Fat mass and obesity-associated (FTO) protein interacts with CaMKII and modulates the activity of CREB signaling pathway

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Polymorphisms in the fat mass and obesity-associated (FTO) gene have been associated with obesity in humans. FTO is a nuclear protein and its physiological function remains largely unknown, but alterations in its expression in mice influence energy expenditure, food intake and, ultimately, body weight. To understand the molecular functions of FTO, we performed a yeast two-hybrid screen to identify the protein(s) that could directly interact with human FTO protein. Using multiple assays, we demonstrate that FTO interacts with three isoforms of calcium/calmodulin-dependent protein kinase II: α, β and γ, which are protein kinases that phosphorylate a broad range of substrates. This interaction is functional; overexpression of FTO delays the dephosphorylation of cAMP response element-binding protein (CREB) in human neuroblastoma (SK-N-SH) cells, which in turn leads to a dramatic increase in the expression of the CREB targets neuropeptide receptor 1 (NPY1R) and brain-derived neurotrophic factor (BDNF), which already are known to regulate food intake and energy homeostasis. Thus, our results suggest that FTO could modulate obesity by regulating the activity of the CREB signaling pathway.

INTRODUCTION

Data from the World Health Organization (WHO) indicate that at least 2.8 million people die each year as a result of being overweight or obese (http://www.who.int), due to the increased risk of several serious health conditions, particularly diabetes, hypertension, cardiovascular disease, breathing difficulties during sleep and some cancers (1,2). Although changes in lifestyle are a root cause of the increased rates of obesity throughout many populations, genetic variation influences the risk of obesity, and several genome-wide association studies have sought the identity of the culprit genes.

One consistent result of genetic association studies of body mass index (BMI) and obesity is the fat mass and obesity-associated (FTO) gene (3–5). This well-conserved gene is present in a single copy in vertebrates and is broadly expressed with particular abundance in the brain and hypothalamus (6–9). Data from mouse models support the role of FTO in the regulation of body mass. Global loss of Fto in mice leads to a significant reduction in weight and fat mass compared with wild-type mice. Although Fto null mice eat more than their wild-type counterparts, they also expend more energy (10). Mice that overexpress Fto, on the other hand, have increased bodyweight and fat mass resulting primarily from significantly increased food intake in the absence of changes in energy expenditure and physical activity compared with wild-type mice (11).

The physiological role of FTO is only beginning to be revealed. Bioinformatics analysis indicated that FTO is a member of the AlkB family of non-heme Fe (II)/2-OG-dependent oxidase DNA/RNA demethylases (12). N6-methyladenosine (m6A) is a major physiological substrate of FTO. FTO efficiently demethylates abundant m6A residues in RNA but has low activity to methylated thymidine (3-meT) and uracil (3-meT) (13). However, how m6A modification is linked to obesity remains to be determined.

In order to better understand the role of FTO in the regulation of body mass, we sought binding partners for FTO using yeast two-hybrid screens. We discovered an interaction between FTO and Calcium/calmodulin-dependent protein kinase II (CaMKII) by yeast hybridization that was confirmed by glutathione S-transferases (GST) fusion protein pull-down assays and by co-immunoprecipitation (co-IP). Furthermore, when

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we overexpress FTO in SK-N-SH cells, the interaction of FTO with CaMKII delays cAMP response element-binding protein (CREB) dephosphorylation, which, in turn, enhances the expression of neuropeptide receptor 1 (NPY1R) and brain-derived neurotrophic factor (BDNF). The RNA demethylase activity of FTO is at least partially required for this effect, as mutant FTO without this activity partially abolishes this induced expression. Overall, our study illustrates a new mechanism by which FTO influences obesity.

RESULTS

SKAR, CaMKIIγ, RFX2 and Dlgap3 are candidate interactors of FTO

To identify the interacting partners of Fto, a human brain cDNA library was screened with a pGBT9-Fto bait construct. Out of 22 positive clones that were sequenced, four candidates were identified: SKAR, RFX2, CaMKIIγ and Dlgap3 (Fig. 1A). In particular, CaMKII isoforms (both beta and gamma) were identified 16 times. To confirm their interactions with FTO, we made a GST-FTO fusion protein to see if it bound to the candidates in pull-down assays. The GST-FTO fusion protein was purified using glutathione-sepharose beads, then incubated with in vitro translated SKAR, CaMKIIγ, RFX2 or Dlgap3. GST-tag alone was included as a negative control. All four candidates bound to FTO in this assay (Fig. 1B), confirming the interactions suggested by the two-hybrid screen.

