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The obesity-associated *Fto* gene is a transcriptional coactivator

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ABSTRACT

The fat mass and obesity associated, *FTO*, gene has been shown to be associated with obesity in human in several genome-wide association scans. *In vitro* studies suggest that *Fto* may function as a single-stranded DNA demethylase. In addition, homologous recombination-targeted knockout of *Fto* in mice resulted in growth retardation, loss of white adipose tissue, and increase energy metabolism and systemic sympathetic activation. Despite these intense investigations, the exact function of *Fto* remains unclear. We show here that *Fto* is a transcriptional coactivator that enhances the transactivation potential of the CCAAT/enhancer binding proteins (C/EBPs) from unmethylated as well as methylation-inhibited gene promoters. *Fto* also exhibits nuclease activity. We showed further that *Fto* enhances the binding C/EBP to unmethylated and methylated DNA. The coactivator role of FTO in modulating the transcriptional regulation of adipogenesis by C/EBPs is consistent with the temporal progressive loss of adipose tissue in the *Fto*-deficient mice, thus suggesting a role for *Fto* in the epigenetic regulation of the development and maintenance of fat tissue. How FTO reactivates transcription from methyl-repressed gene needs to be further investigated.

1. Introduction

The pandemic rise in the incidence of obesity and its associated health problems has significant impact on the cost of global health care [1]. In the United States, approximately two-thirds of the adults are overweight, with one third of these considered obese. Presumably, the epidemic increase in obesity is due to sedentary lifestyle coupled with overconsumption of energy-rich foods, which create a chronic energy imbalance that leads to weight gain in the form of body fat [2,3]. As adiposity increases, the risk of developing comorbidities such as diabetes, hypertension, and cardiovascular disease is also significantly elevated [4–6], thus prompting the search for the underlying genetic and environmental causes for obesity. The alarming rise in the incidence of obesity worldwide in the last two decades has prompted the search for genetic and environmental causes that contribute to this global health crisis [7]. Several genome-wide association scans have identified the association of *FTO* with body mass index [8–12]. *FTO* is a member of the family of non-heme Fe(II)- and 2-oxoglutarate-dependent dioxygenases [13,14], that include the DNA demethylase AlkB (*Escherichia coli*) and ABH (mammalian AlkB homolog) genes, which are involved in the repair of DNA alkylation damage [15–17]. FTO demethylates 3-methylthymine *in vitro* on single-stranded oligonucleotides [13,18], and homologous recombination knockout of *Fto* in mice causes a near-complete loss of adipose tissue and increased energy expenditure [19]. The underlying link between the putative demethylase function of FTO and energy homeostasis is not apparent. The striking loss of fat tissue raises the question whether adipogenesis is impaired in the $Fto^{-/-}$ mice and what the role of *Fto* in this might be.

Some members of the C/EBP family of transcription factors including C/EBPα, β, and δ, and the peroxisome proliferator-activated receptor γ (PPARγ), are considered the master transcriptional regulators of adipogenesis [20,21]. Though transcriptional regulation of adipogenesis has been intensely investigated, the role of *Fto* as a demethylase and epigenetic regulator in this process has not been reported, and epigenetic regulation of adipogenesis through either global or gene-specific DNA methylation and demethylation is underexplored.

Methyl modification at CpG dinucleotide suppresses transcription by modulating DNA–protein interactions and altering the accessibility of transcription factors to the methylated control regions of genes [22,23]. Recent studies suggest that cyclical DNA methylation and demethylation of gene promoters regulates transcription [24,25]. Active DNA methylation and demethylation is important for resetting the epigenetic state of the genome for response to continuous gene environmental interactions resulting from dietary or environmental perturbants exposure. Aberrant methylation is associated with human diseases

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including developmental abnormalities and cancers. The enzymology of DNA methylation including the family of DNA methyltransferases (DNMTs) is well established [26]. In contrast, proteins that demethylate 5-methylcytosine are not well defined and the mechanisms that revert methylation remained controversial [27]. We examined here the ability of *Fto* to reactivate methylation-inhibited promoter reporter gene and modulate transcription factor binding to DNA. Our results showed that FTO is a transcriptional coactivator and enhances transcription through methyl-inhibited DNA. The implications of these findings are discussed.

