Regulation of Fto/Ftm gene expression in mice and humans

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HERITABILITY OF ADIPOSITY, which reflects genetic contribution to the phenotype within a specific environment, is high, and it is variously estimated at 40–60% (28, 49). The search for the underlying genes for obesity—using conventional linkage, association, and candidate gene approaches—has generated a large number of positive findings, many of which have not been replicated (e.g., 19, 25, 32, 37, 55, 67). Among the reasons for lack of consistent replication may be the relatively small population sizes, few markers genotyped, and blunt phenotypes. The recent generation of high-density single nucleotide polymorphism (SNP) and haplotype maps (International HapMap project; http://www.hapmap.org/) has revolutionized the field of human quantitative genetics. Applied to large, suitably phenotyped groups of subjects, whole-genome association studies (GWAS) are implicating novel genes not previously considered based on extant understanding of the molecular physiology of specific phenotypes. The discovery of the “Fat Mass and Obesity Associated gene” (FTO) as a potentially important contributor to human adiposity is such an example.

In two GWAS involving a total of ~42,000 obese and nonobese subjects, dose-dependent highly significant effects of specific SNPs on chr. 16 have been associated with increased body mass index (BMI) (14, 52). In agreement with these results, Dina et al. (8) identified an association between rs1121980 and morbid obesity (BMI ≥ 40 kg/m²) in 8,000 individuals of European ancestry and replicated their findings in another cohort of 4,864 obese and nonobese subjects. Follow-up studies of relatively smaller cohorts confirmed association of the same region with obesity in German and Belgian children and adults (21, 46). However, no significant association was detected in Chinese and oceanic populations (27, 44).

The interval containing the associated SNPs spans ~30 kb and extends by linkage disequilibrium (LD) to a ~47-kb region containing parts of introns 1 and 2 and exon 2 of FTO (Fig. 1). The molecular function(s) of FTO, and the mechanism(s) by which these allelic variations convey effects on adiposity is(are) not clear.

The transcriptional start of RPGRIPL1 (human ortholog of mouse Fm in the human, here referred to as FTM) is ~3.4 kb upstream of FTO in humans (Fig. 1). By virtue of their close proximity to SNPs strongly associated with adiposity (e.g., rs9939609; 13), FTO or FTM, or both, may account for the association of this genetic interval with differences of adiposity in humans.

FTO was originally cloned in the mouse (48) and is part of a contiguous gene deletion in murine Fused toes (64), which has a 1.6-Mb deletion of chr. 8 also containing Ftm, Ft1, and the Iroquois B cluster consisting of Irx3, 5 and 6 (47) (Fig. 1). Homozygous mutants are embryonic lethal and display neural tube defects, left-right asymmetry (16, 20), and polydactyly (17). Homozygous for the Fused toes deletion also have defects in both anteroposterior and dorsoventral patterning of the brain, including a reduction in the size of the hypothalamus that could be due to effects on sonic hedgehog (SHH)-related pathways (2). Heterozygous mice are not obese but have fused digits, as well as hyperplasia of the thymus, possibly because of apparent impairment of programmed cell death (64). In humans, a de novo duplication of the region on chromosome 16q12.2 that includes FTO, FTM, FT1, RBL2 (retinoblastoma-like2) and NET1 [Solute carrier family 6 (neurotransmitter transporter, noradrenaline), member 2] is associ-
ated with anisomastia, somatic dysmorphisms, mental retardation, and obesity (56).

Left-right asymmetry, neural tube patterning, and floor plate defects in the Fused toes mutant may be caused by the absence of Ftm, a regulator of SHH signaling expressed at the basal body of cilia (65). Adult mice lacking cilia throughout the central nervous system and, more specifically, proopiomelanocortin (POMC) neurons display increased food intake that leads to obesity (11). Ftm is homologous to RPGRIP1 (RPGR-interacting protein 1). Mutations of RPGRIP1 result in retinitis pigmentosa in humans (36) due to degeneration of photoreceptor cells, possibly resulting from dysfunction of retinal cilia. (22, 45). In Bardet-Biedl syndrome, a syndromic form of human obesity associated with polydactyly, retinal degeneration, and renal malformations, derangements of ciliary function have been implicated (39).

