Supporting Information

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SI Materials and Methods

Animals. Female 10-week-old C57BLK/ksj *db*/*db* mice were obtained from CLEA Japan Inc. (Tokyo, Japan). Animal experiments were reviewed by the Ethics Committee on Animal Experiments of Nippon Medical School and were carried out in accordance with the Guidelines for Animal Experiments of Nippon Medical School and the guidelines of The Law and Notification of the Government.

In Vitro OGT Assay. HA-tagged IKKβ was recovered from the cell extract by immunoprecipitation, and the precipitant was washed twice with the buffer (25 mM Hepes pH 7.2, 150 mM CH_3COOK, 2 mM EDTA, 0.1% Nonidet P-40 and protease inhibitor mixture) and three times with OGT assay buffer (50 mM sodium cacodylate pH 6.0 and 1 mg/ml BSA [BSA; Nacalai Tesque, Kyoto, Japan]). The crude OGT sample was prepared from the extract of mouse brain tissue. Brain nucleosol was obtained and desalted as previously described (1−2). The desalted nucleosol was dissolved with OGT buffer and incubated with the immune complexes at room temperature for 40 min in the presence or absence of 10 mM UDP-GlcNAc (Sigma, St Louis, MO). After the reaction, the complex was washed twice with OGT buffer and subjected to SDS/PAGE.

RT-PCR and Quantitative Real-Time PCR. Total RNA was extracted using a DNA-free kit (Qiagen) and purified using an RNeasy kit (Qiagen). cDNA was prepared using oligo (dT), random primers and superScript III (Invitrogen). Quantitative real-time PCR analysis was performed using a TaqMan Probe Mix (Applied Biosystems) under the following conditions: 20 sec at 95 °C, followed by 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec using an Applied Biosystems StepOne™. The primer and probe sets used were predesigned primer/probe sets for mouse OGT, β-actin are commercially available (OGT, Mm00507317 m1, β-actin, Mm 00607939 s1), with all primers and probes synthesized. Data show mRNA expression levels relative to those of *β-actin*; the former was then normalized to control expression levels for each experiment.

1. Okuyama R, Marshall S (2003) UDP-N-acetylglucosaminyl transferase (OGT) in brain tissue: Temperature sensitivity and subcellular distribution of cytosolic and nuclear enzyme. *J Neurochem* 86:1271–1280.

2. Marshall S, Duong T, Orbus RJ, Rumberger JM, Okuyama R (2003) Measurement of UDP-N-acetylglucosaminyl transferase (OGT) in brain cytosol and characterization of anti-OGT antibodies. *Anal Biochem* 314:169–179.

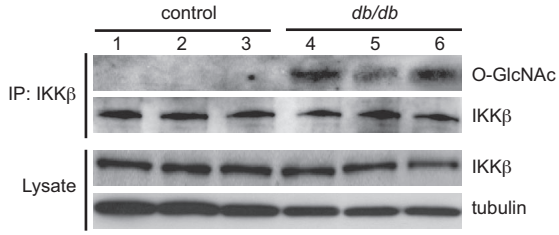

Fig. S1. IKKβ is modified with O-GlcNAc under hyperglycemia. The level of IKKβ O-GlcNAcylation was examined in the liver of diabetic *db/db* mice ($n = 3$; Lanes 4–6) or control C57BL mice ($n = 3$; Lanes 1–3). Tubulin was used as a loading control.

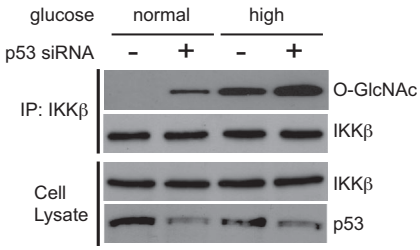

Fig. S2. p53 knockdown in HepG2 cells enhances IKKβ O-GlcNAcylation. HepG2 cells were infected with control- or p53-siRNA-expressing retroviruses. The cells were incubated in normal medium (5.6 mM) or high glucose medium (30 mM) for 3 h. The level of IKKβ O-GlcNAcylation was examined by immunoblot analysis.

