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Review

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 (m^{6}A), 2-*O*-dimethyladenosine (m^{6}A$_m$), N1-methyladenosine (m^{1}A), 5-methylcytosine (m^{5}C), and isomerization of uracil to pseudouridine (Ψ), have the potential to alter RNA processing events and contribute to developmental processes and different diseases. Though the abundance and roles of some RNA modifications remain contentious, the epitranscriptome is thought to be especially relevant in stem cell biology and neurobiology. In particular, m^{6}A occurs at the highest levels in the brain and plays major roles in embryonic stem cell differentiation, brain development, and neurodevelopmental disorders. However, studies in these areas have reported conflicting results on epitranscriptomic regulation of stem cell pluripotency and mechanisms in neural development. In this review we provide an overview of the current understanding of several RNA modifications and disentangle the various findings on epitranscriptomic regulation of stem cell biology and neural development.

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* to make the correct quantities of particular proteins or isomers *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 m^{6}A, has garnered the most attention for its powerful role in regulating mRNA processing (Roundtree et al. 2017).

While the existence of m^{6}A has been known for almost 50 years (Desrosiers et al. 1974; Lavi and Shatkin 1975; Rottman et al. 1974; Wei et al. 1975; Wei and Moss 1977), a combination of events reinvigorated a recent interest in the field. First, Zhong et al. showed in 2008 that m^{6}A is critical for developmental processes in *Arabidopsis thaliana,* which suggested its importance for multicellular eukaryotes, and pushed for the development of m^{6}A mapping methods (Zhong et al. 2008). Next, the discovery of the m^{6}A demethylases, FTO and ALKBH5, in 2011 and 2013, respectively (Jia et al. 2011; Zheng et al. 2013), suggested that the m^{6}A 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 m^{6}A mapping techniques

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were developed and showed that m^6A additions to mRNA are non-random, thus pushing biological interest forward and providing techniques to understand the molecular mechanisms of m^6A 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^6A. 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 epitranscriptome itself is regulated in embryonic stem cells. In addition, we review the role of the epitranscriptome in cortical and cerebellar development, 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^6A

On a global level, 0.2 to 0.5% of all adenines are m^6A 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). Epitranscriptomic detection technologies have been focused on m^6A, making it one of the best-studied modifications to date. m^6A occurs in various types of RNA, including tRNA, rRNA, non-coding RNA (ncRNA), and mRNA. In 2012, two groups independently reported anti-m^6A antibody-based m^6A RIP-Seq (MeRIP-Seq) techniques (Dominissini et al. 2012; Meyer et al. 2012). Subsequent mapping of m^6A 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^6A is especially enriched in the 3'UTR and around the STOP codon (Dominissini et al. 2012; Meyer et al. 2012). While m^6A 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^6A-binding proteins, or readers, have been identified. Individual readers confer unique downstream fates on m^6A-modified mRNA, including altered mRNA stability, translation, localization, and splicing. m^6A methylation patterns in the transcriptome appear to be cell/tissue-specific and species-specific (Liu, 2019). Current m^6A sequencing technologies are not sufficiently sensitive to profile m^6A at the the single-cell level, which would help quell disagreements in the field on whether m^6A is truly different across cell types or dynamic in response to stimuli. For example, Garcia-Campos et al. claimed that m^6A is "hard coded" and largely predictable in the yeast genome based on the extended 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^6A demethylases FTO and ALKBH5 in response to heat stress and hypoxic stress, respectively, suggests that dynamic changes to the m^6A methylome 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^6A sequencing, especially using antibody-based methods, may lead to false conclusions on the variability and dynamic nature of m^6A (McIntyre et al. 2020).

A growing list of proteins has been found to form the methyltransferase complex that adds m^6A onto mRNA (Fig. 1). This complex includes a core heterodimer unit of METTL3 and METTL14 (Liu et al. 2014), with accessory proteins including WTAP (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; Garcias Morales and Reyes 2020).

There are two known m^6A 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^6A demethylase has recently been questioned, with the suggestion that it may instead act on m^6A_m (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 recent study reported that FTO can demethylate m^6A, m^6A_m, and m^1A depending on the nuclear or cytoplasmic localization (Wei et al. 2018). Yet another study argued that since loss of FTO has little effect on cytoplasmic mRNA m^6A or m^6A_m 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^6A; confirming its target is critically important so as not to misattribute a phenotype or biological function to the wrong epitranscriptomic 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^6A can be attributed to its many distinct reader proteins. The central group of readers is the YTH-domain-containing family of proteins, which bind directly to m^6A. 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 promotes 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 remain unclear and may only be functional under special cellular conditions (Patil et al. 2018). In contrast to the notion that each YTHDF reader promotes a unique fate of m^6A-modified mRNA, two recent studies found that YTHDF-1, 2, and 3 are jointly promote mRNA degradation 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^6A reader proteins underscore the vital need for additional research into reader protein function and regulation.

