The Fat Mass and Obesity Associated Gene FTO Functions in the Brain to Regulate Postnatal Growth in Mice

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Abstract

FTO (fat mass and obesity associated) was identified as an obesity-susceptibility gene by several independent large-scale genome association studies. A cluster of SNPs (single nucleotide polymorphism) located in the first intron of FTO was found to be significantly associated with obesity-related traits, such as body mass index, hip circumference, and body weight. FTO encodes a protein with a novel C-terminal α-helical domain and an N-terminal double-strand β-helix domain which is conserved in Fe(II) and 2-oxoglutarate-dependent oxygenase family. In vitro, FTO protein can demethylate single-stranded DNA or RNA with a preference for 3-methylthymine or 3-methyluracil. Its physiological substrates and function, however, remain to be defined. Here we report the generation and analysis of mice carrying a conditional deletion allele of Fto. Our results demonstrate that Fto plays an essential role in postnatal growth. The mice lacking Fto completely display immediate postnatal growth retardation with shorter body length, lower body weight, and lower bone mineral density than control mice, but their body compositions are relatively normal. Consistent with the growth retardation, the Fto mutant mice have reduced serum levels of IGF-1. Moreover, despite the ubiquitous expression of Fto, its specific deletion in the nervous system results in similar phenotypes as the whole body deletion, indicating that Fto functions in the central nervous system to regulate postnatal growth.

Introduction

The mouse gene Fto was first cloned more than a decade ago as one of the several genes deleted in the Fused toes (Ft) mutant mouse created by insertional mutagenesis [1]. However, it did not draw much attention until very recently when its human homolog FTO was implicated in obesity. In 2007, several groups reported that a cluster of SNPs (single nucleotide polymorphism) in the first intron of FTO was highly associated with obesity-related traits and higher obesity risk [2–4]. The association has been further confirmed by other independent studies in different human populations [5–15], thus rendering FTO highly conserved in Fe(II) and 2-oxoglutarate-dependent oxygenase family [25–27]. This enzyme family has diverse biological functions all based on the similar chemical mechanism [28,29]. Indeed, similar to a number of members in this family, FTO can demethylate single-stranded nucleic acids in vitro [25,26,30]. However, the physiological function and in vivo substrates of FTO are not well defined.

The FTO protein contains a double-stranded β-helix fold typical of the members in the Fe(II) and 2-oxoglutarate-dependent oxygenase family [25–27]. This enzyme family has diverse biological functions all based on the similar chemical mechanism [28,29]. Indeed, similar to a number of members in this family, FTO can demethylate single-stranded nucleic acids in vitro [25,26,30]. However, the physiological function and in vivo substrates of FTO are not well defined.

Here we described the generation and characterization of two mouse models with varying Fto deficiencies. The whole body Fto knockout mice displayed growth retardation, with shorter body length, lower body weight, and lower bone mineral density than control mice. However, the mutant mice had relatively normal body composition and were still susceptible to diet induced obesity. In another mouse model, Fto was specifically

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deleted in the neural system. Despite the ubiquitous expression of Fto, its specific deletion in the nervous system results in similar growth retardation phenotypes as the whole body deletion, suggesting that Fto functions in the brain to regulate postnatal growth.

**Results**

**FTO protein expression in mice**

We raised antibodies in rabbit against full-length mouse FTO protein and surveyed the expression in various mouse tissues with immunoblotting. Consistent with the previous RT-PCR results [26], the expression of FTO protein was detected in all of the major mouse tissues examined, with the highest level of expression in the brain and the lowest in the skeletal muscle (Figure 1A). Within the brain, FTO is expressed more or less uniformly in different anatomical structures, such as hypothalamus and hippocampus (Figure 1B). Given the association of FTO with human obesity, we wished to determine if the expression of FTO is influenced by food intake. Adult male C57BL/6 mice were fasted for 24 hrs and the FTO expression was examined in energy metabolism-related tissues including white adipose tissue, brown adipose tissue, liver, pancreas, hypothalamus, and skeletal muscle (Figure 1C). However, no obvious changes in the expression of FTO were detected. We also fed male C57BL/6 mice with high fat diet for 17 weeks starting from 6-week-old, and the FTO protein level did not show noticeable changes either (Figure 1D). Thus, if Fto is regulated by food status or type, the regulation is unlikely at the level of protein expression. Previous studies have shown that the Fto mRNA level in the arcuate nucleus (ARC) of the hypothalamus is reduced by fasting in mice [26], and increased by exposure to high fat diet in rats [33]. It is possible these regional changes cannot be detected in the Western blot of whole hypothalamus.

![Figure 1. FTO protein is ubiquitously expressed in mouse tissues and not affected by nutritional status in mice.](image-url)

(A) Tissues from adult male C57BL/6 mice (except the ovary) were homogenized and immunoblotted. 20 μg total protein was loaded in each lane. (B) Expression of FTO in different regions of the brain. (C) Western blot analysis of FTO in metabolism-related tissues from adult male C57BL/6 mice fed ad libitum or fasted for 24 hrs. (D) Western blot analysis of FTO in tissues from C57BL/6 male mice fed on normal chow or on high fat diet (60 kcal % fat) from 6-week-old for 17 weeks.

