SET7/9 Enzyme Regulates Cytokine-induced Expression of Inducible Nitric-oxide Synthase through Methylation of Lysine 4 at Histone 3 in the Islet β Cell*

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**Background:** SET7/9 regulates expression of NF-κB target genes in non-β cells.

**Results:** SET7/9 knockdown attenuated cytokine-induced expression of inducible nitric-oxide synthase and apoptosis in β cells.

**Conclusion:** SET7/9 regulates Nos2 expression through methylation of H3K4 in β cells.

**Significance:** SET7/9 contributes to proinflammatory cytokine signaling in the pancreatic β cell.

SET7/9 is an enzyme that methylates histone 3 at lysine 4 (H3K4) to maintain euchromatin architecture. Although SET7/9 is enriched in islets and contributes to the transactivation of β cell-specific genes, including Ins1 and Slec2, SET7/9 has also been reported to bind the p65 subunit of nuclear factor κB in non-β cells and modify its transcriptional activity. Given that inflammation is a central component of β cell dysfunction in Type 1 and Type 2 diabetes, the aim of this study was to elucidate the role of SET7/9 in proinflammatory cytokine signaling in β cells. To induce inflammation, βTC3 insulinoma cells were treated with IL-1β, TNF-α, and IFN-γ. Cytokine treatment led to increased expression of inducible nitric-oxide synthase, which was attenuated by the diminution of SET7/9 using RNA interference. Consistent with previous reports, SET7/9 was co-immunoprecipitated with p65 and underwent cytostatic to nuclear translocation in response to cytokines. ChIP analysis demonstrated augmented H3K4 mono- and dimethylation of the proximal Nos2 promoter with cytokine exposure. SET7/9 was found to occupy this same region, whereas SET7/9 knockdown attenuated cytokine-induced histone methylation of the Nos2 gene. To test this relationship further, islets were isolated from SET7/9-deficient and wild-type mice and treated with IL-1β, TNF-α, and IFN-γ. Cytokine-induced Nos2 expression was reduced in the islets from SET7/9 knock-out mice. Together, our findings suggest that SET7/9 contributes to Nos2 transcription and proinflammatory cytokine signaling in the pancreatic β cell through activating histone modifications.

Glucose homeostasis is maintained in response to nutrient intake through finely tuned insulin secretion from the pancreatic β cells that is exquisitely coupled to the demands of insulin-sensitive organs, including muscle, liver, and adipose tissue. Impairments in glucose homeostasis result in the development of hyperglycemia and diabetes mellitus of which there are two main forms. Although the etiologies of Type 1 diabetes (T1D)† and Type 2 diabetes (T2D) are distinct, increasing evidence suggests a primary role for β cell dysfunction arising from inflammation in both disorders. T1D occurs as a result of loss of self-tolerance, activation of innate and adaptive immunity, and immune cell infiltration into islets, all eventually culminating in T cell-mediated destruction of the pancreatic β cell (1, 2). In contrast, T2D arises from impaired peripheral insulin sensitivity and progressive β cell dysfunction. Recently, T2D-associated metabolic dysfunction has been linked to macrophage

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§ The abbreviations used are: T1D, Type 1 diabetes; T2D, Type 2 diabetes; H3K4, histone 3 at lysine 4; iNOS, inducible nitric-oxide synthase; H3, histone 3; TBP, TATA-binding protein; qRT-PCR, quantitative RT-PCR; RIPA, radioimmune precipitation assay.
infiltration of a variety of metabolic tissues, including the pancreatic islets (3–5). Local and systemic elevations of proinflammatory cytokines lead to the activation of deleterious signaling cascades within the β cell, including those regulated by NF-κB, JAK, and STAT1 (6, 7). The Nos2 gene is a key downstream target of NF-κB, leading to translation of inducible nitric-oxide synthase (iNOS) and the catalysis of l-arginine to produce nitric oxide (NO). In both T1D and T2D, NO-supplied reactive oxygen species contribute to mitochondrial dysfunction, impacting cellular energy status, glucose-stimulated insulin secretion, and ultimately β cell survival (8–13). Because the inflammatory response responsible for NO generation could be a potential target to treat diabetes mellitus, an improved understanding of the transcriptional pathways that regulate iNOS production is needed.

