REPORT

Loss-of-Function Mutation in the Dioxygenase-Encoding *FTO* Gene Causes Severe Growth Retardation and Multiple Malformations

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FTO is a nuclear protein belonging to the AlkB-related non-haem iron- and 2-oxoglutarate-dependent dioxygenase family. Although polymorphisms within the first intron of the FTO gene have been associated with obesity, the physiological role of FTO remains unknown. Here we show that a R316Q mutation, inactivating FTO enzymatic activity, is responsible for an autosomal-recessive lethal syndrome. Cultured skin fibroblasts from affected subjects showed impaired proliferation and accelerated senescence. These findings indicate that FTO is essential for normal development of the central nervous and cardiovascular systems in human and establish that a mutation in a human member of the AlkB-related dioxygenase family results in a severe polymalformation syndrome.

Fto was originally identified in mice as one of the genes encoded by the 1.6 Mb deletion causing the fused toes (Ft) mutant, an autosomal-dominant mouse mutation generated by a transgene integration into region D of mouse Chr 8.[1] In addition to *Fto*, the deleted segment also contains three members of the Iroquois gene family (*Irx3*, *Irx5*, and *Irx6*, also known as the IrxB complex) as well as the *Fts/Aktip* and *Ftm/Rpgrip1l* genes. Mice heterozygous for the Ft mutation are characterized by partial syndactyly of forelimbs and thymic hyperplasia.[1] Ft/Ft embryos die in midgestation and present many abnormalities including limb polydactyly and distal truncations, major brain and heart defects, delay or absence of neural tube closure, and facial structure hypoplasia.[2,3]

Interestingly, a human genome-wide search for type 2 diabetes susceptibility genes identified the strong association of a series of single nucleotide polymorphisms (SNPs), in tight linkage disequilibrium with rs9939609 and located in the first intron of the *FTO* gene (MIM 610966), with higher body mass index (BMI) and obesity risk in European cohorts.[4,5] This association was replicated in various European cohorts and several studies have been performed trying to identify association between the obesity risk allele and energy intake/expenditure or eating behavior trait.[6–8] However, how *FTO* variants modulate components of the energy balance remains elusive so far. FTO has been recognized as a member of the AlkB-related family of non-haem iron- and 2-oxoglutarate-dependent dioxygenases.[9] These non-heme iron enzymes, which require Fe^{2+} as a cofactor and 2-oxoglutarate and dioxygen as cosubstrates, reverse alkylated DNA and RNA damages by oxidative demethylation.[10–14] *Fto*-deficient mice have been recently described.[15] Homozygous mutant mice are viable but postnatal death occurs frequently and loss of Fto leads to postnatal growth retardation, significant reduction in adipose tissue, and lean body mass.

We ascertained a large Palestinian Arab consanguineous multiplex family in which nine affected individuals presented with a previously unreported polymalformation syndrome (Figure 1A). The study was approved by the supreme Israeli Helsinki Review Board (33/07-08) and informed consent was obtained from all family members. All affected individuals had postnatal growth retardation, microcephaly, severe psychomotor delay, functional brain deficits, and characteristic facial dysmorphism. In some patients, structural brain malformations, cardiac defects, genital anomalies, and cleft palate were also observed (Table 1). Early lethality resulting from intercurrent infection or unidentified cause occurred at 1–30 months of age. Extensive biochemical, metabolic, and genetic analyses failed to diagnose any previously known inherited disorders. Because the pedigree suggested an autosomal-recessive mode of inheritance, we performed a genome-wide autozygosity screen with the Perkin Elmer Biosystems linkage mapping set (version 1) and identified a unique region of shared homozygosity on chromosome 16q12 (Figure S1 available online). Further genotype and haplotype analyses reduced the critical region to a 6.5 Mb interval between loci D16S411 and D16S3140 (maximum LOD score Zmax = 4.16 at $\theta = 0$ at the D16S3136 locus). No other genomic region showed consistent linkage. This genetic interval encompasses 28 genes that were all systematically

