence of m6A has been known for almost 50 years (Desrosiers et al. 1974; Lavi and Shatkin 1975; Rottman et al. 1974; Wei and Moss 1977), a combination of events reinvigorated a recent interest in the field. First, Zhong et al. showed in 2008 that m6A is critical for developmental processes in Arabidopsis thaliana, which suggested its importance for multicellular eukaryotes, and pushed for the development of m6A mapping methods (Zhong et al. 2008). Next, the discovery of the m6A demethylases, FTO and ALKBH5, in 2011 and 2013, respectively (Jia et al. 2011; Zheng et al. 2013), suggested that the m6A system may be dynamic, which implies that it can be used as a regulatory system to alter mRNA fate in a context-dependent manner. Concurrently, antibody-based m6A mapping techniques

1. Introduction

The central dogma of biology—that information flows from DNA to RNA to protein—has acquired an increasing number of caveats and fine print (He 2019). Beyond direct exceptions to the rule, like non-coding RNAs, the details of the process are marred by questions like how much RNA is made from DNA, and how is this RNA processed at the correct time? How might the system change in response to stimuli, and what regulatory systems drive these dynamics? The first step of the process, DNA to RNA, unfolds into many fields, including chromatin regulation, epigenetics, and transcription. For example, reversible chemical modifications are dynamically added to DNA and histones to alter gene expression and RNA levels (Akichika et al. 2019; Guo et al. 2011; Kohli and Zhang 2013; Strahl and Allis 2000). The next step, making protein from RNA, has largely been focused on the regulation of translation. However, the recent exploration of post-transcriptional modifications on RNA has shown that RNA is much more than a middleman between DNA and protein (Wang and Yi 2019). In fact, over 100 “epitranscriptomic” modifications have been identified, though their biological consequences are largely unknown (Cantara et al. 2011; Machnicka et al. 2013). A particular methylation at the N6 position of adenosine, termed m6A, has garnered the most attention for its powerful role in regulating mRNA processing (Roundtree et al. 2017).

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were developed and showed that m^6^A additions to mRNA are non-
random, thus pushing biological interest forward and providing tech-
niques to understand the molecular mechanisms of m^6^A in the cell
(Dominissini et al. 2013; Meyer et al. 2012). In this review, we first
introduce several epitranscriptomic marks that have garnered the most
attention over recent years, with a focus on m^6^A. We then discuss the
major advancements in our understanding of the epitranscriptome in
stem cell biology, especially embryonic stem cells (ESCs) and induced
pluripotent stem cells (iPSCs). We also touch on how the epitrans-
scriptome itself is regulated in embryonic stem cells. In addition, we
review the role of the epitranscriptome in cortical and cerebellar de-
velopment, as well as in adult neurogenesis. Finally, we discuss the role
of the epitranscriptome in neurological disorders.

2. RNA modifications of particular interest

2.1. N6-methyladenosine: m^6^A

On a global level, 0.2 to 0.5% of all adenines are m^6^A modified
(Geula et al. 2015). The highest levels occur in the brain, where up to
30% of all transcripts are modified (Chang et al. 2017). Epitrans-
scriptomic detection technologies have been focused on m^6^A, making it
one of the best-studied modifications to date. m^6^A occurs in various
types of RNA, including tRNA, RNA, non-coding RNA (ncRNA), and
mRNA. In 2012, two groups independently reported anti-m^6^A antibody-
based m^6^A RIP-Seq (MeRIP-Seq) techniques (Dominissini et al. 2012;
Meyer et al. 2012). Subsequent mapping of m^6^A in the transcriptome
showed that it is most commonly added at a consensus sequence of
DRACH (D = A, U or G; R = G or C; H = A, U, or C). m^6^A is especially
enriched in the 3'UTR and around the STOP codon (Dominissini et al.
2012; Meyer et al. 2012). While m^6^A does not alter Watson-Crick-
Franklin base pairing, it can modify protein binding and affect the
mRNA secondary structure (Farre et al. 2003; Roost et al. 2015). Many
m^6^A-binding proteins, or readers, have been identified. Individual
readers confer unique downstream fates on m^6^A-modified mRNA, in-
cluding altered mRNA stability, translation, localization, and splicing.
m^6^A methylation patterns in the transcriptome appear to be cell/tissue-
specific and species-specific (Liu, 2019). Current m^6^A sequencing
technologies are not sufficiently sensitive to profile m^6^A at the
single-cell level, which would help quell disagreements in the field on
whether m^6^A is truly different across cell types or dynamic in response
to stimuli. For example, Garcia-Campos et al. claimed that m^6^A is “hard
coded” and largely predictable in the yeast genome based on the ex-
tended sequence around the modified site (Garcia-Campos et al. 2019).
In contrast, studies that have profiled multiple mammalian tissues
identified tissue-specific methylation profiles and significant changes
over development (Liu, 2019; Shi et al. 2018; Weng et al. 2018; Yoon
et al. 2017; Zhang et al. 2020). Furthermore, up-regulation of the m^6^A
demethylases FTO and ALKBH5 in response to heat stress and hypoxic
stress, respectively, suggests that dynamic changes to the m^6^A methy-
lome may be a way of modulating cellular responses to stimuli (Zhang
et al. 2016a; Zhang et al. 2016b; Zhou et al. 2015). Nonetheless, a lack of
reproducibility in m^6^A sequencing, especially using antibody-based
methods, may lead to false conclusions on the variability and dynamic
nature of m^6^A (McIntyre et al. 2020).

A growing list of proteins has been found to form the methyl-
transferase complex that adds m^6^A onto mRNA (Fig. 1). This complex
includes a core heterodimer unit of METTL3 and METTL14 (Liu et al.
2014), with accessory proteins including WATP (Liu et al. 2014; Ping
et al. 2014), HAKAI, KIAA1429 (Schwartz et al. 2014b), and RBM15/B
(Patil et al. 2016). This complex has been reviewed elsewhere (Balacco
and Soller 2019; Garcías Morales and Reyes 2020).