To further evaluate whether FTO or FTM might be responsible for the strong association of this genetic region with human adiposity and to assess the molecular physiology of an expression in mouse and human genomes. Single nucleotide polymorphisms (SNPs) associated with body mass index (BMI) are indicated.

MATERIALS AND METHODS

Mouse strains. Lep<sup>ob</sup> (B6.N-Lep<sup>ob</sup>/J), Lepr<sup>eb</sup> (B6.Cg-m +/+ Lepr<sup>eb</sup>/J), Cpe<sup>mut</sup> (B6.Cg-Hrs(BKS)-Cpe<sup>mut</sup>/J), tub (B6.Cg-Tub<sup>mut</sup>/J), A<sup>V</sup> (B6.Cg-A<sup>V</sup>/J), and control +/+ (C57BL/6J) male mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 4 wk of age and killed within 1 day of their arrival. At the Jackson Laboratory, and our laboratory, mice were fed regular chow (6% kcal from fat; NIH 31 6%; Purina Mills, St. Louis, MO). C57BL/6J male diet-induced obese (DIO) mice were raised at the Jackson Laboratory. At 4 wk of age, DIO mice were fed chow containing 10% kcal from fat (cat no. D12450B1; Open Source Diets) for 2 wk, and then switched to high-fat chow (60% kcal from fat; cat no. D12492; Open Source Diets) for an additional 12 wk. DIO control mice were fed chow containing 10% kcal from fat (cat no. D12450B1; Open Source Diets) at 4 wk of age for a period of 14 wk at the Jackson Laboratory. DIO and DIO control mice were killed at 18 wk of age, upon arrival. All mutant mice were killed at 4 wk of age to minimize possible secondary effects of obesity on Fto/Ftm expression.

Fasted mice were not fed for 40 h. For the thermal challenge experiments, mice were placed singly in a 4°C cold room for 30 min with ad libitum access to food and water.

Room temperature was constant at 21°C (unless otherwise stated) on a 12:12-h light-dark cycle (lights were turned off at 7 PM). Mice had ad libitum access to food and water. All protocols were approved by the Columbia University Institutional Animal Care and Use Committee.

Body mass and composition measurements. To examine possible secondary effects of body composition on Fto/Ftm gene expression, body composition of the mice used in these studies was determined by time-domain (TD)-NMR using a Minispec Analyst AD lean fat analyzer (Bruker Optics, Silberstreifen, Germany); the TD-NMR was calibrated according to the manufacturer’s directions. The phenotypes were comparable to those reported in the literature (Table 1). In mice fasted for 40 h, there were anticipated differences in responses of body weight and composition. Wild-type C57BL/6J mice lost ~50% of their total fat mass (P < 0.02). Fasted Lep<sup>ob</sup> mice lost from 5 to 15% of their total fat mass (P < 0.05). Both Lepr<sup>eb</sup> and +/+ mice lost ~20% of their total lean mass during the 40-h period of food restriction (P < 0.01).

Isolation of total RNA and cDNA synthesis. All mice were killed between 2 and 4 PM. All tissues were dissected and immediately flash frozen in liquid N<sub>2</sub>. Each tissue was crushed into a fine powder in a 1.5-ml Eppendorf tube in the presence of liquid N<sub>2</sub> and subsequently lysed in 1 ml of Qiazol (Qiagen, Valencia, CA). Total RNA was extracted and DNase-treated using the RNeasy Lipid Tissue Mini Kit and RNase-free DNase (Qiagen) according to the manufacturer’s instructions. cDNA synthesis from 2.8 µg of total RNA was performed at 42°C for 55 min using the SprintPowerScript PrePrimed Single Shots kit (Clontech, Mountain View, CA) with Oligo (dT)18.
Table 1. Body composition

