LETTER TO THE EDITOR

m⁶A RNA modification controls autophagy through upregulating ULK1 protein abundance

Cell Research (2018) 28:955–957; <https://doi.org/10.1038/s41422-018-0069-8>

Dear Editor,

N⁶-methyladenosine (m⁶A) is the prominent dynamic mRNA modification, governed by methyltransferase complex (“writers”), demethylases (“erasers”) and RNA-binding proteins (“readers”).¹ m⁶A modification directs mRNAs to distinct fates by grouping them for differential processing, translation and decay in the processes such as cell differentiation, embryonic development and stress responses.^{1,2} Owing to a deeper understanding of this modification and the technological advance, functional characterizations of m⁶A in gene regulation have become a hot topic that warrants further dissection.^{3,4}

Autophagy is a fundamental cellular process in eukaryotic cells, which sequesters cytoplasmic material within double-membraned autophagosomes and leads to degradation and recycling of the engulfed substrates.^{5,6} Unc-51-like kinase 1 (ULK1) serves as a protein kinase that is activated upon autophagy stimulation and is critical for the recruitment of other autophagy-related proteins to the autophagosome formation site.⁵ To date, the regulation of autophagy initiation mainly focused on the post-translational modification (PTM) and transactivation of autophagy-related genes.^{5,6} Whether m⁶A modification could regulate the mRNA processing of autophagy-related genes and affect autophagy remains unknown.

To investigate the role of m⁶A modification in autophagy, we performed a screen by using small interfering RNAs (siRNAs) targeting genes encoding the “writers”, “erasers”, and “readers” and identified fat mass and obesity associated (FTO) protein as a positive regulator of autophagy (Supplementary information, Figure S1a). We first confirmed the knockdown efficiency of FTO-specific siRNAs (Supplementary information, Figure S1b). We then examined the puncta formation of autophagy marker light chain 3B (LC3B). Under both basal and rapamycin-induced autophagy conditions, depletion of FTO significantly reduced the formation of GFP-LC3B puncta (Fig. 1a, b). We further monitored the autophagic flux by detecting LC3B in the presence of bafilomycin A1 (Baf A1), a H⁺-ATPase inhibitor that can block the autophagosome turnover. We observed that knockdown of FTO suppressed the accumulation of LC3B II. Consistently, the level of p62/SQSTM1 (an autophagy substrate) was higher in FTO-knockdown cells than that in control cells (Fig. 1c).

To further confirm the role of FTO in autophagy, we ectopically expressed Flag-tagged FTO and found that overexpression of FTO remarkably enhanced LC3B II accumulation and p62 consumption (Supplementary information, Figures S1c and d). Since FTO is a demethylase, we next generated catalytically inactive FTO mutant RQ (R316Q) and observed that cells expressing wild-type (WT) FTO, rather than FTO RQ mutant, displayed an increased number of GFP-LC3B puncta (Fig. 1d, e). Together, our findings reveal that FTO positively regulates autophagy in an enzyme activity-dependent manner.

To reveal the molecular mechanisms underpinning promotion of autophagy by FTO, we overexpressed FTO and observed that ULK1 protein level positively correlated with FTO protein level (Fig. 1f). In addition, FTO-mediated ULK1 stabilization could be potentiated by treatment of Earle’s balanced salt solution (EBSS; an autophagy agonist) (Fig. 1g and Supplementary information, Figure S1e). Moreover, we found that FTO RQ mutant could not stabilize ULK1 (Fig. 1h). These results suggest that FTO-mediated ULK1 stabilization depends on the demethylase activity of FTO. To determine whether the *ULK1* transcripts are bound by FTO, we performed anti-Flag-based RNA immunoprecipitation (IP) coupled to real-time PCR (RIP-PCR) with gene-specific primers. We first verified the efficiency of this immunoprecipitation assay by detecting the expression of Flag-tagged FTO (Supplementary information, Figure S1f). The IP samples from Flag-FTO cells revealed a strong signal for *ULK1* mRNA (Fig. 1i), while *ASB2* and *MYC* were chosen as the positive control.⁷ Collectively, these results indicate that FTO targets the *ULK1* transcripts and positively regulates the abundance of ULK1 protein.

