m^6^A RNA Modification Controls Cell Fate Transition in Mammalian Embryonic Stem Cells

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SUMMARY

N6-methyl-adenosine (m^6^A) is the most abundant modification on messenger RNAs and is linked to human diseases, but its functions in mammalian development are poorly understood. Here we reveal the evolutionary conservation and function of m^6^A by mapping the m^6^A methylome in mouse and human embryonic stem cells. Thousands of messenger and long noncoding RNAs show conserved m^6^A modification, including transcripts encoding core pluripotency transcription factors. m^6^A is enriched over 3’ untranslated regions at defined sequence motifs and marks unstable transcripts, including transcripts turned over upon differentiation. Genetic inactivation or depletion of mouse and human Mettl3, one of the m^6^A methylases, led to m^6^A erasure on select target genes, prolonged Nanog expression upon differentiation, and impaired ESC exit from self-renewal toward differentiation into several lineages in vitro and in vivo. Thus, m^6^A is a mark of transcriptome flexibility required for stem cells to differentiate to specific lineages.

INTRODUCTION

Reversible chemical modifications on messenger RNAs (mRNAs) have emerged as prevalent phenomena that may open a new field of “RNA epigenetics,” where RNA modifications have an impact akin to the diverse roles that DNA modifications play in epigenetics (reviewed by Fu and He, 2012; Sibbritt et al., 2013). N6-methyl-adenosine (m^6^A) is the most prevalent modification of mRNAs in somatic cells, and dysregulation of this modification has already been linked to obesity, cancer, and other human diseases (Sibbritt et al., 2013). m^6^A has been observed in a wide range of organisms, and the methylation complex is conserved across eukaryotes. In budding yeast, the m^6^A methylation program is activated by starvation and required for sporulation. In Arabidopsis thaliana, the methylase responsible for m^6^A modification, MTA, is essential for embryonic development, plant growth, and patterning, and the Drosophila homolog IME4 is expressed in oocytes and testes and is essential for viability (reviewed in Niu et al., 2013). While m^6^A has been suggested to affect almost all aspects of RNA metabolism, the molecular function of this modification remains incompletely understood (Niu et al., 2013). Importantly, m^6^A modifications are reversible in mammalian cells. Two members of the alpha-ketoglutarate-dependent dioxygenases family, fat-mass and obesity associated protein (FTO) and ALKBH5, have been shown to act as m^6^A demethylases (Jia et al., 2011; Zheng et al., 2013). Manipulating global m^6^A levels has implicated m^6^A modifications in a variety of cellular processes, including nuclear RNA export, control of protein translation, and splicing (reviewed in Meyer and Jaffrey, 2014). Recently, m^6^A modification has been suggested to play a role in controlling transcript stability because the YTH domain family of “reader” proteins specifically bind m^6^A sites and recruit the transcripts to RNA decay bodies (Kang et al., 2014; Wang et al., 2014a).

Whereas the DNA methylome undergoes dramatic reprogramming during early embryonic life, the developmental origins and functions of m^6^A in mammals are incompletely understood. Furthermore, the degree of evolutionary conservation of m^6^A sites is not known in ESCs. To date, the functions of m^6^A in mammalian cells have only been examined by RNAi knockdown. Depletion of Mettl3 and METTL14 in human cancer cell lines led to decreased cell viability and apoptosis, leading to the interpretation that m^6^A is important for cell viability (Dominissini et al., 2012; Liu et al., 2014). A recent study reported that depletion of Mettl3 inhibited mouse ESC (mESC) proliferation and led to ectopic differentiation (Wang et al., 2014b). Here we assess the conservation of the m^6^A methylome at the level of gene targets and function in mESCs and human ESCs (hESCs). We...
report the consequences of genetic ablation of Mettl3 in mESCs as well as depletion of METTL3 in hESCs. These experiments led to the unexpected finding that m6A and METTL3 in particular are not required for ESC growth but are required for stem cells to adopt new cell fates.

RESULTS

Thousands of mESC Transcripts Bear m6A
To understand the role of the m6A RNA modification in early development, we mapped the locations of m6A modification across the transcriptome of mESCs and hESCs by m6A RNA immunoprecipitation sequencing (RIP-seq) as described elsewhere (Dominissini et al., 2012; Meyer et al., 2012; Experimental Procedures). For each experiment, libraries were built for multiple biological replicates and concordant peaks for each experiment were used for subsequent bioinformatics analyses.