<table>
<thead>
<tr>
<th>Mouse (n = 5)</th>
<th>Age, wk</th>
<th>Body Weight, g</th>
<th>Fat, g</th>
<th>Lean Mass, g</th>
<th>%Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lep&lt;sup&gt;ob&lt;/sup&gt;</td>
<td>4</td>
<td>24.2 (0.9)</td>
<td>9.4 (0.8)</td>
<td>13.1 (0.3)</td>
<td>39 (2.1)</td>
</tr>
<tr>
<td>Lep&lt;sup&gt;db&lt;/sup&gt;</td>
<td>4</td>
<td>24.5 (2.2)</td>
<td>8.5 (1.3)</td>
<td>14.1 (0.8)</td>
<td>35 (2.7)</td>
</tr>
<tr>
<td>Tub</td>
<td>4</td>
<td>27.0 (0.8)</td>
<td>2.8 (0.3)</td>
<td>22.8 (0.9)</td>
<td>10 (1.2)</td>
</tr>
<tr>
<td>+/+</td>
<td>4</td>
<td>20.5 (1.9)</td>
<td>2.8 (0.5)</td>
<td>17.2 (2.1)</td>
<td>13 (1.4)</td>
</tr>
<tr>
<td>DIO</td>
<td>18</td>
<td>37.0 (2.9)</td>
<td>12.9 (0.91)</td>
<td>21.1 (1.7)</td>
<td>35 (2.4)</td>
</tr>
<tr>
<td>DIO control</td>
<td>18</td>
<td>30.0 (2.0)</td>
<td>7.0 (0.7)</td>
<td>20.1 (1.0)</td>
<td>23 (2.2)</td>
</tr>
</tbody>
</table>

Values are expressed as means (SD). Fat and lean mass and fractional fat content of Lep<sup>ob</sup>, Lep<sup>db</sup>, Cpe<sup>visu</sup> tub, and +/+ (C57BL/6J) mice measured by TD-NMR. Body composition data for the diet-induced obese (DIO) mice and their controls were obtained from The Jackson Laboratory (Bar Harbor, ME; personal communication).

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Four-week-old C57BL/6J or Lep<sup>ob</sup> male mice were perfused with 4% paraformaldehyde, and 13.5 days post coitum (dpc)-old +/- or Lep<sup>ob</sup> embryos were fixed overnight in 4% paraformaldehyde. Tissues were excised, dehydrated in 30% sucrose, frozen, and placed on slides.
as 10-μm medial coronal hypothalamic, sagittal pancreatic, or medial sagittal embroyonic sections.

Probes for in situ hybridization of embryonic and adult tissue were made by amplifying a 792-bp fragment from hypothalamic cDNA of C57BL/6J mice spanning Fto exons 2-4: 5'-CAGGTGGTTCCTTAC- CTTGACC5C, 5'GCTTGGTGAAGAGGATCGATATCCTGTG and a 736-bp fragment spanning Ftm exons 2-6: 5'-GAGGATCGCGATTGAGTACACAGG and 5'-GCCGTCGTTCTGGAAGCAGGAG.

Each DNA fragment was cloned in the Dual Promoter PCR II-TOPO Vector provided with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s specifications. Sense and antisense riboprobes labeled with Digoxigenin (DIG; Roche Diagnostics, Indianapolis, IN) were used. In situ hybridization was performed as previously described (7).

Chromatin immunoprecipitation assay. Primary fibroblast cells (~4×10^6) from human skin heterozygous for rs8050136 (A/C), and rs17817449 (T/G) (Fig. 1) were fixed with formaldehyde, lysed, and sonicated using the chromatin immunoprecipitation (ChIP) assay kit (Millipore, Billerica, MA), according to the manufacturer’s specifications. Halt protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL) were used accordingly to the manufacturer’s specifications. Protein-DNA complexes were incubated with mouse CUTL1 (Advanced Business Research, Carmel, IN), mouse HES1 (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse IgG (Advanced Business Research) antibodies at a 1:400 dilution, and incubated with Protein A (Clontech, Mountain View, CA) or IgG-DNA complexes were reverse cross-linked, and the unbound protein was digested following the protocol provided with the ChIP assay kit (Millipore). The DNA fragments were purified using the QIAquick PCR purification kit (Qiagen). Transcription detection was performed by qPCR in LightCycler 2.0 (Roche Diagnostics) according to the manufacturer’s specifications. Samples were heated at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 55°C for 10 s, 72°C for 15 s. Each 20-μl reaction contained 1× LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) using the recommended amount of the SYBR Green I mix. The following primers were used: rs17817449, 5'-CGTGATTTGATTTCCTTTTGCG and 5'-biotin-GCATTCCATGAGTCCATCTCTACAG; rs17817449, 5'-AGGTCAGGTTCCTCCCTAAACTGG, and 5'-biotin-GGAGTGCCACAAATTCGACC.

The Institutional Review Board at Columbia University Medical Center approved the study, and the participants gave written informed consent.

