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Comparative analysis of glucoprotein profiles of SH-SY5Y cells stably expressing the wild type fat mass and obesity associated proteins

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Keywords

FTO (Fat mass and obesity associated), glycoproteome, SH-SY5Y cell line

ABSTRACT • Background and Aims: Genome wide association studies have shown that fat mass and obesity associated protein, initially associated with obesity, is associated with metabolic diseases, cancer, brain diseases and the cardiovascular system. Fat mass and obesity associated protein is a nuclear protein belonging to the oxidative DNA/RNA demethylase superfamily associated with AlkB-related non-Fe (II)/2-oxoglutarate. *In vitro*, in the presence of Fe^{2+} and 2-oxoglutarate, fat mass and obesity associated protein has been shown to be catalyze demethylation of 3-meT from a single-stranded DNA, 3-meU from a single-stranded RNA and 6-meA from both DNA and RNA. These *in vitro* studies suggest that the physiological role of fat mass and obesity associated protein may be related to nucleic acid repair or modification. However, the exact physiological function of this protein is still unknown. In this study, changes in the level of glucoproteomes after exogenous expression of the wild type wild type fat mass and obesity associated protein were studied. **Materials and Methods:** Protein extracts prepared from SH-SY5Y cells exogenously expressing and non-expressing the wild type fat mass and obesity associated protein were subjected to 2D gel electrophoresis. Gels were first stained with a glycosylated protein-specific dye, ProQ-Emerald-300, and then with a total protein stain SYPRO Ruby. **Results:** After comparative protein spots analysis, there were no changes in the patterns of glycoproteins expressed by the wild type fat mass and obesity associated protein harboring cells. **Conclusion:** The expression of wild type fat mass and obesity associated protein was not a significant effect on the expression patterns of glycoproteins in SH-SY5Y cells.

INTRODUCTION

Fat mass and obesity-associated (FTO) protein is a nuclear protein of the AlkB related non-heme Fe (II)/2-OG-dependent oxidative DNA/RNA demethylases superfamily but the exact physiological function of this gene is not known. Recombinant FTO protein was first discovered to catalyze demethylation of 3-methylthymine in single-stranded DNA, and 3-methyluridine in single-stranded RNA, with low efficiency (1,2). The nucleoside N6-methyladenosine, an abundant modification in RNA, was then found to be a major substrate of FTO (3,4). The internal 6-methyladenin (6mA) modification in mRNA, which it also exists in other RNA species such as tRNA (5) and rRNA (6), was demonstrated to be a dynamic mark (3,4,7,8). These *in vitro* studies suggest that the physiological role of FTO

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may be related to nucleic acid repair or modification (9,10). However, the role of FTO may not be limited to DNA repair or modification.

Initial immunofluorescense studies appeared to show that FTO was localized to the nucleus with no signal detected in the cytoplasm (1,11). Subsequently, FTO was shown to co-localize with nuclear speckles, supporting that 6mA in nuclear RNA could be a relevant substrate for FTO (3). Afterwards, Gulati et al (2013) was able to detect a substantial proportion of FTO residing in the cytoplasmic fraction (12). In a later study, FTO was found to be present in both the nucleus and cytoplasm, with a mobile fraction that shuttles between both cellular compartments, possibly by interaction with exportin 2 (XPO2) (13).

The genome-wide association studies revealed the relationship between SNPs (single nucleotide polymorphisms) of the first intron of the FTO (fat mass and obesity-associated) gene and obesity (14-16). The variant most strongly associated with obesity is found in intron 1 of the FTO gene. In contrast, loss-of-function mutation (R316Q) in the FTO gene, disruption of FTO's demethylase activity, is responsible for a recessive lethal syndrome that includes postnatal growth retardation, microcephaly, psychomotor delay, brain deficits and cardiac defects (17). Caglayan et al (2016) described a patient born of consanguineous union who presented with microcephaly, developmental delay, behavioral abnormalities, dysmorphic facial features, hypotonia, and other various phenotypic abnormalities that carrying a novel homoygous missense mutation in FTO (His271Pro) and concomitant nonsense mutation in cholesterol ester transfer protein (CETP) that is resulting in an early protein truncation. (18).

Initial studies on FTO have focused on the relationship between diabetes, obesity and metabolism (19). Studies in mice and humans have shown that the single nucleotide polymorphisms (SNPs) of FTO gene have been shown to be associated with metabolic diseases (obesity, type 2 diabetes and polycystic ovary), cancers (melanoma, breast, endometrial, pancreatic, prostate, colorectal, lung and kidney), brain diseases (brain volume deficiency and Alzheimer's disease) and the cardiovascular system (20).

Although the involvement of FTO in various diseases have been firmly established there is still a huge amount of space to improve our understanding of molecular functions of FTO in cells. The information regarding the physiological function of FTO mainly comes from activity studies in which demethylase activity of FTO was demonstrated. However those findings do not shed onto the possible effects of FTO's function on cellular interactome, proteome and transcriptome. One of the missing points to comprehensively study is the effect of FTO's action on post-translational modifications (PTMs). In this communication, we focused our attention to protein glycosylation. We asked the question of whether expressed FTO would affect protein glycosylation in neuroblastoma cells. To answer this question, we used a 2D-based glycoproetome analysis approach. Our findings indicated that FTO did not have a pronounced effect on glycoproteome.