In mESCs, m6A-seq revealed a total of 9,754 peaks in 5,578 transcripts (average 2 peaks per transcript), including 5,461 mRNAs (of 9,923 mRNAs) and 117 long noncoding RNAs (lncRNAs). Due to the lower expression levels of lncRNA as a class, our approach likely underestimates the fraction of modified noncoding transcripts (Table S1 available online). Thus, thousands of mESC transcripts, including mRNAs and lncRNAs, are m6A modified.

m6A in mRNAs of mESC Core Pluripotency Factors
We found that mRNAs encoding the core pluripotency regulators in mESCs, including Nanog, Klf4, Myc, Lin28, Med1, Jarid2, and Eed, were modified with m6A (Dunn et al., 2014; Young, 2011), whereas Pou5f1 (also known as Oct4) lacked m6A modification (Figures 1A and 1B). We confirmed m6A-seq results with independent m6A-IP-qRT-PCR. (Figure S1A available online) and m6A-IP followed by Nanostring nCounter analysis (m6A-string)
m6A modification in noncoding RNAs (ncRNAs), which lack stand if there was a topological enrichment or constraint on 1G and S1G–S1H), suggesting that the 3
ncRNAs and toward the 3
long exon processing. The topological enrichment of m6A peaks when the number of peaks per exon was normalized for exon
mechanistically to m6A targeting through as yet unclear systems
gest the possibility that processing of long exons is coupled
surrounding stop codons in mRNAs is a poorly understood
bias for read accumulation around the stop codon in the input
abundance (Figure S1I). Thus, our analysis suggests that m6A
occuring in long internal exons (median exon length of 737 bp
translation within the first 75 amino acids, and immunoblot anal-
ysis confirmed the absence of METTL3 protein (Figures 2A and
KO mESC colonies were consistently larger than WT ESCs
self-renewal.
Contrary to the expectation in the literature, the Mettl3 KO mESCs are viable and, surprisingly, demonstrated improved self-renewal. Mettl3 KO mESCs could be maintained indefinitely over months and exhibited low levels of apoptosis, similar to WT mESCs, as judged by PARP cleavage and Annexin V flow cytom-
retrogenesis is not simply a random modification that occurs on abundant cellular transcripts; rather, m6A preferentially marks transcripts expressed at a medium level.
To further define potential mechanisms of m6A function, we
asked whether m6A-marked transcripts differ from unmodified transcripts at the level of transcription, RNA decay, or translation by leveraging published genome-wide data sets in mESCs. RNA polymerase II (Pol II) occupancy at the promoters encoding both unmodified and m6A-marked RNAs is similar (Figure S1J). In contrast, m6A-marked transcripts had significantly shorter RNA half-life—2.5 hr shorter on average (p < 2.2
m6A modified transcripts have slightly lower translational effi-
ciency than unmodified transcripts (1.32 versus 1.51, respect-
ively) (Ingolia et al., 2011) (Figure S1K). These results suggest that m6A is a chemical mark associated with transcript turnover.
Mettl3 Knockout Decreases m6A and Promotes ESC Self-Renewal
To understand the role of m6A methylation in ESC biology, we chose to inactivate Mettl3, encoding one of the components of the m6A methylase complex. To date no genetic study of Mettl3 has been performed to rigorously define its requirement for m6A modification; all studies have relied on knockdown. We tar-
geted Mettl3 by CRISPR-mediated gene editing and generated several homozygous Mettl3 knockout (KO) mESC lines. DNA sequencing confirmed homozygous stop codons that terminate translation within the first 75 amino acids, and immunoblot anal-
ysis confirmed the absence of METTL3 protein (Figures 2A and
Two-dimensional thin layer chromatography (2D-TLC) showed a significant (~60%) but incomplete reduction of m6A in Mettl3 KO mESC (Figures 2B and S2B). Contrary to a recent publication (Wang et al., 2014b), Mettl3 KO slightly reduced, but did not prevent, the stable accumulation of METTL14 (Fig-
These experiments provide formal genetic proof that METTL3 is a major, but not the sole, m6A methylase in mESCs.
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we injected Mettl3 KO or WT cells subcutaneously into the right or left flank, respectively, of SCID/Beige mice (n = 5). Both WT and Mettl3 KO cells formed tumors consistent in morphology with teratomas. Mutant tumors tended to be larger, in accordance with mutant cell growth curves observed in vitro (Figure 3C). Histological analysis of H&E stained tumor sections revealed consistent differences between the two populations. While both groups of cells formed teratomas that contained some degree of differentiation into all three germ layers, the teratomas derived from KO cells were predominantly composed of poorly differentiated cells with very high mitotic indices and numerous apoptotic bodies, whereas WT cells differentiated predominantly into neuroectoderm (Figure 3D). Analysis of adjacent sections revealed that the mutant teratomas have markedly higher staining of the proliferation marker Ki67 and the ESC protein Nanog, which highlight the poorly differentiated cells (Figures 3E, 3F, and S3A). Mettl3 KO tumors had higher levels of Nanog, Oct4, and Klf7 mRNAs and lower levels of Tuj1, Myh6, and Sox17 mRNAs (Figure S3B). These results suggest that insufficient m6A leads to a block in ESC differentiation and persistence of a stem-like, highly proliferative state.

