FTO mediates cell-autonomous effects on adipogenesis and adipocyte lipid content by regulating gene expression via 6mA DNA modifications

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Abstract  SNPs in the first intron of α-ketoglutarate-dependent dioxygenase (FTO) convey effects on adiposity by mechanisms that remain unclear, but appear to include modulation of expression of FTO itself, as well as other genes in cis. FTO expression is lower in fibroblasts and iPSC-derived neurons of individuals segregating for FTO obesity risk alleles. We employed in vitro adipogenesis models to investigate the molecular mechanisms by which Fto affects adipocyte development and function. Fto expression was upregulated during adipogenesis, and was required for the maintenance of CEBPB and Cebpd/CEBDP expression in murine and human adipocytes in vitro. Fto knockdown decreased the number of 3T3-L1 cells that differentiated into adipocytes as well as the amount of lipid per mature adipocyte. This effect on adipocyte programming was conveyed, in part, by modulation of CCAAT enhancer binding protein (C/ebp)β-regulated transcription. We found that Fto also affected Cebpδ transcription by demethylating DNA N6-methyldeoxyadenosine in the Cebpδ promoter. Fto is permissive for adipogenesis and promotes maintenance of lipid content in mature adipocytes by enabling C/ebpβ-driven transcription and expression of Cebpδ. These findings are consistent with the loss of fat mass in mice segregating for a dominant-negative Fto allele.—Martin Carli, J. F., C. A. LeDuc, Y. Zhang, G. Stratigopoulos, and R. L. Leibel

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SNPs in a region of strong linkage disequilibrium within the first intron of the α-ketoglutarate-dependent dioxygenase (FTO) gene are strongly associated with adiposity; each minor allele is associated with an increase in BMI of ∼0.36 kg/m² (or body weight of ∼1.2 kg) in adults (1–3). The increased body weight in individuals segregating for the FTO risk allele, rs9999609, is attributable primarily to increased food intake (with preference for higher caloric density) rather than decreased energy expenditure (4–7). The mechanism(s) by which this association is conveyed is still unclear. The statistical strength of this association with adiposity and the large number of SNPs within the obesity-associated locus in the first intron of FTO suggest that there may be multiple causal variants contributing to the observed effect. For example, we have demonstrated that two SNPs in the obesity-associated locus (rs8050136 and rs1421085) differentially bind the transcription factor, cut-like homeobox 1 (CUX1). The protective alleles of these SNPs preferentially bind the activating isoform of CUX1.

Abbreviations:  ActD, actinomycin D; ASC, adipose stromal cell; C/ebp, CCAAT enhancer binding protein; CEBPRE, CCAAT enhancer binding protein response element; ChIP, chromatin immunoprecipitation; 2-DG, 2-deoxyglucose; FTO, α-ketoglutarate-dependent dioxygenase; IBMX, isobutylylimidaxanthine; m6A, N6-methyladenosine RNA; m6Am, N6,2′-O-dimethyladenosine; m6A RIP, N6-methyladenosine RNA immunoprecipitation; 6mA, N6-methyldeoxyadenosine DNA; PTPRVP, protein tyrosine phosphatase receptor type V pseudogene; qPCR, quantitative real-time PCR; RNA-Seq, RNA-seq-sequencing; RPRGIP1L, RPRGIP1-like; Runx11L, Runx-related transcription factor 1; SGBS, Simpson-Golabi-Behmel syndrome; SPOV2, spardin 2; SVC, stromal vascular cell.

1 The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Martin Carli et al., 2018) and are accessible through GEO Series accession number GSE113862 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113862).
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p110, proteolytically processed from the p200 isoform by cathepsin L, increasing expression of FTO and neighboring RPRGPI1-like (RPRGPI1L) (8–10).

Mice congenitally null for Fto exhibit a complex phenotype that includes postnatal lethality (11–13). Mice that survive have impaired linear growth; in adulthood, these mice have reduced adipose tissue mass compared to controls and are protected from diet-induced obesity (11, 14). Conversely, Fto-overexpressing mice display increased body weight and adiposity (15). A separate mouse model of ENU-mutagenized Fto, which exhibits partial loss-of-function, is spared the postnatal lethality and impaired skeletal growth seen in constitutive Fto knockout models (16). These mice, free of the confounds of impaired growth, exhibit decreased adiposity when fed both chow and high-fat diet as adults.

Adult mice that are null for Fto have smaller adipocytes (11) and Fto-overexpressing mice have larger adipocytes than controls (15). These phenotypes suggest a role for Fto in enabling facultative adipose tissue expansion, possibly in the context of diet-induced obesity. In support of these inferences, 24-week-old mice segregating for the partial loss-of-function Fto mutation exhibit decreased adiposity, but increased circulating concentrations of fasting glucose, triglyceride, cholesterol, and HDL levels, consistent with a lipodystrophic metabolic profile (16–18).

Humans who are homozygous for null mutations of FTO display severe brain (microcephaly, hydrocephalus, and lisencephaly) and cardiac (ventricular and atrial septal defects, patent ductus arteriosus) malformations that result in growth retardation and death within the first few years of life (19–21). Therefore, effects on adiposity per se cannot be accurately assessed in these individuals. Individuals heterozygous for a variety of FTO mutations have no obvious phenotypic characteristics (22–24). The number of these individuals with variants predicted or proven to have deleterious effects on FTO function is extremely small, however, limiting the strength of inferences regarding the protein’s effects on metabolic homeostasis.

FTO is a member of the ALKB homolog family of non-heme dioxygenases [Fe(II)-dependent dioxygenase and FTO] (25, 26) and exhibits in vitro demethylase activity directed at single-stranded N6-methyladenosine (m6A) RNA and N6-methyldeoxyadenosine (6mA) DNA (27). m6A is an abundant mRNA modification that is thought to play a role in the regulation of mRNA stability, splicing, and/or translation. These modifications are increased around stop codons and 3′UTRs, as well as along long exons (28–30). 6mA is a prevalent DNA modification in prokaryotes, where it plays a part in defense mechanisms designated as “restriction-modification systems,” which detect and degrade foreign (unmethylated) DNA (31). The 6mA also regulates DNA repair and replication as well as transcription in prokaryotes (32). Recently, 6mA has been detected in lower quantities in eukaryotic organisms (33, 34), suggesting an epigenetic role in mammalian cells.