MATERIALS and METHODS

Isolation of RNA from blood and cDNA synthesis

Total RNA was isolated from fresh blood samples according to the instructions provided by the manufacturer (Qiagen, USA) (Code of Ethics Approval: KOU KAEK 2013/25). The quality of each RNA sample was assessed using formaldehyde-agarose gels. The cDNA synthesis reaction was carried out immediately after RNA isolation and the reaction mix consisted of 5 µg of RNA, 1 mM of dNTPs, 0.2 µg of random hexamer primers, 20 units of riboblock inhibitor and 200 units of M-MuLV RT enzyme (Fermentas, USA). An initial 1 min denaturation at 90°C was applied to the RNA, primer, and dNTP mixture, and then the temperature was lowered to 42°C before the addition of M-MuLV RT enzyme plus riboblock inhibitor. The reaction was then allowed to run for 3 h at 42°C.

Amplification of wild type FTO gene by polymerase chain reaction

For the second strand synthesis, polymerase chain reaction (PCR) was performed with a long PCR enzyme mix (Fermentas, USA). The sense and antisense primers With cutting sites at the ends were 5′- AACTTAAGCTTATGAAGCGCACCCCCGACTG and 5′- CTCGAGCTCGAGCTAGGGTTTTGCTTCCAGAAGCTG, respectively. A 25 µL PCR reaction mixture consisted of 1× long PCR buffer, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1.25 mM $MgCl_2$, 1.5 units of long PCR enzyme mix, 2 µL of first strand cDNA reaction (ThermoScientific, USA). An initial 5 min denaturation at 94°C was followed by 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 57°C and 60 s elongation at 72°C. PCR reactions were ended with 10 min final elongation at 72°C (TC-3000X Thermal Cycler (Techne, ABD). PCR products were analyzed by agarose gel electrophoresis, cleaned with a PCR purification kit (Qiagen, USA) and sequenced (Iontek Inc., Istanbul, Turkey).

Creation of plasmid constructs

The PCR product was digested with HindIII and XhoI and ligated into the vectors using routine molecular biology techniques (21). pCDNA4/TO (Invitrogen, USA) was used for stable tetracycline-inducible FTO expression in SH-SY5Y (Invitrogen, USA). Full-length cDNA of human wild type (WT) FTO was cloned into pcDNA4/TO (Life Tech, USA) after reverse transcription. The constructs were sequenced and in-frame FTO sequences were verified (Iontek, Istanbul).

Cell culture and creation of stable cell lines expressing the WT FTO protein

SH-SY5Y cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (Vol/Vol) tetracycline-reduced fetal bovine serum, 100 U/ml penicillin-streptomycin and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. The Tet-R + stabile SH-SY5Y cell line was obtained in our previous study (22) and used in subsequent experiments. Transfection and stable selection of SH-SY5Y cells were performed according to the instructions recommendations (Invitrogen, USA). SH-SY5Y-TetR+ cells were transfected with pcDNA4/TO harboring the WT FTO to create an inducible stable cell line expressing WT FTO protein using the electrotransfection method. Transfected cells were then subjected to a selection in the medium containing 50 µg/ml zeocin. Individual clones were isolated, grown and examined for FTO protein expression by western blotting with an anti-FTO antibody (Santa Cruz, USA).

Preparation of protein extracts

The cells were washed three times with ice-cold phosphate buffer saline (PBS) and homogenized in DIGE lysis buffer (30 mM tris, 7 M urea, 2 M thiourea, 5 mM magnesium acetate, 4% (w/v) CHAPS pH 8.5), by using 0.1 mm glass beads in a mechanical disruption device (Bullet Blender; Next Advance, USA). Homogenates were centrifuged at 15.000×g for 30 min at 4°C to remove cell debris. Protein concentrations were determined by modified Bradford assay (23) and protein extracts were aliquoted, snap-frozen in liquid nitrogen and stored at –80°C.

Western blot analysis

Western blot (WB) analysis was performed using anti-FTO [Clone C-3 (sc-271713), Santa Cruz, USA], and anti-Beta actin (Santa Cruz, USA; ACTBD, sc-81178) antibodies as described by Guzel et al (10).

2DE-based glycoproteomics experiments

In brief, approximately 250 µg of protein was loaded onto an immobilized pH gradient strips (11 cm, pH 3–10, nonlinear) (BioRad, USA) via passive rehydration at 22°C for 16 hr. The strip was run through a stepwise incremental voltage program [250V for 20 min (linear), 10.000 V for 2 hr (linear) and 30.000 V/hr (rapid)] by using Protean IEF system (BioRad,

USA). The strip was then subjected to a two-step equilibration in buffers containing 6 M urea, 2% sodium dodecyl sulphate (SDS), 0.375 M Tris. Cl pH 8.8, 20% glycerol and 2% dithiothreitol (DTT) for the first step and the same buffer without DTT but with iodoacetamide (2.5%) for the second step. The strip was then subjected to second dimension separation using SDS-PAGE (12%). CandyCane™ glycoprotein (Invitrogen, ABD) was used as a molecular weight standard in glycoproteomics studies.

