



# METTLing in Stem Cell and Cancer Biology

John G. Tooley<sup>1</sup> · James P. Catlin<sup>1</sup> · Christine E. Schaner Tooley<sup>1</sup>

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## Abstract

The methyltransferase-like (METTL) family is a diverse group of methyltransferases that can methylate nucleotides, proteins, and small molecules. Despite this diverse array of substrates, they all share a characteristic seven-beta-strand catalytic domain, and recent evidence suggests many also share an important role in stem cell biology. The most well characterized family members METTL3 and METTL14 dimerize to form an N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) RNA methyltransferase with established roles in cancer progression. However, new mouse models indicate that METTL3/METTL14 are also important for embryonic stem cell (ESC) development and postnatal hematopoietic and neural stem cell self-renewal and differentiation. METTL1, METTL5, METTL6, METTL8, and METTL17 also have recently identified roles in ESC pluripotency and differentiation, while METTL11A/11B, METTL4, METTL7A, and METTL22 have been shown to play roles in neural, mesenchymal, bone, and hematopoietic stem cell development, respectively. Additionally, a variety of other METTL family members are translational regulators, a role that could place them as important players in the transition from stem cell quiescence to differentiation. Here we will summarize what is known about the role of METTL proteins in stem cell differentiation and highlight the connection between their growing importance in development and their established roles in oncogenesis.

**Keywords** METTL · Methyltransferase · Stem cell · Cancer · Translation · Transcription · Metabolism

## Introduction

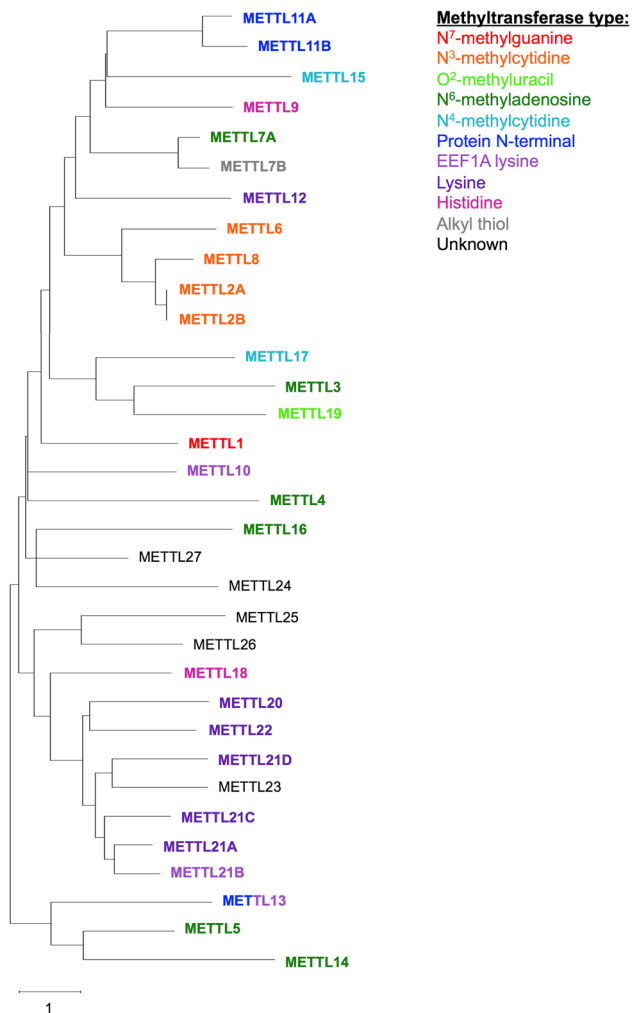
The methyltransferase-like (METTL) family of proteins is a sub-family of seven-beta-strand (7BS) methyltransferases. They, along with the SET-domain (Su(var)3–9, Enhancer-of-zeste, Trithorax) protein methyltransferases, are one of the two major families of methyltransferases [1]. Members of the METTL family are characterized by a conserved S-adenosyl methionine (SAM) binding domain that resides in part of the overall 7BS structure. While members of the SET-domain family only methylate lysine residues in proteins, members of the METTL family are capable of methylating proteins, nucleic acids, and other small molecule metabolites [2]. There are 33 members of the vertebrate METTL family [3]. Fourteen of these methylate DNA or RNA, thirteen methylate protein residues, one methylates alkyl thiol groups, and five remain of unknown function (Fig. 1)

[3–8]. While enzymes modifying similar biomolecules did not evolve from a common ancestor, they do largely group together phylogenetically (Fig. 1).

METTL proteins have a broad substrate range and have been implicated in a wide range of biological processes, including oncogenesis [11], cardiovascular disease [12], and viral replication [13] (Table 1). While their role in cancer progression is currently the best characterized, and seventeen METTL family members already have documented roles as oncogenes or tumor suppressors, eleven METTL family members have also been implicated in regulating stem cell development (Fig. 2, Table 2). Many of these studies are very recent, suggesting that additional METTL family members will be associated with stem cell regulation in the years to come. Additionally, a strong link is emerging between METTL proteins and the regulation of protein translation, a key process central to stem cell differentiation. Taken together, a growing body of literature suggests that the METTL proteins will take their place as important regulators of stem cell biology, and the focus of this review will be to summarize this expanding field.