Although expressed most highly in the brain, Fto is also expressed in adipose tissue (12, 26) where its functions remain unclear. While eQTL studies examining the expression of FTO in individuals segregating for obesity risk or protective alleles have not shown different levels of expression, sample sizes were small and, in some cases, whole adipose tissue was examined, potentially masking effects in specific cell types such as adipocytes or preadipocytes (35–38). In mice, Fto expression is decreased in mesenteric adipose tissue upon fasting (8) and increased in subcutaneous and visceral adipose tissue upon acute exposure to high-fat diets (39), indicating that nutritional status might confound efforts to attribute expression levels of FTO to obesity-risk genotypes.

We assessed the possibility that Fto may be involved in adipocyte differentiation by developmentally timed reduction of Fto gene expression in 3T3-L1 preadipocytes. We also analyzed the effect of FTO knockout on adipogenesis in human adipose tissue-derived preadipocytes. We found that Fto expression was required for adipogenesis; and by using RNA-sequencing (RNA-Seq), we identified CCAAT enhancer binding protein (C/ebp)β activity and Cebpd expression as primary targets of Fto. C/ebpβ and C/ebpα are transcription factors that initiate adipogenesis; C/ebpβ function is required to maintain lipid homeostasis in adipocytes (40). We did not find evidence that these transcripts are differentially methylated or exhibit altered stability or translational efficiency following Fto knockdown, effects that have been proposed for m6A (41) and which could be affected by m6A demethylation by Fto (27). We did find evidence that, in addition to its coactivation activity with C/ebpβ (42), Fto demethylated 6mA DNA at the promoter of Cebpd. By these mechanisms, the downstream targets of C/ebpβ and Cebpd, Pparg and Cebpa (40), are activated.

MATERIALS AND METHODS

Cell culture and transfection

The 3T3-L1 preadipocytes were purchased from ATCC and maintained in sub-confluent cultures. Growth medium consisted of DMEM (with 25 mM glucose, GlutaMAX™, and sodium pyruvate) supplemented with 10% newborn calf serum (both Thermo Fisher). Differentiation was achieved by treating cells 2 days after they reached confluence with differentiation medium containing DMEM plus insulin (from bovine pancreas, 1 μg/ml), dexamethasone (0.1 μM), and isobutylmethylxanthine (IBMX; 0.5 mM; all Sigma-Aldrich) supplemented with 10% FBS (Thermo Fisher). Two days later, differentiation medium was replaced with DMEM containing 1 μg/ml insulin and 10% FBS. Insulin medium was replaced every 2–3 days; cells were filled with lipid and considered to be mature adipocytes by days 8–12.

De-identified human subcutaneous adipose stromal cells (ASCs) were generously provided by the Boston Nutrition Obesity Research Center and were cultured and differentiated as previously described (43). Briefly, cells were maintained in αMEM supplemented with 10% FBS (all Thermo Fisher) to avoid confluence. To differentiate, cells were allowed to reach confluence and 2 days later were treated with a chemically defined serum-free medium [DMEM/F12 containing HEPES and antibiotic-antimycotic (Thermo Fisher), NaHCO3, d-biotin, pantothenate, dexamethasone, human insulin, rosiglitazone, IBMX, T3, and transferrin (Sigma-Aldrich)]. Differentiation medium was replaced every 2–3 days and switched to maintenance medium after 3–7 days (differentiation medium without rosiglitazone, IBMX, T3, and transferrin). Cells were filled with lipid and assayed between 10 and 14 days after initial treatment with differentiation factors.
Transfections were performed prior to differentiation by incubation for 24 h of preconfluent cells with a mix of three Stealth siRNAs targeted against Fto/FTO (designed to span multiple exons) or three nontargeted controls with Lipofectamine 2000 (40 nM siRNA or Lipofectamine 3000 (75 nM siRNA). Fto/protein tyrosine phosphatase, receptor type, V (Ptpro) and Fto/spordin 2 (Spon2) double knockdowns were performed by adding a single Stealth siRNA against Ptpro or Spon2 to the mix of three anti-Fto siRNAs (all pre-designed by Thermo Fisher). Knockdown of Fto in mature adipocytes was achieved by electroporation (44–46) using the same siRNAs after 3T3-L1 cells had been differentiated.

RNA expression studies, RNA-Seq, and m6A RNA immunoprecipitation

Total RNA was isolated using the RNeasy lipid tissue mini kit and columns were treated with DNease (both Qiagen). cDNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis kit (Roche) using both OligoDT and random hexamer primers. Quantitative real-time PCR (qPCR) was performed on a LightCycler 480 using SYBR Green I Master (Roche). Analysis was performed by the LightCycler 480 software (Roche) using the second derivative maximum calculation based on a standard curve. qPCR primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3/) to span exon-exon junctions and are provided in supplemental Tables S1 and S2. Analysis of mRNA in Fto/FTO knockdown experiments in vitro was normalized to RpLp0/RPLP0 unless otherwise indicated.

Sample integrity for RNA utilized for RNA-Seq was assessed with an Agilent 2100 bioanalyzer. All samples had RIN numbers greater than 8.0. mRNA was isolated using poly-A pulldown and reverse transcribed to generate cDNA (47). cDNA was sequenced using single-ended sequencing on a HiSeq2000 according to the manufacturer’s recommendations (Illumina) at the Columbia Genome Center, 1 × 100 bp read length, 30 M read count. The pass filter reads were mapped to mouse reference genome mm9 using TopHat (version 2.0.11; https://ccb.jhu.edu/software/tophat/index.shtml). TopHat infers novel exon-exon junctions and combines them with junctions from known mRNA sequences as the reference annotation. For each read, up to three mismatches and 10 multiple hits were allowed during mapping. FPKM values indicating relative abundance were estimated using Cufflinks software (version 2.2.1; http://cole-trapnell-lab.github.io/cufflinks/). Only transcripts with FPKM values that were verified as acceptable in at least three of four replicates with average FPKM values of all replicates in both conditions (siCtr and siFto) > 1 were considered. Transcripts with average FPKM values below 1 were excluded from data analyses, but included in adjustments for multiple-testings.