Staining of glycoproteins with Pro-Q Emerald-300

The WT-FTO-expressing SH-SY5Y cells were under culture conditions. When cell density reached about 75%, tetracycline (Tet, 1 µg/ml) was added to the cell culture for expression of WT FTO, induction was performed for 24 hours. Before harvesting the cells, a three-time wash was performed to remove media components. The glycosylated proteins were stained with according to the Pro-Q Emerald 300 following the manufacturer's recommendation. In brief, gels were fixed 60 minutes in 50% (v/v) methanol, 5% (v/v) acid. Following wash steps with two changes of 3% acetic acid for 15 min, gels were oxidized with oxidizing reagent (3% acetic acid, 2.5 g periodic acid in 250 ml) for 30 min. Following wash steps with three changes of 3% acetic acid for 15 min, gels were incubated in Pro-Q Emerald 300 fluorescence dye (Invitrogen, USA) for 120 minute in dark. De-staining was achieved with two successive washes using 3% (v/v) acetic acid for 15 min. Images were obtained after washing. The gels were visualized with VersaDoc4000MP (Bio-Rad, USA). Then the same gels were stained with Sypro Ruby (BioRad, USA) for the purpose of displaying all the proteins. Gels were fixed at least 30 minutes in 50% (v/v) methanol, 7% (v/v) trichloroacetic. Following wash steps with four changes of distilled water for 10 min, gels were incubated overnight in SYPRO Ruby fluorescence dye in dark. De-staining was achieved with four successive washes using 10% (v/v) methanol and 7% acetic acid. Images were obtained after washing.

Image analysis

The images were analyzed with PD Quest Advanced software with VersaDoc4000 MP (BioRad, US). For automated spot detection, parameters used were sensitivity (13.8), spot size scale (3), and minimum peak intensity (258). PD Quest Advance 2D-analysis software (BioRad, USA) was used for comparative analysis of protein spots. The outside edges of the image were cropped using the automated crop tool of PD Quest Advance Software. Stain speckles were filtered and the standardized areas of interest from all gels were matched and warped, and the quantity of each spot was normalized using the total valid spot intensity. Total spot numbers and volumes within the normalized area were determined by the automated analyses. Quantity of each spot was normalized by linear regression model. A manual editing tool was used to inspect the determined protein spots detected by the software.

RESULTS

FTO PCR and cloning of the wild type FTO PCR product

Total RNA isolation from leukocytes from a healthy individual was performed with commercially available RNA isolation kit (Qiagen, USA) (Code of Ethics Approval: KOU KAEK 2013/25). PCR products were analyzed by agarose gel electrophoresis (Figure 1).

The wild-type FTO PCR product was cloned into the pcDNA4/TO expression vector. To verify the clone, the insert was excised using double digest with *HindIII* and *XhoI* and the resulting products were analysed using 1% etidium bromide agarose gel (Figure 2). The clone was labeled as pcDNA4/TO-WT-FTO and was sequenced to assess its integrity (Iontek, Istanbul). Prior to transfection into SH-SY5Y cells, endofree plasmid isolation was performed as suggested by the kit (Qiagen). In silico map for pcDNA4/TO-WT-FTO clone was shown in Figure 3.

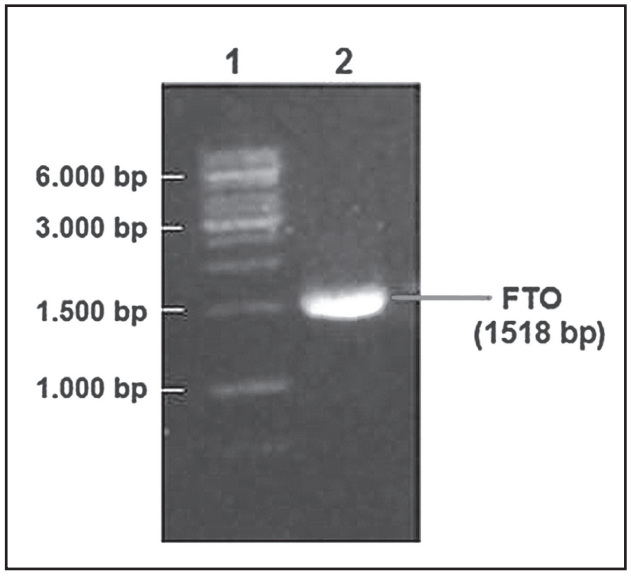

Figure 1 Agarose gel image of the FTO gene amplified by PCR. Lane 1 shows the gene ruler 1 Kb DNA ladder (Thermo Fisher Scientific, USA), Lane 2 shows the FTO PCR product.

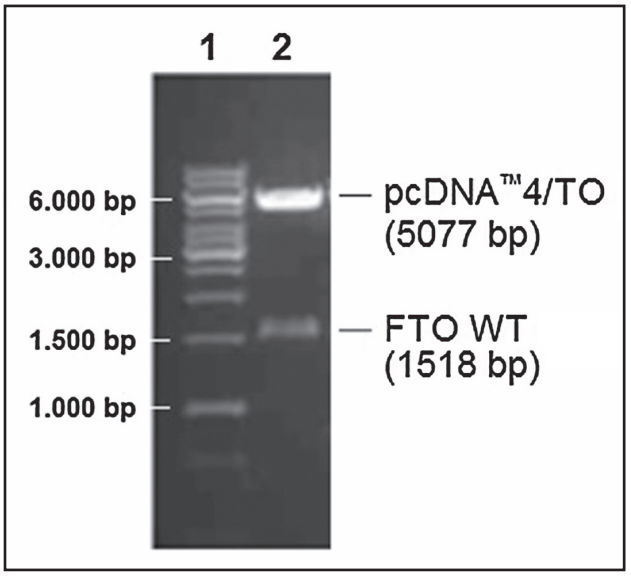

Figure 2 Agarose gel image for insert and vector visualization after double digest with *HindIII* and *XhoI* restriction enzymes. Lane 1 shows the gene ruler 1 Kb DNA ladder (Thermo Fisher Scientific, USA), Lane 2 shows the digested form of pcDNA4/TO-WT-FTO to demostrate the presence of 1518 bp insert.

