WldS Enhances Insulin Transcription and Secretion via a SIRT1-Dependent Pathway and Improves Glucose Homeostasis

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OBJECTIVE—WldS (Wallerian degeneration slow), a fusion protein from a spontaneous mutation containing full-length nicotinamide mononucleotide adenylyltransferase 1, has NAD biosynthesis activity and protects axon from degeneration robustly. NAD biosynthesis is also implicated in insulin secretion in β-cells. The aim of this study was to investigate the effect of WldS on β-cells and glucose homeostasis.

RESEARCH DESIGN AND METHODS—Using the WldS mice, we measured the expression of WldS in pancreas and analyzed the effect of WldS on glucose homeostasis. The direct effect of WldS on insulin transcription and secretion and the related mechanisms was measured in isolated islets or β-cell lines. Silent information regulator 1 (SIRT1), an NAD-dependent protein deacetylase, is involved in insulin secretion. Thus, WldS mice with SIRT1 deficiency were generated to study whether the SIRT1-dependent pathway is involved.

RESULTS—WldS is highly expressed in the pancreas and improves glucose homeostasis. WldS mice are resistant to high-fat diet–induced glucose intolerance and streptozotocin–induced hyperglycemia. WldS increases insulin transcription dependent on its NAD biosynthesis activity and enhances insulin secretion. SIRT1 is required for the improved insulin transcription, secretion, and resistance to STZ-induced hyperglycemia caused by WldS. Moreover, WldS associates with SIRT1 and increases NAD levels in the pancreas, causing the enhanced SIRT1 activity to downregulate uncoupling protein 2 (UCP2) expression and upregulate ATP levels.

CONCLUSIONS—Our results demonstrate that WldS combines an insulinosuppressive effect with protection against β-cell failure and suggest that enhancing NAD biosynthesis in β-cells to increase SIRT1 activity could be a potential therapeutic approach for diabetes. Diabetes 60:3197–3207, 2011

Glucose homeostasis is largely maintained by the pancreatic β-cells, which secrete insulin in response to elevated ATP levels as a result of glucose metabolism (1). β-Cell dysfunction often leads to diabetes (2,3). However, the underlying mechanisms involved in the preservation of β-cell function remain to be fully understood.

Silent information regulator 1 (SIRT1), an NAD-dependent protein deacetylase, regulates various biological processes including glucose homeostasis (4–6). SIRT1 controls the gluconeogenic/glycolytic pathways in the liver (7) and improves insulin sensitivity under insulin-resistant conditions in C2C12 myotubes (8). In β-cells, SIRT1 promotes the expression of NeuroD and Mafa, two factors essential for the transcription of insulin, by deacetylating and activating Foxo1 to protect against oxidative stress (9). Moreover, glucose-stimulated insulin secretion (GSIS) in islets of SIRT1 knockout mice is blunted (10), whereas GSIS is enhanced in β-cell–specific SIRT1-overexpressing mice (11). Nevertheless, how SIRT1 is regulated to modulate insulin secretion and improve glucose homeostasis remains to be further explored.

Wallerian degeneration is an experimental model of axon degeneration. Remarkably, this degeneration is dramatically slowed in Wallerian degeneration slow (WldS) mice, a spontaneous mutant mouse strain (12). The protective function is attributed to a chimeric gene resulting from an 85 kb tandem triplication in chromosome 4 (13). The chimeric gene, WldS, encodes an 85 kb tandem triplication in chromosome 4 (13). The chimeric gene, WldS, encodes an N-terminal 70 amino acids fragment of ubiquitination factor E4B (Ube4b) fused to nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), a crucial enzyme for NAD biosynthesis (14,15). The NMNAT1 activity is pivotal for the neuronal protective function of WldS, which maintains the cellular NAD levels in a steady state and prevents NAD decline in injured axons (16,17). It has been implicated that NAD biosynthesis is also involved in β-cell function. The activity of SIRT1 on GSIS in β-cell–specific SIRT1-overexpressing mice decreases with age, which probably is the result of a decline in systemic NAD biosynthesis (18). The chimeric gene, WldS, encodes an N-terminal 70 amino acids fragment of ubiquitination factor E4B (Ube4b) fused to nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), a crucial enzyme for NAD biosynthesis (14,15). The NMNAT1 activity is pivotal for the neuronal protective function of WldS, which maintains the cellular NAD levels in a steady state and prevents NAD decline in injured axons (16,17). It has been implicated that NAD biosynthesis is also involved in β-cell function. The activity of SIRT1 on GSIS in β-cell–specific SIRT1-overexpressing mice decreases with age, which probably is the result of a decline in systemic NAD biosynthesis (18). 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streptozotocin (STZ)-challenged states and that WldS regulated insulin transcription and secretion dependent on SIRT1. Moreover, WldS associated with SIRT1 and increased NAD levels in the pancreas, which led to the enhanced SIRT1 activity to downregulate uncoupling protein 2 (UCP2) and upregulate ATP levels. Thus enhancing NAD biosynthesis in β-cells to increase SIRT1 activity might be a potential therapeutic approach for diabetes.

