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SIRT6 protects against palmitate-induced pancreatic β-cell dysfunction and apoptosis

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Abstract

Chronic exposure of pancreatic β-cells to abnormally elevated levels of free fatty acids can lead to β-cell dysfunction and even apoptosis, contributing to type 2 diabetes pathogenesis. In pancreatic β-cells, SIRT6 has been shown to regulate insulin secretion in response to glucose stimulation. However, what roles SIRT6 play in β-cells in response to lipotoxicity remain poorly understood. Our data indicated that SIRT6 protein and mRNA levels were reduced in islets from diabetic and aged mice. High concentrations of palmitate also led to a decrease in SIRT6 expression in MIN6 β-cells and resulted in cell dysfunction and apoptosis. Knockdown of *Sirt6* caused an increase in cell apoptosis and impairment in insulin secretion in response to glucose in MIN6 cells even in the absence of high palmitate. Furthermore, overexpression of SIRT6 alleviated the palmitate-induced lipotoxicity with improved cell viability and increased glucose-stimulated insulin secretion. In summary, our data suggest that SIRT6 can protect against palmitate-induced β-cell dysfunction and apoptosis.

Keywords

Sirtuin 6; MIN6 cells; Insulin Secretion; Apoptosis; Palmitate

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DECLARATION OF INTEREST

No potential conflicts of interest relevant to this article were reported.

AUTHOR CONTRIBUTIONS

XX designed and carried out the study, interpreted data, analyzed data, and wrote the manuscript. XS, QW, XQ, YZ, and XP contributed to data collection. XCD conceived the hypothesis, designed the experiments, analyzed and interpreted the data, and wrote the manuscript. All authors approved the manuscript.

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INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM), a chronic metabolic disorder, has been increasing steadily all over the world (Doria, et al. 2008; Kahn, et al. 2006). The pathogenesis of T2DM is multifactorial, but insulin secretory deficiency due to reduced pancreatic β-cell mass or function is a major pathogenic factor (Muoio and Newgard 2008; Prentki and Nolan 2006). In the pre-diabetic stage, because of insulin resistance, islets respond to increased insulin demand with enhanced insulin secretion and expanded β-cell mass in order to maintain euglycemia by compensatory hyperinsulinemia. However, as T2DM ensues, β-cells exhibit decompensation, a defect that has been attributed to both β-cell dysfunction and β-cell death (Weir and Bonner-Weir 2004). A common feature of the pre-diabetic stage is an excess of circulating lipids, especially saturated fatty acids, which impairs not only peripheral insulin sensitivity but also islet β-cell function (Weir and Bonner-Weir 2004). Acute exposure to elevated free fatty acids (FFA) increases β-cell proliferation and insulin secretion, whereas prolonged exposure to FFA inhibits insulin secretion and induces β-cell apoptosis (Biden, et al. 2004; Shimabukuro, et al. 1998). However, the underlying mechanisms responsible for the β-cell lipotoxicity remain incompletely understood.

The sirtuin protein family has seven members in mammals (SIRT1-7) (Dong 2012). SIRT6 is a chromatin-associated deacetylase that specifically deacetylates histone H3 at lysine 9 (H3K9), lysine 18 (H3K18), and lysine 56 (H3K56) residues (Michishita, et al. 2008; Michishita, et al. 2009; Tasselli, et al. 2016; Yang, et al. 2009). In recent years, SIRT6 has been identified to modulate many important cellular processes, such as DNA repair, tumor suppression, anti-inflammation, and metabolism. *Sirt6*-deficient mice display severe hypoglycemia and a multi-systemic aging phenotype and died around 4 weeks after birth (Mostoslavsky, et al. 2006; Xiao, et al. 2010). Interestingly, high-fat diet treated SIRT6 transgenic mice secret more insulin in response to glucose compared with their wild-type littermates (Kanfi, et al. 2010). In our recent study, we have shown that deletion of *Sirt6* in pancreatic β-cells in mice leads to impaired glucose-stimulated insulin secretion (GSIS). We have further identified that SIRT6 regulates insulin secretion by maintaining mitochondrial function and modulating intracellular Ca^{2+} dynamics (Xiong, et al. 2016). However, whether SIRT6 is involved in β-cell lipotoxicity remains unclear. The aim of this study was to illustrate the role of SIRT6 in palmitate (PA)-induced β-cell dysfunction and apoptosis.

