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METTL4 is an snRNA m⁶Am methyltransferase that regulates RNA splicing

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30 **Running title:** U2 snRNA is m⁶Am methylated by human METTL4

Dear editor,

Protein coding mRNAs and non-coding RNAs (e.g., rRNAs, snRNAs and miRNAs) are extensively modified by chemical modifications¹, including methylation of the N⁶ position of adenosine (m⁶A)². While mRNA internal m⁶A methylation is mediated by METTL3 and METTL14, which are members of the MT-A70 family², N⁶-methylation of cap-adjacent N⁶,2'-O-dimethyladenosine (m⁶Am) is catalyzed by PCIF1³⁻⁶. Non-coding RNAs are also m⁶Am methylated internally⁷, but the corresponding enzyme remains unknown. Here we provide evidence that another member of the MT-A70 family, METTL4, mediates m⁶Am methylation of non-coding RNA, specifically, the U2 small nuclear RNA (snRNA).

METTL4, like its paralogs METTL3/14, is conserved from yeast to human (**Fig. 1a**)⁸. METTL4 homologs in *C. elegans* and mouse embryonic stem cells (mESCs) have been suggested to regulate DNA 6mA methylation⁹⁻¹¹, but in both instances, biochemical evidence is lacking to demonstrate that METTL4 has intrinsic DNA 6mA methyltransferase activity. To further investigate the exact roles of METTL4, we first characterized the localization of METTL4 and found that METTL4 carries a well-conserved, putative nuclear localization signal (NLS) in the N-terminal region (**Supplementary information, Fig. S1a**). Consistently, we found mainly nuclear localization of an exogenously introduced METTL4 fused to a FLAG tag at its C-terminus (**Supplementary information, Fig. S1b**). Next, we generated METTL4

knockout (KO) 293T cell lines using CRISPR-Cas9 (**Supplementary information, Fig. S1c**). Unexpectedly, we found that METTL4 KO significantly reduced internal m⁶Am levels in the total RNA (**Fig. 1b**). Importantly, the internal m⁶Am can be readily rescued by wildtype but not the catalytically compromised METTL4 (METTL4^{APPA}: ₂₈₇DPPW₂₉₀ mutated into ₂₈₇APPA₂₉₀), which suggests that this modifying activity is likely to be intrinsic to METTL4 (**Fig. 1c; Supplementary information, Fig. S1d and S1e**). In contrast, METTL4 KO or re-expression of an exogenously introduced METTL4 has no overt impact on 6mA level (near background) in the genomic DNA of 293T cells (**Supplementary information, Fig. S2a**). Furthermore, we failed to identify appreciable levels of 6mA in mitochondrial DNA (mt-DNA) or m⁶Am in mitochondrial RNA (mt-RNA), respectively (**Supplementary information, Fig. S2b and S2c**).

Previous studies demonstrated that PCIF1 is responsible for N⁶-methylation of the cap-adjacent m⁶Am and this m⁶Am is resistant to nuclease P1 cleavage unless the RNA is pre-treated with RNA 5' Pyrophosphohydrolase (RppH) to remove pyrophosphate from the 5' end of the triphosphorylated RNA³⁻⁶. We treated total RNA with or without RppH before subjecting them to nuclease P1 digestion and monitored the changes of m⁶Am levels (**Supplementary information, Fig. S3a**). As shown in **Fig. S3b and S3c**, METTL4 depletion induces a drastic decrease of internal m⁶Am but doesn't affect the level of the cap-adjacent m⁶Am. Conversely, KO of the cap-adjacent m⁶Am methyltransferase, PCIF1, has no impact on the level of the

internal m⁶Am. These findings suggest that METTL4 and PCIF1 represent distinct m⁶Am methyltransferases targeting different RNA types or RNA regions.

We next set out to identify the RNA m⁶Am substrate(s) for METTL4. Interestingly, m⁶Am signal is significantly enriched in RNAs less than 200 nucleotides (nt) in length but not in RNAs longer than 200 nt, including polyA(+) RNA and ribosomal RNAs (18S and 28S) (**Supplementary information, Fig. S4a**). To further identify the type of small RNA that is m⁶Am methylated, we separated small RNAs by electrophoresis and performed Northwestern with m⁶A-specific antibodies. We found that a band migrating at around 150 nt is absent upon METTL4 KO, and importantly this signal can be restored by re-expression of an exogenously introduced METTL4 (**Fig. 1d**, band indicated by an arrow), suggesting that m⁶A(m) methylation of the yet-to-be-identified RNA species is dependent on METTL4. Consistently, LC-MS/MS analysis showed that RNAs in the length range of 125-225 nt are m⁶Am but not m⁶A methylated (**Supplementary information, Fig. S4b and S4c**). As a control, a prominent band around 70 nt was found to be unaffected by METTL4 depletion (**Fig. 1d**). Together, these findings support the hypothesis that METTL4 can mediate internal m⁶Am methylation of certain small RNAs.