There are two known m^6^A demethylases, ALKBH5 and FTO (Jia
et al. 2011; Zheng et al. 2013). ALKBH5 co-localizes with nuclear speckles, indicating that both methylation and demethylation occur in
the nucleus. On the other hand, FTO can act in the nucleus and
cytoplasm. However, the in vivo activity of FTO as an m^6^A demethylase
has recently been questioned, with the suggestion that it may instead
act on m^5^A (Engel et al. 2018; Koh et al. 2019; Linder et al. 2015;
Mauer et al. 2017; Schwartz et al. 2014b). On the other hand, one re-
cent study reported that FTO can demethylate m^5^A, m^6^A, and m^A
depending on the nucleus or cytoplasmic localization (Wei et al. 2018).
Yet another study argued that since loss of FTO has little effect on cy-
toplasmic mRNA m^6^A or m^6^A levels, FTO likely functions primarily in
the nucleus. They further showed that methylation on small nuclear
RNAs (snRNAs) is a substrate for FTO demethylation (Mauer et al.
2019). The specificity of FTO remains a major hurdle in the field of
m^6^A; confirming its target is critically important so as not to mis-
attribute a phenotype or biological function to the wrong epitrans-
scriptomic mark. Finally, full knockouts of either ALKBH5 or FTO are
not lethal in mice, though they appear to be particularly important in
the cellular stress responses (Cao, 2019; Engel et al. 2018; Ma et al.
2018).

The highly variable functions of m^6^A can be attributed to its many
distinct reader proteins. The central group of readers is the YTH-do-
main-containing family of proteins, which bind directly to m^6^A. These
readers have recently been reviewed elsewhere (Patil et al. 2018;
Roundtree et al. 2017). Briefly, YTHDC1 is found in the nucleus and
regulates splicing, while YTHDF1, YTHDF2, YTHDF3, and YTHDC2 are
cytoplasmic and are thought to have distinct functions. YTHDF1 pro-
motes translation, YTHDF2 promotes mRNA degradation, and YTHDF3
seems to promote either translation or degradation in a context-specific
manner. Finally, the binding specificity and function of YTHDC2 re-
main unclear and may only be functional under special cellular con-
ditions (Patil et al. 2018). In contrast to the notion that each YTHDF
reader promotes a unique fate of m^6^A-modified mRNA, two recent
studies found that YTHDF1-2, 3, and 2 are jointly promote mRNA de-
gradation in cell lines (Zaccara and Jaffrey 2020) (BioRxiv: doi: https://
doi.org/10.1101/2020.06.03.131441). These divergent conclusions on
the function of m^6^A reader proteins underscore the vital need for ad-
ditional research into reader protein function and regulation.

2.2. N6,2'-O-dimethyladenosine: m^6^A

Unlike the internal m^A modification, N^6^,2'-O-dimethyladenosine
(m^6^A) occurs in the mRNA terminus at the first nucleotide following
the N^6^-methylguanosine (m^G^) cap. Approximately 0.0036% to
0.0169% of all adenines are m^6^A modified when averaged across
multiple human tissue types, corresponding to 526 to 1028 unique
transcripts, depending on the tissue type (Liu, 2019). The number of
m^6^A-modified transcripts was previously thought to be much higher,
but improved detection sensitivity has led to the view that m^6^A is only
moderately abundant (Frye et al. 2016). Three independent studies
showed that phosphorylated C-terminal domain (CTD)-interacting
factor 1 (PCIF1) is a cap-specific m^6^A methyltransferase that targets
newly transcribed mRNA by associating with RNA Polymerase II
(Akichika et al. 2019; Boulias et al. 2019; Sun et al. 2019). By knocking
out PCIF1 in various cell lines, Boulias et al. found that m^6^A most
strongly correlates with high expression and increased transcript sta-
Bility (Boulias et al. 2019). However, this was not universally true for
all m^6^A-modified transcripts, leaving the regulatory capacity of m^6^A
up for debate. The downstream functions of m^6^A are still unclear, with
multiple conflicting studies reporting opposite effects on mRNA stabili-
and translation (Akichika et al. 2019; Boulias et al. 2019; Liu, 2019;
Sendinc et al. 2020). The field would greatly benefit from identifica-
tion of m^6^A reader proteins that could help disentangle its potential
downstream functions.

2.3. N1-methyladenosine: m^A

The abundance of m^A in cells remains under debate. Some studies
found that 0.015% to 0.16% of adenines are m^A modified,
corresponding with over 4000 mRNA transcripts (about 20% of the transcriptome) with high stoichiometries of m1A (Dominissini et al. 2016; Li et al. 2016). Two independent studies claimed that as few as 15 or 53 total m1A sites exist in mRNA and lncRNA, and that it mostly occurs at low stoichiometries at these sites (Safra et al. 2017; Schwartz 2018). In support of this, antibody cross-reactivity with m7G has been shown to produce false positives in m1A identification, and a more recent study found only one high-confidence, high stoichiometry m1A site using a bioinformatic approach, which was validated with an improved m1A antibody (Grozhik et al. 2019). Though our understanding of the modification is limited, progress was made through identification of putative m1A methyltransferases (Fig. 1), namely, TRMT6/TRMT61A complex in the cytosol and TRMT10C/TRMT61B complex in the mitochondria (Li et al. 2017c). Currently, ALKBH3 is the only known m1A mRNA demethylase, though it also acts on DNA and m3C in RNA (Aas et al. 2003; E et al., 2016; Ougland et al. 2004). m1A primarily exists in the 5'UTR near the translation initiation site, and its positive charge can induce changes in secondary mRNA structure that may promote translation (Dominissini et al. 2016; Li et al. 2017c). No studies of m1A in the brain have been reported, leaving a major gap in knowledge that will undoubtedly be explored in the coming years.

2.4. 5-methylcytosine: m5C

m5C is added to tRNA, rRNA, and mRNA by a variety of methyltransferases with specific RNA targets (Motorin et al. 2010). The reported abundance of m5C on mRNA is extremely variable across different studies, with some reporting up to 10,000 m5C sites in a single tissue or cell type (Amort et al. 2017; Squires et al. 2012; Yang et al. 2017), while others using more stringent detection methods showed that zero to only a few hundred sites are modified in mammalian mRNA (Huang et al. 2019b; Legrand et al. 2017). DNMT2 and especially NSUN2 are the most well-characterized m5C methyltransferases that act on both tRNA and mRNA (Khoddami et al. 2019; Li et al. 2017b; Yang et al. 2017) (Fig. 1). NSUN2-mediated m5C mRNA methylation promotes mRNA nuclear export through ALYREF, a nuclear m5C reader protein (Yang et al. 2017). Additionally, m5C may cooperate with m6A to enhance translation of particular transcripts like p21 (Li et al. 2017b). Finally, m5C addition to a subset of ncRNAs called vault RNAs (vtRNAs) reduces downstream miRNA production (Hussain et al. 2013). For example, NSUN2 regulates the processing of vault RNA, VTRNA1.1, into small-vault RNAs (svRNAs) that function similarly to miRNAs (Sajini et al. 2019). Though no m5C direct demethylases have been identified, ten-eleven translocation (Tet) enzymes can oxidize m5C to 5-hydroxymethylcytosine (hm5C) and then unmodified cytosine (Ito et al. 2011). The frequency of hm5C is about one hm5C per 5000 m5C (Fu et al. 2014). This is slightly enriched in mRNA, with hm5C occurring on ~7 × 10^{-6} of the total cytosines (Xu et al. 2016). In Drosophila, hm5C was shown to preferentially mark mRNAs in coding regions and promote their translation (Delatte et al. 2016; Fu et al. 2014). YBX1 is a recently identified m5C reader protein that promotes stabilization of modified mRNAs in early zebrafish embryos (Yang et al. 2019). One study showed that m5C can impair RBP binding, as is the case for SRSF2, which binds to unmethylated vtRNAs with higher affinity than to methylated RNAs (Sajini et al. 2019).