MATERIALS AND METHODS

Cell culture and treatment

MIN6 cells between passages 15–20 were cultured in Dulbecco's modified Eagle's medium with 25mM glucose supplemented with 15% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine, and 50mM β-mercaptoethanol. Palmitic acid (Sigma-Aldrich) was conjugated with fatty-acid-free BSA prior to addition to cell culture. PA was dissolved in 99% ethanol to a concentration of 100mM, and then mixed with 10% BSA in serum-free high glucose DMEM to make a 4mM PA stock solution. The BSA-conjugated PA was added to MIN6 cells at a final concentration of 0.4mM.

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Adenovirus transduction

Adenoviruses carrying *SIRT6* or *GFP* were generated using pAdEasy system (Agilent) while adenoviral *Sirt6* (mouse) or *GFP* shRNAs were generated using BLOCK-iT system (Invitrogen). Adenoviruses were amplified in HEK293A cells and purified by CsCl gradient centrifugation. The viruses were titered using QuickTiter adenovirus titer immunoassay kit (Cell Biolabs) according to the manufacturer's protocol. Generally, we used 25–50 multiplicity of infection (MOI) for overexpression and 50–100 MOI for shRNA knockdown experiments.

Real-time RT PCR

Total islet RNA samples were prepared by using Trizol reagent (Invitrogen) and converted into cDNA using a cDNA synthesis kit (Applied Biosystems). Real-time PCR analysis was performed using SYBR Green Master Mix (Promega) in an Eppendorf Realplex real-time PCR system.

Western blotting

Protein extracts from cells were made in tissue lysis buffer (50mM Hepes, pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM MgCl2, 1mM EGTA) and an additional protease cocktail tablet from Roche at one tablet/10ml final buffer volume. Protein extracts were resolved on an SDS-PAGE gel and transferred to nitrocellulose membrane (Santa Cruz Biotechnology). The membrane was incubated with the following antibodies: Sirt6 (Sigma-Aldrich), Actinin (Santa Cruz Biotechnology), Ac-H3K9, Cleaved Caspase 3 (Cell Signaling Technology). Detection of proteins was carried out by incubation with HRP-conjugated secondary antibodies, followed by ECL detection reagents (Thermo Fisher Scientific).

Insulin secretion assay

On the day of experiment, MIN6 cells were pre-incubated with secretion assay buffer (SAB; 114mM NaCl, 4.7mM KCl, 1.2mM KH_2PO4, 1.16mM $MgSO_4$, 20mM HEPES, 2.5mM $CaCl_2$, 25.5mM $NaHCO_3$, 0.2% BSA, pH 7.2) containing 2.5mM glucose for 1h. After removal of the incubation medium, cells were then incubated for 1h in SAB containing the indicated glucose concentrations. Incubation medium was then collected and the amount of secreted insulin was analyzed using a mouse insulin ELISA kit (ALPCO). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific) and used to normalize insulin levels.

MTT assay

MIN6 cells were seeded in 96-well plates with 5×10^4 cells per well. The cells were exposed to 0.4mM PA treatment or transduced with indicated adenoviruses for the indicated periods of time. Cell viability was determined by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich) according to the manufacturer's protocol.

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Statistical analysis

All data are presented as means ± SEM. Analysis was performed using 2-tailed unpaired Student's t-test for two-group comparisons and one-way ANOVA for multiple-group comparisons, and $p<0.05$ was considered as significant.