Pyrosequencing. Pyrosequencing was performed as previously described (35). In place of magnetic streptavidin beads, streptavidin coated multiple post hoc testing of differences of means. Levels of statistical significance were set at two-tailed P < 0.05. In all of the figures, error bars show SD.

RESULTS

Comparison of Fto and Ftm expression in various tissues of C57BL/6J (+/+ and −/−) mice. Fto transcript levels were higher than Ftm in all tissues tested (Fig. 2). Fto expression was approximately six-fold higher than Ftm in the hypothalamus (P < 0.001). Expression of Fto in the hypothalamus was approximately two-fold (P < 0.001) and ~25-fold higher (P < 0.001) than mesenteric fat and brown adipose tissue, respectively. Expression levels of Ftm in the hypothalamus and mesenteric fat were at least approximately twofold higher (P < 0.01) than all other fat depots and the liver. Fto expression in the pancreas

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Fig. 2. Comparison of Fto and Ftm expression in various tissue types. Fto and Ftm expression quantified by qPCR in the hypothalamus, mesenteric fat, pancreas, liver, subcutaneous fat, epididymal fat, perirenal fat, and brown adipose tissue from 4-wk +/-C57BL/6J mice (n = 5). Transcript levels were normalized to Gapdh.

CAGCAACTCTAAACCAGGG and 5'-GTAAACATGGGATGTG- GGTTTCTGCTAC.

The same protocol was followed for the assessment of CUTL1 expression. The following CUTL1-specific primers were used to amplify part of exon 18 and 20 and the whole of exon 19: 5'-CTACATGTACAGGAGTGGACACCATG and 5'-CTGCTCGAGAGGCCCAGCAGGAT.

Statistical analysis. Group data are expressed as means ± SD. Statistical analyses were performed using ANOVA and ANCOVA (StatView 5.0, SAS Institute or STATISTICA ver. 6; StatSoft, Tulsa, OK). Where appropriate, P values were Bonferroni corrected for multiple post hoc testing of differences of means.

Comparison of Fto and Ftm expression in various tissues of C57BL/6J (+/+ mice). Fto transcript levels were higher than Ftm in all tissues tested (Fig. 2). Fto expression was approximately six-fold higher than Ftm in the hypothalamus (P < 0.001). Expression of Fto in the hypothalamus was approximately two-fold (P < 0.001) and ~25-fold higher (P < 0.001) than mesenteric fat and brown adipose tissue, respectively. Expression levels of Ftm in the hypothalamus and mesenteric fat were at least approximately twofold higher (P < 0.01) than all other fat depots and the liver. Fto expression in the pancreas
was approximately twofold higher \((P < 0.02)\) than \(Ftm\) expression. By in situ hybridization, \(Fto\) was highly expressed throughout the hypothalamus, but it was relatively higher in the arcuate nucleus (Fig. 3A), while \(Ftm\) expression was restricted to the arcuate nucleus. These findings are consistent with comparable data available in the Allen Brain Atlas (http://www.allenbrainatlas.com). In the pancreas, by in situ hybridization, both \(Fto\) and \(Ftm\) expression was restricted to the islets (Fig. 3B).

Effects of \(Lep^{ob}\), \(Lepr^{db}\), and \(A^{y}\) mutations on \(Fto\) and \(Ftm\) expression. \(Fto\) and \(Ftm\) expression were decreased by approximately two- to three-fold \((P < 0.01)\) in the mesenteric fat and liver of 4-wk-old \(A^{y}\) and \(Lep^{ob}\) mice compared with the \(+/+\) controls (Fig. 4A, B). In subcutaneous, epididymal, and renal and brown fat tissues, \(Fto\) and \(Ftm\) expression followed a similar trend. In the pancreas, \(Fto\) and \(Ftm\) expression did not differ between lean and obese mice (Fig. 4). By in situ hybridization, \(Fto\) and \(Ftm\) expression in sections of \(Lep^{ob}\) pancreata was comparable to that of \(+/+\) mice (data not shown) and was also limited to the islets. As anticipated, expression levels in \(Lepr^{db}\) mice were comparable to those in \(Lep^{ob}\) (Fig. 5B).