RESEARCH DESIGN AND METHODS

Animals. All mice were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committees at the Institute for Nutritional Sciences. WldS mice (C57BL/6 background) were purchased from Harlan. C57BL/6 mice and imprinting control region (ICR) mice were purchased from Slac (Shanghai, China). SIRT1+/− mice (129/ICR background) were obtained previously (20). SIRT1+/− mice were crossed with ICR mice to get SIRT1−/− mice on a outbred genetic background. We then mated WldS mice with SIRT1−/− mice (129/ICR background) and intercrossed the double-heterozygous SIRT1+/− WldS+/−, SIRT1−/− WldS+/−, SIRT1−/− WldS−/−, and SIRT1−/− WldS−/− mice. The genotyping for WldS and SIRT1 was carried out as described previously (20,21).

Islet morphology analysis. The islet area and the islet cell density were determined as described previously (22). All measurements were made using a fluorescence microscope (BX61; Olympus) and Image-Pro Plus software (version 5.0.1; Media Cybernetics). At least four mice and 40 islets per mouse were studied for each group.

Immunofluorescence. Pancreatic fresh-frozen sections of 16-week-old mice or fixed cells were incubated overnight at 4°C with indicated antibodies including anti-mouse insulin antibody (Sigma), anti-WldS antibody (a gift from Dr. Michael P. Coleman, The Babraham Institute), and rabbit anti-Myc antibody (Santa Cruz Biotechnology) and then detected with Alexa Fluor 555 goat anti-rabbit IgG and/or Alexa Fluor 488 goat anti-mouse IgG antibodies (Molecular Probes). DAPI (Sigma) was used to stain the nuclei. Immunofluorescence images were obtained on a Zeiss LSM 510 META confocal microscope or an Olympus IX51 fluorescence microscope.

Glucose, insulin, C-peptide, homeostasis model of insulin resistance, and homeostasis model of β-cell measurements. Fed glucose and insulin levels were measured about 3 h after light on. Fasted glucose, insulin, and C-peptide levels were measured after 17-h fasting. The refed blood glucose levels were measured in the mice reed for 2 h after a 17-h fasting. Tail blood was collected to measure glucose with a glucometer (FreeStyle), to measure C-peptide by an ELISA kit (Millipore). Homeostasis model of insulin resistance (HOMA-IR) and homeostasis model of β-cell function values were obtained using the HOMA Calculator v2.2 (23).

Glucose tolerance and insulin tolerance tests. Glucose tolerance tests were performed on male mice fasted 13 h. Glucose concentrations were measured in blood collected by venous bleeding from the tail vein at the indicated times after an intraperitoneal injection of glucose (2 g/kg body wt). Insulin tolerance tests were performed on male mice fasted for 4 h (9:00–13:00). Glucose levels were likewise measured from tail blood after an intraperitoneal injection at 0.75 units/kg body weight of human insulin (Eli Lilly).

HF diet-induced obesity mouse model. Five-week-old WldS and wild-type mice were randomly assigned and fed with either normal chow containing 10 kcal% fat or HF diet containing 45 kcal% fat (Research Diets). After mice were fed for 12 weeks, fat and lean mass were measured in conscious animals by a minispec mq serial NMR spectrometer (Bruker). Serum total cholesterol, HDL-cholesterol, and LDL-cholesterol levels were determined by enzymatic assays on an Olympus automated analyzer.

Multiple low-dose STZ (MLDS) treatment in vivo. Ten-week-old male mice were injected intraperitoneally for 5 consecutive days with 40 mg/kg STZ (Sigma) freshly prepared in cold 0.1 mol/L citrate buffer (pH 4.5) as described previously (24). Blood glucose was monitored weekly, and the mice were considered diabetic when their blood glucose levels were over 250 mg/dL in 2 consecutive weeks. Pancreata were frozen in liquid nitrogen and processed for frozen sections and immunohistochemistry. The percentage of islet area was quantified as described previously (25).