**Mettl3 Target Genes in mESCs**

The incomplete loss of bulk m6A in Mettl3 KO cells may result because METTL3 is solely responsible for the methylation of a subset of genes or sites and/or because METTL3 functions in a redundant fashion with another methylase on all m6A-modified genes. To distinguish between these possibilities, we mapped the m6A methylome in Mettl3 KO cells. Comparison of the methylomes of WT versus Mettl3 KO mESCs revealed a global loss of methylation across m6A sites identified in WT (Figure 4A). We detected changes in 3,739 sites (in 3,122 genes), including modification sites in Nanog mRNA. Thus, this unbiased analysis suggested a set of targets that rely more exclusively on METTL3, including Nanog and other pluripotency mRNAs (Figures 4B and 4C) (Table S1). Gene set enrichment analysis confirmed that METTL3-target genes significantly overlap functional gene sets important for pluripotency, including targets of Ctnnb1 (4.43 × 10⁻⁶), targets of SMAD2 or SMAD3 (1.03 × 10⁻¹⁸), targets of MYC (9.20 × 10⁻¹⁵), targets of
SOX2 ($4.75 \times 10^{-3}$), and targets of NANOG ($7.18 \times 10^{-6}$) (Figure 4C), and include 5 of 11 core ESC regulators such as Nanog, Rlf1, Jarid2, and Lin28 (Figure 4D). Independent validation by m6A RIP followed by Nanostring detection confirmed loss of m6A in Nanog and other mRNAs in KO versus WT mESCs (Figure 4E). Further, after transcription arrest by flavopiridol treatment, Nanog mRNA showed delayed turnover in Mettl3 KO cells compared to WT, consistent with a requirement for m6A in Nanog mRNA turnover (Figure 4F).

How do you cite this article?

Batista et al., m6A RNA Modification Controls Cell Fate Transition in Mammalian Embryonic Stem Cells, Cell Stem Cell (2014), http://dx.doi.org/10.1016/j.stem.2014.09.019

Cell Stem Cell
m6A Controls Cell Fate Transitions in ESCs

Figure 3. Mettl3 Loss of Function Impairs ESC Ability to Differentiate
(A) Percentage of embryoid bodies with beating activity in Mettl3 KO and WT control cells (right panel). Representative images of bodies stained for MHC and DAPI (center panel) and mRNA levels of Nanog and Myh6, measured by qRT-PCR, in Mettl3 KO cells in relation to WT control cells are also displayed. Error bars, standard deviation of three biological replicates in all panels. *p < 0.05, t test (two-tailed). See also Movie S1 and Movie S2.

(B) Percentage of colonies with TUJ1 projections in Mettl3 KO and WT control cells (right panel). Representative images of bodies stained for TUJ1 and DAPI (center panel) and mRNA levels of Nanog and Tuj1, measured by qRT-PCR, in Mettl3 KO cells in relation to WT control cells are also shown. *p < 0.05, t test (two-tailed).

(C) Weight differences between teratomas generated from WT and Mettl3 KO cells. Tumors are paired by animal (n = 5) *p <0.1, calculated by Wilcoxon matched pair signed ranked test.

(D) Representative sections of teratomas stained with H&E at low magnification. Scale bar represents 1,000 μm. See also Figure S3A.

(E and F) Immunohistochemistry with antibody against KI67 (E) and with antibody against NANOG (F). Scale bar represents 100 μm. See also Figure S3B.

m6A RIP followed by Nanostring detection confirmed loss of m6A in Nanog and other mRNAs in KO versus WT mESCs (Figure 4E). Further, after transcription arrest by flavopiridol treatment, Nanog mRNA showed delayed turnover in Mettl3 KO cells compared to WT, consistent with a requirement for m6A in Nanog mRNA turnover (Figure 4F). However, RNA-seq analysis of Mettl3 KO cells revealed modest perturbations in mRNA steady state levels with only ~300 genes demonstrating...
m^6A Controls Cell Fate Transitions in ESCs

Please cite this article in press as: Batista et al., m^6A RNA Modification Controls Cell Fate Transition in Mammalian Embryonic Stem Cells, Cell Stem Cell (2014), http://dx.doi.org/10.1016/j.stem.2014.09.019
significant changes over 1.5-fold. Collectively, these results suggest that ESC genes are under METTL3 control and that m6A impacts ESO biology.