The m6A RNA immunoprecipitation (m6A RIP) was performed based on previously published protocols (29, 47). Briefly, RNA was isolated (as described above, including DNease treatment) from 2 day postconfluent 3T3-L1 preadipocytes following Fto knockdown and a 5 μg aliquot was treated with RiboMinus (Thermo Fisher) to decrease RNA content. Heat-denatured RNA (300 ng) was pulled down with rabbit anti-m6A antibody (Synaptic Systems) or rabbit IgG negative control coupled to Protein A/G Dynabeads in the presence of an RNAse inhibitor, SUPERase·In (all Thermo Fisher). Eluted RNA was isolated by QIAzol:chloroform extraction and column purification (as described above, without DNase treatment). The resulting RNA and a reserved aliquot containing 10% of the input were reverse transcribed and analyzed by qPCR. No transcript was detectable in the IgG negative control condition. Levels of m6A enriched mRNA transcripts were compared with transcript levels in the input control.

Transcript stability was measured following treatment with actinomycin D (ActD). Cells were treated with 1 μg/ml ActD (stock: 1 mg/ml in DMSO; Thermo Fisher) for the indicated time periods prior to isolation of RNA for analysis by qPCR. Transcript levels were normalized by the amount of 18S transcript present, which remained constant throughout all 8 h of ActD treatment.

**The 6mA DNA immunoprecipitation**

The 6mA DNA immunoprecipitation was performed using the MAGnify chromatin immunoprecipitation (ChIP) system (Thermo Fisher). One hundred and fifty thousand cells per replicate were collected from 2 day postconfluent 3T3-L1 preadipocytes that had been transfected with siRNA against Fto prior to reaching confluence. Sonicated DNA was pulled down with the same rabbit anti-6mA antibody (Synaptic Systems) used in the m6A RIP experiment or a rabbit IgG negative control (Thermo Fisher). Eluted DNA and a reserved aliquot containing 10% of the input were analyzed by qPCR. No template was detectable after pulldown in the IgG negative control condition. Levels of 6mA-enriched DNA content were compared with DNA content of the input control. Primers spanning the Cebpd promoter (493 bases upstream to 200 bases downstream of the transcriptional start site) as well as Gapdh (intron 2) are listed in supplemental Table S1. RpLp0 (intron 2) primers were from Cell Signaling Technologies. A positive control (L1MId-Gf-17) was quantified using primers published in (48).

**Lipid staining**

Mature adipocytes were stained with Oil Red O for lipid content by first fixing cells with 4% paraformaldehyde for 10 min. A freshly mixed solution of 3:2 Oil Red O stock:water (stock: 25 mg Oil Red O in 50 ml isopropanol; Sigma) was prepared and filtered prior to staining for 30 min. Cells were then washed with PBS and imaged. For quantification, Oil Red O was extracted in 300 μl of isopropanol with 4% IGEPAL CA-630 for 5 min and 100 μl were used to measure absorbance at 490 nm (49).

Nile Red staining was performed based on the protocol developed by Smyth and Wharton (50). Mature adipocytes were trypsinized and treated with 4% paraformaldehyde for 10 min. Nile Red (stock: 1 mg/ml in DMSO; Thermo Fisher) was then added for a final concentration of 500 ng/μl and cells were acquired within 30 min by flow cytometry using the PE-TR channel to measure Nile Red fluorescence. Prior to acquisition, equal volumes of 8.0–12.9 μm counting beads (Spherotech) were added to quantify cell numbers per condition. Live cells were gated to include all events except debris (very low FSC and SSC). The Nile Red gate was set on Nile Red-negative preadipocytes.

**Protein isolation and quantification**

Whole cell lysates were collected in RIPA buffer in the presence of Halt protease-phosphatase inhibitor and quantified by BCA assay (all Thermo Fisher). Following denaturing gel electrophoresis, proteins were transferred to nitrocellulose membranes and probed with the following primary antibodies: β-Act (Abcam), Fto (Santa Cruz), αTub, C/ebpβ, pAkt, Akt, pAkt1, Akt1, pAkt2, pFoxo1, and Foxo1 (Cell Signaling). Fluorescent secondary IRDye 680LT and 800CW antibodies were from LI-COR. Blots were imaged using LI-COR’s Odyssey Classic and bands were quantified with Image Studio software (LI-COR).

Leptin ELISA was performed using a kit from R&D Systems. Cell culture supernatants from mature adipocytes were removed 3 days after medium had been changed. Samples (from 12-well plates) were diluted 1:20.

Glucose uptake was measured using a glucose uptake colorimetric assay kit (Sigma) according to the manufacturer’s instructions. Briefly, following Fto knockdown, 2 day postconfluent 3T3-L1 preadipocytes were serum starved overnight and then glucose starved for 40 min. Cells were treated with or without insulin (1 μM) for 20 min and then treated with or without 2-deoxyglucose.
(2-DG) for 20 min. Lysed cells were assayed for 2-DG concentration using the colorimetric assay.

Mice

Mice were group housed by gender and maintained at an ambient temperature of 22–24°C with a 12 h dark-light cycle (lights on at 0700) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee. Fto expression was evaluated in isolated adipocytes and preadipocyte-containing stromal vascular cells (SVCs) from perigonadal and subcutaneous adipose tissue from 4-month-old C57BL/6 mice (derived from Jackson Laboratory, stock number 000664) fed chow (Purina PicoLab 5058). Adipose depots were excised and digested with collagenase (Roche) for 45 min to 1 h prior to separation by centrifugation. The SVC-containing pellet was treated with erythrocyte lysis buffer (0.154 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 3 min before being used for analysis. RNA was isolated, cDNA was generated, and qPCR was performed as described above. mRNA expression was normalized to the arithmetic mean of Rplp0, Actb, Gapdh, and Ppia.