Immunohistochemistry. After being fixed with ice-cold acetone, frozen sections were treated with 3% H2O2 to block the endogenous peroxidase. Anti-mouse insulin antibody (Sigma) was biotinylated goat anti-mouse IgG (Jackson ImmunoResearch) and Streptavidin-HRP (Zymed) were used to amplify the signal, and diaminobenzidine (DAB) was used as chromogen.

Plasmids. pCMV-WldS and pCMV-WldS-F116S were constructed as described previously (26). pCMV-NMNAT1 and pCMV-NMNAT1-F28S were constructed by insertion of MNMAT1 or MNMAT1-F28S cDNA fragment amplified from pCMV-WldS into pEGFP-C3 vector (Clontech) using the primers including CTCGCAAGCTTATGGAGGAGCTGAGCGCTGAC and CTGCTAACTTGCGTGGTGGTTG and CAGCGGATCTATGCTACATCAAGGAAG. pEGFP (enhanced green fluorescent protein)-WldS was constructed by insertion of WldS cDNA fragment amplified from pCMV-WldS into pEGFP-C3 vector (Clontech) using the primers including CTCGCAAGCTTATGGAGGAGCTGAGCGCTGAC and CTGCTAACTTGCGTGGTGGTTG and CAGCGGATCTATGCTACATCAAGGAAG.
FIG. 2. WldS mice show increased serum insulin levels, improved glucose tolerance, normal insulin clearance rate, and normal insulin sensitivity. 

A: Serum insulin levels of WldS mice (n = 9) were significantly higher than those of wild-type (WT) (n = 10) no matter whether they were fed ad libitum or fasted overnight. Except where indicated, in this and all other figures, *P < 0.05 and **P < 0.01.

B: Blood glucose levels of 13-week-old WldS mice (n = 16) were significantly lower than those of wild-type (n = 24) when fasted overnight or refed for 2 h after fasting.

C: HOMA-β (homeostasis model of β-cell function) index of WldS mice (n = 9) was higher than that of wild-type (n = 10).

D: HOMA-IR (homeostasis model of insulin resistance) index of WldS mice (n = 9) was similar to that of wild-type (n = 10).

E: WldS mice showed improved glucose tolerance compared with wild-type as determined by glucose tolerance test (22-week-old; n = 14 to 15 for each group).

F: Area under the curve (AUC) of the glucose tolerance test in (E).

G: Serum insulin levels in WldS mice were higher than those in wild-type mice during the glucose tolerance test (22-week-old;
GTCTGGGATCCCTCCGCTACAGGTAGGATGTGTTG, pEGFP-Wld<sub>5</sub>-H112A was engineered by using Quick Change II Site-Directed Mutagenesis Kit as described by the instruction manual (Stratagene) using the primers CCGGCATCAACGGAATCTCTTTTGG and GCTGGACACGGGCGCTTGG-GGAATCTCTTTTGG and pCMV-Wld<sub>4</sub>-pEGFP, was co-transfected with 300 ng of fragment of rat insulin 1 5′ flanking region into the Snf1 and BγII sites of pGL3-basic vector (Promega) using the primers CCGAGGCTCCCCTCCAAATGTTTCTTCTTG and GCGAGATCGTGGATTCATGCTCACA. pGL3-UCP2-Promoter was constructed by insertion of 800 bp fragment of mouse UCP2 promoter into Xhol and KpnI sites of pGL3-basic vector using the primers CCTGCAGATATCTCTCTCCAGC and GATTTCTCAAAACCGGGAGTACAGC-GGGAGACAGGACGTAGCTCGGAC. pCMV-MyoC-SIRT1 was constructed by insertion of SIRT1 cDNA fragment cut from pCMV-SIRT1 (27) into pCMV-tag3A at the BamHI site. pCMV-PCMV-MyoC-SIRT1 was constructed by insertion of SIRT1 cDNA fragment amplified from pSG5-PPAR<sub>γ</sub>-a (gift from Dr. Ronald M. Evans, the Salk Institute) into pCMV-tag3A at the SfiI and Xhol sites using the primers CCCTGCAGATATCTCTCTCCAGC and GCGCCGACGGACCTGAGACTGTACGATCGTTCAGTATCGA.