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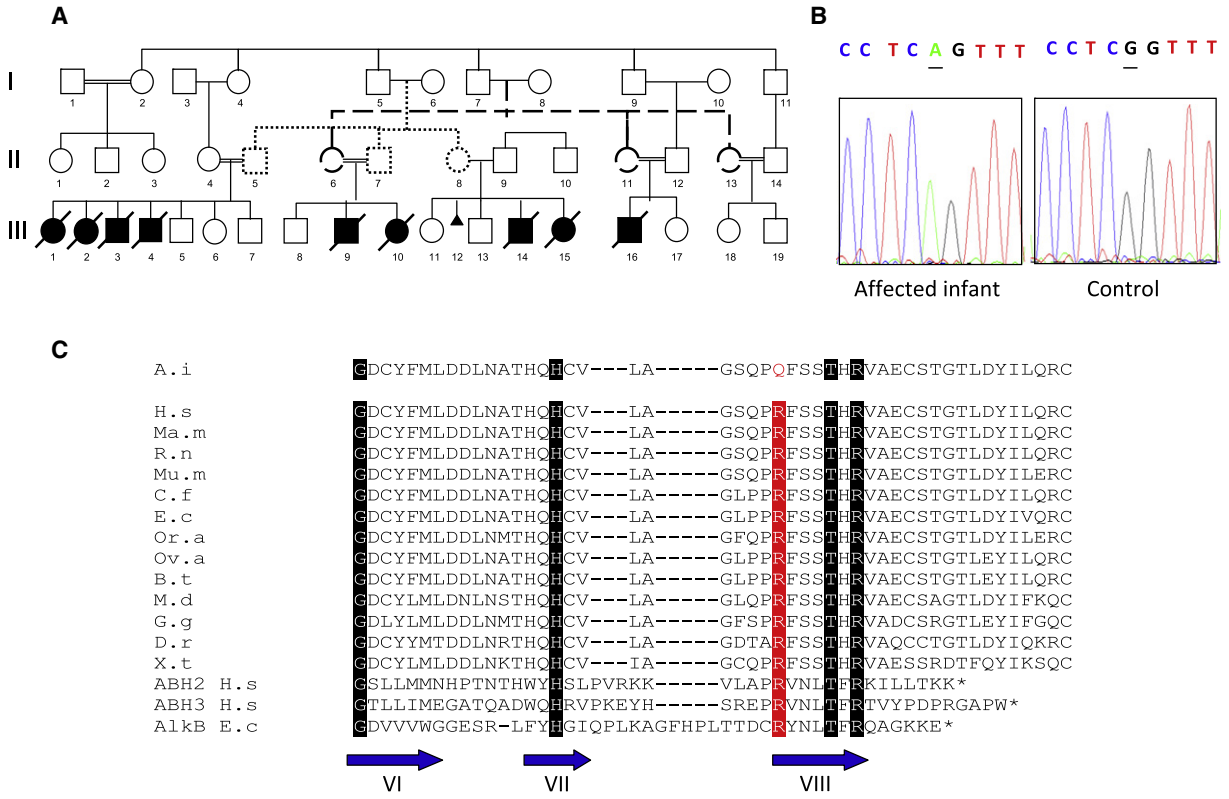