2.5. Pseudouridine: Ψ

While pseudouridine (Ψ) is one of the most abundant modifications in ncRNA, its existence in mRNA is a recent finding (Carlile et al. 2014; Schwartz et al. 2014a). PUS1 and PUS7 enzymes isomerize uridine to pseudouridine (Lovejoy et al. 2014) in an mRNA structure-dependent manner (Carlile et al. 2019) (Fig. 1). Other PUS-family proteins add Ψ to other types of RNA. On the other hand, no direct readers or removal enzymes have been identified, raising the possibility that Ψ is irreversible. Some downstream effects of Ψ include weakening interactions between mRNA and Pumilio family proteins (PUFs) (Vaidyanathan et al. 2017) and stabilizing RNA structure by improved base stacking and increased hydrogen bonding (Carlile et al. 2014; Davis 1995; Spenkuch et al. 2014). Ψ has also been hypothesized to promote

![Fig. 1. Overview of common epitranscriptomic marks.](image-url)
| Mod. | Tissue or cell type | Detection method | # of modified transcripts | Comments | Reference |
|------|--------------------|------------------|---------------------------|----------|-----------|
| m6A  | Mouse ESCs         | m6A RIP-seq      | 8645                      | 3942 annotated; 10,609 peaks are WTAP-dependent (64.3%) | Wang et al., Nat Cell Biology 2015 |
| m6A  | Mouse ESCs         | m6A RIP-seq      | 9754                      | 5461 mRNAs, 117 ncRNAs | Batista et al., Cell Stem Cell 2015 |
| m6A  | Mouse naïve ESCs   | m6A RIP-seq      | 10,431                    | Geula et al., Science 2015 |
| m6A  | Mouse embryonic fibroblasts | m6A RIP-seq | 16,487 | 10,609 peaks are WTAP-dependent (64.3%) | Schwartz et al., Cell Reports 2017 |
| m6A  | Total mouse brain | m6A RIP-seq      | 13,471                    | 4654 residues identified by truncation calling, and 33,157 identified by RIP-seq | Linder et al., Nature Methods 2016 |
| m6Am | Mouse cortex       | m6A RIP-seq      | 18,032                    | 9636 mRNAs | Ke et al., Genes Dev 2017 |
| m6Am | HEK293             | CITS miCLIP      | 18,945                    | 2179 mRNAs | Boulias et al., Molecular Cell 2019 |
| m6Am | Human brainstem    | m6A RIP-seq      | 30,459                    | 12,659 Donor ID 5 | Liu et al., Mol Cell 2019 |
| m6Am | Human cerebellum   | m6A RIP-seq      | 45,975                    | 14,048 Donor ID 5 | Liu et al., Mol Cell 2019 |
| m6Am | Human cerebrum     | m6A RIP-seq      | 28,001                    | 11,770 Donor ID 5 | Liu et al., Mol Cell 2019 |
| m6Am | Human hypothalamus | m6A RIP-seq      | 26,993                    | 11,751 Donor ID 5 | Liu et al., Mol Cell 2019 |
| m1A  | HeLa cells         | m1A RIP-seq      | 4055                      | 2179 mRNAs | Yoon et al., Cell 2017 |
| m1A  | HEK293T            | m1A RIP-seq      | 18,756                    | 5788 | Meyer et al., Cell 2012 |
| m1A  | HEK293T            | m1A-ID-seq       | 901                       | 841 mRNAs | Liet al., Nature Chemical Biology 2016 |
| m1A  | HEK293T            | m1A-CLIP         | 740                       | 28 mitochondrial | Safra et al., Nature 2017 |
| m5C  | HeLa               | RNA-BisSeq       | 5349                      | 105139 | Yang et al., Cell Research 2017 |

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Table 1 (continued)

| Mod. | Tissue or cell type       | Detection method | # of modified sites (peaks) | # of modified transcripts | Comments | Reference                   |
|------|--------------------------|------------------|-----------------------------|----------------------------|----------|------------------------------|
| m⁵C  | Mouse small intestine    | RNA-BisSeq       | 12,201                      | 3722 annotated             | Peaks counted that are modified in at least 10% of m¹A sites in rep 1 | Yang et al., Cell Research 2017 |
| m⁵C  | Mouse heart              | RNA-BisSeq       | 12,997                      | 3915 annotated             | “         | Yang et al., Cell Research 2017 |
| m⁵C  | Mouse muscle             | RNA-BisSeq       | 11,721                      | 3624 annotated             | “         | Yang et al., Cell Research 2017 |
| m⁵C  | Mouse brain              | RNA-BisSeq       | 12,032                      | 3635 annotated             | “         | Yang et al., Cell Research 2017 |
| m⁵C  | Mouse kidney             | RNA-BisSeq       | 12,149                      | 3800 annotated             | “         | Yang et al., Cell Research 2017 |
| m⁵C  | Mouse liver              | RNA-BisSeq       | 11,414                      | 3751                       | “         | Yang et al., Cell Research 2017 |
| m⁵C  | Mouse testis 3 W         | RNA-BisSeq       | 11,853                      | 2727                       | “         | Yang et al., Cell Research 2017 |
| m⁵C  | HeLa                     | RBS-Seq          | 487                         | 340 annotated              | High-threshold cutoff                        | Khoddami et al., PNAS 2019 |
| 5hmC | Drosophila S2 cells      | hMeRIP-seq       | 3058                        | 1597 coding                | Confirmed via dTet KD                          | Delatte et al. Science 2016 |
| 5hmC | HeLa                     | RBS-Seq          | 754                         | 322 mRNA                   | HeLa cells collected with rRNA depleted total RNA, PolyA selected mRNAs & size selected small RNAs | Khoddami et al., PNAS 2019 |
| Ψ    | HeLa                     | Pseudo-seq       | 96 mRNA                     | 88 mRNAs                   |          | Carlile et al., Nature 2014  |
| Ψ    | S. cerevisiae (budding)  | Pseudo-seq       | 260 mRNA                    | 238 mRNA                   | Lower threshold filter shows 466 Ψ on mRNAs.   | Carlile et al., Nature 2014  |
| Ψ    | S. cerevisiae (midlog)   | Ψ-seq            | 74 ncRNA                    | 40 ncRNA                   | Change under heat shock                        | Schwartz et al., Cell 2014  |
| Ψ    | HEK293 and fibroblasts   | Ψ-seq            | 328                         | 168 mRNA                   |          | Schwartz et al., Cell 2014  |
| m⁷G  | mESCs                    | m⁷G MeRIP        | 184                         | 184 mRNA                   | All human sites were combined into one dataset  | Schwartz et al., Cell 2014  |
| m⁷G  | HeLa and HepG2           | m⁷G MeRIP, m⁷G-seq | 801                         | 681 mRNA                   | Functionally confirmed in MetII KO mESCs present in two replicates in both HeLa and HepG2 cells | Zhang et al., Mol Cell 2019 |

