HHS Public Access

Author manuscript

Cancer Cell. Author manuscript; available in PMC 2018 January 09.

Published in final edited form as:

Cancer Cell. 2017 January 09; 31(1): 127–141. doi:10.1016/j.ccell.2016.11.017.

FTO plays an oncogenic role in acute myeloid leukemia as a N^6-methyladenosine RNA demethylase

Zejuan Li[1,7,10], **Hengyou Weng**[1,2,10], **Rui Su**[2,10], **Xiaocheng Weng**[3,8,10], **Zhixiang Zuo**[1,2,9,10], **Chenying Li**[2,4], **Huilin Huang**[2], **Sigrid Nachtergaele**[3], **Lei Dong**[2], **Chao Hu**[1,2,4], **Xi Qin**[2], **Lichuan Tang**[5], **Yungui Wang**[1,2,4], **Gia-Ming Hong**[1], **Hao Huang**[1], **Xiao Wang**[3], **Ping Chen**[1], **Sandeep Gurbuxani**[6], **Stephen Arnovitz**[1], **Yuanyuan Li**[1], **Shenglai Li**[1], **Jennifer Strong**[2], **Mary Beth Neilly**[1], **Richard A. Larson**[1], **Xi Jiang**[1,2], **Pumin Zhang**[5], **Jie Jin**[4], **Chuan He**[3,*], and **Jianjun Chen**[1,2,11,*]

[1]Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, IL 60637, USA

[2]Department of Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, OH 45219, USA

[3]Department of Chemistry, Department of Biochemistry and Molecular Biology, Institute for Biophysical Dynamics, Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637, USA

[4]Key Laboratory of Hematopoietic Malignancies; Department of Hematology, The First Affiliated Hospital Zhejiang University, Hangzhou, Zhejiang 310003, China

[5]Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, USA

[6]Department of Pathology, University of Chicago, Chicago, IL 60637, USA

[7]Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA

[*]Correspondence: chuanhe@uchicago.edu; chen3jj@ucmail.uc.edu.

[10]These authors contributed equally to this work

[11]Lead Contact

ACCESSION NUMBERS

The microarray, m^6A-seq and RNA-seq data have been deposited in the Gene expression Ominibus (GEO) repository with the accession numbers GSE34184, GSE30285, GSE76414, GSE84944 and GSE85008.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Figures and Tables.

AUTHOR CONTRIBUTIONS

Z.L. and J.C. conceived the project. Z.L., R.S., C.H. and J.C. designed the research and supervised experiments conducted in the laboratories. Z.L., H.W., R.S., X.W., Z.Z., C.L., H.H., S.N., L.D., C.H., X.Q., L.T., G.M.H., Y.W., H.H., X.W., P.C., S.G., S.A., Y.L., S.L., J.S., X.J., J.C. performed experiments and/or data analyses; Z.L., H.H., M.B.N., R.A.L., X.J., P.Z., J.J., C.H., and J.C. contributed reagents/analytic tools and/or grant support; Z.L., H.W., R.S., X.W., Z.Z., C.H., and J.C. wrote the paper. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

[8]College of Chemistry and Molecular Sciences, Key Laboratory of Biomedical Polymers of Ministry of Education, Wuhan University, Hubei, Wuhan 430072, PR China

[9]Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, China

SUMMARY

N^6-methyladenosine (m^6A) represents the most prevalent internal modification in mammalian mRNAs. Despite its functional importance in various fundamental bioprocesses, the studies of m^6A in cancer have been limited. Here we show that FTO, as an m^6A demethylase, plays a critical oncogenic role in acute myeloid leukemia (AML). *FTO* is highly expressed in AMLs with t(11q23)/*MLL*-rearrangements, t(15;17)/*PML-RARA*, *FLT3*-ITD and/or *NPM1* mutations. FTO enhances leukemic oncogene-mediated cell transformation and leukemogenesis, and inhibits all-trans-retinoic acid (ATRA)-induced AML cell differentiation, through regulating expression of targets such as *ASB2* and *RARA* by reducing m^6A levels in these mRNA transcripts. Collectively, our study demonstrates the functional importance of the m^6A methylation and the corresponding proteins in cancer, and provides profound insights into leukemogensis and drug response.

eTOC Blurb

Li et al. show that FTO, an N^6-methyladenosine (m^6A) demethylase, is highly expressed in subtypes of AML, promotes leukemogenesis, and inhibits all-trans-retinoic acid-induced leukemia cell differentiation. FTO exerts its oncogenic role by regulating mRNA targets such as *ASB2* and *RARA* by reducing their m^6A levels.

INSTRUCTION

N^6-methyladenosine (m^6A) is the most abundant internal modification in messenger RNA (mRNA) mainly occurring at consensus motif of G[G>A]m^6AC[U>A>C] (Fu et al., 2014; Meyer and Jaffrey, 2014; Niu et al., 2013; Yue et al., 2015). Although m^6A was first discovered in 1970s (Desrosiers et al., 1974; Perry and Kelley, 1974), the lack of technologies to study RNA modifications limited research on m^6A and the field had not advanced for several decades. The identification of the fat mass and obesity-associated protein (FTO) as the first RNA demethylase (Jia et al., 2011) revived RNA methylation research, suggesting that m^6A is a reversible and dynamic RNA modification that may impact biological regulation analogous to the well-studied reversible DNA and histone modifications (Jia et al., 2013). In 2012, two groups reported transcriptome-wide approaches for m^6A RNA immunoprecipitation followed by next-generation sequencing (termed as m6A-seq or MeRIP-seq) and detected m^6A peaks in more than 7,000 mRNA transcripts and hundreds of long non-coding RNAs (lncRNAs) in both human and mouse cells, with many of the m^6A peaks conserved between humans and mice (Dominissini et al., 2012; Meyer et al., 2012). Thus, these studies suggest m^6A methylation in mRNAs is a prevalent modification that likely possesses functional importance.

Recent studies have shown that m^6A modification in mRNAs or non-coding RNAs plays critical roles in tissue development, stem cell self-renewal and differentiation, control of heat

shock response, and circadian clock controlling, as well as in RNA fate and functions such as mRNA stability, splicing, transport, localization and translation, primary microRNA processing, and RNA-protein interactions (Alarcon et al., 2015; Chen et al., 2015; Dominissini et al., 2012; Geula et al., 2015; Liu et al., 2015; Meyer et al., 2015; Meyer et al., 2012; Wang et al., 2014a; Wang et al., 2015; Wang et al., 2014b; Zhao et al., 2014; Zheng et al., 2013; Zhou et al., 2015). FTO and ALKBH5, the second RNA demethylase identified in 2013 (Zheng et al., 2013), both belong to the AlkB family and catalyze m^6A demethylation in a Fe(II)- and α-ketoglutarate-dependent manner, and are referred to as m^6A 'erasers' (Fu et al., 2014; Yue et al., 2015). METTL3 and METTL14 were identified as m^6A 'writers' that form a heterodimer with support of WTAP to catalyze m^6A methylation (Bokar et al., 1997; Liu et al., 2014; Ping et al., 2014; Wang et al., 2014b). YTHDF1/2/3 were identified as m^6A 'readers' that preferentially bind to RNA that contains m^6A at the G[G>A] m^6ACU consensus sequence and lead to different biological consequence (Dominissini et al., 2012; Wang et al., 2014a; Wang et al., 2015), such as inducing RNA decay (Wang et al., 2014a) and promoting mRNA translation (Wang et al., 2015).

FTO was known to be robustly associated with increased body mass and obesity in humans (Dina et al., 2007; Frayling et al., 2007; Scuteri et al., 2007). Animal model studies showed that *Fto* deficiency protected from obesity and caused growth retardation (Fischer et al., 2009; Gao et al., 2010), while overexpression of *Fto* led to increased food intake and obesity (Church et al., 2010). In humans, loss-of-function mutations in *FTO* also caused severe growth retardation and multiple malformations that resulted in premature death (Boissel et al., 2009). As the first identified RNA demethylase that regulates the demethylation of target mRNAs, FTO has been reported to regulate dopaminergic signaling in brain (Hess et al., 2013), and also regulate mRNA splicing of adipogenetic regulatory factors and thus play a critical role in adipogenesis (Ben-Haim et al., 2015; Zhao et al., 2014). However, the impact of FTO, especially as a RNA demethylase, in cancer development and progression has yet to be investigated.

Acute myeloid leukemia (AML) is one of the most common and fatal forms of hematopoietic malignancies with distinct genetic (e.g., t(11q23)/*MLL*-rearranged, inv(16), t(8;21), and t(15;17)) and molecular (e.g., *FLT3*-ITD and *NPM1* mutations) abnormalities and variable response to treatment (Chen et al., 2010; Dohner et al., 2015; Marcucci et al., 2005). With standard chemotherapies, only 35%–40% of younger (aged <60) and 5%–15% of older (aged ≥60) patients with AML survive over 5 years (Dohner et al., 2015). Thus, to develop effective targeted therapies to treat AML is an urgent and significant unmet medical need, which relies on better understanding of the molecular mechanisms underlying the pathogenesis and drug response of AML.

In the present study, we sought to determine the biological function of FTO in the pathogenesis and drug response of AMLs and also investigate the underlying molecular mechanism through identification of critical mRNA targets of FTO.

RESULTS

FTO is highly expressed in certain subtypes of AML

In analysis of our in-house microarray dataset of 100 human AML with t(11q23)/*MLL*-rearranged, t(8;21), inv(16) or t(15;17) and 9 normal control samples, we found that *FTO* was expressed at a significantly higher level in *MLL*-rearranged AML than in both normal controls (p=0.04) and non-*MLL*-rearranged AML samples (p=0.002); amongst non-*MLL*-rearranged AML, *FTO* is expressed at a significantly higher level in t(15;17) AML, also called acute promyelocytic leukemia (APL), than in t(8;21) and inv(16) AMLs (Figure 1A). Our qPCR assay also showed that *FTO* is expressed at a significantly higher level in $CD34^+$ bone marrow (BM) cells isolated from primary *MLL*-rearranged AML patients relative to normal $CD34^+$ BM cells isolated from healthy donors (Figure 1B). In contrast, ALKBH5, another m^6A demethylase (Zheng et al., 2013), was not significantly up-regulated in *MLL*-rearranged AML relative to normal controls (Figures S1A and S1B), although a very recent study suggests involvement of ALKBH5 in breast cancer cell proliferation in vitro (Zhang et al., 2016).

Consistently, in analysis of two large-cohort AML datasets, including GSE37642 set (n=562) and GSE14468 set (n=518), we observed that *FTO* is expressed at a significantly higher level in t(11q23) and t(15;17) AMLs compared to other subtypes of cytogenetically abnormal (CA) AMLs including inv(16) and t(8;21) AMLs (Figures S1C and S1D). The expression level of *FTO* in normal karyotype (NK) AMLs is comparable to that in t(11q23) and t(15;17) AMLs (Figures S1C and S1D). Within NK-AMLs, *FTO* is expressed at a significantly higher level in AML with *FLT3*-ITD and/or *NPM1* mutations (i.e., *NPM1c*) (especially that with both) compared to those without (Figures 1C and 1D). We also observed that FTO was aberrantly up-regulated at the protein level in human primary AML specimens of the above subtypes (Figure 1E).

Expression of *FTO* can be upregulated by the relevant leukemic oncogenes

We then conducted both Western blot and qPCR assays of mouse BM progenitor cell samples transformed by MLL-AF9 (the most common form of MLL-fusions (Chen et al., 2010)), PML-RARA (fusion gene of t(15;17)), and FLT3-ITD/NPM1-mutant, showing that FTO can be upregulated by the oncogenes at both protein and RNA levels (Figure S1E). Moreover, *Fto*, but not *Alkbh5*, was also significantly up-regulated by MLL-fusion in mouse leukemic BM cells collected from primary BM transplantation (BMT) recipient mice, relative to normal control cells; notably, the up-regulation of *Fto* was further enhanced after transplantation of the primary *MLL-AF9* leukemic BM cells into secondary recipients (Figures 1F, S1F and S1G). Fto overxpression is accompanied with mRNA m^6A level decrease in the samples (Figure 1F).

