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Role of GALNT2 in the modulation of ENPP1 expression, and insulin signaling and action
GALNT2: A novel modulator of insulin signaling

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ABSTRACT

Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) inhibits insulin signaling and action. Understanding the mechanisms underlying *ENPP1* expression may help unravel molecular mechanisms of insulin resistance. Recent data suggest a role of *ENPP1*-3′untaslated region (UTR), in controlling *ENPP1* expression. We sought to identify *trans*-acting *ENPP1*-3′UTR binding proteins, and investigate their role on insulin signaling.

By RNA pull-down, 49 proteins bound to *ENPP1*-3′UTR RNA were identified by mass spectrometry (MS). Among these, *in silico* analysis of genome wide association studies and expression profile datasets pointed to N-acetylgalactosaminyltransferase 2 gene (GALNT2) for subsequent investigations. Gene expression levels were evaluated by RT-PCR. Protein expression levels, IRS-1 and Akt phosphorylation were evaluated by Western blot. Insulin receptor (IR) autophosphorylation was evaluated by ELISA.

GALNT2 down-regulation increased while *GALNT2* over-expression reduced ENPP1 expression levels. In addition, *GALNT2* down-regulation reduced insulin stimulation of IR, IRS-1 and Akt phosphorylation and insulin inhibition of phosphoenolpyruvate carboxykinase (*PEPCK*) expression, a key neoglucogenetic enzyme.

Our data point to GALNT2 as a novel factor involved in the modulation of ENPP1 expression as well as insulin signaling and action in human liver HepG2 cells.

1. Introduction

Insulin resistance plays a major role in the pathogenesis of type 2 diabetes, pro-atherogenic dyslipidemia and cardiovascular disease, thus imposing a tremendous burden to morbidity and mortality in developed countries [1].

The molecular mechanisms underlying insulin resistance are mostly unraveled [2]. Among several molecules involved in this process, the class II transmembrane glycoprotein ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) was identified as a putative candidate. ENPP1 binds to [3] and inhibits the insulin receptor (IR) and subsequent downstream insulin signaling and action in both cultured cells [4–10] and animal models [9,11,12]. In addition, ENPP1 is over-expressed in several tissues of insulin-resistant subjects [13–17]. Finally, *in vitro* and *in vivo* studies on a gain of function amino acid substitution (*i.e.* K121Q polymorphism; rs1805101) provided further support to the notion that ENPP1 affects insulin action [7,10,18–22]. Thus, unraveling the mechanisms involved in the modulation of ENPP1 expression might help developing strategies aimed at counteracting and possibly reversing some forms of insulin resistance.

Recent data indicated that the 3′untranslated region (UTR) of *ENPP1* mRNA plays a role in controlling ENPP1 expression, suggesting the existence of *trans*-acting proteins that affect *ENPP1* mRNA stability [23,24]. The aim of this study was to identify specific *ENPP1*-3′ UTR binding proteins by means of RNA pull-down experiments and to investigate their potential role on affecting ENPP1 expression and modulating insulin signaling and action in human liver cells, the utmost target of insulin action on glucose homeostasis.

2. Materials and methods

2.1. RNA preparation

The most conserved region (*i.e.* nucleotide (nt) 2750 to nt 3176 of the ENPP1 cDNA) of *ENPP1*-3′UTR (either wild type or polymorphic-"P" [25])

Abbreviations: ENPP1, ectonucleotide pyrophosphatase phosphodiesterase 1; UTR, untranslated region; GALNT2, N-acetylgalactosaminyltransferase 2 gene; IR, insulin receptor; PEPCK, phosphoenolpyruvate carboxykinase; GWAS, genome wide association studies; HEK293, human embryo kidney; HepG2, human hepatoma cell line; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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was obtained as previously described [24] and then used as template. The antisense sequence of the same region was used as control RNA.

2.2. RNA pull-down

In order to identify those proteins which specifically bind the *ENPP1*-3′UTR, the RNA pull-down with adipic acid dehydrazide bead method was used. Briefly, 15 μg of *ENPP1*-3′UTR, "P" *ENPP1*-3′UTR or control RNA, obtained as previously described [24], was placed in a 400 μl reaction mixture containing 100 mmol/l NaOAc, pH 5.2, and 5 mmol/l sodium m-periodate (Sigma-Aldrich, St. Louis, MO), incubated for 1 h in the dark at room temperature, ethanol precipitated, and resuspended in 100 μl of 100 mmol/l NaOAc, pH 5.2. Then 300 μl of adipic acid dehydrazide agarose beads 50% slurry (Sigma-Aldrich, St. Louis, MO) equilibrated in 100 mmol/l NaOAc, pH 5.2, was added to this mixture and incubated for 12 h at 4 °C on a rotator. The RNA that covalently immobilized onto agarose beads was pelleted, washed twice with 1 ml of 2 mol/l NaCl, equilibrated in washing buffer (5 mmol/l HEPES, pH 7.9, 1 mmol/l $MgCl_2$, 0.8 mmol/l magnesium acetate) and, finally, incubated with 3 mg of cellular protein extract for 30 min at room temperature in 0.6 ml final volume. Heparin was also added to a final concentration of 7 μg/μl. After further washing (*i.e.* four times in 1.5 ml of washing buffer), proteins retained by RNA sequences were eluted in SDS sample buffer and fractionated by a 12.5% SDS-PAGE. Gels were stained with colloidal blue staining kit (Invitrogen Life Technologies, Carlsbad, CA) [26,27].