Interaction of CaMKIIγ and FTO in vivo identified by co-IP

Because the prior experiments were performed in vitro, we next sought to demonstrate that they occur in vivo, using co-IP experiments. Plasmids encoding a FLAG-tagged FTO and an hemagglutinin (HA)-tagged candidate interactor (SKAR, CaMKIIγ, RFX2 or Dlgap3) were co-transfected into human embryonic kidney 293 (HEK293) cells. Anti-FLAG beads were used for the IP, and the resulting proteins were eluted and blotted with an anti-HA antibody. Of the four candidate interactors, only CaMKIIγ was immunoprecipitated in conjunction with FTO. A reverse experiment in which an anti-HA antibody was followed by a western blot with an anti-FLAG antibody also indicated that CaMKIIγ was the only candidate protein that co-immunoprecipitated with FTO (Fig. 2A).

Multiple CaMKII isoforms interact with FTO

CaMKII has two additional isoforms, CaMKIIα and β. To examine the breadth of interaction between CaMKII and FTO, additional co-IP experiments were performed. The coding region for each isoform was cloned into a pCDNA3 vector, which adds a Myc tag to the inserted sequence. Plasmids encoding FLAG-tagged FTO and a Myc-tagged CaMKII isoform were co-transfected into HEK293 cells. FTO was immunoprecipitated using anti-FLAG beads, and immunoblotting of the resulting precipitate was performed with antibody to the Myc tag. The reverse experiment was carried out by immunoprecipitating with the anti-Myc antibody, followed by immunoblotting with the anti-FLAG antibody. These experiments consistently indicated that all three forms of CaMKIIα, β and γ co-IP with FTO (Fig. 2B).

CREB phosphorylation is regulated by FTO

To investigate the consequences of the interaction of FTO with CaMKII, we first explored its role in phosphorylation status of the two interacting proteins. CaMKII did not phosphorylate full-length recombinant mouse Fto (mFto) nor the C-terminal domain (327–502 amino acids) alone (Fig. 3A). Neither did...
excess full-length recombinant mFto affect CaMKII phosphorylation activity significantly (Fig. 3B). Thus, we turned our attention to another known substrate of CaMKII, CREB. We assayed CREB phosphorylation in human neuroblastoma (SK-N-SH) cells in which FTO was overexpressed. A lentivirus containing Fto was prepared in HEK293 cells and used to infect SK-N-SH cells. Forty-eight hours after infection, the cells were serum-starved for 24 h, then stimulated with 20 μM of forskolin for different time periods to stimulate neuronal differentiation. Phosphorylation of CREB was measured by western blotting. An increase in CREB phosphorylation was observed from the initial time point of 5 min of forskolin stimulation (Fig. 4). The effect of forskolin on CREB phosphorylation exhibited different kinetics in the cells infected with lenti-Fto compared with the lenti-GFP control. Whereas in the GFP expressing cells, CREB phosphorylation was weakened with 30 min of forskolin stimulation, it did not begin to go down until 60 min of forskolin stimulation in Lenti-Fto infected cells (Fig. 4). These results suggest that overexpression of FTO delays the dephosphorylation of CREB.

Furthermore, to determine whether FTO-mediated modulation on CREB phosphorylation depends on the FTO demethylation activity, we performed parallel analyses using a previously described mutant FTO protein in which two iron (II) ligands were mutated (H231A, D233A), resulting in complete loss of m6A-demethylation activity (13). We found that the mutant FTO lacking m6A-demethylation activity did not delay CREB dephosphorylation to the same extent as wild-type FTO, suggesting that the m6A-demethylation activity of FTO is involved in the FTO-mediated modulation of CREB phosphorylation.

