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The Akt substrate Girdin is a regulator of insulin signaling in myoblast cells

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ABSTRACT

Akt kinases are important mediators of the insulin signal, and some Akt substrates are directly involved in glucose homeostasis. Recently, Girdin has been described as an Akt substrate that is expressed ubiquitously in mammals. Cells overexpressing Girdin show an enhanced Akt activity. However, not much is known about Girdin's role in insulin signaling. We therefore analyzed the role of Girdin in primary human myotubes and found a correlation between Girdin expression and insulin sensitivity of the muscle biopsy donors, as measured by a hyperinsulinemic–euglycemic clamp. To understand this finding on a cellular level, we then investigated the function of Girdin in C2C12 mouse myoblasts. Girdin knock-down reduced Akt and insulin receptor substrate-1 phosphorylation. In contrast, stable overexpression of Girdin in C2C12 cells strikingly increased insulin sensitivity through a massive upregulation of the insulin receptor and enhanced tyrosine phosphorylation of insulin receptor substrate-1. Furthermore, Akt and c-Abl kinases were constitutively activated. To investigate medium-term insulin responses we measured glucose incorporation into glycogen. The Girdin overexpressing cells showed a high basal glycogen synthesis that peaked already at 1 nM insulin. Taken together, we characterized Girdin as a new and major regulator of the insulin signal in myoblasts and skeletal muscle.

1. Introduction

Activation of the insulin receptor (IR) and the subsequent signaling processes regulate cellular homeostasis and whole body metabolism [1]. The development of insulin resistance, that is the less efficient propagation of the insulin signal, is a hallmark of diabetes. Thus, all signaling processes that affect the efficiency of the insulin signal are of high significance. Insulin signaling starts with binding of insulin to its receptor, leading to receptor auto- and substrate phosphorylation [2]. Along the major axis of signaling the insulin receptor substrate (IRS) proteins are phosphorylated and bind the phosphatidylinositol-3-kinase (PI3K) that then generate phosphatidylinositol-3-phosphate leading to Akt kinase activation and mediating glucose transporter 4 translocation to the cell membrane for glucose uptake into skeletal muscle [3]. Akt activity is mainly regulated by upstream kinases phosphorylating T308 (PDK-1) or S473 (mTORC2) and the dephosphorylation of these residues by PP2a and PHLPP, respectively [4]. Via phosphorylation of the glycogen synthase kinase 3 (GSK3) Akt directly affects glycogen synthesis and via phosphorylation of AS160 it activates glucose transporter 4 translocation. Impairment of this signaling cascade plays a major role in the development of insulin resistance and diabetes mellitus [5].

Recently, Girdin, also named Akt phosphorylation enhancer (APE) or Gα-interacting vesicle-associated protein (GIV), was identified as an Akt or Gα binding protein in yeast two hybrid screens [6–8] or by computer homology search for Hook related proteins (HkRP1) [9]. So far, the main function ascribed to Girdin is to enhance and prolong the Akt kinase activity although the mechanism is still unclear. Two different signaling processes were proposed to be employed by Girdin [10,11]: (i) Girdin was identified as a non-receptor guanine nucleotide exchange factor for $G\alpha_i$ proteins. Through binding and activation of G proteins, Girdin induces the release of the Gβγ subunit which in turn binds and activates p110γ–PI3K. (ii) It was shown that Girdin interacts with activated receptor and non-receptor tyrosine kinases and binds and activates the p85α subunit of PI3K. Girdin is tyrosine phosphorylated at the carboxy-terminus and may stabilize the receptor tyrosine kinase–PI3K complex and amplify the signaling. Ghosh and colleagues also showed that in Hela cells which overexpress Girdin, the epidermal growth factor receptor (EGFR) had an elevated tyrosine phosphorylation but a decreased internalization after ligand binding compared to control cells [12].

Although Girdin was initially characterized by enhancing the activity of Akt upon overexpression, a role in insulin signaling or insulin sensitive tissues has not been clarified. To investigate the role of Girdin for insulin signaling we tested primary myotubes and observed a

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correlation between Girdin expression and insulin sensitivity of the muscle biopsy donors. We then set out to identify the signaling processes behind this finding and turned to C2C12 myoblast cells. Girdin knock-down decreased insulin sensitivity in C2C12 myoblasts but stable overexpression of Girdin led to a massive increase in insulin sensitivity. Our data indicate that Girdin is a critical regulator of insulin sensitivity in the skeletal muscle cell line C2C12 and human muscle.

2. Material and methods

2.1. Ethics statement

The study adhered to the Declaration of Helsinki, and was approved by the Ethics Committee of the Medical Faculty of the Eberhard Karls University Tübingen. All participants gave their written consent to the study.

2.2. Materials

Oligonucleotides were synthesized by Metabion. Commercial antibodies: α-phospho-Akt Thr308 (#9275), α-phospho-GSKα/β Ser21/9 (#9331), α-phospho-Erk1/2 Thr202/Tyr204 (#9106), α-Erk1/2 (#9102), α-phospho-p70/p85 S6K Thr389 (#9206) and p70/p85 S6K (#9202) were purchased from Cell Signaling; α-phospho-IRS-1 Tyr989, α-GSKα/β (#7291/0011-A) and α-c-Abl (#23/24–11) were obtained from Santa Cruz; α-Akt was obtained from BD-Transduction (#610861); α-IRS-1 (#06-248) and α-phosphotyrosine (4G10) were obtained from Millipore; and α-phospho-IR Tyr972 was obtained from Assay Designs. The antibody against Girdin was raised against the coiled coil domain (aa 783–988); IR- and CrkII antibodies against a carboxy-terminal peptide and GST-IRS-1 antibody against a GST-fusion protein containing the carboxy-terminal 300 amino acids.

