ORIGINAL ARTICLE

TUB gene expression in hypothalamus and adipose tissue and its association with obesity in humans

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BACKGROUND/OBJECTIVES: Mutations in the Tubby gene (TUB) cause late-onset obesity and insulin resistance in mice and syndromic obesity in humans. Although TUB gene function has not yet been fully elucidated, studies in rodents indicate that TUB is involved in the hypothalamic pathways regulating food intake and adiposity. Aside from the function in central nervous system, TUB has also been implicated in energy metabolism in adipose tissue in rodents. We aimed to determine the expression and distribution patterns of TUB in man as well as its potential association with obesity.

SUBJECTS/METHODS: In situ hybridization was used to localize the hypothalamic regions and cells expressing TUB mRNA. Using RT–PCR, we determined the mRNA expression level of the two TUB gene alternative splicing isofoms, the short and the long transcript variants, in the hypothalami of 12 obese and 12 normal-weight subjects, and in biopsies from visceral (VAT) and subcutaneous (SAT) adipose tissues from 53 severely obese and 24 non-obese control subjects, and correlated TUB expression with parameters of obesity and metabolic health.

RESULTS: Expression of both TUB transcripts was detected in the hypothalamus, whereas only the short TUB isoform was found in both VAT and SAT. TUB mRNA was detected in several hypothalamic regions involved in body weight regulation, including the nucleus basalis of Meynert and the paraventricular, supraoptic and tuberomammillary nuclei. We found no difference in the hypothalamic TUB expression between obese and control groups, whereas the level of TUB mRNA was significantly lower in adipose tissue of obese subjects as compared to controls. Also, TUB expression was negatively correlated with indices of body weight and obesity in a fat-depot-specific manner.

CONCLUSIONS: Our results indicate high expression of TUB in the hypothalamus, especially in areas involved in body weight regulation, and the correlation between TUB expression in adipose tissue and obesity. These findings suggest a role for TUB in human obesity.

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INTRODUCTION

The TUB gene encodes the Tubby protein, which is found in a wide range of organisms across the animal and plant kingdom, suggesting an evolutionarily conserved biological function.1 TUB was initially discovered through positional cloning of a spontaneous mutation that led to obesity in a mice breeding colony at The Jackson Laboratories.2–5 Mice carrying this mutation (later designated as Tubby mice) have obesity, insulin resistance and neurosensory deficits.2 Compared with other murine models of obesity such as leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice, the obesity in Tubby mice is relatively mild, late in onset and associated with transient alterations in glucose homeostasis.2 The similarity of the Tubby phenotype with common obesity in the human population suggests that the TUB gene may be involved in the pathogenesis of obesity in humans as well.

Despite the TUB gene’s evident physiological relevance to obesity, its exact molecular function remains enigmatic. Based on the protein structure, it has been hypothesized that Tubby is a transcription factor,1 whereas functional data suggest that Tubby is an integrator of insulin and leptin signaling and/or of GPCR (G-protein-coupled receptor) trafficking.6–8 However, so far none of these studies have been completely conclusive. In rodents, TUB is abundantly expressed in several areas of the hypothalamus,9,10 a brain region critical in the regulation of appetite and energy expenditure by the central nervous system (CNS).11 Studies of Tubby mice have reported alterations in the hypothalamic pathways related to feeding behavior12–14 and the defects in
ciliary GPCR trafficking in the central neurons.15,16 From a young age, Tubby mice show reductions in daily food intake and physical activity, which in the end result in a net positive energy balance and weight gain over time, whereas older obese mice develop hyperphagia.17,18 There are also indications of abnormal carbohydrate metabolism in Tubby mice owing to defects in neuronal innervation of the liver14,18 resulting in increased fat deposition.18 Similarly, deletion of the Tubby orthologue tub-1 in Caenorhabditis elegans leads to fat accumulation.19 Taken together, these data indicate an evolutionarily conserved role for TUB in the regulation of energy metabolism. Besides its expression in the CNS, in rodents TUB is also known to be expressed in adipocytes, where it may be involved in controlling peripheral insulin-sensitivity.20 The extent to which the expression of TUB in adipose tissue contributes to the Tubby phenotype is, however, still unclear.

