mTORC1-chaperonin CCT signaling regulates m^{6}A RNA methylation to suppress autophagy

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Mechanistic Target of Rapamycin Complex 1 (mTORC1) is a central regulator of cell growth and metabolism that senses and integrates nutritional and environmental cues with cellular responses. Recent studies have revealed critical roles of mTORC1 in RNA biogenesis and processing. Here, we find that the m^{6}A methyltransferase complex (MTC) is a downstream effector of mTORC1 during autophagy in *Drosophila* and human cells. Furthermore, we show that the Chaperonin Containing Tailless complex polypeptide 1 (CCT) complex, which facilitates protein folding, acts as a link between mTORC1 and MTC. The mTORC1 activates the chaperonin CCT complex to stabilize MTC, thereby increasing m^{6}A levels on the messenger RNAs encoding autophagy-related genes, leading to their degradation and suppression of autophagy. Altogether, our study reveals an evolutionarily conserved mechanism linking mTORC1 signaling with m^{6}A RNA methylation and demonstrates their roles in suppressing autophagy.

mTORC1 | m6A methyltransferase complex (MTC) | chaperonin containing Tailless complex polypeptide 1 (CCT) | m6A RNA methylation | autophagy

Mechanistic Target of Rapamycin Complex 1 (mTORC1), an evolutionarily conserved serine/threonine kinase, is a master regulator of cell growth, metabolism, and proliferation coupling different nutritional and environmental cues, including growth factors, energy levels, cellular stress, and amino acids, with metabolic programs (1). For example, insulin activates PI3K/AKT and inhibits the Tuberous Sclerosis Complex (TSC) 1/2, a negative regulator of mTORC1, thus promoting mTORC1 activation (2). Activated mTORC1 then phosphorylates multiple downstream effectors that control a wide range of anabolic and catabolic processes. Phosphorylation of the ribosomal S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) by mTORC1 promotes protein translation and enhances cell growth and proliferation (3). Moreover, autophagy, an intracellular degradation system that delivers cytoplasmic components to lysosomes, is inhibited by mTORC1 through phosphorylation of Atg13 that, in turn, inhibits ULK1 kinase activity (4).

Recent studies have highlighted a role for mTORC1 in regulating RNA metabolism. Through the phosphorylation of RNA metabolic proteins, mTORC1 modulates various RNA biogenesis and processing events. Phosphorylation of the SR protein kinase SRPK2 by S6K1 promotes its transport into the nucleus where it activates SR proteins and induces splicing of lipogenic pre-messenger RNAs (pre-mRNAs) for de novo synthesis of fatty acids and cholesterol, suggesting that SRPK2 is a critical mediator of mTORC1-dependent lipogenesis (5). In addition, mTORC1 regulates alternative splicing and polyadenylation of autophagic and metabolic genes to control autophagy, lipid, protein, and energy metabolism through the cleavage and polyadenylation complex (6). Furthermore, mTORC1 mediates phosphorylation of the decapping enzyme Dcp2. Phosphorylated Dcp2 associates with RNA helicase RCK family members and binds to transcripts of Autophagy-related genes (Atg) to degrade them, thereby suppressing autophagy (7). Altogether, these studies suggest an essential role for mTORC1 in controlling RNA biogenesis and processing, revealing a major function for mTORC1 in the regulation of protein diversity and in reshaping cellular metabolism and autophagy.

N^6-methyl-adenosine (m^{6}A) is one of the most abundant chemical modifications in eukaryotic mRNA, which is preferentially enriched in 3′ UTRs and around stop codons (8, 9). m^{6}A modification affects almost all aspects of mRNA metabolism, such as splicing, translation, and stability, and plays essential roles in a wide range of cellular processes, including *Drosophila* sex determination and metabolism (10). The m^{6}A methyltransferase complex (MTC) catalyzes m^{6}A formation and is composed of the methyltransferase-like protein 3 (METTL3), the methyltransferase-like protein 14 (METTL14), WTAP (the ortholog of *Drosophila* Fl(2)d), and RBM15/RBM15B (the ortholog of *Drosophila* Nito). Although METTL3 is the only catalytic component of the MTC, its interaction with METTL14

Significance

N6-methyladenosine (m^{6}A) is the most prevalent modification in eukaryotic messenger RNA (mRNA) and affects RNA metabolism including splicing, stability, and translation. The m^{6}A methyltransferase complex (MTC) is responsible for generating the m^{6}A modifications in mRNA; however, the regulation of m^{6}A modification is still unclear. We have identified Mechanistic Target of Rapamycin Complex 1 (mTORC1) as a key regulator of MTC and demonstrate that mTORC1 can stabilize MTC via activation of the chaperonin CCT complex and upregulate m^{6}A modification to promote the degradation of ATG transcripts. Thus, our study unveils an mTORC1-signaling cascade that regulates m^{6}A RNA methylation and autophagy.

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is necessary for RNA substrate recognition and efficient m^{6}A deposition. WTAP stabilizes the interaction between the two METTL proteins, and RBM15/RBM15B have been proposed to recruit the MTC to its target transcripts (10, 11).

Using autophagy as a readout of mTORC1 signaling in *Drosophila*, we identified the MTC as a downstream effector of mTORC1 signaling. From the analysis of high-confidence *Drosophila* and human MTC proteomic data, we further identified the Chaperonin Containing Tailless complex polypeptide 1 (CCT) complex as an MTC interactor that mediates the effects of mTORC1 on m^{6}A modification and autophagy. In mammalian cells, we also found that the CCT complex plays critical roles

