

## Original Article

# Lysosomal integral membrane protein Sidt2 plays a vital role in insulin secretion

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**Abstract:** Abnormal insulin secretion results in impaired glucose tolerance and is one of the causal factors in the etiology of type 2 diabetes mellitus. Sidt2, a lysosomal integral membrane protein, plays a critical role in insulin secretion. Here, we further investigate its regulation in insulin secretion. We show that Sidt2<sup>-/-</sup> mice exhibit weight loss, decreased postnatal survival rate with aging, increased fasting glucose and impaired glucose tolerance. After loading high levels of glucose in their diet, Sidt2<sup>-/-</sup> mice produce notably lower insulin levels at the first-phase secretion compared with Sidt2<sup>+/+</sup> mice. Consistent with the *in vivo* study, INS-1 cells treated with Sidt2 siRNA produced less insulin when loaded with 16.7 mM of glucose. Only 2 of the 13 genes, *synap1* and *synap3* which encode soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, showed significantly decreased expression in Sidt2<sup>-/-</sup> mice. In conclusion, Sidt2 may play a vital role in the regulation of insulin secretion via two SNARE proteins *synap1* and *synap3*.

**Keywords:** Lysosome membrane protein, Sidt2, insulin, secretion, diabetes

## Introduction

Insulin is released by pancreatic-islet  $\beta$  cells in response to elevated blood glucose levels in order to maintain normal glucose homeostasis, and more than 99% of the insulin released from  $\beta$  cells is regulated via a secretory pathway [1]. When extracellular glucose levels rise, regulated insulin-granule exocytosis ensues and plasma glucose enters the  $\beta$  cells via the glucose transporter GLUT2. Subsequently, a series of metabolic processes occur, including a net increase in the ATP: ADP ratio [2, 3], increased intracellular Ca<sup>2+</sup> concentration via the opening of voltage-gated Ca<sup>2+</sup> channels [4, 5], and insulin vesicle fusion with plasma membrane [4]. An impaired secretory response is one of the causal factors in the etiology of type 2 diabetes mellitus (T2DM), hallmarked with hyperglycemia [6].

This type of exocytosis requires synergistic molecular handshakes between three different soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins.

They include vesicle-associated membrane protein (VAMP) which is associated with the vesicle (a v-SNARE), synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin which are associated with the plasma membrane (t-SNAREs) [7]. Neuroendocrine cells, such as pancreatic islet  $\beta$  cells, express SNAREs: VAMP2, SNAP-25b, and syntaxin-1 [8, 9]. Studies have demonstrated that patients with T2DM and animal models of the disease showed reduced expression of all three of these SNAREs [10, 11].

In the last few years, scientists have revealed some of the regulatory processes that control insulin exocytosis. For example, Rab27a, a small GTPase, is involved in insulin secretion by modulating the transport and docking steps of insulin granules via its effector proteins [12-16]. Rab3a, another small GTPase, also regulates insulin exocytotic release [17]. However, the ways in which these proteins regulate insulin secretion remains unclear.

Current evidence shows that the islet lysosome is also involved in the process of insulin secre-

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**Table 1.** The primers used in this project

Gene	Primers	
Insulin-1	sense	5'-CCACCTGGAGACCTTAATGGG-3'
	antisense	5'TACGGATGGACTGTTTGT-3'
Insulin-2	sense	5'-AGCCTATCTTCCAGGTATTGTTTC-3'
	antisense	5'-GGTAGTGGTGGGTCTAGTTGCA-3'
glucagon	sense	5'-CAGCGACTACAGCAAATACC-3'
	antisense	5'-TCCCTGGTGCAAGATTA-3'
Glut 2	sense	5'-AGGACTGTATTGTGGGCTAA-3'
	antisense	5'-GATGGGCTGTCGGTAA-3'
pdx-1	sense	5'-TCCACCAAAGCTCACGC-3'
	antisense	5'-CGAGGTCACCGACAAT-3'
Ampk1	sense	5'-CGTTATCCGCTGGTCT-3'
	antisense	5'-GCTGGTGCATGAAAT-3'
rab27a	sense	5'-CAGTTATGGGACACGG-3'
	antisense	5'-GCCTCCTCCTTTCA-3'
vamp2	sense	5'-CCTCCTCCAAACCTTACT-3'
	antisense	5'-ATGGCGCAGATCACTC-3'
rab3A	sense	5'-ACAAACCTCGTTCCTCTTC-3'
	antisense	5'-ACTCGCTCATCTCCAT-3'
Snap25	sense	5'-TGGCATCAGGACTTTG-3'
	antisense	5'-ATCTGCTCCCGTTCAT-3'
Synap1	sense	5'-TGAGTGCCAGTCGTCC-3'
	antisense	5'-CCAATGATGTCGTGCTT-3'
Synap 3	sense	5'-ACCATCCACCATTTGC-3'
	antisense	5'-GAAGCGGTCGAAGTCA-3'
Synap 4	sense	5'-AGTGAAGTGAAGGGTAA-3'
	antisense	5'-ATCAGATTCGGTAGG-3'
Gapdh	sense	5'-TTCCGTGTTCTACCC-3'
	antisense	5'-GTCGCAGGAGACAACC-3'

tion [18, 19]. Dysfunction of the islet lysosomal system impaired glucose-induced insulin secretion in diabetic Goto-Kakizaki rat [20]. Previously, we described a novel lysosomal integral membrane protein, Sidt2, which belongs to the SID1 transmembrane family [21] and showed that Sidt2 deficiency impaired glucose metabolism [22]. Hypothetically, Sidt2 could promote insulin-secretory exocytosis by affecting some SNARE proteins. Therefore, we explored the role of Sidt2 in insulin secretion *in vivo* and *in vitro*.

