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Phosphoinositide 3-Kinase as a novel functional target for the regulation of the insulin signaling pathway by SIRT1

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Running title: Interplay between SIRT1 and insulin signaling

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ABSTRACT

The protein deacetylase SIRT1, and its activator resveratrol, exert beneficial effects on glucose metabolism. Different SIRT1 targets have been identified, including PTP1B, AMPK, FOXO, PGC-1 α and IRS2. The latter may underscore a tight link between SIRT1 and insulin signaling components. However, whether SIRT1 has a direct effect on insulin resistance and whether resveratrol acts directly or indirectly in this context is still a matter of controversy and this question has not been addressed in muscle cells.

Here, we show that SIRT1 protein expression is decreased in muscle biopsies and primary myotubes derived from type 2 diabetic patients, suggesting a contribution of diminished SIRT1 in the determination of muscle insulin resistance. To investigate the functional impact of SIRT1 on the insulin pathway, the activation of insulin downstream effector PKB was evaluated after SIRT1 inactivation by RNAi, SIRT1 overexpression, or resveratrol treatments. In muscle cells and HEK293 cells, downregulation of SIRT1 reduced, while overexpression increased, insulin-induced PKB activatory phosphorylation. Further molecular characterization revealed that SIRT1 interacts in an insulin-independent manner with the PI3K adapter subunit p85. We then investigated whether resveratrol may improve insulin signaling in muscle cells via SIRT1, or alternative targets. Incubation of muscle cells with resveratrol reverted the insulin-resistant state induced by prolonged TNF α or insulin treatment. Resveratrol-dependent improvement of insulin-resistance occurred through inhibition of serine phosphorylation of IRS1/2, implicating resveratrol as a serine kinase inhibitor. Finally, a functional interaction between PI3K and SIRT1 was demonstrated in *C.elegans*, where constitutively active PI3K - mimicking increased IIS signaling - lead to shortened lifespan, while removal of *sir-2.1* abolished PI3K-induced lifespan shortening. Our data identify SIRT1 as a positive modulator of insulin signaling in muscle cells through PI3K, and this mechanism appears to be conserved from *C.elegans* through humans.

Keywords: Insulin signaling, sirtuins, resveratrol, aging, *C. elegans*

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The abbreviations used are: AMPK, AMP-activated protein kinase; HEK293, human embryo kidney 293 cells; IIS, insulin/insulin-like signaling; IRS, insulin receptor substrate; MAPK, mitogen activated protein kinase; PGC-1 α , PPAR γ general co-activator 1 α ; PI3K, phosphoinositide 3-kinase; PI(4,5)P₂, phosphatidylinositol(4,5)bisphosphate; PKB, protein kinase B; PTP1B, protein tyrosine phosphatase 1B; TNF α , tumor necrosis factor α .

1. INTRODUCTION

The control of lifespan and glucose homeostasis are tightly linked processes in eukaryotes. Caloric restriction, an experimental condition that leads to lifespan prolongation, entails an amelioration of insulin signaling resulting in hypoinsulinaemia and improved glycaemic control. Ample genetic evidence demonstrates that mild inhibition of insulin signaling components including the insulin receptor, insulin receptor substrate proteins (IRS), PI3K; or over-activation of FOXO transcription factors contribute to lifespan extension, which is paralleled by (or consequential to) improved metabolic profile (Holzenberger et al., 2004). PI3K catalyses the phosphorylation of PI(4,5)P₂ to produce PIP₃. The PIP₃ phosphatase PTEN downregulates the insulin signaling pathway. Accordingly, overexpression of the *PTEN* homolog *DAF-18*, by downregulating IIS through termination of PI3K signaling, leads to lifespan extension in *Caenorhabditis elegans* (Mihaylova et al., 1999; Solari et al., 2005). Sir2, a NAD⁺ dependent protein deacetylase (Vaziri et al., 2001), modulates lifespan in lower eukaryotes including *C.elegans* (Tissenbaum and Guarente, 2001) and *Drosophila melanogaster* (Rogina and Helfand, 2004) by acting as calorie-restriction mimetic. It has also been shown that the small polyphenolic molecule resveratrol, acting via Sir2/SIRT1, induces lifespan extension (Baur et al., 2006; Howitz et al., 2003; Wood et al., 2004) and improves the glycaemic profile in rodent models (Baur et al., 2006).

SIRT1 also participates in the control of glucose homeostasis by regulating gluconeogenesis (Rodgers et al., 2005), insulin secretion (Bordone et al., 2006), lipid mobilization from adipocytes (Picard et al., 2004) and fatty acid oxidation in skeletal muscle (Gerhart-Hines et al., 2007). By virtue of these multiple effects on glucose homeostasis, and its capability to protect pancreatic β -cells against cytokine toxicity (Lee et al., 2009), SIRT1 is a promising pharmacological therapeutic target for the treatment of insulin-resistance and subsequent type 2 diabetes (Liang et

al., 2009). Several molecular mechanisms account for metabolic control by SIRT1, including i) deacetylation-dependent activation of PGC1 α (Nemoto et al., 2005), leading to increased mitochondrial function (Rodgers et al., 2008), ii) SIRT1-mediated histone deacetylation on the PTP1B promoter, resulting in PTP1B gene repression (Sun et al., 2007) and iii) deacetylation-dependent nuclear retention of FoxO1, promoting expression of gluconeogenesis-related genes (Frescas et al., 2005). Besides these transcriptionally-related mechanisms, recent research indicates that SIRT1 modulates IRS2 tyrosine phosphorylation (Zhang, 2007) and improves insulin sensitivity in adipocytes (Yoshizaki et al., 2009). In view of these findings, we investigated the effects of SIRT1, and its activator resveratrol, on insulin signaling in human skeletal myotubes, skeletal muscle being the most relevant tissue for glucose disposal. Furthermore, we studied the interplay of IIS - elicited by overexpression of a constitutively active PI3K - and the SIRT1 homolog SIR-2.1 in *C.elegans*, monitoring lifespan as readout, to determine whether the interaction between SIRT1 and insulin signaling is a highly conserved phenomenon.

Here we show that i) SIRT1 protein expression levels are decreased in myotubes obtained from patients with type 2 diabetes, ii) SIRT1 interacts in an insulin-independent manner with the PI3K adapter subunit p85, and modulates insulin signaling at physiological insulin concentrations in skeletal muscle cells, iii) the SIRT1 activator resveratrol protects muscle cells - including human primary myotubes - from insulin-resistance induced by TNF α or prolonged hyperinsulinaemia and iv) lifespan shortening in *C.elegans*, caused by amplification of IIS signaling through expression of a constitutively active PI3K, is reverted by ablation of the *sir-2.1* gene.

2. MATERIALS AND METHODS

2.1 Materials and cell cultures. Resveratrol and recombinant human insulin were from Sigma. Recombinant human TNF α was from PeproTech (London). The following antibodies were used: anti-phospho-Ser-473 PKB, (Cell Signaling Technology), anti-PKB, IRS1, IRS2, SIRT1, and anti-pY (Santa Cruz Biotechnology), anti-p85 α (Upstate Biotechnology). Flag-SIRT1 and MYC-SIRT1 plasmids were from Drs. E. Verdin and B.M. Burgering (North et al., 2003; van der Horst et al., 2004). MYC-SIRT1, Flag-SIRT1 adenovirus were produced as described (Chaussade et al., 2003).

L6 and C2C12 myoblasts and HEK293 cells were cultured in DMEM, containing 4,5 g/l glucose and 10% FCS. Cells were starved in the absence of FCS. Human myotubes were derived from *vastus lateralis* biopsies and cultured as described (Cozzzone et al., 2008). Clinical characteristics of healthy control subjects and patients with type 2 diabetes, from whom *vastus lateralis* biopsies were obtained, are reported in table 1. Experimental protocols handling human biopsies were approved by the Ethical Committees of “Hospices Civils de Lyon” and the Alfred Hospital (Melbourne, Australia).

Insulin-resistance was induced by 24h insulin treatment (1 μ M for L6, C2C12, 100 nM for human myotubes) (Pirola et al., 2003) in the presence or absence of 100 μ M resveratrol. After 24h, insulin was removed for 1h by replacing DMEM, followed by 10-min stimulation with 1 μ M insulin. As TNF α induces insulin-resistance in cultured cells (de Alvaro et al., 2004), we also treated L6 cells with 2 nM TNF α for 48h, followed by medium replacement and insulin stimulation. TNF α treatment was done alone or in parallel with 100 μ M resveratrol.

2.2 Western blotting, Immunoprecipitations, glucose uptake and RNA-interference. Cells lysates were prepared as described (Pirola et al., 2003). When subjected to immunoprecipitation,

cell lysates were incubated with protein A-Sepharose and anti-SIRT1, IRS1, IRS2 antibodies. Standard immunoblotting procedures and ECL detection were employed. Glucose uptake assays were performed as previously described (Frojdo et al., 2007).

siRNAs targeting *SIRT1* and *IRS2* were from Qiagen. Transfection with siRNAs was performed by calcium phosphate precipitation. Sense sequences of siRNAs were as follows: *SIRT1*: 5'-CCCUGUAAAGCUUUCAGAAAdTdT; *IRS2*: 5'-CACUCGGACAGCUUCUUCUdTdT.

2.3 *C. elegans* studies. The strains wild-type N2-bristol and VC199 *sir-2.1* mutant (Caenorhabditis Genetic Center) were used. *sir-2.1* mutants were outcrossed three times with N2.

Constructs and transgenic lines. Vectors driving the expression of PI3K-p110 α catalytic subunit under the control of the daf-18 promoter were obtained by inserting p110 α -wt/KD-CAAX cDNA fragments from pcDNA3-p110 α -CAAX (Khwaja et al., 1998) into pPD95.75-daf-18p (Solari et al., 2005). p110 α -wt/KD-CAAX were also expressed as EGFP-fusion plasmid to verify expression and activity in 293 cells. Wild-type worms were injected with 20 ng/ μ l of daf-18p-p110 α -wt-CAAX or daf-18p-p110 α -KD-CAAX vectors. The injection marker plasmid pRF4(*rol-6*) was co-injected at 100 ng/ μ l, causing a rolling phenotype.

Lifespan assays. *C.elegans* were maintained on nematode growth medium plates and fed with *E.coli* strain OP50. Lifespan assays were performed at 20°C. Animals were grown at 20°C until the L4 stage and then shifted to plates containing 10 μ M 5-fluorodeoxyuracile to prevent growth of progeny. The shift day was counted as day 0 in the lifespan assay. Animals were scored as dead when they ceased moving and responding to prodding.

2.4 Statistical analysis. Experiments were performed at least three times, unless otherwise stated. Values are expressed as means \pm SD (or SEM) as indicated. Statistical tests used (Unpaired

Student's *t* test and Kaplan Meier log-rank for *C. elegans* lifespan comparisons) are indicated in the figure legends. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1 SIRT1 protein expression is decreased in muscle biopsies and primary myotubes derived from type 2 diabetic subjects.