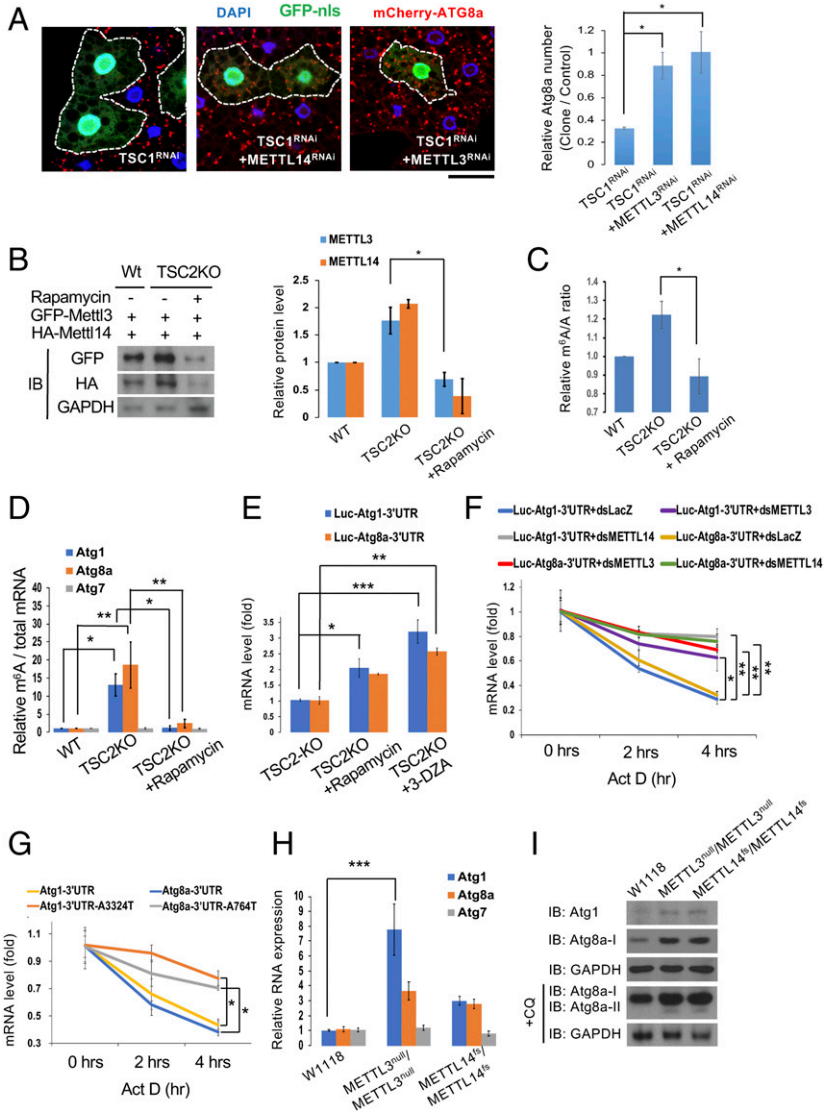

Fig. 1. mTORC1 signaling regulates the MTC to suppress autophagy. (*A*) MTC acts downstream of mTORC1 signaling. Clonal depletion of *TSC1* in GFP-labeled cells suppressed the formation of mCherry-ATG8a puncta under starvation conditions (compared to control cells outside the circled dashed lines). Coexpression of *METTL3*^{RNAi} or *METTL14*^{RNAi} reversed the *TSC1*^{RNAi}-induced effect. Fat body cells were stained with DAPI. (Scale bar, 20 μm.) Quantification of the relative number of mCherry-ATG8a dots per cell. (*B*) mTORC1 activity regulates METTL3 and METTL14 protein levels. Wild-type S2R+ or *TSC2* KO cells transfected with *GFP-METTL3* or *HA-METTL14* were treated with or without rapamycin. The protein levels of METTL3, METTL14, and GAPDH were analyzed by immunoblotting (IB) with antibodies as indicated and quantified. (*C* and *D*) mTORC1 signaling modulates global m^{6}A RNA methylation levels as well as m^{6}A levels in *Atg* transcripts. m^{6}A levels were quantified using LC-MS in S2R+ cells treated as indicated. Compared with wild-type S2R+ cells, *TSC2* KO cells showed enhanced m^{6}A levels in their mRNAs while rapamycin treatment on *TSC2* KO cells reduced it (*C*). Abundance of *Atg1*, *Atg8a*, and *Atg7* transcripts among mRNA immunoprecipitated with anti-m^{6}A antibody from S2R+ cells treated as indicated. m^{6}A-modified *Atg1* and *Atg8a*, but not *Atg7* mRNAs, were increased in *TSC2* KO cells, and rapamycin treatment in *TSC2* KO cells can reduce it (*D*). (*E*) mTORC1 and MTC activities decrease *Atg1* and *Atg8a* mRNA levels through their 3′ UTR regions. *Firefly luciferase* reporters with *Atg1* or *Atg8a* 3′ UTRs were transfected into S2R+ cells. After 48 h, cells were treated with rapamycin (20 nM) or 3-DZA (100 μM) for 48 h and mRNA levels of *Firefly luciferase* were measured by qPCR. (*F* and *G*) m^{6}A methylation is required for *Atg1* and *Atg8a* mRNA degradation. *Firefly luciferase* reporters with the indicated 3′ UTRs were transfected into S2R+ cells treated with or without dsRNA against *LacZ*, *METTL3*, or *METTL14*. After 48 h, cells were treated with actinomycin D (10 μg/mL) for the indicated times to measure mRNA levels of *Firefly luciferase* by qPCR. (*H* and *I*) *METTL3* and *METTL14* mutants exhibit higher *Atg1* and *Atg8a* transcripts as well as ATG1 and ATG8a protein levels in the larval fat body. RNA (*H*) or protein (*I*) extracts from larval fat bodies of wild-type (*w1118*), *METTL3* null mutant (*METTL3*^{null}/*METTL3*^{null}), or *METTL14* mutant (*METTL14*^{fs}/*METTL14*^{fs}), fed with or without CQ, were subjected to qPCR assay (*H*) or Western blot analysis using antibodies as indicated (*I*). One-way ANOVA test was performed followed by Tukey's test to identify significant differences. Measurements shown are mean ± SEM of triplicates; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

in the regulation of MTC protein stability and m^{6}A RNA modification, suggesting that the mTORC1-CCT-MTC axis is conserved from *Drosophila* to mammals. Our studies thus unveil a mechanism linking mTORC1 signaling and the chaperonin CCT complex to RNA methylation and also uncover a layer of mTORC1 regulation of autophagy.

