Supporting Information

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SI Methods

Lysine Deacetylase Inhibitors. Suberoylanilidehydroxamic acid (vorinostat) was synthesized by Italfarmaco Chemical Development Laboratory as described in WO 93/07148, US92 08454 (1) and WO 95/31977, US95 06554 (2). After synthesis, the structure of vorinostat was confirmed by MS and proton NMR spectroscopy. Diethyl-[6-(4-hydroxycarbamoyl-phenylcarbamoyloxymethyl)-naphthalene-2-methyl] ammonium chloride monohydrate (givinostat; WO 97/43251, US60 34096) (3) was also synthesized by Italfarmaco. Purities were ≥99% as assessed by HPLC.

Mice and Drug Administration Protocol. Nonobese diabetic (NOD) mice were housed under semibarrier conditions in a temperature- and humidity-controlled room with 14-h light/10-h dark cycle. All animal experiments were approved by the Institutional Animal Care and Research Committee of the Katholieke Universiteit Leuven.

Lysine deacetylase inhibitors (KDACi; vorinostat or givinostat) were solubilized in 1% 2-hydroxypropyl-β-cyclodextrin (TrappsolHPB; Cyclodextrin Technologies Development) in sterile drinking water, heated to boiling for complete dissolution, and rapidly cooled on ice to room temperature. NOD littermates (female) were randomly divided into three different treatment groups (between 11 and 17 mice per group). Treatment was given continuously each week from weaning until 120 (vorinostat) or 100 (givinostat) d of age. Water bottles were changed twice a week.

Givinostat Determination in Blood Samples. Givinostat (1 mg/kg, via drinking water) was given from weaning until 35 d of age to determine givinostat and its metabolites in blood plasma and to assess the histone H3 acetylation status within various tissues. Determination of givinostat (ITF2357) and its metabolites, ITF2374 and ITF2375, in plasma was carried out using a validated HPLC method. Samples were spiked with an internal standard and extracted with diethyl ether. The organic phase was separated and evaporated to give a residue that was redissolved with the mobile phase. Aliquots were injected into the chromatographic system equipped with a UV detector set at a wavelength of 220 nm.

Syngeneic Islet Transplantation. Daily treatment with vorinostat or givinostat (50 or 1 mg/kg, respectively; via drinking water) was initiated 3–7 d after diabetes onset, and mice were transplanted with 500 syngeneic insulitis-free NOD islets (4). Diabetes recurrence was defined as return to hyperglycemia after an initial period of normoglycemia.

Histone H3 Acetylation Determination. Antibodies recognizing acetylated histone H3 at Lys9 and Lys14 (AcH3) (Bio-connect), and GAPDH (Applied Biosystems) were used for Western blots of whole protein extract as described. Differences in band density were quantified using ImageJ freeware software.

Diabetes Assessment and Tissue Preparation. Body weight was recorded weekly, and diabetes was assessed twice weekly. NOD mice were screened for diabetes onset by evaluating glucose levels in urine (Clinistix; Bayer Diagnostics) and in venous blood (Glucocard Memory 2; Menarini). Mice were considered diabetic when having positive glucosuria and two consecutive blood glucose measurements more than 200 mg/dL, and they were killed by cervical dislocation under CO_2 anesthesia. All remaining mice that did not become diabetic were killed when they reached 250 d of age and exsanguinated by heart puncture. The pancreatic tissue, pancreatic lymph nodes, and spleen were removed under aseptic conditions. For each mouse, the pancreas was snap-frozen in 2-methyl-butane 99+% (ACROS Organics) chilled in liquid nitrogen, and stored at −80 °C. Pancreatic lymph nodes and spleens were either immediately snap-frozen for RNA isolation or placed in RPMI medium 1640 (Invitrogen) supplemented with Glutamax-I, 25 mM Hepes (Cambrex Bio Science), antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL), and 10% (vol/vol) FCS. The pancreatic lymph node and spleen cells were isolated by perfusing the tissue with medium and subsequent shredding. The remaining blood was collected, and the plasma was stored at −20 °C. In a separate experiment, mice (n = 8 per group) were killed at discontinuation of therapy.

Pancreatic Histopathology and Insulin Content Determination. Serial sections were prepared at a thickness of 8 μm using a Microm HM500 cryostat, stained with standard Mayers' H&E, and scored for immune cell infiltration by light microscopy. Insulitis was scored in a blinded fashion for each islet as follows: 0, no insulitis; 1, peri-insulitis; 2, islets with lymphocyte infiltration in less than 50% of the area; 3, islets with lymphocyte infiltration in more than 50% of the area; 4, islets completely destroyed. The mean insulitis score was calculated by dividing the sum of the insulitis scores by the total number of islets (n = 22–87) examined for each mouse. For the calculation of insulitis, three to four sections were used from each pancreas, cut at least 100 μm apart. The remaining half of each pancreas was used for insulin content determination as described previously (5).

Real-Time PCR of Spleen and Pancreatic Lymph Nodes. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol and stored at −80 °C. One microgram of RNA was subjected to reverse transcription using 100 U SuperScript II RT (Invitrogen) at 42 °C for 80 min, in the presence of 5 μM oligodT16. Real-time PCR was performed using MyiQ–Cycler (Bio-Rad). Primers and probes for *IL-2*, *IL-4*, *IL-6*, *IL-10*, *TNF-α*, *TGF-β1*, and *β-actin* as control gene were as described (6, 7). In addition, mRNAs encoding *GATA-3* and *FoxP3* were measured after RNA purification and real-time PCR. mRNA levels were expressed as fold change compared with untreated controls (ratio between the gene of interest and control gene *β-actin*). All samples were run in duplicate.

Flow Cytometry of Spleen and Pancreatic Lymph Nodes. The cell surface phenotype of splenocytes and pancreatic lymph node cells from different treatment groups was analyzed by flow cytometry as described. All cells were incubated in cold PBS supplemented with 2% (vol/vol) FCS. Cells were incubated in 200 μL for 30 min at 4 °C and stained with directly conjugated mAbs against Thy1.2, CD4, CD8, CD25, CD11b, CD11c, L-selectin (CD62L), CD69, B220, and matching isotype controls (eBioscience). Intracellular Foxp3 staining or matching isotype controls was performed according to the manufacturer's instructions. Using a dual- or triple-laser flow cytometer [FACSort; Becton-Dickinson (BD) or Gallios; Beckman Coulter, respectively], 10,000 events were acquired. Cells present in the leukocyte gate as defined by light scatter were analyzed. Results are expressed as percent positive cells.

Cytokine Determination of Spleen Dendritic Cells In Vivo. $CD11c^+$ cells from splenocytes (pooled from two organs per group) were positively enriched by anti–CD11c-labeled MACS microbeads (MiltenyiBiotec) according to the manufacturer's instructions. $CD11c^+$ cells (confirmed by FACS) were cultured (2.5×10^5 cells per well in 200 μL) in triplicate in 96-well plates for

24 h in RPMI medium 1640 with GlutaMAX-I, supplemented with 5% (vol/vol) FCS. Spontaneous release of cytokines was measured in supernatants with a specific ELISA for IL-6, IL-10, IL-12p70, and TNF-α (R&D Systems).