✉ Christine E. Schaner Tooley  
ceschane@buffalo.edu

<sup>1</sup> Department of Biochemistry, Jacobs School of Medicine and Biomedical Sciences, State University of New York at Buffalo, 955 Main St., Buffalo, NY 14203, USA



**Fig. 1 Evolutionary analysis of the METTL family by Maximum Likelihood method:** The evolutionary history was inferred by using the maximum likelihood method and Le/Gascuel model [9]. The tree with the highest log likelihood (-14,505.17) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 33 amino acid sequences. All positions with less than 90% coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 175 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [10]

## METTL Proteins and Embryonic Stem Cells

### METTL3/METTL14

METTL3 and its binding partner METTL14 are currently the most extensively studied of the METTL family members. Together, they act as an N<sup>6</sup>-methyladenosine (m<sup>6</sup>A)

RNA methyltransferase [14]. METTL3 and METTL14 form a stable heterodimer [14], with METTL3 supplying the catalytic activity and METTL14 providing structural support and aiding in substrate recognition [15]. m<sup>6</sup>A marks placed on mRNA targets by the METTL3/14 complex are bound by two families of reader proteins, YTH domain-containing proteins (YTHDF1–3 and YTHDC1–2) and insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1–3) [16, 17]. As m<sup>6</sup>A is one of the most prevalent modifications of mRNA and regulates both its stability and translation efficiency [18], it is not surprising that the METTL3/14 complex has many known roles in cancer progression. It acts as an oncogene in bladder, gastric, colorectal, cervical, pancreatic, blood, liver, and breast cancers and is being developed as both a prognostic marker and therapeutic target [19–26]. This complex also acts as a tumor suppressor in thyroid and endometrial cancer [27, 28].

The roles of METTL3/14 in stem cell biology are also beginning to come to light, but these roles are complex and likely dictated by both stem cell stage and cell type [29]. An early report suggested that the METTL3/14 complex was required for mouse embryonic stem cells (mESCs) to maintain their pluripotent state and self-renewal capabilities, likely by maintaining the expression of stemness genes and repressing the expression of developmental regulators [30]. However, this paper utilized an siRNA-based knockdown (KD) approach in cultured cells, and a later paper generated conflicting results using CRISPR-mediated genetic knockout (KO) of *Mettl3* [31]. Here, the authors showed that *Mettl3* KO in mESCs lead to increased self-renewal capacity and blocked differentiation [31]. These results were supported by subsequent work showing METTL3 methylates transcripts needed to maintain pluripotency, thereby promoting their degradation and entry into differentiation [32]. However, more recent work shows that METTL3 KD promotes both pERK activation and departure from pluripotency and supports the original finding that METTL3/14 is needed for maintenance of pluripotency [33]. These conflicting results may arise from the differences between KD and KO, or from the state the cells existed in (naïve if isolated from the inner cell mass (ICM) of blastocysts or more primed if generated in culture), which would dictate time and degree of METTL3 loss or the predominant transcripts available for modification, respectively [29]. Repetition of these experiments in identical, stage-specific conditions and identification of METTL3/14 targets corresponding to each stage will help resolve these conflicts. The dual roles of METTL3/14 as both an oncogene and tumor suppressor suggest it could be similarly regulating both pluripotency and differentiation in a time/tissue-specific manner.

**Fig. 2 Summary of known oncogenic and stem cell developmental roles of METTL family members:** (A) To date, the majority of METTL proteins exhibit oncogenic activity, with some acting as both oncogenes and tumor suppressors, but none exhibiting only tumor suppressor activity. (B) There is a more even divide when it comes to stem cell development, with the majority promoting differentiation or playing dual roles in the maintenance of pluripotency and transition to a differentiated state

a			
Oncogenes	Both	Tumor Suppressors	
METTL2B	METTL1	<b>Unknown: METTL22</b>	
METTL5	METTL3/14		
METTL6	METTL4		
METTL7B	METTL11A/11B		
METTL8	METTL13		
METTL9	METTL7A		
METTL15	METTL21B		
METTL16			
METTL17			
METTL18			
b			
Pluripotency	Both	Differentiation	
METTL6	METTL1	METTL4	<b>Unknown: METTL2B METTL7B METTL9 METTL13 METTL15 METTL18 METTL21B</b>
METTL8	METTL3/14	METTL7A	
	METTL5	METTL16	
	METTL11A/11B	METTL17	
		METTL22	

## METTL1

During translation, transfer RNAs (tRNAs) serve as adaptor molecules that bridge amino acids with the corresponding mRNA sequence at specific sites in the ribosome. They are subject to numerous post-transcriptional modifications, including methylation, and disruption of these modifications can often lead to disease [34]. *N*<sup>7</sup>-methylguanosine (m<sup>7</sup>G) is one of the most common of these modifications and introduces a positive charge at the modification site. METTL1 was found to be one of the methyltransferases responsible for promoting translation and placing m<sup>7</sup>G on tRNA, mRNA, microRNA (miRNA), and ribosomal RNA (rRNA) [35–37]. METTL1 forms a heterodimer with WD repeat domain 4 (WDR4), and both are essential for m<sup>7</sup>G methylation [35]. While loss of the budding yeast METTL1 homolog, Trm8p, has a modest phenotype [38, 39], the human homolog has been implicated in double-strand DNA repair and shown to act as an oncogene in bladder cancer, hepatocellular carcinoma (HCC), acute myeloid leukemia (AML), and intrahepatic cholangiocarcinoma (ICC) and a tumor suppressor in colon cancer [40–45].

The METTL1/WDR4 complex has also been implicated in stem cell biology. In cultured mESCs, loss of either METTL1 or WDR4 by CRISPR-mediated KO results in partial spontaneous differentiation, failure to self-renew, slower

growth rates, and failure of the ectoderm to differentiate into subsequent neuronal lineages [46]. In *Mettl1* KO mESCs, there is reduced mRNA expression of the pluripotency markers *KLF4* and *Nanog*, in addition to Nanog protein, and a reduction in alkaline phosphatase (AP) positive pluripotent cells [46]. This suggests that the mESCs are failing to properly self-renew and are undergoing partial differentiation. A later paper corroborated the lineage-specification defect by showing that KD of METTL1 in human induced pluripotent stem cells (iPSCs) enhances differentiation down the mesoderm lineage pathway, while suppressing differentiation into neuroectoderm [47]. Similar to *Mettl1* KO mESCs, these cells proliferated more slowly, had a significantly reduced population of AP-positive cells, and appeared morphologically to have prematurely differentiated when cultured in growth media [47]. These data suggest METTL1 is regulating both maintenance of ESC pluripotency and differentiation of neuronal lineages. Accordingly, disruption of m<sup>7</sup>G in humans results in brain malformation, facial dimorphism, and severe encephalopathy with seizures [46, 48, 49].

