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1 **Sirtuin 1 independent effects of resveratrol in INS-1E β -cells**

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Abstract

Resveratrol (Resv), a natural polyphenol, is suggested to have various health benefits including improved insulin sensitivity. Resv activates Sirtuin (Sirt1) in several species and tissues. Sirt1 is a protein deacetylase with an important role in ageing, metabolism and β -cell function. In insulinoma β -cells (INS-1E), Resv is previously shown to improve glucose-stimulated insulin secretion in a Sirt1-dependent mechanism and to protect against β -cell dedifferentiation in non-human primates, while inducing hypertrophy in myoblasts. Mammalian (mechanistic) Target of Rapamycin (mTOR), is a key regulator of cellular metabolism and regulates the cell size, β -cell survival and proliferation.

In order to understand the interaction of Sirt1 and mTOR cascade activity with Resv-induced changes in the INS-1E cell line, we generated stable Sirt1-down-regulated INS-1E cells, and analysed Sirt1-dependent effects of Resv with respect to mTOR cascade activity. Sirt1-knockdown (KD) had a significant increase in cell size compared to negative-control (NEG CTR) cells. Resveratrol treatment increased cell size in both cell types in a dose-dependent manner at 24h (Resv conc: 15-60 μ M), and decreased the cell number (Resv conc: 30-60 μ M). Cell area was increased in NEG CTR cells (Resv conc: 60 μ M) at 24 h and KD cells at 48 h (Resv conc: 15-60 μ M). Rapamycin, a specific mTOR inhibitor, counteracted the Resv-induced cell enlargement (both cell diameter and area). Furthermore, Sirt1-downregulation by itself did not affect the mTOR cascade activities as measured by Western blotting for total and phosphorylated Akt and mTOR. Rapamycin decreased the mTORC1 activity, while increasing the pAkt levels. Resveratrol did not interfere with the mTOR activity or with Sirt1 expression. Altogether, this work indicates that Sirt1 is a negative regulator of cell size. Moreover, the effect of Resv on cell size increase is mTOR-cascade dependent.

1 **Highlights**

- 2 • Sirt1 negatively regulates cell size of clonal insulin secreting β -cells.
- 3 • Resveratrol causes cell enlargement in a dose-dependent manner in both Sirt1
- 4 knockdown and negative-control cells.
- 5 • The cell size increase by resveratrol is mTOR cascade-dependent, while Sirt1
- 6 knockdown does not change the mTOR pathway activity.
- 7 • Cell size is controlled by Sirt1 and mTOR signalling in an independent manner.

8 **Keywords**

9 Sirt1, resveratrol, mTOR, rapamycin, cell size, hypertrophy

10 **Abbreviations:**

11 AMPK, AMP-activated protein kinase
12 mTOR, mammalian (mechanistic) Target of Rapamycin
13 NAD, Nicotinamide adenine dinucleotide
14 Rapa, Rapamycin
15 Resv, Resveratrol
16 SIR2, silent information regulator 2

1. Introduction

Mammalian sirtuins, which belong to the family of silent information regulator 2 (Sir2) proteins [1], are nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases [2, 3] and ADP-ribosyl transferases [4, 5]. They are class III histone deacetylases that deacetylate both histone and non-histone proteins [6]. Reactions conducted by sirtuins occur by hydrolysis of their co-substrate NAD⁺ and the cellular NAD⁺/NADH ratio regulates their activity.

Sirt1 has important roles in cellular metabolism and ageing-associated conditions. Overexpression of Sir2 and its homologues cause increased longevity in lower organisms such as *S. cerevisiae* [7], *C. elegans* [8] and *D. melanogaster*, as well as in mice [9]. In skeletal muscle cells, Sirt1 is proposed to improve mitochondrial biogenesis and function through deacetylation and activation of PGC-1 α [10]. In rat pancreatic β -cells, Sirt1 improves insulin secretion by downregulating UCP2 [11]. Also, Sirt1 knockout mice present with low levels of insulin and constitutively high levels of UCP2 [11].

Sirt1 is activated by the polyphenol resveratrol (3,5,4'-trihydroxy-trans-stilbene) (Resv), which is found in grapes, berries and many other plants [12, 13]. Treatment with Resv improved health, increased insulin sensitivity and mitochondrial number in mice on high calorie diet and mimicked caloric restriction [14]. Low doses (20-30 mg/kg/day) of Resv have been proposed to activate AMP-activated protein kinase (AMPK), cause increased mitogenesis and NAD⁺ levels via Sirt1 activation, whereas high doses (above 215 mg/kg/day) of Resv may activate AMPK in Sirt1-independent manner [15].

The aim of the current study was to characterize the Sirt1-dependent effects of Resv on cell size and growth of a pancreatic β -cell model. This is relevant, because Sirt1 as well as Resv improves β -cell insulin secretion [11, 16]. Resv also protects against high energy diet-induced β -cell dedifferentiation in non-human primates [17], while in skeletal muscle Resv also induces

hypertrophy [18]. Hypertrophy, cell growth and size are to a large extent determined by activity of the mTOR pathway in pancreatic β -cells [19, 20] and we therefore examined β -cell size changes introduced by Sirt1, Resv as well as mTOR activity modulation.

2. Materials and methods

2.1. Cell culture and reagents

INS-1E cells are immortalized rat pancreatic β -cells, and were a kind gift from Claes Wollheim, University of Geneva, Switzerland [21]. Cells were mainly cultured in RPMI1640 with 25 mM Hepes and L-Glutamine (Lonza) or RPMI1640 with GlutaMAXTM-I (used for culturing cells for RNA extraction) (Thermo-Fisher Scientific) supplemented with 10% heat inactivated foetal calf serum (Hyclone), 20 units/mL of penicillin/streptomycin (Sigma-Aldrich) and 50 μ M β -mercaptoethanol at 37 °C under 5 % CO₂ humidified atmosphere. Resveratrol (Resv, Sigma-Aldrich) and rapamycin (Rapa, Sigma-Aldrich) stocks with intended concentrations were prepared in DMSO. Plasticware was from Nunc (Thermo-Fisher Scientific).

2.2. Generation and verification of stable Sirt1 knockdown INS-1E cell lines

Sirt1-knockdown and negative control INS-1E lines were generated using retroviral transduction with plasmids pRetroSuper control and pRetroSuper Sirt1 (a kind gift from Laura Bordone [11]). In brief; retroviral particles were prepared by transient transfection of pRetroSuper plasmids into Phoenix Eco HEK-293 cells and medium containing retroviral particles were harvested 48 hrs post-transfection, centrifuged and filtered through 40 μ M filters. Subsequently, viral particles were mixed with sequabrene (5mg/mL, Sigma-Aldrich) and added to INS-1E cultures (passage 92). 48 hrs after infection, selection was applied (puromycin 0.75 μ g/mL) and pools of clones were established. In total, 3 Negative Control

(NEG CTR.) and 3 Sirt1 knockdown (KD) clone pools were established. Cells were kept under puromycin selection for 7 days, expanded and frozen in aliquots. RNA from selected INS-1E pools were extracted using Triagent (Sigma-Aldrich) using manufacturers' protocol, quantified using a ND1000 spectrophotometer and reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturers' protocol for hexamer primed cDNA. Steady state Sirt1 mRNA levels were measured by Q-PCR using primers 5'-TGCCATCATGAAGCCAGAGA-3' and 5'-GAGAAGACCCAATAACAATGAGGA-3' for Sirt1 and 5'-GTTCTGCTCCAACCTTTGCCT-3' and 5'-TGTGTAGCTGCCATCTGCACTT-3' for TFIIB as reference transcript with QuantiTect Sybr (Qiagen) and standard curve determination of cDNA quantities on an Agilent MX3005P.

