Changes in Gene Expression Associated with FTO Overexpression in Mice

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Abstract

Single nucleotide polymorphisms in the first intron of the fat-mass-and-obesity-related gene FTO are associated with increased body weight and adiposity. Increased expression of FTO is likely underlying this obesity phenotype, as mice with two additional copies of Fto (FTO-4 mice) exhibit increased adiposity and are hyperphagic. FTO is a demethylase of single stranded DNA and RNA, and one of its targets is the m6A modification in RNA, which might play a role in the regulation of gene expression. In this study, we aimed to examine the changes in gene expression that occur in FTO-4 mice in order to gain more insight into the underlying mechanisms by which FTO influences body weight and adiposity. Our results indicate an upregulation of anabolic pathways and a downregulation of catabolic pathways in FTO-4 mice. Interestingly, although genes involved in methylation were differentially regulated in skeletal muscle of FTO-4 mice, no effect of FTO overexpression on m6A methylation of total mRNA was detected.

Introduction

In 2007, a genome-wide association study (GWAS) revealed an association between a single nucleotide polymorphism (SNP) in the first intron of the fat-mass-and-obesity-related gene FTO and increased BMI and adiposity.[1] Subjects carrying two copies of the risk allele were, on average, 3 kg heavier and had a 1.7-fold increased risk for developing obesity.[1] Subsequently, this result has been replicated in various populations and across different age groups (reviewed in [2]). Increased expression of FTO probably underlies the obesity phenotype, as transcripts of the risk allele are produced more abundantly than the non-risk allele.[3,4] Indeed, mice with a constitutive knock-out of Fto[5–7], or with a partial loss-of-function mutation in Fto[8], display a lean phenotype. Conversely, mice carrying one or two additional copies of Fto (FTO-3 and FTO-4 mice) exhibit increased body weight, fat mass and food intake.[9]

FTO is an Fe(II) and 2-oxoglutarate-dependent demethylase of single stranded DNA and RNA.[10] Its main targets appear to be 6-methyladenosine (m6A) (DNA/RNA), 3-methyluracil (m3U) (RNA) and 3-methylthymidine (m3T) (DNA/RNA).[10–12] Special interest is the m6A modification, which is the main substrate of methylation in mRNA. This modification has been suggested to affect RNA processing[13–15], RNA transport[16–18] and translation efficiency[19]. m6A RNA methylation is catalysed by methyltransferases (METTL14 and METTL3) in association with the splicing factor WTAP.[20,21] Silencing of any of these compounds resulted in decreased levels of m6A and increased abundance of target mRNA transcripts.[20] Furthermore, m6A is recognised by the ‘reader’ protein YTHDF2 which causes the mRNA to be localised to mRNA decay sites.[22] This indicates that reversible m6A modifications can influence the stability of mRNA and hence regulate its lifespan, with increased m6A methylation leading to a decrease in translation. Two studies have identified m6A-containing mRNAs in mouse brain[23] and mouse liver.[24] They reported an enrichment of m6A sites around the stop codon, which also suggests a role in translational control and that m6A methylation of mRNA plays a key role in the regulation of gene expression.

In summary, FTO is known to be involved in the regulation of body weight and adiposity, and is able to demethylate single stranded DNA and RNA at m6A, m3U and/or m3T. How this function contributes to the physiological effects of FTO overexpression is still unknown. Alterations in gene expression and m6A methylation have been documented for ghrelin in response to FTO knockout[25] and of genes involved in dopaminergic signalling in response to FTO overexpression[4] and of genes involved in dopaminergic signalling in response to FTO knockout[25]. However, the full range of mRNAs affected by FTO is unknown.

In this study, we aimed to examine the changes in gene expression that occur in FTO-4 mice in order to gain more insight into the underlying mechanisms by which FTO influences body weight and adiposity. Our results indicate an upregulation of anabolic pathways and a downregulation of catabolic pathways in FTO-4 mice. Interestingly, although genes involved in methylation
were differentially regulated in skeletal muscle of FTO-4 mice, no effect of FTO overexpression on m6A methylation levels of total mRNA was detected.

**Materials and Methods**

**Animals**

Mice expressing 2 additional copies (4 in total) of FTO on a C57BL/6j background (FTO-4) were generated as described previously. [9] Wild-type C57BL/6j littersmates were used as controls. Genotyping was performed on DNA extracted by a DNeasy blood and tissue kit (Qiagen, USA). All experiments were carried out on 5-6 week old male mice maintained in a temperature and humidity controlled room on a 12:12 light-dark cycle (lights on at 7am) with ad libitum access to water and food (SDS Rat and Mouse No. 3 Breeding diet (RM3) containing 11.5 kcal% fat, 23.93 kcal% protein and 61.57 kcal% carbohydrate). Mice were killed by cervical dislocation at 1pm. Abdominal white adipose tissue (WAT), skeletal muscle and brain were rapidly dissected and kept on dry ice and subsequently at −80°C until RNA or protein extraction.

**Ethics Statement**

Experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986) and following approval by the University of Oxford local Ethics Review Committee. This study was performed under certificate of designation number 30/2306 and project license 30/2668 following approval by the University of Oxford Departments of Physiology, Anatomy and Genetics and Experimental Psychology Joint Departmental Ethics Review Committee.

