FTO is increased in muscle during type 2 diabetes and its over-expression in myotubes alters insulin signalling, enhances lipogenesis and ROS production, and induces mitochondrial dysfunction.

Short title: Expression and function of FTO in skeletal muscle

Amélie BRAVARD1,2,3,4,5, Etienne LEFAI1,2,3,4,5, Emmanuelle Meugnier1,2,3,4,5, Sandra Pesenti1,2,3,4,5, Emmanuel DISSE5, Julien VOUILLARMET5, Noël PERETTI1,2,3,4,5, Rémi RABAS-LHORET6, Martine LAVILLE1,2,3,4,5, Hubert VIDAL1,2,3,4,5 and Jennifer RIEUSSET1,2,3,4,5.

1 INSERM, U-870, IFR62, Faculté de Médecine Lyon Sud, F-69600 Oullins, France
2 INRA, UMR1235, F-69600 Oullins, France
3 INSA-Lyon, RMND, F-69621 Villeurbanne, France
4 Université Lyon 1, F-69622 Lyon, France
5 Hospices Civils de Lyon, Service de Nutrition et Diabétologie, F-69008 Lyon, France
6 Montreal diabetes research center, Montreal university,

Corresponding author:
Jennifer Rieusset,
Email: jennifer.rieusset@univ-lyon1.fr

Submitted 25 February 2010 and accepted 4 October 2010.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Objectives. A strong association between genetic variants and obesity was found for the fat mass and obesity associated (FTO) gene. However, few details are known concerning the expression and function of FTO in skeletal muscle of patients with metabolic diseases.

Research Design and Methods. We investigated basal FTO expression in skeletal muscle from obese non diabetic subjects and type 1 and type 2 diabetic patients, compared to age-matched control subjects, and its regulation in vivo by insulin, glucose or rosiglitazone. The function of FTO was further studied in myotubes by over-expression experiments.

Results. We found a significant increase of FTO mRNA and protein levels in muscle from type 2 diabetic patients, whereas its expression was unchanged in obese or type 1 diabetic patients. Moreover, insulin or glucose infusion during specific clamps did not regulate FTO expression in skeletal muscle from control or type 2 diabetic patients. Interestingly, rosiglitazone treatment improved insulin sensitivity and reduced FTO expression in muscle from type 2 diabetic patients. In myotubes, adenoviral FTO over-expression increased basal PKB phosphorylation, enhanced lipogenesis and oxidative stress, and reduced mitochondrial oxidative function, a cluster of metabolic defects associated with type 2 diabetes.

Conclusions. This study demonstrates increased FTO expression in skeletal muscle from type 2 diabetic patients, which can be normalized by thiazolidinedione treatment. Furthermore, in vitro data support a potential implication of FTO in oxidative metabolism, lipogenesis and oxidative stress in muscle, suggesting that it could be involved in the muscle defects that characterize type 2 diabetes.

In Genome-wide association studies, polymorphisms in the fat mass and obesity-associated gene (FTO) were strongly associated with an increased risk of obesity (1-3). The FTO gene is expressed in a number of tissues relevant to metabolic diseases, including adipose tissue and skeletal muscle (1). Whereas some investigators found that the FTO mRNA level was increased in adipose tissue from obese subjects (4), others reported a negative correlation between FTO expression and body mass index (BMI) (5), and a reduction in FTO gene expression was observed in adipose tissue depots from rodent models of obesity (6). Nevertheless, recent results obtained either in knock-out mice (7) or in mice expressing a mutated form of FTO (FTOI367F) (8), demonstrated that a loss of function FTO is associated with reduced body weight and fat mass, clearly supporting a role of FTO in fat accumulation.

Some FTO polymorphisms have also been associated with type 2 diabetes (T2D) (9-13), but it could be due to the increased BMI (14). While several reports suggested that FTO expression could be regulated by environmental factors, such as fasting and feeding (6; 15; 16), which are known to impact on insulin responsiveness, only a very few studies have specifically investigated FTO expression in relation to insulin sensitivity. A recent study reported that the age-dependant decline of FTO expression is associated with peripheral defects of glucose and lipid metabolism in adipose tissue and skeletal muscle (17). However, up to date little is known regarding the factors and the mechanisms controlling FTO expression, as
well as its cellular functions, particularly in human skeletal muscle.

Therefore, we investigated FTO gene expression and function in human skeletal muscle and unveiled both in vivo and in vitro a new role of FTO in the pathogenesis of T2D.

RESEARCH DESIGN AND METHODS

**Subject and protocols.** All participants gave their written consent after being informed of the nature, purpose, and possible risks of the study. The protocol was approved by the ethical committees of the Hospices Civils de Lyon and performed according to French Legislation (Huriet Law). The healthy volunteers were divided into two groups based on their age. None had impaired glucose tolerance or a familial or personal history of diabetes, obesity, dyslipidemia, or hypertension. One group of 10 volunteers (Control 50: 4 women and 6 men, age: 55.3±3.5 years, BMI: 23.2±0.5 kg/m^2_, HbA1c: 5.2±0.2 %) was age-matched with 10 non diabetic obese subjects (3 women and 7 men, age: 52.0±1.4 years, BMI: 33.3±0.9 kg/m^2_, HbA1c: 5.5±0.3 %) and 10 obese type 2 diabetic patients (5 women and 5 men, age: 56.2±2.4 years, BMI: 32.1±1.0 kg/m^2_, HbA1c: 10.1±0.7 %). An unrelated group of healthy lean subjects (Control 25: 1 women and 4 men, age: 25.4±1.3 years, BMI: 21.5±0.6 kg/m^2_, HbA1c: 5.2 ±0.3 %) served as the control group for type 1 diabetic patients (1 women and 4 men, age: 28.8±1.5 years, BMI: 24.5±1.3 kg/m^2_, HbA1c: 9.1±0.4 %). Type 1 diabetic patients had no familial antecedent of T2D, and they were all treated with daily injections of insulin (45±5 IU/day) (18). Another group of type 2 diabetic patients (3 women and 3 men, age: 52.6±2.9 years, BMI: 29.5±6.9 kg/m^2_) were treated for 12 weeks with 8 mg per day of rosiglitazone (Avandia®, GlaxoSmithKline France). Patients had a T2D for at least one year and a mean duration of diagnosed diabetes of 4±2 years, and all were treated with metformin alone since at least 3 months (between 1.5 et 2 g/day). They have never been treated with thiazolidinediones or insulin. Metformin treatment was not stopped during the study. They did not have complications and their disease state was of mild severity, as assessed by a mean HbA1c level of 7.0±0.3 %.

After an overnight fast, all subjects underwent a 3-hour euglycemic hyperinsulinemic clamp, as described previously (18; 19). Percutaneous biopsies of the vastus lateralis muscle were performed under local anesthesia, as previously described (18). Another group of six healthy subjects (6 men, age: 21.7±1.5 years, BMI: 22.1±1.4 kg/m^2_, HbA1c: 5.1±0.2) were submitted to a 3h hyperglycemic euinsulinemic clamp with infusion of somatostatin to inhibit endogenous insulin release (20). During this clamp, glycemia roughly doubled (preclamp: 5.1±0.3; postclamp: 9.8±1.2 mmol/l, P < 0.0001) to reach levels classically obtained during post-prandial glycemic incursion in diabetic states.

