Role of Hakai in m⁶A modification pathway in *Drosophila*

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Article

Keywords: Hakai, m6A modification, m6A writer complex, E-cadherin, *Drosophila*

Posted Date: August 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-47812/v1>

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Version of Record: A version of this preprint was published at Nature Communications on April 12th, 2021. See the published version at <https://doi.org/10.1038/s41467-021-22424-5>.

Abstract

N6-methyladenosine (m⁶A), the most abundant internal modification in eukaryotic mRNA, is deposited by a multi-component writer complex. Hakai is an E3 ubiquitin ligase that interacts with several m⁶A writer subunits in proteomic studies, however, its role in m⁶A methylation in animals has not been systematically characterized. Here we show that Hakai colocalizes and interacts with other m⁶A writer components in *Drosophila*, and *Hakai* mutants exhibit typical m⁶A pathway phenotypes, such as lowered m⁶A levels in mRNA, aberrant *Sxl* alternative splicing, wing and behavior defects, common reduced m⁶A peaks and mis-regulated genes with *Mettl3* and *Mettl14* mutants. These results demonstrate that Hakai is a core component of the m⁶A writer complex comprised of seven conserved subunits. Interestingly, our stringent meRIP-seq experiments indicate that the effective m⁶A modification, which depends on the writer complex, is mostly distributed in 5' UTR and near start codon in *Drosophila*, in contrast to the mammalian system. We define a set of high-confident m⁶A methylation sites in 5' UTR in adult flies and it is unlikely the main function of m⁶A modification in *Drosophila* is through RNA degradation. Furthermore, we find that Hakai is required for the protein levels of other m⁶A writer components Fl(2)d and Flacc, but not Nito. Finally, Hakai does not mediate the stability of E-cadherin in wing discs, suggesting its major role as a nuclear protein.

Introduction

There are a variety of chemical modifications on biological macromolecules, such as proteins, nucleic acids, and glycolipids. Like DNA methylation and histone modification, RNA modification represents a new layer of epigenetic regulatory mechanism^{1,2}. More than 150 chemical modifications in RNA have been discovered, and their biological functions are only starting to be revealed³. Chemical modifications of RNA exist in all organisms, including those in tRNA, rRNA, mRNA and long non-coding RNA. Common RNA modifications include N6-methyladenosine (m⁶A), N6,2'-O-dimethyladenosine (m⁶A_m), N1-methyladenosine (m¹A), 5-methylcytosine (m⁵C), N4-acetylcytidine (ac⁴C), 7-methylguanosine (m⁷G), pseudouridine (Ψ), et al^{4,5}. Among them, m⁶A is the most abundant internal modification of mRNA in eukaryotes. Although m⁶A in mRNA was found more than 40 years ago^{6,7}, it was only recently that the field has made extensive progress owing to technological and experimental breakthroughs. By combining m⁶A-specific antibody and high-throughput sequencing, MeRIP-Seq or m⁶A-Seq allows the m⁶A mapping at the whole transcriptome level, thereby providing the possibility to correlate RNA modifications with their biological functions^{8,9}. These and subsequent studies revealed that m⁶A sites contain a consensus motif RRACH (R=G/A; H=U/A/C), and m⁶A peaks are enriched in 3' untranslated region (UTR) and near stop codon in yeast and mammals^{8,9,10}. In *Arabidopsis*, m⁶A is enriched not only in 3'UTR and near stop codon, but also in 5'UTR and around start codon¹¹. In mammalian cells, m⁶A also accumulates in 5'UTR region in response to stress conditions such as heat shock^{12,13}. The distribution of m⁶A is important since it implies the mechanism by which m⁶A modification regulates its mRNA.

Another major breakthrough is the gradual elucidation of the m⁶A modification pathway by biochemical and genetic studies. The m⁶A is deposited by a multicomponent methyltransferase complex (“writers”) ^{14, 15, 16}, mainly recognized by YTH domain-containing “readers” ¹⁷, and can be removed by FTO and ALKBH5 “erasers” ^{18, 19}, although FTO was also indicated as a m⁶A_m demethylase ²⁰. The key catalytic component of the m⁶A writer complex, Mettl3, was purified and cloned in 1990s ^{21, 22}. Since then, studies from yeast, *Arabidopsis*, *Drosophila* and mammalian cells have identified several core components of the writer complex ^{23, 24}, including Mettl14 ^{25, 26}, WTAP (FI(2)d) ^{27, 28, 29}, Virma (Virilizer) ^{30, 31}, Rbm15/15B (Spenito) ^{32, 33}, ZC3H13 (Flacc or Xio) ^{34, 35, 36} and Hakai ³⁷. Interestingly, FI(2)d ³⁸, Virilizer (Vir) ³⁹, Spenito (Nito) ⁴⁰ and Xio ³⁶ were first identified from *Drosophila* sex determination screens and later realized as part of the writer complex. They regulate *Drosophila* sex determination by controlling the alternative splicing of the master regulatory gene *Sex-lethal* (*Sxl*) ^{41, 42, 43, 44, 45, 46}. Recently, Mettl3, Mettl14, as well as the reader YTHDC1, were shown also involved in this process by the same mechanism ^{33, 47, 48}. Thus, *Drosophila* provides a unique system to screen novel components in m⁶A pathway and pinpoints a critical role for m⁶A in regulating splicing. Other than *Sxl* splicing, *Drosophila* m⁶A genes are highly expressed in the nervous system and exhibit similar wing and behavior defects when mutated ^{33, 36, 47}. Mutants of several fly m⁶A factors are viable and thus provide an ideal model to study other processes, such as metabolism and immunity, in the future.

