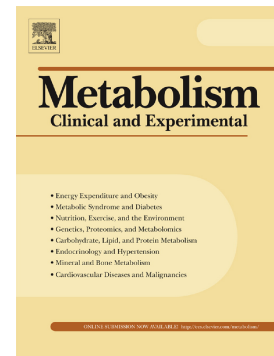


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GNIP1 E3 ubiquitin ligase is a novel player in regulating glycogen metabolism in skeletal muscle

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ABSTRACT

Background

¹This study is dedicated to the memory of Anna M^a Gomez-Foix, who passed away on 18 March 2015.

Glycogenin-interacting protein 1 (GNIP1) is a tripartite motif (TRIM) protein with E3 ubiquitin ligase activity that interacts with glycogenin. These data suggest that GNIP1 could play a major role in the control of glycogen metabolism. However, direct evidence based on functional analysis remains to be obtained.

Objectives

The aim of this study was 1) to define the expression pattern of glycogenin-interacting protein/Tripartite motif containing protein 7 (GNIP/TRIM7) isoforms in humans, 2) to test their ubiquitin E3 ligase activity, and 3) to analyze the functional effects of GNIP1 on muscle glucose/glycogen metabolism both in human cultured cells and *in vivo* in mice.

Results

We show that GNIP1 was the most abundant GNIP/TRIM7 isoform in human skeletal muscle, whereas in cardiac muscle only TRIM7 was expressed. GNIP1 and TRIM7 had autoubiquitination activity *in vitro* and were localized in the Golgi apparatus and cytosol respectively in LHCN-M2 myoblasts. GNIP1 overexpression increased glucose uptake in LHCN-M2 myotubes. Overexpression of GNIP1 in mouse muscle *in vivo* increased glycogen content, glycogen synthase (GS) activity and phospho-GSK-3 α/β (Ser21/9) and phospho-Akt (Ser473) content, whereas decreased GS phosphorylation in Ser640. These modifications led to decreased blood glucose levels, lactate levels and body weight, without changing whole-body insulin or glucose tolerance in mouse.

Conclusion

GNIP1 is an ubiquitin ligase with a markedly glycogenic effect in skeletal muscle.

Keywords

GNIP1: TRIM7 isoform 1

TRIM7: TRIM7 isoform 4

Skeletal muscle

Glycogen metabolism

Abbreviations

Akt: Protein kinase B

CM: Cardiac muscle

GP: Glycogen phosphorylase

GS: Glycogen synthase

GSK-3: Glycogen synthase kinase 3

GST: Glutathione S-transferase

GYG1: Glycogenin 1

SKM: Skeletal muscle

1. INTRODUCTION

Glycogen metabolism is crucial for skeletal muscle (SKM) activity in humans, since glycogen hydrolysis supplies glucose to the muscle cells during muscle contraction. Glycogen synthesis requires a series of reactions that include glycogenin 1 (GYG1) self-glucosylation, which forms an oligosaccharide primer that serves as substrate for glycogen synthase (GS) [1].

Recently, in the search for proteins that interact with GYG1, the cDNA for a protein designated glycogenin-interacting protein (GNIP) was isolated from the human SKM cDNA library [2]. The gene for GNIPs was shown to generate four different transcripts: three transcripts that were designated GNIP1, GNIP2, and GNIP3 and another one identical to previously identified tripartite motif containing protein 7 (TRIM7). TRIM7 cDNA was initially identified and cloned from mouse and human [3], and was found to be mainly expressed in SKM in adults.

The tripartite motif (TRIM) protein family is defined by the presence of a tripartite motif composed of a RING domain, one or two B-box motifs and a coiled-coil region [4]. The structure-function analysis of GNIP/TRIM7 proteins revealed that GNIP1, GNIP2, and GNIP3 have in common (from the N- to C-terminus), a predicted coiled-coil region and a B30.2-like domain. GNIP2 and GNIP3 are truncated so that a shorter fragment of the coiled-coil domain is N-terminal. GNIP1 has a predicted N-terminal RING finger domain and a B box domain. The N-terminal 206 amino acids of TRIM7 are identical to those of GNIP1, including the predicted RING and a B box domain, and the C-terminus of TRIM7 bears a coiled-coil domain that is short and unique [5]. Thus, GNIP1 and TRIM7 have in common the N-terminal end, and are members of the TRIM protein family. TRIM proteins have been implicated in a broad range of biological processes and metabolic disorders, including obesity and insulin resistance in mouse [6] and human [7]. The activity of most E3 ligases is specified by a RING domain, which plays a critical role in ubiquitination [8]. Ubiquitination is a post-translational modification mechanism that controls protein levels through proteasome-mediated proteolysis and may also regulate protein activity and subcellular localization [9, 10]. Protein ubiquitination requires the participation of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. GNIP1 was recently characterized as an ubiquitin ligase [11], but the potential ubiquitin E3 ligase activity of TRIM7 (predicted by the RING domain) has not been demonstrated. Moreover, it has been found by yeast two-hybrid assay that GNIP2, which is not a member of the TRIM protein family, and GNIP1 interact with GYG1. Furthermore, GNIP2 was shown to stimulate GYG1 self-glucosylation *in vitro* [2], and the B30.2 domain was reported to mediate GNIP2 interaction with GYG1 [5]. All these data suggest that GNIP1 is an ubiquitin ligase that plays a role in glycogen metabolism in muscle, but its putative role has never been analyzed. In the present study, we described the pattern of expression of the GNIP/TRIM7 isoforms, determined the subcellular location of TRIM7 and GNIP1, and addressed the role of GNIP1 in glycogen metabolism by means of overexpression in SKM.

2. MATERIALS AND METHODS

2.1. Gene expression analyses

Total RNA from cultured cells was extracted with the RNeasy Minikit (Qiagen) and total RNA from gastrocnemius mouse muscle was extracted with TRIreagent (Bioline), following the manufacturer's instructions. Human SKM total RNA was from Applied Biosystems (FirstChoice Human Skeletal Muscle Total RNA #AM7982). Total RNAs were used to perform an RT-PCR using specific primers for human: TRIM7 (TRIM7 isoform 4), GNIP1 (TRIM7 isoform 1), GNIP2 (TRIM7 isoform 3), GNIP3 (TRIM7 isoform 2) or mouse: interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α). Human β -2 microglobulin or mouse adenine phosphoribosyltransferase (APRT) were used as a reference gene. Primer sequences used for RT-PCR are displayed in the Supplementary Methods (SM) S1.1.1.

2.2. GST-GNIP1, GST-TRIM7 and GST-GNIP2 recombinant proteins and the *in vitro* autoubiquitination assay

GNIP1 and TRIM7 cDNAs were cloned into the pGEX4T1 vector (Amersham) (cloning site: *XhoI* for GNIP1 and *EcoRI-SalI* for TRIM7), and GNIP2 was cloned into the pGEX4T2 vector (Amersham) (cloning sites: *XhoI-NotI*), to produce the GST recombinant proteins.

The pGEX-GNIP/TRIM7 constructions were transformed in BL21 codon plus (DE3) RP *Escherichia coli*. Protein expression was induced and *in vitro* ubiquitination reactions were carried out as described in [12].

The samples were subjected to immunoblotting and membranes were hybridized with appropriate antibodies. For further details, please refer to the SM S1.1.2.

2.3. Cytolocalization of the fusion construct pGNIP1-EGFP and pTRIM7-EGFP

Human GNIP1 and TRIM7 cDNA were cloned into the pEGFP-C3 (cloning site: *XhoI*) or pEGFP-C2 (cloning sites: *EcoRI-SalI*) vectors (Clontech) respectively to obtain pGNIP1-EGFP and pTRIM7-EGFP constructs. Colocalization analysis of GNIP1-GFP

and TRIM7-GFP fusion proteins with Golgi apparatus in LHCN-M2 myoblasts was performed as described in [13]. For further details, please refer to the SM S1.1.3.