**Quantification of mRNAs.** Total RNA samples from muscles and cell cultures were purified as previously described (18; 20). mRNA levels were measured by reverse transcription followed by real-time PCR using a Rotor-Gene™ 6000 (Corbett Research, Mortlake, Australia), as previously described (21; 22). Primers were listed in Supplementary Table 1 in the online appendix available at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org). Values were normalized using Hypoxanthine guanine phosphoribosyl transferase (HPRT) or TATA box binding protein (TBP) mRNA, measured as reference genes, which were similar between subjects or culture conditions.

**Western blot.** Muscle biopsies were lysed in PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail, and myotubes in a buffer containing 200mM NaF, 20mM NaH2PO4,
150mM NaCl, 50mM HEPES, 4mM NaVO4, 10mM EDTA, and 2mM PMSF, with 1% Triton X, 10% glycerol and protease inhibitor cocktail. Primary antibodies used were FTO (abcam, ab65366), total Akt (Cell Signaling, #9272), phospho-Akt (Cell Signaling, #9271), OXPHOS cocktail (Mitosciences, MS604), with Tubulin (Santa Cruz, sc-8035) or actin (Sigma A5060) as a loading control.

**Protein carbonylation:** Total protein carbonylation was detected, using the Oxidized Protein Detection Kit (Chemicon), as previously described (23).

**FTO expression vector and recombinant adenovirus.** The cDNA sequence encoding full-length human FTO was PCR-amplified from IMAGE clone 100016381 (Geneservice UK Ltd.). The amplified PCR product was cloned into the pGEM®-T Easy vector (Promega) and fully sequenced. It was then excised by digestion with EcoRV/NotI and cloned into the pcDNA3 vector (Invitrogen), generating the pcDNA3/FTO plasmid. Recombinant adenoviral genome encoding human FTO was generated by homologous recombination and amplified as described previously (24; 25).

**Cell culture.** HEK293 cells were grown in Dulbecco modified Eagle’s medium (DMEM, PAA, Germany) supplemented with 10% fetal bovine serum (FBS). Cells were transfected in six well plates for 48h with 3µg expression plasmid for the FTO gene or with the empty vector as control, using EXGEN 500 transfecting reagent (Invitrogen), generating the pcDNA3/FTO plasmid. Recombinant adenoviral genome encoding human FTO was generated by homologous recombination and amplified as described previously (24; 25).

**Lipogenesis.** Post-infected myotubes were cultured for 24 hours in a serum-free medium containing 5 mM glucose. The rate of lipogenesis from [2-¹⁴C]acetate (0.75µCi per flask; Perkin Elmer NEC-553) was then measured in the presence of 1mM acetate during a 24h period. Labelled fatty acids were extracted according to the Folch Method (27) and then quantified by liquid scintillation counting. Data were normalised by protein concentrations.

**ROS production:** Post-infected human myotubes were treated with BSA (0.1%) or palmitate (750µM) for 16 hours, and ROS production was detected by the nitroblue tetrazolium assay, as previously reported (23).

**Microarray analysis.** The procedure used to obtain microarray data has previously been described (7). Briefly, total RNA extracted from GFP and FTO over-expressing myotubes from four different healthy subjects were hybridized on oligonucleotide microarrays produced by the French Genopole network (RNG), consisting of 25 342 oligonucleotides of 50-mers printed on glass slides. The signal intensities of the microarray spots were loaded to R (version 2.9.2), background corrected with a offset of 50 (28), lowess normalized within arrays, quantile normalized between arrays and log transformed using the limma package from BioConductor (29). Only spots with a signal to noise ratio for Cy3 and Cy5 above 2 and presents on the four slides were selected for statistical analysis. The call for differential expression was done fitting a linear model to each probe (30). The data set is available from the GEO database (GSE22857).
**Expression and function of FTO in skeletal muscle**

**ATP synthesis:** Mitochondrial ATP synthesis in C₂C₁₂ cells was measured according to the protocol of Vives-Bauza C et al (31).

**Statistical analysis.** All data are presented as means ± SEM. Normality was tested and all variables had a Gaussian distribution. Statistical significance was determined using ANOVA when comparing age-matched groups of subjects (lean, obese and type 2 diabetic subjects). Student’s paired t-test was used when comparing mRNA levels before and after the clamp or rosiglitazone treatment in the same group of subjects. Correlations were analyzed using Spearman’s rank correlation test. Statistical significance of *in vitro* results was calculated according to unpaired Student’s t-test. The threshold for significance was set at P < 0.05.

**RESULTS**

**Metabolic characteristics of the subjects.** The metabolic characteristics of the subjects are presented in Table 1. After an overnight fast, insulin, nonesterified fatty acid and triglyceride plasma concentrations were higher in obese individuals, with or without diabetes, than in lean control subjects. Type 2 diabetic patients also had a higher fasting glycemia. The insulin-stimulated glucose disposal rate was profoundly reduced both in obese and type 2 diabetic patients. The type 1 diabetic patients had fasting hyperglycemia compared to age-matched control subjects and displayed a slight, but non significant, reduction in glucose disposal rate during the clamp, indicating that they were not insulin-resistant in contrast to obese and type 2 diabetic patients.

**Regulation of FTO expression in skeletal muscle.** Type 2 diabetic patients had significantly higher skeletal muscle FTO mRNA levels compared either to age-matched lean controls or to obese non diabetic subjects (Figure 1A). However, there was no difference between lean controls and obese non-diabetic subjects (Figure 1A). It should be noted that the FTO mRNA levels in skeletal muscle were positively correlated with HbA1c (r=0.62, p=0.04) and glycemia (r=0.5, p=0.02), and a tendency observed with the basal rate of lipid oxidation (r=-0.48, p=0.06). However, we did not observe correlation of FTO mRNA levels with glucose disposal during the clamp (r=0.22 p=0.43). In addition, increased FTO expression was also demonstrated at the protein level in muscle from type 2 diabetic patients (Figure 1B). Interestingly, there was no difference in FTO mRNA levels between type 1 diabetic subjects and age-matched control subjects (Figure 1A), suggesting that the up-regulation of FTO expression observed in type 2 diabetics was not related to chronic hyperglycemia (Figure 1A).

To get more insight into the regulatory mechanisms of FTO expression, we investigated the effect of 3 hours of hyperinsulinemia on FTO mRNA levels in muscle from control and type 2 diabetic patients. FTO expression was not modified during the euglycemic hyperinsulinemic clamp, in both groups of subjects (Figure 1C). To further test whether FTO expression could be regulated by hyperglycemia, we investigated samples from healthy subjects, submitted to an acute rise of glycemia, independently from insulin (20). Once again, we did not observe significant changes in FTO expression in muscle samples taken before and after a 3-hour hyperglycemic euinsulinemic clamp (Figure 1D).

Since impaired FTO expression was observed only in tissue from type 2 diabetic patients, we investigated the effects of the insulin-sensitizer rosiglitazone on FTO expression in another group of type 2 diabetics. Rosiglitazone treatment for 12 weeks reduced serum insulin levels and significantly improved glucose utilization and oxidation rates (Supplementary Table 2). The treatment significantly reduced FTO mRNA levels in skeletal muscle (Figure 1E). There
was no correlation between the observed changes in FTO expression and improved insulin sensitivity in this small group of subjects (data not shown).

Effect of FTO over-expression on insulin signaling. To determine whether the observed up-regulation of FTO in muscle from type 2 diabetic patients could contribute to altered insulin action, we transiently over-expressed FTO in HEK293 cells and investigated the consequences on insulin signaling. A 2-fold increase of FTO protein was associated with a marked increase of basal PKB phosphorylation on both Ser473 and Thr308 residues (Figure 2). Furthermore, the insulin-stimulated phosphorylation of PKB was inhibited in FTO over-expressing cells (Figure 2).