Figure 1. Genetic Analysis of a Family with Ante- and Postnatal Growth Retardation and a Severe Polymalformative Syndrome
(A) Pedigree of the family. Filled symbols and slashes indicate affected and deceased infants.
(B) Electrophoregrams showing the variation of *FTO* gene sequence in an affected infant and a healthy control.
(C) Part of the multiple sequence alignment of FTO representative orthologs (H.s, *Homo sapiens*; Ma.m, *Macaca mulatta*; R.n, *Rattus norvegicus*; Mu.m, *Mus musculus*; C.f, *Canis familiaris*; E.c, *Equus caballus*; Or.a, *Ornithorhynchus anatinus*; Ov.a, *Ovis aries*; B.t, *Bos taurus*; M.d, *Monodelphis domestica*; G.g, *Gallus gallus*; D.r, *Danio rerio*; X.t, *Xenopus tropicalis*; ABH2 H.s, human ABH2; ABH3 H.s, Human ABH3; AlkB E.c, *E. coli* AlkB). The conserved residues are highlighted and the amino acid highlighted in red is the absolutely conserved arginine involved in the R316Q mutation in patients (Ai, affected infant). Blue strands labeled with roman numerals identify three of the eight β strands that form the conserved double-stranded β-helix of the 2OG-oxygenases.

analyzed at both DNA and/or RNA levels (Table S1). We identified a homozygous single-nucleotide variation at cDNA position 947 (c.947G→A) within the *FTO* gene (Figure 1B). The c.947G→A transition predicts a p.R316Q substitution. Notably, this amino acid residue is absolutely conserved across all known FTO paralogs and AlkB orthologs (Figure 1C) and is involved in 2-oxoglutarate coordination by forming stabilizing salt bridges with the carboxylates of this cosubstrate.[9] This variant cosegregated with the disease and was not found in 730 control chromosomes, including 378 chromosomes from individuals of Palestinian Arab origin. We subsequently undertook to estimate the frequency of *FTO* sequence variants within the general population. *FTO* coding exons and intron-exon boundaries sequence was determined in 1492 controls of European descent without overt disease or syndromic features. No nonsense variant was identified and the prevalence of missense variants was 0.87%. In addition, neither homozygous missense mutation nor composite heterozygous mutations were detected in this large series of control subjects (data not shown), further supporting the hypothesis that the R316Q mutation is the disease-causing defect. To estimate the prevalence of *FTO* mutations, 27 unrelated children with partially overlapping clinical features were tested but no mutation in the *FTO* gene was identified, strengthening the idea that the new syndrome described here is a very rare condition.

FTO belongs to the AlkB-related protein family and was shown to localize in the nucleus.[9] Immunofluorescence experiments performed on patient and control fibroblasts demonstrated that the mutation did not affect its nuclear localization (data not shown). Murine and human Fto have been shown to demethylate 3-methylthymine and 3-methyluracil residues in single-stranded DNA and RNA in vitro, albeit with a relatively low efficiency. Fto was less active on the more common forms of methylated DNA base damage, 1-methyladenine and 3-methylcytosine.[9–14,16] Moreover, the recombinant murine Fto protein mutated in the residue corresponding to human R316 amino acid displays a much reduced or absent DNA demethylation activity. To investigate the functional consequences of the R316Q mutation, we tested the catalytic activity of the wild-type and mutant R316Q FTO proteins in vitro. Two different assays were used: the first followed the conversion of the cosubstrate 2-oxoglutarate to succinate and the second monitored the ability of FTO to demethylate

Table 1. Major Clinical Features in Affected Individuals with c.947G→A Mutation in *FTO*

Clinical Features	Number of Patients Presenting the Symptom/Number of Patients Examined
Survival	
Death before 3 years of age	8/8
Build	
Intrauterine growth retardation	3/7
Failure to thrive (severe)	8/8
CNS	
Developmental delay (severe)	8/8
Postnatal microcephaly (severe)	8/8
Hypertonicity	6/6
Hydrocephalus	4/8
Lissencephaly	3/8
Seizures	3/8
Dandy walker malformation	2/8
Brain atrophy	1/8
Heart	
Ventricular septal defect, atrio ventricular defect, atent ductus arteriosus	6/8
Hypertrophic cardiomopathy	4/8
Dysmorphism	
Ante verted nostrils	7/7
Thin vermilion	7/7
Prominent alveolar ridge	6/6
Retrognathia	7/7
Coarse face	7/7
Protruding tongue	3/7
Other	
Short neck	7/7
Cutis marmorata	7/7
Drumstick fingers	6/6
Brachydactyly	6/6
Toenail hypoplasia	6/6
Skull asymmetry	6/6
Neuro sensory deafness	5/5
Weak cry	4/6
Umbilical hernia	4/6
Hypertrophy of labia/genital ambiguity/undescending testis	4/7
Cleft palate/Bifid uvula	3/6
Optic disk abnormality	3/7

Medical information was available from 8/9 patients.