Establishment of stably transfected cell lines. MIN-6 cells were transfected with pEGFP-C3, pEGFP-Wld<sub>5</sub>, pEGFP-Wld<sub>5</sub>-H112A, pCMV-tag3A, pCMV-Wld<sub>4</sub>, and pCMV-Wld<sub>5</sub>-pF116S, respectively, using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were treated with 280 μg/mL G418 (Sigma). Days (4–7) later, the living cells were diluted to 96-well plates and monolocional MIN6 cells with stable expression of the indicated proteins were then amplified and confirmed by Western blot and immunofluorescence.

Measurement of NMMAT enzyme activity and Luciferase assay. The NMMAT enzyme activity was measured as described previously (26). To measure insulin promoter activity, INS-1 cells (a generous gift from Dr. Christopher B. Newgard, Duke University Medical Center) in 24-well plates were cotransfected with 0.1 μg pGL3-Insulin Promoter, 0.1 μg pCMV-Wld<sub>5</sub>-Insulin Promoter, 0.8 μg pSV40-β-gal, and 0.8 μg indicated plasmids per well using Lipofectamine 2000 (Invitrogen). To evaluate UCP2 promoter activity, 293T cells in 24-well plates were transiently cotransfected with 0.1 μg pGL3-UCP2 Promoter, 0.1 μg pSV40-β-gal, and 0.8 μg indicated plasmids. The transfected plasmids were balanced with empty vector, and the total amount of transfected plasmids was 1 μg per well. After transfection for 40 h, cells were harvested and measured with a luciferase assay kit (Promega) and normalized to β-galactosidase activity as described previously (25).

Islet isolation, glucose-stimulated insulin secretion, insulin, and ATP content. Islets were isolated by collagenase P (Roche) digestion as described previously (11). Ten handpicked islets per well with similar average size were cultured in 24-well plates overnight in RPMI 1640 containing 11 mM glucose, 2.5 mM L-glutamine, and 10% FBS. The islets were then preincubated in Krebs–Ringer bicarbonate buffer for 1 h at 37°C and then stimulated with 2 mM glucose, 20 mM glucose, or 20 mM L-arginine plus 2 mM L-glutamate, and 20 mM L-arginine glucose in Krebs–Ringer bicarbonate buffer in triplicate for 2 h. The supernatant was collected for insulin measurements.

The remaining islets were washed twice with PBS and then extracted with ethanol-water-concentrated HCl (750:25:15) overnight at 4°C to measure insulin content and then extracted with radioimmunoprecipitation assay buffer and then lyophilized. The supernatant was subjected to radioimmunoprecipitation assay with 20 μg of protein in 100 μL of 1% sodium dodecyl sulfate (SD)-adenine sulfate-10% glycerol and 2 µL of peroxidase-conjugated antibody (Cell Signaling). The supernatant was analyzed with anti-insulin, tyrosine-phosphorylated insulin receptor, and β-tubulin antibodies using Western blot and immunochemical detection of mRNA (n = 8 for each group). H: AUC of the insulin secretion in g. I: Serum insulin levels after intraperitoneal injection of 10 units/kg human insulin to chase Wld<sub>5</sub> and Wld<sub>5</sub>-H112A mice at the indicated time points (17 mice for each group). J: The fasting plasma insulin molar ratio was similar between wild-type and Wld<sub>5</sub> mice (18-week-old; n = 7 for each group). K: Insulin tolerance in 23-week-old wild-type and Wld<sub>5</sub> mice was similar as determined by insulin tolerance test (n = 8 for each group). L: AUC of the insulin tolerance test in K. M and N: Insulin sensitivity was similar in 24-week-old Wld<sub>5</sub> and wild-type mice as determined using the insulin clamp method with the muscle (M) or liver (N) samples. O: Stimulation of INS-1 cells (17 mice) with 10 nM insulin. P: Serum insulin levels in 15-week-old wild-type and Wld<sub>5</sub> mice were fasted for 16 h, anesthetized, and injected with PBS or human insulin (5 units/kg) through their inferior vena cava. The liver and muscle samples were collected 5 and 10 min after injection of insulin, respectively, and snap-frozen in liquid nitrogen for subsequent Western blot analysis. O–R: Quantification of the muscle and liver phospho-Tyr1150/1151-InsR and phospho-Ser473-Akt protein levels corresponding to M and N.
serum insulin and fasted blood glucose levels were also observed in mice with a mixed genetic background (C57/129/ICR), and the effects in heterozygous WldS mice were weaker than the homozygous ones (Supplementary Fig. 2A and B). Furthermore, homeostasis model of β-cell function of 13-week-old WldS mice was significantly upregulated (Fig. 2C), and HOMA-IR was similar (Fig. 2D). WldS mice showed markedly improved glucose tolerance (Fig. 2E and F), and these effects were dependent on the gene dose of WldS and decreased with age (Supplementary Fig. 2C–E). Serum insulin levels of WldS mice during the glucose tolerance test were significantly higher than those of wild-type mice (Fig. 2G and H), suggesting the improved β-cell function in WldS mice. Insulin clearance rate was not markedly altered (Supplementary Fig. 2C–E).