**RNA extraction**

Total ribonucleic acid (RNA) from abdominal white adipose tissue (WAT), skeletal gastrocnemius muscle, hypothalamus and cerebellum of wild-type (n = 4) and FTO-4 (n = 4) mice was extracted using an RNeasy Mini Kit (brain), RNeasy fibrous Mini Kit (skeletal muscle) or RNeasy Lipid Tissue Mini Kit (WAT) (Qiagen, USA) according to the manufacturer’s protocol. The RNA concentration was measured in triplicate with a NanoDrop 2000 with access to water and food ad libitum for 10 min at 95°C, followed by 40 cycles of 95°C for 15sec and 60°C for 60 seconds. Threshold cycle (Ct) values were calculated using ABI SDS 3000 software (Applied Biosystems).

**Microarray**

Labelling sense ssDNA for hybridization was generated from 300 ng (cerebellum, hypothalamus, muscle) or 500 ng (WAT) starting RNA with the Ambion WT Expression Kit, the Affymetrix WT Terminal Labeling and Controls Kit and the Affymetrix Hybridization, Wash, and Stain Kit, according to the manufacturer’s instructions. Sense ssDNA was fragmented and the distribution of fragment lengths measured on a BioAnalyzer. The fragmented ssDNA was labeled and hybridized to the Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000.

**Cell culture**

Mouse embryonic fibroblasts (MEFs) of wild-type and FTO-4 C57BL/6j mouse embryos were cultured at 37°C and 7% CO2 in Dulbecco’s Modified Eagles Medium Glutamax (Gibco, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 1x non-essential amino acids (Gibco, Paisley, Scotland) and 50 M betamercaptoethanol (Gibco, Paisley, Scotland). Total RNA was isolated using an RNeasy Mini Kit (Qiagen, USA) according to the manufacturer’s instructions. An aliquot of total RNA was taken for subsequent analysis. The remainder of the total RNA was stored in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2331.

**RT-qPCR experiments**

For each sample, 1 μg of total RNA was reverse transcribed in a 20 μl final volume using the High Capacity complementary DNA (cDNA) Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s protocol. As a control, another 1 μg of total RNA was processed identically but without reverse transcriptase (non-RT control). cDNA samples were subsequently diluted to 4 ng/μl in nuclease-free water (Sigma) and stored at −80°C until further use.

Reverse transcription quantitative PCR (RT-qPCR) was carried out with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reactions were performed in triplicate and contained 12.5 μl Power SYBR Green Master Mix, 300 nM forward and reverse primers, 20 ng of cDNA template (or non-RT control or nuclease-free water) and nuclease-free water to a total volume of 25 μl. The RT-qPCR reaction consisted of an initial denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 seconds. Threshold cycle (Ct) values were calculated using ABI SDS 3000 software (Applied Biosystems).

Primers for targets to be validated (see Table S1 in File S1) and for three reference genes (HSPA8, HPRT1, ACTB) were designed with Geneious Pro software (Biomatters Inc) and NCBI sequence information. Primer specificity was confirmed by BLAST searches, the appearance of a single band on gel electrophoresis and melt curve analysis. Primer efficiencies were calculated as described previously [26] and used to calculate relative quantities of transcripts. Normalisation factors (the geometric mean [27]) were calculated with the geNorm applet for Microsoft Excel based on the transcript levels of the three reference genes. These were used to normalize the expression levels of the transcripts for differences in the amount of input material. Ct values for amplification of the transcripts of interest were significantly higher for non-RT controls (32.07 ± 0.70) than for cDNA templates (22.39 ± 0.68). The minimum difference in Ct values we observed between non-RT controls and cDNA samples was 5.63 cycles. Hence, contamination by genomic material was unlikely to influence the RT-qPCR data presented here.

To examine whether m6A might influence primer annealing and hence our microarray and qPCR results, PCR products with or without m6A were generated with 3 primer sets (HSPA8, HPRT1, ACTB). Equal amounts of these DNA templates were used to perform qPCR as described above.
was used for mRNA extraction with the polyATtract system (Promega).

Hydrolysis of RNA and isolation of nucleosides by solid phase extraction

RNA was incubated with 2U of nuclease P1 (Sigma) in a solution containing 25 mM NaCl and 2.5 mM of ZnCl₂. After 1 hour incubation at 37°C, 10 U alkaline phosphatase (NEB) and NH₄HCO₃ (to an end concentration of 0.05 M) were added, followed by an additional 1 hour incubation at 37°C. Subsequently, 20 mM acetic acid was added to the reaction mixture to a total volume of 1 ml. A strong cation exchange column (Telos SCX 50 mg/1 ml) was equilibrated with 1 ml methanol and washed with 1 ml 20 mM acetic acid, after which the samples were loaded onto the column. Following a washing step with 1 ml 20 mM acetic acid, nucleosides were eluted with 1 ml methanol containing 2.8% ammonium hydroxide. Finally, samples were dried using a speed-vac, and the pellet was dissolved in buffer A containing 95% water, 5% acetonitrile and 2.5 mM ammonium.

