A neural m^6^A/Ythdf pathway is required for learning and memory in *Drosophila*

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Epitranscriptomic modifications can impact behavior. Here, we used *Drosophila melanogaster* to study N^6^-methyladenosine (m^6^A), the most abundant modification of mRNA. Proteomic and functional analyses confirm its nuclear (Ythdc1) and cytoplasmic (Ythdf) YTH domain proteins as major m^6^A binders. Assays of short term memory in m^6^A mutants reveal neural-autonomous requirements of m^6^A writers working via Ythdf, but not Ythdc1. Furthermore, m^6^A/Ythdf operate specifically via the mushroom body, the center for associative learning. We map m^6^A from wild-type and Mettl3 mutant heads, allowing robust discrimination of Mettl3-dependent m^6^A sites that are highly enriched in 5' UTRs. Genomic analyses indicate that *Drosophila* m^6^A is preferentially deposited on genes with low translational efficiency and that m^6^A does not affect RNA stability. Nevertheless, functional tests indicate a role for m^6^A/Ythdf in translational activation. Altogether, our molecular genetic analyses and tissue-specific m^6^A maps reveal selective behavioral and regulatory defects for the *Drosophila* Mettl3/Ythdf pathway.
About 45 years ago, pioneering studies led by Seymour Benzer identified *dunce*, the first learning mutant in any animal, and established *Drosophila* as an important model to elucidate mechanisms of learning and memory. Although flies execute a broad repertoire of learned behaviors, associative odor learning remains the most widely studied type of learning in this organism. In the aversive olfactory-conditioning paradigm, flies are presented with a pair of neutral odors in succession, one in the presence of electric shock and the other without. Subsequently, when flies encounter this odor again later in the absence of shock, the shock-paired odor elicits an avoidance response. A single training trial is sufficient to induce short-term odor-avoidance memory, which can last several hours. Studies during the past decades identified dozens of protein-coding genes and a half-dozen microRNAs that are required for normal short-term memory (STM) formation.

Along with advances in *Drosophila* memory genetics, substantial progress has been made in deciphering the neuronal anatomy and circuits that underlie memory. The mushroom bodies (MB) have been revealed as the higher-order brain center for associative learning; intrinsic MB neurons called Kenyon cells (KC) receive olfactory signals from primary olfactory sensory neurons via the antennal lobe. As in some vertebrate systems, neuronal ensembles in the *Drosophila* MB are thought to represent odor-memory engrams that are continuously modified by the animal’s experience. More recent studies provided a higher functional resolution of MB compartments (lobes) with regards to various types of olfactory memory. Moreover, clusters of dopaminergic neurons, such as PPL1 and PAM, were found to innervate distinct MB lobes and provide instructive value to the perceived olfactory stimulus.

In our effort to identify additional factors that regulate memory, we were enticed by the “epitranscriptome”, the multitude of modified bases that exceed the standard RNA nucleotides. The most abundant and most well-studied internal modification of mRNA is N6-methyladenosine (m6A). While m6A has been recognized to exist in mRNA since the 1970s, its functional significance has been elusive until recently. Key advances included (1) techniques to determine individual methylated transcripts, and in particular specific methylated sites, and (2) mechanistic knowledge of factors that install m6A (“writers”) and mediate their regulatory consequences (“readers”). The core m6A methyltransferase complex acting on mRNA consists of the Mettl3 catalytic subunit and its heterodimeric partner Mettl14. These associate with other proteins that play broader roles in splicing, mRNA processing and gene regulation, but that are collectively required for normal accumulation of m6A.

Downstream of the writers, various readers are sensitive to the presence or absence of m6A, and thereby mediate differential regulation by this mRNA modification. The most well-characterized readers contain YTH domains, for which atomic insights reveal how a tryptophan-lined pocket selectively binds methylated adenosine and discriminates against unmodified adenosine. In addition, some other proteins were proposed as m6A readers, based primarily on preferential in vitro binding to methylated vs. unmethylated RNA probes. In mammals, m6A readers confer diverse regulatory fates onto modified transcripts, including splicing and nuclear export via the nuclear reader YTHDC1, and RNA decay via cytoplasmic readers Ythdf1-3. Certain YTHDF3 and YTHDC2 were also reported to regulate translation via m6A under specific contexts.

Despite intense efforts into m6A mechanisms and genomics using cell systems, genetic analyses of the m6A pathway have only begun in earnest in the past few years, mostly in vertebrates. Notably, many studies have revealed sensitivity of the mammalian nervous system to manipulation of m6A factors. Mutants in writer (Mettl3 and Mettl14), reader (primarily ythdf1), and eraser (FTO) factors have collectively been shown to exhibit aberrant neurogenesis and/or differentiation. Moreover, these mutants impact neural function and behavior, including during learning and memory paradigms. Overall, these observations may reflect some heightened requirements for m6A in neurons, perhaps owing to their unique architectures and/or regulatory needs.

Amongst invertebrates, *Caenorhabditis elegans* lacks the core m6A machinery, but the presence of a *Drosophila* ortholog of Mettl3 (originally referred to as IME4) opened this model system. While mammals contain multiple members of both nuclear and cytoplasmic YTH pathway domains, the fly system is simplified in containing only one of each, referred to as Ythdc1 (YT-521B or CG12076) and Ythdf (CG6422), respectively. Recently, the Soller, Rognant and Lai labs established biochemical, genetic, and genomic foundations for studying the m6A pathway in *Drosophila*. Surprisingly, these studies jointly reported that knockout of all core m6A writer factors in *Drosophila* is compatible with viability and largely normal exterior patterning. Nevertheless, mutants of Mettl3, Mettl14, and Ythdc1 exhibit a common suite of molecular and phenotypic defects. These include several behavioral abnormalities as well as aberrant splicing of the master female sex determination factor *Sex lethal* (Sx). The suite of locomotor and postural defects in *Drosophila* mutants was again consistent with the notion that the nervous system might be especially sensitive.

However, a major open question from these studies concerns the regulatory and biological roles of the sole *Drosophila* cytoplasmic YTH factor, Ythdf. In contrast to other core m6A factors, we did not previously observe overt defects in our Ythdf mutants, nor did it seem to exhibit robust m6A-specific binding activity. Here, we use proteomic analyses to reveal Ythdc1 and Ythdf as the major m6A-specific binders in *Drosophila*, and focused biochemical tests show that Ythdf prefers a distinct sequence context than tested previously. Hypothesizing that the nervous system might exhibit particular needs for the m6A pathway, we utilized a paradigm of aversive olfactory conditioning to reveal an m6A/Ythdf pathway that is important for STM in older animals. We complement these phenotypic data with high-stringency maps of methylated transcript sites from fly heads, and show that m6A does not impact transcript levels but is preferentially deposited on genes with lower translational efficiency. Nevertheless, functional tests reveal that Mettl3/Ythdf can enhance protein output. Finally, we show that physiological Mettl3/Ythdf function is explicitly required within mushroom body neurons to mediate normal conditioned odor memory during aging. Overall, our study provides insights into the in vivo function of this mRNA modification pathway for normal behavior.

**Results**

*Drosophila* Ythdc1 and Ythdf bind m6A in A-rich contexts. In mammals, two general classes of m6A-binding proteins (“readers”) are recognized, based on whether they contain or lack a YTH domain. Although evidence has been shown for preferential association to m6A vs. A for non-YTH proteins, the YTH domain is the only module for which the structural basis of selective m6A binding is known.

The *Drosophila* genome encodes single orthologs of nuclear (Ythdc1) and cytoplasmic (Ythdf) YTH factors. We previously tested capacities of their isolated YTH domains to associate preferentially with m6A, using RNA probes bearing GGm6ACU vs. GGACU contexts. This motif represents the favored binding site for mammalian YTHDC1, which has explicitly been shown to prefer G and disfavor A at the −1 position. Of note, however,
m6A probe: AA[m6A]C-5DzU-GUAC-biotin
A probe: AAAC-5DzU-GUAC-biotin

Fig. 1 Ythdc1 and Ythdf are the major m6A-binding proteins in Drosophila. A RNA photoaffinity probes used in crosslinking assays. B and C Both nuclear (Ythdc1) and cytoplasmic (Ythdf) Drosophila YTH factors specifically recognize m6A within the AAm6ACU context. Point mutations of aromatic residues that line the m6A cage (3A variants) abolish selective binding to m6A probes. FT Flow through. Shown is one representative result from two repeats. D Proteomic profiling of S2 cells using m6A and A RNA photoaffinity probes reveals Ythdc1 and Ythdf as the only preferentially bound (reader) proteins, and no strongly repelled proteins were found in these conditions. Background proteins are clustered together around the plot origin; threshold interquartile range. Two biological replicates were plotted. E Selected values of fly homologs of mammalian m6A readers and repelled proteins expressed in S2 cells, as well as candidates of novel bound/repelled factors. Source data are provided as a Source Data file and Supplementary Dataset 1.