RESULTS

Fto knockdown impairs adipogenesis in mouse and human adipocytes

To determine whether Fto participates directly in adipogenesis and, by that process, in energy storage capacity, we treated 3T3-L1 preadipocytes with siRNA targeted against Fto prior to in vitro adipogenesis. The decrease in mRNA transcript and protein levels of Fto was maintained during the entire differentiation period in the Fto knockdown condition (Fig. 1A, supplemental Fig. S1A, B). Knockdown of Fto impaired the ability of 3T3-L1 cells to develop into mature adipocytes, as demonstrated by Oil Red O staining of intracellular lipid (Fig. 1B, supplemental Fig. S1D). These preadipocytes showed decreased (~57%) expression of Cebpd and a modest (~14%), but statistically nonsignificant, decrease in Cebpb (Fig. 1C). Expression of other preadipocyte developmental and functional genes (Pparg, Cebp, Fabp4, Glut4, Plin1, Lpl, Adipoq, and Lep) was also decreased in mature adipocytes in the Fto-knockdown condition (Fig. 1D, supplemental Fig. S1C). The total amount of leptin secreted into the culture media was decreased as well (supplemental Fig. S1E); however, when normalized to the number of adipocytes in each well (supplemental Fig. S1F), there was no difference in the amount of leptin secreted per cell (supplemental Fig. S1G), suggesting that Fto does not directly regulate leptin synthesis or release by adipocytes on a per-cell basis, consistent with our previous observations (46). The decrease in lipid content and adipocyte gene expression in bulk culture could have been caused by a decrease in the number of cells that could fully develop into adipocytes and/or by a limitation of differentiation causing cells to arrest at a less mature stage. We analyzed Nile Red staining for neutral lipids by flow cytometry and found that both factors contributed to the decrease in lipid per well; there were fewer Nile Red-positive cells as a percentage of total cells analyzed (Fig. 1E); and of the cells that were Nile Red positive, there was less lipid in each cell, as indicated by the median fluorescence intensity of the Nile Red+ adipocyte population (supplemental Fig. S1H).

To confirm the effects of Fto on adipogenesis observed in 3T3-L1 cells, we manipulated FTO expression in primary human ASCs (43). Using the same experimental paradigm (FTO knockdown prior to differentiation) with cells from four adult individuals (two males and two females), we found that knockdown of FTO expression (Fig. 1F; supplemental Fig. S1J, K) impaired adipogenesis. We observed the same decrease in Oil Red O (Fig. 1G) staining and percentage of Nile Red-positive adipocytes (Fig. 1J), although there was no difference in Nile Red fluorescence in mature cells (supplemental Fig. S1L) in the FTO knockdown condition. In preadipocytes, expression of both CEBPB and CEBPD was decreased (Fig. 1H), and in mature adipocytes, expression of adipocyte developmental and functional genes (PPARG, CEBPA, FABP4, GLUT4, PLIN1, LEP, and ADIPOQ) (Fig. 1I) was decreased in the FTO knockdown condition compared with nontargeted siRNA controls.

Fto has recently been implicated in the differentiation of adipocytes (51–53). In addition to loss-of-function studies consistent with those detailed here, Fto overexpression studies using mouse embryonic fibroblasts and 3T3-L1 cells demonstrate increased adipogenesis (51, 53). In particular, Fto demethylase activity is required for adipogenesis, as overexpression of a mutant Fto construct lacking demethylase activity acted as a dominant negative, inhibiting adipogenesis (53), possibly by regulating mRNA splicing (52). One possible mediator of Fto’s demethylase function in adipocytes is an alternatively spliced isoform of Runt-related transcription factor 1 (Runx1t1) lacking exon 6. Inclusion of exon 6 may be due to enhanced binding of m6A-methylated RNA by serine/arginine-rich splicing factor 2 (SRSF2) in 3T3-L1 cells lacking Fto (with increased methylation) (51, 52). Exon 6 skipping, possibly due to the lack of methylated SRSF2 binding sites in cells with functional Fto demethylase activity, may generate a short isoform of Runx1t1 that is pro-adipogenic in 3T3-L1 cells and mouse embryonic fibroblasts (51, 52). We measured long (containing exon 6) and short (missing exon 6) splice variants of Runx1t1 by qPCR and did not detect any significant difference in total amounts or the ratio of the isoforms between control and Fto-knockdown conditions in either the preadipocyte or mature adipocyte stage in 3T3-L1 cells (supplemental Fig. S1M–O). We also did not detect changes in long or short splice variants of RUNXIT1 in human ASCs (data not shown).

From these findings, we infer that FTO is required for the development, in vitro, of human and murine adipocytes. This functional consequence is conveyed during the preadipocyte stage, when Cebpd expression is decreased in Fto-knockdown 3T3-L1 cells and CEBPB and CEBPD expression is decreased in FTO-knockdown human ASCs. We were unable to confirm, however, previous findings regarding an alternative splicing effect attributed to Fto (51, 52).
RNA-Seq analysis identifies Fto-targeted genes as candidates for regulation of adipogenesis

To identify a mechanism for Fto’s adipogenic functions in 3T3-L1 cells, we analyzed the gene expression profile following Fto knockdown by RNA-Seq. We examined 2 day postconfluent preadipocytes prior to the induction of differentiation in order to focus on primary targets of Fto, minimizing secondary effects produced by an altered adipogenic program. There were 20 genes significantly (\( P < 0.05 \)) up- or downregulated following Bonferroni correction (see Fig. 2D); with effect sizes of 1.40- to 2.14-fold and 0.22- to 0.84-fold, respectively. We identified four additional genes (Clec1, Rhob, Tusc5, and Klf2) with differential expression that was not as statistically significant (uncorrected \( P < 1 \times 10^{-5} \); Bonferroni corrected \( P < 0.23 \)), but with effect sizes of \( \geq 2 \)-fold or \( \leq 0.5 \)-fold (Fig. 2A). One other notable change was the decrease in Pten expression (\( P = 0.1 \) following Bonferroni correction, 0.8-fold). We confirmed the effect of Fto knockdown on these genes by measuring transcript levels in Fto-knockdown preadipocytes.
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in two independent experiments by qPCR. We verified the effect of *Fto* on 20 of 24 genes identified by RNA-Seq (Fig. 2D).

Using qPCR, we examined the effects of *FTO* knockdown on human homologs of the most highly regulated targets by RNA-Seq in 3T3-L1 preadipocytes (>3-fold increase or <0.3-fold decrease after *Fto* knockdown in 3T3-L1 cells; protein tyrosine phosphatase receptor type V pseudogene (*PTPRVP*), *TMED5*, and *SPON2*), as well as genes known to be associated with adipogenesis (*GREM2*, *KLF2*, and *WNT4*).
and Pten, an inhibitor of insulin signaling (58). Murine homologs of these transcripts (measured by RNA-Seq) have been marked by open circles in Fig. 2A. These studies were conducted in both human ASC-derived preadipocytes (Fig. 2E) and mature adipocytes (Fig. 2F) to identify molecules that might convey FTO’s effects on adipogenesis.

