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**The obesity-associated SNPs in intron 1 of the *FTO* gene affect
primary transcript levels.**

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Short running title – *FTO* expression and obesity

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Abstract

As shown by genome-wide association studies single nucleotide polymorphisms (SNPs) within intron 1 of the *FTO* gene are associated with the body mass index (BMI) and type 2 diabetes, although the functional significance of these SNPs has remained unclear. Using primer extension assays, we have determined the ratio of allelic *FTO* transcript levels in unspliced heterogeneous nuclear RNA (hnRNA) preparations from blood of individuals heterozygous for SNP rs9939609. Allelic expression ratios of the neighbouring *RPGRIP1L* gene were investigated in individuals who were heterozygous for SNP rs4784319 and heterozygous or homozygous for rs9939609. In each of five individuals, the *FTO* transcripts containing the A (risk) allele of rs9939609 were more abundant than those with T allele (mean 1.38; 95% confidence interval 1.31 to 1.44). Similar results were obtained in a fibroblast sample. We also observed skewed allelic expression of the *RPGRIP1L* gene in blood, but skewing was independent of the *FTO* genotype. Our data suggest that increased expression of *FTO* is associated with increased body mass.

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Key words: *FTO*, SNP, allelic expression

Introduction

Recent genome-wide association studies have revealed a strong association between a block of SNPs in the fat mass and obesity associated (*FTO*) gene, BMI and other obesity-related traits in children and adults of different populations^{1,2}. The obesity-associated SNPs are located in intron 1 of the *FTO* gene, which contains 9 exons (Figure 1). Using bioinformatic analysis, Gerken et al. (2007) have proposed that the FTO protein is a Fe(II) and 2-oxoglutarate-dependent oxygenase³. Loss-of-function mutation in *FTO* causes severe growth retardation and multiple malformations in homozygotes⁴ whereas loss of one functional copy of this gene appears to be compatible with both lean and obese phenotype⁵. Leanness, postnatal growth retardation and a higher metabolic rate were observed in *Fto* knockout mice⁷ and in mice with a missense mutation in exon 6 (ref. 8).

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The *FTO* gene shares a CpG island with the adjacent *RPGRIP1L* gene, which is transcribed in the opposite direction, suggesting that the two genes are co-regulated. The RPGRIP1L protein is located in cilia and centrosomes⁶. Loss-of-function mutations in *RPGRIP1L* cause Joubert syndrome type 7 or Meckel syndrome type 5. Of note, *FTO* and *RPGRIP1L* genes are ubiquitously expressed and show similarity of expression profile both in fetal and adult tissues (data not shown).

Because the obesity-associated SNPs in the *FTO* gene are intronic, their functional significance is unclear. It is possible that one of the SNPs is located in a regulatory sequence and that the risk allele increases or decreases the transcription rate, but strong

linkage disequilibrium (LD) of these SNPs makes it difficult to identify the functionally relevant SNP. In 2008, Stratigopoulos et al. reported that the risk allele (A) of SNP rs8050136 preferentially bound the transcription factor CUTL1 in human fibroblast DNA and that an siRNA knock-down of *CUTL1* by 70% decreased *FTO* and *RPGRIP1L* expression by 90% and 65%, respectively⁹. However, these findings are not consistent with their model in which *FTO* and/or *RPGRIP1L* mediate suppressive effects on energy intake. To fit the physiological model, CUTL1 should preferentially bind the non-risk allele of rs8050136. Furthermore, several studies have failed to reveal any influence of the *FTO* genotype on total mRNA level of *FTO* or *RPGRIP1L*^{10,11,12}. Recently, an association between intronic variation of the *FTO* gene and transcript levels of the retinoblastoma-like 2 (*RBL2*) gene, which maps 270 kb upstream of *FTO*, was reported¹³. One problem in interpreting the previous findings is that the CCAAT-displacement activity of CUTL1 was implicated in the transcriptional repression of several genes, whereas some CUTL1 isoforms were found to participate in the transcriptional activation¹⁴. Another problem is that studies on *cis*-regulatory effects on gene transcription in humans are hampered by the fact that the tested individuals unavoidably differ in genetic background, age, life events and environment. In order to detect subtle differences in transcript levels, very large numbers of individuals would have to be tested. These problems can be circumvented by determining the ratio of allelic transcript levels in heterozygous individuals, where each allele serves as an internal control for the other^{15,16,17,18}. For this approach, only few subjects are needed.