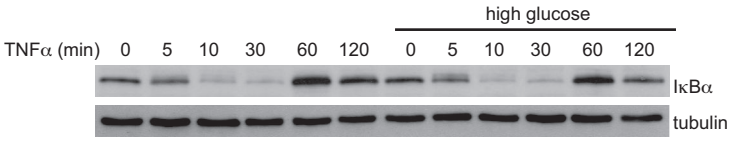

Fig. S3. Re-synthesis of IκBα is remarkably induced at 60 min after stimulation in HepG2 cells. HepG2 cells were incubated in normal medium (5.6 mM) or high glucose medium (30 mM) for 3 h and stimulated with TNFα (100 ng/ml) for the indicated periods. The cell extracts were subjected to immunoblot analysis with an anti-IκBα antibody (*Top*). Tubulin was used as a loading control (*Bottom*).

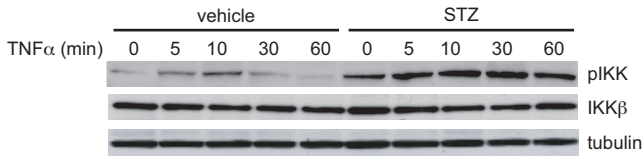

Fig. S4. TNFα-induced activating phosphorylation of IKK is prolonged by STZ. HepG2 cells were pretreated with streptozotocin for 3 h and stimulated with TNFα (100 ng/ml) for the indicated periods. The cell extracts were subjected to immunoblot analysis with an anti-phospho-IKK (pIKK) antibody.

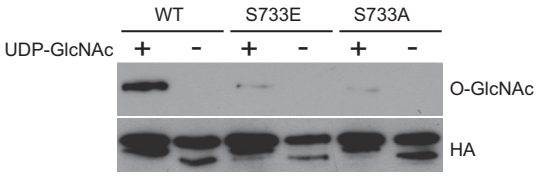

Fig. S5. IKKβ at Ser 733 is O-GlcNAcylated in vitro. NIH 3T3 cells infected with mouse IKKβ siRNA-expressing retrovirus were superinfected with retroviruses encoding HA-tagged human IKKβ WT, S733E, or S733A mutants. The in vitro OGT assay was performed using crude OGT from mouse brain nucleosol, which was reacted with immunoprecipitated HA-IKKβ as a substrate in the presence of UDP-GlcNAc.

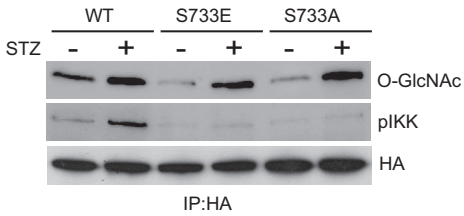

Fig. S6. Activating phosphorylation of IKKβ Ser 733 mutants is not induced by STZ. NIH 3T3 cells infected with mouse IKKβ siRNA-expressing retrovirus were superinfected with retroviruses encoding HA-tagged human IKKβ WT, S733E, or S733A mutants. The cells were treated with STZ (5 mM) for 3 h. The levels of IKKβ O-GlcNAcylation and IKKα/β phosphorylation were examined by immunoblot analysis.