In human ASC-derived cells, upregulation in adipocytes of SPON2, a gene not previously associated with adipogenesis, and downregulation of PTEN (phosphatase and tensin homolog) in preadipocytes were consistent with the results of the RNA-Seq and qPCR analyses in 3T3-L1 preadipocytes. We were unable to detect mRNA expression of PTPRV, consistent with a report indicating that it is a pseudogene in humans (59). In murine cells, however, Ptprv is dramatically upregulated in conditions of p53-regulated G1 cell cycle arrest, and loss of this gene restores cell cycle progression in a manner similar to loss of p53 (60, 61). Expression of other p53-induced cell cycle arrest effectors (MADD4, MDM2, and CDKN1A) was upregulated in human ASC preadipocytes (Fig. 2G) and mature adipocytes (Fig. 2H).

We analyzed our 3T3-L1 preadipocyte Fto knockdown RNA-Seq dataset using Enrichr [http://amp.pharm.mssm.edu/Enrichr/ (62, 63)], a publicly available tool for gene list enrichment analysis. We analyzed genes that were up- or downregulated by more than 10% with P < 0.05 (without correction; 2,469 genes). The most striking results came from the ChEA 2016 analysis (62, 63), which queries a database of ChIP-seq datasets for transcription factor target enrichment (supplemental Fig. S2). The Fto-knockdown condition was highly enriched for C/ebpβ and C/ebpδ targets identified in ChIP-seq studies performed in 3T3-L1 cells, with Enrichr’s calculated combined scores of 94.09 for C/ebpβ (adjusted P = 2.7E-25; 410/2,000 overlap) (Fto-affected transcripts/all C/ebpβ-regulated transcripts) and 87.8 for C/ebpδ (adjusted P = 1.36E-22; 358/1,735 overlap) targets. This result is consistent with the decreased expression of Cebpβ (~57%) demonstrated in Fig. 1. We observed a non-statistically significant (~14%) decrease in Cebpδ expression in 3T3-L1 Fto-knockdowns, but consistent significant downregulation of both CEBPB and CEBPD in FTO-knockdown human ASCs. Peak expression of Cebpβ (after 2 h) and Cebpδ (after 1 h) immediately after the addition of differentiation medium in 3T3-L1 preadipocytes displayed the same pattern (i.e., lower Cebpδ expression in the Fto knockdown condition, with no difference in Cebpβ expression between groups; Fig. 2B, C). The downregulation of C/ebpβ-driven transcription in the Fto-knockdown condition (supplemental Fig. S2) is also consistent with evidence that Fto can act as a transcriptional coactivator of C/ebpβ (42). Furthermore, RNA-Seq analysis identified candidate effectors for the molecular mechanism of Fto’s effects on adipocyte development (Pten, Ptprv, and Spn2).

**Fto regulates Akt signaling phosphorylation in adipocytes**

The decrease in Pten expression associated with Fto knockdown (identified by RNA-Seq and illustrated in Fig. 2) was unexpected, as Pten is a well-characterized tumor suppressor (64), and its decreased expression after Fto knockdown would be predicted to increase cell proliferation, in contrast to the decreased cell proliferation observed. Pten also inhibits insulin signaling by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to PtdIns(4,5)P2 (PIP2), thereby inhibiting Akt phosphorylation (58, 65). A previous report (66) indicated that Fto knockdown increased Akt phosphorylation of Ser473 in 3T3-L1 cells. This finding is consistent with the decreased Pten expression we observed following Fto knockdown. We also observed increased phosphorylation of Akt at Ser473 in 3T3-L1 preadipocytes after 4 h serum starvation and after serum starvation plus exposure to 1 μg/ml insulin for 15 min (Fig. 3A, B). This effect was seen in both the Akt1 and Akt2 isoforms (Fig. 3C, D; supplemental Fig. S3A, B), and elevated pAkt/Akt response to insulin in the Fto knockdown condition was sustained until the cells became mature adipocytes (Fig. 3E, supplemental Fig. S3C). Foxo1 is also phosphorylated via the Akt signaling pathway (67, 68), and pFoxo1/Foxo1 ratios were elevated in 3T3-L1 adipocytes in response to insulin (Fig. 3F, supplemental Fig. S3D).

Increased Akt signaling in the Fto knockdown condition was not associated, however, with an increase in glucose uptake in either the serum/glucose-starved or insulin-treated state. Uptake of 2-DG, a nonmetabolizable glucose analog, into 3T3-L1 preadipocytes was decreased in the Fto knockdown condition (Fig. 3G). This result is consistent with the decrease in Glut4 expression described above (Fig. 1D). Additionally, expression of the non-insulin-sensitive glucose transporter, Glut2 (Scl2a1), was decreased by Fto knockdown in both 3T3-L1 preadipocytes and mature adipocytes (Fig. 3H).

The unexpected reduction of Pten expression as a consequence of Fto knockdown is accompanied by increased insulin sensitivity. Evidently, this increase in insulin sensitivity is not sufficient to rescue glucose uptake and subsequent lipogenesis in adipocytes in which differentiation is impaired, as the glucose uptake machinery (i.e., decreased Glut4 and Glut1 expression in response to decreased Pparg and Cebpα expression) is not available to convey effects (i.e., Glut4 translocation) of the activated insulin response.

**Ptprv and Spn2 contribute to Fto’s role in adipogenesis in 3T3-L1 cells**

We evaluated the importance of candidate genes identified as Fto targets by RNA-Seq to the adipogenesis phenotypes we observed. Simultaneous knockdown of both Fto and Ptprv (Fig. 4A, B) restored adipogenesis lost when Fto alone was knocked down. Pparg expression (Fig. 4C) and Oil Red O staining (Fig. 4D, supplemental Fig. S4A) were increased in these double knockdown cells. Fto/Ptprv double knockdown also increased the percentage of Nile Red staining (Fig. 4F) and fluorescence of Nile Red+ cells (supplemental Fig. S4B) compared with Fto knockdown alone. Elevated expression of Spn2 after Fto knockdown (Fig. 4E) is attributable to Ptprv, as Fto/Ptprv knockdown restored Spn2 expression close to control levels. However, Fto/Ptprv knockdown did not increase Cebpβ (Fig. 4H) or Cebpδ (Fig. 4I) expression, indicating that Ptprv is either downstream of
C/ebpβ and C/ebpδ or functioning in a different pathway from these critical regulators of adipogenesis. Fto/Ptprv knockdown did not restore Pten expression (supplemental Fig. S4C), suggesting that Pten is not regulated by Fto by the same mechanism as Ptprv and Spon2. Knockdown of Spon2 in addition to Fto also restored adipogenesis, but to a lesser degree (supplemental Fig. S4A–G) and, like Fto/Ptprv knockdown, did not increase Cebpb (supplemental Fig. S4H) or Cebpd expression (supplemental Fig. S4I).

