Sirtuin 2–mediated deacetylation of cyclin-dependent kinase 9 promotes STAT1 signaling in type I interferon responses

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Edited by Luke O’Neill

Type I interferons (IFNs) induce expression of multiple genes that control innate immune responses to both antiviral and antineoplastic activities. Transcription of these interferon-stimulated genes (ISGs) occurs upon activation of the canonical Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling pathways. Phosphorylation and acetylation are both events crucial to tightly regulate expression of ISGs. Here, using mouse embryonic fibroblasts and an array of biochemical methods including immunoblotting and kinase assays, we show that sirtuin 2 (SIRT2), a member of the NAD-dependent protein deacetylase family, is involved in type I IFN signaling. We found that SIRT2 deacetylates cyclin-dependent kinase 9 (CDK9) in a type IIFN–dependent manner and that the CDK9 deacetylation is essential for STAT1 phosphorylation at Ser-727. We also found that SIRT2 is subsequently required for the transcription of ISGs and for IFN-driven antiproliferative responses in both normal and malignant cells. These findings establish the existence of a previously unreported signaling pathway whose function is essential for the control of JAK–STAT signaling and the regulation of IFN responses. Our findings suggest that targeting sirtuin activities may offer an avenue in the development of therapies for managing immune-related diseases and cancer.

The interferons (IFNs) are multifunctional cytokines that regulate diverse cellular biological responses, including important antitumor, antiviral, and immunomodulatory effects. IFNs have been used widely in the treatment of many human diseases, including viral infections, autoimmune disorders, and malignancies. Type I IFNs engage their specific receptor complex, IFNAR, and initiate a sequence of events that lead to Janus-activated kinase–signal transducer and activator of transcription (JAK–STAT) signaling engagement and transcriptional activation of IFN-stimulated genes (ISGs). Activation of JAKs results in tyrosine phosphorylation of STAT1 and STAT2. Once phosphorylated, these STATs heterodimerize and bind to interferon regulatory factor 9 to assemble the transcription complex ISGF3 (interferon-stimulated gene factor 3), which binds to IFN-stimulated response elements to initiate gene expression in the nucleus. IFNs also invoke formation of STAT1 homodimers that translocate to the nucleus and bind to distinct IFNγ-activated site (GAS) elements. IFN–dependent nuclear translocation of STAT proteins requires STAT tyrosine phosphorylation, although the serine phosphorylation of STAT1 is essential for maximum transcriptional activation of ISGs. Beyond JAK–STAT activation, several other signaling cascades are activated during engagement of IFNAR and these are essential for IFN responses, associated with both transcriptional regulation and mRNA translation of ISGs.

Beyond phosphorylation, acetylation plays a crucial role in post-translational regulation of STAT1, affecting its activity. Moreover, histone deacetylases (HDACs) are essential for transcription of IFN-responsive genes. Sirtuin 2 (SIRT2) is a class III HDAC, which requires NAD+ as a cofactor. SIRT2 is predominantly localized in the cytoplasm.

1 Both authors contributed equally to this work.
2 Supported in part by National Institutes of Health, NCI Grant T32 CA080621.
3 Supported by Narodowe Centrum Nauki (NCN) 2016/22/M/NZ2/0054.
4 Tier 1 Canada Research Chair.
5 Supported by National Institutes of Health, NCI Grant R01CA182506.
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This work was supported in part by National Institutes of Health Grants CA77816 and CA155566 and Grant I01CX000916 from the Department of Veterans Affairs. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The NCBI GEO accession number for the microarray data reported in this paper is GSE66033.
where it deacetylates lysine (Lys) residues on several targets including tubulin. SIRT2 shuttles between the cytosol and nucleus, where, during mitosis, it regulates key components of the mitotic machinery (15–20). SIRT2 participates in the modulation of numerous biological processes, including cell cycle control, metabolism, differentiation, and has been described to regulate important oncogenes, such as Myc and KRAS (21–27). Mice deficient in Sirt2 develop various malignancies, including breast and liver cancer, suggesting that SIRT2 functions as a tumor suppressor (18, 21, 28). The tumor suppressor function of SIRT2 can be mechanistically explained in part through deacetylation of specific targets including Cdc20/Cdh1 (cell-division cycle protein 20/CDC20 homologue 1) of the APC/C (anaphase-promoting/cyclosome) complex, H4K16 (histone H4 lysine 16), and the cyclin–dependent kinase 9 (CDK9) (21, 22, 29).