2.3. Identification and characterizations of ENPP1-3′UTR binding proteins

Protein bands were excised from the destained gel, reduced, alkylated and digested with trypsin [28]. Peptide mixtures were extracted from the gel and analyzed by nano-chromatography tandem MS (nanoLC–MS/MS) on a CHIP MS Ion Trap XCT Ultra equipped with a capillary 1100 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400 to 2000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Raw data from nanoLC–MS/MS analyses were employed to query a non-redundant protein database using in house MASCOT software (Matrix Science, Boston, MA). Proteins identified in both the control and the sample lane were discarded, whereas those proteins solely identified in the sample and absent in the control were selected as putative *ENPP1*-3′UTR interactors.

2.4. In silico analysis

In order to prioritize proteins that were specifically bound to *ENPP1*-3′UTR and identified by mass spectrometry analysis, we interrogated and cross-checked genome wide association studies (GWAS) and expression profile datasets. Only proteins coded by genes that satisfied the following criteria were selected for further analyses:

1. Genes containing or being nearby single nucleotide polymorphisms (SNPs) showing genome-wide association with one or more of the following traits: adiposity measures (*i.e.* BMI and waist circumference), insulin resistance (*i.e.* homeostatic model assessment of insulin resistance – HOMA-IR index – and fasting insulin), glucose homeostasis (*i.e.* fasting and 2 h glucose at OGTT and type 2 diabetes), and pro-atherogenic dyslipidemia (*i.e.* triglycerides and HDL-cholesterol).
2. Genes whose expression in human and animal tissues is relevant for glucose homeostasis (*i.e.* liver, skeletal muscle and adipose tissue) was significantly ($p < 0.05$) associated with at least one of the above-mentioned trait.

The *in silico* analysis was carried out by browsing:

- HuGENavigator/GWAS Integrator (www.hugenavigator.net/HuGENavigator/AHitStartPage.do), a bioinformatics tool that provides robust lookup and analytic functionalities for all published GWAS.
- NextBio (NextBio, www.nextbio.com) that allows exploring experimental data from an extensive set of public sources, covering a broad range of techniques and therapeutic areas.

2.5. Cell culture

HEK293 (human embryo kidney) or HepG2 (human hepatoma cell line) cells (ECACC, Salisbury, UK) were maintained at 37 °C and 5% CO_2 in DMEM/F12 containing 10% FBS (EuroClone S.p.A., Milano, Italy). Before experiments, HepG2 cells were seeded in six-well plates and grown in DMEM/F12 complete medium for 48 h.

2.6. GALNT2 siRNA and cDNA transfections

HepG2 cells were either transfected with 10 nmol/l of small interfering RNA (siRNA) targeted against *GALNT2* mRNA (ON-TARGETplus SMARTpool Human GALNT2 L-011865-01-0005 Thermo-Scientific Dharmacon Lafayette, CO) or with 10 nmol/l of scrambled siRNA (ON-TARGETplus non-targeting Pool D-001810-10-20 Thermo-Scientific Dharmacon Lafayette, CO) by using Interferin™ Transfection Reagents (Polyplus, Illkirch, France), according to the manufacturer's instructions. After 48 or 96 h treatment, followed by cell lysis, equal amount of proteins was analyzed by Western blot as described below.

Otherwise, HepG2 were transiently transfected either with empty vector (mock cells) or with *GALNT2* cDNA Myc/DDK tagged, cloned in pCMV6 Entry vector TrueORF Gold (OriGene Technologies, Rockville, MD) (GALNT2 cells), by using TransIT Reagent (Mirus, Madison, WI), according to the manufacturer's instructions.

2.7. RNA extraction, cDNA synthesis, and gene expression analysis

Total RNA was isolated from cells using RNeasy Mini kit (Qiagen S.r.l., Milan, Italy). cDNA was generated by reverse transcription with iScript™ Reverse Transcription (Biorad, Hercules, CA) according to the manufacturer's instructions and used as template in the subsequent analyses. Gene Expression Assay on Demand Kit Reagents (Applera Life Technologies, Carlsbad, CA) or PrimeTime Std qPCR Assay (IDT, San Jose, CA) were used to quantify relative gene expression levels of *ENPP1*, *PEPCK* (phosphoenolpyruvate carboxykinase) and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) on ABI-PRISM 7500 (Applera Life Technologies, Carlsbad, CA). Expression levels of *ENPP1* and *PEPCK* were calculated by using the comparative ΔCT method. Briefly, the amount of *ENPP1* and *PEPCK* were normalized to *GAPDH* as endogenous reference ($2^{-\Delta CT}$) in experiments run in triplicate and expressed as percentage of control cells of the first of several experiments.

2.8. Antibodies

Antibody directed against GALNT2 was obtained from Abcam (Cambridge, UK). Anti-ENPP1 anti-GAPDH and anti-IR β-subunit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Phospho-IRS-1 Tyr^{895}, anti-IRS-1, anti-Phospho-Akt Ser^{473} and anti-Akt, antibodies were purchased from Cell signaling (Boston, MA).