RESULTS

SIRT6 is decreased in both diabetic and aged pancreatic islets

Our recent study has shown that *Sirt6* deletion in β-cells results in β-cell dysfunction (Xiong et al. 2016), so we were curious about the SIRT6 status under diabetic and aging conditions. The leptin receptor mutant *db/db* mouse is a widely used type 2 diabetic mouse model. We analyzed SIRT6 protein levels in isolated islets from the *db/db* and control wild-type mice, and the data showed that SIRT6 protein levels were decreased in the *db/db* islets as compared to controls (Fig. 1A). As a substrate of SIRT6, acetylated H3K9 was elevated in the *db/db* islets (Fig. 1A). Since SIRT6 has been recently identified as an important regulator of aging, we also checked whether SIRT6 is altered in the aged pancreatic islets. Indeed, SIRT6 protein levels were decreased in aged mouse islets as compared to young controls (Fig. 1B). Consistently, acetylated H3K9 was also elevated in the aged islets as expected (Fig. 1B).

Palmitate decreases *Sirt6* expression in mouse islets and MIN6 cells

As shown in Fig. 1, *Sirt6* expression is down-regulated in islets from diabetic or aged mice. Since hyperlipidemia is one of the common features of type 2 diabetes, we also examined *Sirt6* expression in mouse islets exposed to PA (0.4mM) for 72hrs. SIRT6 protein levels in the mouse islets were decreased after 72hrs of PA treatment relative to the controls (Fig. 2A). To further assess the effect of PA on SIRT6 gene expression in pancreatic β-cells, MIN6 cells were treated with 0.4mM PA. As shown in Fig. 2B, *Sirt6* mRNA levels were significantly reduced in MIN6 cells exposed to PA for 48hrs. The western blot data also confirmed that PA treatment decreased SIRT6 protein in a time-dependent manner (Fig 2C, D). As expected, acetylation of the H3K9 residue was elevated after the PA treatment (Fig. 2C, D). It is known that lipotoxicity can ultimately lead to β-cell dysfunction and apoptosis (Janikiewicz, et al. 2015; Sharma and Alonso 2014). To investigate the lipotoxic effect of PA on MIN6 cells, we further examined glucose-stimulated insulin secretion (GSIS) and cell viability in MIN6 cells in the presence or absence of PA. As shown in Fig. 2E, MIN6 cell viability was significantly decreased after exposure to PA for 48hrs. As expected, a significant induction of cleaved caspase 3 by PA was observed. Consistent with the previous studies, although PA treatment did not reduce the basal insulin secretion in MIN6 cells, high glucose-stimulated insulin secretion was remarkably attenuated. As shown in Fig. 2F, PA-treated MIN6 cells secreted ~30% less insulin compared with BSA-treated MIN6 cells when stimulated with 16.7mM glucose.

Sirt6 knockdown in MIN6 cells leads to cell apoptosis and insulin secretory impairment

To verify whether *Sirt6* deficiency can lead to apoptosis in MIN6 cells, we performed *Sirt6* knockdown by transduction with *Sirt6* shRNA adenoviruses. *Sirt6* knockdown was

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confirmed by western blot analysis (Fig. 3A, B). As expected, *Sirt6* knockdown in MIN6 cells increased the levels of Ac-H3K9 and cleaved caspase 3 (Fig. 3A, B). Moreover, PA treatment further exaggerated the elevation of Ac-H3K9 and cleaved caspase 3 induced by *Sirt6* knockdown (Fig. 3A, B). Consistently, *Sirt6*-deficient MIN6 cells exhibited decreased cell viability and impaired GSIS even without PA treatment (Fig. 3C, D).

SIRT6 protects MIN6 cells against palmitate induced cell apoptosis and insulin secretory defects

To examine whether SIRT6 can protect MIN6 cells from lipotoxicity, we overexpressed SIRT6 in MIN6 cells by adenoviral transduction. Overexpression of SIRT6 in MIN6 cells under regular culture conditions exhibited no effect on cell survival and insulin secretory capacity (Fig. 4A–D). However, after 48hrs of PA treatment, SIRT6 overexpression resulted in reduced caspase 3 activation and enhanced cell viability (Fig. 4A–C). Moreover, overexpression of SIRT6 improved insulin secretory capacity in response to glucose stimulation in PA treated MIN6 cells compared to control GFP (Fig. 4D).