To date, only a few studies have been done in humans regarding TUB’s function and whether or not it might be involved in obesity. We have previously shown that common variation in the TUB gene is associated with body weight21,22 and macro-nutrient intake (that is, higher carbohydrate consumption),22 whereas others have reported the TUB mutation to cause retinal dystrophy and syndromic obesity.23 So far, the precise role of TUB in metabolic regulation in humans is still undefined.

In an attempt to better understand the role for TUB in energy homeostasis and disease in humans, we determined the expression and distribution patterns of TUB in the hypothalamus and in adipose tissue, two tissues that are highly relevant in the context of metabolic regulation and obesity. We also examined whether and how TUB expression correlates with several parameters of metabolic health and body weight in obese and healthy control individuals. Finally, we assessed TUB expression during adipogenesis in vitro and investigated whether TUB expression in human adipocytes can be modulated by metabolic hormones.

**MATERIALS AND METHODS**

Hypothalamic tissues from human subjects

Post-mortem hypothalamic material was obtained by autopsy. The first subset of formalin-fixed and paraffin-embedded hypothalamic samples from the subjects without neurological or psychiatric disease (n = 6) was used to assess TUB mRNA distribution using in situ hybridization. A second subset containing the frozen hypothalamic material of 12 pairs of obese (body mass index (BMI) > 30) and normal-weight control subjects (BMI < 25), matched for sex, age, clinical diagnosis and Braak stage of Alzheimer progression,24 was used to examine TUB mRNA expression levels. The samples were processed within a time range of 3:15–19:35 h of post-mortem delay. All brain samples were obtained from The Netherlands Brain Bank (NBB), Netherlands Institute for Neurosciences, Amsterdam (director Dr Inge Huizinga, open access: www.brainbank.nl). All material had been collected from donors for or from whom the NBB had obtained written informed consent for a brain autopsy and the use of the material and clinical information for research purposes.

**TUB in situ hybridization**

LNA probe. A locked nucleic acid (LNA) LNA-2′-O-methyl-RNA probe specific for the human TUB mRNA was designed. LNA-2′-O-methyl-RNA nucleic acid analogs were used because of their stability and high hybridization affinity, and because of their successful application in the human brain.25–27 An antisense probe was used (sequence: 5′-TtUmAaCmUaTtAaUmUAaGmCtTmGmGmAmGmG-GG-3′) that is complementary to bases 1035–1053 of the long isoform of the human TUB gene (RefSeq NM_003320.4) and to bases 778–796 of the short TUB isoform (RefSeq NM_177972.2), wherein ‘m’ = 2′-O-methyl-RNA and ‘l’ = LNA bases (Supplementary Figure 1). Probe specificity was assessed using a scrambled probe containing the same nucleotides but in random order (sequence: 5′-lGmCtTmUaUmAaGmAaTmGmGmGmGmUmAmAT-A-3′), and by testing a concentration gradient ranging from 25 nM to 500 nM. Probes were FAM tagged at the 5′-end and custom ordered (Ribotask, Denmark).

In situ hybridization. Hypothalami were dissected during autopsy and fixed in 10% (v/v) phosphate-buffered formalin at room temperature (RT). The dissection borders of the hypothalamus were as follows: frontal cut before the optic chiasm and behind the mammillary bodies, lateral cuts 1.5 cm from the midline, horizontal cut at the level of the anterior commissure. After dehydration in graded ethanol series, tissues were cleared in toluene and embedded in paraffin. Coronal serial sections (6 μm) were cut over the entire rostro-caudal axis. Each 100th section was collected, mounted on Superfrost Plus slides (Menzel Glaser, Braunschweig, Germany) and subsequently dried for 2 days at 37°C. The in situ hybridization procedure was performed as described in detail in Supplementary Text. In total, 120 s were included in the experiment. Ten in situ hybridization runs were performed, with 12 sections per run.