2.4. Human tissues

Myoblast cell populations were isolated from a cryopreserved collection of human SKM biopsies from Hospital Sant Joan de Déu (Barcelona, Spain). Immunoblot analysis was performed using post-mortem muscle tissues from one subject. Immediately after excision, all tissue samples were frozen in liquid N₂. When stated, tissue samples were powdered in a mortar under liquid N₂ and thereafter homogenized in the appropriate buffer. For further details, please refer to the SM S1.1.4.

2.5. Ethics statement

The animal protocols were approved by the Universitat de Barcelona Animal Care and Use Committee (permit number: DMAH-5444). Animal sacrifice was performed under CO₂ anesthesia. The human tissue extraction protocol was approved by the ethics committee of University Hospital Joan XXIII (Tarragona, Spain) and Hospital Sant Joan de Déu (Barcelona, Spain) according to Spanish legislation at that time. The Ethics Committee of Hospital Sant Joan de Déu approved the waiver of consent for research. For deceased patients, relatives have given signed informed consent.

2.6. Glucose and insulin tolerance test

The glucose (GTT) or insulin (ITT) tolerance tests were performed on 15- or 12-week-old conscious mice respectively after 4 hours of fasting. The mice were injected intraperitoneally with a glucose bolus (2 g/kg of body weight) or an insulin bolus (1 unit/kg of body weight). Glucose was monitored via the tail before and at 15, 30, 60 and 120 min after glucose administration, as well as at 15, 30, 60 and 90 min after insulin administration. Animals were re-fed *ad libitum* after the test.

2.7. Cultured cells

Primary cultured myotubes came from a cryopreserved collection of human SKM biopsies described in 2.4. Myotubes were derived from confluent myoblast as described in [13]. 48 hours before RNA isolation, human myotubes were depleted of insulin and 24 hours before, they were depleted of FBS. The human SKM cell line LHCN-M2 was used as undifferentiated (myoblast) or differentiated (myotubes) for 5 days as described in [14]. 24 hours before the experiments, LHCN-M2 cells were incubated with basal medium (DMEM(25mM glucose) /M199 (4:1), 0.02 M Hepes and antibiotics). The human AC16 cardiac muscle (CM) cell line was grown as previously described in [15]. 293 cell line was grown in DMEM (25mM glucose) with 10% (v/v) FBS at 37°C in a humid atmosphere of 5% CO₂/95% air.

2.8. Transduction with recombinant adenoviruses

LHCN-M2 myotubes were transduced with recombinant adenoviruses expressing the cDNA of human GNIP1 (Ad-GNIP1), TRIM7 (Ad-TRIM7) (ABM Inc.) or GFP (Ad-GFP) [16] under the control of the CMV cytomegalovirus promoter at a multiplicity of infection of 20 for 2 hours, and then incubated for 24 hours.

For delivery to mice, Ad-GFP or Ad-GNIP1 were purified and injected in two gastrocnemius muscles of ICR-CD1 male mice from two separate batches (6 mice for Ad-GFP and 6 mice for Ad-GNIP1), as described in [17]. Thereafter, mice were fed with standard laboratory chow diet (2014 Tekland Global 14% Protein Rodent Maintenance Diet, Harlan Iberica) and water *ad libitum*. Animals were sacrificed at 16 weeks of age after an overnight fast. A blood sample was collected and tissues were excised and immediately frozen in liquid nitrogen. For further details, please refer to the SM S1.1.5.

2.9. Enzyme activities, immunoblotting and glycogen quantification.

The measure of GS and GP activities, immunoblotting, and glycogen content of gastrocnemius and LHCN-M2 samples was assessed as described in [18] by supplementing homogenization buffers with 10 mmol/l iodoacetamide to inactivate

deubiquitylases and 20 $\mu\text{mol/l}$ proteasome inhibitor MG132 (Sigma-Aldrich), except for glycogen measurement.

293 cell monolayers or human tissue extracts were subjected to immunoblot analysis as described in [13]. For human tissues, the homogenization buffer was supplemented with 40% (v/v) glycerol and 100 mmols/l dithiothreitol. Commercial antibodies used for membranes hibridation are displayed in the SM S1.1.6.

2.10. Glucose uptake

2-Deoxy-d-[1-3H] glucose uptake was assessed in LHCN-M2 myotubes. After adenovirus transduction, cells were incubated with basal medium without glucose for 3 hours. Then, glucose uptake was assayed as described in [19].

2.11. Statistical analysis

All data are given as means $\pm$ SEM, and the significance of the difference was analyzed by the student's t test. Values were considered significant at $p < 0.05$.

3. RESULTS

3.1. Expression pattern of GNIP/TRIM7 isoforms in human muscle tissues and cultured cells

The relative abundance of GNIP/TRIM7 transcripts was assessed in samples of human muscle tissue and cultured cells using $\beta 2$ -microglobulin as a reference gene (Figure 1A). In all tested SKM cultured cells, the GNIP1 mRNA level was always much higher than TRIM7. In contrast, in the cardiomyocyte AC16 cell line, GNIP1 mRNA was undetectable, whereas mRNA levels of TRIM7 were comparable to the ones measured in SKM. The mRNA levels of GNIP2 and GNIP3 were undetectable in all measured samples (data not shown).

The GNIP/TRIM7 protein isoforms profile was analyzed in human muscle tissues by immunoblot, using an antibody generated against a peptide sequence that is present in all GNIP/TRIM7 isoforms. As shown in Figure 1B, the strongest signal corresponding to a protein band of the same size as GNIP1 overexpressed in LHCN-M2 cells (Figure 1C) was observed for SKM tissue. In contrast, the intensity of a protein band of the predicted molecular size for TRIM7 (23.7 kDa) and the same size of TRIM7 overexpressed in LHCN-M2 cells (Figure 1D) was higher in CM. However, these bands were not observed in smooth muscle tissue (Figure 1B).

Therefore, these data suggest that, among the different GNIP/TRIM7 variants, GNIP1 (isoform 1) is the most abundant form in human SKM, whereas TRIM7 (isoform 4) is predominant in CM, both at the mRNA and protein levels.

3.2. Autoubiquitination activity of GNIP/TRIM7 proteins

Enzymatic activity of E3 ubiquitin ligases can be assayed through autoubiquitination of the protein *in vitro* in the absence of identified substrate. First, in order to find the optimum E2 enzyme for GNIP1/TRIM7 proteins, we performed the *in vitro* autoubiquitination assay on GST-TRIM7 using different E2 enzymes. As shown in Figure 2A, high molecular weight TRIM7 species indicative of autoubiquitination were primarily formed in the presence of UbCH5a and UbCH6 enzymes. Removal of the E2 enzyme abolished the reaction, which indicates that these high molecular weight TRIM7 forms were the products of a polyubiquitination reaction, catalyzed by TRIM7 E3 and UbCH5a or UbCH6 E2 enzymes.

Then, we tested the activity of GST fusion proteins of GNIP1 and GNIP2 (as a putative negative control), as well as TRIM7, for autoubiquitination with UbCH5a. We found that high molecular weight TRIM7 and GNIP1 species were formed in the presence of UbCH5a, while we did not observe the high molecular weight GST-GNIP2 species (Figure 2B). Membranes were stained with GNIP/TRIM7 antibody and an ubiquitin antibody. Both antibodies detect the same protein bands indicating that the specific bands are ubiquitinated species of GNIP1 or TRIM7 (Figure 2A and 2B). Together, these data indicate that TRIM7 and GNIP1 have autoubiquitination activity *in vitro*, whereas GNIP2 lacks this activity.