Materials and methods

Study cohort

The study was approved by the ethics committee of the University Hospital Essen. Blood samples and skin biopsies from normal weight individuals (BMI 18.5 - 25) were obtained after informed consent was given.

Genotyping

Subjects were genotyped by sequence analysis of genomic DNA (gDNA) extracted from whole blood with EZ1 DNA Blood Kit (Qiagen, Hilden, Germany). Sequence reactions were performed with Big Dye Terminators (BigDye® Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA). Reaction products were analyzed with an ABI 3100 Genetic Analyzer and Sequencing Analysis software (Applied Biosystems, Foster City, CA, USA).

Preparation of hnRNA and total RNA

For hnRNA extraction, lymphocytes were isolated with Ficoll-Paque PLUS (GE Healthcare, USA) from fresh blood collected in EDTA tubes. Skin fibroblasts were cultured in AmnioMAX+M-C100 medium containing 20% AmnioMAX- Supplement (Gibco, Invitrogen, CA, USA) at 37⁰C in a humidified atmosphere containing 5% CO₂. Both lymphocytes and fibroblasts were subjected to hnRNA extraction with the Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corporation, Thorold, Canada). DNase treatment was carried out in solution followed by cleaning-up on spin

columns (Qiagen Hilden, Germany). To minimize losses of hnRNA, all steps were carried out as quickly as possible. Isolated hnRNA was dispensed in several aliquots and frozen in liquid nitrogen. For testing SNP rs4784319, we used total RNA extracted from blood with the PAXgene blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland).

Allelic expression assays

Reverse transcription and primer extension assays were performed with kits from Applied Biosystems, Foster City, CA, USA. Total RNA from blood was reverse transcribed with random hexamers, whereas cDNA from hnRNA was primed with sequence specific primers FTO R1 or FTO R2 (all primer sequences and annealing temperatures are given in the Table S1). For amplification the GoTaq DNA Polymerase Kit (Promega, Madison, USA) was used. *FTO* genomic DNA and cDNA were amplified with primers FTO F1 and FTO R1. *RPGRIP1L* genomic DNA was amplified with primers gRP F and gRP R, cDNA was amplified with cRP F and cRP R. SNPs in the primer binding sites were excluded by sequencing across these regions in all individuals.

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Samples were heated at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 55°C (*FTO* gDNA and cDNA), 60°C (*RPGRIP1L* gDNA) and 62°C (*RPGRIP1L* cDNA) for 30 sec, and 72°C for 40 sec, finally 72°C for 5 min. Using equal amount of amplicons from cDNA and genomic DNA, primer extension assays were carried out with Snapshot R

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primers and ABI Prism SNaPshot Kit. Reaction conditions were as follow: 96°C for 3 min, 25 cycles of 96°C for 10 sec, 53°C (*FTO*, rs9939609) and 41°C (*RPGRIP1L*, rs4784319) for 5 sec, 60°C for 30sec. The reaction products were analyzed by gel capillary electrophoresis on ABI 3700 DNA Analyzer and the electropherograms were

analyzed with the Gene Mapper 4.0 software. Allelic DNA ratios were used to normalize the cDNA ratios. Means and confidence intervals were calculated with JMP7 (SAS, Cary, NC, USA).

Results

To measure the allelic ratios of *FTO* and *RPGRIP1L* transcripts, we developed fluorescence-tagged single nucleotide primer extension assays. This approach has been successfully used by us and others for detecting skewed and non-skewed allelic expression of many genes (see for example ^{18, 19}). For *FTO*, we used unspliced hnRNA because (i) all obesity-associated SNPs are of intronic location and (ii) no expressed polymorphism in LD with these SNPs is known. We chose the clinically associated SNP rs9939609 within intron 1 of *FTO* as a marker (Figure 1), because it was tested in many independent studies of large Caucasian populations. SNP rs9939609 is in complete LD with SNP rs8050136, and the A (risk) allele of rs9939609 is associated with the A allele of rs8050136 (data not shown). For *RPGRIP1L*, we used an expressed SNP located in the 3' untranslated region (rs4784319) (Figure 1). Since SNPs in this gene are not in LD with the obesity-associated *FTO* SNPs and there is no easy way of establishing phase in double heterozygotes, we decided to determine the allelic expression pattern of the *RPGRIP1L* gene in *RPGRIP1L* heterozygous individuals with different *FTO* genotypes. If the obesity-associated *FTO* SNPs affected *RPGRIP1L* expression, we should observe allelic expression imbalance of *RPGRIP1L* in *FTO* heterozygotes, but not in *FTO* homozygotes.