FIG. 3. WldS mice show improved glucose tolerance when fed HF diet. A: Weekly body weight of wild-type (WT) and WldS mice fed chow or HF diet from 5 to 17 weeks old. Except where indicated, n = 6–8 for each group in all panels of Fig. 3. Error bars indicate SEM. *P < 0.05 vs. WldS mice fed chow; #P < 0.01 vs. wild-type fed HF diet. B: Fat and lean mass of 16-week-old wild-type and WldS mice fed chow or HF diet. C and D: Fat content was increased and lean content was decreased in both wild-type and WldS mice after feeding with 12-week HF diet. E–G: Serum total cholesterol, HDL-cholesterol, and LDL-cholesterol levels were elevated in both groups after feeding with 12-week HF diet, and no significant difference was observed between wild-type and WldS mice. H and I: WldS mice showed significantly decreased fasting blood glucose (H) and increased serum insulin levels (I) compared with wild-type mice in a similar extent (n = 4 for each genotype). (A high-quality color representation of this figure is available in the online issue.)
affected by WldS when monitored by in vivo insulin clearance assay and fasting C-peptide-to-insulin molar ratio (Fig. 2I and J), which indicated that the elevated serum insulin levels in WldS mice were not a result of altered insulin clearance. Insulin tolerance test confirmed that WldS and wild-type mice had similar insulin sensitivity as measured by HOMA-IR (Fig. 2K and L). Insulin-induced phosphorylation of insulin receptor and AKT in muscle and liver was also indistinguishable between WldS and wild-type mice (Fig. 2M-R). In addition, the protein level of SIRT1, an important regulator in hepatic metabolism, and NAD levels in the liver were similar in wild-type and WldS mice (Supplementary Fig. 3A–C). These data show that WldS improves β-cell function and glucose homeostasis but does not affect insulin sensitivity.

**WldS mice show improved glucose tolerance when fed HF diet.** The body weight and fat mass were moderately increased in WldS mice compared with wild-type when fed chow, and the differences were more prominent when fed HF diet (Fig. 3A and B). As expected, HF diet caused increased fat content and decreased lean content in both groups (Fig. 3C and D). Interestingly, when compared with wild-type mice, the fat content was increased in WldS mice fed HF diet (Fig. 3C). Serum total cholesterol, HDL-cholesterol, and LDL-cholesterol levels were elevated in both groups fed HF diet for 12 weeks, and no differences were observed between wild-type and WldS mice (Fig. 3E–G). In WldS mice fed with either chow or HF diet, fasting blood glucose levels were decreased, and serum insulin levels but not insulin sensitivity was remarkably increased, compared with corresponding wild-type controls (Fig. 3H–K). Notably, wild-type mice fed HF diet exhibited impaired glucose tolerance, and this was strikingly improved in WldS mice (Fig. 3L and M). The islet area of WldS and wild-type mice fed HF diet was enlarged to a similar extent (Fig. 3N and O). These data suggest that WldS alleviates HF diet-induced glucose intolerance by enhancing insulin production.

**WldS ameliorates STZ-induced hyperglycemia in mice.** To study the direct effect of WldS on β-cells, mice were administered STZ, which selectively destroys β-cells (32). After MLDS treatment, blood glucose levels in both groups increased gradually, but were much lower in WldS mice than those in wild-type (Fig. 4A). The diabetes incidence in WldS mice was only about 18% compared with 100% in wild-type 21 days after MLDS treatment, and these trends were maintained thereafter for at least 14 days (Fig. 4B). After MLDS treatment, WldS mice preserved more insulin producing β-cells and remarkably higher serum insulin levels than wild-type (Fig. 4C–E). These data show that WldS protects against pancreatic β-cell failure and hyperglycemia induced by MLDS.

