

Mettl5 coordinates protein production and degradation of PERIOD to regulate sleep in *Drosophila*

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eLife Assessment

In this manuscript, the authors present **useful** findings demonstrating that the RNA modification enzyme Mettl5 regulates sleep in *Drosophila*. Through transcriptome- and proteome-wide analyses, the authors identified downstream targets affected in heterozygous mutants and proposed that Mettl5 regulates the translation and degradation of clock genes to maintain normal sleep function. However, the mechanisms by which Mettl5 achieves these functions, and whether they are direct or indirect, remain **incomplete** and would benefit from further analysis.

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Abstract

Summary

Sleep is crucial for animal physiology, primarily governed by the brain, and disruptions are prevalent in various brain disorders. *Mettl5*, associated with intellectual disability (ID) often accompanied by sleep disturbances, remains poorly understood in its role causing these disorders. Previous research demonstrated that Mettl5 forms a complex with Trmt112, influencing rRNA methylation. In our study, we explored sleep phenotypes due to *Drosophila* *Mettl5* mutations. Rescue experiments pinpointed *Mettl5*'s predominant role in neurons and glia marked by *Mettl5*-Gal4 in sleep regulation. Notably, a *Trmt112* mutation mirrored these sleep disturbances, implicating translational regulation via the Mettl5/Trmt112 complex. Subsequent RNA-seq and Ribo-seq analyses unveiled downstream events from *Mettl5*^{1bp} mutations, revealing altered expression levels of proteasome components and Clock genes. Rescue experiments confirmed that the net increased PERIOD protein is responsible for the sleep phenotype. This investigation sheds light on ribosome, clock genes, and proteasome interplay in sleep regulation, underscoring protein synthesis and degradation's integrative role. These findings could potentially provide an example of *in vivo* study of the function of rRNA methylation, expand our understanding of the role of protein homeostasis in sleep and inspire explanations on the ID related sleep phenotypes.

Introduction

Sleep serves critical functions for animals. Investigating the molecular mechanisms underlying sleep holds profound importance for both fundamental research and practical applications. While previous studies have identified several sleep regulators (Du et al., 2021 [DOI](#)), gaps persist in comprehending fundamental cellular processes like protein synthesis and degradation in relation to sleep.

Evidence suggests a correlation between sleep regulation and protein synthesis and degradation, both of which control protein levels dynamically. Protein synthesis is particularly active during sleep (Lyons et al., 2023 [DOI](#)). Sleep deprivation targets translational initiation, impacting this process (Costa et al., 2019 [DOI](#)). The proteasome, crucial for ubiquitination-mediated protein degradation, plays a key role in synapse homeostasis by modulating ribosomal components (Costa et al., 2019 [DOI](#)), thereby influencing synaptic function. Additionally, proteasome components exhibit a circadian expression pattern at the transcriptional level in human cells (Desvergne et al., 2016 [DOI](#)). A recent study in *Drosophila* showed that mutation of a proteasome component alters sleep patterns (Fernández et al., 2020 [DOI](#)). The roles of protein synthesis and degradation in sleep regulation, as well as their interactions, remain open questions.

Several factors influencing proteasome assembly and subunit transcription have been identified (Kapetanou et al., 2022 [DOI](#)). However, there remains a significant gap in research regarding the translational control of proteasome subunits. Furthermore, it has been reported that proteasome inhibition can lead to changes in ribosome function (Galimberti et al., 2016 [DOI](#); Costa et al., 2019; Palanca et al., 2014 [DOI](#)). An open question persists as to whether the proteasome is regulated by the status of the translation machinery. Exploring this relationship could provide deeper insights into the interplay between proteasome function and translation.

Drosophila Mettl5 exhibits 18S ribosomal RNA m⁶A methyltransferase activity (Leismann et al., 2020 [DOI](#)). In both *Drosophila* and human, Mettl5 interacts with Trmt112 to facilitate this function (Leismann et al., 2020 [DOI](#); van Tran et al., 2019 [DOI](#)). Depletion of *Mettl5* results in the absence of m⁶A modification on 18S rRNA, yet does not affect rRNA maturation. The loss of *Mettl5*-mediated m⁶A modification influences fly orientation. In mammalian cells, this gene was found mediating 18S rRNA N6-methyladenosine (m⁶A) modification and controlling stem cell fate determination and neural function (Wang et al., 2020 [DOI](#)). However, it remains intriguing whether *Mettl5* impacts global or transcript-specific translation profile, especially in tissues *in vivo* instead of cell lines. Furthermore, *Mettl5*, associated with intellectual disability (ID) often accompanied by sleep disturbances, remains poorly understood in its role causing these disorders. Thus, test whether the loss of *Mettl5* loss affect behavior such as sleep in *Drosophila* would shed light on this problem.

We discovered that *Mettl5* regulates sleep. To investigate the underlying mechanism, we used RNA-seq and Ribo-seq to reveal downstream events associated with *Mettl5* mutations. We identified multiple clock genes and proteasome components regulated by *Mettl5*, suggesting a coordinated regulation of protein production and degradation, crucial for protein homeostasis. Further experiments showed that the protein level of Period was upregulated, contributing to the sleep phenotype. This study highlights how ribosome defects can impact proteasome function, demonstrating a mechanism that coordinates protein degradation with protein production. Additionally, by mapping the genome-wide profile of an rRNA methylation modifier, this study offers insights into the gene-specific roles of ribosome function.

Results

***Mettl5* is a regulator of *Drosophila* sleep**

Mettl5 is predicted to have a N6 adenine specific nucleic acids methylase domain. In order to study the function of this gene in sleep regulation, we generated mutants by CRISPR-Cas9 deletion. Allele *Mettl5*^{1bp}, which deleted 1bp in the coding region results in a truncated version (Figure 1A) and down regulation of *Mettl5* at mRNA level (Figure 1B). Another allele *Mettl5*^{9bp}, which results in 3 amino acids deleted version of *Mettl5* (Figure 1A) does not cause significant change at mRNA level (Figure 1B). We found that the heterozygous *Mettl5*^{1bp} mutation results in decreased total sleep during the night time, which is resulted from the effect of decrease of sleep at early night time (Figure 1C-F). The sleep curve showed a significant down regulation of sleep at ZT12 to ZT16 (Figure 1C). The calculation of the awake percentage at ZT14 indicated a significant increase of this parameter in *Mettl5*^{1bp} (Figure 1G). In addition, *Mettl5* expression was significantly up regulated during the sleep recovery period after mechanical sleep deprivation (Figure 1M-N). Consistently, the sleep recovery was down regulated in the *Mettl5* mutant (Figure 1O), indicating the sleep homeostasis was affected by *Mettl5*. In conclusion, the truncated mutation of *Mettl5* resulted in sleep phenotypes.

To verify the specificity of the sleep phenotype caused by *Mettl5*^{1bp}, we did the rescue experiment. The results showed that supplying of one copy of *Mettl5* to *Mettl5*^{1bp}rescued the sleep phenotype in sleep amount and awake percentage at ZT14 (Figure 1H-L). These results verified the specificity of the phenotype.

To verify in which cells did *Mettl5* function for the sleep phenotype, we checked the expression pattern of *Mettl5*-Gal4. The results indicated that a portion of the *Mettl5*-Gal4 marked cells colocalized with Repo antibody staining (Figure S1A-C). Another portion of the *Mettl5*-Gal4 marked cells colocalized with ELAV antibody staining (Figure S1D-F). In addition, we also tested the climbing ability of *Mettl5* mutation which showed an increase (Figure S1G). The sleep arousal assay also indicated an increased sleep arousal (Figure S1H). Moreover, the lifespan increased in the mutant (Figure S1I).

***Mettl5* regulation on *Drosophila* sleep was dependent on its methyltransferase activity**

Previous studies have shown that *Mettl5* interacts with *Trmt112* to regulate 18S rRNA m⁶A modification (Leismann et al., 2020; van Tran et al., 2019). Consistently, we found that the methylation level in both total and 18S rRNA decreased in heterozygous *Mettl5*^{1bp}. We tested the m⁶A level using LC-MS/MS experiment. The m⁶A level decreased in heterozygous *Mettl5*^{1bp} either in both total RNA and 18S rRNA (Figure 2A-B). To investigate the mechanism of *Mettl5* regulation on sleep. We examined the phenotype of *Trmt112* knockdown. We found that the *Mettl5*-Gal4 driven *Trmt112* RNAi resulted in a similar phenotype to *Mettl5*^{1bp} (Figure 2C-G), suggesting that *Mettl5* regulates sleep through its 18S rRNA m⁶A modification activity. The efficiency of the *Trmt112* RNAi line was tested in previous study (López-Varea et al., 2021). More important, we performed a rescue experiment with a mutated form of *Mettl5* that lacks the NPPF amino acids required for its methyltransferase activity. We observed that this mutant *Mettl5* failed to rescue the sleep phenotype caused by *Mettl5*^{1bp} (Figure 2H-L). These results collectively indicated that the methyltransferase activity of *Mettl5* is essential for its role in *Drosophila* sleep.