Suppressor Assay in Spleen In Vivo. Spleens (pooled from two to four mice per group) were processed to single-cell suspensions and separated into effector T cells ($CD4^+CD25^-$) and regulatory T cells ($CD4^+CD25^+$) using magnetic beads (MiltenyiBiotec). Carboxy-fluorescein diacetatesuccinimidyl ester (CFSE; Invitrogen)–labeled effector T cells isolated from 14-wk-old NOD mice were incubated in 96-well round-bottom plates (Nunc) at 5×10^4 cells $well^{-1}$ with 1×10^5 irradiated autologous splenocytes (as feeder cells) in the presence of varying amounts of $CD4^+CD25^+$ regulatory T cells (Tregs) from different groups. Cell cultures were stimulated with 0.25 μg/mL anti-CD3 (clone 145–2C11). After 5 d, cells were harvested, incubated for 30 min at 4 °C with 7-amino actinomycin D (7-AAD) and fluorophore-conjugated Abs specific for CD4. Viable cells were gated based on negative 7-AAD staining and carboxy-fluorescein diacetatesuccinimidyl ester (CFSE) signal of gated $CD4^+$ T cells was analyzed by flow cytometry.

Mixed Lymphocyte Culture and Cytokine Determination in Spleen In Vivo. Purified $CD4^+CD25^-$ T cells isolated from spleen, using magnetic beads (MiltenyiBiotec), were labeled by CFSE according to the manufacturer's instructions before culture in an allogeneic mixed lymphocyte culture (MLC). The MLC was set up in triplicate in 96-well flat-bottomed plates by culturing the CFSE-labeled (NOD) effector T cells (5×10^5 cells per well) with irradiated (C57BL/6) stimulator cells (5×10^5 cells per well) at a 1:1 volume ratio or culturing the CFSE-labeled effector T cells (5×10^5 cells per well) with medium alone (unstimulated condition). Spontaneous release of cytokines was measured in supernatants with a specific ELISA for IL-2, IL-6, and IFN-γ (R&D Systems).

Islet Cell Culture and Treatments In Vitro. The insulin producing cell line INS-1 was a kind gift from C. Wollheim (Department of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland) (8) and was maintained as previously described (9). Primary rat islets were isolated and cultured in RPMI medium 1640 containing 11 mM glucose as previously described (9). After counting and before the experiment, islets were left for at least 2 h to reduce handling stress.

Human islets were obtained from Lorenzo Piemonti, Diabetes Research Institute, San Raffaele Scientific Institute, Milan and Reid Alexander Aikin, Department of Surgery, McGill University, Montreal and cultured in RPMI 1640 containing 5.6 mM glucose, penicillin, streptomycin, and 10% (vol/vol) FCS 5–6 d before experimentation. Experiments were performed in RPMI 1640 containing 5.6 mM glucose, penicillin, streptomycin, and 2% human serum. After counting and before the experiment, islets were left for at least 2 h to reduce handling stress. For cell death (and additional *iNOS* mRNA levels) islets were preexposed for 1 h to givinostat (500 nM) and vorinostat (1 μM) before a 6-d (8 h for additional *iNOS* mRNA) exposure to 150 pg/mL IL-1β + 10 ng/mL hIFN-γ + 50 ng/mL hTNF-α. For *iNOS* mRNA vorinostat-only experiments, islets were preexposed to vorinostat (10 μM) for 1 h before exposure to 2 ng/mL IL-1β + 100 ng/mL hIFN-γ + 100 ng/mL hTNF-α for 6 h.

Cell death. The percentage cell death was performed as previously described (9).

Real-time RT-PCR and Western blot. mRNA expression was determined from total RNA at indicated time points as previously described (9). Individual probe set IDs (all from Applied Biosystems) are given as follows: *Tnf-α*: Rn99999017_m1, *Il-1α*: Rn00566700_m1, *Il-1β*: Rn99999009_m1 *Cxcl2*: Rn00586403_m1, *Cxcl10*: Rn00594648_m1, *Fas*: Rn00685720_m1, *Il-6*: Rn01410330_m1, *Socs1:* Rn00595838_s1, *Socs3:* Rn00585674_s1, *Sirt1*: Mm00490762_m1, *inos*: Rn00561646_m1 and *hprt*: Rn01428093_m1. Human *iNOS* (*NOS2*): Hs00167248_m1 and human *HPRT*: Hs01003267_m1. Each cDNA sample in triplicate was subjected to two individual amplifications either targeting the gene of interest (GOI) or the reference gene *HPRT1*.

Immunoblot analysis was performed according to standard protocols. Chemiluminescence using LumiGLO (Cell Signaling) was suitable for detection of phosphorylated JNK, whereas the fluorescence based Q-Dot technology (Invitrogen) was suitable for detection of p65/Rel A, phosphorylated ERK, and phosphorylated p38. In both cases, light emission was captured digitally with the Flourchem Q BioImager System (Kem-En-Tac). Q-Dot 605 and 705 were detected using 605 ± 30- and 705 ± 30-nm bandwidth filters (Kem-En-Tec).

Dual luciferase assay. INS-1 cells were transiently transfected using Dharmafect 1 (Thermoscientific Dharmacon) according to manufacturer's instructions with 1.2 μg total DNA per reaction: 1.1 μg NF-κB reporter plasmid (PathDetect NF-κB *cis*-reporting system; Stratagene) and 0.1 μg Renilla reniformis luciferase reporter gene/Cytomegalovirus (hRluc/CMV) vector (Promega). Following transfection, cells were incubated in complete medium with or without givinostat (125 nM) for 1 h before adding cytokine mixture (150 pg/mL IL-1β + 5 ng/mL IFN-γ) for 6 h. Luciferase signals were measured according to manufacturer's instructions with the Dual Luciferase Reporter Assay System (Promega). NF-κB reporter signal was normalized to hRluc/CMV signal for each individual well.

Separation of nuclear and cytosolic protein. INS-1 cells were pretreated 1 h with givinostat (125 nM) before cytokine (150 pg/mL IL-1β + 5 ng/mL IFN-γ) exposure for the indicated time periods before lysing cells. Lysates were centrifuged at 2,500 × *g* for 5 min at 4 °C, and the supernatant was used for assessment of cytosolic protein. The pellet was resuspended in lysis buffer with 400 mM NaCl and incubated 30 min on ice before centrifuging at 20,000 × *g* for 30 min at 4 °C. The supernatant was used for assessment of nuclear protein. Samples were analyzed by SDS/PAGE and immunoblotting against p65 as described (9).

Rat islet microarray. Total RNA, isolated with the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions from ∼1,400 rat islets exposed to cytokines (150 pg/mL IL-1β + 2 ng/mL IFN-γ) for 1, 6, and 24 h with or without preculture for 1 h with vorinostat (1 μM) was spotted onto an AffymetrixGeneChip Rat Genome 230 2.0 Array. Visual and outlier control, normalization, and gene expressions were calculated by the robust multi-array average (RMA) algorithm. Network analyses were done with the Ingenuity Pathway Analysis program. Before Network analyses, data were subjected to selection criteria dictating a fourfold up- or down-regulation vs. control in response to exposure to cytokines and a subsequent twofold reversion when exposing to cytokines in the presence of vorinostat.

ChIP assay.

Preparation of chromatin. One percent formaldehyde (JT Baker) in PBS (without Ca^{2+} and Mg^{2+}) was added to cells for 10 min at room temperature before adding 0.125 M (final concentration) freshly prepared glycine (Merck) for 10 min at room temperature, followed by rinsing twice with PBS, after which lysis buffer [0.1% SDS (VWR), 1% Triton X-100 (Sigma), 0.15 M NaCl (Sigma), 1 mM EDTA (Sigma), 20 mM Tris (Sigma), pH 8, and protease inhibitor cocktail (Roche)] was added. Cells were lifted and transferred to Eppendorf tubes. Samples were sonicated at high intensity for a total of 10 min in pulses of 30 s (Diagenode Bioruptor). The presence of specific DNA coprecipitated with p65 was determined by real-time PCR directed against a proximal and distal p65-binding site in the iNOS promoter: proximal forward primer, *GTCCATCGCGAATGAGCTA*, reverse primer, *TATACCCATCCACGCTCTGC*; distal forward primer, *GCTGAGCTGAATTTGGGAAC*, reverse primer, *GGGGAAAAGGA-*

ACAAACAGA. Background was assessed by the average signal from two no gene controls: β-globin forward primer, *TTTCCCCACAGGCCATTAAC*, reverse primer, *TCAGTCGCTCCTCACATTTGC* and 1q32A forward primer, *TTAAGCACATAGCCAAAACATCTATGA*, reverse primer, TTGTTCTTAATTCAGGCAAATACTTCA.