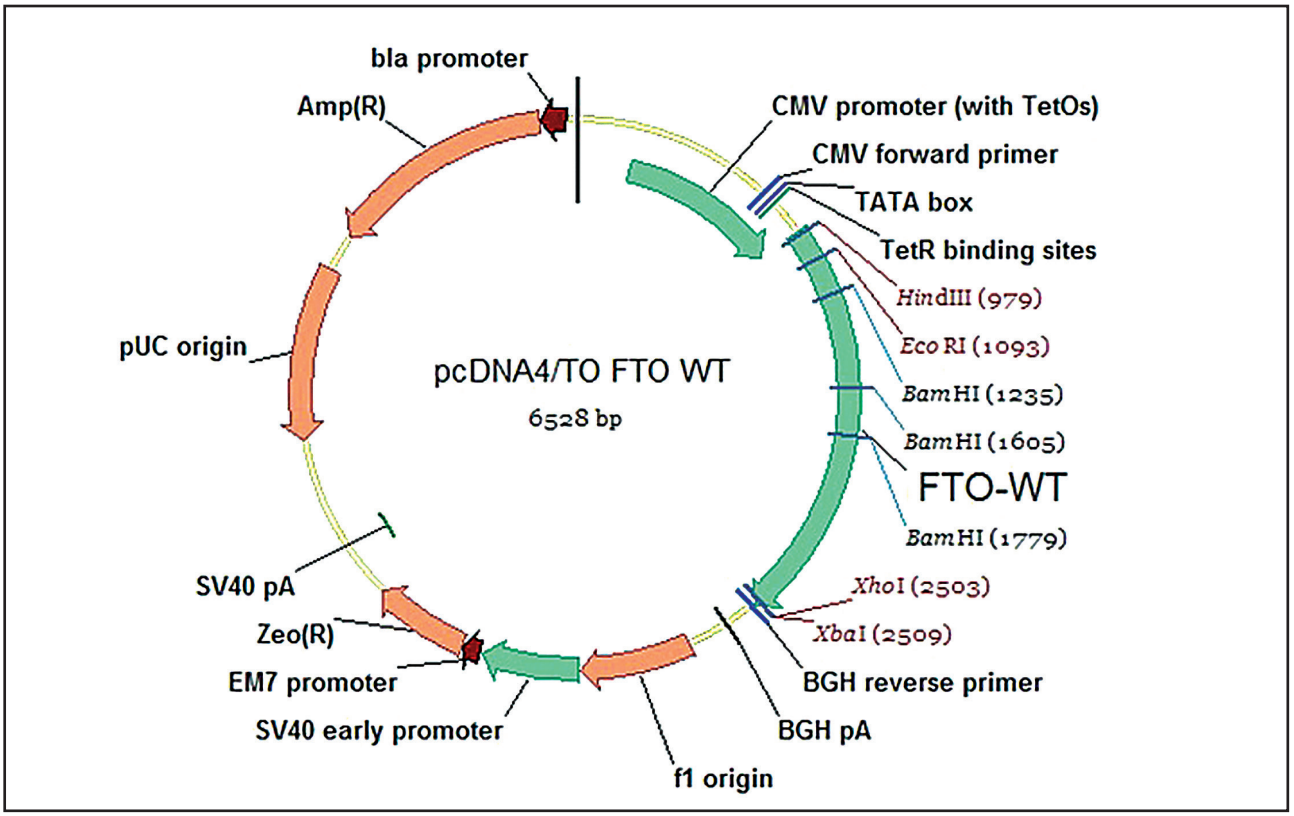

Figure 3 In silico map for pcDNA4/TO-WT-FTO clone. The map was prepared with Vector NTI (Invitrogen, USA).

Establishment of stable cell lines capable of expressing the wild type FTO

SH-SY5Y cell lines expressing the WT FTO under the control of Tet induction were created using pcDNA4/TO-WT-FTO clone (Figure 4). Several of these cell lines were screened for FTO expression using western blotting (Figure 5). Three of the screened colonies were selected based on their tight controlled expressing of the FTO protein and one of those colonies (colony #5) was used in subsequent studies.