To confirm this effect in muscle cells, we constructed adenoviruses for the over-expression of either GFP (control) or FTO in differentiated myotubes. Over-expression of FTO in C2C12 myotubes (5 fold increase) induced a significant increase of basal PKB phosphorylation (Figure 3A). In addition, the phosphorylation of PDK1, the kinase mediating the phosphorylation of PKB on Thr308, was also increased after FTO over-expression. Next, we investigated the effect of FTO on insulin-stimulated PKB phosphorylation. At a maximal concentration of insulin (10^{-7} M), despite a marked reduction in the amplitude of insulin to phosphorylate PKB (3 fold vs 11 fold increase in GFP-over-expressing cells), insulin was still able to increase PKB phosphorylation in FTO-over-expressing cells (Figure 3B). In addition, FTO over-expression induced basal phosphorylation of both PKB and p70/85-S6 kinase in human myotubes (Figure 3C), indicating also an activation of some PKB down-stream events. Silencing FTO using a specific RNAi, (Figure 3D) did not significantly modify basal Ser473 PKB phosphorylation in human myotubes (Figure 3E).

Effect of FTO over-expression on lipogenesis and oxidative stress. Increased lipids in muscle from type 2 diabetic patients has been associated with insulin resistance (32). In agreement with its implication in obesity, a role for FTO in lipid accumulation has been previously hypothesized (7; 8). We therefore investigated the role of FTO on lipid accumulation in human myotubes. FTO over-expression significantly increased the rate of lipogenesis compared to GFP-over-expressing myotubes (Figure 4A). In addition, the expression of key genes involved in this pathway were induced by FTO over-expression (Figure 4B), confirming a role for FTO in the control of lipogenesis.

Lipids are known to induce the production of reactive oxygen species (ROS) in skeletal muscle (23), and ROS have been implicated in insulin resistance and diabetes (33). We therefore investigated whether FTO could modify lipid-induced ROS production in human myotubes. As shown in Figure 4C, we did not observe a palmitate-induced ROS production in GFP over-expressing myotubes, under our experimental conditions. In contrast, a robust increase of palmitate-induced ROS production was observed in cells over-expressing FTO (Figure 4C). A tendency to increased basal ROS production was also noticeable in response to FTO over-expression (p=0.07). Silencing FTO did not modify palmitate-induced ROS production, whereas it tended to reduce basal ROS production (Figure 4C).

Effect of FTO over-expression on mitochondria function. Increased of both lipogenesis and ROS production in myotubes could be related to reduced mitochondria function. This hypothesis is supported by the in vivo negative correlation of FTO expression with lipid oxidation observed in the present study and already reported by others (17). To test this hypothesis, we first performed a transcriptomic analysis to identify genes regulated by FTO over-
Expression and function of FTO in skeletal muscle

expression in human myotubes. Microarrays analysis led to the identification of 1,103 regulated spots (for adjusted p-value<0.05 and fold change>1.2) coding for 1,080 unique genes (542 upregulated and 538 downregulated genes). Using David (http://david.abcc.ncifcrf.gov/) to analyze this set of genes, the Gene Ontology class “mitochondrion” showed significant over-representation compared to its representation in the human genome (GO:0005739, adjusted P value 2.13 e-3). The corresponding genes (n=76) are listed in Table 2. Then, we further performed structural and functional analysis of mitochondria. Electronic microscopy analysis of human myotubes over-expressing FTO showed no major structural modification, compared to GFP (Figure 5A). Analysis of protein levels of individual OXPHOS complexes revealed a significant reduction in complex II 30 kDa subunit, whereas the expression of NDUF8, CIICore2, and CV subunit alpha (characteristics of complexes I, III, and V respectively) was not altered in human myotubes (Figure 5B). In addition, PPARγ coactivator 1 (PGC1α) mRNA levels were not significantly modified in myotubes over-expressing FTO compared to GFP (PGC1α/TBP (fold vs GFP): 1.0±0.3 vs. 1.1±0.1 , p=0.64, respectively). Lastly, we investigated the repercussion of FTO over-expression on in vitro substrate-mediated ATP synthesis. As shown in Figure 5C, FTO over-expression reduced complex I-mediated ATP synthesis in C2C12 cells, whereas ATP synthesis mediated by complex II substrates was not significantly modified.

**Decreased expression of FTO-regulated genes in muscle of type 2 diabetic patients.** To confirm in vivo the relevance of our in vitro observations, we measured some FTO-regulated mitochondria genes in skeletal muscle of control and type 2 diabetic patients. We measured the expression of 3 FTO-regulated genes, selected in Table 2 on their implication on oxidative metabolism (ATP5B, UQCR) and oxidative stress (SOD2). As observed in vitro, we found a significant reduction of ATP5B and SOD2 in muscle of type 2 diabetic patients compared to controls (Figure 6A), whereas a tendency to reduction was observed for UQCR (p=0.06). In addition, PGC1α expression is significantly reduced in muscle of type 2 diabetic patients (Figure 6A). Lastly, protein carbonylation is induced in muscle of type 2 diabetic patients (Figure 6B), indicating an increase of oxidative stress.

**DISCUSSION**

While variations of the FTO gene are associated with obesity and T2D, the in vivo regulation of FTO and its function are still largely unknown. Here, we demonstrate that FTO expression is increased in skeletal muscle from type 2 diabetic patients and that rosiglitazone treatment partially reversed this defect. In addition, FTO over-expression in cultured myotubes enhanced basal PKB phosphorylation, increased lipogenesis and lipid-induced ROS production, and reduced mitochondria oxidative function. Since in vitro effects of FTO were partially confirmed in muscle of type 2 diabetic patients, we suggest that increased FTO expression in these patients may contribute to reduced mitochondria oxidative capacities, excessive lipid accumulation and oxidative stress, a cluster of metabolic defects known to be associated with T2D.

We report for the first time a significant increase of both FTO mRNA and protein levels in vastus lateralis muscle from type 2 diabetic patients, compared either to healthy lean control subjects or to BMI-matched obese non-diabetic individuals. However, we found no correlation between FTO expression and insulin sensitivity as estimated by glucose disposal rate during the hyperinsulinemic clamp. This finding may indicate that FTO induction was related to diabetes per se rather than insulin resistance, a
Expression and function of FTO in skeletal muscle

Conclusion also supported by the fact that FTO mRNA levels were not altered in muscle of insulin resistant obese subjects. Concerning the regulation of FTO expression, previous data in mice demonstrated that FTO expression could be affected by environmental factors, such as fasting and feeding (6; 15; 16), suggesting a possible regulation by hormones and/or nutrients. However, we did not observe rapid regulation of FTO mRNA by either insulin or glucose during specific clamp studies, suggesting that FTO expression is not acutely regulated by insulin or glucose in human skeletal muscle, as recently reported (17; 34). The unaltered expression of FTO in skeletal muscle from type 1 diabetic patients is in agreement with the absence of a direct regulation of FTO during hyperglycaemic clamp. This observation reinforces the concept that increased level of FTO in muscle is a characteristic of T2D, independently from obesity and whole-body insulin resistance and also from hyperglycemia. Interestingly, therapy with the antidiabetic agent rosiglitazone produced a significant reduction in the expression of FTO in muscle of type 2 diabetic patients. Unfortunately, up to now, the relationship between insulin sensitivity and loss of FTO function has not been systematically examined in mice or in humans (7; 8; 35; 36), although it is interesting to point out that invalidation of FTO in mice has been associated with a mild improvement of insulin sensitivity (7).