To determine the identities of the m⁶Am-methylated RNA species, we carried out RNA-seq of the RNAs in the size range of 125 nt to 225 nt purified from the gel slice,

and found that 5.8S rRNA, SNORA73 snoRNA and U2 snRNA, were ranked as the top three most abundant RNAs migrating at around 150 nt (**Fig. 1e**). In order to determine which RNA(s) carries the internal m⁶Am, we purified 5.8S rRNA, SNORA73 snoRNA, U2 as well as U1 snRNAs (which was suggested to be m⁶Am methylated by a recent study¹²) with biotin-labeled synthetic complementary DNA oligos, respectively, and quantified the amounts of m⁶Am carried by these four different RNAs (detailed procedure in **Supplementary information, Fig. S4d**, U2 snRNA was shown as an example). **As shown in Fig. 1f, a considerable amount of m⁶Am was detected only in the U2 snRNA, and there is essentially no m⁶Am signal detectable in the 5.8S rRNA, SNORA73 snoRNA or U1 snRNA, suggesting that U2 snRNA is modified by internal m⁶Am and that METTL4 may be the responsible enzyme *in vivo*.** This finding is consistent with a four-decades-old discovery that adenosine of U2 snRNA at position 30 is possibly decorated by m⁶Am modification¹³, and a more recent transcriptomic single-nucleotide resolution mapping of m⁶Am suggesting that U2 snRNA may carry internal m⁶Am modification⁷.

To interrogate the hypothesis that METTL4 may mediate internal m⁶Am methylation on U2 snRNA, we investigated the enzymatic activity of the recombinant human METTL4 by carrying out *in vitro* assays using a 35 nt long RNA of human U2 as a substrate (nt 6 to 40). Recombinant METTL4 protein was incubated with multiple RNA oligos representing different substrates (detailed in Methods) in the presence of stable isotope-labeled S-adenosylmethionine (*d3*-SAM) as a methyl group donor

(**Supplementary information, Fig. S5a and S5b**), and then the RNA oligos were isolated for LC-MS/MS analysis. We found that the enzymatic activity of METTL4 for the U2 snRNA Am30 probe was approximately 100-fold higher than that for the A30 probe, suggesting that 2'-O-methylation on the ribose ring is necessary for METTL4 to mediate methylation on the N⁶ position (compare probe A30 and Am30 in **Fig. 1g; Supplementary information, Fig. S5b**). Interestingly, this methylation event is drastically reduced when the neighboring A29 or G31 was changed to other bases (refer to probes AAmA and GAmG in **Fig. 1g**). In addition, only low, near background level of methylation was observed when DNA oligos, Cap-Am or GACU RNA oligos (optimal substrates of METTL3/14) were used as substrates (Cap-Am and GACU probe in **Fig. 1g**; DNA probe in **Supplementary information, Fig. S5c**). Consistent with the *in vivo* results, this enzymatic activity is intrinsic to METTL4 as mutation of the catalytic motif in METTL4 abrogated its ability to methylate the U2 snRNA probe (**Supplementary information, Fig. S5d**). We next determined the K_m (Michaelis constant) value of recombinant METTL4 methylating probe Am30 to be around 0.43 μM (**Fig. 1h**). These results suggest that U2 snRNA is a substrate of METTL4, which can mediate U2 m⁶Am30 methylation, with a preferred sequence motif of AAG, and a requirement for pre-deposited 2'-O methylation on adenosine.

U2 snRNA is an essential component of the major spliceosome and is involved in branch point selection and catalysis during pre-mRNA splicing¹⁴. Interestingly, m⁶Am30 is localized immediately upstream of the branch point recognition sequence

(BPRS) (**Supplementary information, Fig. S6a**), suggesting that this modification may regulate splicing activity of the spliceosomal complex¹⁴. To test this possibility, we performed RNA-seq and investigated splicing changes in WT and METTL4 KO cells using rMATS software. A total of 1,402 significantly altered splicing events (delta PSI: 637 up-regulated vs 765 down-regulated) were identified after comparing the transcriptomes of WT and METTL4 KO cells (**Fig. 1i; Supplementary information, Fig. S6b**) and validated by RT-PCR for *PPP3CB*, *TTC28* and *TJP1* (**Supplementary information, Fig. S6c and S6d**), demonstrating that METTL4 is likely involved in splicing regulation.