Gene expression analysis. Frozen human hypothalami were homogenized in liquid nitrogen. For further details see Supplementary Text. RNA quantity was measured on a NanoDrop. RNA metrics such as peak heights, peak areas and concentration are used to determine an RNA Quality Score (RQS) number for each sample using LabChip GX (Caliper, PerkinElmer, Waltham, MA, USA). The RQS has been validated to correlate well with Agilent’s RIN (RNA Integrity Number) and follows the same 0–10 scale rating (with 10 being the highest). TUB expression was normalized to the 36B4 gene (also known as RPLP0), which was selected from our test of five different housekeeping genes previously reported to be the most stable in human CNS post-mortem tissue.28 Relative expression was calculated using the Δ[ΔCt] method.29

Primers. mRNA reference sequences were obtained from the NCBI (www.ncbi.nlm.nih.gov), and the mRNA levels for the long and short isoforms of the TUB gene were determined using a primer set designed by Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi): the forward primers were 5′-CACGAGAAGTACCTGAAGG-3′ for the long isoform and 5′-ATGACTTCAAGCCCGCATT-3′ for the short isoform; the reverse primer was 5′-TTCTGGTGCCGTACGGTCT-3′ for both isoforms.

Adipose tissue material

Control samples. The control samples included in this study were originally obtained from 24 Caucasian women who had undergone surgery owing to benign gynecological problems.30 The subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) biopsies were taken by means of scissors at the place of the incision, midline lower abdomen above the symphysis and under the umbilicus (VAT) and at the lower edge of the omentum (SAT). The AT samples were snap-frozen in liquid nitrogen and stored at −80°C. For this study, the collection of SAT (n = 18) and VAT (n = 21) biopsies was used (for 15 subjects both SAT and VAT tissue was available). Research subjects were in good health with no history or symptoms of type 2 diabetes or inflammatory diseases. Subjects were between 30 and 45 years of age, with BMI ranging from 23 to 29 kg m⁻².

Samples from obese individuals. The 53 severely obese individuals (BMI ranging from 30 to 74 kg m⁻²) included in this study had undergone elective bariatric surgery at the Department of General Surgery, Maastricht University Medical Centre.31 We excluded samples taken from subjects with acute or chronic inflammatory diseases, or degenerative diseases, and those reporting an alcohol intake exceeding 10 g per day or the use of anti-inflammatory drugs. The sampling of VAT (n = 46) and SAT (n = 41) tissues (n = 34 with both VAT and SAT) obtained during bariatric surgery has been described before.31 In short, venous blood samples were obtained on the morning of surgery, whereas wedge biopsies of VAT (omentum majus) and SAT (abdominal) were taken during surgery. In both controls and obese patients, fasting blood samples were collected for biochemical measurements.

Gene expression analysis. RNA was isolated using the Qiagen Lipid Tissue Mini Kit (#74804, Qiagen). RNA concentration was determined with NanoDrop. cDNA synthesis was performed from total RNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. 20 ng cDNA was used for subsequent RT–PCR analysis using SYBR Green (Biorad, Veenendaal, The Netherlands) and 7900HT real-time PCR System (ThermoFisher, Etten-Leur, The Netherlands). Data were analyzed with SDS 2.0 software (Applied Biosystems). TUB mRNA levels were expressed relative to those of the beta-2 microglobulin housekeeping gene (B2M) and the 18S ribosomal RNA (18S rRNA) to correct for differences in RNA loading using the ΔΔCT method.30
gene (B2M), as the geNorm VBA applet for Microsoft Excel determined B2M to be more stable than other common housekeeping genes.

ASC experiments
ASC isolation, culture and differentiation. Human subcutaneous liposaprate samples were obtained from healthy human subjects (BMI < 30, non-diabetic) during liposuction surgery (Bergman Clinics, Heerenveen and Zwolle, The Netherlands). The details on adipose-derived stromal cells (ASCs) isolation, culture and differentiation are provided in Supplementary Text.

Gene expression analysis. Total RNA was isolated using Trizol according to the manufacturer's protocol. cDNA was synthesized and RT–PCR was performed as described for the hypothalami. Gene expression levels were normalized to 36B4.

All participants provided written informed consent and all procedures were performed in accordance with national and institutional guidelines and with the ethical guidelines of the Declaration of Helsinki.