We also used *MLL*-rearranged leukemia as a model to further investigate whether *FTO* is directly up-regulated by the oncogenic proteins. Through chromatin immunoprecipitation (ChIP) assays, we found that MLL (see MLL-C binding) and particularly MLL-fusion proteins (see the portion of MLL-N binding exceeding that of MLL-C) are enriched at the CpG area (CpG sites), but not the distal upstream site (Control site), of *FTO* locus in human

MONOMAC-6 (a *MLL-AF9* AML line) and KOCL-48 (a *MLL-AF4* AML line) cells; the locus also shows a significant enrichment of H3K79 methylation (H3K79me2/3), a mark of active transcription (Bernt et al., 2011; Okada et al., 2005) (Figure 1G). No such significant enrichments were observed in a control cell line, K562 (a human erythroleukemic cell line) (Figure 1G). Thus, our data suggest that *FTO* is likely a direct target of MLL fusions. In addition, we observed that *Fto* (but not *Alkbh5*) expression was significantly ($p<0.01$) down-regulated in MLL-ENL-ERtm mouse myeloid cells carrying tamoxifen-inducible *MLL-ENL* (Zeisig et al., 2004) when expression of *MLL-ENL* was depleted after withdrawal of 4-Hydroxy-tamoxifen (4-OHT) (Figure 1H), indicating that *Fto* expression in MLL-ENL-ERtm cells relies on the presence of MLL-ENL.

FTO promotes cell proliferation/transformation and suppresses apoptosis in vitro

Both gain- and loss-of-function studies were performed to investigate the pathological role of *FTO* in AML. As shown in Figures 2A–F, lentivirally transduced wild-type *FTO*, but not *FTO* mutant (carrying two point mutations, H231A and D233A, which disrupt the enzymatic activity of FTO (Jia et al., 2011; Lin et al., 2014)), promoted cell growth/proliferation and viability, while decreasing apoptosis and the global mRNA m^6A level, in two *MLL*-rearranged AML cell lines (MONOMAC-6 and MV4-11); the opposite is true when endogenous expression of *FTO* was knocked down by *FTO* shRNAs. Similar phenomena were observed when FTO was overexpressed by a retroviral construct or knocked down by siRNAs (Figures S2A–D). In contrast, while forced expression of FTO exhibited moderate (though significant) effects, *FTO* knockdown exhibited no significant effects on cell growth/proliferation, growth or apoptosis in K562 AML cells (a control line) (Figures S2E–I); thus, FTO is less functionally essential in K562 cells than in *MLL*-rearranged AML cells, likely due to its much lower endogenous expression in K562 cells (Figure S2J). We next performed mouse BM colony-forming/replating assays (CFAs) to investigate the function of FTO in MLL-AF9-mediated cell transformation. As expected, co-overexpression of FTO, but not FTO-Mut, significantly increased colony numbers after replating, compared to the MLL-AF9 alone group; knockdown expression of *Fto* led to the opposite (Figures 2G and S2K).

Similar patterns were observed in the *PML-RARA*/t(15;17) and *FTL3*-ITD/*NPM1* leukemic cell models when both gain- and loss-of-function studies of FTO/Fto were conducted (see Figures 3 and S3), demonstrating the oncogenic role of FTO in these AML subtypes.

Fto significantly enhances leukemogenesis in vivo

To investigate the role of Fto in vivo, we conducted mouse BM transplantation (BMT) assays. We found that forced expression of Fto significantly ($p<0.05$; log-rank test) accelerated MLL-AF9-induced leukemogenesis in recipient mice (Figures 4A and S4), and this pattern is repeatable (Figure 4B). Notably, forced expression of *Fto* significantly increased c-Kit^+ immature blast cell proportion (Figure 4C) and decreased global m^6A level in leukemic BM cells (Figure 4D). Conversely, the knockdown of *Fto* by shFto-1 or shFto-2 significantly delayed *MLL-AF9*-mediated leukemogenesis in mice (Figure 4E). Similarly, *MLL-AF9*-tranduced BM progenitor cells from $Fto^{+/-}$ mice (Gao et al., 2010) caused leukemia in recipient mice significantly slower than did *MLL-AF9*-tranduced BM progenitor cells from wild-type ($Fto^{+/+}$) mice (Figure 4F), and were associated with a

decrease in c-Kit$^+$ cell proportion (Figure 4G) and an increase in m^{6}A abundance in leukemic BM cells (Figure 4H).

Transcriptome-wide m^{6}A-seq and RNA-seq assays to identify potential targets of FTO

To identify potential mRNA targets of FTO whose m^{6}A levels are reduced by FTO in AML cells, we retrovirally transduced MSCV-PIG-FTO (i.e., FTO) or MSCV-PIG (i.e., Ctrl/Control) into human MONOMAC-6 AML cells and then selected individual stable clones under selection of puromycin (0.5ug/ml). Two *FTO*-overexpressing (namely FTO_1 and _2) and two control (namely Ctrl_1 and _2) stable cell lines were selected for transcriptome-wide m^{6}A-sequencing (m^{6}A-seq) and RNA-sequencing (RNA-seq) assays. The *FTO*-overexpressing stable lines exhibit a 4–5 fold increase in FTO protein level (Figure 5A) and a noticeable decrease in m^{6}A level (Figures 5B and 5C), compared to the control lines. As shown in Table S1, 17.7 million – 83.1 million reads were generated from each m^{6}A-seq or RNA-seq (also serving as the input control of the corresponding m^{6}A-seq) library. A total of 13,278 m^{6}A peaks were identified by both MACS (Zhang et al., 2008) and exomePeak (Meng et al., 2013) methods from at least two of the four m^{6}A-seq libraries (Figure S5A). Consistent with previous studies (Chen et al., 2015; Dominissini et al., 2012; Meyer et al., 2012), the most common m^{6}A motif GGAC is significantly enriched in our 13,278 m^{6}A peaks (Figure S5B); the m^{6}A peaks are mostly located in exons (>87%; Figure S5C) and are especially enriched in the vicinity of the stop codon (Figures S5D and S5E).

Potential target genes of FTO tend to be negatively regulated by FTO

We next compared the abundance (normalized to input) of the 13,278 m^{6}A peaks between *FTO*-overexpressing cells (i.e., FTO_1/2) and the control cells (i.e., Ctrl_1/2). A total of 2,785 and 3,180 m^{6}A peaks showed a significant decrease and increase ($p<0.005$; fold change ≥1.2), respectively, in abundance, in FTO_1/2 cells relative to Ctrl_1/2 cells, and thereby they were termed as hypo- and hyper-methylated m^{6}A peaks, respectively (Figure S5F). Through analysis of the RNA-seq data, we identified 322 hypo-methylated m^{6}A peaks whose mRNA transcripts were significantly ($p<0.005$; fold change ≥1.2) down-regulated (275; Hypo-down) or up-regulated (47; Hypo-up) in FTO_1/2 cells relative to Ctrl_1/2 cells (Figure 5D). Notably, 85% of the 322 hypo-methylated m^{6}A peaks are associated with down-regulated mRNA transcripts in *FTO*-overexpressing cells, significantly more frequent ($p<0.0001$; χ^2-test) than the rate (54%) in the 308 hyper-methylated m^{6}A peaks (Figure 5D). Thus, mRNA transcripts carrying hypo-methylated m^{6}A peaks, which are likely potential targets of FTO since FTO is an m^{6}A demethylase, tend to be down-regulated in *FTO*-overexpressing AML cells.

Through searching the Molecular Signature Database (MSigDB) of GSEA (Subramanian et al., 2005), we found that the Hypo-up transcripts were significantly enriched with target genes of SOX2, NANOG and LEF1, key transcription factors for embryonic stem cell pluripotency or WNT signaling activation (Figure S5G). Hypo-down transcripts were significantly enriched with genes involving the interferon signaling and immune system; interestingly, the most enriched genes are a set of potential direct target genes of PML-RARA, a fusion protein resulting from t(15;17) in APL (Figure S5G). The enriched genes sets in Hyper-up or Hyper-down transcripts are shown in Figure S5H. We next investigated

the correlation between *FTO* and the above genes in expression across four large cohorts of AML patient datasets (see Table S2). We found that 8 Hypo-down (i.e., *ASB2*, *KCNG1*, *PPARD*, *RAB17*, *RARA*, *SLC11A1*, *SLCO4A1*, and *TBC1D9*) and 3 Hypo-up (*C21orf59*, *MZF1*, and *TXLNA*) genes exhibited a significantly negative and positive correlation, respectively, with *FTO* in expression across all the four datasets (Table S2). We next conducted gene-specific m^6A qPCR assays for five such m^6A-Hypo genes (*ASB2*, *PPARD*, *RARA*, *SLC11A1*, and *TXLNA*) and confirmed the m^6A-level decrease in 4 out of the 5 genes (80%; Figure 5E), demonstrating the reliability of our transcriptome-wide m^6A-seq data.

We then conducted m^6A-seq and RNA-seq of *MA9/FLT3*-ITD leukemic cells (i.e., *MLL-AF9* plus *FLT3*-ITD-transformed human $CD34^+$ cord blood cells (Wunderlich et al., 2013)) with or without *FTO*-shRNA knockdown. Remarkably, more than 90% m^6A-Hypo transcripts identified from *FTO*-overexpressing MONOMAC-6 cells turned into m^6A-Hyper in *FTO*-knockdown *MA9/FLT3*-ITD cells (Figure 5F), with approximately 85% of the Hypo-down and 47% of Hypo-up transcripts became Hyper-up and Hyper-down, respectively (see Figures 5F and 5G), which might be genuine targets of FTO. Overall, the vast majority of potential targets of FTO are likely negatively regulated by FTO. Eight out of the 11 m^6A-Hypo genes listed in Table S2 showed expected patterns in the m^6A and RNA level changes in *FTO*-knockdown cells (Table S3).

ASB2 and *RARA* are functionally important target genes of FTO in AML

Interestingly, amongst the genes listed in Tables S2 and S3, *ASB2* and *RARA* have been reported to be up-regulated during normal hematopoiesis and in ATRA-induced differentiation of leukemia cells and function as key regulators during the processes (Glasow et al., 2005; Guibal et al., 2002; Kohroki et al., 2001; Sakamoto et al., 2014; Wang et al., 2012; Zhu et al., 2001). Notably, ASB2 can also degrade MLL during hematopoietic differentiation via ubiquitination (Wang et al., 2012). Consistent with their down-regulation in *FTO*-overexpressing AML cells, *ASB2* and *RARA* exhibit a significant inverse correlation with *FTO* in expression across all four independent AML cohorts (see Table S2 and Figure 5H). Our m^6A-seq data indicates that FTO targets the 3′UTR of *ASB2* and both 3′UTR and 5′UTR of *RARA* transcripts; FTO overexpression and knockdown causes a significant decrease and increase, respectively, in the m^6A level of the UTR(s) (Figures 5I and 5J). We then focused on these two FTO potential targets for further studies.

As expected, we showed that in both MONOMAC-6 and NB4/t(15;17) AML cells, forced expression of wild-type FTO, but not mutant FTO, substantially reduced expression of ASB2 and RARA, while increasing expression of MLL, a negative downstream target of ASB2 (Wang et al., 2012) (Figures 6A and S6A). The opposite phenomena were observed when we knocked down endogenous expression of *FTO* (Figures 6B, 6C, and S6B). Forced expression or depletion of FTO affected expression of *RARA*, but not that of other *RAR* genes such as *RARG* (Figure S6C). In addition, we observed similar changes in *RARA* and *ASB2* expression after overexpression or knockdown of *FTO* in nuclear and cytoplasm (Figure S6D).

We then cloned *ASB2* and *RARA* coding regions (CDS) or shRNAs into lentiviral vectors and investigated their functions in AML cells. As expected, forced expression of either *ASB2* or *RARA* largely recapitulated the phenotypes caused by *FTO* knockdown in both MONOMAC-6 and NB4 cells (Figures 6D–E *vs.* Figures 2B and 3B). Moreover, the effects of *FTO* overexpression can be largely rescued by forced expression of *RARA* or *ASB2* (Figures S6E–J). Conversely, knockdown of *ASB2* or *RARA* significantly enhanced AML cell growth and viability, which mimics the effect of *FTO* overexpression, and was sufficient to rescue the inhibitory effect of *FTO* knockdown on AML cell growth and viability (Figures 6F–I). Collectively, our data demonstrate that *ASB2* and *RARA* are functionally important targets of FTO.

