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LETTER TO THE EDITOR

METTL4 is an snRNA m^{6}Am methyltransferase that regulates RNA splicing

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Dear Editor,

Protein coding mRNAs and non-coding RNAs are extensively modified by chemical modifications,[1] including methylation of the *N*^{6} position of adenosine (m^{6}A).[2] While mRNA internal m^{6}A methylation is mediated by METTL3 and METTL14, which are members of the MT-A70 family,[2] *N*^{6}-methylation of cap-adjacent *N*^{6},2′-*O*-dimethyladenosine (m^{6}Am) is catalyzed by PCIF1.[3–6] Non-coding RNAs are also m^{6}Am methylated internally,[7] but the corresponding enzyme remains unknown. Here we provide evidence that METTL4 mediates internal m^{6}Am methylation of U2 small nuclear RNA (snRNA).

METTL4, like its paralogs METTL3/14, is conserved from yeast to human (Fig. 1a).[8] *C. elegans* and mouse METTL4 homologs have been shown to regulate DNA 6mA methylation in vivo,[9–11] but are only considered as candidate DNA methyltransferases because of lack of biochemical evidence demonstrating that METTL4 has intrinsic DNA 6mA methyltransferase activity. To further investigate METTL4, we first characterized the localization of METTL4 and found that METTL4 carries a well-conserved, putative nuclear localization signal (NLS) (Supplementary information, Fig. S1a). Consistently, we found mainly nuclear localization of an exogenously introduced METTL4 (Supplementary information, Fig. S1b). Next, we generated METTL4 knockout (KO) 293T cell lines (Supplementary information, Fig. S1c) and found that METTL4 KO significantly reduced internal m^{6}Am levels in the total RNA (Fig. 1b). Importantly, the internal m^{6}Am can be readily rescued by wild-type but not the catalytically compromised METTL4 (METTL4^{APPA}) (Fig. 1c; Supplementary information, Fig. S1d and e). In contrast, METTL4 KO or re-expression of an exogenously introduced METTL4 has no overt impact on 6mA level in the genomic DNA (Supplementary information, Fig. S2a). Furthermore, we failed to identify appreciable levels of 6mA in mitochondrial DNA or m^{6}Am in mitochondrial RNA in 293T cells, respectively (Supplementary information, Fig. S2b and c).

Previous studies demonstrated that PCIF1 is responsible for *N*^{6}-methylation of the cap-adjacent m^{6}Am, which is only sensitive to nuclease P1 cleavage when pre-treated with the RNA 5′ pyrophosphohydrolase, RppH.[3–6] We treated total RNA with or without RppH before nuclease P1 digestion and monitored the changes of m^{6}Am levels (Supplementary information, Fig. S3a). As shown in Supplementary information, Fig. S3b and c, METTL4 depletion induces a drastic decrease of internal m^{6}Am but does not affect the level of the cap-adjacent m^{6}Am. Conversely, KO of the cap-adjacent m^{6}Am methyltransferase, PCIF1, has no impact on the level of the internal m^{6}Am. These findings suggest that METTL4 and PCIF1 represent distinct m^{6}Am methyltransferases targeting different RNA types/regions.

We next set out to identify the m^{6}Am substrate(s) for METTL4. Interestingly, m^{6}Am 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 m^{6}Am methylated, we separated small RNAs by electrophoresis and performed Northwestern with m^{6}A-specific antibodies. We found that a 150-nt band 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 m^{6}Am 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 m^{6}Am but not m^{6}A methylated (Supplementary information, Fig. S4b and c). As a control, a prominent band around 70 nt is unaffected by METTL4 depletion (Fig. 1d, marked by an asterisk). Together, these findings support the hypothesis that METTL4 can mediate internal m^{6}Am methylation of certain small RNAs.

To determine the identities of the m^{6}Am-methylated RNA species, we carried out RNA-seq of the RNAs in the size range of 125–225 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 (Fig. 1e). In order to determine which RNA(s) carries the internal m^{6}Am, we purified 5.8S rRNA, SNORA73 snoRNA, U2 as well as U1 snRNAs (which was suggested to be m^{6}Am methylated[12]) with biotin-labeled synthetic complementary DNA oligos, respectively, and quantified m^{6}Am carried by these four different RNAs (detailed procedure in Supplementary information, Fig. S4d). As shown in Fig. 1f, a considerable amount of m^{6}Am was detected only in U2 snRNA, and there is essentially no m^{6}Am signal detectable in the 5.8S rRNA, SNORA73, or U1 snRNA, suggesting that U2 snRNA is modified by internal m^{6}Am and that METTL4 may be the responsible enzyme in vivo. This finding is consistent with a four-decades-old finding that adenosine of U2 snRNA at position 30 is possibly decorated by m^{6}Am modification,[13] and a more recent transcriptomic single-nucleotide resolution mapping of m^{6}Am suggesting that U2 snRNA may carry internal m^{6}Am modification.[7]