Hakai, also known as CBLL1, was found as an interacting protein with several m⁶A writer components in proteomic studies ^{23, 31, 49}. It encodes a RING finger type E3 ubiquitin ligase and was originally identified as an E-cadherin binding protein in human cell lines ⁵⁰. It was proposed that Hakai ubiquitinates E-cadherin at the plasma membrane and induces its endocytosis, thus playing a negative role post-translationally. Due to the key role of E-cadherin in tumor metastasis, especially epithelial-mesenchymal transition, Hakai has been extensively studied mainly using cell culture and overexpression system ⁵¹, but a previous study using *Drosophila* model did not observe an increase of E-cadherin in *Hakai* mutants ⁵². In *Arabidopsis*, *Hakai* mutants show partial reduced m⁶A levels and the mutant phenotypes are weaker than other writer components ³⁷. Importantly, the in vivo role of Hakai as a core m⁶A writer component has not been demonstrated in any animal species. Here we analyzed the role of Hakai in the *Drosophila* m⁶A modification pathway. Our results demonstrate that Hakai is a bona fide member of the m⁶A writer complex, with its mutants showing reduced global m⁶A levels, typical m⁶A mutant phenotypes and commonly-regulated gene sets. We also obtained a high-quality fly m⁶A methylome using stringent meRIP-seq, characterized the mechanism of Hakai in m⁶A modification, and finally revisited the function of Hakai in E-cadherin regulation.

Results

Hakai interacts and co-localizes with known m⁶A writer complex subunits

Since Hakai was found as an interaction protein with other m⁶A writer components in mammalian proteomic study²³, we searched the large-scale *Drosophila* Protein interaction Map (DPiM) database⁵³. Hakai as a bait can pull down Fl(2)d, Vir, and Nito in affinity purification and mass spectrometry experiments; on the other hand, Flacc as a bait can pull down Hakai (Supplemental Fig. 1B). Similarly, our own previous mass-spec study using Fl(2)d or Nito as a bait can reciprocally pull down Hakai (Supplemental Fig. 1A)³⁶. To confirm these interactions, we performed both co-localization and co-immunoprecipitation (Co-IP) experiments. GFP-Hakai localized to nucleus in live S2 cells and co-localized well with mRFP-Mettl3, mRFP-Mettl14, mRFP-Fl(2)d, mRFP-Nito, and mRFP-Flacc (Fig. 1A-E). Next, we transfected GFP-Hakai and different HA-tagged constructs in S2 cells, and used myc-GFP as a control. In Co-IP experiments, GFP-Hakai, but not myc-GFP, was able to pull down HA-Mettl3, HA-Mettl14, HA-Fl(2)d, HA-Nito, and HA-Flacc (Fig. 1F-J). Interestingly, the pull-down between Hakai and Fl(2)d is particularly strong compared to other factors, suggesting that Hakai may directly interact with Fl(2)d (Fig. 1H). Together, these data suggest that Hakai is a conserved core component of the m⁶A writer complex.

Hakai is required for m⁶A methylation in *Drosophila*

Hakai is located on the second chromosome and its transcript is alternatively spliced, producing long and short protein isoforms, both of which containing the RING finger domain (Fig. 2A). *Hakai* transcript shows a similar expression pattern to those of other m⁶A writers and readers³⁶, with high expression in CNS, ovary, fat body and imaginal discs (Supplemental Fig. 2; modENCODE developmental and tissue expression database⁵⁴). During development, its expression is high in early embryos, decreases during larval stages and rises again at pupal stages (Supplemental Fig. 2), which coincides with the reported m⁶A levels³³. To monitor endogenous protein expression, we raised an antibody against Hakai (Fig. 2A) and found that it is a ubiquitously-expressed nuclear protein that strongly co-localizes with Fl(2)d (Fig. 2C-C").

To study Hakai function, we generated a sgRNA and constructed or obtained three non-overlapping shRNA lines (Fig. 2A)^{55, 56}. By crossing the *U6:3-Hakai sgRNA* with *nanos-Cas9*⁵⁷, we generated a series of *Hakai* mutants with various small deletions and/or insertions. We chose two alleles, *Hakai*^{SH2} and *Hakai*^{SH4} for further analysis, since they represent different early frameshift mutations that are expected to disrupt translation (Fig. 2B). Indeed, only background level of Hakai antibody staining remained in *Hakai* homozygous mutant wing discs compared to wildtype (Fig. 2D, E, compared to 2C), suggesting these are null or strong loss-of-function alleles. *Hakai* homozygous mutants are semi-lethal and only produce viable adult flies in non-crowded conditions, which have delayed developmental time, smaller size, and reduced lifespan (data not shown).

We then measured N6-methyladenosine levels in *Hakai* mutant adults by quantitative liquid chromatography–mass spectrometry (LC-MS) and used *yw* and *w*¹¹¹⁸ as wild-type controls. We used an external calibration curve prepared with A and m⁶A standards to determine the absolute quantities of each ribonucleoside (Supplemental Fig. 3). After two rounds of polyA selection, m⁶A levels drop to around

30% in *Mettl3* or *Mettl14* mutant flies, while m⁶A levels reduce more than half in *Hakai*^{SH2} or *Hakai*^{SH4} mutants (Fig. 2F). These results clearly indicated a critical role of Hakai in m⁶A methylation.