**WldS enhances insulin transcription and secretion.** We next investigated how WldS enhanced insulin production. As shown in Fig. 5A and B, islets isolated from WldS mice released more insulin under basal, glucose-, or KCl-stimulated conditions than those from wild-type mice (Fig. 5D). WldS mice treated with glucose or KCl had higher glucose levels than those of the corresponding wild-type controls (Fig. 5C). The percentage of insulin positive area in islets of WldS mice was higher than that of wild-type corresponding to C. E: Serum insulin levels of WldS mice were higher than those of wild-type 35 days after vehicle or MLDS treatment. n = 8–13 for each group. *P < 0.05 and **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)
The effect of WldS on the upregulation of insulin content in islets was not significant (Fig. 5C), which might be because of the very high insulin levels in normal islets. Insulin expression was increased in MIN6 cells stably expressing EGFP-fused WldS or WldS with Myc-tag, whereas the increase was attenuated in cells stably expressing WldS-H112A or WldS-F116S, in which the NAD biosynthesis activity of WldS was abolished (Fig. 5D–F). The protein levels of WldS and WldS/NMNAT1 protein ratio in the cell lines were even higher than those in the islets of WldS mice (Supplementary Fig. 5). Moreover, increased NAD biosynthesis activity was observed in the pancreas of WldS mice (11-week-old male mice, n = 3 for each genotype). I: WldS heightened insulin mRNA levels dependent on its enzyme activity as determined by real-time PCR with MIN6 cells stably transfected with the indicated plasmids. J and K: WldS enhanced insulin promoter activity in a dose-dependent manner (J) and required its enzyme activity (K). INS-1 cells transfected with pGL3-Insulin-Promoter, and the indicated plasmids were used for luciferase assay to measure insulin promoter activity. *P < 0.05 and **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)

FIG. 5. WldS enhances insulin transcription and secretion. A and B: Insulin secretion of islets isolated from WldS mice was upregulated compared with wild-type (WT) when treated with the indicated concentration of glucose or KCl (n = 7 for glucose stimulation and n = 4 for KCl stimulation). The protein concentration (A) and the insulin content (B) were measured as internal control, respectively. C: Insulin content in the islets of WldS mice (n = 4). D: NMNAT enzyme activity in MIN6 cells stably transfected with the indicated plasmids. In this and other panels of this figure, WldS-H112A stands for EGFP-WldS-H112A. E: WldS upregulated insulin expression dependent on its enzyme activity. Insulin expression in MIN6 cells stably transfected with the indicated plasmids were stained with anti-insulin antibody. In the right panel anti-WldS antibody was added to visualize the WldS protein. DAPI was used to stain the nuclei. Scale bar, 5 μm. F: Insulin content in MIN6 cells stably transfected with the indicated plasmids.
Increased cellular NAD levels have been shown to enhance insulin transcription and secretion (10). The body weight and fat content of SIRT1−/− mice compared with wild-type (Fig. 7A–C). Moreover, SIRT1−/− mice increased much faster than those of SIRT1+/+ WldS+/+ mice (Fig. 6G). Therefore, SIRT1 is necessary for the enhancement of insulin transcription, secretion, and the resistance to STZ-induced hyperglycemia caused by WldS.

**WldS downregulates UCP2 expression and upregulates ATP levels through SIRT1.** Next, we explored how WldS exerts its function through SIRT1. NMNAT1 was reported to interact with and regulate the activity of SIRT1 in breast cancer cells (33), suggesting WldS containing the full-length NMNAT1 has similar effects. As expected, we found WldS downregulated UCP2 promoter activity like SIRT1 (Fig. 7G) and repressed UCP2 protein and mRNA levels dependent on its NAD biosynthesis activity (Fig. 7C and H). The effect of WldS on UCP2 mRNA and protein levels also depended on SIRT1 (Fig. 7I–K).

**DISCUSSION**

Over the past decade, numerous studies have focused on the axon protective function of WldS and its potential application in neuronal diseases (16). The effect of WldS in nonneuronal cells would also probably shed light on the understanding and treating of other diseases. However, there were few reports concerning this. In this study, we demonstrate that WldS enhances insulin transcription and secretion as well as improves glucose homeostasis, and SIRT1 is required in these processes.