Effects of \(Cpe^{fat}\) and \(tub\) mutations and DIO on \(Fto\) and \(Ftm\) expression. \(Fto\) and \(Ftm\) expression was assessed in mesenteric fat, liver, subcutaneous fat, and hypothalamus of 4-wk-old \(Cpe^{fat}\), \(tub\), and DIO mice. Expression of \(Fto\) and \(Ftm\) was reduced approximately twofold in the mesenteric fat of \(Cpe^{fat}\) and \(tub\) mice. There was no statistically significant difference in the expression of either gene in any organ from DIO mice (Fig. 5A). \(Fto\) expression was also reduced by 30\% \((P < 0.03)\) in the liver of \(tub\) mice, and \(Ftm\) expression followed the same trend (Fig. 5C). There was no significant difference in \(Fto\) or \(Ftm\) expression in subcutaneous fat or liver of \(Cpe^{fat}\), \(tub\), and DIO compared with lean mice.

\(Fto\) and \(Ftm\) expression in hypothalami and mesenteric fat of fed, fasted, and 4°C ambient animals. In the hypothalamus, \(Fto\) and \(Ftm\) expression did not differ among ad libitum fed \(A^{y}\), \(Lep^{ob}\), \(Lepr^{db}\), \(Cpe^{fat}\), \(tub\) or DIO compared with fed control mice (Figs. 5D, 6A). By in situ hybridization, \(Fto\) and \(Ftm\) expression in hypothalamic sections of \(Lep^{ob}\) mice was iden-
tical to that of +/+ mice (data not shown). To assess other possible regulatory functions of Fto/Ftm, their expression was measured in fasted animals and mice exposed to 4°C for 30 min. Fto and Ftm expression levels were decreased in hypothalami of fasted Lepob mice compared with fed Lepob (Fto -20%, *P* < 0.03; Ftm -40%, *P* < 0.001) and fed +/+ mice (Fto -20%, *P* < 0.04; Ftm -45%, *P* < 0.01). Fasting had no effect on Fto or Ftm expression in mesenteric fat of Lepob mice (Fig. 6B). Fasting was associated with a twofold (*P* < 0.01) decrease in Fto expression in mesenteric fat of +/+ mice but had no effect on Ftm.

Fto and Ftm expression decreased by 30% (*P* < 0.01) and 40% (*P* < 0.04), respectively, in hypothalami of +/+ mice exposed to 4°C for 30 min (Fig. 6A). In the same mice, Fto expression was reduced twofold in mesenteric fat (*P* < 0.001); there was a similar trend in Ftm expression (Fig. 6A).
Comparison of Fto/Ftm expression in adipocytes and stromal vascular cells. To determine whether Fto/Ftm are expressed in both adipocytes and stromal vascular cells (SVC), cDNA specific to each cell fraction obtained from epididymal fat of +/+ and Lepr<sup>db</sup> mice (68) was used as a template for qPCR analysis. Fto and Ftm were expressed in both adipocytes and SVC, although both genes were expressed at ~36% (P = 0.005) and ~26% (P = 0.01) higher levels in SVC, respectively (Fig. 7). The expression of both genes was decreased in both adipocytes (Fto, ~60%, P = 0.007; Ftm, ~45%, P = 0.01) and SVC (Fto, ~67%, P < 0.001; Ftm, ~69%, P < 0.001) obtained from Lepr<sup>db</sup> compared with +/+ mice (Fig. 7).

Effects of adiposity per se on Fto/Ftm expression. To assess the respective contributions to Fto/Ftm expression of 1) obesity mutation(s), per se, and 2) the degree of adiposity resulting from such mutations, we assessed body fat content by TD-NMR in Lepr<sup>ob</sup>, Lepr<sup>db</sup>, Cpe<sup>flit</sup>, and tub animals at 4 wk of age and obtained body composition data for 18-wk-old DIO and DIO control animals from The Jackson Laboratory. Data grouped by tissue or mutation/DIO showed statistical significance (ANOVA; P < 0.001). Significant tissue × mutation/DIO interactions were also present for both Fto and Ftm (ANOVA; P < 0.001). We used absolute and fractional fat content to adjust (by ANCOVA) expression levels of Fto/Ftm in all tissues, by genotype. Levels of adiposity, per se, had no significant effect on these expression differences. Hence, expression differences are generally attributable to the respective obesity mutations or DIO.