Hakai controls *Sxl*/alternative splicing and adult fly phenotypes

In *Drosophila*, *Sxl* pre-mRNA is the best characterized example of m⁶A modified transcripts. *Sxl*/transcripts are alternatively spliced. While male form includes exon 3 that contains a stop codon and leads to early termination of Sxl protein, the female form skips exon 3 and thus produces a functional Sxl protein (Fig. 3A)⁵⁸. Previously multiple m⁶A sites have been mapped in introns on both sides of exon 3 and these modifications are proposed to facilitate the alternative splicing of *Sxl* in female flies⁴⁸. Indeed, the switch from female form to male form of *Sxl*/splicing occurs in all m⁶A mutants, including *Mettl3*, *Mettl14*, *Ythdc1*, *Fl(2)d*, *Vir*, *Nito*, *Flacc*, thus representing a gold standard to validate new components in this pathway^{33, 34, 36, 38, 41, 43, 47, 48}. To monitor *Sxl*/splicing pattern, we used a pair of primers flanking exon 3 that detects the small female and large male spliced *Sxl* products in RT-PCR (Fig. 3A, B, lane1, 2)⁵⁹. As positive controls, *Sxl*/splicing is partially shifted from the female form to the male form in *Mettl3* or *Mettl14* mutant females (Fig. 3B, lane 3-6). In *Hakai*^{SH2} or *Hakai*^{SH4} female flies, a large band corresponding to the male-specific spliced form was clearly detected (Fig. 3B, last 4 lanes), similar to *Mettl3* or *Mettl14* mutants.

Disruption of several m⁶A components *Fl(2)d*, *Vir*, *Nito* or *Flacc* leads to not only aberrant *Sxl*/splicing, but also strongly reduced Sxl protein levels, thus generating a striking female-to-male transformation phenotype in adult flies^{34, 36, 38, 41, 43, 60}. Mutation of other factors *Mettl3*, *Mettl14* or *Ythdc1* alone does not affect Sxl protein levels and does not exhibit the transformation phenotype^{33, 47, 48}. We found that Sxl protein is not reduced in *Hakai*^{SH2} or *Hakai*^{SH4} female discs (Supplemental Fig. 4A-D). Furthermore, we expressed three *Hakai* RNAi using *dome-Gal4* and did not observe any transformation phenotype in females, as evidenced before (data not shown)^{36, 41}.

Other than *Sxl*/splicing, m⁶A writer and reader mutants show characteristic adult defects. The most prominent ones are the held-out wings and flightless phenotypes in *Mettl3*, *Mettl14*, *Ythdc1*, or *Flacc* mutants^{36, 61, 62}, likely due to m⁶A functions in the nervous system⁶³. Wild-type flies normally keep their wings in a folded position (Fig. 3C, G), however, majority of *Mettl3* mutant flies cannot fold their wings correctly and exhibit held-out wings (Fig. 3D, G), and 100% *Mettl3* mutant flies cannot fly (Fig. 3H). Interestingly, *Hakai*^{SH2} and *Hakai*^{SH4} mutants phenocopy *Mettl3* adult defects in terms of strong held-out wing (Fig. 3E-G) and 100% flightless phenotypes (Fig. 3H). Together, these data suggest that Hakai plays an essential role in m⁶A modification pathway in vivo.

Effective m⁶A modification occurs in 5'UTR and around start codon in *Drosophila*

Previously, only two studies were reported to map global m⁶A methylation pattern in *Drosophila*, one MeRIP-seq in S2R+ cells and another miCLIP in embryos^{33, 47}. They found m⁶A enrichment in CDS³³, in

5'UTR and around start codon⁴⁷, as well as in 3'UTR and around stop codon⁴⁷, but these experiments were not controlled against m⁶A writer mutants. To obtain a high-stringent m⁶A methylome in adult flies, we performed methylated RNA immunoprecipitation sequencing (MeRIP-seq) in *yw* control, *Mettl3*, *Mettl14* and *Hakai* mutants (Supplemental Data 1). In wild-type flies, m⁶A is mostly enriched in 3'UTR and near stop codon, and to a lesser extent enriched in 5' UTR and near start codon (Fig. 4A, B). *De novo* motif analysis using HOMER identified the consensus sequence RRACH (Fig. 4C), consistent with those in mammalian system^{8,9}. There are fewer m⁶A peaks in *Mettl3*, *Mettl14* or *Hakai* mutant compared to wild-type (Fig. 4D), and more peaks are reduced in 5' UTR than 3' UTR region (Fig. 4A, B). We then filtered differential peaks with more stringency ($p < 0.05$ and fold change ≥ 2 or ≤ 0.5) (Supplemental Data 2). Interestingly, many more m⁶A peaks are reduced (*Mettl3*, 2745; *Mettl14*, 2615; *Hakai*, 2036) than increased (*Mettl3*, 330; *Mettl14*, 260; *Hakai*, 278) in these mutants, validating their role as m⁶A writers (Fig. 4E, F). Only in reduced m⁶A peaks, a significant overlap between the three mutants was found (1345 common peaks), suggesting that *Hakai* plays a similar role to *Mettl3* and *Mettl14* in m⁶A methylation. In addition, the common reduced peaks in three writer mutants likely represent a high-confident m⁶A modification sites in *Drosophila*.