3.3. GNIP1 and TRIM7 intracellular location

We constructed a fusion protein of GNIP1 or TRIM7 with a C-terminal EGFP flag (GNIP1-EGFP or TRIM7-EGFP). Western blotting confirmed the correct expression and size of the fusion proteins (Figure 3A, B).

We analyzed the subcellular location of GNIP1 and TRIM7 proteins in LHCN-M2 myoblasts. TRIM7-EGFP protein appeared throughout the cytosol with a diffuse pattern, and was occasionally located in the nucleus (Figure 3C). In contrast, the GNIP1-EGFP protein showed a pattern clustered around the nucleus that was collocated with the Golgi marker. As a control, we transfected LHCN-M2 cells with a plasmid encoding EGFP, and we observed that this protein was located in the cytoplasm and nucleus and was not collocated with Golgi-targeted RFP.

3.4. Effect of GNIP1 overexpression on glycogen metabolism in mouse SKM

To analyze the possible impact of GNIP1 protein on glycogen metabolism, gastrocnemius mouse muscle was treated with the Ad-GNIP1 adenovirus to overexpress human GNIP1 or Ad-GFP adenovirus, as a transduction control. In an immunoblot analysis, the antibody against GNIP/TRIM7 proteins detected a band in Ad-GFP-treated muscle between 60-75 kDa (Figure 4A), which was much more intense in mouse muscle treated with Ad-GNIP1 (1320 ± 265 expressed as a percentage of the value obtained in gastrocnemius muscle of GFP-mice. $p \leq 0.001$ ($n=12$)). Importantly, human GNIP1 and GFP protein levels were undetectable in the liver of GNIP1- or GFP-mice respectively (data not shown). Therefore, we concluded that no systemic distribution of GNIP1 or GFP had occurred.

We analyzed changes in body weight and blood glucose levels at various time points after adenovirus transduction. To analyze whole-body insulin sensitivity in relation to GNIP1 overexpression, we also performed an ITT and a GTT. The body weight of GNIP-1 mice was statistically reduced by about 8% after day 85 post-adenoviral infection (Figure 4B). In the same way, from day 44, the blood glucose levels were about 15% lower in GNIP1-mice than in GFP-mice (Figure 4C). In contrast, whole-

body insulin and glucose tolerance were unaffected by GNIP1 gene delivery (Figures 4D, E). After overnight fasting, blood glucose levels remained lower in GNIP1-mice than in GFP-mice, although this difference did not reach statistical significance (Table 1A). Lactate levels were clearly decreased by GNIP1, whereas insulin levels were unaffected. Body weight was lower in GNIP1-mice than in GFP-mice (Table 1B) but we did not observe differences between groups in body weight/liver or adipose tissue ratios.

We next studied the effect of GNIP1 overexpression on GS and GP activities and its impact on glycogen accumulation and GYG1 protein content in gastrocnemius muscle. The activity of the total and active form of GS in GNIP1-mice gastrocnemius muscle increased about 34% (Figure 5A), and 22% (Figure 5B) respectively compared to control muscles, whereas GS activity ratio (-G6P/+G6P) was unaffected by GNIP1 (GFP-mice = 0.14 ± 0.02 ; GNIP1-mice = 0.12 ± 0.01 ; $p = 0.2$). No effects of GNIP1 overexpression were observed on GP activity (Figure 5C, D). In concordance with the GS activation, the glycogen levels in gastrocnemius muscle were 40% higher in GNIP1-mice than in GFP-mice (Figure 5E), whereas no significant changes were observed in GYG1 protein content (Figure 5F).

To decipher the molecular mechanism underlying GS activation, we investigated possible GNIP1-mediated regulation of the protein kinase B (Akt)-glycogen synthase kinase-3 (GSK-3)-GS signaling cascade. No changes were observed in total Akt, GSK-3 or GS protein content in relation to GNIP1 overexpression (Figure 6), but a significant increase in phospho-Akt/total Akt (47%) and phospho-GSK-3/total GSK-3 (53%) protein content ratios was observed. Accordingly, phosphorylation of GS in Ser 640/total GS ratio was about 30% lower in GNIP1 overexpressed muscle.

To determine whether GNIP1 can regulate pro-inflammatory mediators, the mRNA levels of TNF- α and IL-6 in gastrocnemius muscle from GFP- and GNIP1-mice were measured by real-time PCR. No changes in TNF- α ($2^{-\Delta\Delta C_t} = 1.05 \pm 0.16$, $p=0.89$ (n=9)) or IL-6 ($2^{-\Delta\Delta C_t} = 1.08 \pm 0.13$, $p=0.84$ (n=9)) gene expression were observed in GNIP1-mice compared to GFP-mice.

Thus, together these data suggest that GNIP1 overexpression in mouse muscle *in vivo* leads to decreased blood glucose levels, lactate levels and body weight, and activates

the Akt-GSK-3-GS signaling pathway and glycogen accumulation without altering the inflammatory status of gastrocnemius muscle.

3.5. Effect of GNIP1 on glucose transport and GS activity in LHCN-M2 myotubes

We analyzed whether these effects were accompanied by an increase in glucose transport through the cell membrane. To achieve this, we performed an adenoviral gene transfer of GNIP1, TRIM7 and GFP in cultured LHCN-M2 myotubes and determined 2-deoxyglucose uptake in the presence or absence of insulin.

Human GNIP1 and TRIM7 overexpression was confirmed by immunoblot, showing bands in Ad-GNIP1- and Ad-TRIM7-treated cells of about 60-75 kDa and 20-25 kDa respectively (Figure 7A), which were not found in cells treated with Ad-GFP adenovirus.

Next, we confirmed that basal glucose uptake was increased in response to GNIP1 (30%) and TRIM7 (20%) overexpression (Figure 7B). As expected, insulin increased 2-deoxyglucose uptake by 25% in myotubes treated with Ad-GFP. In the presence of insulin, GNIP1 overexpression further stimulated 2-deoxyglucose uptake (27%), whereas no effect of TRIM7 was observed under this condition. This indicates that TRIM7 increases glucose transport through the cell membrane, less than GNIP1 and in the absence of insulin only. However, GNIP1 can enhance glucose uptake additively to insulin.

To analyze whether Akt-GSK-3-GS pathway activation observed in GNIP1-mice gastrocnemius muscle is dependent on the phosphoinositide 3-kinase (PI3K), GS activity was measured in the presence and absence of insulin or/and LY294 (an inhibitor of PI3K) in LHCN-M2 cultured myotubes. Insulin increased GS activity ratio (-G6P/+G6P) similarly in Ad-GFP- and Ad-GNIP1-treated cells (Figure 8). As expected, activation of GS by insulin was blocked by LY294. GNIP1 overexpression caused a significant GS activation that did not reach statistical significance in LY294 treated cells. These data suggest that GS activation by GNIP1 is partially dependent on PI3K.

4. DISCUSSION

It is well known the importance of the interaction between GYG1 and GS for glycogen biosynthesis in muscle, and GNIPs have recently emerged as a proteins with a novel putative role in glycogen metabolism.