### Materials and methods

#### Animals

Sidt2<sup>-/-</sup> mice were generated using the Cre-LoxP system and described in our previous study [22]. All animals were bred under conventional pathogen-free conditions. Animals were fed

with food and water *ad libitum*. All animal experimental protocols were approved by the Animal Ethics Committee of Yijishan Hospital of Wannan Medical College and performed according to their recommendations.

#### Cell culture

The insulin-secreting cell line INS-1 was purchased from the Shanghai Institute of Biochemistry and Cell Biology, Academy of Sciences of China. INS-1 cells were cultured in RPMI-1640 media (Gibco®, Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and routinely grown at 5% CO<sub>2</sub>/95% air at 37°C. The cells were passaged weekly by performing trypsin-EDTA detachment. All of the studies were performed in INS-1 between passages 5 and 20.

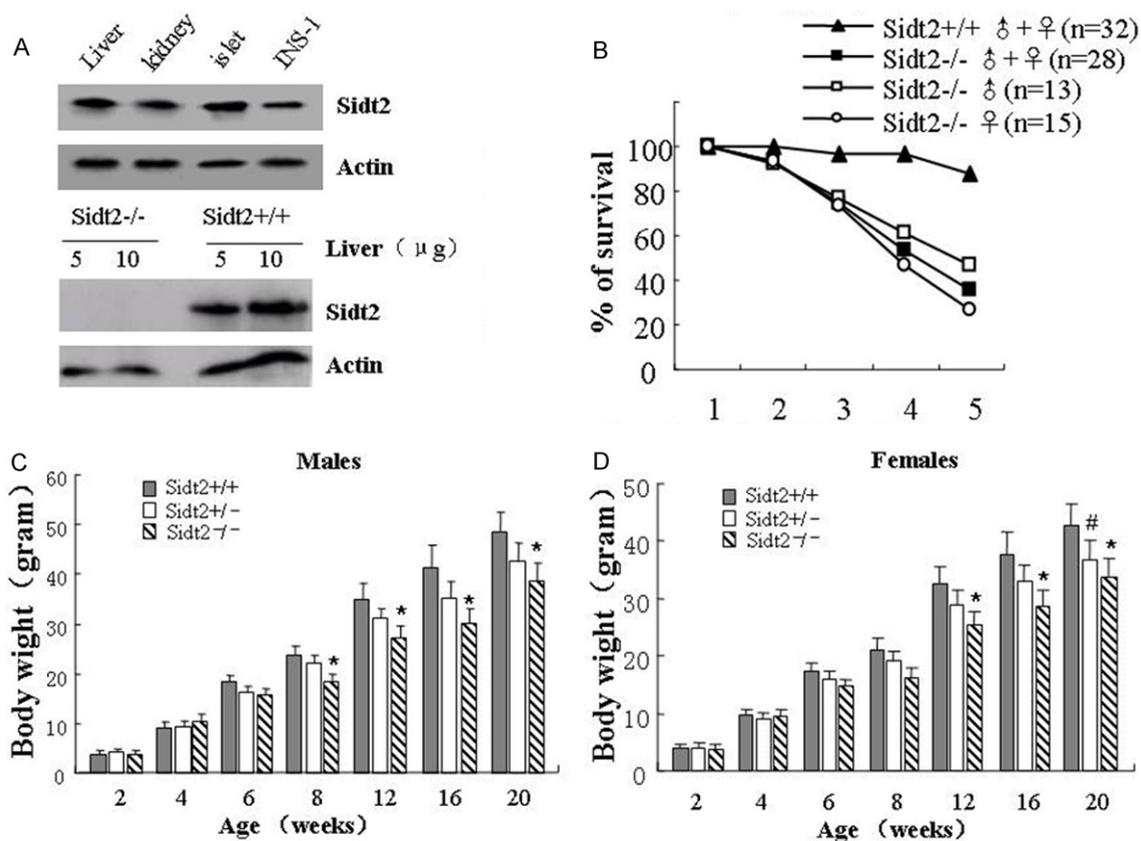
#### Insulin secretion assay

Insulin secretion was measured as previously described [23]. Briefly, 5×10<sup>5</sup> insulinoma cells were placed in 12-well plates in growing medium. The cell lines were treated with siRNA (1 nM or 10 nM, respectively) for 48 h. Subsequently, fresh medium containing 4.0 mM glucose was added after 24 h. And then, cells were cultured for an additional 48 h. The medium was removed on the day of the experiment, and the cell lines washed three times with warm Krebs-Ringer buffer (KRB). The cells were incubated with 1 ml of KRB at 37°C for 1 h, and then incubated with KRB containing two different glucose concentrations (4 and 16.7 mM) for 1 h at 37°C. The supernatant was collected and the insulin content was assayed by ELISA (Millipore, Charles, MO). Cells were then treated with acidified ethanol (75% ethanol, 1.5% HCl), and the total protein was measured using the Bio-Rad Dc Protein assay (Hercules, CA, USA). The amount of insulin secretion was corrected by comparing to the amount of total protein in each well.