To interrogate the hypothesis that METTL4 may mediate internal m^{6}Am 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 snRNA as a substrate (nt 6–40). Recombinant METTL4 protein was incubated with multiple RNA substrates (detailed in Supplementary information, Materials and Methods) in the presence of stable isotope-labeled S-adenosylmethionine (*d3*-SAM) (Supplementary information, Fig. S5a and b). The 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 is necessary for METTL4 to mediate methylation on the *N*^{6} 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 a low level of methylation was observed when DNA oligos, Cap-Am, or GACU RNA oligos (optimal substrates of METTL3/14) were used as

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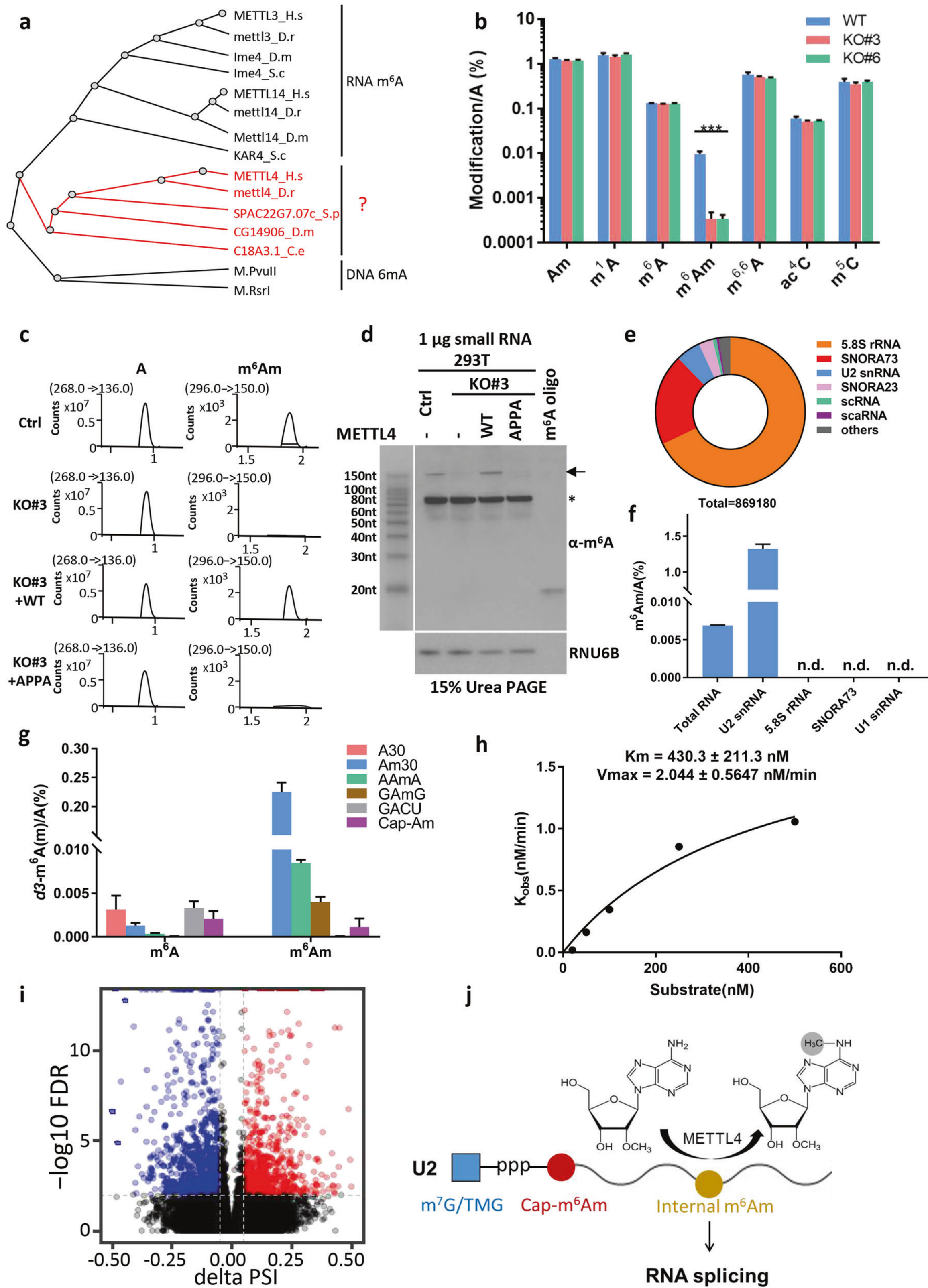

substrates (Cap-Am and GACU probes in Fig. 1g; DNA probe in Supplementary information, Fig. S5c). Consistent with the in vivo results, this enzymatic activity is intrinsic to METTL4 as catalytic mutation abrogated its ability to methylate the U2 snRNA probe (Supplementary information, Fig. S5d). We next 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 mediates U2 m^6Am30 methylation, with a preferred sequence motif of AAG, and a requirement for pre-deposited 2′-*O*-methylation.