Next we focused on reduced m⁶A peaks for further analysis. Surprisingly, around 90% of these peaks are located in 5' UTR and near start codon, and only very small portion occurs in 3' UTR (Fig. 4G, Supplemental Fig. 5A-C). A few examples of loss of m⁶A peaks in the three mutants are shown in Fig. 4H and Supplemental Fig. 6, which almost always happen in 5' UTR, while m⁶A peaks in 3' UTR are generally not changed. These results indicate that although most m⁶A sites map to 3' UTR in *Drosophila*, the peaks responding to the loss of m⁶A writers, and thus the effective ones, are distributed in 5' UTR and this is very different from the mammalian system. Majority peaks in 3' UTR may be mediated by another methyltransferase or come from non-specific background. To demonstrate the categories of genes modified by m⁶A, we performed GO and KEGG enrichment for genes with reduced m⁶A peaks. GO analysis revealed important regulatory mechanisms such as signal transduction, kinase activity, transcription factor activity, as well as biological processes such as nervous system development and open tracheal system development (Fig. 4I, Supplemental Fig. 5D, F, H). KEGG analysis found most key signaling pathways, including mTOR, FoxO, Wnt, Hedgehog, TGF- β , Hippo, MAPK, Notch, Toll and Imd, Autophagy, et al (Fig. 4J, Supplemental Fig. 5E, G, I). Since so many signaling pathways are enriched for m⁶A targets, it is not surprising that this modification is involved in numerous biological processes.

m⁶A modification does not reduce mRNA levels in *Drosophila*

We further analyzed gene expression regulated by m⁶A writer components by RNA-seq (Supplemental Data 3). In *Mettl3*, *Mettl14* and *Hakai* mutant adult flies, 987, 954 and 886 genes were differentially expressed ($p < 0.05$ and fold change ≥ 2 or ≤ 0.5), respectively (Fig. 5A) (Supplemental Data 4). These genes substantially overlap with each other (Fig. 5B) and common differentially expressed genes generally change in the same direction in the three mutants (Fig. 5C), arguing they act in the same

pathway. Interestingly, differentially expressed genes were strongly enriched for immune response genes, as well as chitin binding genes in GO analysis (Fig. 5D, Supplemental Fig. 7). KEGG analysis revealed enrichment for metabolic pathways, including carbohydrate, amino acid and lipid metabolism, possibly reflecting an indirect regulation by m⁶A pathway (Fig. 5E, Supplemental Fig. 7). By exhibiting MeRIP-seq together with RNA-seq data, we observed the reduction of m⁶A peaks in *Mettl3*, *Mettl14* or *Hakai* mutant, and no obvious trend for change of mRNA expression correlated with differential m⁶A peaks (Fig. 5F-H, Supplemental Data 5). We further divided genes into m⁶A targets or non-targets, and cumulative plot show that there is no obvious difference between m⁶A targets and non-targets in *Hakai* or *Mettl3* mutant flies (Fig. 5I, J), while there is a slight positive effect of m⁶A on mRNA levels in *Mettl14* mutant (Fig. 5K), similar to a previous report³³. These findings are consistent with the notion that effective m⁶A modifications are located in 5' UTR in *Drosophila*, and thus do not mediate mRNA degradation as in mammalian system.

Hakai modulates the stability of other m⁶A writer components

We aim to look further the mechanisms of Hakai in m⁶A methylation. Since Hakai is a potential E3 ubiquitin ligase, we examined the protein distribution of other m⁶A writer factors in the absence of *Hakai* using available antibodies. In wild-type wing discs, Fl(2)d, Nito and Flacc are ubiquitous nuclear proteins that co-localizes with each other (Fig. 6A-B). Expression of *Hakai* RNAi in the dorsal half of the wing disc using *ap-Gal4* leads to no effect on Nito protein level (Fig. 6C'), but a strong reduction of Fl(2)d and Flacc levels (Fig. 6C, D). In addition, we crossed *actin-Cas9* with *U6-Hakai-sgRNA* flies to generate random *Hakai* loss-of-function clones. These clones are marked by the loss of Hakai staining and Fl(2)d level is reduced in these clones as well (Fig. 6E-E'). Overall, Hakai is required for the protein levels of Fl(2)d and Flacc in the m⁶A pathway.

Hakai does not mediate E-cadherin levels in wing discs

Hakai was first demonstrated as an E-cadherin interaction protein and its role in E-cadherin endocytosis and down-regulation was extensively studied in cell culture^{50, 51}. However, a previous study in *Drosophila* failed to observe a major role for Hakai in E-cadherin regulation⁵². Thus, we addressed this question using our new genetic toolset. In wild-type wing disc epithelia, E-cadherin shows a membrane distribution and accumulates in the adherens junction (Fig. 7A, A'). First, we used *ap-Gal4* to drive the expression of *Hakai* RNAi in the dorsal half of the wing disc. Although Hakai level is effectively knocked down (Fig. 7C'), E-cadherin level does not change in either the apical or the lateral section (Fig. 7C, C"). Second, in *Hakai* mutant clones generated by crossing *actin-Cas9* with *U6-Hakai-sgRNA*, E-cadherin level is not affected as well (Fig. 7D-D"). Finally, we examined the localization of E-cadherin and Hakai in detail using the large tracheal cells. As shown in Fig. 7B-B", most E-cadherin is in the cell membrane, while most Hakai is in the nucleus, and we did not observe co-localization between these two proteins. In conclusion, Hakai is not important for E-cadherin levels in *Drosophila* and its major function likely happens in the nucleus.

Discussion

m⁶A modification has been known for more than 40 years⁶ but recently gained great attention due to emergence of technologies to map m⁶A methylome^{8, 9, 64}, as well as the identification of the writers, readers and erasers in this pathway^{4, 14, 15, 16}. Since the initial purification of the key methyltransferase Mettl3²¹, other components of the writer complex were gradually identified through biochemical experiments and genetic screens. We now know that m⁶A writer complex is comprised of multiple components including Mettl3, Mettl14, WTAP, Virma, Rbm15/15B, ZC3H13. Hakai was first indicated as a WTAP interaction protein²³ and was shown later required for full m⁶A methylation in *Arabidopsis*³⁷, however, its role in m⁶A pathway in animals has not been studied. Here, we show that Hakai interacts with other m⁶A writer subunits, and *Hakai* mutants exhibit characteristic m⁶A pathway phenotypes, such as lowered m⁶A levels in mRNA, aberrant *Sxl* alternative splicing in females, held-out wings and flightless flies, as well as reduced m⁶A peaks shared with *Mettl3* and *Mettl14* mutants in MeRIP-seq. Altogether, these data unambiguously argue that Hakai is the seventh, and likely last core component of the conserved m⁶A writer complex (Fig. 6F).