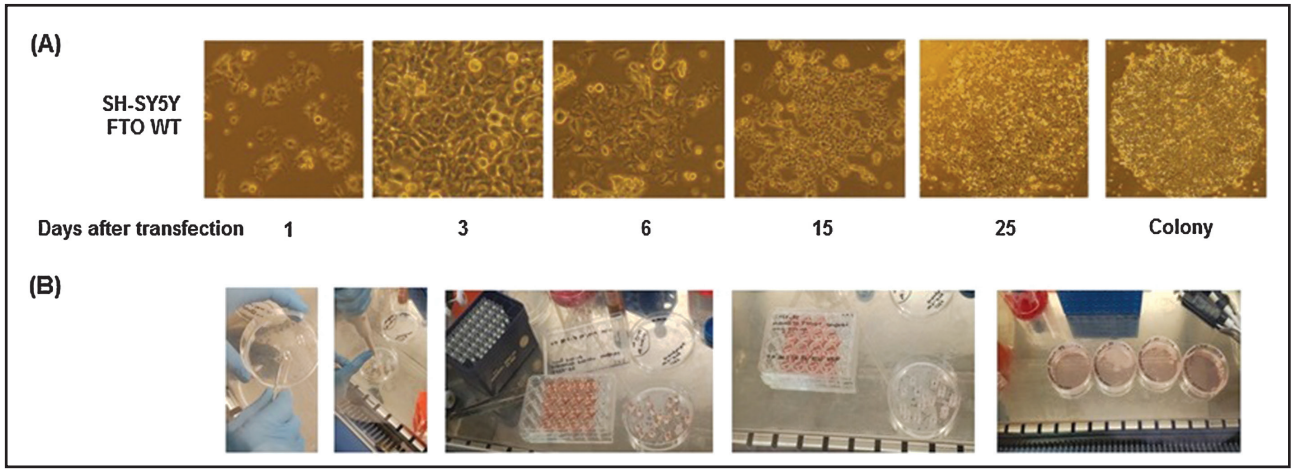

Figure 4 **(A)** Images of the cells to demonstrate the selection process of SH-SY5Y cells **(B)** Photos taken during colony "pick-up" process.

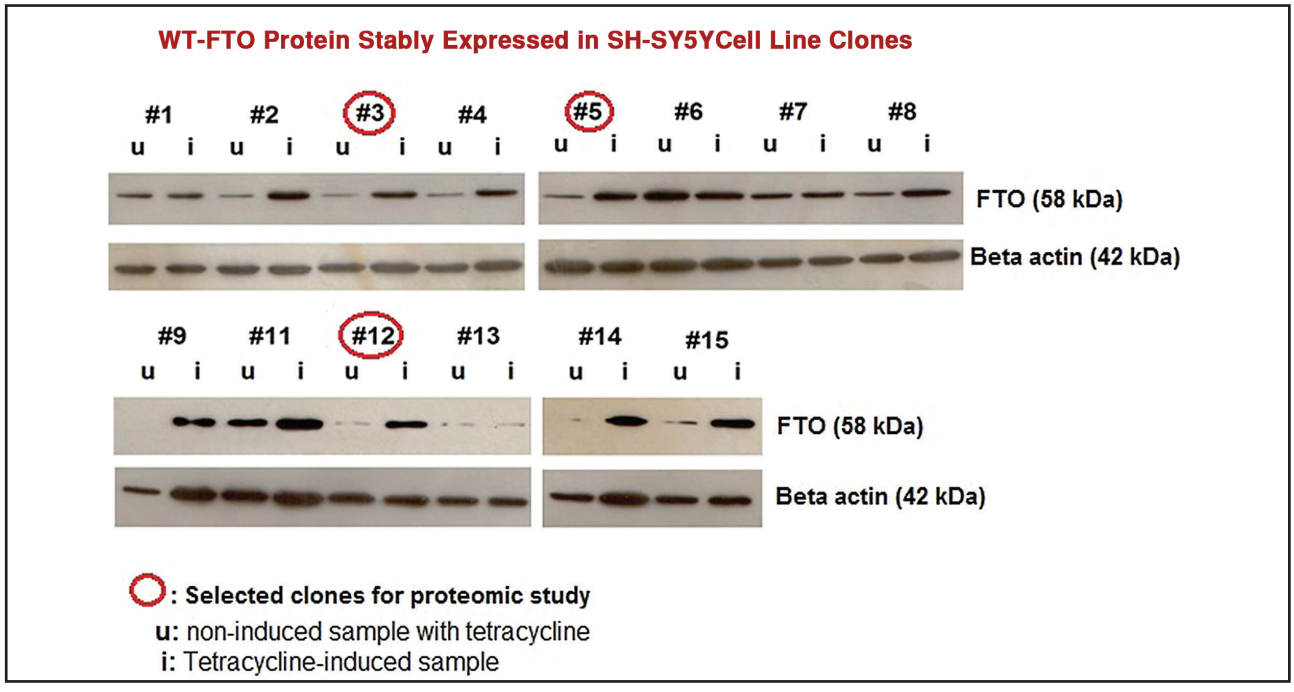

Figure 5 Western blots analysis for colony screening to determine the wild- type FTO expression under tetracycline induction. 20 µg proteins per lane were loaded from each sample. The blot was probed with a monoclonal anti-FTO antibody (SantaCruz, USA), stripped and then reprobed with a monoclonal anti-beta actin antibody (SantaCruz, USA).

To compare FTO levels before and after induction, we performed WB analysis in which 20 µg/lane of FTO negative and positive protein extracts were used. Three different exposure times were used (2 min, 15 min and overexposed) to detect any endogenous FTO protein expression. While there was a high level of exogenous FTO expression in induced cells within a short period of exposure time, a relatively weak band belonging to endogenous FTO protein was observed after a prolonged time of exposure (Figure 6). The expression profiles at the basal level observed in *in vitro* expression studies are not problematic in interpreting the obtained data. In similar studies in the literature, basal level expression was overlooked (22).