As our previous mapping suggested that Drosophila m6A modifications are biased to have upstream adenosines55, we next compared AAm6ACU/AACU probes. Interestingly, both Ythdc1 and Ythdf exhibited clearly preferential binding to methylated adenosine in this context (Fig. 1B, C). Next, we tested variants in which three critical tryptophan/leucine residues in the m3A-binding pocket were mutated to alanine (Supplementary Fig. 1). Although “3A” mutant proteins accumulated to similar levels as their wild type counterparts, both Ythdc1-3A and Ythdf-3A failed to bind m6A (Fig. 1B, C), indicating that their specificity for methylated RNA requires intact YTH domains. Thus, Drosophila YTH proteins, in particular Ythdf, may prefer an A-rich context.

Ythdc1 and Ythdf are the dominant Drosophila proteins specifically bound to AAm6ACU probes. Having clarified that both fly YTH factors specifically discriminate between m6A and A, we sought to identify differential binders using an unbiased approach. Proteomic studies in mammalian cells reveal YTH factors as dominant proteins that preferentially associate with mammalian YTHDF1 does not share this discriminatory feature25,56. We previously observed the YTH domain of Drosophila Ythdc1 exhibits robust and selective binding to this methylated probe, but the corresponding domain of Ythdf had only modest activity. From these tests, it was not clear whether the isolated YTH domain might not be fully functional, or perhaps prefers a distinct target site. We tested both of these notions.

We compared the binding of full-length Ythdc1 and Ythdf proteins to m6A vs. A using biotinylated RNA photoaffinity probes. These probes contain diazirine-modified uridine (5-DzU) that can be cross-linked to protein upon UV irradiation (Fig. 1A). We have shown that 5-DzU does not interfere with protein binding at the modified nucleotide, and therefore enables high-efficiency detection of associated proteins57. We incubated cell lysates expressing tagged YTH proteins with beads conjugated to GGm6ACU/GGACU RNA probes, immunoprecipitated complexes with streptavidin, and performed Western blotting for YTH factors. We observed modestly enhanced association of Ythdc1 to GGm6ACU vs. GGACU, while Ythdf did not crosslink preferentially to this methylated probe (Supplementary Fig. 1).
m6A compared to unmethylated probes, along with some other proteins (e.g. FMRP and LRPPRC), and reciprocally some factors that are repelled by this modification (e.g. stress granule factors such as G3BP1/2, USP10, CAPRIN1, and RBM42 57,58. As well, other methods were used to identify mammalian factors that appear to bind preferentially to m6A, such as G3BP1/2, USP10, CAPRIN1, and RBM42. Overall, while conceivable that other target sequences or lysate sources might reveal additional differential binders, we subsequently focused on YTH domain factors as the major direct readers for m6A biology in Drosophila.

Neural autonomous function of m6A supports olfactory learning. The expression of several m6A factors is elevated in the Drosophila nervous system, and mutants of m6A factors are viable, but some exhibit locomotor defects55–57. As this suggested preferential sensitivity of the nervous system to m6A, we examined phenotypic requirements of neural m6A in greater detail.

Recent studies reported that the m6A pathway is required for learning and memory in mice34,46. To investigate whether this is also true for flies, we used a classical aversive conditioning paradigm to test Drosophila m6A mutants for deficits in STM. To obtain time-resolved performance measurements, we employed a conditioning apparatus that we named the multi-olfactory training (MOT) chamber (Supplementary Fig. 1). Briefly, during olfactory training one odor is administered in the presence of a shock stimulus while the other odor is subsequently delivered in the absence of foot shock (Fig. 2B). Because shock is innately aversive, Drosophila will associate the odor given in the presence of shock with harm and will tend to avoid it during later encounters. During the test phase the flies are presented with both odors and the avoidance of the conditioned odor is quantified as a measure of aversive shock-odor memory.

We used the respective heterozygotes as controls in following tests. To minimize background genetic effects, a frequent confound of behavioral assays, we compared these to trans-heterozygous or hemizygous (over deficiency) allelic combinations. In young flies, both writer mutants were essentially normal: we observed only a modest STM reduction in 10-day-old Mettl3[null] flies. While similarly aged Mettl4 mutants showed no impairment (Supplementary Fig. 3). However, at 20 days, both Mettl3[null] and Mettl3[∆cat] hemizygous nulls displayed a substantially stronger (API −0.2 to −0.3) STM impairment (Fig. 2C, D). Consistent with the role of Mettl14 as a cofactor for Mettl3, hemizygote Mettl14[sk1] mutants also exhibited comparable STM impairments in 20-day-old flies (Fig. 2E, F). Assays of whole animal mutants did not resolve if the nervous system per se was involved in these behavioral defects.
addressed this using tissue-specific knockdown and rescue experiments. We first generated Mettl3[null] hemizygote animals bearing elav-Gal4 and UAS-Mettl3 transgenes, to drive their expression in all neurons. In this genetic background, all non-neuronal cells of the intact animal lack Mettl3. Strikingly, these flies exhibited normal STM (Fig. 2G), providing stringent evidence that the odor avoidance behavioral defect of m6A knockouts is strictly due to a cell-autonomous function of Mettl3 in neurons.

**Ythdf, but not Ythdc1, is the functional effector of m6A during STM.** We sought to elaborate the regulatory pathway underlying m6A in learning and memory. Prior genetic assays linked m6A writers Mettl3/Mettl14 in a pathway with nuclear reader Ythdc1 in neurons. However, this was measured using a different assay in which the effect of neuronal loss of Mettl3 or Mettl3-RNAi-dependent phenotypes was assessed. Since a subset of these called regions contained clear local m6A peaks (Fig. 4C), suggesting they are functionally less relevant. Therefore, we applied stringent filtering to focus our attention on the rich set of clearly Mettl3-dependent peaks (Fig. 4C). In addition, as we employed strong selection for polyadenylated transcripts for input, we prioritized studies of annotated genes. Altogether, our analyses (see the "Methods" section) yielded 3874 Mettl3-dependent m6A peaks (Fig. 4B, Supplementary Dataset 3). These might conceivably represent transcript regions modified by another factor, but we did not identify such factors.

**Normal locomotor response and olfactory acuity in Mettl3 and Ythdf mutants.** Since STM performance depends on locomotion and olfaction, we asked if the defects in Mettl3 and Ythdf mutants were specific to memory-related behavior. We noted that a previous study reported adult walking defects in m6A mutants, but we did not detect such defects in Mettl3 and Ythdf mutants before, during, and after a shock stimulus in the MOT. Both Mettl3 and Ythdf locomotor responses to shock were very similar to controls (Supplementary Fig. 6a), indicating that m6A mutants do not exhibit shock sensitivity or locomotion defects in the MOT setup that might affect the memory measurements. Second, we tested the olfactory acuity of aged m6A mutants. Given a choice between the MCH odor and clean air (in the absence of conditioning), wildtype, Mettl3, and Ythdf avoidance scores were all comparable (Supplementary Fig. 6b, c). This was true for both the same MCH concentration used in conditioning experiments, and a four-fold higher concentration. Thus, the olfactory acuity of m6A mutants appears normal.

Neither Mettl3 nor Ythdf can cross-rescue each other's memory defects. We next asked whether overexpression of Ythdf in Mettl3 mutants, or the reciprocal genetic manipulation, would affect STM. Successful rescue could, for example, suggest that the reading function of Ythdf is not fully dependent on Mettl3 methylation, i.e. may somehow involve a parallel pathway. However, Mettl3 mutants supplemented with pan-neuronal Ythdf overexpression did not show STM improvement (Supplementary Fig. 7a). Similarly, overexpression of Mettl3 did not improve the STM impairment in Ythdf mutants (Supplementary Fig. 7b). Beyond serving as stringent negative controls for the cognate rescue experiments (Figs. 2 and 3), these results provide further credence to the notion that a linear, directional Mettl3/14 → Ythdf pathway underlies m6A-mediated function for STM.