## METTL5

METTL5 was recently identified as the rRNA methyltransferase responsible for placing the m<sup>6</sup>A<sub>1832</sub> mark on the eukaryotic 18S ribosomal subunit [50, 51]. Similar to other

**Table 1** Known METTL family member substrates and loss of function phenotypes

METTL Enzyme	Known substrates	Loss of function phenotype
METTL1	Internal mRNA, tRNA, miRNA, rRNA (34,35)	Ribosome pausing, protein translation (34), radiosensitivity, DNA ds-break repair (43)
METTL2A/B	tRNAThr (62)	Unknown
METTL3/14	RRACH motifs in mRNA, tRNA, rRNA, snRNA, miRNA, lncRNA (129)	Altered mRNA expression/translation (16), altered RNA stability, decay, splicing and export (171)
METTL4	snRNA, miRNA, mtDNA (110, 111, 112)	Mitochondrial function, glycolysis, Complex III activity, mtDNA copy number (112)
METTL5	18S rRNA at A <sub>1832</sub> (49)	Cardiomyocyte hypertrophy (53), proliferation, apoptosis, MMR (54), polysome number (52)
METTL6	tRNA <sup>Ser</sup> (62)	Colony formation, proliferation, migration (63)
METTL7A	lncRNA (117)	Odontogenic differentiation (119)
METTL7B	hydrogen sulfide, captopril, 7 $\alpha$ -thiospiroolactone, L-penicillamine (4)	Cell proliferation, migration, cell cycle arrest at G0/G1, apoptosis (164)
METTL8	mRNA (61) mt-tRNA <sup>Ser(UCN)</sup> , mt-tRNA <sup>Thr</sup> at C <sub>32</sub> (70)	Mitochondrial translation (70), respiratory chain activity (69), tumor growth, migration (71)
METTL9	HxH motif substrates (159) (e.g., S100A9, NDUFB3, ARMC6)	Growth, colony formation, anti-tumor immunity (154)
METTL10	eEF1A K318 (141)	Virus RNA replication (142)
METTL11A/B	X-P-K N-terminal motif (88) (e.g., RB, SET, RCC1)	Multi-polar spindles (88), altered proliferation and migration, sensitivity to DNA damage (94), fertility, mitochondrial function, liver degeneration (93), neurodegeneration (97), myogenesis (100)
METTL12	Citrate synthase K395 (160)	Unknown
METTL13	eEF1A (N-terminal and K55) (140)	Altered translation dynamics, changed translation rates of specific codons (140), cells in G0/G1, cells in S phase (149)
METTL15	mt-rRNA (m <sup>4</sup> C <sub>839</sub> ) (156)	Muscle performance, learning capabilities (103), translation, mitochondrial respiration (155), mitochondrial ribosome assembly (156)
METTL16	mRNA, lncRNA, snRNA U6 (132)	Transcriptome dysregulation (133), proliferation (135)
METTL17	mt-rRNA (m <sup>4</sup> C <sub>840</sub> , m <sup>5</sup> C <sub>842</sub> ) (64)	Translation of mitochondrial genes, oxygen consumption rate, TCA metabolites (64)
METTL18	RPL3 His245 (174)	Polysome formation, codon-specific changes in mRNA translation and pre-RNA processing (174)
METTL19	U <sub>44</sub> of tRNA <sup>Ser</sup> (5)	Unknown
METTL20	ETF $\beta$ K199 K202 (157)	Oxygen consumption (157)
METTL21A/D	A - HSP70 proteins (6) D - VCP K315 (7)	Unknown
METTL21B	eEF1A K165 (143) MKK7 K296 (175)	Invasiveness and migration (175), large ribosomal subunit production (143), altered mRNA translation (139)
METTL21C	AARS1 K943 (8) HSPA8 K561 (104) p97 K315 (105)	MEF2 protein levels in slow muscle (104), endurance capacity, autophagic vacuoles (105), myocyte fusion, osteocyte survival (106)
METTL22	KIN17 K135 (122)	Unknown

Green text indicates activation, red text indicates inhibition

METTL family members, METTL5 requires a stabilizing co-factor, TRM112, for its stability and function [50, 52, 53]. To date, the m<sup>6</sup>A<sub>1832</sub> site on the 18S subunit is the only identified substrate of METTL5 [50], though disruption of this mark has the potential to affect downstream translation of many proteins. Mechanistically, modification of the m<sup>6</sup>A<sub>1832</sub> site on 18S rRNA brings a conformational change to the decoding center of the ribosome that favors increased RNA binding, and loss of METTL5 decreases polysome number [50, 54]. Loss of METTL5 also promotes cardiomyocyte hypertrophy, apoptosis, and mismatch repair [55, 56].

Shortly following its identification, functions were ascribed to METTL5 in both promoting oncogenic growth and mESC pluripotency [54, 57]. Originally identified as promoting breast cancer growth, recent studies have shown METTL5 also promotes pancreatic cancer progression [54, 58]. mESCs with CRISPR/Cas9-mediated KO of METTL5 displayed decreased mRNA and protein levels of several core pluripotency genes, including *Klf4*, *Nanog*, *Sox2*, and *Rex1/Zfp42* [57]. Contrastingly, they also exhibited a reduced differentiation potential, specifically in the neuroectodermal and mesodermal lineages [57]. A later

