Dear Editor,

N6-methyladenosine (m6A) is an abundant epitranscriptomic modification that regulates messenger RNA (mRNA) biology. The m6A modification regulates mRNA splicing, transport, stability, and translation through coordinated activities by methyltransferases (writers), binding proteins (readers), and demethylases (erasers) (Huang et al., 2020; Wu et al., 2020). Among m6A regulators, fat mass of obesity-associated protein (FTO), is the first discovered eraser with RNA m6A demethylation activity (Jia et al., 2011). Since then, FTO has been reported to play m6A-dependent roles in a variety of physiological processes including adipogenesis, neurogenesis and tumorigenesis (Fischer et al., 2009; Li et al., 2017; Huang et al., 2020). Consequently, FTO deficiency in mice leads to dramatic phenotypes, such as decreased fat mass and impaired brain development (Fischer et al., 2009; Li et al., 2017). Similarly, inhibition of FTO reduces tumorigenesis in multiple types of cancer models, while FTO is highly expressed in many cancers (Huang et al., 2020).

Although FTO is primarily linked with biological functions related to demethylation of internal m6A, recent work indicates a more complex role in FTO-mediated epitranscriptional regulation. Specifically, FTO was reported to demethylate other substrates, such as N6,2′-O-dimethyladenosine (m6A2′) and N′-methyladenosine (m1A), and, based on its varied cellular distribution across cell types, proposed to be afforded differential access to RNA substrates (Wei et al., 2018; Sun et al., 2021). Indeed, in recent work, we discovered that m6A RNA methylation is reduced in human stem cell models of senescence, and that m6A-dependent mRNA stabilization of MIS12, a kinetochore component, protects against senescence (Wu et al., 2020). However, whether and how FTO regulates human stem cell homeostasis remain largely unexplored. Here, using CRISPR/Cas9-based strategy, we generated FTO-deficient human stem cell models and demonstrated an m6A-independent way by which FTO stabilizes MIS12 and antagonizes human stem cell senescence.

To investigate the role of FTO in regulating human stem cell homeostasis, we first generated FTO-deficient human embryonic stem cells (hESCs) through CRISPR/Cas9-based gene editing (Fig. 1A and 1B). FTO depletion was verified by western blotting and immunofluorescence staining (Figs. 1C, 1D and S1A). Furthermore, we did not detect any off-target effects resulting from FTO deletion (Fig. S1B). Although a previous study reported potential large structural variations near the target site induced by CRISPR-editing in hESCs (Bi et al., 2020), karyotype and copy number variation analyses revealed that the genomic integrity of FTO-deficient (FTO+/−) hESCs was well maintained (Fig. S1C–F). Relative to wild type (WT, FTO+/+) hESCs, DNA hypomethylation at the OCT4 promoter region and the expression of typical pluripotency markers, including OCT4, SOX2 and NANOG, were comparable in FTO−/− hESCs (Figs. 1E, S1G and S1H). Immunofluorescence staining and flow cytometry analysis further showed comparable Ki67-positive cell numbers and cell cycle status between FTO+/+ and FTO−/− hESCs (Figs. 1F and 1G). Similarly, we did not detect any remarkable differences in the expression of nuclear lamina-associated protein LAP2 and heterochromatin relevant proteins HP1α and H3K9me3, whose downregulation was associated with stem cell senescence (Shan et al., 2021; Li et al., 2022), between FTO+/+ and FTO−/− hESCs (Figs. 1H and S1I–K). Altogether, these data indicate that FTO is dispensable for maintaining hESC self-renewal and the expression of pluripotency genes.

Next, we differentiated FTO+/+ and FTO−/− hESCs into human mesenchymal progenitor cells (hMPCs) for addressing the effects of FTO deficiency on human adult stem cells (Fig. 1A). Both FTO+/+ and FTO−/− hMPCs expressed typical hMPC surface markers including CD73, CD90, and CD105 (Fig. S1L). We confirmed the loss of FTO protein by western blotting and immunofluorescence staining (Figs. 1I, 1J and S1M). Compared with FTO+/+ hMPCs, FTO−/− hMPCs were able to differentiate into adipocytes, chondrocytes, and osteoblasts, although with a differentiation bias towards the osteoblast fate at the expense of differentiation towards the adipocyte fate (Fig. S1N–P). Strikingly, and different from what we observed in hESCs, self-renewal capacity was impaired in FTO−/− hMPCs, as indicated by early-onset growth arrest, restricted clonal expansion, decreased Ki67-positive cells, reduced S phase and prolonged G0/G1 phase (Fig. 1K–N). In addition, FTO ablation led to accelerated senescence, as manifested by elevated senescence-associated β-galactosidase (SA-β-gal) activity.
FTO stabilizes MIS12 and counteracts senescence

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

FTO stabilizes MIS12 and counteracts senescence

© The Author(s) 2022

Protein & Cell
activity, shortened telomere length, downregulated expression of LAP2, HP1α, and H3K9me3, as well as increased nuclear abnormalities and size (Fig. 1O–T). Collectively, these findings indicate that FTO deficiency accelerates hMPC senescence.