2.3. Cell size, number and impedance measurements

INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 1×10^5 cells/well in 24-well plates. After o/n attachment cells were treated with Resv, Rapa or combinations of Resv and Rapa for 24-48 hr with indicated concentrations. Cells were detached by trypsination (0.05 % trypsin with 0.02 % EDTA for 20 min.) and cell number and cell diameter were measured on Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter). Cell size measurements were then analysed according to best fit with Gaussian distribution log-transformed counts to obtain the mean cell diameter. Microscopy images were obtained for each indicated time point and analysed with ImageJ software.

For impedance measurements, INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 6×10^4 cells/well in E-plate L-8 (Acea Biosciences, Inc.). As the first step, basal readings were obtained with 300 μ L cell culture medium for 10 x 1 min. Then, 200 μ L of cell suspension

with desired cell density was added into the wells. During the attachment period for 24 h, cell index measurements were obtained every 10 min. for 4 h. and then every 30 min. After the attachment period, medium was gently removed and cells were treated with Resv, Rapa or combinations of Resv and Rapa. Cell index measurements were obtained every 10 min. for 4 h. and then every 30 min.

2.4. Cell cycle analysis

INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 1×10^6 cells/well in 6-well plates. After o/n attachment period, cells were treated either with vehicle (DMSO) or Resv (30 μ M) for 6-24 h. Cells were collected by trypsination and washed with cold PBS. The cell number and cell diameter were measured on Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter). 500,000 cells/sample were collected in 200 μ L cold PBS. After fixation with ice-cold ethanol (70% v/v) for 30 min on ice, samples were preserved at -20 °C until staining and flow cytometry analysis.

For flow cytometry analysis of cell cycle phase distribution, the fixed sample was centrifuged and supernatant discarded. After washing with PBS, cells were resuspended in 400 μ L PBS and treated with RNase A (100 μ g/mL) and propidium iodide (40 μ g/mL) at room temperature in the dark for 30min. The supernatant was discarded by centrifugation and sample was collected in 500 μ L PBS, and analyzed by flow cytometry (BD FACS Calibur). The obtained results were analysed by using ModFit LT 4.1 (Verity Software House).

2.5. Antibodies and western blotting

All primary antibodies were used at 1:1000 dilution in 5% skim milk-TBS-T (Sigma-Aldrich). Anti-phospho-mTOR (Ser2448) antibody (2971), anti-mTOR antibody (2972), anti-phospho-Akt (Ser473) antibody (4060), anti-Akt (pan) antibody (4685), anti- β -Actin antibody (4967), anti-phospho-S6 ribosomal protein (Ser235/236) antibody (2211) were obtained from Cell

1 Signalling Technology. Anti-Sirt1 antibody (07-131) was purchased from Merck Millipore,
2 anti-GAPDH (ab8245) was purchased from Abcam, and anti-Deptor antibody (SAB4200241)
3 was purchased from Sigma-Aldrich. HRP conjugated anti-rabbit IgG (P0217) and anti-mouse
4 IgG (P0260) was purchased from Dako (Agilent Technologies Denmark), and used at 1:2000
5 dilution in 5% skim milk.

6 For immunoblotting from total lysate, 2×10^6 cells were seeded in 6 cm dishes. Cells were lysed
7 in in lysis buffer (1% Triton x-100, 0.5% NP-40, 75 mM NaCl, 50 mM Tris at pH=7.4, 10 %
8 glycerol) supplemented with phosphatase inhibitor mix (Halt Phosphatase Inhibitor Cocktail,
9 Thermo Scientific) and protease inhibitor mix (cOmplete, EDTA-free protease inhibitor
10 cocktail, Roche). Total protein concentrations were determined by using Bio-Rad protein assay
11 reagent and samples were stored at -80 °C until use. 30 µg of total cell lysates were separated
12 on 4-12 % SDS-PAGE gels (Expedeon). Proteins were transferred to 0.45 µm PVDF
13 membrane (GE Healthcare) using semi-dry transfer with Towbin transfer buffer (25 mM Tris,
14 192 mM Glycine, 20% methanol) at 22 V for 1 h at 4 °C. The membrane was blocked with 5
15 % skim milk for 1h. Phospho-specific antibodies were always used in the first round of probing.
16 Membrane was stripped by using Restore™ PLUS Western Blot Stripping Buffer (Thermo
17 Scientific) and probed with total protein specific antibodies. Immunoblot images were acquired
18 and bands were quantified by using BioSpectrum® AC Imaging System (UVP).

19 **2.6. Statistical analysis**

20 Statistical analyses were carried out using the GraphPad Prism® 6. All results are shown as
21 mean and standard deviation. Statistical analyses methods were indicated within the figure texts
22 and the p values equal to or lower than 0.05 were accepted as significantly different.

3. Results

3.1. Assessment of Sirt1 mRNA and protein levels in Sirt1 KD and NEG CTR INS-1E β -cells

To analyse the level of Sirt1 reduction in INS-1E cells, we measured the mRNA and protein from Sirt1-Negative Control (NEG CTR) and Sirt1-Knockdown (KD) cells. Sirt1 mRNA levels were downregulated $43 \pm 16\%$ ($P=0.0003$) in KD cells compared to NEG CTR cells (Figure 1a). In accordance with this, Sirt1 protein levels were found to be down-regulated in KD cells by $59 \pm 19\%$ ($p=0.0004$) (Figure 1b and c).

Figure 1

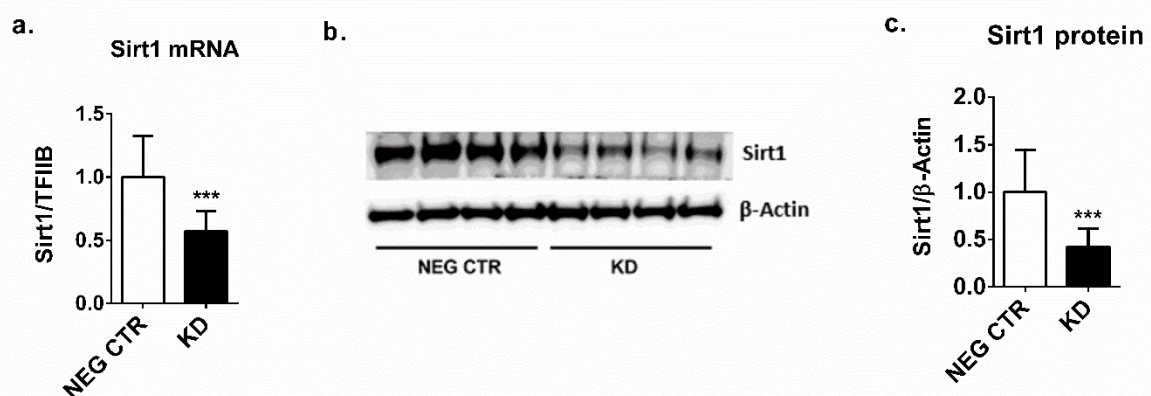


Figure 1. Assessment of Sirt1 knockdown. (a) Sirt1 mRNA levels ($n=16$ and 12 for NEG CTR and KD INS-1E cells, respectively), (b) Representative immunoblot image, and (c) Sirt1 protein levels ($n=12$) of in NEG CTR (White bars) and KD (Black bars) cells. Data are mean \pm S.D. and are normalized against the NEG CTR cells. Statistical analysis: Unpaired t test *** $p<0.001$.