DISCUSSION

Hyperlipidemia is known to cause β-cell dysfunction, which is characterized by impaired GSIS and increased apoptosis (Janikiewicz et al. 2015; Sharma and Alonso 2014). Individuals with T2DM have elevated levels of palmitate, which is one of the most prevalent saturated fatty acids in the circulation and has been linked to functional impairment of pancreatic β-cells (Kharroubi, et al. 2004). Several molecular processes associated with lipotoxicity in β-cells have been reported, including endoplasmic reticulum (ER) stress, mitochondrial dysfunction, increased reactive oxygen species (ROS), elevated ceramide, and impaired autophagy (Janikiewicz et al. 2015; Sharma and Alonso 2014).

SIRT6 is primarily characterized as a nuclear NAD^+-dependent deacetylase of histone H3K9, H3K18, and H3K56 (Michishita et al. 2008; Michishita et al. 2009; Tasselli et al. 2016; Yang et al. 2009). SIRT6 has been implicated in diverse cellular functions including anti-inflammation, metabolic homeostasis, stress resistance and tumor suppression (Kugel and Mostoslavsky 2014). In this study, we have examined the role of SIRT6 in protection against PA-induced β-cell lipotoxicity. Our data reveal that SIRT6 expression is significantly decreased not only in PA-treated MIN6 cells but also in diabetic and aged mouse islets. Knockdown of *Sirt6* in MIN6 cells increases cell apoptosis and impairs GSIS whereas overexpression of SIRT6 protects β cells from apoptosis and improves insulin secretory capacity in response to glucose.

We have demonstrated in our previous work that *Sirt6* knockout in pancreatic β-cells reduces ATP production and increases mitochondrial damage (Xiong et al. 2016). Mitochondrial oxygen consumption rates (OCR) are significantly decreased in Sirt6 knockdown MIN6 cells as well (Xiong et al. 2016). Consistent with our findings, mitochondrial defects have been also observed in *Sirt6* knockout mouse ES cells and *Sirt6*-knockout breast cancer cells (Choe, et al. 2015; Zhong, et al. 2010). Chronic PA exposure induced β-cell dysfunction and apoptosis is partly mediated by mitochondrial dysfunction (Janikiewicz et al. 2015). Thus, it

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is likely that SIRT6 may exert its protective effect against the PA-induced β-cell dysfunction through regulation of mitochondrial function.

Oxidative stress generated by fatty acid oxidation has been considered to be involved in the pathogenesis of PA-induced β-cell dysfunction and apoptosis (Janikiewicz et al. 2015). A recent report has shown that SIRT6 can protect human mesenchymal stem cells from oxidative stress through regulation of nuclear factor erythroid 2-related factor 2 (NRF2) (Pan, et al. 2016). *SIRT6*$^{-/-}$ hMSCs exhibit elevated ROS, dysregulated redox metabolism and increased sensitivity to oxidative stress. Mechanistically, SIRT6 acts as a trans-activator of transcription factor NRF2 to modulate the expression of genes involved in antioxidant pathway (Pan et al. 2016). However, whether SIRT6 also plays an anti-oxidative stress role in pancreatic β-cells is unclear.

Autophagy, a self-degradative process, has been shown to play a protective role in the PA-induced death of β-cells (Lee 2014; Watada and Fujitani 2015). Interestingly, several studies have demonstrated that SIRT6 functions as a positive regulator of autophagy in some cell types, such as bronchial epithelial cells, cardiomyocytes and neurons. In these cells, SIRT6 regulates the autophagy process via inhibiting the AKT activity (Lu, et al. 2016; Shao, et al. 2016; Takasaka, et al. 2014). Considering a critical role of autophagy in PA-induced β-cell dysfunction, it is reasonable to postulate that SIRT6 may exert a protective effect against the PA-induced β-cell dysfunction and apoptosis through modulation of autophagy.