The number of modified sites (peaks) and total number of unique transcripts that contain at least one modification varies greatly by tissue or cell type, detection method, and lab.
translational efficiency, though this remains to be proven (Carlile et al. 2014).

While many modifications have been identified, accurately mapping modifications has been difficult. Not only are detection strategies largely limited to antibody-based methods, but different labs mapping modifications in the same cell or tissue type have obtained largely variable results (Table 1). Ongoing efforts to improve reproducibility will be crucial for understanding the biological function of each modification.

3. Epitranscriptomics in stem cell biology

Epirtranscriptomics appears to be especially important in stem cell biology, as it contributes to self-renewal and differentiation capacity. m6A is by far the most studied RNA modification in stem cells, particularly in ESCs and iPSCs.

3.1. m6A in ESCs

Early reports of m6A in ESCs were somewhat conflicting. One study reported that knockdown of Mettl3 and Mettl14 reduces m6A abundance and impairs stem cell self-renewal (Wang et al. 2014b), whereas another study reported that Mettl3 knockout in mESCs improves self-renewal but blocks differentiation (Batista et al. 2014). However, both of these studies examined mESCs in vitro, which muddies our understanding of the exact stage the ESCs are in and what m6A might do to drive embryonic development in vivo. This gap was addressed by Geula et al., who examined m6A in naïve pluripotent mouse ESCs. Naïve mESCs exist in a distinct molecular state compared to more advanced, “primed” epiblast stem cells (EpiSC). By knocking out Mettl3, they identified m6A as a key driver of termination of the naïve state and entry into the primed state, which is necessary for proper lineage differentiation at the post-implantation embryonic stage. The effects of impaired differentiation are so drastic that loss of m6A causes early embryonic lethality (Geula et al. 2015). Importantly, this study further clarified that m6A regulates the genes governing both naïve and primed states, and that loss of m6A causes upregulation of whichever genes are modified in that particular stem cell state. Naïve mESCs show enhanced pluripotency upon Mettl3 knockdown, whereas primed EpiSCs show increased stability of lineage-commitment genes upon loss of m6A (Geula et al. 2015; Zhao and He 2015). Mechanistically, this study and others determined that m6A primarily functions in development by reducing mRNA stability, which allows for the clearance of key naïve pluripotency-promoting transcripts or pro-differentiation transcripts, depending on the stem cell stage (Fig. 2) (Batista et al. 2014; Geula et al. 2015; Wang et al. 2014a; Wang et al. 2014b).

In addition to the traditional Mettl3/Mettl14-mediated addition of m6A to mRNA, there are several other m6A methyltransferases. In particular, Mettl16 was identified in human cells as an m6A methyltransferase that primarily targets small nuclear RNA (snRNA), specifically U6 snRNA, and other non-coding RNAs (Warda et al. 2017). Additionally, Mettl16 regulates expression of the SAM synthetase MAT2A (Pendleton et al. 2017), which is highly consequential for all modifications that use SAM as a methyl donor (Fig. 2). Accordingly, Mendel et al., 2018 found that METTL16-mediated modification of Mat2a mRNA is necessary for proper embryonic development of mouse blastocysts, and homozygous knockout of Mettl16 is embryonic lethal. Analysis of E2.5 Mettl16 KO mouse blastocysts showed that only 20 genes are differentially expressed relative to the wildtype, with Mat2a showing the most significant downregulation. However, by E3.5 the global transcriptome was massively dysregulated (Mendel et al. 2018). While the more common METTL3/METTL14-mediated pathway has garnered the most attention, understanding the complexities of the epitranscriptome and the consequences of mediating highly consequential genes like Mat2a will be necessary to accurately characterize the many roles of m6A.

3.2. m5C in mESCs

While the overwhelming focus of research in mESCs has been centered around m6A, m5C has also been examined. In one study, 12,492 m5C sites were identified in nuclear mESC mRNA. Modified mRNAs were enriched for gene ontologies corresponding to cell cycle, RNA processing, chromatin modification, and developmental processes. Though the functionality of m5C in mESCs was not shown, a correlation between m5C sites and RBP sites was identified (Fig. 2). Approximately 29% of m5C sites in mESCs overlapped with known RBP sites. More specifically, the largest overlaps correspond to UPF1 binding, which regulates nonsense-mediated RNA decay. Additionally, SR5F3 and SR5F3 splicing factors and the PRC2 subunit EZH2 have binding sites that significantly overlap with m5C sites. This led to the hypothesis that m5C may contribute to RBP binding and functionality, though this requires further validation (Amort et al. 2017). Finally, a recent study showed that during the maternal-to-zygotic transition in zebrafish development, the m5C reader, YBX1, binds to m5C-modified mRNA to promote stabilization of maternal mRNAs during the transition (Yang et al. 2019).

As detection of diverse mRNA modifications continues to improve and orphan methyltransferase targets are identified, we expect our understanding of epitranscriptomic regulation of stem cells to grow rapidly. Notably, the low stoichiometry of some modifications relative to m6A should not decrease their perceived importance, as the power of the modification is derived from the strength of its downstream effects, which vary widely among reader proteins.