doi:10.1371/journal.pone.0014005.g001
Generation of Fto deletion mice

In order to investigate the physiological function of Fto in mice, we generated a conditional knockout (cko) line by flanking exon 3 with two loxP sites (Figure 2A). Exon 3 encodes about 40% of the protein. Deletion of exon 3 would result in frame-shift of the downstream exons and early termination in translation (Figure 2B). Germline transmission of the cko allele (Fto\(^{cko}\)) was obtained and confirmed by Southern blot analysis (Figure 2C). F1 Fto\(^{cko}\) mice were either directly bred to Meox2-Cre [34] mice to generate a knockout allele (Fto\(^{*D}\)) still bearing the neo selection cassette, or first bred to Flpase-expressing mice [35] to generate a floxed allele (Fto\(^{flox}\)). Fto\(^{+}\)/flox mice were then crossed to Meox2-Cre mice to generate the clean deletion of exon 3 allele (Fto\(^{\#D}\)) (Figure 2A). Both Fto\(^{*D}\) and Fto\(^{\#D}\) were used as knockout in this study and will be referred together as Fto\(^{D}\) from hereon, since there is no phenotypic difference between Fto\(^{*D}\) and Fto\(^{\#D}\) mice. Homozygous knockout mice (Fto\(^{D/D}\)) were obtained by crossing of heterozygous pairs, and the absence of FTO protein expression in Fto\(^{D/D}\) mice was confirmed by Western blot analysis (Figure 2D).

Complete depletion of Fto in mice results in postnatal growth retardation

Fto\(^{D/D}\) mice are viable, but only about 50% of them could survive to postnatal day 10–14 (Table S1) as has been reported in

Figure 2. Generation of Fto knockout mice. (A) Strategy to generate various Fto alleles. Hind III indicates the enzyme cutting sites for Southern blot analysis in (C). (B) Schematic representation of predicted Fto wildtype and knockout coding sequences (CDS). (C) Southern blot analysis of wildtype and cko alleles. The genomic DNA was digested by Hind III, separated on agarose gel, blotted, and hybridized with the indicated probes as shown in (A). (D) Western blot analysis of different tissues isolated from wildtype (+/+) heterozygous (+/Δ) and homozygous (Δ/Δ) mice.

doi:10.1371/journal.pone.0014005.g002
another Fto knockout mouse model [31,36]. The death mostly happened within a few days after birth (Table S2). It is unclear what causes the death at present. However, near term Fto<sup>+/A</sup> embryos were recovered at the Mendelian ratio (Table S3), indicating that Fto is not required for embryogenesis in mice and the death mostly likely happened after the birth. Surviving Fto<sup>+/A</sup> mice displayed immediate growth retardation in both males and females (Figure 3A). Within the first few days after the birth, the Fto<sup>+/A</sup> mice were already significantly lighter in weight comparing to their wildtype and heterozygous littermates, and the difference increased over time (Figure 3B). This is unlikely a result of that the mutant pups were born smaller than normal, because there was no weight difference among embryos of all three genotypes at E18.5 (Figure 3C). At the time of weaning, both male and female Fto<sup>+/A</sup> mice were about 65% the weight of wildtype and heterozygous littermates (Figure 3D). While the male Fto<sup>+/A</sup> mice remained behind throughout adulthood, the females showed a trend of catching up in weight later on (Figure 3D). Fto<sup>+/A</sup> mice were also significantly shorter in body length throughout lifetime (Figure 3E), and they had much lower bone mineral density (Figure 3F). Consistent with the growth retardation phenotype, Fto<sup>+/A</sup> mice had significantly lower serum levels of IGF-1 (insulin-like growth factor 1) than the controls at the time of growth spurt (Figure 3G).

**Fto<sup>+/A</sup> mice do not display a lean phenotype**

To determine whether Fto-deficiency affects body fat mass, the body compositions of 16-week-old mice fed on normal chow were measured by DEXA (dual energy X-ray absorptiometry). Despite the general decrease in size, Fto<sup>+/A</sup> mice did not show a specific reduction in fat. Among the 16-week-old males, Fto<sup>+/A</sup> mice had about 25% less lean mass than Fto<sup>+/+</sup> or Fto<sup>+/+</sup> mice (Figure 4A) and slightly less fat mass (statistically not significant) (Figure 4B). When normalized to the total tissue weight (lean mass plus fat mass) (Figure 4C), male mice of all three genotypes had similar fat content (fat mass/total tissue mass %) (Figure 4D). On the other hand, at the age of 16 weeks, female Fto<sup>+/A</sup> mice had even more fat mass on average than their wildtype and heterozygous littermates (Figure 4B), while their lean mass were still less than the controls (about 12% less, not as dramatic as the difference in males) (Figure 4A). As a result, female Fto<sup>+/A</sup> mice contained higher proportion of fat (fat mass/total tissue mass %) than wildtype or heterozygous mice (Figure 4D). The increased fat mass in mutant females compensated for their slight deficit in lean mass, and made it the total tissue mass even (Figure 4C), which also explains the trend of catching-up in whole body weight as the mice got older (Figure 3D).

**Fto<sup>+/A</sup> mice are susceptible to diet-induced-obesity**

To determine if the status of Fto has an effect on the response to diet, both male and female mice of three genotypes (Fto<sup>/+</sup>, Fto<sup>+/A</sup> and Fto<sup>+/+</sup>) were fed on high fat diet (60 kcal % fat) from 4-week-old on for 10 weeks. Unexpectedly, about half of the Fto<sup>+/A</sup> mice on high fat diet developed dermatitis (Figure S2A, Table S4) at the late stage of the diet regimen, and they became very lean due to the illness (Figure S2B). The reason of this illness is unclear. The dermatitis-free Fto<sup>+/A</sup> mice did respond to high fat diet and developed DIO (diet-induced-obesity) (Figure S2B). At the end of the diet regimen, all the mice free of dermatitis were measured by DEXA (dual energy X-ray absorptiometry). The Fto<sup>+/+</sup> mice still had significantly less lean mass than wildtype and heterozygotes (Figure 5A), while they had the similar amount of fat mass (Figure 5B). When normalized to total tissue mass (Figure 5C), the Fto<sup>+/+</sup> mice had relatively higher percentage of fat mass than controls, though not statistically significant in females (Figure 5D).