**Table 2** METTL family members with known roles in cancer and/or stem cell regulation

METTL Family member	Enzymatic function	Cancer Type (s)	Stem Cell Type(s)
METTL1	N7-methylguanine	Bladder (38), HCC (39), AML (41), Colon (40), ICC (42)	mESC (44), Neuroectoderm/mesoderm (44, 45)
METTL2B	N3-methylcytidine	Gastric (138)	
METTL3/METTL14	N6-methyladenosine	Bladder (18), Gastric (19), Colorectal (20), Cervical (17), Thyroid (25), Pan- creatic (22), Leukemia (23), HCC (24), Breast (21), Endometrial (26)	mESC (28,29), HSC (77,78), NSC (82,83), Muscle (84), Dental pulp (86), Mesenchymal (85)
METTL4	N6-methyladenosine	Sarcoma (115), Melanoma (116)	Adipose (114)
METTL5	N6-methyladenosine	Breast (52), Pancreatic (56)	mESC (55)
METTL6	N3-methylcytidine	HCC (63)	mESC (60)
METTL7A	N6-methyladenosine	Choriocarcinoma (120), Myeloma (117), Breast (121)	Bone (118), Dental pulp (119)
METTL7B	alkyl thiol methylation	Glioma (162), Thyroid (163), ccRCC (164), Breast (165), NSCLC (166)	
METTL8	N3-methylcytidine	Lung (71)	mESC (72)
METTL9	Histidine methylation	HCC (153)	
METTL11A/B	N-terminal protein methylation	Breast (94), Cervical (95), Colon (96)	Neural (97), Muscle (100), Bone (101)
METTL13	eEF1A methylation N-terminal and lysine)	ccRCC (148), Bladder (149), HNSCC (145), Pancreatic and Lung (147)	
METTL15	N4-methylcytidine	Lung (173)	
METTL16	N6-methyladenosine	Gastric (135), HCC (136)	mESC (133)
METTL17	N4-methylcytidine	Breast (67)	mESC (64)
METTL18	Histidine methylation	HCC (172)	
METTL21B	eEF1A methylation	Glioma (146), Gastric (175)	
METTL22	Lysine methylation		HSC(123)

*HCC*, hepatocellular carcinoma; *AML*, acute myeloid leukemia; *ICC*, intraphepatic cholangiocarcinoma; *mESC*, mouse embryonic stem cells; *HSC*, hematopoietic stem cell; *ccRCC*, clear cell renal cell carcinoma; *NSCLC*, non-small cell lung cancer; *HNSCC*, head and neck squamous cell carcinoma

paper supported the differentiation phenotype, showing *Mettl5* KO mESCs did not properly respond to differentiation signals [59]. They ascribed this phenotype to a ribosomal defect that reduced translation of the differentiation regulator FBXW7 [59]. In the absence of FBXW7, *c-Myc* expression remained elevated even when cells were induced to differentiate [59]. These data indicate that, similar to METTL3/14 and METTL1, METTL5 is playing a role in both pluripotency and differentiation.

Interestingly, *Mettl5*-deficient mice are subviable, as they exhibit non-Mendelian birth rates [53, 57]. They also exhibit reduced body weight, eye and craniofacial abnormalities, male infertility, intellectual disabilities, and hyperactivity [57, 60]. It is thought the intellectual disabilities result from abnormal myelination [60]. In zebrafish, knockdown of METTL5 results in reduced brain size [61], and human patients with frameshift variants of METTL5 have been shown to have intellectual disabilities, microcephaly, and facial dimorphisms [61].

### METTL6, METTL17, and METTL8

METTL6 is an N<sup>3</sup>-methylcytidine (m<sup>3</sup>C) methyltransferase that methylates tRNA and regulates tumor growth and pluripotency [62–64]. In HCC cells, METTL6 promotes oncogenic phenotypes by increasing expression of cell adhesion-related genes [65]. In mESCs, deletion of *Mettl6* limits pluripotency, resulting in flat, differentiated-like cells and a decrease over time of AP-positive cells [62]. Not surprisingly, RNA-seq analysis of these cells demonstrated a decrease in pluripotency gene expression (*Rex1*, *Klf4*, *Esrrb*, *Tbx3*, *Dppa3*) [62]. Concomitantly, there was an increase in gastrulation (*Fgf5*, *Lefty1*, *Nodal*), mesoderm (*Brachyury*), and ectoderm (*Nestin*) markers [62]. *Mettl6* KO mice, while found to have decreased liver weight, altered glucose homeostasis and metabolic imbalance, were viable and exhibited no gross morphological phenotypes [62], indicating the role of METTL6 in stem cell development may ultimately be tissue-specific or redundant with that of other family members.

METTL17 is an N<sup>4</sup>-methylcytidine (m<sup>4</sup>C) methyltransferase in the mitochondria that methylates C<sub>840</sub> and C<sub>842</sub> of 12S mt-rRNA to promote its stability [66]. Loss of METTL17 results in defective mitochondrion assembly and decreased protein translation [66]. A recent study has shown METTL17 is required for mESC differentiation [66]. *Mettl17* KO in mESCs negatively affects their ability for oxidative phosphorylation (oxphos) and inhibits their differentiation [66]. Specifically, *Mettl17* KO mESCs have a significant decrease in basal oxygen consumption rate (OCR) and reduced levels of the TCA intermediate metabolites citrate and isocitrate [66]. These cells also have a dramatic increase in the ratio of reduced to oxidized forms of  $\gamma$ -glutamyl-cysteinyl-glycine (GSH) [66]. It is hypothesized that the protein translation defects seen in *Mettl17* KO mESCs lead to the disruption of oxphos because components of the electron transport chain (ETC) are translated by the mitochondrial ribosome [66]. Genes that control cellular metabolism are commonly found to regulate stem cell development, as ESCs rely heavily on anaerobic glycolysis and somatic cells favor aerobic metabolism, indicating the transition from glycolysis to oxphos is imperative for the transition from ESC to differentiated cell [67]. *Mettl17* was also identified as a gene essential for oxphos in human myelogenous leukemia K562 cells [68], and its knockdown in breast cancer cells reduces proliferation [69].