Gene transcription is regulated epigenetically through alterations in patterns of DNA methylation and covalent histone modifications that either promote or restrict the accessibility of components of the transcriptional machinery to gene promoters (14, 15). SET7/9 is a SET (Su(var)3–9, Enhancer-of-zeste, Trithorax) domain-containing enzyme that exhibits methyltransferase activity and promotes open chromatin architecture and target gene expression through methylation of histone 3 at lysine 4 (H3K4) (16). In addition to its activity as a histone methyltransferase, SET7/9 is also known to methylate lysine residues of non-histone proteins, including TAF10, pRb, p53, and the estrogen and androgen receptors, where SET7/9-mediated methylation has been shown to regulate target protein stability and/or activity (17–21). Previously, we have shown that SET7/9 is enriched in rodent and human islets and methylates H3K4 in a number of β cell-specific genes, including Ins1 and Slc2a2. Furthermore, mice with attenuated islet SET7/9 expression exhibited glucose intolerance and impaired glucose-stimulated insulin secretion (22, 23). However, several groups have also shown that SET7/9 contributes to inflammation through interactions with components of the NF-κB signaling cascade. In monocytes, SET7/9 forms a complex with NF-κB and is recruited specifically to the CCL2 and TNFA promoters where it methylates H3K4 (24). In mouse embryonic fibroblast cells, SET7/9 has also been shown to methylate Lys-37 of the p65 subunit of NF-κB and up-regulate NF-κB transcriptional activity (25). In contrast, in human osteosarcoma cells, p65 is methylated at lysine residues 314 and 315, leading to its ubiquitination and degradation and subsequent down-regulation of NF-κB activity (26). Therefore, the effects of SET7/9 on NF-κB activity remain controversial. Moreover, at present, the role of SET7/9 in the pathogenesis of islet inflammation has not been explored.

In this report, we investigate the role of SET7/9 in cytokine-induced inflammatory gene expression and β cell apoptosis. Our results show that SET7/9 interacts with NF-κB and is recruited to and enhances cytokine-induced H3K4 methylation of the Nos2 promoter. Diminution of SET7/9 attenuates cytokine-induced iNOS expression as well as apoptosis in a murine insulinoma cell. Furthermore, we show that cytokine-induced Nos2 expression was reduced in islets isolated from SET7/9 knock-out mice compared with wild-type mice. Together, these data suggest a novel role for SET7/9 in the regulation of proinflammatory β cell gene expression.

**Experimental Procedures**

**Antibodies and Materials**—Monoclonal antibodies against SET7/9 were obtained from Epitomics (5131-1) and LifeSpan BioSciences (LS-C138726). Polyclonal antibodies against di-methyl-H3 Lys-4 (07-030), monomethyl-H3 Lys-4 (07-436), and iNOS (06-573) were obtained from Millipore. Polyclonal antibodies against p65 (ab7970) and TATA-binding protein (TBP) (ab63766) were obtained from Abcam. A polyclonal antibody against cleaved caspase-3 (9661) and a monoclonal antibody for p53 (2524) were from Cell Signaling Technology. Anti-FLAG® M2 affinity gel was obtained from Sigma-Aldrich. Mouse TNF-α, mouse IL-1β, and mouse IFN-γ were obtained from PeproTech.

**Cell Culture and Cytokine Treatment**—BTC3 mouse insulinoma cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 15% horse serum, 2.5% fetal bovine serum (FBS), and 1% penicillin/streptomycin. MIN6 mouse insulinoma cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 15% FBS, 10 mM HEPES, and 1% penicillin/streptomycin. BTC3 cells were treated with or without a mixture of cytokines that included 5 ng/ml IL-1β, 10 ng/ml TNF-α, and 100 ng/ml IFN-γ. 

**RNA Interference**—Stealth RNAi™ siRNAs against Setd7/9 (si-Set7/9) or non-targeting sequences (si-scramble) were purchased from Life Technologies and transfected into BTC3 cells and MIN6 cells using Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer’s instructions. Ninety-six hours after transfection, cells were treated with or without a cytokine mixture for the indicated times. siRNA sequences used were as follows: si-Set7/9, 5’-CCUGGACGGAGAGACGUACAUUAU-3’; si-scramble, 5’-UAAAGUACUGCGGUAGGAAA-3’.