3.2. Sirt1 KD and Resveratrol treatment independently increase cell size

Cell size analysis of Sirt1 KD vs. NEG CTR showed that KD cells had a significant increase in cell diameter ($4.6 \pm 3.6\%$ and $2.1 \pm 0.7\%$ at 24 h and 48 h, respectively; Figure 2a and b). The increase in cell size was more pronounced after 24 h of exposure than 48 h, most likely because

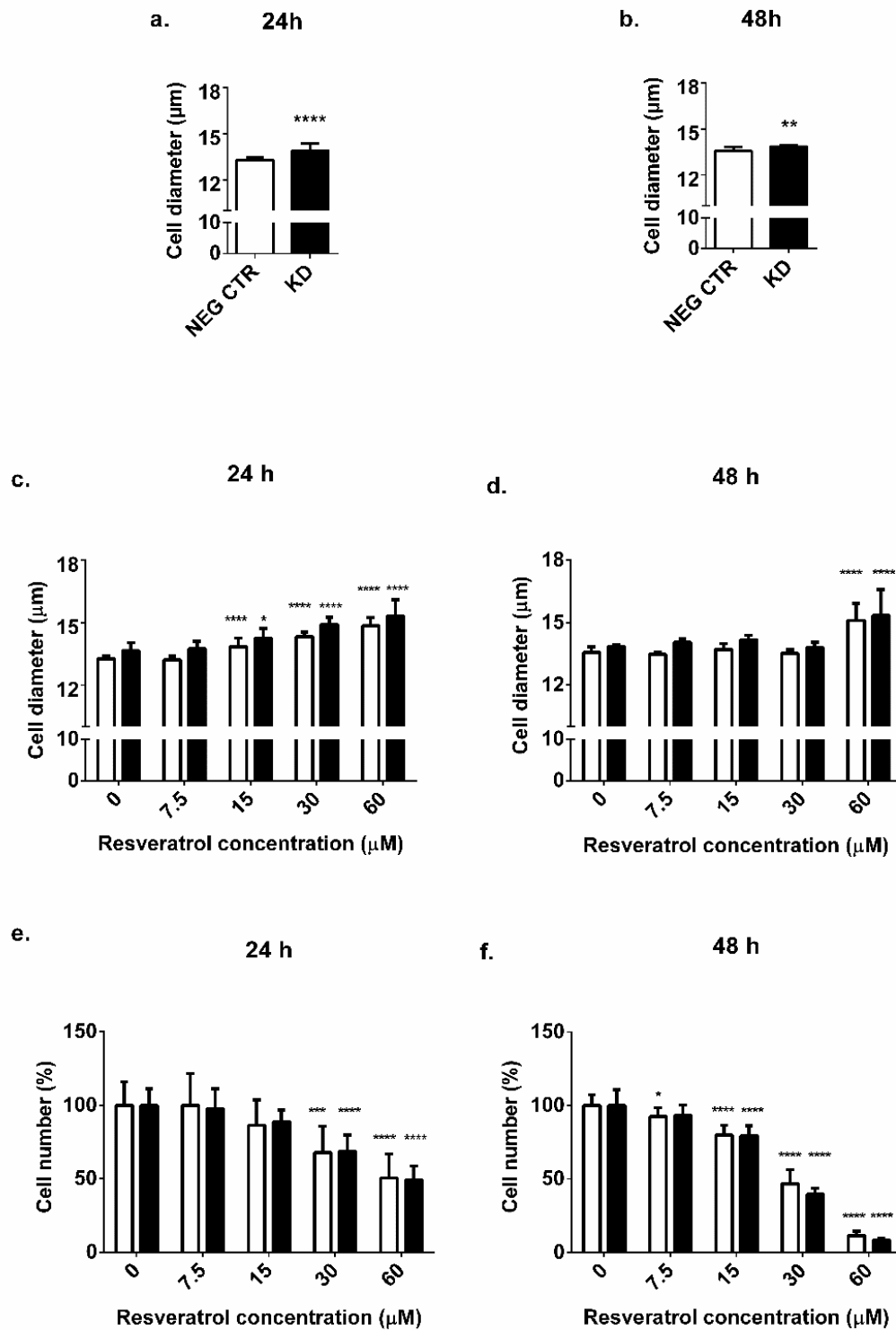
the confluency of cells at 48 h limits cell size expansion. In other cell types, Resv has been found to increase cell size and in order to analyse the interaction between Resv and Sirt1 activity on cell number and cell size, we treated NEG CTR and Sirt1 KD INS-1E cells with Resv in a dose-dependent manner.

Resv caused a dose-dependent increase in cell diameter in both NEG CTR and KD cells at 24 and 48 h (Figure 2c and d). A significant increase in cell diameter was observed at 15, 30 and 60 μ M Resv treatment at 24 h, but only at 60 μ M Resv at 48 h. The effect of Resv on cell diameter increase did not differ in response to the Sirt1 deficiency. In NEG CTR cells, 15, 30 and 60 μ M Resv for 24 hrs resulted in $4.3\pm2.9\%$, $8.0\pm2.1\%$ and $11.9\pm2.8\%$ increase in cell diameter, respectively (Figure 2c). In KD cells, 15, 30 and 60 μ M Resv for 24 h increased the cell diameter with $4.6\pm3.5\%$, $9.4\pm1.9\%$ and $12.4\pm3.5\%$, respectively (Figure 2c). Moreover, 48 hrs treatment with 60 μ M Resv resulted in $11.4\pm6.0\%$ and $10.8\pm9.0\%$ increase in cell diameter of NEG CTR and KD, respectively (Figure 2d).

Change in cell size should be seen as change in cell area as well, but measurements indicated no difference between non-treated NEG CTR and KD cells at both 24 and 48 h (data not shown). Also, cell area was found unaffected by resveratrol treatment in NEG CTR cells after 24 and 48 h (Figure S1a and b), whereas 60 μ M at 24 h and 15, 30 and 60 μ M Resv significantly increased the area in KD cells after 48 h treatment (Figure S1a and c).

Resv treatment for 24 h caused a significant dose-dependent decrease in cell counts at 30 and 60 μ M Resv in both NEG CTR and KD cells (Figure 2e), whereas 48 hrs treatment led to a significant cell number reduction at lower concentrations (at 7.5 and 15 μ M treatment in NEG CTR and at 15 μ M in KD) and further reduction at 30 and 60 μ M treatments in both NEG CTR and KD cells (Figure 2f).

Figure 2



1 **Figure 2. Sirt1 KD increases cell diameter, while Resv decreases the cell number and increasing the cell**
2 **diameter in a dose-dependent manner. (a, b):** Effect of Sirt1 KD on cell diameter at **(a)** 24 h and **(b)** 48 h. Data
3 are mean \pm S.D. (n=23 at 24 h and n=12 at 48 h). Statistical analyses: Unpaired t test **p=0.0022 and
4 ****p<0.0001. **(c, d):** Cell diameter comparison of NEG CTR and KD cells in response to resveratrol treatment

at (c) 24 h and (d) 48 h. Data are mean \pm S.D. (n=3). (e, f): Dose-dependent effect of resveratrol on cell counts for NEG CTR and KD cells at (e) 24 h and (f) 48 h. Cell counts were normalized against the 0 μ M Resv treatment for each cell line. Data are mean \pm S.D. (n=3). Statistical analyses for c-f: One-way ANOVA followed by Dunnett's multiple comparison test against 0 μ M Resv treatment within each cell line. *p<0.05, ***p<0.001 and ****p<0.0001. White and black bars belong to the measurements for NEG CTR and KD cells, respectively.

3.3. Sirt1-KD and resveratrol induces accumulation of the cells in different phases

In order to analyse the effect of Sirt1 activity and Resv on cell cycle distribution, we treated the NEG CTR and KD cells with Resv for 6, 12 and 24 h. Knock-down of Sirt1 led to a significant increase in G₂/M phase accumulation (Figure 3). Treatment with 30 μ M Resv caused an increased number of NEG CTR cells in S phase reaching a statistical significantly higher level at 12 h, while there was no change in KD cells. Besides the cell cycle analyses, we also analysed the effect of Resv on apoptotic events in both cell lines. Resv did not induce apoptosis neither in NEG CTR nor KD cells (Figure S2).