Liquid chromatography- mass spectrometry (LC-MS) for quantification of nucleosides

Samples were analysed by LC-MS on a Ultimate 3000 high performance liquid chromatography (HPLC) system (Dionex, Camberley, UK) coupled via the standard electrospray ionisation (ESI) interface to an Amazon ETD ion trap mass spectrometer (Bruker Daltonics) equipped with a nitrogen generator (Dominick Hunter, LCMS20-1). The HPLC system consisted of a solvent rack and degasser (SRD-3600), a dual gradient pump (DGP-3600M), a flow manager (FLM-3100) and a well plate autosampler (WPS-3000T). The mass spectrometer was controlled by Trap-Control software version 7.0 and the LC-MS/MS system was controlled by HySphere software version 3.2.

RNA hydrolysates were separated by HPLC and a 1.0 µl aliquot of the sample was injected per run. The chromatographic separation was carried out at a flow rate of 250 µl/min on a Thermo Accucore HILIC column (2.1 x 100 mm, 2.6µm particle size, Hemel Hempstead, UK) at 35°C with isotropic elution using 95% acetonitrile, 5% water 2.5 mM ammonium formate, pH 3.8 as solvent. The mass spectrometer was operated in full scan MS mode (75 to 500 m/z scan range) and ionization was achieved at a capillary voltage of 4500 V, a nitrogen gas flow of 8 l/min and 15 psi pressure and a temperature of 300°C. The ion trap was set to an ion charge control (ICC) target of 250,000 and a maximum accumulation time of 200 ms. The instrument was calibrated at least once a month using an ESI-T tuning mix (Agilent) by performing scan, isolation and fragmentation calibrations (Bruker Daltonics).

Data analysis

Analysis was performed using Quantanalyse software (version 2.0, Bruker Daltonics) and chromatograms of mass values for analytes (adenosine (A), N6-methyl adenosine (m6A); [M-H]+ m/z 268.0 Da(A) and 282.0 Da(m6A)) and internal standard (L-C5-adenosine (13C5-A), [M-H]+ m/z 273.0 Da). A Gaussian smoothing algorithm (smoothing width 1s, 1 cycle) was applied to the chromatograms and automatic peak detection parameters for analyte and internal standard were: retention time (A, 13C5-A: 3.8 min, m6A: 2.9 min) and retention time window (0.3 min). Peak area ratios of nucleoside to internal standard (y axis) were plotted against the plasma concentrations (x-axis). A linear regression analysis of the calibration standards was carried out using the least squares method. Calibration, QC and study samples were analysed by triplicate injection on the LC-MS/MS system and the determined value was taken as the arithmetic mean of the three measurements.

Results

Previous studies have shown that the body weights of wild-type (WT) mice and those overexpressing FTO (FTO-4 mice) start to diverge at 5 weeks of age. [9] We therefore used 5-week-old male mice for this study. We anticipate changes in gene expression underlying the FTO overexpression phenotype will be present at this age, but not changes that occur as a secondary consequence of increased body weight. We found no significant difference in body weight between 5-week-old FTO-4 and WT mice (FTO-4, 18.1 ± 1.9 g; WT, 14.2 ± 1.2 g; p = 0.13).

Overexpression of FTO in FTO-4 mice was confirmed by qPCR at 5 weeks of age. We found the highest expression of FTO in WAT (5.4-fold greater than WT), followed by cerebellum (2.1-fold), gastrocnemius muscle (1.5-fold) and hypothalamus (1.5-fold). Differences in the extent of overexpression between tissues of FTO-4 mice were also described previously, being greatest in muscle, but also significant in brain and white adipose tissue (WAT). [9]

Influence of m6A on DNA amplification

Because it has been reported that FTO demethylates 6-methyladenosine (m6A) in mRNA [10,12], we first examined whether m6A might affect primer annealing and subsequent amplification, and hence potentially influence both the qPCR and microarray analysis. We generated PCR products (ACTB, HPRT1, HSPA8) with and without m6A and used these as templates for qPCR analysis. As shown in Figure 1, no differences in threshold cycle (Ct) were observed with ACTB (A), HPRT1 (B) or HSPA8 (C) at any of the template concentrations used.

Gene expression changes

Differences in gene expression between FTO-4 and WT mice were examined by microarray at 5 weeks of age in cerebellum, hypothalamus, gastrocnemius muscle and WAT. An overview of all differentially expressed genes per tissue can be found in File S2. Tables 1 and 2 list the top 10 genes that showed the greatest increase and decrease in expression per tissue. Table 3 lists all genes that showed altered expression in FTO-4 mice which have previously been associated with obesity. We selected several of these for validation by qPCR (Figure 2). We focused on genes with the highest fold-change, genes that showed altered expression in multiple tissues, and genes that have been previously implicated in obesity. All genes showed equivalent changes in both microarray and qPCR experiments (Table S2 in File S1).

Changes in gene expression common to several tissues

Several genes showed same-direction changes in expression in multiple tissues. For example, Glo1 was upregulated in all four tissues examined. Glo1 encodes an enzyme involved in the detoxification of methylglyoxal (a metabolic by-product of glycolysis) and glutathione, and has been linked to dietary obesity in various QTL studies in mice [28,29]. Wdfy1, also known as FENS-1, showed markedly increased expression in hypothalamus, cerebellum and gastrocnemius muscle. FENS-1 protein has been reported to localise to early endosomes within the cell [30] and it contains a phosphatidylinositol-phosphate (PI3)-binding FYVE domain potentially allowing it to participate in the PI3 kinase signal transduction cascade.
Nothing is known of the function of this gene but as it contains a phosphatidylinositol-phosphate (PI3)-binding FYVE domain it may participate in the PI3 kinase signal transduction cascade.