**Quantitative RT-PCR (qRT-PCR)**—BTC3 cells (7 × 10^5) were seeded in 6-well plates, transfected with si-Set7/9 or si-scramble, and treated with cytokines 96 h after transfection. Total RNA was isolated from BTC3 cells using the RNeasy® kit (Qiagen) and subjected to cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR mixtures were prepared using Fast SYBR Green Master Mix (Applied Biosystems). Forward and reverse primers used for quantitative RT-PCR are listed in Table 1.

**Immunoblot Assays**—For isolation of total protein, BTC3 cells were washed twice with PBS and then lysed by adding RIPA buffer containing 140 mM NaCl, 20 mM Tris, 1 mM EDTA, 50 mM NaF, 2 mM MgCl₂, 1 mM PMSF, 25 units/ml Benzonase, and 1× Halt™ protease-phosphatase inhibitor mixture. Cytoplasmic and nuclear fractions were prepared as described previously (27). Approximately 20 μg of total protein, 20 μg of the cytosolic fraction, or 10 μg of the nuclear fraction were separated in 12% SDS-polyacrylamide gels, transferred to a polyvinyllidene fluoride membrane, and incubated with antibodies against SET7/9, iNOS, p65, cleaved caspase-3, p53, β-actin, or TBP. Bound primary antibodies were detected with peroxidase-
coupled secondary mouse or rabbit antibodies and IRDye®-conjugated secondary mouse or rabbit antibody. Immunoblots were visualized using the Odyssey (LI-COR Biosciences), LAS-3000 (Fuji Film) with ECL-Plus™ (Amersham Biosciences), or conjugated secondary mouse or rabbit antibody. Immunoblots were generated using trypsin/EDTA, and washed twice with PBS. Apoptosis analysis was performed using an Annexin V-FITC/PI tomycin. Islets were treated with or without a mixture of cytokines that included 10 ng/ml TNF-α, 5 ng/ml IL-1β, and 100 ng/ml IFN-γ. Consistent with previous reports using rodent islets and rat insulinoma cell lines (30, 31), cytokine treatment led to increased expression of NF-κB target genes, including Nos2, Tnfa, and Sod2 (Fig. 1B). Diminution of SET7/9 expression attenuated cytokine-induced Nos2 mRNA expression as well as elevations in iNOS protein levels (Fig. 1, B and C). Interestingly, SET7/9 knockdown had no effect on the NF-κB target genes Tnfa and Sod2. To rule out potential off-target

### Table 1

| Gene   | Forward sequences | Reverse sequences |
|--------|-------------------|-------------------|
| Tdy    | GCTGGACTACATCGAAGTGAAAG     | CACCATGGTTCTGATGCTTGAAGT |
| Setd7  | GCCAGTCCATCCGCTTCCCTCC     | GCCAGTCCCTGGCATCCTTTA   |
| Nos2   | GCCAGGACCAAGCCATCGATT    | GCCAGCCTGGATGCTCATTTGG |
| Tnfa   | GCGGCTGCTCTGCTGCTTGC    | GCGGCTGCTCTGCTGCTTGC   |
| Sod2   | TCTTGCGGCAATGGCTGAGT    | TCTTGCGGCAATGGCTGAGT   |
| Traf6  | AGAGATGCAATCCTGAGGAA    | AGGATGCAATCCTGAGGAA    |
| Cdkn1a | CCTCTGACAGTGGAGTCAAGG | CCTCTGACAGTGGAGTCAAGG |

**Primers for RT-PCR**

- **Tdy**: GCTGGACTACATCGAAGTGAAAG
- **Setd7**: GCCAGTCCATCCGCTTCCCTCC
- **Nos2**: GCCAGGACCAAGCCATCGATT
- **Tnfa**: GCGGCTGCTCTGCTGCTTGC
- **Sod2**: TCTTGCGGCAATGGCTGAGT
- **Traf6**: AGAGATGCAATCCTGAGGAA
- **Cdkn1a**: CCTCTGACAGTGGAGTCAAGG

**Primers for ChIP assay**

- **β-gal**: TTCATTCCGGTCTTTCCCCCAT
- **Setd7**: GCCGAGCAAGGAGGAGGAGG
- **Sod2**: AGGTGCTCAGAACCCTAAGCC
- **Tnfa**: TGGACAGGGCTTCTGCTGCTTGC
- **Cdkn1a**: TCCAGATCAAATCCTGAGGAA