Results

Components of the MTC Modify *TSC1^{RNAi}*-Inhibited Autophagy. Inhibition of either *TSC1* or *TSC2* leads to mTORC1 overactivation which impairs autophagy (12). To identify downstream effectors of mTORC1 signaling, we performed an RNA interference (RNAi) screen for autophagy modifiers by generating flip-out clones expressing *TSC1-RNAi* and the autophagosomal marker *mCherryAtg8a* in larval fat bodies. While starvation induced autophagosome formation, clonal expression of *TSC1-RNAi* abolished starvation-induced mCherry-ATG8a punctae formation (Fig. 1*A*). Interestingly, RNAi lines against *METTL3* or *METTL14*, components of the MTC, suppressed the *TSC1-RNAi*–induced effects, indicating that METTL3 and METTL14 are required for the effect of *TSC1*-inhibited autophagy (Fig. 1*A*). Furthermore, mCherryATG8a punctae were induced by depletion of either *METTL3* or *METTL14* (*SI Appendix*, Fig. S1*A*). Consistently, *METTL3* null mutants also exhibited higher autophagy levels with or without chloroquine (CQ) treatment which blocks autophagosome degradation (13) (*SI Appendix*, Fig. S1*B*). Together, these results suggest that the MTC acts downstream of mTORC1 signaling to regulate autophagy.

mTORC1 Positively Regulates METTL3 and METTL14 Protein Levels to Control m^{6}A RNA Methylation and Autophagy. To investigate how mTORC1 regulates the MTC, we first tested whether METTL3 and/or METTL14 protein levels are affected by mTORC1 activity. As shown in Fig. 1*B*, both METTL3 and METTL14 levels were increased in *TSC2* knockout (KO) cells, an effect that was inhibited by rapamycin (Fig. 1*B*). The time-course treatment of rapamycin further showed that METTL3 and METTL14 protein levels were significantly decreased after 48 h of rapamycin treatment (*SI Appendix*, Fig. S1 *C* and *D*). Consistent with this result, as METTL3 and METTL14 are essential for m^{6}A RNA methylation, liquid chromatography-tandem mass spectrometry (LC-MS) analysis showed that global m^{6}A RNA levels were enhanced in *TSC2* KO cells and that the *TSC2* KO-induced effect was suppressed by rapamycin (Fig. 1*C*). Together, these data show that mTORC1 activity induces METTL3 and METTL14 protein expression to increase m^{6}A RNA methylation levels. In contrast, the protein levels of Nito, another MTC component, were not affected by rapamycin treatment (*SI Appendix*, Fig. S1*D*), indicating that METTL3 and METTL14 are specifically controlled by mTORC1 signaling.

Several *Autophagy-related gene* (*Atg*) transcripts have been shown to possess m^{6}A methylation, which promotes mRNA degradation of these transcripts and suppresses autophagy (14, 15). We therefore examined whether mTORC1 regulation of METTL3 and METTL14 controls autophagy through this mechanism. m^{6}A RNA immunoprecipitation (MeRIP) assays using an anti-m^{6}A antibody revealed that *Atg1* and *Atg8a* mRNAs from *TSC2* KO cells show higher levels of m^{6}A methylation than those from wild-type cells (8) (Fig. 1*D*). Consistently, rapamycin significantly reduced m^{6}A levels in *Atg1* and *Atg8a* transcripts from *TSC2* KO cells (Fig. 1*D*), demonstrating that mTORC1 activity controls m^{6}A methylation of *Atg* transcripts.

Next, to analyze the effects of the mTORC1-dependent m^{6}A methylation of *Atg* transcripts, we tested whether m^{6}A affects the *Atg1* and *Atg8a* mRNA turnover. We generated *luciferase* reporter constructs with the short 3′ UTR region of either *Atg1* or *Atg8a* and expressed them in S2R+ cells. Cells were then treated with either rapamycin or 3-DZA (the m^{6}A inhibitor). qPCR analysis revealed that the amounts of *luciferase* mRNAs with either the *Atg1* or *Atg8a* 3′ UTRs in 3-DZA- or rapamycin-treated cells were higher than those in wild-type cells, indicating that mTORC1-dependent m^{6}A methylation reduces *Atg1* and *Atg8a* mRNA levels through their 3′ UTRs (Fig. 1*E*). Moreover, double-stranded RNA (dsRNA)-mediated depletion of either *METTL3* or *METTL14* increased the half-life of *luciferase* mRNA with either *Atg1* or *Atg8a* 3′ UTRs (Fig. 1*F*). Further, mutation of the m^{6}A sites in the *Atg1* and *Atg8a* 3′ UTR regions identified by miCLIP enhanced mRNA stabilities (16) (Fig. 1*G*). These results demonstrate that m^{6}A RNA modification is required for *Atg1* and *Atg8a* mRNA degradation. Consistent with these results, both mRNA and protein levels of *Atg1* and *Atg8a* were increased in larval fat bodies of *METTL3* and *METTL14* null mutants, compared to wild type (Fig. 1 *H* and *I*). However, no significant changes of m^{6}A and mRNA levels of *Atg7* were detected in *TSC2* KO cells or *MTC* mutants (Fig. 1 *D* and *H*). Taken together, these results indicate that mTORC1-regulated m^{6}A methylation enhances degradation of specific *Atg* transcripts, which in turn inhibits autophagy.

Proteomic Identification of m^{6}A RNA Methyltransferase Complex Protein–Protein Interaction Networks. As mTORC1 increases protein expression through transcriptional, posttranscriptional, or translational regulation (5, 6, 17), we further investigated how mTORC1 up-regulates METTL3 and METTL14 protein levels. qPCR analysis of *METTL3* and *METTL14* revealed no significant changes in their transcript levels in *TSC2* KO cells with or without rapamycin treatment (*SI Appendix*, Fig. S1*E*), indicating that the increase in METTL3 and METTL14 protein levels by mTORC1 signaling is not at the mRNA level. In addition, even though inhibition of protein translation by cycloheximide (CHX) reduced MTC protein levels, cotreatment of CHX with rapamycin further induced a dramatic decline of METTL3 and METTL14 levels (*SI Appendix*, Fig. S1*F*). In contrast, Nito protein levels were the same in these two conditions (*SI Appendix*, Fig. S1*F*). Together, these results suggest that mTORC1 enhances METTL3 and METTL14 protein levels in a transcription- and translation-independent manner.