As FTO is an m⁶A “eraser” of RNA methylation, we next investigated the m⁶A modification of *ULK1* transcripts. N⁶-methyladenosine-sequencing (m⁶A-seq) is an immunocapturing approach for the unbiased transcriptome-wide localization of m⁶A in high resolution.^{8,9} To assess the distribution of m⁶A within *ULK1* transcripts, we performed m⁶A-seq and found three distributions of m⁶A in the 3′-untranslated region (3′-UTR) of *ULK1* transcript (Fig. 1j). To elucidate the function of FTO in the m⁶A modification of *ULK1* mRNA, we generated an FTO-low-expressing (sgFTO) HEK293T cell line by using the CRISPR/Cas9 genome-editing tool (Supplementary information, Figure S1g). We immunoprecipitated the total mRNA with anti-m⁶A antibody and found that mRNA isolated from sgFTO cells showed a much higher enrichment of anti-m⁶A-bound *EEF1A1* gene than that from control (sgGFP) cells (Supplementary information, Figure S1h), indicating that *EEF1A1* gene can be m⁶A modified as described by the instructions of Magna MeRIP™ m⁶A Kit (Merck Millipore, Cat# 17-10499) and FTO could demethylate it. We further used the same samples for real-time PCR analysis to detect the potential *ULK1* m⁶A-bound fraction using gene-specific primers and observed that the anti-m⁶A-bound fraction showed greater enrichment for *ULK1* at three sites in sgFTO cells (Fig. 1k). Furthermore, we performed luciferase reporter assay and found that WT FTO could not promote the luciferase activity of the reporter construct bearing *ULK1*-3′-UTR with the mutations at these three m⁶A sites (Fig. 1l, m). Together, these results confirm that the m⁶A sites on *ULK1* transcripts are the direct substrates of FTO-catalyzed demethylation.

Increasing evidence suggests that m⁶A modification affects many aspects of gene expression, including nuclear export, mRNA decay, alternative pre-mRNA splicing, 3′-end processing and translation.⁴ We investigated the effect of FTO on the intracellular

Received: 3 April 2018 Revised: 26 June 2018 Accepted: 27 June 2018
Published online: 25 July 2018

distribution of the *ULK1* transcripts and found that FTO did not affect the translocation of *ULK1* mRNA from the nucleus to the cytoplasm (Supplementary information, Figures S1i and j). We then investigated whether FTO-mediated demethylation is implicated in *ULK1* mRNA decay. We observed that the decay

rate of *ULK1* mRNA was much slower in FTO-overexpressing cells, compared to that in control cells, when the transcription was halted with actinomycin D (Act D; Fig. 1n). Consistently, the protein level of *ULK1* was also higher in FTO-overexpressing cells than in control cells (Supplementary information, Figure S1k).