Our in vitro over-expression experiments showed that FTO increased the basal phosphorylation of major actors of the insulin signaling cascade, such as PDK1, PKB and p70/85S6K. In muscle cells however, FTO did not appear to inhibit the effect of insulin on PKB activation, suggesting that the upstream steps of the IRS1/PI3-kinase pathway are not acutely affected by FTO in muscle cells. However, FTO inhibited insulin-induced PKB phosphorylation in HEK293 cells, supporting the concept that FTO is able to affect, directly or indirectly, the signaling pathways. While the increased phosphorylation of PKB on Thr308 is probably related to induced PDK1 activity, it is more difficult to explain increased Ser473 PKB phosphorylation since several PKB-Ser473 kinases, including the rictor-mTOR complex, are known. Alternatively, one cannot exclude that PKB-specific phosphatases were inhibited in FTO over-expressing cells.

A new interesting finding is that FTO over-expression induced lipid accumulation, oxidative stress and mitochondrial dysfunction in myotubes. Alterations of these biological processes have clearly been associated with T2D (33; 37). Interestingly, expression of multiple components of the mitochondrial respiratory chain were altered by FTO over-expression, including 6 complex I, 2 complex III, 1 complex IV, and 3 complex V. Whereas no genes of complex II were altered by FTO over-expression in our transcriptomic analysis, we found that FTO induced a significant reduction, at protein level, of the iron sulfur subunit of complex II, indicating that complex II was also controlled by FTO over-expression. We sought to determine whether these changes might be occurring via PGC1α, a transcriptional co-activator that regulates mitochondrial biogenesis and plays a crucial role in the regulation of genes involved in oxidative phosphorylation in muscle (38). Interestingly, FTO encodes a 2-oxoglutarate-dependent nucleic acid demethylase (4) and methylation of the promoter of PGC1α was altered in muscle of type 2 diabetic patients (39). However, we found no modification of PGC1α mRNA levels following FTO over-expression in human myotubes. Nevertheless, PGC1α mRNA levels are barely detectable in myotubes, we cannot exclude that subtle change of PGC1α expression occurred. Nevertheless, we observed, as previously
reported (40-42), a significant reduction of PGC1α expression in muscle of type 2 diabetic patients compared to control subjects, indicating that PGC1α and FTO are regulated in an opposite manner in these patients. In a recent study, a positive correlation between FTO and PGC1α mRNA levels were observed in young and elderly twins (17). This correlation was obtained with non-diabetic subjects and thus cannot be compared with our study. All together, our data, suggest that PGC1α is probably not involved in FTO-induced alterations of mitochondria genes under our experimental conditions. Interestingly, the FTO-induced modifications of mitochondria transcriptome is associated with a reduction of oxidative metabolism, as suggested by the decrease of complex I-mediated ATP synthesis. Coupled with the increase of lipid synthesis, these data suggest that FTO induces a shift from oxidation of lipid towards their accumulation in myotubes. These data are in accordance with a recent study showing a negative association between muscle FTO expression and lipid oxidation in a large cohort of young and old subjects (17). It was also recently demonstrated that mice with a dominant mutation in the mouse FTO gene (FTO<sup>1367F</sup>) showed increased fatty acid (FAS) gene expression in skeletal muscle (8). While this data could suggest enhanced lipid synthesis, this latter was not measured and no conclusion could be drawn regarding the relationship between FTO and lipid storage in muscle. In contrast, our data clearly indicated that increased expression of FTO into myotubes is associated with enhanced lipogenesis.

Strong similarities between FTO function in myotubes and alterations in skeletal muscle of T2D exist. Diabetic muscle was characterized by decreased expression of oxidative phosphorylation genes (41; 42), increased lipid accumulation (37) and increased oxidative stress (33). All these metabolic alterations are reproduced by over-expression of FTO in human myotubes. Interestingly, we found a reduction of OXPHOS and antioxidant genes and an increase of protein carbonylation in muscle of type 2 diabetic patients, confirming partially <em>in vitro</em> data. Taken together, these data suggest that the increased FTO in muscle of type 2 diabetic patients may contribute to altered oxidative metabolism and increased oxidative stress, which characterize the muscle of these patients. Additional studies are clearly needed now to more precisely investigate the <em>in vivo</em> repercussions of skeletal muscle FTO expression.

In conclusion, we demonstrate that T2D is associated with increased FTO expression, reduced expression of OXPHOS and antioxidant genes, and increased oxidative stress in human skeletal muscle. Over-expression of FTO in myotubes induces basal PKB phosphorylation, increases lipogenesis and oxidative stress, and reduces mitochondria oxidative function. Therefore, we propose that FTO may contribute in the muscle defects that characterize T2D.

**Author Contributions.** A.B. researched data, contributed to discussion, reviewed/edited manuscript. E.L. contributed to discussion. E.M. researched data. S.P. researched data. E.D., J.V., and N.P. contributed to discussion. R.R.L., M.L. and H.V. contributed to discussion, reviewed/edited manuscript. J.R. researched data, contribute to discussion, wrote manuscript.

**ACKNOWLEDGEMENTS**

This work was supported by grants of INSERM and ANR (grant to J. R., n° ANR-09-JCJC-0116-01). A.B. is a recipient of a grant from Servier Laboratories and the ANRT. GlaxoSmithKline sponsored the Rosiglitazone treatment study. We acknowledge the editorial assistance of Elisabeth Harley, Servier Laboratories.
REFERENCES

1. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JR, Elliott KS, Lango H, Rayner NW, Shields B, Harries LW, Barrett JC, Ellard S, Groves CJ, Knight B, Patch AM, Ness AR, Ebrahim S, Lawlor DA, Ring SM, Ben-Shlomo Y, Jarvelin MR, Sovio U, Bennett AJ, Melzer D, Ferrucci L, Loos RJ, Wareham NJ, Karpe F, Owen KR, Cardon LR, Walker M, Hitman GA, Palmer CN, Doney AS, Morris AD, Smith GD, Hattersley AT, McCarthy MI: A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* 316:889-894, 2007

2. Dina C, Meyre D, Gallina S, Durand E, Korner A, Jacobson P, Carlsson LM, Kiess W, Vatin V, Lecoeur C, Delplanque J, Vaillant E, Pattou F, Ruiz J, Weill J, Levy-Marchal C, Horber F, Potoczna N, Hercberg S, Le Stunff C, Bougnères P, Kovacs P, Marre M, Balkau B, Cauchi S, Chevre JC, Froguel P: Variation in FTO contributes to childhood obesity and severe adult obesity. *Nat Genet* 39:724-726, 2007

3. Scuteri A, Sanna S, Chen WM, Uda M, Albai G, Strait J, Najjar S, Nagaraja R, Orru M, Usala G, Dei M, Lai S, Maschio A, Busonero F, Mulas A, Ehret GB, Fink AA, Weder AB, Cooper RS, Galan P, Chakravarti A, Schlessinger D, Cao A, Lakatta G, Abecasis GR: Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet* 3:e115, 2007

4. Wahlen K, Sjolin E, Hoffstedt J: The common rs9939609 gene variant of the fat mass- and obesity-associated gene FTO is related to fat cell lipolysis. *J Lipid Res* 49:607-611, 2008