In summary, our data suggest that SIRT6 plays a critical role in the protection of pancreatic β-cells from lipotoxicity-induced cellular dysfunction or even cell death. These findings are significant in T2DM as dyslipidemia is often associated with the pathogenesis of this type of diabetes. A decrease in SIRT6 protein or activity in β-cells may contribute to the T2DM development. Targeting SIRT6 may be useful for therapeutic development in the treatment of T2DM.

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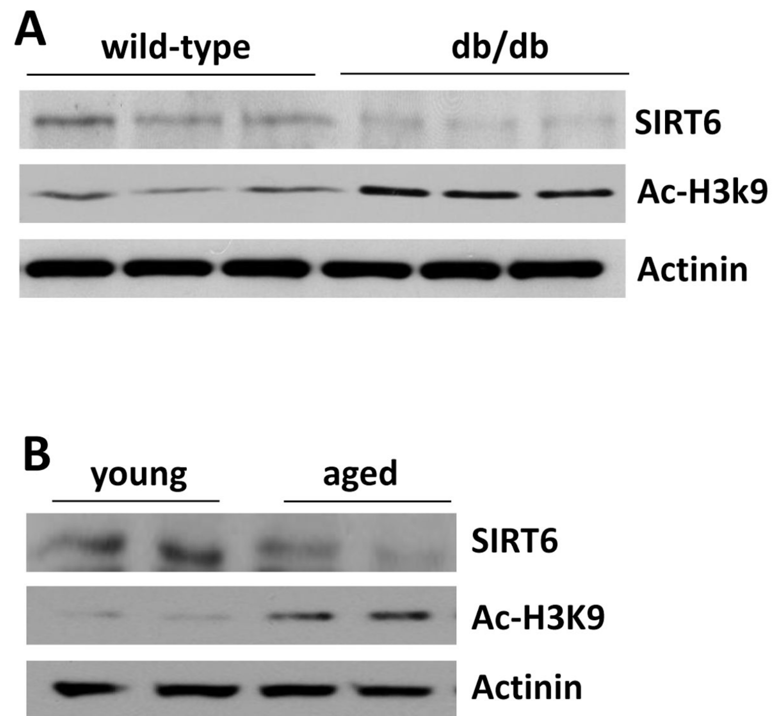

Figure 1. SIRT6 is decreased in diabetic and aged islets

A: Western blot analysis of SIRT6 and Ac-H3K9 in islet extracts of 5-month-old wild-type and *db/db* mice (n=3 per group, islets were pooled from 3 mice per genotype). B: Western blot analysis of SIRT6 and Ac-H3K9 in islet extracts of young (2-month-old) and aged (18-month-old) mice (n=2 per group, islets were pooled from 3 mice per genotype).

