Prevalence of Loss-of-Function FTO Mutations in Lean and Obese Individuals

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OBJECTIVE—Single nucleotide polymorphisms (SNPs) in intron 1 of fat mass– and obesity-associated gene (FTO) are strongly associated with human adiposity, whereas Fto<sup>-/-</sup> mice are lean and Fto<sup>+/-</sup> mice are resistant to diet-induced obesity. We aimed to determine whether FTO mutations are disproportionately represented in lean or obese humans and to use these mutations to understand structure-function relationships within FTO.

RESEARCH DESIGN AND METHODS—We sequenced all coding exons of FTO in 1,433 severely obese and 1,433 lean individuals. We studied the enzymatic activity of selected non-synonymous variants.

RESULTS—We identified 33 heterozygous nonsynonymous variants in lean (2.3%) and 35 in obese (2.4%) individuals, with 8 mutations unique to the obese and 11 unique to the lean. Two novel mutations replace absolutely conserved residues: R322Q in the catalytic domain and R96H in the predicted substrate recognition lid. R322Q was unable to catalyze the conversion of 2-oxoglutarate to succinate in the presence or absence of 3-methylthymidine. R96H retained some basal activity, which was not enhanced by 3-methylthymidine. However, both were found in lean and obese individuals.

CONCLUSIONS—Heterozygous, loss-of-function mutations in FTO exist but are found in both lean and obese subjects. Although intron 1 SNPs are unequivocally associated with obesity in multiple populations and murine studies strongly suggest that FTO has a role in energy balance, it appears that loss of one functional copy of FTO in humans is compatible with being either lean or obese. Functional analyses of FTO mutations have given novel insights into structure-function relationships in this enzyme. Diabetes 59:311–318, 2010

Genome-wide association studies have revealed that single nucleotide polymorphisms (SNPs) within the first intron of fat mass– and obesity-associated gene (FTO) are strongly associated with adiposity (1–4). These associations have been largely consistent across multiple different ethnicities including Europeans (5–8), Asians (9–11), Hispanics (12,13), and Africans (13,14). At present, it remains unclear whether these SNPs influence the expression or splicing of the FTO gene and/or whether the unequivocal association with obesity is causally related to alterations in expression or function of FTO. If the effect of the intron 1 SNPs is through FTO, it also remains unclear whether the obesity risk SNPs result in a loss or a gain of FTO function. It remains possible that the SNPs influence the expression and/or function of neighboring genes and that such an effect might underlie the association with adiposity. There are several examples in metabolic disease where common variants close to a particular gene are associated with alterations in risk of common phenotypes such as fat mass or risk of obesity, whereas rare loss- or gain-of-function mutations in the same gene are associated with a more severe version of the same metabolic phenotype (e.g., MCR4 [5,15–19], POMC [20,21], BDNF [22–24], and PCSK1 [25,26]). When information is available from both types of variants, clarity of mechanistic understanding is greatly enhanced. To establish whether nonsynonymous variants of FTO might be enriched in either lean or obese subjects, we sequenced the FTO coding region and intron-exon boundaries in a large group of subjects with severe obesity and a similarly sized group of individuals with lifelong leanness. We have recently established that FTO encodes a 2-oxoglutarate (2-OG) Fe<sup>2+</sup>-dependent dioxygenase (27). In this study, we have examined the functional enzymatic properties of naturally occurring variants to gain further insights into structure-function relationships in the human enzyme.

RESEARCH DESIGN AND METHODS

FTO was sequenced in 1,433 severely obese subjects. All adults had BMI >40 kg/m<sup>2</sup>, and all obese children had a BMI >97th percentile for sex and age (Table 1). Three hundred forty-five French morbidly obese adults were recruited by the CNRS UMR8900 at Lille and the Department of Nutrition of Paris Hotel Dieu Hospital. Thirty-three French morbidly obese adults were patients of the CHRU Lille hospital. We also sequenced 287 Swiss morbidly obese adults, all of them recruited after gastric surgery in Zurich, Switzerland. Eighty-eight morbidly obese adult patients were recruited at the Antwerp University Hospital. Three hundred ninety-nine French obese children were recruited in CNRS UMR8900, and 281 obese children were recruited as part of the Genetics of Obesity Study (GOOS) cohort (28). A total of 1,221 lean adult subjects were part of the French DESIR (Data from an Epidemiological Study