At the same time, the Fto<sup>+/A</sup> mice on high fat diet retained other aspects of the growth retardation phenotype, such as shorter body length (Figure 5E) and lower bone mineral density (Figure 5F).

**Fto<sup>+/A</sup> mice have higher metabolic rates**

To determine whether the deletion of Fto would affect the metabolism in mice, the O<sub>2</sub> consumption and CO<sub>2</sub> production of 16–17-week-old male mice were measured using indirect calorimetry. As there was no phenotypic difference in wildtype and heterozygous mice, only Fto<sup>+/A</sup> and Fto<sup>+/+</sup> mice were used here due to the availability of mice at the time of experiment. When the absolute amount of O<sub>2</sub> consumption (VO<sub>2</sub>) and CO<sub>2</sub> production (VCO<sub>2</sub>) were measured, Fto<sup>+/A</sup> mice showed relatively lower rates of O<sub>2</sub> consumption and CO<sub>2</sub> production than Fto<sup>+/+</sup> mice (Figure 6A, C), during both light and dark periods (though the difference of VO<sub>2</sub> during light period was not statistically significant). When divided by lean mass (conventional normalization), the Fto<sup>+/A</sup> mice displayed significantly higher metabolic rates (Figure 6B, D), possibly due to their much-reduced lean mass. When the data was analyzed by ANCOVA using lean mass as a covariant, the Fto<sup>+/A</sup> mice still showed higher O<sub>2</sub> consumption and CO<sub>2</sub> production rates during both light and dark period (Figure S3A–D), suggesting the deletion of Fto increases the metabolic rate, even after its effect on body mass has been taken into account.

While the oxygen consumption was measured, we also measured the food intake of Fto mutant mice. In a period of 72 hours, Fto<sup>+/A</sup> and Fto<sup>+/+</sup> mice consumed similar amount of food (Figure 6E). However, Fto<sup>+/A</sup> mice appeared to have consumed more food when the amount of food intake was normalized to lean mass (Figure 6F).

Taken together, these data suggest that Fto affects the food intake and energy expenditure at the same time. In its absence, both are increased.

**Neural-specific Fto knockout mice are phenotypically similar to the complete knockout**

Before we generated the Fto conditional knockout line, we had made another genetrap mouse line (Figure S1A, B) which still had residual expression of wildtype FTO (Figure S1C). It is interesting that homozygous genetrap mice (Fto<sup>gt/gt</sup>) displayed none of the phenotypes (Figure S1D–F) of Fto<sup>+/A</sup> mice. It is possible the residual FTO in the peripheral tissues may be enough for its function, considering Fto heterozygous knockout mice, as well as humans heterozygous for the FTO loss of function mutations are generally normal [37]. On the other hand, since there was only minimal reduction of FTO in the brain tissues of the gene-trap mice comparing to other tissues (Figure S1C), we reasoned that Fto might function in the brain. To explore this possibility, we deleted Fto in the nervous system by crossing Fto<sup>+/+</sup> with the Nestin-Cre transgenic mice [38] (Figure 7A). Western blotting analysis of Fto<sup>+/+</sup>/Nestin-Cre (will be referred to as Fto<sup>N</sup>) hereafter, N denotes neural) mice confirmed the efficient reduction of FTO protein in the brain, but not the peripheral tissues (Figure 7B). These mice displayed growth retardation, similar to the complete knockout mice (Figure 7C, D). Both male and female Fto<sup>N</sup> mice had shorter body length (Figure 7E) and lower bone mineral density (Figure 7F). Fto<sup>+/</sup> mice also had much lower serum levels of IGF-1 during growth spurt (Figure 7G).

**Neural-specific deletion of Fto affects body composition of mice in a similar way as the whole body knockout**

Similar to the whole body knockout, the body composition of 16-week old Fto<sup>N</sup> mice didn't show any deficits in fat.
Figure 3. Complete knockout of Fto results in postnatal growth retardation. (A) Representative pictures of wildtype and Fto knockout mice at different ages. (B) Growth curves of Fto+/+, Fto+/Δ, and FtoΔ/Δ mice from postnatal day 1 to 7. n = 16/16/14 (Fto+/+. Fto+/Δ/Δ), P-values from day 1 to 7: 0.29, 0.062, 0.011, 0.0069, <0.0001, and <0.0001. (C) Body weights of Fto+/+, Fto+/Δ and FtoΔ/Δ embryos at E18.5. n = 11/19/12 (Fto+/+, Fto+/Δ/Δ, FtoΔ/Δ). P = 0.68. (D) Growth curves of male and female Fto+/+, Fto+/Δ and FtoΔ/Δ mice. For each genotype (Fto+/+. Fto+/Δ/Δ, FtoΔ/Δ), n = 26/31/33 (male), n = 27/37/49 (female). **P<0.01 for all the time points, except in females at 12 weeks old, P = 0.024. (E) Body length of adolescent and adult
accumulation. The \( Fto^{+/+} \) mice had similar fat mass as to the controls, with a trend of slightly less fat mass in males, and more in females (Figure 8A). Here the difference in the fat mass in females was not statistically significant as in the complete knockout (possibly due to the incomplete depletion of \( Fto \), the genetic background introduced by the \( Nestin-Cre \) transgene, or a sampling issue of individual variance). The deficit in lean mass of \( Fto^{+/+} \) mice remained the same as the complete knockout (Figure 8B). Taken together, the \( Fto^{+/+} \) mice had less total tissue mass than the controls (Figure 8C). When normalized to the total tissue mass, \( Fto^{+/+} \) mice had relatively higher fat content (fat mass/total tissue mass %) (Figure 8D), which was more obvious in the case of females, similar to the complete knockout mice (Figure 4D). Thus, the general trend of body composition (increasing fat content) in neural-specific \( Fto \) knockout is similar to that in the complete knockout.