2.3. Cell culture and transfection

C2C12 murine myoblasts (ATCC, CRL-1772) were cultivated in DMEM (Lonza) containing 4.5 g/L glucose, 2 mM glutamine and 10% FCS (Thermo Scientific). Cells were plated and grown until approximately 80–90% confluence, serum starved (DMEM 0.5% FCS) overnight and stimulated for 15 min with different concentrations of insulin (Eli Lilly). STI-571 experiments were performed by pretreating the cells with 50 μM STI-571 (Cayman) for 24 h or 48 h. For glycogen synthesis and receptor internalization assay cells were seeded at a density of 1–2 × 10^5 cells/6-well and cultivated for 24 h followed by individual protocols (see below). Primary human skeletal muscle cells were obtained from needle biopsies of the vastus lateralis muscle of thirty-two mostly young healthy volunteers. Cells were grown as described [13]. Measurement of gene expression was done in first-pass cells after growth to 80–90% confluence and five days of differentiation to myotubes [14]. The differentiation media contained 2% fetal calf serum and 1 mg/L glucose.

Human Girdin cDNA was cloned into the expression vector pRK (Clontech) and cotransfected with the pSV2neo vector into C2C12 cells using a calcium phosphate precipitation method adapted from Chen and Okayama [15]. SiRNA transfection with DharmaFECT was performed according to the manufacturer's instructions (Dharmacon).

2.4. Cell lysis and Western blot analysis

Cells were washed in PBS and lysed in lysis buffer as described [16]. After protein measurement, SDS loading buffer [3% (w/v) SDS, 3% (v/v) β-mercaptoethanol, 10 mM EDTA (pH 8), 20% (v/v) glycerol, 0.05% (w/v) bromphenol blue] was added and samples were boiled for 5 min at 95 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filter. The membranes were blocked for minimum 1 h in Net-G [150 mM NaCl, 5 mM EDTA (pH 8), 50 mM Tris–HCl (pH 7.5), 0.05% (v/v) Triton X-100, 0.25% (w/v) gelatine] and incubated with primary antibody overnight at 4 °C. Proteins were detected by horseradish peroxidase conjugated secondary antibody and using an ECL reagent (Thermo Scientific). For quantification by densitometric scanning we used ImageJ software (1.46r).

2.5. Glycogen synthesis and IR internalization assay

Glycogen synthesis in C2C12 cells was done as described [17]. To determine IR internalization, C2C12 cells were incubated with 0.03 μCi 125-I-Insulin (Perkin Elmer) at 4 °C for 1 h in HEPES buffered saline, washed three times with cold HEPES buffer and incubated for 10 and 30 min at 37 °C. Cells were then placed on ice, washed two times with acetic acid (0.2 M) and the acid washes collected. Afterwards, cells were lysed with 30% (w/v) NaOH and the radioactivity of acid washes and lysates was determined by gamma counting. Receptor internalization was expressed as percentage of total insulin (bound and internalized) at time point zero.

2.6. RNA isolation, RT-PCR and real-time quantitative PCR analysis

Cells were washed once with PBS, lysed with RLT buffer and homogenized using the QIAshredder (Qiagen) according to the manufacturer's instructions. Reverse transcription of total RNA (1 μg) was performed in a volume of 20 μl using random hexamers with the First Strand cDNA Synthesis Kit for RT-PCR (Roche). Aliquots of 2 μl of the reverse transcription reactions were used for quantitative PCR with the Roche LightCycler 1.5 with SYBR® Green. The following primers were used: IR sense: TCTGAAATTCACAAGATGGAAGAA and antisense: CCGAATGAAAGAAAATTTAAGCA. The quantitative PCR was performed in triplicate in a total volume of 20 μl containing 2 μl FastStart DNA-Master SYBR Green I (Roche), 4 mM $MgCl_2$, and primers at a concentration of 1 μM. After denaturing at 95 °C for 10 min, amplification was performed by denaturing at 95 °C for 15 s, annealing at 60 °C for 10 s, and elongation at 72 °C for 6 s. The number of cycles was 50. The mRNA content is given in relative arbitrary units.

For qPCR analysis of Girdin expression in primary human skeletal muscle myotubes, RNA was extracted and transcribed as mentioned before. Then, qPCR was performed in duplicate with fluorescence-labeled probes from the Roche Universal Probe Library on a Roche LightCycler 480 according to the manufacturer's instruction. The following primers were used: Girdin sense: CAAAAGGCTGCAACAA GAGA and antisense: CTGCTTTCTCTCGAAGTGCAT and RPS13 sense: CCCCACTTGGTTGAAGTTGA and antisense: ACACCATGTGAATCTCT CAGGA. These quantitative mRNA data were normalized to the housekeeping gene *RPS13* using the ΔC_t method.

2.7. Statistical analysis

Statistics were done by analysis of the standard deviation, followed by Student's *t* tests. For the statistical analysis of the human data, all variables with non-normal distribution were $\log_e$-transformed before linear regression analysis. The least-squares method was used for a multiple linear regression analysis. In the regression model, insulin sensitivity was chosen as outcome variable, Girdin mRNA expression as independent variable, and gender, age, and BMI as possible confounding variables. The significance threshold was set to $p < 0.05$. The statistical software package JMP 8.0 (SAS Institute) was used.

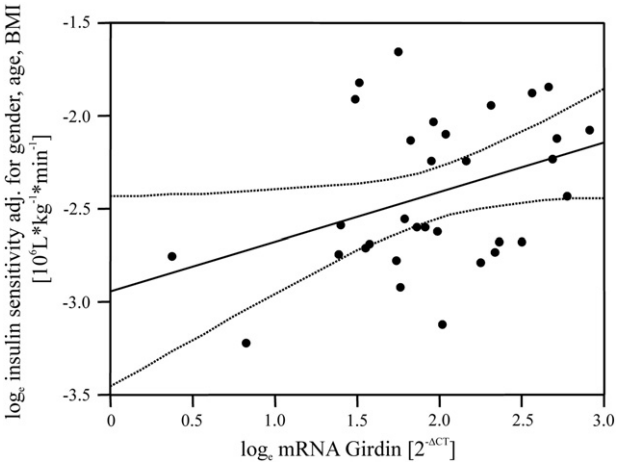

Fig. 1. Girdin expression correlates with insulin sensitivity. Analysis of mRNA expression of Girdin in primary human myotubes was measured by real time quantitative PCR. Insulin sensitivity of participants of the study (n = 32; male 20/female 12) was assessed by a hyperinsulinemic–euglycemic clamp and adjusted to gender, age (median = 25 y) and BMI (median = 22.88 kg/m^2). All parameters were log$_e$-transformed before linear regression analysis (r = 0.369; p = 0.0378).