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DIABETES, VOL. 59, JANUARY 2010 311
TABLE 1
Clinical characteristics for obese and lean subjects

|          | n (male/female) | Age (years) | BMI (kg/m²) | zBMI     |
|----------|-----------------|-------------|-------------|----------|
| Obese    |                 |             |             |          |
| adults   | (BMI >40 kg/m²) |             |             |          |
| children | BMI >97th percentile | | | |
| French adults | 378 (79/299) | 43.15 ± 11.52 | 51.63 ± 9.17 | —        |
| Swiss adults | 287 (79/208) | 41.56 ± 11.21 | 52.32 ± 5.80 | —        |
| Belgian adults | 88 (36/52) | 45.45 ± 11.21 | 54.79 ± 5.20 | —        |
| French children | 390 (188/211) | 10.56 ± 3.23 | —          | 5.01 ± 1.04 |
| English children | 281 (135/146) | 11.5 ± 2.40 | —          | 4.6 ± 1.80  |
| Lean      | (BMI <23 kg/m²) |             |             |          |
| adults   | BMI <90th percentile | | | |
| French adults | 1,221 (378/843) | 43.26 ± 9.37 | 20.42 ± 1.28 | —        |
| French children | 212 (96/116) | 11.77 ± 2.42 | —          | 0.10 ± 0.98 |

Data are means ± SD unless otherwise indicated.

RESULTS
Prevalence of nonsynonymous mutations in lean and obese subjects. To identify potential loss-of-function mutations, we screened the nine coding exons of FTO by direct nucleotide sequencing in 1,433 extremely obese subjects (753 adults with a BMI >40 kg/m² and 680 children with a BMI >97th percentile) and in 1,433 lean subjects (1,221 adults with a BMI <23 kg/m² and 212 children recruited with a zBMI <90th percentile) according to the ECOG (31), with an average zBMI close to the 50th percentile (zBMI = 0.1 ± 0.98) (Table 1). Additionally, we also genotyped the FTO rs9939609 SNP (2) in all of the patients. Genotype call rate was >99% in both lean and obese subjects, and genotypic distribution obeyed the Hardy-Weinberg equilibrium in the lean control subgroup (P = 0.58). We genotyped 363 duplicated DNA samples and observed a concordance rate of 100%. In the lean subgroup, genotype counts were 532 (TT), 668 (TA), and 224 (AA). In the obese subgroup, genotype counts were 367 (TT), 665 (TA), and 390 (AA). As expected, the obesity risk A allele frequency was 11.6% higher in obese vs lean obesity subjects (obese: 50.8%; lean: 39.2% OR 1.60 [1.44 – 1.78]; P = 1.2 × 10⁻¹⁸).

A total of 26 different rare (frequency <1% in the present cohort) nonsynonymous variants were identified in FTO (Table 2). The prevalence of nonsynonymous variants was similar between the two groups, with 33 lean (2.3%) and 35 obese (2.4%) individuals carrying buffer (20 mmol/l HEPES, pH 8, 5% glycerol, 50 mmol/l NaCl) was added to the concentrated fraction, which was concentrated again down to 100 μl by centrifugation at 2500g. FTO preparations were aliquoted and then stored at −80°C. FTO proteins were visualized by SDS–10% PAGE and stained with Coomassie blue.