**Mapping the Mettl3-dependent m6A methylome in Drosophila.** To link these brain-function defects to the underlying molecular landscape of RNA methylation, we sequenced m6A sites from polyadenylated transcripts using miCLIP. Although we previously reported miCLIP datasets from Drosophila embryos and larvae, we recognized that there can be background association in such data. Thus, individual sequencing “peaks” need to be interpreted cautiously. To provide a stringent basis to infer the existence of m6A at given sites, we analyzed companion input and miCLIP libraries from dissected heads, which are highly enriched for neurons, comparing wild-type and deletion mutants of Mettl3, which encodes the catalytic methyltransferase subunit essential for mRNA modification (e.g. Fig. 1 and Supplementary Dataset 2). The miCLIP libraries from Mettl3 mutants proved especially valuable, because they allowed us to distinguish m6A-IP loci that were clearly genetically dependent on endogenous Mettl3 (Fig. 4A, Supplementary Fig. 8a). Reciprocally, numerous regions of the transcriptome were significantly enriched in miCLIP libraries compared to input, but whose signals persisted in Mettl3 mutants (Fig. 4B, Supplementary Fig. 8b). These might conceivably represent transcript regions modified by another factor, but we did not identify such factors. In general, the Mettl3-independent peaks were globally present in weaker m6A peaks (Fig. 4C), suggesting they are functionally less relevant. Therefore, we applied stringent filtering to focus our attention on the rich set of clearly Mettl3-dependent peaks (Fig. 4A–C). In addition, as we employed strong selection for polyadenylated transcripts for input, we prioritized studies of annotated genes. Altogether, our analyses (see the "Methods" section) yielded 3874 Mettl3-dependent m6A peaks from 1635 genes. Since a subset of these called regions contained clear local minima, we applied PeakSplitter to arrive at 4686 head m6A peaks (Supplementary Dataset 3).

**Drosophila m6A is highly enriched in 5′ UTRs within adenine-rich contexts.** Characterization of Drosophila Mettl3-dependent m6A peaks revealed fundamental similarities and differences with m6A patterns in other organisms. Mammalian (e.g. human and mouse) m6A is well-known to dominate at stop codons and 3′ UTRs. In fish, m6A is also highly enriched at...
**Fig. 3** Ythdf, but not Ythdc1, mediates the role of m^6^A in *Drosophila* STM. A–C Lifespan measurements of m^6^A writer and reader mutants. Mutants of Mettl3 (A) and nuclear reader Ythdc1 (B) exhibit severely shortened lifespan, but mutants of cytoplasmic reader Ythdf (C) shows only a minor lifespan reduction. Mettl3[null]/Df (n = 72) vs. w[1118] (n = 106) = −34.2[95CI −42.1, −26.3] p < 1*10^{-4}. Mettl3[null]/Mettl3[Δcat] (n = 88) vs. w[1118] (n = 106) = −43.3[95CI −50.66, −35.82] p < 1*10^{-4}. Ythdc[ΔN]/Df (n = 91) vs. w[1118] (n = 106) = −50.5[95CI −57.53, −42.81] p < 1*10^{-4}. Ythdf[NP3]/+ (n = 113) vs. w[1118] (n = 133) = −12.9[95CI −6.57, +4.24] p = 0.431. Ythdf[NP3]/Df (n = 133) vs. w[1118] (n = 133) = −14.7[95CI −19.37, −9.96] p < 1*10^{-4}. D–G STM measurements in 20-day flies. D Despite gross behavioral defects and short lifespan, Ythdc1 mutants exhibit normal STM. Ythdc[ΔN]/Df (n = 180) vs. Ythdc1[ΔN]/+ (n = 180) = +0.04[95CI −0.18, +0.25] p = 0.709. E Pan-neuronal knockdown of Ythdc1 using elav-Gal4 (elav-G4) also yields normal STM. Ythdc1[RNAi]/+ (n = 216) vs. elav-G4/+(n = 168) = −0.03[95CI −0.25, +0.14] p = 0.717. Elav-G4/Ythdc1[RNAi] (n = 216) vs. elav-G4/+ (n = 168) = −0.09[95CI −0.26, +0.08] p = 0.568. F Ythdf hemizygotes recapitulate age-induced STM impairment seen in m^6^A writer mutants. Ythdf[NP3]/Df (n = 168) vs. Ythdf[NP3]/+(n = 168) = −0.27[95CI −0.49, −0.04] p = 0.023. G Pan-neuronal knockdown of Ythdf compromised STM similar to the impairment observed in whole animal Ythdf mutants. Ythdf[RNAi]/+(n = 144) vs. elav-G4/+(n = 144) = +0.06[95CI −0.14, +0.28] p = 0.707. Elav-G4/Ythdf[RNAi] (n = 144) vs. elav-G4/+(n = 144) = −0.21[95CI −0.4, −0.02] p = 0.053. STM assays in D–G were conducted in 20-day-old flies. Bars in D–G represent mean values. All control-test differences are displayed as effect sizes with error curves and 95% confidence intervals, two-tailed Mann–Whitney P values are shown for legacy purposes only. Source data are provided as a Source Data file.
Fig. 4 High stringency mapping of the Drosophila m6A methylome reveals new features. A IGV screenshots of genes that exemplify archetypal 5′ UTR miCLIP enrichment and the utility of wild type vs. Mettl3 mutant comparisons. The IGV tracks above the transcript model depict miCLIP and input libraries from wild type and Mettl3 female heads. Note the 5′ UTRs of all three genes (Atpalphα, nrv3, and fre) contain prominent Mettl3-dependent peaks (blue box). Other exonic regions sequenced in miCLIP libraries are not enriched above input libraries (e.g. gray box). B IGV screenshot of Non2 illustrates a 5′ UTR Mettl3-independent peak (red box). C Heatmaps of Mettl3-dependent and -independent m6A peaks. Heatmaps of input normalized miCLIP signals at Mettl3-dependent and independent peaks. Each line represents miCLIP enrichment over input across a MACS2-called peak, as well as 500 nt flanking regions. Heatmaps on the left are all peaks including Mettl3-dependent and independent m6A peaks. Mettl3-independent m6A peaks are displayed in the right panels. Note that the scales are different for the heatmap panels. Approximate locations of Mettl3-independent m6A peaks within all peaks are indicated by a dashed box. D Metagene profiles of miCLIP and input signals along a normalized transcript using genes that contain high-confidence m6A peaks in head libraries. An overwhelming 5′ UTR enrichment is observed. E Metagenes of enrichment at high-confidence m6A peaks that have been group as 5′ UTR/start codon and 3′ UTR/CDS regions. Metagenes are generated by averaging signals from input normalized miCLIP from WT head. On average, stronger signals are observed at 5′ UTRs/start codons. Peak start and end are specified on the x-axis. Dashed lines include the start and end locations of peaks. F Pie chart depicting the fraction of m6A peaks in different transcript segments. G Nucleotide content surrounding CIMs located within the top 1000 Mettl3-dependent m6A peaks in head. Source data are provided as a Source Data file and Supplementary Dataset 3.

stop codons, but the predominant Mettl3-dependent signals localize to 5′ UTRs.65 Previous work in Drosophila was conflicting, since low-resolution meRIP-seq suggested mostly CDS modification with a small minority in UTRs,54 while our prior miCLIP data indicate dominant UTR modifications, preferentially in 5′ UTRs.55 However, these maps were generated with different technologies, and neither was controlled against mutants.

Our miCLIP data provide a clearer perspective. Strikingly, while found at some level throughout the transcriptome, m6A predominates in 5′ UTRs in Drosophila. This can be observed at numerous individual loci (Fig. 4A and Supplementary Fig. 8) and via miCLIP metagene profiles (Fig. 4D). Overall, while we do observe some Mettl3-dependent coding sequence (CDS) and 3′ UTR miCLIP peaks (Fig. 4E, and Supplementary Fig. 8), these were overall rare, of generally lower ranks than 5′ UTR and start codon peaks (Fig. 4F), and not appreciably enriched in metagene profiles over companion mutant datasets (Fig. 4D).

We examined C-to-T crosslinking-induced mutations following adenosine residues (CIMs), which have been taken to represent individual m6A site in miCLIP data.61,66 In particular, we focused on CIMs located within Mettl3-dependent m6A-IP peaks, which we took as bearing high-confidence RNA methylation sites (Supplementary Dataset 4). Within these, the sequence context of CIMs in Drosophila roughly resembles the DRAC context that has been observed in other species.19 However, while a majority of sites fall into a GGACH context in vertebrates,19, m6A sites in Drosophila prefer AAAACD (Fig. 4G), correlating with the preferred binding sites of Ythdc1 and Ythdf in our assays of photocrosslinking-activated m6A probes (Fig. 1).