FTO-mediated regulation of *ASB2* and *RARA* depends on its m^{6}A demethylase activity and the m^{6}A modifications in the target mRNA transcripts

To elucidate the molecular mechanism underlying FTO-mediated regulation of expression of its targets such as *ASB2* and *RARA*, we first validated effects of FTO expression changes on the m^{6}A levels in target mRNA transcripts. Using gene-specific m^{6}A qPCR assays, we demonstrated that forced expression and knockdown of *FTO* reduced and increased, respectively, the m^{6}A levels on *ASB2* and *RARA* mRNA transcripts (Figure 7A and data not shown). More importantly, to assess the requirement of the target mRNA m^{6}A modifications for FTO-mediated gene regulation, we conducted Luciferase reporter and mutagenesis assays (Figure 7B). As expected, compared with mutant FTO or empty vector, ectopically expressed wild-type FTO substantially reduced luciferase activity of the individual reporter constructs bearing wild-type *ASB2* 3′UTR, *RARA* 3′UTR or *RARA* 5′UTR that has intact m^{6}A sites, while mutations in the m^{6}A sites abrogated the inhibition (Figure 7B). To validate whether the effects observed above are related to m^{6}A modifications, we conducted gene-specific m^{6}A qPCR of the cloned wild-type and mutant 3′UTR fragments of *ASB2* or *RARA* in HEK-293T cells that were used for the Luciferase reporter assays. As expected, while the wild-type 3′UTR fragments have high abundance of m^{6}A modifications, the mutant 3′UTR fragments contain no or minimal level of m^{6}A modifications; co-tansfected wild-type FTO, but not FTO mutant, significantly reduced the m^{6}A abundance on the wild-type 3′UTR fragments of *ASB2* or *RARA* in HEK-293T cells (see Figure 7C). Together, our data demonstrate that FTO-mediated gene regulation relies on its demethylase activity and m^{6}A modifications in the target mRNA transcripts.

A set of m^{6}A-modified transcripts could be recognized by YTHDF2, which exhibits a shorter half-life than non-methylated ones (Wang et al., 2014a). Thus, it is surprising that most of the FTO potential targets we identified tend to be negatively regulated by FTO in AML cells (Figures 5D, 5F and 5G). To analyze the effect of m^{6}A level decrease on the stability of potential target transcripts of FTO, we conducted RNA stability assays (Wang et al., 2014a) and showed that forced expression and knockdown of *FTO* shortened and prolonged, respectively, the half-life of *ASB2* and *RARA* mRNA transcripts in AML cells (Figures 7D, S7A and S7B). Thus, FTO-induced repression of *ASB2* and *RARA* expression is at least in part due to the decreased stability of *ASB2* and *RARA* mRNA transcripts upon FTO-mediated decrease of m^{6}A level in their mRNA transcripts. Consistent with the effect of *FTO* overexpression, we found that knockdown of *METTL3* and especially of *METTL14*,

two m^{6}A writers, also resulted in the down-regulation of *ASB2* and *RARA* levels (Figure S7C).

Our results suggest that alternative m^{6}A reading processes may exist which recognize m^{6}A sites in FTO target transcripts and promote their stability. YTHDF1 and YTHDF2 are two well-established m^{6}A readers with each recognizing a few thousands of methylated transcripts in mammalian cells (Wang et al., 2014a; Wang et al., 2015). Nonetheless, knockdown of either of them did not lead to a significant reduction at the mRNA levels of *ASB2* or *RARA* (Figures 7E and 7F), indicating that YTHDF1 and YTHDF2 are unlikely the readers that can promote the stability of *ASB2* and *RARA* mRNAs. While *YTHDF1* knockdown resulted in a minor decrease in the protein levels of ASB2 and RARA, *YTHDF2* knockdown showed no effect on their protein levels at all (Figure 7G). Furthermore, neither forced expression nor knockdown of *FTO* affected expression of *METTL3*, *METTL14*, *YTHDF1* or *YTHDF2* (Figure 7H). Thus, the effects of *FTO* overexpression or knockdown on *ASB2* and *RARA* mRNA stability are unlikely due to the changes in the above m^{6}A writers or readers. The reader(s) that promotes the mRNA stability of FTO target transcripts (e.g., *ASB2* and *RARA*) has yet to be identified.

The FTO⊣ASB2/RARA axis contributes to the response of APL cells to ATRA treatment

While ATRA-based differentiation therapy has transformed APL from a highly fatal disease to a highly curable one (Huang et al., 1988; Wang and Chen, 2008), it is still important to better understand the underlying molecular mechanism(s). As shown in Figure 8A, upon ATRA treatment, *FTO* is significantly down-regulated in NB4 APL cells, associated with a significant up-regulation of *RARA* and especially *ASB2*. To investigate the potential role of FTO in ATRA-induced APL cell differentiation, we compared *FTO*-overexpressing NB4 cells and control NB4 cells on their response to ATRA treatment through flow cytometry assays. As ATRA can induce APL cells towards granulocytic and monocytic differentiation (Arteaga et al., 2013; Carlesso et al., 1999; Gocek et al., 2014), anti-CD11b (a granulocytic differentiation marker) and anti-CD14 (a monocytic differentiation marker) antibodies were used in the flow cytometric assays. As shown in Figure 8B, forced expression of *FTO*, but not *FTO* mutant, noticeably increased the undifferentiated NB4 cell population (i.e., CD11b$^-$/CD14$^-$ cells) after 48 hours of 500 nM ATRA treatment. We then conducted a loss-of-function study, and found that depletion of *FTO* expression by two individual different shRNAs could substantially enhanced ATRA-induced cell differentiation, resulting in a striking decrease in undifferentiated population of NB4 cells after 48 hours of treatment with a lower concentration of ATRA (100 nM) (Figure 8C). Figure 8D shows a summary of the results from three independent experiments. Consistent with the phenotype caused by *FTO* knockdown, forced expression of either *RARA* or *ASB2* could also substantially enhance NB4 cell differentiation (Figures 8E and 8F). Collectively, FTO inhibits ATRA-induced differentiation of APL cells likely through post-transcriptionally repressing expression/function of *RARA* and *ASB2*, and such function of FTO relies on its m^{6}A demethylase activity.

DISCUSSION

In the present study, we demonstrate that *FTO*, an obesity risk-associated gene and the first m^{6}A eraser identified, plays a critical oncogenic role in hematopoietic cell transformation and leukemogenesis. Briefly, FTO expression can be up-regulated by certain oncogenic proteins (e.g., MLL-fusion proteins, PML-RARA, FLT3-ITD and NPM1 mutant) and thereby FTO is aberrantly up-regulated in certain subtypes of AMLs, e.g., t(11q23)/*MLL*-rearranged, t(15;17), *FLT3*-ITD and/or *NPM1*-mutanted AMLs. In vitro, we show that forced expression of *FTO* significantly enhances the viability and proliferation/growth of human AML cells, while inhibiting the apoptosis of the cells, and also significantly enhances leukemic oncogenes-mediated transformation/immortalization of normal hematopoietic stem/progenitor cells; the opposite is true when expression of *FTO* is knocked down. In vivo, we show that forced and depleted expression of *Fto* significantly promotes and inhibits, respectively, MLL-fusion-mediated leukemogenesis in mice. Our transcriptome-wide m^{6}A-seq assay and the subsequent validation and functional studies suggest that *ASB2* and *RARA* are two critical target genes of FTO. As an m^{6}A RNA demethylase, FTO reduces the m^{6}A levels of *ASB2* and *RARA* mainly at untranslated regions (UTRs), which in turn leads to the down-regulation of these two genes at the RNA level and especially at the protein level. Mechanistically, we demonstrate that the biological function of FTO relies on its m^{6}A demethylase activity as mutations in the FTO catalytic domain sufficiently abrogate the function of FTO. Moreover, our luciferase reporter/mutagenesis assays indicate that the m^{6}A sites in the UTRs of its critical target genes such as *ASB2* and *RARA* are essential for FTO to post-transcriptionally regulate their expression. Our data and previous studies (Glasow et al., 2005; Guibal et al., 2002; Kohroki et al., 2001; Zhu et al., 2001) demonstrate the anti-leukemic effects of ASB2 and RARA. Thus, the FTO⊣ASB2/RARA axis likely plays a critical role in the pathogenesis of AMLs. Furthermore, we provide evidence that this axis likely also plays an essential role in mediating the response of leukemic cells to ATRA treatment. A schematic model summarizing our discoveries is shown in Figure 8G.

Epidemiology studies demonstrate a strong association between FTO single nucleotide polymorphisms (SNPs) or overweight/obesity and the risk of various types of cancers, such as breast, prostate, kidney and pancreatic cancers, as well as hematopoietic malignancies including myeloma, lymphoma and leukemia (Castillo et al., 2012; Li et al., 2012a; Soderberg et al., 2009). Therefore, it is possible that increased expression of *FTO*, caused by its obesity-associated SNPs (Berulava and Horsthemke, 2010; Church et al., 2010), may contribute (to some extent) to the higher risk of individuals with overweight and obesity in developing various types of cancers. If so, FTO may play an oncogenic role also in other cancers, besides leukemia.

Previous studies suggest that mRNA transcripts with m^{6}A modifications tend to be less stable (Schwartz et al., 2014; Wang et al., 2014a), largely due to the relocation of such mRNAs by YTHDF2 to RNA decay sites (Wang et al., 2014a). Surprisingly, herein we show that over 80% of FTO potential targets tend to be negatively regulated by FTO in AML cells. We show that forced and depleted expression of *FTO* substantially shortens and prolongs, respectively, the half life of its critical targets such as *ASB2* and *RARA*, suggesting that

FTO-mediated repression of *ASB2* and *RARA* expression is at least in part due to the decreased stability of *ASB2* and *RARA* mRNA transcripts. Our data further suggest that additional reading process may exist which controls the stability of FTO target transcripts. Neither YTHDF2 nor YTHDF1 target all m^6A sites in mammalian cells. Other reading processes have been suggested (Liu et al., 2015). Although certain m^6A-sites on *ASB2* or *RARA* could be affected by YTHDF2 or YTHDF1, our data indicate that the FTO-targeted sites exhibit effects on mRNA distinct from these known reading processes. It will be very interesting to uncover such an alternative reading process in the future.

Interestingly, all the subtypes of AMLs with high levels of endogenous *FTO* expression, such as those carrying t(11q23), t(15;17), *NPM1* mutations, and/or *FLT3*-ITD, are more sensitive to ATRA than the other AML subtypes (Dos Santos et al., 2013; El Hajj et al., 2015; Hu et al., 2009; Huang et al., 1988; Iijima et al., 2004; Jiang et al., 2016; Lo-Coco et al., 2013; Martelli et al., 2015; Niitsu et al., 2001; Schlenk et al., 2009). It is possible that the survival/proliferation of these subtypes of AML cells relies more on the FTO signaling, and thus they are more responsive to ATRA treatment, as ATRA can release the expression/function of ASB2 and RARA, two negative targets of FTO, and thereby trigger cell differentiation.

In summary, here we provide compelling in vitro and in vivo evidence demonstrating that FTO, an m^6A demethylase, plays a critical oncogenic role in cell transformation and leukemogenesis as well as in ATRA-mediated differentiation of leukemic cells, through reducing m^6A levels in mRNA transcripts of its critical target genes such as *ASB2* and *RARA* and thereby triggering corresponding signaling cascades. Our study highlights the functional importance of the m^6A modification machinery in cancer, and provides profound insights into the molecular mechanisms underlying tumorigenesis by revealing a previously unrecognized mechanism of gene regulation in cancer. In addition, given the functional importance of FTO in leukemogenesis and drug response, targeting FTO signaling by selective inhibitors may represent a promising therapeutic strategy to treat leukemia, especially in combination of ATRA treatment. As FTO has also been implicated in other types of cancers, our discoveries may have a broad impact in cancer biology and cancer therapy.

EXPERIMENTAL PROCEDURES

Leukemic patient samples

The leukemia patient samples were obtained at the time of diagnosis or relapse and with informed consent at the University of Chicago Hospital (UCH) or other collaborative hospitals, and were approved by the institutional review board of the institutes/hospitals.

The care and maintenance of animals

It was approved by Institutional Animal Care and Use Committee (IACUC) of the University of Chicago or University of Cincinnati.

Cell Culture and Transfection, Chromatin Immunoprecipitation (ChIP), qPCR, In vitro Functional Study Assays, and in vivo Bone Marrow Transplantation Studies

These assays were performed as we did previously (Huang et al., 2013; Jiang et al., 2012; Li et al., 2012b) with some modifications.