**Assays of FTO activity.** Conversion of ¹⁴C-2-oxoglutarate to ¹⁴C-succinate has been assayed previously (33). This method was modified and optimized to assay FTO as previously described (32). To measure the uncoupled reaction (no prime substrate present), 1.5 μmol/l FTO was assayed in 10 μl reaction mixture containing 50 mmol/l HEPES-KOH, pH 7, 50 μg/ml BSA, 4 mmol/l ascorbate, 75 μmol/l Fe(NO₃)₃(SO₄)₂, and 20 μmol/l [5⁻¹⁴C]-2-oxoglutarate (30 mCi/mmol from Moravek Biochemicals) and incubated at 37°C for various times. To measure stimulation of this activity by 3-methylthymidine, 1 mmol/l 3-methylthymidine (Moravek Biochemicals) was included in the assay mix. The reaction was stopped by adding 5 μl stop solution containing 20 μmol/l succinate, 20 μmol/l 2-OG, followed by 5 μl 100 mmol/l dinitrophenylhydrazine, which precipitates 2-OG. This mix was incubated at room temperature for 30 min. An additional 10 μl 1 M 2-OG was added and incubated for a further 30 min. The precipitate was removed by centrifugation. Clear supernatant (10 μl) was scintillation counted to monitor the ¹⁴C-succinate generated.

**Human wild-type and mutant FTO protein production.** Human wild-type and mutant FTO protein production was carried out as previously described (32). Briefly, expression plasmids were transformed into Esherichia coli BL21-Gold (DE3; Stratagene) and cultured in 2 l Luria-Bertani broth and 50 μg/ml carbenicillin to A600 1.0 at 37°C. Expression of the cloned gene was induced by addition of 0.5 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) at 15°C for 4 h. The cells were harvested and washed in PBS, and pellets were stored at −80°C. Cells were resuspended in 40 ml of lysis buffer containing 50 mmol/l HEPES-KOH, pH 8, 2 mmol/l β-mercaptoethanol, 5% glycerol, and 300 mmol/l NaCl, prior to addition of lysozyme (2 mg/ml). After 30 min of incubation on ice, cells were sonicated and the lysate was clarified by centrifugation at 15000g for 20 min. The lysate was supplemented with imidazole (10 mmol/l) and loaded onto a 1.5-Ml Ni-NTA (nickel–nitrilotriacetic acid)–agarose column (Qiagen) previously equilibrated in lysis buffer containing 10 mmol/l imidazole. The column was washed with 30 ml of lysis buffer containing 10 mmol/l imidazole, followed by 7.5 ml of lysis buffer containing 40 mmol/l imidazole. The FTO protein was eluted in lysis buffer containing 250 mmol/l imidazole. The eluate was then loaded onto a 30-kDa concentrator (Sartorius Stedium Biotech) and centrifuged at 2500g for 30 min. Then 2 ml of

on the insulin resistance syndrome) general prospective study (29). Selection criteria were BMI <23 kg/m² at inclusion. We included 212 lean French children selected from the Stanislas study (30). Inclusion criteria was BMI z score (zBMI) <90th percentile for sex and age according to the Eastern Cooperative Oncology Group (ECOG) (31). All patients were of European ancestry. The study protocol was approved by all local ethics committees, and informed consent was obtained from each subject before participating in the study.

Genotyping of the FTO rs9939609 SNP. Genotyping of the FTO rs9939609 SNP was performed using a Taqman SNP Genotyping Assay (Applied Biosystems) on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions.

Sequencing. We screened the nine exons of the FTO by direct nucleotide sequencing. We amplified overlapping PCR fragments according to primers and PCR-optimized conditions (available from the authors upon request). PCR amplifications were inspected for single bands of expected sizes on agarose gels before purification with Agencourt AMPure on Biomek NX (Beckman Coulter). Sequencing was achieved using the automated ABI Prism 3730x1 DNA Sequencer in combination with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems), and purification of sequencing reaction was performed with Agencourt CleanSEQ on Biomek NX (Beckman Coulter). Sequences were assembled and analyzed using Sequencher software (GeneCodes).

Statistical analysis. The comparison of prevalence has been tested with Fisher exact test, and the reported P values are two-sided. P values of <0.05 were considered to indicate statistical significance.