METTL8 was originally identified as the first methyltransferase to place the m<sup>3</sup>C modification on mRNA in mammalian systems [63]. However, subsequent studies have shown it can also act as a mitochondrial tRNA methyltransferase that performs m<sup>3</sup>C<sub>32</sub> methylation of mt-tRNA<sup>Thr</sup> and mt-tRNA<sup>Ser(UCN)</sup> [70–72]. Through its mtRNA m<sup>3</sup>C<sub>32</sub> methylation activity, METTL8 promotes mitochondrial translation and respiratory chain activity similar to METTL17, and as such, acts as an oncogene [71, 73]. METTL8 has high expression in multiple lung cancer cell lines and lung squamous cell carcinomas and promotes proliferation [73]. In contrast, during mESC development, METTL8 promotes pluripotency, and this seems dependent on its mRNA methylation activity [74]. *Mettl8* transcripts are positively regulated by STAT3, a transcription factor that has been shown to be crucial for maintaining the pluripotency of ES cells [75–78]. METTL8 promotes pluripotency by negatively regulating the JNK signaling pathway and impeding differentiation [74]. Specifically, METTL8 binds the mRNA of MAPKBP1, a key component of the JNK signaling cascade, and prevents its translation [74]. Levels of *Mapkbp1* mRNA translation and lineage marker expression are increased following METTL8 loss, though whether this results directly from methylation of *Mapkbp1* mRNA by METTL8 remains to be determined [74]. Increased expression of METTL8 alone is not strong enough to promote pluripotency, indicating it too may be acting as part of a larger complex [74].

## METTL Proteins and Tissue-specific Stem Cells

### METTL3/METTL14

In addition to its role in ESC regulation, the METTL3/14 complex has also been shown to be critical in later postnatal stem cell development. One of the best-studied examples is in the hematopoietic system. Arising from the endothelial lineage, hematopoietic stem cells (HSCs) are lineage-committed and give rise to all cell types of the blood. Conditional KO of *Mettl3* in mouse adult bone marrow causes HSCs to prematurely exit the quiescent state, indicating METTL3 is needed to retain quiescence [79]. Similarly, METTL14 loss in primed hematopoietic stem and progenitor cells (HSPCs) promotes their terminal differentiation [80]. Mechanistically, it is hypothesized that m<sup>6</sup>A methylation by METTL3/14 promotes degradation of transcripts needed for differentiation, and it has been recently shown that long-term HSCs have a subset of mRNAs involved in pluripotency (*Kit*, *Hoxa5*, *Gata2*, *Cttnb1*, *Ldb1*, *Hoxb4*) with nearly undetectable levels of m<sup>6</sup>A modification and high mRNA expression [81]. Conversely, they also have a subset of mRNAs involved in differentiation (*Eomes*, *Bcl6*, *Prmt6*) with high levels of m<sup>6</sup>A modification and low levels of mRNA expression [81], indicating METTL3/14 helps retain HSC quiescence by preventing transcription of differentiation factors.

The METTL3/14 complex is also strongly implicated in neural stem cell (NSC) biology, as the brain contains the highest abundance of m<sup>6</sup>A RNA among human tissues [82–84]. Similar to HSCs, transcripts involved in NSC differentiation and highly expressed in late intermediate progenitor cells (IPCs) and post-mitotic neurons have high m<sup>6</sup>A methylation [85]. However, in contrast to HSCs, many transcripts involved in NSC pluripotency are also subject to m<sup>6</sup>A methylation [85]. Accordingly, conditional KO of *Mettl14* during embryogenesis using the Nestin-Cre promoter (*Mettl14*<sup>flox/flox</sup>; *Nestin-Cre*, referred to as *Mettl14* cKO) results in prolonged retention of a pluripotent NSC pool that abnormally expresses neuronal proteins [85]. These data indicate both NSC pluripotency and differentiation are affected by loss of m<sup>6</sup>A. In addition, *Mettl14* cKO animals are much smaller than their control littermates and have enlarged lateral ventricles [85]. *Mettl3* conditional KO mice (*Mettl3*<sup>flox/flox</sup>; *Nestin-Cre*, referred to as *Mettl3* cKO) have similar phenotypes, including significantly smaller brains, and ventricle enlargement [84]. These animals also exhibit tremors, balance problems, uneven gait, feet and hand clapping in tail hold test, and decreased foliation and disorganization of the layers contained within the cerebellum [84].

New evidence indicates METTL3/14 is also important for retaining quiescence in skeletal muscle myoblasts,

dental pulp stem cells, and human mesenchymal stem cells (hMSCs) [86–88]. In myoblasts, levels of METTL3 have been shown to markedly decline as they enter the differentiation process (both in C2C12 cultured myoblasts and primary myoblasts) [86]. Functionally, the loss of METTL3 causes premature differentiation in C2C12 myoblasts, with increases in myogenic differentiation 1 (*MyoD*) (~ twofold) and myogenin (~ 18-fold) mRNA in cells grown in high serum media [86]. In pulp cells, KD of METTL3 results in an increase in senescence and apoptosis [88]. Similarly, in hMSCs, KO of *Mettl3* causes an acceleration of senescence and premature aging phenotypes [89]. This is due, at least in part, to loss of the m<sup>6</sup>A mark on the cell cycle regulator *Mis12* [89]. Taken together, these data indicate m<sup>6</sup>A methylation by METTL3/14 generally protects the quiescent state of tissue-specific stem cells but may promote differentiation in a subset of tissues as well. This is likely controlled by the availability of substrates in each tissue at each developmental stage.

### METTL11A/METTL11B

METTL11A, also known as N-terminal RCC1 methyltransferase 1 (NRMT1), is the first identified eukaryotic N-terminal methyltransferase [90]. It is a ubiquitously expressed trimethylase that acts on the N-alpha (N $\alpha$ ) amino group of proteins with a defined consensus sequence [91]. Based on this consensus sequence, it is predicted that METTL11A methylates more than 300 targets [91]. N $\alpha$ -methylation has been shown to regulate protein/DNA interactions, and many of its targets are involved in chromatin assembly, transcription, or DNA damage repair [90, 92–94]. Accordingly, loss of METTL11A results in a variety of oncogenic and developmental phenotypes [95, 96]. METTL11A acts as a tumor suppressor in breast cancer cells, as its KD results in increased cell proliferation, migration, and xenograft growth [96]. It can also serve as an oncogene, promoting migration of cervical cancer cells and growth of colorectal cancer cells [97, 98]. Developmentally, *Mettl11A* KO mice demonstrate premature aging phenotypes, including early graying, kyphosis, neurodegeneration, and impaired mitochondrial function [95, 99].