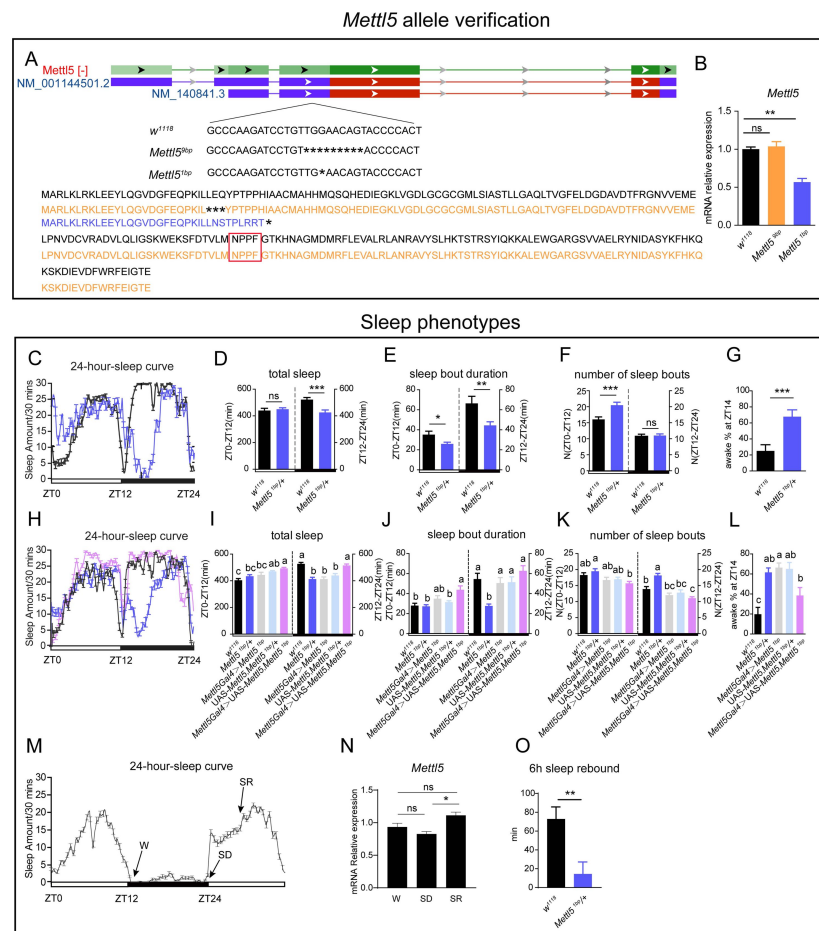


Figure 1

Mettl5 is a regulator of *Drosophila* sleep.

(A) Diagram illustrating CRISPR-Cas9 knockout of 1 or 9 bases in the *Mettl5* gene. (B) Relative expression of *Mettl5* mRNA in *Mettl5*^{1bp} and *Mettl5*^{9bp} mutant male flies compared to control flies. (C) Sleep curve throughout the day for *Mettl5* mutant male flies and control flies. (D) Total sleep of *Mettl5* mutant male flies and control flies. (E) Sleep bout duration of *Mettl5* mutant male flies and control flies. (F) Number of sleep bouts of *Mettl5* mutant male flies and control flies. (G) Percentage of awake for *Mettl5* mutant flies and control flies. (H) Sleep curve throughout the day for *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. (I) Total sleep of *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. (J) Sleep bout duration of *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. (K) Number of sleep bouts of *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. (L) Percentage of awake for *Mettl5* mutant flies, *Mettl5* over expression male flies and control flies. (M) Sleep curve is tracked throughout the entire day prior to sleep deprivation and during the daytime sleep rebound period. (N) *Mettl5* mRNA expression level at different time points. W(wake), SD (sleep deprivation), SR (sleep recovery). (O) The daytime sleep recovery during the six hours following sleep deprivation in *Mettl5* mutant male flies. For * stands for p<0.05, ** stands for p<0.01, *** stands for p<0.001, ns stands for not significant.

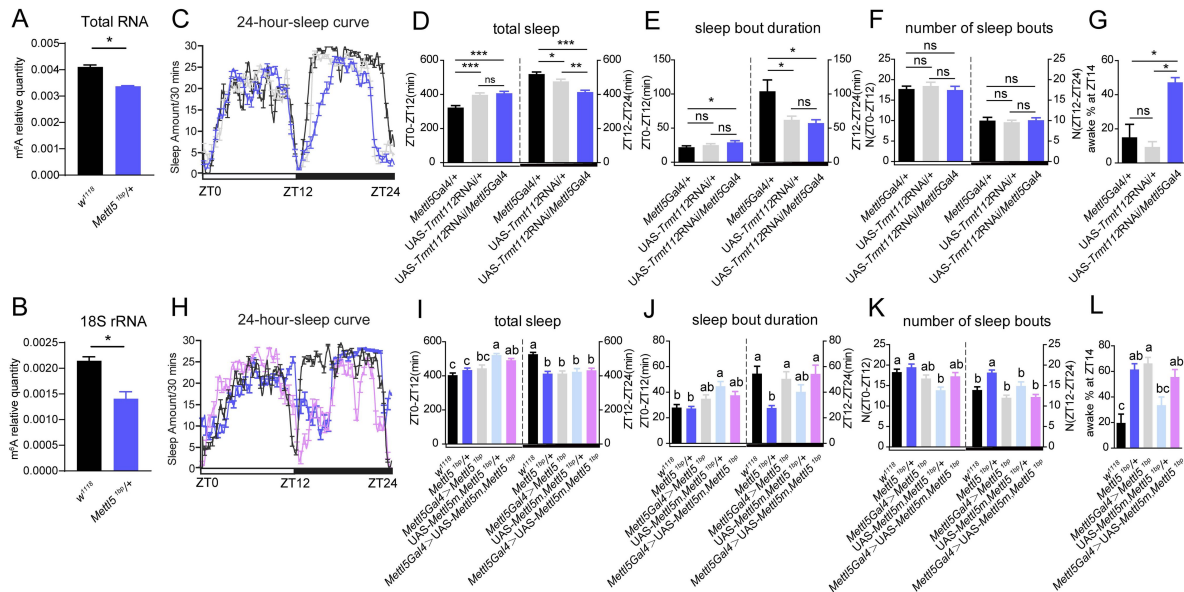


Figure 2

***Mettl5* regulation of *Drosophila* sleep was dependent on its methyltransferase activity.**

(A) The m⁶A level in the total RNA of *Mettl5* mutant male flies. (B) The m⁶A level in the 18S rRNA of *Mettl5* mutant male flies. (C) The sleep curve throughout the day shows the sleep pattern of induced *Trmt112* RNAi male flies and control flies. (D) Total sleep of induced *Trmt112* RNAi male flies and control flies. (E) Sleep bout duration in induced *Trmt112* RNAi male flies and control flies. (F) Number of sleep bouts in induced *Trmt112* RNAi male flies and control flies. (G) Percentage of awake in *Trmt112* RNAi and control flies. (H) Sleep curve throughout the day for *Mettl5* mutant male flies, induced *Mettl5m* over expression male flies and control flies. (I) Total sleep of *Mettl5* mutant male flies, induced *Mettl5m* over expression male flies and control flies. (J) Sleep bout duration in *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. (K) Number of sleep bouts in *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. (L) Percentage of awake in *Mettl5* mutant male flies, induced *Mettl5* over expression male flies and control flies. For * stands for p<0.05, ** stands for p<0.01, *** stands for p<0.001, ns stands for not significant.

RNA-seq and Ribo-seq revealed the downstream gene profile of *Mettl5*

To better understand the downstream events of *Mettl5*, we analyzed RNA-seq and Ribo-seq data to evaluate the effects of *Mettl5*^{1bp} on gene expression. The analysis of the RNA-seq and Ribo-seq data followed by the Principal Coordinates Analysis (PCoA) showed a clear separation between the two groups (Figure S2A, B, D, E). High correlation was found between the three biological repeats for each sample (Figure S2C, F), indicating that RNA-seq and Ribo-seq quality was high. Differentially expressed gene analysis of RNA-seq showed that 1053 genes ($|\log_2(\text{Fold Change})| \geq 1$ & $p^{\text{adj}} < 0.05$) were significantly changed at the transcription level (Figure 3A), including 217 up-regulated and 836 down-regulated genes (Figure 3B, Table S1). Differentially expressed gene analysis of Ribo-seq showed that 258 genes were significantly changed at the translation level ($|\log_2(\text{Fold Change})| \geq 0.265$ & $p^{\text{adj}} < 0.05$), including 149 up-regulated and 150 down-regulated genes (Figure 3C, Table S1). The top 100 differentially expressed genes found in RNA-seq (Figure S3A) and Ribo-seq (Figure S3B) were shown in a heatmap generated according to the rlog transformed values.