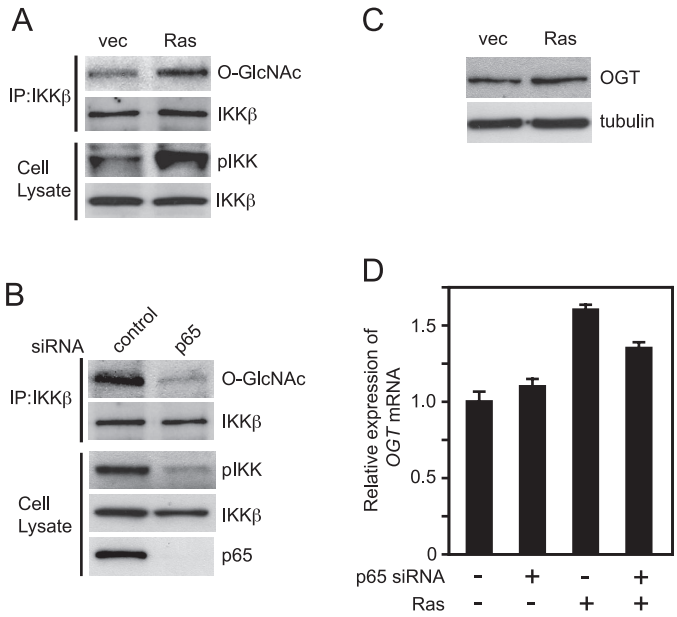

Fig. S7. O-GlcNAcylation of IKKβ in *p53*$^{-/-}$MEFs is enhanced by Ha-RasV12-introduction. (*A*) The level of IKKβ O-GlcNAcylation was examined in *p53*$^{-/-}$MEFs infected with retroviruses encoding activated Ha-RasV12 (Ras) or empty vector (vec). (*B*) The cell extracts were prepared from *p53*$^{-/-}$MEFs infected with Ha-RasV12- and control or p65 siRNA-expressing retroviruses and subjected to immunoprecipitation (IP) with an anti-IKKβ antibody, followed by immunoblot analysis with an anti-O-GlcNAc antibody. The cell extracts were subjected to immunoblot analysis with an anti-phospho-IKK (pIKK) antibody. (*C*) The cell extracts from *p53*$^{-/-}$MEFs infected with control or an Ha-RasV12-expressing retrovirus were subjected to immunoblot analysis with an anti-OGT antibody. (*D*) *p53*$^{-/-}$MEFs were infected with control- or p65-siRNA-expressing retroviruses together with an Ha-RasV12-expressing retrovirus. The expression of *OGT* was examined by quantitative real-time PCR.

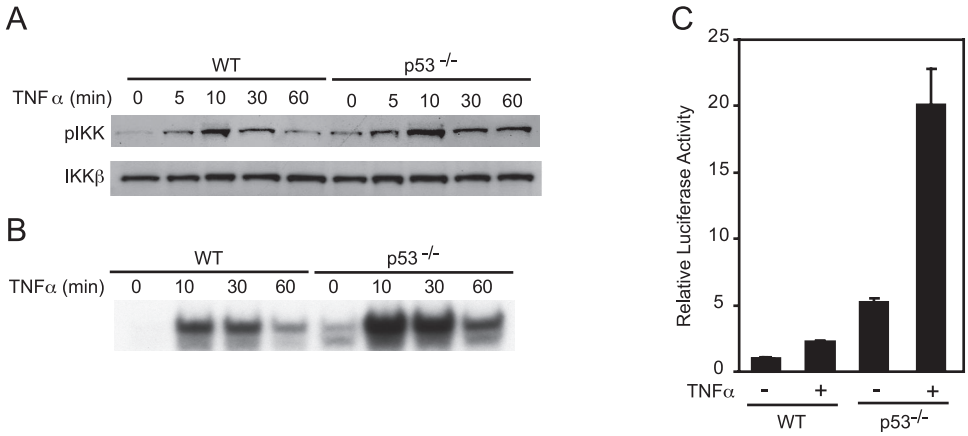

Fig. S8. *p53*$^{-/-}$MEFs exhibit increased NF-κB activity induced by TNFα. (*A* and *B*) Wild-type and *p53*$^{-/-}$MEFs were treated with TNFα (100 ng/ml) for the indicated periods. (*A*) The phosphorylation of IKKα/β was examined by immunoblot analysis. (*B*) Nuclear extracts were subjected to EMSA using a radiolabeled-κB oligonucleotide probe. (*C*) Wild-type and *p53*$^{-/-}$MEFs were transfected with the NF-κB Luc reporter plasmid and phRL-TK. The transfected cells were treated with TNFα for 6 h before harvesting. The luciferase activity is shown normalized to Renilla luciferase activity. Data are means ± SD. from three independent experiments.