In the present study, we examined the potential involvement of SIRT2 in type I IFN signaling and its role in the induction of IFN responses. We report that SIRT2 controls IFN-driven gene transcription via a novel mechanism involving control of phosphorylation of STAT1 on serine 727. Furthermore, we provide evidence that SIRT2-dependent STAT1 phosphorylation is important for transcriptional activation of ISGs.

**Results**

**SIRT2 is required for type I IFN-induced STAT1 serine 727 phosphorylation**

At the outset we examined whether IFNβ treatment of Sirt2−/− mouse embryonic fibroblasts (MEFs) leads to phosphorylation of STAT1 on Ser-727. Whereas IFNβ treatment of Sirt2+/+ MEFs resulted in phosphorylation of STAT1 on Ser-727, IFNβ-
induced phosphorylation of STAT1 on Ser-727 was defective in Sirt2−/− MEFs (Fig. 1A). By contrast, there was no SIRT2-dependent requirement for tyrosine phosphorylation of STAT1, as both Sirt2+/+ and Sirt2−/− MEFs exhibited IFNβ-inducible phosphorylation of STAT1 on Tyr-701 (Fig. 1B). To evaluate the potential involvement of other sirtuins in the control of IFNα/β–dependent STAT1 serine phosphorylation, we employed Sirt1−/− and Sirt6−/− MEFs. As shown in Fig. 1, C and D, IFNα/β–dependent serine phosphorylation of STAT1 occurs in both Sirt1−/− and Sirt6−/− MEFs, to similar levels observed in their WT counterparts, Sirt1+/+ and Sirt6+/+ MEFs.

To further establish the role of SIRT2 in the regulation of type I IFN–dependent STAT1 serine phosphorylation, we conducted studies in U937 hematopoietic cells, stably expressing control shRNA or SIRT2 shRNA (Fig. 1E). As shown in Fig. 1F, IFNβ–dependent phosphorylation of STAT1 on Ser-727 was substantially impaired in cells expressing SIRT2 shRNA.

### SIRT2 regulates CDK9-mediated phosphorylation of STAT1 in a type I IFN–dependent manner

To define the mechanisms by which SIRT2 activity may regulate type I IFN–dependent phosphorylation of STAT1 on Ser-727, we examined whether this event requires deacetylation of CDK9 on Lys residue 48. Previous studies have established that Lys-48 in CDK9 is a major SIRT2 downstream target and that SIRT2 regulates CDK9-mediated phosphorylation of STAT1, as both Sirt2+/+ and Sirt2−/− MEFs exhibited IFNβ-inducible phosphorylation of STAT1 on Tyr-701 (Fig. 1B). To evaluate the potential involvement of other sirtuins in the control of IFNα/β–dependent STAT1 serine phosphorylation, we employed Sirt1−/− and Sirt6−/− MEFs. As shown in Fig. 1, C and D, IFNα/β–dependent serine phosphorylation of STAT1 occurs in both Sirt1−/− and Sirt6−/− MEFs, to similar levels observed in their WT counterparts, Sirt1+/+ and Sirt6+/+ MEFs.