Research on cultured cell lines indicated that SIRT1 is required to achieve full insulin-induced IRS2 tyrosine phosphorylation in hepatocytes (Zhang, 2007). As peripheral insulin-resistance in type 2 diabetes is characterised by decreased insulin action in skeletal muscle (Fig. 1A and (Cozzone et al., 2008)), the tissue prominently contributing to glucose disposal, we investigated whether alterations of SIRT1 expression occur in muscle biopsies and primary myotubes derived from patients with type 2 diabetes. A significant reduction in SIRT1 protein content in both primary skeletal muscle cells (Fig. 1B) and muscle biopsy samples (Fig. 1C) was observed in the patient cohort relative to control subjects. This difference was likely due to post-transcriptional modifications, as no differences in SIRT1 mRNA levels were observed between controls and type 2 diabetic patients (data not shown). Consistent with these data, a recent study demonstrated that SIRT1 protein stability can be promoted by phosphorylation-dependent regulation (Ford et al., 2008).

3.2 SIRT1 modulates proximal insulin signaling by interacting with p85 α PI3K

The observation that SIRT1 protein expression is diminished in muscle biopsies and primary myotubes from insulin-resistant type 2 diabetic subjects prompted us to study the impact of SIRT1 on downstream insulin signaling skeletal muscle-derived cell lines, and HEK293 cells. We hypothesized that, since skeletal muscle contributes prominently to glucose disposal, functional interaction between SIRT1 and insulin signaling in these tissue is relevant for the control of glucose homeostasis.

Firstly, we evaluated whether modulation of SIRT1 expression levels had an impact on the insulin responsiveness of cells. Downregulation of *SIRT1* by RNAi resulted in a decrease of insulin-induced PKB-Ser473 phosphorylation after 10-minutes, 10 nM insulin treatment in HEK293 cells (Fig. 2A), while no difference was seen at saturating insulin concentrations (100-1000 nM), suggesting a modulatory function for SIRT1 at submaximal insulin concentrations. Similarly, in L6 myoblasts treated with siRNAs to *SIRT1*, insulin-induced phosphorylation of PKB was reduced, with the most prominent effect at 10 and 100 nM insulin (Fig. 2B). In L6 cells, RNAi to *IRS2* was used as a positive control for insulin signaling downregulation (Pirola et al., 2003). Next, we investigated whether SIRT1 overexpression could improve insulin responsiveness. Overexpression in 293 cells of SIRT1 by calcium phosphate transfection resulted in increased phosphorylation of PKB in response to 10 nM insulin stimulation for 10 minutes as compared to untransfected or GFP-transfected cells (Fig. 3A). Adenoviral-mediated overexpression of SIRT1 in L6 myoblasts (Fig. 3B) and primary human myotubes (Fig. 3C) also resulted in increased phosphorylation of PKB, in response to stimulation with 1 μ M (in L6) or 100 nM (in primary myotubes) insulin. Thus, increased SIRT1 expression level improved the insulin-induced phosphorylation of PKB in multiple cell types.

Collectively, these results show that modulation of SIRT1 levels have a direct impact on insulin action, with increased levels of SIRT1 giving a stronger response to insulin and decreased SIRT1 levels reducing the response. These data suggest the existence of a direct interplay between SIRT1 and the insulin signaling pathway. To further explore the molecular mechanisms involved, HEK293 cells were transfected with either *IRS2* or *SIRT1* and stimulated with insulin. Cell lysates were immunoprecipitated with antibodies to *IRS2* or *SIRT1* and probed with anti-phosphotyrosine antibodies (Fig. 4A, upper panel). *SIRT1* immunoprecipitates from insulin-stimulated cells displayed associated tyrosine-phosphorylated proteins at >170kDa, likely

representing tyrosine phosphorylated IRS1/2, as the same signal was also present in IRS2 immunoprecipitates (Figure 4A, upper panel, lanes 2,4). Furthermore, we detected tyrosine-phosphorylated proteins between the 72-95 kDa molecular weight markers. Therefore, we performed an anti-PI3K-p85 α western blot (Fig. 4A, lower panel), showing that p85 α co-immunoprecipitates with IRS2 and SIRT1. These results suggest the putative existence of interactions between SIRT1, the p85-PI3K adapter and IRS proteins. To further demonstrate such interaction, SIRT1, IRS1 and IRS2 were co-overexpressed in HEK293 followed by immunoprecipitation of SIRT1. Western-blotting to p85 α -PI3K (Fig. 4B,C) indicated that p85 α co-immunoprecipitates with SIRT1, but not with control IgG (Fig. 4D). SIRT1-p85 α co-immunoprecipitation occurred independently from insulin stimulation, pointing to the existence of a constitutive complex between SIRT1 and PI3K that could be observed both upon SIRT1 (or IRS and SIRT1) overexpression (Fig. 4B,C, lanes 3 to 6), as well as on endogenous proteins (Fig. 4B,C, lanes 1-2). Further western blotting to IRS1 and IRS2 (Fig. 4E,F) on SIRT1 immunoprecipitates confirmed, as previously reported (Zhang, 2007), an insulin-induced interaction between SIRT1 and IRS1 (Fig 4E), and between SIRT1 and IRS2 (Fig. 4F). Overall, considering that SIRT1 co-immunoprecipitates tyrosine phosphorylated proteins and the PI3K adapter p85 α , it is likely that the IRS-PI3K-SIRT1 complex built upon insulin stimulation, from a pre-existing SIRT1-p85 complex, potentiates the insulin signaling cascade.

3.3 Resveratrol reverts insulin- and TNF α -induced insulin-resistance by inhibiting IRS protein serine/threonine phosphorylation

Resveratrol has been shown to activate SIRT1 (Borra et al., 2005; Howitz et al., 2003) and has subsequently been used to demonstrate the beneficial effects of SIRT1 on longevity and metabolic control (Borra et al., 2005; Howitz et al., 2003; Lagouge et al., 2006). However,

resveratrol has also been shown to inhibit short term insulin action independently from SIRT1 (Zhang 2006), likely through PI3K inhibition (Frojdo et al., 2007). Therefore, we investigated the effects of resveratrol on insulin signaling in muscle cell lines in the light that resveratrol may act either via SIRT1 activation or PI3K inhibition.

Firstly, we studied the impact of resveratrol treatment on IRS1 tyrosine phosphorylation and IRS1Ser307/IRS2Thr348 (equivalent to IRS1Ser307) phosphorylation (Solinas et al., 2006), which mirror the proteins' activatory potential. L6 myoblasts overexpressing IRS1 or IRS2 were pre-treated with resveratrol (5-100 μ M) prior to insulin stimulation. Serine/threonine and tyrosine phosphorylation of immunoprecipitated IRS1/2 and associated PI3K-p85 α were monitored with the respective antibodies. We observed that resveratrol pre-treatment did not affect tyrosine phosphorylation levels of either IRS1 or IRS2, nor the interaction of IRS proteins with PI3K-p85 α (Fig. 5A). However, resveratrol reduced the phosphorylation of IRS1Ser307 and IRS2Thr348 (Fig. 5A), which are known to be markers of insulin resistance (Draznin, 2006). Decreased IRS1/2 serine/threonine phosphorylation also correlated to inhibition of JNK (Fig. 5A) and, as previously reported (Frojdo et al., 2007), PKB phosphorylations. As JNK is responsible for IRS1Ser307 and IRS2Thr348 phosphorylations, we propose that resveratrol affects IRS-Ser/Thr phosphorylation status via inhibition of a protein kinases cascade, with PKB being putatively upstream of JNK. That resveratrol acts as a protein kinase inhibitor, rather than SIRT1 activator, in this experimental setup, is further confirmed by the observation that SIRT1 overexpression – expected to mimic resveratrol activation - did not decrease insulin-induced IRS1 phosphorylation on Serine307 (Fig. 5B) and SIRT1 knock-down did not affect the inhibitory action of resveratrol on insulin-stimulated PKB phosphorylation (Fig. 5C). Furthermore, the potentiation of insulin-induced PKB phosphorylation by SIRT1 overexpression

independently from changes in IRS proteins tyrosine phosphorylation (Fig. 5B) further confirms that the relevant molecular event resides in the p85-SIRT1 interaction, which occurs downstream of IRS1/2 tyrosine phosphorylation events.

We next asked whether resveratrol-dependent inhibition of IRS1/2 serine phosphorylation could, over the long-term, improve the insulin sensitivity of cells subjected to chronic treatment with insulin or TNF α , which both lead to insulin-resistance. In the three muscle cell lines tested (L6, C2C12 and primary myotubes), the 24h chronic insulin treatment induced marked insulin-resistance, as monitored by reduced PKB-Ser473 phosphorylation following acute insulin stimulation (Fig. 6A). Within this experimental system, we observed that resveratrol co-administration during chronic hyperinsulinemia alleviated the induction of insulin-resistance in the three cell lines used, leading to increased phosphorylation of PKB upon acute insulin stimulation (Fig. 6A). However, 24h simultaneous treatment with resveratrol slightly inhibited glucose transport, in L6 myotubes (Fig. 6E), indicating that amelioration of a distal insulin signaling response such as glucose uptake by resveratrol may require longer incubation times. Interestingly, resveratrol also prevented insulin-resistance as induced by TNF α (Fig. 6C), indicating a beneficial action in multiple models of induction of insulin resistance. Improvement of PKB responsiveness upon prolonged resveratrol treatment was independent from changes in SIRT1 expression, as assessed in L6 cells in which SIRT1 protein levels were unaltered after 24 hours exposure to insulin or insulin and resveratrol (Fig. 6B). Furthermore, the independency from SIRT1 of cell responses to resveratrol have also been verified in SIRT1 knock-down L6 cells, in which the alleviating effect of resveratrol on insulin-induced insulin resistance was retained (Fig. 6D). Collectively, these results indicate that multiple inducers of insulin-resistance can be counteracted by resveratrol and support the idea that amelioration of insulin sensitivity by resveratrol is a consequence of the inhibition of protein kinases targeting IRS1Ser307 (and

IRS2Thr348) including PI3K-mTOR (Frojdo et al., 2007; Ozes et al., 2001) and JNK (Fig. 5A), rather than SIRT1 activation.

3.4 In *C.elegans*, PI3K action is dependent on SIR-2.1

To study the interplay between insulin signaling and SIRT1 in an entire organism, we used *C.elegans* as a model system in which IIS and SIRT1 actions can be monitored by assessing lifespan. To fully induce IIS we generated transgenic worms expressing a membrane-targeted constitutively active PI3K(wt) and, as a control, worms expressing a membrane-targeted, but catalytically inactive PI3K (herein referred to as kinase dead, KD). PI3K(wt) worms displayed body size defects, in that they appeared shorter but larger than wild-type worms (Fig. 7A), while PI3K(KD) and wild-type controls were indistinguishable (data not shown). Furthermore, expression of PI3K(wt) reduced the average lifespan by 15% in 3 independent transgenic lines compared with wild-type N2 and PI3K(KD) worms (Fig. 7B). Thus, constitutive IIS activation via PI3K expression decreases *C.elegans* lifespan, in agreement with previous genetic reports showing that mild downregulation of IIS by mutations in the PI3K homologue *age-1* (Ayyadevara et al., 2008) or overexpression of the PTEN homologue DAF-18 (Solari et al., 2005) conversely prolong lifespan.