5. Kloting N, Schleinitz D, Ruschke K, Berndt J, Fasshauer M, Tonjes A, Schon MR, Kovacs P, Stumvoll M, Bluher M: Inverse relationship between obesity and FTO gene expression in visceral adipose tissue in humans. *Diabetologia* 51:641-647, 2008

6. Stratigopoulos G, Padilla SL, LeDuc CA, Watson E, Hattersley AT, McCarthy MI, Zeltser LM, Chung WK, Leibel RL: Regulation of Fto/Ftm gene expression in mice and humans. *Am J Physiol Regul Integr Comp Physiol* 294:R1185-1196, 2008

7. Fischer J, Koch L, Emmerling C, Vierkotten J, Peters T, Bruning JC, Ruther U: Inactivation of the Fto gene protects from obesity. *Nature* 458:894-898, 2009

8. Church C, Lee S, Bagg EA, McTaggart JS, Deacon R, Gerken T, Lee A, Moir L, Mecinovic J, Quwailid MM, Schofield CJ, Ashcroft FM, Cox RD: A mouse model for the metabolic effects of the human fat mass and obesity associated FTO gene. *PLoS Genet* 5:e1000599, 2009

9. Herder C, Rathmann W, Strassburger K, Finner G, Grillert H, Huth C, Meisinger C, Gieger C, Martin S, Giani G, Sarbaser WA, Wichmann HE, Illig T: Variants of the PPARC, IGF2BP2, CDKAL1, HHEX, and TCF7L2 genes confer risk of type 2 diabetes independently of BMI in the German KORA studies. *Horm Metab Res* 40:722-726, 2008

10. Yajnik CS, Janipalli CS, Bhaskar S, Kulkarni SR, Freathy RM, Prakash S, Mani KR, Weedon MN, Kale SD, Deshpande J, Krishnaveni GV, Veena SR, Fall CH, McCarthy MI, Frayling TM, Hattersley AT, Chandak GR: FTO gene variants are strongly associated with type 2 diabetes in South Asian Indians. *Diabetologia* 52:247-252, 2009

11. Ng MC, Park KS, Oh B, Tam CH, Cho YM, Shin HD, Lam VK, Ma RC, So WY, Cho YS, Kim HL, Lee HK, Chan JC, Cho NH: Implication of genetic variants near TCF7L2, SLC30A8, HHEX, CDKAL1, CDKN2A/B, IGF2BP2, and FTO in type 2 diabetes and obesity in 6,719 Asians. *Diabetes* 57:2226-2233, 2008

12. Legry V, Cottel D, Ferrieres J, Arveiler D, Andrieux N, Bingham A, Wagner A, Ruidavets JB, Ducimetiere P, Amouyal P, Meirhaeghe A: Effect of an FTO polymorphism on fat mass,
Expression and function of FTO in skeletal muscle

11

obesity, and type 2 diabetes mellitus in the French MONICA Study. *Metabolism* 58:971-975, 2009

13. Sanghera DK, Ortega L, Han S, Singh J, Ralhan SK, Wander GS, Mehra NK, Mulvihill JJ, Ferrell RE, Nath SK, Kamboh MI: Impact of nine common type 2 diabetes risk polymorphisms in Asian Indian Sikhs: PPARG2 (Pro12Ala), IGF2BP2, TCF7L2 and FTO variants confer a significant risk. *BMC Med Genet* 9:59, 2008

14. Freathy RM, Timpson NJ, Lawlor DA, Pouta A, Ben-Shlomo Y, Ruokonen A, Ebrahim S, Shields B, Zeggini E, Weedon MN, Lindgren CM, Lango H, Melzer D, Ferrucci L, Paolizzo G, Neville MJ, Karpe F, Palmer CN, Morris AD, Elliott P, Jarvelin MR, Smith GD, McCarthy MI, Hattersley AT, Frayling TM: Common variation in the FTO gene alters diabetes-related metabolic traits to the extent expected given its effect on BMI. *Diabetes* 57:1419-1426, 2008

15. Fredriksson R, Hagglund M, Olszewski PK, Stephansson O, Jacobsson JA, Olszewska AM, Levine AS, Lindblom J, Schioth HB: The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. *Endocrinology* 149:2062-2071, 2008

16. Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, Hewitson KS, Yeo GS, McDonough MA, Cunliffe S, Neill LA, Galvanovskis J, Rorsman P, Robins P, Coll AP, Ma M, Jovanovic Z, Farooqi IS, Sedgwick B, Barroso I, Lindahl T, Ponting CP, Ashcroft FM, O’Rahilly S, Schofield CJ: The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science* 318:1469-1472, 2007

17. Grunnet LG, Nilsson E, Ling C, Hansen T, Pedersen O, Groop L, Vaag A, Poulsen P: Regulation and function of FTO mRNA expression in human skeletal muscle and subcutaneous adipose tissue. *Diabetes*, 2009

18. Ducluzeau PH, Perretti N, Laville M, Andreelli F, Vega N, Riou JP, Vidal H: Regulation by insulin of gene expression in human skeletal muscle and adipose tissue. Evidence for specific defects in type 2 diabetes. *Diabetes* 50:1134-1142, 2001

19. Laville M, Auboeuf D, Khalfallah Y, Vega N, Riou JP, Vidal H: Acute regulation by insulin of phosphatidylinositol-3-kinase, Rad, Glut 4, and lipoprotein lipase mRNA levels in human muscle. *J Clin Invest* 98:43-49, 1996

20. Meugnier E, Rome S, Vidal H: Regulation of gene expression by glucose. *Curr Opin Clin Nutr Metab Care* 10:518-522, 2007

21. Cozzone D, Frojdo S, Disse E, Debard C, Laville M, Pirola L, Vidal H: Isoform-specific defects of insulin stimulation of Akt/protein kinase B (PKB) in skeletal muscle cells from type 2 diabetic patients. *Diabetologia* 51:512-521, 2008

22. Cozzone D, Debard C, Dif N, Ricard N, Disse E, Vouillarmet J, Rabasa-Lhoret R, Laville M, Prunet D, Rieuasset J, Lefai E, Vidal H: Activation of liver X receptors promotes lipid accumulation but does not alter insulin action in human skeletal muscle cells. *Diabetologia* 49:990-999, 2006

23. Bonnard C, Durand A, Peyrol S, Chaumeame E, Chauvin MA, Morio B, Vidal H, Rieuasset J: Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest* 118:789-800, 2008