To explore the biological implication of the differentially expressed genes at transcriptional and translational levels, Gene ontology (GO) enrichment was performed. At the transcriptional level (Figure S4A, C), the most significantly enriched biological processes GO terms were related to cellular response, biosynthetic process and metabolic process, such as cellular response to chemical stimulus, small molecule biosynthetic process, fatty acid metabolic process (Figure S4A). Several term related gene network also be shown (Figure S4C). At the translation level (Figure S4B, D), the most significantly enriched biological processes GO terms were related to organic acid metabolic process, amino acid metabolic process, lipid catabolic process, cellular respiration, and transport mechanisms. Additionally, we found prominent terms related to circadian rhythm genes, including circadian regulation of gene expression and entrainment of the circadian clock (Figure S4B, D).

The GO results usually contain a long list of enriched terms that have highly redundant information and are difficult to summarize. So, we performed a simplify Enrichment analysis that visualizes the summaries of clusters by word cloud for the GO enrichment result at transcriptional and translational levels. We found that the main biological process was metabolic, the response signaling stimulus; the development of wing disc; imaginal et.al system; the homeostasis and transport for ion-channel; the cycle of cytokinesis and transition in transcriptional significant DEG (Figure S5A). Except for the above main biological progress, the significant translated genes also showed enrichment for the sleep wake, cycle related behavior circadian rhythm and neuron death (Figure S5D). For cellular components, the main enrichments are chromosome structures, complexes, membranes, and vesicles (Figure S5B). Molecular functions primarily include enzymatic activities, ion and protein binding, receptor activities, and transcription processes (Figure S5C). The RNA-seq results are very similar to the Ribo-seq results (Figure S5E-F).

Gene Set Enrichment Analysis (GSEA) revealed pathways that were enriched in differentially expressed genes at both transcriptional and translational levels. As the significant genes enrichment analysis will lose some key important message which are caused by unreasonable screening parameters, these gene sets ranked by $\log_2$ -fold-change only (not screened by p values) were considered in the GSEA. The GSEA-GO and GSEA-KEGG enrichment results of the transcriptional and translational genes were sorted according to the Normalized enrichment score (NES) value (Figure S6A-D). We found some important pathway such as proteasome pathway that were suppressed at both transcriptional and translational levels ($P_{\text{adjust}} < 0.05$) (Figure 3E, I). Arginine and proline metabolism and insect hormone biosynthesis were significantly affected at

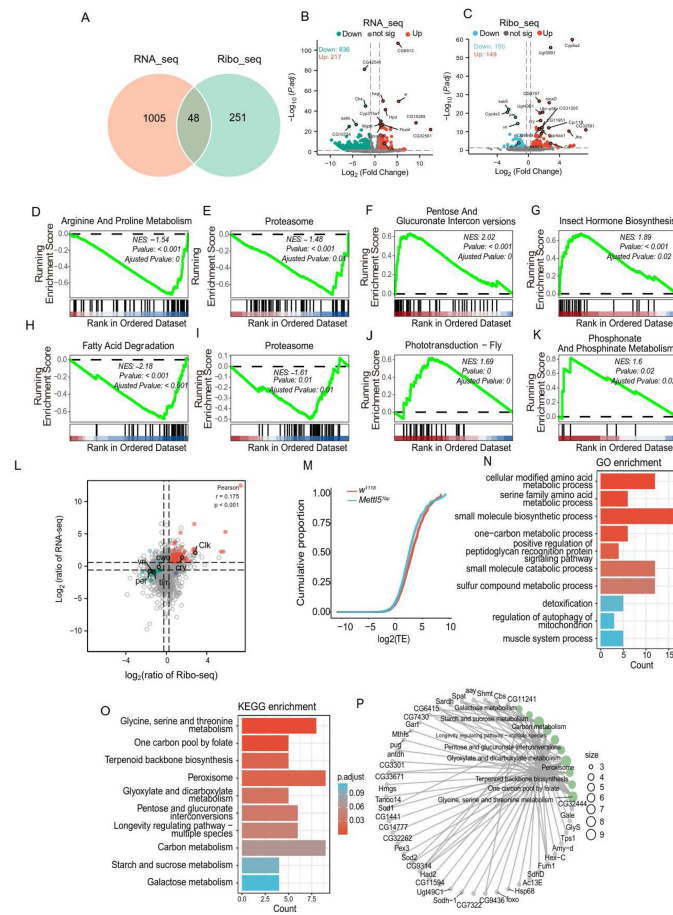


Figure 3

RNA-seq and Ribo-seq analysis revealed changes in the gene profile of *Mett15*^{1bp}.

(A) Venn diagram depicting the number of significant differentially expressed genes revealed by RNA-seq and Ribo-seq. (B) Volcano plot representing the differentially expressed genes identified by RNA-seq. Genes that met the criteria of $|\log_2(\text{fold-change})| \geq 1$ and $p.\text{adjust} < 0.05$ were considered significantly expressed, marked in orange for downregulation and green for upregulation. (C) Volcano plot representing the differentially expressed genes identified by Ribo-seq. Candidates that satisfied the criteria of $|\log_2(\text{fold-change})| \geq 0.265$ and $p.\text{adjust} < 0.05$ were regarded as significantly expressed, marked in red for downregulation and blue for upregulation, respectively. (D-K) Gene set enrichment analysis of differentially expressed genes revealed by RNA-seq (D-G) and Ribo-seq (H-K). All the plots are generated using the KEGG gene set database. The bar chart at the bottom of each panel shows the distribution of target genes for each pathway according to their rank position. Each vertical line represents a gene. Genes on the left show positive correlation with *Mett15*^{1bp}, while, genes on the right show negative correlation with *Mett15*^{1bp}. The green line indicates the enrichment score (ES), and NES stands for normalized enrichment score. (L) Distribution of the differentially expressed genes revealed by both RNA-seq and Ribo-seq. (M) Cumulative distribution of TE frequencies among *w*¹¹¹⁸ and *Mett15*^{1bp} (N, O) GO and KEGG enrichment of significantly changed TE-related genes between *w*¹¹¹⁸ and *Mett15*^{1bp}. The color of the bar indicates the enrichment $p.\text{adjust}$ value. (P) KEGG network showing the top 5 pathway and associated genes. The size of the dots represents the number of genes in the pathway.

transcriptional level ($P_{\text{adjust}} < 0.05$) (Figure 3D, G [↗](#)). While, fatty acid degradation, Phosphonate and Phosphinate metabolism, etc. were significantly affected at translational level ($P_{\text{adjust}} < 0.05$) (Figure 3H-K [↗](#)).

In particular, GSEA analysis showed some interesting results. Gene ontology analysis (GSEA-GO) of the GSEA gene set showed a suppression of mitochondria related activities both at transcriptional and translational levels (Figure S6A, C). The aromatic amino acid metabolic and catabolic process were activated at transcriptional level (Figure S6A). While, the signaling receptor, molecular transducer activity related term was activated at translational level (Figure S6C). The KEGG analysis (GSEA-kegg) indicated that at transcriptional level, we found that the pathway Pentose and glucuronate interconversions, Insect hormone biosynthesis, Drug metabolism -cytochrome P450 et.al were activated (Figure S6B). While, arginine and proline metabolism, Proteasome, Phagosome, Oxidative phosphorylation etc. pathway were suppressed (Figure S6B). *Mettl5^{1bp}* mainly affected the physiological activities through activating Phototransduction, Phosphonate metabolism, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis etc. pathways at translational level (Figure S6D). At the same time, it suppressed Peroxisome, amino acid metabolism, Fatty acid degradation, proteasome etc. pathways at the translational level (Figure S6D).

To better understand the correlation between transcription and translation levels, we performed correlation analysis of differentially expressed genes based on fold change of DEGs (Figure 3L [↗](#)). we found 977 genes which had opposite tendency of change at transcriptional vs. translational levels, 24 genes which had same tendency of change at transcriptional vs. translational levels., 240 genes which changed only at translational level and 3309 genes which changed only at transcriptional level. These four gene clusters were analyzed by enrichment analysis (Figure S7A-H). The expression of important clock gene *Clk*, *tim* etc. were significantly changed at both transcriptional and translational levels (Figure 3L [↗](#)).