2.9. Western blot analysis

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blotting with specific antibodies described above, immunocomplexes were detected with the Super Signal West Pico (Thermo Fisher

Scientific Pierce, Rockford, IL). Gel images were acquired by Molecular Imager ChemiDoc XRS (Biorad, Hercules, CA) and band intensities were measured by Kodak Molecular Imaging Software 4.0 as Optical Density (OD) values. ENPP1 protein expression was evaluated by ENPP1/GAPDH OD ratio and expressed as means ± SD. IRS-1-Tyr^{895} and Akt-Ser^{473} phosphorylations were calculated as IRS-1-Tyr^{895} phosphorylation/IRS-1 or Akt-Ser^{473} phosphorylation/Akt OD ratio respectively and expressed as means ± SD.

2.10. IR β-subunit autophosphorylation

After 18 h starvation, both GALNT2 silenced and GALNT2 transfected as well as their respective control HepG2 cells were stimulated with 100 nmol/l insulin for 5 min and lysed. IR β-subunit autophosphorylation was analyzed by using CST's PathScan Phospho-Insulin Receptor β (Tyr1150/1151) solid phase sandwich enzyme-linked immunosorbent assay (ELISA) Kit according to manufacturer's instruction (Cell signaling, Boston, MA). Briefly, equal amount of protein was incubated with a coated anti-Insulin Receptor β mouse antibody in order to capture both phospho- and nonphospho-insulin receptor proteins. Following extensive washing, anti-phospho Insulin Receptor β (Tyr1150/1151) rabbit antibody is added to detect the captured phospho-insulin receptor protein. Anti-rabbit IgG, HRP-linked antibody is then used to recognize the bound detection antibody. OD values were used as a measure of IR β-subunit autophosphorylation. Data are means ± SD.

2.11. IRS-1-Tyr^{895} and Akt-Ser^{473}phosphorylation

After 18 h starvation, GALNT2 silenced or not silenced HepG2 cells were stimulated with 100 nmol/l insulin for 5 min and lysed; then, equal amount of proteins was analyzed by Western blot and probed with anti-Phospho-IRS-1 Tyr^{895}, anti-Phospho-Akt Ser^{473}, anti IRS-1 or anti-Akt. IRS-1 and Akt phosphorylation levels were normalized against IRS-1 or Akt content and expressed as described above.

2.12. PEPCK mRNA expression levels

Insulin action on glucose metabolism was assessed by studying mRNA level of the gluconeogenetic enzyme *PEPCK* in HepG2 cells. After 18 h starvation, silenced or not silenced HepG2 cells were stimulated with 100 nmol/l insulin for 10 h. Total RNA extraction and cDNA synthesis and *PEPCK* mRNA level measurement were performed as previously reported [10] and calculated as described above.

2.13. Statistical analyses

Differences between the mean values were evaluated by Student's *t* test. Data are presented as means ± SD. SPSS 13 software package was used for all analyses.

3. Results

3.1. Identification and characterization of GALNT2 as an ENPP1-3′UTR binding proteins

Proteins that specifically bind the *ENPP1*-3′UTR were identified by a functional proteomic approach using affinity capture and tandem MS. Fig. 1 shows the corresponding gel stained with colloidal blue Coomassie. The entire sample and control lanes of the gel were cut in slices and proteins occurring in each slice were identified by tandem-MS. Common proteins identified in both the sample and the control gel slices were eliminated, thus greatly decreasing the number of false positives. A total of forty-nine putative candidate proteins were identified (Supplementary Table 1).

A screening procedure was applied to the list of identified proteins to select the most promising 3′UTR interactors by interrogating GWAS and expression profile datasets using the selection criteria described in Materials and methods. Among the forty-nine putative protein interactors, ten matched the inclusion criteria at the GWAS dataset, whereas only two proteins were selected when using the expression profile datasets. When data from the two screening procedures were cross-checked, only *GALNT2* matched inclusion criteria at both datasets and was, then, selected for subsequent investigation. The *GALNT2* gene codes for an UDP-N-acetyl-alpha-D-galactosamine polypeptide N-acetylgalactosaminyltransferase (O-GalNAc) which contributes to the initiation of mucin-type O-linked glycosylation [29].

3.2. Confirmation of GALNT2-ENPP1-3′UTR interaction

The interaction of GALNT2 with *ENPP1*-3′UTR RNA was confirmed by an additional RNA pull-down assay from HEK293 total protein extract followed by Western-blot analysis (Fig. 2). By using anti-GALNT2 specific antibody, a clear band was detected in RNA pulled-down human HEK293 cells (Fig. 2 panel A lane 2, n = 2), thus confirming that GALNT2 binds to *ENPP1*-3′UTR. The interaction between *ENPP1* and GALNT2 was further confirmed in typical human insulin target cells, namely liver HepG2 cells (Fig. 2 panel B). Such binding was not influenced by the polymorphic "P" 3′UTR haplotype (Supplementary Fig. 1), reported to affect *ENPP1* mRNA expression [25].

3.3. Role of GALNT2 downregulation on ENPP1 expression and on insulin signaling and action

Exposure of HepG2 cells to *GALNT2* siRNA for both 48 and 96 h almost abolished GALNT2 expression as compared to scrambled siRNA transfected cells (Fig. 3, panel A: lanes 2 *vs.* 1, n = 3 and lanes 4 *vs.* 3, n = 5, respectively). As compared to scrambled siRNA transfected cells, in GALNT2 downregulated cells (Fig. 3, panel B), *ENPP1* expression tended to be increased at 48 h (*i.e.* 43% increase, p = 0.05–0.1), and was significantly increased at 96 h (116% increase, $p < 0.05$). Coherently, although to a lesser extent, also ENPP1 protein level was increased at 96 h in GALNT2 downregulated as compared to control cells (27% increase, $p < 0.01$, Fig. 3, panel C).