**Widespread m6A Modification of hESCs**

The identification of thousands of m6A sites raises the challenge of defining the functional importance of each and every one of the sites. We reasoned that evolutionary conservation provides a powerful and comprehensive metric of function. To this end, we mapped m6A sites in hESCs and during endoderm differentiation to elucidate the patterns and potential conservation of m6A methylome (Figure 5A). In basal state hESCs [Time (T) = 0], m6A-seq identified 16,943 peaks in 7,871 genes representing 7,530 coding and 341 noncoding RNAs. Upon hESC differentiation toward endoderm (T = 48, “endoderm differentiation” thereafter), m6A-seq identified 15,613 m6A peaks in 7,195 genes representing 6,909 coding and 286 noncoding RNAs (Table S3). As shown in Figure 5B, 11,322 peaks (6,004 genes) were common between the undifferentiated and differentiated hESCs, while 5,348 (3,979 genes) versus 4,087 peaks (3,024 genes) were unique, respectively.

**Many Master Regulators of hESC Maintenance and Differentiation Are Modified with m6A**

As we observed for mESC, transcripts encoding many hESC master regulators, including human NANOG, SOX2, and NR5A2, were m6A modified. As in mESCs, the transcripts for OCT4 (POU5F1) in hESCs did not harbor an m6A modification (Figure 5D). These results show that in both organisms the core pluripotency/maintenance genes are under the regulatory influence of the m6A pathway. We also identified human-specific lncRNAs with known roles in hESC maintenance, such as LINC-ROR and MEGAMIND/TUNA, to contain m6A modifications (Figures 5D and S4A) (Lin et al., 2014; Loewer et al., 2010). Upon induction of hESC differentiation, we observed transcripts encoded by several key regulators of endodermal differentiation, including EOMES and FOX2A (Figure 5D), to also have m6A modifications. Gene ontology (GO) analyses of methylated genes in undifferentiated hESCs, and after endodermal differentiation, were significantly enriched in biological functions such as regulation of transcription (FDR = 1.2 × 10⁻¹¹), chordate embryonic development (FDR = 1.1 × 10⁻⁴), and regulation of cell morphology (FDR = 0.01).

Upon hESC differentiation toward endoderm, 1,356 peaks in 1,137 genes showed quantitative differences of at least 1.5-fold in m6A intensity, after normalization for input transcript abundance (Figures 5E and 5F and Table S4). The majority of these differential m6A sites represented quantitative differences at existing sites (i.e., 59.1% of the peaks were called in both time points), rather than state-specific de novo appearance or erasure of modification (Figure 5G). This is consistent with the observation that 74.9% of sites in the hESCs overlapped those observed in HEK293T data (Meyer et al., 2012) and the minimal changes in m6A sites observed in a recent survey of m6A pattern across cell types (Schwartz et al., 2014). We suggest that transcripts exhibit dynamic differential peak m6A methylation intensity largely at “hard-wired sites” during differentiation under the conditions examined and when compared to other tissue types.

**Conserved Features of m6A Modifications Spanning Different Species**

We found that three salient features of the m6A methylome are conserved in hESCs. First, m6A sites in hESCs are also dominated by the RRACU motif seen in mESC and somatic cells (Dominissini et al., 2012; Meyer et al., 2012) (Figure 5C). There was also a strong preference for the methylome to target long-internal exons at the RRACU motif even after we normalized for exon length and number of m6A motifs (Figure 5H). Second, there was a significant enrichment in m6A peaks at the 3′ end of transcripts, near the stop codon of coding genes or the last exon in noncoding RNAs (Figures 5I and S4B–S4D). Furthermore, the topology of m6A modification is preserved upon endodermal differentiation (Figure 5I). As in mESCs, moderately to lowly expressed genes have higher probabilities of becoming methylated (Figure S4E). Lastly, hESC m6A is not correlated with transcription rate as judged by Global Run-On sequencing (GRO-seq) (Sigova et al., 2013), but it is strongly anticorrelated with measured mRNA half-life in human pluripotent cells (Neff et al., 2012), strongly suggesting that m6A modification also marks RNA turnover in hESCs (Figures 5J, S4F, and S4G).

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**Figure 5. m6A-seq Profiling of hESCs during Endoderm Differentiation**

(A) m6A-seq was performed in resting (undifferentiated) human H1-ESCs (T0) and after 48 hrs of Activin A induction toward endoderm (mesoendoderm) (T48).

(B) Venn diagram of the overlap between high-confidence T0 and T48 m6A peaks. The number of genes in each category is shown in parenthesis. See also Table S3 and Table S4.

(C) Sequence motif identified after analysis of m6A enrichment regions.

(D) UCSC Genome browser plots of m6A-seq reads along indicated RNAs. Gray reads are from non-IP control input libraries and red (T0) or blue (T48) reads are from anti-m6A IP libraries. y axis represents normalized number of reads; x axis is genomic coordinates. Key regulators of stem cell maintenance (left) and master regulators of endoderm differentiation (right) are represented. See also Figure S4A.

(E) Scatter plot of m6A peak intensities between two different time points (T0 versus T48) of the same biological replicate with only “high-confidence” T0 or T48 specific peaks supported by both biological replicates highlighted.