Quantification. Accumulated area under the curve (AAUC) was calculated for the two binding sites and background. AAUCs for proximal and distal binding from four independent experiments were subtracted background signal.

NF-κB nuclear translocation assay. INS-1 cells were seeded in eight-chamber cover slides 2 d before transfection using the Superfect reagent (Qiagen) according to the manufacturer's instructions. Cells were incubated for 4 h with transfection reagent before changing medium and incubating cells overnight at standard cell culture conditions before treating cells with IL-1β (160 pg/mL) for 30 min. Cells were transfected with three p65-GFP constructs: p65 WT, p65 K221R, and p65 KR. All three constructs have been described previously (10). Nuclear localization was assessed by evaluating the percentage of GFP signal present in the nucleus.

p65 acetylation cross-immunoprecipitation. INS-1 cells were exposed to givinostat (250 nM) for 1 h before cytokine exposure (600 pg/mL IL-1β + 5 ng/mL IFN-γ) for 90 min. Cells were lysed with immunoprecipitation (IP) buffer (50 mM Tris·HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) by a 30-min incubation at 4 °C with agitation. Protein concentration was measured by Bradford assay, and equal amounts of protein (400 μg) were subjected to IP against acetylated lysines (1:100 #05–515; Millipore) and p65 (5 μg #sc372-X; Santa Cruz Biotechnology). Protein A Sepharose beads were prepared by washing three times in IP buffer and incubated with 0.1% BSA at 4 °C for 2 h with rotation. Prepared beads were gently added to a mixture of protein and antibody and incubated overnight at 4 °C with rotation. Beads were washed three times with IP buffer before adding 2× SDS/PAGE loading solution and heating at 95 °C for 5 min. Samples were subjected to SDS/PAGE and immunoblotting as previously described. Immunoblotting against acetylated lysines was done with a 1:100 antibody solution.

Peritoneal Macrophage Cultures and Treatments In Vitro. Macrophages were isolated by peritoneal lavage from female 8- to 10-wk-old diabetes-prone NOD (5). Macrophages from each group were preincubated for 1 h with KDACi (givinostat, 100 nm; or vorinostat, 1 μM; in medium with 0.01% DMSO) and stimulated for different time periods with LPS (100 μg/mL; Sigma).

Immunostaining and Cell Viability Assay. After incubation with KDACi for 1 h and LPS stimulation for either 30 min (p65 staining) or 6 h (AcH3 staining), macrophages were fixed with 4% (vol/vol) formalin, and slides were washed with Tris-buffered saline solution containing 0.1% Triton-X-100 and incubated with the following primary antibodies: a rat anti-mouse monoclonal IgG2b against F4/80 antigen (Abcam), a rabbit polyclonal IgG against the p65/RelA component of the NF-κB complex (Santa Cruz Biotechnologies), and a rabbit polyclonal IgG against AcH3 (Bio-connect). After washing, cells were incubated with the appropriate AlexaFluor-conjugated IgG (Invitrogen, Molecular Probes), used at a 1:500 dilution, for 60 min in the dark. After aspiration of secondary antibodies, coverslips were mounted on slides with ProLong Gold mounting medium with DAPI (Invitrogen, Molecular Probes) and then visualized on a Radiance 2001 confocal laser-scanning microscope (Bio-Rad), coupled to an inverted Olympus microscope, and images were acquired on a Laser Sharp 2000 (Bio-Rad) and processed with ImageJ freeware software. Macrophage cell viability was assessed after 6 h of culture using Hoechst (HO) 33342 and propidium iodide (PI) (Invitrogen, Molecular Probes). Viability was evaluated by at least two independent observers, one of whom was unaware of sample identity.

mRNA Analysis. After incubation with KDACi for 1 h and LPS stimulation for 6 and 24 h, 1×10^6 cells were harvested and used immediately for RNA extraction. The remaining cells (without KDACi preexposure) were bathed in warm RPMI 1640 containing 0.01% DMSO and 10% (vol/vol) FCS (37 °C) and served as controls.

Total RNA was extracted using the High pure RNA Isolation Kit (Roche) according to the manufacturer's instructions and stored at −80 °C. One microgram of RNA was reverse transcribed using 100 U SuperScript II RT (Invitrogen) at 42 °C for 80 min, in the presence of 5 μM oligodT16. Real-time PCR was performed using MyiQ-Cycler (Bio-Rad) as described. Primers and probes for *Il1*, *Il6*, *Cox2*, *Ccl2*, *Tnfα*, and *Gapdh* as a control gene were as described previously (6, 7).

Statistics. Values are expressed as means ± SEM. One-way ANOVA analysis followed by Tukey's or Student *t* test was used to calculate significance levels between groups. Dunnett's and Bonferroni's correction were used to correct *P* values for multiple comparisons against a single group or against multiple groups, respectively. For analysis of insulitis and diabetes incidence data, Kaplan–Meier survival curves, the log-rank test, and the χ^2 test were used. Statistical analysis was performed with NCSS 2000 software.

1. Breslow R, Marks PA, Rifkind RA, Jursico B (1993) World Intellectual Property Organization publication WO 93/07148 (April 15, 1993) and US patent publication US92 08454 (October 5, 1992).
2. Breslow R, Marks PA, Rifkind RA (1995) World Intellectual Property Organization publication WO 95/31977 (November 30, 1995) and US patent publication US95 06554 (May 19, 1995).
3. Breslow R, Marks PA, Rifkind RA (1997) World Intellectual Property Organization publication WO 97/43251 (November 20, 1997) and US patent publication US60 34096 (March 7, 2000).
4. Gysemans C, et al. (2002) Treatment of autoimmune diabetes recurrence in non-obese diabetic mice by mouse interferon-beta in combination with an analogue of 1alpha,25-dihydroxyvitamin-D3. *Clin Exp Immunol* 128(2):213–220.
5. Gysemans C, et al. (2008) Unaltered diabetes presentation in NOD mice lacking the vitamin D receptor. *Diabetes* 57(1):269–275.
6. Overbergh L, Gysemans C, Mathieu C (2006) Quantification of chemokines by real-time reverse transcriptase PCR: Applications in type 1 diabetes. *Expert Rev Mol Diagn* 6(1):51–64.
7. Overbergh L, et al. (2003) The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech* 14(1):33–43.
8. Asfari M, et al. (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130(1):167–178.
9. Lundh M, et al. (2010) Lysine deacetylases are produced in pancreatic beta cells and are differentially regulated by proinflammatory cytokines. *Diabetologia* 53(12): 2569–2578.
10. Chen LF, Mu Y, Greene WC (2002) Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB. *EMBO J* 21(23):6539–6548.