Given the well established inhibitory role of ENPP1 on insulin signaling and action [10], we tested the effect of GALNT2 downregulation on such pathways. After GALNT2 downregulation, insulin-induced IR

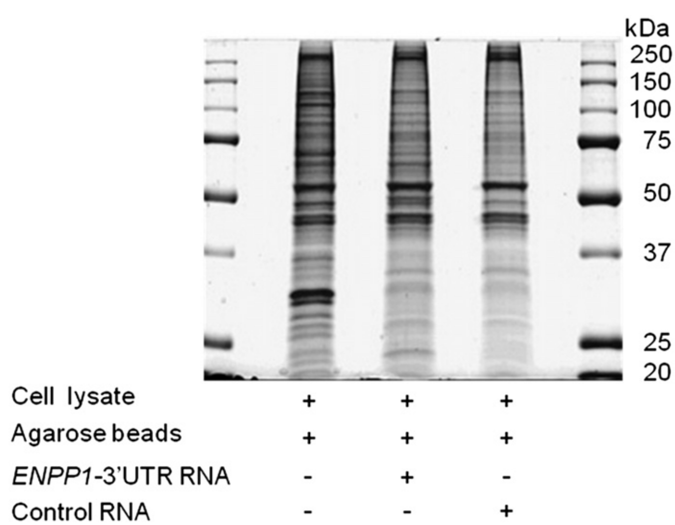

Fig. 1. SDS-PAGE of RNA protein pulled-down. HEK293 total protein extract was incubated with either agarose beads alone (lane 1) or *ENPP1*-3′UTR RNA (lane 2) or control RNA (lane 3) covalently linked to agarose beads. The complex were eluted with SDS sample buffer, loaded on 12.5% SDS-PAGE, separated by electrophoresis and stained with colloidal blue staining kit. Entire lanes observed in the gel were cut in slices and submitted to the identification procedure as described in Materials and methods.

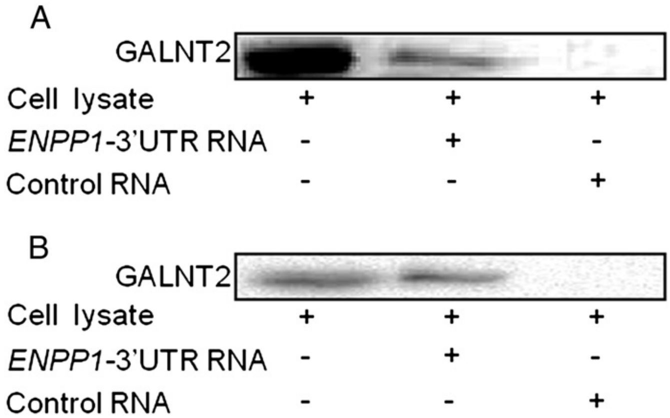

Fig. 2. *GALNT2*/*ENPP1*-3′UTR interaction in HEK293 and HepG2 cells. RNA pull-down was performed as described in Materials and methods. Following incubation with 3 mg of HEK293 (panel A), or HepG2 (panel B) total cell lysates, pulled-down proteins with either *ENPP1*-3′UTR, or control RNA were analyzed by Western blot with anti-GALNT2 specific antibody. A clear band was evident in *ENPP1*-3′UTR (lane 2, panels A and B) pulled-down proteins while no band was detectable in control RNA pulled-down proteins (lane 3, panels A and B). GALNT2 expression in 50 μg of total cell lysate is shown in lane 1, panels A and B.

β-subunit autophosphorylation (Fig. 4 panel B) was significantly reduced as compared to insulin-stimulated scrambled siRNA cells (23% reduction, $p < 0.05$). IR protein content was superimposable across all different experimental conditions (Fig. 4 panel A). In GALNT2 siRNA cells also IRS-1 Tyr^{895} (Fig. 5, panel A) and $Akt\text{-}Ser^{473}$ (Fig. 5, panel B) phosphorylations were significantly reduced as compared to what observed in insulin-stimulated scrambled siRNA cells (25% reduction $p < 0.01$ and 57% reduction $p < 0.05$ respectively).

Insulin significantly suppressed gluconeogenetic enzyme *PEPCK* mRNA levels in scrambled siRNA cells (54% reduction, $p < 0.01$). This effect was partially lost in *GALNT2* siRNA HepG2 cells in which only a 33% reduction of *PEPCK* mRNA levels was found (p = not significant *vs.* non insulin stimulated cells; Fig. 6). In addition, *PEPCK* mRNA levels in scrambled siRNA insulin-stimulated cells were significantly lower than those in *GALNT2* siRNA insulin-stimulated cells ($p < 0.05$, Fig. 6). Thus, the observed deleterious effect of GALNT2 downregulation on *ENPP1* expression is paralleled by a similar effect on downstream insulin signaling and action on glucose metabolism.

