Recent studies reported that METTL4 regulates DNA 6mA in vivo and therefore is a candidate DNA m6A methyltransferase. However, the enzymatic activity of METTL4 in vitro has not been demonstrated in part due to the difficulties of obtaining well-folded proteins. Here we show that mettl4 is a major methyltransferase responsible for m6A methylation of U2 snRNA both in vitro and in vivo in fly, and identify adenosine at 29th position as the site of m6A methylation. This study answered a long-standing question regarding the enzymatic activity of METTL4, and thus paved the way for further investigating...
the functions of METTL4 in different biological settings.

Introduction

While the eukaryotic candidate m6A methyltransferases belong to multiple distinct methylase lineages, the most widespread group belongs to the MT-A70 family exemplified by the yeast mRNA adenine methylase complex Ime4/Kar4. At the structural level, all of these enzymes are characterized by a 7-β-strand methyltransferase domain at their C-terminus, fused to a predicted alpha-helical domain at their N-terminus and require S-adenosyl-L-methionine (SAM) as a methyl donor. The catalytic motif, [DSH]PP[YFW] , shown to be critical for METTL3/METTL4-mediated mRNA m6A methylation[1]. The high degree of amino acid sequence conservation among the predicted N6-Methyladenosine methyltransferases motivates further explorations into their potential functional conservation. METTL4 is a member of the MT-A70-like protein family, which is conserved during evolution (Fig.1a) [2].

Previous studies suggested that METTL4 regulates DNA 6mA in vivo and therefore is a candidate DNA m6A methyltransfease [3-5]. However, the enzymatic activity of METTL4 in vitro has not been demonstrated.

Identification of potential substrates by eCLIP-seq

To identify the substrate(s) for METTL4, we purified his-tagged, wildtype as well as a catalytic mutant (DPPW mutated to NPPW) (Fig. S1) of Drosophila melanogaster mettl4 from E. Coli strain BL21 (DE3). In order to unbiasedly identify potential substrates of mettl4, we performed in vitro enzymatic assays using various substrates including both DNA and RNA with and without secondary structures. We used deuterated S-Adenosyl methionine (SAM-d3) in the in vitro enzymatic assays in order to identify m6A mediated by mettl4. Although we detected a weak enzymatic activity on DNA substrates composed of previously published sequence motifs, mettl4 appears to prefer RNA substrates with secondary structures (Fig.S2). We next performed eCLIP-seq, which was originally developed to map binding sites of RNA Binding Proteins on their target RNAs [6], to identify the RNA type that is targeted by mettl4 in vivo. Since there are no commercial antibodies available for fly mettl4, we generated a Drosophila KC cell line with a FLAG-tagged mettl4 for the eCLIP-seq experiment [7]. In total, we generated two biological replicates for immunoprecipitation (IP) samples, and their respective input samples, together with one IP-control and Input-control sample for the quality control and enrichment analysis [8]. The two replicates showed a strong correlation with a Spearman correlation coefficient of 0.97, indicating great consistency between the replicates (Fig.S3). Thus, we merged the two replicates to increase the sequencing depth and power for downstream analyses, which showed that mettl4 captured RNA molecules, mostly tRNA and snRNA, including U2, U4
and U6atac (Fig.1b, c).

**Mettl4 catalyze U2 m6A in vitro**

We next investigated whether the RNAs identified by the eCLIP experiments are indeed substrates of METTL4 by carrying out in vitro enzymatic assays. We synthesized oligonucleotides containing tRNA and snRNA sequences and various controls including DNAs with the same sequences. The in vitro enzymatic activity of mettl4 on each candidate substrate and control sequences was measured by LC-MS/MS. These in vitro experiments led to the identification of U2 as the best substrate among all the snRNA subtypes (Fig.1d). Next, we wished to identify the adenosine residues in U2 that are methylated by METTL4. Previous studies documented that the adenosine at the 30th position of U2 is frequently methylated in vertebrate U2 snRNA [9], with a sequence motif of AA-G as opposed to 28AAAG31 in fly. To identify which adenosine within the motif is essential for the enzyme activity in fly, we generated point mutations and deletions of adenosine within and close to this motif and measured the enzymatic activity of mettl4 on these substrates. We found that when the 29th position adenosine is mutated or deleted, no m6A methylation was detected by LC-ms/ms, whereas other point mutations or deletions (26th and 31st positions) did not affect substrate methylation or only decreased methylation partially (i.e., the 30th position). These results indicate that adenosine at position 29 is the adenosine in U2 that is methylated by mettl4 in fly (Fig.1d). In order to better characterize the enzymology of mettl4, we next investigated the kinetics of mettl4 and determined that mettl4 was able to methylate U2 with a Michaelis-Menten constant (K\textsubscript{m}) of 5.298 µM and a catalytic rate constant (k\textsubscript{cat}) of 46.566 min\textsuperscript{-1} (Fig.1e). In addition, the enzyme is inhibited by the substrate at higher concentrations (Fig.1e).