Global m^{6}A Quantitative Assays, m^{6}A-seq, RNA-seq, Gene-Specific m^{6}A qPCR and RNA Stability Assays

These assays were conducted as described previously (Dominissini et al., 2013; Jia et al., 2011; Liu et al., 2014; Wang et al., 2014a) with some modifications (see Supplemental Information for details).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Michelle M. Le Beau for providing primary AML patient samples and James Mulloy for providing the *MA9/FLT3*-ITD AML cell line. This work was supported in part by the National Institutes of Health (NIH) R01 Grants CA178454 (J.C.), CA182528 (J.C.) and GM071440 (C.H.), Leukemia & Lymphoma Society (LLS) Special Fellowship (Z.L.), LLS Translational Research Grant (J.C.), American Cancer Society (ACS) Research Scholar grant (J.C.), ACS-IL Research Scholar grant (Z.L.), Gabrielle's Angel Foundation for Cancer Research (J.C., Z.L., X.J. and H.H.), China Scholarship Council (CSC) visiting scholar (X.W.), and Foundation of Innovation Team for Basic and Clinical Research of Zhejiang Province (Grant 2011R50015) (J.J.). S.N. is an HHMI Fellow of the Damon Runyon Cancer Research Foundation (DRG-2215-15). C.H. is an investigator of the Howard Hughes Medical Institute (HHMI).

References

Alarcon CR, Lee H, Goodarzi H, Halberg N, Tavazoie SF. N6-methyladenosine marks primary microRNAs for processing. Nature. 2015; 519:482–485. [PubMed: 25799998]

Arteaga MF, Mikesch JH, Qiu J, Christensen J, Helin K, Kogan SC, Dong S, So CW. The histone demethylase PHF8 governs retinoic acid response in acute promyelocytic leukemia. Cancer Cell. 2013; 23:376–389. [PubMed: 23518351]

Ben-Haim MS, Moshitch-Moshkovitz S, Rechavi G. FTO: linking m6A demethylation to adipogenesis. Cell research. 2015; 25:3–4. [PubMed: 25475057]

Bernt KM, Zhu N, Sinha AU, Vempati S, Faber J, Krivtsov AV, Feng Z, Punt N, Daigle A, Bullinger L, et al. MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. Cancer Cell. 2011; 20:66–78. [PubMed: 21741597]

Berulava T, Horsthemke B. The obesity-associated SNPs in intron 1 of the FTO gene affect primary transcript levels. Eur J Hum Genet. 2010; 18:1054–1056. [PubMed: 20512162]

Boissel S, Reish O, Proulx K, Kawagoe-Takaki H, Sedgwick B, Yeo GS, Meyre D, Golzio C, Molinari F, Kadhom N, et al. Loss-of-function mutation in the dioxygenase-encoding FTO gene causes severe growth retardation and multiple malformations. Am J Hum Genet. 2009; 85:106–111. [PubMed: 19559399]

Bokar JA, Shambaugh ME, Polayes D, Matera AG, Rottman FM. Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N6-adenosine)-methyltransferase. Rna. 1997; 3:1233–1247. [PubMed: 9409616]

Carlesso N, Aster JC, Sklar J, Scadden DT. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. Blood. 1999; 93:838–848. [PubMed: 9920832]

Castillo JJ, Mull N, Reagan JL, Nemr S, Mitri J. Increased incidence of non-Hodgkin lymphoma, leukemia, and myeloma in patients with diabetes mellitus type 2: a meta-analysis of observational studies. Blood. 2012; 119:4845–4850. [PubMed: 22496152]

Chen J, Odenike O, Rowley JD. Leukaemogenesis: more than mutant genes. Nat Rev Cancer. 2010; 10:23–36. [PubMed: 20029422]

Chen T, Hao YJ, Zhang Y, Li MM, Wang M, Han W, Wu Y, Lv Y, Hao J, Wang L, et al. m(6)A RNA methylation is regulated by microRNAs and promotes reprogramming to pluripotency. Cell Stem Cell. 2015; 16:289–301. [PubMed: 25683224]

Church C, Moir L, McMurray F, Girard C, Banks GT, Teboul L, Wells S, Bruning JC, Nolan PM, Ashcroft FM, Cox RD. Overexpression of Fto leads to increased food intake and results in obesity. Nat Genet. 2010; 42:1086–1092. [PubMed: 21076408]

Desrosiers R, Friderici K, Rottman F. Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. Proc Natl Acad Sci USA. 1974; 71:3971–3975. [PubMed: 4372599]

Dina C, Meyre D, Gallina S, Durand E, Korner A, Jacobson P, Carlsson LM, Kiess W, Vatin V, Lecoeur C, et al. Variation in FTO contributes to childhood obesity and severe adult obesity. Nat Genet. 2007; 39:724–726. [PubMed: 17496892]

Dohner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015; 373:1136–1152. [PubMed: 26376137]

Dominissini D, Moshitch-Moshkovitz S, Salmon-Divon M, Amariglio N, Rechavi G. Transcriptome-wide mapping of N(6)-methyladenosine by m(6)A-seq based on immunocapturing and massively parallel sequencing. Nat Protoc. 2013; 8:176–189. [PubMed: 23288318]

Dominissini D, Moshitch-Moshkovitz S, Schwartz S, Salmon-Divon M, Ungar L, Osenberg S, Cesarkas K, Jacob-Hirsch J, Amariglio N, Kupiec M, et al. Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. Nature. 2012; 485:201–206. [PubMed: 22575960]

Dos Santos GA, Kats L, Pandolfi PP. Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. J Exp Med. 2013; 210:2793–2802. [PubMed: 24344243]

El Hajj H, Dassouki Z, Berthier C, Raffoux E, Ades L, Legrand O, Hleihel R, Sahin U, Tawil N, Salameh A, et al. Retinoic acid and arsenic trioxide trigger degradation of mutated NPM1, resulting in apoptosis of AML cells. Blood. 2015; 125:3447–3454. [PubMed: 25800051]

Fischer J, Koch L, Emmerling C, Vierkotten J, Peters T, Bruning JC, Ruther U. Inactivation of the Fto gene protects from obesity. Nature. 2009; 458:894–898. [PubMed: 19234441]

Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JR, Elliott KS, Lango H, Rayner NW, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. 2007; 316:889–894. [PubMed: 17434869]

Fu Y, Dominissini D, Rechavi G, He C. Gene expression regulation mediated through reversible m(6)A RNA methylation. Nat Rev Genet. 2014; 15:293–306. [PubMed: 24662220]

Gao X, Shin YH, Li M, Wang F, Tong Q, Zhang P. The fat mass and obesity associated gene FTO functions in the brain to regulate postnatal growth in mice. PLoS ONE. 2010; 5:e14005. [PubMed: 21103374]

Geula S, Moshitch-Moshkovitz S, Dominissini D, Mansour AA, Kol N, Salmon-Divon M, Hershkovitz V, Peer E, Mor N, Manor YS, et al. Stem cells. m6A mRNA methylation facilitates resolution of naive pluripotency toward differentiation. Science. 2015; 347:1002–1006. [PubMed: 25569111]

Glasow A, Prodromou N, Xu K, von Lindern M, Zelent A. Retinoids and myelomonocytic growth factors cooperatively activate RARA and induce human myeloid leukemia cell differentiation via MAP kinase pathways. Blood. 2005; 105:341–349. [PubMed: 15339853]

Gocek E, Marchwicka A, Bujko K, Marcinkowska E. NADPH-cytochrome P450 reductase is regulated by all-trans retinoic acid and by 1,25-dihydroxyvitamin D3 in human acute myeloid leukemia cells. PLoS ONE. 2014; 9:e91752. [PubMed: 24642534]

Guibal FC, Moog-Lutz C, Smolewski P, Di Gioia Y, Darzynkiewicz Z, Lutz PG, Cayre YE. ASB-2 inhibits growth and promotes commitment in myeloid leukemia cells. J Biol Chem. 2002; 277:218–224. [PubMed: 11682484]

Hess ME, Hess S, Meyer KD, Verhagen LA, Koch L, Bronneke HS, Dietrich MO, Jordan SD, Saletore Y, Elemento O, et al. The fat mass and obesity associated gene (Fto) regulates activity of the dopaminergic midbrain circuitry. Nat Neurosci. 2013; 16:1042–1048. [PubMed: 23817550]

Hu J, Liu YF, Wu CF, Xu F, Shen ZX, Zhu YM, Li JM, Tang W, Zhao WL, Wu W, et al. Long-term efficacy and safety of all-trans retinoic acid/arsenic trioxide-based therapy in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci USA. 2009; 106:3342–3347. [PubMed: 19225113]

Huang H, Jiang X, Li Z, Li Y, Song CX, He C, Sun M, Chen P, Gurbuxani S, Wang J, et al. TET1 plays an essential oncogenic role in MLL-rearranged leukemia. Proc Natl Acad Sci USA. 2013; 110:11994–11999. [PubMed: 23818607]

Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhoa L, Gu LJ, Wang ZY. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood. 1988; 72:567–572. [PubMed: 3165295]

Iijima K, Honma Y, Niitsu N. Granulocytic differentiation of leukemic cells with t(9;11)(p22;q23) induced by all-trans-retinoic acid. Leukemia & lymphoma. 2004; 45:1017–1024. [PubMed: 15291362]

Jia G, Fu Y, He C. Reversible RNA adenosine methylation in biological regulation. Trends Genet. 2013; 29:108–115. [PubMed: 23218460]

Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, Yi C, Lindahl T, Pan T, Yang YG, He C. N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat Chem Biol. 2011; 7:885–887. [PubMed: 22002720]

Jiang X, Hu C, Arnovitz S, Bugno J, Yu M, Zuo Z, Chen P, Huang H, Ulrich B, Gurbuxani S, et al. miR-22 has a potent anti-tumor role with therapeutic potential in acute myeloid leukemia. Nat Commun. 2016; 7:11452. [PubMed: 27116251]

Jiang X, Huang H, Li Z, Li Y, Wang X, Gurbuxani S, Chen P, He C, You D, Zhang S, et al. Blockade of miR-150 maturation by MLL-fusion/MYC/LIN-28 is required for MLL-associated leukemia. Cancer Cell. 2012; 22:524–535. [PubMed: 23079661]

Kohroki J, Fujita S, Itoh N, Yamada Y, Imai H, Yumoto N, Nakanishi T, Tanaka K. ATRA-regulated Asb-2 gene induced in differentiation of HL-60 leukemia cells. FEBS Lett. 2001; 505:223–228. [PubMed: 11566180]

Li G, Chen Q, Wang L, Ke D, Yuan Z. Association between FTO gene polymorphism and cancer risk: evidence from 16,277 cases and 31,153 controls. Tumour Biol. 2012a; 33:1237–1243. [PubMed: 22396042]

Li Z, Huang H, Chen P, He M, Li Y, Arnovitz S, Jiang X, He C, Hyjek E, Zhang J, et al. miR-196b directly targets both HOXA9/MEIS1 oncogenes and FAS tumour suppressor in MLL-rearranged leukaemia. Nat Commun. 2012b; 2:688.