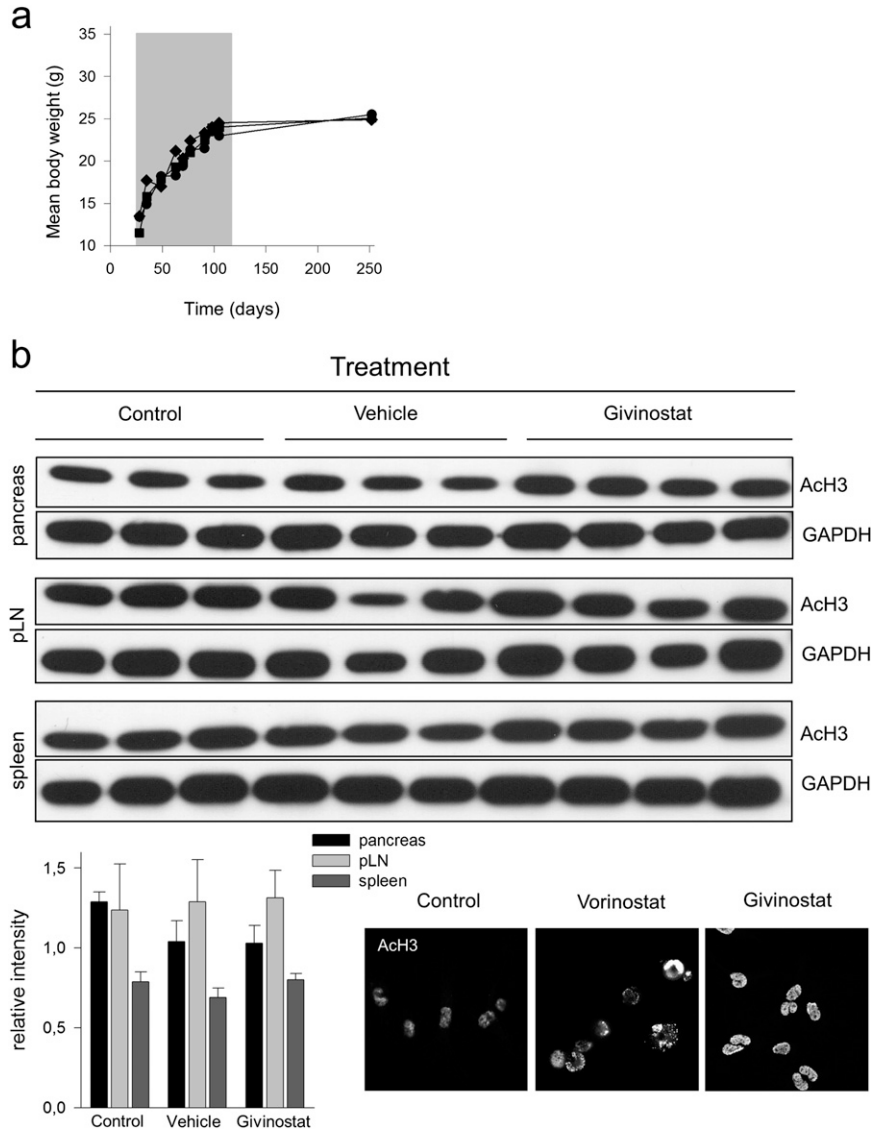


Fig. S1. (*A*) KDACi does not cause significant changes in mean body weight between groups in diabetes-prone NOD mice. Body weight (g) was monitored during (shaded area) and after treatment and is shown here for untreated mice (n = 11; ●), mice treated with vehicle (n = 14; ■), and mice treated with vorinostat (50 mg/kg, via drinking water; n = 17; ◆). All mice were monitored until more than 250 d of age. (*B*) Histone acetylation status within pancreas, pancreatic lymph nodes (pLN), and spleen of diabetes-prone NOD mice was not affected by givinostat. Givinostat (1 mg/kg, via drinking water) was given from weaning until 35 d of age to assess the histone acetylation status within various tissues. Total protein levels of acetylated histone H3 were detected by Western blots with the anti-acetyl histone H3 (AcH3) antibody (17 kDa; *Upper*). GAPDH (*Lower*) was used as loading control. Differences in band intensity were quantified using ImageJ freeware software (*Lower Left*). Data are mean + SEM of three to four samples per group. (*Lower Right*) Peritoneal macrophages from diabetes-prone NOD mice were cultured on chamber slides, adhered for 2 h at 37 °C, and left untreated [incubated with medium alone; control or pretreated with vorinostat (1 μM) or givinostat (100 nM)] for 1 h. After an additional 6 h of culture, the cells (F4/80, red) were fixed, and local acetylation of histone H3 was determined by confocal immunofluorescence microscopy. Representative images of AcH3 intensity in peritoneal macrophages from untreated and KDACi-treated cultures (three independent experiments).