**Co-immunoprecipitation**—A/G Mag Sepharose (GE Healthcare) was preincubated with anti-SET7/9 antibody or mouse IgG, transferred into 500 μl of RIPA buffer containing 500 μg of total protein, incubated at 4 °C for 2 h, and washed three times with RIPA buffer. Approximately 1 × 10⁷ TC3 cells were seeded in a 10-cm dish for 24 h before transfection. Cells were next transfected with 10 μg of an expression plasmid for FLAG-SET7/9 (16) or an empty vector using Effectene (Qiagen) according to the manufacturer’s instructions. Total protein was obtained using RIPA buffer, immunoprecipitated with anti-FLAG M2 affinity gel at 4 °C for 2 h, and washed three times with RIPA buffer. Eluted proteins were subjected to immunoblotting as described previously.

**Chromatin Immunoprecipitation Assays**—Chromatin immunoprecipitation (ChIP) assays were performed as described previously (28). Briefly, ~2 × 10⁷ βTC3 cells were treated with 1% formaldehyde to cross-link protein to DNA. Chromatin was next fragmented by sonication using a Q500 Sonicator (QSonica) and then immunoprecipitated with anti-monomethyl-H3 Lys-4 or anti-dimethyl-H3 Lys-4 antibodies or normal rabbit serum. DNA fragments extracted from immunoprecipitated protein and DNA complexes were subjected to quantitative PCR as described. Forward and reverse primers used for PCR are listed in Table 1.

**Mouse Experiments and Islet Isolation**—Setd7-deficient mice were established and provided by the laboratory of Dr. Danny Reinberg (New York University). Animal studies were performed under protocols approved by the Indiana University School of Medicine Animal Care and Use Committee. Islets were isolated following collagenase digestion of the pancreas as described previously (29) and maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Islets were treated with or without a mixture of cytokines for 4 h and then harvested for mRNA isolation.

**Statistical Analysis**—All data are presented as the means ± S.E. Student’s t tests were used for comparisons involving two conditions. Differences between groups were analyzed for significance using one-way analysis of variance with multiple comparisons and a Tukey-Kramer post-test. Statistical significance was assumed at p < 0.05.

**Results**

**SET7/9 Regulates Cytokine-induced iNOS Expression and Apoptosis in β Cells**—To investigate the role of SET7/9 in inflammatory signaling in the β cell, the gene encoding SET7/9 (Setd7) was first knocked down using RNA interference. Setd7 mRNA levels were reduced by ~75%, whereas protein levels were reduced by 60% in βTC3 cells treated with an siRNA against Setd7 (si-Setd7/9) compared with those treated with an siRNA for a nonspecific target (si-scramble) (Fig. 1, A and B). Next, βTC3 cells were treated with a mixture of proinflammatory cytokines that included 10 ng/ml TNF-α, 5 ng/ml IL-1β, and 100 ng/ml IFN-γ. Consistent with previous reports using rodent islets and rat insulinoma cell lines (30, 31), cytokine treatment led to increased expression of NF-κB target genes, including Nos2, Tnfa, and Sod2 (Fig. 1B). Diminution of SET7/9 expression attenuated cytokine-induced Nos2 mRNA expression as well as elevations in iNOS protein levels (Fig. 1, B and C). Interestingly, SET7/9 knockdown had no effect on the NF-κB target genes Tnfa and Sod2.
**SET7/9 Regulates iNOS Expression via Histone Modification**