Lin L, Hales CM, Garber K, Jin P. Fat mass and obesity-associated (FTO) protein interacts with CaMKII and modulates the activity of CREB signaling pathway. Hum Mol Genet. 2014; 23:3299–3306. [PubMed: 24488767]

Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, Jia G, Yu M, Lu Z, Deng X, et al. A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. Nat Chem Biol. 2014; 10:93–95. [PubMed: 24316715]

Liu N, Dai Q, Zheng G, He C, Parisien M, Pan T. N(6)-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. Nature. 2015; 518:560–564. [PubMed: 25719671]

Lo-Coco F, Avvisati G, Vignetti M, Thiede C, Orlando SM, Iacobelli S, Ferrara F, Fazi P, Cicconi L, Di Bona E, et al. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. N Engl J Med. 2013; 369:111–121. [PubMed: 23841729]

Marcucci G, Mrozek K, Bloomfield CD. Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. Curr Opin Hematol. 2005; 12:68–75. [PubMed: 15604894]

Martelli MP, Gionfriddo I, Mezzasoma F, Milano F, Pierangeli S, Mulas F, Pacini R, Tabarrini A, Pettirossi V, Rossi R, et al. Arsenic trioxide and all-trans retinoic acid target NPM1 mutant oncoprotein levels and induce apoptosis in NPM1-mutated AML cells. Blood. 2015; 125:3455–3465. [PubMed: 25795919]

Meng J, Cui X, Rao MK, Chen Y, Huang Y. Exome-based analysis for RNA epigenome sequencing data. Bioinformatics. 2013; 29:1565–1567. [PubMed: 23589649]

Meyer KD, Jaffrey SR. The dynamic epitranscriptome: N6-methyladenosine and gene expression control. Nature reviews Molecular cell biology. 2014; 15:313–326. [PubMed: 24713629]

Meyer KD, Patil DP, Zhou J, Zinoviev A, Skabkin MA, Elemento O, Pestova TV, Qian SB, Jaffrey SR. 5′ UTR m(6)A Promotes Cap-Independent Translation. Cell. 2015; 163:999–1010. [PubMed: 26593424]

Meyer KD, Saletore Y, Zumbo P, Elemento O, Mason CE, Jaffrey SR. Comprehensive analysis of mRNA methylation reveals enrichment in 3′ UTRs and near stop codons. Cell. 2012; 149:1635–1646. [PubMed: 22608085]

Niitsu N, Hayashi Y, Sugita K, Honma Y. Sensitization by 5-aza-2′-deoxycytidine of leukaemia cells with MLL abnormalities to induction of differentiation by all-trans retinoic acid and 1alpha,25-dihydroxyvitamin D3. Br J Haematol. 2001; 112:315–326. [PubMed: 11167824]

Niu Y, Zhao X, Wu YS, Li MM, Wang XJ, Yang YG. N6-methyl-adenosine (m6A) in RNA: an old modification with a novel epigenetic function. Genomics Proteomics Bioinformatics. 2013; 11:8–17. [PubMed: 23453015]

Okada Y, Feng Q, Lin Y, Jiang Q, Li Y, Coffield VM, Su L, Xu G, Zhang Y. hDOT1L links histone methylation to leukemogenesis. Cell. 2005; 121:167–178. [PubMed: 15851025]

Perry RP, Kelley DE. Existence of methylated messenger RNA in mouse L cells. Cell. 1974; 1:37–42.

Ping XL, Sun BF, Wang L, Xiao W, Yang X, Wang WJ, Adhikari S, Shi Y, Lv Y, Chen YS, et al. Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. Cell Res. 2014; 24:177–189. [PubMed: 24407421]

Sakamoto K, Imamura T, Yano M, Yoshida H, Fujiki A, Hirashima Y, Hosoi H. Sensitivity of MLL-rearranged AML cells to all-trans retinoic acid is associated with the level of H3K4me2 in the RARalpha promoter region. Blood Cancer J. 2014; 4:e205. [PubMed: 24769646]

Schlenk RF, Dohner K, Kneba M, Gotze K, Hartmann F, Del Valle F, Kirchen H, Koller E, Fischer JT, Bullinger L, et al. Gene mutations and response to treatment with all-trans retinoic acid in elderly patients with acute myeloid leukemia. Results from the AMLSG Trial AML HD98B. Haematologica. 2009; 94:54–60. [PubMed: 19059939]

Schwartz S, Mumbach MR, Jovanovic M, Wang T, Maciag K, Bushkin GG, Mertins P, Ter-Ovanesyan D, Habib N, Cacchiarelli D, et al. Perturbation of m6A writers reveals two distinct classes of mRNA methylation at internal and 5′ sites. Cell Rep. 2014; 8:284–296. [PubMed: 24981863]

Scuteri A, Sanna S, Chen WM, Uda M, Albai G, Strait J, Najjar S, Nagaraja R, Orru M, Usala G, et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet. 2007; 3:e115. [PubMed: 17658951]

Soderberg KC, Kaprio J, Verkasalo PK, Pukkala E, Koskenvuo M, Lundqvist E, Feychting M. Overweight, obesity and risk of haematological malignancies: a cohort study of Swedish and Finnish twins. Eur J Cancer. 2009; 45:1232–1238. [PubMed: 19091543]

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005; 102:15545–15550. [PubMed: 16199517]

Wang J, Muntean AG, Hess JL. ECSASB2 mediates MLL degradation during hematopoietic differentiation. Blood. 2012; 119:1151–1161. [PubMed: 22174154]

Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G, et al. N6-methyladenosine-dependent regulation of messenger RNA stability. Nature. 2014a; 505:117–120. [PubMed: 24284625]

Wang X, Zhao BS, Roundtree IA, Lu Z, Han D, Ma H, Weng X, Chen K, Shi H, He C. N(6)-methyladenosine Modulates Messenger RNA Translation Efficiency. Cell. 2015; 161:1388–1399. [PubMed: 26046440]

Wang Y, Li Y, Toth JI, Petroski MD, Zhang Z, Zhao JC. N6-methyladenosine modification destabilizes developmental regulators in embryonic stem cells. Nat Cell Biol. 2014b; 16:191–198. [PubMed: 24394384]

Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008; 111:2505–2515. [PubMed: 18299451]

Wunderlich M, Mizukawa B, Chou FS, Sexton C, Shrestha M, Saunthararajah Y, Mulloy JC. AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. Blood. 2013; 121:e90–97. [PubMed: 23349390]

Yue Y, Liu J, He C. RNA N6-methyladenosine methylation in post-transcriptional gene expression regulation. Genes Dev. 2015; 29:1343–1355. [PubMed: 26159994]

Zeisig BB, Milne T, Garcia-Cuellar MP, Schreiner S, Martin ME, Fuchs U, Borkhardt A, Chanda SK, Walker J, Soden R, et al. Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization. Mol Cell Biol. 2004; 24:617–628. [PubMed: 14701735]

Zhang C, Samanta D, Lu H, Bullen JW, Zhang H, Chen I, He X, Semenza GL. Hypoxia induces the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated m6A-demethylation of NANOG mRNA. Proc Natl Acad Sci U S A. 2016; 113:E2047–2056. [PubMed: 27001847]

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008; 9:R137. [PubMed: 18798982]

Zhao X, Yang Y, Sun BF, Shi Y, Yang X, Xiao W, Hao YJ, Ping XL, Chen YS, Wang WJ, et al. FTO-dependent demethylation of N6-methyladenosine regulates mRNA splicing and is required for adipogenesis. Cell Res. 2014; 24:1403–1419. [PubMed: 25412662]

Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, Vagbo CB, Shi Y, Wang WL, Song SH, et al. ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. Mol Cell. 2013; 49:18–29. [PubMed: 23177736]

Zhou J, Wan J, Gao X, Zhang X, Jaffrey SR, Qian SB. Dynamic m(6)A mRNA methylation directs translational control of heat shock response. Nature. 2015; 526:591–594. [PubMed: 26458103]

Zhu J, Heyworth CM, Glasow A, Huang QH, Petrie K, Lanotte M, Benoit G, Gallagher R, Waxman S, Enver T, Zelent A. Lineage restriction of the RARalpha gene expression in myeloid differentiation. Blood. 2001; 98:2563–2567. [PubMed: 11588055]

SIGNIFICANCE

The identification of FTO as the first N^6-methyladenosine RNA demethylase and the high prevalence of m^6A methylation demonstrated in mammalian mRNA transcriptomes have spurred immense interest in the function of m^6A modifications in post-transcriptional regulation. However, little is known about the function of FTO or m^6A modifications in cancer. Here we show that FTO functions as an oncogene that promotes leukemic oncogene-mediated cell transformation and leukemogenesis, and inhibits all-trans-retinoic acid (ATRA)-mediated leukemia cell differentiation. Mechanistically, FTO exerts its oncogenic role as an m^6A demethylase by targeting a set of critical transcripts such as *ASB2* and *RARA*. Thus, our study reveals a previously unrecognized mechanism of gene regulation in tumorigenesis and highlights functional importance of FTO and m^6A modification in cancer.

HIGHLIGHTS

- *FTO* is highly expressed in certain subtypes of AMLs such as *MLL*-rearranged AML
- FTO promotes leukemic oncogene-mediated cell transformation and leukemogenesis
- FTO targets a set of genes (e.g. *ASB2* and *RARA*) in AML as an m^6A RNA demethylase
- The FTO⊣ASB2/RARA axis mediates AML cell growth and (ATRA-induced) differentiation

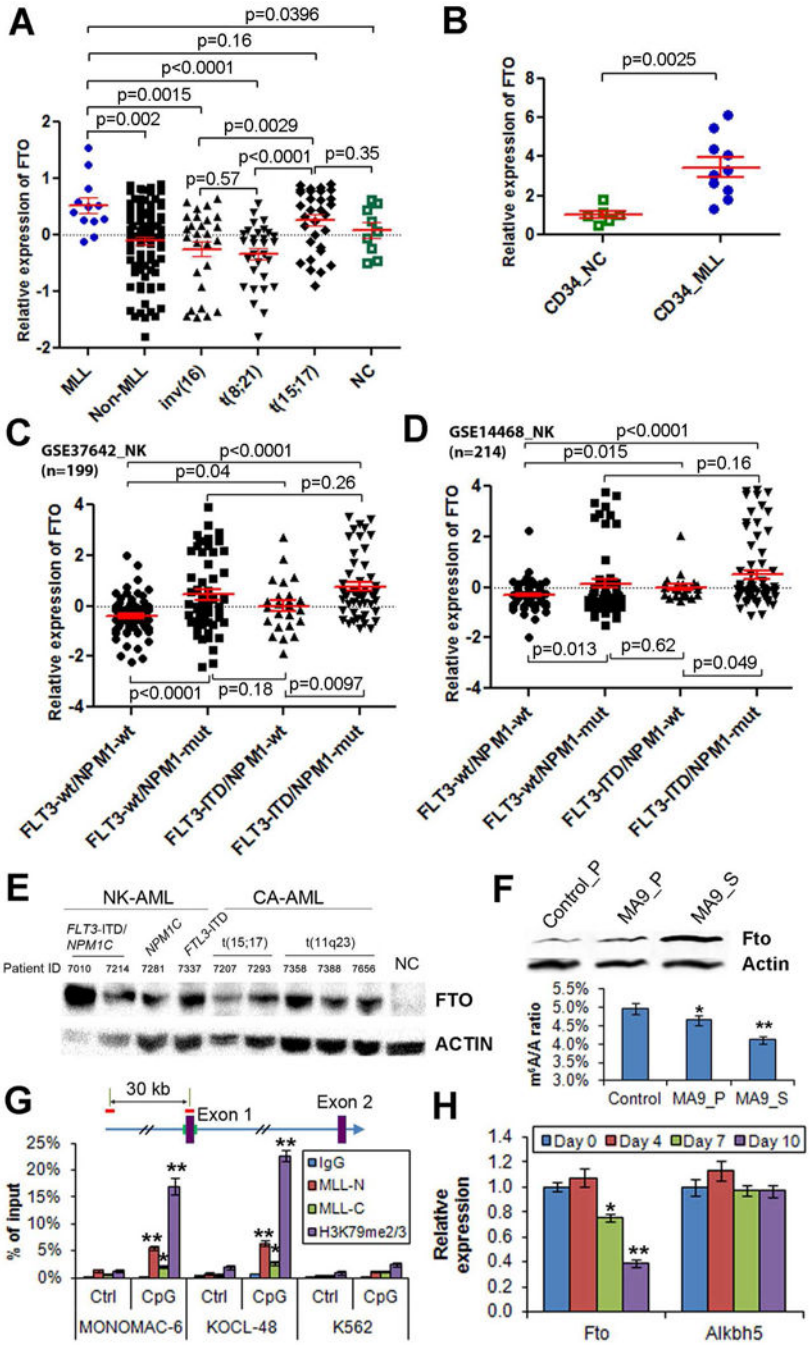

Figure 1. *FTO* is highly expressed in certain AML subtypes

(A) Comparison of *FTO* expression between human primary AML cases with *MLL* rearrangements/t(11q23) (MLL) and those without *MLL* rearrangements (non-MLL), or AML cases with inv(16), t(8;21) or t(15;17), or normal controls (NC). The expression values were detected by Affymetrix exon arrays (Huang et al., 2013). The expression values were log2-transformed and mean centered. **(B)** qPCR analysis of *FTO* expression in human $CD34^+$ AML BM cells isolated from 10 primary *MLL*-rearranged AML patients (CD34_MLL) and normal $CD34^+$ BM cells isolated from 6 healthy donors (CD34_NC). The

average expression level of *FTO* in the CD34_NC samples was set as 1. **(C,D)** The expression patterns of *FTO* across cytogenetically normal (or normal karyotype; NK) AMLs within GSE37642 set (n=562) and GSE14468 set (n=518) AML datasets. **(E)** Western blot assay of FTO expression in human primary AML specimens with different fusion genes or mutant oncogenes and a healthy donor sample (NC). Mononuclear cells isolated from primary AML patients and the healthy donor were used for the assay. **(F)** The protein level of Fto (upper panel) and m^6A level (lower panel; by QQQ-MS) in the representative samples of the control group (from a primary BMT recipient) or MA9 leukemic group (one each from primary and secondary BMT recipients). **(G)** ChIP-qPCR assays of the enrichment of MLL-N (i.e., MLL N-terminal, representing both wild-type MLL and MLL-fusion proteins), MLL-C (i.e., MLL C-terminal, representing wild-type MLL only), and H3K79me2/3 at the promoter region of *FTO* (CpG site) and a distal upstream region (Control site) in MONOMAC-6, KOCL-48 and K562 cells. IgG was used as a negative control. **(H)** qPCR analysis of *Fto* and *Alkbh5* expression in mouse MLL-ENL-ERtm cells after withdrawal of 4-OHT. *, $p<0.05$; **, $p<0.01$; *t*-test. See also Figure S1.