Expression of BDNF and NPY receptor 1 are increased in FTO-overexpressing cells

CREB phosphorylation can either stimulate or inhibit its target genes by binding to the regulatory CREs (14). Because we observed that the dephosphorylation of CREB was delayed in the presence of excess FTO, we next asked whether the expression levels of known CREB target genes were affected. Using real-time quantification PCR, we examined three known CREB target genes: NPY, NPY1R and BDNF. Whereas the expression levels of NPY1R and BDNF were significantly increased after 30 min of forskolin stimulation in Lenti-Fto infected cells, expression levels of CREB and NPY were unchanged (Fig. 5). Differences in the activation of NPY1R and BDNF expression between Lenti-GFP and Lenti-Fto infected cells are likely a result of the delayed dephosphorylation of CREB that was demonstrated in the previous experiments. Consistent with this notion, the expression of mutant Fto induced significantly less expression of NPY1R and BDNF after 30 min of forskolin stimulation (Fig. 5).

DISCUSSION

The association of FTO with obesity is well established. The initial report identified a single-nucleotide polymorphism
SNP) in the first intron of FTO (rs9939609) that was strongly associated with type 2 diabetes and increased BMI in a UK population (7). Following this report, associations between additional SNPs in FTO and human BMI have been reported in various populations (3,15–17). The link between FTO risk alleles and obesity has been supported by both loss-of-function and overexpression mouse models (10,11). The identification of m6A residues in RNA as a physiological target that is oxidatively demethylated has increased our understanding of the activity of this protein (13), but the mechanism by which this influences obesity has largely been unknown. We report here a functional pathway that links FTO with obesity: FTO → CaMKII → CREB → NPY. Increased FTO expression delays CREB dephosphorylation by interacting with CaMKII, resulting in the increased gene expression of NPY1R and BDNF. These two proteins, in turn, are already known to regulate food intake and energy homeostasis, thereby connecting the pathway with BMI and obesity.

CaMKII is a serine/threonine protein kinase family encoded by four genes (α, β, γ and δ) in mammals (18). CaMKIIα and β are highly expressed in neurons (19–22). CaMKII is regulated by multiple phosphorylation sites that alter its enzymatic activity and induce its interaction with other proteins (23). CaMKII phosphorolytates a broad range of substrates, which are involved in numerous physiological pathways, including cell development, proliferation, cellular transport and neuronal function (24–28).
CaMKII has also been reported to regulate hepatic glucose homeostasis in obesity (29).

Interactions between signaling molecules and various protein-binding partners are critical for cells to respond appropriately to their physiological status. CaMKII is an essential cellular kinase that binds to and phosphorylates a variety of targets in response to stimuli. Numerous binding proteins of CaMKII have been identified via binding or IP assays. For example, CaMKII binds to and phosphorylates tau and amyloid precursor protein, which are key proteins in the pathogenesis of Alzheimer’s disease (AD) (30,31). At the same time, CaMKII auto-phosphorylation is altered in the brains of AD patients (32). CaMKII also binds to the cyclin-dependent kinase 5 activators p35 and p39 (33), suggesting that CaMKII is involved in a signaling pathway that contributes to synaptic plasticity, memory and learning. Targets of CaMKII may achieve a functional effect in one of two ways; the interaction might alter the kinase activity of CaMKII through an allosteric mechanism, or it might direct CaMKII to its substrates, thereby leading to faster and more efficient phosphorylation of key proteins. In our case, we found that FTO did not alter the kinase activity of CaMKII. Therefore, a plausible explanation is that binding of FTO to CaMKII induces CaMKII to phosphorylate CREB, in that way resulting in the prolongation of CREB phosphorylation.

CREB is a transcriptional activator and promotes adipogenesis by regulating glucose homeostasis (34). It has been reported that CREB can be phosphorylated by CaMKII (35,36). Phospho-CREB, by binding to CRE, leads to stimulation or inhibition of target genes, such as NPY and BDNF, indicating that CREB is a regulator of NPY (37). NPY is a 36-amino acid peptide with widespread distribution in the brain (38–41). It mediates food intake and decreases physical activity by acting through its receptors (40,42–44). Five known NPY receptors (Y1 through Y5) have been found in mammals (45–47). It has been shown that hypothalamic NPY expression is up-regulated by fasting, and the blockade of NPY receptors leads to the inhibition of NPYergic activity, and a resulting reduction in food intake (48–51). BDNF also is regulated by CREB. BDNF is a member of the neurotrophin family and is widely expressed throughout the central nervous system and skeletal muscles (52–55). BDNF plays a critical role in normal neural development (56–61); knockout of BDNF in mice results in developmental defects in the brain, and the mice die soon after birth (62). BDNF regulates energy homeostasis through binding to tropomyosin-related kinase B receptor (63–67). Central injection of BDNF gradually reduces food intake and causes body weight loss in rats (68,69). Genome-wide associated studies found that SNPs in BDNF are associated with BMI and obesity (70,71).