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

Unlike the internal m^6A modification, N^6,2'-*O*-dimethyladenosine (m^6A_m) occurs in the mRNA terminus at the first nucleotide following the N^7-methylguanosine (m^7G) cap. Approximately 0.0036% to 0.0169% of all adenines are m^6A_m 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^6A_m-modified transcripts was previously thought to be much higher, but improved detection sensitivity has led to the view that m^6A_m 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^6A_m 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^6A_m most strongly correlates with high expression and increased transcript stability (Boulias et al. 2019). However, this was not universally true for all m^6A_m-modified transcripts, leaving the regulatory capacity of m^6A_m up for debate. The downstream functions of m^6A_m are still unclear, with multiple conflicting studies reporting opposite effects on mRNA stability and translation (Akichika et al. 2019; Boulias et al. 2019; Liu, 2019; Sendinc et al. 2019). The field would greatly benefit from identification of m^6A_m reader proteins that could help disentangle its potential downstream functions.

2.3. N1-methyladenosine: m^1A

The abundance of m^1A in cells remains under debate. Some studies found that 0.015% to 0.16% of adenines are m^1A modified,

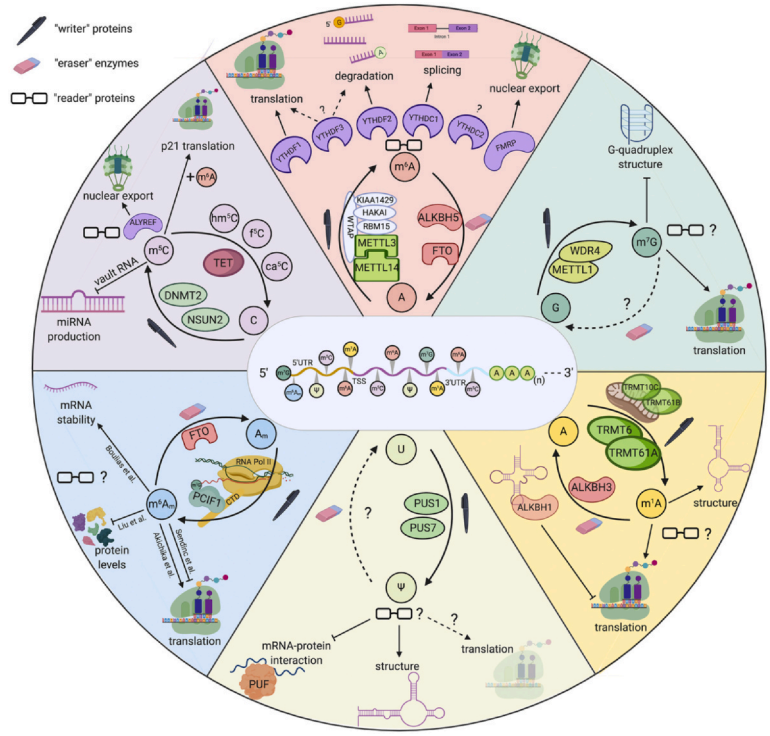

Fig. 1. Overview of common epitranscriptomic marks.
This pinwheel shows the current knowledge for m^6A, m^7G, m^1A, Ψ, m^6A_m, and m^5C (from top, clockwise). Each slice shows the known writer proteins, known eraser enzymes, known reader proteins and downstream functions of the epitranscriptomic mark.

corresponding with over 4000 mRNA transcripts (about 20% of the transcriptome) with high stoichiometries of m^1A (Dominissini et al. 2016; Li et al. 2016). Two independent studies claimed that as few as 15 or 53 total m^1A sites exist in mRNA and lnRNA, 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 m^7G has been shown to produce false positives in m^1A identification, and a more recent study found only one high-confidence, high stoichiometry m^1A site using a bioinformatic approach, which was validated with an improved m^1A antibody (Grozhik et al. 2019). Though our understanding of the modification is limited, progress was made through identification of putative m^1A 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 m^1A mRNA demethylase, though it also acts on DNA and m^3C in RNA (Aas et al. 2003; E et al., 2016; Ougland et al. 2004). m^1A 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 m^1A 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: m^5C

m^5C is added to tRNA, rRNA, and mRNA by a variety of methyltransferases with specific RNA targets (Motorin et al. 2010). The reported abundance of m^5C on mRNA is extremely variable across different studies, with some reporting up to 10,000 m^5C 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 m^5C methyltransferases that act on both tRNA and mRNA (Khoddami et al. 2019; Li et al. 2017b; Yang et al. 2017) (Fig. 1). NSUN2-mediated m^5C mRNA methylation promotes mRNA nuclear export through ALYREF, a nuclear m^5C reader protein (Yang et al. 2017). Additionally, m^5C may cooperate with m^6A to enhance translation of particular transcripts like p21 (Li et al. 2017b). Finally, m^5C 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 m^5C direct demethylases have been identified, ten-eleven translocation (Tet) enzymes can oxidize m^5C to 5-hydroxymethylcytosine (hm^5C) and then unmodified cytosine (Ito et al. 2011). The frequency of hm^5C is about one hm^5C per 5000 m^5C (Fu et al. 2014). This is slightly enriched in mRNA, with hm^5C occurring on $\sim 7 \times 10^{-6}$ of the total cytosines (Xu et al. 2016). In *Drosophila*, hm^5C 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 m^5C reader protein that promotes stabilization of modified mRNAs in early zebrafish embryos (Yang et al. 2019). One study showed that m^5C 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

Table 1
Compilation of RNA modifications mapped in various cell types.