**The metabolic parameters of neural-specific \( Fto \) knockout mice are increased similarly as in the complete knockout mice**

The metabolic rates and food intake were also measured in 16~17-week-old male \( Fto^{+/+} \) and \( Fto^{+/+} \) mice. Similarly, when the raw VO\(_2\) and VCO\(_2\) values were compared, \( Fto^{+/+} \) mice showed relatively lower O\(_2\) consumption and CO\(_2\) production rate than the control mice (Figure 9A, C), during both light and dark periods. Again, when normalized to lean mass by the simple division, the \( Fto^{+/+} \) mice seemed to have relatively higher metabolic rates (Figure 9B, D). As the set of data of neural-specific knockout mice did not show a significant linear relationship to lean mass within groups (Figure S3E, F), ANCOVA analyses could not be applied here. \( Fto^{+/+} \) mice also had similar food consumption as of \( Fto^{+/+} \) mice during a period of 72 hours (Figure 9E), and appeared to have consumed more food when normalized to the lean mass (Figure 9F).

**Discussion**

We report here the analysis of two mouse models of \( Fto \) deficiencies. The whole body deletion of \( Fto \) resulted in postnatal growth retardation manifested as reduced body weight and length, lower bone mineral density and lower serum IGF-1 levels, but did not affect fat accumulation when fed on either normal or high fat diet. Interestingly, the deletion of \( Fto \) in the nervous system resulted in the similar growth retardation phenotypes as the whole

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**Figure 4. \( Fto \) knockout mice do not display a lean phenotype.** (A, B, C) Lean mass (A), fat mass (B) and total tissue mass (C) of 16-week-old mice of each genotype. (D) Body composition (fat mass/total tissue mass %) of 16-week-old mice of each genotype. \( P = 0.35 \) (male), and \( P < 0.0001 \) (female). In (A) to (D), all mice were fed on normal chow after weaning at 3-week-old and were 16-week-old at the time of DEXA (dual energy X-ray absorptiometry) measurement. For each genotype (\( Fto^{+/+}/Fto^{+/+}/Fto^{+/+} \)), \( n = 14/13/13 \) (male), 17/25/32 (female). Statistical analyses were performed by one-way ANOVA. **\( P < 0.001 \). All values are mean ± s.e.m.


doi:10.1371/journal.pone.0014005.g004
body knockout, indicating that Fto functions in the brain to regulate growth.

The strong growth retardation phenotype in our Fto deletion mice is consistent with that in the previously reported knockout mouse model generated by a slightly different strategy [31]. However, we did not observe a strong lean phenotype in our Fto-deficient mice as reported previously [32,36]. Instead, we detected a trend of increasing fat mass content (fat mass/total tissue mass %) in the mutants when the mice were fed on normal chow, especially in the females. Both male and female knockout mice also responded well to high fat diet and developed obesity. The male mutants were even more susceptible to diet-induced-obesity than the controls. We did recognize that the individual variation in body fat mass was relatively large, even in the control groups, while the lean mass was quite constant among individuals. This variation might have come from both genetic and environmental factors and likely underlie the difference with the previous reports.

In the initial report of Fto-deficient mice, the mutant mice were stated to have increased energy expenditure when compared to controls, which was cited as the reason for the reduction in adiposity in spite of the relative hyperphagia [31]. This explanation was recently challenged based on the lack of statistic

Figure 5. Fto<sup>Δ/Δ</sup> mice are susceptible to high fat diet-induced obesity. (A, B, C) Lean mass (A), fat mass (B) and total tissue mass (C) of Fto<sup>+/+</sup>, Fto<sup>+/Δ</sup> and Fto<sup>Δ/Δ</sup> mice on high fat diet. (D) Body composition (fat mass/total tissue mass %) of Fto<sup>+/+</sup>, Fto<sup>+/Δ</sup> and Fto<sup>Δ/Δ</sup> mice on high fat diet. P = 0.0055 (male), P = 0.1 (female). (E, F) Body length (E) and femur bone mineral density (F) of Fto<sup>+/+</sup>, Fto<sup>+/Δ</sup> and Fto<sup>Δ/Δ</sup> mice on high fat diet. In (A)–(F), all mice were fed on 60 kcal% fat diet for 10 weeks starting from 4-week-old. DEXA measurements were performed at the end of the 10-week period. For each genotype (Fto<sup>+/+</sup>/Fto<sup>+/Δ</sup>/Fto<sup>Δ/Δ</sup>), n = 14/14/12 (male), 11/9/8 (female). Statistical analyses were performed by one-way ANOVA. **P < 0.01. All values are mean ± s.e.m. doi:10.1371/journal.pone.0014005.g005
power and the oversimplification of normalization method [39,40]. We also measured the metabolic rate (in terms of O2 consumption and CO2 production) using indirect calorimetry. While the unnormalized O2 consumption and CO2 production rates were decreased in Fto knockout mice, the correction for lean mass using either direct division or ANCOVA reversed the result, pointing to an increase of metabolic rates in the mutant mice. On the other hand, both ours and the previous study all indicated an increase in food intake caused by Fto deficiency [31]. A more recent report demonstrated that manipulating the FTO levels in the arcuate nucleus of hypothalamus in rats could affect the food intake [33]. Thus, it appears that the status of Fto has an impact on both the energy expenditure and food intake. In the absence of Fto, both of the two parameters are increased. Given the trend of more fat accumulation in our Fto knockout mice, the increase in the energy expenditure seemed overwhelmed by the increase in food intake.