3.3. Induced pluripotent stem cells (iPSCs)

The understanding that m6A contributes to pluripotency and differentiation drove studies of its regulatory capacity in iPSCs. In 2015, Chen et al. showed that high abundance of m6A increases the reprogramming efficiency of mouse embryonic fibroblasts (MEFs) to pluripotent stem cells, in part by altering expression of key pluripotency factors like Oct4, Sox2, and Nanog. This study further found an interplay between microRNA (miRNA) binding to mRNA and enhanced Mettl3 binding to mRNA to promote de novo addition of m6A (Chen et al. 2015). This concept of m6A interplay with noncoding RNAs has been explored with contrasting conclusions, and has been reviewed in-depth elsewhere (Fazi and Fatica 2019). Furthermore, Chen et al. found that Mettl3 knockdown reduces iPSC colony formation (Chen et al. 2015). However, Geula et al. found that Mettl3 knockdown does not impair reprogramming efficiency, but rather slows proliferation of iPSCs in early reprogramming (Geula et al. 2015). Finally, Wu et al. found that Mettl3 knockdown decreases the proliferation rate of porcine iPSCs (piPSCs) and impairs expression of key pluripotency genes, though they did not test for reprogramming efficiency. This study further identified that m6A promotes YTHDF1-mediated translation of JAK2 in piPSCs, while promoting degradation of SOCS3 via YTHDF2 (Wu et al. 2019). Both of these mechanisms lead to upregulation of the JAK2-STAT3 signaling pathway, which is known to promote stem cell self-renewal by increasing expression of the core pluripotency genes Oct4 and Sox2 (Niwa et al. 1998; Wu et al. 2019). In parallel, YTHDF2 has been shown to be upregulated in iPSCs to destabilize m6A-modified mRNAs related to neural development and thereby promote pluripotency (Heck et al. 2020).

Overall, m6A clearly regulates the pluripotency of iPSCs, but its role in reprogramming likely depends on the cellular context of the starting material or the stage of reprogramming. As was the case in ESCs, m6A may alter expression of the gene transcripts already present. Still, further investigation is needed to identify the fate of m6A-modified transcripts. While m6A-mediated mRNA degradation appears to be a major mechanism, expression of other reader proteins suggests a more complex system. Understanding how m6A reader proteins selectively bind particular mRNA targets will be a major step forward in further
elucidating the mechanisms of $m^6$A action in iPSCs.

3.4. Regulation of stem cell epitranscriptomes

Upstream regulation of $m^6$A deposition or differential expression of the writers, readers, and erasers contributes to the function of $m^6$A in stem cells. For example, Aguilo et al. showed that zinc finger protein 217 (ZFP217) coordinates epigenetic regulation with $m^6$A deposition. More specifically, ZFP217 is a transcription factor that directly activates transcription of several key pluripotency genes, then blocks $m^6$A modification of these genes by sequestering METTL3 in mESCs and iPSCs. ZFP217 knockdown causes global increases in $m^6$A levels, which correlates with a decreased half-life of Nanog, Sox2, c-Myc, and Klf4 mRNA transcripts. This in turn impairs pluripotency and reprogramming (Aguilo et al. 2015).

Wen et al. found that another zinc-finger protein, Zc3h13, is critical for $m^6$A deposition, and Zc3h13 knockdown significantly impairs self-renewal and maintenance of pluripotency in mESCs (Knuckles et al. 2018; Wen et al. 2019). Zc3h13 can form a complex with WTAP, Virilizer (Kiaa1429), and Hakai, which also contribute to the METTL3-METTL14 $m^6$A methylation complex (Horiuchi et al. 2013; Wan et al. 2015). Wen et al. then showed that Zc3h13 knockdown in mESCs decreases global $m^6$A levels to about 30–40% as in the control, and confirmed $m^6$A dependency on Zc3h13 through MeRIP-seq. More specifically, Zc3h13 promotes $m^6$A deposition by localizing the Zc3h13-WTAP-Virilizer-Hakai complex to nuclear speckles; loss of Zc3h13 causes these complex components, as well as METTL3/METTL14, to significantly shift to localization in the cytoplasm. Functionally, Zc3h13 knockdown impairs mESC self-renewal, decreases expression of pluripotency genes, and increases expression of differentiation markers in correlation with differential $m^6$A modifications of these gene transcripts (Wen et al. 2018). The conclusion that $m^6$A promotes self-renewal is consistent with previous studies (Wang et al. 2014b), and the consequences on pluripotency correspond to studies performed under similar conditions in mESCs (Geula et al. 2015). These two studies on zinc finger proteins are important examples of how $m^6$A may be
regulated or targeted to individual transcripts in stem cells. This connection between transcription factors and epitranscriptomic regulation remains an interesting avenue for further research.

Finally, one study has found a direct connection between histone methylation and sites of m6A deposition. Huang et al. showed that histone H3 trimethylation at lysine-36 (H3K36me3) drives m6A methylation by recruiting and promoting interactions between the m6A methyltransferase complex and its target mRNA. More specifically, METTL14 binds to H3K36Me3, chromatin, and RNA, thereby promoting the co-transcriptional addition of m6A to genes with H3K36Me3 epigenetic marks. Knockdown of the H3K36Me3 methyltransferase, SETD2, impairs binding of the m6A methyltransferase complex to sites that lose H3K36Me3, and globally reduces m6A levels. In mESCs, SETD2 knockdown induces higher expression of pluripotency factors (OCT4, SOX2, NANOG) and prevents increased m6A methylation during differentiation. This suggests that H3K36Me3 drives m6A modifications to destabilize pluripotency genes and promote differentiation, and loss of either H3K36Me3 or METTL14 promotes pluripotency over differentiation (Huang et al. 2019a). This corresponds with previous reports that m6A is necessary for proper differentiation of mESCs (Batista et al. 2014; Geula et al. 2015), and provides the first evidence that m6A addition may be directed by epigenetic marks.

While a few studies have identified how the epitranscriptome may be regulated, over 100 putative METTL3 or METTL14 binding proteins have been identified, suggesting that there is much left to be learned about upstream regulation of m6A (Malovannaya et al. 2011). A better understanding of how the methyltransferase complex and demethylases target specific gene transcripts, as well as how writer, reader, and eraser expression is regulated, will drive the field forward.

4. Epitranscriptomics in neural development

Recent work has shown that the epitranscriptome, in particular m6A, is especially important for neural development and brain function (Livneh et al. 2020; Yoon et al. 2018). (Lence et al., 2016) performed one of the first studies of m6A in the brain, using Drosophila melanogaster as a model organism. This study showed that m6A is enriched in the nervous system and that knockout of the methyltransferase components causes reduced lifespan, severe behavioral defects, and global changes in neural gene expression (Lence et al. 2016). While this work was important for understanding m6A in vivo, it contrasted with mammalian studies in that loss of m6A methyltransferases is not lethal in flies. The next major advances came from studies of conditional knockout of the m6A methyltransferase complex in mice to examine epitranscriptomic regulation during mammalian brain development. Below we provide an in-depth overview of the epitranscriptome in mammalian neural development (Fig. 3).