We validated our map by testing m6A-IP to IgG-IP samples for enrichment of m6A target transcripts using rt-qPCR (Supplementary Fig. 9). We validated a number of top m6A targets (e.g. agq, Syx1A, fl(2)d, prosap, pup, futsch, gish) from whole female fly RNA (Fig. 5A). Still, recognizing that m6A-IP-qPCR evaluates the presence of entire transcripts in pulldowns, we performed parallel experiments from Mettl3[null] female flies. All of these binding events, even loci with very modest enrichment in wild type (e.g. sky, Fig. 5A), were found to be Mettl3-dependent. By contrast, control loci lacking m6A peaks (five and CG7970) showed very little m6A-dependent IP signals, and these were
Drosophila YTH factors associate with m6A targets in a YTH-dependent manner. We next assessed association of m6A targets with YTH factors using transfected constructs in S2-S cells, a derivative of S2 cells lacking viruses67. Although overexpression may affect the localization properties of YTH domain proteins31, we showed that ectopic Ythdc1 and Ythdf localize to the nucleus and cytoplasm of cultured cells, respectively35. In these tests, it is also relevant to consider that we are evaluating the association of the test proteins and target RNAs, which may or may not occur directly through the modified nucleotides. However, we can compare these to YTH-"3A" point-mutant counterparts that disrupt m6A selectivity (Fig. 1).

We immunoprecipitated tagged YTH wild-type or “3A” mutant factors and performed qPCR for validated m6A targets or negative control transcripts. By comparison to control GFP-IP, we observed preferential binding of Ythdc1/Ythdf on multiple m6A targets, compared to non-m6A transcripts (Fig. 5B, C). By testing companion “3A” mutant factors, we gained evidence for direct association of YTH factors on m6A targets. However, a clear picture of target selectivity did not emerge (Fig. 5B, C). Syx1A and aqu exhibited the most clearly differential association between wt and 3A forms of both Ythdc1 and Ythdf. We observed potentially selective association with other loci, in that gish was preferentially bound only by wt Ythdc1 while fl(2)d was preferentially bound only by wt Ythdf.

We bear in mind these were ectopic experiments, and thus cannot rule out non-physiological associations. Even though we observed many cases of YTH-dependent target association, both YTH-3A proteins still exhibited apparent enrichment compared to GFP. If these mutant YTH proteins are still capable of incorporating into RNA granules, this may conceivably indicate indirect interactions with transcripts. Nevertheless, these data provide evidence that YTH domain proteins, including Ythdf, associate with specific m6A target transcripts via their m6A-binding pocket in Drosophila cells.

m6A does not globally influence mRNA levels in Drosophila. There is diverse literature on linking mammalian YTHDF homologs to RNA decay and/or translational activation, while the function of Drosophila m6A/Ythdf has been little studied. The only prior study integrated MeRIP-seq peaks from S2R+ cells with RNA-seq data from m6A pathway deletions, and concluded that m6A correlated with slightly elevated levels of target mRNAs at steady state54. With our high-stringency m6A map from heads, we generated RNA-seq data from one- and three-week old heads using Mettl3, Ythdf heterozygotes, and transheterozygotes. The heterozygote samples provide matched genetic backgrounds for comparison, and the temporal series assesses CNS stages including an advanced setting during which behavioral phenotypes were apparent (Figs. 2 and 3).

Transcriptome analyses revealed scores of differentially expressed genes in one- and three-week old mutants (Supplementary Dataset 5), a majority of which were uniquely misexpressed (Supplementary Fig. 10a, b). Most affected genes were not found to be common between m6A writer (Mettl3) and reader (Ythdf) mutants, although there were mild changes that gradually increased with tissue age (Supplementary Fig. 10c and d). Thus, there did not appear to be a clear signature of m6A/ Ythdf regulation revealed by bulk gene expression.

We examined this more closely by directly examining the behavior of m6A targets. We reasoned that targets with systematically higher levels of methylation—that is, genes with increasing proportions of methylated transcripts—would be more sensitive to loss of the m6A pathway. However, while our miCLIP libraries provide Mettl3-dependent peaks and single nucleotide resolution mapping of m6A sites in the transcriptome, it is not possible to infer overall methylation levels. A solution to this limitation, grouping targets by number of sites/peaks, has been adopted by others31,68 and proposes that targets with increasing numbers of peaks/sites may have more individual transcripts with at least one m6A modification. Therefore, we binned genes by numbers of Mettl3-dependent m6A peaks.
In contrast to prior association of *Drosophila* m\(^6\)A with increased steady-state levels of targets\(^5\), we did not observe many changes in our high-confidence m\(^6\)A targets in *Mettl3* (Fig. 6A) or *Ythdf* mutant CNS from any stage (Supplementary Fig. 10f–h). Paradoxically, even though all bins of m\(^6\)A targets clustered closely with a log\(_2\) fold change of 0, Kolmogorov–Smirnov (KS) tests indicated statistical significance when comparing sets of m\(^6\)A targets and background. While statistically different, our analyses failed to detect any directional gene expression changes in methylated transcripts under writer or reader loss (Fig. 6A and Supplementary Fig. 10).

Since it was conceivable that some expression trends were masked in steady-state measurements, we examined a published dataset of in vivo mRNA decay rates generated using dynamic TU-tagging from the *Drosophila* CNS, obtained by pulse-chase labeling of *pros-Gal4 > UAS-UPRT* cells with 4-thiouridine\(^6\). We

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### Figure Legends

**Figure 2**

**A** m\(^6\)A vs. steady state RNA levels

**B** m\(^6\)A vs. mRNA stability

**C** m\(^6\)A vs. translational efficiency (TE)

**D** m\(^6\)A vs. m\(^5\)C

**E** m\(^6\)A vs. m\(^5\)U

**F** mRNA Decay Assay

**G** Head TE

**H** Neural mRNA halflife

**I** mRNA Decay Assay

**J** aqz-wt (aqz)

**K** aqz-M

**L** ptc-gal4; UAS-HA-YTHDF / tub-GFP

**M** ptc-gal4 / UAS-DsRed ; tub-aqz-5'UTR-GFP

**N** ptc-gal4; UAS-HA-YTHDF / tub-aqz-5'UTR-GFP

**O** ptc-gal4; UAS-HA-YTHDF-3A / tub-aqz-5'UTR-GFP

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### Notes

- In contrast to prior association of *Drosophila* m\(^6\)A with increased steady-state levels of targets\(^5\), we did not observe many changes in our high-confidence m\(^6\)A targets in *Mettl3* (Fig. 6A) or *Ythdf* mutant CNS from any stage (Supplementary Fig. 10f–h).
- Paradoxically, even though all bins of m\(^6\)A targets clustered closely with a log\(_2\) fold change of 0, Kolmogorov–Smirnov (KS) tests indicated statistical significance when comparing sets of m\(^6\)A targets and background. While statistically different, our analyses failed to detect any directional gene expression changes in methylated transcripts under writer or reader loss (Fig. 6A and Supplementary Fig. 10).
- Since it was conceivable that some expression trends were masked in steady-state measurements, we examined a published dataset of in vivo mRNA decay rates generated using dynamic TU-tagging from the *Drosophila* CNS, obtained by pulse-chase labeling of *pros-Gal4 > UAS-UPRT* cells with 4-thiouridine\(^6\). We
Fig. 6 m6A mediates translational activation in Drosophila. A Steady-state RNA levels of m6A targets are not affected by loss of m6A. Differential gene expression analysis of 1-week-old Mettl3 heterozygote and mutant heads. No directional change was observed in any group of m6A targets, binned by increasing numbers of Mettl3-dependent m6A peaks. B m6A targets are not biased in their mRNA stability. The half-life of neural genes obtained from TUD-decay measurements is plotted as cumulative distribution grouping target genes as in (A), based on number of peaks per target. C CRISPR/Cas9 mutagenesis of Mettl3 in S2-5 cells. D Western blotting shows lines #3-5 and #4-3 lack Mettl3 protein; #3-3 was used as a control line. E Quantitative liquid chromatography-mass spectrometry (LC-MS) and absolute quantification confirms specific lack of m6A in Mettl3-KO cells. Error bars, mean ± SD; n = 3 biological replicates, two-tailed t-test, ***p < 0.001. m6A p = 5.87e-06. n.s. = non-significant, m6C p = 0.604, m5U p = 0.612. F qPCR of m6A-modified mRNAs shows they have similar half-lives in Mettl3-KO and wild-type cells. Error bars, mean ± SD; n = 3 biological replicates, two-tailed t-test, ns, not significant. G Translation efficiency (TE) measurements plotted as cumulative fractions for targets with different numbers of m6A peaks. m6A is preferentially deposited on genes with low TE. For panels A, B, and G, a bootstrap method generated the background distribution (None) using genes that lacked m6A peaks. To generate p-values, two-sided Kolmogorov-Smirnov (KS) tests were performed comparing the background distribution and each group of m6A target genes. Number of targets are included in parentheses. H Puromycin labeling (5′ in 5 ug/ml media, 5′0 recovery) shows reduced global protein synthesis in Mettl3-KO cells. I Quantification of nascent protein synthesis in independent Mettl3-KO S2-5 cells. Error bars, mean ± SD; n = 4 biological replicates, Two-tailed t-test, **p < 0.01, **p = 0.00441, #4-3 p = 0.00496. J aqz, a model m6A target. We assayed a wildtype aqz 5′ UTR reporter and a variant with two point mutations of m6A sites within the strongest Mettl3-dependent peak (aqz-M). K aqz-wt-Luc generates more reporter output than aqz-M-Luc in wildtype cells, but these have equivalent output in Mettl3-KO cells. Error bars, mean ± SD; n = 3 replicates (Luciferase), n = 5 replicates (qPCR). Two-tailed t-test, **p < 0.01, *p = 0.0006, p = 0.0089. n.s.-significant, F-Luc/R-Luc p = 0.278, mRNA F-Luc/R-Luc p = 0.326. This is one representative result from three independent repeats. L-Wild type and 3A mutants or UAS-DSRed (HA/DSRed in red) and DAPI (blue). L Ecotropic Ythdf has only marginal effects on the parental tub-GFP reporter. M DsRed does not affect the tub-aqz-S'UTR-GFP reporter. N-O Wildtype Ythdf (N) but not Ythdf-3A (O) enhances GFP production from tub-aqz'-UTR-GFP. Numbers of disks analyzed are labeled, and representative results are shown. Scale bar is 100 μM. Source data are provided as a Source Data file.