A number of human studies suggested that the risk SNPs in FTO associate with increases in food intake [16–21], and rejected an effect on energy expenditure [18–20,22]. It should be noted that the effect of those SNPs on FTO itself is still unknown. Thus, the phenotypes of Fto knockout mice do not necessarily contradict with the results from the human studies.

The SNPs in FTO that are associated with obesity in humans reside in the intronic region and their effect on the function of FTO remains elusive. The limited expression studies in humans

Figure 6. Metabolic parameters in Fto complete knockout mice. (A) Average hourly oxygen consumption of 16–17-week-old male Fto+/Δ and FtoΔ/Δ mice during the light and dark period. P = 0.1044 (light), and P = 0.0098 (dark). (B) Average hourly oxygen consumption divided by lean mass. P<0.0001. (C) Average hourly carbon dioxide production of 16–17-week-old male Fto+/Δ and FtoΔ/Δ mice during the light and dark period. P = 0.0463 (light), and P = 0.011 (dark). (D) Average hourly carbon dioxide production divided by lean mass. P<0.0001. (E) Accumulative food intake over a period of 72 hours of 16–17-week-old male Fto+/Δ and FtoΔ/Δ mice. P = 0.3015. (F) Food intake divided by lean mass. P = 0.0001. The number of mice used was 7 for each genotype. Statistical analyses were performed by unpaired t-test. **P<0.01. All values are mean ± s.e.m. doi:10.1371/journal.pone.0014005.g006
found no clear association between obesity-related SNPs and the FTO mRNA expression levels in adipose tissue and skeletal muscle [41–43]. In fact, both negative and positive correlations have been observed [41,43], complicating the effort in deciphering the effect of these SNPs on FTO expression. Recently, heterozygous loss-of-function FTO mutations have been identified in both lean and obese humans [37]. Moreover, patients carrying homozygous loss-of-function mutations in FTO show severe growth retardation and multiple malformations, but no record of obesity [44]. Thus, the growth retardation phenotype is shared between human and mouse when FTO is not functional, indicating a primary function of FTO in the regulation of linear growth.

Figure 7. Neural-specific Fto knockout mice are growth retarded. (A) The breeding scheme to generate neural-specific Fto knockout mice. (B) Western blot analysis of different tissues of Fto<sup>N/+</sup> and Fto<sup>N/Δ</sup> mice. (C) Body weights of 7-day-old Fto<sup>N/+</sup>, Fto<sup>N/Δ</sup> and Fto<sup>N/Δ</sup> mice. For each genotype (Fto<sup>N/+</sup>/Fto<sup>N/+</sup>/Fto<sup>N/Δ</sup>/Fto<sup>N/Δ</sup>), n = 17/7/9 (male), and 14/7/8 (female). (D) Growth curves of male and female Fto<sup>N/+</sup> and Fto<sup>N/Δ</sup> mice. For each genotype (Fto<sup>N/+</sup>/Fto<sup>N/Δ</sup>), n = 30/25(male), and 24/20(female). (E, F) Body length (E) and femur bone mineral density (F) of 16-week-old Fto<sup>N/+</sup> and Fto<sup>N/Δ</sup> mice measured by DEXA. For each genotype (Fto<sup>N/+</sup>/Fto<sup>N/Δ</sup>), n = 19/21(male), 19/17(female). (G) Relative serum IGF-1 levels of 4-week-old Fto<sup>N/+</sup> and Fto<sup>N/Δ</sup> mice. For each genotype (Fto<sup>N/+</sup>/Fto<sup>N/Δ</sup>), n = 6/6(male), and n = 5/4(female). Statistical analyses were performed by one-way ANOVA (C) or unpaired t-test (D–G). **P<0.01. All values are mean ± s.e.m.

doi:10.1371/journal.pone.0014005.g007
growth. However, whether FTO plays a role in obesity needs further investigation.

The reduced serum IGF-1 levels and a significant decrease in bone mineral density in both whole body and neural Fto knockout models argue for a function of Fto in the hypothalamus-pituitary axis. This axis controls the expression and secretion of IGF-1 by the liver through the action of growth hormone (GH), and produces a host of other endocrine factors involved in the regulation of many aspects of normal physiology including mineral metabolism in the bone [45,46]. GH deficiency is associated with obesity in humans [47] and the loss of GH function due to a missense mutation in mice results in disproportional increases in body fat despite an overall reduction in body size and weight [48]. It is possible that the Fto mutant mice suffer some degrees of GH deficiency. Determining whether or not FTO regulates GH and/or other hormones secreted by the hypothalamus-pituitary axis will greatly facilitate the elucidation of FTO’s physiological function in future.

Our neural-specific knockout mice recapitulated essentially all of the phenotypes in the complete knockout mice. This indicates that FTO function in the brain is crucial in spite of its ubiquitous expression. The analysis of mice with sub-regional deletion of Fto in the brain, especially, the hypothalamus-specific deletion, will pinpoint where exactly Fto functions.

As a member of the Fe (II) and 2-oxoglutarate (2-OG)-dependent oxygenase superfamily [25–27], FTO belongs to the AlkB subfamily [25–27,30]. AlkB proteins are important enzymes catalyzing the removal of alkyl adducts from DNA in both prokaryote and eukaryote organisms [49]. More recent biochemical analysis suggested that FTO might be a RNA demethylase [30], and recent structure analysis of FTO also supported its preference for single-stranded over double-stranded nucleic acids [25]. This is more consistent with a role of FTO in regulating gene expression (at the posttranscriptional level) than in repairing DNA. Likewise, a role of FTO in the regulation of gene expression is more fitting with the phenotypes of Fto mutant mice than a role in DNA repair. Knowing where exactly FTO functions will assist greatly in the identification of its physiological substrates. The conditional Fto knockout mice we have generated will be helpful in that regard.