*A* SET7/9

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**FIGURE 1.** SET7/9 knockdown attenuates cytokine-induced Nos2 expression in mouse βTC3 cells. A, βTC3 cells were transiently transfected with a non-targeting siRNA (si-scramble) or si-RNA against Setd7 (si-Set7/9) and then harvested for immunoblot analysis. The expression level of SET7/9 was normalized to β-actin expression (n = 4), 96 h after transfection with si-scramble or si-Set7/9, βTC3 cells were stimulated with or without a mixture of cytokines (5 ng/ml IL-1β, 10 ng/ml TNF-α, and 100 ng/ml IFN-γ) for the indicated times. Total RNA was isolated, and expression of NF-κB target genes was determined by qRT-PCR. The expression level of mRNA was normalized to 18S expression. Data represent the average of four independent transfections. B, 96 h after transfection with si-scramble or si-Set7/9, βTC3 cells were stimulated with or without cytokines for 4 h. Total protein was isolated and subjected to immunoblotting for SET7/9, iNOS, p65, p53, and β-actin, and the expression level of each protein was normalized to β-actin protein levels. The expression level of p53 and its downstream targets was unaffected by SET7/9 knockdown (Fig. 3C). Because p53 is a crucial regulator of apoptosis that has been shown to be stabilized by SET7/9-induced lysine methylation (19, 32), we next evaluated the expression of p53 and its downstream targets. Although SET7/9 knockdown decreased cleaved caspase-3 levels, expression of p53 (Fig. 1C) or p53 target genes, including Pten, Fas (gene symbol Fasl), p21 (gene symbol Cdkn1a), and p53 target genes, including Bax, Fas, Tnfrsf6, and Bax, was increased (Fig. 3C).  

**SET7/9 Translocates into the Nucleus in Response to Cytokine Treatment—Yang et al. (26) have shown that SET7/9 methy-lates the p65 subunit of NF-κB, leading to p65 ubiquitination and degradation. To evaluate whether SET7/9 regulates NF-κB protein stability in β cells, total protein from βTC3 cells was isolated before and after cytokine treatment, and immunoblotting for the p65 subunit of NF-κB was performed. SET7/9 knockdown had no effect on total p65 protein levels in βTC3 cells (Fig. 1C), indicating that SET7/9 does not regulate NF-κB protein stability in β cells.**

SET7/9 has also been shown to shuttle between the nucleus and cytosol in response to various stimuli (33, 34). To investigate the effect of cytokines on SET7/9 intracellular distribution, SET7/9 content was evaluated in total, nuclear, and cytosolic fractions isolated from βTC3 cells before and after cytokine treatment. Consistent with a previous study performed in THP1 cells (24), p65 translocated into the nucleus in response to cytokine treatment, and this was unaffected by SET7/9 knockdown (Fig. 4, A and B). In response to cytokine treatment, SET7/9 significantly increased in the nuclear fraction and decreased in the cytosolic fraction, indicating that proinflammatory cytokines induced translocation of SET7/9 into the nucleus (Fig. 4, A and C). SET7/9 is known to form a complex with p65 in non-β cells (24–26). To test this relationship in β cells, immunoprecipitation experiments were performed. Results showed that p65 was co-immunoprecipitated using anti-SET7/9 antiserum in βTC3 cells (Fig. 5A).
Furthermore, p65 was similarly detected in immunoprecipitated fractions by an anti-FLAG antibody in \( \beta TC3 \) cells transfected with a construct encoding FLAG-SET7/9 (Fig. 5B).

**SET7/9 Contributes to the Methylation of Histone 3 at Lysine 4 at the Nos2 Promoter**—Given the observed effects on Nos2 expression, we hypothesized that SET7/9 may regulate chromatin structure in the Nos2 gene promoter. Using ChIP analysis, we evaluated the H3K4 methylation status of three distinct regions of each NF-κB target gene. These regions included the proximal promoter region containing consensus sequences for NF-κB-binding (Fig. 6A), the distal promoter region (−5 kb), and the 3′ region of the transcriptional start site (+1 kb) that lacked the consensus sequences. Treatment of \( \beta TC3 \) cells with cytokines augmented mono- or di-methylation of the proximal promoter and the +1 kb region of Nos2 as well as the proximal promoter region of Tnfa but had no effect on H3K4 monomethylation across the Sod2 gene (Fig. 6B and D). Interestingly, SET7/9 knockdown partially inhibited cytokine-induced H3K4 monomethylation of the Nos2 but not the Tnfa or Sod2 proximal promoters (Fig. 7, A–C), consistent with qRT-PCR results (Fig. 1B). Although we also evaluated the −5 kb region and the +1 kb region of each gene, SET7/9 knockdown did not impair monomethylation of these regions (Fig. 7, A–C).

Histone lysine residues may undergo mono-, di-, or trimethylation. These modifications may be regulated independently (35), and di- and trimethylation of H3K4 are strongly associated with actively transcribed genes (36, 37). To demonstrate further evidence of cytokine-induced changes in the chromatin architecture of the Nos2 promoter, H3K4 dimethylation levels were next evaluated. Cytokine treatment led to increased levels of H3K4 dimethylation of the Nos2 proximal promoter. Moreover, this effect was prevented by SET7/9 knockdown (Fig. 8, A and B).