Pyruvate dehydrogenase kinase 4 (PDK4), which plays an important role in the regulation of glucose utilization and lipid metabolism [31], was downregulated in the hypothalamus, WAT and gastrocnemius muscle of FTO-4 mice. The expression of xanthine dehydrogenase (XDH), which is involved in the oxidative metabolism of purines, was reduced in hypothalamus, cerebellum (1.46 fold) and gastrocnemius muscle.

D14Ertd449e (human C10orf57), Plac9 and Duxbl were all downregulated in both hypothalamus and cerebellum of FTO-4 mice. Duxbl was downregulated 1.42 fold and 1.35 fold in hypothalamus and cerebellum respectively. In addition, D14Ertd449e was downregulated in WAT and Plac9 in gastrocnemius muscle.

Changes in gene expression specific to certain tissues

Several genes associated with inhibition of growth and/or proliferation were downregulated in FTO-4 mice. This included the cell cycle inhibitors CDKN1A and CDKN1B, the protein synthesis inhibitor SGK1, and TXNIP (which is involved in unfolded protein response, and whose activation leads to apoptosis).

In WAT, major urinary protein (MUP) genes were highly upregulated in FTO-4 mice. MUPs have been implicated in many other physiological processes, including the regulation of glucose and lipid metabolism [32] and energy expenditure [33]. Adiponectin was upregulated in WAT and its receptor AdipoR2 was downregulated 1.38 fold in the hypothalamus of FTO-4 mice. Expression of the anorexigenic hormone leptin was also increased. Leptin is expressed in proportion to body fat content [34], and as FTO-4 mice increase their adipose tissue mass, leptin expression is likely to rise. CYP2E1, an enzyme that participates in drug metabolism and oxidation of several fatty acids, was also upregulated in adipose tissue. Its activity results in the production of reactive oxygen species and CYP2E1 KO mice are protected against diet induced obesity. [35] Furthermore, CYP2E1 protein levels are increased in rodents on a high-fat diet. [36]

The most striking feature relating to gastrocnemius muscle is that the fold-increases in gene expression were generally larger than those in other tissues. NMRK2, which is involved in myogenesis and is largely muscle-specific, was upregulated in FTO-4 mice. In addition, the transcriptional activator CITED4 and the mitochondrial translational activator ZMYND17 were upregulated. These changes are consistent with the small expansion of muscle mass observed in FTO-4 mice [9]. In addition, both the peroxisome proliferator-activated receptor gamma...
coactivator-1B (PPARGC1B) and the retinoid X receptor RXRG were upregulated. PPARGC1B is a transcriptional co-activator that coordinates metabolic gene programs in response to environmental cues, and in muscle it stimulates mitochondrial biogenesis. The PPARGC1-family acts as a co-activator for the transcription of retinoid X receptors (RXRs). [37]

| Hypothalamus | Gene name | Gene symbol | Entrez ID | Fold change |
|--------------|-----------|-------------|-----------|-------------|
| Glyoxalase 1 | Glo1      | 109801      | 2.17      |
| WD repeat and FYVE domain containing 1 | Wdfy1 | 69368 | 1.91 |
| Ribonuclease, RNase A family, 1 (pancreatic) | Rnase1 | 19752 | 1.91 |
| Expressed sequence A413582 | A413582 | 106672 | 1.67 |
| Solute carrier family 38, member 5 | Slc38a5 | 209837 | 1.67 |
| Transmembrane protein 242 | Tmem242 | 70544 | 1.65 |
| Ermin, ERM-like protein | Ermin | 77767 | 1.63 |
| Carbonic anhydrase 2 | Car2 | 12349 | 1.63 |
| Epithelial membrane protein 1 | Emp1 | 13730 | 1.59 |
| Cholinergic receptor, nicotinic, alpha polypeptide 3 | Chna3 | 110834 | 1.57 |

| Cerebellum | Gene name | Gene symbol | Entrez ID | Fold change |
|------------|-----------|-------------|-----------|-------------|
| WAP four-disulfide core domain 10 | Wdfc10 | 629756 | 4.01 |
| WD repeat and FYVE domain containing 1 | Wdfy1 | 69368 | 1.99 |
| Solute carrier family 38, member 5 | Slc38a5 | 209837 | 1.84 |
| Ribonuclease, RNase A family, 1 (pancreatic) | Rnase1 | 19752 | 1.82 |
| Expressed sequence A413582 | A413582 | 106672 | 1.78 |
| Predicted gene 3435 | Gm3435 | 100041621 | 1.73 |
| Transmembrane protein 242 | Tmem242 | 70544 | 1.66 |
| Epithelial membrane protein 1 | Emp1 | 13730 | 1.64 |
| Predicted gene 13931 | Gm13931 | 668825 | 1.62 |
| Predicted gene 7168 | Gm7168 | 635895 | 1.57 |