3. Results

3.1. Girdin — a parameter for insulin sensitivity in humans

Since Girdin was identified as a protein enhancing Akt activity and Akt is a crucial enzyme of the insulin signaling cascade, we investigated the relevance of Girdin expression for insulin sensitivity in primary human myotubes. To this end, we performed a hyperinsulinemic–euglycemic clamp in mostly young healthy volunteers according to the method described by Stefan et al. [13]. Additionally, the vastus lateralis muscle of the participants was biopsied. The primary human skeletal muscle cells obtained from the biopsies were grown and differentiated into myotubes. Girdin expression was measured by quantitative PCR and normalized to the housekeeping gene RPS13. Fig. 1 shows the association of Girdin expression with the participants' insulin sensitivity adjusted for gender, age and BMI. Increased insulin sensitivity correlated with an increased expression of Girdin in human skeletal muscle. These data suggest that Girdin expression is an important parameter of insulin sensitivity in skeletal muscle cells.

3.2. Girdin knock-down decreases insulin sensitivity in C2C12 cells

To better understand how Girdin may improve insulin sensitivity we used C2C12 myoblasts to study consequences of changes in Girdin expression for insulin signaling. To verify the importance of Girdin in these cells we first knocked down endogenous Girdin by transfecting cells with Girdin specific siRNA. Western blot analysis of the lysates of transfected cells indicated a reduction of Girdin protein by 60% whereas the control siRNA transfected cells showed no Girdin protein decrease (Fig. 2). Analysis of the IRS-1 protein revealed that a decrease in Girdin expression also led to a reduction in IRS-1 protein, and upon insulin stimulation tyrosine phosphorylation was reduced as well. Consequently, Akt phosphorylation at T308 was reduced, although the Akt protein level was not affected. The reduced Akt phosphorylation reflected the Akt kinase activity, also leading to a diminished phosphorylation of GSK3α at S21 compared to control cells. Looking at the IR as a possible cause for the reduced IRS-1 phosphorylation, we did not find an effect of siRNA treatment on IR expression or phosphorylation (Fig. S1). Girdin was not only important for members of the PI3K-pathway, but also the

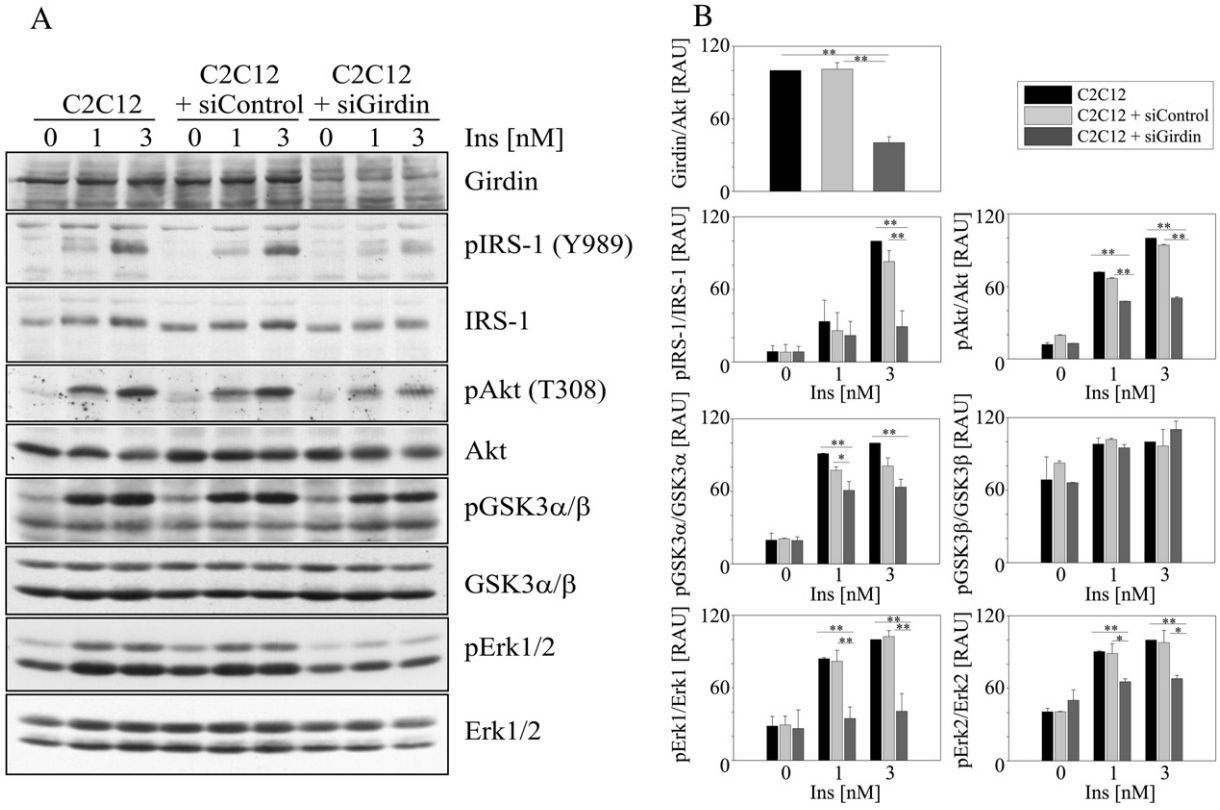

Fig. 2. Girdin knock-down decreases insulin sensitivity in C2C12 cells. (A) C2C12 cells were transfected with control or mouse Girdin siRNA or used untransfected. After serum starvation the cells were stimulated for 15 min with the indicated concentrations of insulin. The cells were then lysed and proteins were separated by SDS-PAGE and analyzed by Western blotting with protein- and phospho-specific (p) antibodies as indicated. The antibody detecting IRS-1 was from Millipore. (B) Densitometric scans of the Western blot results shown in (A). RAU — relative arbitrary units; *p < 0.05; **p < 0.005.