Mod.	Tissue or cell type	Detection method	# of modified sites (peaks)	# of modified transcripts	Comments	Reference
m^6A	Mouse ESCs	m^6A RIP-seq	8645	3942 annotated	6667 and 6159 peaks in Mettl3 KD and Mettl14 KD cells.	Wang et al., *Nat Cell Biology* 2015
m^6A	Mouse ESCs	m^6A RIP-seq	9754	5461 mRNAs, 117 ncRNAs	Average 2 peaks per transcript, including on core pluripotency factors	Batista et al., *Cell Stem Cell* 2015
m^6A	Mouse naïve ESCs	m^6A RIP-seq	10,431	6412		Geula et al., *Science* 2015
m^6A	Mouse embryonic fibroblasts	m^6A RIP-seq	16,487	–	10,609 peaks are WTAP-dependent (64.3%)	Schwartz et al., *Cell Reports* 2017
m^6A	Total mouse brain	m^6A RIP-seq	13,471	4654 coding 236 non-coding		Meyer et al., *Cell* 2012
m^6A	Mouse cerebellum	m^6A RIP-seq with bioinformatic correction for m^6A_m	18,594	10,483 mRNAs	16,576 peaks shared with cortex, and 8924 mRNAs shared with cortex	Chang et al., *Open Biology* 2017
m^6A	Mouse cortex	m^6A RIP-seq with bioinformatic correction for m^6A_m	18,032	9636 mRNAs	16,989 peaks shared with cerebellum, and 8924 mRNAs shared with cerebellum	Chang et al., *Open Biology* 2017
m^6A	E13.5 mouse cortex	m^6A RIP-seq	4055	2179 mRNAs		Yoon et al., *Cell* 2017
m^6A	PCW11 human cortex	m^6A RIP-seq	10,980	5960 mRNAs		Yoon et al., *Cell* 2017
m^6A	Mouse P7 cerebellum	m^6A RIP-seq	19,459	10,449 total 9879 mRNA	Increases to 20,722 peaks on 11,215 RNAs upon hypobaric hypoxia exposure	Ma et al., *Genome Biology* 2018
m^6A/m	Mouse cortex	m^6A RIP-seq	14,656	7982	25 m^6A/m peaks (in 20 genes) were significantly modified in response to 4 h stress	Engel et al., *Neuron* 2018
m^6A	Human brainstem	m^6A RIP-seq	30,459	12,659	Donor ID 5	Liu et al., *Mol Cell* 2019
m^6A	Human cerebellum	m^6A RIP-seq	45,975	14,048	Donor ID 5	Liu et al., Mol Cell 2019
m^6A	Human cerebrum	m^6A RIP-seq	28,001	11,770	Donor ID 5	Liu et al., Mol Cell 2019
m^6A	Human hypothalamus	m^6A RIP-seq	26,993	11,751	Donor ID 5	Liu et al., Mol Cell 2019
m^6A	HEK293	CITS miCLIP, mutation calling	9536	–	12,051 residues identified by truncation calling, and 33,157 identified by RIP-seq	Linder et al., *Nature Methods* 2016
m^6A	HEK293T	m^6A RIP-seq	18,756	5768	43% conserved transcripts with mouse brain	Meyer et al., *Cell* 2012
m^6A	HeLa cells	m^6A-CLIP	37,557	–	~90% peaks are deposited at pre-mRNA stage, 99% are constant between nucleus and cytoplasm	Ke et al., *Genes Dev* 2017
m^6A_m	HEK293	CITS miCLIP	487	–		Linder et al., *Nature Methods* 2016
m^6A_m	HEK293	miCLIP	2350	–	Compared miCLIP in *PCIF1* KO cells and WT cells to distinguish m^6A_m and m^6A	Boulias et al., *Molecular Cell* 2019
m^6A_m	MEL624	m^6A_m-Exo-Seq	–	1521	Compared *PCIF1* WT and KO cells	Sendinc et al., *Molecular Cell* 2019
m^6A_m	Human brainstem	m^6A RIP-seq peaks at TSS	511	497 annotated	Donor ID 5; one peak per transcript	Liu et al., Mol Cell 2019
m^6A_m	Human cerebellum	m^6A RIP-seq peaks at TSS	601	583 annotated	Donor ID 5; one peak per transcript	Liu et al., Mol Cell 2019
m^6A_m	Human cerebrum	m^6A RIP-seq peaks at TSS	513	494 annotated	Donor ID 5; one peak per transcript	Liu et al., Mol Cell 2019
m^6A_m	Human hypothalamus	m^6A RIP-seq peaks at TSS	472	455 annotated	Donor ID 5; one peak per transcript	Liu et al., Mol Cell 2019
m^1A	HeLa	m^1A RIP-seq	7154	4151 coding 63 non-coding	Average of 1.4 peaks per methylated gene	Dominissini et al., *Nature* 2016
m^1A	HEK293T	m^1A RIP-seq	2129	–		Dominissini et al., *Nature* 2016
m^1A	HEK293T	m^1A-ID-seq	901	841 mRNAs 46 ncRNAs	1989 peaks identified in ALKBH3 KO cells	Li et al., *Nature Chemical Biology* 2016
m^1A	HEK293T	m^1A RIP-seq	277	226 tRNA 28 mitochondrial 8 rRNA 15 mRNA/other		Safra et al., *Nature* 2017
m^1A	HEK293T	m^1A-MAP	740	473 mRNA and lncRNAs	Subset of 53 sites is strongly enriched fro GUUCRA motif	Li et al., *Molecular Cell* 2018
m^1A	HeLa	RBS-Seq	91	88 tRNA 2 rRNA 1 snoRNA	mRNA peaks identified only if less stringent filters used	Khoddami et al., *PNAS* 2019
m^5C	HeLa	RNA-BisSeq	5399	1995 mRNAs 336 ncRNAs	45% in CDS	Yang et al., *Cell Research* 2017