GNIP1 and GNIP2 are known to interact with GYG1 and GNIP2 to stimulate GYG1 self-glucosylation *in vitro* [2]. The B30.2 domain, which is present in GNIP1 and GNIP2, mediates interaction with GYG1 [5]. Autoglucosylation of GYG1 leads to the formation of a maltosaccharide chain that acts as the primer for GS and branching enzyme to accomplish glycogen synthesis [20]. We demonstrated here that GNIP1 overexpression in mouse SKM increased the glycogen accumulation and did not change GYG1 protein content. Thus, our data, together with previous studies, point to a likely interaction between GNIP1 and GYG1 that improve GYG1 self-glucosylation *in vivo*, which promotes glycogen synthesis without altering GYG1 protein content. Skurat et al. proposed that the stimulatory effect of GNIP2 on GYG1 self-glucosylation *in vitro* occurs via a change in the conformation of the GYG1 dimer that creates a better condition for attachment of glucose to the polysaccharide chain [2]. Thus, GNIP1 could exert this same action on GYG1 *in vivo*.

On the other hand, our results confirm that GNIP1 has E3 ubiquitin ligase activity that GNIP2 does not have. GNIP1 and TRIM7-deduced proteins have been found to contain an N-terminal RING domain [3], which suggests they could have E3 ubiquitin ligase activity. Recently, Chakraborty et al. characterized human GNIP1 as an ubiquitin ligase with a role in activator protein 1 (AP-1) activation via Ras, through an *in vivo* ubiquitination assay in 293T cells [11]. Here, for the first time, we demonstrate enzymatic activity as an E3 ubiquitin ligase for TRIM7, and confirm this activity for GNIP1 through *in vitro* autoubiquitination. Initially, ubiquitination was defined as a tag to promote protein degradation by the proteasome. However, other roles have emerged for ubiquitination, such as regulation of binding properties, subcellular localization and activity [9, 10, 21].

It is known that the coiled-coil domain of TRIM family proteins promotes the definition of discrete subcellular compartments within the cell [22]. Previous data suggest possible involvement of the TRIM proteins and their alternatively spliced isoforms in the regulation of compartmentalization [3]. Moreover, regulation by ubiquitination can occur via the subcellular localization of the enzymes, the substrates or via binding to

“auxiliary” proteins [23]. In this regard, we show that TRIM7 is mostly present in the cytosol and occasionally in the nucleus of cultured human SKM cells. This is in agreement with previous data, which determined TRIM7 localization in a diffuse cytoplasmatic and nuclear pattern in HeLa and U2OS cells [3]. Otherwise, the cellular localization of GNIP1, which has never before been described, was in the Golgi apparatus. Thus, we speculate that the different structure domain of GNIP1 and TRIM7 is presumably responsible for their different intracellular localization, and probably for their different function in SKM.

The human tissue expression profile of the GNIP/TRIM7 gene using a partial coding sequence for TRIM7 is reported to be most prominent in SKM [2]. However, the tissue distribution of GNIP/TRIM7 proteins in mouse extracts analyzed using antibodies generated against the C-terminal B30.2 domain present in GNIP1, GNIP2, GNIP3 (not in TRIM7) revealed a 55 kDa species (the same size as GNIP1 expressed in COS cells) in several tissues, and relatively low levels in SKM [5]. In the present study, using an antibody against a sequence in the N-terminal part of GNIP1, GNIP2 and GNIP3 and the C-terminal part of TRIM7, we detected stronger signals for these proteins in human SKM and CM samples than in smooth muscle tissue. Specifically, GNIP1 showed the strongest signal in SKM, whereas for TRIM7 the highest content was observed in CM. In agreement with these results, GNIP1 mRNA levels were higher than TRIM7 in human SKM cultured cells, whereas in human cardiomyocytes only TRIM7 was expressed.

The main glycogen stores are confined to SKM and liver in humans, although SKM is the major tissue where insulin stimulates glucose uptake to store glycogen and remove glucose from the blood [24]. Insulin regulates glucose metabolism by the phosphorylation of the insulin receptor substrate 1 (IRS1), which causes PI3K activation and a subsequent phosphorylation cascade that activates and phosphorylates Akt. The activated phospho-Akt then inactivates GSK-3 by phosphorylation, which is involved in GS regulation by phosphorylation at site 3a [25, 26]. We observed an increment in GS activation by dephosphorylation in ser 640, accompanied by an activating effect on the insulin Akt-GSK-3 signaling cascade in GNIP1-mice, and an increment in glucose uptake in GNIP1-overexpressed cultured LHCN-M2 myotubes. It is known that some ubiquitination processes are involved in the regulation of glycogen accumulation. For instance, the RING-type E3 ubiquitin ligase malin ubiquitinates GS

and GP in cultured cells, and regulates expression and phosphorylation levels of GP in mouse [27]. However, it has never been demonstrated that malin directly regulates glycogen metabolism by controlling the GS activation state or phospho-GS or total GS protein content levels in mouse muscle *in vivo* [28]. Our results showed that GNIP1, on the contrary, did not elicit any effect on GP activity, but increased GS activation as well as Akt and GSK-3 phosphorylation levels in mouse. Furthermore, our findings in LHCN-M2 cultured cells suggest that GNIP1-mediated activation of GS is partially dependent on PI3K. This upregulation of the insulin signaling pathway by GNIP1 might be occurring through an ubiquitination process of a protein directly or indirectly involved in this pathway. Other ubiquitin ligases located in Golgi apparatus can regulate metabolic signaling pathways by ubiquitin-mediated modification of molecules involved in up- or down-regulation of these pathways [29, 30]. Currently, RACO-1 is the only substrate described for GNIP1, which stimulates c-Jun/AP-1 activation [11]. c-Jun N-terminal kinases have been considered signaling molecules, linking inflammation and insulin resistance [31], and have been identified as a substrate of GSK-3 [32, 33]. It has been described that some E3 ubiquitin ligases regulate insulin signaling through targeting pro-inflammatory mediators involved in insulin resistance (review [34]). In this regard, although more pro-inflammatory markers should be assessed to confirm our results, they might suggest that pro-inflammatory mediators expression is not modulated by GNIP1 in mouse muscle.

Recently, TRIM family proteins were found to be implicated in the regulation of insulin pathway. An example is the muscle-specific MG53 (also called TRIM72) that mediates the degradation of IRS1 and, when upregulated in mouse, causes metabolic syndrome featuring insulin resistance, obesity, hypertension and dyslipidemia [6]. Our results showed that GNIP1, even though it was only overexpressed in gastrocnemius muscle, decreased mouse body weight and blood circulating glucose and lactate levels. We propose that this effect is due, in part, to GNIP1-mediated activation of the Akt-GSK-3-GS signaling cascade and subsequent glycogen synthesis in muscle. In a recent study, muscle-specific GS deletion in adult mice showed increased lactate levels, decreased muscle glycogen content and glucose intolerance [35]. However, in other studies, modification of GS activity and muscle glycogen stores in muscle resulted in normoglycemic animals [36, 37]. Our findings showed no effects of GNIP1 on glucose tolerance in mouse. Therefore, other mechanisms might mediate the reduced blood

glucose and lactate levels in Ad-GNIP1 treated-mice. Previous studies in human have found that muscles break down glycogen to lactate during intense exercise, and during the recovery period, muscle glycogen reappears while lactate disappears [38]. Our results showed that GNIP1 is mainly expressed in SKM in humans and Zhai et al. [5] have proposed that GNIP1 might be a factor responsible for controlling the rate of reassociation of GYG1 and GS during glycogen-resynthesis in muscle. Therefore, we speculate that GNIP1 overexpression in gastrocnemius mouse muscle could be mimicking certain processes associated to the recovery period after exercise.