#### Sidt2 siRNA transfection

Cells were transfected with two siRNAs: a negative control and Sidt2 siRNA (siRNA transfection kit, sc-45064, from Santa Cruz Biotechnology, CA, USA). Sidt2 siRNA or non-targeting negative control duplex (sc-37007, Santa Cruz

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**Figure 1.** Sidt2<sup>-/-</sup> mice characterized with weight loss and postnatal survival rate. A: Sidt2 expression in multiple tissues and INS-1 cell line (on the top), and in the liver from Sidt2<sup>-/-</sup> mice (on the bottom). B: Analysis of survival rate in offspring of sidt2<sup>-/-</sup> mice. C: Changes in body weight in male sidt2<sup>-/-</sup> mice. D: Changes in body weight in female Sidt2<sup>-/-</sup> mice. Results are expressed as mean ± SD (n = 15-20). Compared with sidt2<sup>+/+</sup> mice as control, #P<0.05, \*P<0.01.

Biotechnology) and transfection reagent were mixed with transfection medium. After 6 h of transfection, the culture media was replenished with fresh medium containing 10% fetal bovine serum (FBS). After an additional 48 h in culture, the cells were harvested for further analysis.

### Glucose tolerance test

Intraperitoneal glucose tolerance tests (IPGTTs) were performed as described in our previous study [22]. Briefly, after a 12 h fasting period, a blood sample from Sidt2<sup>-/-</sup> or Sidt2<sup>+/+</sup> mice was collected from the tail vein at time 0, 15, 30, 60 and 120 min after intraperitoneal injection (i.p.) of 1.5 g/kg body weight glucose. Blood glucose concentrations were measured using a blood glucose meter (Johnson & Johnson). Insulin ELISA Kit (Millipore) was used to analyze insulin levels according to the manufacturer's instructions.

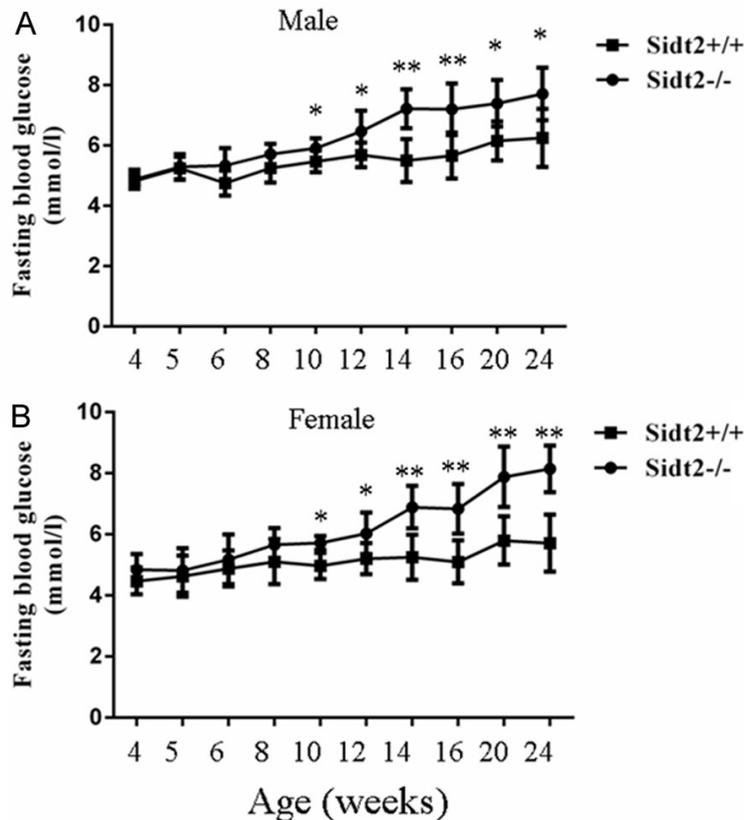
### Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using an RNA extraction kit (Sangon Biotech Co. LTD) according to the manufacturer's instructions. RT-PCR was performed as previously described in our literature [21]. The primers used in this study were designed by Primer 5.0 software and synthesized by Sangon Biotech Co. LTD. The primer sequences are listed in **Table 1**.