observed that, in aggregate, m6A-modified transcripts had identical mRNA half-lives as the background distribution (Fig. 6B). Thus, we were not able to discern global m6A regulatory impacts on transcript properties.

To test this further, we used CRISPR/Cas9 to delete the Mettl3 locus from S2-S cells. Western blotting validated absence of Mettl3 protein in multiple independent cell lines (Supplementary Fig. 11), including two lines that we used for further analysis (#3-5 and #4-3, Fig. 6C, D). Because #4-3 retained genomic material internal to the confirmed deletion, despite absence of Mettl3 protein (Supplementary Fig. 11), we generally relied on line #3-5 (Mettl3-KO). Moreover, we directly measured Nm6A levels in Mettl3-KO cells using quantitative liquid chromatography–mass spectrometry (LC–MS). External calibration curves prepared with A and m6A standards determined the absolute quantities of each ribonucleoside. The mRNA m6A methylation levels in knockout cells were <5% of those in wild-type cells, whereas other modified ribonucleosides were unaffected (Fig. 6E).

Using Mettl3-KO cells, we performed RNA decay assays of validated m6A targets and control transcripts. Following inhibition of transcription using actinomycin D, we observed a range of transcript levels across different loci, but none of these were significantly different between wild-type and m6A-deficient cells (Fig. 6F). Overall, our analyses using S2 cells and intact nervous system indicate that mRNA stability of m6A-containing transcripts is neither substantially nor directionally influenced by loss of m6A in Drosophila, in contrast to m6A in mammals.

m6A is preferentially deposited on fly transcripts with lower translational efficiency. In light of these data, we examined the alternate possibility of m6A-dependent translational control. For this purpose, we utilized ribosome-profil ing datasets from Drosophila heads to assess translational efficiencies of transcripts with or without m6A modiﬁcations. Strikingly, we found that genes with m6A had lower translational efficiency than the background distribution (Fig. 6G). The functional relevance of this observation was strengthened by the fact that the number of m6A peaks per transcript exhibited a progressive, inverse correlation with translational efficiency and contrasted with the lack of correlation of m6A modification with either steady-state transcript levels or RNA stability. Altogether, these results suggest that m6A mediates translational control. Moreover, our miCLIP maps were generated from highly DT-selected RNAs, we infer that this may reflect modifications that are mostly present in cytoplasmic transcripts available for binding to Ythdf.

Drosophila m6A enhances target protein output. The dominant location of Drosophila m6A in 5′ UTRs, contrasting with the preferred residence of mammalian m6A in 3′ UTRs, is suggestive of a role in influencing translation. However, the above genomic analyses are correlational in nature, and do not directly connect m6A to gene regulation. One scenario is that m6A, being enriched amongst poorly translated mRNAs, is a suppressive mark. However, an alternative logic is that m6A is a positive mark that is preferentially deposited on transcripts that are inefficiently translated, which might make the potential impact of enhancement more overt. Such logic was proposed for mammalian YTHDC2 to enhance translation of low efficiency translated m6A targets.

We first evaluated if m6A might exert global impact on translation. We exploited our Mettl3-KO S2-S cells and monitored newly synthesized proteins using puromycin incorporation. Interestingly, we observe a difference between steady-state protein accumulation and nascent protein synthesis in wild-type and m6A-mutant cells. In particular, when analyzing similar amounts of total cellular protein, we observed that both Mettl3-KO cells (#3-5 and #4-3) consistently generated less newly synthesized bulk proteins than did control S2-S cells (Fig. 6H, I). Western blotting for tubulin, a non-m6A target, veriﬁed similar steady-state accumulation between wild-type and knockout cells, while Mettl3 blotting conﬁrmed knockout cell status. These data suggested that m6A may enhance protein output.

We tested this further using a reporter assay. One of the most prominent m6A targets was aequetuzali (aqz), whose 5′ UTR bore highly Mettl3-dependent miCLIP peaks with multiple CIMS (Fig. 6J), and whose modiﬁcation we had validated (Fig. 5). Aqz is required for cell polarity and neural development. We cloned its ptc-Gal4 > UAS-HA-Ythdf wild type or 3A mutants or UAS-DSRed (HA/DSRed in red) and DAPI (blue). L Ecotropic Ythdf has only marginal effects on the parental tub-GFP reporter. M DsRed does not affect the tub-aqz-S'UTR-GFP reporter. N-O Wildtype Ythdf (N) but not Ythdf-3A (O) enhances GFP production from tub-aqz'-UTR-GFP. Numbers of disks analyzed are labeled, and representative results are shown. Scale bar is 100 μM. Source data are provided as a Source Data file.
Drosophila Ythdf enhances output of an m6A reporter in a YTH-dependent manner. To test if Ythdf might be an effector of m6A-mediated target activation, we implemented a transgenic assay. We used a reporter backbone consisting of GFP under control of the tubulin promoter (tub-GFP), a transgene that is broadly and relatively evenly expressed in the animal. In this genetic background, we coexpress factors in a spatially defined subpattern, to assess regulatory impact on the transgene. When we stain wing imaginal disks bearing a naive reporter, and expressing UAS-HA-Ythdf along the anterior–posterior boundary (using ptc-Gal4), we do not observe substantially different GFP protein accumulation in cells co-expressing Ythdf, compared to non-Gal4 cells as internal control territories (Fig. 6L).

Since we had validated aag as both an m6A target (Fig. 6K) and a Ythdf target (Fig. 5), we transferred its 5’ UTR into the tub-GFP reporter. The tub-aag-5’UTR-GFP transgene expressed GFP broadly and the levels were not noticeably different from the parent transgene. Its accumulation was not affected by coexpression of a UAS-DsRed transgene (Fig. 6M). However, when we introduced into the ptc > HA-Ythdf background, GFP was elevated specifically within the Ythdf-expressing domain (Fig. 6N). This was consistent with a role for Ythdf in enhancement of this m6A target.

To test if this was due to specific activity of Ythdf, we generated an HA-tagged transgene containing the three YTH pocket mutations, which we showed abrogates association to m6A in vitro (Fig. 1C) and to validate m6A-bearing transcripts in cells (Fig. 5C). HA-Ythdf-3A protein accumulated to a similar level as wild type, and was also similarly neutral as its wild-type counterpart when tested on the parent tub-GFP reporter. Moreover, mutant Ythdf-3A was unable to enhance GFP protein output from the tub-aag-5’UTR-GFP transgenic reporter (Fig. 6O). These data support the notion that Ythdf recognizes m6A-bearing 5’UTR targets for translational enhancement.

An autonomous, m6A-dependent, neural function for Ythdf in memory. The availability of wild-type and mutant Ythdf transgenes allowed us to conduct further genetic tests of the connection between m6A readout and STM. Overexpression of either Ythdf transgene, (e.g. with ptc-Gal4, ap-Gal4) did not substantially impair viability or developmental patterning; and pan-neuronal expression of Ythdf using elav-Gal4 did not affect lifespan (Supplementary Fig. 4). Therefore, even though we could detect a selective m6A-dependent impact of Ythdf on reporter transgenes, elevated Ythdf expression does not interfere with normal developmental programs, or has effects that are otherwise within the range of developmental compensation. This mirrors the lack of substantial consequences of removing Ythdf.