While the human homolog of Ptprv is a pseudogene, and therefore not transcribed, we anticipate that Ptprv is one of a number of p53-mediated cell cycle arrest effectors that likely contribute to impaired proliferation in these cells. In support of this inference, we saw decreased SPON2 expression in human ASCs, a target of Ptprv in murine 3T3-L1 cells, indicating activation of the same pathway in both species. A pathway independent of Ptprv (and the associated cell cycle arrest) could also mediate the inhibition of differentiation, as Cebpd expression was not rescued by double knockdown of Fto and Ptprv.

**DNA demethylation capacity of Fto can account for its effects on gene expression**

As noted, Fto acts as a demethylase of m6A (26, 27). We looked for changes in RNA methylation of the candidate genes mentioned above (Cebpb, Cebpd, Ptprv, Spon2, and Pten). RNA was immunoprecipitated using an anti-m6A antibody (m6A RIP) and transcript levels were quantified by

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**Fig. 3.** Fto knockdown increases pAkt in 3T3-L1 cells. Western blot (A) and quantification (B) of phospho-Akt (Ser473) normalized to total Akt in 3T3-L1 preadipocytes after 4 h of serum starvation followed by treatment with or without 1 μg/ml insulin for 15 min. Quantification of Western blots for phospho-Akt1/total Akt1 (C) and phospho-Akt2/total Akt2 (D) in serum-starved preadipocytes treated with insulin for 15 min. Quantification of Western blots for phospho-Akt/total Akt (E) and phospho-Foxo1 (Ser256)/total Foxo1 (F) in mature 3T3-L1 adipocytes treated with 1 μg/ml insulin for 15 min. G: 3T3-L1 preadipocytes (2 days postconfluence) were serum starved overnight and then glucose starved for 40 min prior to stimulation with or without insulin (1 μM) for 20 min. This was followed by addition of 2-DG for 20 min (n = 3 for 2-DG–treated conditions, n = 1 for 2-DG–untreated negative controls). Cells were lysed and 2-DG concentrations were assayed. H: mRNA expression of Slc2a1 in 3T3-L1 preadipocytes and mature adipocytes, mRNA expression was normalized to Rplp0. pAkt (total, pAkt1, and pAkt2), Akt (total, Akt1, and Akt2), pFoxo1, and Foxo1 protein bands were first normalized to βTub before determining the ratios of Phospho/total protein levels. Adiponectin bands were normalized to βAct (n = 3–6 per condition except where indicated; unpaired t-test). *P < 0.05, **P < 0.01, ***P < 0.001.

**Fig. 4.** Knockdown of siFto upregulated Ptprv restores adipogenesis. qPCR analysis of Fto (A), Ptprv (B), Spon2 (E), Cebpb (H), and Cebpd (I) expression in 3T3-L1 preadipocytes, and Pparg2 expression in mature adipocytes following single Fto (solid gray bars) or double Fto/Ptprv knockdown (open gray bars) prior to confluence and differentiation (C). Mature adipocytes stained for Oil Red O (D) and percent of live cells positive for Nile Red staining analyzed by flow cytometry (F). G: Total number of live cells collected by flow cytometry after differentiation. All plots represent the mean ± SEM for n = 3–4 per condition; unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
qPCR to assess whether any of these transcripts might be a direct target of Fto’s demethylase function. When normalized to the amount of input transcript (prior to m6A immunoprecipitation), there were no changes in m6A status in any of the transcripts evaluated, with the exception of Pten (Fig. 5A, B). This finding suggests that these transcripts are secondary targets of an upstream target of Fto’s m6A demethylase function or are direct targets of Fto via a mechanism independent of m6A RNA demethylation, such as transcriptional activation. A recent report indicates that the preferred target of Fto’s demethylase function is actually N6,2’-O-dimethyladenosine (m6Am), a subset of m6A found only at the 5’ end of mRNA transcripts (69). Loss of specific 5’ m6Am demethylation could have been masked by precipitating transcripts with an antibody that detects all m6A modifications.

Demethylation of m6A and, more specifically, m6Am by Fto has been associated with altered transcript stability (69, 70). Thus, if Cebpb, Cebpd, Ptprv, Spon2, and/or Pten mRNAs are direct targets of Fto despite the unchanged m6A pattern in the majority of these transcripts, the altered transcript levels observed may be the result of changes in message stability. To test this possibility, we inhibited nascent transcription with ActD following knockdown of Fto in 3T3-L1 preadipocytes (Fig. 5B–F, H–L). We did not observe any change in transcript stability of Cebpb (Fig. 5B, H), Cebpd (Fig. 5C, I), Ptprv (Fig. 5D, J), Spon2 (Fig. 5E, K), or Pten (Fig. 5F, L) that might account for the altered expression levels observed following Fto knockdown. Methylation of m6A residues may also contribute to changes in translation from the RNA transcript (71). We anticipated that this reaction might contribute to the altered expression of C/ebpβ target genes observed by RNA-Seq despite the absence of a decrease in Cebpb transcript levels, as it is possible that Cebpb was not being properly translated or was being translated from alternative start sites. Western blotting

![Diagram](image-url)
for C/ebpβ protein in 3T3-L1 preadipocytes showed slightly (but not statistically significant) decreased levels of all isoforms in the Fto knockdown condition compared with controls (Fig. 5M, N), consistent with the mRNA expression data described. These data indicate that Cebpβ, Cebpδ, Ptpov, and Spon2 mRNAs may not be direct targets of Fto’s m6A demethylase function, even though they are critically regulated by Fto.