Cloning of human FTO. Wild-type human FTO cDNA was amplified (Expand DNA polymerase; Roche) using Xhol-tagged primers with the first strand of the reverse-transcribed cDNA (Superscript II; Invitrogen) from human brain total RNA as a template. Using the Xhol sites, the resulting PCR product was cloned into an NH₄-terminal 6× His-tagged bacterial expression vector (pET302/NT-His; Invitrogen). The FTO mutants were generated from cloned wild-type FTO using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s protocols. The mutations were confirmed by DNA sequencing using Big Dye terminator chemistry (Applied Biosystems) and electrophoresis on an ABI 3730 automated DNA sequencer. Sequences were assembled and examined using Sequencher software (GeneCodes). All primer sequences are available upon request.

Lean (adults: BMI >40 kg/m²; children: BMI >97th percentile) were considered to indicate statistical significance.

DNA polymerase; Roche) using P Statistical analysis. (Beckman Coulter). Sequences were assembled and analyzed with Se-
mutations, and no significant differences in prevalence were observed between children and adults (Table 2). We found that a subset of those was unique to each group. Eight (A134T, G187A, M223V, A241T, H419R, E471G, I492V, and V493F) nonsynonymous mutations were identified uniquely in the obese cohort, whereas 11 (P5L, E24K, R80P, P93R, V94I, N143S, I148R, D189N, E234D, R316Q, and P399H) were identified uniquely in the lean cohort (Table 3). G187A (\(n/H11005\)), A241T (\(n/H11005\)), and V493F (\(n/H11005\)) were identified in multiple obese subjects (Table 3). Seven variants (R96H, L146M, A163T, V201I, S256N, R322Q, and A405V) were present in both cohorts. The prevalence of synonymous mutations was not significantly different in obese compared with lean subjects (0.8% vs. 0.4%, \(P = 0.05\); Table 4). All mutations were identified in heterozygous form, and no nonsense variants were reported in the studied populations. Thus, there is no obvious enrichment of nonsynonymous mutations in lean or obese individuals.

### Functional properties of nonsynonymous mutations

FTO is a member of the AlkB homologue (ABH) protein family, which numbers nine homologues in humans (27). Most 2-OG and Fe\(^{2+}\)-dependent dioxygenases slowly catalyze the conversion of 2-OG to succinate even in the absence of their prime substrate (33), and this so-called uncoupled reaction may be stimulated by substrates or their analogues. We have previously reported that murine Fto is capable of demethylating the base 3-methylthymine in the context of single-stranded DNA (27), but that reaction proceeds rather slowly. Human FTO can also accomplish this reaction, but again at a very slow turnover rate (34). We have previously exploited the ability of wild-type human FTO to efficiently catalyze the conversion of 2-OG to succinate in a manner that is

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**Table 2**

Summary of all nonsynonymous mutations found in obese and lean subjects

| Nonsynonymous mutations | Obese children | Obese adults | All obese subjects | Lean children | Lean adults | All lean subjects |
|-------------------------|----------------|--------------|-------------------|--------------|------------|------------------|
| P5L                     | 1              | 1            | 1                 | 1            | 1          | 1                |
| E24K                    | 1              | 1            | 1                 | 1            | 1          | 1                |
| R80P                    | 1              | 1            | 1                 | 1            | 1          | 1                |
| P93R                    | 1              | 1            | 1                 | 1            | 1          | 1                |
| V94I                    | 1              | 1            | 1                 | 1            | 1          | 1                |
| R96H                    | 1              | 1            | 1                 | 1            | 1          | 1                |
| A134T                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| N143S                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| L146M                   | 1              | 1            | 2                 | 1            | 2          | 3                |
| I148R                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| A163T                   | 2              | 5            | 7                 | 1            | 4          | 5                |
| G187A                   | 2              | 2            | 2                 | 1            | 1          | 1                |
| D189N                   | 2              | 2            | 2                 | 1            | 1          | 1                |
| V201I                   | 2              | 2            | 2                 | 1            | 1          | 1                |
| M223V                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| E234D                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| A241T                   | 2              | 2            | 2                 | 1            | 1          | 1                |
| S256N                   | 3              | 5            | 8                 | 3            | 6          | 9                |
| R316Q                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| R322Q                   | 2              | 2            | 2                 | 1            | 1          | 1                |
| P399H                   | 3              | 3            | 3                 | 1            | 1          | 1                |
| A405V                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| H419R                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| E471G                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| I492V                   | 1              | 1            | 1                 | 1            | 1          | 1                |
| V493F                   | 1              | 3            | 4                 | 1            | 1          | 1                |

Prevalence of mutation carriers (%) 1.32 3.45 2.44 3.77 2.05 2.31

Data in boldface indicate combined adult and children numbers.