We moved to perform cell-type-specific transgenic rescue assays. Building on our observation that neural-knockdown of Ythdf phenocopied the STM defects seen in whole-animal mutations (Fig. 3G), we introduced elav-Gal4 and UAS-Ythdf-wt or UAS-Ythdf-3A transgenes into Ythdf hemizygous null backgrounds. The Ythdf mutants carrying elav-Gal4 had defective STM, indicating the Gal4 transgene does not improve this behavioral output (Fig. 7A). This was important to rule out, since at least some other Drosophila neuronal phenotypes are modified by Gal4 alone47,53. With this control background as reference, we found that the STM deficit of Ythdf nulls could be rescued by pan-neuronal restoration of Ythdf, thereby restoring normal STM (Fig. 7A). In contrast, elav-Ythdf-3A transgenes did not rescue normal STM capacity to Ythdf mutants (Fig. 7A). Thus, m6A binding is critical for neural Ythdf function during STM formation.

An m6A/Ythdf pathway functions specifically in MB to promote memory. In Drosophila, associative olfactory memory is stored in the intrinsic neurons of the MB9,76. Bearing in mind that Ythdf-mediated regulation has particular impact on STM, we evaluated the effects of its gain-of-function on wild-type MB neurons using MB247-Gal4. Strikingly, MB247-Gal4 > Ythdf flies exhibited compromised STM formation at 20 days, while MB247>Gal4-Ythdf-3A flies were normal (Fig. 7B). This behavioral defect was relatively specific flies overexpressing wild-type and mutant Ythdf exhibited similar locomotor activity when quantified over 60 s of tracking (Fig. 7C). Thus, ectopic Ythdf disrupts STM in an m6A-dependent manner.

These data prompted us to investigate the endogenous m6A/Ythdf pathway with respect to the MB more rigorously. Flies depleted of Mettl3 in the MB exhibited STM impairment (Fig. 7D), comparable to whole-animal Mettl3 null mutants (Fig. 2). Similarly, MB247-Gal4 > UAS-Ythdf[RNAi] flies exhibited substantial STM defects (Fig. 7E), comparable to Ythdf null animals (Fig. 3F, G). Thus, Mettl3 and Ythdf are specifically required in MB neurons to facilitate normal STM.

It was possible that these m6A factors act not only in MB neurons, but also in other neural populations, to promote STM. To address the sufficiency of the m6A/Ythdf pathway in MB neurons, we conducted further transgenic rescue tests. Recall that we were able to rescue Mettl3 null animals (Fig. 2G) and Ythdf null animals (Fig. 7A) by pan-neuronal expression of these factors. Now, we tested whether we could do so by expressing them only using MB247-Gal4. Indeed, this fully rescued the STM defects of Mettl3 mutants (Fig. 7F) and substantially improved those of Ythdf mutants (Fig. 7G). Therefore, even though m6A regulation undoubtedly impacts most cell types in the animal, the m6A/Ythdf pathway plays a cell-autonomous role in MB neurons to mediate STM.

To investigate whether this might be potentially associated with effects on MB structure, we introduced a UAS-GFP transgene into the MB247-Gal4, Mettl3[null] mutant background, which facilitated visualization of the mushroom-body lobes. In Mettl3 heterozyotes, the MB adopts its characteristic morphology: the left and right MB horizontal lobes are distinct (Fig. 7H, top left). However, a majority of Mettl3 mutants exhibit fusion of the MB α-lobes, indicating a spatially restricted effect. We initially conducted these tests at 3 weeks, to align with our functional tests of learning and memory. However, preferential fusion of Mettl3 mutant MB β-lobes was also detected at 1 week, even though their behavioral performance was normal at this time (Supplementary Fig. 3). Accordingly, we tested Ythdf heterozygous and hemizygous mutants, and similarly observed qualitatively similar fusion of MB β-lobes in mutants (Fig. 7I), at both 1 and 3 weeks. Our MB staining data of the two mutants at both timepoints are quantified in Fig. 7J.
Fig. 7 An m6A/Ythdf pathway acts in the mushroom body to mediate STM. A–C In vivo function of Ythdf during learning and memory requires m6A-binding capacity. A Pan-neuronal expression (using elav-Gal4, =G4) of wild-type Ythdf, but not Ythdf-3A, rescued STM defects in Ythdf hemizygote mutants at 20 days. Elav-G4; Ythdf[NP3]/Df vs. elav-G4/+ = 0.4295CI −0.53, −0.31 p < 1*10−4. Elav-G4; UAS-Df; Ythdf[NP3]/Df vs. elav-G4/+ = 0 [95CI −0.13, +0.14] p = 0.684. Elav-G4; UAS-Df-3A; Ythdf/NP3/Df vs elav-G4/+ = −0.2795CI −0.41, −0.17 p < 1*10−4. B Mushroom body (MB) expression (using MB247-Gal4) of Ythdf, but not Ythdf-3A, impairs STM in 20-day-old flies. MB247-G4; UAS-Ythdf −0.2495CI −0.37, −0.12 p = 0.001. MB247-G4/+ vs. MB247-G4; UAS-Ythdf-3A (vs. MB247-G4/+ = +0.0495CI −0.05, +0.15) p = 0.415. C Expression of Ythdf-wt/3A proteins does not affect locomotion behavior. D Depletion of Mettl3 in the MB phenocopied STM impairment seen in whole animal Mettl3 mutants (see Fig. 2). Mettl3[RNAi]/+ vs. MB247-G4/+ = +0.0195CI −0.26, +0.27 p = 0.654. MB247-G4; UAS-Mettl3[RNAi] vs MB247-G4/+ = −0.2195CI −0.38, −0.02 p = 0.034. E MB-specific knockdown of Ythdf compromised STM, similar to what has been observed in whole animal Ythdf mutants (see Fig. 3). Ythdf[RNAi]/+ vs. MB247-G4/+ = +0.0495CI −0.11, +0.2 p = 0.835. MB247-G4; UAS-Ythdf[RNAi] vs MB247-G4/+ = −0.2195CI −0.38, −0.02 p = 0.002. F Re-expression of Mettl3 only in MB neurons rescued STM defects in Mettl3 trans-heterozygous mutants. MB247-G4; UAS-Mettl3/+ vs. MB247-G4/+ = +0.0495CI −0.23, +0.12 p = 0.86. Mettl3[null]/Mettl3[Δcat] vs. MB247-G4/+ = −0.2895CI −0.45, −0.13 p = 0.004. MB247-G4/UAS-Mettl3; Mettl3[null]/Mettl3[Δcat] vs. MB247-G4/+ = −0.0295CI −0.12, +0.17 p = 0.766. G Re-expression of Ythdf only in MB neurons improves STM defects in Ythdf hemizygous mutants. MB247-G4; Ythdf[NP3]/Df vs. MB247-G4/+ = −0.2595CI −0.41, −0.09 p = 0.005. MB247-G4/UAS-Ythdf; Ythdf[NP3]/Df vs. MB247-G4/+ = −0.1295CI −0.47, +0.03 p = 0.572. STM and locomotion assays were conducted in 20-day-old flies. Each dot in the STM scatter plots represents a PI average of 12 flies. All control-test differences are displayed as effect sizes with error bars and 95% confidence intervals. No null-hypothesis significance testing was performed; two-tailed Mann–Whitney P values are shown for legacy purposes only. For multiple comparisons, several test groups were compared against a common control group. H and I MB structural defects in Mettl3 (H) and Ythdf (I) mutants at 3 weeks. All panels depict GFP driven by MB247-Gal4. H Representative Mettl3 heterozygote (left) and trans-heterozygote mutant (right), showing fusion of β-lobes in the latter. I Representative Ythdf heterozygote (left) and hemizygote mutant (right), showing fusion of β-lobes in the latter. J Quantification of genotypes analyzed in H and I at 1 and 3 weeks; n sizes are labeled. Source data are provided as a Source Data file.

Discussion
Distinct local contexts, genic location, and regulatory impact for m6A in different metazoans. Despite tremendous interests in the regulatory utilities and biological impacts of mRNA methylation, there has been relatively little study from invertebrate models. Given that the m6A pathway seems to have been lost from C. elegans, Drosophila is an ideal choice for this. Since the
Roles for the m6A/DF1 pathway in learning and memory.