3-methylthymine in DNA. Most 2-oxoglutarate- and Fe^{2+}-dependent dioxygenases slowly catalyze the conversion of 2-oxoglutarate to succinate even in the absence of their prime substrate,[16] and this uncoupled reaction may be stimulated by substrates or their analogs. We optimized conditions to assay the ability of human recombinant FTO protein to convert ^{14}C-2-oxoglutarate to ^{14}C-succinate and found that this activity was stimulated 6- to 8- fold by free 3-methylthymidine (Figure 2A). Other nucleosides such as 1-methyldeoxyadenosine and thymidine or the base 3-methylthymine did not stimulate the activity (data not shown). Interestingly, the R316Q FTO mutant protein had no detectable ability to catalyze the uncoupled reaction and also was not stimulated by 3-methylthymidine (Figure 2A). The ability of FTO to demethylate ^{14}C-labeled 3-methylthymine in single-stranded poly(dT) substrate was assayed by measuring the release of ^{14}C-formaldehyde. The optimum pH for the demethylation of 3-methylthymine in DNA by AlkB, ABH2 (MIM 610602), and ABH3 (MIM 610603) is 6 to 6.5 and this is also the case for human FTO. Wild-type FTO[11,16,17] actively but slowly demethylated this substrate whereas the mutant R316Q protein was inactive in the assay (Figure 2B). The defective activity of FTO, as observed in both assays, is most likely due to the inability of the mutant protein to interact with the cosubstrate 2-oxoglutarate.

To gain insight into the function of the *FTO* gene product and the pathophysiology of the disease, we investigated the expression of *FTO* in human embryos and adult tissues. RT-PCR showed an ubiquitous expression in all fetal and adult tissues tested, as has been observed in mice (data not shown).[18] Human embryo sections were hybridized in situ at stages Carnegie (C)15, 33–36 days postfertilization (dpf) and C18 (44–46 dpf) with antisense probes against *FTO* and sense probes as negative controls. We observed a nearly ubiquitous expression of *FTO*, with higher expression in the central nervous system and the liver. In the heart, the mitral and semilunar valves and the wall of the pulmonary trunk stained as did the ventricular myocardium, and a strong and uniform expression was observed in the developing pituitary and the frontonasal and mandibular mesenchyme (Figure S2). It is worth noting that this wide spatiotemporal pattern of expression is consistent with the broad spectrum of clinical manifestations of the disease (Table 1).

Interestingly, the cultured skin fibroblasts of patient III.15 displayed an altered morphology (including hypertrophy), an increased number of vacuoles and cellular debris (Figure 3A), a reduced life span, and decreased proliferative abilities when compared to controls (Figure 3B). Cellular expression of the senescence-associated β-galactosidase (SA-β-Gal) is thought to be a reliable indicator of the switch mechanism that occurs when cells enter the senescent phenotype.[19] Notably, a significantly elevated number of SA-β-Gal-positive cells, increasing with passage numbers, was found in patient cultured cells compared to passage-matched control fibroblasts (Figure 3C). Although further analyses on skin fibroblasts of additional patients