Statistical analysis
Continuous variable data were presented as mean ± s.e. unless stated otherwise. Spearman's rho test was used to examine correlations. Expression differences between the groups were statistically evaluated by the nonparametric Mann–Whitney U-test or by the paired Student's t-test (between SAT and VAT). Graphs were computed with GraphPad Prism software for Windows, Version 5. Statistical analyses were conducted with SPSS (version 22, SPSS corporation, Chicago, IL, USA). Tests were two-tailed. The level of nominal significance was set at \( P < 0.05 \).

RESULTS
Distribution of TUB mRNA in the human hypothalamus
The expression pattern of TUB in the human hypothalamus was determined by in situ hybridization using an LNA-2'-O-methyl-RNA probe detecting both the short and the long transcripts of TUB. Probe specificity was confirmed by the absence of staining with the control-scrambled probe (Figure 1). To localize regions and cells expressing TUB mRNA, consecutive sections of six hypothalami (three men, three women) were analyzed (for subject characteristics see Supplementary Table 1). Although considerable inter-individual differences were observed in TUB mRNA staining intensity, the staining distribution in the hypothalamus was similar among individuals. No obvious effects of sex, age, fixation time (up to 130 days) and post-mortem delay (up to 19:30 h) were observed (data not shown). The distribution of TUB mRNA expression in the hypothalamus was widespread with strongest staining in the NBM (nucleus basalis of Meynert), neurons of the PVN (paraventricular nucleus), SON (supraoptic nucleus) and TMN (tuberomammillary nucleus) and less-intense staining in the IFN (infundibular nucleus) (Figure 1, Supplementary Figure 2).

Hypothalamic expression of TUB in obese individuals and controls
We were not able to distinguish between the expression patterns of the long and the short TUB isoforms using the in situ hybridization approach. The expression of the two individual TUB isoforms was therefore determined by RT–PCR using hypothalami from 12 obese subjects and 12 controls (Supplementary Tables 1 and 2). Both TUB transcripts were

Figure 1. Representative images of TUB mRNA expression in human hypothalamus. Tuberomammillary nucleus (TMN) neurons show intense staining, whereas there is no specific staining with the control-scrambled probe. Staining is also visible in neurons in the infundibular nucleus (IFN), nucleus basalis of Meynert (NBM), paraventricular nucleus (PVN), supraoptic nucleus (SON) and suprachiasmatic nucleus (SCN). Samples derived from male (patient #97157). Scale bar = 25 μm.
expressed in the hypothalamus, whereby the levels of the short TUB isoform were markedly higher than those of the long TUB isoform (Figure 2a). In addition, the expression levels of both isoforms were highly correlated with each other ($r = 0.58$, $P = 0.003$).

Because central defects in TUB function have been associated with obesity in mice, we next examined the potential association between human TUB mRNA levels in the hypothalamus and obesity. For both TUB isoforms, however, there were no significant differences in expression between obese and control subjects (Figures 2b and c). As the quality of RNA taken from post-mortem material may influence the gene expression profile, we assessed whether the RQS values were affected by confounding factors. No differences in the mean RQS values between obese individuals (6.37 ± 0.24) and controls (6.48 ± 0.24) were found, indicating that the quality of the RNA was similar in both groups (Supplementary Figure 3).

**TUB expression in adipose tissue**

Because it is unknown whether TUB is expressed in human adipose tissue, we assessed TUB mRNA levels in VAT and SAT in two study (clinical characteristics in Table 1). In both fat depots, only the short isoform of TUB was detected. In paired samples of VAT and SAT taken from 49 subjects (15 controls and 34 severely obese), we first looked at differences in TUB gene expression between the two fat depots and observed higher TUB mRNA levels in SAT than in VAT in both controls ($n = 15$ paired samples) and obese individuals ($n = 34$ paired samples) as well as in the pooled sample of both groups (mean ± s.e.: SAT: 0.72 ± 0.07 vs VAT: 0.51 ± 0.08, $P = 0.018$) (Figure 3). To examine a potential effect of gender on gene expression, we compared TUB mRNA levels between men and women (in obese group) and found no difference in either fat depot (data not shown). Next, in both SAT and VAT, we found a clear indication of lower TUB expression in obese subjects relative to controls ($P < 0.05$) (Figure 4). This difference was still observed, when the control group was compared to the sex-matched obese group of women only (Supplementary Figure 4).