In order to characterize how mTORC1 regulates MTC, we generated Protein-Protein Interaction Networks (PPINs) centered on the *Drosophila* and human MTC by affinity purification and mass spectrometry (AP/MS) from *Drosophila* S2R+ and human HEK293T cells (Fig. 2*A*). We identified unfiltered networks of 1,462 and 1,504 interactions in *Drosophila* and human Methyltransferase Complex Protein–Protein Interaction Networks (MTC-PPINs), respectively, with more than 95% of the preys being evolutionarily conserved, and used the Significance Analysis of Interactome (SAINT) algorithm to evaluate the networks obtained with AP/MS (18). By comparing MTC-PPIN with published interactions (*SI Appendix*, Fig. S2 *A* and *B*), we further generated high-confidence MTC-PPIs with a SAINT score (SS) $\geq$ 0.2 (130 interactions for *Drosophila* as and 230 interactions for humans) (Fig. 2*A*). (See Dataset S1 for the full list of interactions and Dataset S2 for the list of interaction pairs with SS $\geq$ 0.2). Eleven known biochemical interactions were recovered in the *Drosophila* high-confidence MTC-PPIN (Fig. 2*A*, red and yellow edges). Moreover, the *Drosophila* MTC-PPIN was significantly enriched for hits from the published m^{6}A RNA pull-down analysis (19) (*SI Appendix*, Fig. S2*C*), revealing that it is of high quality.

The CCT Complex Directly Interacts with and Stabilizes METTL3 and METTL14. To gain further insights into the organization of the MTC-PPIN, we used COMPLEAT to perform a protein-complex enrichment analysis and identified several protein complexes involved in posttranscriptional regulation and modification (Fig. 2*B*) (20). Physical interactions of those complexes with MTC were confirmed by coimmunoprecipitation (CoIP) (Fig. 2*C* and *SI*