In human muscle cultured cells GNIP1 increased glucose uptake additively to insulin. However, a lack of effect of GNIP1 on whole-body insulin tolerance was observed in mice. The reason for this discrepancy may be that some processes associated to the *in vivo* model could not be happening in cultured cells. We find several similarities between our findings and the results obtained by Ruisheng S. et al. [6]. In their study, MG53 deficiency markedly attenuated high-fat diet-induced metabolic syndrome. However, no effects were observed in animals fed with a standard chow diet, except for a reduction in blood glucose concentration. So, we should also consider that dietary intervention could be important to detect the potential effects of GNIP1 on insulin or glucose response in mouse.

We observed a GNIP1-mediated increase of GS activity ratio (-G6P/+G6P) in human LHCN-M2 cells that was not detected in mouse. However, the activity of the total (+G6P) and active (-G6P) form of GS was increased in GNIP1-mice, without altering GS protein content. An unusual disconnection between total GS activity and GS protein content has been already observed by other authors [39, 40]. The reason for this effect is unclear but it may reflect a mechanism of GNIP1 to modify the total enzyme activity. It is also possible that other mechanisms may make GS more sensitive to G6P in GNIP1-mice muscle.

The activation of the insulin signaling pathway by GNIP1 is in accordance with the observed increment in glucose uptake in cultured cells. It is known that Akt plays a role in directing GLUT4 vesicles to the plasma membrane and thus promotes glucose transport in 3T3-L1 adipocytes [41]. Constitutive activation of Akt in L6 muscle cells, also stimulates the uptake of glucose and promotes the inactivation of GSK-3 and glycogen synthesis [42]. Therefore, our data suggest that GNIP1 can enhance glucose transport and, at least in part, GS activity by increasing phospho-Akt levels. However,

since GNIP1 was capable of stimulating glucose uptake in LHCN-M2 cells, both in the presence and absence of insulin, we cannot discard a possible non-insulin dependent action of GNIP1 on basal glucose uptake, more dependent of GLUT1 in SKM [43].

Together, our results suggest that defects in GNIP1 in skeletal muscle could be involved in pathogenesis and insulin resistance. However, further studies are needed to determine whether GNIP1 could be considered a treatment option for diabetes and obesity.

In summary, the present study demonstrates that GNIP1 can stimulate glycogen synthesis and promote the insulin signaling pathway activation and glucose uptake in SKM. This indicates that GNIP1 is an E3 ubiquitin ligase enzyme with a new, emerging role in glycogen metabolism, that could be a potential therapeutic target for the treatment of obesity or type 2 diabetes.

FIGURE LEGENDS

FIGURE 1. GNIP/TRIM7 expression in human tissues. (A) The relative expression of TRIM7 and GNIP1 genes was analyzed in human SKM, primary cultured myotubes (PCMT), LHCN-M2 (MB= myoblast, MT=myotube) and AC16 cells by RT-PCR. Percentage of mean values of $2^{-\Delta Ct} \pm SEM$ are shown. The significance of differences versus SKM: * $p < 0.05$; ** $p < 0.01$ and versus GNIP1 in the same tissue or cell type: † $p < 0.05$; ‡ $p < 0.01$. (B) Immunoblot analysis was performed on extracts from the indicated human muscle tissues or (C) from human skeletal muscle and cultured LHCN-M2 myoblasts treated with Ad-GFP or Ad-GNIP1 or (D) from human cardiac muscle and cultured LHCN-M2 myoblasts treated with Ad-TRIM7. Membranes were hybridized with antibodies against GNIP/TRIM7 (B, C, D) and α -actinin (C, D). GNIP1 and TRIM7 (B) protein bands are indicated by an arrow.

FIGURE 2. Autoubiquitination assay on TRIM7, GNIP1 and GNIP2. (A) GST-TRIM7 or (B) GST-GNIP1, GST-GNIP2 and GST-TRIM7 were incubated in the ubiquitination reaction mix, without (-) or with the indicated human E2 enzymes. Immunoblot was performed and membranes were hybridized with indicated antibodies. A representative image is shown.

FIGURE 3. Cytolocalization of GFP-GNIP1 and GFP-TRIM7. 293 cells were transfected with pEGFP, pEGFP-GNIP1 or pEGFP-TRIM7 and immunoblot analysis was performed using (A) anti-GNIP/TRIM7 or (B) anti-GFP antibodies. (C) LHCN-M2 myoblasts were co-transfected with pRFP1-N1-GalT (Golgi marker) and (a to d) pEGFP (e to h) pEGFP-TRIM7 or (i to l) pEGFP-GNIP1. The image shows the fluorescent signal of Hoechst (a, e, i), EGFP (b, f, j), RFP (c, g, k) and the colocalization of the signal of Hoechst, EGFP and RFP (d, h, l).

FIGURE 4. Effect of GNIP1 on mouse. (A) Immunoblot analysis was performed on gastrocnemius extracts from GFP- and GNIP1-mice using antibodies against GNIP/TRIM7 and actin. Body weight (B) and blood glucose levels (C) were measured on the days indicated. An (D) ITT or (E) GTT was performed. Data are mean $\pm$ SEM (n=6). Significance of differences versus GFP-mice: *p < 0.05; **p < 0.01.

TABLE 1. Body and tissue weight, blood metabolites and insulin levels in GFP- and GNIP1-mice. (A) Blood glucose, lactate and insulin levels and (B) body (Bw), liver (Lw) and peritoneal white adipose tissue (Aw) weight were measured in overnight fasted mice at 113 days of age. Data are mean $\pm$ SEM (n=6). Significance of differences versus GFP-mice: *p < 0.05.

FIGURE 5. Effects of GNIP1 on overexpressed mouse muscle. Gastrocnemius muscle extracts from GFP- and GNIP1-mice were used to perform total (A) GS or (C) GP activity and active (B) GS or (D) GP activity measures, (E) glycogen content and (F) immunoblot analysis with anti- GYG1 and anti-actin antibodies. Data are means $\pm$ SEM (n=12). Significance of differences versus GFP-mice muscle: *p < 0.05; **p < 0.01.

FIGURE 6. GNIP1 effects on the Akt-GSK-3-GS signaling cascade. Immunoblot analysis was performed on gastrocnemius muscle extracts from GFP- and GNIP1-mice using antibodies against actin, GS, Akt, GSK-3 α/β and phospho-GS (Ser641/0), phospho-GSK-3 α/β (Ser21/9) and phospho-Akt (Ser473). Data are mean $\pm$ SEM of phospho-protein /total protein percentage of GNIP1-mice versus GFP-mice band intensity ratio (n=12). Significance of differences versus GFP-mice muscle: *p < 0.05; **p < 0.01.

FIGURE 7. Effect of GNIP1 and TRIM7 on glucose uptake. Overexpressed cultured LHCN-M2 myotubes were used to (A) perform an immunoblot analysis using antibodies against GNIP/TRIM7 and GAPDH or α -actinin or (B) a glucose uptake measure in the presence or absence of insulin. Data are mean $\pm$ SEM (n=8). Significance of differences versus cells treated with Ad-GFP under the same incubation conditions: *p < 0.05; **p < 0.01; or without insulin under the same transduction conditions: † p < 0.05.

FIGURE 8. Effect of GNIP1, insulin and LY294 on GS activity in LHCN-M2 cells. LHCN-M2 myotubes were transduced with Ad-GFP or Ad-GNIP1 and incubated with 25 mM glucose for 24h. Then, cells were incubated with glucose-deprived medium for 3 h. Finally cells were incubated with 10 mM glucose and with DMSO(control) or 10 μ M LY294 inhibitor (Sigma-Aldrich) and/or 100 nM insulin during 30 min. GS activity ratio (without glucose 6-phosphate / with glucose 6-phosphate) was measured in cell extracts. Data are means $\pm$ SEM (n=5). Significance of differences versus cells treated with Ad-GFP (DMSO): *p < 0.01; **p < 0.001; or versus cells treated with insulin under the same transduction conditions: †p < 0.05; ‡p<0,01

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Conflicts of interest

The authors declare that there are no conflicts of interest associated with this manuscript.