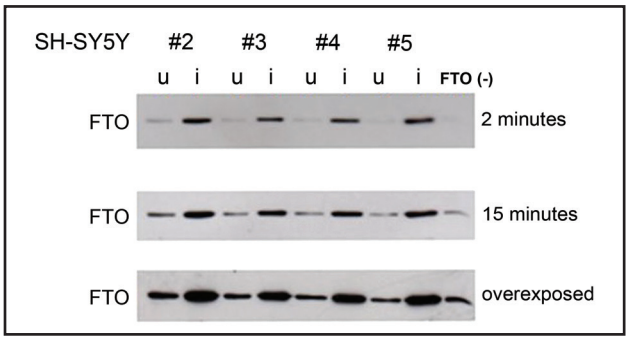

Figure 6 Western blots images to demonstrate FTO expression at different exposure times. The lanes were loaded with proteins (20 µg) extracted from uninduced (u) and tetracycline-induced (i) cells. The blot was probed with a monoclonal anti-FTO antibody (SantaCruz, USA). FTO (-) lane is for the protein sample obtained from SH-SY5Y cells nontransfected with pcDNA4/TO-WT-FTO.

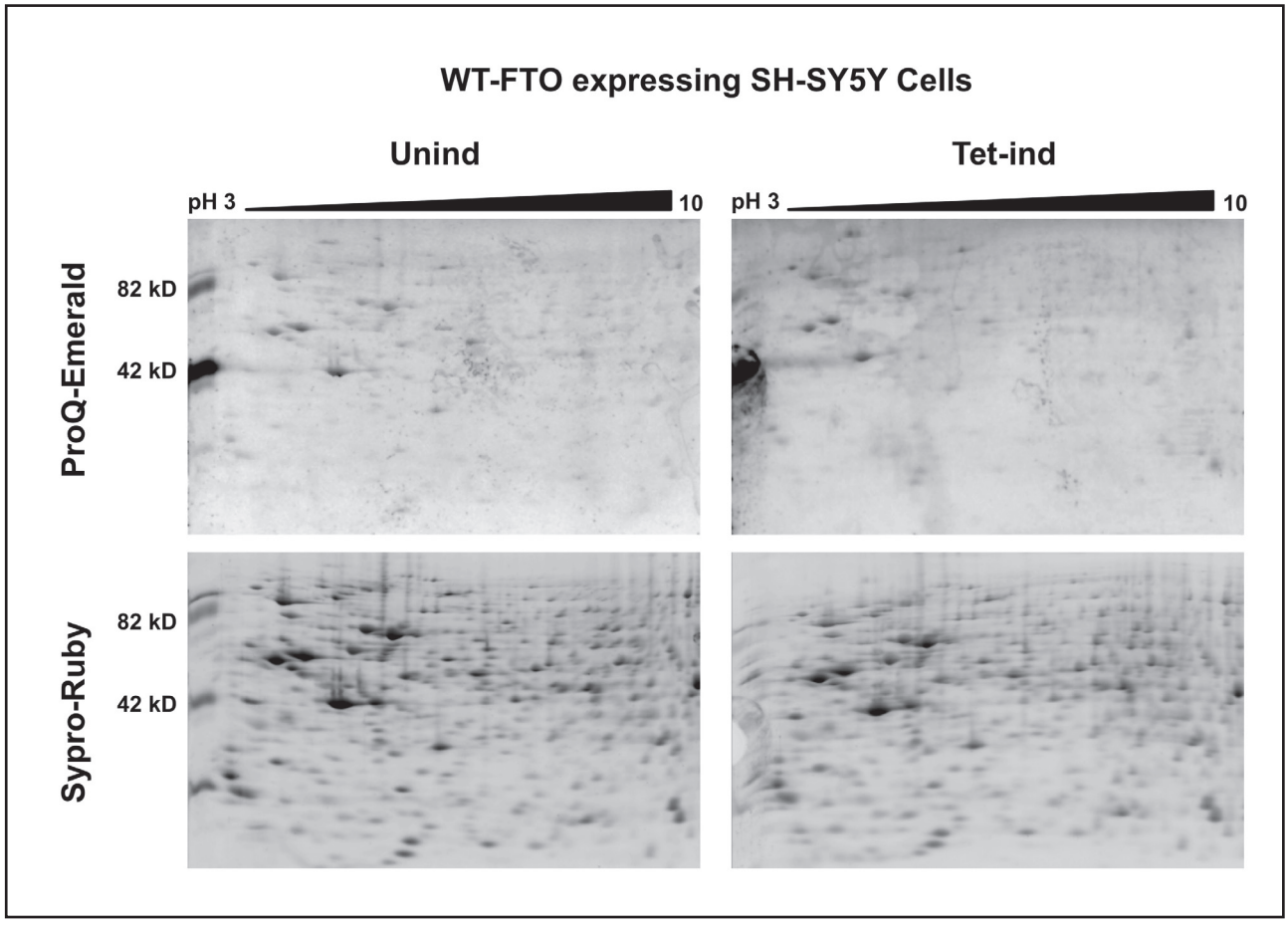

Figure 7 ProQ-Emerald and SYPRO Ruby stained 2D gel images of proteins from Tet-induced (ind) and uninduced (unind) SH-SY5Y cells.