As 6mA DNA modifications have recently been discovered in both mouse and human DNA (34, 48) and because Fto is capable of demethylating this modification (27), we tested to determine whether Fto affects 6mA methylation by immunoprecipitating DNA using an anti-6mA antibody. We quantified DNA content at specific regions by qPCR. We analyzed the region surrounding the Cebpδ promoter, as Cebpδ expression is decreased following Fto knockdown (Fig. 1C). Alkbh1 has been described as a 6mA DNA methylase, particularly of evolutionarily young LINE elements (48). As a potential positive control, we measured 6mA methylation of an L1 LINE element (L1Md-Gf-17) that is a target of Alkbh1. This L1 element was enriched (23%) after pulldown with anti-6mA (Fig. 6A, C) in the control siRNA condition. The 6mA enrichment of L1Md-Gf-17 was increased in the Fto knockdown condition, suggesting overlap in 6mA targeting between Fto and Alkbh1 and confirming that our assay can capture changes in methylation status induced by Fto. We observed enrichment of the region surrounding the Cebpδ promoter (6–8% of input, compared with ~4–5% in the siCtrl condition) that was increased (1.2- to 1.9-fold) after Fto knockdown (Fig. 6B, D). These results are consistent with an increase in 6mA in the absence of the demethylase function of Fto, and may account for the inhibition of transcription of Cebpδ seen after Fto knockdown.

**Fto knockdown in mature adipocytes also leads to impaired adipocyte lipid handling**

We and others have demonstrated an impairment in the ability of cells to undergo adipogenesis when Fto expression is decreased prior to differentiation (51, 52). Fto expression was increased as 3T3-L1 and human ASC preadipocytes differentiated into mature adipocytes (Fig. 1A, F). We isolated mature adipocytes and preadipocyte-containing SVCs from perigonadal and subcutaneous adipose tissue of 4-month-old male C57BL/6 mice by collagenase digestion. Fto expression in mature adipocytes was approximately 3-fold higher than that in the SPCs (Fig. 7A) suggesting that Fto may play a more substantial role in mature lipid-filled cells. We assessed knockdown of Fto in mature (8–10 days post differentiation) 3T3-L1 adipocytes (Fig. 7B) to understand whether Fto affects adipocyte function after cells have undergone differentiation. Expression of developmental (Cebpβ, Pparγ2, and Cebpδ) and functional (Fabp4, Glut4, Plin1, and Adipon) adipocyte genes (Fig. 7C) was decreased, including most lipogenic and lipolytic genes assayed (Dgat1, Lpl, Hsl, Atgl, and Dgflb; Fig. 7D) in the Fto knockdown condition, with no change in Cebpδ expression (Fig. 7C); however, there was no detectable difference in lipid content by staining with Oil Red O (Fig. 7E). We did not observe any change in the percentage (Fig. 7F) of Nile Red-positive cells reported by flow cytometry. There was, however, a modest decrease in the amount of lipid per cell (Fig. 7G). We speculate that this change was detectable by Nile Red staining because flow cytometric analysis of a single cell suspension is a more sensitive measure of lipid content than quantification of bulk Oil Red O uptake.

While our in vitro Fto knockdowns in early adipogenesis show that Fto is necessary to enable C/ebpβ- and C/ebpδ-driven adipogenesis (Fig. 8A), C/ebpβ is also critical for maintaining adipocyte identity and function in differentiated cells, by functioning as a coactivator of Pparγ and C/ebpα (72). The upregulation of Fto during adipogenesis suggests that its functions with regard to adipose tissue may be more critical after differentiation has occurred (Fig. 8B). These data suggest that Fto plays a role in the maintenance of adipocyte lipid storage capacity. Thus,
**DISCUSSION**

The molecular bases for the strong association of sequence variants in intron one of *FTO* with human adiposity are complex and remain unclear. *FTO* itself, and nearby *RPGRIP1L*, *IRX3*, and *IRX5* have all been implicated by mechanisms relating to food intake, adipocyte differentiation, and energy expenditure. Here, we provide evidence of the mechanism by which *FTO* plays a permissive role in vitro adipogenesis in both murine 3T3-L1 cells and human ASCs by demethylating 6mA DNA in mature adipocytes and allowing transcription, particularly of *Cebpd* and its transcriptional targets, to proceed.

In humans, the effects of intronic alleles of *FTO* on adiposity are conveyed primarily by effects on food intake (4–7), though not necessarily by the *FTO* molecule itself (38, 73, 74). Adipose tissue mass is determined by complex interactions of cell autonomous, metabolic, and behavioral processes. Adipose tissue may affect metabolic and behavioral phenotypes by hormonal secretions (leptin, adiponectin, TNF-α), effects on circulating metabolites (glucose, triglycerides, and cholesterol) consistent with restricted adipose tissue storage capacity phenotype (16). These effects are consistent with growing evidence, including a recent meta-analysis, that there is a contribution of *FTO* locus to diabetes beyond its effect on adiposity (76–79).

We identified *Ptprv* and *Spon2* as effectors of Fto’s effect on proliferation of 3T3-L1 cells during adipogenesis. C/ebpβ inhibits expression of the tumor suppressor, p53 (80); p53 drives expression of *Ptprv* (61). The upregulation of *Ptprv* in response to Fto knockdown is possibly an effect of impaired C/ebpβ function, reflecting a broader impairment of cell cycle progression (Fig. 8, supplemental Fig. S11). Although the human homolog of murine *Ptprv*, *PTPRVP*, is a pseudogene (59), other effectors of cell cycle arrest (*GADD45A*, *MDM2*, and *CDKN1A*) are upregulated in human ASCs following Fto knockdown (Fig. 2). Furthermore, a decrease in *Ptprv* expression has previously been demonstrated during differentiation of 3T3-L1 cells into mature adipocytes (81), consistent with our observations of increased Fto expression during adipogenesis, which we suggest could be responsible for the downregulation of *Ptprv*. Previous studies have not implicated FTO as a primary regulator of adipogenesis in human cells (82). However, the cells used in these experiments, Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes, were derived from a patient with X-linked SGBS (83). SGBS is characterized as an overgrowth syndrome caused by mutations in the gene encoding glypican-3, which regulates signaling in response to growth factors such as Wnts, Hedgehogs, FGFs, and BMPs (84, 85). Our data, and those of others (51, 86, 87), indicate that Fto positively regulates *Glut4* and fatty acid- and lipid-uptake (*Fabp4* and *Lpl*) genes in addition to decreased lipid content. Mice segregating for a dominant-negative mutation of Fto have reduced fat mass, are protected from diet-induced obesity, and display metabolic phenotypes (increased circulating glucose, triglycerides, and cholesterol) consistent with restricted adipose tissue storage capacity phenotype (16). These effects are consistent with growing evidence, including a recent meta-analysis, that there is a contribution of the *FTO* locus to diabetes beyond its effect on adiposity (76–79).