2. Materials and methods

2.1. Fto cDNA and C/EBP response-element reporter constructs

Full-length cDNA of murine *Fto* was obtained from American Type Culture Collection (ATCC), PCR-amplified and subcloned into either pGEX-6P-2 vector (GE Healthcare) or the expression vector pcDNA3-FLAG (Addgene), to generate either a glutathione-*S*-transferase (GST)-FTO fusion protein or an N-terminal FLAG tag-FTO hybrid, respectively. The GST-FTO hybrid was batched purified using GST beads and FTO was recovered after cleaving from the fusion protein using PreScission Protease (GE Healthcare). Oligonucleotides containing three tandem repeats of the C/EBP-response elements were ligated into pGL3-Luc reporter vector and sequence-verified to produce the reporter plasmid.

2.2. Plasmid methylation

HhaI restriction site is present in each of the C/EBP response element, seven in the coding region within the luciferase gene, and 19 more scattered in the remaining portion of the vector. CEBPRE plasmid was methylated *in vitro* using HhaI methyltransferase according to manufacturer's specification (New England Biolab), and extent of methylation assessed by resistance to HhaI endonuclease restriction. CEBPRE reporter demethylation was performed in the presence of 1 μg recombinant FTO in a reaction mixture of 50 mM TRIS–HCl, pH 7.5, 1 mM 2-oxoglutarate, 2 mM ascorbate, 75 μM ferrous ammonium sulfate [$(NH_4)_2Fe(SO_4)_2$], 50 μM BSA, 5 mM DTT, 1 mM MgCl2, and 150 mM NaCl, in 100 μl total volume, at 37 °C for 2 h. Reaction was terminated with 10 mM EDTA (final concentration) and plasmid recovered using the Plasmid Mini Kit (Qiagen). Isolated DNA was subjected to restriction analysis using HhaI endonuclease on 1% agarose gel. Alternatively, plasmid was methylated with radiolabelled *S*-adenosyl-L-[methyl-^{3}H]methionine, and demethylated by FTO as described above. Methylation was monitored by scintillation counting of eluted plasmid DNA and flow through from column.

2.3. Chromatin immunoprecipitations (ChIPs)

ChIPs were performed as before [28]. In brief, after formaldehyde crosslinking, nuclei isolated in a buffer containing 50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 0.25% TritonX, and protease inhibitors, were washed, lysed, immunoprecipitated, followed by five washes in RIPA buffer (50 mM HEPES, 500 mM LiCl, 0.1 mM EDTA, 1.0% NP-40 and 0.7% Na-deoxycholate) and once with TE containing 50 mM NaCl. Eluted immune complexes were subjected to real-time PCR analysis.

2.4. Cell culture and reporter assay

HEK293 cells (ATCC) were cultured at 37 °C in 5% CO_2 with DMEM (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen) and antibiotics accordingly. OP9 stromal preadipocytes (a gift of Dr. Perry Bickel, University of Texas Health Science Centre, Houston) were cultured and differentiated with 15% Knock-Out SR (Invitrogen) as described [29]. Transfection was performed in HEK293 and OP9 cells typically with 25 ng of reporter plasmids mixed with 10 ng of expression constructs for *Fto*, C/EBPs, and CBP using the LTX transfection reagent (Invitrogen). *Renilla* luciferase reporter was used as internal control and relative luciferase activities were determined 24 h following transfection and normalized to the *Renilla* luciferase activity.

2.5. Statistical analyses

Single factor ANOVA or Student's *t*-tests were used for all statistical analysis using PRISM (GraphPad Software, Inc.). P values *of less than or equal to 0.05* were considered statistically significant.