(F) UCSC Genome browser plots of m6A-seq reads along indicated mRNAs in undifferentiated (T0) versus differentiated (T48) cells. The gray reads are from non-IP control input libraries. The red and blue reads are from the anti-m6A RIP of T = 0 and T = 48 samples, respectively.

(G) Differential intensities of m6A peaks (DMPIs) identify hESC cell states T0 versus T48 hrs. Z score scaled log2 peak intensities of DMPIs are color-coded according to the legend. The peaks and samples are both clustered by average linkage hierarchical clustering using 1-Pearson correlation coefficient of log2 peak intensity as the distance metric.

(H) Number of peaks per exon normalized by the number of motifs (on sense strand) in the exon. The error bars represent standard deviations from 1,000 times of bootstrapping.

(I) The normalized distribution of m6A peaks across the 5′ UTR, CDS, and 3′ UTR of mRNAs for T0 and T48 m6A peaks. See also Figures S4B–S4D.

(J) Box plot representing the half-life for transcripts, with transcripts separated according to enrichment score. See also Figures S4E, S4F, and S4G.
Evolutionary Conservation and Divergence of the m6A Epi-transcriptomes of Human and Mouse ESCs

Previous studies suggested significant conservation of m6A modified genes between mouse and human in somatic cell types, but the comparisons are limited by nonmatched tissue types (Dominissini et al., 2012; Meyer et al., 2012). We were thus interested in examining the evolutionary conservation of hESC and mESC m6A methylomes. At the gene level, 69.4% (3,609 of 5,204) of hESC genes are also m6A modified in the orthologous mouse gene (p = 8.3 × 10^{-179}; Fisher exact test) (Figure 6A; Figure 6. Evolutionary Conservation and Divergence of the m6A Epi-transcriptomes of hESCs and mESCs

(A) Venn diagram showing a 62% overlap between methylated genes in Mus musculus (purple) and Homo sapiens (red) ESCs (p = 3.5 × 10^{-13}; Fisher exact test). See also Table S5 and Table S6.

(B) The m6A peaks that could be mapped to orthologous genomic windows between mouse and human were identified. The intensities of m6A-seq signals in hESCs and mESCs were shown for m6A peaks found to be unique in mouse (blue), unique in human (red), and conserved between human and mouse (black).

(C) Box plot of peak intensities of m6A sites conserved (“common”) or not conserved (“specific”) in mESCs and hESCs. (p = 1.3 × 10^{-15} and 8.7 × 10^{-23}, respectively, Wilcoxon test).

(D–F) UCSC Genome browser plots of m6A-seq reads along indicated mRNAs. The gray reads are from non-IP control input libraries and the purple and red reads are from the anti-m6A RIP of mESCs and hESCs (T0), respectively. (D) Mouse-specific m6A modifications are represented. (E) Human-specific m6A modifications of ESCs are represented. (F) Conserved m6A modifications at the gene and site level are represented. Genes such as CHD6 have a conserved m6A peak location at its 3’ UTR as well as mouse- and human-specific m6A peaks at conserved but distinct exons.
Table S5). Furthermore, we identified 632 conserved m^6^A peak sites (46.1%) between hESCs and mESCs (Table S6). Notably, conserved sites tend to have higher m^6^A peak intensities compared to m^6^A peak sites that were not conserved (Figures 6B and 6C, p = 1.3 x 10^−15 and 8.7 x 10^−3^3 for hESCs or mESCs, respectively; Wilcoxon test). Commonly methylated genes can demonstrate m^6^A modification sites at identical sites (as in the case of GLI1), similar but not identical locations (as in the case of SOX2), or m^6^A sites at different exons (as in the case of CHD8) (Figures 6D–6F and Table S4). Our data thus reveal a substantial overlap at the gene level, suggesting broad functional significance of m^6^A modification in ESCs in both species. At the same time, we also observed numerous species-specific m^6^A patterns that may contribute to specific aspects of ESC biology (Schnerch et al., 2010).

**METTL3 Is Required for hESC Differentiation**

To address the function of m^6^A in hESCs, we generated hESC colonies with stable knockdown of METTL3 and shRNA control (Figure 7A). Knockdown of METTL3 in hESCs resulted in reduction in METTL3 mRNA levels and reduction in m^6^A level (Figures 7B and 7C and Figures S5B and S5C). METTL3-depleted hESCs could be stably maintained, suggesting the dispensability of METTL3 for hESC self-renewal or viability. Strikingly, differentiation of METTL3-depleted hESCs into neural stem cells (NSCs) by dual inhibition of SMAD signaling, using Dorso morphin and SB431542, revealed a block in neuronal differentiation (Experimental Procedures). While 44% (± 3.5% SD) of the control cells were SOX1+, only 10% (± 3.1% SD) of the METTL3-depleted were SOX+ (Figure S5A).