For determining allelic *FTO* transcript levels, we investigated hnRNA from blood of five individuals heterozygous for rs9939609. All assays were performed in sextuplicate. There was no evidence for DNA contamination of the hnRNA samples (Suppl. Figure 1). As shown in Figure 2A, there was very little inter-assay variation of allelic ratios. In each of the five individuals, relative transcript levels were skewed to similar degrees in favour of the A (risk) allele (mean 1.38; 95% confidence interval 1.31 to 1.44). In skin fibroblasts from individual 18 we observed a ratio of 1.31 (95% confidence interval 1.23 to 1.39, data not shown).

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To investigate the effect of the obesity-associated *FTO* SNPs on allelic *RPGRIP1L* transcript levels, we tested blood RNA of six individuals who were heterozygous for the *RPGRIP1L* SNP rs4784319. Of these, four were heterozygous for the *FTO* SNP rs9939609, one was homozygous for the A (risk) allele of this SNP, and one was homozygous for the T allele. As shown in Figure 2B, we observed similar degrees of skewing in favour of the T allele in all six individuals. These results suggest that *RPGRIP1L* gene expression is not affected by the *FTO* genotype, but by *cis*-regulatory variation which is in LD with rs4784319.

Discussion

Using single nucleotide primer extension assays we have determined allelic expression ratios of the *FTO* and *RPGRIP1L* genes. We demonstrate for the first time (i) that the obesity associated *FTO* SNPs affect *FTO* and not *RPGRIP1L* expression and (ii) that the

primary *FTO* transcript made from the risk allele is more abundant than the transcript made from the non-risk allele, at least in blood cells and skin fibroblasts. The observed skewing cannot be due to the presence of genomic DNA in our hnRNA preparations, because (i) there was no evidence for DNA contamination (Suppl. Fig. 1) and (ii) allelic PCR products from genomic DNA would be present in equal amounts.

Our observation that allelic expression imbalance of the *RPRGIPIL* gene is not dependent on the *FTO* genotype is consistent with previous studies reporting no association between *RPRGIPIL* variation and obesity²⁰. The degree of skewing of allelic *FTO* expression is remarkably similar in our subjects, suggesting that most of the variation in *FTO* expression is due to *cis*-regulatory variation in intron 1 of this gene. Since the determination of allelic expression with intronic SNPs gives very similar estimates with those obtained with exonic SNPs¹⁸, we can assume that allelic mRNA levels are skewed, also. Higher mRNA levels might translate into higher protein levels, but, of course, we have no proof for this assumption.

One limitation of our study is the fact that we could analyze easily accessible cell types only. Blood cells and fibroblasts are most probably not involved in body weight regulation, but we do not know the cell type through which *FTO* exerts its effect, and this cell type may not be accessible. On the other hand, *FTO* is ubiquitously expressed. Therefore, it not unreasonable to assume that the allelic expression ratios found in blood cells and fibroblasts are similar to those in many other cells.

Based on our results we propose that increased rather than decreased expression of *FTO* is causally evolved in increased BMI measures and obesity-associated traits. This interpretation is consistent with mouse models, in which decreased levels of *Fto* cause leanness^{7,8}. Increased expression of the risk allele as shown here is compatible with a role of CUTL1 in activating *FTO*, as originally suggested by Stratigopoulos et al. (2008), although other transcriptional activators may play a role. It is tempting to speculate that subtle reduction of *FTO* expression by pharmacological intervention may contribute to the prevention of obesity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Web resources

The URLs used for this study are as follows:

UCSC Human Genome Browser Gateway at <http://genome.ucsc.edu/>

NCBI Single Nucleotide Polymorphism at <http://www.ncbi.nlm.nih.gov/projects/SNP/>

HapMap Genome Browser at

http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r2_B36/

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Titles and Legends for figures

Figure 1. Physical map of the *FTO* and *RPGRIP1L* loci.