Each component in the m⁶A writer complex is essential for m⁶A methylation in mRNA but the exact roles of them are not fully understood. Mettl3 is the only subunit that has the methyltransferase enzymatic activity, while Mettl14 contains an RNA-binding site and allosterically activates the catalytic activity of Mettl3^{65, 66, 67}. WTAP is a key adaptor to interact with Mettl3 and Mettl14^{25, 27, 29} and is required for their nuclear speckle localization^{28, 68}. Virma preferentially mediates m⁶A methylation in 3' UTR and near stop codon in human cells and recruits polyadenylation cleavage factor CPSF5 and CPSF6 to regulate alternative polyadenylation³¹. Rbm15 and Rbm15B contain three RRM domains and bind positions adjacent to m⁶A sites, thus may provide specificity for the m⁶A writer complex³². ZC3H13 is important for the nuclear localization of several writer components, and has also been proposed to link Rbm15/Nito to WTAP/FI(2)d³⁴. Here, we show that Hakai is required for the stability of FI(2)d and Flacc, but not Nito, in *Drosophila* tissues. Overall, more mechanistic studies including super-resolution imaging, biochemical analysis and crystal structures of the complete writer complex will be required to fully elucidate the activity of the m⁶A writer complex.

Recent emerging studies suggest that m⁶A is involved in numerous developmental processes and human diseases^{16, 69, 70}, mainly by controlling mRNA stability, translation or splicing. *Drosophila* has all seven writer complex components, one homolog of YTHDC1 and one homolog of YTHDF, representing a simple and genetically tractable model system, since mammals have three YTHDF readers with specific or redundant functions¹⁷. Pioneer work from three labs have established the framework for m⁶A pathway in *Drosophila*^{33, 47, 48}. However, only published *Drosophila* m⁶A methylome was performed in S2R+ cells or embryos^{33, 47}. It is not clear which genomic regions are enriched for effective m⁶A modification since these experiments were not done against writer mutants. Other than *Sxl*, few m⁶A target loci have been firmly mapped. By performing MeRIP-seq in wild-type adult flies, as well as *Mettl3*, *Mettl14* and *Hakai*

mutants, we demonstrated that although m⁶A peaks are distributed mostly in 3' UTR region, the functional peaks responding to the loss of m⁶A writers are almost all located in 5' UTR region and near start codon. This finding indicates a major difference between *Drosophila* and mammalian m⁶A methylome, which mainly occurs in 3' UTR, and is in agreement with a recently submitted manuscript using miCLIP⁷¹. In zebrafish, more m⁶A peaks were decreased in 5' UTR than 3' UTR region in *Mettl3* morpholino knockdown⁷². Since m⁶A modification in 3' UTR usually causes mRNA instability and m⁶A in 5' UTR is linked to translation enhancement, our results imply that the major role of m⁶A modification in *Drosophila* is not mRNA degradation, but likely translation upregulation. Indeed, we did not observe an increase in the mRNA expression of m⁶A targets in *Mettl3*, *Mettl14* or *Hakai* mutants compared to wild-type flies.

By screening for m⁶A peaks commonly reduced in *Mettl3*, *Mettl14* and *Hakai* mutants, we identified a set of high-confident m⁶A modification genes and peaks in *Drosophila* adults. Interestingly, these genes are enriched for most key signaling pathways, thus explaining the involvement of m⁶A modification in numerous developmental and physiological processes. RNA-seq analysis revealed strong enrichment in immune response genes, as well as various metabolic pathways. The involvement of m⁶A modification in fly immune response has not been reported before, and is likely mediated by direct regulation since Toll and Imd signaling are also enriched in meRIP-seq. The involvement in metabolism might be a combined effect caused by signaling such as mTOR, FoxO, autophagy, et al, as indicated by meRIP-seq. We have observed several developmental and metabolic defects associated with m⁶A mutants, and are currently working on the underlying mechanisms.

Hakai was initially identified as an E-cadherin binding protein to downgrade its levels post-translationally⁵⁰ and the role of Hakai in cell proliferation and tumor progression have been extensively studied in cell culture⁵¹. However, our in vivo analysis using various genetic tools did not find a requirement of Hakai in E-cadherin regulation. In addition, we found that Hakai is a ubiquitous nuclear protein showing little co-localization with E-cadherin in the membrane. Consistently, Hakai was shown to interact with PTB-associated splicing factor (PSF), a nuclear protein, and to affect its RNA-binding ability⁷³. Together, we think that the major function of Hakai occurs in the nucleus and its role in E-cadherin regulation needs to be further investigated using knockout mouse model.