### Western blotting analysis

For protein expression studies, tissues or insulinoma cells were homogenized in lysis buffer (Beyotime, Beijing, China). Homogenates were centrifuged at 12,000×g at 4°C for 10-20 min. Western blot analysis was performed as described previously [24]. Briefly, 5 and 10 μg of total soluble proteins were separated on 12.5% SDS-PAGE and transferred onto PVDF

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**Figure 2.** The levels of fasting glucose (FG) in *Sidt2*<sup>-/-</sup> mice. A: The levels of FG in male *Sidt2*<sup>-/-</sup> mice; B: The levels of FG in female *Sidt2*<sup>-/-</sup> mice. Results are expressed as mean  $\pm$  SD (n = 10-15 for each measurement). \*P<0.05; \*\*P<0.01 versus control.

membranes. Membranes were incubated with rabbit anti-*Sidt2* specific antibody (Sigma-Aldrich® Co. LLC., St. Louis, MO, USA) and then with a horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody (Sangon Biotech Co. LTD, Shanghai, China). Antibodies specific to actin (Sangon Biotech Co. LTD) served as an internal control. The target proteins were visualized using an enhanced chemiluminescence detection system (Sangon Biotech Co. LTD).

### Statistical analysis

Generally, data from independent experiments were averaged. Error bars represent standard deviations (SD). The data was analyzed using SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA). Differences between groups were performed using unpaired Student t-test or one-way analysis of variance (ANOVA) with a significance limit set at P<0.05.

## Results

*Sidt2*<sup>-/-</sup> mouse shows weight loss and lower postnatal survival rate

Previously, we used RT-PCR to show that the *Sidt2* gene was widely expressed in various organs, including heart, liver, kidney, pancreas and brain [22]. In the present study, the expression of the *Sidt2* protein was confirmed in liver, kidney, pancreatic islet, as well as in the ISN-1 cell line using western blot analysis (Figure 1A, on the top). Furthermore, in the *Sidt2*<sup>-/-</sup> mouse, the protein was not detected by western blot (Figure 1A, on the bottom).

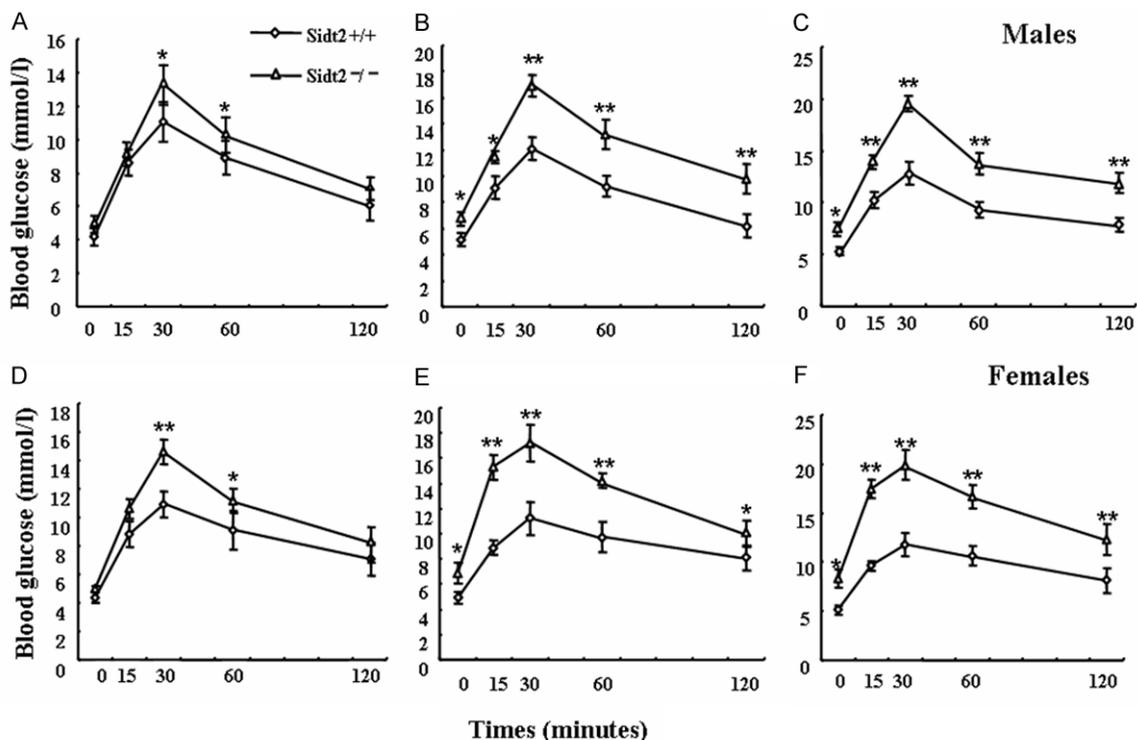
In order to determine the effect of *Sidt2* on the postnatal survival rate and weight, we measured the survival rates and weights of the *Sidt2*<sup>-/-</sup> mice. The results showed that the survival rates of the *Sidt2*<sup>-/-</sup> mice declined significantly from 3 weeks to 4 weeks of age, compared with their

*Sidt2*<sup>+/+</sup> littermates (Figure 1B). Moreover, the *Sidt2*<sup>-/-</sup> mice had thinner and smaller physiques (data not shown). After assessing body mass, we found that the male *Sidt2*<sup>-/-</sup> mice lost the majority of their weight at 8 to 20 weeks of age, compared with those of *Sidt2*<sup>+/+</sup> littermates (Figure 1C). However, the female *Sidt2*<sup>-/-</sup> mice lost their weights at 12 to 20 weeks of age, compared with their *Sidt2*<sup>+/+</sup> littermates (Figure 1D).