Fig. 1 FTO-mediated demethylation of *ULK1* transcripts promotes autophagy flux by upregulating *ULK1* protein abundance. **a** Representative images of GFP-LC3B puncta in HeLa-GFP-LC3B cells transfected with scrambled siRNA or *FTO*-specific siRNA after rapamycin (250 nM) treatment for 18 h. Scale bar, 20 μ m. **b** Quantification of GFP-LC3B puncta in HeLa-GFP-LC3B cells transfected with scrambled siRNA or *FTO*-specific siRNA. Bars represent mean $\pm$ SEM of triplicate samples (20 cells per sample). ****P* < 0.001. **c** HEK293T cells transfected with control or *FTO*-specific siRNA were treated with rapamycin (250 nM) for 18 h. The lysates were subjected to immunoblot analysis with the indicated antibodies. **d** HeLa cells stably expressing GFP-LC3B were transfected with pcDNA3.1 empty vector or plasmids expressing wild-type or the mutant form of FTO (RQ, short for R316Q). Scale bar, 20 μ m. **e** Average GFP-LC3B puncta per cell were calculated. Bars represent mean $\pm$ SEM of triplicate samples (20 cells per sample). ****P* < 0.001; NS, not significant. **f** Immunoblot analysis of extracts of 293T cells transfected with the increasing doses of Flag-FTO expression vector (wedge). **g** 293T cells were transfected with control or *FTO*-specific siRNA, followed by EBSS treatment for the indicated times. The protein expression levels of Beclin-1 and *ULK1* were detected by immunoblot. **h** 293T cells were transfected with plasmids encoding Flag-FTO or Flag-FTO RQ, and the cellular extracts were analyzed by immunoblot. **i** Confirmation of FTO binding to *ULK1* mRNA by RNA immunoprecipitation. *ABSI2* and *MYC* were used as the positive control. Data are normalized to negative control that is set to a value of 1. The relative Flag-FTO/Flag-EV ratios (log₁₀) are plotted. Data are shown as means $\pm$ SEM of three independent experiments. ****P* < 0.001. **j** Schematic representation of the position of m⁶A motifs within *ULK1* transcript. **k** Abundance of *ULK1* transcripts among mRNA immunoprecipitated with anti-m⁶A antibody from sgGFP-expressing and sgFTO-expressing cells. Data are expressed as means $\pm$ SEM of three independent experiments. ****P* < 0.001. **l** Wild-type or m⁶A consensus sequence mutant (A-to-T mutation) *ULK1*-3'-UTR was fused with *Renilla* luciferase reporter. **m** Relative luciferase activity of *ULK1*-3'-UTR with wild-type or mutated m⁶A sites after co-transfection with empty vector, FTO, or FTO RQ into 293T cell. *Renilla* luciferase activity was measured and normalized to firefly luciferase activity. **n** 293T cells transfected with plasmids encoding Flag-FTO were treated with Act D (5 μ g/mL) for the indicated times. The expression of *ULK1* was analyzed by real-time PCR. Data are expressed as means $\pm$ SEM of three independent experiments. ***P* < 0.01 and ****P* < 0.001. **o** 293T cells were co-transfected with control or *FTO*-specific siRNA and *YTHDF2*-specific siRNA, followed by Act D (5 μ g/mL) treatment for 18 h. The lysates were subjected to immunoblot analysis with the indicated antibodies. **p** A proposed working model to illustrate how FTO mediates the m⁶A modification of *ULK1* transcripts and positively regulates autophagy

Furthermore, we found that the decay of *ULK1* transcripts was enhanced in 293T cells transfected with *FTO*-specific siRNA than that with scramble siRNA (Supplementary information, Figure S1l). As YTHDF2 is the major m⁶A reader for the decay of m⁶A-modified mRNA, we next determined whether YTHDF2 targets cellular m⁶A-bound *ULK1* transcripts and found that the reduction of *ULK1* protein by FTO impairment was almost abolished in *YTHDF2*-knockdown cells (Fig. 1o and Supplementary information, Figure S1m). Together, our data suggest that the m⁶A changes caused by FTO influence the stability of *ULK1* transcripts, likely through a YTHDF2-dependent manner.

Our results sheds light on how FTO specifically upregulates the *ULK1* protein abundance, thereby promoting the initiation of autophagy. *ULK1* mRNA undergoes m⁶A modification in the 3'-UTR and the m⁶A-marked *ULK1* transcripts can further be targeted for degradation by YTHDF2. FTO reverses the m⁶A mRNA modification of *ULK1* transcripts, thus prolonging the half-life time of *ULK1* transcripts (Fig. 1p). Actually, since a great number of genes are involved in autophagy, we cannot exclude the possibility that m⁶A modification regulates autophagy by targeting other autophagic genes. As previous studies indicated that FTO functioned as an oncogenic m⁶A demethylase in leukemia and brain tumor,^{7,10} considering the importance of autophagy in tumorigenesis, we should not ignore the oncogenic roles of FTO via autophagy. Our study for the first time reveals a crosstalk of mRNA m⁶A modification and autophagy, which broadens the multilayer regulation of autophagy and expands our understanding of such interplays that are essential for therapeutic application.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31700760, 31522018, 91629101, 91753137, 31771462, 31471252, 31500813), the National Key Basic Research Program of China (2015CB859800, 2014CB910800),

Science and Technology Planning Project of Guangzhou, China (201804010385, 201604020003, 201604046001), and China Postdoctoral Science Foundation (2017M622862).

ADDITIONAL INFORMATION

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41422-018-0069-8>.

Competing interests: The authors declare no competing interests.

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