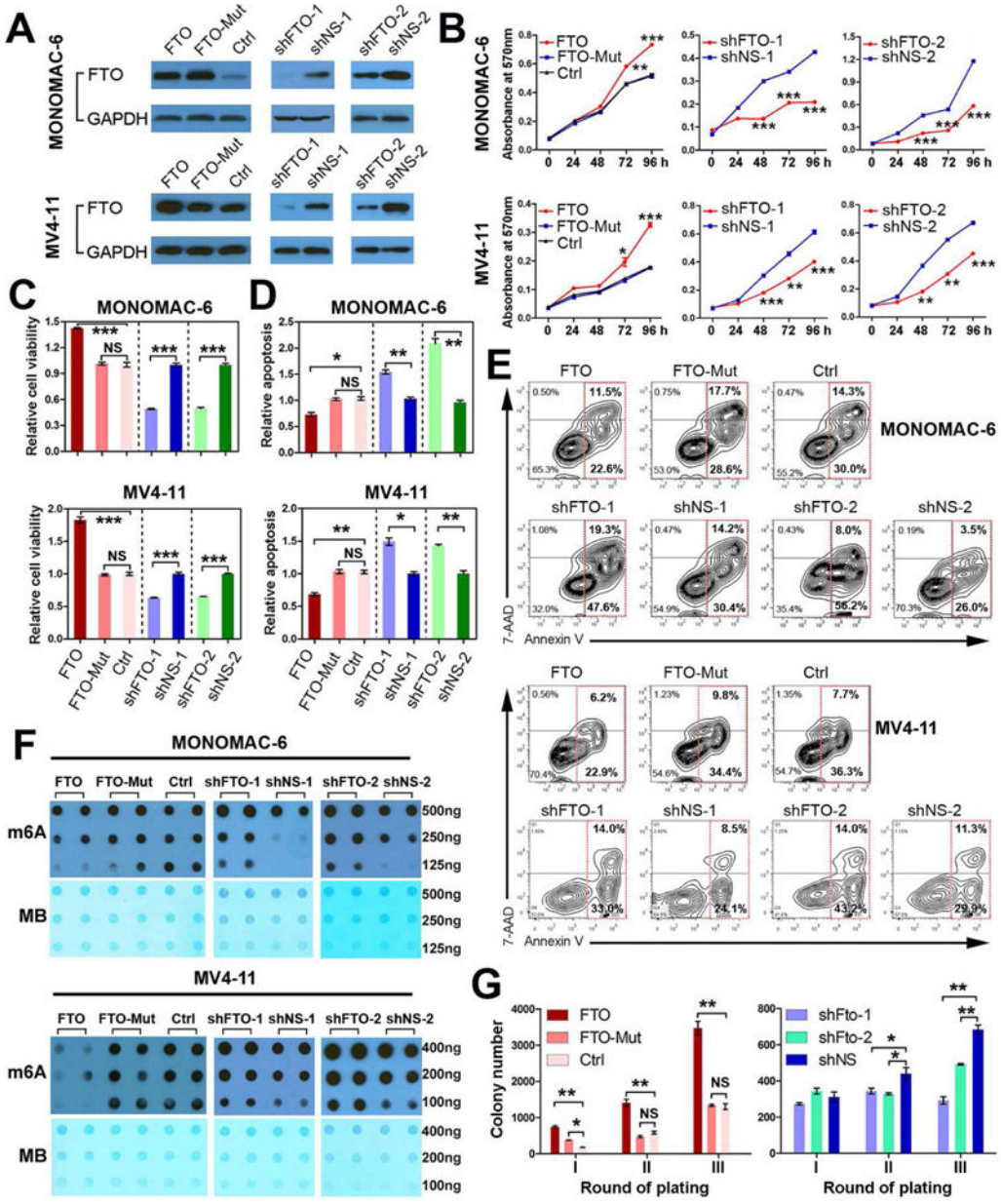

Figure 2. Biological effects of forced expression or knockdown of *FTO/Fto* expression in *MLL*-rearranged AML

(**A**) Western Blotting confirmation of forced expression and knockdown of FTO by lentiviral constructs in MONOMAC-6 and MV4-11 cells. FTO, pMIRNA1-FTO; FTO-Mut, pMIRNA1-FTO-Mut; Ctrl, control vector (empty pMIRNA1); shFTO-1, pLKO.1-shFTO-1; shNS-1, pLKO.1-shNS-1; shFTO-2, pGFP-C-shLenti-shFTO-2; shNS-2, pGFP-C-shLenti-shNS-2. **(B–E)** Effects of forced expression or knockdown of *FTO* expression on cell growth/proliferation (**B**), viability (**C,D**), and apoptosis (**E**) in MONOMAC-6 and MV4-11 cells. h, hour. (**F**) m^6A dot blot assays of MONOMAC-6 and MV4-11 cells with or without forced expression or knockdown of *FTO*. MB, methylene blue staining (as loading control). (**G**) Effects of forced expression of FTO or FTO-Mut (with the above pMIRNA1 constructs) or knockdown of *Fto* expression (with pGFP-V-RS-Fto shRNAs) on colony-forming/

replating capacity of mouse normal BM progenitor cells transduced by MSCVneo-MLL-AF9 (MA9). Colony cells were replated every 7 days. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS, not significant ($p>0.05$); *t*-test. See also Figure S2.

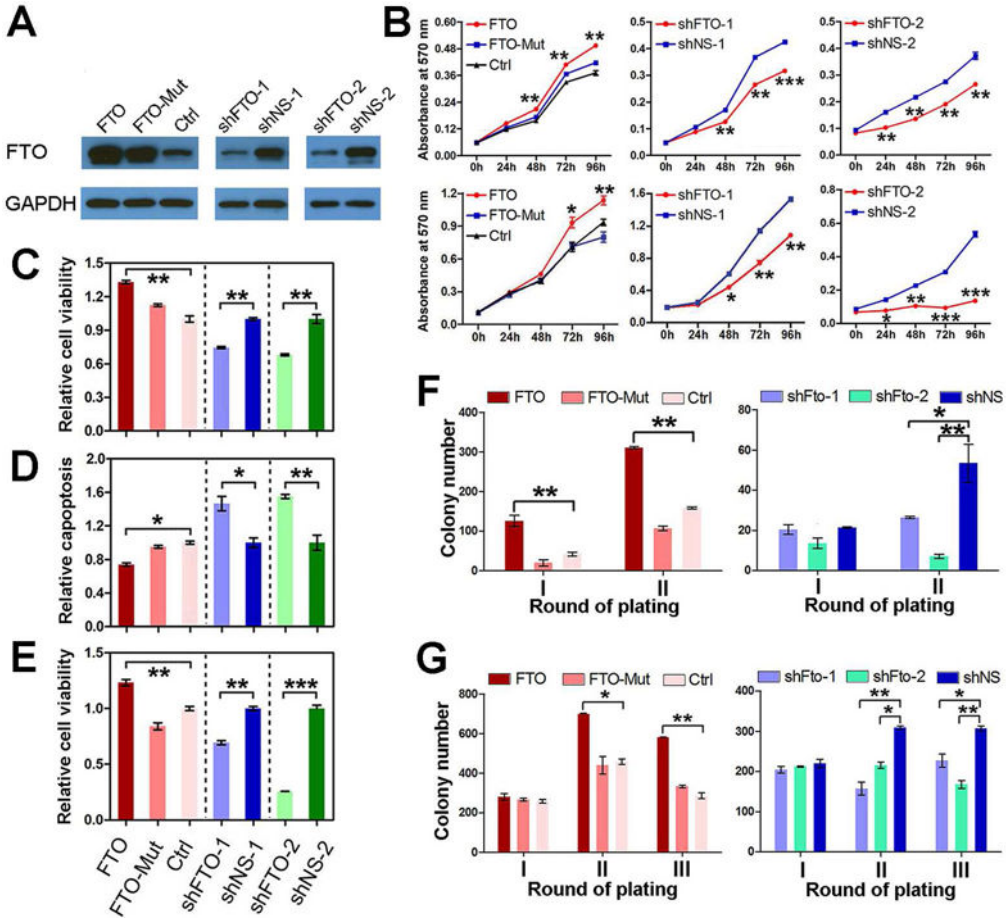

Figure 3. Effects of forced expression or knockdown of *FTO/Fto* in *PML-RARA* and *FLT3*-ITD/*NPM1*-mutant AML

(**A**) Confirmation of overexpression and knockdown of FTO by Western blotting in PL-21/t(15;17) AML cells. (**B**) Effects of forced expression or knockdown of *FTO* expression on cell growth/proliferation in PL-21 (upper panels) and NB4/t(15;17) (lower panels) AML cells. (**C–E**) Effects of forced expression or knockdown of *FTO* expression on cell viability in PL-21 (**C**) and NB4 (**E**), and apoptosis in PL-21 (**D**) cells. (**F, G**) Effects of forced expression of FTO or FTO-Mut, and knockdown of *Fto* expression on colony-forming/replating capacity of mouse BM progenitor cells carrying *PML-RARA* (**F**) or *FLT3*-ITD/*NPM1*-mutant (**G**). Colony cells were replated every 7 days. *, p<0.05; **, p<0.01; ***, p<0.001. See also Figure S3.