*Sidt2*<sup>-/-</sup> mice have a high level of fasting glucose (FG) during aging

In order to investigate the effect of *Sidt2* deficiency on glucose metabolism, the levels of fasting glucose (FG) were assessed in *Sidt2*<sup>-/-</sup> mice and *Sidt2*<sup>+/+</sup> mice (as control) from 4 to 24 weeks after birth. The results showed that the levels of FG in male *Sidt2*<sup>-/-</sup> mice were significantly higher from 10 to 24 weeks-old than those in male wild type littermates (Figure 2A).

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**Figure 3.** *Sidt2*<sup>-/-</sup> mice characterized with a progressively impaired glucose tolerance with aging. (A-C) Glucose tolerance test (2 g/kg body weight) was performed with an i.p. injection of glucose into male *Sidt2*<sup>+/+</sup> (open diamonds) and *Sidt2*<sup>-/-</sup> mice (open triangles) at age of 2 (A), 4 (B) and 6 (C) months. (D-F) Glucose tolerance test (2 g/kg body weight) was performed with i.p. injection into female *Sidt2*<sup>+/+</sup> (open diamonds) and *Sidt2*<sup>-/-</sup> mice (open triangles) at age of 2 (D), 4 (E) and 6 (F) months. Results are expressed as mean  $\pm$  SD (n = 10-15 for each measurement). \*P<0.05; \*\*P<0.01 versus *Sidt2*<sup>+/+</sup> mice.

A similar trend was also observed in female *sidt2*<sup>-/-</sup> mice (**Figure 2B**). These findings suggested that the KO mice had a diminished ability to use the lower levels of insulin for glucose uptake or were less insulin-sensitive.

### *Sidt2*<sup>-/-</sup> mice exhibit impaired glucose tolerance

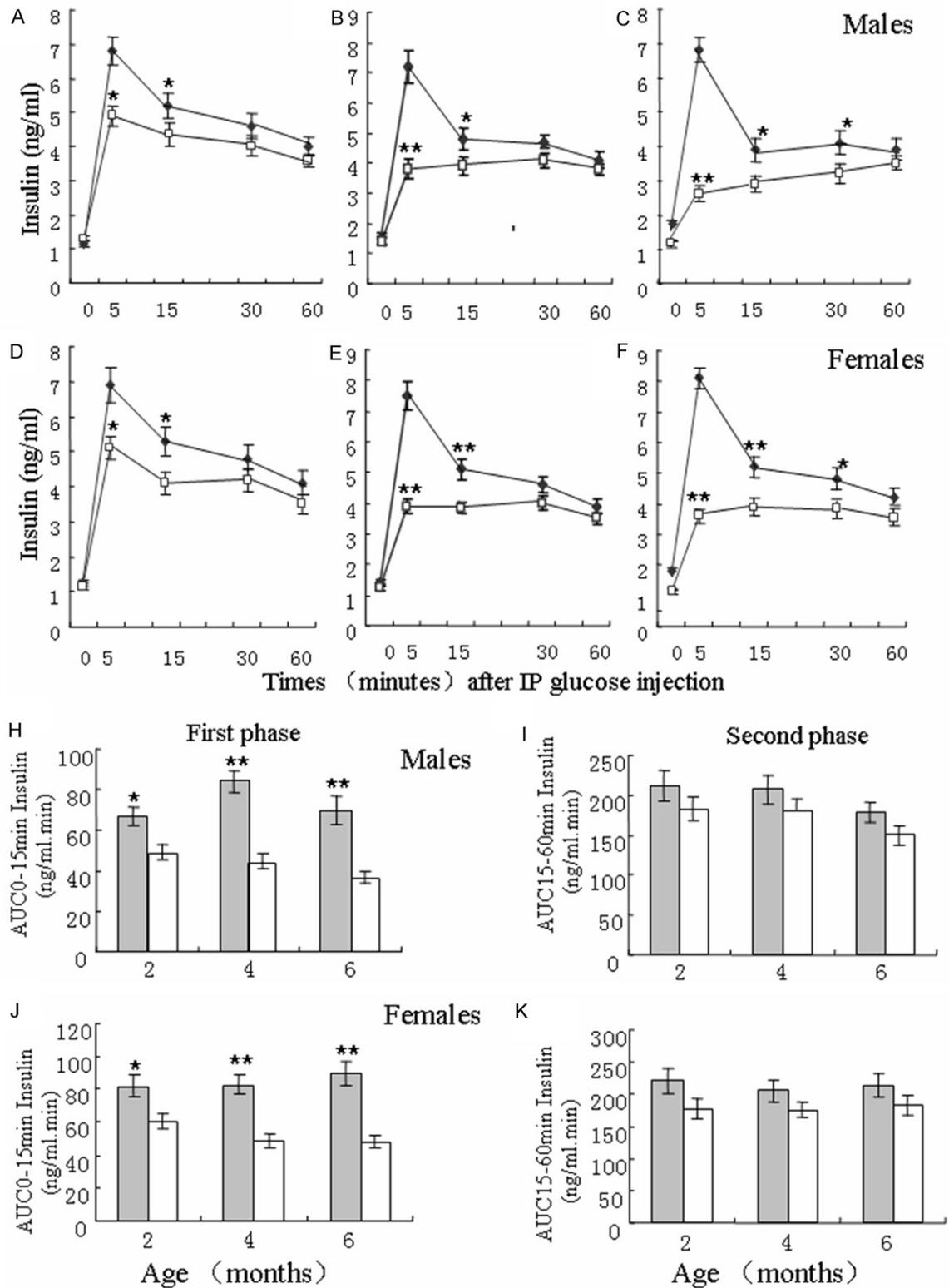
To determine whether *Sidt2* knockout causes insulin insensitivity, glucose tolerance tests were performed. Animals were fasted overnight, injected i.p. with 2 g/kg (body weight) glucose and their blood glucose was assessed. In two month-old mice, the levels of blood glucose in both male and female *Sidt2*<sup>-/-</sup> mice were significantly higher than those in the wild-type littermates, 30 and 60 min after challenge with glucose (**Figure 3A** and **3D**). Furthermore, in aged mice that were 4 and 6 months old, after loading glucose, both male (**Figure 3B** and **3E**) and female (**Figure 3C** and **3F**) KO mice at different points in time 0, 15, 30, 60 and 120 min cleared the glucose significantly slower than