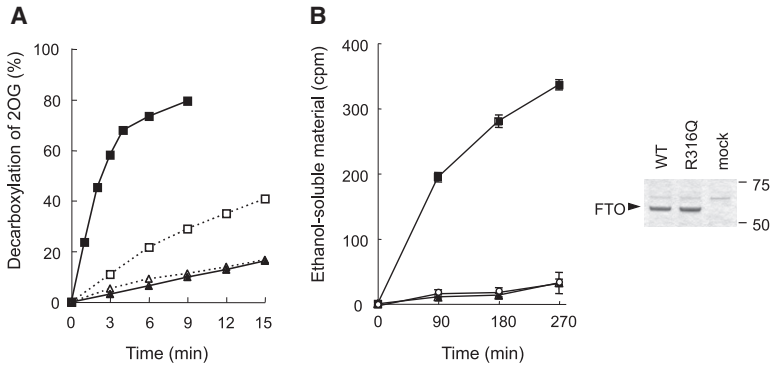

Figure 2. Biochemical Analyses of the Wild-Type and Mutant FTO Proteins

(A) Purified FTO was added to 10 μl reaction mixture containing 50 mM HEPES.KOH (pH 7), 50 μg/ml BSA, 4 mM ascorbate, 75 μM $Fe(NH_4)_2(SO_4)_2$, and 20 μM [5-^{14}C]-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 mM 3-methylthymidine (Moravek Biochemicals) was included in the assay mix. The reaction was stopped by adding 5 μl stop solution containing 20 mM succinate, 20 mM 2-oxoglutarate followed by 5 μl 160 mM dinitrophenylhydrazine, which precipitates 2-oxoglutarate. This mix was incubated at room temperature for 30 min. An additional 10 μl 1M 2-oxoglutarate 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 ^{14}C-succinate generated. Time course of activity of 1.5 μM FTO: open and closed squares, wild-type FTO protein; open and closed triangles, mutant R316Q FTO protein. Open symbols and dotted line: without 3-methylthymidine; closed symbols and solid line: with 3-methylthymidine (1 mM).

(B) A DNA substrate containing ^{14}C-3-methylthymine was prepared by treating poly(dT) with [^{14}C]-methyl iodide (54 Ci/mmole, Amersham Biosciences) as previously described[11] and had a specific activity of 1580 cpm/μg poly(dT). FTO was added to the ^{14}C-methylated poly(dT) substrate (1000 cpm) in a 100 μl reaction mix containing 50 mM MES-HCl (pH 6), 75 μM $Fe(NH_4)_2(SO_4)_2$, 100 μM 2-oxoglutarate, 2 mM ascorbate, 10 μg/ml bovine serum albumin and incubated at 20°C for various times. All assays were performed in triplicate. To stop the reaction, EDTA was added to a final concentration of 10 mM. The polynucleotide substrate was then ethanol precipitated in the presence of carrier calf thymus DNA. Two-thirds of the ethanol-soluble radioactive material was monitored by scintillation counting. Equal volumes of the protein preparations were assayed. Standard error of the mean is shown for each time point. Closed square, 2 μM wild-type FTO protein; closed triangle, 2 μM mutant R316Q FTO protein; open circle, "mock prep" prepared in the absence of recombinant FTO expression. Equal volumes of the protein preparations were also examined by SDS-10% PAGE.

are necessary to validate these findings, taken together these data could yet suggest that the defective FTO activity may cause premature senescence-like phenotype.

Although repair of alkylated nucleic acid by oxidative demethylation is crucial to maintain genome integrity, no AlkB-related protein mutations have been hitherto identified in human. The results reported here provide therefore the first example of a human disorder related to the defect of an AlkB-related protein. The *Escherichia coli* AlkB protein catalyzes the oxidative demethylation of 1-methyladenine, 3-methylcytosine, and 3-methylthymine in DNA and RNA. Eight mammalian orthologs of AlkB have been previously identified (ABH1 to 8),[20] and FTO is the ninth member of this family. Of this family, only ABH2 and ABH3 have been demonstrated to be highly active in repairing methylation damage to DNA.[13,14] In comparison, ABH1 (MIM 605345) has a modest activity on 3-methylcytosine in single-stranded DNA or RNA[21] whereas FTO has a similar low activity on 3-methylthymine in these substrates.[9,16] Mice lacking either Abh2 or Abh3 have no overt phenotype but the *Abh2*$^{-/-}$ mice accumulate significant levels of 1-methyladenine in their genomes.[22] Notably, *Abh1*$^{-/-}$ mice are viable but have intrauterine growth retardation and defects in placental differentiation.[23]