4.1. Cortical development

In 2017, our laboratory showed that conditional knockdown (cKO) of Mettl14 in mice and subsequent loss of m6A in neural progenitor cells (NPCs) drastically impairs brain development in vivo (Yoon et al. 2017). Loss of m6A impairs NPC differentiation, slows cell cycle progression, and elongates the timing of cortical neurogenesis into postnatal stages. Mechanistically, m6A-modified genes are significantly enriched for gene ontologies that correlate with regulation of transcription, neuron differentiation, cell cycle, and stem cell differentiation. These modified transcripts have a shorter half-life than their corresponding unmodified transcripts in Mettl14 cKO mouse NPCs, suggesting that m6A normally destabilizes mRNAs in the developing brain. By modifying both multipotency and differentiation-promoting transcripts, the m6A system may contribute to harmonious changes in gene expression that are necessary for the progression of NPCs through the distinct phases of embryonic cortical neurogenesis. To this end, we found that Mettl14 cKO NPCs co-express stem cell and neuronal markers, and that rapid degradation of neuronal markers in wildtype NPCs allow for pre-patterning of differentiation by allowing transcription of pro-neuronal genes but preventing significant protein production (Yoon et al. 2018). Finally, we used iPSC-derived human brain organoids to confirm that m6A also regulates NPC cell cycle progression in humans (Yoon et al. 2017). We then compared m6A-seq analysis among human brain organoids, human post-conception week 11 embryonic brain tissue and E13.5 mouse brains. While many gene transcripts are m6A-modified in both species, the human-specific modifications correlated strongly with disease ontologies for human-specific mental disorders like autism and schizophrenia. This work provided the first in vivo analysis of m6A in mammalian brain development and highlighted the possibility that m6A may contribute to psychiatric or neurodevelopmental disorders in humans.

Shortly thereafter, an independent study by Wang et al. knocked out Mettl14 in the developing forebrain, and also found that loss of m6A slows NPC cell cycle progression. In vitro analysis of Mettl14 cKO NPCs showed that loss of m6A can cause premature differentiation, and in vivo analysis showed that Mettl14 cKO mice had reduced numbers of Pax6 + NPCs and reduced numbers of Satb2 + late-born neurons. This led the authors to suggest that depletion of the NPC pool causes a reduction in neurogenesis (Wang et al. 2018b). This contrasted with our study, which showed an increase in Pax6 + cells in Mettl14 cKO forebrains, but a similar decrease in late-born neurons; we therefore proposed that m6A is necessary for the timely differentiation of NPCs, and loss of m6A causes a build-up of Pax6 + NPCs (Yoon et al. 2017). These differences may stem from different methodologies or antibodies. Nonetheless, the studies agree that m6A regulates mRNA stability to alter gene expression and NPC fate.

Next, Wang et al. identified genome-wide changes in histone modifications upon Mettl14 knockout. Specifically, cKO NPCs show increases in histone H3 acetylation at lysine 27 (H3K27ac), histone H3 trimethylation at lysine 4 (H3K4me3), and histone H3 trimethylation at lysine 27 (H3K27me3). Chemically blocking these epigenetic marks partially rescues cKO NPC proliferation defects. The changes in histone modification were partially attributed to m6A-mediated destabilization of CBP and p300 transcripts, which are stabilized upon loss of m6A. However, this did not apply to transcripts in the PRC2 complex, suggesting there are other mechanisms at play (Wang et al. 2018b). Overall, the connection between the epitranscriptome and epigenetics in the developing brain is highly intriguing. As single-transcript m6A editing techniques are developed (Wilson et al. 2020), it would be pertinent to edit only CBP and p300 mRNA to quantify the degree to which their methylation contributes to the Mettl14 cKO phenotype, as opposed to the sum of many modified transcripts.

Finally, a third study conditionally knocked out Ythdf2 in the developing forebrain to show that m6A largely functions through YTHDF2-mediated mRNA degradation during cortical development. In this study, Li et al. showed that Ythdf2 KO mice have a very similar phenotype to Mettl14 cKO mice. In particular, loss of Ythdf2 impairs NPC proliferation and differentiation, and causes delays in cortical neurogenesis. They also found that Ythdf2 -/- NPCs create fewer primary neurites per neuron and shorter neurites overall when differentiated in vitro, suggesting that m6A also regulates neuron maturation during the differentiation process (Li et al. 2018b). This study was necessary to confirm that m6A regulation of cortical development functions primarily through YTHDF2-mediated mRNA degradation and that m6A promotes NPC proliferation and differentiation.

Beyond m6A, m5C is also crucial for proper neurodevelopment. Flores et al. showed that the m5C methyltransferase NSUN2 is essential for neural stem cell differentiation (Flores et al. 2017). Loss-of-function mutations in Nsun2 caused neurodevelopmental defects such as microcephaly in mouse and human models. In a conditional Nsun2 knockout in the developing mouse brain, there were signs of decreased sizes of the cerebral cortex, hippocampus, and striatum compared to wildtype mouse brains. In addition, Nsun2 cKO mice had lower levels of
global protein production and increased cellular stress compared to wildtype brains. These phenotypes are thought to arise due to differentiation delays, lack of neural lineage commitment, and a reduction in upper-layer neurons (Flores et al. 2017). This interesting study broadens the importance of the epitranscriptome in neural development beyond m6A. Indeed, as detection methods for other modifications become more reliable, it will be worthwhile to explore a greater diversity of epitranscriptomic marks in neural development.