the controls, respectively. Moreover, the glucose tolerance was progressively attenuated with aging (4-month-old mice versus 6-month-old mice, **Figure 3B-F**).

### Dysfunction of insulin secretion in *Sidt2* knockout mice

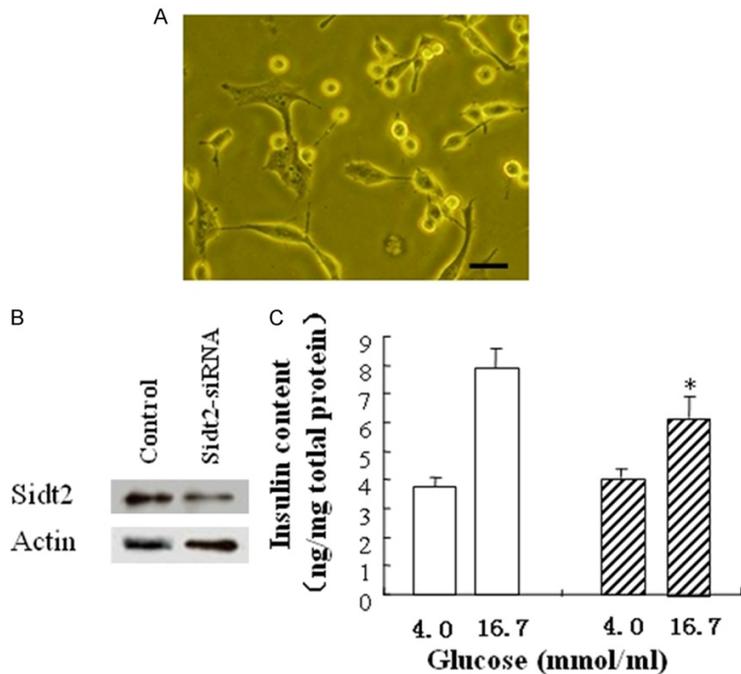
To investigate the role of *Sidt2* in insulin secretion, blood insulin were measured in *Sidt2*<sup>-/-</sup> mice at 2-, 4- and 6-months old; as well as in *Sidt2*<sup>+/+</sup> mice. Compared with the male littermate controls at 2-months old, male *Sidt2*<sup>-/-</sup> mice had much lower blood levels of insulin in the first-phase (0-15 min; **Figure 4A**). Moreover, the peak of insulin secretion occurred 5 min after injection and then decreased until the 60 min time point. However, insulin blood levels were not evident at the first- and second-phase (15-60 min) in 4 and 6 month-old *Sidt2*<sup>-/-</sup> mice; although the difference was also observed in which case insulin levels were significantly lower than those in the controls, respectively (**Figure 4B** and **4C**). A similar trend was also

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**Figure 4.** Changes in insulin secretion in the first- and second-phases in 2-, 4- and 6-month-old Sidt2<sup>+/+</sup> and Sidt2<sup>-/-</sup> mice. (A-C) Insulin levels in male 2-, 4- and 6-month-old Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice (A-C, respectively). (D-F) Insulin levels in female 2-, 4- and 6-month-old Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice (D-F, respectively). (H-K) Area Under the Curve (AUC) for insulin in the first phase (H, J) and second phase (I, K) calculated from (A-F) in male (H, I) and female (J, K) mice. n = 8-12 for each measurement, \*P<0.05 versus control, \*\*P<0.01 versus control.

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**Figure 5.** Impact of Sidt2 deficiency for insulin secretion in INS-1 cells. A: Cultured INS-1 cells. B: The changes in expression of Sidt2 protein in INS-1 cells transfected with Sidt2-siRNA or non-targeting control siRNA. C: Induction of insulin secretion in INS-1 cells treated with the non-target siRNA (open bars, as control) or Sidt2-siRNA (diagonal grid bars) after loading with 4 mM or 16.7 mM glucose. Experiments were done in triplicate, \*P<0.05, compared with the controls.

noted in female littermate Sidt2<sup>-/-</sup> mice (**Figure 4D-F**).