Surprisingly, although homozygous *Fto*$^{-/-}$ mice have postnatal growth retardation, significant reduction in adipose tissue, and lean body mass, they are not, however, reported to have any developmental abnormalities in the central nervous or cardiovascular systems.[15] Thus, humans homozygous for a catalytically inactive FTO and *Fto* null mice share the growth retardation phenotype but both mutations differ in the impact on development of central nervous and cardiovascular systems. There are several possible reasons for the different findings in our patients versus the *Fto*$^{-/-}$ mouse. First, major phenotypic differences between rodent models and humans harboring identical mutations are well described. For example, maturity-onset diabetes of the young subtype 3 (MODY3 [MIM 600496]) is caused by heterozygous mutations in the transcription factor hepatocyte nuclear factor (*HNF*)-*1α* (MIM 142410).[24] By contrast, mice with heterozygous mutations in *Hnf-1α* gene are phenotypically normal.[25] Second, it is possible that the presence, in the nucleus, of a mutant catalytically inactive FTO might have biochemical consequences over and above that seen with complete deficiency of Fto. These could include toxic "gain-of-function" effects or dominant-negative interference with other related family members. Finally, it is formally possible that the phenotype seen in the human subjects is due to a combination of the mutant FTO and a second, as yet undetected, mutation in the 6 Mb critical region. The fact that no mutations were detected on sequencing of the coding regions and splice junctions of all 28 known and putative genes in that linked region makes the chances of such a second mutation very small.

A cluster of variants located within the first intron of *FTO* has recently been strongly and reproducibly associated with human adiposity.[4,5] Yet, whether these variants influence the risk of obesity directly, via the altered expression of FTO or through other mechanisms, remains questionable. In this regard, it is worth noting that homozygosity for the R316Q FTO mutation caused severe growth retardation. Detailed anthropometric measurements are not available on unaffected family members but, noticeably, none of