FIGURE 3

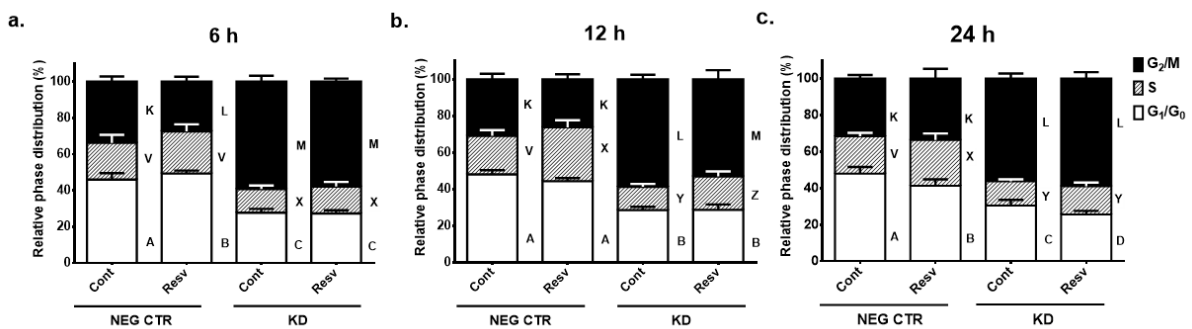


Figure 3. Sirt1 KD induces the G₂/M phase accumulation, and Resv (30 μ M) did only slightly induce the S-phase accumulation. Cell cycle distribution of NEG CTR and KD cells at (a) 6 h, (b) 12 h and (c) 24 h following exposure to vehicle or 30 μ M Resveratrol (Resv). Statistical analyses: 2-way ANOVA followed by Tukey's multiple comparison test within each column (Treatment groups), comparing the rows (Cell cycle phase; simple effects within columns). Data are mean \pm S.D, n=3 independent experiments. Different letters in each cell cycle phase (a-d, v-z and k-m) indicate statistical differences between the treatment groups. p<0.05.

3.4. Inhibition of mTOR activity reverses the cell-size increase by resveratrol

Because the mTOR pathway is a known regulator of cell size, we analysed the effect of the mTOR inhibitor rapamycin on cell counts and diameter. In order to determine the relevant rapamycin concentration, we screened the dose dependent effect of rapamycin (Conc: 2.4-300 nM) on cell number and diameter for 24 h in our preliminary experiments. In NEG CTR cells, up to 60 nM Rapa, while in KD cells up to 12 nM Rapa ($p=0.04$; Figure S3a), did not affect the cell number counts, whereas 300 nM led to significant reduction compared to control in both NEG CTR and KD cells ($p<0.0001$; Figure S3a). Rapa significantly reduced the cell diameter at all concentrations tested, in both NEG CTR and KD cells (Figure S3b).

The mTOR-dependent effect of Resv-induced cell enlargement was tested with the combination of 30 μ M Resv and 12 nM Rapa on cell counts and cell size in NEG CTR and KD cells. Treatment with 12 nM rapamycin alone did not reduce the cell counts compared to control group in both cell lines (Figure 4a), whereas it reduced the cell diameter significantly (5.5 % in NEG CTR and 6.4 % in KD cells; Figure 4b). Cell counts were significantly reduced in 30 μ M Resv treated cells (Figure 4a). Resv increased the cell size significantly in both NEG CTR (8.5 %) and KD (3.7 %) cells and this increase was reversed with treatment in combination with Rapa (Figure 4b). 10 μ M Resv either alone or in combination with Rapa did not interfere either with cell number or diameter in both cell lines (data not shown). These results suggest that the Resv-induced cell size is mTOR dependent. Cell area analyses revealed a similar pattern with diameter measurements (Figure S4a and b). Resv significantly increased the cell area in both cell lines and Rapa reversed this increase. However, Rapa alone did not influence the cell area.

1 Measurement of the impedance in cultured NEG CTR and KD cells is an indication of cell
2 attachment (adherence), cell area and cell number. During attachment, KD cells showed
3 increased impedance relative to NEG CTR cells, indicating a higher degree of cell attachment
4 or bigger cells (Figure 5a). During the first 1-4 h of treatment with Rapa and combined
5 treatment with Resv and Rapa, the impedance increase was significantly higher in both cell
6 lines (Figure 5b). Also, a small but significant increase in impedance was observed for Resv,
7 but only in NEG CTR cells. These effects were transient, and no differences were observed in
8 the following 24 hr (data not shown).

Figure 4

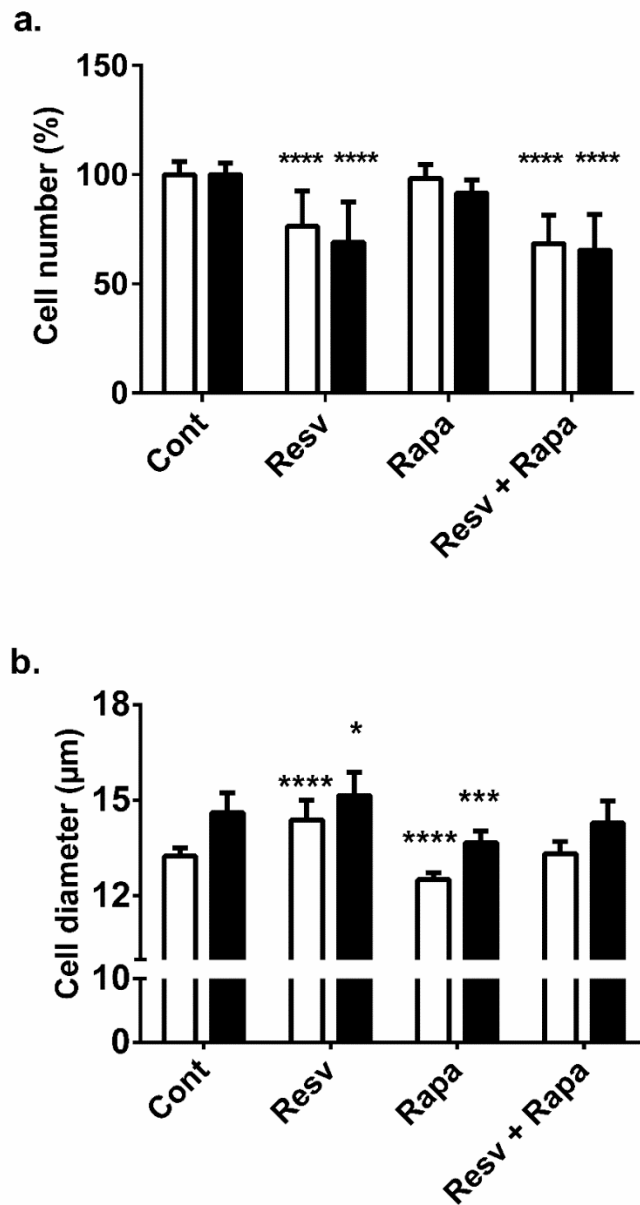


Figure 4. Rapa (12 nM) reverses the cell size increase induced by Resv (30 μM) but does not influence the cell number. (a) Relative cell number measurements and **(b)** cell diameter for NEG CTR (white bars) and KD (black bars) cells at 24 h. Data are mean ± S.D. (n=4). Statistical analyses: One-way ANOVA followed by Dunnett's multiple comparison test against control group of the respective cell line. *p<0.05, ***p<0.001 and ****p<0.0001. White and black bars in belong to the measurements for NEG CTR and KD cells, respectively.