Having demonstrated that PI3K(wt) expression shortens lifespan, we crossed PI3K(wt) transgenic worms with worms mutated for the *SIRT1* homolog *sir-2.1*, with the rationale that if SIRT1 directly contributes to IIS signaling, its ablation may affect the short-lived phenotype induced by PI3K(wt) expression. Indeed, while *sir-2.1* null worms had lifespan comparable to N2 wild-type worms (Figure 7C), the *sir-2.1* null allele crossed within the constitutively active PI3K(wt) strain showed normal body morphology and reverted the reduced lifespan phenotype (Fig. 7D). Thus, SIR-2.1 is required to mediate the effects of PI3K upon body morphology and lifespan reduction,

likely by exerting a permissive function in driving 3'-phosphoinositides lipid messenger production by PI3K.

Overall, these data indicate that, in a whole organism, SIRT1 mediates the effects of constitutive activation of PI3K. The data also demonstrate that the functional interaction between SIRT1 and insulin signaling is highly conserved between human cells and invertebrate species and underscore the close interplay of the two pathways in the control of metabolism and longevity.

4. DISCUSSION

As SIRT1 exerts positive metabolic effects through several mechanisms (reviewed in (Chaudhary and Pfluger, 2009), and in view of the decreased SIRT1 protein expression observed by us (Figure 1) and others (de Kreutzenberg et al., 2010) in muscle and peripheral blood mononuclear cells from insulin resistant patients, we sought to investigate the role of SIRT1, and its activator resveratrol, on the insulin signaling cascade in muscle cells, since in this cell type insulin signaling is quantitatively relevant for the disposal of circulating glucose. Furthermore, we studied the interplay of IIS and the SIRT1 homolog SIR-2.1 in the model organism *C. elegans*, monitoring lifespan as readout, to define the interaction between IIS and SIRT1 in an entire organism.

Downregulation of SIRT1 expression levels by RNAi diminished insulin-stimulated PKB phosphorylation when sub-maximal insulin concentrations were used (10 nM in 293 cells and 10-100 nM in L6). Conversely, at saturating insulin concentrations (1 μ M in L6 and 100 nM in 293 cells), PKB responsiveness was unaltered. This may indicate that SIRT1 plays a modulatory role within the insulin signaling cascade at submaximal insulin concentrations. Reciprocally, we observed increased insulin-stimulated PKB phosphorylation upon overexpression of SIRT1 in muscle-derived cell lines, including human primary myotubes, and HEK293. Thus, our data suggest that SIRT1 positively modulates the activity of upstream components in the insulin pathway. In contrast to our observations, Yoshizaki and colleagues reported that SIRT1 overexpression in 3T3-L1 adipocytes was not sufficient to increase insulin-stimulated PKB and MAPK phosphorylations (Yoshizaki et al., 2009). These contrasting data may be explained on the basis of the different cell lines analyzed, the different adenoviral constructs and multiplicities of infection employed to achieve SIRT1 overexpression and the different insulin concentrations employed – up to 17 nM in the Yoshizaki study and 10-1,000 nM in our study, with the higher

concentration in adenoviral-infected myotubes to overcome a higher basal PKB phosphorylation induced by adenoviral overexpression .

To investigate whether the SIRT1 modulation of insulin-stimulated PKB phosphorylation depends on a direct interaction of SIRT1 with the insulin signaling pathway, we performed co-immunoprecipitation experiments which showed that SIRT1 interacts with tyrosine phosphorylated proteins, and with the PI3K-p85 α adapter in a constitutive manner, leading to the formation of a SIRT1-p85-IRS1/2 ternary complex upon insulin stimulation in an overexpression system (Fig. 4, E,F). The existence of a modulatory role of SIRT1 in receptor tyrosine kinase dependent signaling pathways has previously been shown in neurons (Li et al., 2008) and human cancer cell lines (Ota et al., 2006). In these studies, inhibition of SIRT1 by sirtinol or nicotinamide decreased IGF-1 and EGF induced Ras-MAPK signaling, leading to neuroprotection and senescence-like growth arrest, respectively (Li et al., 2008; Ota et al., 2006). In contrast, numerous studies indicate that SIRT1 activation exerts a beneficial role in the control of metabolism (reviewed in (Elliott and Jirousek, 2008)). In accordance with these previous studies, the SIRT1 knock-down and overexpression experiments in muscle cells, demonstrating that SIRT1 positively participates in the activation of insulin signaling, underscore the importance of SIRT1 action in increasing insulin signaling via its constitutive association to PI3K-p85 α adapter. Our observations add a further mechanism of direct action of SIRT1 on the insulin signaling pathway that complements the previously reported mechanism by Sun *et al.* implicating transcriptional downregulation of PTP1B (Sun et al., 2007). As pointed out above, the observation that SIRT1 protein expression is diminished in insulin-resistant primary myotubes derived from type 2 diabetic patients and in *vastus lateralis* biopsies from patients with type 2 diabetes (Fig. 1) strengthens the clinical relevance of SIRT1 action on insulin signalling.

SIRT1 downregulation in T2D subjects is likely dependent on accelerated degradation, as decreased protein amounts were observed in spite of unaltered SIRT1 mRNA levels. This observation is reminiscent of increased fasting-induced SIRT1 protein levels in liver in the absence of variation of the corresponding mRNA (Rodgers et al., 2005).

Besides its action on SIRT1 (Howitz et al., 2003) - which is controversial (Kaeberlein et al. 2005; Pacholec et al., 2010) - resveratrol has been shown to act on a plethora of different molecular targets (reviewed in (Harikumar and Aggarwal, 2008)), including insulin signaling enzymes (Zhang, 2006). Since several of the biological effects mediated by resveratrol, such as increased insulin sensitivity and prolonged lifespan, could also be explained by modulation of insulin signaling, we sought to define more precisely the implication of the insulin signaling pathway in the beneficial metabolic effects of resveratrol. In L6 myoblasts, short pre-treatment with resveratrol did not affect insulin induced tyrosine phosphorylation of IRS1/2, nor their interaction with p85 α (Fig. 5A). In contrast, IRS1Ser307, and the homologous IRS2Thr348, phosphorylations were totally blocked by >50 μ M resveratrol. We suggest that inhibition of IRS1/2 Ser/Thr phosphorylation depends on direct inhibition of protein kinases by resveratrol rather than an indirect action initiated by SIRT1 activation, inasmuch SIRT1 overexpression, as opposed to resveratrol treatment, did not affect insulin-stimulated IRS1Ser307 phosphorylation (Fig. 5B).

Given that phosphorylations on IRS1Ser307 and IRS2Thr348 relay a feedback inhibition of the insulin signaling pathway (Lee et al., 2003; Solinas et al., 2006) and are known to be augmented in insulin resistance (Morino et al., 2005), we evaluated whether induction of insulin-resistance in muscle cell lines by chronic hyperinsulinemia or TNF α treatment could be reverted, or alleviated,

by resveratrol co-administration (Fig. 6). In all the muscle cells studied, we observed that co-incubation of resveratrol during chronic insulin treatment resulted in improved insulin responsiveness. Similarly, resveratrol diminished the extent of insulin-resistance induced by TNF α treatment in L6 myoblasts. A previous study by Sun *et al.* (Sun et al., 2007) demonstrated that palmitate- and glucosamine-induced insulin-resistance was accompanied by decreased SIRT1 protein levels in hepatocyte-derived cell lines. In our experiments, insulin-resistance induced by TNF α in L6 or insulin in L6, C2C12 and human primary myotubes did not coincide with decreased SIRT1 protein expression as observed in other studies (Sun et al., 2007; Yoshizaki et al., 2009). This discrepancy is likely explained by the different treatment used to induce insulin-resistance (insulin/TNF α versus palmitate/glucosamine) and by the different cell types used. In spite of these differences, our results showing that resveratrol treatment ameliorates PKB phosphorylation in insulin resistant cells support the data by Sun *et al.* (Sun et al., 2007) and are in line with a recent report showing SIRT1 depletion in peripheral blood monocytes cells from insulin resistant subjects but lack of SIRT1 downregulation in insulin-treated THP-1 monocytes (de Kreutzenberg et al., 2010).

Overall, the above findings suggest that direct interaction between SIRT1 and insulin signaling does exist in cell culture models. To obtain insight on the occurrence of IIS and SIRT1 interplay in a whole organism we used *C. elegans* as model system. We constructed *C.elegans* transgenic worms overexpressing constitutively active PI3K(wt). Since mild inhibition of key components of IIS signaling in *C. elegans* - i.e. DAF-2, and AGE-1; homologous to insulin-receptor and PI3K respectively - leads to lifespan prolongation (Tatar et al., 2003), we reasoned that chronic activation of IIS signaling by expression of constitutively active PI3K would shorten the worm's lifespan. Indeed, mean lifespan of transgenic worms expressing constitutively active PI3K(wt), was decreased compared to wild-type or PI3K(KD) control worms (Fig. 7B). To further explore

the interaction between IIS and SIRT1 we crossed PI3K(wt) transgenic worms with *sir-2.1* null worms, to define whether SIR-2 was required for lifespan shortening induced by constitutively active PI3K. Interestingly, while lifespan of *sir-2.1(-)* worms was comparable to that of N2 wild-type *C. elegans* (Figure 7C), lifespan of PI3K(wt);*sir-2.1(-)* worms was reverted to normal (Figure 7D), indicating that SIR-2.1 mediates the life-shortening action of PI3K(wt) and supporting the existence of a specific regulatory network encompassing SIRT1 and PI3K in the determination of lifespan. These observations are unexpected in light of data reported by Tissenbaum *et al.* showing that SIR-2.1 overexpression prolongs *C.elegans* lifespan in a DAF-16/FOXO dependent manner (Tissenbaum and Guarente, 2001). We, therefore, expected that SIR-2.1 inactivation should have been deleterious rather than beneficial for lifespan. Furthermore, recent results from Berdichevsky *et al.* (Berdichevsky *et al.*, 2006) showed that SIR-2.1 activates DAF-16 via deacetylation under stress conditions. However, they also demonstrated that SIR-2.1/DAF-16 interaction is unrelated to lifespan regulation by the IIS pathway. In this latter context, our results show that when PI3K is constitutively activated, SIR-2.1 potentiates - rather than antagonizes - the PI3K role in lifespan control. This functional interaction involves - rather than a FOXO/DAF-16 pathway - a SIR-2.1 interaction with other upstream components of the IIS pathway. The recovery of normal lifespan in PI3K(wt); *sir-2.1(-)* worms can be explained by postulating that SIR-2.1 acts either downstream, or - as our experiments in mammalian cells suggest - in parallel to PI3K (Fig. 8). Overall, lifespan data in *C.elegans* suggest that the results obtained in cell culture, demonstrating a functional interaction between SIRT1 and insulin signaling, are transposable to an entire organism.