**Table 3**

Summary of nonsynonymous mutations unique to the lean or the obese group

| Nonsynonymous mutations | \(n\) |
|-------------------------|-------|
| Obese subjects          |       |
| A134T                   | 1     |
| G187A                   | 2     |
| M223V                   | 1     |
| A241T                   | 2     |
| H419R                   | 1     |
| E471G                   | 1     |
| I492V                   | 1     |
| V493F                   | 4     |
| Prevalence of mutations (%) | 0.91 |

| Lean subjects           |       |
|-------------------------|-------|
| P5L                     | 1     |
| E24K                    | 1     |
| R80P                    | 1     |
| P93R                    | 1     |
| V94I                    | 1     |
| N143S                   | 1     |
| I148R                   | 1     |
| D189N                   | 1     |
| E234D                   | 1     |
| R316Q                   | 1     |
| P399H                   | 1     |
| Prevalence of mutations (%) | 0.77 |
TABLE 4
Summary of all synonymous mutations found in lean and obese subjects

| Synonymous mutations | Obese subjects (n) | Lean subjects (n) |
|----------------------|-------------------|------------------|
| T6T                  | 5                 | 1                |
| P33P                 | 2                 | —                |
| L44L                 | 1                 | —                |
| P93P                 | 1                 | —                |
| A174A                | 1                 | —                |
| E217E                | 1                 | —                |
| E334I                | —                 | 1                |
| Q336Q                | —                 | 1                |
| V374V                | 1                 | —                |
| C456C                | —                 | 1                |
| L464L                | —                 | 1                |
| I492I                | —                 | 1                |
| Prevalence of mutations (%) | 0.84 | 0.42 |

stimulated by the presence of the free nucleoside 3-methylthymidine (32), and have used this assay throughout this study.

Analysis based on a three-dimensional (3D) homology model (27) comparing FTO with the known 3D structure of three members of the family (ABH2, ABH3, and AlkB) (35) suggests that FTO can be divided in several structural and functional regions (Fig. 1). The more conserved NH2-terminal segment contains 1) a putative nuclear localization signal present only in FTO (36), 2) a double-stranded \( \beta \)-helix in a “jellyroll fold” containing all the catalytic apparatus that is well conserved in all ABHs, 3) a substrate recognition lid that is very different in each ABH member but phylogenetically well conserved among species within each paralog, and 4) two long insertions, absent in other ABH members, that are of variable lengths in different species and are substantially less conserved than the rest of the protein. The COOH-terminal domain, whose structure and function remain unknown, is absent in all other ABH members but contains several residues that are absolutely conserved among highly diverse species.

Two of the naturally occurring mutations that we detected, namely R316Q (found in one lean subject) and R322Q (found in one lean and one obese), replace absolutely conserved residues in the catalytic domain (Fig. 2). The model predicts R316 and R322 to be involved in 2-OG coordination, by forming stabilizing salt bridges with the carboxylates of this cosubstrate. We have previously studied the enzymatic property of R316Q (32), and in this study we have examined R322Q compared with wild-type FTO (Fig. 3A). As predicted, R322Q, like R316Q (32), was completely unable to convert 2-OG to succinate in either the absence or presence of 3-methylthymidine.

One mutation, R96H (detected in one lean and one obese subject) occurs at an absolutely conserved residue within the putative “substrate recognition lid” of the catalytic machinery. 

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**FIG. 1.** Predicted structural and functional regions of FTO based on a 3D homology model of FTO (27).
protein (Fig. 1). In other members of the ABH family, this region of the molecule is known to be required for binding of the primary substrate but is not involved in interactions with the cosubstrate 2-OG. Notably, in contrast to R322Q, R96H retained some ability to convert 2-OG to succinate, but this activity was not enhanced by the presence of 3-methylthymidine (Fig. 3B), a finding consistent with the hypothesis that R96 functions as part of the primary substrate recognition lid.