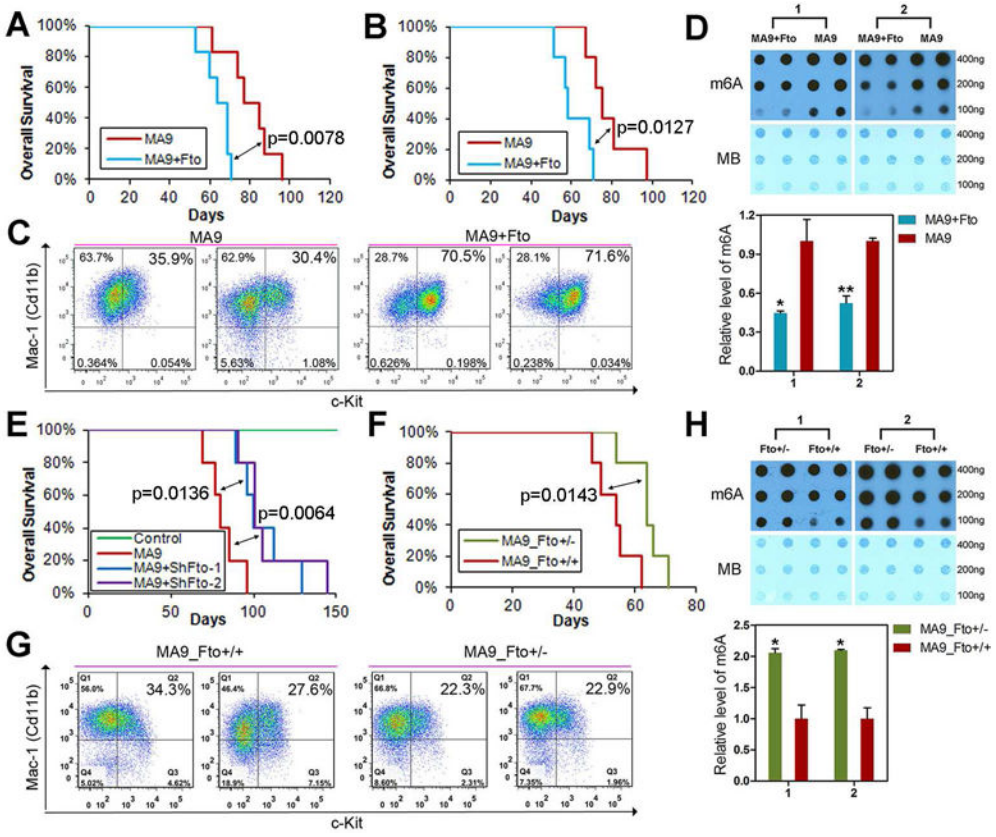

Figure 4. The role of Fto in leukemogenesis mediated by MLL-AF9

(**A,B**) Effect of forced expression of *Fto* on MLL-AF9 (MA9)-induced leukemogenesis. Kaplan-Meier curves are shown for two cohorts of transplanted mice including MSCVneo-*MA9*+MSCV-PIG (MA9) and MSCVneo-*MA9*+MSCV-PIG-Fto (MA9+Fto) from two independent BM transplantation (BMT) assays. Six mice per group in plot A and 5 mice per group in plot B. The *p* values were calculated by log-rank test. **(C,D)** Flow cytometry analysis of Mac-1$^+$ and c-Kit$^+$ cell populations (**C**) and m^{6}A dot blot analysis (**D**) in BM cells of the representative leukemic mice from the BMT assay shown in Figure 4A. **(E)** Effect of depleted expression of *Fto* by shRNAs on MA9-induced leukemogenesis. Kaplan-Meier curves are shown for four cohorts of transplanted mice including MSCVneo+MSCV-PIG (Control), MSCVneo-*MA9*+MSCV-PIG (MA9), MSCVneo-*MA9*+pGFP-V-RS-shFto-1 (MA9+shFto-1) and MSCVneo-*MA9*+pGFP-V-RS-shFto-2 (MA9+shFto-2). Five mice were studied per group. **(F)** Effect of depleted expression of *Fto* by genetic knockout (heterozygous) on MA9-induced leukemogenesis. Kaplan-Meier curves are shown for two cohorts of recipient mice transplanted with MSCVneo-*MA9* transduced wild-type donor cells (MA9_Fto+/+) and MSCVneo-*MLL-AF9* transduced Fto+/− donor cells (MA9_Fto+/−). Five mice were studied per group. **(G, H)** Flow cytometry analysis of Mac-1$^+$ and c-Kit$^+$ cell populations (**G**) and m^{6}A dot blot analysis (**H**) in BM cells of the representative leukemic mice from the BMT assay shown in Figure 4F. *, p<0.05; **, p<0.01; *t*-test. See also Figure S4.

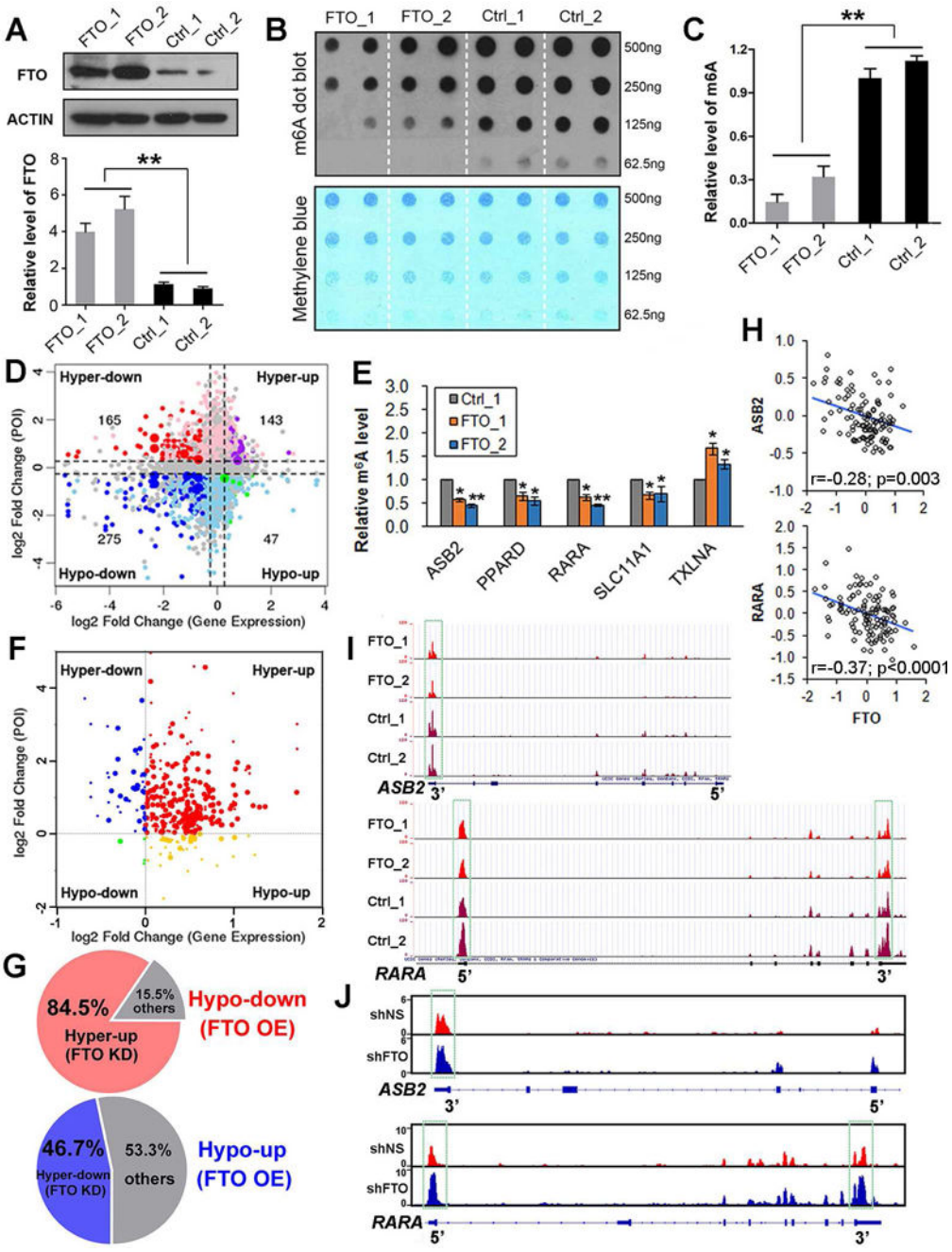

Figure 5. Identification of potential targets of FTO in AML via transcriptome-wide m^{6}A-seq and RNA-seq assays

(**A**) Western blot assay of FTO expression in human MONOMAC-6 AML cell lines with or without forced expression of *FTO*, including FTO_1/2 and Ctrl_1/2 cell lines. Upper panel shows the image and the lower panel shows the relative quantitative information of FTO expression at the protein level in different AML cell lines. **(B,C)** The m^{6}A dot blot assay (**B**) and relative quantitative information (**C**) of global m^{6}A abundance in transcriptomes of the above four cell lines. (**D**) Distribution of genes with a significant change in both m^{6}A level and overall transcript (i.e., expression) level in *FTO*-overexpressing (FTO_1 and FTO_2)

compared to control (Ctrl_1 and Ctrl_2) MONOMAC-6 AML cells. **(E)** Gene-specific m^6A qPCR validation of m^6A level changes of five representative m^6A-Hypo genes in MONOMAC-6 cells. **(F)** Distribution of the FTO-induced hypo-genes (including the hypo-down and hypo-up groups shown in Figure 5**D**) in *FTO*-knockdown *MA9/FLT3*-ITD AML cells relative to the control AML cells. **(G)** Up to 84.5% Hypo-down genes in *FTO*-overexpressing (*FTO* OE) MONOMAC-6 AML cells display Hyper-up pattern in *FTO* knockdown (*FTO* KD) *MA9/FLT3*-ITD AML cells; 46.7% Hypo-up genes in the *FTO* OE cells display Hyper-down pattern in the *FTO* KD cells. (**H**) Correlation of expression between *FTO* and *ASB2* or *RARA* across the 109 (100 AML and 9 normal control) samples shown in Figure 1A. **(I, J)** The m^6A abundances in *ASB2* and *RARA* mRNA transcripts in *FTO*-overexpressing (FTO_1 and FTO_2) and control (Ctrl_1 and Ctrl_2) MONOMAC-6 AML cells (**I**), and in *FTO*-knockdown (shFTO-1) and control (shNS) *MA9/FLT3*-ITD AML cells (**J**), as detected by m^6A-seq. The m^6A peaks shown in the green rectangles are those have a significant reduced abundance ($p<0.005$; fold change>1.2) in FTO_1/2 than in Ctrl_1/2 cells. *, $p<0.05$; **, $p<0.01$; *t*-test. See also Figure S5 and Tables S1–3.

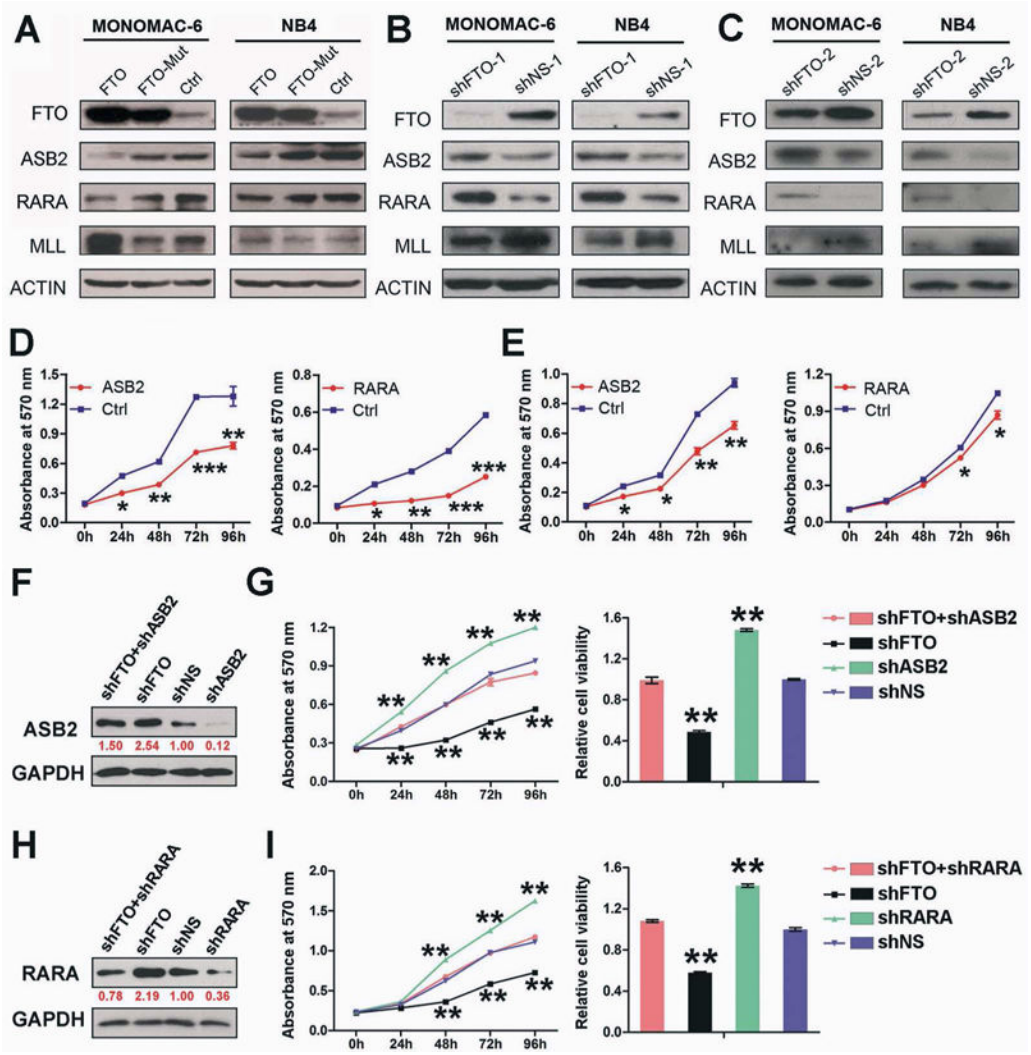

Figure 6. *ASB2* and *RARA* are two critical target genes of FTO in AML

(**A–C**) Western blot assays of FTO, ASB2, RARA and MLL in MONOMAC-6 or NB4 AML cells with lentivirally transduced *FTO* (pmiRNA1-*FTO*), *FTO* mutant (H231A and D233A; pmiRNA1-*FTO-Mut*) or control (Ctrl; pmiRNA1) construct (**A**), as well as shFTO-1/shNS-1 (**B**) and shFTO-2/shNS-2 (**C**). ACTIN was used as the endogenous control protein for loading control. **(D, E)** Effects of forced expression of *ASB2* and *RARA* on cell growth/proliferation in MONOMAC-6 (**D**) and NB4 (**E**) AML cells. **(F)** Western blot assays of ASB2 in MONOMAC-6 cells transduced with sh*FTO*+sh*ASB2* (pLKO.1-sh*FTO*-1+ pTRIPZ-sh*ASB2*), sh*FTO* (pLKO.1-sh*FTO*-1 + pTRIPZ), shNS (pLKO.1-shNS + pTRIPZ) or sh*ASB2* (pLKO.1-shNS-1 + pTRIPZ-sh*ASB2*). **(G)** Effects of *FTO* and/or *ASB2* knockdown on cell growth/proliferation (left panel) and viability (right panel) in MONOMAC-6 cells. **(H)** Western blot assays of RARA in MONOMAC-6 AML cells transduced with sh*FTO*+sh*RARA* (pLKO.1-sh*FTO*-1+ sh*RARA*), sh*FTO* (pLKO.1-sh*FTO*-1+ shNS), shNS (pLKO.1-shNS + shNS) or sh*RARA* (pLKO.1-shNS-1 + sh*RARA*). **(I)** Effects of *FTO* and/or *RARA* knockdown on cell growth/proliferation (left panel) and

viability (right panel) in MONOMAC-6 cells. *, p<0.05; **, p<0.01; ***, p<0.001; *t*-test. See also Figure S6.