(*continued on next page*)

Table 1 (*continued*)

Mod.	Tissue or cell type	Detection method	# of modified sites (peaks)	# of modified transcripts	Comments	Reference
m^5C	Mouse small intestine	RNA-BisSeq	12,201	3722 annotated	Peaks counted that are modified in at least 10% of m^1A sites in rep 1	Yang et al., *Cell Research* 2017
m^5C	Mouse heart	RNA-BisSeq	12,397	3915 annotated	"	Yang et al., *Cell Research* 2017
m^5C	Mouse muscle	RNA-BisSeq	11,721	3624 annotated	"	Yang et al., *Cell Research* 2017
m^5C	Mouse brain	RNA-BisSeq	12,032	3635 annotated	"	Yang et al., *Cell Research* 2017
m^5C	Mouse kidney	RNA-BisSeq	12,149	3800 annotated	"	Yang et al., *Cell Research* 2017
m^5C	Mouse liver	RNA-BisSeq	11,414	3751	"	Yang et al., *Cell Research* 2017
m^5C	Mouse testis 3 W	RNA-BisSeq	11,853	2727	"	Yang et al., *Cell Research* 2017
m^5C	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
Ψ	HeLa	RBS-Seq	754	322 mRNA 44 pseudogenes 55 rRNA 246 tRNA 24 snRNA 41 snoRNA 22 ncRNA	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 12 ncRNA	88 mRNAs 9ncRNA		Carlile et al., *Nature* 2014
Ψ	*S. cerevisiae* (budding)	Pseudo-seq	260 mRNA 74 ncRNA	238 mRNA 40 ncRNA	Lower threshold filter shows 466 Ψ on mRNAs.	Carlile et al., *Nature* 2014
Ψ	*S. Cerevisiae* (midlog)	Ψ-seq	328	168 mRNA 24 ncRNA 46 tRNA	Change under heat shock	Schwartz et al., *Cell* 2014
Ψ	HEK293 and fibroblasts	Ψ-seq	396	322 mRNA 28 ncRNA	All human sites were combined into one dataset	Schwartz et al., *Cell* 2014
m^7G	mESCs	m^7G MeRIP	184	184 tRNa	Functionally confirmed in *Mettl1 KO* mESCs	Lin et al., *Mol Cell* 2019
m^7G	HeLa and HepG2	m^7G meRIP, m^7G-seq	801	681 mRNA	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.

translation 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

Epitranscriptomics appears to be especially important in stem cell biology, as it contributes to self-renewal and differentiation capacity. m^6A is by far the most studied RNA modification in stem cells, particularly in ESCs and iPSCs.

3.1. m^6A in ESCs

Early reports of m^6A in ESCs were somewhat conflicting. One study reported that knockdown of *Mettl3* and *Mettl14* reduces m^6A 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 m^6A might do to drive embryonic development *in vivo*. This gap was addressed by Geula et al., who examined m^6A 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 m^6A 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 m^6A causes early embryonic lethality (Geula et al. 2015). Importantly, this study further clarified that m^6A regulates the genes governing both naïve and primed states, and that loss of m^6A 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 m^6A (Geula et al. 2015; Zhao and He 2015). Mechanistically, this study and others determined that m^6A 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 m^6A to mRNA, there are several other m^6A methyltransferases. In particular, METTL16 was identified in human cells as an m^6A 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 m^6A.

3.2. m^5C in mESCs

While the overwhelming focus of research in mESCs has been centered around m^6A, m^5C has also been examined. In one study, 12,492 m^5C 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 m^5C in mESCs was not shown, a correlation between m^5C sites and RBP sites was identified (Fig. 2). Approximately 29% of m^5C sites in mESCs overlap with known RBP sites. More specifically, the largest overlaps correspond to UPF1 binding, which regulates nonsense-mediated RNA decay. Additionally, SRSF3 and SRSF3 splicing factors and the PRC2 subunit EZH2 have binding sites that significantly overlap with m^5C sites. This led to the hypothesis that m^5C 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 m^5C reader, YBX1, binds to m^5C-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 m^6A 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 m^6A contributes to pluripotency and differentiation drove studies of its regulatory capacity in iPSCs. In 2015, Chen et al. showed that high abundance of m^6A 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 m^6A (Chen et al. 2015). This concept of m^6A 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 m^6A 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 *Klf4* and *Sox2* (Niwa et al. 1998; Wu et al. 2019). In parallel, YTHDF2 has been shown to be upregulated in iPSCs to destabilize m^6A-modified mRNAs related to neural development and thereby promote pluripotency (Heck et al. 2020).