Figure 5

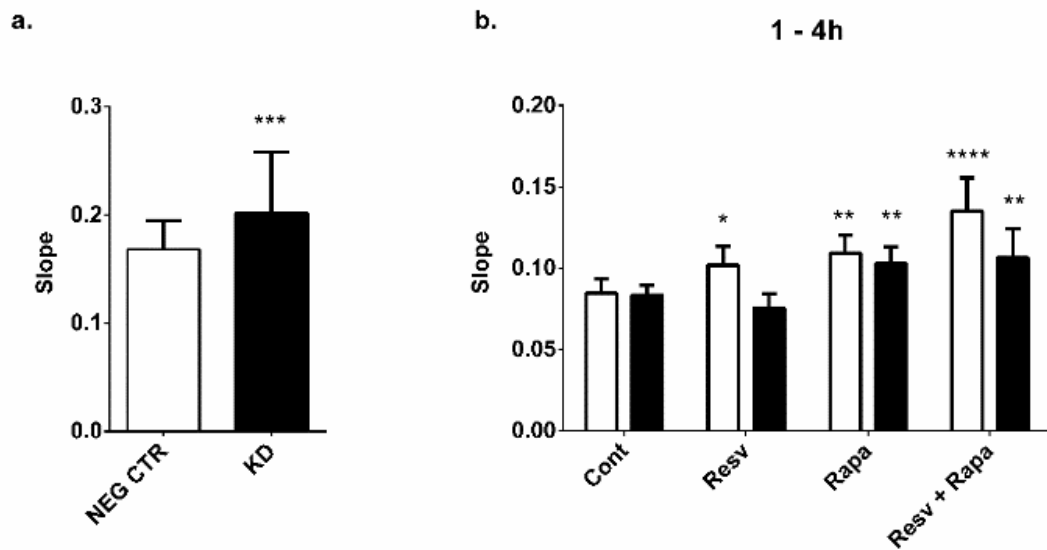


Figure 5. Effects of Sirt1 KD, Resv (30 μ M) and Rapa (12 nM) on impedance measurements. (a) Effect of Sirt1 KD on the impedance increase during the attachment period. Statistical analysis: Unpaired t test *** $p=0.001$. **(b)** Effect of Resv, Rapa and their combinations on the impedance increase between 1 and 4 h in NEG CTR (white bars) and KD (black bars) cells. Data are mean \pm S.D. ($n=5$) and normalized against NEG CTR cells control treatment. Statistical analysis: One-way ANOVA followed by Dunnett's multiple comparison test against control group of the respective cell line. * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$.

3.5. Effect of resveratrol on mTOR pathway activity

The effect of Resv on the proteins of Sirt1 and mTOR pathway in NEG CTR and KD cells was analysed by western blotting (Figure 6). Sirt1 protein levels were reduced in KD cells compared to NEG CTR cells (solvent treated cells). Neither Resv nor Rapa changed Sirt1 protein levels in NEG CTR (Figure 6a and b). The level of phosphorylated mTOR (pmTOR) was not changed by the Sirt1 KD and treatments that included Rapa reduced the pmTOR level by $\sim 50\%$ (Figure 6a and c). Phosphorylation of Akt or mTOR was not affected by Resv treatment. Furthermore, in both NEG CTR and KD cells levels of phosphorylated Akt were upregulated when the cells were treated with Rapa for 24 h (Figure 6a and d).

Figure 6

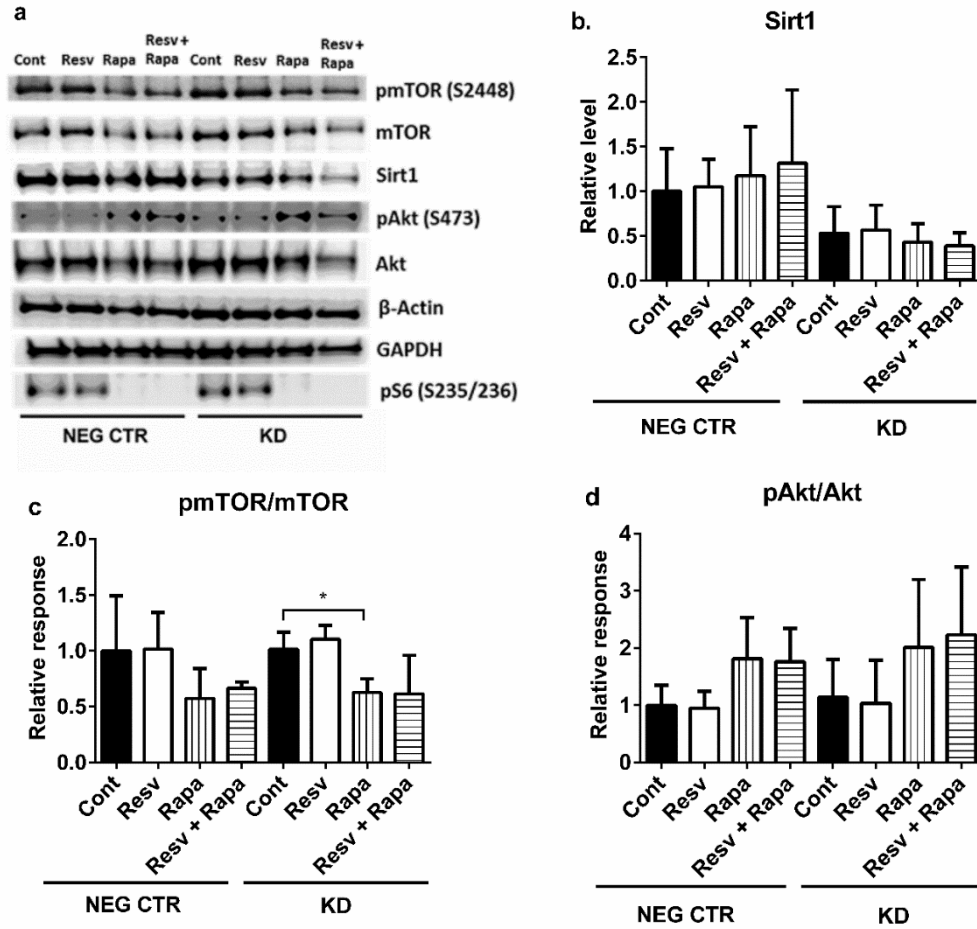


Figure 6. Resv (30 μ M) and/or Sirt1 deficiency does not interfere with mTOR pathway activity.

Representative western blot image of (a) mTOR pathway proteins, (b) Relative Sirt1 levels, (c) Relative phosphorylation of mTOR, (d) Relative phosphorylation of Akt for NEG CTR and KD cells at 24 h. Data are mean \pm S.D. (n=3) and normalized against NEG CTR cells control treatment. *p<0.05. Statistical analysis: two tailed Student's t-test.

Previously, it has been suggested that Resv inhibits mTORC1 by strengthening the physical interaction between mTOR and its inhibitor protein DEPTOR via a Sirt1-independent mechanism [22]. In order to test the effect of Sirt1 downregulation and treatment with Resv and/or Rapa, DEPTOR western blots were prepared for NEG CTR and KD cells (Figure 7). Sirt1 KD as well as Resv treatment did not affect levels of DEPTOR protein levels. Rapa treatment significantly downregulated the DEPTOR levels in both cell lines (p<0.05), but the

effect of Rapa was blunted in Sirt1 KD cells; DEPTOR levels in NEG CTR: 41% and KD: 58% of control treated, respectively. Moreover, combined treatment with the Resv and Rapa increased the DEPTOR protein level only significantly in NEG CTR cells ($p=0.05$) relative to the Rapa treated cells (Figure 7b).