3.4. Role of GALNT2 upregulation on ENPP1 expression and on insulin signaling

Exposure of HepG2 cells to *GALNT2* cDNA for 96 h, which almost doubled GALNT2 expression (Fig. 7, panel A), reduced *ENPP1* mRNA expression levels by 30% as compared to mock HepG2 control cells ($p < 0.01$, Fig. 7, panel B), thus mirroring the data obtained by GALNT2 downregulation. In contrast, GALNT2 upregulation did not affect ENPP1 protein content as shown in Fig. 7, panel C. This lack of effect is observed despite the maintained ability of transfected GALNT2 to bind *ENPP1*-3′UTR (Supplementary Fig. 2). Conversely, these apparently discordant results may be due to a high ENPP1 protein stability in HepG2 cells which makes it difficult to detect any effect of *ENPP1* mRNA reduction due to GALNT2 overexpression. To test this hypothesis, we exposed cells to *ENPP1* siRNA for 96 h and then measured both *ENPP1* mRNA and protein content. While *ENPP1* mRNA expression levels were decreased by 39 ± 12% as compared to scrambled siRNA HepG2 transfected cells (n = 3 experiments,

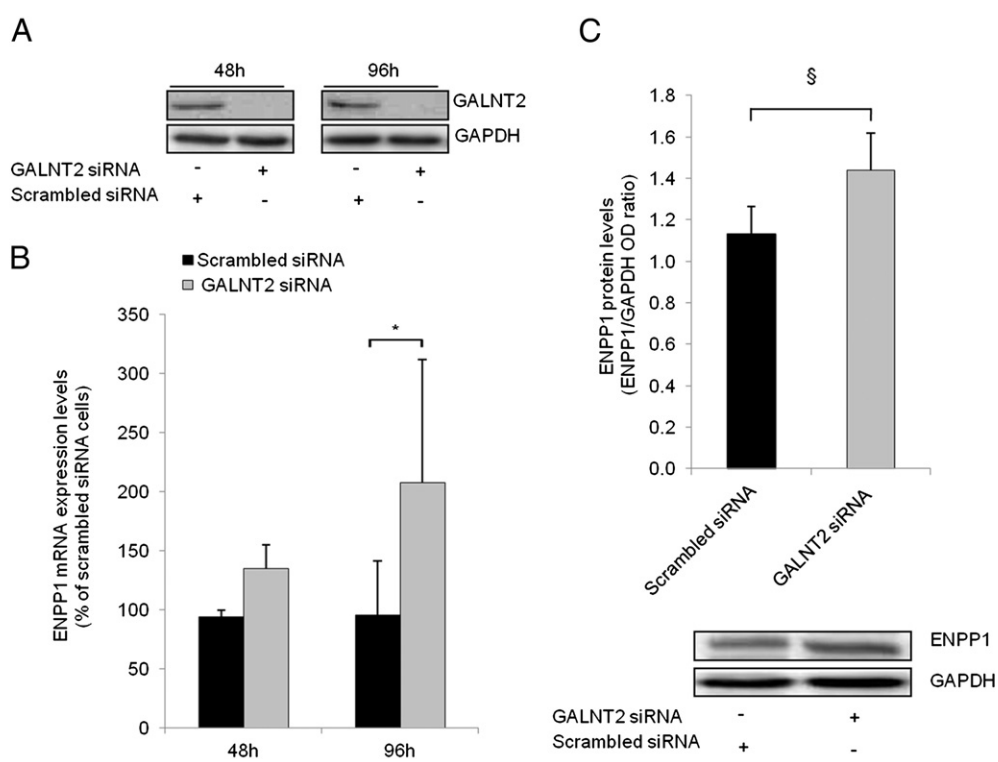

Fig. 3. Effect of *GALNT2* siRNA on *ENPP1* expression. A. HepG2 cells were transfected with either scrambled siRNA or *GALNT2* siRNA for 48 or 96 h, as described in Materials and methods. Expression of GALNT2 (upper blot) and GAPDH (lower blot) was evaluated by Western blot by using anti-GALNT2 or anti-GAPDH specific antibody respectively. A representative blot for each condition is shown. B. HepG2 cells were either treated with scrambled siRNA (black bar) or *GALNT2* siRNA (gray bar) and *ENPP1* mRNA expression levels were measured by Real-Time PCR as described in Materials and methods. Data are expressed as percentage of scrambled siRNA cells of the first experiment at 48 h (n = 3) and 96 h (n = 5) respectively. Data are means ± SD. *$p < 0.05$. C. HepG2 cells were either transfected with scrambled siRNA or *GALNT2* siRNA for 96 h, as described in Materials and methods. Equal amount of protein from cell lysates was separated by SDS-PAGE and probed with anti-ENPP1 and anti-GAPDH specific antibody. Bars (upper panel) represent ENPP1/GAPDH OD ratio (representative immunoblots are shown in lower panel). Data are means ± SD of 6 experiments in separate times. §$p < 0.01$.