Similarly, knockdown of METTL3, in three independently generated hESC colony clones selected for METTL3 knockdown, led to a profound block in endodermal differentiation at day 2 and day 4 based on their failure to express the endoderm markers EOMES and FOXA2 compared to either two shRNA control colony clones (Figure 7D) or WT hESCs (Figure S5D). Consistently, METTL3-depleted ESCs retain high levels of expression of the master regulators NANOG and SOX2 throughout the differentiation time course in contrast to their diminishing expression in WT cells (Figures 7E and S5E). These results indicate that METTL3 and m^6^A control differentiation of hESCs.

**DISCUSSION**

**m^6^A Methyome in ESCs**

Our analysis of the ESC m^6^A methyome in mouse and human cells reveals extensive m^6^A modification of ESC genes, including most key regulators of ESC pluripotency and lineage control. However, this observation does not mean that m^6^A is uniquely tied to the pluripotency network. Because m^6^A marks moderately expressed transcripts that need to be turned over in a timely fashion, such genes in ESCs likely include many regulators of pluripotency and lineage determination. The pattern and sequence motif associated with ESC m^6^A are similar if not identical to those previously reported in somatic cells, suggesting a single mechanism that deposits m^6^A modification in early embryonic life. This invariant mechanism for m^6^A contrasts with the complexity of 5-methyl-cytosine in DNA and histone lysine methylations that undergone extensive reprogramming with distinct rules in pluripotent versus somatic cells.

We identified a general and conserved topological enrichment of m^6^A sites at the 3’ end of genes among single-exon and multiple-exon mRNAs as well as ncRNAs. Thus, neither the stop codon nor the last exon-exon splice junction can alone explain the observed m^6^A topology in RNA. However, all species examined to date including Saccharomyces cerevisiae and A. thaliana exhibit a strong 3’ bias in m^6^A localization, suggesting an evolutionary constraint that may target the m^6^A modification to the 3’ ends of genes regardless of gene structure or coding potential. This bias may be achieved by preferential m^6^A methylase recruitment to 3’ sites or preferential action of demethylases in upstream regions of the transcript. Although the role of demethylases cannot be excluded, the observation of 3’ end m^6^A bias in S. cerevisiae, which lacks known m^6^A demethylases, argues against the latter mechanism (Bodi et al., 2012; Jia et al., 2011; Schwartz et al., 2013; Zheng et al., 2013). The functional importance of m^6^A location versus its specific molecular outcome needs to be addressed in future studies.

**Mettl3 Selectively Targets mRNAs, Including Pluripotency Regulators**

While several studies had approached Mettl3 function by RNAi knockdown (Dominissini et al., 2012; Fustin et al., 2013; Liu et al., 2014; Wang et al., 2014b), genetic ablation of Mettl3 allowed us to examine the true loss-of-function phenotypes. The importance of using definitive genetic models is highlighted by recent studies in the DNA methylation field where shRNA experiments led to misassigned functions of Ten-eleven translocation (TET) proteins that were later recognized in genetic KOs (Dawlaty et al., 2011, 2013). We found that both Mettl3 KO and depletion led to incomplete reduction of the global levels of m^6^A in both mESCs and hESCs, demonstrating redundancy in m^6^A methylases. However, m^6^A profiling in Mettl3 KO cells revealed a subset of targets, approximately 33% of m^6^A peaks, that are preferentially dependent on METTL3, and these included Nanog, Sox2, and additional pluripotency genes. A second m^6^A methylase, METTL14, was described during the preparation of this manuscript.

RNAi knockdown of Mettl3 in somatic cancer cells led to apoptosis (Dominissini et al., 2012), and one study reported ectopic differentiation of mESCs with Mettl3 depletion (Wang et al., 2014b). In contrast, we found that Mettl3 KO does not affect mESC cell viability or self-renewal, and in fact mESCs renewed at an improved rate. The differences in phenotype observed could potentially be explained by different dependency on m^6^A modified RNAs in different cell types, acute versus chronic inactivation, or RNAi off-target effects. m^6^A methyolne analysis in different cell types with Mettl3 inactivation may shed light on these differences in the future.

**Conservation of m^6^A Methyolme in Mammalian ESCs**

The conserved methylation patterns of many ESC master regulators and the shared phenotype observed upon inactivation of Mettl3 suggest that METTL3 operates to control stem cell differentiation. It is known that hESCs and mESCs are not equivalent (Schnerch et al., 2010) and are cultured in different conditions. By focusing in on orthologous genes, we were able to
Figure 7. METTL3 Is Required for Normal hESC Endoderm Differentiation: A Model of METTL3 Function

(A) hESC cells were transfected with anti-METTL3 shRNA (KD) as well as control shRNA and stable hESC colonies were obtained after drug selection. Two independent clones were subjected to endodermal differentiation with Activin A and examined at various indicated time points. A schematic of the trends of gene expression for indicated markers of stem maintenance and endoderm differentiation is also shown. See also Figure S5A.