To assess net translation alterations and exclude the effects of transcription changes, we analyzed translation efficiency (TE). We found a change of TE in *Mettl5^{1bp}* compared to *w¹¹¹⁸* (Figure 3M [↗](#), Table S1). We obtained 1204 genes that had significantly different TE. We performed GO and KEGG enrichment analysis for these genes. GO enrichment indicated that these genes mainly enriched in amino acid metabolic process and small molecule biosynthetic process etc. (Figure 3N [↗](#)). The KEGG enrichment analysis showed that the main pathway are Glycine, serine and threonine metabolism, one carbon pool by folate, etc. (Figure 3O, P [↗](#)).

Ribo-seq revealed the *Mettl5^{1bp}* led to changes of some global translation features

Ribo-seq revealed the global translation features in two groups, *w¹¹¹⁸* and *Mettl5^{1bp}*, respectively. The correlation coefficient of TE between the three biological replicates of two groups varied from 0.87 to 0.93, indicating a high correlation between the samples of the same genotype (Figure S8A-F). We found that the length of ribosome-protected RNA fragments (RPFs) was approximately 28nt (Figure S10A). The RPFs exhibited a significant 3-nt periodicity. Metagene analysis of individual 28nt reads revealed the distribution of RPFs across the gene locus in *w¹¹¹⁸* and *Mettl5^{1bp}*, respectively (Figure S8, S9). The starting point of translation is 12nt upstream of start-codon and gradually disappears 15nt from stop-codon (Figure S8G-L). RPFs on the metagene plot distribution around the translation start and translation stop site (Figure S9A-F), different coding frames on CDS, 3'-UTR and 5'-UTR, respectively in two groups of samples all showed the periodicity (Figure S9G-L). As expected, all plots show an enrichment of P-sites in the first frame on the coding sequence but not the UTRs, in accord with ribosome protected fragments from protein coding mRNAs (Figure S9G-L).

Analysis of the proportion of the different types of open reading frame (ORF) indicated an alteration of the percentage of some types of ORFs. Compared to w^{1118} , we found that *Mettl5*^{1bp} have more overlapped dORFs and less non-overlapped dORFs (Figure S10B). Both of the two groups showed the shorter length of translated uORF compared to untranslated uORF ($P < 0.05$) (Figure S10C). *Mettl5*^{1bp} had less dORF and uORF read count (Figure S10D-E). According to the translation potential of uORF, the translated uORF and untranslated uORF can be divided from different sample. Motif analysis was performed on untranslated uORF and translated uORF respectively (Figure S10F-G).

Mutation of *Mettl5* altered the codon preference. When comparing the codon occupancy (A-site) correlation between two groups, we find two codons (GAC and GAU) were preferred by *Mettl5*^{1bp} and UCC were preferred by w^{1118} (Figure S10H). The cumulative frequency of these three codons also demonstrated the same trend (Figure S10I-K). The Asp codon GAC and GAU, so we compared the amino acid occupancy of Asp. The result indicated that the frequency of Asp in w^{1118} was significantly enriched in translation (Figure S10L).

Metagene plot comparing RPFs between *Mettl5*^{1bp} and w^{1118} revealed the differential translation. The CDS and region around it was divided into 100 equal bins, and the average RPF density was calculated for each bin. The plot visually depicting the differences in ribosome occupancy patterns between *Mettl5*^{1bp} and w^{1118} along the CDS (Figure S10M), around the translation start site (Figure S10N) and around the translation end site (Figure S10O). The plot highlights the divergent ribosome occupancy patterns between *Mettl5*^{1bp} and w^{1118} along the CDS and near the translation start site, indicated potential variations in translation dynamics and disparities in translation initiation (Figure S10M, O).

Clock gene regulatory loop regulating circadian rhythm was affected by *Mettl5*^{1bp}

We found that multiple clock genes had significant changes in both transcriptional and translational levels. *Cry* and *Clock* was significantly upregulated at both transcriptional and translational levels (Figure 4A-B). *Tim*, *per*, *vri* and *pdp1* were significantly downregulated at both transcriptional and translational levels (Figure 4A-B). More important, *per*, *vri*, and *pdp1* were significantly downregulated in translational efficiency (Figure 4C). These genes are components of the clock gene regulatory network which controls the circadian rhythm. The clock neuron morphology was not affected by the mutant (Figure S11).

According to the relationship between these genes in the clock regulatory circuit, if *Per* was downstream of *Clock* in mediating the function of *Mettl5*, we should have seen the change of both genes to the same direction. However, we detected changes to opposite for *Clock* and *Per*. Therefore, we propose that *Per* was upstream of *Clock* in mediating the function of *Mettl5*. Then, we examined the protein level of *Per*. Surprisingly, we found that the protein level of *Per* was upregulated in the mutant detected by both immunostaining and western blot (Figure 4D-G). This is confirmed by the circadian rhythm phenotype showed by *Mettl5* mutant, which showed a significant increased period length (Table 1). An increased period length was reported in previous case of inactive kinase activity which results in a stabilized Period (Philpott et al., 2023). Consistently, genetic evidence indicated that both of *Clock* and *Per* genes were downstream of *Mettl5*, because the double mutant showed the phenotype of clock genes (Figure 4H-M, Table 1). This result indicated that other mechanism regulating *Per* protein was affected by *Mettl5*^{1bp}.

Then we ask what's the factors mediated *Per* protein level changes in *Mettl5* mutant. By examining the RNA-seq, Ribo-seq data and translation efficiency, we found that multiple components of the proteasome pathways were significantly downregulated in *Mettl5*^{1bp} (Figure 3E, I, 4N). Based on the above results, we proposed a working model in which *Mettl5* directly regulates proteasome,

Figure 4.

Clock genes expression mediated the sleep phenotype caused by *Mettl5* mutation.

(A-C) Fold changes in clock genes with significant expression level differences between *w¹¹¹⁸* and *Mettl5^{1bp}* were observed in RNA-seq, Ribo-seq, and translation efficiency analyses. (D-E) PER protein immunofluorescence staining in small LNvs (ventral lateral neurons) and its immunofluorescence intensity statistics. (F-G) Western blot analysis of PER protein and Tubulin protein, along with their grayscale quantification. (H) Sleep curves throughout the day for *Mettl5^{1bp}*, *CLK^{RR}*, double mutant and control flies. (I) Total sleep for *Mettl5^{1bp}*, *CLK^{RR}*, double mutant and control flies. (J) Percentage of awake time in *Mettl5^{1bp}* flies, partially rescued by double mutant flies. (K) Sleep curve throughout the day for *Mettl5^{1bp}*, *per⁰¹*, double mutant and control flies. (L) Total sleep for *Mettl5^{1bp}*, *per⁰¹*, double mutant and control flies. (M) Percentage of awake time in *Mettl5^{1bp}* flies, partially rescued by double mutant flies. (N) Fold changes in proteasome subunits with significant expression level differences between *w¹¹¹⁸* and *Mettl5^{1bp}* were observed in RNA-seq, Ribo-seq, and translation efficiency analyses. For statistical significance, * stands for $p < 0.05$, ** stands for $p < 0.01$, *** stands for $p < 0.001$, ns stands for not significant.

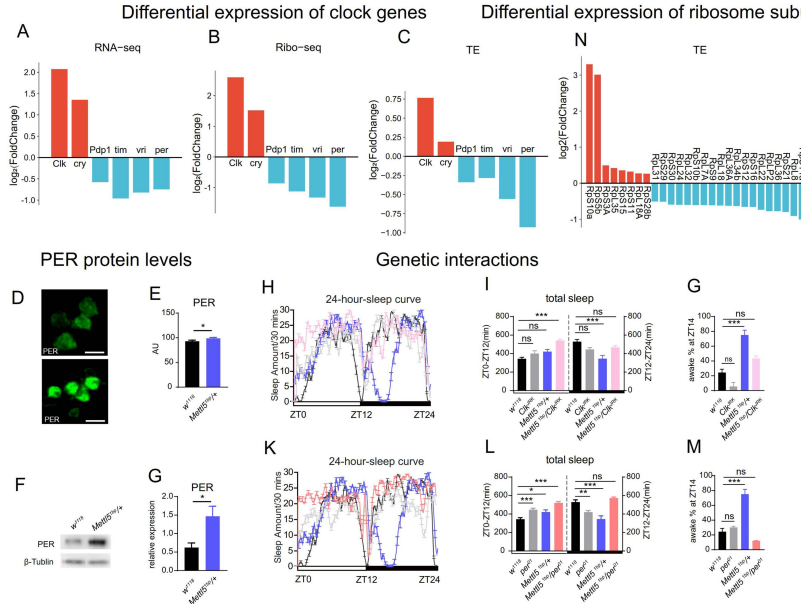


Table 1

Circadian rhythm phenotypes of various mutants.