Authors' contributions

MM carried out the gene expression and protein content analysis of human samples, the transduction with recombinant adenoviruses; the plasmids construction, the glucose uptake and enzyme activity assays and the glycogen and lactate content measurement. MM also designed and coordinated the work of RP and ZB, carried out the statistical analysis, drew up figures, drafted the manuscript and participated in ubiquitination studies and experiments in mouse. RP carried out the protein content analysis of mouse samples and participated in mouse experiments, activity assays and figures. ZB carried out the preparation of pEGFP-GNIP1 construct and performed the analysis of cytolocation. IL carried out the ubiquitination studies and participated in plasmid construction. AO carried out the primary cell cultures and cell lines growth. JC and JCJ carried out the *in vivo* metabolite measures and performed insulin and glucose tolerance test and tissue excision in mouse. CG carried out the transduction with recombinant adenoviruses *in vivo*. OO carried out the preparation of human tissue extracts for immunoblot analysis. MRC carried out the human tissue excision. JV coordinated human tissue sample supply. MVC carried out the AC16 cell growth and mRNA extraction. SD carried out the gene expression analysis design and participated in the ubiquitination studies. AMG conceived the study and carried out its design and coordination until March 2015. CG, JCJ, MVC and SD collaborated in drafting the manuscript.

All authors have read and approved the final manuscript.

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Table 1

A

	Glucose (mg/dl)	Lactate (mg/dl)	Insulin (ng/ml)
GFP-mice	49.17±3.06	27.90±2.14	0,71±0.18
GNIP1-mice	41.6±5.13	20.50±1.80*	0,79±0.12

B

	Body weight (g)	Liver weight (g)	Adipose weight (g)	tissue Bw/Lw (%)	Bw/Aw(%)
GFP-mice	36.91±0.80	1.33±0,11	0.30±0.09	3.59±0.23	0.82±0.22
GNIP1-mice	33.46±1.16*	1.17±0.04	0.23±0.06	3.51±0.19	0.70±0.18

Highlights

- GNIP1 protein content is higher than TRIM7 in human skeletal muscle.
- GNIP1 and TRIM7 have autoubiquitination activity *in vitro*.
- GNIP1 and TRIM7 localize in the Golgi apparatus and cytosol respectively.
- GNIP1 is an ubiquitin ligase with a glycogenic effect in skeletal muscle.

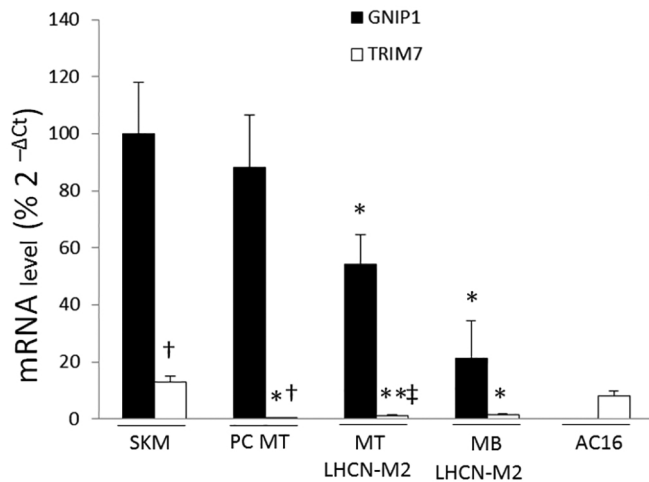
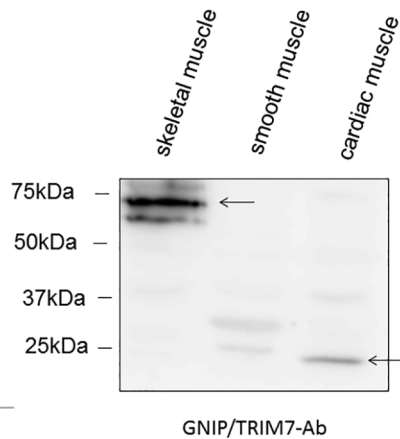
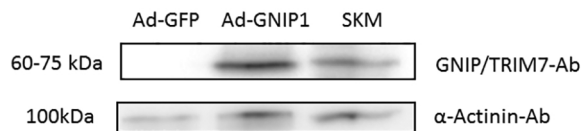
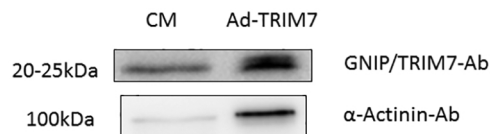
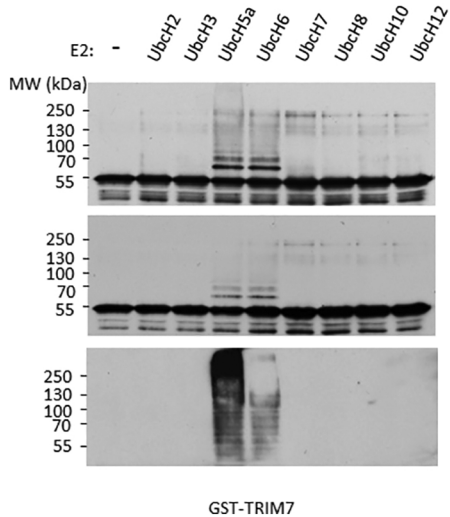
A**B****C****D**

Figure 1

A



B

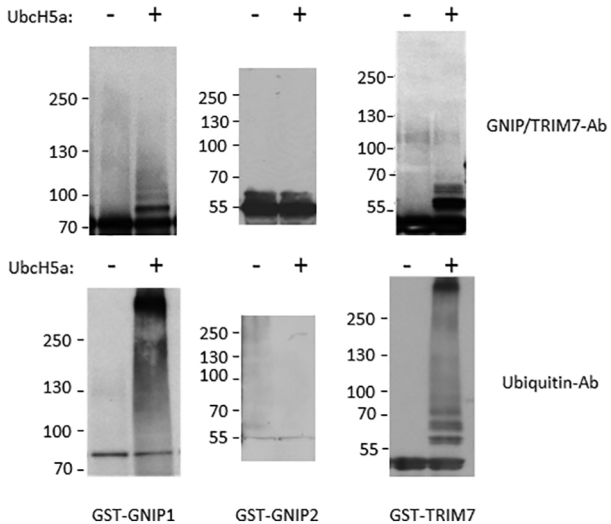
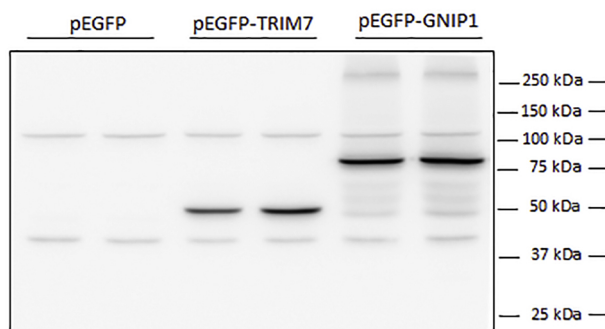
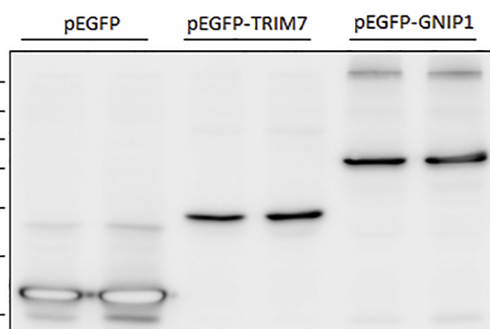