Next, to assess for the presence of SET7/9 on the Nos2 proximal promoter, \( \beta TC3 \) cells were transfected with an expression vector encoding FLAG-SET7/9 or an empty vector control. ChIP analysis was performed using anti-FLAG antibody. As shown in Fig. 8C, recovery of the proximal promoter of Nos2 was increased in \( \beta TC3 \) cells transfected with SET7/9 compared with cells transfected with the control...
vector, indicating occupancy of SET7/9 on the Nos2 promoter. Together, these results suggest that SET7/9 regulates Nos2 transactivation through histone methylation of the Nos2 gene.

Cytokine-induced Nos2 Expression Was Attenuated in Islets Isolated from SET7/9 Knock-out Mice—Finally, to investigate the role of SET7/9 in Nos2 expression in pancreatic islets, the Setd7 locus was deleted to generate total body SET7/9 knock-out mice. Previously, our group demonstrated that SET7/9 was expressed in liver, muscle, and islets (22). Therefore, we confirmed the absence of SET7/9 in those tissues (Fig. 9A). Islets were isolated from SET7/9 knock-out mice or wild-type controls and then treated with the mixture of cytokines. In response to cytokines, islets isolated from wild-type mice
SET7/9 Regulates iNOS Expression via Histone Modification

FIGURE 4. SET7/9 translocates from the cytosol into the nucleus in response to cytokine treatment. After transfection with si-scramble or si-Set7/9, βTC3 cells were stimulated with or without cytokines for 4 h and subjected to nuclear and cytosolic fractionation. A, panels show a representative immunoblot using antisera for SET7/9, p65, β-actin, and TBP in the cytosolic and nuclear fractions. B and C, cytosolic protein expression was normalized to β-actin levels, and nuclear protein expression was normalized to TBP levels. Relative protein levels are displayed as the means ± S.E. (error bars) (n = 5). * indicates a statistical difference (p < 0.05) compared with βTC3 cells treated without cytokines.

FIGURE 5. SET7/9 forms a complex with p65. A, after stimulation with or without cytokines for 4 h, βTC3 cells were harvested and immunoprecipitated with either rabbit serum or SET7/9 antiserum. Immunoprecipitated proteins were subjected to immunoblotting (IB) using a p65 antibody. B, βTC3 cells were transfected with either an empty vector or a vector encoding FLAG-SET7/9. Cells were harvested and immunoprecipitated (IP) with anti-FLAG antibody. Eluates were analyzed with antisera for p65 or SET7/9. n = at least 3 independent experiments.

exhibited significant augmentation of Nos2 expression, and this was partially attenuated in islets isolated from SET7/9 knock-out mice. In contrast, expression of Tfα and Sod2 was not different in wild-type and SET7/9 knock-out islets, consistent with results in MIN6 cells (Figs. 1B and 9B).

Discussion

SET7/9 is a monomethyltransferase that maintains euchromatin architecture through modification of H3K4 (16). We have previously demonstrated a role for SET7/9 in the regulation of H3K4 methylation at β cell-specific genes, including Ins1 and Slc2a2 (22). Several groups have also recently suggested a specific role for SET7/9 in inflammatory signaling in non-β cells wherein SET7/9 has been shown to physically interact with components of the NF-κB complex, undergo recruitment to specific NF-κB targets, and regulate target gene transcription (24–26). Inflammation is increasingly appreciated as a key contributor to islet β cell death and dysfunction in both T1D and T2D; however, the role of SET7/9 in β cell inflammatory signaling pathways has not been defined. In this study, we tested the hypothesis that SET7/9 modifies targets of NF-κB in the β cell. Our results showed that SET7/9 forms a complex with the p65 subunit of NF-κB and translocates into the nucleus to increase inflammatory gene transcription through H3K4 mono- and dimethylation of the Nos2 promoter. SET7/9 also regulated cytokine-induced β cell apoptosis, whereas cytokine-induced Nos2 expression was attenuated in islets isolated from SET7/9 knock-out mice.