**Materials and Methods**

**Ethics Statement**

All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine. The Protocol number is AN-5002.

**Western blot analysis and antibodies**

For Western blot analysis, tissues were lysed in RIPA buffer and the protein concentration was determined with Bradford method. We raised rabbit anti-mFTO antisera and affinity-purified the antibodies for the use in immunoblotting. Anti-α-TUBULIN antibodies were obtained from Sigma (T 5168).

**Generation of Fto conditional knockout mice**

The genomic DNA of targeting mouse Fto sequence containing exon 3 (about 9 kb) was isolated from a BAC clone (Sanger Center, UK) by gap repair. The construction of the conditional targeting vector was carried out via homologous recombination in E. coli [50]. The vector was linearized and introduced into E14Tg2a.4 ES cells. Recombinant ES clones were identified by Southern blot analysis and used to produce chimeric mice.

The F1 Fto conditional knockout mice (Fto<sup>−/cko</sup>) were bred with Meox2-Cre mice (JAX 003755) expressing Cre recombinase in all the epiblast-derived tissues to delete exon3. The progenies were

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**Figure 8. The body composition of Fto neural-specific knockout mice.** (A) Fat mass of 16-week-old Fto<sup>+/N</sup> and Fto<sup>−/N</sup> mice. P = 0.4551(male), 0.1681(female). (B) Lean mass of 16-week-old Fto<sup>+/N</sup> and Fto<sup>−/N</sup> mice. (C) Total tissue mass of 16-week-old Fto<sup>+/N</sup> and Fto<sup>−/N</sup> mice. P<0.0001(male), and P = 0.0175(female). (D) Body composition (fat mass/total tissue mass %) of 16-week-old Fto<sup>+/N</sup> and Fto<sup>−/N</sup> mice. P = 0.0118(male), 0.0024(female). In (A)–(D), for each genotype (Fto<sup>+/N</sup> vs Fto<sup>−/N</sup>), n = 19/21(male), 19/17(female). Statistical analyses were performed by unpaired t-test. *P<0.05, **P<0.01. All values are mean ± s.e.m.

doi:10.1371/journal.pone.0014005.g008
backcrossed to C57BL/6 mice for at least two more generations. The heterozygotes (F3 and beyond) were then intercrossed to generate complete knockout mice.

F1 Fto+/cko mice were also bred with mice expressing FLP1 recombinase (JAX 003946) to first delete the selection cassette flanked by the Frt sites. The mice with one floxed Fto allele (Fto+/flox) were then bred with Meox2-Cre mice (JAX 003755) to delete exon 3. Similarly, the progenies with one Fto knockout allele (having no selection cassette now) were backcrossed to C57BL/6 mice for at least three more generations. The heterozygotes were then intercrossed to generate knockout mice.

To generate brain specific Fto deletion, Fto+/flox mice were crossed with Nestin-Cre mice (JAX 003771) to generate Fto+/flox/Tg(Nes-Cre) mice, which were then crossed to Fto+/flox mice to generate FtoN+/FloX+/flox and FtoN+/flox, for control and FtoN–/FloX+/flox/Nes-Cre) mice.

**Animal experiments**

Animals were housed in a specific pathogen free facility at 22±2°C under a cycle of 12 hr light (7:00 am light on) and 12 hr dark (7:00 pm light off). They have free access to water and food (normal chow: 2920X Teklad Rodent Diet). For 24 hr fasting experiments, the food was removed from the cages at 6:00 pm, but

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**Figure 9. The metabolic parameters of Fto neural-specific knockout mice.** (A) Average hourly oxygen consumption of 16–17-week-old male FtoWT and FtoNX mice during the light and dark period. \( P = 0.0105 \) (light), and \( P = 0.0044 \) (dark). (B) Average hourly oxygen consumption divided by lean mass. \( P = 0.00556 \) (light), and \( P = 0.0233 \) (dark). (C) Average hourly carbon dioxide production of 16–17-week-old male FtoWT and FtoNX mice during the light and dark period. \( P = 0.004 \) (light), and \( P = 0.0196 \) (dark). (D) Average hourly carbon dioxide production divided by lean mass. \( P = 0.0131 \) (light), and \( P = 0.0142 \) (dark). (E) Accumulative food intake over a period of 72 hours of 16–17-week-old male FtoWT and FtoNX mice. \( P = 0.4601 \). (F) Food intake divided by lean mass. In (A)–(F), the number of animals used were 7 (FtoWT) and 9 (FtoNX). Statistical analyses were performed by unpaired t-test. *\( P < 0.05 \), **\( P < 0.01 \). All values are mean ± s.e.m.

doi:10.1371/journal.pone.0014005.g009
the mice had free access to water. For high fat diet challenge, normal chow was substituted by 60 kcal% fat diet (Research Diets, Inc. D12492).

Body composition and bone mineral density were measured with Lunar PIXImus dual energy x-ray absorptiometry (DEXA) densitometry following the standard protocol.

Measurement of the rates of metabolism and food consumption
The metabolic rates of mice were measured by indirect calorimetry using the Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments). The food intake was also monitored by the system. 16–17-week-old mice were housed individually in the monitoring cage with free access to water and food for 3 days for acclimatization. Then the lean mass of the mice was measured by MRI (EchoMRI), before the animals were put back into the monitoring cages and monitored for the next 3 days for oxygen consumption, carbon dioxide production and food intake.