Methods

Fly strains and genetics

Flies were grown on standard cornmeal food and experiments were performed at 25°C. The following stocks were used: *w¹¹¹⁸* (used as wild-type, WT), *yw*, *Mettl3^{SK2}*, *Mettl14^{SK1 61}*, *Df(3R)Exel6197* (*Mettl3* deficiency, Bloomington 7676), *ap-Gal4*, *Hakai* shRNA (VDRC 330548), *actin-Cas9* (Bloomington 54590)⁵⁵, *nanos-Cas9* (Bloomington 78782)⁵⁷. To generate *U6-Hakai-sgRNA*, target sequence "ACGTCCGCGCCCGCGAGCCC" was picked by DRSC Find CRISPRs⁵⁷ and cloned into pCFD3 vector⁵⁵

(Addgene 49410). The construct was inserted at attP40 site by standard PhiC31-mediated transformation to make transgenic flies in UniHuaii. *U6-Hakai-sgRNA* was crossed with *nanos-Cas9* flies to generate a series of indel mutations and *Hakai*^{SH2} and *Hakai*^{SH4} were chosen for further analysis. Two additional Hakai shRNAs were made based on the new pNP vector by Tsinghua Fly Center⁵⁶. TH14422.S with target sequence “GAGCTCGACAAGGACGGCGAA” was inserted at attP2 site and TH14423.S with target sequence “CGGCCGCATGATACCCTGCAA” was inserted at attP40 site. The three *Hakai* shRNAs show similar knockdown efficiency as examined by Hakai antibody staining. TH14422.S was used in Fig. 6C-C’, TH14423.S was used in Fig. 6D, and VDRC330548 was used in Fig. 7C-C”. To test adult flight ability, cohorts of 10 male flies were tapped down into a petri dish and the number of flies that flew away within 2 minutes was recorded.

Immunostaining

Larval wing discs were stained as previously described⁴⁰. Briefly, tissues were dissected in PBS and fixed in 4% formaldehyde (Sigma) in PBST (PBS + 0.1% Triton X-100). After blocking in 1% normal donkey serum (Jackson Immuno) in PBST for 1 hour, the samples were incubated with the primary antibody in the same solution at 4°C overnight. After three washes in PBST, samples were incubated with the secondary antibody for 2 hours at room temperature, washed in PBST three times, and subsequently mounted in Antifade Mounting Medium (Beyotime). All images were taken on a Zeiss LSM 880 microscope.

The following antibodies were used: mouse anti-Fl(2)d (1:10) (9G2, DSHB), rat anti-Ecad (1:5) (DCAD2, DSHB), mouse anti-Sxl (1:10) (M18, DSHB), rabbit anti-Flacc (1:200)³⁶, rabbit anti-Hakai (1:200); Alexa 488- (1:1000) (ThermoFisher) or Cy3- (1:400) (Jackson Immuno) conjugated secondary antibodies and DAPI (1:1000) (Beyotime). Hakai antibody was generated in rabbits against a recombinant protein containing amino acids 1-120 and affinity-purified at ABclonal.

Molecular cloning and co-immunoprecipitation

To generate the GFP-, mRFP- or HA-tagged plasmids, full-length cDNAs for Hakai (the long isoform amplified from a cDNA library), Flacc (GH14795), Nito (GH11110), Fl(2)d (LD21616), METTL3 (AT20169), and METTL14 (LD06016) were cloned into the pENTR vector (Invitrogen), and transferred into the *Drosophila* Gateway vector pAGW, pARW and pAHW. GFP was cloned into pAWM as a control.

Drosophila S2 cells were maintained in Schneider’s medium (Gibco) supplemented with 10% FBS (Gibco) at 25°C. 2 µg of total DNA was transfected into S2 cells in a 60 mm dish with Effectene (QIAGEN). After 48 hours, cells were lysed in IP lysis buffer (Beyotime, 50mM Tris (pH7.4), 150mM NaCl, 1% NP-40, 1 x protease inhibitor) on ice for 30 mins, and cleared at 20000g for 10 mins at 4 °C. Supernatants were incubated with anti-GFP nanobody agarose beads (Allele Biotechnology) for 2 hours at 4 °C. The beads were washed 3-4 times with 1 ml lysis buffer and resuspended in 2 x SDS sample buffer. Eluted proteins were detected by Western blotting using rabbit anti-GFP (1:1000, A6455, Molecular Probes) or rat anti-HA (1:1000, 3F10, Roche) primary antibodies, and HRP-conjugated secondary antibodies (1:3000, Santa Cruz

Biotech). For co-localization, S2 cells were grown on Lab-Tek chamber slides and imaged in live conditions 2 days after transfection.

RT-PCR

Total RNAs were extracted using TRIzol (Invitrogen) and cDNAs were generated from 0.5 µg of RNA using Hifair II 1st Strand cDNA Synthesis Kit with gDNA digester plus (Yeaston). 2xHieff PCR Master Mix (Yeaston) was used for regular PCR. Sx/primers used in Fig. 3B are GTGGTTATCCCCCATATGGC and GATGGCAGAGAATGGGAC, and PCR conditions were described in ⁵⁹.

Analyzing m⁶A levels by LC-MS

Total RNAs were extracted from *yw*, *w¹¹¹⁸*, *Mettl3^{SK2}/Df*, *Mettl14^{SK1}*, *Hakai^{SH2}* and *Hakai^{SH4}* male adult flies about 1-2 days old using TRIzol (Invitrogen), and then subjected to two rounds of poly(A) selection using the GenElute mRNA Miniprep kit (Sigma). Before LC-MS analysis, all RNA samples were hydrolysed enzymatically to ribonucleosides. Briefly, 600ng RNA of each sample was digested by nuclease P1 (Sigma) and snake venom phosphodiesterase (Sigma) at 37°C for 2 hours, and followed by digestion with fast alkaline phosphatase (Thermo Fisher) for another 1 hour. Then, the digested samples were used for the following LC-MS analysis.