4.2. Cerebellar development

The complexity of the brain suggests that epitranscriptomic regulatory systems may have distinct functions in different parts of the brain. Indeed, Chang et al. showed that m6A levels are increased in the adult mouse cerebellum compared to the cerebral cortex, and that there are region-specific methylation patterns (Chang et al. 2017). Even within the cerebellum, methylation patterns change over development. Ma et al. showed that methylation targets change across postnatal day 7 (P7), P14, P21, and P60 mouse cerebella. There are 12,452 m6A peaks that are turned “ON” (emerge at a later stage) over time, and 11,192 that are turned “OFF” (disappear in later stages). The groups of transcripts methylated at each time point correspond with the developmental processes happening at that time. For example, gene transcripts in which m6A is turned OFF from P7 to P14 have gene ontologies enriched for cell cycle. On the other hand, gene transcripts in which m6A is turned ON at P14, P21, or P60 have gene ontologies enriched for signal transduction, cell adhesion, learning, and synaptic plasticity. Overall, m6A modification patterns strongly correlate with the progression from proliferating cells at P7 to mature neuronal activities at P60. This study also examined changes in expression of METTL3, METTL14, WTAP, FTO, and ALKBH5. Though cerebellar expression of all of these genes decreased on average over time, there was a specific reduction in internal granular layers but elevated expression in Purkinje cells. Lentiviral Metl3 knockdown at P7 lowers the number of Purkinje cells and impairs their organization along the outer surface of the inner granule cell layer. On the other hand, Alkbh5-KO mice had no observable phenotype in the cerebellum under normal conditions, which may be due to redundant actions by FTO. After stressing the developing brain with hypobaric hypoxia, Alkbh5-KO mice had significantly smaller cerebella and significantly more proliferating cells. This suggests that ALKBH5 is critical for promoting cerebellar neurogenesis under stress. Finally, this study showed that several important gene transcripts are differentially localized in the cytoplasm over nucleus in Alkbh5-KO cerebella, indicating that m6A promotes nuclear export in this tissue (Ma et al. 2018).

In contrast, Wang et al. used a Metl3 cKO mouse model to show that m6A promotes mRNA degradation and alternative splicing in the cerebellum. Metl3 cKO mice have drastically smaller cerebella, significantly fewer cerebellar granule cells (CGCs) in the internal granular layer (IGL), and disordered Purkinje cell organization relative to

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**Fig. 3. m6A in neural development.**

Top: In the cortex, loss of m6A elongates the timeframe of cortical neurogenesis such that the postnatal brain is still generating upper-layer neurons. This is accomplished through altered mRNA degradation rates of key pluripotency and fate-determining gene transcripts. Middle: In the cerebellum, loss of m6A causes disorganization of the Purkinje cell layer (PCL) into the inner granule cell layer (IGL). IGL cells also exhibit a higher rate of apoptosis, resulting in fewer total IGL cells. Mechanistically, m6A has been shown to promote alternative splicing and mRNA degradation in the cerebellum. Bottom: In hippocampal adult neurogenesis, *in vitro* studies found that loss of m6A impairs self-renewal and neurogenesis, but the mechanism of action remains unclear.
wildtype controls. Furthermore, loss of m^6^A causes significantly increased levels of apoptosis of newborn granule cells, which explains the depletion of GGCs. Again, loss of m^6^A increases mRNA stability; m^6^A modifications on apoptosis-associated transcripts normally restrict their expression. Notably, m^6^A-mediated regulation of apoptosis appears to be specific to the cerebellum, as these transcripts are not stabilized in the cortex of Mettl3 cKO mice. Finally, Wang et al. identified an additional mechanism of m^6^A-mediated alternative splicing in the cerebellum. Exon exclusion occurs more frequently upon m^6^A depletion, especially in transcripts that are normally methylated in the wildtype. These alternatively spliced transcripts are enriched for gene ontologies in synapase-associated pathways and neurotransmitter receptors. Further analysis showed that increases in intracellular calcium concentration in Mettl3 cKO GGCs contributes to their increased apoptosis (Wang et al. 2018a). This work highlights the fact that epitranscriptomic regulation is highly cell-type specific with unique roles in different parts of the brain. How this specificity is regulated will be an interesting avenue of future research.

4.3. Adult neurogenesis

The m^6^A demethylase FTO has been implicated in numerous pathways in the mature brain, from cancer (Cui et al. 2017), to psychiatric and neurodegenerative diseases (Choudhry et al. 2013; Hess et al. 2013; Keller et al. 2011; Li et al. 2018a; Widagdo et al. 2016), to regulation of adult neural stem cells (Gao et al. 2010; Li et al. 2017a). However, understanding the role of FTO remains difficult due to its multiple functions in DNA and RNA demethylation. In fact, the first study on FTO in neurogenesis was published in 2010, before FTO was even identified as an m^6^A demethylase (Gao et al. 2010; Jia et al. 2011). Gao et al. generated whole-body and neural-specific Fto KO mice and found that the two have very similar phenotypes, indicating that the majority of FTO functions occur in the nervous system (Gao et al. 2010). In 2017, it was shown that FTO is expressed in adult NSCs (aNSCs) and in mature neurons and its expression increases over postnatal time. Fto KO mice show reduced proliferation and differentiation of aNSCs, which functionally impairs learning and memory. Furthermore, loss of FTO results...
in slightly higher (~15%) levels of m^6^A, though only 363 genes are both m^6^A modified and differentially expressed upon loss of FTO (out of 5635 m^6^A-modified genes and 1862 FTO-dependent genes) (Li et al. 2017a). While FTO does seem to regulate adult neurogenesis, the degree to which this is enacted through m^6^A remains in question, especially considering that FTO can act on multiple targets in vivo.

Next, Chen et al. found that Mettl3 knockdown impairs both proliferation and differentiation of aNSCs cultured in vitro. m^6^A sequencing showed that the m^6^A landscape is dynamic between proliferating and differentiating cultured aNSCs; transcripts modified only in proliferating aNSCs correlate with cell cycle, while transcripts modified only in differentiating aNSCs are enriched for protein localization, signaling, and synapse organization (Chen et al. 2019). This study is slightly more direct in studying m^6^A in adult neurogenesis by knocking down Mettl3, but the use of cultured aNSCs limits the conclusions that can be drawn; aNSCs exist in highly specialized niches in vivo that are difficult to recapitulate in vitro (Ming and Song 2011; Song et al. 2012).

Finally, a 2019 study found that Fto cKO in aNSCs decreases aNSC proliferation and differentiation into NeuN^+^ neurons at 4 weeks after FTO knockout. While the fate of m^6^A-modified transcripts was not tested, individual mRNA transcripts in the Stat3 signaling pathway, Socs5 and Pdgfra, were shown to play important roles in FTO-mediated regulation of aNSCs. However, Socs5 mRNA and protein decrease in Fto cKO aNSCs, while Pdgfra mRNA and protein increase (Cao, 2019). Therefore, the involvement of m^6^A and mechanisms of m^6^A-mediated regulation in aNSC remain unclear. In multiple studies, effects of Fto or Mettl3 KD appear stronger in in vitro cultured cells than in vivo aNSCs. The highly dynamic nature of m^6^A in response to signaling and stress stimuli suggest that culturing systems need to be incredibly carefully controlled to maintain an accurate representation of the epitranscriptome in in vivo aNSCs.