In conclusion, our experiments support the existence of a functional interplay between insulin signaling/IIS and SIRT1 both in muscle derived cell lines and *C.elegans*, leading to positive modulation of insulin responsiveness. These findings add a further level of understanding as to

how SIRT1 regulates insulin action and IIS. Furthermore, we provide evidence that resveratrol can reverse insulin resistance through targets other than SIRT1, as demonstrated by the inhibition of protein serine kinases that result in decreased phosphorylation of IRS1/2, indicating that the pleiotropic actions of resveratrol should be taken into account when studying its effects on metabolism.

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FIGURE LEGENDS

Figure 1: SIRT1 protein levels are lower in myotubes and muscle biopsies from type 2 diabetic patients compared to control subjects. (A) Myotubes derived from control subjects (control) or type 2 diabetic patients (T2D) were starved overnight and stimulated 10 minutes with 100 nM insulin. PKB pS473 phosphorylation was measured as a readout for the insulin resistant state observed in primary myotubes derived from type 2 diabetic patients, as previously reported (Cozzone et al., 2008). (B, Upper part) SIRT1 protein expression levels were evaluated by immunoblotting in myotubes derived from controls and T2D patients. Separation bars indicate non-contiguous lanes. Tubulin loading controls were as previously published (Cozzone et al., 2008). As no difference in SIRT1 expression level was seen after the 10 minutes insulin stimulation, both conditions were analysed together to quantify SIRT1 expression level (B, lower part). Quantification is based on 10 samples. Controls, filled bar; T2D patients, empty bar. * $p < 0.05$ versus SIRT1 protein levels in controls. (C, Upper part) SIRT1 protein expression levels in muscle biopsies from controls (C) and T2D patients (T2D). (B, lower part). Quantification is based on 7 controls biopsies and 9 T2D biopsies and was adjusted to tubulin expression levels. Controls, filled bar; T2D patients, empty bar. Data in (B,C) are expressed as means \pm SEM. * $p < 0.05$ versus SIRT1 protein levels in controls.

Figure 2. SIRT1 modulates insulin signaling in 293 cells and L6 myoblasts. (A) 293 cells were transfected with increasing concentrations (0 nM, 16.6 nM and 50 nM) of siRNA directed to SIRT1 (as depicted graphically). 48 hours post-transfection cells were starved overnight and stimulated for 10 min with the indicated concentrations of insulin. Upper panel: SDS-PAGE

separated proteins were immunoblotted with antibodies to IRS2 and PKB, to monitor protein loading, SIRT1 and PKB pS473 as indicated. Lower panel : quantification of PKB pS473 ; results are expressed as means \pm SEM (n=4). * PKB pS473 levels in SIRT1 knock-down cells upon 10 nM insulin stimulation are significantly different from the respective control ($p < 0.05$ at Student's t test). **(B)** L6 myoblasts were transfected with 50 nM siRNA to SIRT1 and IRS2 as indicated. 48 hours post-transfection myoblasts were starved overnight and stimulated for 10 min with the indicated concentrations of insulin. SDS-PAGE separated proteins were immunoblotted with antibodies to IRS2, SIRT1, PKB pS473, and PKB (to monitor protein loading), as indicated. ** Non-specific band.

Figure 3. SIRT1 overexpression increases insulin action in 293 cells, L6 myoblasts and primary human myotubes. **(A)** 293 cells were transfected with increasing amounts of pCDNA3<MYC-SIRT1> or pEGFP-C2 (1 to 5 μ g plasmid DNA). 48 hours post-transfection cells were starved overnight and stimulated for 10 min with the indicated concentrations of insulin. Separated proteins were immunoblotted with antibodies to SIRT1 and PKB pS473 as indicated. L6 myoblasts **(B)** and differentiated human myotubes **(C)** were infected with adenoviruses coding for GFP, Flag-SIRT1 and MYC-SIRT1. 48 hours post-infection cells were starved overnight and stimulated for 10 min with 1000 nM (L6) or 100 nM insulin (primary myotubes). Separated proteins were immunoblotted with antibodies to SIRT1, PKB pS473 and total PKB as indicated. Lanes of the pPKB blot from primary myotubes, from the same PVDF membrane, were rearranged to maintain loading consistency with L6 cells.

Figure 4. SIRT1 is bound to the p85 PI3K adapter, and insulin stimulation induces SIRT1-p85 association to tyrosine phosphorylated proteins and IRS1/2. **(A)** 293 cells were

transfected with expression plasmids for MYC-SIRT1 and IRS2 as indicated. 48 hours post-transfection cells were starved overnight and stimulated or not for 10 min with 1000 nM insulin. Cell lysates were immunoprecipitated with antibodies directed to IRS2 or SIRT1. Immunoprecipitates were probed for phosphotyrosine content with anti-PY antibodies (upper panel) and associated PI3K with anti p85 α antibody (lower panel). * indicates a non specific (or unidentified) band. **(B,C)** 293 cells were co-transfected with expression plasmids for MYC-SIRT1, IRS1 and IRS2 as indicated. 48 hours post-transfection cells were starved overnight and stimulated or not for 10 min with 1000 nM insulin. p85, IRS1, IRS2 and SIRT1 expression levels were verified on total lysates. Cell lysates were then immunoprecipitated with antibodies directed to SIRT1 and immunoprecipitates were probed with antibodies directed to p85 α . The constitutive SIRT1-p85 α association pattern has been observed in two independent experiments. **(D)** To monitor immunoprecipitation specificity, lysates from unstimulated and insulin-stimulated SIRT1-overexpressing 293 cells were immunoprecipitated with antibodies directed to SIRT1 or IgG. p85 α and SIRT1 expression levels were verified on total lysates (upper blots) and SIRT1-associated p85 α was visualized by immunoblotting with anti-p85 antibodies (lower blot). **(E,F)** 293 cells were co-transfected with expression plasmids for MYC-SIRT1 and IRS1 or IRS2. 48 hours post-transfection cells were starved overnight and stimulated or not for 10 min with 1000 nM insulin. The expression of each protein was verified with the relevant antibody on total lysates. Cell lysates were then immunoprecipitated with antibodies directed to SIRT1. SIRT1 immunoprecipitates were probed with antibodies directed to IRS1 **(D)** and IRS2 **(E)**. IP, immunoprecipitation; WB, western blot.

Figure 5: Resveratrol inhibits insulin-stimulated serine phosphorylation of IRS proteins and PKB/JNK phosphorylation independently from SIRT1 (A, upper part) L6 myoblasts were infected with adenoviruses expressing IRS1 (left panels) and IRS2 (right panels). 48 hours post-infection, myoblasts were serum-starved overnight, pre-treated for 20 min with the indicated concentrations of resveratrol and subsequently stimulated 10 min with 1000 nM insulin. IRS1 and IRS2 were immunoprecipitated with the respective antibodies and immunoprecipitates were immunoblotted with anti-PY, anti IRS-1 pS307 (that cross react with the homologous phosphorylation site in IRS2) and anti p85 α antibodies. **(A, lower part)** Phospho-JNK, PKB pS473 and SIRT1 were detected in total lysates. **(B)** L6 myoblasts were infected with IRS1 and SIRT1 adenoviruses as indicated and transgene expression levels as well as PKB pS473 and total PKB as loading control were visualized by immunoblotting (upper blots on total lysates). 48 hours post-infection, myoblasts were serum-starved overnight and subsequently stimulated with insulin. IRS1 was immunoprecipitated and immunoblotted with anti phospho-tyrosine and anti IRS-1 phosphoS307 (Lower blots). **(C)** 293 cells were transfected with siRNA directed to SIRT1 as indicated. 48 hours post-transfection cells were starved overnight, pretreated with 100 μ M RSV for 20 min and stimulated for 10 min with the indicated concentrations of insulin. Total cell lysates were immunoblotted with antibodies to GAPDH, to monitor protein loading, SIRT1, total PKB and PKB pS473 as indicated.

Figure 6: Resveratrol prevents insulin- and TNF α - induced insulin-resistance in muscle cells without interfering on SIRT1 expression. Insulin-resistance was induced by incubation for 24 hours with 1000 nM insulin in L6, C2C12 myoblasts and with 100 nM in primary myotubes **(A)**; or by incubation with 2 nM TNF α for 48 hours in L6 myoblasts **(C)**.

Simultaneously to the insulin-resistance inducing stimulus, cells were co-incubated with 100 μ M resveratrol where indicated. At the end of the 24/48 hours incubation period, cells were placed in fresh DMEM medium for 1 hour and subsequently stimulated for 10 min with 1000 nM (L6, C2C12) or 100 nM (primary myotubes) insulin as indicated. **(A)**, Resveratrol-mediated protection from insulin-induced insulin-resistance in L6 myoblasts, primary human myotubes and C2C12 cells. Upper panel, evaluation of Ser473 phosphorylation of PKB together with total PKB and/or α -tubulin as loading controls in the three muscle cell lines. Lower panel, quantification of PKB pS473 levels, adjusted to total PKB, in L6 from 6 independent experiments, results are means \pm SEM. PKB pS473 levels are expressed as arbitrary units. * PKB pS473 levels are significantly diminished in the insulin-resistant condition versus non-insulin resistant cells $p < 0.05$. ** PKB pS473 levels are significantly increased in insulin resistant-cells co-treated with resveratrol, $p < 0.05$, Student's t-test. **(B)** SIRT1 protein expression levels in L6 were quantified by western blot and normalized for the tubulin loading control. ns: not statistically significant versus the control condition. **(C)**, Resveratrol-mediated protection from TNF- α induced insulin-resistance in L6 myoblasts. Upper panel, evaluation of pPKB S473 and pMAPK levels. Lower panel, quantification of PKB pS473 levels. Each bar represents the ratio between the basal and the insulin-stimulated condition. Results are means \pm SEM from 3 independent experiments. * PKB pS473 fold activation is significantly diminished in the insulin-resistant condition versus non-insulin resistant cells $p < 0.05$. ** PKB pS473 fold activation is significantly increased in insulin resistant-cells co-treated with resveratrol. $p < 0.05$, Student's t-test. **(D)** L6 myoblasts transfected with siRNA to GFP or SIRT1 were treated and analyzed as in panel A. Resveratrol-induced amelioration from insulin resistance was observed also in SIRT1 knock-down cells. **(E)** Prolonged resveratrol treatment slightly affects glucose uptake in L6 myotubes and 3T3-L1

adipocytes. L6 myoblasts were left untreated or treated for 24 hours with 1000 nM insulin and 100 μ M resveratrol as indicated. Prior to performing glucose uptake, cells were switched to glucose free DMEM medium for 60 minutes. * Glucose uptake significantly increased by insulin treatment versus untreated cells $p < 0.05$. ** Glucose uptake significantly decreased by insulin/RSV treated cells as compared to insulin-treated cells. $p < 0.05$, Student's t-test.