It is well established that β-cells show a variety of similarities with neuronal cells (34), which implicates that WldS, a protein functional in neuronal cells, may also function in β-cells. As expected, we found that WldS was highly expressed in the pancreas including insulin-producing β-cells (Fig. 1A–C) and enhanced insulin transcription and secretion (Fig. 5). In addition, WldS mice exhibited increased serum insulin levels (Figs. 2A and 3I), even though their blood glucose levels were normal in the fed state (Fig. 2B). Similarly, SIRT4 knockout mice, for example, also show high insulin and normal fed blood glucose levels (35), which might be as a result of the existence of factors that increase blood glucose levels, including glucagon and gluconeogenesis. These factors acted as a counterbalance to neutralize the glucose-lowering effect of insulin and thus to maintain the blood glucose at a stable level. WldS mice showed improved glucose tolerance (Fig. 2E). Similarly, β-cell-specific SIRT1-overexpressing mice also show improved glucose tolerance as a result of enhanced GSIS (11). Comparably, we found that SIRT1 was required for the function of WldS in insulin secretion (Fig. 6C and D). Increased cellular NAD levels have been shown to enhance

**FIG. 6. SIRT1 is required for the enhancement of insulin transcription, secretion, and the resistance to STZ-induced hyperglycemia caused by WldS.** A: Sirtinol attenuated the activation of insulin promoter induced by WldS. INS-1 cells were transfected with p633 Insulin-Promoter and the indicated plasmids and treated with or without 60 μM Sirtinol for 24 h for luciferase assay. B: Upregulation of insulin transcription and islet secretion by WldS required SIRT1. Islets with the indicated genotypes were used for determination of insulin mRNA levels by real-time PCR. C: Insulin secretion and serum insulin levels of SIRT1−/− and SIRT1+/+ WldS+/+ mice were decreased compared with SIRT1+/+ WldS+/+ mice (Fig. 6E and F), probably resulting from their increased energy expenditure (Supplementary Fig. 6D–H). After MLDS treatment, the blood glucose levels of SIRT1−/− WldS+/+ and SIRT1+/+ WldS−/− mice increased much faster than those of SIRT1+/+ WldS+/+ mice (Fig. 6G). And the diabetes incidence of SIRT1−/− WldS+/+ mice was much higher than that of SIRT1+/+ WldS+/+ mice (Fig. 6H). Therefore, SIRT1 is necessary for the enhancement of insulin transcription, secretion, and the resistance to STZ-induced hyperglycemia caused by WldS.
FIG. 7. WldS downregulates UCP2 expression and upregulates ATP levels through SIRT1. A: WldS colocalized with SIRT1 in MIN6 cells. The stable cell lines expressing EGFP-WldS were transiently transfected with pCMV-myc-SIRT1 and stained with anti-Myc antibody and DAPI. Scale bar, 5 μm. B: WldS was communoprecipitated with SIRT1 from the pancreatic lysates of WldS mice. C: WldS and its enzyme-dead mutant WldS-H112A coimmunoprecipitated with SIRT1. The MIN6 cells stably expressing EGFP, EGFP-WldS, or EGFP-WldS-H112A were used for immunoprecipitation. UCP2 protein levels were also detected by Western blot. D and E: Liquid chromatography-tandem mass spectrometry analysis of NMN, NADP, NADPH, NAD, NADH, NA, and NAM extracted from pancreas of 9-week-old wild-type (WT) or WldS mice (* with significant difference). F: Quantification of the small molecules corresponding to D and E showed that NAD and NMN levels were upregulated in the pancreas of WldS mice (n = 4 for each genotype). G: WldS repressed UCP2 promoter activity like SIRT1. UCP2 promoter activity was measured by luciferase assay in 293T cells transfected with pGL3-UCP2-Promoter and the indicated plasmids. H: WldS downregulated UCP2 mRNA levels dependent on its enzyme activity. UCP2 mRNA level was measured by real-time PCR with MIN6 cell lines stably expressing EGFP, EGFP-WldS, and EGFP-WldS-H112A. I: WldS downregulated UCP2 mRNA levels via SIRT1. UCP2 mRNA levels were determined by real-time PCR using islets isolated from mice with the indicated genotype (n = 4 for each genotype). J: WldS downregulated UCP2 protein levels via SIRT1. The protein levels in brown fat tissue with the indicated genotype were detected with SIRT1, WldS, UCP2, and tubulin antibodies (n = 3 for each genotype). K: Quantification of the UCP2 protein levels corresponding to J. L: WldS increased ATP levels in primary cultured islets at the indicated glucose concentration (n = 3). M: WldS upregulated ATP level in islets via SIRT1. ATP levels were measured in islets with indicated genotypes at 2 mmol/L or 20 mmol/L glucose (n = 3). *P < 0.05 and **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)
SIRT1 activity (6). Consistently, we found NAD and its precursor NMN were upregulated in the pancreas of WldS mice (Fig. 7D–F), and WldS enhanced insulin transcription dependent on its NAD biosynthesis activity (5). These data suggest that NAD plays a key role in SIRT1-mediated enhancement of GSIS induced by WldS. It is noteworthy that NMNAT2, an enzyme catalyzing NAD biosynthesis, is highly expressed in the islets of Langerhans (36), which also suggests the importance of NAD biosynthesis in insulin secretion. It has been reported that UCP2 knockout mice show improved GSIS (37), and β-cell-specific SIRT1-overexpressing mice show improved GSIS by decreasing UCP2 expression and elevating ATP levels (11). Analogously, we found WldS downregulated UCP2 expression and upregulated ATP levels via SIRT1 (Fig. 7), which further confirmed that WldS regulates insulin secretion through a SIRT1-dependent pathway.