Genotype	NumTotal	%Rhythmic	Period	Power
<i>w¹¹¹⁸</i>	32	92.6	23.9± 0.05	127.9± 7.62
<i>Mettl5^{1bp}/+</i>	32	92.3	28.3± 0.4 ***	114.3± 6.76 ns
<i>Mettl5^{1bp}/+</i> ; UAS- <i>Mettl5</i> / <i>Mettl5</i> Gal4	32	96.9	24± 0.02 ns	127.1± 5.42 ns
<i>Mettl5^{1bp}/Clk^{RR}</i>	44	29.5	24± 0.04 ns	68.5± 8.85 ***
<i>Mettl5^{1bp}/Per⁰¹</i>	54	9.3	24.2± 0.2 ns	43.8± 13.41 ***

Per and Clock in transcription and translation. The proteasome regulates Per at post translational level. And in turn Per inhibits Clock at protein level. As a result, in *Mettl5*^{1bp}, the down regulation of proteasome results in the upregulation of Per protein and as a result up regulation of Clock protein activity and its downstream gene transcription (**Figure 5** [↗](#)).

The axon complexity was affected by *Mettl5*^{1bp}

Previous results have indicated a close correlation between sleep need and synaptic complexity (Bushey et al., 2011 [↗](#)). Moreover, it has been observed that synaptogenesis stimulates proteasome activity in axons (Costa et al., 2019 [↗](#)). Considering the loss of sleep rebound after deprivation and the expression level changes of proteasome subunits resulting from *Mettl5*^{1bp}, we were interested in investigating whether synaptic complexity is affected.

To quantify synaptic complexity, we employed a method previously reported (Bushey et al., 2011 [↗](#)). Syt-GFP, a protein product that colocalizes with native synaptic vesicles, was used to visualize changes in presynaptic morphology. As controls, UAS-*Fmr1* and *Fmr1* mutation were utilized, resulting in a decrease and increase of syt-eGFP, respectively (**Figure 6A-C** [↗](#)). Interestingly, we found that the *Mettl5*^{1bp} led to an increase in syt-eGFP staining in presynaptic terminals (**Figure 6D** [↗](#)).

Discussion

In this study, we found that *Mettl5*, a previously identified rRNA methylase, regulates sleep through its RNA methylation activity. RNA-seq and Ribo-seq of the *Mettl5*^{1bp} identified downstream genes of *Mettl5* at transcriptional and translational levels. Further study identified *Mettl5* can affect clock gene network and proteasome pathway which mediated *Mettl5* regulation of sleep. By elucidated the molecular mechanism for *Mettl5* function on sleep, this study highlighted an integrative role of protein synthesis and degradation in sleep regulation. The Ribo-seq analysis in this study indicated that global features of translation, such as uORF translation efficiency and codon choices, were affected in the *Mettl5*^{1bp}, suggesting a role of rRNA methylation in these processes.

The ortholog of *Mettl5* in humans is METTL5, mutation of which causes intellectual disability (ID) (Richard et al., 2019 [↗](#)). This study expanded our understanding of the molecular mechanism of *Mettl5* function, which potentially provides new insights for the potential treatment of ID. Previous clinical studies found that the ID patients have sleep problems, including sleep duration shortness. This symptom is consistent with what we found for the *Mettl5*^{1bp}. The mechanism for this phenotype found in this study is potentially important. More studies need to be done in vertebrates to verify if this mechanism is conserved for sleep disorders.

The RNA-seq and Ribo-seq of *Mettl5*^{1bp} revealed the gene network that is potentially directed by *Mettl5*. The direct or immediate effect of *Mettl5* may lie in the regulation of translation. Indeed, we found that the codon occupancy was significantly changed for GAC, GAU, UCC. Moreover, the occupancy on dORF were significantly downregulated in the *Mettl5*^{1bp}. The translated uORF were significantly downregulated by the *Mettl5*^{1bp}. These may lead to fluctuations in certain protein production, which may eventually lead to altered downstream gene expression. This result indicated that small perturbations in translation process have a significant impact on downstream events, highlighting the importance of maintaining the accuracy of the translation process.

We observed a significant decrease in total RNA and 18S rRNA methylation levels in individuals with the heterozygous allele of *Mettl5*. Interestingly, this finding differs slightly from studies conducted in mice, where heterozygous knockouts still exhibited normal levels of 18S rRNA m⁶A

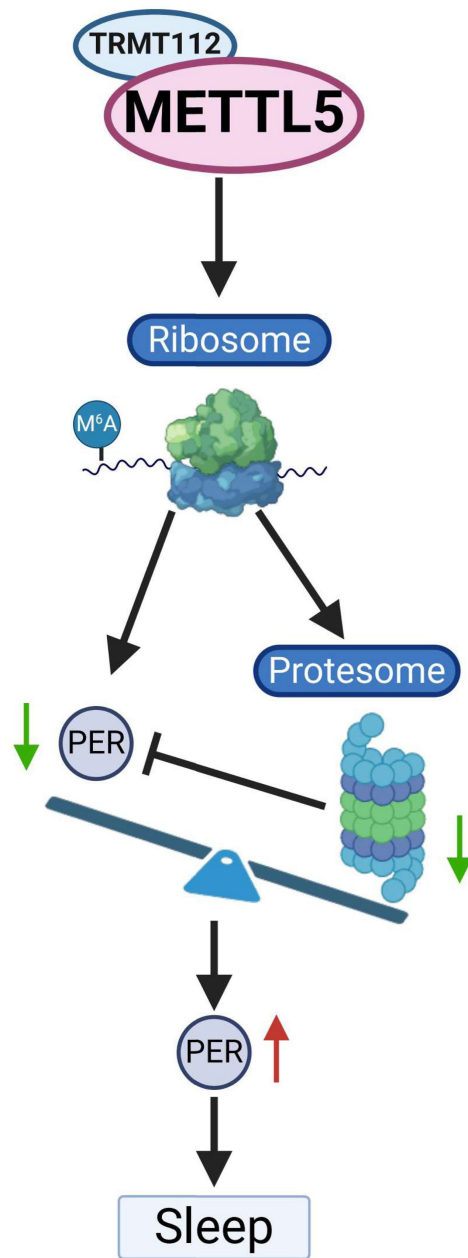


Figure 5

A working model illustrating the role of *Mettl5* in *Drosophila* sleep was presented.

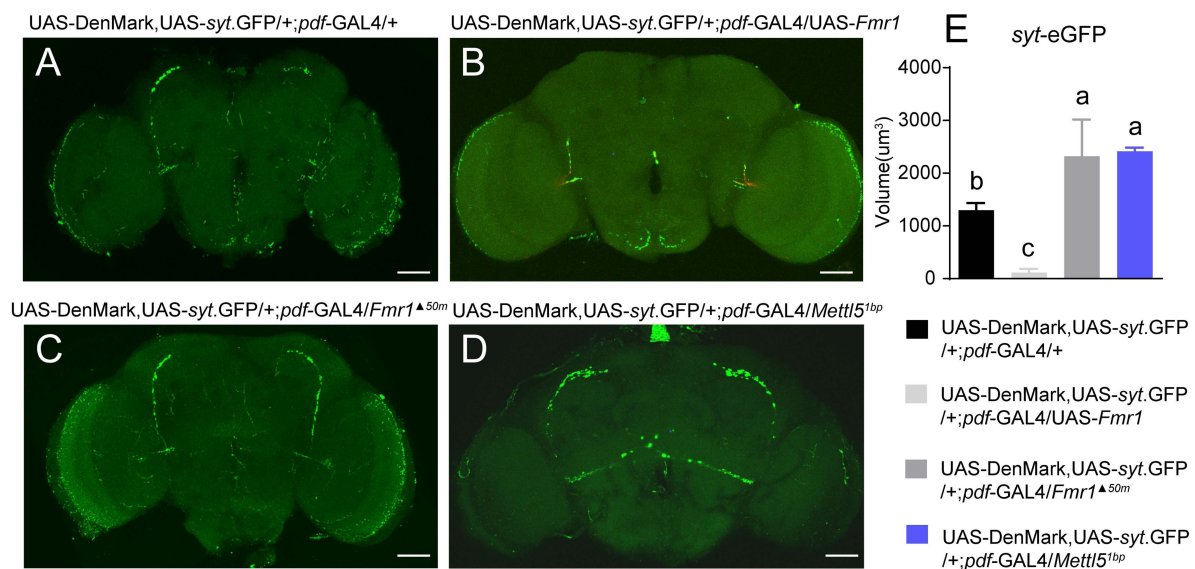


Figure 6

The axon complexity was found to be affected by *Mettl5*^{1bp}

(A-D) Examples of small LNv axonal terminals labeled by syt-eGFP (green). A rectangular box indicates the location where LNv axons exit the posterior optic tract and the first axonal bifurcation to the tip of the terminal region, whose volume was measured. (E) Mean volume measurements of small LNv axonal terminals labeled by syt-eGFP.

methylation (Sepich-Poore et al., 2022 [DOI](#)). It may attribute to potential species-specific regulatory differences between *Drosophila* and mice. Another plausible explanation may lie in the distinct nature of the knockdown compared to knockouts. Notably, prior research has highlighted compensation effects arising from complete knockouts (Teng et al., 2013 [DOI](#); Rossi et al., 2015 [DOI](#); Vu et al., 2015 [DOI](#); Ma et al., 2019 [DOI](#); El-Brolosy et al., 2019 [DOI](#)).