Figure 7: Constitutively active PI3K decreases *C. elegans* lifespan in a SIR-2 dependent manner (A) Light microscopy pictures (20x magnification) of *C. elegans* wild-type N2 strain and PI3K(wt) overexpressing strain, the latter displaying shortened and enlarged body shape. (B) Expression of constitutively active PI3K in N2 worms (PI3K(wt)) reduced the lifespan of wild-type worms (mean lifespan 13.3 ± 0.7 , log-rank $p < 0.05$ versus N2) compared to wild-type N2. Worms expressing the kinase dead PI3K (PI3K(KD)) had a lifespan comparable to wild-type (mean lifespan 15.3 ± 0.7 and 15.6 ± 0.9 , respectively, log-rank $p = \text{NS}$ versus N2). Lifespan tests have been repeated four times with three independent transgenic strains and gave similar results. The survival curve show results obtained for one representative experiment. (C) wild-type N2 and *sir-2.1* null *C. elegans* display comparable lifespan (mean lifespan 16.96 ± 0.63 and 16.90 ± 0.40 , respectively, log-rank $p = 0.334$) (D) *sir-2.1* mediates reduction of lifespan induced by constitutive activation of PI3K. PI3K(wt);*sir-2.1*(-) worms show an extension of lifespan compared to wild-type worms expressing constitutively active PI3K (PI3K(wt)) (mean lifespan 20.0 ± 0.6 and 14.5 ± 0.7 , respectively, log-rank $p < 0.0001$). The average lifespans of *sir-2.1*(-) mutants and PI3K(wt);*sir2.1*(-) worms are similar (19.7 ± 0.6 and 20.0 ± 0.6 , respectively, log-rank $p = \text{NS}$).

Figure 8: Model for functional interaction between SIRT1 and the insulin pathway in mammalian muscle cells and in *C.elegans*. (A) SIRT1 is required for physiological activation of the insulin pathway; (B) Downregulation of SIRT1 expression lowers insulin-induced PKB Ser473 phosphorylation and may contribute to insulin-resistance; (C) Constitutive activation of PI3K shortens lifespan, through PKB-AKT activation and inhibition of DAF-16/FOXO transcription factor; (D) Absence of SIR-2.1 suppresses the effect of constitutive activation of PI3K and worms recover a normal lifespan.

Table 1 Clinical characteristics of *vastus lateralis* biopsy donors

	Control subjects, (n=7)	Type 2 diabetic subjects, (n=9)
Age (years)	51±11	54±6
BMI (kg/m ²)	27.1±2.8	30.3±2.8 *
Fasting glucose (mmol/L)	4.9±0.4	10.4±3.3 *
HbA1c (%)	n.d.	8.6±1.7 **
Plasma triacylglycerol (mmol/L)	1.1±0.6	3.0±2.0 *
Fasting insulin (pmol/L)	n.d.	176±198 **

Data are mean values ± SD. * denotes $p < 0.05$ for type 2 diabetic patients versus control subjects, unpaired t -test. ** denotes clinically elevated parameters. High SD for fasting insulin is due to 2 study participants with high values (369 and 633 pmol/L).

Table 1 Clinical characteristics of *vastus lateralis* biopsy donors

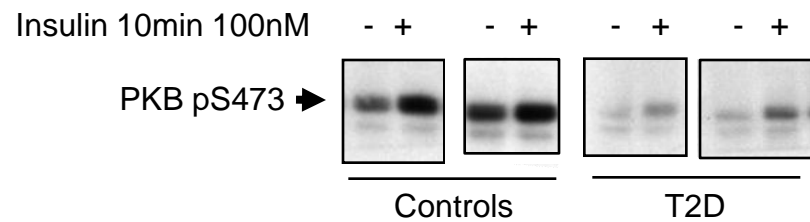
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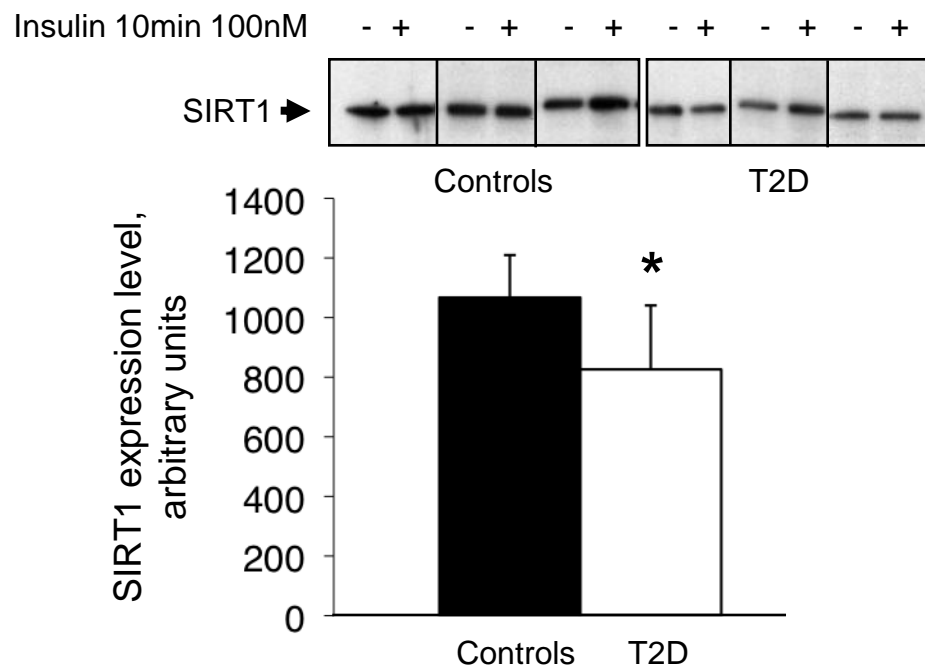
Figure 1

Figure 1

A



B



C

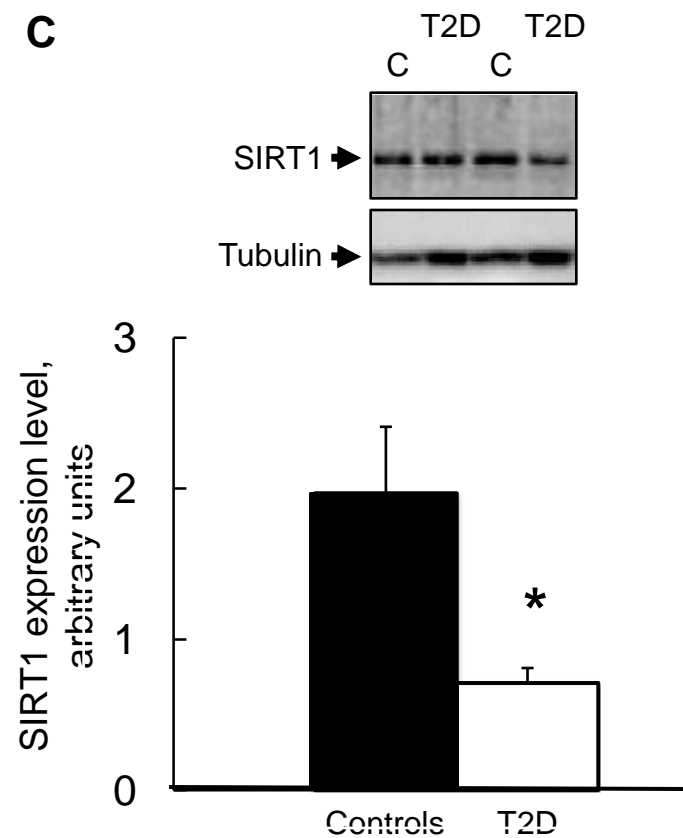
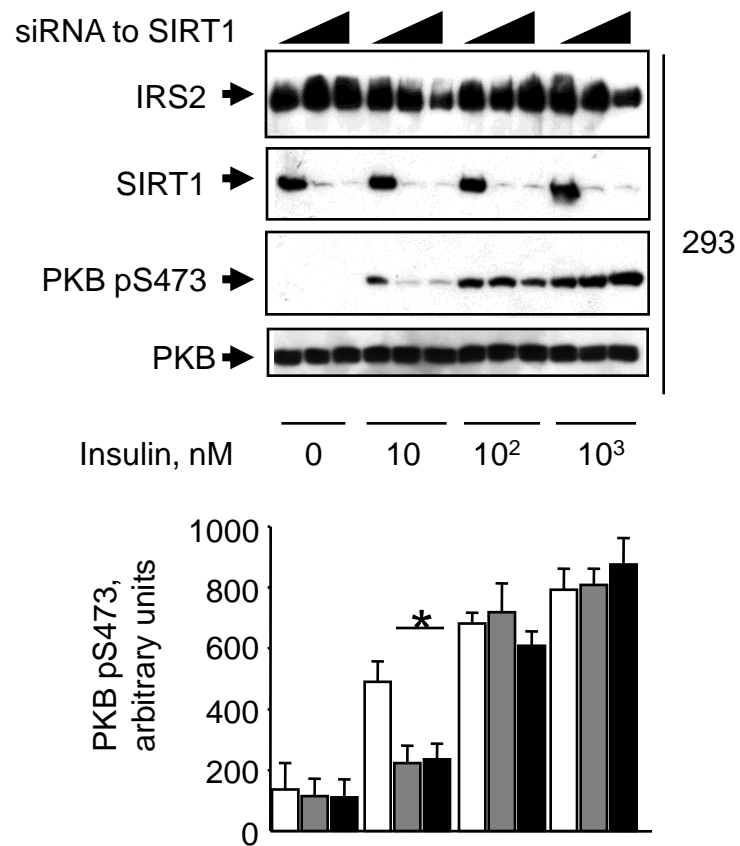