Overall, m^6A 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, m^6A may alter expression of the gene transcripts already present. Still, further investigation is needed to identify the fate of m^6A-modified transcripts. While m^6A-mediated mRNA degradation appears to be a major mechanism, expression of other reader proteins suggests a more complex system. Understanding how m^6A reader proteins selectively bind particular mRNA targets will be a major step forward in further

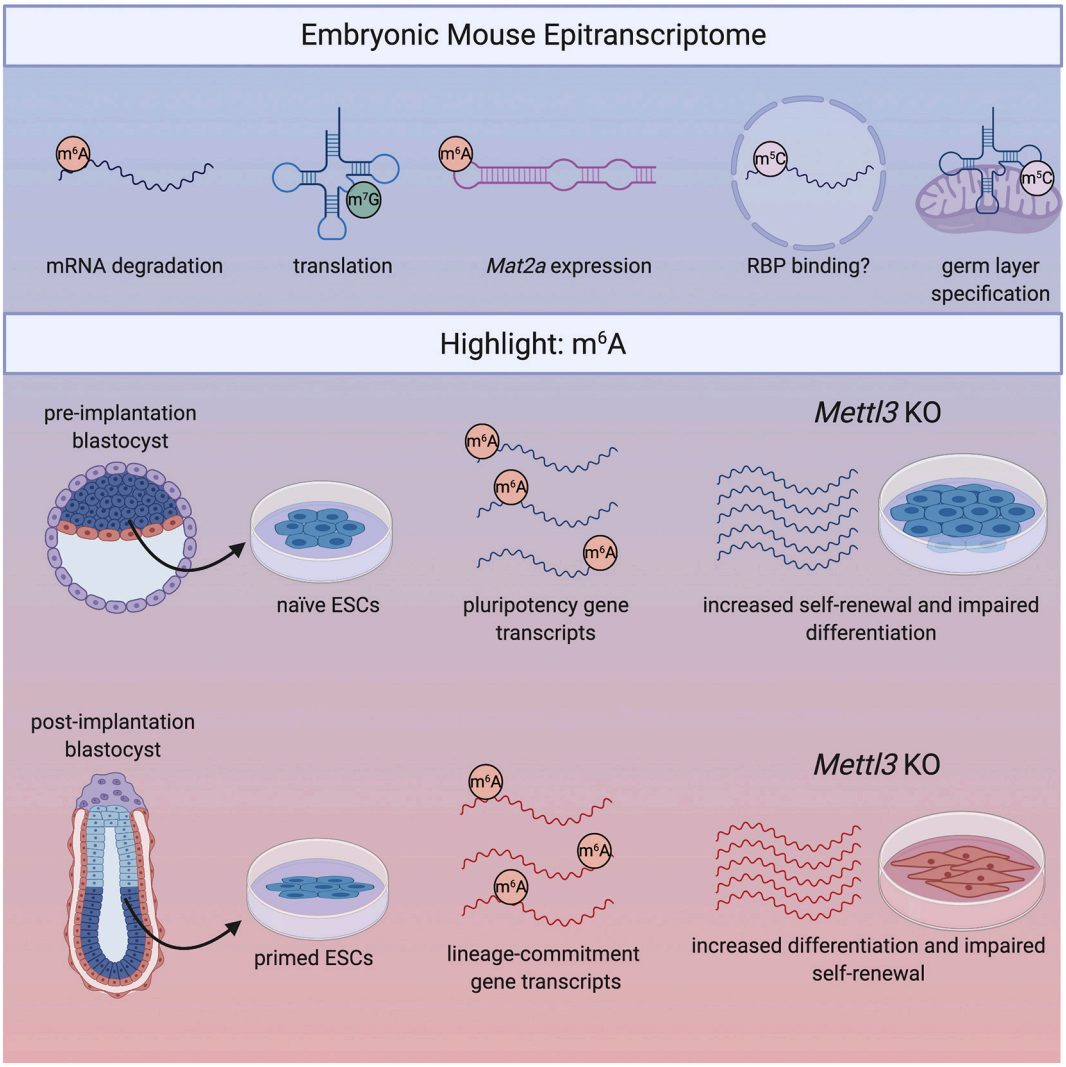

Fig. 2. The epitranscriptome in embryonic development and ESCs.
Top: Marks that have been studied in the developing embryo include m^6A on mRNA to promote mRNA degradation, m^7G on tRNA to promote translation, METTL16-mediated addition of m^6A to ncRNA like *Mat2a*, m^5C on mRNA to promote RNA Binding Protein (RBP) mRNA binding, and m^5C on mitochondrial tRNA to regulate germ layer specification. Bottom: The most-studied modification in embryonic stem cells (ESCs) is m^6A. While several conflicting studies have reported different phenotypes after *Mettl3* knockdown, the current consensus is that m^6A functions to destabilize whichever gene transcripts are modified at the time. In naïve ESCs purified from pre-implantation blastocysts, m^6A is primarily added to pluripotency-promoting gen transcripts. Therefore loss of m^6A improves self-renewal and impairs differentiation. In contrast, in primed ESCs derived from post-implantation blastocysts, m^6A is primarily added to lineage-commitment gene transcripts, so loss of m^6A promotes differentiation and impairs self-renewal.

elucidating the mechanisms of m^6A action in iPSCs.