Map-kinase pathway was affected. As shown in Fig. 2, the insulin stimulated phosphorylation of Erk1 was almost abolished in Girdin-siRNA treated cells compared to control siRNA. Phosphorylation of Erk2 and its protein level were also reduced. Taken together, Girdin expression is an important regulatory mechanism for cellular signaling processes in C2C12 cells.

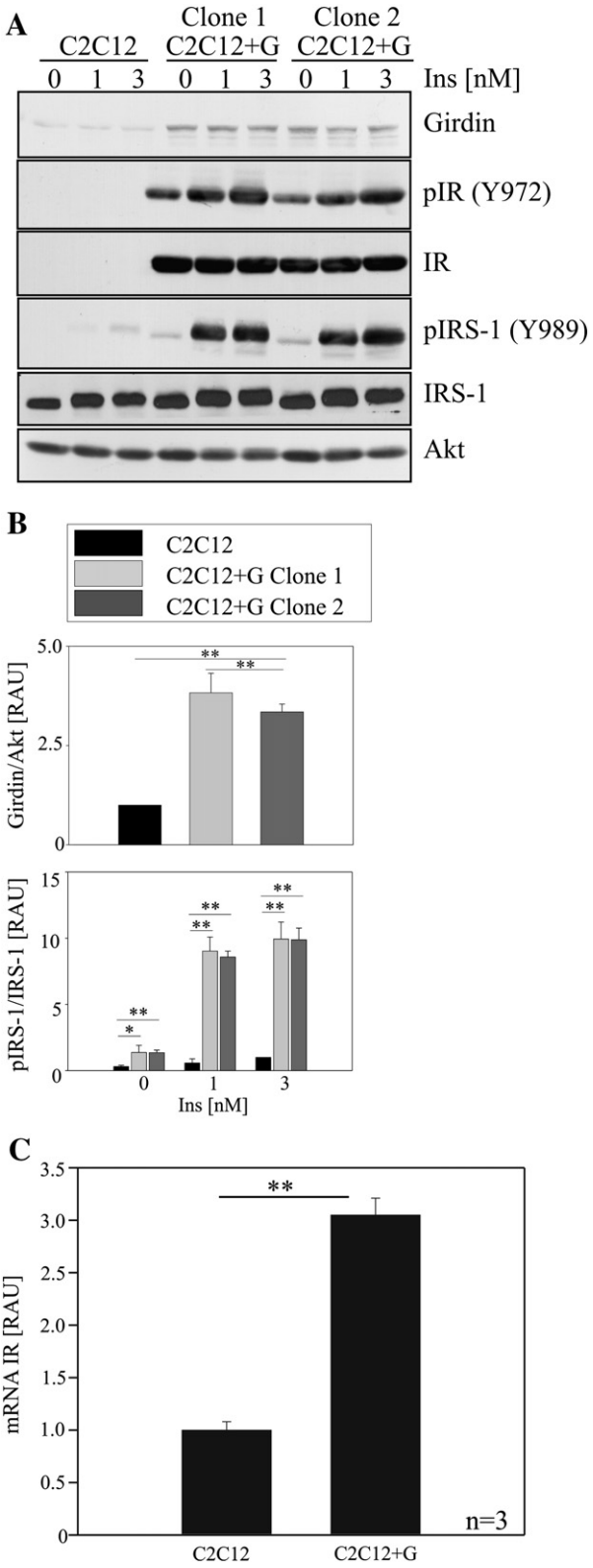

Fig. 3. Girdin overexpression in C2C12 cells causes manifold changes in protein level and phosphorylation status. (A) Parental C2C12 cells and C2C12 cells stably overexpressing Girdin were serum starved and stimulated for 15 min with the indicated concentrations of insulin. Cells were lysed and the phosphorylation (p) and protein pattern of the lysates analyzed by SDS-PAGE and Western blotting. (B) Densitometric scans of the Girdin protein and IRS-1 phosphorylation shown in part (A). (C) mRNA analysis of the IR by real time quantitative PCR. RAU — relative arbitrary units; G — Girdin; *p < 0.05; **p < 0.005.

3.3. Overexpression of Girdin in C2C12 cells causes manifold changes

To investigate the influence of a permanent increase in Girdin protein, C2C12 myoblasts were stably transfected with full length Girdin. As shown in Fig. 3A/B, the approximately fourfold increase in Girdin protein in both cell lines, as determined by densitometric scanning, led to a massive change in quantity and phosphorylation of the IR, here detected as phosphorylation at Y972. Of note, the tyrosine phosphorylation was already apparent in the absence of insulin, as is seen frequently when cells express a high amount of a receptor tyrosine kinase. The level of IR in parental C2C12 cells was too low to be detected under the conditions used. To get an idea for the reason of the increased IR expression we isolated RNA from parental and overexpressing cells and performed real time quantitative PCR (Fig. 3C). We found an approximately threefold higher amount of IR specific mRNA in Girdin overexpressing cells. However, this would only in part explain the higher amount of IR protein found in these cells (see below).

We next looked at the phosphorylation of Y989 of the IRS-1 protein that was also remarkably increased in Girdin overexpressing cells without changes in IRS-1 protein levels (Fig. 3A/B). We then turned to analyze IR distal signaling and started with the Akt kinase that is a central mediator of cellular insulin effects. Activation of the Akt kinase, determined by its phosphorylation at T308, became insulin-independent upon Girdin overexpression (Fig. 4). As a loading control, another aliquot of the same lysate was blotted with an antibody directed against the Akt protein. Akt activation was confirmed by the finding that the