| White adipose tissue | Gene name | Gene symbol | Entrez ID | Fold change |
|----------------------|-----------|-------------|-----------|-------------|
| Major urinary protein family | Mup | - | 5.27 |
| Eosinophil-associated, ribonuclease A family, member 11 | Ear11 | 93726 | 4.88 |
| Carbonic anhydrase 3 | Car3 | 12350 | 4.20 |
| Cytochrome P450, family 2, subfamily e, polypeptide 1 | Cyp2e1 | 13106 | 3.83 |
| UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2 | B3galt2 | 26878 | 3.50 |
| Adiponectin, C1Q and collagen domain containing | Adipoq | 11450 | 3.26 |
| Carboxylesterase 1D | Ces1d | 104158 | 3.19 |
| Regulator of G-protein signaling 13 | Rgs13 | 246709 | 3.17 |
| Galactosidase, beta 1-like 2 | Gbs1l2 | 244757 | 3.06 |
| Myeloblastosis oncogene-like 1 | Mybl1 | 17864 | 3.06 |

| Gastrocnemius muscle | Gene name | Gene symbol | Entrez ID | Fold change |
|----------------------|-----------|-------------|-----------|-------------|
| Nicotinamide riboside kinase 2 | Nmrk2 | 69564 | 13.93 |
| Zinc finger, MYND domain containing 17 | Zmynd17 | 74843 | 6.12 |
| Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal Domain, 4 | Cited4 | 56222 | 4.86 |
| Transferrin receptor | Tfrc | 22042 | 4.41 |
| Potassium voltage-gated channel, subfamily G, member 4 | Kcnj4 | 66733 | 4.32 |
| Dual specificity phosphatase 10 | Dusp10 | 63953 | 3.44 |
| Parkinson disease (autosomal recessive, juvenile) 2, parkin | Park2 | 50873 | 3.24 |
| Integrin beta 6 | Itgb6 | 16420 | 3.04 |
| Inhibin alpha | Inha | 16322 | 2.90 |
| Ret proto-oncogene | Ret | 19713 | 2.83 |

Table 1. Top 10 genes that showed the highest amount of overexpression in FTO-4 mice per tissue.

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Pathway analysis

To examine pathways that might contribute to the phenotype observed in FTO-4 mice, we carried out pathway analysis using GO-Elite. Pathways in which more than 20% of the genes showed changes in expression in FTO-4 mice are listed in Tables S3 (GO-

Table 2. Top 10 genes that showed the highest amount of downregulation in FTO-4 mice per tissue.

| Tissue                      | Gene name                                      | Gene symbol | Entrez ID | Fold change |
|-----------------------------|------------------------------------------------|-------------|-----------|-------------|
| Hypothalamus                | Growth hormone                                | Gh          | 14599     | −14.89      |
|                             | Cyclin-dependent kinase inhibitor 1A (P21)     | Cdkn1a      | 12575     | −2.19       |
|                             | Cyclin-dependent kinase inhibitor 1B           | Cdkn1b      | 12576     | −2.00       |
|                             | Thioredoxin interacting protein                | Tnlp        | 56338     | −1.92       |
|                             | Zinc finger and BTB domain containing 16      | Zbtb16      | 235320    | −1.91       |
|                             | DNA segment, Chr 14, ERATO Doi 449, expressed | D14Ertd449e | 66039     | −1.8        |
|                             | Sulfotransferase family 1A, phenol-prefering, member 1 | Sult1a1 | 20887 | −1.79 |
|                             | RIKEN cDNA 6720456H20 gene                     | 6720456H20Rik | 218989 | −1.78 |
|                             | Serum/glucocorticoid regulated kinase 1        | Sgk1        | 20393     | −1.74       |
|                             | FKS06 binding protein 5                        | Fkbp5       | 14229     | −1.68       |
| Cerebellum                  | Zinc finger and BTB domain containing 16      | Zbtb16      | 235320    | −2.44       |
|                             | Nuclear receptor interacting protein 2         | Nrip2       | 60345     | −2.28       |
|                             | Glutathione peroxidase 3                      | Gpx3        | 14778     | −2.23       |
|                             | Prolactin family 2, subfamily c, member 3      | Prlc3       | 18812     | −2.14       |
|                             | Alanine-glyoxylate aminotransferase 2-like 1   | Agt2l1      | 71760     | −2.13       |
|                             | Sulfotransferase family 1A, phenol-prefering, member 1 | Sult1a1 | 20887 | −2.05 |
|                             | Solute carrier family 15 (H+/peptide transporter), member 2 | Slc15a2 | 57738 | −2.03 |
|                             | Connective tissue growth factor                | Ctgf        | 14219     | −1.99       |
|                             | DNA segment, Chr 14, ERATO Doi 449, expressed | D14Ertd449e | 66039 | −1.97 |
|                             | Contactin 4                                    | Cntn4       | 269784    | −1.96       |
| White adipose tissue        | Predicted gene 10002                           | Gm10002     | 791405    | −4.98       |
|                             | Killer cell lectin-like receptor subfamily B member 1B | Krb1b | 80782 | −3.7 |
|                             | Predicted gene 2663                            | Gm2663      | 100040208 | −3.23 |
|                             | Pyruvate dehydrogenase kinase, isoenzyme 4     | Pdk4        | 27273     | −2.88       |
|                             | Solute carrier family 15 (H+/peptide transporter), member 2 | Slc15a2 | 57738 | −2.75 |
|                             | Arginase type II                               | Arg2        | 11847     | −2.64       |
|                             | CDS antigen-like                               | Cds1        | 11801     | −2.48       |
|                             | Cysteinyl leukotriene receptor 2               | Cystlr2     | 70086     | −2.48       |
|                             | Transient receptor potential cation channel, subfamily V, member 6 | Tprv6 | 64177 | −2.47 |
|                             | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 | Hmgcs2 | 15360 | −2.47 |