Quantitative LC-MS analyses of m⁶A and adenosine were achieved using a Waters UPLC coupled to Thermo Q Exactive mass spectrometer in positive ion mode using dynamic multiple reaction monitoring. The ribonucleosides in the hydrolysed RNA samples were resolved on an acquity UPLC HSS T3 column (1.8 µm particle size, 100Å pore size, 2.1 mm X 100 mm, 30°C) at 300 µl min⁻¹ using a solvent system of 0.1% formic acid in H₂O (A) and acetonitrile (B). The elution profile was 2% B for 2min, 2-11% B over 4 min, then to 11-80% B over 4min, followed by a column washing at 80% B and column equilibration. The quantification of a ribonucleoside can be achieved using m/z of the parent ribonucleoside ion and m/z of the deglycosylated ion product. Nucleosides were quantified based on the transition of the parent ribonucleoside to the deglycosylated base ion: m/z 282.1-150.1 for m⁶A and m/z 268.1-136.1 for A. Absolute quantities of each ribonucleoside were determined using an external calibration curve prepared with A standards (Sigma) and m⁶A standards (Selleck).

meRIP-seq

MeRIP-seq was performed as described in ^{74,75}. Total RNA was isolated from *yw*, *Mettl3^{SK2}/Df*, *Mettl14^{SK1}*, and *Hakai^{SH4}* male adult flies about 1-2 days old using TRIzol (Invitrogen). The RNA amount of each sample was quantified using NanoDrop ND-1000 and the RNA integrity was assessed by Bioanalyzer 2100 (Agilent) with RIN number >7.0, and confirmed by electrophoresis with denaturing agarose gel. Poly(A) RNA was purified from 50µg total RNA using Dynabeads Oligo (dT)25 (Thermo Fisher) by two rounds of purification and then was fragmented into small pieces using Magnesium RNA Fragmentation Module (NEB) under 86°C for 7 minutes. The cleaved RNA fragments were incubated for 2

hours at 4°C with Dynabeads (Dynabeads Antibody Coupling Kit, Thermo Fisher) coupled with m⁶A-specific antibody (202003, Synaptic Systems) in IP buffer (50 mM Tris-HCl, 750 mM NaCl and 0.5% Igepal CA-630). Then the IP RNA fragments and untreated input control fragments are converted to final cDNA library in accordance with a strand-specific library preparation by dUTP method. The average insert size for the final cDNA library was 200±50 bp. At last, we performed the 2×150bp paired-end sequencing (PE150) on an illumina Novaseq 6000 (LC-Bio Technology) following the vendor's recommended protocol.

Bioinformatic analysis

fastp software was used to remove the reads that contained adaptor contamination, low quality bases and undetermined bases with default parameters. Then sequence quality of IP and Input samples were also verified using fastp. We used HISAT2⁷⁶ to map reads to the genome of *Drosophila_melanogaster* (Version: v96) with default parameters. Mapped reads of IP and input libraries were provided for R package exomePeak⁷⁷, which identifies m⁶A peaks with bed or bigwig format that can be adapted for visualization on the IGV software. HOMER⁷⁸ was used for de novo and known motif finding followed by localization of the motif with respect to peak summit. Called peaks were annotated by intersection with gene architecture using R package ChIPseeker⁷⁹. A common peak was picked if the peaks from different groups overlap in the genome > 50% of the smallest of the peaks. StringTie⁸⁰ was used to perform expression level for all mRNAs from input libraries by calculating FPKM. The differentially expressed mRNAs were selected with log2 (fold change) >1 or log2 (fold change) <-1 and p value < 0.05 by R package edgeR⁸¹

Declarations

Acknowledgements

We thank Eric Lai, Bloomington Drosophila Stock Center, TsingHua Fly Center and Vienna Drosophila Resource Center for fly stocks; Developmental Studies Hybridoma Bank for antibodies; the mass-spec and imaging core facility of Institute of Plant Physiology and Ecology for technical support. This work is supported by the National Natural Science Foundation of China (31970786, 91857114, 31771586) and National Key R&D Program of China (2018YFA0800100, 2018YFA0800102) to D.Y..

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Figures

Figure 1

Hakai co-localizes and interacts with other m6A writer components (A-E) GFP-Hakai and mRFP-Mettl3, mRFP-Mettl14, mRFP-FI(2)d, mRFP-Nito or mRFP-Flacc were co-transfected into S2 cells and their subcellular localization examined in live conditions. All the proteins are predominantly nuclear and GFP-Hakai shows strong co-localization with other factors. Scale bars: 5 μ m. (F-J) GFP-Hakai or myc-GFP and HA-Mettl3, HA-Mettl14, HA-FI(2)d, HA-Nito, HA-Flacc were co-transfected into S2 cells. Cell lysates were immunoprecipitated using GFP nanobody and analyzed by Western blot. myc-GFP is used as a control. GFP-Hakai can pull down HA-Mettl3, HA-Mettl14, HA-FI(2)d, HA-Nito and HA-Flacc. Note that much more HA-FI(2)d was co-IPed than other factors.

Figure 2

Hakai is required for m⁶A levels in mRNA (A) Flybase JBrowse view of the Hakai gene locus. Hakai has four transcripts due to alternative splicing that generate two long and two short protein isoforms. The positions of three independent shRNAs, Hakai sgRNA, and the protein region used to generate a Hakai antibody are indicated. (B) Sequencing results showing frameshift indels in Hakai^{SH2} and Hakai^{SH4} flies generated by CRISPR/Cas9 mediated mutagenesis. The targeted genomic DNA sequence is

underlined and the NGG PAM sequence is in bold type. (C-C") Hakai and Fl(2)d antibody staining in WT wing discs showing a high degree of co-localization. Hakai antibody staining is strongly reduced in HakaiSH2 (D) or HakaiSH4 (E) homozygous mutant wing discs. (F) Quantifications of m6A relative to A in mRNA extracted from male adult flies. Compared to yw and w1118 controls, m6A levels drop to about 30% in Mettl3 or Mettl14 mutants and to less than 50% in HakaiSH2 or HakaiSH4 mutants. Error bars represent standard deviation.