Fto and Ftm expression in the mouse embryo. Fto and Ftm expression was also examined by in situ hybridization and qPCR in the mouse embryo. At 13.5 dpc, Fto was expressed throughout the whole embryo, especially in the brain and spinal cord (Fig. 3C). In the midbrain, Fto expression was relatively high in the developing arcuate nucleus and mamillary area. Ftm was expressed at lower levels in fewer tissues than Fto. The Ftm expression pattern in the midbrain was comparable to that of Fto (Fig. 3C). In whole brain, Ftm expression levels were approximately twofold less than Fto (P < 0.02) (Fig. 3D).

The role of leptin during embryonic development is not clear. Leptin receptor isoforms are expressed in the mouse brain as early as 13.5 dpc and seem to induce differentiation of neuronal lineage cells (62). Fto and Ftm expression were assessed in Lepr<sup>ob</sup> embryos. The Lepr<sup>ob</sup> mutation was not associated with differences in Fto or Ftm expression in the whole brain of embryos at 13.5 dpc compared with +/+ controls (Fig. 3D). Similarly, no differences in patterns of Fto or Ftm expression were observed between Lepr<sup>ob</sup> and +/+ embryos at 13.5 dpc by in situ hybridization (data not shown).

**CUTL1 controls FTO and FTM expression.** All FTO SNPs reported to be strongly associated with BMI (8, 14, 51, 52), and all 46 SNPs in linkage disequilibrium with those associated SNPs, were analyzed using MatInspector (matrix CDPRCR3.01. Genomatix, Munich, Germany; http://www.genomatix.de) to identify canonical cis transcriptional regulatory elements containing these SNPs. rs17817449 (Matrix similarity 0.78) and rs8050136 (Matrix similarity 0.82) (Fig. 1), were predicted to be located in CUTL1 binding sites (Fig. 8A). In ChIP of DNA from human fibroblasts using a CUTL1-specific antibody, a ~90-bp fragment that included rs8050136 was precipitated (Fig. 8B). Because the fibroblasts were heterozygous for rs8050136 (A/C), it was possible to determine whether CUTL1 displayed a binding preference for either of these alleles. Only 20% of the rs8050136 fragments isolated by ChIP with the CUTL1 antibody carried the “C” allele (Fig. 8C). When CUTL1 expression in the fibroblasts was reduced by 70% with siRNA, FTO expression was decreased by 90% and FTM by 65% (P < 0.001) (Fig. 8D).
In all of the obesity mutations studied, Fto and Ftm expression was lower in the mesenteric fat than any other fat depot. The reasons for preferential effects (vs. other adipose tissue depots) in the mesenteric adipose tissue or for the higher levels of Fto and Ftm expression in SVC than adipocytes are not clear. In a recent study of human adipose tissue, FTO expression was reported to be about twofold higher in isolated adipocytes than whole subcutaneous adipose tissue, implying that in humans, expression in adipocytes is greater than SVC adipocytes. Further studies will be needed to determine whether relative rates of expression in adipose tissue subfractions, and effects of obesity on expression, differ between mice and humans. Downregulation of Fto, but not Ftm, in the mesenteric fat of fasted +/+ animals is effectively the only qualitative difference in expression pattern between the two genes. The decline in Fto expression in this depot with fasting (a response not seen in other fat depots) suggests that levels of FTO/FTM expression may be influenced by substrates coming at high concentrations from the small bowel.

In situ hybridization suggests that Ftm and Fto are coexpressed in a limited region of the arcuate nucleus. In the Allen Brain Atlas (http://www.brainatlas.org/aba/), Cutl1 spatial expression is comparable to that of Ftm in the arcuate nucleus of the adult mouse. In addition, the expression pattern in fasted animals is consistent with downregulation of Fto and Ftm expression observed in the hypothalamus of +/+ mice housed at 4°C, in that cold exposure increases food consumption in mice (9). These findings are consistent with centrally mediated effects on energy homeostasis. Such influence could be conveyed by developmental/structural effects of FTO/FTM and/or participation, as suggested by responses to environmental manipulation, in intercurrent metabolic/behavioral homeostasis. The salience of effects in the genetic models with direct interruptions of the leptin axis (Lepob, Leprdb) are intriguing and could point to specific neurophysiological roles for Fto and Ftm in canonical neuroregulation of energy metabolism. The site-related differences in adipose tissue expression could reflect cell-autonomous differences in Fto/Ftm expression and/or neurally mediated effects on these depots. The apparent role of FTO/FTM in cell cycle and developmental aspects of the hypothalamus might suggest that they would be unlikely to respond to intercurrent metabolic or environmental changes. However, there is ample precedent for just such a dual role (in brain development and metabolic homeostasis) by hormone leptin (5).