Figure 2

A



B

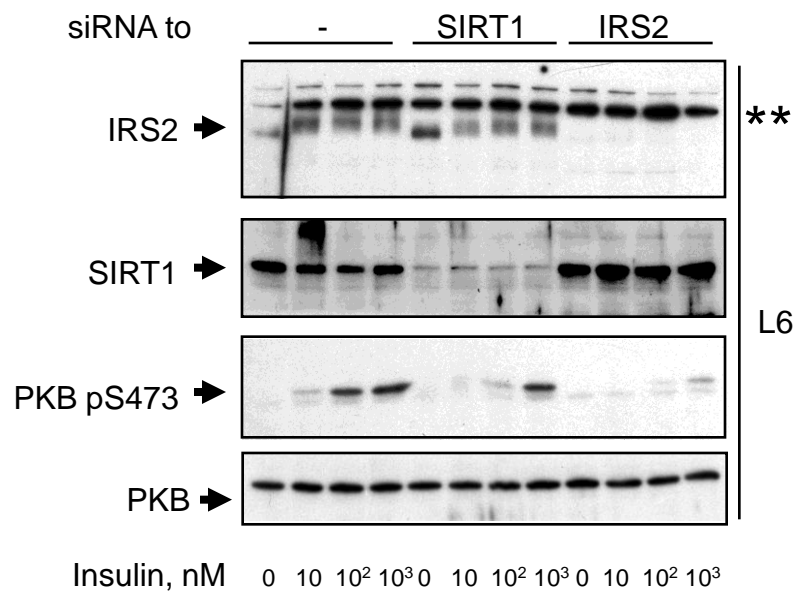


Figure 3
Figure 3

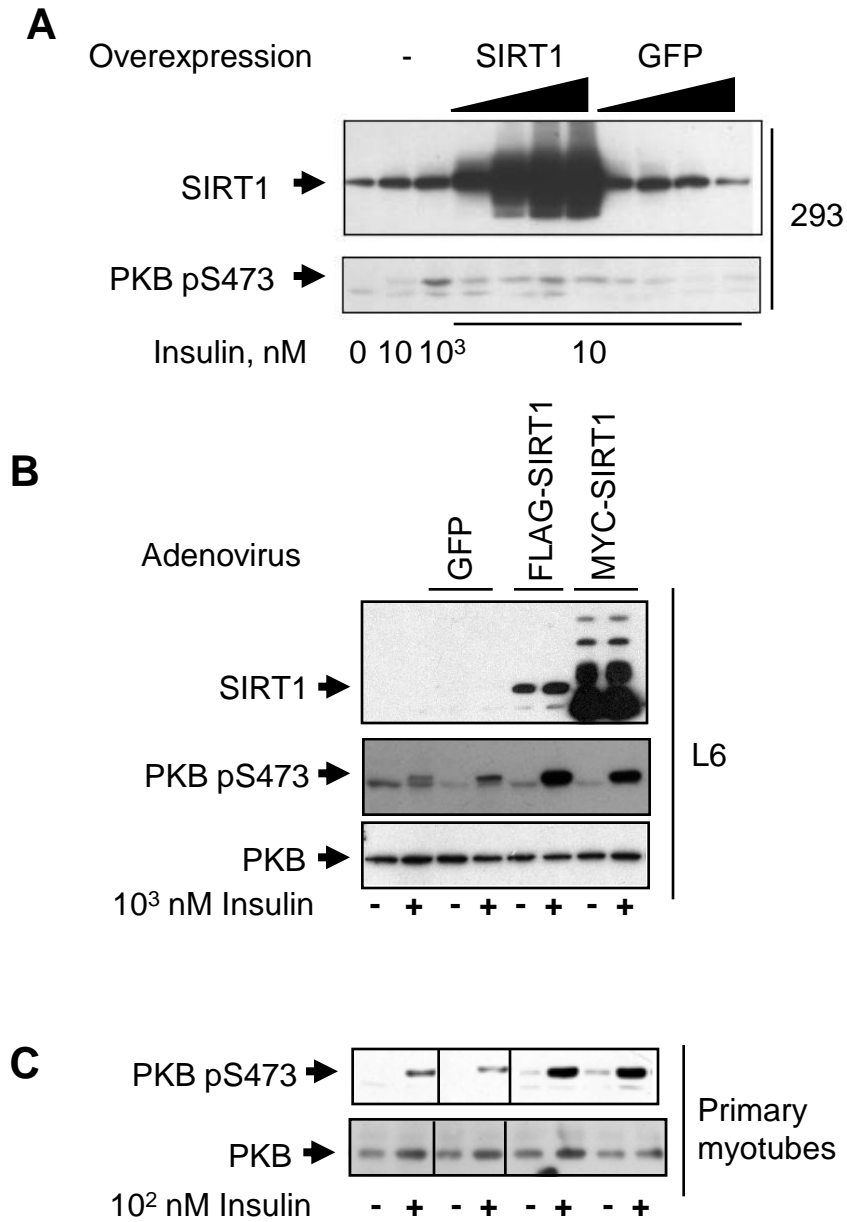
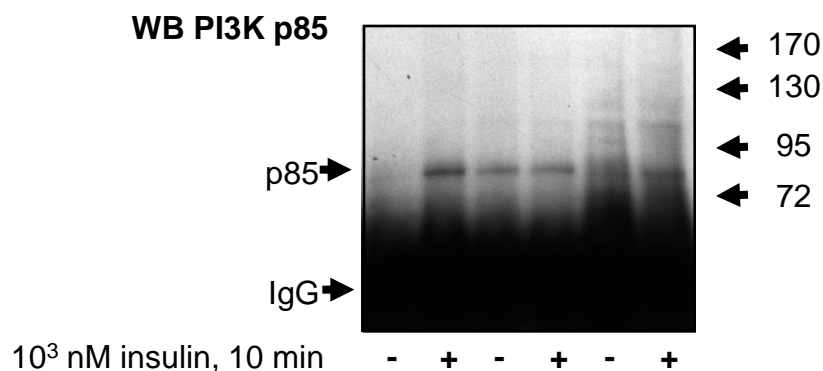
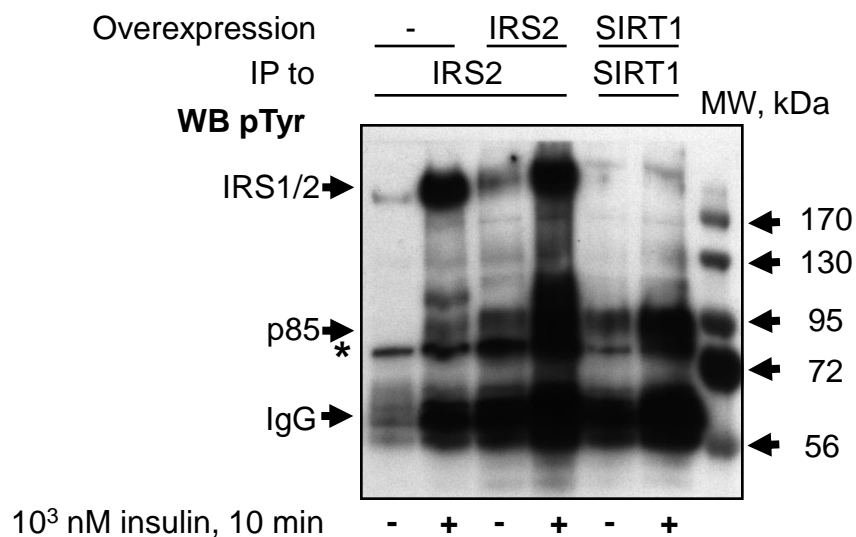
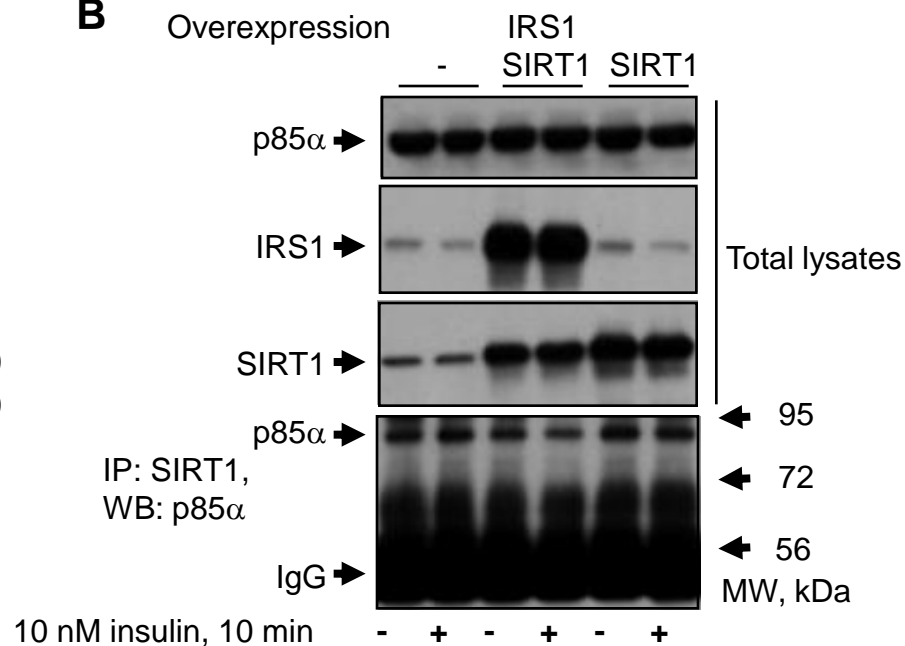


Figure 4

A



B



C

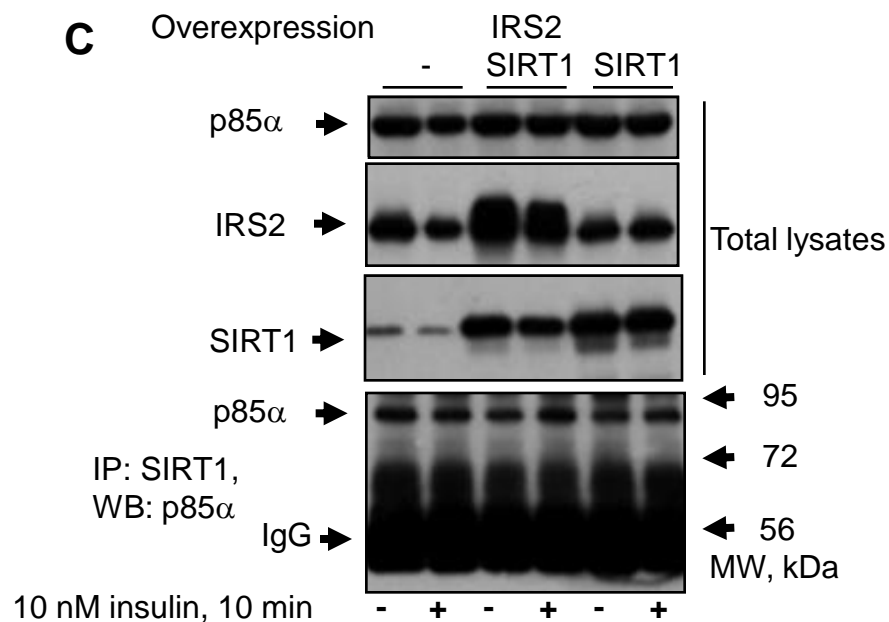
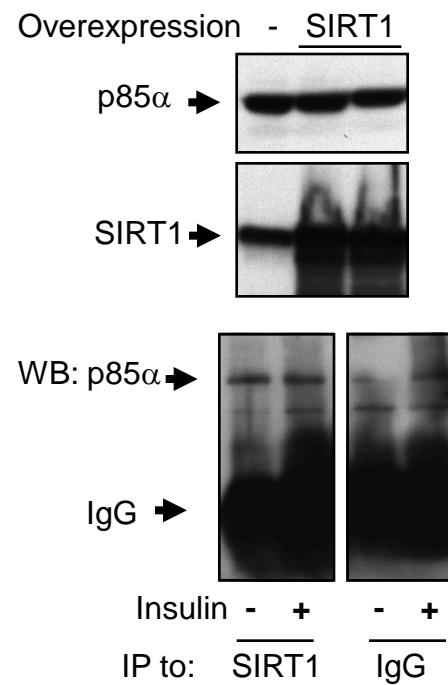
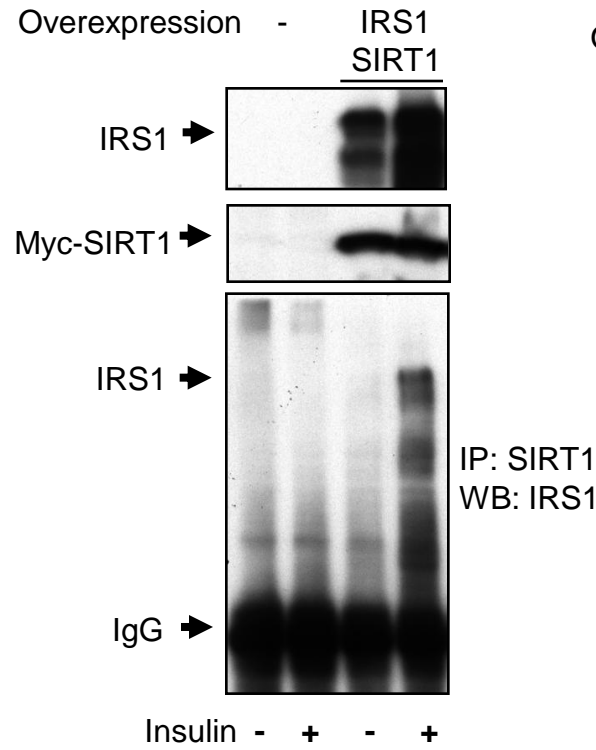