3.4. Regulation of stem cell epitranscriptomes

Upstream regulation of m^6A deposition or differential expression of the writers, readers, and erasers contributes to the function of m^6A in stem cells. For example, Aguilo et al. showed that zinc finger protein 217 (ZFP217) coordinates epigenetic regulation with m^6A deposition. More specifically, ZFP217 is a transcription factor that directly activates transcription of several key pluripotency genes, then blocks m^6A modification of these genes by sequestering METTL3 in mESCs and iPSCs. *ZFP217* knockdown causes global increases in m^6A 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^6A deposition, and *Zc3h13* knockdown significantly impairs self-renewal and maintenance of pluripotency in mESCs (Knuckles et al. 2018; Wen et al. 2018). Zc3h13 can form a complex with WTAP, Virilizer (Kiaa1429), and Hakai, which also contribute to the METTL3-METTL14 m^6A methylation complex (Horiuchi et al. 2013; Wan et al. 2015). Wen et al. then showed that *Zc3h13* knockdown in mESCs decreases global m^6A levels to about 30–40% as in the control, and confirmed m^6A dependency on Zc3h13 through MeRIP-seq. More specifically, Zc3h13 promotes m^6A 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^6A modifications of these gene transcripts (Wen et al. 2018). The conclusion that m^6A 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^6A 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 m^{6}A deposition. Huang et al. showed that histone H3 trimethylation at lysine-36 (H3K36me3) drives m^{6}A methylation by recruiting and promoting interactions between the m^{6}A methyltransferase complex and its target mRNA. More specifically, METTL14 binds to H3K36Me3, chromatin, and RNA, thereby promoting the co-transcriptional addition of m^{6}A to genes with H3K36Me3 epigenetic marks. Knockdown of the H3K36Me3 methyltransferase, SETD2, impairs binding of the m^{6}A methyltransferase complex to sites that lose H3K36Me3, and globally reduces m^{6}A levels. In mESCs, SETD2 knockdown induces higher expression of pluripotency factors (OCT4, SOX2, NANOG) and prevents increased m^{6}A methylation during differentiation. This suggests that H3K36Me3 drives m^{6}A 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 m^{6}A is necessary for proper differentiation of mESCs (Batista et al. 2014; Geula et al. 2015), and provides the first evidence that m^{6}A 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 m^{6}A (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 m^{6}A, 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 m^{6}A in the brain, using *Drosophila melanogaster* as a model organism. This study showed that m^{6}A 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 m^{6}A *in vivo,* it contrasted with mammalian studies in that loss of m^{6}A methyltransferases is not lethal in flies. The next major advances came from studies of conditional knockout of the m^{6}A 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 m^{6}A in neural progenitor cells (NPCs) drastically impairs brain development *in vivo* (Yoon et al. 2017). Loss of m^{6}A impairs NPC differentiation, slows cell cycle progression, and elongates the timing of cortical neurogenesis into postnatal stages. Mechanistically, m^{6}A-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 m^{6}A normally destabilizes mRNA in the developing brain. By modifying both multipotency and differentiation-promoting transcripts, the m^{6}A 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 m^{6}A also regulates NPC cell cycle progression in humans (Yoon et al. 2017). We then compared m^{6}A-seq analysis among human brain organoids, human post-conception week 11 embryonic brain tissue and E13.5 mouse brains. While many gene transcripts are m^{6}A-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 m^{6}A in mammalian brain development and highlighted the possibility that m^{6}A 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 m^{6}A slows NPC cell cycle progression. *In vitro* analysis of *Mettl14* cKO NPCs showed that loss of m^{6}A 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 m^{6}A is necessary for the timely differentiation of NPCs, and loss of m^{6}A 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 m^{6}A 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 m^{6}A-mediated destabilization of CBP and p300 transcripts, which are stabilized upon loss of m^{6}A. However, this did not apply to transcripts in the PRC2 complex, suggesting there are also 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 m^{6}A 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 m^{6}A 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 m^{6}A also regulates neuron maturation during the differentiation process (Li et al. 2018b). This study was necessary to confirm that m^{6}A regulation of cortical development functions primarily through YTHDF2-mediated mRNA degradation and that m^{6}A promotes NPC proliferation and differentiation.

Beyond m^{6}A, m^{5}C is also crucial for proper neurodevelopment. Flores et al. showed that the m^{5}C 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