When concentrations of cell cultures reached 75%, addition of 1 µg/ml tetracycline was performed in order to synthesize WT FTO protein in SH-SY5Y-TetR+ -WT-FTO (clone #5) cells. After 24 hours of incubation, the tetracycline-induced and uninduced cells were washed 3 times with cold PBS. For the glycoproteomics study, protein extracts were made using DIGE buffer, 0.1 mm steel beads. Routine 2DE protocol was followed for differences in the first and second dimensions. To display only the glycosylated proteins, the gels were stained according to the Pro-Q Emerald 300 process the manufacturer's recommendation. Then the same gels were stained with Sypro Ruby for the purpose of displaying all the proteins. The images were analyzed with PD Quest Advanced software with VersaDoc4000 MP (BioRad, US). For automated spot detection, parameters used were sensitivity (13.8), spot size scale (3), and minimum peak intensity (258). A comparative analysis of the gels after staining with Pro-Q Emerald 300 was performed for the samples with and without wild-type FTO expressed. 33 glycosylated protein spots were matched in the ind and unind gels. As a result of the analysis, there was no change in the regulation of these proteins. More than 500 protein spots were analyzed as a result of SYPRO staining of the same subjects. Analysis by the PDQuest Advance program revealed no regulation of phosphoprotein levels in SH-SY5Y cells, with or without the WT FTO proteins (Figure 7).

DISCUSSION

In the present study, we investigated the possible effects of exogenously expressed wild type FTO on the glycoproteom of SH-SY5Y cells, a well-known human brain-derived neuroblastoma cell line. This cell line is often used to study the changes in neuronal system as a model. FTO plays a particularly important role in adipocytes and in hypothalamic neurons (24). FTO is highly expressed in the hypothalamus (25), an area of the brain strongly linked to eating behavior. To mimic the role of FTO on neuronal system, we also used this cell line.

The effect of FTO may be elucidated in several ways. For instance, FTO may behave as a hub to control or dictate changes occurring at the genomic level. Studies already provided some evidence for this type of role FTO harbors. In addition, a transcriptomic aspect of FTO may provide a second control mechanism for cells to control their FTO associated pathways. Indeed, a BioGrid search to elucidate the interaction partners of FTO easily retrieved twenty eight interactors (Figure 8). (https://thebiogrid.org/122520/summary/homo-sapiens/fto.html). A recent study established a link between FTO and HNRNPK, a seemingly to unrelated proteins, via ubiquitin C (10). The authors of that study commented on their findings by predicting that FTO may dictate the metabolic changes occurring in cells by partnering with other multifunctional proteins. The observation of the involvement of ubiquitin C may well be presented as a putative evidence for such interaction networks. A BioGrid search with ubiqutin C indicated the presence of 1579 unique interactions creating various possible metabolic roads to connect distantly related proteins.

FTO itself is post-translationally modified (26). Therefore, a prediction of its involvement in various post translations modifications (PTMs) may be logical. However, the involvement of FTO in PTM associated networks has not been studied in detail. Therefore in this study we wished to scrutinize the changes on glycosylation patterns upon FTO expression. Detailed search for the proteins retrieved by BioGrid search predicted that SDC1, OR5F1, FIBB, MPZL1, CTSA (PPGB) proteins were glycosylated (Supplementary Material). (https://thebiogrid.org/122520/summary/homo-sapiens/fto.html). However, in our hands, SH-SY5Y cells expressing FTO protein did not display any significant change in their glycosylation pattern. However, this does not necessarily mean that FTO is certainly not involved in changes occurring in glycosylation of var-