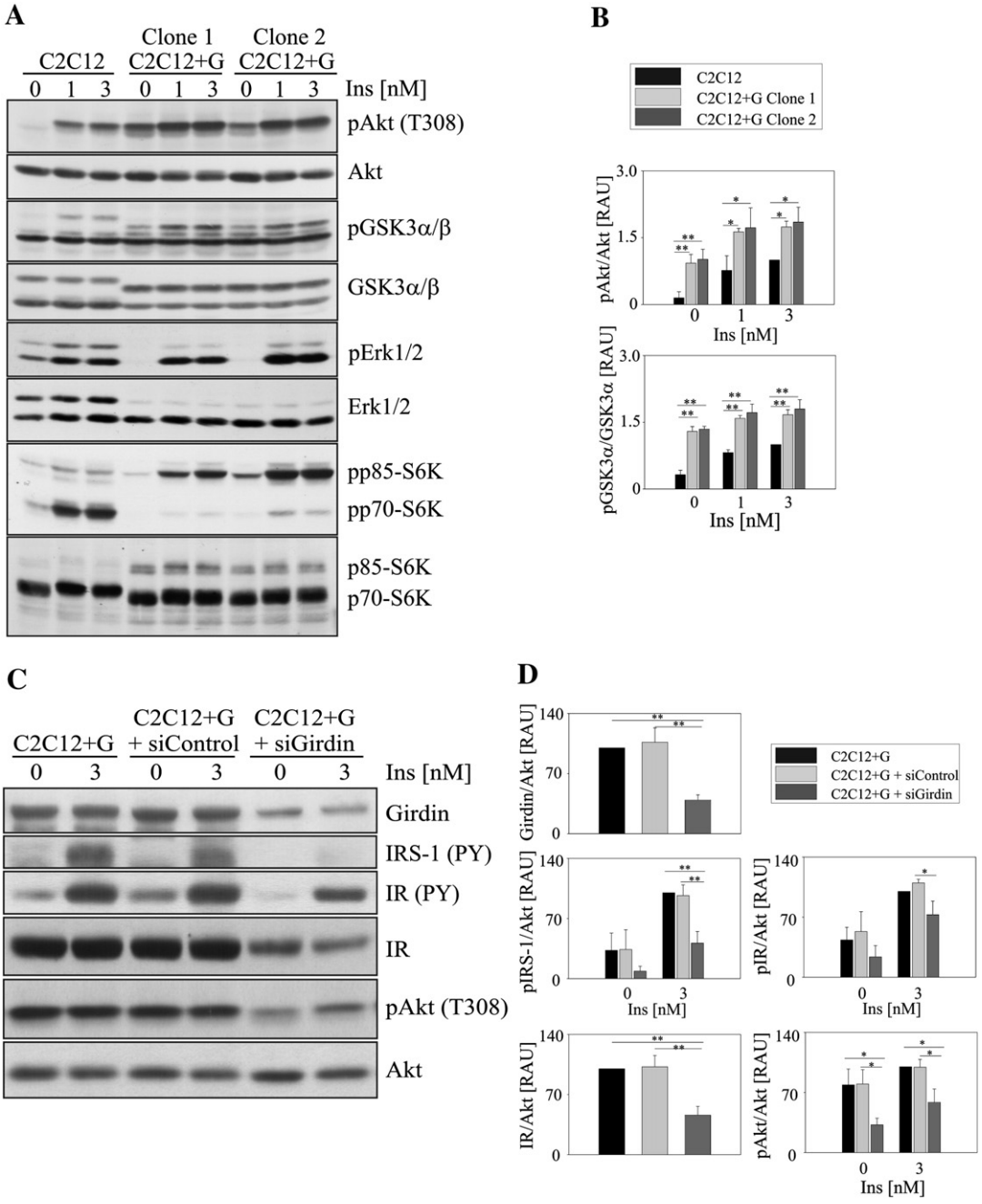

Fig. 4. (A) Effects on IR distal signaling by Girdin overexpression in C2C12 cells. Cells were treated as described in Fig. 2A and analyzed with antibodies directed against phosphopeptides or proteins. (B) Densitometric scans of Akt and GSK3α phosphorylation shown in part (A). (C) Reversal of Girdin overexpression by transfection with siRNA specific for human Girdin. Cells were left untreated or transfected with control or specific siRNA, stimulated as indicated and aliquots of the cell lysates analyzed as before. (D) Densitometric scans of the Western blot results shown in part (C). RAU — relative arbitrary units; G — Girdin; *$p < 0.05$; **$p < 0.005$.

downstream substrate GSK3α was constitutively phosphorylated at S21 in overexpressing cells. Strikingly, the GSK3α exhibited a lower apparent molecular weight in Girdin overexpressing cells. Two forms of GSK3α with molecular weights of 53 and 51 kD have been described by Nikoulina et al. [18] and may arise from different posttranslational processing. By contrast, GSK3β phosphorylation did not change with insulin stimulation but expression was slightly reduced.

When testing insulin signaling pathways aside from the PI3K-pathway, we first looked at Erk activity. Expression and insulin stimulated phosphorylation were reduced for Erk1, whereas for Erk2 the protein level was unchanged, but Girdin overexpression led to a reduced basal phosphorylation and a stronger response to insulin (Fig. 4A). Finally, phosphorylation at T389 of the kinase of the ribosomal protein S6 (p70-S6K) reflects activity of the mTOR kinase, another important insulin activated kinase regulating transcription, translation and signaling events. It was strongly reduced whereas protein amount and phosphorylation of the p85 isoform were enhanced. To check for physiological consequences of the S6K isoform change, we looked at IRS-1 degradation that correlates with phosphorylation of IRS-1 at S636 by S6K. We found that compared to parental cells Girdin overexpression enhanced IRS-1 degradation after insulin stimulation (Fig. S2). This indicates that the p85 isoform can act as potent as the p70 isoform of the S6K. On the other hand, IRS-1 degradation was not as strong as in IR overexpressing C2C12 cells, indicating that additional mechanisms are involved. These complex findings indicate that Girdin overexpression has many consequences for IR proximal and distal signaling events in C2C12 cells.

3.4. Downregulation of overexpressed human Girdin reverses the effects on insulin signaling

To warrant that the in part drastic changes in C2C12 cell physiology are really due to the increased level of Girdin protein we transfected the cells with an siRNA specific for human Girdin and investigated whether this would reverse the changes. As shown in Fig. 4C/D, 48 h after transfection the Girdin protein level was reduced, and in parallel, tyrosine phosphorylation of IRS-1 and the IR was lower as well. In addition, the expression of the IR was decreased. For IR distal signaling, activation of the Akt kinase was tested and found to be reduced whereas expression of the protein was unchanged. We conclude that the changes observed in C2C12 cells overexpressing Girdin are reversible and thus represent true Girdin dependent effects.