Figure 4

D



E



F

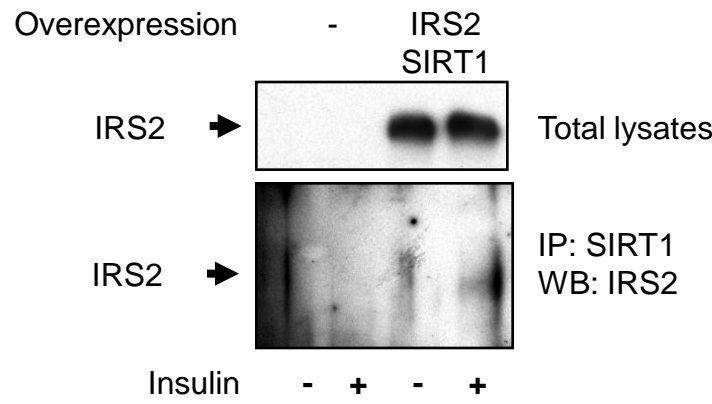


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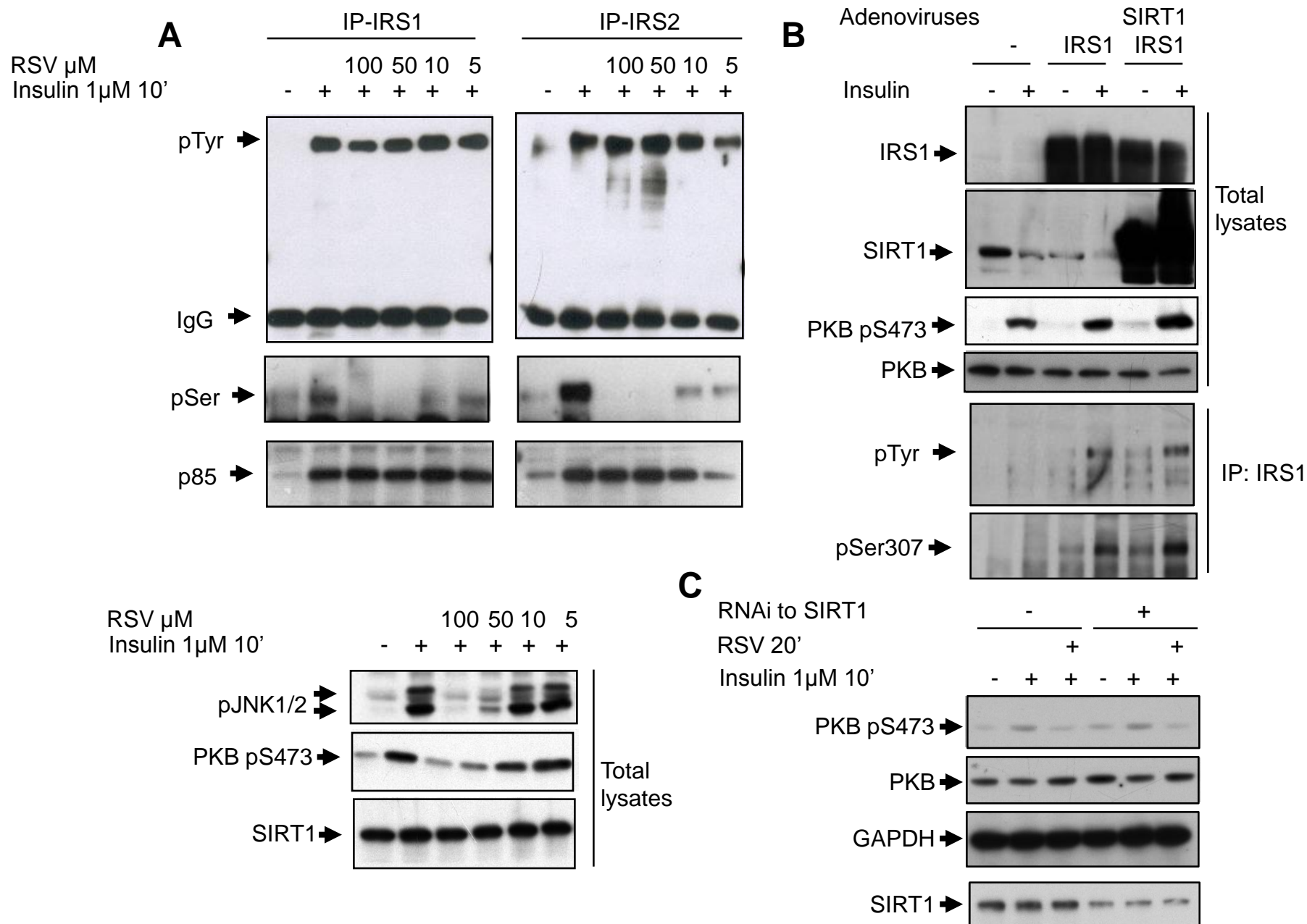


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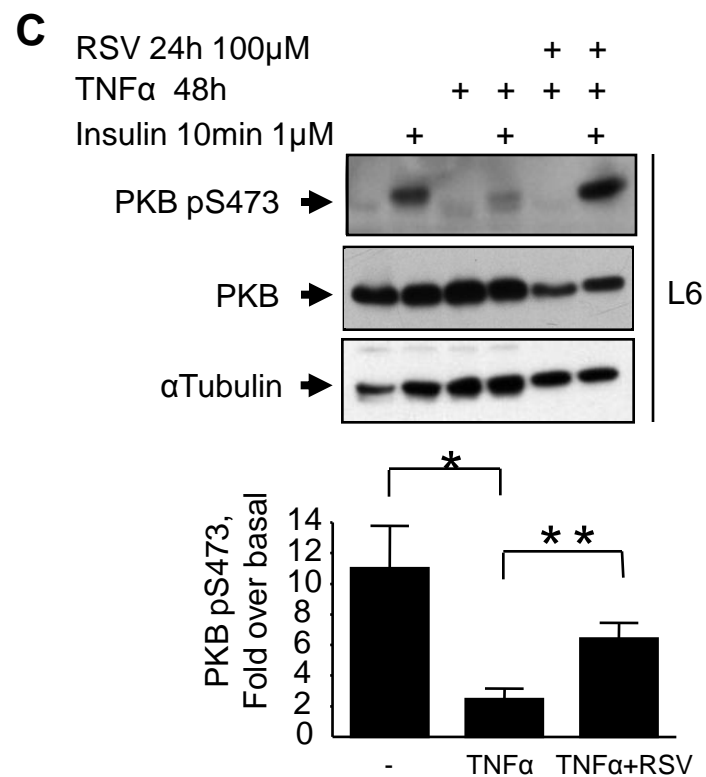
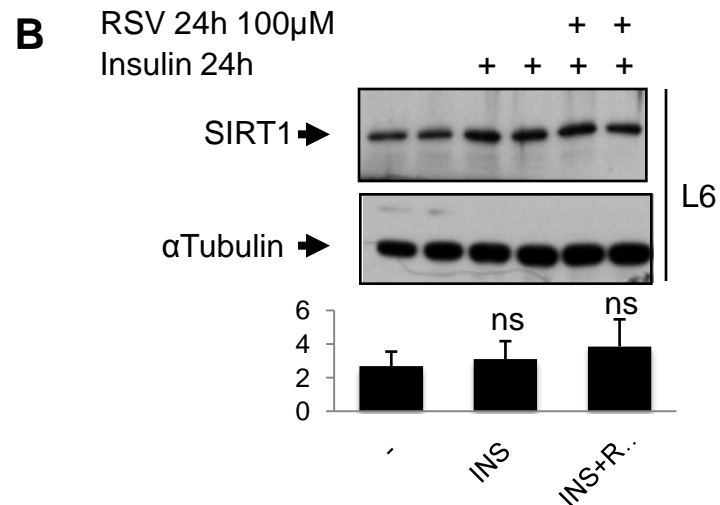
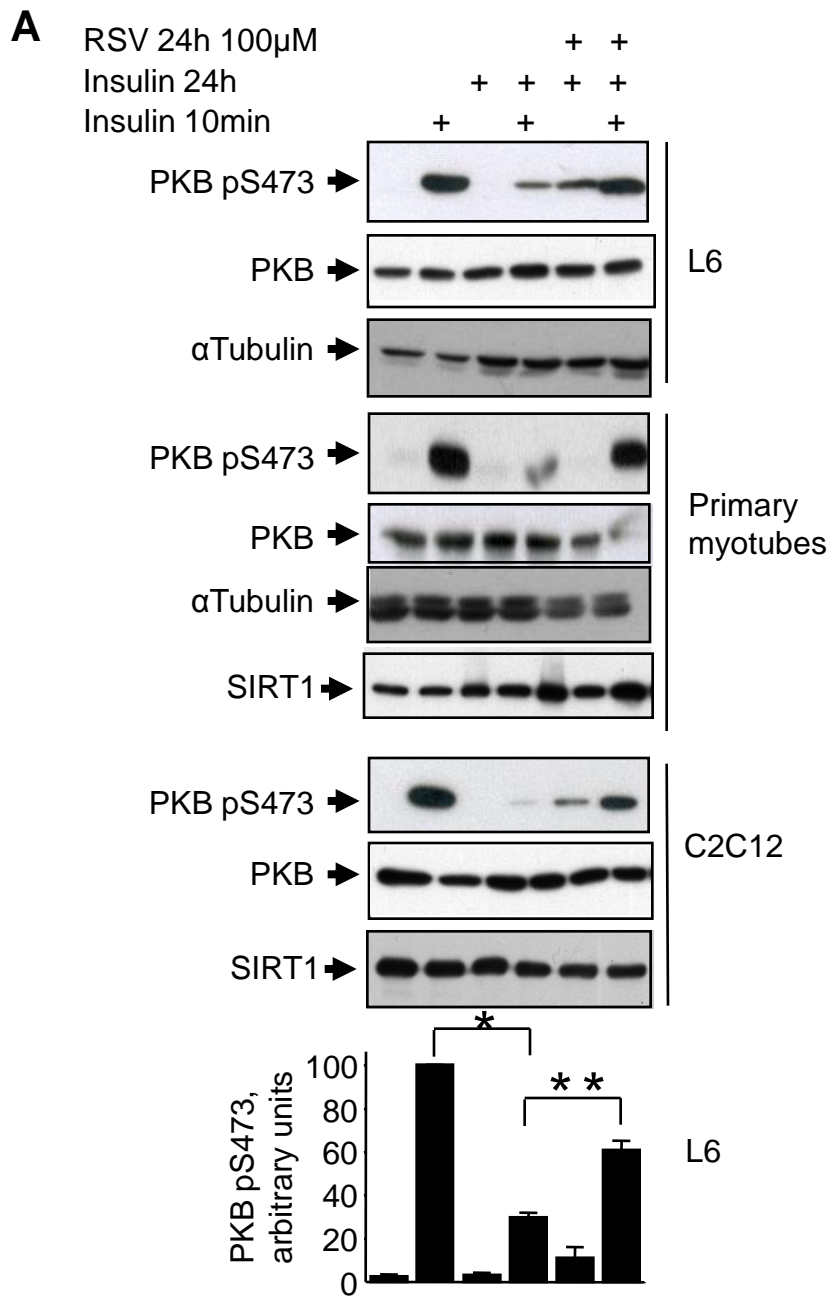