Recent studies have highlighted neuronal functions of mammalian m6A pathway factors. There is a growing appreciation that mouse mutants of multiple components in the m6A RNA-modification machinery affect learning and memory. Here, we provide substantial evidence that, in Drosophila, neural m6A is critical for STM. We specifically focused on STM as this paradigm has been extensively characterized in Drosophila. Mouse studies have almost exclusively examined effects on LTM, and these two memory phases are mechanistically distinct. One main distinction is that LTM requires protein synthesis after training, while STM does not. So, while direct comparisons between the two systems are not possible, it is nevertheless instructive to consider the parallels and distinctions of how m6A facilitates normal memory function in these species. This is especially relevant given that both mouse and fly central nervous systems require a cytoplasmic YTH factor for memory.

In mice, the m6A writer Mettl3 enhance long-term memory consolidation, potentially by promoting the expression of genes such as Arc, c-Fos and others. Another study found that Mettl14 is required for LTM formation and neuronal excitability. Conversely, knockdown of the m6A demethylase FTO in the mouse prefrontal cortex resulted in enhanced memory consolidation. Amongst mammalian YTH m6A readers, YTHDF1 was shown to induce the translation of m6A-marked mRNA specifically in stimulated neurons. In cultured hippocampal neurons, levels of YTHDF1 in the PSD fraction were found to increase by ~30% following KCl treatment. This suggests that YTHDF1 concentration at the synapse could be critical for regulating the expression levels of proteins (such as CaMK2a) involved in synaptic plasticity. Taken together, these studies suggest the m6A pathway is a crucial mechanism of LTM consolidation in mammals that optimizes animal behavioral responses.

Of note, the genetics and sample sizes possible in Drosophila permit comprehensive, stringent, and anatomically resolved analyses. Thus, in our study, we systematically analyze all writer and reader factors, and reveal a notable functional segregation, suggesting that the cytoplasmic reader Ythdf is a major effector of Mettl3/Mettl14 m6A in memory. Given that Ythdf mutants otherwise exhibit few overt developmental or behavioral defects in normal or sensitized backgrounds (while Ythdc1 mutants generally phenocopy Mettl3/Mettl14 mutants) its role in STM is a surprising insight into the contribution of Ythdf to a critical adaptive function. Moreover, we can pinpoint the spatial requirements of m6A for STM, by showing that (1) neuronal-specific and MB-specific depletion of Mettl3/Ythdf can induce defective STM, and (2) neuronal and MB-specific restoration of Mettl3 or Ythdf to their respective whole-animal knockouts restores normal STM. Moreover, the fact that Ythdf gain-of-function in the MB can also disrupt STM, but does not generally alter other aspects of development or behavior, points to a homeostatic role of m6A regulation in Drosophila learning and memory.

We observed that STM defects in fly m6A mutants are age-dependent, which has not been reported in mammals. Although many physiological capacities decline with life history, the observed STM defects seem to be decoupled from other age-related phenotypes, since mutation of Ythdf or neural over-expression of Ythdf can interfere with STM but does not substantially impact lifespan or locomotion. In this regard, Mettl3 and Ythdf are different from classical memory genes such as rutabaga because STM impairment in m6A mutants was absent in young flies and only became apparent with progressing age.

One interpretation is that there is a cumulative effect of deregulated m6A networks that has a progressive impact specific to mushroom-body neurons. To gain further mechanistic insights, future studies will need to examine age-related changes in gene expression and/or translation, in a cell-specific manner. It remains to be seen whether specific deregulated targets downstream of Ythdf have large individual effects, or whether the STM deficits arise from myriad small effects on translation. Ythdf-CLIP and ribosome profiling from the CNS may prove useful to decipher this. Assuming that loss of translational enhancement of m6A/Ythdf targets mediates STM defects, one possibility, to be explored in future studies, is that some targets may already be known from prior genetic studies of memory.
Method

mA reader constructs. We obtained full-length cDNAs obtained by PCR from a cDNA library for Yhdh1c (encoding Yhdh1c-PA, 271aa), and Yhdh (encoding Yhdh-PA, 700aa) into pENTR vector. We then used site-directed mutagenesis (primers listed in Supplementary Dataset 6) to generate pENTR-Yhdh1c-3A (w276a w327a L338A), and pENTR-Yhdh-3A (w404a W459a w464a). These were transferred into the Drosophila Gateway vector pAWG (N-terminal GFP fusion) and pAHW (N-terminal HA). The reactions were then irradiated with 365 nm UV light and “3A” mutant variants for expression in Drosophila cell culture. We also cloned the Yhdh sequences into pTHW (N-terminal HA fusion) to generate UAS-HA-Yhdh and UAS-HA-Yhdh-3A for transgene. For mammalian expression, we cloned wild-type and “3A” mutant variants of Yhdh1c and Yhdh into pcDNA5/FRT/TTO with an N-terminal 3xFlag tag.

mA probe pulldown with fly Yhdh and Yhdh1c. As we were unable to purify sufficient amounts of full-length Drosophila Yhdh and Yhdh1c proteins from Drosophila cell culture in vitro characterization, we instead turned to purifying from mammalian cells. For initial interaction tests we used wild-type Flag-Yhdh1c/DF with mA’mA RNA probes, we seeded 4 million HEK293T cells in a 10 cm dish 24 h prior to calcium phosphate transfection, and used 10 µg of plasmid DNA (pcDNA5/FRT/TTO) for each transfection. The RNA-bound proteins were then purified by boiling the beads in 50 µL of 1× Laemmli sample buffer (80 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% B-mercaptoethanol, 0.02% bromophenol blue) at 95 °C for 5 min. The input, flow-through, and eluates were separated by SDS–PAGE and analyzed by Western blotting using mouse α-Flag M2 (Sigma #F1804).

Proteomic profiling of the fly mA interactome

Mass spectrometry analysis. For proteomics experiments with Drosophila flies, we adapted our previously described method23. S2 cells were lysed by cryomilling. The resulting cell pellet was first extracted with 1.5 mL of lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM MgCl2, EDTA-free protease inhibitor tablet (Roche), 1 mM PMSF) on ice. Clarified lysate was then incubated with A or mA containing ampicillin competent S. aureus 29303 (1 x 1 mL). The RNA-bound proteins were then purified by boiling the beads in 50 µL of 1× Laemmli sample buffer (80 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% B-mercaptoethanol, 0.02% bromophenol blue) at 95 °C for 5 min. The input, flow-through, and eluates were separated by SDS–PAGE and analyzed by Western blotting using mouse α-Flag M2 (Sigma #F1804).

In the experiments mA mutants of Yhdh and Yhdh1c were tested, cells from two 10 cm dishes and 1.2 mL of lysis buffer were used per condition. Cells were lysed in NP-40 lysis buffer (50 mM Tris 150 mM NaCl, 0.5% NP-40, 5 mM MgCl2, EDTA-free protease inhibitor tablet (Roche), 1 mM PMSF) on ice. Clarified lysate was then incubated with A or mA containing ampicillin competent S. aureus 29303 (1 x 1 mL). The RNA-bound proteins were then purified by boiling the beads in 50 µL of 1× Laemmli sample buffer (80 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% B-mercaptoethanol, 0.02% bromophenol blue) at 95 °C for 5 min. The input, flow-through, and eluates were separated by SDS–PAGE and analyzed by Western blotting using mouse α-Flag M2 (Sigma #F1804).

For mA mutants of Yhdh and Yhdh1c were tested, cells from two 10 cm dishes and 1.2 mL of lysis buffer were used per condition. The cross-linking and pull-down were performed as described above. AAACU and mA–CU sequences were used for this experiment.

Odo delivery and odor concentrations. Odo delivery in the MOT was done as previously described14, with some protocol modifications. The rack with stacked conditioning chambers was connected to an olfactometer that was used to deliver precisely timed odor stimuli (Supplementary Fig. 2b). The conditioning odors methylcyclohexanol (MCH) and 3-octanol (OCT) were carried by dry, compressed air and routed through mass flow controllers (MFC; Sensiron AG). Carrier air flow was controlled with two 2 L/min MFCs and purifying gas cylinders. The carrier air was passed through a gas bottle containing distilled water (Schott Duran) at 0.6 L/min. Odo streams were controlled with 500 mL/min MFCs and pushed through glass vials containing pure liquid odorants (either MCH or OCT, respectively). Prior to conditioning, the odor concentrations were adjusted to ensure that flies did not display a strong preference for one of the odors over the other prior to training. Odo administration was carried out with the following MFC settings: OCT left side 25–35 mL/min; OCT right side 30–40 mL/min; MCH left side 50–60 mL/min;
m6A individual-nucleotide-resolution cross-linking and immunoprecipitation (iCLIP) was performed using a custom humidity sensor with a custom LabVIEW code (National Instruments, USA).

miCLIP bioinformatic analyses. Read processing, mutation calling, and annotation of CIMs was performed as described40. Briefly, to prepare libraries for mapping, adapters and low-quality reads were trimmed using flexbar v2.5. Next, the FASTQ files were de-multiplexed using the pyBarcodeFilter.py script from the pyCRAC suite. Random barcodes were removed from sequencing reads and appended to sequence IDs using an awk script and PCR duplicates were removed using samtools. The resulting bam files were also grouped using DMI Filter and mapped to the Drosophila reference genome sequence (BDGP Release 6/dmd) using Novoalign (Novocraft) with parameters -s 85 and -l 16.