3.5. Overexpression of Girdin in C2C12 cells increases glycogen synthesis

To investigate the consequences of activation of the PI3K-pathway for midterm insulin signaling, we turned to analysis of glycogen synthesis. This assay is a readout for glucose uptake and incorporation into glycogen in response to insulin. Compared to parental C2C12 cells, Girdin overexpressing cells showed significantly higher glycogen synthesis even without stimulation (Fig. 5). In addition, the insulin sensitivity was elevated with glycogen synthesis peaking at 1 nM. However, the increased sensitivity was lost at 3 nM and even decreased at higher, not physiological concentrations of insulin. Thus, Girdin overexpression is improving glycogen synthesis and insulin sensitivity of muscle cells at low insulin concentrations.

3.6. Girdin overexpression reduces insulin receptor internalization

Girdin overexpression led to an increased expression of endogenous IR that was in part based upon a higher transcription (Fig. 3). Since Ghosh et al. [12] found a reduced internalization of EGFR in Girdin overexpressing Hela cells, we investigated IR internalization in C2C12 cells. Measurements of binding and incorporation of 125-I-labeled insulin were done in Girdin overexpressing cells and in C2C12 cells which stably overexpress a similar amount of human IR (Fig. 6). Our

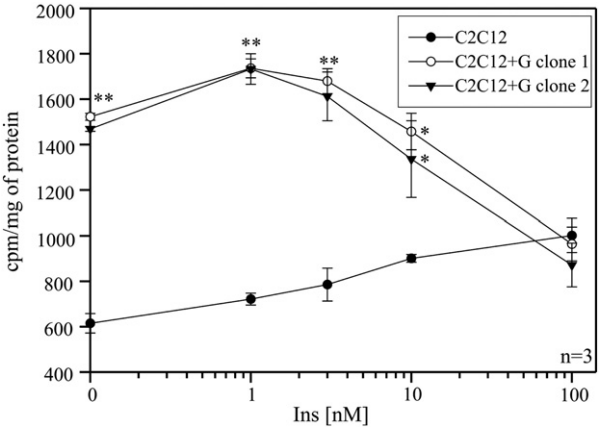

Fig. 5. Overexpression of Girdin in C2C12 cells increases glycogen synthesis. 14-C-glucose incorporation into glycogen was carried out in C2C12 wild type (filled circle) and Girdin overexpressing cells (circle: Girdin overexpressing clone 1/triangle: Girdin overexpressing clone 2) and measured by liquid scintillation counting. Protein concentrations for each sample were determined and results are presented as counts per minutes (cpm) per mg of protein plotted against the insulin concentration. *$p < 0.05$; **$p < 0.005$.

data reveal that Girdin overexpression led to a significantly reduced receptor internalization that is only half of that of the control cells. We conclude that similar to what has been described for the EGFR, the IR internalization is also affected by Girdin.

3.7. Girdin overexpression leads to constitutively active Abl kinase

Since Girdin overexpression in C2C12 cells upregulated the IR we explored the insulin-induced changes in the general tyrosine phosphorylation pattern of these cells. Parental and overexpressing cells were treated with the indicated amounts of insulin and cell lysates probed for phosphotyrosine (Fig. 7A). A protein of about 120 kD was strongly phosphorylated in parental but not in Girdin overexpressing cells. By immunoprecipitation, it was identified as the FAK kinase (data not shown) that is well known to be affected by the IR in a cell line dependent manner [19]. By contrast, the IR at 97 kD was strongly and in part insulin independently phosphorylated, as was the case for IRS-1 at approximately 180 kD. In addition, proteins at about 140, 66 and around 50 kD were stronger phosphorylated. The latter two proteins were identified to be members of the Shc family of proteins by immunoprecipitation experiments (data not shown) whereas the 140 kD protein was identified as the Abl kinase (Fig. 7B). Of note, Abl protein expression was similar for parental and transfected cells but it was constitutively phosphorylated and thus active only in transfected cells. Since the Abl kinase has been implicated in mediating aspects of insulin signaling, we investigated its contribution in this cell line by treatment with the Abl-inhibitor STI-571/gleevec. First, we confirmed the kinase inhibition by immunoprecipitating the Abl substrate CrkII from STI-571 treated and untreated cells. As shown in Fig. 7C, CrkII phosphorylation was strongly reduced under these conditions. We then analyzed lysates from cells treated for 24 or 48 h and found that phosphotyrosine in general was reduced but phosphotyrosine of the 140 kD protein was abolished (Fig. 7D). Further, the IR running at 97 kD was less expressed and contained less phosphotyrosine. The slightly lower apparent molecular weight also indicates loss of other posttranslational modifications. IR downstream signaling was affected at the level of Akt that was better insulin stimulable after 24 h and less activated after 48 h although the protein level was not changed (Fig. 7D/E). Little effects were seen for Erk- or p85-S6K-activation. By contrast, the residual T389-phosphorylation of the p70-S6K isoform was abolished by STI-571 treatment, as had been shown earlier by Parmar et al. [20]. To test a direct effect of Girdin on c-Abl phosphorylation, we treated the cells with siRNA against human Girdin and looked at Abl-phosphorylation in total cell lysates (Fig. S3). The phosphorylated protein at 140 kD,