In our study, mRNA levels of NPY1R and BDNF were significantly increased in FTO-overexpressing cells. The time at which NPY1R and BDNF mRNA levels are maximal matches perfectly with the prolongation of CREB phosphorylation that occurs in conjunction with FTO overexpression. Thus, we suggest that expression levels of NPY1R and BDNF are affected by CREB through NPY and that FTO plays a role in obesity by regulating the CREB signaling system. The RNA demethylase activity of FTO contributes to this activity but does not fully explain the effects we see; a mutant form of FTO has an intermediate effect on CREB phosphorylation and induction of NPY1R and BDNF expression compared with overexpression of wild-type FTO and to the control samples.

In sum, we propose a cellular mechanism by which FTO functions in obesity. The interaction of FTO with CaMKII triggers the prolongation of CREB phosphorylation, which ultimately affects the expression levels of at least two key genes that
regulate energy balance, BDNF and NPY1R. These results provide new targets for the development of therapeutic approaches to obesity.

MATERIALS AND METHODS

Plasmid construction

Full-length mFto complementary DNA was cloned using mouse brain cDNA library. The coding region was then cloned into the pGEX-2TK vector or the pCDNA3 vector or the Lenti-GFP vector (with Flag tag). The full-length Fto coding region with mutation at H231A and D233A (kindly provided by Dr Chuan He at University of Chicago) was cloned into the Lenti-GFP vector. Full-length cDNAs of SKAR, CaMKIIγ, RFX2 andDlgap3 were cloned into the pCDNA3 vector (with HA tag). Full-length CaMKIIβ, β and γ cDNAs were cloned into the pCDNA3 vector (with Myc tag). The sequences of all constructs were confirmed by DNA sequencing.

Yeast two-hybrid screen

The full-length mFto cDNA was fused in frame with the GAL4 DNA-binding domain of the pGBT9 vector to generate pGBT9-Fto bait construct. Yeast two-hybrid screening was provided by Duke University Genomic Core. Briefly, PGBT9-Fto construct was co-transfected with human cDNA keratinocyte libraries in Saccharomyces cerevisiae. Positive clones were identified by TRP1 selection at 0 mM 3-aminotriazol. All positive clones were sequenced by Sanger sequencing. The details of the pGBT9-Fto bait construct and yeast two-hybrid screening protocols are available upon request.

GST fusion protein purification and GST pull down assay

BL21 (DE3) pLysS Competent Cells (Promega) were transformed with GST constructs (both GST and GST-Fto), and single colonies were grown in 10 ml of Lysogenic Broth (LB)/ampicillin (100 μg/ml) overnight at 37°C. 500 ml of LB were inoculated with 10 ml of overnight culture at 37°C with shaking for ~2 h until absorbance at 600 nm reached between 0.6. Culture was induced with 16 μM isopropyl-β-D-1-thiogalactopyranoside and grown at 28°C for 6 h. Cells were pelleted by centrifugation at 3000 g for 15 min and washed twice with ice-cold 1× phosphate buffered saline (PBS). Cells were resuspended in 1 ml of ice-cold Tris-buffered saline and 1 ml of cold 1× phosphate buffered saline (PBS). Cells were resuspended in 1 ml of ice-cold Tris-buffered saline and 1 ml of Profound Lysis buffer (Pierce) with 1× Complete ethylene-diamine-tetra-acetic acid (EDTA)-free protease inhibitor complex (Roche) and inverted to mix immediately. The cellular resuspension was incubated on ice for 30 min. Lysate was centrifuged at 12 000g for 10 min. Supernatant was collected and stored at -80°C until use. Glutathione-sepharose beads (Amersham-Pharmacia) were washed and resuspended in 1× PBS, to make a 50% bead slurry. 200 μl of bead slurry was incubated with 200 μl bacterial lysate for 1 h at 4°C. Beads were washed twice with 1× PBS with 0.5% Triton X-100. In a 1.5-ml tube, 200 μl of GST/or GST-FTO beads were incubated with 22 μl of the appropriate in vitro translated protein (SKAR, CaMKIIγ, RFX2 or Dlgap3; TnT Coupled Reticulocyte Lysate Systems, Promega) diluted with 180 μl of ice-cold binding buffer (1× PBS; 0.2% Triton X-100; Protease Inhibitor) at 4°C for 2 h. Beads were then washed twice with 1 ml of binding buffer. The beads were resuspended with protein loading buffer and denatured at 100°C for 5 min. Supernatants were separated by 6% polyacrylamide gel electrophoresis (PAGE) for analysis.