Taken together, we demonstrated that the MT-A70 family member METTL4 is a novel internal m⁶Am methyltransferase, which mediates N⁶-methylation of Am30 on U2 snRNA in the context of an AAG motif *in vivo* and *in vitro*. Our findings support the notion that most if not all RNA internal m⁶Am modification is mediated by METTL4, but whether METTL4 has additional substrates besides U2 snRNA remains to be explored in the future. Additionally, analysis of RNA alternative splicing in WT and METTL4 KO cells shows that METTL4 loss impacts splicing (**Fig. 1j**). Our genetic rescue experiments, which examined a few candidate splicing events using both wildtype and catalytically compromised METTL4 mutant, suggested that the enzymatic activity of METTL4 and therefore likely U2 m⁶Am methylation is important for splicing regulation. However, we cannot formally exclude the possibility that METTL4 exerts its splicing regulation via regulation of different

substrate(s) other than U2 snRNA, or in an enzymatic activity independent manner. Future experiments including transcriptome-wide mapping of internal m⁶Am and CLIP-seq of METTL4 are necessary to address these questions further. Though we didn't observe any significant defects (e.g. cell proliferation) of 293T cells when METTL4 is abrogated, HepG2 cell growth is compromised upon METTL4 knockdown ([Supplementary information, Fig. S6e and S6f](#)), suggesting that METTL4's biological roles may be context-dependent. Interestingly, a previous study demonstrated that Mettl4-deficient mice display craniofacial dysmorphism and abnormalities in the immune system¹⁰. Our discovery of METTL4 as a novel RNA m⁶Am methyltransferase not only identifies a potential epitranscriptomic mechanism to regulate RNA alternative splicing, but also provides a basis for future mechanistic investigations of biological functions of METTL4 in embryonic development and human diseases.

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Contributions

Y.S. and H.C. conceived and designed the project. H.C., L.G., J.G. and Q.L. carried out most of the biochemical and cellular experiments. Y.W. performed the bioinformatic analysis under supervision of Y.X.. E.A.O. performed m⁶A Northwestern analysis under supervision of R.I.G.. Y.S. supervised the project throughout. Y.S. and H.C. co-wrote the manuscript and all authors contributed to manuscript writing.

Competing financial interests

R.I.G. is a co-founder, scientific advisory board member, and equity holder of 28-7

Therapeutics. Y. X is a scientific cofounder of Panorama Medicine. Y.S. is a consultant/Advisor for the Institutes of Biomedical Sciences, Fudan University, Shanghai Medical School. YS is a co-founder and equity holder of Constellation Pharmaceuticals, Inc, a consultant of Guangzhou BeBetter Medicine Technology Co., LTD and an equity holder of Imago Biosciences. All other authors declare no competing financial interests.

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Figure Legends

Fig. 1 METTL4 is an internal m⁶Am methyltransferase regulating RNA splicing.

a. Phylogenetic analysis of MT-A70 family proteins indicating that METTL4 is likely an evolutionarily conserved N⁶-methylation-related enzyme from yeast to human. Hs: *Homo sapiens*, Dr: *Danio rerio*, Dm: *Drosophila melanogaster*, Ce: *Caenorhabditis elegans*, Sp: *Schizosaccharomyces pombe*, Sc: *Saccharomyces cerevisiae*; M.pvuII and M.rsrl refer to two DNA 6mA methyltransferases derived from *Proteus vulgaris* and *Rhodobacter sphaeroides*, respectively. **b.** Changes of distinct types of RNA modification in total RNA upon METTL4 KO in 293T cells, measured by quantitative LC-MS/MS. KO#3 and KO#6 denote two different METTL4 knockout clones. Data were analyzed by Student's t-test. Error bars indicate mean \pm s.d. (n =3). **c.** LC-MS/MS chromatograms of total A and m⁶Am in total RNA extracted from indicated cell lines. **d.** m⁶A(m) level changes were determined by m⁶A Northwestern blot in WT, KO and rescued cell lines. RNU6B was included as an internal loading control and a small RNA oligo containing m⁶A was used as a positive control. The black arrow indicates the migrating position of the most significantly decreased RNA(s). **e.** Pie chart of relative percentage of each identified RNA in the gel region showing a decreased m⁶A(m) signal. **f.** LC-MS/MS results showing that internal m⁶Am modification was only readily detected in U2 snRNA but not in 5.8S rRNA, SNORA73 snoRNA or U1 snRNA. n.d.= not detected. **g.** The enzymatic activity of recombinant METTL4 towards different RNA substrates *in vitro*. **h.** Michaelis–Menten kinetics of recombinant METTL4 was determined using the Am30

257 probe as a substrate *in vitro*. **i.** Genome-wide alternative splicing changes upon
258 METTL4 KO were identified by rMATS. PSI: Percent Spliced In; delta PSI: PSI (KO
259 - WT). Red indicates significant upregulation while blue indicates downregulation of
260 PSI relative to wildtype cells. $|\text{delta PSI}| > 0.05$ and $\text{FDR} < 0.01$ were used as
261 threshold value. **j.** A working model for METTL4 methylating U2 snRNA. METTL4
262 is a novel internal m⁶Am methyltransferase, which can mediate N⁶-methylation of
263 m⁶Am30 on U2 snRNA within an AAG motif. Loss of METTL4 induces global
264 splicing changes. m⁷G: 7-Methylguanosine, TMG: 2,2,7-trimethylguanosine.

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