This study revealed a new aspect on the connection of clock genes and proteasome. The clock regulation on proteasome was important in several different contexts. Previous studies showed that the circadian clock rhythmically controls the daily expression of multiple components of the proteasome, which responds to dietary restriction in *Drosophila* fat body (Hwangbo et al., 2023 [DOI](#)). In this study, we found *Mettl5* can regulate clock protein synthesis and degradation in clock neurons via affecting proteasome function. The role played by *Mettl5* in this process may be through affecting the methylation of ribosomes in clock neurons, affecting the proteasome degradation pathway (Costa et al., 2019 [DOI](#)), and ultimately leading to changes in clock proteins in clock neurons.

The function of *Mettl5* highlights an integrative regulation of rRNA methylation and the proteasome. This provides an opportunity to coordinate protein synthesis and protein degradation, which would be a feasible way to keep balance for the total protein level. Interestingly, we observed specificity in the downstream affected genes both at transcriptional and translational levels. For the proteasome subunits, we also detected level changes for some of them, indicating a specificity of *Mettl5* function. The specificity for the proteasome subunits changes could be attributed to gene specificity of *Mettl5* on translation. Another possibility is that there are other layers of regulation on protein degradation pathway by *Mettl5* protein interaction or other mechanisms. These factors may form a regulatory network to coordinate with each other. It is an intriguing question how the downstream specificity is achieved. More studies are needed to elucidate these mechanisms.

This result indicates the complex relationship between clock genes and sleep homeostasis regulation. The data in this study are consistent with previous reports on the involvement of clock genes in the regulation of sleep homeostasis. It has been reported that loss of function alleles of *Per* and *Clock* exhibit a more pronounced sleep rebound after sleep deprivation (Shaw et al., 2002 [DOI](#)). Conversely, the *Mettl5* allele, which results in increased PER protein, leads to reduced sleep rebound. Furthermore, data in this study suggested that the regulatory mechanism of *Clock* has tissue specificity. According to the expression patterns of *Mettl5* based on *Mettl5*-Gal4, it does not express in clock neurons. Instead, it is expressed in *Mettl5*-Gal4 positive cells, which are composed of a part of neurons and glia. *Clock* is expressed not only in clock neurons of the *Drosophila* brain (Patop et al., 2023 [DOI](#)). The combinational expression of these proteins constitutes the specificity of clock upstream regulators. Our study indicates that these *Mettl5* expressing neurons and glia are important in sleep regulation. It would be interesting to find out the *Mettl5* dependent sleep homeostasis regulatory circuits.

Evidence indicates that sleep functions in development, metabolism and neuronal plasticity (Anafi et al., 2019 [DOI](#)). Sleep exerts effects on neuronal plasticity by modifying synapses. The synaptic homeostasis theory proposes that sleep has a role in downscale synaptic strength (Tononi and Cirelli, 2006 [DOI](#)). Indeed, synapse markers progressively decrease during sleep in both mammals and *Drosophila* brains (López-Varea et al., 2021 [DOI](#); Vyazovskiy et al., 2008 [DOI](#); Gilestro et al., 2009 [DOI](#); Liu et al., 2010 [DOI](#)). More importantly, evidence indicates that sleep need and protein levels in synapse are tightly linked. Studies of the presynaptic active zone have shown that synaptic plasticity regulates sleep homeostasis (Huang et al., 2020 [DOI](#)). Genome-wide proteomic studies of synapse in mouse brain indicate that the synaptic proteins peak around dusk and dawn (Noya et al., 2019 [DOI](#); Brüning et al., 2019 [DOI](#)). Sleep deprivation experiments have demonstrated that the sleep drive is significantly dependent on the cycling of proteins and phosphoproteins in synapses, in contrast to mRNAs (Noya et al., 2019 [DOI](#); Brüning et al., 2019 [DOI](#)). These results suggest that the

gene expression regulation at the protein level is crucial for sleep. Consistently, we detected changes in the quantity of axons in *Mettl5*^{1bp}, suggesting a possible regulation of neural circuits by *Mettl5*.

This study revealed gene-specific functions of ribosome components. Previous study suggested a context dependent component and function of ribosomes (Simsek et al., 2017 [\[1\]](#)). We observed changes in the translational efficiency of genes in the *Mettl5* mutant. One possible mechanism is that rRNA modifications influenced by *Mettl5* affect the binding affinity of ribosomes to specific RNA sequences. Alternatively, the rRNA modifications influenced by *Mettl5* may be responsible for certain ribosome types that impact the translation of this gene profile. Further evidence is needed to elucidate these possibilities.

Material and methods

Drosophila Strains

Fly stocks used in this study were maintained under standard culture conditions. We used the *w*¹¹¹⁸ as the control strain. The following flies were obtained from Bloomington Stock Center: *w*¹¹¹⁸ (Bl: 5905), *Mettl5*-Gal4 (Bl: 19514), *Repo*-Gal4 (Bl: 7415), *nSyb*-Gal4 (Bl: 51941), *pdf*-Gal4 (Bl: 41286), *Clk*^{JRK} (Bl: 80927), *per*⁰¹ (Bl: 80917), *Fmr1*^{Δ50M} (Bl: 6930), UAS-*Trmt112*RNAi (VDRC: 101515), UAS-DenMark, UAS-syt.eGFP (Bl: 33064), UAS-GFPstinger (from Yi Rao's lab), UAS-*Fmr1* (Bl: 6931), UAS-*Mettl5* (FlyORF: F000760) are from Fly ORF collection. UAS-*Mettl5m*-3HA (generated in this study), *Mettl5*^{1bp} and *Mettl5*^{9bp} were generated by the CRISPR/Cas system.

Sleep behavior assays

Sleep assays were performed in an incubator at a temperature of 25 ± 1 °C and a relative humidity of 60% ± 5%. Lights were turned on at ZT0 (06:30 am) and off at ZT12 (18:30 pm). The locomotor activity was recorded using Drosophila Activity Monitoring System (Trikinetics, Waltham, MA). Flies were acclimated to the experimental conditions for 2 days before data collection. Locomotor activity data were collected at 1-minute intervals for 3 days, and analyzed by pysolo as described previously (Gilestro and Cirelli, 2009 [\[2\]](#)). Sleep was defined as periods of 5 min or more of behavioral immobility.

Sleep deprivation is applied by mechanical stimuli using a timer-controlled rotating shaker. The intensity of stimuli was 1500 rpm once every minute (2 s/1 min) (Shimizu et al., 2008 [\[3\]](#)). After acclimating to the experimental conditions for 2 days, the flies were sleep deprived for 12 h (ZT12-ZT24). Mechanical sleep deprivation was performed using the SNAP method to keep flies awake for 12 h overnight (Shaw et al., 2002 [\[4\]](#)). Flies were sleep deprived from ZT12 (beginning of the dark phase) to ZT0 (beginning of the light phase) by a vortex at the lower intensity setting for 3 s every minute. Sleep lost and recovery were calculated for each fly by using the 24-h period preceding deprivation as the baseline. Sleep recovery values were calculated by subtracting 6 h sleep amount during night (ZT12-ZT18) after deprivation from the sleep amount of the corresponding time of the previous day (baseline sleep amount).

Sleep arousal is measured at ZT14. Flies are given a uniform, gentle mechanical stimulus, and any activity within the next five minutes is recorded. If no activity is observed during this period, the fly is considered not to have awakened in response to the stimulation. The number of flies that awaken due to the stimulus is counted, and their percentage is calculated.