Although 198 genes were differentially expressed upon IFNβ treatment in both Sirt2+/+ and Sirt2−/− MEFs (Fig. 3, B and C), 44 of those genes showed higher expression in Sirt2+/+ MEFs compared with Sirt2−/− MEFs (Table S1, genes expressed higher are highlighted in red). Moreover, 172 genes were found to be differentially expressed only in IFNβ-treated Sirt2+/+ MEFs (Fig. 3, C and D, and Table S2), whereas 42 genes were differentially expressed only in IFNβ-treated Sirt2−/− MEFs (Fig. 3, C and E, and Table S3). To further characterize the differentially expressed genes found only in IFNβ-treated Sirt2+/+ MEFs, gene set pathways and process enrichment analyses were performed. The group of genes whose transcriptional induction by IFNβ is Sirt2-dependent (Table S2) were associated with pathways involved in the defense response to virus, lymphocyte migration, inflammatory responses, regulation of innate immune responses, regulation of erythrocyte differentiation, regulation of growth, and positive regulation of the extrinsic apoptotic signaling pathway (Fig. 4A and Table S4), suggesting that SIRT2 might play an important role in type I IFN–dependent biological functions. In further studies, we confirmed the requirement for SIRT2 activity in the expression of several key ISGs using quantitative RT-PCR (Fig. 4A). As anticipated, expression of several ISGs was defective in the absence of Sirt2. Specifically, induction of Oasl2, Cxcl10, Isg15, and Isg54 by IFNβ was significantly defective in Sirt2−/− MEFs compared with Sirt2+/+ MEFs (Fig. 4A). We also determined whether SIRT2 activity is required for expression of ISG protein products. IFNβ treatment of Sirt2+/+ MEFs resulted in induction of Isg15 protein, however, this induction was defective in Sirt2−/− MEFs (Fig. 4B, left panel). Similar results were obtained with human U937 stably expressing control shRNA or SIRT2 shRNA (Fig. 4B, right panel).

### Role of sirtuins in the generation of type I IFN–dependent biological responses

To determine the potential effects of SIRT2 on IFNα– mediated antiproliferative responses, we assessed cellular proliferation of Sirt2+/+ and Sirt2−/− MEFs treated with increasing doses of IFNα, using the viability WST-1 assay. Depletion of Sirt2 in MEFs resulted in decreased cellular sensitivity to type I IFN–induced antiproliferative responses (Fig. 5A). Next, we examined the role of SIRT2 in the generation of type I IFN–dependent anti-leukemic activity in cells expressing the JAK2(V617F) mutation, which is a critical pathogenic mutation in myeloproliferative neoplasms (31). For this purpose, we performed studies involving siRNA-mediated knockdown of SIRT2 in human erythroleukemia HEL cells and in SET-2 cells derived from an essential thrombocytopenia patient at the megakaryoblastic stage.
leukemic transformation phase that express the JAK2 (V617F) mutation (32) and are sensitive to type I IFN–mediated growth inhibition (Fig. S1). As shown in Fig. 5B, IFNα treatment significantly reduced proliferation of SET-2 cells, however, siRNA-mediated knockdown of SIRT2 (Fig. S2A) decreased the sensitivity of these cells to the antiproliferative effects of IFNα. Similarly, IFNα treatment suppressed the growth of primitive malignant hematopoietic precursors from HEL cells transfected with control siRNA, but this inhibition was suppressed by SIRT2 knockdown (Fig. 5C and Fig. S2B), indicating a requirement for SIRT2 in the generation of the inhibitory effects of type I IFNs on JAK2(V617F)-transformed cells. We also determined the potential involvement of SIRT2 in the generation of type I IFN anti-leukemic responses in the BCR-ABL expressing KT-1 leukemic cell line (33). IFNα treatment suppressed growth of KT1-derived primitive leukemic progenitors (CFU-L) in clonogenic assays in methylcellulose (Fig. 5D). However, these suppressive effects were blocked by siRNA-mediated SIRT1/2 knockdown (Fig. S2C), indicating that SIRT1/2 activity is required for IFNα–dependent anti-leukemic effects. In further studies, we examined whether engagement of SIRT activity is necessary for the generation of the suppressive effects of IFNα on normal hematopoiesis. Normal human bone marrow-derived CD34+ cells were transfected with siRNA to knockdown the expression of SIRT1/2 and the inhibitory effects of IFNα were determined. As anticipated,
Figure 3. SIRT2 activity is essential for ISG transcription. A and B, volcano plots of differentially expressed genes after IFNβ treatment are shown for (A) Sirt2^{+/+} MEFs and (B) Sirt2^{+/−} MEFs. C, Venn diagram showing the gene expression overlap between differentially expressed genes in Sirt2^{+/+} MEFs (blue ellipse) and Sirt2^{+/−} MEFs (red ellipse) after treatment with IFNβ. D, hierarchical clustering of differentially expressed genes identified only in IFNβ-treated Sirt2^{+/+} MEFs. E, hierarchical clustering of differentially expressed genes identified only in IFNβ-treated Sirt2^{−/−} MEFs. F, bar graph showing enriched ontology clusters of the uniquely differentially expressed genes in IFNβ-treated Sirt2^{+/+} MEFs (see also Table S4).
treatment with IFNα resulted in significant suppression of normal myeloid (CFU-GM) and early erythroid (BFU-E) hematopoietic progenitors growth in colony formation assays (Fig. S3). Together, these data indicate that SIRT2 activity is required for induction of type I IFN-driven antiproliferative effects in both normal and malignant cells.