Figure 6

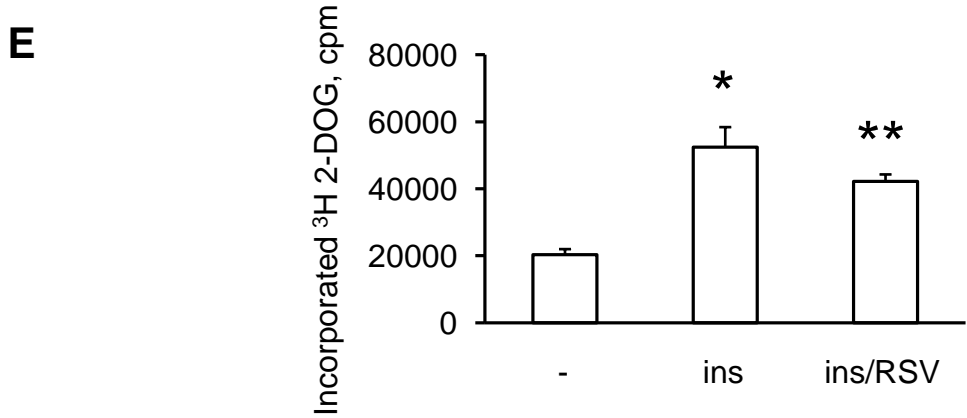
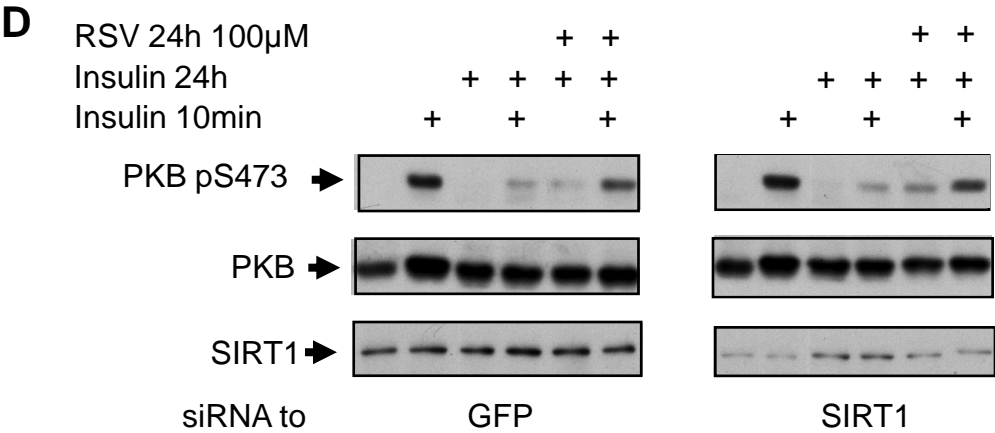


Figure 7

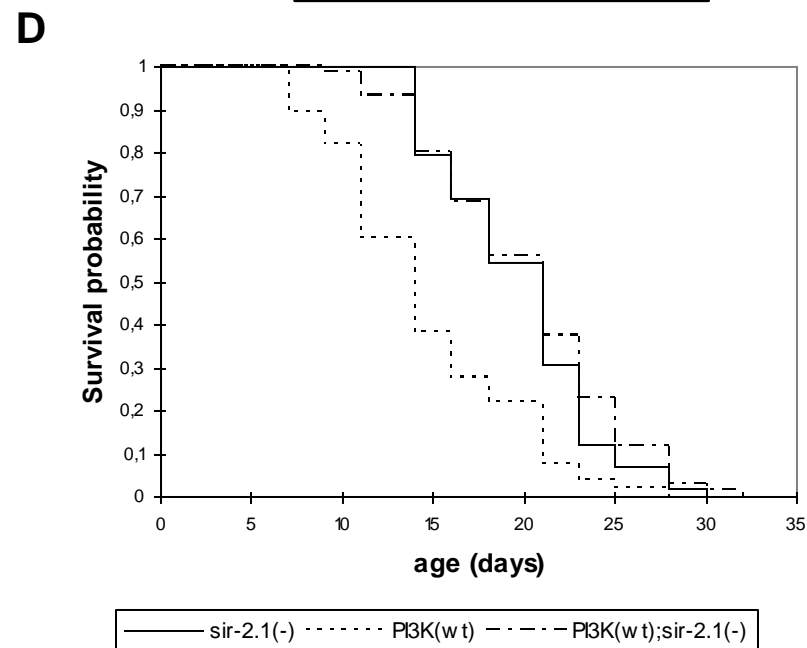
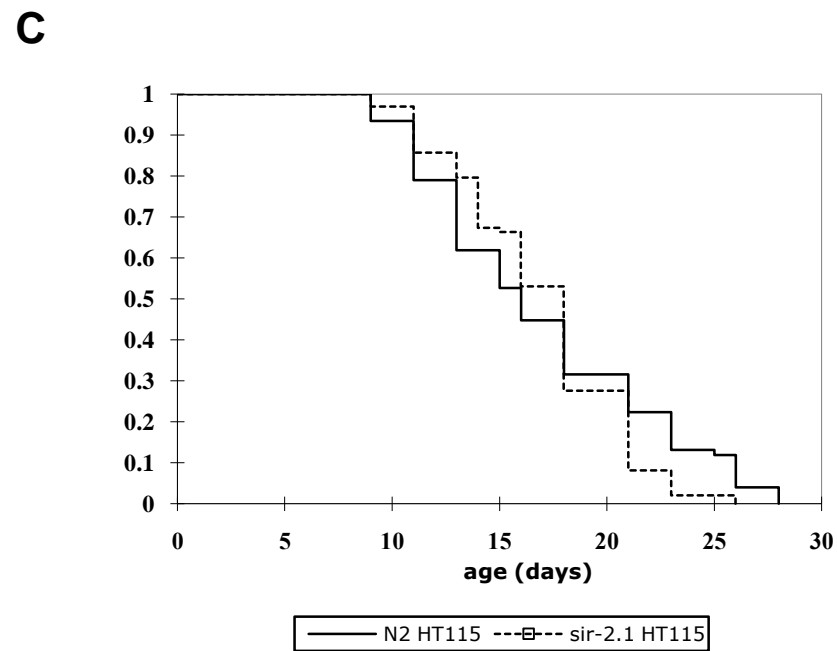
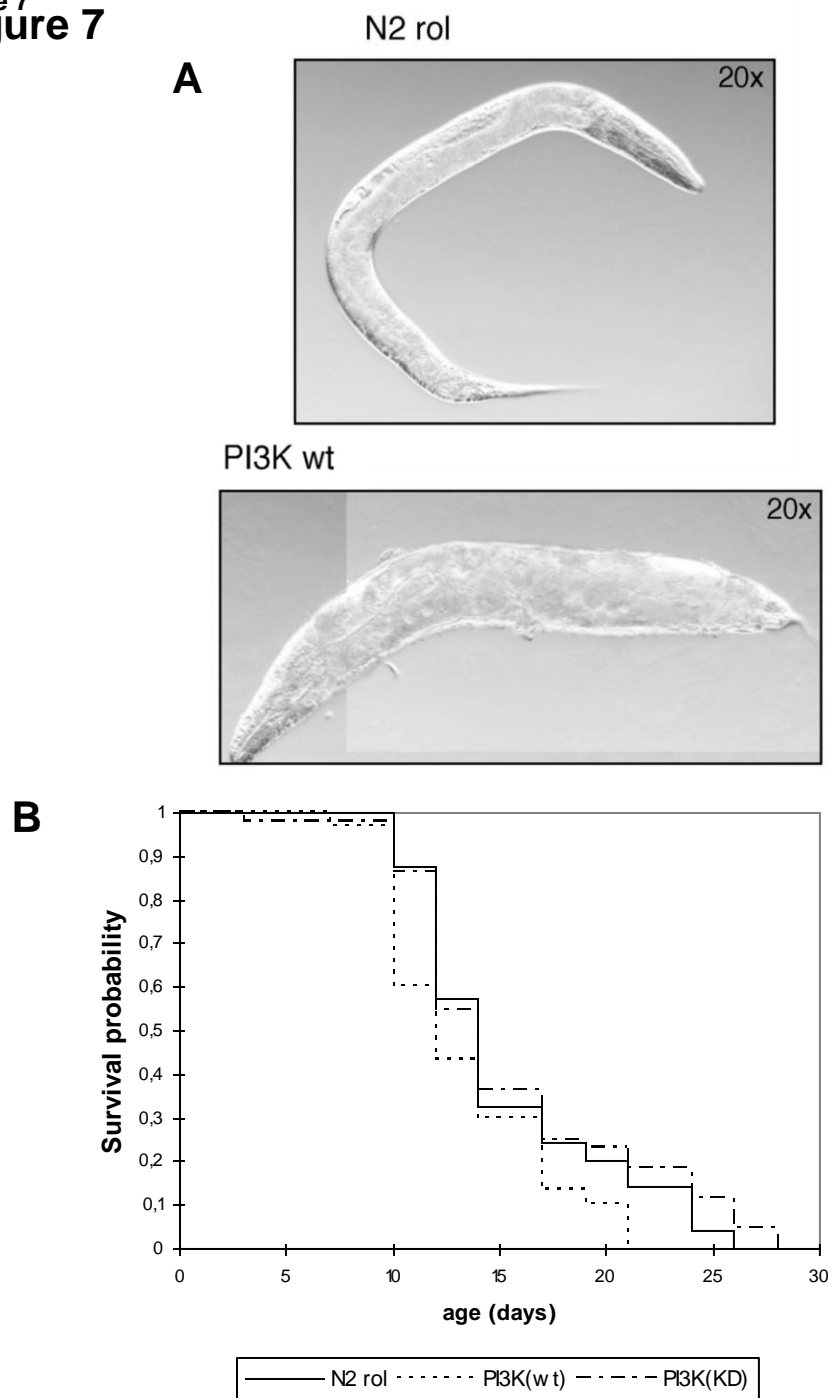


Figure 8

Figure 8