Peaks were called by adapting the model-based analysis for Chip-Seq (MACS) algorithm105. Mettl3-dependent peaks for head libraries were determined using the MACS command line type (either MCH or OCT) and two half PI OCT genomes were compared against each other. For OCT in the chambers, a relative humidity of 70% was maintained via regulation of the flow. This was monitored (ad hoc, between experiments) by using a custom humidity sensor with a custom LabVIEW code (National Instruments, USA).

Behavioral data analysis. Analyses of STM and survival experiments were performed with estimation statistics95. The rationale for the estimation framework was described previously91,96. For data analysis and visualization, the individual values of full PIs were plotted with standard-deviation lines; to describe the differences in expression of the genes of interest, a two-tailed t-test was performed with comparisons as listed in Supplementary Dataset 5 using the R packages DESeq2107 and applying a strict adjusted p-value cutoff of 0.05. Gene expression analysis was performed with the TruSeq Stranded Total RNA Sequencing Kit (Illumina) following the manufacturer’s protocol. Sequencing was performed on a HiSeq 2500 System in paired end read mode, with 100 bases per read at the Integrated Genomics Operation (IGO) at Memorial Sloan Kettering Cancer Center.

CRISPR/Cas9 deletion of Mettl3 in S2-S cells. We used CRISPR/Cas9-mediated mutagenesis as described26 to generate Mettl3−/− S2 cell lines. Guide RNA sequences are listed in Supplementary Dataset 6. We analyzed 11 candidate clonal lines obtained from subcloning of two initial low-complexity mixed cell populations, and kept the deletions #3-5 and #4-3 as described in Supplementary Fig. 11.

m6A-RIP-PCR and RIP-HIPCR. We adapted a protocol from our recent study35. Plasmids of 5 µg were transfected into 6 x 10^6 S2 cells using Effectene (Qiagen) and incubated for 3 days. Cells were washed with PBS and lysed with IP lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40) supplied with Complete, EDTA-free Protease Inhibitor and 40 U mL^-1 SUPERase-In RNase Inhibitor (Ambion) on ice for 30 min, followed by 2 x 10 min
centrifugation at 20,000g at 4 °C. 10% of the cleared cell lysate were kept as input and the rest was incubated with 15 μL Dynabeads Protein G (Thermo Fisher, cat#10004D) (with HA or GFP antibody) for 4 h at 4 °C. RNAse I (Invitrogen, AM2294) was added to the sample for RNAse treatment at 0.2 U final concentration. The beads were washed three times using IP lysis buffer and then resuspended in 100 μL lysis buffer. To elute RNA, the beads were mixed with 900 μL of Trizol, vortexed for 1 min and incubated at RT for 50 min with rotation. RNA extracted and treated were Turbo DNase (Ambion) for 30 min then cDNA synthesized using SuperScript III (Life technology) with random hexamers. PCRs were done using fusion high-fidelity polymerase (ThermoFisher Scientific).

For m6A-RIP-qPCR, the mRNAs were immunoprecipitated using a- m6A antibody according to the procedure shown above. The IP-mRNAs were then reverse transcribed and amplified following the same protocol. The enrichment of m6A was quantified using qPCR as reported. The sequences of qPCR primers are listed in Supplementary Dataset 6.

RNA degradation assay. S2 cells were seeded as 3 x 10^6 cells per well. Actinomycin-D (Gibco, 11-805-017) was added to a final concentration of 5 μM, and cells were collected before or 5 h after adding actinomycin-D. Then the cells were processed as described in ‘RT-qPCR’, except that the data were normalized to the t = 0 time point.

SunSET assay and Western blotting. For each assay, we incubated 3 x 10^6] cells in 1 mL Schneider’s medium including 10% FBS for 5 min at 25 °C with or without 5 μg/mL puromycin (Gibco) and 1 μL puromycin (2A4, 1:1000) and mouse anti-puromycin (1 μg/mL) then incubated on ice for 30 min, centrifuged at 16,000g x 10 min at 4 °C. Protein concentration was measured using Bio-Rad Protein Assay Dye (50-0006) and 2 μg proteins were separated on SDS-PAGE and transferred to Immobilon-P membrane. Membranes were blocked for 1 h in TBS containing 5% nonfat milk and 0.1% Tween-20, followed by incubation with mouse α-puromycin (1:1000) overnight at 4 °C. Appropriate secondary antibodies conjugated to HRP (Jackson) were used at 1:5000 for 1 h at room temperature, then visualized using chemiluminescence detection (Amersham ECL Prime Western Blotting Detection Reagent). Mouse α-puromycin (2A4, 1:1000) and mouse α-β-tubulin (E7, 1:1000) were from DSHB; guinea pig α-M-pol (1:5000) was gifted from Cintia Hongay.

Luciferase sensory assays. To generate the pAc5.1-aqz-wt-luc reporter, we cloned the aqz 5′ UTR (chr3R8.818,731–8,820,682) into the 5′ UTR pAc5.1-luc vector encoding Firefly Luciferase (Kpl/EcoRI). We then used site-directed mutagenesis (primers listed in Supplementary Dataset 6) to generate pAc5.1-Maqz-M-luc (8823087A, 8820401A into T). To assay luciferase reporter activity and mRNA levels, 10^5 S2 cells were seeded per well of 12-well plate and transfected with 150 ng pAc5.1-Renilla, and 150 ng pAc5.1-aqz-wt-luc, or pAc5.1-aqz-M-luc constructs. Luciferase activities were measured 3 days after transfection using Dual Glo luciferase assay system (Promega) and CyQuant® (BioTek) from 100 μL cell from each well of the 12-well plate. We calculated the ratio between Firefly and Renilla luciferase activities. The remaining cells in the 12-well plate were processed to extract total RNA (DNase I digested) by TRizol® reagent (Invitrogen), followed by RT-qPCR quantification. The level of F-luc mRNA was normalized by that of R-luc mRNA.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All transgenic strains and plasmids generated for this study are available upon request. All of the raw miCLIP and RNA-seq data generated in this study were deposited in NCBI-GEO under GSE147230. Source data are provided with this paper. All of the raw and summarized STX, locomotion, and survival data are available from the zenodo repository under the following DOI: 10.5281/zenodo.4446416. Source data are provided with this paper.

Received: 10 March 2020; Accepted: 28 January 2021; Published: 05 March 2021

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Acknowledgements
We thank the Developmental Studies Hybridoma Bank (DSHB) and Cintia Hongay for antibodies, Jean-Yves Rognant, Matthias Soller and the Bloomington Drosophila Stock Center (BDSC) for fly stocks, Stefan Ameres for the S2-S line and advice on cell mutagenesis. Sara Zaccara and Samie Jaffrey provided helpful advice on generation of miCLIP libraries and discussion about m6A. A.C.-C. and S.O. were supported by grants from the Singapore Ministry of Education (MOE2013-T2-2-054 and MOE2017-T2-1-089) and by Duke-NUS Medical School. S.O. was supported by a Khoo Postdoctoral Fellowship Award (Duke-NUS-KPFA/2017/0015). Work in the REK lab was supported by NIH grants R01-GM083300 and R01-NS083833, and by the MSK Core Grant P30-CA08748.

Author contributions
L.K. generated all constructs and transgenic animals; constructed the miCLIP and RNA-seq libraries; performed SUnSET, RNA degradation, and reporter assays, and all disc and CNS immunostainings. B.I. performed all the bioinformatic analyses. E.S.P. generated mettl3-KO cell lines and conducted co-IP and RIP tests. W.D. analyzed m6A-YTH factor interactions, performed proteomic analyses, and quantified modified nucleosides. A.C.-C., R.E.K., and E.C.L. supervised experiments in their respective laboratories, obtained funding, helped interpret analyses, and wrote the manuscript with input from all other authors. E.C.L. and A.C.-C. conceived the project.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-21537-1.

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Peer review information Nature Communications thanks Alex Keene and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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