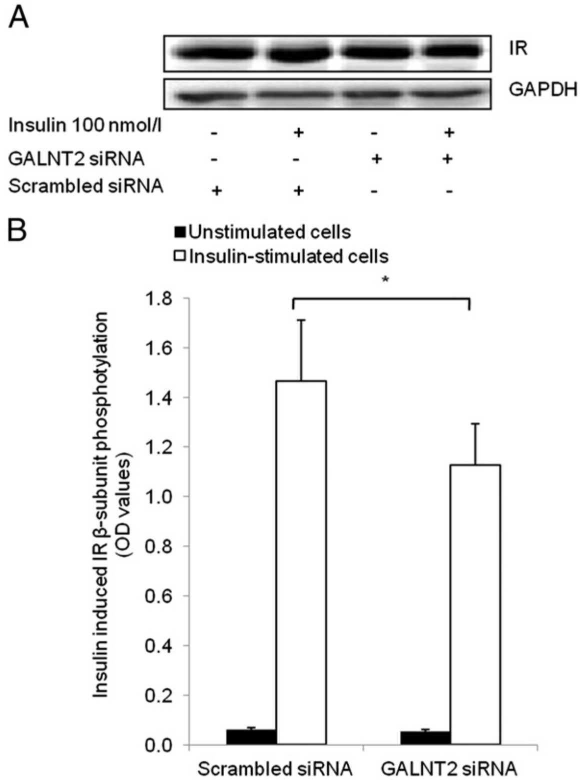

Fig. 4. Effect of *GALNT2* siRNA on IR β-subunit autophosphorylation. HepG2 cells were either transfected with scrambled siRNA or *GALNT2* siRNA for 96 h and then stimulated with insulin as described in Materials and methods. A. Equal amount of protein from cell lysates was separated by SDS-PAGE. IR and GAPDH protein content was evaluated by Western blot by using anti-IR β-subunit or anti-GAPDH specific antibody, respectively (a representative blot out of 3 experiments for each condition is shown). B. Bars represent OD values of IR β-subunit autophosphorylation as assessed by ELISA (see Materials and methods). Data are means ± SD of 3 experiments in separate times. $^*p < 0.05$.

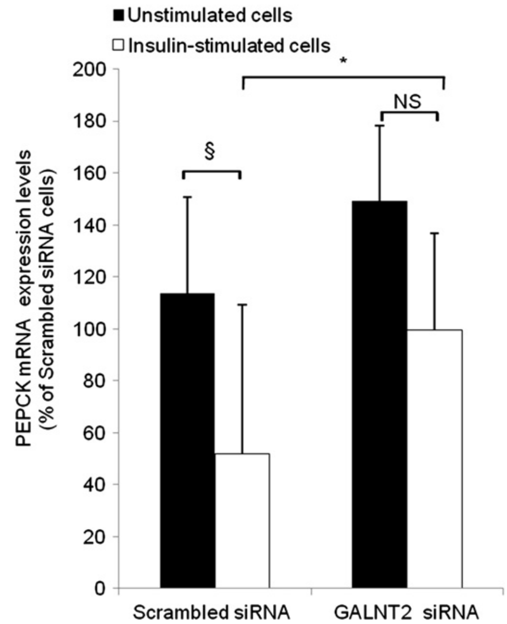

Fig. 6. Effect of *GALNT2* siRNA on *PEPCK* expression. HepG2 cells were either transfected with scrambled siRNA or *GALNT2* siRNA for 96 h and stimulated with insulin as described in Materials and methods. *PEPCK* mRNA expression levels were measured by Real-Time PCR, as described in Materials and methods and expressed as percentage of unstimulated scrambled siRNA cells of the first experiment. Data are means ± SD of 4 experiments in separate times. $^*p < 0.05$ and §p < 0.01.

$p < 0.05$), ENPP1 protein content was totally unaffected (110 ± 15% as compared to scrambled siRNA cells, n = 3 experiments, $p < 0.05$), thus reinforcing the hypothesis of a high ENPP1 protein stability, which does not allow to appreciate variation in protein level, at least under these experimental conditions.