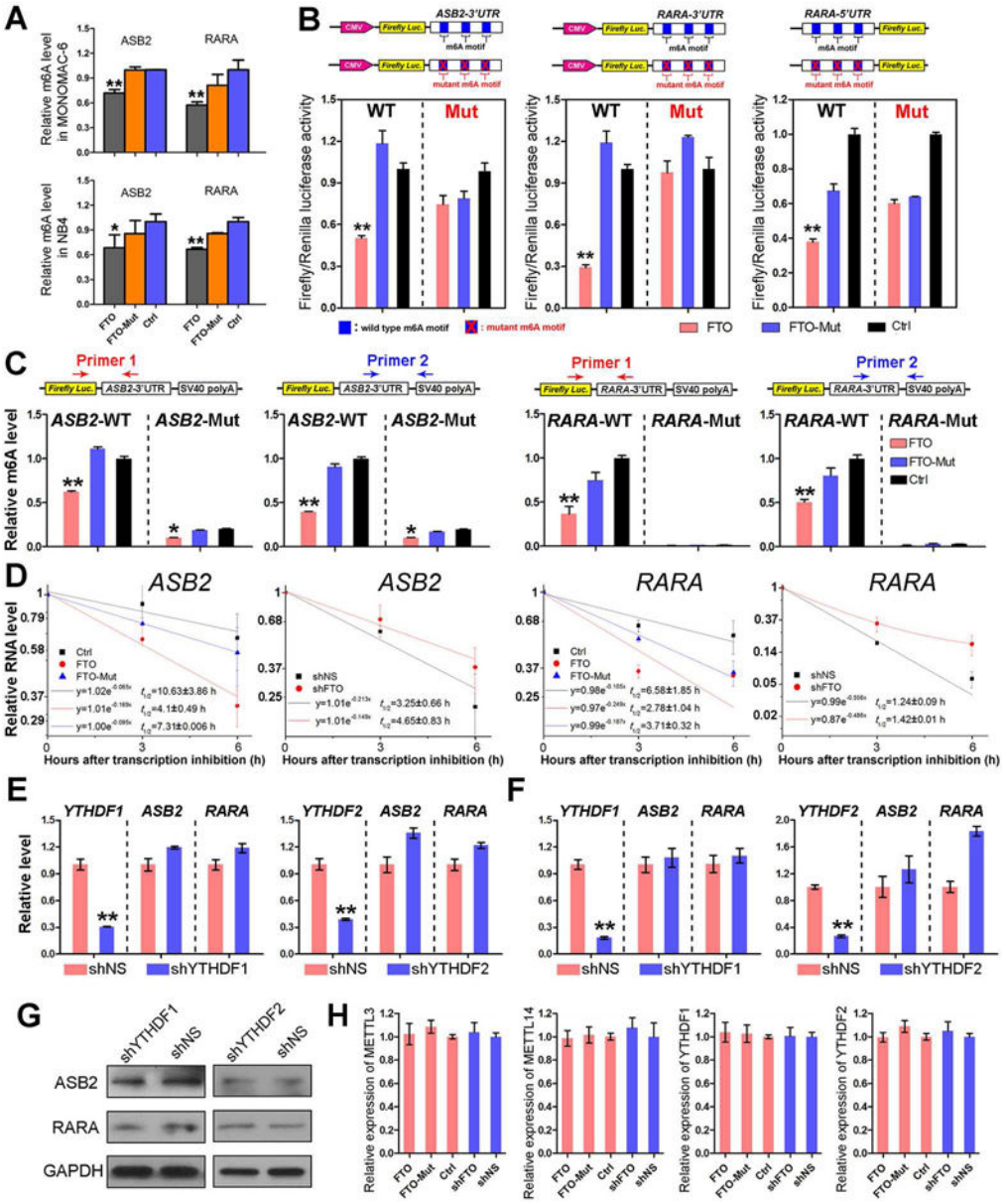

Figure 7. FTO-mediated regulation of expression of *ASB2* and *RARA* relies on its m^{6}A demethylase activity and the m^{6}A modifications in target mRNAs

(**A**) Gene-specific m^{6}A qPCR analysis of m^{6}A level in mRNA transcripts of each gene in MONOMAC-6 and NB4 cells transduced with *FTO* (FTO), *FTO* mutant (FTO-Mut) or control vector (Ctrl). **(B)** Relative luciferase activity of pMIR-REPORT-*ASB2*-3′UTR (left panel), pMIR-REPORT-*RARA*-3′UTR (middle panel) and pGL3-basic-*RARA*-5′UTR (right panel) with either wild-type or mutant (A-to-T mutation) m^{6}A sites after co-transfection with *FTO* (FTO), *FTO* mutant (FTO-Mut) or control vector (Ctrl) into HEK-293T cell. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. **(C)** Luciferase reporter assay related gene-specific m^{6}A qPCR analysis of m^{6}A levels in exogenous mRNA transcripts of Firefly Luc-*ASB2* 3′UTR or Firefly Luc-*RARA* 3′UTR in HEK-293T cells. For each luciferase reporter construct, we designed two

different pairs of primers crossing the inserted *ASB2* or *RARA* 3′UTR fragment and pMIR-Report vector fragment. Primer 1 covers the joint of Firefly Luc and *ASB2*-3′UTR or *RARA*-3′UTR. Primer 2 covers the joint of *ASB2*-3′UTR or *RARA*-3′UTR and SV40 poly A. (**D**) The mRNA half-life ($t_{1/2}$) of *ASB2* or *RARA* in NB4 cells transduced with *FTO* (FTO), *FTO* mutant (FTO-Mut) or control vector (Ctrl), or with depleted expression of *FTO* (shFTO-1) or not (shNS-1). **(E, F)** Relative expression of *ASB2* and *RARA* after knockdown of m^6A readers, *YTHDF1* or *YTHDF2*, in MONOMAC-6 (**E**) and NB4 (**F**) cells. **(G)** Western blot assay of ASB2 and RARA expression after depletion of m^6A readers in MONOMAC-6 cells. **(H)** Relative expression of m^6A writers and readers with forced or depleted expression of *FTO* in MONOMAC-6 cells. *, $p<0.05$; **, $p<0.01$; *t*-test. See also Figure S7.

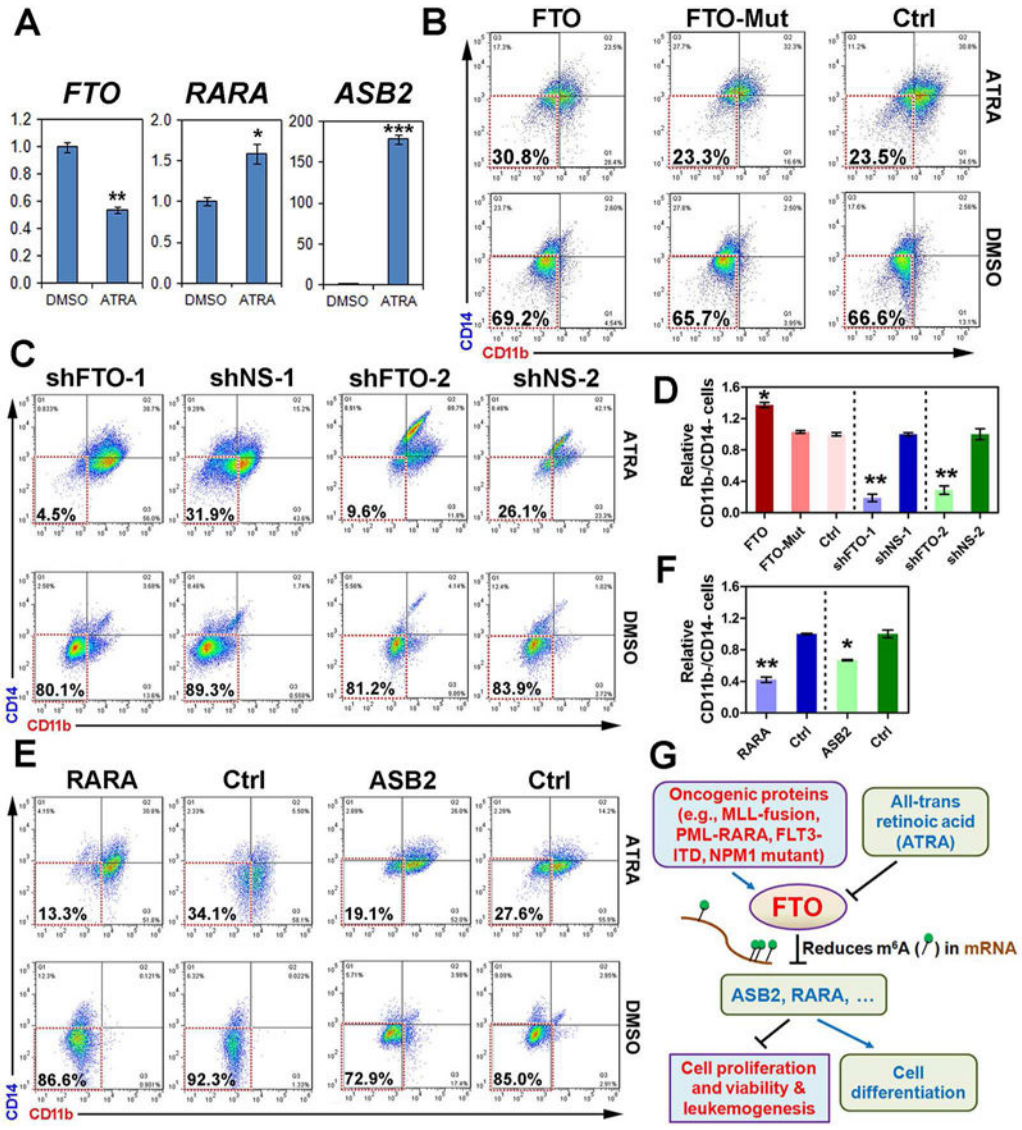

Figure 8. The potential role of the FTO⊣RARA/ASB2 axis in ATRA-induced NB4 cell differentiation and a schematic model of FTO signaling in AML

(**A**) Expressional changes of *FTO*, *RARA*, and *ASB2* in NB4 cells 48 hours post treatment with 100 nM ATRA as detected by qPCR. *, p<0.05; **, p<0.01; ***, p<0.001; *t*-test. **(B)** Flow cytometric analyses of NB4 cells transduced with *FTO* (FTO), *FTO* mutant (FTO-Mut) or control vector (Ctrl) 48 hours post treatment with 500 nM ATRA or DMSO. **(C)** Flow cytometric analyses of NB4 cells with knockdown of *FTO* (shFTO-1 or shFTO-2) 48 hours post treatment with 100 nM ATRA or DMSO. (**D**) The summary of results from three independent experiments with those shown in Figure 8B or 8C as representatives. (**E, F**) The representative (**E**) or summary (**F**; from triplicates) results of flow cytometric analyses of NB4 cells with forced expression of *RARA* or *ASB2* after exposed to 100 nM ATRA or DMSO for 48 hours. (**G**) The schematic model of the role and underlying mechanism of FTO in leukemogenesis and ATRA-induced differentiation of leukemic cells.