We then asked whether the accelerated senescence of FTO−/− hMPCs is caused by alterations in the substrate abundance of FTO. Through dot blotting and liquid chromatography assays coupled with tandem mass spectrometry (LC-MS/MS) analysis, we found comparable m^6^A levels in both total RNA and mRNA between FTO+/+ and FTO−/− hESCs (Fig. S2A–C), and between FTO+/+ and FTO−/− hMPCs (Fig. S2D–F). Meanwhile, m^6^A abundance, as detected by fluorometric assays, was also similar between FTO+/+ and FTO−/− hESCs or hMPCs (Fig. S2G and S2H).

We then went on to conduct m^6^A-methylated RNA immunoprecipitation sequencing (MeRIp-seq) (Fig. S2I and S2J), and identified similar motif enrichment and distribution patterns of m^6^A methylation between FTO+/+ and FTO−/− hESCs or hMPCs (Fig. 2A–D). There was also no increase in the whole-transcriptome m^6^A peak intensity in FTO-deficient hESCs or hMPCs (Fig. S2K and S2L). Finally, LC-MS/MS analysis of the m^6^A^m_ modification in mRNA and dot blot analysis of the m^1^A modification in transfer RNA (tRNA) revealed no statistical difference between FTO+/+ and FTO−/− hESCs, and between FTO+/+ and FTO−/− hMPCs (Fig. S2M–P). In all, these data demonstrate that FTO depletion exerts minimal impact on global m^6^A, m^6^A^m_ and m^1^A levels in both hESCs and hMPCs, suggesting that FTO may regulate the homeostasis of hMPCs in a demethylation-independent manner.

Previous studies reported that FTO interacts with downstream proteins to enhance their biological activity (Tao et al., 2020). To identify such a potential mechanism for FTO in regulating hMPC senescence, we ectopically expressed FLAG-tagged FTO proteins in HEK293T cells and conducted co-immunoprecipitation (co-IP) assay with an anti-FLAG antibody, followed by LC-MS/MS analysis (Fig. 2E and Table S1). We identified a panel of potential FTO-interacting proteins (Table S1) that were found to be mostly enriched for "mitotic cell cycle process" through Gene Ontology (GO) enrichment analysis (Fig. 2F). Among these was MIS12 (Fig. S2Q and S2R), a key regulator of cell cycle distribution that we had reported in the regulation of hMPC senescence (Wu et al., 2020). Here, we validated the interaction between FTO and MIS12 through co-IP assay (Fig. 2G). Then, we
FTO stabilizes MIS12 and counteracts senescence