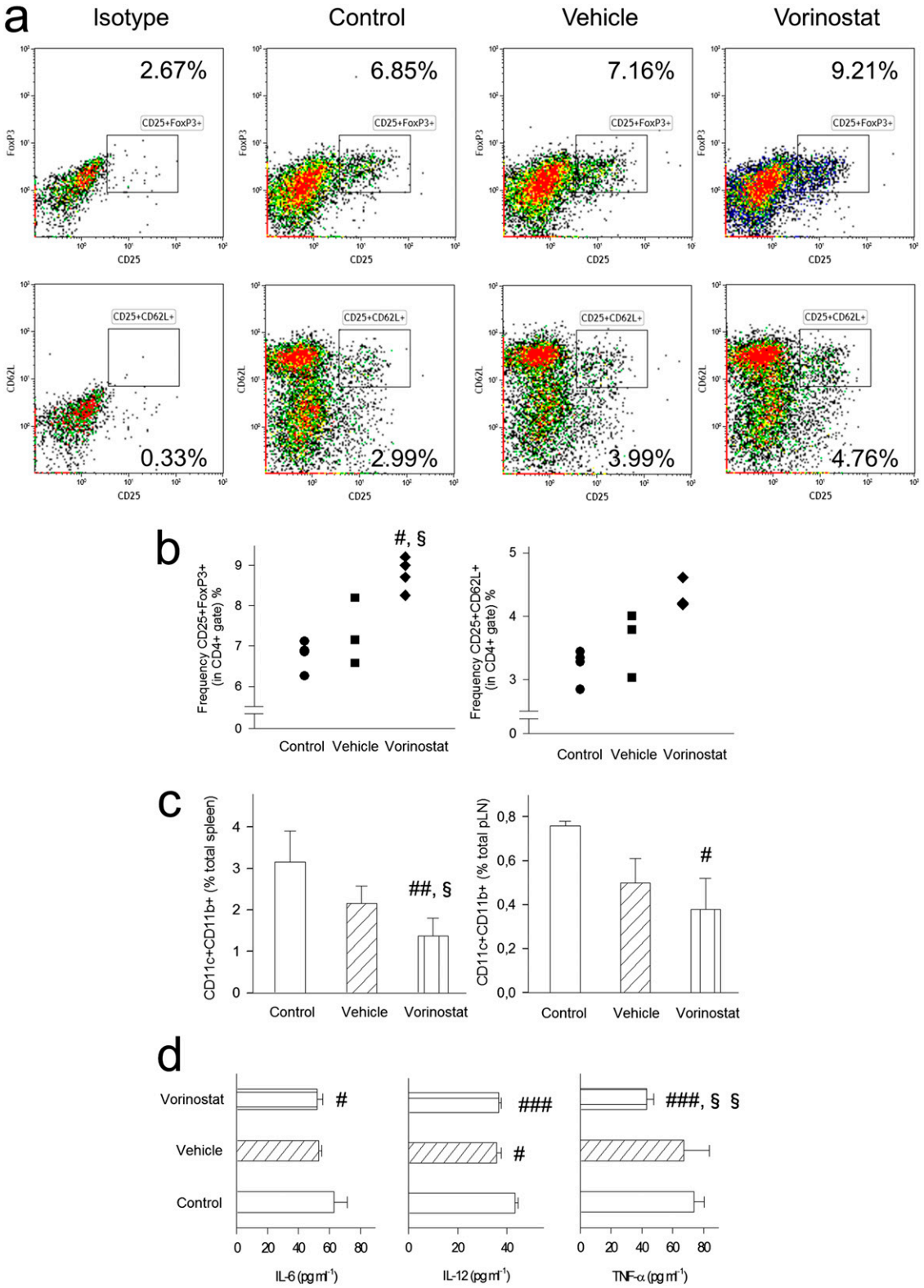


Fig. S2. KDACi treatment increases expression of Treg markers in spleen of NOD mice and moderately decreases markers of DCs and their cytokines. (*A* and *B*) For FACS analysis, lymphocytes from spleen of untreated (●), vehicle-treated (■), or vorinostat-treated (◆) mice (at ~120 d of age) were stained to identify Treg subsets with antibodies to CD4, CD25, CD62L, FoxP3, and isotype controls for mouse IgG. Intracellular Foxp3 staining was performed according to the manufacturer's instructions. The frequency of each population is indicated, and data are representative of three to four mice per group. (*C*) Frequency of $CD11c^{+}CD11b^{+}$ DCs in spleen and pancreatic draining lymph nodes (pLN) from vorinostat-, vehicle-, and untreated (Control) NOD mice. The mean frequency + SEM of each population is indicated (three to eight mice per group). (*D*) Cytokine production from >80% pure $CD11c^{+}$ DCs isolated from spleen of vorinostat-, vehicle-, and untreated NOD mice (at 120 d of age) and cultured for 24 h. Mean cytokine concentration + SEM from three independent experiments (with two to four mice per group). [#]vs. control; [§]vs. vehicle. One symbol, $P < 0.05$; two symbols, $P < 0.01$; three symbols, $P < 0.001$.

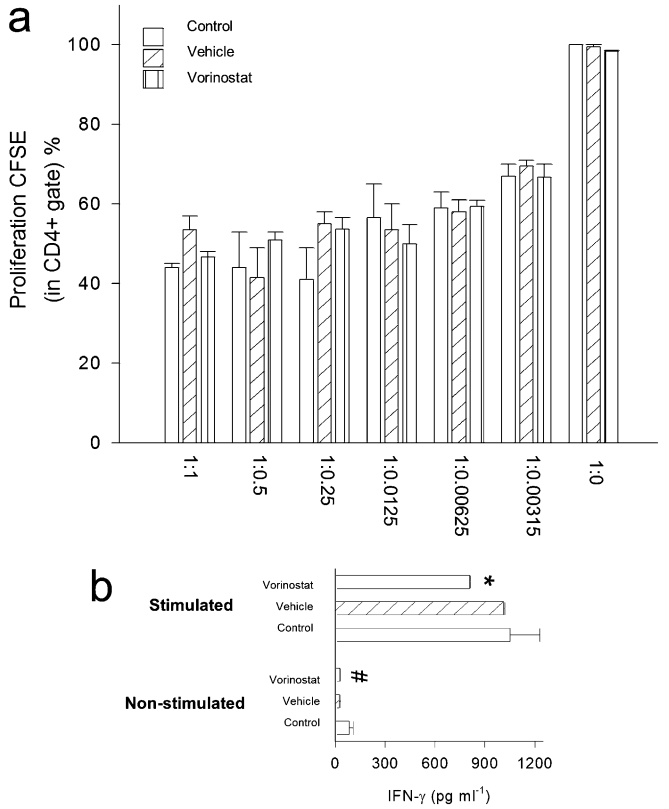


Fig. S3. KDACi increases percentages of functional $CD4^+$ Tregs and decreases IFN-γ production. (*A*) In vitro Treg suppressor assay. Splenic $CD4^+CD25^+$ Tregs from vorinostat-, vehicle-, and untreated NOD mice were cultured at indicated dilutions with $CD4^+CD25^-$ T effector cells isolated from spleen of prediabetic NOD mice in anti-CD3 and irradiated antigen-presenting cell cultures. Proliferation is shown as percentage of dividing cells (measured by CFSE labeling). Means + SEM from three independent experiments (with two to four mice per group). (*B*) $CD4^+CD25^-$ T cells isolated from spleen of untreated, vehicle-treated, and vorinostat-treated NOD mice were stimulated with irradiated splenocytes isolated from C57BL/6 mice and analyzed for cytokine production after 4 d of culture. The responder-to-stimulator ratio was 1:1. Data are mean + SEM from three independent experiments (with two to four mice per group). #vs. nonstimulated control; *vs. stimulated control. $P \leq 0.05$.