Fig. 1 METTL4 is an internal m^{6}Am methyltransferase regulating RNA splicing. a Phylogenetic analysis of MT-A70 family proteins indicating that METTL4 is likely an evolutionarily conserved N^6-methylation-related enzyme from yeast to human. **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 ± SD ($n = 3$). **c** LC–MS/MS chromatograms of total A and m^{6}Am in total RNA extracted from indicated cell lines. **d** m^{6}Am level changes were determined by m^{6}A Northwestern blot in WT, KO, and rescued cell lines. RNU6B was included as an internal loading control and a small RNA oligo containing m^{6}A 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 m^{6}Am signal. **f** LC–MS/MS results showing that internal m^{6}Am modification was only readily detected in U2 but not in 5.8S rRNA, SNORA73 or U1. n.d., not detected. **g** The activity of recombinant METTL4 towards different RNA substrates in vitro. **h** Michaelis–Menten kinetics of recombinant METTL4 was determined using the Am30 probe as a substrate in vitro. **i** Genome-wide alternative splicing changes upon METTL4 KO were identified by rMATS. Red indicates upregulation while blue indicates downregulation. **j** A working model for METTL4 methylating U2 snRNA. METTL4 is a novel internal m^{6}Am methyltransferase, which can mediate N^6-methylation of m^{6}Am30 on U2 snRNA within an AAG motif. Loss of METTL4 induces global splicing changes. m^{7}G: 7-methylguanosine, TMG: 2,2,7-trimethylguanosine.

U2 snRNA is an essential component of the major spliceosome and is involved in branch point selection and catalysis during pre-mRNA splicing.[14] Interestingly, m^{6}Am30 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 complex.[14] To test this possibility, we performed RNA-seq and investigated splicing changes in WT and METTL4 KO cells using rMATS software. A total of 1402 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* genes (Supplementary information, Fig. S6c and d), demonstrating that METTL4 is likely involved in splicing regulation.

Taken together, we demonstrated that the MT-A70 family member METTL4 is a novel internal m^{6}Am methyltransferase, which mediates N^6-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 m^{6}Am 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 suggested that the enzymatic activity of METTL4 and therefore likely U2 m^{6}Am 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 transciptome-wide mapping of internal m^{6}Am and CLIP-seq of METTL4 are necessary to address these questions further. Additionally, it will be interesting to investigate the enzymatic properties of METTL4 in different species as 2′-*O*-methylation, which is necessary for human METTL4, is absent in lower species such as yeast and fruitfly. Lastly, though we did not 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 f), suggesting that the role of METTL4 may be context-dependent. Interestingly, a previous study demonstrated that Mettl4-deficient mice display craniofacial dysmorphism and abnormalities in the immune system.[10] Our discovery of METTL4 as a novel RNA m^{6}Am 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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AUTHOR 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 m^{6}A Northwestern analysis under supervision of R.I.G. L.W. and Z.S. contributed to characterization of enzymatic activity under supervision of H.W. Y.S. supervised the project throughout. Y.S. and H.C. co-wrote the manuscript and all authors contributed to manuscript writing.

ADDITIONAL INFORMATION

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Competing 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 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.

Hao Chen[1], Lei Gu[1], Esteban A. Orellana[2], Yuanyuan Wang[3], Jiaojiao Guo[4], Qi Liu[2], Longfei Wang[5,6], Zhangfei Shen[1,5,6], Hao Wu[5,6], Richard I. Gregory[2], Yi Xing[7,8] and Yang Shi[1]

[1]Division of Newborn Medicine and Program in Epigenetics, Boston Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA; [2]Stem Cell Program, Division of Hematology/Oncology, Boston Children's Hospital, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA; [3]Bioinformatics Interdepartmental Graduate Program, University of California, Los Angeles, Los Angeles, CA 90095, USA; [4]College of Science, Northeastern University, 360 Huntington Ave., Boston, MA 02115, USA; [5]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA 02115, USA; [6]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA; [7]Center for Computational and Genomic Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA and [8]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

These authors contributed equally: Hao Chen, Lei Gu

Correspondence: Yang Shi (yshi@hms.harvard.edu)

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