Following completion of the present study, Gerken et al. (15) reported that the FTO protein has sequence similarity to Fe(II)- and 2-oxoglutarate-dependent oxygenases, localizes to the nucleus, and demethylates single-stranded DNA in vitro in the presence of Fe(II) and ascorbate, suggesting a possible role for FTO in regulation of gene transcription or DNA damage repair. They reported a 60% decrease of Fto expression by qPCR in laser-dissected murine arcuate nuclei of fasted animals but no alteration in ventromedial or paraventricular nuclei of these same mice. In our study, we also observed a trend toward reduced Fto expression levels in whole hypothalami of fasted +/+ mice and found a statistically significant decrease (∼20%) in fasted Lepob compared with fed Lepob mice (Fig. 6A). Our data are consistent with those of Gerken et al. (15) and suggest that leptin does not mediate these declines of Fto expression in fasted animals.

Shh expression is reduced in the limb buds of Fm−/− mouse embryos (65). In chick limb buds, Shh overexpression causes ectopic expression of Cux2, a Cutl1 ortholog (58). Thus, a positive feedback loop may exist between Cutl1, Ftm, and Shh signaling. Misregulation of SHH signaling and cilia dysfunction are implicated in obesity, retinal degeneration, right-left asymmetry, renal dysplasia, and polydactyly that characterize the Bardet-Biedl syndrome (10, 57, 59). Ftm is a basal body protein of cilia that affects SHH signaling (65), suggesting that Ftm may contribute to aspects of hypothalamic development. Reminiscent of aspects of the Bardet-Biedl syndrome, humans with mutations in RPGRIP, the FTM homolog, develop renal dysplasia (43, 57). Additionally, Cutl1−/− and Fused toes homozygous mutants display left-right asymmetry. There is striking similarity of gene structure and order of Fto, Ftm, Fis, and the Irx genes between human and mouse (Fig. 1). Moreover, the large Fto intronic regions (up to
FTO/FTM EXPRESSION IN MICE

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Perspectives and Significance

The recent availability of very high-density molecular maps of intrinsic single nucleotide pair variation within the human genome has enabled a new means of looking for genes that account for quantitative (adiposity) or qualitative (diabetes) human phenotypic variation. In such genome-wide scans (GWAS), no prior information regarding the type of gene or its physical location is required. Statistical signals correlating phenotype with SNPs are used to implicate genetic regions and constituent genes. These approaches, while inherently more sensitive than older parametric linkage techniques, can (and do) generate large numbers of authentic “candidates” whose functional activity depends upon proteolytic cleavage, resulting in a truncated isoform, p110 (including the C-terminal “Cut repeats” 2, 3 and the homeodomain), which upregulates DNA pol a (60). p110 and the DNA pol a promoter interact during the G1/S phase transition (50, 60). In the present study, the ChIP assay was performed using an antibody that recognizes both CUTL1 and p110 (60). The siRNA knock-down experiment indicates that CUTL1 (or p110) is needed for transcriptional activation. The finding that FTO and FTM are coregulated by CUTL1 is consistent with the expression data in mouse organs.

CUTL1 acts as a transcriptional repressor by displacing DNA pol a and the p110 (60). The siRNA knock-down experiment indicates that CUTL1 (or p110) is needed for transcriptional activation. The finding that FTO and FTM are coregulated by CUTL1 is consistent with the expression data in mouse organs.

~177.5 kb) show extended sequence conservation with their human counterparts (Genomic Sequence Alignment function; Ensembl). SNP rs8050136 and the CUTL1 binding site in which it is located are part of a highly conserved 1.2-kb intronic region (58% identity with the mouse). CUTL1 belongs to the CDP/Cut family of homeoproteins (40). It consists of a Cut homeodomain and 3 “Cut repeat” DNA-binding domains (18). Members of the CDP/Cut family have the capacity to bind to a wide range of DNA sequences (1, 3, 7, 18, 64). In the present study, CUTL1 interacted only with the predicted binding sequence “aggtcagatatt(g/t)ATTGc” (rs8050136) and not with the predicted binding sequence “cacacaGAaac(g/t)gttttaa” (rs17817449) (Fig. 8A). Moreover, CUTL1 preferentially bound to DNA fragments carrying the “C” allele of rs8050136. Using data from the Tübingen Family Study, Tschritter et al. (61) identified a dose-dependent increase of ~5 kg in body mass.