To begin to understand the molecular mechanism behind the neurodegeneration seen in *Mettl11A* KO mice, molecular analysis of the subventricular zone (SVZ) NSC niche was performed [99]. It was found that, at birth, *Mettl11A* KO mice had significant expansion of the quiescent NSC pool in the SVZ [99]. However, by postnatal day 14 (P14), this NSC pool became depleted due to premature differentiation into intermediate progenitor cells (IPCs) and neuroblasts [99]. The resulting neuroblasts differentiated into mature neurons, but many of these neurons were unable to completely exit the cell cycle and ultimately underwent apoptosis [99]. The

observed NSC differentiation phenotypes were reminiscent of those seen with loss of the METTL11A target retinoblastoma protein (RB) [100, 101], and it was shown that RB phosphorylation and function are impaired in *Mettl11A* KO mice [99]. These data indicate that, in neuronal cells, METTL11A is necessary for inhibiting the cell cycle during both quiescence and terminal differentiation.

A separate study identified cAMP-response element binding protein 1 (CREB1) as one of the primary transcription factors driving *Mettl11A* expression [102]. Screening for conditions of CREB1-induced *Mettl11A* expression identified serum starvation and C2C12 mouse myoblast differentiation [102]. Accordingly, CRISPR/Cas9-mediated *Mettl11A* KO in C2C12 cells inhibited myoblast differentiation. In growth media, the *Mettl11A* KO cells failed to upregulate expression of the master regulator of postnatal myogenesis, *Pax7*. After three days of culturing in low-serum differentiation media, they also failed to upregulate the downstream myogenesis markers *MyoD* and myogenin [102]. As myoblasts are derived from a mesenchymal stem cell progenitor pool, and could potentially transdifferentiate into osteoblasts, adipocytes, or chondrocytes, markers of these lineages were assayed. While expression of *Sox9* (chondrocytes) and *Ppar $\gamma$*  (adipocytes) was unchanged in the KO cells, the osteoblastic markers alkaline phosphatase, *Runx2*, osteocalcin, osterix, and type 1 collagen were significantly increased [102]. These data indicate that, in myoblasts, METTL11A is necessary for responding to differentiation cues and undergoing myogenesis. The closest METTL11A homolog, METTL11B, is a tissue-specific N $\alpha$ -monomethylase that is upregulated during osteocytic and myogenic differentiation [103]. Similar to METTL3/METTL14, METTL11A and METTL11B complex together and METTL11B activates METTL11A activity [104], indicating METTL11A and METTL11B could be working synergistically to regulate mesenchymal stem cell differentiation. Interestingly, loss of METTL15 and METTL21C produce similar muscle phenotypes to loss of METTL11A [105–108], indicating they too could be METTL11A interactors.

Similar to METTL3/14, METTL11A appears to regulate pluripotency or differentiation differently in different tissues, and this could also be due to substrate availability. Though studies in NSCs indicate that loss of methylation of the METTL11A target RB is contributing to the observed phenotypes, METTL11A has numerous other targets that may predominate in other tissues. Many of these are transcription factors, but interestingly, the budding yeast homolog of METTL11A, TAE1, was initially identified in a screen for genes that alter protein synthesis rates [109]. Deletion of *Tae1* in yeast results in decreased polysome formation and downstream impairment of translation efficiency [109], and METTL11A is known or predicted to methylate numerous ribosomal proteins, including RPL23a, RPS25, and RPL12

[90, 110]. These data indicate METTL11A and METTL11B could also be regulating stem cell development through translational regulation, like many of the above-mentioned METTL family members.

### METTL4, METTL7A, and METTL22

METTL4 is an additional m<sup>6</sup>A methyltransferase that is conserved from yeast to humans [111]. Though, to date, human METTL4 has only been shown to methylate small nuclear RNA (snRNA), miRNA, and mitochondrial DNA (mtDNA) [112–114], the *C. elegans* and *M. musculus* homologs of METTL4 also have genomic DNA m<sup>6</sup>A activity [115, 116]. In humans, m<sup>6</sup>A methylation of snRNA by METTL4 is important for regulation of RNA splicing, m<sup>6</sup>A methylation of miRNA affects mRNA repression efficacy, and m<sup>6</sup>A methylation of mtDNA promotes mitochondrial function [111, 113, 114]. METTL4 promotes melanoma cell growth, but is predicted to act as a tumor suppressor in soft tissue sarcomas [117, 118]. In mice, m<sup>6</sup>A DNA methylation by METTL4 has been shown to be important for adipogenesis [116]. Loss of METTL4 in 3T3-L1 mouse preadipocytes inhibits their ability to properly differentiate, as they have lowered expression of the major adipogenic factors and decreased lipid production [116]. It also resulted in decreased glucose uptake and consumption, due to decreased expression of the insulin receptor gene (*Insr*) [116]. It is hypothesized that m<sup>6</sup>A DNA methylation of promoter regions is necessary to promote transcription of genes that drive differentiation [116].

METTL7A is an m<sup>6</sup>A methyltransferase known to methylate both DNA and long non-coding RNAs (lncRNAs) [119, 120]. It was identified in an RNA-seq screen as a gene associated with enhanced osteogenesis and cell survival in human bone marrow stem cells (hBMSCs) [120]. Knockdown of METTL7A reduced osteogenic differentiation as determined by Alizarin Red staining, while overexpression of METTL7A could reverse the differentiation defects seen in glucose-free conditions [120]. Similarly, METTL7A knockdown in human dental pulp stem cells (DPSCs) inhibited their differentiation, while overexpression enhanced odontogenic differentiation [121]. It is hypothesized that DNA methylation by METTL7A promotes osteogenic and odontogenic differentiation by upregulating expression of genes that promote differentiation and survival [120]. METTL7A has also been shown to act as an oncogene in multiple myeloma and choriocarcinoma cells and a tumor suppressor in breast cancer cells [119, 122, 123].