24. Chaussade C, Pirola L, Bonnafous S, Blondeau F, Brenz-Verca S, Tronchere H, Portis F, Rusconi S, Payrastre B, Laporte J, Van Obberghen E: Expression of myotubularin by an adenoviral vector demonstrates its function as a phosphatidylinositol 3-phosphate [PtdIns(3)P] phosphatase in muscle cell lines: involvement of PtdIns(3)P in insulin-stimulated glucose transport. *Mol Endocrinol* 17:2448-2460, 2003
25. Dif N, Euthine V, Gonnet E, Laville M, Vidal H, Lefai E: Insulin activates human sterol-regulatory-element-binding protein-1c (SREBP-1c) promoter through SRE motifs. Biochem J 400:179-188, 2006
26. Bouzakri K, Roques M, Gual P, Espinosa S, Guebre-Egziabher F, Riou JP, Laville M, Le Marchand-Brustel Y, Tanti JF, Vidal H: Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 52:1319-1325, 2003
27. Folch J, Lees M, Sloane Stanley GH: A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509, 1957
28. Silver JD, Ritchie ME, Smyth GK: Microarray background correction: maximum likelihood estimation for the normal-exponential convolution. Biostatistics 10:352-363, 2009
29. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang YJ, Zhang J: Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5:R80, 2004
30. Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:Article3, 2004
31. Vives-Bauza C, Yang L, Manfredi G: Assay of mitochondrial ATP synthesis in animal cells and tissues. Methods Cell Biol 80:155-171, 2007
32. Hegarty BD, Furler SM, Ye J, Cooney GJ, Kraegen EW: The role of intramuscular lipid in insulin resistance. Acta Physiol Scand 178:373-383, 2003
33. Rosen P, Nawroth PP, King G, Moller W, Triteschler HJ, Packer L: The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. Diabetes Metab Res Rev 17:189-212, 2001
34. Grunnet LG, Brons C, Jacobsen S, Nilsson E, Astrup A, Hansen T, Pedersen O, Poulsen P, Quistorff B, Vaag A: Increased recovery rates of phosphocreatine and inorganic phosphate after isometric contraction in oxidative muscle fibers and elevated hepatic insulin resistance in homozygous carriers of the A-allele of FTO rs9939609. J Clin Endocrinol Metab 94:596-602, 2009
35. Boissel S, Reish O, Proulx K, Kawagoe-Takaki H, Sedgwick B, Yeo GS, Meyre D, Golzio C, Molinari F, Kadhom N, Etchevers HC, Saudek V, Farooqi IS, Froguel P, Lindahl T, O'Rahilly S, Munnich A, Colleaux L: Loss-of-function mutation in the dioxygenase-encoding FTO gene causes severe growth retardation and multiple malformations. Am J Hum Genet 85:106-111, 2009
36. Meyre D, Proulx K, Kawagoe-Takaki H, Vatin V, Gutierrez-Aguilar R, Lyon D, Ma M, Choquet H, Horber F, Van Hul W, Van Gaal L, Balkau B, Visvikis-Siest S, Pattou F, Farooqi IS, Saudek V, O'Rahilly S, Froguel P, Sedgwick B, Yeo GS: Prevalence of loss-of-function FTO mutations in lean and obese individuals. Diabetes 59:311-318
37. Schrauwen P, Hesselink MK: Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. Diabetes 53:1412-1417, 2004
38. Puigserver P: Tissue-specific regulation of metabolic pathways through the transcriptional coactivator PGC1-alpha. Int J Obes (Lond) 29 Suppl 1:S5-9, 2005
39. Barres R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, Krok A, Zierath JR: Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. *Cell Metab* 10:189-198, 2009

40. Debard C, Laville M, Berbe V, Loizon E, Guillet C, Morio-Liondore B, Boirie Y, Vidal H: Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of Type 2 diabetic patients. *Diabetologia* 47:917-925, 2004

41. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267-273, 2003

42. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A* 100:8466-8471, 2003

### Table 1. Clinical and metabolic characteristics of the subjects

|                      | Control 50 | Obese | Type 2 diabetes | Control 25 | Type 1 diabetes |
|----------------------|------------|-------|-----------------|------------|-----------------|
| **n**                | 10         | 10    | 10              | 5          | 5               |
| **Men/Women**        | 6/4        | 7/3   | 5/5             | 4/1        | 4/1             |
| **Age (years)**      | 55.3 ± 3.5 | 52.0 ± 1.4 | 56.2 ± 2.4     | 25.4 ± 1.3 | 28.8 ± 1.5      |
| **BMI (kg/m²)**      | 23.2 ± 0.5 | 33.3 ± 0.9‡ | 32.1 ± 1.0 ‡   | 21.5 ± 0.6 | 24.5 ± 1.3      |
| **HbA1C**            | 5.2 ± 0.2  | 5.5 ± 0.3 | 10.1 ± 0.7 ‡*  | 5.2 ± 0.3  | 9.1 ± 0.4       |

#### Basal

|                      | Control 50 | Obese | Type 2 diabetes | Control 25 | Type 1 diabetes |
|----------------------|------------|-------|-----------------|------------|-----------------|
| Glucose (mmol/L)     | 4.8 ± 0.1  | 5.3 ± 0.1 | 10.5 ± 0.7 ‡   | 4.6 ± 0.2  | 13.5 ± 0.9†     |
| Insulin (pmol/L)     | 34.5 ± 2.9 | 55.9 ± 10.7 † | 68.2 ± 6.6 ‡  | 32.3 ± 1.5 | nd              |
| Triglycerides (mmol/L) | 0.8 ± 0.1  | 1.8 ± 0.2 † | 1.9 ± 0.3 ‡    | 0.4 ± 0.1  | 0.5 ± 0.02      |
| Non esterified fatty acid (µmol/L) | 472.1 ± 46.4 | 644.5 ± 41.3 † | 619.5 ± 49.1† | 492 ± 48 | 519 ± 97         |
| Glucose disposal rate (mg/(kg.min)) | 2.4 ± 0.1 | 1.8 ± 0.1 † | 2.3 ± 0.3      | nd         | nd              |
| Glucose oxidation rate (mg/(kg.min)) | 1.1 ± 0.1 | 1.0 ± 0.2 | 1.3 ± 0.2      | 1.6 ± 0.3  | 1.5 ± 0.2       |
| Lipid oxidation rate (mg/(kg.min)) | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.6 ± 0.1      | nd         | nd              |

#### Clamp Study

|                      | Control 50 | Obese | Type 2 diabetes | Control 25 | Type 1 diabetes |
|----------------------|------------|-------|-----------------|------------|-----------------|
| Glucose (mmol/L)     | 5.0 ± 0.2  | 5.2 ± 0.4 | 5.4 ± 0.2       | 4.5 ± 0.1  | 5.6 ± 0.2 †     |
| Insulin (pmol/L)     | 810.0 ± 122.5 | 835.7 ± 150.4 | 1184.0 ± 67.9 ‡* | 875 ± 48  | 949 ± 115       |
| Non esterified fatty acid (µmol/L) | 25.7 ± 3.5 | 77.3 ± 23.8 | 93.7 ± 16.7 †   | nd         | nd              |
| Glucose disposal rate (mg/(kg.min)) | 10.6 ± 0.9 | 3.4 ± 0.5 ‡ | 3.7 ± 0.5 ‡    | 9 ± 1.4    | 8.2 ± 1.4       |
| Glucose oxidation rate (mg/(kg.min)) | 3.2 ± 0.2 | 2.1 ± 0.1 ‡ | 2.2 ± 0.1‡      | 3.5 ± 0.2  | 3.4 ± 0.2       |
| Lipid oxidation rate (mg/(kg.min)) | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 †    | 6.9 ± 0.7  | 5.3 ± 0.8       |

a: parameters measured after an overnight fast. The clamp study refers to a euglycemic hyperinsulinemic clamp as detailed in Methods.