Gastrocnemius muscle

| Indolethylamine N-methyltransferase | Inmt | 21743 | −7.33 |
| RIKEN cDNA 8430408G22 gene         | 8430408G22Rik | 213393 | −6.05 |
| Chemokine (C-X-C motif) ligand 13  | Cxcl13 | 55985 | −5.57 |
| Chac, cation transport regulator 1 | Chac1 | 69065 | −5.51 |
| CCAAT/enhancer binding protein (C/EBP), delta | Cebp | 12609 | −4.85 |
| F-box protein 32                   | Fbxo32 | 67731 | −4.79 |
| Arrestin domain containing 2       | Arrdc2 | 70807 | −4.40 |
| Sestrin 1                         | Sesn1 | 140742 | −4.31 |
| Solute carrier family 43, member 1 | Slc43a1 | 72401 | −4.30 |
| DNA-damage-inducible transcript 4 | Ddit4 | 74747 | −4.18 |

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Pathway analysis
Pathway analysis showed that anabolic pathways in WAT and skeletal muscle were significantly associated with FTO-4 mice, which is in line with the increased body weight of these mice. Furthermore, analysis revealed that a general methylation pathway contained a high percentage of genes (4 out of a total of 9 genes) that showed altered expression in gastrocnemius muscle (Table S4 in File S1). These included Tpmt (thiopurine S-methyltransferase) and Mat2a (methionine adenosyltransferase II alpha), which were upregulated, and Inmt (indolethylamine N-methyltransferase) and Pnmt (phenylethanolamine N-methyltransferase), which were downregulated.

Influence of FTO on m6A levels

Because genes involved in methylation showed altered expression levels in the gastrocnemius muscle of FTO-4 mice and FTO is a known demethylase of single stranded DNA and RNA, we investigated whether FTO affected m6A levels. Comparing MEFs from wild-type to FTO-4 mice, FTO was clearly upregulated

### Table 3. Obesity-associated genes that show altered expression in FTO-4 mice.

| Hypothalamus | Gene name | Gene Symbol | Entrez ID | Fold change |
|--------------|-----------|-------------|-----------|-------------|
| Carbonic anhydrase 2 | Car2 | 12349 | 1.59 |
| Growth hormone | Gh | 14599 | -14.89 |
| Pyruvate dehydrogenase kinase, isoenzyme 4 | Pdk4 | 27273 | -1.58 |
| Cerebellum | Delta-like 1 homolog (Drosophila) | Dlk1 | 13386 | -1.55 |
| Uncoupling protein 2 (mitochondrial, proton carrier) | Ucp2 | 7351 | -1.50 |
| White adipose tissue | Adiponectin, C1Q and collagen domain containing | Adipoq | 11450 | 3.26 |
| Adrenergic receptor, beta 3 | Adrb3 | 11556 | 2.59 |
| Androgen receptor | Ar | 11835 | 1.70 |
| Carbonic anhydrase 3 | Car3 | 12350 | 4.20 |
| Caveolin 1, caveole protein | Cav1 | 12389 | 1.89 |
| Cytochrome P450, family 2, subfamily e, polypeptide 1 | Cyp2e1 | 13106 | 3.83 |
| Leptin | Lep | 16846 | 2.72 |
| Monoamine oxidase B | Maob | 109731 | 1.68 |
| Pyruvate dehydrogenase kinase, isoenzyme 4 | Pdk4 | 27273 | -2.88 |
| Perilipin 1 | Plin1 | 103968 | 2.64 |
| Gastrocnemius muscle | Adrenergic receptor, beta 2 | Adrb2 | 11555 | -1.78 |
| CCAAT/enhancer binding protein (C/EBP), beta | Cebpbb | 12608 | -1.96 |
| Eukaryotic translation initiation factor 4E binding protein 1 | Eif4ebp1 | 13685 | -2.74 |
| Heparin-binding EGF-like growth factor | Hbegf | 15200 | 1.91 |
| Interleukin 6 receptor, alpha | I6ra | 16194 | -3.61 |
| Insulin receptor substrate 1 | Irs1 | 16357 | 1.70 |
| Insulin receptor substrate 2 | Irs2 | 384783 | -2.10 |
| Low density lipoprotein receptor | Ldr | 16835 | 1.66 |
| Lipin 2 | Lpin2 | 64898 | -1.80 |
| Neuropeptide Y receptor Y1 | Npy1r | 18166 | 1.60 |
| nuclear receptor subfamily 4, group A, member 3 | Nr4a3 | 18124 | 2.01 |
| Phosphodiesterase 4A, cAMP specific | Pde4a | 18577 | 2.15 |
| Phosphodiesterase 7A | Pde7a | 18583 | -1.98 |
| Phosphodiesterase 7B | Pde7b | 29863 | 1.72 |
| Pyruvate dehydrogenase kinase, isoenzyme 4 | Pdk4 | 27273 | -1.56 |
| Paternally expressed 3 | Peg3 | 18616 | -1.69 |
| Peroxisome proliferative activated receptor, gamma, coactivator 1 beta | Ppargc1b | 170826 | 2.10 |
| Retinoid X receptor gamma | Rrg | 20183 | 2.09 |
| Serine (or cysteine) peptidase inhibitor, clade E, member 1 | Serpine1 | 18787 | -1.62 |
| Vascular endothelial growth factor A | Vegfa | 23399 | 1.71 |
Subsequently, the ratio of m6A to A was determined in both total RNA and mRNA in wild-type and FTO-4 MEFs. This was accomplished by RNA extraction, enzymatic hydrolysis to free nucleotides using nuclease P1 and dephosphorylation to nucleosides by alkaline phosphatase. Concentrations of individual nucleosides were determined by LC-MS using HILIC chromatography with isocratic elution and positive mode electrospray mass spectrometry with $^{13}$C5-adenosine as an internal standard. From the obtained concentrations, ratios of m6A to A were calculated and used as a measure of m6A level for comparison between different samples. FTO overexpression was found to have no effect on m6A levels in both total RNA and mRNA (Figure 3B).