IGF-1 ELISA
Blood was collected from 4-week-old mice by cardiac puncturing. The sera were aliquoted and snap-frozen in liquid nitrogen. Before ELISA, the sera were extracted by acid-ethanol with cryo-precipitation to release IGF-1 from its binding proteins [51]. IGF-1 ELISA was performed using commercial kits (Signosis EA-2204, and AssayPro EMI1001-1) according to the manufacturer’s protocols. The results were presented as the read-out from the microplate reader (spectrometer).

Statistic analysis
All statistic analyses were performed by one-way ANOVA (three sample sets) or unpaired t-test (two sample sets). ANCOVA was used when indicated.

Supporting Information
Figure S1 Generation and characterization of Fto gene-trap mice. (A) Schematic representation of Fto gene-trap strategy. (B) Schematic representation of predicted Fto wildtype and gene-trap coding sequence (CDS). (C) Western blot analysis of tissues from mice of all genotypes. (D) Growth curves of Fto+/+ and Fto+/D mice. For each genotype (Fto+/+ /Fto+/+), n = 16/15 (males); 12/16 (females). All values are mean ± s.e.m. (E) The body length of adult Fto+/+ and Fto+/D mice. At the time of the measurement, males were 13–14.5-week-old, and females 13–16-week-old. For each genotype (Fto+/+ /Fto+/+), n = 10/9 (males); 7/7 (females). All values are mean ± s.e.m. (F) Body composition (fat mass/total tissue mass %) of Fto+/+ and Fto+/D mice fed on normal chow or high fat diet. Body composition was measured by DEXA (dual energy X-ray absorptiometry). For normal chow group, males were 13–14.5-week-old, and females 13–16-week-old. For each genotype (Fto+/+ /Fto+/+), n = 10/9 (males); 7/7 (females). For the high fat diet group, at the time of the measurement, the mice had been fed on high fat diet (60 kcal % fat) for 12 weeks from 6-week-old.

For each genotype (Fto+/+ /Fto+/+), n = 11/6 (males); 8/8 (females). Statistical analyses were performed by unpaired t-test. All values are mean ± s.e.m.

Found at: doi:10.1371/journal.pone.0014005.s001 (0.74 MB TIF)

Figure S2 Dermatitis in Fto knockout mice after high fat diet regimen. (A) A representative picture of an Fto+/A mouse suffering dermatitis around the neck area. (B) A representative picture of Fto+/+ and Fto+/A mice after the high fat diet. The asterisk denotes the one with dermatitis.

Found at: doi:10.1371/journal.pone.0014005.s002 (0.74 MB TIF)

Figure S3 Metabolic rate of Fto mutant mice analyzed by ANCOVA. (A, C) Average hourly O2 consumption (A) and average hourly CO2 production (C) in relation to lean mass of 16–17-week-old male Fto+/A and Fto+/D mice during light and dark period. (B, D) Average hourly O2 consumption (B) and average hourly CO2 production (D) of 16–17-week-old male Fto+/+ and Fto+/A mice adjusted by ANCOVA using an average lean mass. In (A, B), n = 7/7 (Fto+/A/Fto+/D). In (B) and (D), statistical analyses were performed by unpaired t-test using adjusted data. **P<0.01. All values are mean ± s.e.m. (E, F) Average hourly O2 consumption (E) and CO2 production (F) in relation to lean mass of 16–17-week-old male Fto+/+ and Fto+/D mice during light and dark period. n = 7/9 (Fto+/+/Fto+/D). No significant linear relationship was detected.

Found at: doi:10.1371/journal.pone.0014005.s003 (0.50 MB TIF)

Table S1 Genotypes of 10–14-day-old pups from heterozygote intercrosses.

Found at: doi:10.1371/journal.pone.0014005.s004 (0.04 MB PDF)

Table S2 Genotypes of 1–3-day-old pups from heterozygote intercrosses that were found dead or missing.

Found at: doi:10.1371/journal.pone.0014005.s005 (0.03 MB PDF)

Table S3 Genotypes of E14.5~18.5 embryos from heterozygote intercrosses.

Found at: doi:10.1371/journal.pone.0014005.s006 (0.03 MB PDF)

Table S4 Number of mice that developed dermatitis on high fat diet.

Found at: doi:10.1371/journal.pone.0014005.s007 (0.04 MB PDF)

Acknowledgments
We thank Drs. B. Lee, L. Chan, J. Qin, L. Chen, T. Yang, W. Chen, J. Mao, H. Zheng of BCM for discussion and sharing of reagents. We thank Dr. Y. Chen of BCM Nutrition Center for technical help.

Author Contributions
Conceived and designed the experiments: XG YHS QT PZ. Performed the experiments: XG YHS ML FW. Analyzed the data: XG. Wrote the paper: XG PZ.

References
1. Peters T, Ausmefier K, Ruthen U (1999) Cloning of Fato (Fto), a novel gene deleted by the Fused toes (ft) mouse mutation. Mamm Genome 10: 983–986.
2. Dina C, Meyre D, Gallina S, Durand E, Korner A, et al. (2007) Variation in FTO contributes to childhood obesity and severe adult obesity. Nat Genet 39: 724–726.
3. Freyling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, et al. (2007) A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316: 889–894.
4. Scuteri A, Sanna S, Chen WM, Uda M, Albai G, et al. (2007) Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet 3: e115.
5. Heyne A, Nguyen TT, Scherag A, Friedel S, Brionner G, et al. (2007) Genome-wide association (GWA) study for early onset extreme obesity supports the role of fat mass and obesity associated gene (FTO) variants. PLoS ONE: 2: e1361.
6. Ohashi J, Naka I, Kimura R, Natsukawa K, Yamauchi T, et al. (2007) FTO polymorphisms in oceanic populations. J Hum Genet 52: 1031–1033.
26. Gerken T, Ivancic D, Girard CA, Tung YC, Webby CJ, Saudek V, et al. (2007) The FTO gene and obesity in a large European population sample: the HAPPEI study. Obesity (Silver Spring) 16: 2764–2766.