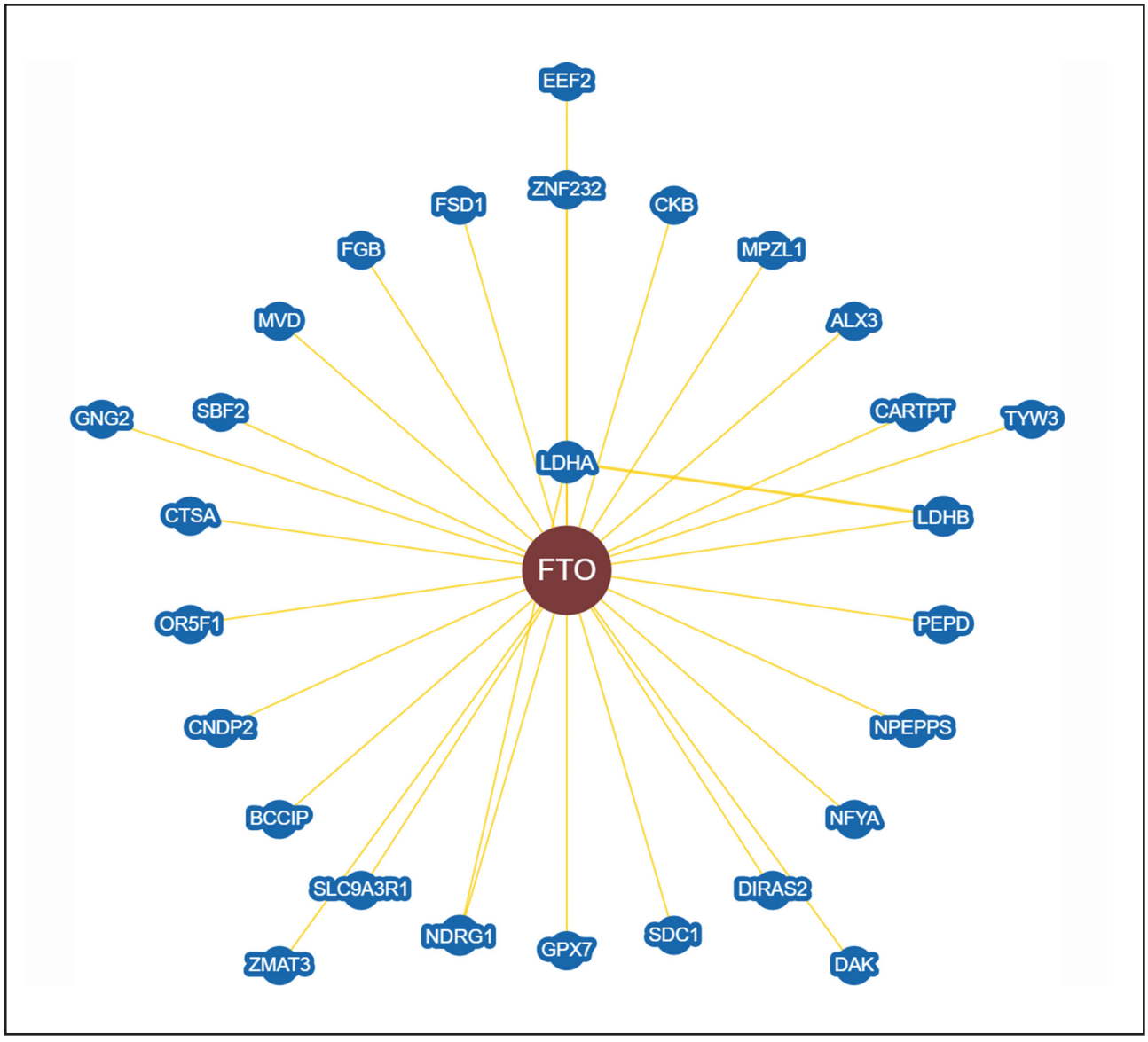

Figure 8 The interaction partners of FTO as predicted by BioGrid analysis.

ious proteins. We are aware that the experimental approaches sometimes are not able to reflect the complexity of cellular changes. The 2D-based glycoproteomic approach, although useful did not provide us with valuable information to dwell on. Therefore it is our conclusion to suggest that alternative approaches besides 2D-based glycoproteomics should be utilized to elucidate the effect of FTO on glycoproteomics.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary material. Analysis of the modifications of BioGrid retrieved FTO interacting proteins to demonstrate glycosylation.

Accesion Numbers	Best Protein Accesion	Best Protein Description	Post Translational Modifications (Amino acid modifications) Feature Key	Position(s)	Description Action
P18827	SDC1_HUMAN	Syndecan-1	Glycosylation	37	O-linked (Xyl...) (chondroitin sulfate) serine
			Glycosylation	43	N-linked (GlcNAc...) asparagine
			Glycosylation	45	O-linked (Xyl...) (heparan sulfate) serine
			Glycosylation	47	O-linked (Xyl...) (heparan sulfate) serine
			Glycosylation	206	O-linked (Xyl...) (chondroitin sulfate) serine
			Glycosylation	216	O-linked (Xyl...) (chondroitin sulfate) serine
			Modified residue	285	Phosphoserine
P10619	PPGB_HUMAN	Lysosomal protective protein (CTSA)	Disulfide bond	88 ↔ 362	
			Glycosylation	145	N-linked (GlcNAc...) asparagine
			Disulfide bond	240 ↔ 256	
			Disulfide bond	241 ↔ 246	
			Disulfide bond	281 ↔ 331	
			Glycosylation	333	N-linked (GlcNAc...) asparagine
O95297	MPZL1_HUMAN	Myelin protein zero-like protein 1	Glycosylation	50	N-linked (GlcNAc...) asparagines
			Disulfide bond	58 ↔ 135	PROSITE-ProRule annotation
			Glycosylation	130	N-linked (GlcNAc...) asparagines
			Modified residue	204	Phosphoserine
			Modified residue	206	Phosphoserine
			Modified residue	208	Phosphoserine
			Modified residue	210	Phosphoserine
			Modified residue	219	Phosphoserine
			Modified residue	221	Phosphoserine
			Modified residue	241	Phosphotyrosine
			Modified residue	260	Phosphoserine
			Modified residue	263	Phosphotyrosine
O95221	OR5F1_HUMAN	Olfactory receptor 5F1	Glycosylation	5	N-linked (GlcNAc...) asparagine
			Disulfide bond	97 ↔ 189	PROSITE-ProRule annotation
P02675	FIBB_HUMAN	Fibrinogen beta chain	Modified residue	31	Pyrrolidone carboxylic acid
			Disulfide bond	95	Interchain (with C-55 in alpha chain)
			Disulfide bond	106	Interchain (with C-68 in alpha chain)
			Disulfide bond	110	Interchain (with C-45 in gamma chain)
			Disulfide bond	223	Interchain (with C-184 in alpha chain)
			Disulfide bond	227	Interchain (with C-161 in gamma chain)
			Disulfide bond	231 ↔ 316	Combined sources
			Disulfide bond	241 ↔ 270	Combined sources
			Glycosylation	394	N-linked (GlcNAc...) asparagines
			Disulfide bond	424 ↔ 437	Combined sources

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