The *FTO* and *RPGRIP1L* genes are located on the long arm of chromosome 16, share a CpG island with 51 CpG dinucleotides, and are transcribed in opposite directions. The obesity-associated SNPs are in strong LD and located within intron 1 of the *FTO* gene. The variants used in this work as well as the CUTL1 binding site identified by Stratigopoulos et al.⁹ are indicated.

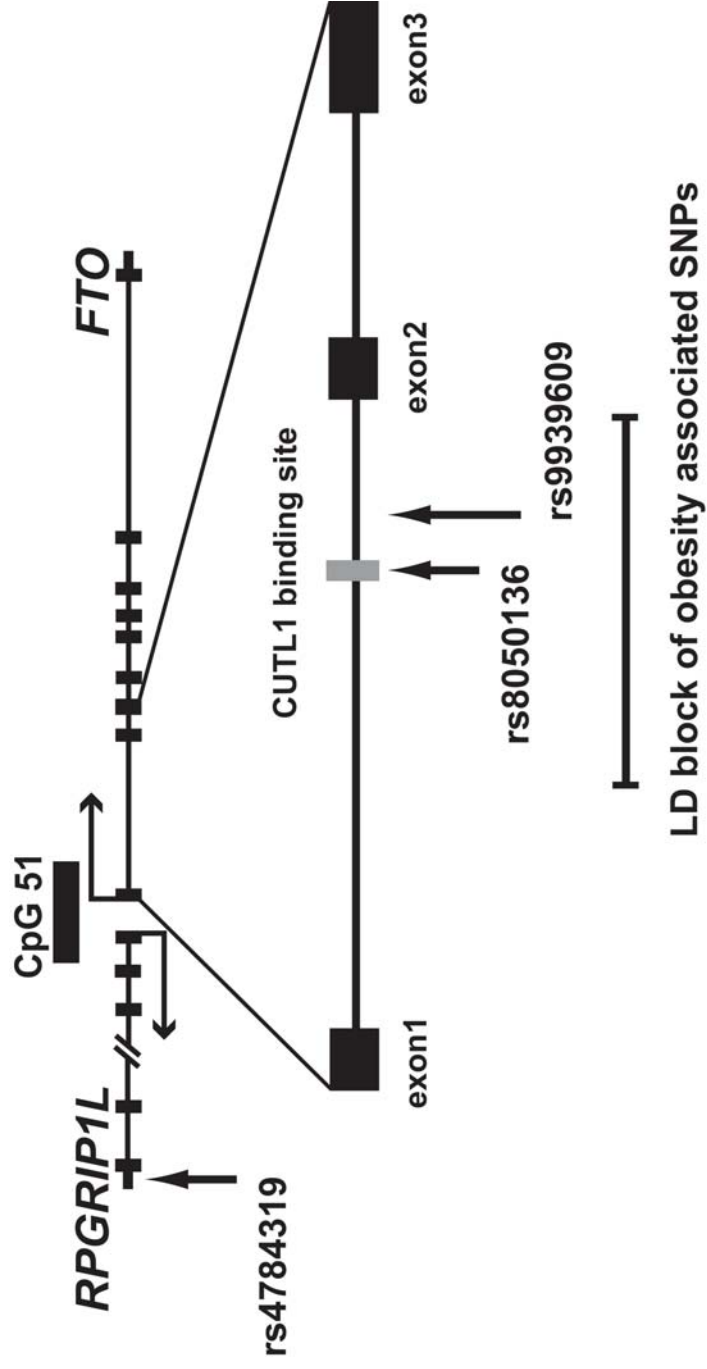
Figure 2. Allelic expression studies.

A) Allelic expression of the *FTO* gene in five individuals. The ratio of the two alleles of SNP rs9939609 is skewed in favour of the A (risk) allele in all individuals investigated. Results are displayed as diamonds, where the horizontal line represents the mean value and the top and bottom of the diamonds represent the 95% confidence interval.