We also tested the enzymatic activity of several other variants representing the different regions of FTO (P5L, V94I, I148R, M223V, E234D, A241T, A405V, I492V, and V493F) (Fig. 2). None of these variants, all of which are located in less conserved positions, had any significant impact on enzymatic activity (Fig. 3C and D and data not shown).

We have previously demonstrated that murine Fto localizes to the nucleus (27). Bioinformatic analysis suggests that a sequence of 18 amino acid residues in the NH2-terminal of FTO encodes a putative nuclear localization signal (Fig. 1). P5L affects a residue within this putative nuclear localization domain. COS-7 cells were transfected with a construct expressing WT FTO-GFP (wild-type FTO green fluorescent protein), P5L FTO-GFP mutant protein, or GFP alone (mock-GFP). WT FTO-GFP and P5L FTO-GFP colocalized with DAP1, whereas GFP alone was seen in both the cytoplasm and the nucleus (supplementary Fig. 1, available in an online appendix at http://diabetes.

![Mutants assayed: highlighted in red or blue](diabetes.diabetesjournals.org DIABETES, VOL. 59, JANUARY 2010 315)

| Mutants assayed: highlighted in red or blue |
|--------------------------------------------|
| Red = inactive                             |
| Blue = active                              |

![Putative nuclear localization signal (NLS)](diabetes.diabetesjournals.org DIABETES, VOL. 59, JANUARY 2010 315)

![Catalytic domain](diabetes.diabetesjournals.org DIABETES, VOL. 59, JANUARY 2010 315)

![COOH-terminal domain of unknown function](diabetes.diabetesjournals.org DIABETES, VOL. 59, JANUARY 2010 315)

**FIG. 2.** Distribution of nonsynonymous mutations along FTO exons and functional domains. Highlighted mutants are those that have been assayed in the uncoupled reaction in the absence and presence of 3-methylthymidine. Mutants that were inactive in the uncoupled reaction assay with 3-methylthymidine are shown in red, whereas those that did not affect this reaction are shown in blue.
FIG. 3. Activity of wild-type (WT) and mutant FTO proteins. Decarboxylation of \(^1\text{C}\)-2-oxoglutarate to \(^1\text{C}\)-succinate by 1.5 mmol/l FTO in the absence or presence of 3-methylthymidine was measured at various time intervals. A: WT1 and R322Q FTO proteins. B: WT1 and R96H FTO proteins. C: WT2, P5L, V94I, and A241T FTO proteins. D: WT3 and V493F FTO proteins. WT1, 2, and 3 are three different wild-type FTO preparations made at the same time as the mutant proteins with which they are assayed in A, B, C, or D. Open symbols indicate without 3-methylthymidine; closed symbols indicate with 1 mmol/l 3-methylthymidine (also indicated by – and +). E: SDS–10% PAGE of FTO protein preparations stained with Coomassie blue. Per lane, 3 μg total protein was loaded. FTO and size markers are indicated.

diabetesjournals.org/cgi/content/full/db09-0703/DC1). Thus, P5L does not appear to impair the nuclear localization of the protein.