Cutl1 was originally cloned in Drosophila and was shown to be a downstream effector of Notch (24, 34, 38, 42). Lack of functional Cutl1 is embryonic lethal, while flies with viable mutations display developmental defects in various organs, including limbs, Malpighian tubules, and external sensory organs (4, 23, 30). Cutl1 was originally identified in vertebrates for its CCAAT displacement activity and was later linked by homology to its Drosophila paralog (41). In the mouse, disruption of Cutl1 results in generalized somatic growth retardation (12, 31, 53), while Cutl1 overexpression leads to multior gan cellular hyperplasia and organomegaly (26). CUTL1 plays an apparent role in cell cycle progression, as an accelerator of entry into S phase (50).

CUTL1 acts as a transcriptional repressor by displacing activators (54) and/or recruitment of histone deacetylase 1 (33) and has been proposed to inhibit gene expression in terminally differentiated cells (13). However, CUTL1 has also been implicated as a transcriptional activator. This activity depends upon proteolytic cleavage, resulting in a truncated isoform, p110 (including the C-terminal “Cut repeats” 2, 3 and the homeodomain), which upregulates DNA pol a (60). p110 and the DNA pol a promoter interact during the G1/S phase transition (50, 60). In the present study, the ChIP assay was performed using an antibody that recognizes both CUTL1 and p110 (60). The siRNA knock-down experiment indicates that CUTL1 (or p110) is needed for transcriptional activation. The finding that FTO and FTM are coregulated by CUTL1 is consistent with the expression data in mouse organs. The discovery of the genetic interaction between CUTL1 and FTO/FTM at rs8050136 does not exclude the possibility that regulation by CUTL1 may involve other CUTL1 binding sites. In the implicated (by LD) ~47-kb region, MatInspector (Genomatix; http://www.genomatix.de) identified 12 CUTL1 binding sites (matrix CLOX/CDPCR3.01; Matrix similarity >0.8) all present in the first intron of FTO. Except rs8050136, only SNP rs7202296 (~6 kb downstream of rs8050136) is located in a putative CUTL1 binding site.
functional relevance with regard to the dependent phenotype is largely or entirely unknown. *Fto* and *Ftm* are good examples. One or both of these genes is clearly implicated (statistically) in the control of human adiposity (BMI), but the molecular physiology of such effects is unknown. FTO is apparently an Fe(II)- and 2-oxoglutarate-dependent oxygenase, and FTM a cilary basal body component. The former might operate as a DNA demethylase, the latter in what is now appreciated, by virtue of the obesity phenotype in the Bardet-Biedl and Alstrom syndromes, as an important pathway in human energy homeostasis. To get an idea of how these newly implicated genes might operate in energy homeostasis, we examined their organ distributions (ubiquitous), their changes in expression in the context of classical monogenic and dietary obesities (reduced) and in response to food restriction and cold exposure (decreased), and the distribution of their transcripts within the arcuate of the hypothalamus. Although these findings are consistent with a role for these genes in canonical molecular pathways related to energy homeostasis, the specific mechanisms/pathways by which one or both genes influence adiposity are not revealed by our experiments. The study of mice with conditional, organ-specific knockouts/overexpression of these genes (alone and together) will be needed, as will studies of the expression of *Fto/Ftm* in relevant tissues obtained from humans with 0, 1, or 2 of the risk alleles for rs8050136. More interesting at this point is the possibility that both genes are regulated by a single transcription factor (CUTL1) via a single regulatory site in the first intron of *FTO*. Although our paper provides circumstantial evidence in this regard, more definitive analysis will require the study of mice with underactive/overactive alleles of *Cutf*, and, hopefully, the identification of humans with aberrant alleles of *CUTL1*. Whereas the mouse has been heavily relied upon to identify candidate genes for phenotypes such as obesity and diabetes, in the era of the GWAS, their role will now be expanded to the vetting of traits such as obesity and slow-onset cystic kidney disease. *Curr Biol* 17: 1586–1594, 2007.


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