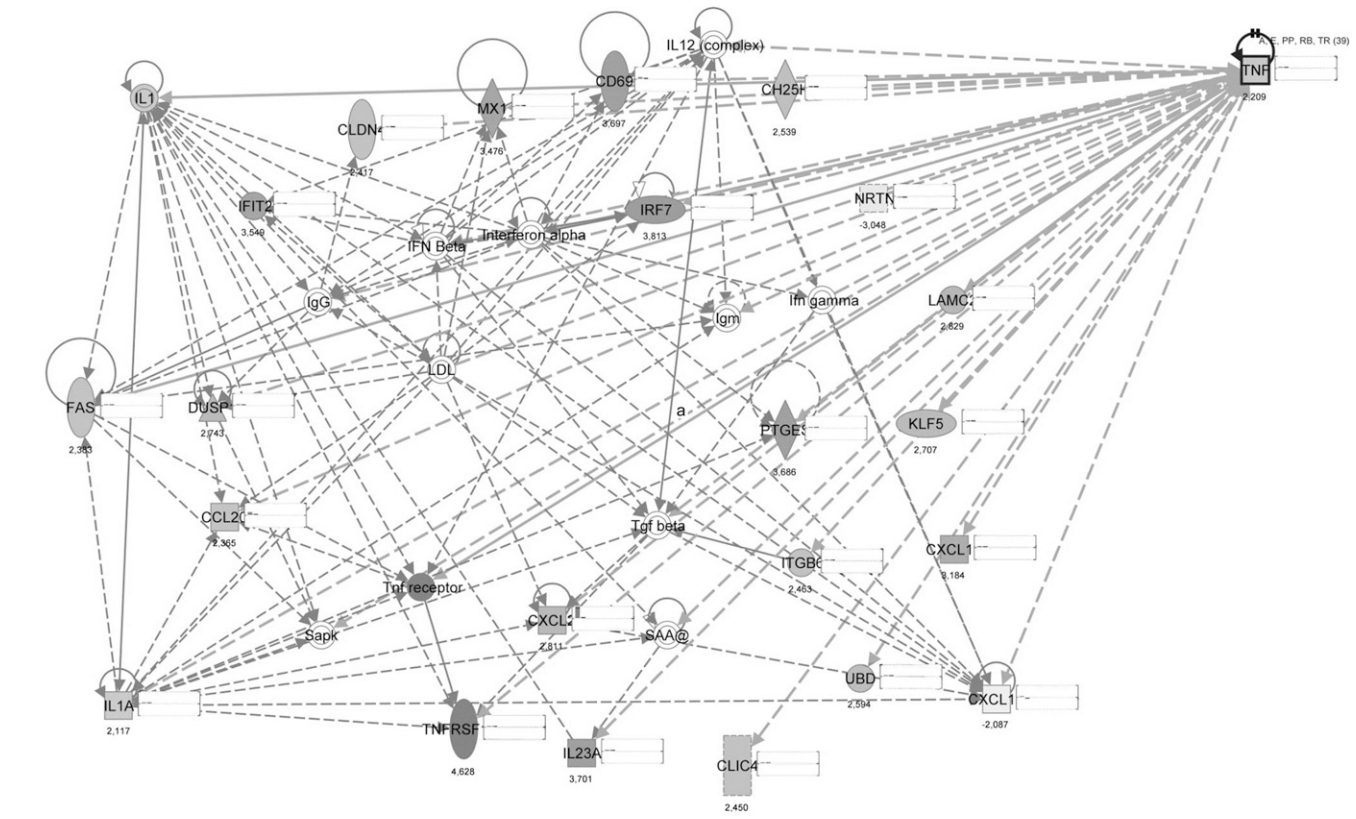
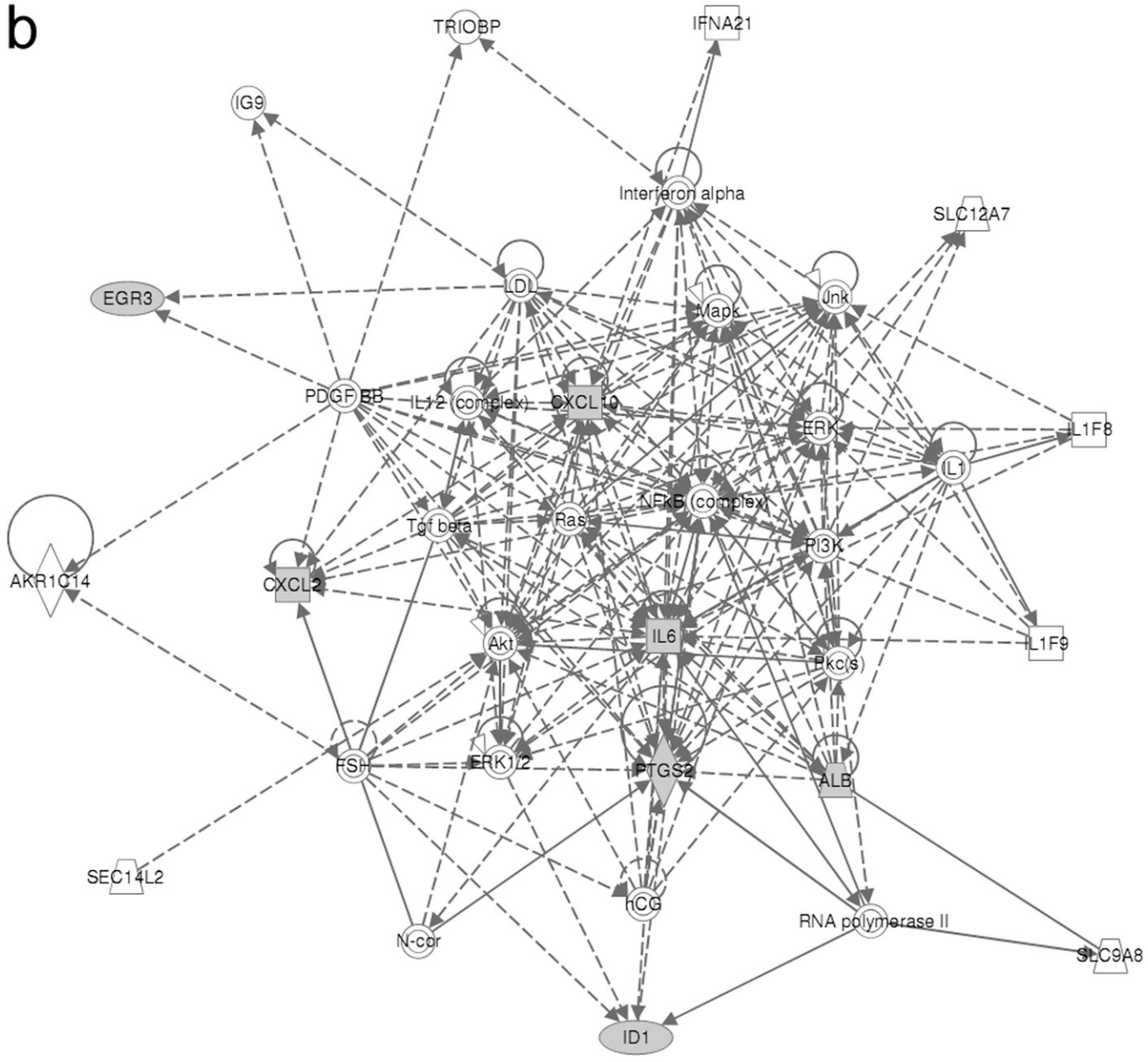


Fig. S4. (Continued)



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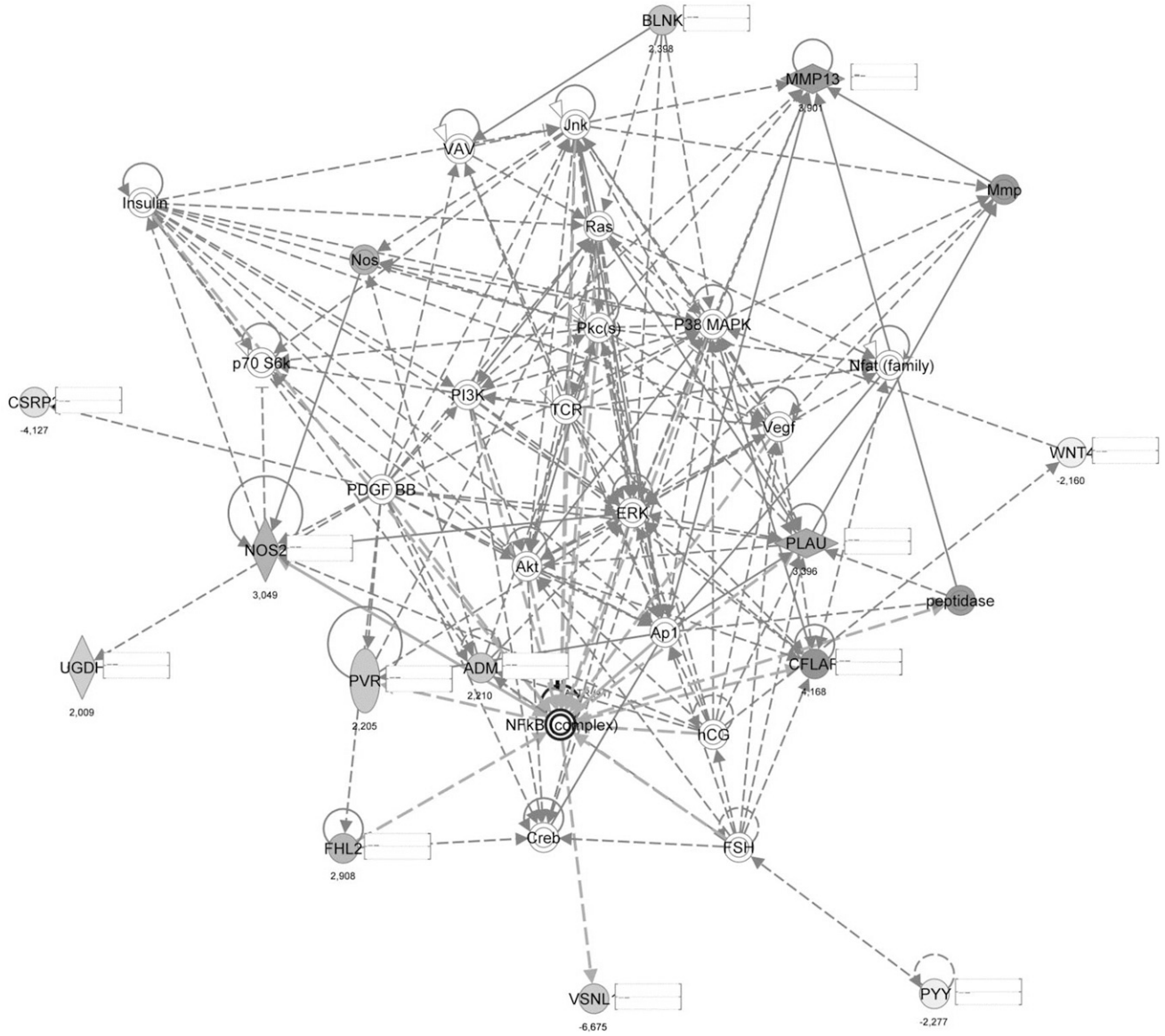