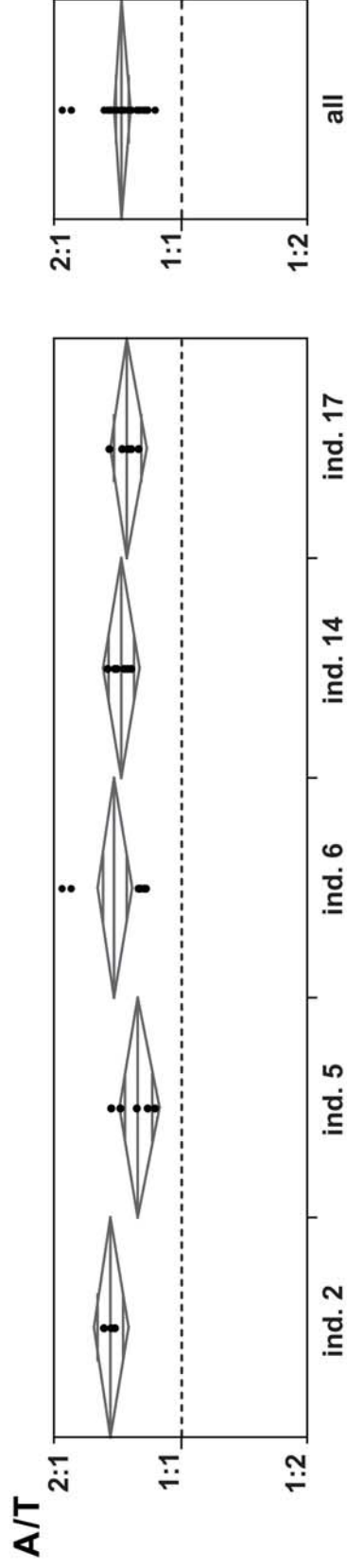
B) Allelic expression of the *RPGRIP1L* gene in six individuals with different *FTO* genotypes. In each individual, the ratio of the two alleles of SNP rs4784319 is skewed in favour of the T allele, independently of the *FTO* genotype.

Supplementary Figure 1. RT-PCR of hnRNA from blood.

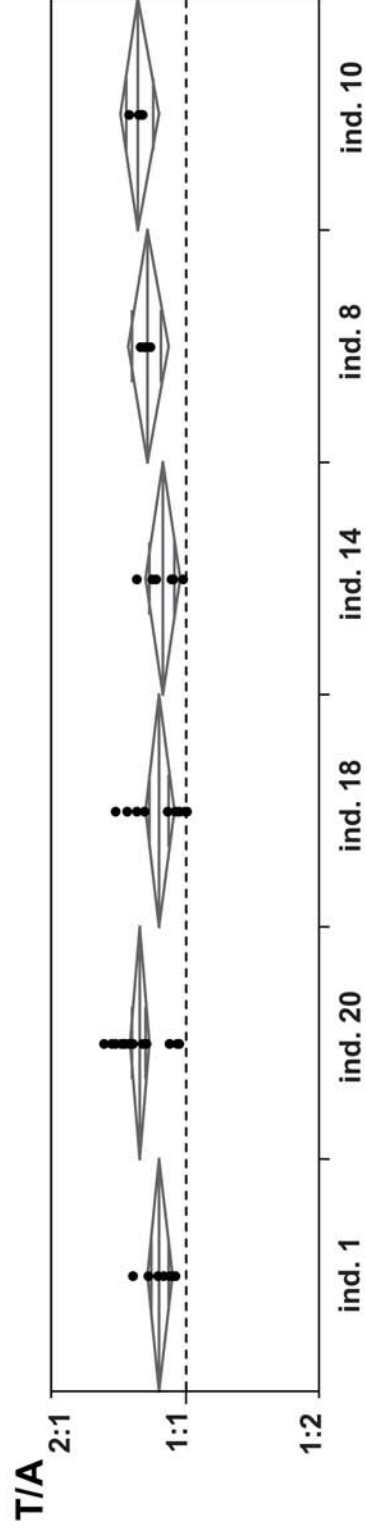
RT-PCR of hnRNA from lymphocytes of five individuals. The specific product is 256 bp. Even after 60 cycles of amplification, no product is observed in the –RT lanes, indicating absence of DNA contamination. M, size marker; RT, reverse transcriptase.



A. *FTO* (rs9939609)



B. *RPGRIP1L* (rs4784319)



FTO genotype
at rs9939609

AT

AT

AT

AT

AA

TT