**LETTER**

1. Transfection
2. Immunoprecipitation
3. Elution
4. SDS-PAGE
5. Gel digestion
6. LC-MS/MS analysis
7. Bioinformatic analysis
Figure 2. FTO deficiency impairs MIS12 protein stability to accelerate hMPC senescence in an m6A-independent manner. (A) Identification of the m6A motif by MeRIP-seq analysis in FTO+/+ and FTO−/− hESCs. (B) Distribution of m6A peaks along the 5′UTR, CDS, and 3′UTR regions of mRNA from FTO+/+ and FTO−/− hESCs. (C) Identification of the m6A motif by MeRIP-seq analysis in FTO+/+ and FTO−/− hMPCs. (D) Distribution of m6A peaks along the 5′UTR, CDS, and 3′UTR regions of mRNA from FTO+/+ and FTO−/− hMPCs. (E) A schematic diagram showing the workflow of co-immunoprecipitation (co-IP) assay followed by mass spectrometry analysis. (F) Gene Ontology (GO) enrichment analysis of FTO-interacting proteins identified by mass spectrometry. (G) Co-IP analysis to verify the interaction between MIS12 and FLAG-FTO in HEK293T cells. (H) Western blot analysis of MIS12 and FTO in FTO+/+ and FTO−/− hMPCs. GAPDH was used as the loading control. Data are presented as the means ± SEM. n = 3 biological replicates. *, P < 0.05. (I) Protein stability analysis of MIS12 in FTO+/+ and FTO−/− hMPCs. Protein levels of MIS12 at indicated time points after treatment with a protein synthesis inhibitor cycloheximide (CHX) were determined by western blotting. GAPDH was used as the loading control. Data are presented as the means ± SEM. n = 6 biological replicates. ns, not significant; *, P < 0.05; ***, P < 0.01. (J) Western blot analysis of MIS12 in FTO−/− hMPCs with or without the treatment of CHX and MG132. GAPDH was used as the loading control. Data are presented as the means ± SEM. n = 3 biological replicates. *, P < 0.05. (K) Western blot analysis of MIS12 in control (sgNTC) and MIS12-knockout (sgMIS12) hMPCs. GAPDH was used as the loading control. (L) Clonal expansion analysis of control and MIS12-knockout hMPCs. Data are presented as the means ± SEM. n = 3 biological replicates. *, P < 0.05. (M) Immunofluorescence analysis of Ki67 in control and MIS12-knockout hMPCs. Scale bars, 20 μm. White arrows indicate Ki67-positive cells. Data are presented as the means ± SEM. n = 3 biological replicates. **, P < 0.01. (N) SA-β-gal staining of control and MIS12-knockout hMPCs. Scale bars, 50 μm. Data are presented as the means ± SEM. n = 3 biological replicates. **, P < 0.01. (O) Western blot analysis of Lamin B1 in control and MIS12-knockout hMPCs. GAPDH was used as the loading control. Data are presented as the means ± SEM. n = 3 biological replicates. *, P < 0.05. (P) Immunofluorescence analysis of LAP2 in control and MIS12-knockout hMPCs. Scale bars, 20 μm. White dashed lines indicate the nuclei with diminished signals of LAP2. Data are presented as the means ± SEM. n > 300 cells from three biological replicates. ***, P < 0.001. (Q) Immunofluorescence analysis of HP1α in FTO+/+ and FTO−/− hMPCs. Scale bars, 20 μm. White dashed lines indicate the nuclei with diminished signals of HP1α. Data are presented as the means ± SEM. n > 300 cells from three biological replicates. **, P < 0.01. (R) Immunofluorescence analysis of Lamin A/C in FTO+/+ and FTO−/− hMPCs. Scale bars, 20 μm. White arrows indicate abnormal nuclei. Data are presented as the means ± SEM. n = 3 biological replicates. *, P < 0.05. (S) Telomere length analysis of control and MIS12-knockout hMPCs. Data are presented as the means ± SEM. n = 3 biological replicates. *, P < 0.05.
FTO-deficient hMPCs, previous studies have unveiled that inhibition of FTO led to impeded cell cycle progression and compromised cell proliferation in myoblasts and a variety of cancer cells (Huang et al., 2020; Deng et al., 2021). Moreover, FTO knockdown in human ovarian granulosa cells also resulted in accelerated senescence (Jiang et al., 2021). Collectively, these reports and our study demonstrate cell-type-specific roles of FTO.

Cell cycle arrest is a common feature of cellular senescence (Wu et al., 2020). Consistently, we also detected aberrant cell cycle progress in FTO-deficient hMPCs. Furthermore, LC-MS/MS analysis identified a panel of cell cycle-related FTO-interacting proteins, indicating a potential role of these cell cycle factors in regulating hMPC senescence upon FTO deficiency. Subsequently, we noticed MIS12, a cell cycle regulator that interacts with DSN1, NSL1, and PMF1, to form the kinetochore complex, thus promoting chromosome segregation during mitosis and being involved in the regulation of cell proliferation (Abe-Kanoh et al., 2019). Indeed, our previous study has demonstrated that down-regulation of MIS12 due to m6A reduction decreased cell proliferation and accelerated senescence in hMPCs (Wu et al., 2020). Coincidently, we also observed a decrease in the protein level of MIS12 in FTO-deficient hMPCs, and that knockout of MIS12 led to compromised self-renewal and premature senescence in WT hMPCs, mimicking majority of the senescent phenotypes caused by FTO deficiency. Discordantly, we did not detect any visible alterations in the amounts of both m6A modification and mRNA level of MIS12 upon FTO depletion, indicating an m6A-independent way by which FTO regulates the expression of MIS12. Apart from MIS12, NSL1, another component of the kinetochore complex (Abe-Kanoh et al., 2019), was also identified as a candidate protein interacting with FTO (Fig. S2O and S2R; Table S1), supporting the implication of cell cycle regulators in FTO-mediated biological process. Our further results demonstrated that FTO deficiency facilitated the degradation of MIS12 through the proteasome-mediated pathway. Although previous work supported that MIS12 can be involved in this process. For example, RB1 and CDKN2A, two well-known senescence regulators, were also identified as potential FTO-interacting proteins (Fig. S2O and Table S1). Whether RB1 or CDKN2A partially contributes to hMPC senescence caused by FTO deficiency needs to be explored.