Figure 7

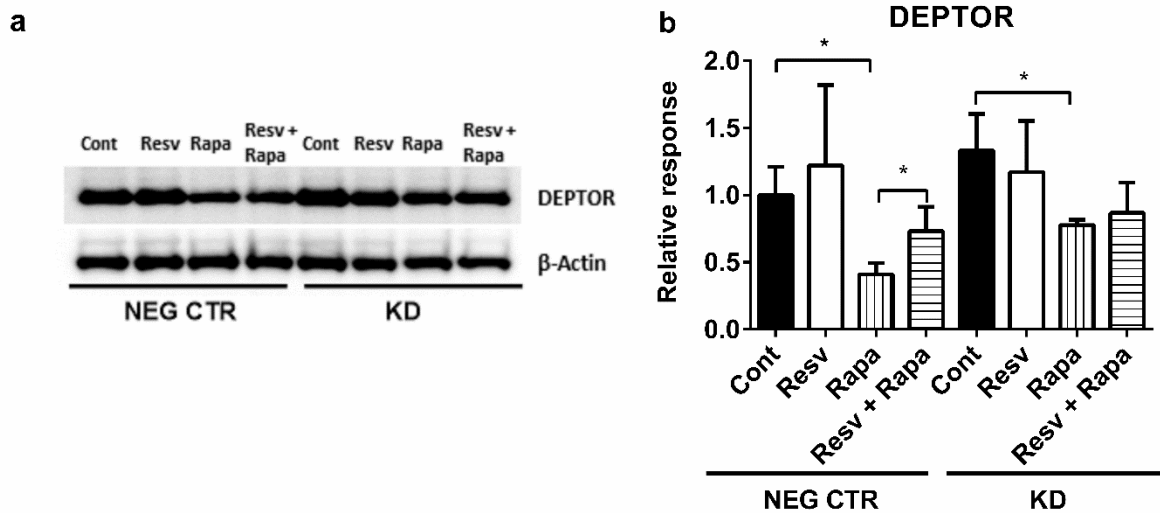


Figure 7. Resv (30 μ M) and Rapa (12 nM) decreases the total DEPTOR protein levels, whereas Resv (30 μ M) does not have an effect. Representative western blot image for DEPTOR (a) and relative DEPTOR levels (b) for NEG CTR and KD cells at 24 h. Data are mean \pm S.D. ($n=3$) and normalized against NEG CTR cells control treatment. $*p \leq 0.05$. Statistical analysis: two tailed Student's t-test.

4. Discussion and conclusion

The present work demonstrates that Sirt1 negatively regulates cell size in INS-1E β -cells and that the effect of Resv on cell size is mTOR activity-dependent, because the mTOR inhibitor Rapa reverses the effect of Resv and mTOR is known as a key regulator of cell size [23, 24]. Previous studies reported that inhibition of mTOR by Rapa leads to reduced cell size in different cell lines and primary β -cells [25]. Further, in this work, we show that chronic Sirt1 deficiency increases the cell diameter.

1 Resv increases the cell size in both Sirt1 negative control and knock-down cells, which
2 indicates that Sirt1 is not the primary target of Resv in cell size regulation. Increased cell size
3 by Resv may be a consequence of slower cell cycle progression. In pancreatic β -cell lines,
4 others have shown that Resv induces apoptosis and cell cycle arrest at 50 μ M [26]. Resv also
5 induces cell cycle accumulation in G₁ / S phases in various cancer cell lines, and inducing
6 apoptosis [27, 28]. Our results are consistent with the hypothesis that the cell enlargement could
7 be due to cell cycle arrest without halting protein synthesis. Sirt1 reduction caused an
8 accumulation in G₂/M phases whereas Resv-treatment caused a transient accumulation in the
9 S-phase. Moreover, the observed dose-dependent decrease in cell numbers after Resv
10 treatment, which is not dependent on Sirt1, could possibly be due to the induction of apoptosis
11 in INS-1E cells, but in our hands, 30 μ M Resv does not cause apoptosis in INS-1E cells (Figure
12 S2). Recently, direct molecular targets of Resv, including proteins in metabolism, such as
13 carbonyl reductase 1, fatty acid synthase and quinone 2, cell cycle, i.e. Pol α and δ , and
14 posttranslational modifications, i.e. histone deacetylases, have been reviewed by Britton *et al.*
15 [29]. However, because of the wide range of possible molecular targets of resveratrol in the
16 cell, a clear mode of action of Resv is not yet fully characterised.

17 Sirt1 [30, 31] and Resv have both been reported to inhibit the mTOR cascade [22, 32] in a
18 dose- and time-dependent manner [33]. In our study, neither Sirt1 downregulation nor Resv
19 treatment or a combination of those two interfered with mTOR pathway activity in β -cells.
20 Moreover, a more recent study showed that Resv has a nonlinear dose-response effect in mouse
21 adenoma cells, where low concentrations of Resv (0.01-1 μ M) inhibit mTOR pathway activity,
22 whereas the activity was unaltered at 10 μ M concentration [34]. This suggests that the mode
23 of action of Resv might be concentration dependent. Additionally, in our experiments, Rapa
24 completely reversed the cell enlargement induced by Resv.

1 We observed that Rapa non-significantly increased phosphorylation of Akt, an mTORC1
2 upstream factor, in a Sirt1-independent manner. Akt plays an activator role on mTORC1 [35].
3 A previous study showed that phosphorylation of Akt is induced by Rapa treatment in a
4 concentration-dependent manner [36]. In INS-1E cells, Akt regulates insulin secretion and is
5 regulated by activity of both mTOR complexes, and inhibition of mTORC1 by Raptor siRNA
6 transfection leads to activation of Akt [37].

7 DEPTOR is an mTOR complex-associated protein found in both mTORC1 and mTORC2 and
8 inhibits their activities [38]. Here, we observed that Rapa significantly downregulates the
9 DEPTOR protein levels independently of Sirt1. mTOR plays a key role in mRNA translation
10 via regulation of translational initiation and elongation, and ribosomal biogenesis [35].
11 Inhibition of mTOR by Rapa might lead to a decrease in *Deptor* mRNA translation caused by
12 inhibition of protein synthesis. Furthermore, mTOR was found to interact with YY1 *in vitro*
13 [39] and several other transcription factors *in silico* [40]. Although Rapa treatment did not
14 interfere with the mTOR-YY1 interaction [39], inhibition of mTOR by Rapa might decrease
15 the interaction of mTOR with other transcription factors that positively regulate the *Deptor*
16 promoter activity, thereby decreasing DEPTOR protein levels. The quantitatively lower effect
17 of Rapa treatment on DEPTOR levels in Sirt1 KD β -cells could be due to a feedback
18 mechanism regulating DEPTOR following the increase in mTOR activity associated with the
19 cell diameter and cell size increase.

20 Epigenetic changes, such as histone acetylation/deacetylation and DNA methylation, are
21 important for regulating gene expression. Histone deacetylation leads to transcriptional
22 inactivation caused by heterochromatin [41]. Sirt1 does not only deacetylate histone proteins,
23 but also deacetylates a number of non-histone proteins including transcription factors, such as
24 p53 [42], NF- κ B [43], the forkhead transcription factor Foxo3a [44] and DNA
25 methyltransferase DNMT1 [45]. In our study, Sirt1 knockdown resulted in increased size of

1 INS-1E β -cells. c-Myc is one of the transcription factors that regulate cell and organ size [46,
2 47], and its stability and activity is regulated by Sirt1 through deacetylation [48]. Thus, one
3 mechanism by which Sirt1 down-regulation leads to increased cell size may be via increased
4 levels or activity of c-Myc.

5 Impedance measurements may determine the cell proliferation non-invasively in real time, but
6 also the changes in cell size and cell adherence/attachment may be determining factors. The
7 increased cell size observed for KD will likely cause an increased cell index [49] during cell
8 establishment, but enhanced cell attachment by the Sirt1-KD cells relative to NEG CTR may
9 also play a role. An increased attachment may be a result of an increased expression of
10 adhesion-related proteins in response to the KD of Sirt1. Cortactin, an F-actin binding protein,
11 is a Sirt1 substrate [50], and a reduced Sirt1 activity may have an impact on the cell
12 cytoskeleton observed as an increased cell attachment. However, this hypothesis needs to be
13 further elaborated. Treatment with Rapa alone and combinations of Resv and Rapa enhanced
14 the impedance increase at short term (1-4 h). The cell size increase by Resv is only seen as a
15 small impedance increase in NEG CTR cells, but the reduced cell size by Rapa observed for
16 the present INS-1E β -cells did not reduce the impedance reading. A short-term increase of
17 impedance in Rapa-treated Keloid cells was observed [51], but the molecular basis for this
18 observation is not known. Combination of Resv and Rapa, did increase the impedance much
19 more than that is observed for the two compounds alone, indicating an additive effect.