Usually, β-cells will secrete more insulin to overcome the reduced insulin sensitivity, which is often related with obesity (38). When their compensate mechanisms are impaired, type 2 diabetes occurs (1). WldS mice show increased serum insulin levels no matter whether mice were fed chow or HF diet without altering insulin sensitivity (Fig. 3F–K), which indicates enhanced β-cell function in WldS. Furthermore, when fed HF diet, the glucose tolerance of WldS mice was strikingly improved (Fig. 3L and M). Similarly, ghrelin knockout mice or GPR40 β-cell-specific transgenic mice also show increased insulin secretory capacity without altering insulin sensitivity and improved glucose tolerance when fed HF diet (39,40). In addition, WldS promoted insulin secretion and downregulated UCP2 via SIRT1 (Figs. 6C and 7K–I), which consisted with the studies that both β-cell-specific SIRT1-overexpressing mice and UCP2 knockout mice showed enhanced insulin secretion and resistance to HF diet–induced glucose intolerance (18,41). Taken together, our findings suggest that enhanced insulin secretory capacity by upregulating NAD biosynthesis activity in β-cells would be beneficial to overcome reduced insulin sensitivity induced by HF diet.

Type 1 diabetes is a chronic autoimmune disease, during which β-cells are selectively destroyed (2). MLDS has been extensively used to generate β-cell destruction to mimic type 1 diabetes (25). In this study, we found that MLDS-induced hyperglycemia was alleviated in WldS mice, which also demonstrated increased serum insulin levels (Fig. 4). It is noteworthy that UCP2 knockout mice, which showed enhanced insulin secretory capacity, had accelerated hyperglycemia after MLDS treatment as a result of stronger inflammation (24). In this scenario, WldS mice were superior to UCP2 knockout mice, probably resulting from some different underlying mechanisms. It has been reported that WldS shows protective effects in some neurodegenerative disease models (12,16). It is likely that amelioration of MLDS-induced hyperglycemia and attenuation of neurodegenerative diseases by WldS could share some common underlying mechanisms. Additionally, the resistance to MLDS-induced hyperglycemia was abolished in WldS mice with SIRT1 deficiency (Fig. 6G and H), which is consistent with the report that intrarterial targeted islet-specific expression of SIRT1 protects β-cells from STZ-induced apoptosis in mice (42). Thereby, the SIRT1-dependent WldS pathway is a potential target not only to enhance insulin secretory capacity, but also to protect against β-cell failure.

In this study, our results demonstrate that WldS combines an insulinotropic effect with protection against β-cell failure and suggest that upregulation of the NAD biosynthesis to increase SIRT1 activity in β-cells will be beneficial for diabetes.

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J.W. designed the research, performed research, analyzed data, and wrote the manuscript. F.Z., M.Y., D.W., Q.Y., Y.Z., and B.Z. performed research. M.W.M. provided the data, and wrote the manuscript. F.Z., M.Y., D.W., Q.Y., Y.Z., and B.Z. performed research. M.W.M. provided the data, and wrote the manuscript.

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