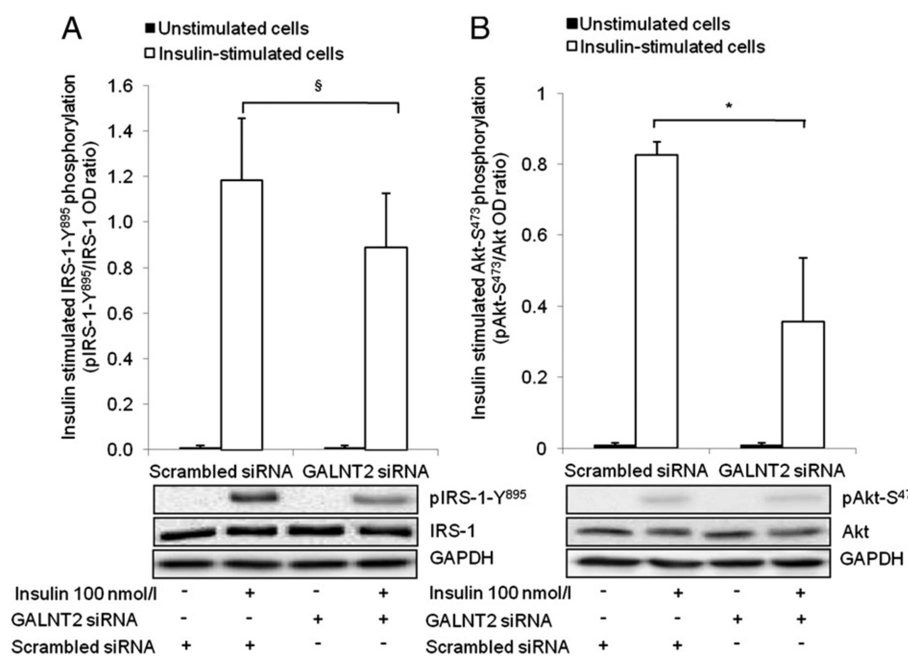

Fig. 5. Effect of *GALNT2* siRNA on IRS-1-Y^{895} and Akt-S^{473} phosphorylation. HepG2 cells were either transfected with scrambled siRNA or *GALNT2* siRNA for 96 h and then stimulated with insulin as described in Materials and methods. Equal amount of protein from cell lysates was separated by SDS-PAGE. A. Insulin stimulated IRS-1-Y^{895} phosphorylation and both IRS-1 and GAPDH protein content were evaluated by Western blot analysis by using anti phospho-IRS-1-Y^{895}, anti-IRS-1 or anti-GAPDH specific antibody, respectively. Bars (upper panel) represent quantitative analysis of IRS-1-Y^{895} phosphorylation calculated as IRS-1-Y^{895} phosphorylation/IRS-1 OD ratio. Data are means ± SD of 4 experiments in separate times. §p < 0.01. B. Insulin stimulated Akt-Ser^{473} phosphorylation and both Akt and GAPDH protein content were evaluated by Western blot analysis by using anti phospho-Akt-Ser^{473}, anti-Akt or anti-GAPDH specific antibody, respectively. Bars (upper panel) represent quantitative analysis of Akt-Ser^{473} phosphorylation calculated as Akt-Ser^{473} phosphorylation/Akt OD ratio. Data are means ± SD of 3 experiments in separate times. $^*p < 0.05$.

Coherently with the unchanged ENPP1 protein content, insulin-induced IR β-subunit autophosphorylation was similar in GALNT2 overexpression and mock HepG2 control cells (Fig. 8 panel B). IR protein content was superimposable across all different experimental conditions (Fig. 8 panel A). Based upon these negative results no further experiments on downstream insulin signaling were carried out.

4. Discussion

Functional [4,7,10] metabolic [13,14,16] and genetic [18,23,25,30–38] studies suggest that ENPP1 acts as a negative modulator of insulin signaling and action. Thus, addressing the mechanisms underlying ENPP1 expression may help unravel novel molecular mechanisms of insulin resistance.

Our study on human liver HepG2 cells shows that GALNT2 binds to *ENPP1*-3′UTR mRNA and inhibits ENPP1 transcript and protein levels. This finding adds to previous functional [24,25] and genetic [23,25] reports suggesting that, in fact, the 3′UTR is involved in the regulation of ENPP1 expression in human liver cells, the utmost target of insulin action on glucose homeostasis. Of note, a previously reported 3′UTR polymorphic haplotype did not impair GALNT2 binding affinity, thus suggesting that its effect on *ENPP1* mRNA levels [25] is not mediated by GALNT2. Whether other SNPs associated with metabolic traits [23] which are located outside the most conserved 3′UTR we used for our experiments, affect GALNT2 binding may be interested to be addressed by further investigations. Also of note is that HSP70 was not identified as a binding protein of *ENPP1*-3′UTR, a finding which is in contrast with our previous reports [24]. We like to speculate that the different methods utilized in our present and previous studies (RNA-pull-down *vs.* RNA Mobility Shift Analysis) underline the different results we obtained.

A second important finding of this study is that GALNT2 downregulation affects insulin-induced IR β-subunit autophosphorylation, IRS-1 Tyr^{895} and Akt Ser^{473} phosphorylations and PEPCK expression, four key steps of insulin signaling and gluconeogenetic

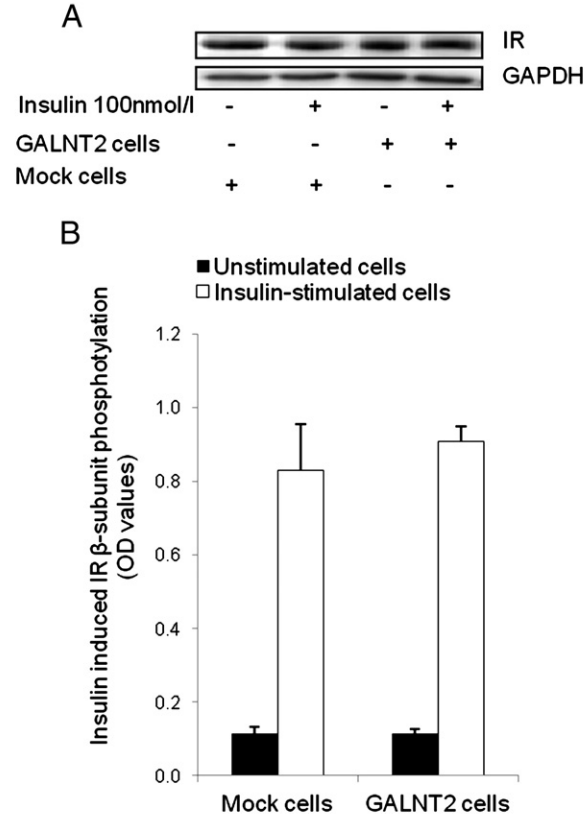

Fig. 8. Effect of *GALNT2* transfection on IR β-subunit autophosphorylation. HepG2 cells were either transfected with mock or with *GALNT2* cDNA and then stimulated with insulin as described in Materials and methods. A. Equal amount of protein from cell lysates was separated by SDS-PAGE. IR and GAPDH protein content was evaluated by Western blot by using anti-IR β-subunit or anti-GAPDH specific antibody, respectively (a representative blot out of 3 experiments for each condition is shown). B. Bars represent OD values of IR β-subunit autophosphorylation as assessed by ELISA (see Materials and methods). Data are means ± SD of 3 experiments in separate times.