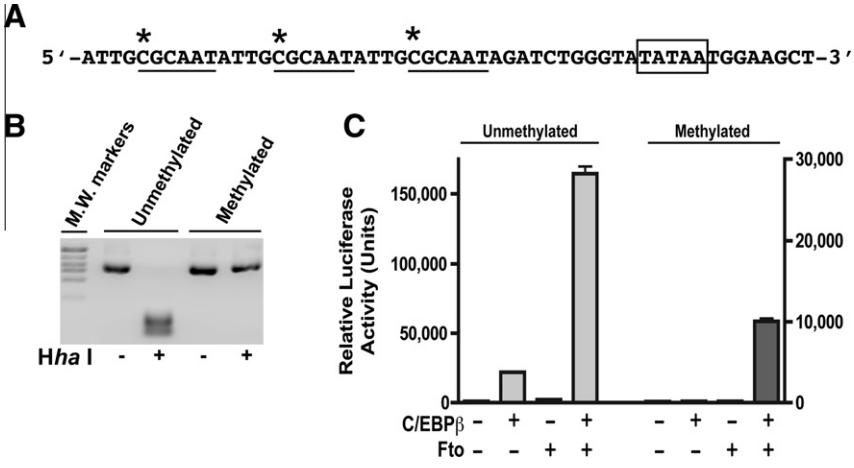

Fig. 1. *Fto* is a transcriptional coactivator. (A) Nucleotide sequence of the minimal promoter of CEBPRE reporter that includes a TATA binding site (boxed) and three C/EBP response elements (underlined) in tandem with each containing a CpG dinucleotide (asterisk) targeted for methylation by the HhaI methyltransferase (consensus site: GCGC). (B) Methylation status of the CEBPRE reporter plasmid assessed by HhaI endonuclease restriction analysis shows that *in vitro* methylated reporter was resistant to endonuclease cleavage. (C) *Fto* and C/EBPβ were ectopically expressed in OP9 preadipocytes by transfection and show that *Fto* synergistically coactivated C/EBPβ-mediated transactivation from the unmethylated CEBPRE luciferase reporter, and reactivated C/EBPβ transactivation from the methyl-inhibited reporter. All luciferase assays were performed in triplicates and statistics were conducted as student *t*-test. Results are means ± s.e.m., normalized to *Renilla* luciferase activity.

3. Results

3.1. Reactivation of methyl-inhibited reporter gene expression by Fto

To investigate *Fto* demethylase activity, we first examined its effect on the expression of a methylated cytomegalovirus (CMV) early promoter-driven luciferase reporter. The unmethylated reporter showed constitutive luciferase activity and methylation by HhaI DNA methyltransferase inhibited HhaI endonuclease restriction and luciferase expression (Fig. S1). Coexpression with *Fto*, but not the vector, reactivated the methyl-repressed reporter expression, thus suggesting that *Fto* may epigenetically regulate gene expression.

In light of the progressive and gradual loss of white adipose tissue in *Fto* knockout mice [19], it is conceivable that *Fto*-deficiency could disrupt adipogenesis. The C/EBPs and PPARγ transcription factors are the master regulators of adipogenesis, hence we considered the effect of *Fto* demethylase on their transactivation potential. Since PPARγ response elements, NNNAGGTCANAGGTCA [30,31], do not contain CpG dinucleotide, therefore, we focused on the transactivation of the C/EBP response element (CEBPRE)-driven promoter reporter, which contained three CEBPREs in tandem, each with a CpG site for methylation (Fig. 1A). The vector contained additional CpG sites. Methylation of CEBPRE-reporter inhibited HhaI cleavage (Fig. 1B) and C/EBPβ transactivation from the promoter (Fig. 1C). Expression of *Fto* alone had no effect on either the unmethylated or the methylated reporters. Cotransfecting C/EBPβ with *Fto* synergistically activated transcription from the unmethylated promoter. Albeit attenuated, *Fto* enhanced C/EBPβ transactivation from the methyl-inhibited promoter (Fig. 1C). Moreover, reactivation of C/EBPβ transactivation was markedly reduced with an *Fto* deletion mutant (Δ274–317), which lacked the Fe(II)- and carboxylate-binding residues (Fig. 2A and B). We further compared *Fto* to the transcriptional coactivator CREB binding protein (CBP), a histone acetyltransferase [32], and found that overexpression of CBP enhanced the transcriptional activity of C/EBPβ from an unmethylated-reporter, but failed to reactivate transcription from the methylation-inhibited reporter (Fig. 2C). These results were recapitulated with C/EBPα and δ (Fig. S2).