METTL22 is a lysine methyltransferase with one known target, the DNA-binding protein Kin17 [124]. Methylation of lysine 135 in Kin17 by METTL22 impairs the binding of Kin17 to chromatin and promotes its cytoplasmic localization [124]. Though little is known of its targets and biological function, METTL22 emerged as one

of the top candidates in a genome-wide RNAi screen for genes required for the differentiation of ESCs into HSPCs and is involved in later steps of HSPC specification [125]. Knockdown of METTL22 in zebrafish reduces the expression of *Runx1* and *c-myb* during embryonic HSC development, while METTL22 knockdown in human cord blood CD34+ cells led to a marked reduction in HSPCs [125]. It remains uncertain whether the role of METTL22 in HSPC specification is through its methylation of Kin17 or additional unknown protein targets [125].

### METTL Proteins and Translation in Stem Cells

In recent years there has been a growing appreciation of the role translational control plays in stem cell biology [126, 127]. Differentiation of pluripotent stem cells represents a state change for the cell and often requires a rapid response. As transcriptional change is not immediate, cells often rely on post-transcriptional (translational) control of protein levels to quickly respond to differentiation cues [128]. As far back as 2008, it was appreciated that mESCs, in addition to upregulating transcription of genes during differentiation, also greatly upregulated translation [129]. Protein levels remain low in undifferentiated, pluripotent mESCs, but upon removal of LIF many notable changes were observed [130]. The majority of genes studied (76%) showed an increase in both transcriptional and translational upregulation, and a small subset (~2%) that showed no increase in transcript abundance did show upregulated translation [130]. Moreover, very few transcripts showed translational downregulation during differentiation [130]. Taken together, this study indicated that ESCs seem to inhibit translation of nascent transcripts as a way to remain poised to rapidly respond to different differentiation cues.

Many METTL proteins play a role in translational regulation, and this could contribute to their role in stem cell development. m<sup>6</sup>A methylation is one of the most prominent and well-studied modifications made on RNA. It occurs on RRACH motifs and in mRNA is mostly enriched in the coding sequences (CDS) of long internal exons, 3' untranslated regions (UTRs), and near stop codons [82, 131, 132]. It is thought to promote mRNA degradation when in non-coding regions, and alternatively, promote translation when in coding regions [18, 82]. m<sup>6</sup>A methylation promotes translation by facilitating the recruitment of translation initiation factors through its YTHD readers [18, 133]. In addition to those discussed already (METTL3/14, METTL4, METTL5 and METTL7A), METTL16 is also an m<sup>6</sup>A methyltransferase that methylates mRNA, lncRNA, and snRNA [134]. It is essential for embryonic development in mice [135], and its expression is induced as mESCs are differentiated into



cardiomyocytes [136]. It also works as an oncogene in gastric cancer and hepatocellular carcinoma [137, 138].

m<sup>3</sup>C modification has also been implemented in translational control, hypothesized to be one of the modifications promoting translation efficiency by maintaining the conformation of the anticodon loop, enhancing codon interactions, and preventing frameshifts [139]. *Mettl8* KO in HCT116 cells alters the polysome to monosome ratio, which suggests increased ribosomal stalling with loss of METTL8, and loss of METTL6 also affects translation [63, 139]. In addition to METTL8 and METTL6, METTL2A and METTL2B are also m<sup>3</sup>C methyltransferases [64]. Little is known about the biological functions of METTL2A and METTL2B, though METTL2B is a predicted prognostic marker in gastric cancer [140]. Given their similar enzymatic functions to METTL6 and METTL8, it is likely their part in translational regulation and stem cell development will eventually be uncovered.

It is also interesting to note that three of the METTL family proteins (METTL10, METTL13, METTL21B) have now been identified as enzymes that methylate human eukaryotic elongation factor 1 alpha (eEF1A), the second most abundant protein in the cell and a critical player in translational control [141–143]. METTL10 trimethylates eEF1A on lysine 318 [143, 144], and METTL21B methylates lysine 165 [141, 145]. METTL13 methylates both the N $\alpha$ -amino group and lysine 55 (K55) of eEF1A [142]. While the role of eEF1A in stem cells is largely unstudied, it has been shown to regulate primordial germ cells (PGCs) in the sea urchin embryo [146]. Here, eEF1A mRNA is regulated in its 3'UTR by the translational repressor Nanos, in a manner that excludes it from the PGCs early in development and keeps translation rates low [146]. Moreover, cancer cells are often thought to hijack the machinery used to maintain stem cell populations, and both METTL13 and METTL21B are overexpressed in certain cancer types [147, 148]. It has been shown that methylation of eEF1A by METTL13 upregulates protein translation to sustain the needs of rapidly proliferating cancer cells, but METTL13 has also been shown to act as a tumor suppressor [149–151]. It is possible METTL13 will also play dual roles during stem cell development.

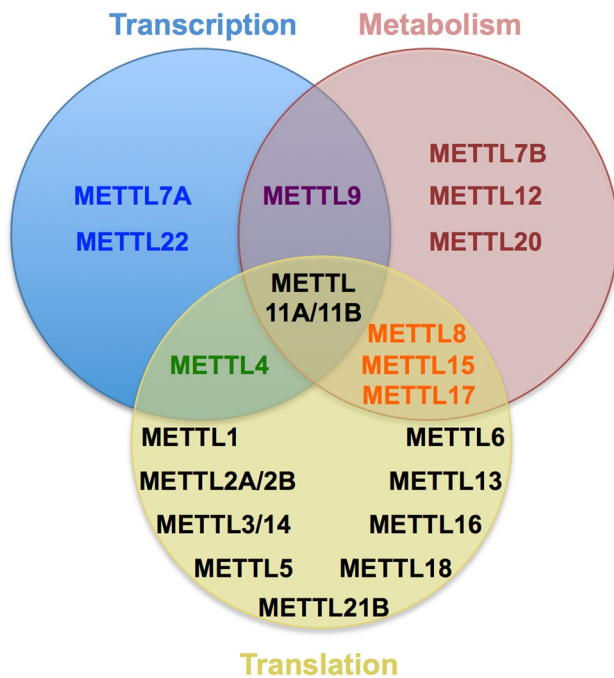
## Conclusions

Given the extensive roles of METTL family members in cancer progression, it is not surprising that corresponding roles in stem cell biology are beginning to emerge (Table 2). Both cancer cells and stem cells are unique in being able to retain the ability to re-enter the cell cycle from a quiescent state and undergo proliferation [152]. Therefore, proteins that regulate cellular process that support and/or repress proliferation are strong candidates for playing a role