20 It is remarkable that Resv and Sirt1 both increased β -cell size independently from the mTOR
21 cascade. Our results clearly confirm that rapamycin dependent inhibition of mTOR causes β -
22 cell size decrease. Cell size regulation by Sirt1 of pancreatic β -cells has not been shown
23 previously, while Sirt1 KD in the 3T3-L1 adipocytes leads to hyperplasia and smaller
24 adipocytes [52]. These observations are interesting given that rat β -cells increase in size during
25 aging [53], while Sirt1 is reported decreased by aging in several other tissues [54]. Sirt1

increases rat islet glucose-stimulated insulin secretion [11, 16] and it would be relevant to determine the relationship between Sirt1 levels, β -cell size and age in islets from suitable rodent models or human donor islets.

Our study demonstrates that Sirt1 negatively regulates cell size in INS-1E β -cells, while the effect of Resv on the cell number reduction and increased size is Sirt1-independent. Moreover, although the cell size effect of Sirt1 KD was remarkable and stable, neither Sirt1 down-regulation nor the Resv concentration used in our study affected mTOR cascade activity in INS-1E β -cells. These results suggest that cell enlargement caused by Resv occurs through other factors than direct mTOR activity interference but a crosstalk between mTOR pathway and the other Resv target factors in cell size regulation.

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Supplementary information

1. Supplementary material and methods

1.1. Cell area measurements

Microscopy images were obtained for each indicated time points and analysed with ImageJ software. In brief, cells were grown in 24-well plates and treated with Resv (0-60 μ M) either for 24 or 48 h. At the indicated time points, microscopy images were obtained with 20X magnification. For each experiment, one random image was obtained from each well. Using the ImageJ software, the conversion from pixels to μ m was made using a 20x picture of a 100 μ m scale. In each image, 5-6 cells were covered by the “Freehand selection” tool and the selected area was determined, and the mean of area/cell was calculated.

1.2. Assessment of Apoptosis

INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 1×10^6 cells/well in 6-well plates. After o/n attachment period, cells were treated either with vehicle (DMSO) or Resv (30 μ M) for 24 h, and collected by trypsination, washed with PBS and stained with Annexin V-FITC and propidium iodide according to the manufacturer’s instructions (MEBCYTO[®] Apoptosis Kit; Cat. No. 4700, MBL International).

Supplementary Figures

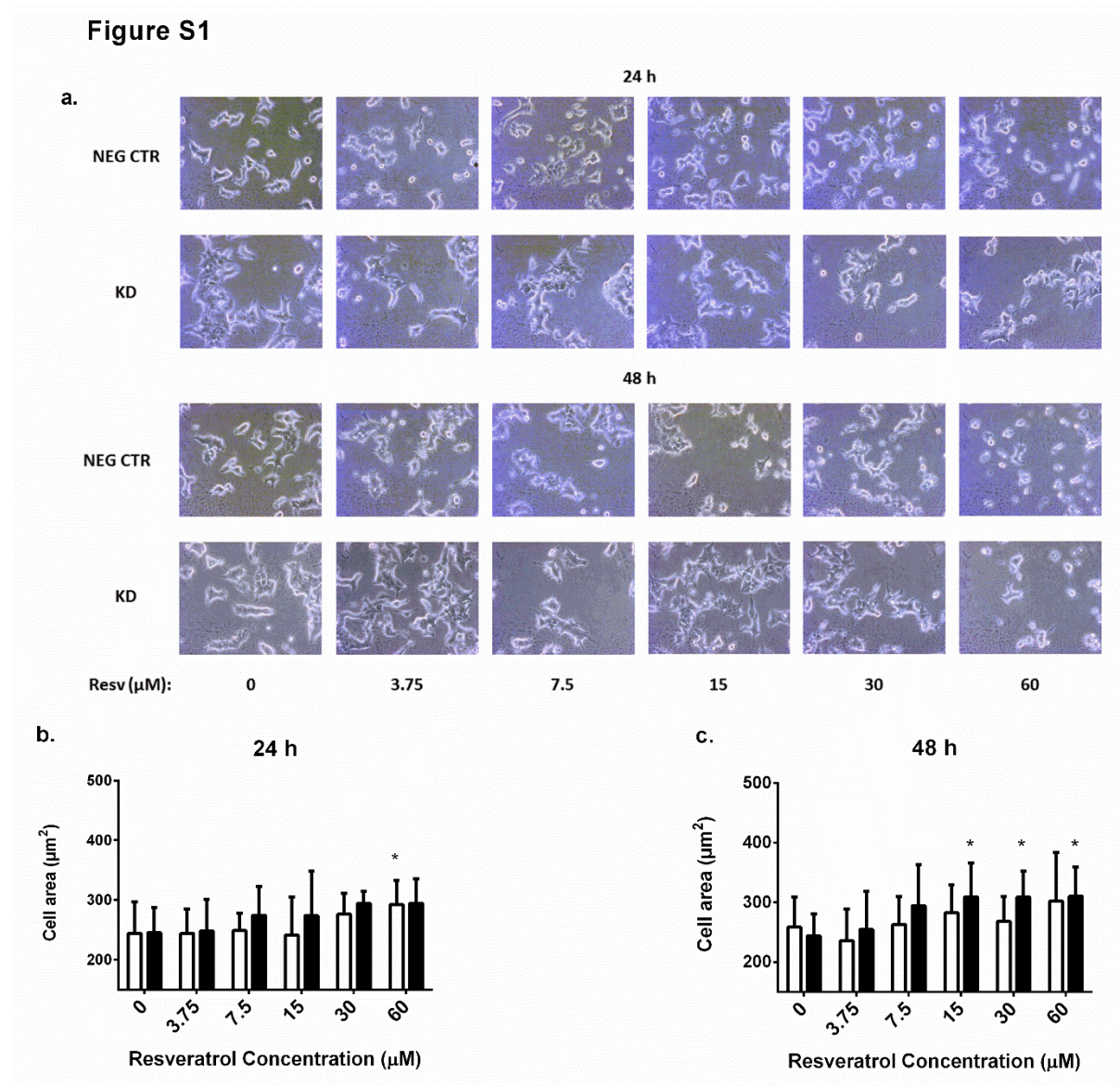


Figure S3. Effects of Sirt1 KD and resveratrol on cell area at 24 (a and b) and 48 h (a and c). (a) Representative microscopy images for 24 and 48 h treatments. Quantification of the mean cell area at (b) 24 and (c) 48 h. Data are mean \pm S.D. (n=12). Statistical analyses: One-way ANOVA followed by Dunnett's multiple comparison test against 0 μM Resv treatment within each cell line. * $p < 0.05$. White and black bars belong to the measurements for NEG CTR and KD cells, respectively.