† p<0.05 and ‡ p<0.001 vs. the respective control subjects; *p < 0.05 when comparing type 2 diabetic with obese subjects; nd, not determined.
Table 2: Mitochondria genes regulated by FTO over-expression in human myotubes.

| Symbol | Gene Name | GeneID | Fold change |
|--------|-----------|--------|-------------|
| ACOT9  | acyl-CoA thioesterase 9 | 23597  | -1.25 |
| ACSL4  | acyl-CoA synthetase long-chain family member 4 | 2182  | -1.56 |
| AK2    | adenylate kinase 2 | 204  | -1.47 |
| ALDH7A1| aldehyde dehydrogenase 7 family, member A | 501  | 1.2 |
| ATP5B  | ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide | 506  | -1.47 |
| ATP5I  | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit E | 521  | 1.35 |
| ATP5J2 | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F2 | 9551  | 1.25 |
| Bnip3  | BCL2/adenovirus E1B 19kDa interacting protein 3 | 664  | 1.36 |
| CABC1  | chaperone, ABC1 activity of bc1 complex homolog (S. pombe) | 56997 | -1.32 |
| CDC56  | coiled-coil domain containing 56 | 28958 | -1.43 |
| CKB    | creatine kinase, brain | 1152  | -1.54 |
| COQ2   | coenzyme Q2 homolog, prenyltransferase | 27235 | 1.2 |
| COX18  | COX18 cytochrome c oxidase assembly homolog | 285521 | 1.32 |
| COX5B  | cytochrome c oxidase subunit Vb | 1329 | -1.37 |
| CTsb   | cathepsin B | 1508 | -1.35 |
| CYB5B  | cytochrome b5 type B(outer mitochondrial membrane) | 80777 | 1.22 |
| CYBA   | cytochrome b-245, alpha polypeptide | 1535 | -1.27 |
| CYC1   | cytochrome c-1 | 1537 | -1.27 |
| Dguk   | deoxyguanosine kinase | 1716 | 1.2 |
| Dypsl2 | dihydropyrimidinidase-like 2 | 1808 | 1.24 |
| Fam82a2| family with sequence similarity 82, member A2 | 55177 | 1.21 |
| Gatm   | glycine amidinotransferase (L-arginine:glycine amidinotransferase) | 2628 | 1.37 |
| Gpx1   | glutathione peroxidase 1 | 2876 | -1.43 |
| H ebp1 | heme binding protein 1 | 50865 | 1.27 |
| Hibch  | 3-hydroxyisobutryl-Coenzyme A hydrolase | 26275 | -1.41 |
| Hsd17b10| hydroxysteroid (17-beta) dehydrogenase 10 | 3028 | -1.32 |
| Idh3b  | isocitrate dehydrogenase 3 (NAD+) beta | 3420 | 1.3 |
| Idh3g  | isocitrate dehydrogenase 3 (NAD+) gamma | 3421 | 1.2 |
| Isca1  | iron-sulfur cluster assembly 1 homolog (S. cerevisiae) | 81689 | -1.27 |
| Isoc2  | isochorismatase domain containing 2 | 79763 | -7.69 |
| Kif1b  | kinesin family member 1B | 23095 | -1.56 |
| Lactb  | lactamase, beta | 114294 | -1.25 |
| Lonn1  | lon peptidase 1, mitochondrial | 9361 | 1.23 |
| Mcat   | malonyl CoA:ACP acyltransferase | 27349 | 1.28 |
| Mlycd  | malonyl-CoA decarboxylase | 23417 | 1.23 |
| Mrpl13 | mitochondrial ribosomal protein L13 | 28998 | 1.24 |
| Mrpl20 | mitochondrial ribosomal protein L20 | 55052 | -1.32 |
| Mrpl27 | mitochondrial ribosomal protein L27 | 51264 | 1.2 |
| Mrpl30 | mitochondrial ribosomal protein L30 | 51263 | 1.26 |
| Mrpl44 | mitochondrial ribosomal protein L44 | 65080 | 1.25 |
| Mrpl47 | mitochondrial ribosomal protein L47 | 57129 | 1.25 |
| Mrps22 | mitochondrial ribosomal protein S22 | 56945 | 1.32 |
| Mt-coi | cytochrome c oxidase | 4512 | 1.24 |
| Mtif2  | mitochondrial translational initiation factor 2 | 4528 | 1.35 |
| Napi   | N-ethylmaleimide-sensitive factor attachment protein, gamma | 8774 | 1.23 |
| Ndufa11| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11, | 126328 | 1.22 |
| Ndufa12| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12 | 55967 | 1.2 |
| Ndufa3 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, | 4696 | -1.41 |
| Ndufa6 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, | 4700 | 1.21 |
Expression and function of FTO in skeletal muscle

| Gene        | Description                              | Value   | Log2 Fold Change |
|-------------|------------------------------------------|---------|------------------|
| NDUFC1      | NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 | 4717    | -1.25            |
| NDUFS5      | NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa | 4725    | -1.35            |
| PARS2       | prolyl-tRNA synthetase 2, mitochondrial (putative) | 25973   | -1.43            |
| PHB2        | prohibitin 2                              | 11331   | 1.2              |
| PMPCB       | peptidase (mitochondrial processing) beta | 9512    | 1.22             |
| PPDF        | peptidylprolyl isomerase F               | 10105   | 1.38             |
| PRDX4       | peroxiredoxin 4                          | 10549   | -1.33            |
| PTCD3       | Pentatricopeptide repeat domain 3        | 55037   | 1.2              |
| PTRF        | polymerase I and transcript release factor| 284119  | 1.3              |
| RAB32       | RAB32, member RAS oncogene family        | 10981   | 1.23             |
| RTN4IP1     | reticulon 4 interacting protein 1        | 84816   | 1.23             |
| SARS2       | seryl-tRNA synthetase 2, mitochondrial   | 54938   | 1.88             |
| SCP2        | sterol carrier protein 2                 | 6342    | -1.25            |
| SLC27A3     | solute carrier family 27 (fatty acid transporter), member 3 | 11000   | -1.28            |
| SOD2        | superoxide dismutase 2, mitochondrial    | 6648    | -1.28            |
| SPNS1       | spinster homolog 1 (Drosophila)          | 83985   | 1.22             |
| SUPV3L1     | suppressor of var1, 3-like 1 (S. cerevisiae) | 6832   | 1.22             |
| TACO1       | translational activator of mitochondrially encoded cytochrome c oxidase I | 51204 | 1.36 |
| TIMM10B     | racture callus 1 homolog (rat)           | 26515   | 1.24             |
| TIMM23      | translocase of inner mitochondrial membrane 23 homolog (yeast) | 10431 | -1.32 |
| TIMM9       | translocase of inner mitochondrial membrane 9 homolog (yeast) | 26520 | 1.23 |
| TIMMLHE     | trimethyllysine hydroxylase, epsilon     | 55217   | 1.2              |
| TOMM34      | translocase of outer mitochondrial membrane 34 | 10953 | 1.32 |
| UQCR        | ubiquinol-cytochrome c reductase         | 10975   | -1.27            |
| VHL         | von Hippel-Lindau tumor suppressor        | 7428    | 1.24             |

Figure Legends

**Figure 1:** Regulation of FTO expression in human skeletal muscle. A) Basal FTO mRNA levels were measured by real-time RT-PCR in skeletal muscle of age-matched control, obese, type 2 diabetic (T2DM) patients and in type 1 diabetic (T1DM) subjects. Values are means ± SEM (n=5-10). * p<0.05 vs. age-matched controls. The mRNA level of the reference gene HPRT did not differ among groups (3.9 ± 0.5, 2.9 ± 0.1, 3.8 ± 0.5, 3.3 ± 0.3 and 4.2 ± 0.5 amo/µg total RNA, in control 50, obese, T2DM patients, control 25 and T1DM patients, respectively, NS). B) Representative Western-blot illustrating FTO protein levels in skeletal muscle of age-matched control, obese, and T2DM patients. Data of the histogram are means ± SEM (n=3). * p<0.05 vs. age-matched controls. C-D) FTO mRNA levels were measured by real-time RT-PCR in skeletal muscle of age-matched control and T2DM patients, before and after a 3h-euglycemic hyperinsulinemic clamp (C) or a 3h-hyperglycemic euinsulinemic clamp (D). Values are means ± SEM (n=6). E) FTO mRNA levels were measured by real-time RT-PCR in skeletal muscle of T2DM patients, before and after a 12 weeks rosiglitazone treatment. Values are means ± SEM (n=6). * p<0.05 vs. before treatment. The mRNA level of the reference gene HPRT did not differ before and after rosiglitazone treatment (5 ± 0.6 and 6.6 ± 0.5, amo/µg total RNA, respectively, NS). NS: non significant.