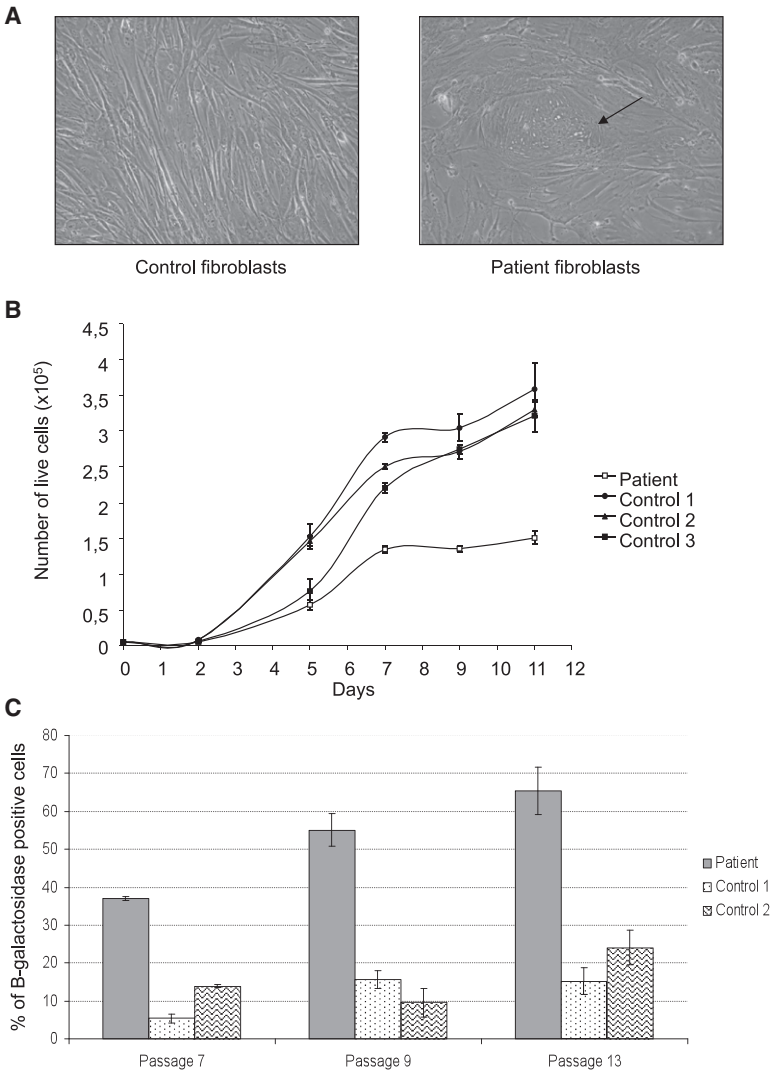

Figure 3. Cell Morphology and Proliferative Abilities of Cultured Skin Fibroblasts Harboring the R316Q *FTO* Mutation

(A) Fibroblast morphology. The arrow shows altered cell morphology and enlarged cell size of a patient fibroblast.

(B) Fibroblast growth curves. Fibroblasts were seeded at a density of 5000 cells/well in 12-well dishes. The number of cells per dish was determined with a CASY cell counter on days 2, 5, 7, 9, and 11 after seeding. Filled and open symbols correspond to patient and control fibroblasts, respectively. Data correspond to the mean of three replicates. Standard error of the mean is shown for each point.

(C) Senescence-associated β-galactosidase assay. Fibroblasts were seeded on 6-well dishes at a density of 130,000 cells/well. Senescent fibroblasts were stained with the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology). Percentages of β-galactosidase-positive cells for passages 7, 9, and 13 were calculated on the basis of approximately 100 cells. Data correspond to the mean of three replicates. Standard error of the mean is shown for each point.

the parents were clinically obese. Given the results from the Fto-deficient mice, it might be hypothesized that humans heterozygous for a loss-of-function mutation in FTO might actually be relatively resistant to becoming obese. Future studies of the body composition of carrier and noncarrier relatives will be required to clarify the relationship between FTO variant heterozygosity and adiposity.

The molecular mechanisms whereby the mutant FTO leads to the severe phenotype observed in our patients remain unknown. The fact that (1) FTO is a member of the Alk-B-related family of dioxygenases, (2) at least two of the mammalian members of this family have established roles in DNA repair, and (3) FTO can demethylate 3-methylthymine in the context of DNA might suggest that FTO plays a role in the maintenance of genome integrity. If that were the case, one could easily understand how FTO defect relates to developmental malformations and the accelerated senescence observed in cultured fibroblasts derived from the patients. However, it should also be pointed out that (1) several members of the mammalian Alk-B-related family have no established role in DNA repair, (2) FTO demethylates 3-methylthymine slowly and inefficiently, suggesting that this is not its true physiological substrate, (3) 3-methylthymine is a very rare lesion in vivo, and (4) defective repair of rare methylated bases in DNA can hardly be related to the striking increase in energy expenditure observed in Fto-deficient mice. Taken together, this suggests that FTO roles in the nucleus are presumably not restricted to DNA repair.

Because of its association with obesity, FTO is currently the subject of intense interest. The finding of obesity resistance in Fto-deficient mice has led to the suggestion that interference with Fto enzymatic function might be a novel and interesting antiobesity therapeutic strategy. The discovery that humans homozygous for an enzymatically inactive mutant form of FTO have multiple congenital abnormalities suggests that any program of research exploring the therapeutic utility of FTO inhibitors should incorporate a particularly careful assessment of teratogenicity and other toxic effects.

Supplemental Data

Supplemental data include two figures and one table and can be found with this article online at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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