After analyzing the Area Under the Curve (AUC) s of the first-and second-phases, respectively, the results showed that the AUCs of the first-phase of insulin secretion in male Sidt2<sup>-/-</sup> mice were significantly decreased compared with the male littermate controls (**Figure 4H**). However, the AUCs at the second-phase of insulin secretion were not markedly changed between male Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice (**Figure 4I**). Similar trends were also observed in female Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice (**Figure 4J and 4K**).

### *Sidt2 drives glucose-induced insulin secretion in cultured cells*

In order to address the role of Sidt2 of insulin secretion *in vitro*, the pancreatic  $\beta$ -cell line INS-1 was employed. INS-1 cells were grown in culture (**Figure 5A**). INS-1 cells were transfected with Sidt2 siRNA or a negative control for 48 h, and the western blot analysis showed that the expression level of Sidt2 protein in Sidt2-

siRNA group was notably decreased compared to the control (**Figure 5B**). The cells were treated with glucose (4.0 mM or 16.7 mM) for 48 h, and analysis indicated that the control cells showed robust induction of insulin secretion by these two glucose concentrations (**Figure 5C**). However, the Sidt2-siRNA-treated cells displayed a significantly reduced insulin secretion after treatment with 16.7 mM glucose, compared with the control (**Figure 5C**).

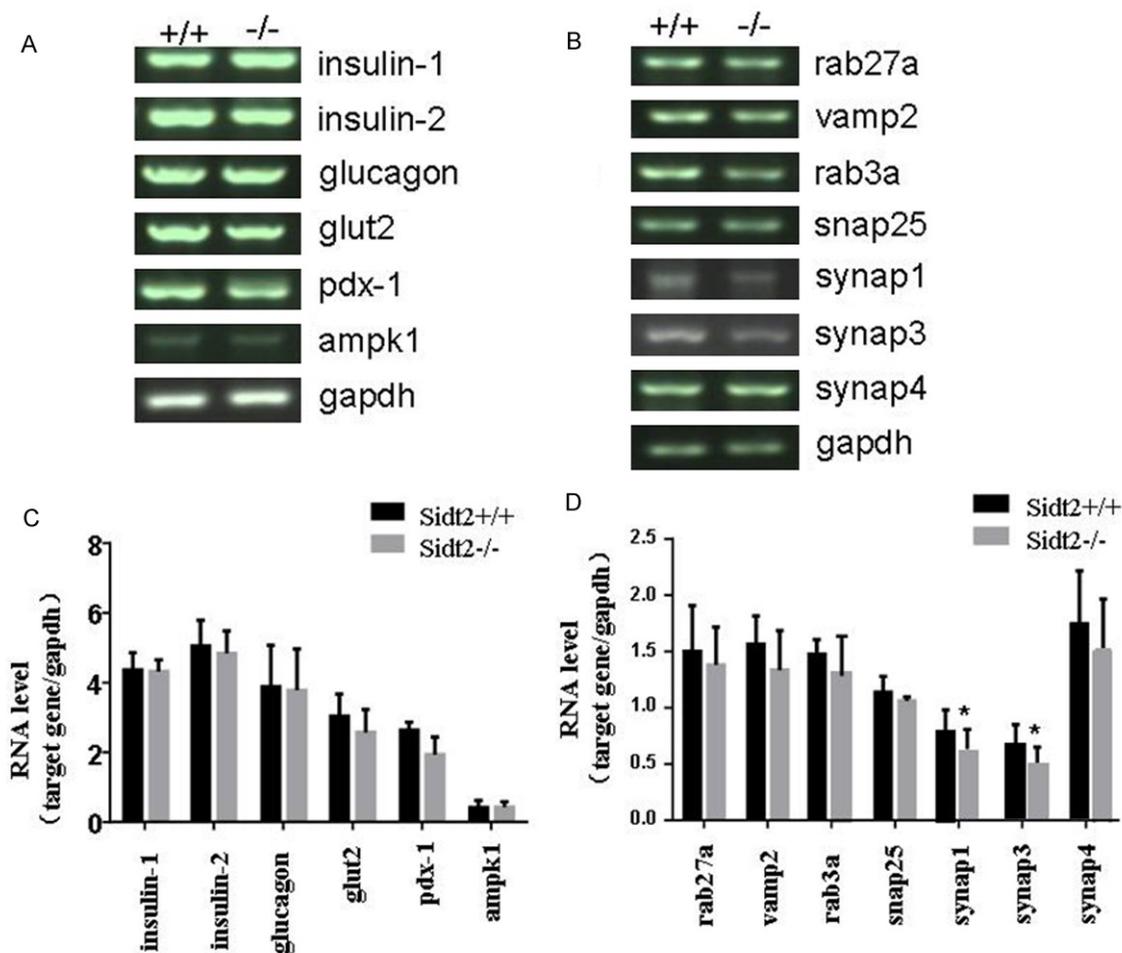
### *Impact of Sidt2 expression levels on islet and $\beta$ -cell gene expression*