**Figure 2:** Effect of FTO over-expression on insulin signalling in HEK293 cells. HEK293 were transiently transfected with pcDNA3-FTO or empty pcDNA3 vector (control). Forty-eight
hours post-transfection, cells were depleted in serum for 3 hours and stimulated with insulin (10^{-7}M, 10 minutes). A) Representative Western blots of FTO, pSer473PKB, pThr308PKB and total PKB. B) Histogram illustrates the quantification and normalisation of the phosphorylation of PKB in control and FTO-over-expressing cells. Values are means ± SEM (n=3). * p<0.001 vs. control cells, # p<0.001 FTO vs. GFP.

**Figure 3:** Adenoviral over-expression of FTO in differentiated myotubes. Human myotubes or C2C12 cells were infected with recombinant adenovirus encoding human FTO or GFP (control) for 48h. A) Representative Western blots of FTO, pSer473PKB, PKB, pPDK1, PDK1 and tubulin, in GFP- or FTO-over-expressing C2C12 myotubes. B) Representative Western blots of pSer473PKB and PKB total, in GFP- or FTO-over-expressing C2C12 myotubes (210^{7} iuf/well), under basal conditions or after insulin stimulation. Histogram represents means ± SEM (n=4). * p<0.05 vs. basal situation, # p<0.05 FTO vs. GFP. C) Representative Western blots of FTO, pSer473PKB, PKB, p70/85S6K and actin in human myotubes over-expressing either GFP or FTO (210^{7} iuf/well). D-E) Myotubes were transfected with siRNA control or specific for FTO (48h). D) Validation of FTO silencing in muscle cells. E) Representative Western blots of pSer473PKB and PKB in myotubes silencing for FTO (50nM of siRNA). Histogram illustrates the quantification and normalisation of the phosphorylation of PKB in myotubes silenced for FTO. Values are means ± SEM (n=3).

**Figure 4:** Effect of FTO over-expression on de novo lipogenesis. Human myotubes were infected with recombinant adenovirus encoding human FTO genome or GFP (control) for 48h (210^{7} iuf/well). A) De novo lipogenesis was measured with [2-{^{14}}C]acetate during 24h. Values are means ± SEM (n=3). * p<0.05. B) mRNA levels of FAS, ACC1, GPAT and PPARγ were measured by real-time RT-PCR. Data represent means ± SEM (n=4).* p<0.05. C) Palmitate-induced-ROS production either in human myotubes over-expressing either GFP or FTO, or in myotubes silencing for FTO (siRNA: 50nM). After 48h of infection/transfection, myotubes were incubated with BSA or palmitate (750µM) for 16h, and ROS production was measured using NBT assay. Values represent means ± SEM (n=3).* p<0.05. FAS: fatty acid synthase, ACC1: Acetyl-CoA carboxylase 1, GPAT: glycerol-3 phosphate acyltransferase, PPARγ: peroxisome proliferators-activated receptor gamma.

**Figure 5:** Effect of FTO over-expression on mitochondria structure and function in human myotubes. Human myotubes were infected with recombinant adenovirus encoding human FTO genome or GFP (control) for 48h (210^{7} iuf/well). A) Electronic microscopy analysis of human myotubes over-expressing GFP or FTO. B) Analysis of the expression of respiratory chain complexes in human myotubes overexpressing GFP or FTO. It should be noted that complex IV was not detected in our conditions. Histograms represent the means ± SEM (n=4). * p<0.05. C) Analysis of complex I (pyruvate + malate) and complex II (succinate + rotenone)-mediated ATP synthesis in GFP and FTO overexpressing myotubes. Histograms represent the means ± SEM (n=4). * p=0.02.

**Figure 6:** Reduced OXPHOS and antioxidant genes and increased oxidative stress in skeletal muscle of type 2 diabetic patients. A) mRNA levels of ATP5B, UQCR, SOD2 and PGC1α were measured by real-time RT-PCR in skeletal muscle of control and type 2 diabetic patients. Data represent means ± SEM (n=10). * p<0.05. B) Immunoblot showing total protein
Expression and function of FTO in skeletal muscle

carbonylation in skeletal muscle of control and type 2 diabetic patients (T2DM). Histogram represents means ± SEM (n=4).* p<0.05. ATP5B: ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide, UQCR: ubiquinol-cytochrome c reductase, SOD2: superoxide dismutase 2, PGC1α: PPARγ coactivator 1.

Figure 1

A

B

C

D

E
Expression and function of FTO in skeletal muscle

**Figure 2**

**A**

|              | Empty vector | FTO |
|--------------|--------------|-----|
| + Ins        | + Ins        |     |

- FTO
- pSer473PKB
- pThr308PKB
- PKB

**B**

- Control
- Insulin

![Graph showing pSer73 PKB/PKB levels for Empty vector and FTO conditions with control and insulin treatments.](image)
Figure 3

A

Expression and function of FTO in skeletal muscle

B

Adenovirus (IU/well)

2.10^7

2.10^8

GFP  FTO  GFP  FTO

FTO  pSer473PKB  PKB  pPDK-1  PDK-1  Tubulin

pSer473PKB

PKB

pPDK-1

PDK-1

Tubulin

B

Insulin

pSer473PKB

PKB

Ad-GFP

Ad-FTO

*  *

*  *

#  #

C

Adenovirus (IU/well)

2.10^7

2.10^8

GFP  FTO  GFP  FTO

FTO  pSer473PKB  PKB  p70S6K  Actin

D

RNAi control

RNAi FTO

25nM  50nM

FTO  Actin

E

RNAi control  RNAi FTO

pSer473PKB

PKB

pSer473PKB

PKB

RNAi control  RNAi FTO
Figure 4

A

Expression and function of FTO in skeletal muscle

B

C

Expression and function of FTO in skeletal muscle
Expression and function of FTO in skeletal muscle

Figure 5

A

Ad-GFP

Ad-FTO

B

Subject: 1 2 3 4

Adenoviruses: GFP FTO GFP FTO GFP FTO GFP FTO

C

Protein/actin levels

(CI vs. GFP) N.S

N.S

N.S

C1 CII CIII CV

C

ATP synthesis (a.u.)

| Condition         | Ad-GFP | Ad-FTO |
|-------------------|--------|--------|
| Succinate+Rothone | 0.5    | 2.0    |
| Succinate         | 0.5    | 2.0    |
Expression and function of FTO in skeletal muscle

Figure 6

A

B

Control  T2DM

Carboxylated protein levels (a.u.)

0.0  0.5  1.0  1.5  2.0  2.5  3.0

Control  T2DM

Figure 6