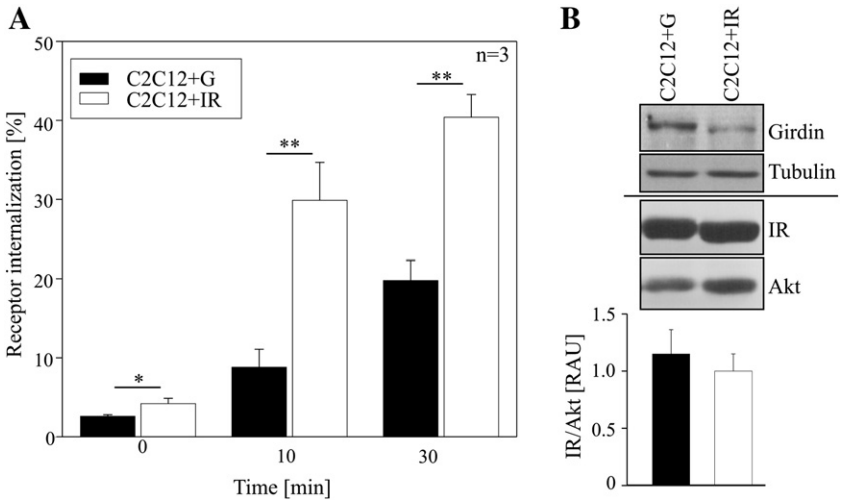

Fig. 6. Girdin overexpression reduces insulin receptor internalization. (A) An IR internalization assay was performed using C2C12 cells which overexpress human IR or Girdin. (B) In parallel, cells were lysed and lysates were separated by SDS-PAGE and immunoblotted for Girdin, tubulin, IR and Akt. IR expression was evaluated by scanning densitometry and expressed as the relation of IR to Akt protein. RAU — relative arbitrary units; *$p < 0.05$; **$p < 0.005$.

previously identified as c-Abl, was no longer phosphorylated. Further, we looked at the consequences of inhibition of c-Abl for IR internalization and glycogen synthesis (Fig. S4). After treatment with the inhibitor, IR was internalized more efficiently whereas glycogen synthesis decreased to about half. We conclude that activity of the Abl kinase is an important feature in Girdin overexpressing cells that contributes to the complex phenotype.

4. Discussion

Akt is a regulator of the actin cytoskeleton organization, and this activity is mediated via its substrate Girdin [7]. Especially in cancer cells Girdin is expressed at a higher level, is required for migration and may be involved in cancer progression and metastasis [21]. Besides the activation of the Akt kinase and its downstream signaling, not much is known about Girdin's role in insulin signaling. In this work we identified a correlation of Girdin expression in myotubes and whole body insulin sensitivity and subsequently investigated the function of Girdin in a myoblast cell line.

4.1. Girdin expression regulates insulin sensitivity in myoblasts

Knock-down of Girdin expression in C2C12 cells led to decreased insulin sensitivity. Cells displayed a slightly reduced IRS-1 protein level and strongly reduced IRS-1 tyrosine phosphorylation after insulin treatment. Furthermore, these cells showed an impaired activation of Akt, as was also reported in similar knock-down experiments using HepG2 or Hela cells [6,22]. In contrast, C2C12 cells stably overexpressing Girdin showed an increased insulin signaling. The IR was strongly upregulated and constitutively phosphorylated. This constitutive activity may be a consequence of its enhanced expression and is also found in C2C12 cells overexpressing a similar amount of the human IR (C2C12-IR cells in Fig. 6B; data not shown). Insulin stimulation led to an enhanced tyrosine phosphorylation of IRS-1 but there was no IRS-1 phosphorylation in the absence of insulin. Nevertheless, the cells exhibited a constitutively active Akt kinase in the absence of insulin, as demonstrated by phosphorylation of the downstream substrate GSK3. This important effect was also reported upon transient overexpression of Girdin in HepG2 and COS7 cells [6] and may in part be based on the guanine nucleotide exchange factor activity of Girdin that frees Gβγ subunits and activates p110β of the PI3K [10,23]. Lin et al. [11] reported that Girdin can be tyrosine phosphorylated and directly binds to the p85 subunit of the PI3K as well as the EGFR and in this way enhances the signaling. Unfortunately, we were not able to show tyrosine phosphorylation of Girdin or any interaction between Girdin and IR or p85 (PI3K) either in C2C12-Girdin or transiently overexpressing cells (data not shown). Of note, the C2C12-IR cells displayed a similar pattern of IR and IRS-1 phosphorylation but Akt was only active after insulin stimulation (not shown).

An important negative regulator of the PI3K-pathway, p70-S6K, differed between wild type and Girdin expressing cells. The phosphorylation of p70-S6K was reduced and the p85-S6K isoform was upregulated and phosphorylated in C2C12-Girdin cells. Initially, the p85 isoform was thought to be nuclear and involved in cell division [24,25]. However, later reports found both isoforms in the nucleus and the cytoplasm [26,27], or the p85 isoform only in the cytoplasm [28]. Although the activation mechanism differs between the isoforms, as only p85 can be phosphorylated independent of mTOR and the PI3K-pathway, a functional difference is not clear [27,29]. But since IRS-1 degradation after insulin stimulation was rather enhanced in C2C12-Girdin compared to parental cells, the p85-S6K is at least as potent in phosphorylating IRS-1 as the p70 isoform.

4.2. Girdin overexpression and increased insulin receptor expression

C2C12-Girdin cells showed an upregulated IR expression that contributed to the enhanced downstream signaling after insulin stimulation. As possible reasons for IR overexpression we found an approximately threefold rise of the IR mRNA level and a reduced IR internalization rate. Ghosh et al. [12] proposed a direct interaction of the EGFR with Girdin and a direct and negative effect of Girdin on internalization in Hela cells. Although intracellular trafficking of EGFR and IR is only initially similar [30] this finding points to a general effect of Girdin on receptor internalization. Our experiment showing that the Abl-inhibitor STI-571 improves internalization indicates a causal role of c-Abl in this process. Balaji et al. [31] also recently described c-Abl together with Rin1 and Rab5 as important regulators for EGFR internalization. However, we did not find a difference for expression of Rab5 and could not detect Rin1 in C2C12 with the antibodies available.