We identified *Ptprv* and *Spon2* as effectors of Fto’s effect on proliferation of 3T3-L1 cells during adipogenesis. C/ebpβ inhibits expression of the tumor suppressor, p53 (80); p53 drives expression of *Ptprv* (61). The upregulation of *Ptprv* in response to Fto knockdown is possibly an effect of impaired C/ebpβ function, reflecting a broader impairment of cell cycle progression (Fig. 8, supplemental Fig. S11). Although the human homolog of murine *Ptprv*, *PTPRVP*, is a pseudogene (59), other effectors of cell cycle arrest (*GADD45A*, *MDM2*, and *CDKN1A*) are upregulated in human ASCs following Fto knockdown (Fig. 2). Furthermore, a decrease in *Ptprv* expression has previously been demonstrated during differentiation of 3T3-L1 cells into mature adipocytes (81), consistent with our observations of increased Fto expression during adipogenesis, which we suggest could be responsible for the downregulation of *Ptprv*. Previous studies have not implicated FTO as a primary regulator of adipogenesis in human cells (82). However, the cells used in these experiments, Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes, were derived from a patient with X-linked SGBS (83). SGBS is characterized as an overgrowth syndrome caused by mutations in the gene encoding glypican-3, which regulates signaling in response to growth factors such as Wnts, Hedgehogs, FGFs, and BMPs (84, 85). Our data, and those of others (51, 86, 87), indicate that Fto positively regulates...
C/ebp development and function. Fto regulates the transcriptional activity of C/ebpα and the expression of Cebpd, possibly by different mechanisms. A: During adipogenesis, Fto-driven C/ebpβ activity promotes cell cycle progression by suppressing p53 activity and, thereby, Ptpo and Spon2 expression. The 6mA demethylation of the Cebpd promoter by Fto promotes Cebpd transcription, driving Ptpo and Cebpα expression, which coordinately initiate and sustain adipocyte differentiation. There may be other mechanisms for Fto’s effects on adipogenesis that are independent of the actions of C/ebpα and C/ebpβ (direct arrow to adipogenesis). B: In mature adipocytes, Fto coactivates C/ebpβ-mediated transcription and demethylates 6mA in the Cebpd promoter, increasing Cebpd expression. This combination facilitates maintenance of adipocyte activities, including lipid and glucose uptake. There may be other mechanisms acting independently of Fto’s effects on C/ebpα and Cebpd in mature adipocytes as well.

adipocyte proliferation. The proliferative decrease in the FTO knockdown condition might have been masked in SGBS cells, which are hyper-responsive to growth signals.

We performed RNA-Seq in preadipocytes to identify primary regulators of Fto’s effect on adipogenesis and found that the C/ebpα and C/ebpβ-mediated transcription that is required for adipogenesis is dramatically altered. Fto has been identified as an RNA demethylase of m6A modifications (26, 27) and the locations and functions of m6A modifications have been increasingly well-described (28, 29, 47, 71). Although the demethylase function of Fto is clearly required for its effects on adipogenesis (51–53, 88), we did not identify any changes by m6A RIP in the m6A methylation status of candidate transcripts most highly regulated by Fto knockdown. We did not observe any effects on transcript stability or translational differences in Fto-regulated targets. Contrary to others, we did not find differential splicing of Runx111 in response to Fto knockdown (52, 89), suggesting that demethylation of m6A RNA in adipocytes may not be Fto’s critical function. We identified Cebpd and Cebpd as targets of Fto. These are monoexonic genes that lack splice variants.

We find that Fto may alter 6mA DNA methylation at the promoter of Cebpd, expression of which is downregulated by Fto knockdown. Additionally, our RNA-Seq results indicating impaired C/ebpβ activity are consistent with the report by Wu et al. (42) identifying Fto as a transcriptional coactivator, facilitating C/ebpβ transactivation of the C/ebp-response element (CEBPRE; Fig. 8).

Whether Fto demethylates 6mA in CEBPREs directly, affecting C/ebpβ binding, remains unclear; using electrophoretic mobility shift assays, Wu et al. (42) did not detect direct binding of Fto to CEBPREs. The effects of Fto in regulating C/ebpβ activity and Cebpd expression may be mediated by similar (i.e., 6mA DNA demethylation in CEBPREs and the Cebpd promoter) or independent mechanisms. 6mA DNA demethylation may play a role in transcriptional silencing (53, 48). Our data imply that Fto plays a functional role in adipogenesis by affecting 6mA DNA demethylation at the Cebpd promoter (48, 90). Although in vitro experiments indicate that single stranded 6mA DNA is the primary target of Fto (double stranded 6mA is demethylated to a lesser extent), FTO knockdown increased the genomic 6mA DNA content in human cell lines, and FTO overexpression decreased 6mA DNA content (91). While current understanding of 6mA biology in eukaryotes is in its infancy, 6mA modifications of DNA are widely distributed and may be distinct among different tissues. There are likely species-specific functions of 6mA (34). Furthermore, 6mA is enriched in stem cells compared with differentiated cells (48), consistent with our observations that Fto expression, which may demethylate 6mA, is elevated in mature adipocytes.

Genetic variations in noncoding portions of the FTO locus are associated with modest, but statistically very significant, effects on human adiposity. The high prevalence of the relevant “risk” alleles means that these variants account for a literal large burden of excess fat in virtually all human populations. SNPs in intron 1 of FTO have been implicated (by cis effects on vicinal genes) in both food intake (7) and energy expenditure (38, 74). Some of these variants also affect the expression of the FTO gene itself (9, 10). Human obesity is primarily a disorder of energy balance, not a storage disease due to cell-autonomous characteristics of adipocytes (92). However, the capacity and anatomic disposition of adipose tissue have important effects on the metabolic consequences of the storage of excess somatic fat (17, 18). Here, we show that FTO protein modulates the generation of adipocytes and their filling with triglyceride by cell-autonomous mechanisms that appear to reflect effects of FTO on 6mA DNA demethylation. These effects could be important in conveying susceptibility to the adverse metabolic consequences of obesity. Thus, FTO may be implicated not only in the energy imbalance that causes deposition of excessive fat but also in the medical consequences of that deposition.

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