in both development and oncogenesis. One obvious prerequisite for altering proliferative growth is transcriptional regulation of genes that control the cell cycle, and a variety of METTL proteins target transcription factors or DNA directly. METTL11A methylates RB and inhibits cell cycle progression [99]. METTL22 promotes the DNA-binding of KIN17, and KIN17 has recently been found to activate p38 MAPK signaling, a pathway known to regulate cell cycle [124, 153, 154]. METTL9, a histidine methyltransferase overexpressed in HCC [155], methylates the zinc transporter SLC39A7, and its loss impairs zinc homeostasis and alters gene expression [156], indicating diminished function of zinc-responsive transcription factors. DNA methylation by METTL7A is thought to regulate the transcription of genes involved in pluripotency, and in mice, DNA methylation by METTL4 is thought to regulate transcription of differentiation factors [116, 120].

Another prerequisite for proliferative growth is increased uptake and catabolism of nutrients [152], and there are also METTL genes that regulate metabolism. Loss of METTL8, METTL17, METTL15, METTL20, METTL11A, and METTL9 all decrease mitochondrial respiration. METTL8 and METTL17 regulate respiration by promoting translation of ETC complex members [66, 71]. METTL15 methylates 12S mt-rRNA, promoting both mitochondrial ribosome assembly and respiration [157, 158]. METTL20 methylates the  $\beta$ -subunit of the electron transfer flavoprotein (ETF $\beta$ ) and promotes oxygen consumption, presumably by regulating the interaction of ETF $\beta$  with acyl-CoA dehydrogenase [159]. METTL11A regulates transcription of liver metabolic genes [95, 160], and methylation by METTL9 activates NDUFB3 activity, a subunit of mitochondrial respiratory Complex I [161]. METTL12 methylates K395 of citrate synthase [162], and METTL7B is an alkyl thiol methyltransferase [4]. METTL7B is upregulated in many cancers and involved in hydrogen sulfide metabolism, which regulates both EGFR/ERK/MMP-2 and PTEN/AKT signaling [4, 163–168]. These pathways also regulate stem cell development, providing a potential role for METTL7B in this area as well [169–171].

Finally, as mentioned above, translational upregulation is necessary for both oncogenic growth and the onset of differentiation, and many METTL family proteins have roles in the regulation of translation. The m<sup>6</sup>A activity of METTL3/14, METTL4, METTL5, and METTL16 is necessary for many aspects of RNA metabolism, including translation, degradation, splicing, export, and folding [172], and all have been found to be misregulated in human cancers (Table 2) [20, 54, 118, 122, 138, 173]. METTL1, METTL6, METTL8, METTL11A/11B, METTL13, METTL15, METTL17, METTL18, and METTL21B are all also involved in protein translation and play a role in oncogenic growth (Table 2) [65, 73, 96, 148, 149, 158, 174–177]. While the primary role of many METTL proteins is translational regulation,



**Fig. 3 Mechanisms shared by cancer and stem cells to exit quiescence and begin proliferation:** To re-enter the cell cycle both cancer and stem cells have to alter their transcriptional programs, activate metabolism, and increase protein translation. METTL family members play roles in all three of these processes, and some have overlapping roles in more than one. These roles are substrate-specific and are likely to expand as additional METTL family targets are identified

some also have overlapping roles in transcriptional and/or metabolic regulation (Fig. 3). These roles appear to be substrate-specific and are likely to expand as additional targets are identified. Taken together, it is not surprising that the diverse family of METTL proteins is playing roles in both stem cell development and cancer growth, but it is important to recognize and more comprehensively understand these dual roles to better understand the mechanism, efficacy, and safety of METTL-targeted therapeutics.

### Future Directions

Until recently, focus in the METTL field has been directed at assigning specific activities to each methyltransferase. Now that only five members remain unclassified, focus will begin to shift to a more comprehensive analysis of substrate identity, and this will help better clarify the functions of the METTL family members in retaining pluripotency and promoting differentiation. Many METTL methyltransferases still have only one known target, which limits understanding of how they affect these processes. It is likely that over time the substrate list of each enzyme will greatly expand, and the challenge in the field will be to discern which substrate is most important at each

developmental stage and in each tissue type. For example, we have shown that loss of METTL11A in the brain results in depletion of the neural stem cell pool and this correlates with misregulation of the cell cycle and the NRMT1 target RB [178]. However in the liver, loss of NRMT1 impairs the function of the transcription factor ZHX2 and increases Gpc3 expression, which could promote expansion of the hepatic stem cell pool [179]. Therefore, the effects of METTL11A loss could differ and produce opposing results due to the specific substrate driving each phenotype. It is also likely that additional METTL11A complex members could be tissue-specific and the exchange of interactors, regulators, and readers could fine-tune downstream signaling targets to result in different phenotypic outcomes.

Finally, in addition to tissue-specific functions, there is new evidence that METTL proteins also have cell compartment-specific functions. METTL16, which was initially identified as a nuclear m<sup>6</sup>A writer with hundreds of mRNA targets, is now known to have an additional cytoplasmic role promoting translation by facilitating the assembly of the translation-initiation complex [138]. This function is independent of its methylation activity [138]. The dual substrate specificity of METTL8 has been explained by isoforms that are specific to either the mitochondria or the nucleolus [180], and we have recently found that METTL11A, previously thought to be primarily a nuclear protein, interacts with and regulates METTL13 in the cytoplasm (unpublished data). We are currently working to better understand the phenotypic consequences of this new cytoplasmic role of METTL11A. In summary, production of a more comprehensive and detailed “road map” of METTL substrates, interactions, and localization will provide the bigger context in which METTL proteins are acting and should help resolve current data conflicts in the field.

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### Declarations

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