Figure S2

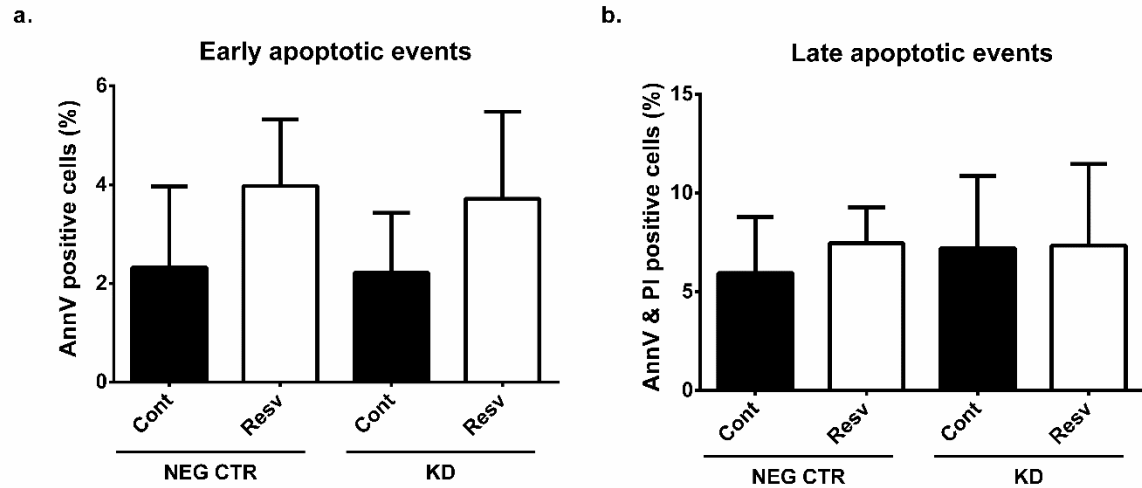


Figure S2. Resv (30 μ M) does not induce (a) early apoptotic and (b) late apoptotic events in NEG CTR and KD cells at 24 h. Data are mean \pm S.D, n=3 independent experiments. Treatment groups are not statistically different from each other. AnnV: Annexin V; PI: Propidium iodide.

Figure S3

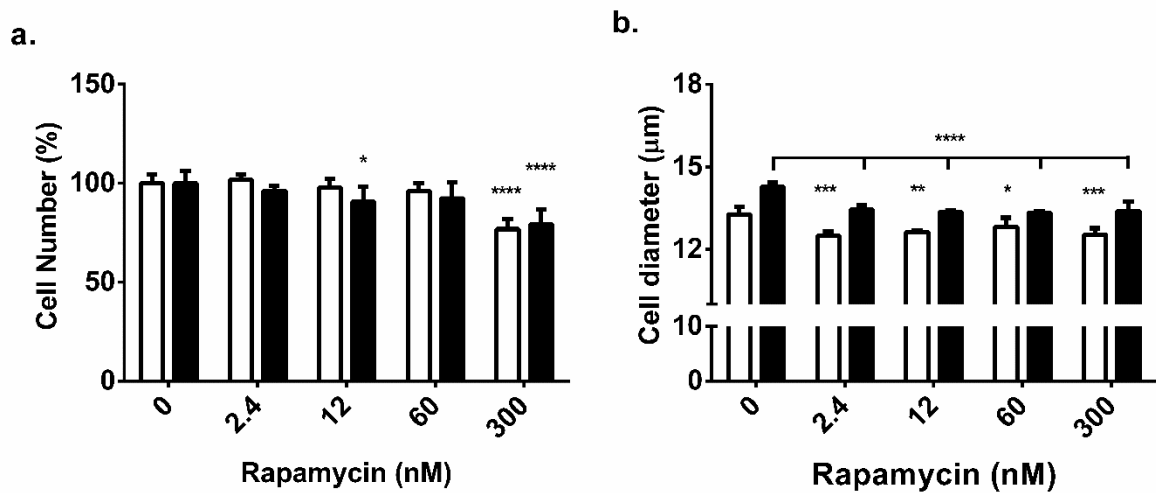


Figure S3. Effect of Rapa on (a) cell number and (b) diameter at 24 h. Data are mean \pm S.D. (n=8 for Rapa-untreated and n=4 for Rapa-treated groups). Statistical analyses: One-way ANOVA followed by Dunnett's multiple comparison test against untreated group within each cell line. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. White and black bars belong to NEG CTR and KD cells, respectively.

Figure S4

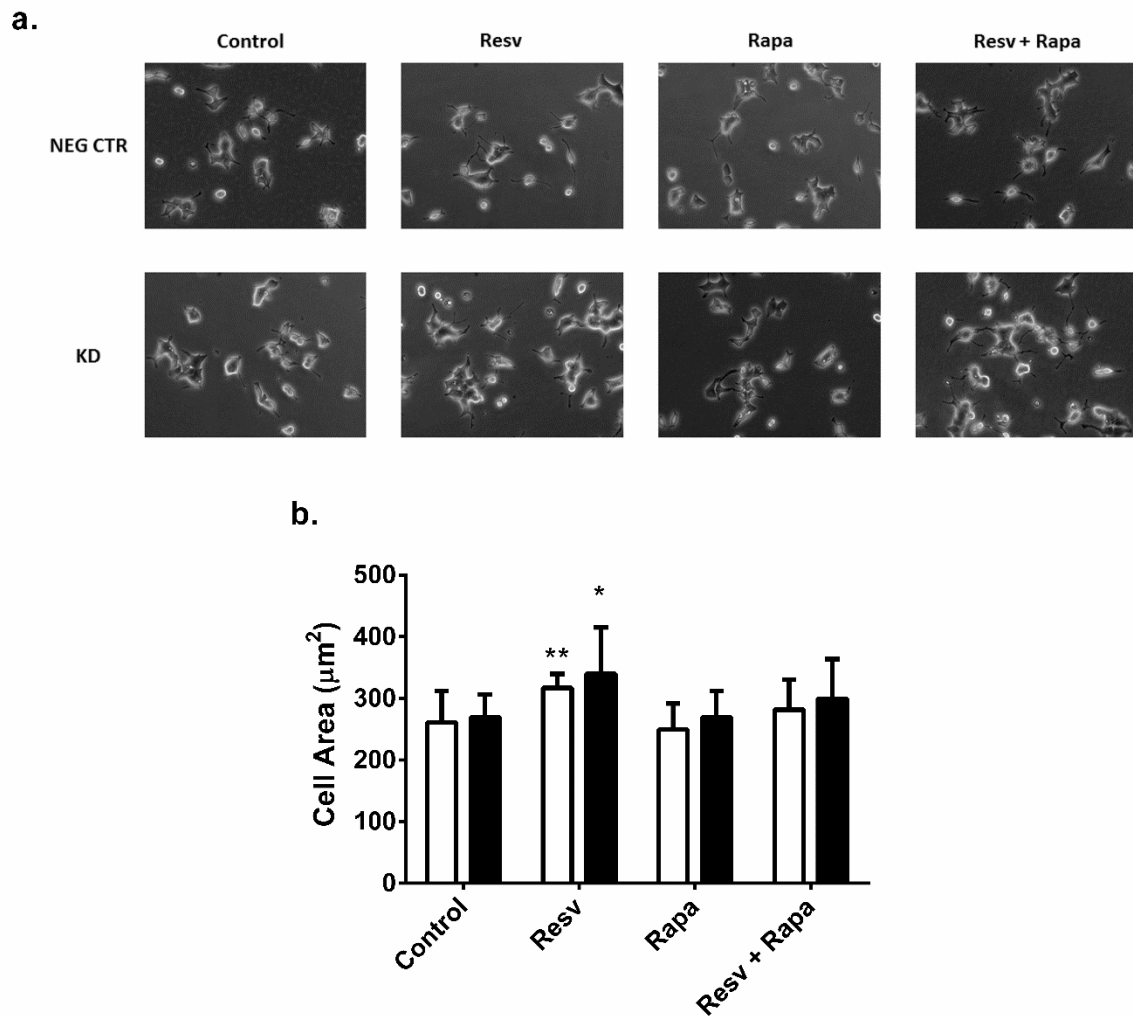


Figure S4. Effect of Resv (30 μM) and Rapa (12 nM) on cell area at 24 h. Data are mean \pm S.D. (n=12). Statistical analyses: One-way ANOVA followed by Dunnett's multiple comparison test against untreated group within each cell line. *p<0.05 and **p<0.01. White and black bars belong to the measurements for NEG CTR and KD cells, respectively.