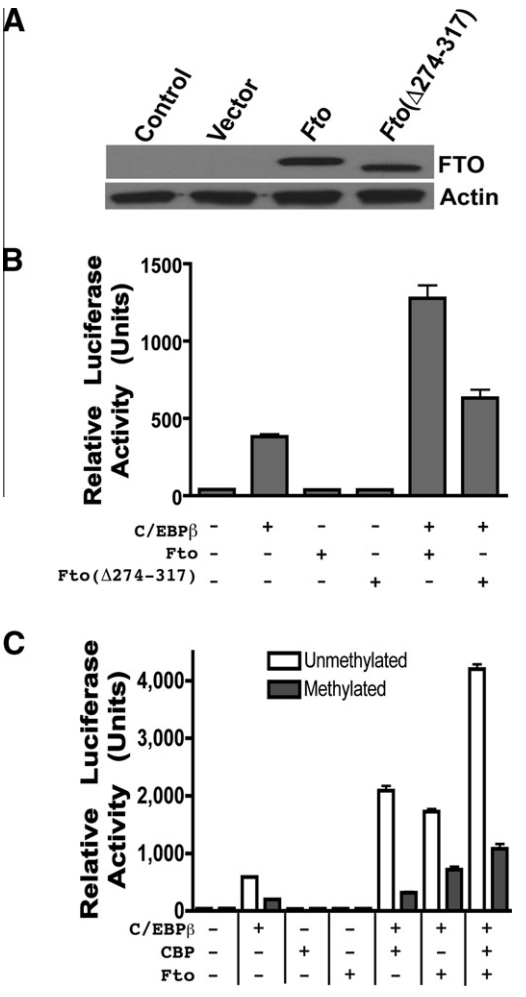

Fig. 2. Transactivation of CEBPRE reporter by mutant *Fto* and CBP. (A) OP9 preadipocytes were transfected with expression vector for FLAG-tagged *Fto* and *Fto*(Δ274–317). Immunoblot with anti-FLAG antibody shows the expression of FTO and FTO(Δ274–317). (B) OP9 preadipocytes ectopically expressing *Fto* and *Fto*(Δ274–317) show reactivation of CEBPRE reporter by *Fto* and attenuated reactivation by *Fto*(Δ274–317). (C) OP9 preadipocytes co-expressing either *Fto* or CBP with C/EBPβ shows CBP failed to reactivate methylation-inhibited CEBPRE reporter compared to *Fto*. All studies were performed in triplicates. Results are means ± s.e.m., normalized to *Renilla* luciferase activity.