DISCUSSION
Recent genome-wide association efforts have been highly successful in identifying a large number of common genetic variants reliably associated with important human diseases and quantitative traits (2,32,37). Understanding the precise biological mechanisms underlying such associations will provide a major scientific challenge that will require a multiplicity of approaches in humans and in model organisms. FTO exemplifies many of these challenges. Although SNPs in intron 1 of FTO are unequivocally associated with adiposity in multiple populations, there is still no evidence that those SNPs influence the expression or splicing of FTO itself. FTO is located adjacent to other genes, the expression or function of which could conceivably influence energy balance (10,36,38). Recent findings in mice rendered null for Fto support the notion that Fto itself has an important influence on energy balance. Fto-null mice are small, are lean, have an increased metabolic rate, and are hyperphagic, whereas Fto\(^{+/−}\) mice are resistant to diet-induced obesity (39). Given that information, it is reasonable to speculate that loss-of-function mutations in FTO might be more common in lean rather than obese humans. To test this hypothesis, we sequenced FTO in a large number of lean and obese subjects and found that 1) nonsynonymous mutations were equally common in both the obese and lean cohorts and 2) heterozygous mutations that severely impaired enzymatic activity of FTO were found in both lean and obese individuals with no other obvious major clinical phenotypes. Our findings illustrate the importance, when sequencing “candidate” genes whose candidacy derives largely from genome-wide association studies in extreme phenotypes, of studying both ends of the spectrum of a quantitative trait. (If we had considered FTO to be an “obesity gene” and sequenced only obese subjects, the finding of loss-of-function mutations would have been misleading.) From our human genetic data, we can conclude that heterozygosity for a severely dysfunctional FTO allele is compatible with being either lean or obese in humans. Understanding the relationship between SNPs in intron 1 of FTO with human adiposity will require other approaches. In collaboration with Dr. Laurence Colleaux from Hopital Necker Paris and others, we have recently found a consanguineous Israeli-Arab family in which nine siblings were homozygous for the R316Q mutation in FTO (32). All homozygous carriers were severely growth retarded, had multiple congenital malformations, and died in infancy. Although heterozygous parents of these children had no obvious metabolic phenotype, ongoing efforts are directed at establishing measures of adiposity in heterozygous versus wild-type relatives (32).

Our studies have brought new insights into aspects of the structure-function relationship of the human FTO enzyme. Human FTO can catalyze the uncoupled reaction (2-OG to succinate) and is stimulated by 3-methylthymidine (32), and we have used this as a robust test of FTO’s catalytic activity. As predicted, we found that the arginine at position 322 is essential for the catalytic activity of human FTO, and we have made similar observations for R316Q (32). Mutation of the arginine corresponding to human 316 in mouse Fto (R313A) also generated an inactive enzyme (27). Perhaps more interestingly, we have demonstrated that mutation of R96 to histidine in human FTO produces an enzyme that is capable of some basal conversion of 2-OG to succinate but is insensitive to the effects of 3-methylthymidine. These findings are consistent with R96 being part of the so-called substrate recognition lid that is responsible for substrate fixation and selectivity. Thus, the lid is well conserved among species orthologues, but has diverged substantially in the various paralogs of ABH to accommodate different substrates. For instance, R131 at this position in the lid of ABH3 is positioned to interact with 1-meA N3, and R131A in ABH3 abolishes the activity toward 1-me-A containing single-stranded DNA (27).

Although all of the other nonsynonymous variants we tested appeared to have normal enzymatic activity, we should express caution about categorically stating that these are fully wild type in function. First, we do not know what the true natural primary substrate(s) of FTO is (are), and it is entirely possible that mutations that affect FTO’s actions on this elusive substrate will not necessarily
impair the uncoupled reaction. Second, although we have demonstrated that FTO, in vitro, possesses dioxygenase activity, whether it has any other biological role, enzymatic or otherwise, is yet to be determined. This could potentially be of relevance to V493F, a COOH-terminal mutation that was found only in the obese population but was found in four different individuals (Fig. 2), and yet did not impact on the ability of FTO to convert 2-OG to succinate or its stimulation by 3-methylthymidine (Fig. 3D). The COOH-terminal region of FTO, although highly conserved across species, is not shared with other members of the ABH family, and its structure and function are unknown.

We noted two potentially interesting observations regarding the COOH-terminal region of the protein. First, on a “mutations per nucleotide” basis, nonsynonymous mutations are found ~3 times less frequently in the COOH-terminus than in the rest of the molecule. Second, although nonsynonymous variants elsewhere in the molecule are found equally in obese and lean subjects, eight such variants found in the COOH-terminus were detected in obese subjects and only two in lean (Fig. 2). These preliminary observations are of potential interest and might lead to a better understanding of FTO function and its role in energy homeostasis localization.

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