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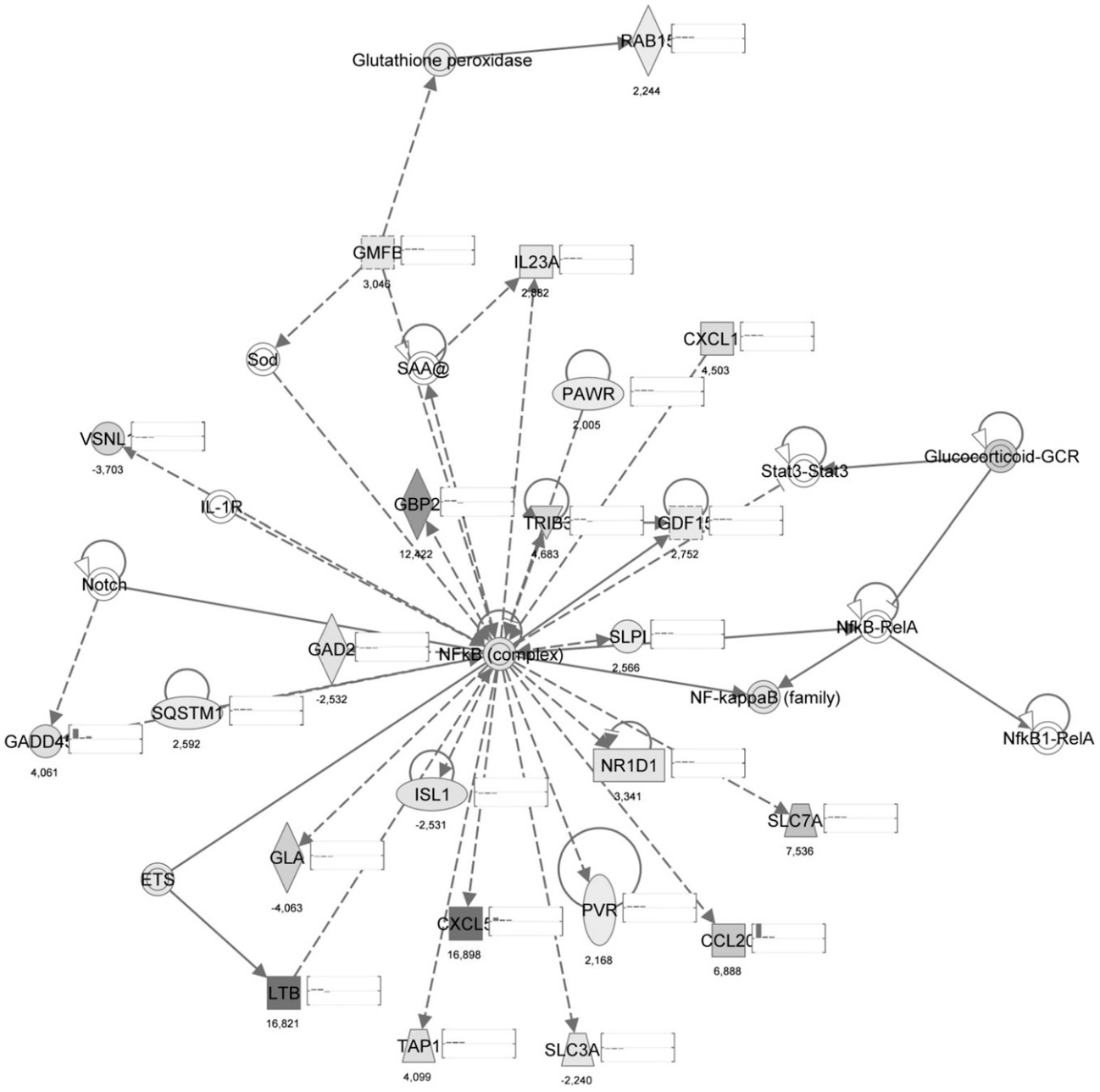

Fig. S4. KDACi resets aberrant islet cell proinflammatory gene mRNA expression induced by IL-1β+ IFN-γ. Top-rated networks from Ingenuity Pathway Analysis [the networks were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com)] of rat islet microarray. Highest rated networks from 6 (*A*), 1 (*B*), and 24 h (*D*) and second highest rated network from 6 h (*C*). RNA isolated from rat islets exposed to cytokines (150 pg/mL IL-1β + 2 ng/mL IFN-γ) with or without prior exposure to the KDACi vorinostat (1 μM) was spotted onto an Affymetrix gene array chip. Array data were subjected to visual and outlier control and normalized, and gene expressions were calculated by the RMA algorithm. Network analyses were performed using the Ingenuity Pathway Analysis program. Before Network analyses, genes were selected for a minimum of fourfold expressional change on cytokine exposure and a subsequent minimum of twofold KDACi-mediated reversion of the cytokine effect. This resulted in a list of 5, 65, and 237 genes at 1, 6, and 24 h, respectively. Output from the analyses are networks rated by the Ingenuity Pathway Analysis program according to least possibility of being combined by chance. Gray coloring indicates an expressional regulation (minimum fourfold cytokine-induced up/down-regulation followed by a minimum of twofold reversal by KDACi). White nodes are not expressionally regulated.

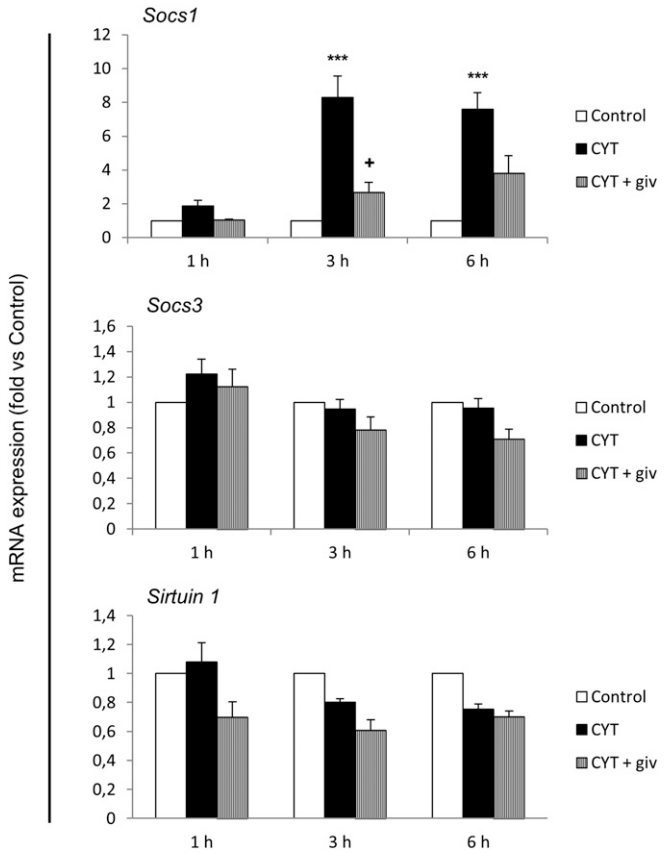

Fig. S5. KDACi does not increase SOCS-1 or -3 or Sirt1 mRNA in INS-1 cells. INS-1 cells were cultured for 1, 3, and 6 h in the presence (CYT) or absence (Control) of IL-1β (150 pg/mL) + IFN-γ (5 ng/mL). Givinostat (giv) was added (125 nM) 1 h before cytokine exposure. Data from six independent experiments are presented as fold change compared with controls. Results are shown as means + SEM. $^{***}P < 0.001$ vs. Control and $^{++}P < 0.01$ (ANOVA with Tukey's correction for multiple comparisons).