Statistical analysis

Statistical analysis was carried out in GraphPad Prism 5. The statistical tests for each experiment are shown in the figures. The significance of difference for sleep parameters was analyzed using Nonparametric test, including two-tailed Mann-Whitney test or one-way ANOVA with Tukey's multiple comparison test. Unpaired Student's t-test analyses were carried out for qPCR data and Log-rank (Mantel-Cox) test analyses were carried out for lifespan data using GraphPad Prism software. For all statistical tests, $p < 0.05$ was considered significant.

Quantitative PCR

Total RNA was extracted from cells and tissues using the TRNzol Universal Reagent (Tiangen #DP4-02). RNA reverse transcription was performed by using PrimeScript RT reagent Kit with gDNA Eraser (TAKARA #RR047A). Quantitative PCR was carried out using SuperReal PreMix Plus (SYBR Green) (Tiangen #DP4-02). *RP49* were used to normalize results between groups. Three independent biological replicates were performed for each experiment. The primers used in this experiment are *RP49*-F: CGGTTACGGATCGAACAAGC; *RP49*-R: CTTGCGCTTCTGGAGGAGA; *Mettl5*-F: CAACACGGTCGTACATTCAG; *Mettl5*-R: AGTCCACCTC AATATCCTTCG.

Imaging and analysis of axon volume

To quantify the axon volume of small LNvs, the plugin Object 3D counter in ImageJ was used to quantify number of pixels from the raw images. The images were processed according to a threshold in which an intact axon can be observed in controls. Axon span from the first axonal bifurcation to the tip of the terminal which was labeled by white rectangular box in Figure 8 was quantified.

Sample collection and library construction for Ribo-seq and RNAseq

For *Drosophila* samples, the *w¹¹¹⁸* and *Mettl5^{1bp}* at ZT15 were transferred to centrifuge tubes and quick-frozen by immersion in liquid nitrogen. The fly heads were separated and collected after quick-frozen with liquid nitrogen. Three biological replicates were collected for *w¹¹¹⁸* and *Mettl5^{1bp}*, respectively. Half of each sample was used for RNA-seq and the other half for Ribo-seq.

For Ribo-seq library construction, samples were treated with specific lysis buffer with cycloheximide (50mg/mL) to acquire the lysate (Novogen, China). To digest the RNA other than ribosome-protected fragments (RPF), fly tissue lysate was treated with unspecific endoribonuclease, RNase I. Isolation of monosomes was performed by size-exclusion chromatography using MicroSpin S-400 HR columns. The RNA samples were then treated with rRNA depletion kit to remove as much rRNA contamination as possible before performing PAGE purification of the relatively short (20~38 nt) RPFs. Following PAGE purification, both ends of the RPF were phosphorylated and ligated with 5' and 3' adapters, respectively. Then the fragments were reversely transcribed to the cDNAs, amplified by PCR, and subjected to library construction after quality test. Ribo-seq libraries were sequenced using PE150 (Illumina).

For RNA-seq library construction, total RNA was extracted using a standard TRIzol RNA extraction protocol. The cDNA library was built and sequenced using illumina PE 150 (Illumina). The RNAseq analysis was performed by Novogen.

Ribo-seq and RNA-seq data were analyzed as follows. Raw data (raw reads) of FASTQ format were first processed. Clean reads were obtained by removing reads containing adapters, reads containing N bases and low-quality reads from the raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated.

RNA-seq analysis

Index of the *Drosophila melanogaster* reference genome (dm6) was built using Hisat2 v2.0.5 (Kim et al., 2019) and paired-end clean reads were aligned to the *Drosophila melanogaster* reference genome (dm6) using Hisat2 v2.0.5. The mapped reads of each sample were assembled by StringTie (v1.3.3b) (Pertea et al., 2015) in a reference-based approach. FeatureCounts (v1.5.0) (Liao et al., 2014) was used to count the reads numbers mapped to each gene. Differential expression analysis of *w¹¹¹⁸* and *Mettl5^{1bp}* was performed using the DESeq2 R package (1.20.0) (Pertea et al., 2015). Genes with an adjusted P-value < 0.05 and $|\log_2\text{fc}| \geq 1$ were assigned as significant differentially expressed gene.

Ribo-seq analysis

First, the clean reads were aligned to a FASTA file containing rRNA, tRNA, snoRNA, and snRNA from Flybase Release 6.13 annotations using Bowtie (Liao et al., 2014) with a maximum of two mismatches (-v 2) by default. Successful alignments were discarded, and all unaligned reads were collected. Unaligned clean reads (ribosome-protected fragments, RPF) were then mapped to the FlyBase D. melanogaster reference genome (Release 6.13) using STAR (v2.7.3a) (Dobin et al., 2013) with default options. The unique genome-mapped reads are then mapped against protein coding transcripts using Bowtie (v1.2.2) (Langmead et al., 2009) with parameters “-a -v 2” by default. The featureCounts program in Subread package v1.6.3 is used to count the number of RPFs uniquely mapped to CDS regions based on fly genome mapping file (-t CDS -g gene_id), which were then normalized as RPF Per Kilobase per Million mapped RPFs (RPKM). DESeq2 R package (1.14.1) (Love et al., 2014) was used for differential expression analysis. The threshold for significantly differential expression is $|\log_2(\text{Fold Change})| \geq 0.265$ & $p^{\text{adj}} < 0.05$. Differential TE analysis was performed using RiboDiff. Coding frame distribution and 3-nt periodicity analyses for Ribo-seq quality evaluation were performed using R package riboWaltz (v1.1.0) (Lauria et al., 2018). The Ribocode package (Xiao et al., 2018) was used to analyze the P-site and the motif of translated/untranslated uORFs. Gene Ontology (GO) and KEGG pathway analysis, Gene Set Enrichment Analysis (GSEA) analysis was performed using the clusterProfiler package (v4.5.2.002) (Wu et al., 2021). Volcano plots, veen plots a were plotted with the ggplot2 package. SimplifyEnrichment package (Gu and Hübschmann, 2022) was used to simply the GO enrichment results.

Immunofluorescence experiments

Adult flies that were 7-15 days old (unless otherwise noted) were anaesthetized with CO₂ and dissected in 0.03% PBST (1 × PBS with 0.03% Triton X-100; Sigma, T9284) on ice. After a 55 min fixation of samples in 2% paraformaldehyde (PFA) at room temperature (RT), samples were washed four times for 15 min in 0.03% PBST at RT, blocked in 10% Normal Goat Serum (NGS; diluted with 1 × PBS with 2% Triton) overnight at 4°C and incubated with primary antibodies for 24 h at 4°C. The samples were washed again four times with 1 × PBS with 1% Triton for 15 min at RT and incubated with secondary antibodies overnight at 4 °C. And then the samples were washed with 1 × PBS with 1% Triton again 4 times for 15 min at RT and mounted on a slide using anti-fading Mounting medium (with DAPI; Solarbio, S2110). The primary antibodies we used were anti-Elav rat (DSHB, 7E8A10, 1:200) and anti-Repo mouse (Developmental Studies Hybridoma Bank, 8D12; 1:200). The secondary antibodies were Alexa Fluor™ 568 (Thermo Fisher Scientific, A11004; 1:200) and Alexa Fluor™ 647 (Thermo Fisher Scientific, A21247; 1:200). The primary and secondary antibodies were diluted in dilution buffer (1.25% PBST, 1% NGS). Images were taken using confocal microscopy (Leica SP8) with LAX software with auto Z brightness correction to generate a homogeneous signal where it seemed necessary and were formatted using Adobe Photoshop CS6. Figures were generated using Adobe Illustrator 2020. For the immunofluorescence

experiment of PDF, the method is similar with the description above. All immunofluorescence experiments were done with at least 3 repeats, each repeat with a sample size of more than 10 individual flies. ImageJ was used to analyze immunofluorescence brightness.

Western Blotting

Total protein was extracted from approximately 30 fly heads using RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0). Protease inhibitor cocktail (CW2200S) and phosphatase inhibitor cocktail (CW2383S) were added according to the manufacturer's instructions. An equal volume of 2x SDS loading buffer was added to the samples, followed by heating in a water bath at 100 °C for 5 minutes, and then cooling on ice for several minutes.

Primary antibodies (Rabbit anti-PER, Gift from Dr. Jeffrey Price's laboratory at University of Missouri at Kansas City, U.S.) were diluted 1:5000 and incubated with the membranes overnight at 4°C, while HRP-labeled secondary antibodies (HRP Goat Anti-Rabbit IgG (H + L) (ABclonal, AS014)) were diluted 1:1500 and incubated for 4 hours at room temperature. Signals were detected using ECL (ABclonal, RM00021P) and visualized with the Amersham ImageQuant 800 (GE Healthcare, Sweden). Signal intensity was quantified using ImageJ software. Three biological replicates were conducted for this experiment.