(B) Levels of METTL3 mRNA in hESC cells with control shRNA versus anti-METTL3 shRNA (KD) across the three indicated time points during endodermal differentiation (n = 2 independent generated ESC knockdown and control clones shown). In all panels, error bars represent standard deviation across three replicates per time point; *p < 0.05 t test (two-tailed) between different clones. See also Figure S5B.

(C) Anti-m6A dot-blot was performed on 10× fold dilutions of poly(A)-selected RNA from hESCs derived from control shRNA versus anti-METTL3 shRNA clones. See also Figure S5C.

(D and E) mRNA levels of endodermal and stem maintenance/marker genes. qRT-PCR was performed on indicated genes and time points (n = 2 independently generated ESC knockdown and control clones shown). See also Figure S5D.

(F) Model: m6A marks transcripts for faster turnover. Upon transition to new cell fate, m6A marked transcripts are readily removed to allow the expression of new gene expression networks. In the absence of m6A, the unwanted presence of transcripts will disturb the proper balance required for cell fate transitions.
catalog both shared and species-specific methylation sites. The observation that certain methylation sites are modified whenever a target transcript is expressed in both species, despite cell state or culture differences, argues that these modification events have been preserved under strong purifying selection during evolution. Our comparative genomic analyses also pave the way to further understand potential biological differences between hESCs and mESCs at the level of m6A epi-transcriptome, given the unique patterns of some methylation sites between the species.

RNA “Antiepigenetics:” m6A as a Mark of Transcriptome Flexibility
Stem cell gene expression programs need to balance fidelity and flexibility. On the one hand, stem cell genes need sufficient stability to maintain self-renewal and pluripotency over multiple cell generations, but on the other hand, gene expression needs to change dynamically and rapidly in response to differentiation cues. It has been proposed that ESC gene expression programs are in constant flux between competing fates, and pluripotency is a statistical average (Loh and Lim, 2011; Montserrat et al., 2013; Shu et al., 2013). We found that mRNAs with m6A tend to have a shorter half-life, and Nanog and Sox2 mRNAs could not be properly downregulated with differentiation in METTL3-deficient mESCs and hESCs. However, METTL3 deficiency has only modest effects on steady state gene expression, which could arise from the nonstoichiometric nature of the m6A modification. The application of methods that can determine the level of modification of each RNA species will allow us to answer these questions (Harcourt et al., 2013; Liu et al., 2013). Mettl3 KO mESCs have enhanced self-renewal but hindered differentiation, concomitant with a decreased ability to downregulate ESC mRNAs. WTAP, a conserved METTL3 interacting partner from yeast to human cells (Horichu et al., 2013; Schwartz et al., 2011), is also required for endodermal and mesodermal differentiation (Fukusumi et al., 2008). The observed phenotypes in ESCs and teratomas are all the more notable because we have significantly reduced, but not eliminated, m6A.

Our findings suggest a model where m6A serves as the necessary flexibility factor to counterbalance epigenetic fidelity—an RNA “antiepigenetics” measure (Figure 7F). m6A marks a wide range of transcripts, including ESC fate determinants to limit their level of expression and ensure their continual degradation so that cells can rapidly transition between gene expression programs. In ESCs, m6A is required for cells to rapidly exit the pluripotent state upon differentiation. The inability to exit the stem cell state and continued proliferation upon insufficient m6A offers a potential explanation for the association of FTO with human cancers (Loos and Yeo, 2014). METTL3 depletion also leads to elongation of the circadian clock (Fustin et al., 2013), suggesting a role for m6A in resetting the transcriptome. In yeast, m6A is active during meiosis (Clancy et al., 2002), where diploid gene expression programs are reset to generate haploid offspring. We propose that m6A makes the transition between cell states possible by facilitating a reset mechanism between stages, as occurs in ESCs and likely other cell types. In contrast to epigenetic mechanisms that provide cellular memory of gene expression states, m6A enforces the transience of genetic information, helping cells to forget the past and thereby embrace the future.

EXPERIMENTAL PROCEDURES
For full details, see the Supplemental Experimental Procedures.