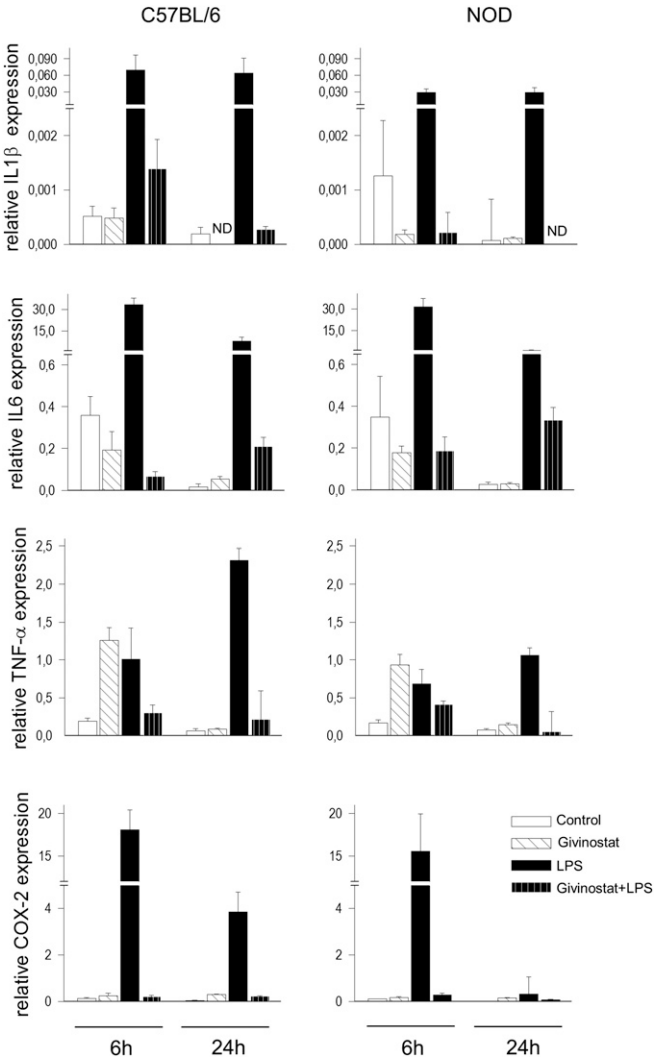

Fig. S6. KDACi reduce inflammatory gene expression. Peritoneal macrophages from non–diabetes-prone C57BL/6 mice and diabetes-prone NOD mice were preexposed to givinostat (100 nM) for 1 h and then stimulated with LPS (100 μg/mL) for different time periods. mRNA levels for *IL-1β*, *IL-6*, *TNF-α*, and *COX-2* after 6- and 24-h LPS exposure by real-time qPCR (*SI Methods*). Levels were quantified and normalized to *GAPDH*. Data are means + SEM of three independent experiments.

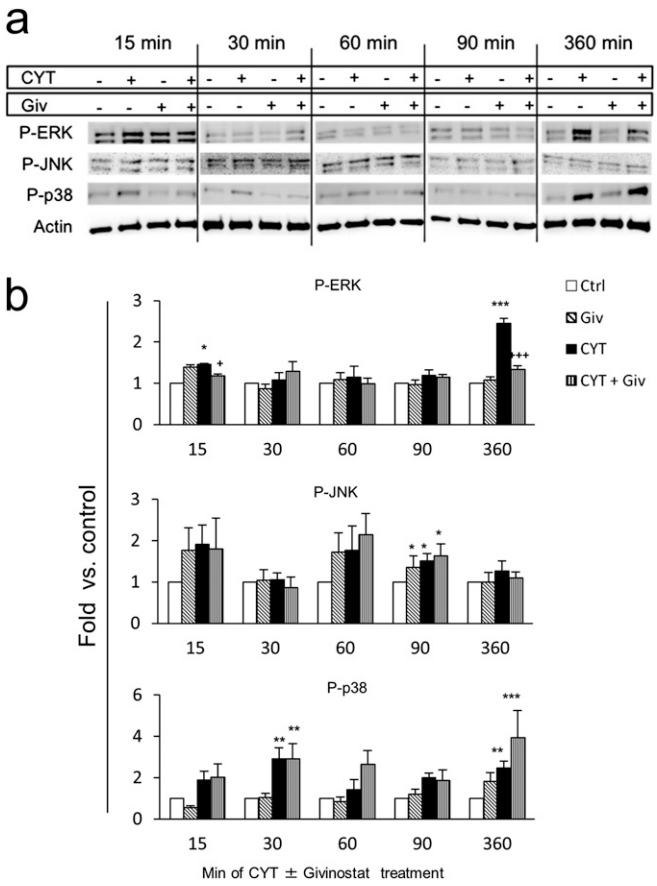

Fig. S7. Givinostat prevents IL-1β+ IFN-γ–induced ERK phosphorylation. Representative blots shown in (*A*) INS-1 cells were cultured for 15, 30, 60, 90, and 360 min in the presence (CYT) or absence [Control (Ctrl)] of IL-1β (150 pg/mL) + IFN-γ (5 ng/mL). Givinostat (giv, 125 nM) was added 1 h before cytokine exposure (CYT+Giv) or as a control without cytokine exposure (Giv). Cells were lysed, and total protein was isolated and subjected to SDS/PAGE and Western blot analysis. (*B*) Quantification by densitometry, data were normalized to β-actin as a loading control. Data from four independent experiments are presented as fold means + SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. Ctrl and $^{+++}P < 0.05$ vs. CYT.

Table S1. Syngeneic graft survival in spontaneously diabetic NOD mice

Groups	Mean graft survival time (d)	Range (d)
Control ($n = 7$)	8.6 ± 2.5	4–15
Vehicle ($n = 3$)	11.3 ± 0.7	10–12
Vorinostat ($n = 8$)	9.4 ± 4.1	4–19
Givinostat ($n = 4$)	6.3 ± 4.1	5–7

Treatment with vorinostat or givinostat (50 or 1 mg/kg, respectively, daily via drinking water) was started 3–7 d after diabetes onset, and mice were transplanted with 500 syngeneic insulitis-free NOD islets. Vorinostat and givinostat were unable to prevent diabetes recurrence in diabetes-prone NOD mice after syngeneic islet transplantation. Data are mean survival time [time posttransplantation at which hyperglycemia recurred (blood glucose levels >200 mg/dL)] of three to eight mice per group. Kaplan–Meier statistics were used to compute mean survival times, and the log-rank test was used to compare survival times with $P \leq 0.05$ considered significant.

Table S2. Givinostat (1 mg/kg, via drinking water) was given to weaning NOD mice until 35 d of age to determine ITF2357 (givinostat) and its metabolites, ITF2374 and ITF2375, in plasma

	Metabolites		
Groups	ITF2357 ($nmol^{-1}$)	ITF2374 ($nmol^{-1}$)	ITF2375 ($nmol^{-1}$)
Control ($n = 3$)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Vehicle ($n = 3$)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Givinostat ($n = 4$)	2.9 ± 0.6	8.4 ± 1.1	17.4 ± 3.9

Givinostat yields two main metabolites in vivo deriving from the biotransformation of the hydroxamate moiety into a carboxylate (ITF2375) or into an amide group (givinostat). The metabolites block KDAC enzymes from high micromolar to low millimolar concentrations and not in the nanomolar range, in which ITF2357 inhibits these enzymes. Data are mean ± SEM of three to four samples per group.