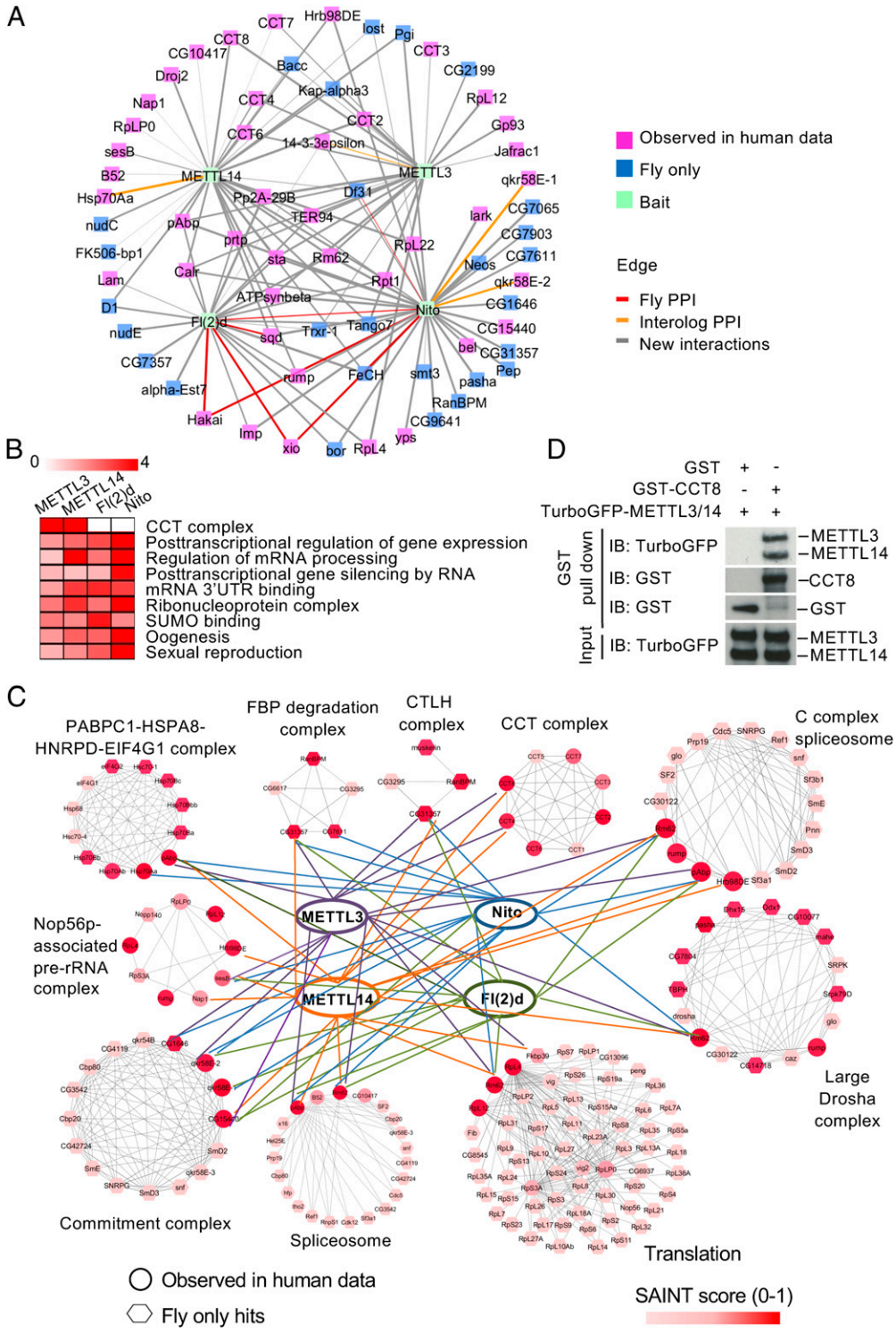

Fig. 2. Identification of CCT complex as an interactor of MTC. (*A*) Network representation of the *Drosophila* MTC-PPIN. We collected the published PPIs deposited in public repositories from MIST (35) as well as the full LC-MS/MS datasets from two relevant studies (32, 36) and selected the interactions that are supported by at least two of three resources as the positive control set (*SI Appendix*, Fig. S2 *A* and *B*). Based on the analysis, comparing fly MTC-PPIN with interactions in the positive control set, we defined the SAINT cutoff and selected 130 and 230 high-confident interactions from fly and human MTC-PPINs, respectively, for follow up. PPIs with high confidence are shown. The pink node represents hits that can be found in both *Drosophila* and human MTC-PPINs, while the blue node indicates that the hits were found only in *Drosophila* MTC-PPIN. Green node indicates the bait. Red or yellow edges indicate the known interactions based on *Drosophila* data or data mapped from orthologous genes (interologs) annotated at MIST. Gray edges indicate interactions from the PPINs. The thickness of edges corresponds to the SAINT score. (*B*) Heatmap, based on COMPLEAT analysis, displaying interaction between baits (*Top*) and the $-\log_{10}$ (*P* value) for selected cellular processes (*Right*). Color represents the strength of significance. (*C*) Validated *Drosophila* MTC-PPIN with complexes involved in protein folding, RNA processing, and translation. Co-IP data are shown in *SI Appendix*, Fig. S3. (*D*) METTL3 and METTL14 directly interact with CCT8. In-vitro–translated *Drosophila* TurboGFP-METTL3, TurboGFP-METTL14, and GST-CCT8 proteins were subjected to GST pull-down assay. Pull-down fractions and input were analyzed by immunoblotting with antibodies as indicated.

Appendix, Fig. S3). Surprisingly, we found that only METTL3 and METTL14, but not other MTC components, specifically associate with the CCT complex, a chaperonin complex that facilitates protein folding and complex assembly (Fig. 2 *B* and *C* and *SI Appendix*, Fig. S3) (21). To determine whether this interaction is direct, we performed GST pull-downs with in-vitro–translated GST-tagged CCT8 and TurboGFP-tagged METTL3 and METTL14. As shown in Fig. 2*D*, METTL3 and METTL14 directly interacted with CCT8 (Fig. 2*D*), revealing the direct interaction between MTC and CCT.

The interaction of the CCT complex with METTL3 and METTL14 raised the possibility that METTL3/14 may be substrates of chaperonin CCT complex and that CCT can help their folding and promote their protein stabilities. Indeed, we found that knockdown of *CCT1-8* significantly reduced METTL3 and METTL14 protein levels, but not Nito, suggesting that CCT specifically stabilizes METTL3 and METTL14 proteins (Fig. 3*A* and *SI Appendix*, Fig. S4). Consistently, LC-MS analysis showed that m^6A RNA levels are decreased in CCT2- or CCT7-depleted cells, similar to the effect of mTORC1 depletion (Fig. 3*B*). In conclusion, these results suggest that the CCT complex impacts METTL3 and METTL14 to enhance their protein stabilities and regulate m^6A methylation.

The Effects of mTORC1 on m^6A Modification and Autophagy Are Mediated by the CCT Complex. Previous studies have reported that mTORC1 activates the CCT complex. A recent study in *Drosophila* provides in vivo evidence showing that the CCT complex is regulated by mTORC1 signaling (22). Moreover, p70 ribosomal S6 kinase (S6K), a downstream effector of mTORC1, phosphorylates CCT2, suggesting that mTORC1 plays critical roles in regulating the CCT complex (23). Thus, we investigated whether the CCT complex mediates the mTORC1-dependent regulation of autophagy and m^6A methylation. As shown in Fig. 3*C*, *TSC1-RNAi*–induced mTORC1 activation inhibited autophagy upon starvation in fat body, while coexpression of *CCT8-RNAi* suppressed this effect (Fig. 3*C*). LC-MS and MeRIP assays further revealed that the *TSC2* KO-enhanced global m^6A and methylated *Atg1* and *Atg8a*, but not *Atg7*, mRNA levels were significantly reduced by depletion of CCT4 or CCT7 (Fig. 3 *D* and *E*), suggesting that mTORC1-dependent m^6A methylation and autophagy are mediated through CCT. Consistent with these results, depletion of the CCT complex alone was able to reduce METTL3 and METTL14 protein levels (*SI Appendix*, Fig. S5*A*), increase *Atg1* and *Atg8a* transcripts (*SI Appendix*, Fig. S5*B*), and enhance ATG1 and ATG8 protein levels (*SI Appendix*, Fig. S5*C*), resulting in an induction of autophagy (*SI Appendix*, Figs. S1*A* and S5*C*). Thus, these results suggest that the CCT complex, which acts downstream of mTORC1, is a positive regulator of m^6A RNA methylation and suppresses autophagy.

mTORC1-CCT-MTC Signaling Regulates Autophagy in Mammalian Cells. To determine whether the mTORC1-CCT-MTC axis is conserved in mammals, we treated MCF7 cells with rapamycin or transfected *TSC2* short hairpin RNA (shRNA) to manipulate mTORC1 activity. Similar to previous results in *Drosophila*, rapamycin reduced both METTL3 and METTL14 protein levels (Fig. 4*A*). m^6A levels were increased in *TSC2*-knocked down cells, an effect that was abrogated by rapamycin (Fig. 4*B*). Furthermore, depletion of human *METTL3* increased the number of LC3B puncta as well as the conversion of cytosolic LC3B (LC3B-I) to the lipidated form (LC3B-II) (Fig. 4*C* and *SI Appendix*, Fig. S6*A*). The mCherry-EGFP-LC3B reporter assay also revealed increases in both autophagosomes (mCherry +; GFP + vesicles) and autolysosomes (mCherry +; GFP- vesicles) in *METTL3*-depleted cells, compared to control cells, suggesting that *METTL3* knockdown increases autophagy flux (*SI Appendix*, Fig. S6*B*). Together, these results show that the regulation of MTC by mTORC1 to modulate autophagy is evolutionarily conserved.

Next, we examined the function of the human CCT complex in regulating MTC and autophagy. The physical interaction between the MTC and CCT complexes was observed in the human MTC-PPIN and confirmed in HEK293T cells (Fig. 4 *D* and *E*). A GST pull-down assay also revealed the direct interaction between human METTL3, METTL14, and TCP1 (also known as CCT1) (Fig. 4*F*). Depletion of CCT8 resulted in reduced METTL3 and METTL14 protein levels and increased autophagy (Fig. 4*G* and *SI Appendix*, Fig. S6 *A* and *B*), suggesting that the CCT complex can directly bind to and regulate METTL3 and METTL14 protein stabilities and their downstream functions. Furthermore, MCF7 cells expressing *shTSC2* exhibited higher METTL3 and METTL14 protein levels, enhanced m^6A levels, and reduced autophagy (Fig. 4 *H* and *I*). Depletion of CCT8 markedly suppressed an increase in m^6A levels induced by mTORC1 activation (Fig. 4*H*). Similarly, HSF1A, an inhibitor of CCT (24), reversed the *shTSC2*-induced effects, including up-regulation of METTL3 and METTL14 and inhibition of autophagy (Fig. 4*I*). Therefore, these findings demonstrate that the mTORC1-CCT-MTC-autophagy axis is conserved between *Drosophila* and mammals (Fig. 4*J*).