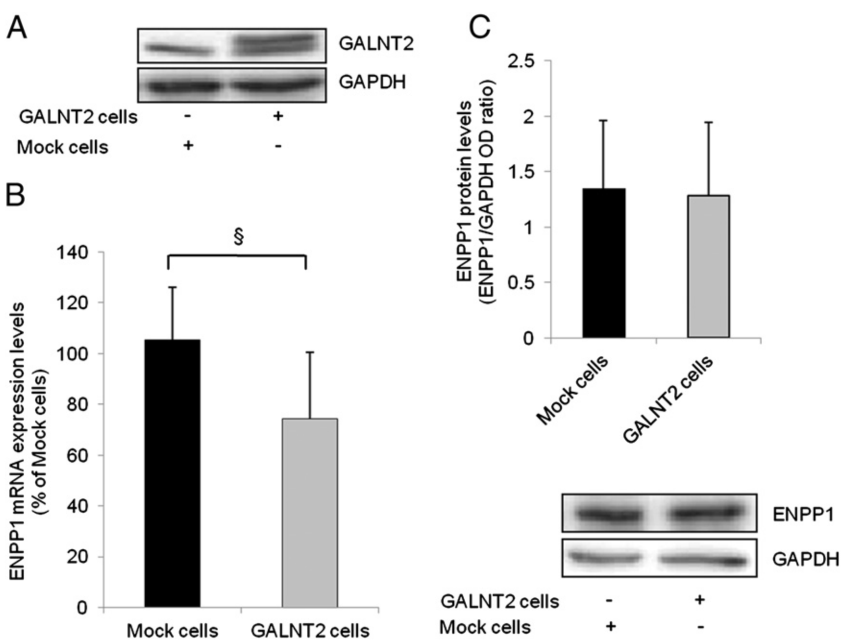

Fig. 7. Effect of *GALNT2* transfection on *ENPP1* expression. A. HepG2 cells were either transfected with mock or with *GALNT2* cDNA as described in Materials and methods. Equal amount of protein from cell lysates was separated by SDS-PAGE and probed with anti-GALNT2 (upper blot) or anti-GAPDH (lower blot) specific antibody. A representative blot out of 3 experiments for each condition is shown. B. *ENPP1* expression levels were measured by Real-Time PCR as described in Materials and methods and expressed as percentage of mock HepG2 cells of the first experiment. Data are means ± SD of 3 experiments in separate times. §$p < 0.01$. C. HepG2 cells were either transfected with mock or with *GALNT2* cDNA as described in Materials and methods. Equal amount of protein from cell lysates was separated by SDS-PAGE and probed with anti-ENPP1 and anti-GAPDH specific antibody. Bars (upper panel) represent ENPP1/GAPDH OD ratio (representative immunoblots are shown in lower panel). Data are means ± SD of 3 experiments in separate times.

activity. These effects are very likely mediated, by ENPP1 protein up-regulation. In fact, GALNT2 overexpression, which down-regulates *ENPP1* mRNA levels but not ENPP1 protein content, is not paralleled by any change of insulin-induced IR β-subunit autophosphorylation. This finding reinforces the hypothesis that ENPP1 up-regulation, mediates, at least partly, GALNT2 down-regulation effects on insulin signaling and action. We acknowledge that further experiments using cellular models lacking both ENPP1 alleles, are needed to definitively confirm such hypothesis.

It is entirely possible that other mechanisms also underlie such GALNT2 effects. As a matter of fact, GalNAc-T2 coded by GALNT2 is responsible for the O-linked glycosylation, allowing the transfer of *N*-acetylgalactosamine from UDP-GalNAc to the hydroxyl group of a serine or threonine residue [29]. Such glycosylation has been reported to play an important role on insulin resistance and diabetes, either by competing for phosphorylation on insulin-stimulated sites on effector molecules, or by directly regulating components of insulin signaling, including IRS1 and Akt [39–42].

Regardless the mechanism of action, also data from the few studies so far published on *GALNT2* expression levels in several models of metabolic abnormalities related to insulin resistance suggests that it modulates insulin resistance traits. While *GALNT2* expression is reduced in liver of insulin resistant Goto-Kakizaki diabetic rats [43], it is increased in adipose tissue of obese, insulin resistant non-diabetic PIMA Indians [44]. In addition, *GALNT2* expression in mice causes an inverse modulation of serum HDL-c levels, an established marker of insulin sensitivity [45]. All in all, expression data in both humans and rodents, although somehow variable, clearly point to GALNT2 as a potential mediator of abnormalities related to insulin resistance. Further support to this hypothesis comes from studies showing that genetic variability at the *GALNT2* locus (SNP rs4846914) is associated with decreased HDL-c and increased triglyceride levels [46–48], two main components of the insulin resistance/metabolic syndrome.

5. Conclusions

In conclusion, our data identified GALNT2 as a new potential modulator of insulin signaling and action in human liver cells through the modulation of ENPP1 expression. Additional experiments in other cell types and tissues relevant to glucose metabolism are needed to confirm this finding, thus deeper addressing the role of GALNT2 as a potential target of novel treatments of insulin resistance and abnormal glucose homeostasis. Finally, because of the very restrictive prioritizing criteria we used to identify GALNT2, the list of *ENPP1*-3′UTR binding proteins may, in fact, contain additional important candidates to be investigated in further studies.

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Disclosure statement

The authors declare that they do not have any actual or potential conflict of interest.

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