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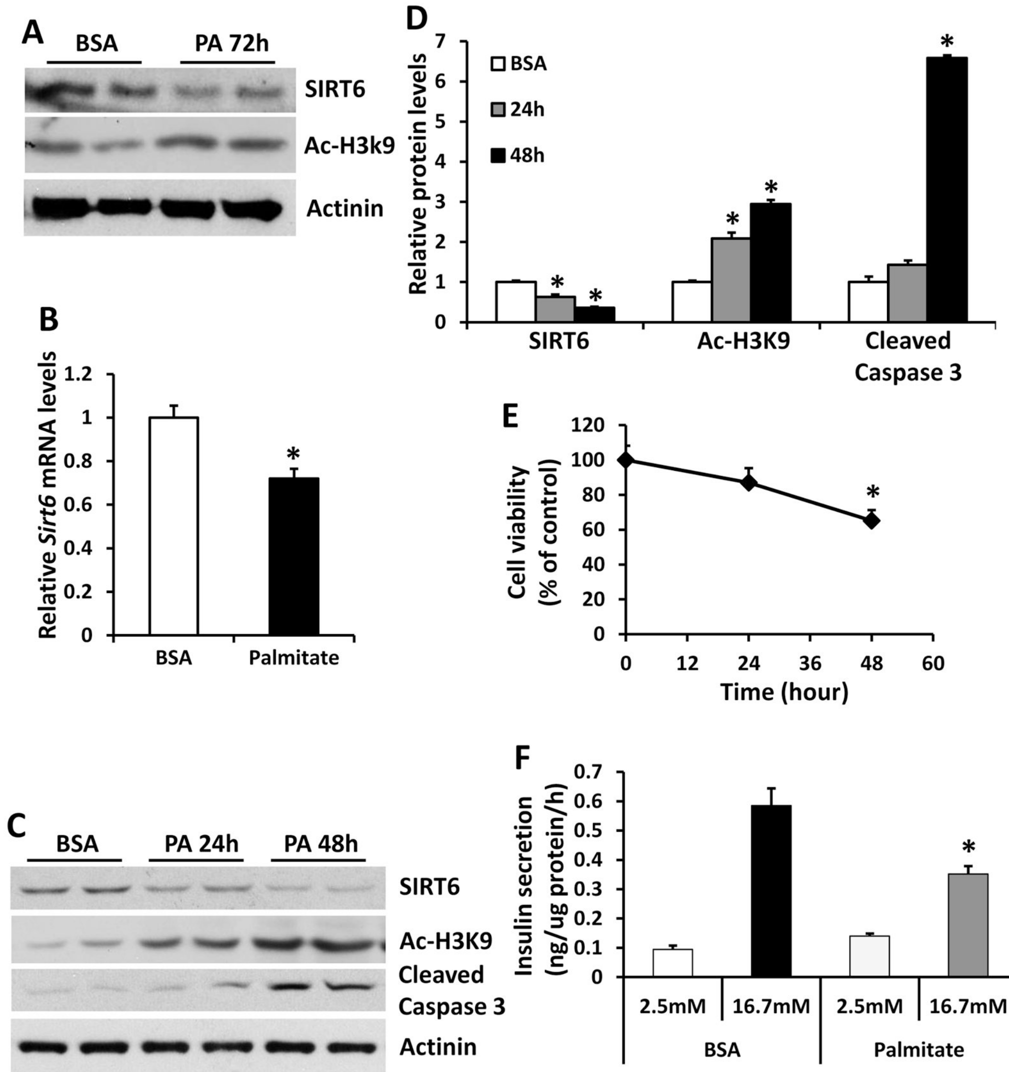

Figure 2. Palmitate exposure decreases *Sirt6* expression in islets and MIN6 cells and leads to cell dysfunction and apoptosis

Mouse islets or MIN6 cells were treated with 1% BSA (BSA) or 0.4mM palmitate (PA) complexed to 1% BSA. A: Western blot analysis of SIRT6 and Ac-H3K9 in mouse islets exposed to PA for 72hrs (n=2 per group, islets were pooled from six 2-month-old C57BL/6J male mice). B: Real-time PCR analysis of *Sirt6* mRNA in MIN6 cells treated with PA for 48hrs. C and D: Western blot (C) and densitometric analysis (D) of SIRT6, Ac-H3K9 and cleaved caspase 3 in MIN6 cells exposed to PA at indicated time points. E: MIN6 cell viability upon PA treatment for various time periods was analyzed by MTT assay. F: MIN6 cells were incubated with PA for 48hrs, and then GSIS was analyzed. n=3 per group for all the experiments. Data are presented as mean ± SEM. *p<0.05 vs (B, D) BSA, vs (E) 0hr, vs (F) 16.7mM BSA.