Collectively, our study disclosed an uncharacterized role of FTO in maintaining the protein stability of MIS12, independent of its m6A demethylation activity, and in counteracting hMPC senescence. These new findings further increase the complexity of FTO in the regulation of cellular physiology. Yet, more efforts need to be integrated to figure out the possible explanation for the globally unchanged m6A abundance in FTO-deficient human stem cells.

**FOOTNOTES**

We are grateful to Lei Bai, Ruijun Bai, Qun Chu, Shikun Ma, Jing Lu, Luyang Tian and Ying Yang for their administrative assistance. We thank Mengyi Sun, Jiayin Wang from Prof. Keqiong Ye’s lab (IBP, CAS) for their technical supports on RNA electrophoresis, Xueying Wang (Tsinghua University), Jifeng Wang (IBP, CAS), Zhensheng Xie (IBP, CAS) for their help in LC-MS/MS assays, Junying Jia (IBP, CAS), Shu Meng (IBP, CAS) and Shuang Sun (IBP, CAS) for their assistance in flow cytometry analyses. We also thank Chuanqian Liang and Wang Kang for their technical supports. This work was supported by the National Key Research and Development Program of China (2019YFA0110100), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA16010000), the National Natural Science Foundation of China (Grant Nos. 31900524, 31907097, 81921006, 81625009, 91749202, 8186118034, 91949209, 92049304, 82125011, 82120201, 92049116, 82071588, 82122024, 32100937, 92149301, and 92168201), the National Key Research and Development Program of China (2018YFC2000100, 2020YFA0804000, 2017YFA0103300, 2017YFA0102800, 2018YFA0107200, 2019YFA0110900, 2020YFA0112200, 2020YFA0803401, and 2019YFA0802202), Beijing Natural Science Foundation (Z190019), the Key Program Research of the Chinese Academy of Sciences (KFKZSW-221), the 14th Five-year Network Security and Informatization Plan of Chinese Academy of Sciences (WX145XQ07-18), K. C. Wong Education Foundation (GJTD-2019-06, GJTD-2019-08), Youth Innovation Promotion Association of CAS (E1CAZW0401), CAS Special Research Assistant (SRA) Program, the State Key Laboratory of Membrane Biology, the State Key Laboratory of Stem Cell and Reproductive Biology, the State Key Laboratory of Brain and Cognitive Science, the Tencent Foundation (2021-1045), and the Milky Way Research Foundation (MWRF).

Sheng Zhang, Zeming Wu, Yue Shi, Si Wang, Jie Ren, Zhiyu Yu, Daoyuan Huang, Kaowen Yan, Yifang He, Xiaoqian Liu, Qianzhao Ji, Beibei Liu, Zunpeng Liu, Jing Qu, Guang-Hui Liu, Weimin Ci, Xiaojun Wang and Weiqi Zhang declared no conflict of interest.

Sheng Zhang1,2,3, Zeming Wu2,4,5, Yue Shi6,7, Si Wang8,9,10, Jie Ren3,4,6,7,11, Zhiyu Yu3,4,7, Daoyuan Huang8, Kaowen Yan2,4,5, Yifang He2,3,4,5, Xiaoqian Liu4,5,12, Qianzhao Ji2,3, Beibei Liu8,7, Zunpeng Liu3,12, Jing Qu3,4,5,12, Guang-Hui Liu2,3,4,5,8,9, Weimin Ci3,4,6,7,10, Xiaojun Wang1,3,4,13, Weiqi Zhang9,14.

1 State Key Laboratory of Brain and Cognitive Science, CAS Center for Excellence in Brain Science and Intelligence Technology, Institute of Brain-Intelligence Technology (Shanghai), Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
2 State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
3 University of Chinese Academy of Sciences, Beijing 100049, China
4 The Author(s) 2022

© The Author(s) 2022

995
Supplementary Information  The online version contains supplementary material available at https://doi.org/10.1007/s13238-022-00914-6.