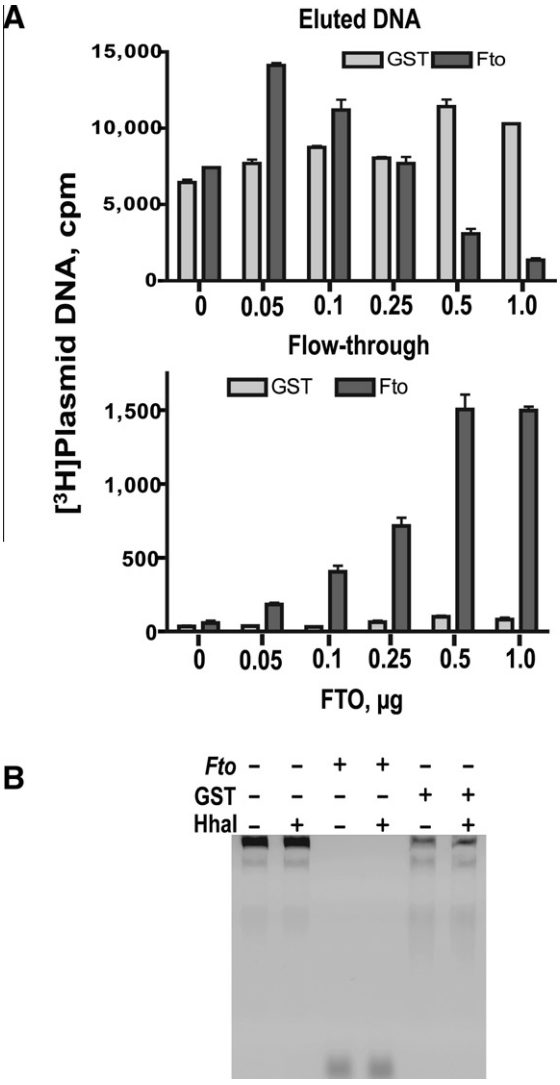

Fig. 3. FTO is a demethylase and endonuclease. (A) CEBPRE reporter plasmids were methylated and then incubated with purified FTO, followed by extraction with DNA affinity column as described in Experimental. Decrease in eluted [^{3}H] methylated-DNA and increase in [^{3}H]-count in flow through occur in an FTO dose-dependent manner. All studies were performed in triplicates and results are means ± s.e.m. (B) Purified FTO, but not GST, exhibits endonucleolytic activity and caused cleavage of methylated CEBPRE reporter.

3.2. Demethylation of 5-methylcytosine and DNA cleavage by Fto

These observations are intriguing given that FTO does not demethylate 5-methylcytosine *in vitro* [13]. The mechanism of 5-methylcytosine demethylation is unclear and base excision repair via the excision of methylated base by DNA glycosylases [33], or direct reversal of the methyl group by dioxygenases and photolyases has been suggested [17]. To determine if FTO demethylase targets 5-methylcytosine in intact cells, we methylated the CEBPRE reporter with radiolabelled coenzyme *S*-adenosyl methionine (SAM) and assessed the ability of FTO to demethylate the reporter by monitoring changes in the radiolabelled reporter plasmid retained on affinity purification cartridge. If FTO removes 5-methylcytosine by direct oxidative demethylation, plasmid DNA would remain intact and be retained on the cartridge. In contrast, base excision by FTO would result in DNA cleavage, producing DNA fragments due to the presence of multiple methylated sites on the plasmid that would elute from the cartridge into the flow through. Our results showed that ^{3}[H]-SAM-methylated CEBPRE reporter was resistant to HhaI (Fig. S3). Incubation with purified FTO caused a dose-dependent decrease in radiolabelled methylated plasmid with a concomitant increase in radioactivity in the flow-through from the purification column, whereas treatment with GST did not affect the incorporated radioactivity levels either in the eluted DNA or cartridge flow through (Fig. 3A). Further, treatment with FTO resulted in DNA cleavage but not with GST (Fig. 3B), thus suggesting an excision of 5-methylcytosine by FTO. It is also intriguing that both unmethylated and methylated reporters were susceptible to cleavage following exposure to FTO (Fig. S3).