**Mettl4 catalyze U2 m6A in vivo**

Next, we investigated whether mettl4 catalyzes U2 m6A in vivo. To accomplish this goal, we generated mettl4 KO and rescue cell lines (rescued by either wildtype or catalytic mutant of mettl4) (Fig.S4 and S5). Indeed, the U2 m6A level is decreased dramatically in the mettl4 KO cells and restored in the wt mettl4 rescued cells, but not in the catalytic mutant mettl4 rescued cells (Fig.1f, Fig.S7 and Fig.S8a). Furthermore, the same reduction of U2 m6A level was also seen in KO flies (Fig.1g, Fig.S6, Fig.S7 and Fig.S8b). The low DNA 6mA levels between WT and KO fly cells for both nuclear and mitochondrial DNA showed no significant differences (Fig.S9). These findings suggest that it is mettl4 that mediates U2 methylation in vivo. Interestingly, the U2 m6A level in wildtype female flies is significantly higher than in males, suggesting that mettl4 might play sex-specific roles (Fig.1g and Fig.S8b), which will be interesting to investigate in the future. Given U2 snRNA is involved in
pre-mRNA splicing[10], we performed RNA-seq using both wild type and knockout Drosophila KC cell lines to determine if RNA splicing is affected as a result of mettl4 loss. In total, we identified 2,366 transcripts with differential alternative splicing events, which cover 1,771 genes. Gene Ontology Enrichment analysis suggests that mettl4 affects a broad set of biological processes, including differentiation, development, growth, and response to stimulus (Fig.1h).

Discussion

Since U2 is an essential component of the major spliceosomal complex, which plays an important role in pre-RNA splicing, loss of mettl4 might have broad impacts through altered RNA splicing. However, whether the altered RNA splicing events are regulated by mettl4 through methylation of U2 snRNA or other yet-to-be-identified substrates, or whether mettl4 regulates splicing in an enzymatic activity-independent manner, remain to be determined in the future.

In summary, we demonstrated that mettl4 catalyzes U2 m6A in fly both in vitro and in vivo and identified adenosine 29 in U2 snRNA as the site of methylation by mettl4. Furthermore, whole transcriptome profiling revealed that loss of mettl4 broadly impacts various biological pathways. Our work answered a long-standing question regarding the enzymatic activity of mettl4, and thus paved the way for further investigating the functions of mettl4 in different biological settings.

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AUTHOR CONTRIBUTIONS

L.G., H.C. and Y.S. conceived, designed and coordinated the project. L.G. performed data analysis. L.G. and L.W., performed most of the in vitro experiments. J.H. generated mettl4 KO cell line and performed in vivo analysis. A.D. performed kinetics analysis. Z.S., Z. W., and Y. X. helped the in vitro and in vivo experiments under the supervision of L.G., L.W., D. C., H.C., T. L., Z. L. and H.T. J.C. generated mettl4 KO fly. N.P., D. R., H.W., and Y.S. supervised
the project in general. L.G. and Y.S. wrote the manuscript with support from all authors.

Competing interests

Y.S. is a co-founder and equity holder of Constellation Pharmaceuticals, Inc, a co-founder of Athelas Therapeutics, 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 CG14906 (mettl4) methylates U2 snRNA in Drosophila melanogaster. a. Cladogram of mettl4 in model organisms based on their sequence similarity, the pink rectangle indicates the MT-A70 domain. b. Enrichment analysis of eCLIP-seq data for different RNA types. tRNA and snRNA are enriched among all RNA types in general and snRNA is the top enriched RNA molecules targeted by mettl4 in vivo. c. Enrichment analysis of eCLIP-seq data for subgroups of snRNA. U2, U4, and U6atac are the top enriched subgroups. d. In vitro enzymatic activity is measured by LS-MS/MS using substrates including U2, U4, U6atac, U2 with different point mutations and deletions, tRNA and DNA with U2 sequences. Results show that U2 is the best substrate for fly mettl4 and that adenosine at position 29 in U2 is methylated by METTL4. e. Michaelis-Menten kinetics of recombinant mettl4 was determined using U2 probes as substrate by LC-MS/MS analysis. f. U2 m6A analysis in WT, KO and rescued (wt: wild type mettl4; mut: catalytic dead mutant mettl4, DPPW→NPPW) cells by LC-MS/MS. g. In vivo U2 m6A analysis by LC-MS/MS of WT and KO flies. Error bars indicate mean ± s.d. (n=3). h. Genes with differential alternative splicing were used for the GO analysis. The top 20 enriched biological processes are shown in the bar plot. Statistical significance is determined as: ns = p > 0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001.