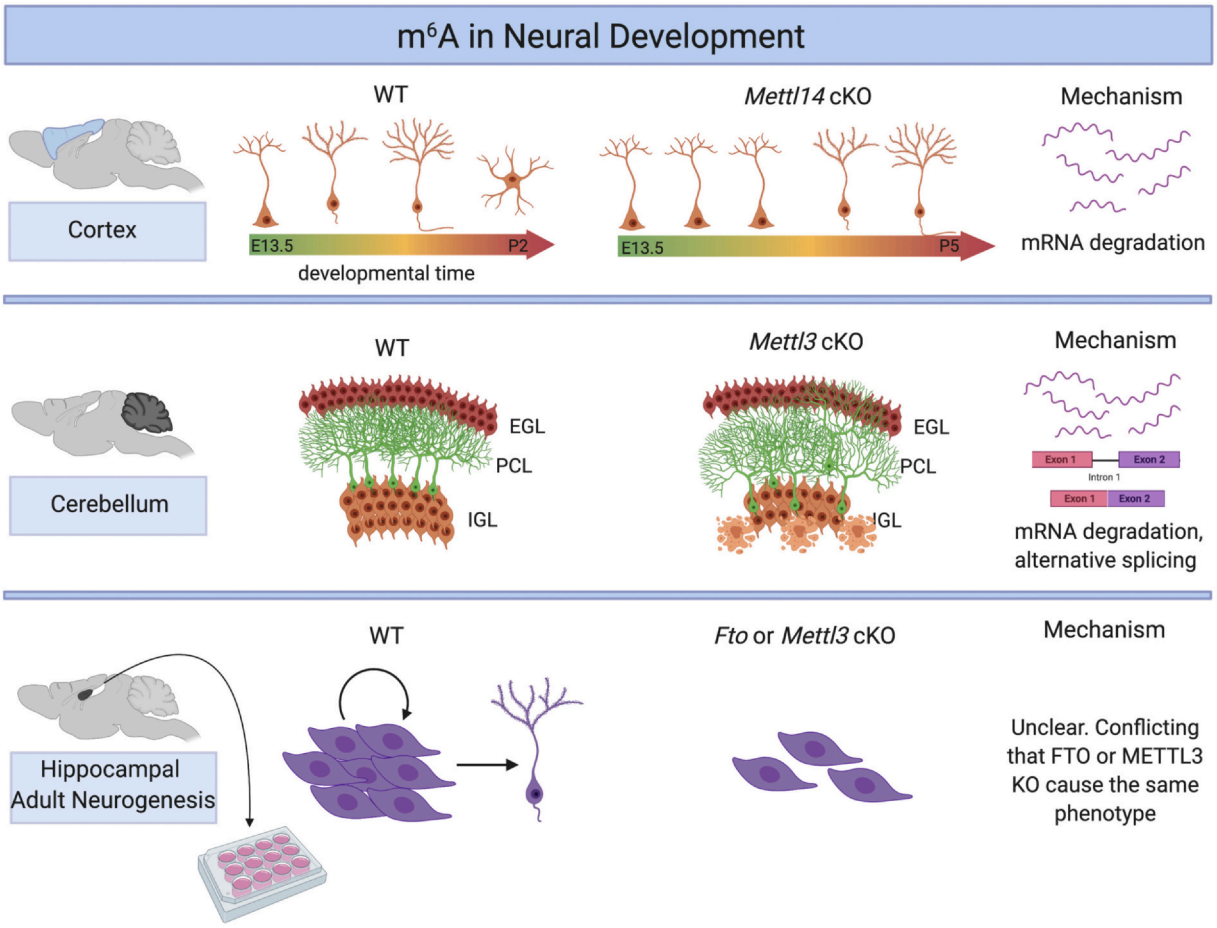

Fig. 3. m^{6}A in neural development.
Top: In the cortex, loss of m^{6}A elongates the timeframe of cortical neurogenesis such that the postnatal brain is still generating upper-layer neurons. This is accomplished through altered mRNA degradation rates of key pluripotency and fate-determining gene transcripts. Middle: In the cerebellum, loss of m^{6}A 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, m^{6}A 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 m^{6}A impairs self-renewal and neurogenesis, but the mechanism of action remains unclear.

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 m^{6}A. 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 m^{6}A 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 m^{6}A 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 m^{6}A is turned OFF from P7 to P14 have gene ontologies enriched for cell cycle. On the other hand, gene transcripts in which m^{6}A is turned ON at P14, P21, or P60 have gene ontologies enriched for signal transduction, cell adhesion, learning, and synaptic plasticity. Overall, m^{6}A 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 *Mettl3* 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 fewer mature neurons, yet 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 m^{6}A promotes nuclear export in this tissue (Ma et al. 2018).

In contrast, *Wang* et al. used a *Mettl3* cKO mouse model to show that m^{6}A promotes mRNA degradation and alternative splicing in the cerebellum. *Mettl3* 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