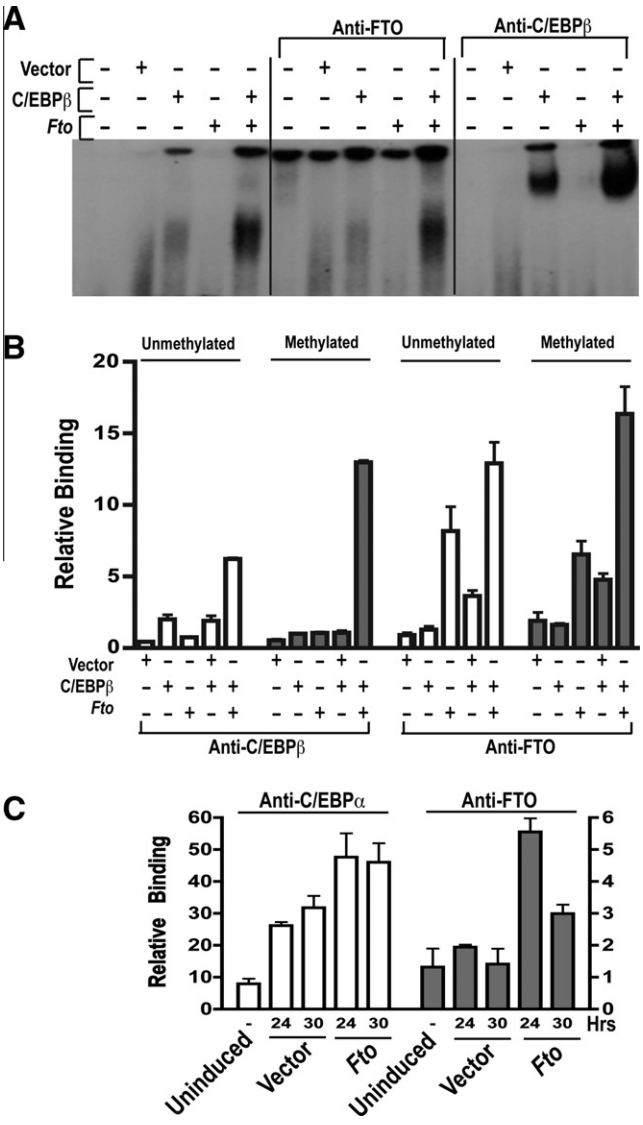

Fig. 4. FTO binding to unmethylated and methylated DNA. (A) EMSA using nuclear extracts from HEK293 cells transfected with either C/EBPβ or FLAG-*Fto* to monitor protein-DNA interaction using the CEBPRE oligonucleotides (Fig. 1A) shows FTO enhanced C/EBPβ binding to response elements that was supershifted with anti-C/EBPβ but not *Fto* antibody. ChIP analysis of: (B) HEK293 cells transfected with either unmethylated or methylated CEBPRE reporter plasmid, and in the presence or the absence of C/EBPβ and *Fto* as indicated; or (C) PPARγ gene promoter in C/EBPα and *Fto*-transfected OP9 preadipocytes undergoing differentiation at either 24 or 30 h compared to undifferentiated or vector transfected controls. ChIP analysis was performed in triplicates for C/EBPα and β, and FTO; and immunoprecipitates were normalized to β-actin; and results are means ± s.e.m.

3.3. Recruitment of Fto coactivator to target gene promoter

To characterize the transcriptional coactivation role of *Fto*, electrophoretic mobility shift assay (EMSA) using nuclear extracts from *Fto* and C/EBPβ transfected HEK293 cells showed that FTO did not bind to methylated-CEBPRE oligonucleotides, but enhanced C/EBPβ binding to the methylated-DNA. The DNA–protein complex was supershifted by an anti-C/EBPβ, but not anti-FTO antibody (Fig. 4A). ChIP assay confirmed that FTO increased the association of C/EBPβ with both unmodified and methyl-modified CEBPRE (Fig. 4B). Since C/EBPα and PPARγ positively regulate each other's expression in adipogenesis [34], we further demonstrated the association of *Fto* with C/EBPβ at the PPARγ gene promoter in OP9 preadipocytes undergoing differentiation, compared to an undifferentiated and vector transfected controls (Fig. 4C), thus suggesting that *Fto* is recruited to both unmethylated and methylated promoters and enhances C/EBPs binding to DNA.

4. Discussion

We show here that *Fto* is a transcriptional coactivator that facilitates transcription from unmethylated and also methyl-inhibited gene. In contrast, the ubiquitous transcriptional coactivator, CBP, fails to potentiate C/EBPs-mediated transactivation from the methyl-inhibited promoter, thus revealing the unique dual role of *Fto* to regulate non-epigenetic and epigenetic transcription.