In control subjects, we further observed negative correlations between VAT TUB mRNA and weight ($r = -0.64$, $P = 0.002$), BMI ($r = -0.56$, $P = 0.009$), waist circumference ($r = -0.66$, $P = 0.002$) and hip circumference ($r = -0.54$, $P = 0.017$) (Table 2, Supplementary Figure 5). Similar correlations were found for SAT TUB mRNA and the same anthropometric characteristics but these were not as strong. In contrast, in obese subjects we detected correlation between BMI and SAT TUB mRNA ($r = -0.44$, $P = 0.004$), but not VAT TUB mRNA ($r = -0.15$, $P = 0.32$). The analysis also revealed significant relationships between TUB expression and different metabolic traits in severely obese individuals, but not in controls (Table 2): levels of SAT TUB mRNA were negatively correlated with insulin levels ($r = -0.31$, $P = 0.047$) and positively correlated with high-density lipoprotein (HDL) cholesterol ($r = 0.48$, $P = 0.002$). We also observed opposite trends in the direction of the correlations between TUB mRNA in VAT and blood glucose in severely obese patients ($r = -0.30$, $P = 0.046$) and controls ($r = 0.16$, $P = 0.50$) (Table 2). The sex-stratified analysis revealed that the observed relationships between TUB expression in VAT and blood glucose and TUB expression in SAT and HDL cholesterol were driven by the stronger correlations in women ($r = -0.36$, $P = 0.035$ and $r = 0.44$, $P = 0.012$, respectively) as well as identified a new correlation between TUB mRNA level and LDL cholesterol in men ($r = 0.81$, $P = 0.014$) (Supplementary Table 3).

**Expression of TUB in ASCs**

To establish whether the expression of TUB in human adipocytes is regulated by adiposity and/or adipose development, ASCs were cultured in vitro and induced to differentiate into adipocytes. The expression of both TUB isoforms was determined, as well as TUB

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**Table 1. Clinical characteristics of study population used to study TUB gene expression in adipose tissue**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls ($N = 24$)</th>
<th>Severely obese individuals ($N = 53$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N$</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24</td>
<td>45.2 ± 10.8</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>0/24</td>
<td>–/12/41</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>24</td>
<td>74.1 ± 11.2</td>
</tr>
<tr>
<td>BMI (kg m$^{-2}$)</td>
<td>24</td>
<td>25.5 ± 3.3</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>21</td>
<td>85.1 ± 9.6</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>22</td>
<td>103.1 ± 8.5</td>
</tr>
<tr>
<td>Glucose (mmol l$^{-1}$)</td>
<td>23</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td>Insulin (pg ml$^{-1}$)</td>
<td>23</td>
<td>304.5 ± 129.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>–/ –</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol l$^{-1}$)</td>
<td>23</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>HDL cholesterol (mmol l$^{-1}$)</td>
<td>23</td>
<td>0.9 ± 1.7</td>
</tr>
<tr>
<td>LDL cholesterol (mmol l$^{-1}$)</td>
<td>23</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

Abbreviations: HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TUB, Tubby gene.
Figure 3. Measurement of TUB expression in two different adipose tissue depots. Expression of the TUB short isoform in paired samples from visceral (VAT) and subcutaneous (SAT) adipose tissue: (a) In controls. Mean ± s.e. for VAT: 0.72 ± 0.18; for SAT: 0.96 ± 0.16; P = 0.20 (n = 15). (b) In obese individuals. Mean ± s.e. for VAT: 0.42 ± 0.08; for SAT: 0.62 ± 0.06; P = 0.05 (n = 34). (c) In pooled sample of controls and obese individuals (n = 49). Mean ± s.e. for VAT: 0.51 ± 0.08; for SAT: 0.72 ± 0.07; P = 0.018.