25. Peeters A, Beckers S, Verrijken A, Roevers P, Peeters P, et al. (2008) Variants in the FTO gene are associated with common obesity in the Belgian population. Mol Genet Metab 93: 481–484.

24. Villalobos-Comparan M, Teresa Flores-Dorantes M, Teresa Villarreal-Molina M, Rodriguez-Cruz M, Garcia-Ulloa AC, et al. (2008) The FTO gene is associated with adulthood obesity in the Mexican population. Obesity (Silver Spring) 16: 2296–2301.

23. Zhang F, Xu L, Jin L, Wang X (2008) A common variant in the FTO gene is associated with obesity in the Uygur population. J Endocrinol Invest 31: 1043.

22. Berentzen T, Kring SI, Holst C, Zimmermann E, Jess T, et al. (2008) Lack of effect of interaction between an FTO variant (rs9939609) and physical activity on obesity in 15,925 Swedish and 2,511 Finnish adults. Diabetologia 52: 1334–1338.

21. Timpson NJ, Emmett PM, Frayling TM, Rogers I, Hattersley AT, et al. (2008) Association of fatness-related FTO gene variants with energy expenditure or diet. Diabetes 57: 2241–2252.

20. Haupt A, Thamer C, Staiger H, Tschritter O, Kirchhoff K, et al. (2009) Variation in the FTO gene influences food intake but not energy expenditure. Obesity (Silver Spring) 16: 2196–2201.

19. Speakman JR, Tautz D, Zanetti D, Diederich D, Chu S, et al. (2008) Replication of the association of common rs9939609 variant of FTO with increased BMI in an Australian adult twin population but no evidence for gene by environment (G x E) interaction. Int J Obes (Lond) 33: 75–79.

18. Cornes BK, Lind PA, Medland SE, Montgomery GW, Nyholt DR, et al. (2009) Expression of the FTO gene is associated with obesity in the Uyghur population. J Endocrinol Invest 32: 953–956.

17. Grunnet LG, Nilsson E, Ling C, Hansen T, Pedersen O, et al. (2009) Regulation and function of FTO mRNA expression in human skeletal muscle and subcutaneous adipose tissue. Diabetes 58: 215–220.

16. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, et al. (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet 22: 99–103.

15. Gonzalez-Sanchez JL, Zabena C, Martinez-Larrad MT, Martinez-Calatrava MJ, Perce-Barba M, et al. (2009) Variant c8939609 in the FTO gene is associated with obesity in an adult population from Spain. Clin Endocrinol (Oxf) 70: 390–393.

14. Jia G, Yang CG, Yang S, Jian X, Yi C, et al. (2008) Oxidative demethylation of 5-methylthymine and 5-methyluracil in single-stranded DNA and RNA by mouse and human FTO. FEBS Lett 582: 3313–3319.

13. Molina M, Rodriguez-Cruz M, Garcia-Ulloa AC, et al. (2008) The FTO gene encodes a 2-oxoglutarate-dependent nucleic acid modifier. Nature 457: 1205–1209.

12. Kloting N, Schleinitz D, Ruschke K, Berndt J, Fasshauer M, et al. (2008) Inverse relationship between obesity and FTO gene expression in visceral adipose tissue. Diabetes 57: 311–318.

11. Loenarz C, Schofield CJ (2008) Expanding chemical biology of 2-oxoglutarate dioxygenases. Chem Rev 108: 215–220.

10. Boissel S, Reish O, Proulx K, Kawagoe-Takaki H, Sedgwick B, et al. (2009) Inactivation of the Fto gene protects from obesity. Nature. 458: 894–898.

9. Tung VC, Tsao E, Raffe CE, O’Rahilly S, et al. (2010) Hypothalamic-specific manipulation of Fto, the ortholog of the human obesity gene FTO, affects food intake in rats. PLoS ONE 5: e1000599.

8. Chang YC, Liao YC, Wang CL, Yang SW, Lai KY, et al. (2008) Differentiation of the fat mass and obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 318: 1469–1472.

7. Cha SW, Choi SM, Kim KS, Park BK, Kim JR, et al. (2008) Replication of genetic effects of FTO polymorphisms on BMI in a Korean population. Obesity (Silver Spring) 16: 2187–2189.

6. Chang YC, Liu PH, Lee WJ, Chang TJ, Jang YD, et al. (2008) Common variation in the fat mass and obesity-associated (FTO) gene confers risk of obesity and modulates BMI in the Chinese population. Diabetes 57: 2241–2252.

5. Hakanen M, Raitakari OT, Lehtimaki T, Peltonen N, Pahkala K, et al. (2009) Variations in the FTO gene are associated with severe obesity in the Japanese. J Hum Genet 55: 546–553.

4. Sanchez-Pulido L, Andrade-Navarro MA (2007) The FTO (fat mass and obesity associated) gene codes for a novel member of the non-heme dioxygenase superfamily. BMC Biochem 8: 23.

3. Ozer A, Bruck KK (2007) Non-heme dioxygenases: cellular sensors and regulators. J Biol Chem 282: 144–153.

2. Cornes BK, Lind PA, Medland SE, Montgomery GW, Nyholt DR, et al. (2009) Assessing the FTO gene and obesity in 15,925 Swedish and 2,511 Finnish adults. Diabetologia 52: 1334–1338.

1. Grunnet LG, Nilsson E, Ling C, Hansen T, Pedersen O, et al. (2009) Regulation and function of FTO mRNA expression in human skeletal muscle and subcutaneous adipose tissue. Diabetes 58: 215–220.