The cleavage of DNA following incubation with FTO is curious because previous *in vitro* study under similar condition did not result in the degradation of oligonucleotides [13]. Moreover, the GST protein serving as control, purified in parallel with FTO under similar experimental conditions did not exhibit DNA cleavage. AlkB, ABH2 and ABH3 have been shown to directly reverse alkylation damage by oxidative demethylation of single-stranded DNA [16]. It is possible that the differences in the DNA templates used (single-stranded oligonucleotides versus double-stranded plasmid DNA) as well as substrate specificity (1-alkyladenine and 3-alkylcytosine versus 5-methylcytosine) may account for the differences in the observed activity of FTO in our study. Nevertheless, the DNA cleavage observed in our study is reminiscent of XPF and XPG, which are involved in nucleotide excision repair, serving to excise the modified or damaged nucleotides [35], and resulting in the appearance of small nucleolytic DNA fragments, thus further raises the possibility that 5-methylcytosine is excised from DNA in a step analogous to either nucleotide excision or base excision repair.

The precise mechanism of DNA demethylation is still controversial [36] and the notion that FTO demethylates CpG by excision is provocative. That FTO increases C/EBPs binding to and potentiates transcription from both unmethylated and methylated DNA also seems counterintuitive to its role as a demethylase. Such a mechanism is not inconceivable given that nucleotide excision

repair factors including XPB and XPD are also part of the basal transcription initiation machinery [37,38]; and moreover, transcription coupled repair of oxidative damage requires an XPG function distinct from its nucleotide excision repair endonuclease activity [39].

More likely, it is conceivable that the partially purified FTO may contain impurities including nucleases or the association and co-purification of DNA glycosylases with FTO that caused the observed DNA cleavage. Hence, further investigation with homogeneously purified FTO or its enzymatically inactive mutants will yield insights into the precise mechanisms of FTO as a transcriptional coactivator. Therefore, we speculate that *Fto* may serve as a transcriptional coactivator and also in recruiting DNA demethylase, a mechanism that is similar to XPB and XPD in DNA repair and transcription initiation. We envision that during epigenetic reactivation of gene expression, within the transcription initiation complex, *Fto* functions to recruit associated factors that recognize 5-methylcytosine, which then excises the methyl-modified base from the promoter. Following excision, cellular DNA polymerase and ligase presumably would fill in and seal the gap resulting the excision, thereby restoring the DNA for further cyclical epigenetic regulation.

In addition to the striking loss of adipose tissue, $Fto^{-/-}$ mice also exhibit an increase in energy expenditure that may be a result of elevated sympathetic activity. Therefore, it is possible that *Fto* may have a critical role in the central control of energy homeostasis but not adipogenesis. It has been shown that C/EBPβ and δ are expressed in the hypothalamus and paraventricular nucleus in the brain [40,41], which seemed to overlap with the localization of *Fto* [13]. Therefore, the notion that coactivation of C/EBPs by *Fto* that results in the dual regulation of energy homeostasis and adipogenesis cannot be ruled out.

It is also unclear how the present finding relates to the obesity-associated single nucleotide polymorphisms [11]. It is possible that these intronic genetic variants may influence the tissue-specific expression of FTO and differentially modulate satiety and adipogenesis in the brain and adipose tissues, respectively. Taken together, our results point to an epigenetic role for *Fto* as a transcriptional coactivator in regulating adipogenesis, and its absence causes decreased adiposity, which is consistent with the disappearance of white adipose tissue in *Fto*-deficient mice.

5. Conclusions

Our results revealed that *Fto* is a transcriptional coactivator for the C/EBP family of transcriptional regulator, thus providing the first evidence that *Fto* may play a role in the epigenetic regulation of adiposity. Hence targeting the *Fto* pathway may be a novel mechanistic approach for the development of anti-obesity pharmacotherapeutics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.064.

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