### Discussion

Beginning at around 5 weeks of age, FTO-4 mice exhibit increased body weight, increased fat mass and slightly increased lean mass.[9] It is therefore of interest that significant changes in expression of a number of genes associated with growth and/or proliferation were observed in the hypothalamus. Likewise, in gastrocnemius muscle, genes potentially involved in the proliferation of muscle mass were upregulated. Several genes whose expression was altered were also associated with metabolism and/or obesity. Furthermore, pathway analysis revealed the association of several anabolic pathways in skeletal muscle and WAT with FTO-4. Thus the changes in gene expression we observe may underlie the increase in body weight observed in FTO-4 mice that is initiated at around 5 weeks of age and becomes significant in later life.

### Changes in gene expression

D14Ertd449e (human C10orf57), Plac9 and Duxbl were downregulated in several tissues. Interestingly, all three of these genes are located close to one another on mouse chromosome 14. In C57/BL6 mice, D14Ertd449e, Plac9 and Duxbl exist as three tandem repeats; but in other mouse strains, the number of repeats varies [38]. In humans, C10orf57 and Plac9 are also located in close vicinity to one another on chromosome 10, although not as tandem repeats.

In WAT, gene expression of two important hormones was altered. Leptin mRNA was increased, in line with the increase in fat mass in FTO-4 mice. Interestingly, leptin was found to mediate FTO expression via STAT3 in the brain [39] as well as in the liver [40]. Moreover, overexpression of FTO in the liver reduced leptin-induced phosphorylation of STAT3, which impaired leptin-induced signaling pathways and resulted in downregulation of leptin-regulated genes [40]. In addition, liver FTO overexpression...
in mice led to increased levels of leptin. These data suggest that the increase in leptin mRNA might be influenced by both an increase in fat mass and by a link between FTO and leptin signaling.

Adiponectin was upregulated in WAT and its receptor AdipoR2 was downregulated 1.38 fold in the hypothalamus of FTO-4 mice. The hormone adiponectin is exclusively secreted from adipose tissue and has been implicated in several metabolic processes, such as improvement of insulin sensitivity and fatty acid oxidation. In contrast to the increase in gene expression of adiponectin we observed at 5 weeks of age, 20-week-old male FTO-4 mice showed a decrease in plasma adiponectin levels. This reduction in plasma adiponectin levels was also observed in rodents on a high fat diet and in humans with obesity and insulin resistance. However, the reduction in adiponectin levels might take longer to occur, as the stimulatory effect of caloric restriction on adiponectin gene expression was only detectable after long-term caloric restriction and not after short-term restriction.

In WAT, major urinary protein (MUP) genes were highly upregulated. MUPs are encoded by 20 discrete isoforms in mice, which show >85% identity in DNA sequences. They are members of the lipocalin family of proteins and are abundantly expressed in the liver. In urine, MUPS act as pheromones. They have also been implicated in many other physiological processes, such as the regulation of glucose and lipid metabolism and energy expenditure. MUP gene expression is likely to be regulated by metabolism as MUP genes are down-regulated in liver in response to dietary restriction. On the other hand, MUP gene expression is also decreased in WAT and liver of obese mice and in the liver in response to refeeding.

In gastrocnemius muscle, several genes and pathways related to muscle differentiation and growth were differentially regulated in FTO-4 mice, which is in line with their small increase in muscle mass. In future studies, it would be interesting to examine the effect of FTO overexpression on proliferation and differentiation of myotubes.

Interestingly, pathway analysis revealed that a methylation pathway that includes methyl transferases and enzymes involved in the biosynthesis of S-adenosylmethionine was significantly represented in genes differentially expressed in gastrocnemius muscle of FTO-4 mice. Of the four genes with altered expression levels in this pathway, Mat2a is of most interest. It catalyzes the production of S-adenosylmethionine, which is the major biological methyl donor in cellular processes, including the formation of m6A. The increased expression of Mat2a in FTO-4 mice might serve as a compensatory mechanism to counteract the potential demethylation by FTO.