Co-immunoprecipitation

HEK293 cells were cultured and transfected with 10 μg of the each relevant plasmid using Lipofectamine 2000 (Invitrogen) on 10-cm plate, according to the instructions of the manufacturer. After 48 h, cells were harvested and lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol and 0.5% Triton X-100 supplemented with complete protease inhibitor cocktail]. Lysates were kept on ice for 30 min and cell debris was removed by centrifugation. Following the addition of 50 μl Protein A Agarose (Invitrogen), supernatants were incubated with mouse monoclonal anti-myc or anti-HA antibody overnight at 4°C. [Alternatively, supernatants were incubated with anti-FLAG beads (Sigma).] The beads were pelleted by centrifugation; the supernatant was removed, and the pelleted beads were washed three times with 1 ml of washing buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5% glycerol and 0.1% Triton X-100]. Proteins were eluted with 50 μl of mouse protein loading buffer for 5 min at 100°C. Inputs and co-IPs were subjected to sodium dodecyl sulfate–PAGE and transferred to polyvinylidene difluoride membranes (Millipore).

Western blotting

For analysis of the IPs, western blots carrying inputs and immunoprecipitated samples were first incubated with primary antibody (mouse anti-HA or mouse anti-FTO or mouse anti-MYC) followed by appropriate horseradish peroxidase-conjugated antibodies. Membranes were processed following the HyGLO Quick Spray western blotting protocol (Denville). For loading controls, membranes were stripped and re-probed with the antibody against GAPDH (Ambion).

CaMKII activity assay

CaMKII activity assay was performed using CaM Kinase II Assay kit (Upstate) following the manufacturer’s instructions. To determine whether CaMKII can phosphorylate FTO, the substrates were used 25 μM of purified recombinant full-length mouse FTO or the C-terminal domain of mouse FTO. Bovine serum albumin (BSA) was used as a negative substrate. The positive control was a control peptide from the kit. HEK293 cells were transfected with Myc-CaMKIIα, β and γ and cells lysate were immunoprecipitated with an anti-MYC antibody. The Protein A Agarose and corresponding IPs were added to the reactions. To determine whether FTO can affect CaMKII phosphorylation activity, the IPed Protein A Agarose and 100 ng of recombinant full-length mouse FTO or BSA were added to the reactions. Peptide from the kit was used as the substrate.

Virus infection and forskolin treatment

Lenti-GFP, Lenti-Fto and Lenti-mutant-Fto viruses were packaged according to the method described previously (72).
SK-N-SH cells were cultured and infected 72 h by Lenti-GFP or Lenti-Fto viruses. After starving for 24 h in FBS free medium, cells were treated with 20 μM of forskolin for 0, 5, 15, 30, 60 and 180 min, then media were removed and cells were harvested in ice-cold PBS and separated into two parts, one for total RNA purification and another one for western blotting.

**Reverse transcription and real-time PCR**

Total RNAs were purified using the standard TRIzol method (Invitrogen). Reverse transcription was carried out using SuperScript III Reverse Transcriptase (Invitrogen) according to the instructions of the manufacturer. Messenger RNA quantities were determined via the CT method (74). All relative quantities were calibrated to control samples. Three biological replicates were assayed.

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**Conflict of Interest statement.** None declared.

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