Figure 3

Xio regulates Sxl alternative splicing and adult fly behavior (A) Diagram showing the alternative splicing event that produces male- or female-specific Sxl transcripts. The arrows indicate primers used for RT-PCR. (B) Sxl splicing was analyzed by RT-PCR using RNA extracted from adult flies of indicated genotypes. Note the appearance of male-specific bands in Mettl3SK2/Df, Mettl14SK1, HakaiSH2, HakaiSH4 females. Male-specific bands: 2-3-4. Female-specific bands: 2-4. (C) *yw* flies have their wings properly folded. (D) Mettl3SK2/Df, (E) HakaiSH2, (F) HakaiSH4 flies cannot fold their wings and exhibit a held-out wing phenotype (marked by the double arrows). The frequency of flies showing held-out wings were quantified in (G); error bars represent standard deviation. (H) Flies of the indicated genotypes were tested for their flight abilities and the number of flightless flies were quantified; error bars represent standard deviation. All flies used from (C) to (H) are males.

Figure 4

Effective m6A modification in *Drosophila* is distributed in 5' UTR and around start codon (A) MeRIP-seq experiments show the normalized density of m6A peaks across 5' UTR, CDS, and 3' UTR of mRNA in yw, Mettl3, Mettl14, and Hakai mutant male adult flies. (B) Pie charts depicting m6A peak distribution in different transcript segments in yw, Mettl3, Mettl14, and Hakai flies. (C) Sequence motif identified from m6A peaks in yw flies by HOMER program. (D) Cumulative distribution function of log2 peak density of

m6A sites in yw, Mettl3, Mettl14, and Hakai flies. (E) Venn diagram showing overlap of reduced m6A peaks ($p < 0.05$ and fold change ≤ 0.5) between Mettl3, Mettl14, and Hakai flies versus yw control. (F) Venn diagram showing overlap of increased m6A peaks ($p < 0.05$ and fold change ≥ 2) between Mettl3, Mettl14, and Hakai flies versus yw control. (G) Pie chart depicting the distribution of 1345 common reduced m6A peaks in different transcript segments. (H) Integrative Genomics Viewer (IGV) tracks displaying MeRIP-seq (lower panels, IP) and RNA-seq (upper panels, input) reads along S6k mRNA in yw, Mettl3, Mettl14, and Hakai flies. Two replicates are shown. Note the reduced peak in 5' UTR (shaded in yellow) and peaks in 3' UTR (shaded in purple) are not changed. (I) GO term and (J) KEGG pathway analysis of genes with common reduced m6A peaks. The top 20 terms are displayed.

Figure 5

Hakai regulates common transcripts with Mettl3 and Mettl14 (A) Numbers of differentially expressed genes ($p < 0.05$ and fold change ≥ 2 or ≤ 0.5) in Mettl3, Mettl14, and Hakai mutant male adult flies versus yw control. (B) Venn diagram showing common differentially expressed genes between them. (C) Heat map showing the relative expression of 250 common differentially expressed genes in Mettl3, Mettl14, Hakai and yw flies. (D) GO term and (E) KEGG pathway analysis of 250 common differentially expressed

genes between Mettl3, Mettl14, and Hakai. The top 20 terms are displayed. (F-H) Four quadrant plots showing differential m6A peaks together with differential mRNA expression in Mettl3 (G), Mettl14 (H), and Hakai (F) mutants versus yw. Hyper- or Hypo- means increased or decreased m6A peaks; Up or down means increased or decreased mRNA expression levels. (I-K) Cumulative distribution function of fold changes (log2) of RNA expression in Mettl3 (J), Mettl14 (K), and Hakai (I) mutants over yw control separated between m6A targets and non-targets.

Figure 6

Hakai controls the protein levels of other m6A writer components. In wild-type wing discs, FI(2)d (A), Nito (A'), and Flacc (B) show ubiquitous nuclear staining pattern. (C-D) Expressing Hakai RNAi in the dorsal half of the disc (below the dashed line) using *ap-Gal4* results in strong reduction of FI(2)d (C) and Flacc (D) levels, but not Nito staining (C'). (E-E') FI(2)d staining is similarly reduced in Hakai mutant clones generated by *actin-Cas9/U6-Hakai-sgRNA* (E), which are marked by the loss of Hakai staining (E'). (F) A model of the m6A writer complex comprised of seven core components.

Figure 7

Hakai does not mediate E-cadherin levels in wing discs (A-A') Lateral (A) and apical (A') section of E-cadherin staining in wild-type wing disc epithelia. (B-B'') E-cadherin is mainly localized in cell membrane (B), while Hakai is mostly in the nucleus (B') in the large tracheal cells. (C-C'') Expression of Hakai RNAi in the dorsal half of the disc (below the dashed line) using ap-Gal4 leads to strong reduction of Hakai (C'), but E-cadherin staining is not affected either laterally (C) or apically (C''). (D-D'') Lateral (D) and apical (D'') E-cadherin distribution is not changed in Hakai mutant clones marked by the absence of Hakai staining (D'), when crossing actin-Cas9 with U6-Hakai-sgRNA.

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