Discussion

The role of mTORC1 signaling in RNA metabolism is just emerging. In this study, we demonstrate that the MTC acts as a downstream effector of mTORC1 to regulate m^6A RNA methylation of *Atg* transcripts, inducing their degradation and thus suppressing autophagy. Furthermore, we identified the CCT complex as a link between mTORC1 and MTC. CCT downstream of mTORC1 signaling can stabilize METTL3 and METTL14 to up-regulate m^6A levels and inhibit autophagy. Accordingly, depletion of either mTORC1, CCT, METTL3, or METTL14 compromises m^6A RNA methylation and promotes autophagy. Importantly, the role of mTORC1-CCT-MTC signaling in regulating autophagy is conserved from *Drosophila* to mammals. Thus, our study discovered a function of mTORC1 in regulating m^6A RNA methylation during autophagy (Fig. 4*J*).

mTORC1 Regulates m^6A Methylation to Control mRNA Turnover and Autophagy. mTORC1 inhibition suppresses protein translation but also affects gene expression at different levels. Here, we identify an epitranscriptomic mechanism by which mTORC1 activates m^6A RNA methylation to promote *Atg* mRNA turnover and inhibits autophagy. This m^6A-mediated mRNA degradation represents a layer of gene regulation by mTORC1. Moreover, as mTORC1 activity regulates global m^6A levels, it is likely that the MTC also mediates additional physiological functions of mTORC1. We noted that depletion of METTL3, METTL14, or CCT8 cannot fully rescue TSC1-induced effects. Although these results could be caused by partial RNAi knockdown, they may also indicate that other pathways contribute to mTORC1 regulation of autophagy. Indeed, studies have reported that mTORC1 suppresses autophagy through modulation of transcription factors, RNA-processing complexes, and mRNA degradation machinery, further highlighting that mTORC1 utilizes multiple RNA biogenesis processes to control autophagy (6, 17, 25, 26).

Interspecies MTC-PPINS. The catalytic core components of the MTC, METTL3/METTL14, have a substrate sequence specificity for a DRA*CH motif (D = G/A/U, R = G/A, A* = methylated adenosine, H = A/U/C) (27). However, only a subset of consensus sites across the mRNA transcriptome are methylated. Thus, it has been speculated that other factors in the MTC specify METTL3/METTL14 methylation patterns. Our proteomic results combined with biochemical validation in both

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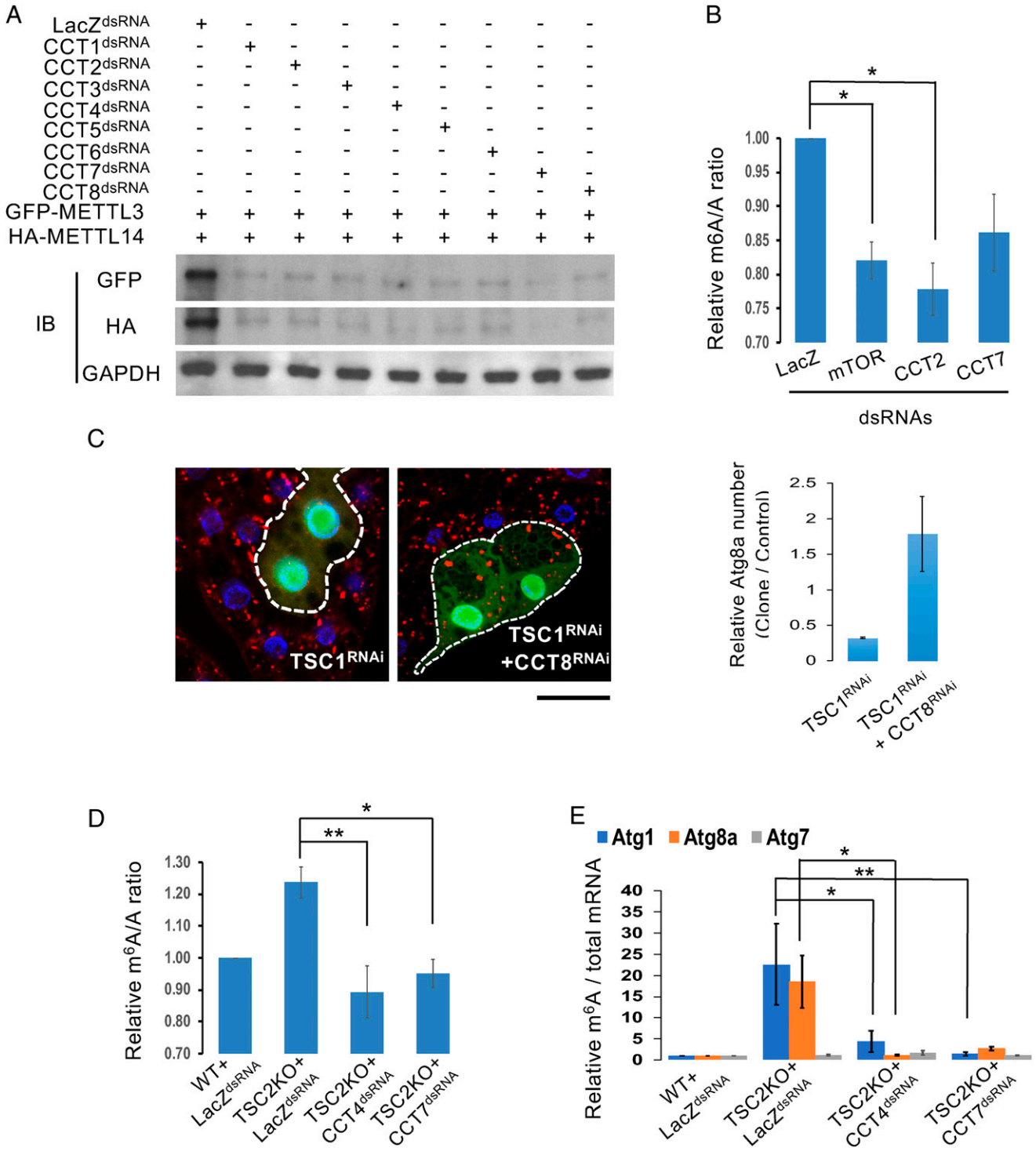