5. Epitranscriptomics in neurodevelopmental diseases

In accordance with its powerful role in neural development, m^6^A has been linked to neurodevelopmental defects as well. To date, m^6^A in Fragile X Syndrome is the best-characterized interaction. Additionally, emerging genome-wide association studies and human genetics studies have linked mutations in epitranscriptomic enzymes with intellectual disability (Fig. 4).

5.1. Fragile X syndrome

Fragile X mental retardation protein (FMRP), encoded by FMR1, is an RNA-binding protein that is best known for negatively regulating the translation of its target mRNAs (Darnell et al. 2011; Richter et al. 2015) and trafficking mRNA granules (De Diego Otero et al. 2002). Loss-of-function mutations in FMR1 cause Fragile X Syndrome, which is marked by intellectual disability and delayed development. In 2017, Arguello et al. identified FMRP as an mA binding protein in vitro (Arguello et al. 2017). Zhang et al. then showed that FMRP binds to YTHDF2 and that FRMP target genes are enriched for mA marks in the mouse cerebral cortex (Zhang et al. 2018). A knockout of Fmr1 resulted in the down-regulation of some mA mRNA FMRP target transcripts, suggesting that FMRP stabilizes these mA-modified mRNAs (Zhang et al. 2018). Next, Edens et al. showed that FMRP promotes nuclear export of mA-modified mRNA by interacting with CRM1, a nuclear export protein. Additionally, Fmr1 KO mice phenocopy Mettl14 cKO mice in terms of delayed embryonic cortical neurogenesis and prolonged NPC cell cycle progression. In both of these mouse knockout models, FMRP target mRNAs are retained in the nucleus (Edens et al. 2019). The binding affinity of FRMP for mA-modified mRNA and its role in nuclear export was recently confirmed by another study (Hsu, 2019).

5.2. Intellectual disability

Recent studies identified correlations between epitranscriptomic modifications and intellectual disability. First, Shaheen et al. found that mutations in human PUS3, a pseudouridylation enzyme, correlates with intellectual disability and microcephaly in three affected siblings (Shaheen et al. 2016). The affected individuals also have a significant reduction in Ψ-modified tRNA relative to healthy controls in purified lymphoblastoid cells. The PUS3 deficiency phenotype in humans is largely brain-specific, suggesting that PUS3-mediated tRNA Ψ modification is especially important for cognitive function.

Next, both de Brouwer et al., 2018 and Shaheen et al., 2019 identified mutations in PUS7, a tRNA and mRNA pseudouridylation enzyme, that cause intellectual disability, microcephaly, speech delay, and aggressive behavior (de Brouwer et al. 2018; Shaheen et al. 2019). Ψ at position 13 in tRNA and PUS7 target mRNAs were significantly reduced in affected individuals compared to healthy controls. Additionally, Pus7 knockout in Drosophila recapitulates the cognitive impairment phenotype and the molecular loss of Ψ at particular target sites (de Brouwer et al. 2018). This provides exciting evidence that Ψ modifications of mRNA and tRNA are not only highly conserved across species, but are critical in neural development. Additional studies using mouse models to investigate the exact mechanism of Ψ in neural development will be an exciting next step.

A 2012 study by Abbasi-Moheb et al. showed that homozygous loss of the m^6^C methyltransferase, NSUN2, due to nonsense or splicing mutations in the transcript causes memory and learning deficits in humans (Abbasi-Moheb et al. 2012). Further experiments with a knockout of the NSUN2 ortholog, CG6133, in Drosophila showed short-term-memory deficits that could be rescued when the wild type protein was expressed. The fact that humans with NSUN2 mutations show intellectual disabilities and facial dysmorphism, combined with the finding that similar phenotypes are found in Drosophila suggests that m^6^C may be a fundamental regulator of neural function across species (Abbasi-Moheb et al. 2012). These data strongly suggest that m^6^C writers are important in neural development and function, but further studies are necessary to understand the mechanistic role of m^6^C in the brain.

Finally, Richard et al. identified frameshift mutations in METTL5, which adds mA to 18S rRNA (van Tran et al. 2019), that cause autosomal-recessive intellectual disability and microcephaly. METTL5 is expressed in the human brain from early development and into adulthood, particularly in the cerebellar cortex, hippocampus, and striatum. Analysis in rodents confirmed ubiquitous METTL5 expression in the brain, with increased staining in neural soma and nuclei, as well as in pre- and post-synaptic regions. Finally, Mettl5 knockout in zebrafish recapitulates the microcephaly phenotype and specifically causes a decrease in forebrain and midbrain size (Richard et al. 2019). While mechanistic studies of METTL5 action have only recently begun, this genetic evidence suggests that it is yet another epitranscriptomic modifier that is crucial for proper brain development.

6. Concluding remarks and future outlook

The field of epitranscriptomics has reached a point where the power of various mRNA modifications has become widely accepted, but the specific mechanisms of their action remain under debate. It is becoming increasingly important to perform extremely careful experiments to detect and validate epitranscriptomic marks to prevent further confusion regarding their downstream functions. Furthermore, expression of multiple reader proteins and multiple published functions of mA in a single cell type suggest that mA may differentially regulate various gene transcripts within a single cell. Several important strategies to further elucidate the regulatory capacities of mA in stem cells and neural development include (1) improved detection techniques for higher sensitivity and accuracy, (2) studies on how reader proteins
selectively bind a subset of m\(\text{6}A\)-modified mRNAs, and (3) careful analyses and conservative interpretations of data to prevent over-confident conclusions that will hinder future studies.

In addition to clarifying studies on m\(\text{6}A\), we are particularly excited by the prospects of other epitranscriptomics marks in neural development and disease. Careful mapping of m\(\text{6}A\), m\(\text{5}C\), m\(\text{5}G\), m\(\text{6}Am\) and \(\text{W}\) in the brain alongside generating animal knockouts of their respective modifying enzymes will greatly expand the breadth of knowledge in the field of epitranscriptomics. With an increasing number of scientists working in this field, we expect the next five years to be full of new discoveries with profound implications for basic and translational science.

Declaration of Competing Interest
None.

Acknowledgement
We thank K. M. Christian for comments. The work in the authors’ laboratories was supported by the National Institutes of Health (R35NS116843 to H.S., and R35NS097370 to G.L.M.), the SAFRI (#575050 to H.S.), and the Sheldon G. Adelson Medical Research Foundation (to G.L.M.).

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