Figure 2

A



B



C

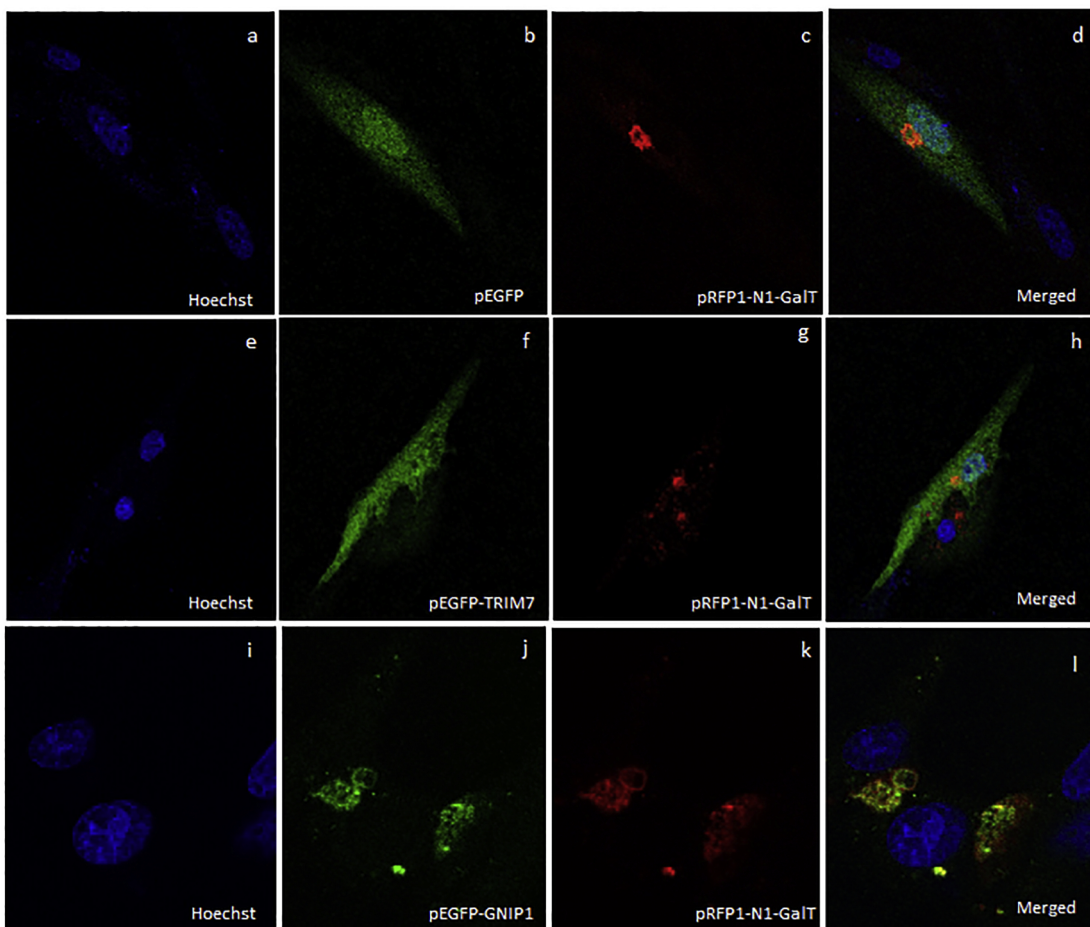


Figure 3

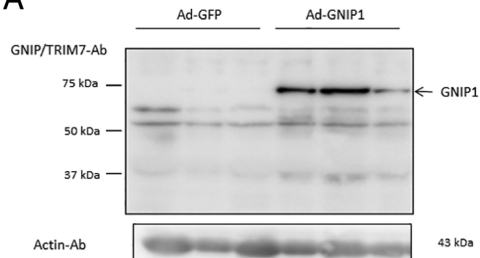
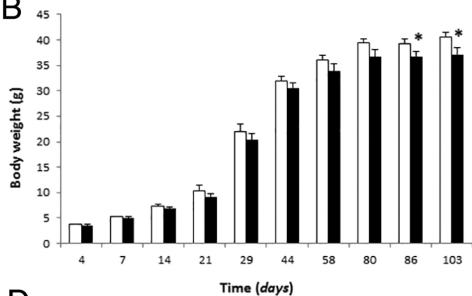
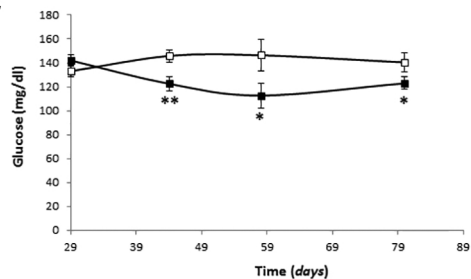
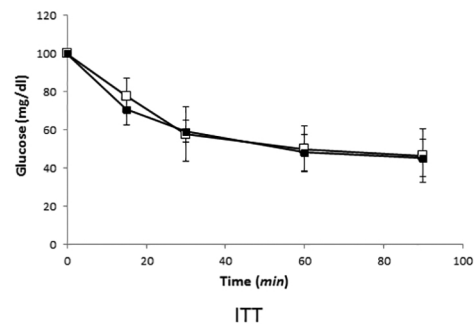
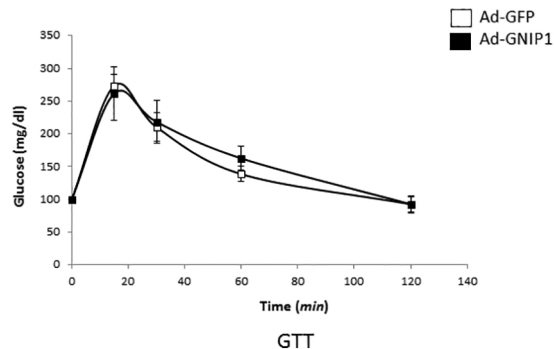
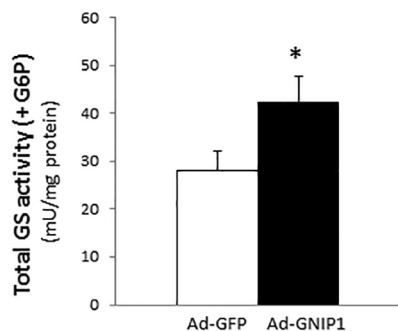
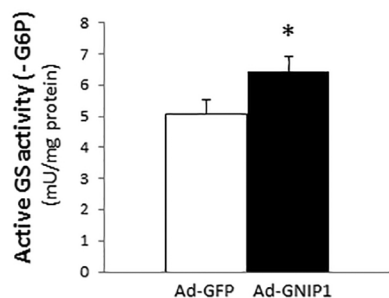
A**B****C****D****E**