Several groups have shown that SET7/9 also recognizes non-histone protein substrates such that SET7/9 methylation may modulate both protein activity and/or stability. The p65 subunit of NF-κB contains several lysine residues that could serve as potential SET7/9 targets. In this regard, Ea and Baltimore (25) reported previously that SET7/9 methylation of the Lys-37 residue of p65 led to increased NF-κB-dependent gene expression. In contrast, Yang et al. (26) demonstrated that SET7/9-mediated methylation of the Lys-314 and Lys-315 residues of p65 triggers its ubiquitination and degradation, leading to decreased NF-κB function. To test the possibility that SET7/9 regulates inflammatory signaling in the β cell through p65 methylation, we endeavored to detect methylated lysine in immunoprecipitated p65 using an anti-pan-methylated lysine antibody. Using this approach, we could not find evidence that SET7/9 directly methylates p65 protein in βTC3 cells (data not shown). This finding is consistent with studies performed in monocytes demonstrating a primary role for SET7/9-mediated methylation of H3K4 at NF-κB gene targets (24). These discrepancies may certainly arise from differences in cell type or from the use of different provocative stimuli. For example, although HEK293 cells have the necessary machinery to produce COX-2 in response to proinflammatory cytokines, they fail to produce iNOS (38). Therefore, we cannot exclude the possibility that p65 methylation might contribute in part to cytokine-induced Nos2 expression; mass spectrometric approaches could be used to investigate this in future studies.

Whereas the treatment of β cells with cytokines up-regulated the expression of a number of NF-κB target genes, including Nos2, Tfα, and Sod2, deletion of SET7/9 uniquely attenuated...
SET7/9 Regulates iNOS Expression via Histone Modification

A  
proximal Nos2  
-0.5 kb  \[\text{Exon1}\]  +1  
PCR product  

proximal Tnfa  
-0.4 kb  \[\text{Exon1}\]  +1  
PCR product  

proximal Sod2  
-0.3 kb  \[\text{Exon1}\]  +1  
PCR product  

Consensus Sequences for NF-kB binding

B

\begin{tabular}{ccc}
 & Nos2 -5 kb & proximal Nos2 & Nos2 +1 kb \\
Recovery ratio & 0.01 & 0.10 & 0.08 \\
\end{tabular}

\begin{tabular}{ccc}
Cytokine & - & + & + \\
4 hours & & & & \\
\end{tabular}

C

\begin{tabular}{ccc}
 & Tnfa -5 kb & proximal Tnfa & Tnfa +1 kb \\
Recovery ratio & 0.02 & 0.06 & 0.06 \\
\end{tabular}

\begin{tabular}{ccc}
Cytokine & - & + & + \\
2 hours & & & & \\
\end{tabular}

D

\begin{tabular}{ccc}
 & Sod2 -5 kb & proximal Sod2 & Sod2 +1 kb \\
Recovery ratio & 0.01 & 0.04 & 0.04 \\
\end{tabular}

\begin{tabular}{ccc}
Cytokine & - & + & + \\
2 hours & & & & \\
\end{tabular}

E

\begin{tabular}{ccc}
 & Alb & \\
Recovery ratio & 0.02 & 0.04 \\
\end{tabular}

\begin{tabular}{ccc}
Cytokine & - & + + \\
4 hours & & & & \\
\end{tabular}
the expression of Nos2 in islets and cell lines. In agreement with these results, SET7/9 knockdown decreased methylation of H3K4 at the proximal promoter region of Nos2 but not Tnfa or Sod2. Interestingly, the time frame of peak cytokine-induced up-regulation was different between each gene. For example, Nos2 expression peaked about 4 h after cytokine treatment, whereas the maximal change in expression of Tnfa and Sod2 was noted after 2 h after cytokine exposure. These results suggest that mechanisms regulating the expression of cytokine-responsive genes may be different. Indeed, methyltransferases other than SET7/9 may control chromatin architecture in the promoters of Tnfa and Sod2, and this could likewise be tested in future studies.