Figure 4. TUB expression in adipose tissue depots of controls and obese individuals. (a) Expression of the TUB short isoform in subcutaneous adipose tissue (SAT). Mean ± s.e. for controls (n = 18): 0.99 ± 0.16; for obese individuals (n = 41): 0.64 ± 0.06; P = 0.0068. (b) Expression of TUB in visceral adipose tissue (VAT). Mean ± s.e. for controls (n = 21): 1.10 ± 0.28; for obese individuals (n = 46): 0.45 ± 0.07; P = 0.01. Data plotted as mean ± s.e.

expression over time during differentiation. To assess the putative effects of prolonged culture time on gene expression patterns, undifferentiated cells were cultured alongside the differentiated cells. Differentiation was assessed by visual assessment of lipid droplets and quantification by assessing the expression of the adipogenesis marker adipocyte protein 2 (aP2, FABP4) (Supplementary Figures 6A and B). In line with the findings for TUB expression in primary adipose tissue, ASCs (both differentiated and undifferentiated) only expressed the short variant of TUB (Supplementary Figure 7A). However, neither differentiation of ASCs into mature adipocytes nor treatment with the metabolic hormones insulin and triiodothyronine (T3) had an effect on the expression levels of TUB in undifferentiated or differentiated ASCs (Supplementary Figures 7B–D).

DISCUSSION

Defects in TUB gene function are known to cause metabolic disturbances and the development of obesity in mice, yet the relevance of TUB in human obesity is unclear. The results of this study demonstrate that high levels of TUB are expressed in the hypothalamus, especially in areas involved in body weight regulation. We also show that TUB expression correlates negatively with indices of body weight and obesity in a fat-depot-specific manner. However, TUB expression in human adipocytes does not appear to be modulated by metabolic hormones such as insulin. These findings are discussed in more detail below.

TUB expression in the hypothalamus

Most of available knowledge on the mechanisms underlying the phenotype of the Tubby mouse points toward aberration in the control of body weight by the CNS. In rodents, TUB is abundantly expressed in several areas of the hypothalamus, particularly in the arcuate, PVN and ventromedial nuclei—regions that are involved in the regulation of satiety and appetite. We observed similar distribution pattern of TUB expression in the human hypothalamus. The highest levels of TUB mRNA were detected in the neurons of the IFN—the human orthologue of the rodent arcuate—and the PVN. Both the IFN/arcuate and the PVN are hypothalamic regions necessary for the integration of homeostatic signals that regulate energy balance. We also detected expression of TUB in the suprachiasmatic nucleus and TMN, two nuclei involved in the circadian control of energy metabolism in humans; in the NBM, which is important for memory; and the SON, which regulates water reabsorption. These expression patterns for TUB are similar to those reported in the human hypothalamus for three major regulators of energy homeostasis, namely neuropeptide Y, agouti-related protein and the melanocortin-4 receptor. Our observations are also in line with the role of Tubby in GPCR trafficking in the neuronal cilia, a cellular compartment widely present in hypothalamic neurons and involved in energy homeostasis. Altogether, these data support the hypothesis that TUB is involved in the central regulation of energy metabolism. The expression of TUB in NBM, SON, suprachiasmatic nucleus and TMN, which has not been described for the mouse orthologues of these nuclei, suggests species-specific differences in hypothalamic TUB localization between mice and humans.

We did not find evidence for an association between TUB expression levels in the hypothalamus and obesity. This could be due to the relatively small sample size, caused by the limited availability of high-quality frozen brain material from obese individuals and well-matched controls. Also, since we used whole hypothalami to assess gene expression levels, spatial differences in expression could not be discerned. Finally, it is known that obesity is a complex condition with a multifactorial background.
Whereas mutations in TUB are known to cause syndromic obesity in humans, moderate alterations in TUB function are not necessarily a common feature of obesity. This notion is supported by the observation that in three different mouse models of obesity the expression of TUB does not significantly differ from that in normal-weight controls. Further studies are needed to determine whether hypothalamic TUB contributes to the development of obesity in a subpopulation of patients and to what extent.