Fig. 3. mTORC1-CCT–signaling pathway acts as a positive regulator of MTC in *Drosophila*. (*A* and *B*) Depletion of CCT complex reduces METTL3 and METTL14 protein levels as well as m^{6}A RNA methylation. S2R+ cells were treated with dsRNAs against *LacZ* or *CCT1-8*. After 48 h, cells were transfected with *GFP-METTL3* or *HA-METTL14* and then subjected to immunoblotting (IB) with antibodies as indicated (*A*). Quantification of m^{6}A in S2R+ cells treated with dsRNAs against *LacZ*, *mTOR*, *CCT2*, or *CCT7*. One-way ANOVA followed by Tukey's multiple comparisons test was performed to identify significant differences. Data are expressed as mean ± SEM of three independent experiments; **P* < 0.05 (*B*). (*C*) The CCT complex acts downstream of mTORC1. Clonal depletion of *TSC1* in GFP-labeled cells suppressed the formation of mCherry-ATG8a puncta under starvation condition, compared to wild-type cells outside the circled dashed line. Coexpression of *CCT8^{RNAi}* reversed the *TSC1^{RNAi}*-induced effect. Fat-body cells were stained with DAPI. (Scale bar, 20 μm.) Quantification of the relative number of mCherry-ATG8a dots per cell. (*D* and *E*) mTORC1 activates m^{6}A RNA methylation in a CCT-dependent manner. Quantification of m^{6}A in wild-type or *TSC2* KO S2R+ cells treated with dsRNAs against *LacZ*, *CCT4*, *or CCT7*. Compared with wild-type S2R+ cells, *TSC2* KO cells showed enhanced m^{6}A levels in their mRNAs while *TSC2* KO cells treated with *CCT* dsRNAs had reduced m^{6}A levels (*D*). Abundance of *Atg* transcripts among mRNA immunoprecipitated with anti-m^{6}A antibody from S2R+ cells treated as indicated. m^{6}A-modified *Atg1* and *Atg8a*, but not *Atg7* mRNAs, were increased in *TSC2* KO cells, and knockdown of *CCT4* or *CCT7* in *TSC2* KO cells reduced m^{6}A-modified *Atg1* and *Atg8a* (*E*). One-way ANOVA followed by Tukey's multiple comparisons test was performed to identify significant differences. Data are expressed as mean ± SEM of three independent experiments; **P* < 0.05; ***P* < 0.01.