FIGURE 7. SET7/9 knockdown inhibits cytokine-induced augmentation of H3K4 monomethylation of the Nos2 gene. BTC3 cells were transfected with si-scramble or si-Set7/9 and then treated with cytokines. ChIP assays were performed using an antibody for H3K4 monomethylation or normal rabbit serum. ChIP-enriched samples were analyzed by quantitative PCR using primers that amplified the proximal promoter region and the region −5 and +1 kb from the transcriptional start site of the Nos2, Tnfa, and Sod2 genes. The proximal promoter region of the Alb gene was used as a negative control. Data represent the recovery ratio to input level, and n = at least 6 for each panel. * indicates statistical difference (p < 0.05) compared with BTC3 cells transfected with si-scramble. Results are displayed as the means ± S.E. (error bars).

FIGURE 6. Cytokine treatment augments H3K4 methylation of the Nos2 gene. A, schematic depiction of the proximal regions of the Nos2, Tnfa, and Sod2 genes. The transcriptional start site of each gene is indicated as +1. The arrows indicate regions amplified by quantitative PCR in each ChIP assay. The gray boxes indicate consensus sequences for NF-κB binding. B−E, BTC3 cells were treated with or without cytokines for the indicated times, and ChIP assays were performed using an antibody for H3K4 monomethylation or normal rabbit serum. ChIP-enriched samples were analyzed by quantitative PCR using primers that amplified the proximal promoter region and the region −5 and +1 kb from the transcriptional start site of the Nos2, Tnfa, and Sod2 genes. The proximal promoter region of the Alb gene was used as a negative control. Data represent the recovery ratio to input level, and n = at least 6 for each panel. * indicates statistical difference (p < 0.05) compared with BTC3 cells treated without cytokines. Results are displayed as the means ± S.E. (error bars).
Notwithstanding these limitations, our data show that SET7/9 knockdown attenuates cytokine-induced apoptosis in a β cell line. NO production by iNOS leads to a number of detrimental actions in the β cell, including mitochondrial dysregulation, Fas activation, dysregulated endoplasmic reticulum calcium, and activation of proapoptotic endoplasmic reticulum stress responses (8–10, 39–42). SET7/9 modulation of p53 signaling could contribute to this effect as p53 has been shown to be a protein target of SET7/9 in an osteosarcoma cell line (19, 32). Methylated p53 is stabilized and remains localized in the nucleus, preserving expression of downstream targets. We evaluated the expression of p53 and its target genes in βHC cells, including Cdkn1a, Pten, Tnfrsf6, and Bax. However, no alterations in their expression levels were observed with SET7/9 knockdown, suggesting that p53 methylation does not play a primary role in cytokine-induced apoptosis in our system.

Therefore, we propose a model for the role of SET7/9 in the regulation of Nos2 expression in Fig. 10. As part of this model, SET7/9 binds p65 and is translocated into the nucleus in response to cytokine treatment. SET7/9 is recruited to the Nos2 gene promoter where it methylates H3K4, induces an open chromatin conformation, and facilitates proinflammatory gene transcription. Previous studies have shown that SET7/9 has a variety of different targets, suggesting that SET7/9 could exert control of distinct pathways in the βHC cell depending on available binding partners. Previous studies have shown that SET7/9 forms a complex with PDX1 to induce euchromatin at genes related to glucose-stimulated insulin secretion (43, 44). Whereas SET7/9 maintains β cell function through activation of Ins1 and Slc2a2 via this pathway, SET7/9 also modulates the inflammatory response to cytokine signaling through an interaction with p65 and H3K4 methylation of the Nos2 promoter. Whether SET7/9 should be activated or inhibited may therefore depend on the cellular context and prevailing conditions. A potential weakness of our study is that we were not able to recapitulate effects on apoptosis in SET7/9-deficient islets (data not shown), suggesting that germ line deletion of SET7/9 in a mouse model may not fully recapitulate the effects of acute SET7/9 knockdown. However, additional studies are needed to fully understand the therapeutic potential of targeting SET7/9...
as a means to regulate β cell inflammation in either T1D or T2D. Such studies may be facilitated by recent efforts to identify small molecule inhibitors of SET7/9 (45).

Author Contributions—T. O., C. E.-M., and H. W. designed the study. K. F., D. L. M., and H. I. performed and analyzed the experiments. H. O. contributed to the establishment of the SET7/9 knockout mouse. T. O., C. E.-M., H. W., Y. F., H. O., and R. G. M. analyzed the data. K. F., T. O., C. E.-M., and H. W. wrote the paper. All authors reviewed and approved the final version of the manuscript.

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SET7/9 Regulates iNOS Expression via Histone Modification

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