Figure S1 SDS-PAGE of recombinant proteins encoded by CG14906 (mettl4). His-tagged recombinant Drosophila melanogaster METTL4 proteins (wildtype and a catalytic mutant with a point mutation in the DPPW motif: DPPW to NPPW) were purified from the E.coli strain BL21 (DE3).

Figure S2 In vitro enzymatic activity of Drosophila METTL4 on DNA and RNA substrates with different sequences. Both DNA and RNA substrates, upon incubation with the recombinant METTL4, were measured for the m6A level by LC-MS/MS. A low level of m6A was observed in the DNA substrates and a relatively high level of m6A was observed in structured RNA substrates. Error bars indicate mean ± s.d. (n=3).

Figure S3 Correlation between biological replicates of the eCLIP-seq samples. Reads aligned to fly reference genome (dm6) were normalized in reads per million and the reads density for each 1-kb window was used for the Spearman correlation coefficient calculation.

Figure S4 Generation of a knockout Kc cell line. mettl4 in fly Kc cell line was knocked out by CRISPR-Cas9. Small indels produced by a single guide RNA caused frame shift mutations and the editing efficiency was over 90% based on the ICE CRISPR Analysis Tool.
Figure S5 Rescue of m6A level of U2 snRNA by overexpressing mettl4 in the mettl4 KO cells. The Pentry-PAWF Gateway system was used to over-express mettl4 in the fly Kc KO cells. The expression level of mettl4 was measured by qPCR and normalized to Kc pAWF.

Figure S6 Generation of mettl4 knockout fly. mettl4 knockout flies were generated using the CRISPR/Cas9 system and verified by PCR.

Figure S7 MS spectra of U2 in mettl4 WT, KO and the rescued cells. A, m6A and m6Am levels were measured by LC-MS/MS and compared among WT, KO and rescued cells. Standard peaks indicate the retention time for each modification.

Figure S8 LC-MS/MS results for other independent KO cell lines and flies. a. m6A levels for the other two independent KO cell lines and b. one KO fly line were measured by LC-MS/MS. Error bars indicate mean ± s.d. (n=3). Statistical significance is determined as: ns = p > 0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001.

Figure S9 LC-MS/MS results for m6A on nuclear and mitochondrial DNA from WT and KO fly cells. m6A levels for both nuclear and mitochondrial DNA from WT and KO fly cells were measured by LC-MS/MS. Error bars indicate mean ± s.d. (n=3). Statistical significance is determined as: ns = p > 0.05.
Figure 1 CG14906 (mettl4) methylates U2 snRNA in *Drosophila melanogaster*

- **a**
  - METL4, House mouse
  - METL4, Norway rat
  - METL4, Human
  - METL4, Chicken
  - METL4, Zebrafish
  - C18A3.1, C.elegans
  - CG14906, Fruit fly
  - N/A, Fission yeast
  - N/A, Thale cress

- **b**
  - Normalized Enrichment Score
  - Inputs: tRNA, mRNA, snRNA, snoRNA, miRNA, lncRNA

- **c**
  - Normalized Enrichment Score
  - Substrates: U1, U2, U2sta, U6sta, U12, U12sta, LikeU

- **d**
  - m6A level (%)
  - Conditions: Blank, dsDNA, RNA, U2-1, U2-1 DNA, U2-2, U2-2 DNA, U2-2 deQ26, U2-2 deQ26 DNA, U2-2 deQ92, U2-2 deQ92 DNA, U2-2 deQ92 AG, U2-2 deQ92 AG DNA, U2-2 deQ92 AGG, U2-2 deQ92 AGG DNA, U2-2 deQ92 AGGG, U2-2 deQ92 AGGG DNA, U2-2 deQ92 AGGGG

- **e**
  - Km = 5.298 μM
  - Kcat = 46.566/min
  - Substrate (%)

- **f**
  - m6A level (%)
  - Conditions: WT, KO, Rescue (wt mettl4), Rescue (mut mettl4)

- **g**
  - m6A level (%)
  - Conditions: WT, KO, Rescue (wt mettl4), Rescue (mut mettl4)

- **h**
  - GO terms: neuron differentiation, regulation of developmental process, locomotion, growth, cytoskeleton organization, regulation of signal transduction, imaginal disc development, cell-cell signaling, regulation of localization, import into cell, positive regulation of response to stimulus, regulation of anatomical structure size, learning or memory, cell recognition, pattern specification process, developmental growth involved in morphogenesis, biological adhesion, cell proliferation, regulation of multi-organism process, sensory organ development