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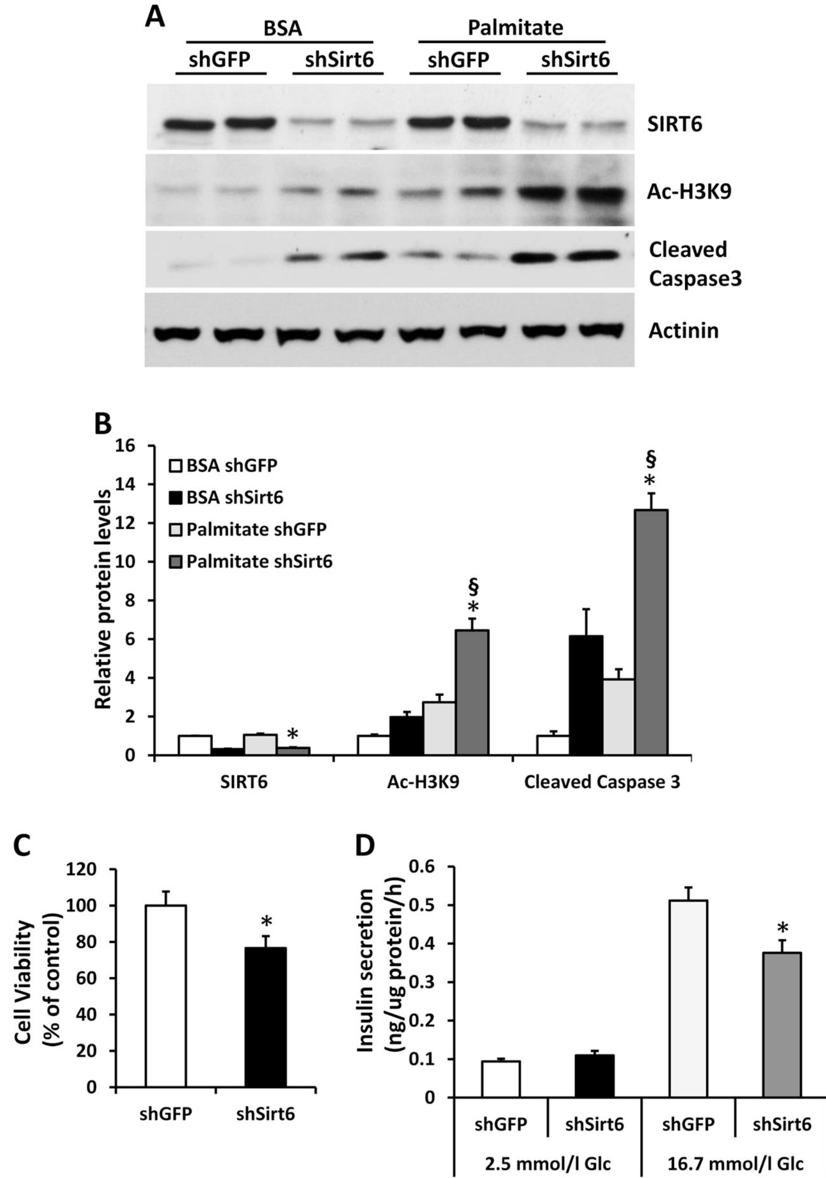

Figure 3. Knockdown of *Sirt6* increases cell apoptosis and impairs glucose stimulated insulin secretion in MIN6 cells

MIN6 cells were transduced with *shSirt6* or control *shGFP* adenoviruses for 48hrs. A and B: Western blot (A) and densitometric analysis (B) of SIRT6, Ac-H3K9 and cleaved caspase 3 in MIN6 cells. C: MIN6 cell viability was measured by MTT assays. D: GSIS was analyzed in control and Sirt6 knockdown MIN6 cells. n=3 per group for all experiments. Data are presented as mean ± SEM. *$p<0.05$ vs palmitate + *shGFP*, § $p<0.05$ vs BSA + *shSirt6*.