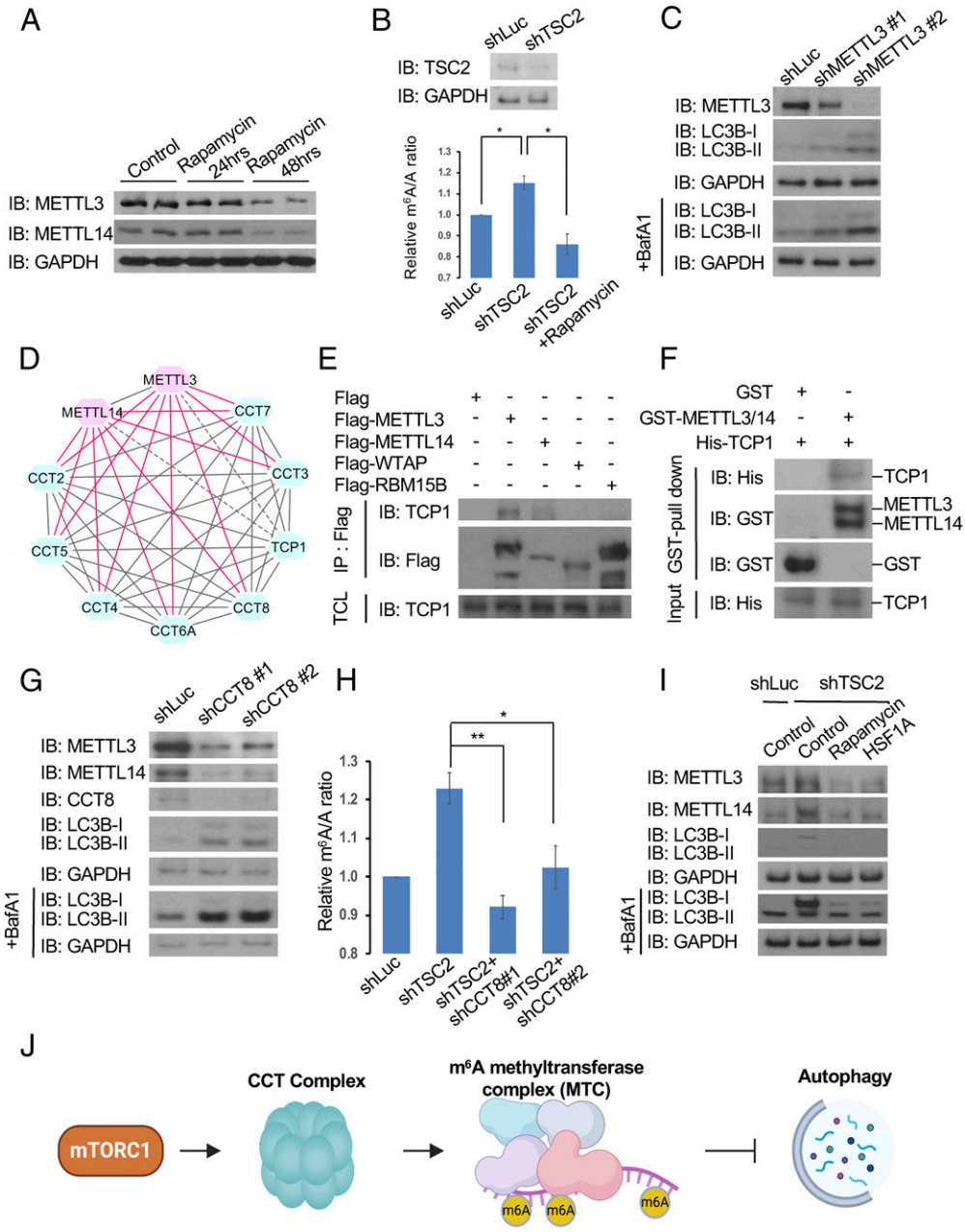

Fig. 4. Regulation of autophagy by mTORC1-CCT-MTC signaling is conserved in mammals. (*A*–*C*) mTORC1-MTC signaling regulates autophagy in human cells. MCF7 cells treated with 20 nM rapamycin for 24 or 48 h were subjected to immunoblotting with antibodies as indicated (*A*). Quantifications of m^{6}A relative to A in MCF7 cells stably infected with lentivirus expressing control (shLuc) or *TSC2* shRNA (shTSC2) with or without 20 nM rapamycin and immunoblot analysis was performed to determine the level of *TSC2* knockdown (*B*). MCF7 cells stably infected with lentivirus expressing control (shLuc) or *METTL3* shRNAs in the presence or absence of the lysosomal inhibitor Bafilomycin A1 (BafA1) were subjected to immunoblotting with antibodies as indicated (*C*). (*D* and *E*) CCT complex physically interacts with METTL3 and METTL14 in mammalian cells. Recovered PPIs between CCT complex, METTL3, and METTL14 from the human MTC-PPIN. Gray edges indicate the known interactions. Red edges suggest new interactions while gray dashed edges indicate the insignificant interactions (SAINT score < 0.2) (*D*). HEK293T cells, transfected with plasmids as indicated, were subjected to immunoprecipitations with anti-Flag antibody. Immunoprecipitated proteins (IP) and total cell lysates (TCL) were analyzed by immunoblotting with antibodies as indicated (*E*). (*F*) Human METTL3 and METTL14 directly interact with TCP1. Recombinant human GST-METTL3, GST-METTL14, and His-TCP1 proteins were subjected to GST pull-down assay. Pull-down fractions and input were analyzed by immunoblotting with antibodies as indicated. (*G*) Depletion of *CCT8* reduces METTL3 and METTL14 protein levels and induces autophagy. MCF7 cells stably infected with lentiviruses expressing control (shLuc) or *CCT8* shRNA in the presence or absence of BafA1 were subjected to immunoblotting with antibodies as indicated. (*H* and *I*) Inhibition of the CCT complex suppresses the effects induced by mTORC1 hyperactivation in human cells. Quantification of m^{6}A in MCF7 cells expressing *shLuc*, *shTSC2*, or *shTSC2* along with *shCCT8* (*H*). MCF7 cells stably expressing *shLuc* or *shTSC2* were treated with dimethylsulfoxide (control), 20 nM rapamycin, 200 μM HSF1A, or 100 nM BafA1 for 48 h and subjected to immunoblotting with antibodies as indicated (*I*). (*J*) Model showing that mTORC1 activates CCT complex to assist METTL3 and METTL14 proteins in folding, in turn enhancing m^{6}A RNA methylation, degrading *Atg* transcripts, and thus suppressing autophagy. Created with BioRender.com. One-way ANOVA test was performed followed by Tukey's test. Measurements shown are mean ± SEM of triplicates; *$P < 0.05$; **$P < 0.01$.

Drosophila and mammalian cells identified multiple splicing factors that interact with known MTC components. Future work will be needed to confirm whether these factors are directly involved in the regulation of m^6A methylation and how they coordinate with the m^6A machinery to affect RNA processing. It will also be interesting to investigate whether mTORC1 controls other regulators of RNA m^6A methylation, in addition to METTL3 and METTL14. Moreover, our proteomics data revealed that multiple components of E3 ubiquitin ligase complex interact with MTC, suggesting that they may be involved in ubiquitination of MTC. Ubiquitination of METTL3 has also been observed, but its function and related E3 ubiquitin ligases remain unclear (28).

mTORC1 Activates CCT Complex Transcriptionally and Posttranslationally to Stabilize METTL3 and METTL14. Previous genetic analyses showed that the CCT complex functions downstream of mTORC1 and that mTORC1 positively regulates the transcriptional levels of the CCT complex (22). Another study identified CCT2 as a substrate of S6 kinase, a downstream effector of mTOR, in mammalian cells (23), suggesting that both transcriptional and posttranslational regulations contribute to CCT complex activation by mTORC1. However, the phosphorylation site (Ser-260) of mammalian CCT2 is not conserved in *Drosophila* and how this phosphorylation modulates CCT function is not clear. Multiple phosphorylation sites have been detected in CCT components (29, 30). Interestingly, our previous study showed that CCT8 was phosphorylated following insulin stimulation (31), suggesting that other phosphorylation sites are involved in mTORC1-regulated CCT activation. Future studies are needed to comprehensively map the phosphorylation sites on CCT components and investigate their physiological roles.

The CCT complex is a highly conserved complex that assists the folding of about 10% of the eukaryotic proteome (21). The interactions of the CCT complex with METTL3 and METTL14 were observed in a previous study using AP/MS in human cells (32). Consistently, our genetic and biochemical data further confirmed their interactions and characterized the functions of CCT in stabilizing METTL3 and METTL14 and controlling m^6A RNA methylation. Our findings thus further expand the impact of the CCT complex on RNA metabolism.

Multiple studies have reported that CCT complex protein levels dramatically increase in autophagy mutants (33, 34), proposing that CCT is one of the substrates of autophagy. Future studies will be needed to test whether autophagy is able to degrade the CCT complex and whether autophagy feedback inhibits CCT.

Materials and Methods

Details on the fly strains, plasmids, and antibodies used in this study, as well as methods used for antibody staining, RNA interference, immunoprecipitation, RT-PCR, and analyzing m^6A levels, can be found in *SI Appendix, SI Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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