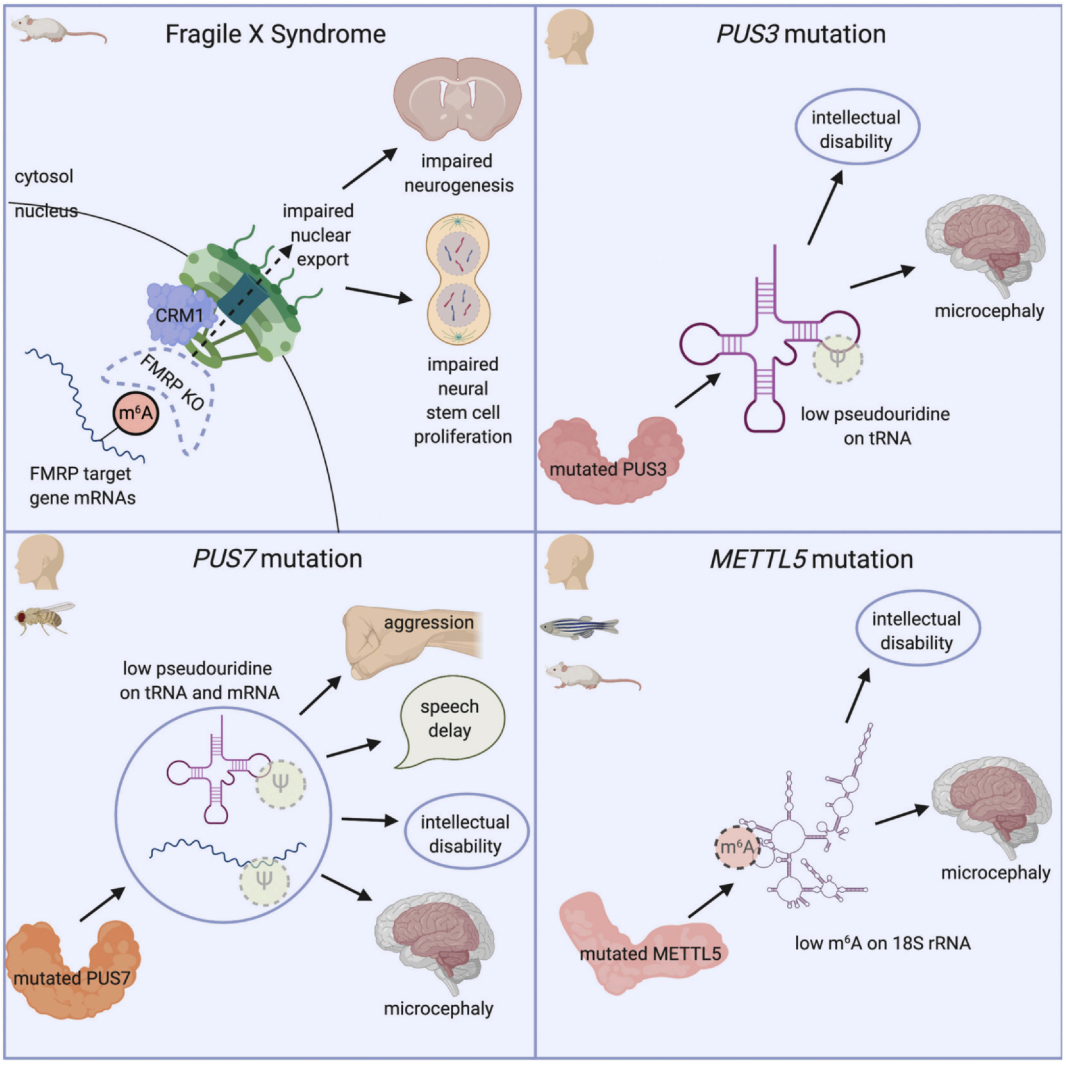

Fig. 4. The epitranscriptome in neural disorders.
Top left: Fragile X Syndrome is correlated with m^{6}A in that the central protein involved in Fragile X, FMRP, can bind m^{6}A to promote nuclear export of modified mRNAs through interaction with CRM1, a component of the nuclear pore complex. Loss of FMRP in mice impairs this export and causes decreased levels of embryonic neurogenesis and NSC proliferation. Top right: *PUS3* mutations in humans significantly correlate with intellectual disability and microcephaly. Though the exact mechanism is unknown, mutations in *PUS3* cause significantly lower levels of pseudouridine addition on tRNA relative to wildtype controls. Bottom left: *PUS7* mutations identified in humans and validated in drosophila cause increased aggression, speech delay, intellectual disability, and microcephaly through decreased levels of pseudouridine on both tRNA and mRNA. Bottom right: *METTL5* mutation in humans and validated in zebrafish and mice cause intellectual disability and microcephaly through decreased levels of m^{6}A on 18S ribosomal RNA.

wildtype controls. Furthermore, loss of m^{6}A causes significantly increased levels of apoptosis of newborn granule cells, which explains the depletion of CGCs. Again, loss of m^{6}A increases mRNA stability; m^{6}A modifications on apoptosis-associated gene 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 synapse-associated pathways and neurotransmitter receptors. Further analysis showed that increases in intracellular calcium concentration in *Mettl3* cKO CGCs 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 *Pdgfrα*, were shown to play important roles in FTO-mediated regulation of aNSCs. However, *Socs5* mRNA and protein decrease in *Fto* cKO aNSCs, while *Pdgfrα* 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 m^{6}A 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 m^{6}A marks in the mouse cerebral cortex (Zhang et al. 2018). A knockout of *Fmr1* resulted in the down-regulation of some m^{6}A mRNA FMRP target transcripts, suggesting that FMRP stabilizes these m^{6}A modified mRNAs (Zhang et al. 2018). Next, Edens et al. showed that FMRP promotes nuclear export of m^{6}A-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 m^{6}A-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 pseudouridinylation 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 pseudouridinylation 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^{5}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^{5}C may be a fundamental regulator of neural function across species (Abbasi-Moheb et al. 2012). These data strongly suggest that m^{5}C writers are important in neural development and function, but further studies are necessary to understand the mechanistic role of m^{5}C in the brain.

Finally, Richard et al. identified frameshift mutations in *METTL5*, which adds m^{6}A 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 m^{6}A in a single cell type suggest that m^{6}A may differentially regulate various gene transcripts within a single cell. Several important strategies to further elucidate the regulatory capacities of m^{6}A 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^6A-modified mRNAs, and (3) careful analyses and conservative interpretations of data to prevent overconfident conclusions that will hinder future studies.

In addition to clarifying studies on m^6A, we are particularly excited by the prospects of other epitranscriptomics marks in neural development and disease. Careful mapping of m^1A, m^5C, m^7G, m^6A_m, and Ψ 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.

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