To elucidate the possible mechanisms of Sidt2 effects on insulin secretion, we examined changes in the expression of 13 genes involved in glucose-stimulated insulin exocytosis and glucose metabolism by RT-PCR analysis. The results showed there were no differences in the expression of 6 genes related to glucose metabolism between Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice, including *insulin-1*, *insulin-2*, *glucagon*, *glut2*, *pk-1* and *ampk1* (**Figure 6A and 6C**). However, 2 of the 7 genes involved in insulin secretion, including *synap1* and *synap3*, were significantly decreased in Sidt2<sup>-/-</sup> mice, compared with controls (p<0.05, **Figure 6B and 6D**). The expression of the other SNARE proteins, such as *rab27a*, *vamp2*, *snap25* and *synap4*, showed no significant changes compared with controls.

## Discussion

In this study, we show that Sidt2 is expressed in the liver and kidneys, tissues that have abundant lysosomes. Gluconeogenesis in these tissues contributes to an increase in plasma glucose level after overnight fasting [25, 26]. Sidt2 expression was also detected in islets and INS-1 cells. However, there was no expression of Sidt2 in the liver from Sidt2-deficient mice. It has been postulated that the liver is the main source of glucose under physiologic circum-

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**Figure 6.** Changes in the expression of 13 genes involved in glucose-stimulated insulin exocytosis and glucose metabolism in Sidt2-deficient mouse islet cells. (A) Expression of hormones (insulin, glucagon), glucose transporter 2 and insulin signaling molecules Ampk1 and Pdx-1. (B) Key genes involved in insulin granule exocytosis. (C) Data calculated from (A), (D) Data calculated from (B). Total RNA was isolated as described in the Materials and Methods section, and the gene expressions were assessed by RT-PCR. Data are displayed as means  $\pm$  SD,  $n = 3$  for each measurement. \* $P < 0.05$ , versus Sidt2<sup>+/+</sup> control.

stances [25]. Hepatic abnormalities contribute to impaired glucose tolerance [27]. Our results showed that the postnatal survival rate of Sidt2 KO mice was significantly decreased compared with controls. Both the male and female Sidt2<sup>-/-</sup> mice also showed increased weight loss with age, suggesting that Sidt2 might be involved in glucose metabolism.

In order to determine the role of Sidt2 in glucose metabolism, we measured the levels of FG in Sidt2<sup>-/-</sup> mice. The results showed that Sidt2<sup>-/-</sup> mice had a higher level of FG compared with the controls. In other words, the depletion of Sidt2 might cause impaired glucose tolerance and this may be aggravated with age.

Glucose tolerance tests further confirmed that Sidt2 was involved in normal glucose tolerance.

Rapid insulin exocytosis stimulated by elevated blood glucose levels occurs within the initial 5-10 min after stimulation, referred as the first phase. Subsequently, the second-phase secretion is sustained for up to several hours when increased plasma glucose levels persist [28, 29]. To demonstrate that Sidt2 exerts an effect on insulin secretion, blood insulin was measured in Sidt2<sup>-/-</sup> mice. The Sidt2<sup>-/-</sup> mice released lower blood levels of insulin during the first and second phases compared with the controls. However, the AUC analysis confirmed that there

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was no significant difference during the second phase of insulin release between Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice. Our data shows that Sidt2 plays a vital role on the insulin secretion during the first-phase.

To address the role of Sidt2 on insulin secretion, Sidt2 siRNA was transfected into INS-1 cells. The results showed that Sidt2-siRNA-treated cells released lower insulin levels after injection with 16.7 mM glucose, compared with the control untreated cells. In order to further analyze the deficit in insulin secretion in the Sidt2 KO mice, we analyzed the expression of 13 genes involved in insulin release and glucose metabolism. Insulin, encoded by nonallelic *insulin-1* and *-2* genes, plays a vital role in glucose homeostasis and is a negative regulator of islet cell growth [30]. Pdx1, also known as IPF1 in human, is thought to play essential roles in insulin gene expression [31, 32]. AMP-activated protein kinase (AMPK) in the liver is a master regulator of glucose homeostasis mainly through the inhibition of gluconeogenic gene expression and hepatic glucose production [33]. Our results showed that the expression levels of these genes have no statistical changes between Sidt2<sup>-/-</sup> and Sidt2<sup>+/+</sup> mice. The data indicates that Sidt2 is not related to the process of glucose metabolism. The expression two insulin-exocytosis related SNARE genes, *synap1* and *synap3*, was decreased in Sidt2 KO mice. It implies that insulin secretion is regulated by Sidt2 via *synap1* and *synap3* proteins instead of the other SNARE proteins and regulators, such as *synap 4*, VAMP2, SNAP-25, *rab3a* and *rab27a*.

In general, our study confirms that Sidt2, a lysosomal integral membrane protein, plays a key role in insulin exocytosis via two SNARE proteins, *synap1* and *synap3*. Depletion of Sidt2 results in weight loss, elevated postnatal survival rate, and impaired glucose tolerance. However, the precise mechanism of insulin exocytosis regulation by Sidt2 needs further investigation.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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