LC-MS/MS analysis of m⁶A levels

Total RNA was extracted from cells and tissues using TRNzol Universal Reagent (Tiangen #DP4-02). 18S rRNA was obtained through polyacrylamide gel electrophoresis and gel recovery of total RNA. Ribonucleoside standards included rA and N6 mA. The fluid phase used in this experiment was a mixture of methanol and ddH₂O. For each biological replicate, 1 µg of total RNA or 18S rRNA was degraded to single nucleotides using Nucleoside Digestion Mix (NEB #M0649), followed by the addition of a 4-fold volume of methanol and precipitation at -20°C for 2 hours to remove proteins. Multiple reaction monitoring transitions used in this study were 268.10275 → 136.0621 (rA), 282.11835 → 150.0774 (N6 mA).

Lifespan and Negative geotaxis RING assay

Mated males were collected and maintained at a density of 20 flies per vial. The flies were kept at a temperature of 25 °C with 60% humidity and a 12-hour light/12-hour dark cycle. Every other day, the flies were transferred to new vials, and their survival rate was recorded. Statistical analysis and survival curve plotting were performed using GraphPad Prism 5.0. Each experiment was conducted with three biological replicates, using a total of 300 flies for lifespan assays.

For climbing assays, flies were collected under CO₂ anesthesia and allowed to recover overnight. Typically, 200 flies (distributed among 10 vials, each containing 20 flies and marked at 90 mm from the bottom) were used for each genotype. To initiate the climbing assay, vials were tapped sharply on the table three times to elicit negative geotaxis responses. A 15-minute rest period was observed between each trial. Three trials were conducted for each group, and five groups were used for each genotype. The number of flies attempting to reach the mark within 10 seconds was counted for each trial. Videos were recorded during the assay, and the positions of the flies were captured. The camera and vials were separated by a 20 cm distance, and a white open-faced box placed behind the setup provided uniform lighting.

Author contributions

XW and TF designed and performed the experiments, analyzed the data and wrote the manuscript. XY performed the bioinformatics analysis, analyzed the data and edited the manuscript. YR analyzed data and edited the manuscript. JD designed experiments, analyzed data, wrote and edited the manuscript. All authors have read and approved the final manuscript.

Data availability

The RNA-seq and Ribo-seq data from this publication have been deposited to the NCBI bioproject database <https://www.ncbi.nlm.nih.gov/bioproject/> and assigned the identifier PRJNA994860.

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Supplementary information

Supplementary Figures S1-S11

Supplementary Table1

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Reviewer #1 (Public review):

Summary:

Here the authors attempted to test whether the function of Mettl5 in sleep regulation was conserved in *Drosophila*, and if so, by which molecular mechanisms. To do so they performed sleep analysis, as well as RNA-seq and ribo-seq in order to identify the downstream targets. They found that the loss of one copy of Mettl5 affects sleep and that its catalytic activity is important for this function. Transcriptional and proteomic analyses show that multiple pathways were altered, including the clock signaling pathway and the proteasome. Based on these changes the authors propose that Mettl5 modulate sleep through regulation of the clock genes, both at the level of their production and degradation.

Strengths:

The phenotypical consequence of the loss of one copy of Mettl5 on sleep function is clear and well-documented.

Weaknesses:

The imaging and molecular parts are less convincing.

- The colocalization of Mettl5 with glial and neuronal cells is not very clear
- The section on gene ontology analysis is long and confusing
- Among all the pathways affected the focus on proteasome sounds like cherry picking. And there is no experiment demonstrating its impact in the Mettl5 phenotype
- The ribo seq shows some changes at the level of translation efficiency but there is no connection with the Mettl5 phenotypes. In other words, how the increased usage of some codons impact clock signalling. Are the genes enriched for these codons?
- A few papers already demonstrated the role of Mettl5 in translation, even at the structural level (Rong et al, Cell reports 2020) and this was not commented by the authors. In Peng et al, 2022 the authors show that the m6A bridges the 18S rRNA with RPL24. Is this conserved in *Drosophila*?
- The text will require strong editing and the authors should check and review extensively for improvements to the use of English.

Conclusion

Despite the effort to identify the underlying molecular defects following the loss of Mettl5 the authors felt short in doing so. Some of the results are over-interpreted and more experiments will be needed to understand how Mettl5 controls the translation of its targets. References to previous works was poorly commented.

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Reviewer #2 (Public review):

Summary:

The authors define the m6A methyltransferase Mettl5 as a novel sleep-regulatory gene that contributes to specific aspects of *Drosophila* sleep behaviors (i.e., sleep drive and arousal at early night; sleep homeostasis) and propose the possible implication of Mettl5-dependent clocks in this process. The model was primarily based on the assessment of sleep changes upon genetic/transgenic manipulations of Mettl5 expression (including CRISPR-deletion allele); differentially expressed genes between wild-type vs. Mettl5 mutant; and interaction effects of Mettl5 and clock genes on sleep. These findings exemplify how a subclass of m6A

modifications (i.e., Mettl5-dependent m6A) and possible epi-transcriptomic control of gene expression could impact animal behaviors.

Strengths:

Comprehensive DEG analyses between control and Mettl5 mutant flies reveal the landscape of Mettl5-dependent gene regulation at both transcriptome and translatome levels. The molecular/genetic features underlying Mettl5-dependent gene expression may provide important clues to molecular substrates for circadian clocks, sleep, and other physiology relevant to Mettl5 function in *Drosophila*.

Weaknesses:

While these findings indicate the potential implication of Mettl5-dependent gene regulation in circadian clocks and sleep, several key data require substantial improvement and rigor of experimental design and data interpretation for fair conclusions. Weaknesses of this study and possible complications in the original observations include but are not limited to:

(1) Genetic backgrounds in Mettl5 mutants: the heterozygosity of Mettl5 deletion causes sleep suppression at early night and long-period rhythms in circadian behaviors. The transgenic rescue using Gal4/UAS may support the specificity of the Mettl5 effects on sleep. However, it does not necessarily exclude the possibility that the Mettl5 deletion stocks somehow acquired long-period mutation allelic to other clock genes. Additional genetic/transgenic models of Mettl5 (e.g., homozygous or trans-heterozygous mutants of independent Mettl5 alleles; Mettl5 RNAi etc.) can address the background issue and determine 1) whether sleep suppression tightly correlates with long-period rhythms in Mettl5 mutants; and 2) whether Mettl5 effects are actually mapped to circadian pacemaker neurons (e.g., PDF- or tim-positive neurons) to affect circadian behaviors, clock gene expression, and synaptic plasticity in a cell-autonomous manner and thereby regulate sleep. Unfortunately, most experiments in the current study rely on a single genetic model (i.e., Mettl5 heterozygous mutant).

(2) Gene expression and synaptic plasticity: gene expression profiles and the synaptic plasticity should be assessed by multiple time-point analyses since 1) they display high-amplitude oscillations over the 24-h window and 2) any phase-delaying mutation (e.g., Mettl5 deletion) could significantly affect their circadian changes. The current study performed a single time-point assessment of circadian clock/synaptic gene expression, misleading the conclusion for Mettl5 effects. Considering long-period rhythms in Mettl5 mutant clocks, transcriptome/translatome profiles in Mettl5 cannot distinguish between direct vs. indirect targets of Mettl5 (i.e., gene regulation by the loss of Mettl5-dependent m6A vs. by the delayed circadian phase in Mettl5 mutants).

(3) The text description for gene expression profiling and Mettl5-dependent gene regulation was very detailed, yet there is a huge gap between gene expression profiling and sleep/behavioral analyses. The model in Figure 5 should be better addressed and validated.

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Reviewer #3 (Public review):

Xiaoyu Wu and colleagues examined the potential role in sleep of a *Drosophila* ribosomal RNA methyltransferase, mettl5. Based on sleep defects reported in CRISPR generated mutants, the authors performed both RNA-seq and Ribo-seq analyses of head tissue from mutants and compared to control animals collected at the same time point. While these data were subjected to a thorough analysis, it was difficult to understand the relative direction of differential expression between the two genotypes. In any case, a major conclusion was that the mutant showed altered expression of circadian clock genes, and that the altered

expression of the period gene in particular accounted for the sleep defect reported in the *mettl5* mutant. As noted above, a strength of this work is its relevance to a human developmental disorder as well as the transcriptomic and ribosomal profiling of the mutant. However, there are numerous weaknesses in the manuscript, most of which stem from misinterpretation of the findings, some methodological approaches, and also a lack of method detail provided. The authors seemed to have missed a major phenotype associated with the *mettl5* mutant, which is that it caused a significant increase in period length, which was apparent even in a light: dark cycle. Thus the effect of the mutant on clock gene expression more likely contributed to this phenotype than any associated with changes in sleep behavior.

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