Mouse Cell Culture and Differentiation
J-1 murine ESCs were grown under typical feeder-free ESC culture conditions. For CM formation, mESCs were differentiated in CM differentiation media and scored on day 12. For neuron formation, mESCs were differentiated in MEF and ITSFn medium and scored after 10 days in ITSFn medium. For the cell proliferation assay 5,000 cells were cultured in 24-well plates and the assay was performed according to the manufacturer’s protocol (MTT assay, Roche). For the single-colony assays and Nanog staining, 1,000 cells were cultured per well, on a six-well plate. For alkaline phosphatase staining, cells were stained according to the manufacturer’s protocol (Vector Blue Alkaline Phosphatase Substrate Kit).

hESC Cell Culture, Transfection, and Differentiation
H1 (WA01) cells were cultured in feeder-free conditions as described elsewhere (Sigova et al., 2013). Stable hESC lines were created that expressed shMETTL3 RNA or scrambled shRNA by transfection of hESCs with plasmids encoding shMETTL3 or scrambled shRNA and a puromycin resistance gene. Cells were treated with puromycin for 6 days beginning 2 days after transfection. For scrambled shRNA and METTL3 shRNA, two and three independent puromycin-resistant colonies were picked and expanded, respectively. Endodermal differentiation was then induced by Activin A, as described elsewhere (Sigova et al., 2013). Day 2 and Day 4 of differentiation were measured from the time that Activin A was added. Puromycin was removed from the media 1 day prior to endodermal differentiation.

DNA Binding Assays
DNA binding assays were performed using the DSF assay (Dawson et al., 2011). Bacterial extracts were prepared as previously described, and 5 μg of extract was used for each reaction. DNA binding was characterized by measuring the change in melting temperature of the DNA-DNA binding protein complex in the presence of increasing amounts of protein. For single-colony assays and Nanog staining, 1,000 cells were cultured per well, on a six-well plate. For alkaline phosphatase staining, cells were stained according to the manufacturer’s protocol (Vector Blue Alkaline Phosphatase Substrate Kit).

miRNA Analysis
miRNA expression analysis was performed using RT-qPCR with hairpin miRNA primers. Samples were run in triplicate on three independent PCR reactions.

RNA Methylation Analysis
RNA methylation analysis was performed using Mass Spec with the m6A2010 kit. RNA was converted to labeled cDNA and sequenced using MiSeq. The reads were aligned to the UCSC mm9 sequence using TopHat (Trapnell et al., 2009). We performed the search for enriched peaks by scanning each gene using 100-nucleotide sliding windows and calculating an enrichment score for each sliding window. The peaks with the highest enrichment score were chosen. For single-colony assays, 1,000 cells were cultured per well, on a six-well plate. For alkaline phosphatase staining, cells were stained according to the manufacturer’s protocol (Vector Blue Alkaline Phosphatase Substrate Kit).

CRISPR-Mediated Mettl3 KO
Plasmids for guide RNA (design with CRISPR design tool; Hsu et al., 2013) and CRISPR-Cas9 vectors were cotransfected into mouse embryonic stem cells. After puromycin selection, cells were expanded in the presence of puromycin. For the single-colony assays and Nanog staining, 1,000 cells were cultured per well, on a six-well plate. For alkaline phosphatase staining, cells were stained according to the manufacturer’s protocol (Vector Blue Alkaline Phosphatase Substrate Kit).

analysis, slides were stained with hematoxylin and eosin (H&E) or stained by immunohistochemistry (IHC) with VECTASTAIN ABC Kit and DAB Peroxidase Substrate Kit following the manufacturer’s instructions. Analyses were performed by a boarded veterinary pathologist (D.M.B.).

ACCESSION NUMBERS

The accession numbers for the RNA sequencing data are GSE52681 (mouse) and GSE52600 (human).

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes five figures, Supplemental Experimental Procedures, six tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.09.019.

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank C. He, C. Mason, S. Schwartz, A. Regev, J.M. Claycomb, N. Van Wittenbergh, B.D. Howard, and members of the Chang and Giallourakis labs for discussions and assistance. We thank H.E. Arda and S.K. Kim for help with FACS analysis and A. Mammelar for his expertise in graphic arts. This work was supported by the California Institute for Regenerative Medicine and NIH R01-CA118750 (H.Y.C.), the MGH Start-Up Funds and MGH ECOG grant 2013A051178 (C.C.G.), NIH grant DK090122 (A.C.M.), and the Eli and Edythe L. Broad Center of Regenerative Medicine and Stem Cell Research at UCLA (Y.X.). P.J.B. is the Kenneth G. and Elaine A. Langone Fellow of the Damon Runyon Cancer Research Foundation. Y.X. is an Alfred Sloan Research Award (Y.X.). P.J.B. is the Kenneth G. and Elaine A. Langone Fellow of the Damon Runyon Cancer Research Foundation. Y.X. is an Alfred Sloan Foundation Research Fellow. H.Y.C. is an Early Career Scientist of the Howard Hughes Medical Institute.

Received: April 3, 2014
Revised: August 14, 2014
Accepted: September 30, 2014
Published: October 16, 2014

REFERENCES


Cell Stem Cell

m6A Controls Cell Fate Transitions in ESCs