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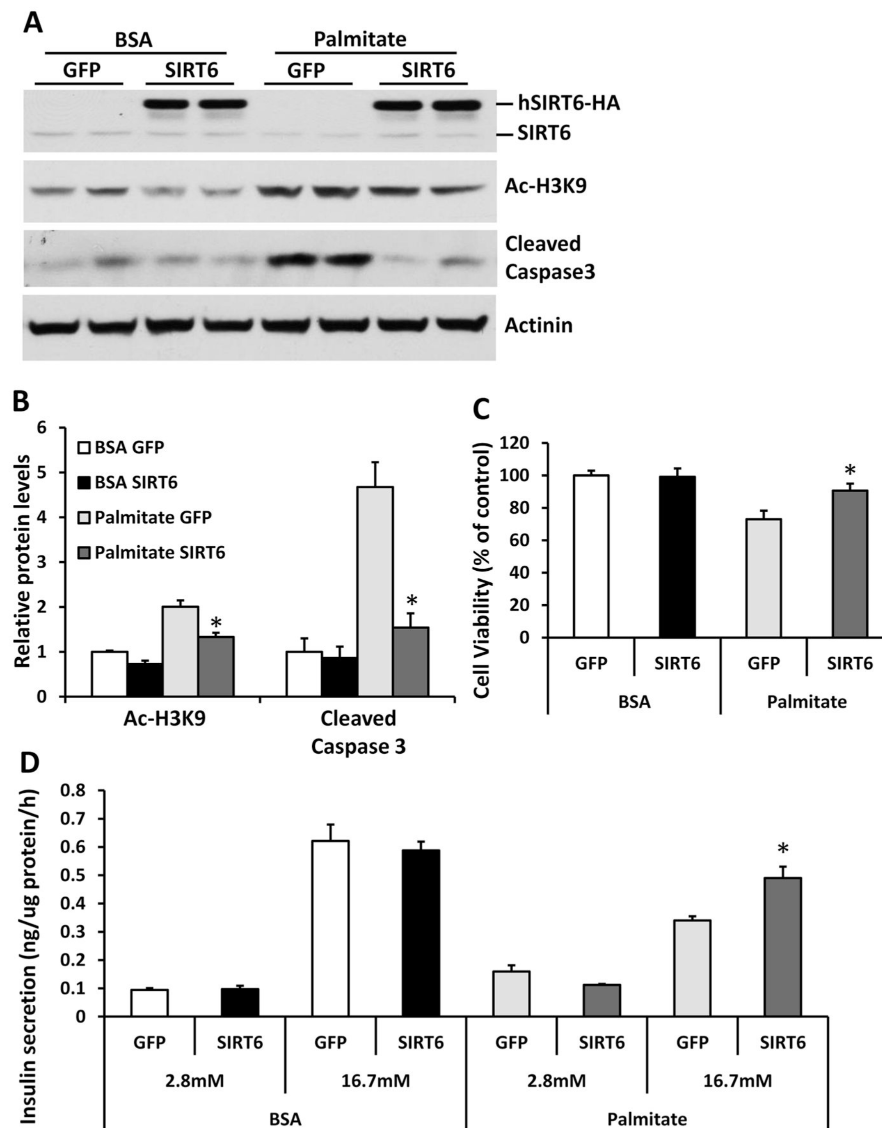

Figure 4. Overexpression of SIRT6 protects against PA-induced cell apoptosis and GSIS impairment

MIN6 cells were transduced with SIRT6 or control GFP adenoviruses. 4hrs later, medium was changed and cells were treated with 1% BSA or 0.4mM PA for 48hrs. A: Western blot of SIRT6, Ac-H3K9 and cleaved caspase 3. B: Quantification of relative levels of Ac-H3K9 and cleave caspase. C: MIN6 cell viability was measured by MTT assays. D: GSIS was analyzed in the absence or presence of 0.4mM PA. n=3 per group for all the experiments. Data are presented as mean ± SEM. *p<0.05 vs palmitate + GFP.