**TUB expression in adipose tissue**

In mice, TUB is expressed in a variety of insulin-sensitive peripheral tissues, which suggests that the Tubby protein, besides its role in the CNS may also have a peripheral function in energy homeostasis. We chose to analyze TUB expression in adipose tissue as it is the main depot for energy storage, and because it has an important role in obesity pathophysiology. Although both TUB splice variants were expressed in the hypothalamus, only the short isoform of TUB was found in adipose tissue, suggesting that TUB expression might be regulated in a tissue-specific manner. Previous studies identified that the two Tubby isoforms are similar in their COOH-terminal DNA binding domain, while are distinct owing to the alternatively spliced NH2-terminal regions that induce transcription activation. Boggon et al. identified further that such alternative splicing does not affect the overall character of Tubby protein but results in the difference to activate transcription, a common mechanism of control in transcriptional modulators. Together with these data, our findings indicate differential regulation of transcription by the different TUB isoforms in CNS and in adipose tissue.

We also observed fat-depot-specific differences in TUB expression, with TUB being expressed at higher levels in SAT than in VAT. A similar expression pattern in adipose tissues has also been reported for the FTO gene, a gene strongly implicated in human obesity, suggesting similarity in fat-depot-specific properties between the genes. Notably, we found a negative correlation between TUB mRNA levels in adipose tissue and BMI in both obese and non-obese individuals. However, in control subjects the levels of TUB mRNA in VAT correlated the strongest with parameters of body weight, whereas in obese individuals a stronger relationship was found in SAT. More detailed analysis revealed a significant reduction in TUB expression in the VAT of severely obese individuals relative to controls. This is of particular interest as excess VAT has been linked to a higher risk of metabolic derangements.

Next, in severely obese patients we found significant and directionally consistent correlations between TUB mRNA levels and blood glucose and lipid-related traits. Our data also suggest gender-specific pattern in correlations between TUB expression and metabolic profile of adipose tissue depots. This may indicate a peripheral role for TUB in the regulation of metabolic homeostasis. It should be noted, however, that from the present study design it is not possible to determine whether the detected relationships contribute to the causes of these metabolic changes or whether they represent the obesity-driven consequences. Taking into account our observations on the TUB expression in adipose tissue and previously reported associations between the TUB common variants and increased risk of obesity, as well as the obesity phenotypes linked to the loss-of-function mutation in Tubby mice or in C. elegans, we hypothesize the following: that metabolic aspects of obesity can be affected by peripheral alterations in the function of TUB.

Our experiments in human ASC-derived adipocytes showed that TUB mRNA levels remain stable during differentiation. These results suggest that adipocyte development is not a driver of TUB expression in humans, whereas Stretton et al. reported a differentiation-linked reduction in TUB expression in murine 3T3-L1 adipocytes. Next, previous studies in rodent adipocytes and neuronal cells have reported on the regulation of TUB expression by insulin and T3, two important hormones in the regulation of metabolism, but we were unable to reproduce these results in human ASC-derived adipocytes. This discrepancy reflects the complex aetiology of obesity, and that there are distinctive differences between humans and rodents, as shown by similar functional studies. Also, we cannot exclude the possibility that a decrease in TUB expression increases adiposity, because of the negative correlation between TUB mRNA levels and body weight parameters observed in our study.

This work has several limitations. First, in this expression study we used whole adipose tissue that is known to consist of multiple cell types such as mature and pre-adipocytes, endothelial cells, fibroblasts and a range of immune cells. Next, owing to limitations in the availability of both adipose and hypothalamic tissues we were not able to determine the levels of the Tubby protein in the same sample sets. Therefore, we used a proteomics approach to validate the TUB expression in human adipocytes. In the available data of the human adipocyte proteome obtained using a combination of different proteomics techniques Tubby protein was not identified. This may be owing to a low abundance of the protein and/or insufficient sensitivity of the used mass.