In addition and not yet investigated, the 3′-untranslated region of the IR mRNA could differ from that in parental C2C12 cells. This would affect mRNA stability and translation efficiency, as described by Lee

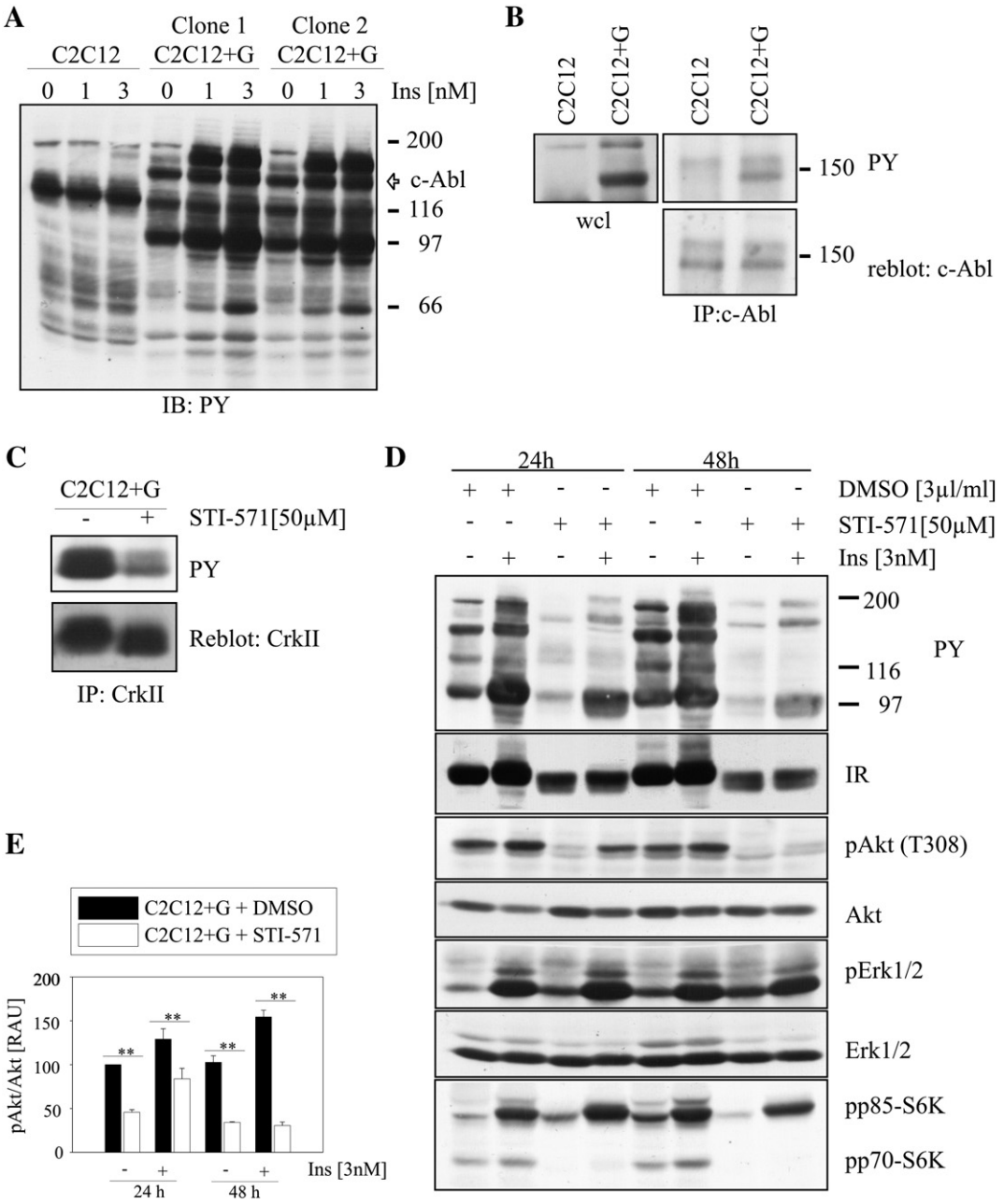

Fig. 7. Girdin overexpression leads to constitutive phosphorylation of c-Abl. (A) Parental C2C12 and Girdin overexpressing cells were starved and treated with the indicated concentrations of insulin. The cells were lysed and lysates were separated by SDS-PAGE and immunoblotted for phosphotyrosine (PY). (B) Cell lysates were also used for immunoprecipitation of c-Abl with a specific antibody. After separation by SDS-PAGE and immunoblotting for phosphotyrosine, the membrane was stripped and reblotted with c-Abl antibody. (C) Wild type and Girdin overexpressing C2C12 cells were pretreated with STI-571 (50 µM) for 4 h and lysed. CrkII was immunoprecipitated with a specific antibody and the samples analyzed by SDS-PAGE and Western blotting with the indicated antibodies. (D) Pretreatment of C2C12 cells which stably overexpress Girdin with STI-571 (50 µM) or DMSO for 24 h or 48 h. The cells were starved overnight, stimulated with insulin, lysed and proteins analyzed by SDS-PAGE and Western blotting with specific antibodies (right panel). (E) Densitometric scan of the Akt phosphorylation shown in part (D). wcl — whole cell lysate; RAU — relative arbitrary units; $^{**}p < 0.005$.

and Gorospe [32]. Since C2C12-Girdin cells overexpress the endogenous IR, they provide a unique tool to further dissect these regulatory events.

4.3. Girdin stimulates the metabolic pathway by activating c-Abl in C2C12 cells

Our data show that c-Abl was constitutively active in Girdin overexpressing cells, and inhibition of c-Abl reduced IR expression and internalization, Akt activation and glycogen synthesis. The role of c-Abl for the insulin signal was previously investigated by Frasca et al. [33]. Here, treatment with the inhibitor STI-571 also attenuated Akt activation and glycogen synthesis but enhanced Erk activation in different cell types. Frasca and colleagues concluded that c-Abl signaling is important for restraining the mitogenic and enhancing the metabolic effects of insulin. These findings suggest that Girdin expression regulates insulin signaling also by shifting the cellular homeostasis from mitogenic to metabolic signals. It would therefore be of high interest to see whether in tumor derived cell lines with a higher metastatic potential and expressing an elevated amount of Girdin c-Abl activity is affected.

Overall, an increased expression of Girdin led to a plethora of changes in C2C12 cells. These are in part due to higher IR expression

and activation of the Abl-kinase. In addition, we found changes in caveolin-1 expression and miRNA pattern (data not shown) that may also contribute to insulin independent signaling events.

In conclusion, we have provided evidence that regulation of Girdin expression may be an important parameter of insulin sensitivity in humans. These findings encourage further investigations on the role of Girdin in other insulin sensitive tissues and in the development of insulin resistance and diabetes mellitus.

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