Figure 4

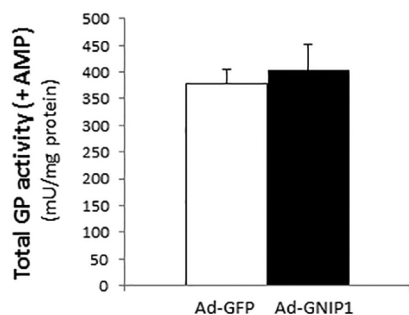
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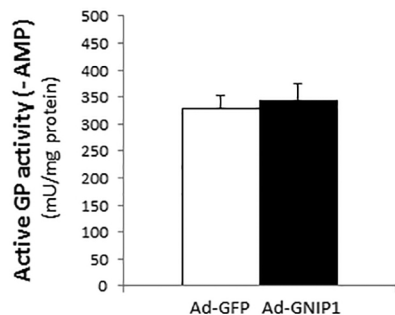
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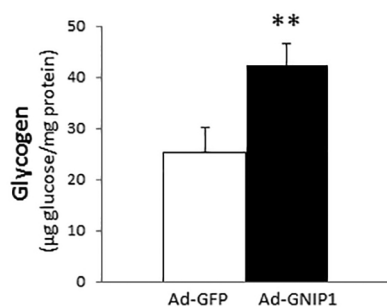
C



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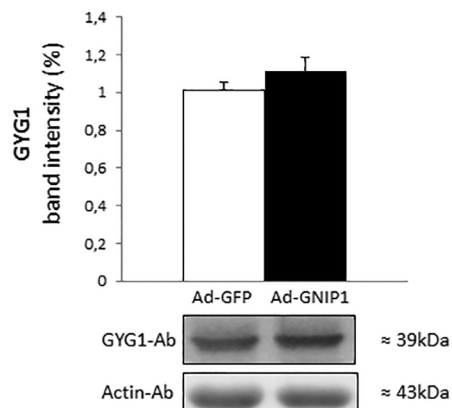


Figure 5

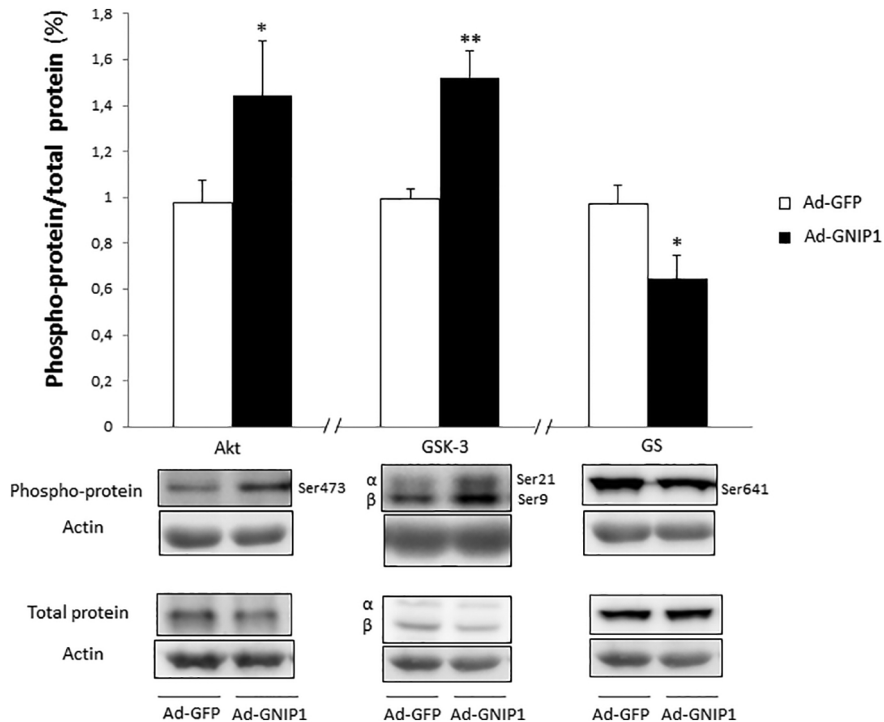
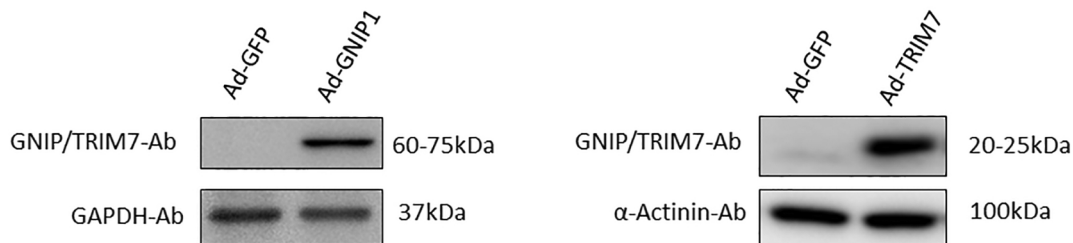


Figure 6

A



B

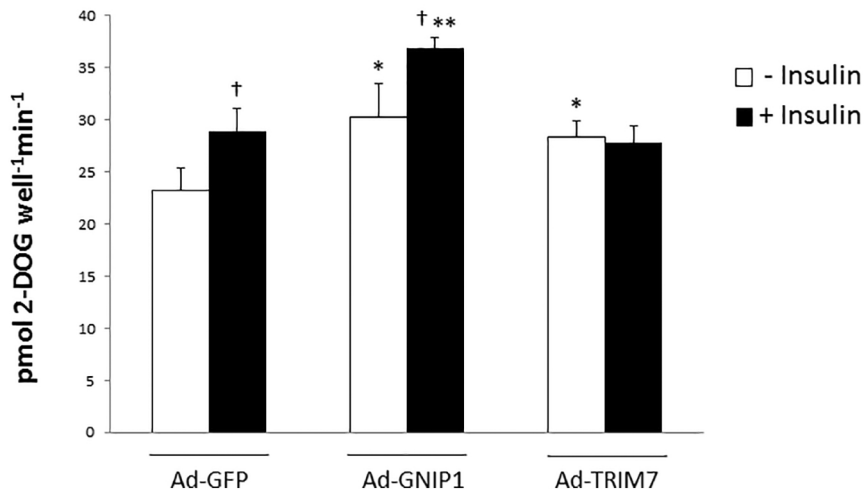
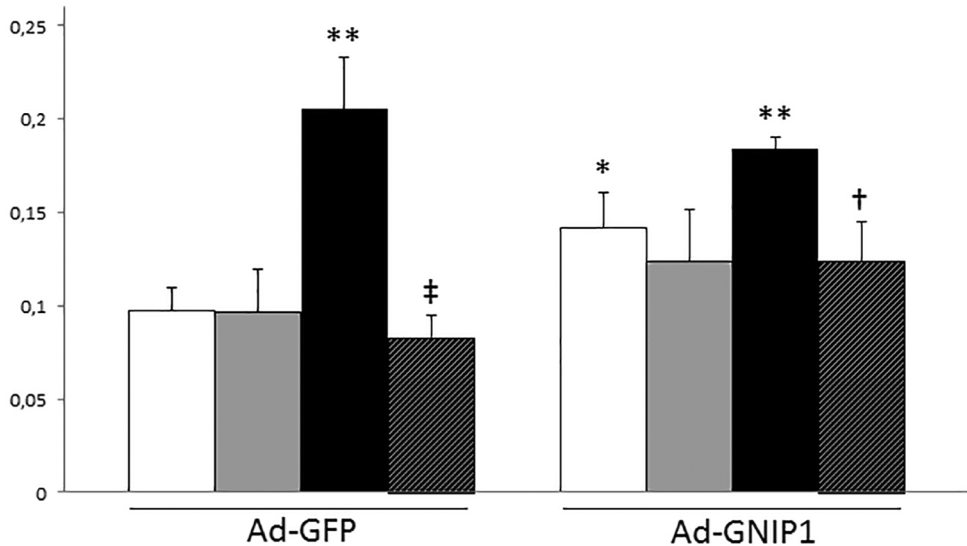


Figure 7

GS activity ratio

(-G6P/+G6P)



Insulin

- - + +

LY294

- + - +

- - + +

- + - +

Figure 8