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**Table 2. Correlations between clinical parameters and TUB gene expression in visceral and subcutaneous adipose tissues**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Visceral AT</th>
<th>Controls</th>
<th>Subcutaneous AT</th>
<th>Severe obese individuals</th>
<th>n</th>
<th>r_s</th>
<th>P-value</th>
<th>n</th>
<th>r_s</th>
<th>P-value</th>
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<th>P-value</th>
</tr>
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<tbody>
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<td>Weight</td>
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<td>0.002</td>
<td>21</td>
<td>-0.42</td>
<td>0.08</td>
<td></td>
<td>46</td>
<td>-0.15</td>
<td>0.32</td>
<td>41</td>
<td>-0.44</td>
<td>0.004</td>
</tr>
<tr>
<td>BMI</td>
<td>21</td>
<td>-0.56</td>
<td>0.009</td>
<td>18</td>
<td>-0.37</td>
<td>0.13</td>
<td></td>
<td>46</td>
<td>-0.15</td>
<td>0.32</td>
<td>41</td>
<td>-0.44</td>
<td>0.004</td>
</tr>
<tr>
<td>Waist</td>
<td>19</td>
<td>-0.66</td>
<td>0.002</td>
<td>16</td>
<td>-0.48</td>
<td>0.06</td>
<td></td>
<td>46</td>
<td>-0.30</td>
<td>0.046</td>
<td>41</td>
<td>-0.08</td>
<td>0.64</td>
</tr>
<tr>
<td>Hip</td>
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<td>-0.54</td>
<td>0.017</td>
<td>17</td>
<td>-0.38</td>
<td>0.13</td>
<td></td>
<td>46</td>
<td>-0.16</td>
<td>0.29</td>
<td>41</td>
<td>-0.31</td>
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<tr>
<td>Glucose</td>
<td>20</td>
<td>-0.26</td>
<td>0.27</td>
<td>17</td>
<td>0.09</td>
<td>0.72</td>
<td></td>
<td>45</td>
<td>-0.26</td>
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<td>40</td>
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<tr>
<td>Insulin</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>HbA1c</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total cholesterol</td>
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<td>0.20</td>
<td>0.40</td>
<td>17</td>
<td>0.06</td>
<td>0.83</td>
<td></td>
<td>43</td>
<td>0.08</td>
<td>0.62</td>
<td>39</td>
<td>0.16</td>
<td>0.34</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>20</td>
<td>0.11</td>
<td>0.65</td>
<td>17</td>
<td>-0.01</td>
<td>0.96</td>
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<td>44</td>
<td>0.28</td>
<td>0.07</td>
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<td>0.48</td>
<td>0.002</td>
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<tr>
<td>LDL cholesterol</td>
<td>20</td>
<td>0.06</td>
<td>0.79</td>
<td>17</td>
<td>0.00</td>
<td>0.99</td>
<td></td>
<td>44</td>
<td>0.19</td>
<td>0.21</td>
<td>40</td>
<td>0.11</td>
<td>0.48</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>20</td>
<td>-0.31</td>
<td>0.19</td>
<td>17</td>
<td>-0.23</td>
<td>0.37</td>
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<td>44</td>
<td>-0.28</td>
<td>0.07</td>
<td>40</td>
<td>-0.26</td>
<td>0.11</td>
</tr>
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</table>

Abbreviations: AT, adipose tissue; BMI, body mass index; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TUB, Tubby gene, rs, Spearman regression coefficient. P-values < 0.05 are indicated in bold.
spectrum analyses/sample processing to detect Tubby. Further research investigating cell-specific expression of TUB and its protein expression are needed to determine the exact role of TUB in adipose and hypothalamic tissue metabolism and function.

CONCLUSIONS

Together, our findings are consistent with a role for TUB in energy metabolism, and support the involvement of TUB in obesity in humans, thereby providing insights into the enigmatic function of the Tubby protein. Further molecular studies should reveal how TUB affects body weight regulation, which will help to improve preventive therapies and aid in the development of new drugs and in the identification of subgroups of patients with increased risk for obesity, and help to improve preventive therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

JVJMN conceived and designed the experiments. VJMM, JVVO performed the experiments. JVJVO, VJMN, MGMW, TPvdM, UU, DFS analyzed the data. DFS, BHRW, JWJ critically revised the article for important intellectual content. SSR, ES, DFS, BHRW, JWJ, MGMW, TPvdM, UU, DFS, BHRW, JWJ conceived and designed the experiments. VJMN, JVvVO wrote the paper.

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