Comparison with other studies

Changes in gene expression in mice carrying a loss-of-function point mutation in the FTO gene have been reported. Several of these genes showed a significant change in the opposite direction to those seen when FTO is overexpressed (this study).
These included D14Ertd449s, C6, Pkl2, Fat3 and Gm4841. For example, D14Ertd449s was upregulated in hypothalamus and WAT of mice carrying the FTO mutation, but downregulated in the same tissues in FTO-4 mice. This gene encodes a transmembrane protein of unknown function. Similarly, expression of C6, Pkl2 and Fat3 in WAT and Gm4841 in gastrocnemius muscle was reduced in FTO mutant mice, but increased in FTO-4 mice. The role of these genes in body weight regulation remains to be explored. C6 is part of the complement system, its enhanced expression potentially may relate to the increased inflammatory response in obesity. Pkl2 is a serine-threonine protein kinase required for centriole duplication, and may therefore be related to the observed expansion of lean mass in FTO-4 mice. Fat3 may be involved in cell adhesion, via its cadherin domains: the role of Gm4841 is unknown.

Microarray studies have also examined changes in gene expression when FTO is overexpressed in tissue-cultured HEK293 cells [56] and in human myotubes [57]. In the HEK293 cells, only one gene showed a change in expression in the same direction as in our in vivo study. This was the Cnbdl, a cysteine hydrolase, which was upregulated in both HEK293 cells and in gastrocnemius muscle. The differences between the two studies may relate to the fact they were carried out under very different conditions (48-hours post-transfection in tissue culture, versus our in vivo study). FTO overexpression in human myotubes resulted in an overrepresentation of mitochondrial genes that changed expression. Three of these genes were significantly upregulated in FTO overexpressing myotubes as well as in skeletal muscle of FTO-4, namely Cyb5b, Ppif, and Taco1. Interestingly, our study found an overrepresentation of genes involved in positive regulation of mitochondrial depolarization in gastrocnemius muscle of FTO-4 mice (Table S3 in File S1).

Comparison with m6A studies

Two studies have identified m6A-containing mRNAs in mouse liver [24] and brain [23]. As FTO is known to demethylate mRNA at m6A [10,12], we compared whether mRNAs that alter their expression in FTO-4 mice have been reported to contain m6A (Table S5 in File S1). Most FTO-regulated genes in gastrocnemius muscle that are known to be associated with obesity were found to contain m6A (Figure 4). In the hypothalamus, all FTO-regulated genes that are known to be associated with obesity were described to contain m6A sites. However, only 3 FTO-regulated genes have been associated with obesity in the hypothalamus, compared to 20 genes in gastrocnemius muscle. Although some genes that alter their expression in cerebellum and WAT of FTO-4 mice also contained m6A, the numbers were not as dramatic as in gastrocnemius muscle. Nevertheless, the data are compatible with the idea that FTO might influence the expression of at least some genes via regulating the amount of m6A in their mRNA.

To examine the effect of FTO overexpression on m6A levels, MEFs were isolated from wild-type and FTO-4 mice. Quantitative nucleoside analysis revealed that overexpression of FTO did not attenuate m6A levels in either total RNA or mRNA. Similarly, others have failed to see an effect of FTO knockdown on m6A levels in total RNA in mouse brain. [56]. A previous study reported a decrease in m6A levels in mRNA of HeLa cells in response to FTO overexpression and an increase in mRNA m6A levels following silencing of FTO in both HeLa and 293FT cells.[12] This study differed from ours in that FTO overexpression was higher (6 to 9-fold on protein level) and only acute effects of overexpression (24 hrs) were examined.[12] MEFs taken from FTO-4 embryos might have been adapted to high levels of the demethylase FTO, which could mask the effect of FTO on m6A levels. m6A methylation is a very dynamic process, and small differences in conditions could affect m6A levels. By using littermates for generating the MEFs, and also in the microarray study, we tried to avoid as much variation as possible. Our results do not provide evidence that under physiological conditions, overexpression of FTO lead to changes in total and mRNA m6A levels.

Conclusion

This study examined the changes in gene expression in FTO-4 mice before the onset of obesity at 5 weeks of age. Our data revealed that anabolic pathways and genes are already activated at this time-point, consistent with the increased body weight and adiposity observed in these mice at an older age [9]. FTO is known to demethylate single-stranded DNA and RNA. Interestingly, a methylation pathway in skeletal muscle was associated with FTO overexpression. Moreover, the majority of FTO-regulated genes that were previously associated with obesity in this tissue are likely to contain m6A sites in their mRNA transcripts. Hence regulation of expression of these genes potentially could be mediated by FTO. However, we failed to detect an effect of FTO overexpression on general m6A levels in either mRNA or in total RNA derived from MEFs. This suggests any effect of FTO on m6A methylation is gene specific and involves only a small subset of mRNAs. This stresses the importance of investigating the direct mRNA targets of FTO, as has been done in the case of ghrelin [4] and genes involved in dopaminergic signalling. [25]

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Author Contributions

Conceived and designed the experiments: MM JST SL FMA. Performed the experiments: MM JST SL HBK LB ML. Analyzed the data: MM JST SL HBK. Contributed reagents/materials/analysis tools: RDC FM. Wrote the paper: MM JST SL FMA.

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