Discussion

Control of innate and adaptive immunity by IFN-activated signaling effectors involves JAK–STAT signaling (34) and the subsequent regulation of ISGs with antiviral and antineoplastic properties (1, 2, 35, 36). Optimal transcription of ISGs requires STAT1 phosphorylation on Ser-727, an event dependent on prior IFN-induced phosphorylation of STAT1 on tyrosine 701 (1, 6, 37). There is also evidence that acetylation/deacetylation events are important for induction of expression of ISGs (11, 12, 38). In a previous study, siRNA-mediated knockdown of HDAC3, a member of class I HDACs, was shown to reduce STAT1 phosphorylation on tyrosine 701 upon IFN treatment (12). Similarly, the class I HDAC inhibitors, valproic acid and entinostat (MS-275), were shown to impair IFN-induced phosphorylation of STAT1 (11). Additionally the class I, IIa, and IIb, inhibitor, trichostatin A, was shown to block IFN-induced gene expression (5, 12–14). These studies support a potential role for deacetylases in type I IFN signaling. However, the IFN–dependent effectors that control STAT1 function via deacetylation are not well understood.

In the present study, we provide the first evidence implicating SIRT2, a class III HDAC (16), in the control of IFN-induced transcriptional and biological responses. Employing cells with targeted disruption of the Sirt2 gene we found that IFNβ-induced phosphorylation of STAT1 on tyrosine 701, but not tyrosine 701, depends on the presence of Sirt2, implicating SIRT2 in the control of IFN responses.
HDAC activity is critical for chromatin remodeling to allow for transcriptional induction of ISGs (13, 14, 39, 40). HDACs are required to recruit RNA polymerase II (RNAP II) to the promoter regions of ISGs (5). CDK9 is a component of the positive transcription elongation factor b (P-TEFb), which phosphorylates the C-terminal domain of the large subunit of RNAP II (29, 30). SIRT2 promotes deacetylation of lysine 48 of CDK9, which is required for induction of its kinase activity (29). Treatment with flavopiridol, a CDK inhibitor with increased specificity for CDK8 and CDK9, blocks type I and type II IFN-induced phosphorylation of STAT1 on Ser-727 (30). In the present study, we demonstrate that CDK9 is required for phosphorylation of STAT1 on Ser-727 in a type I IFN–dependent manner. Notably, our work suggests that SIRT2 may regulate type I IFN–dependent STAT1 phosphorylation on Ser-727 via deacetylation of CDK9 on lysine 48. Additionally, we provide evidence that SIRT2 activity is required for transcription of ISGs and the generation of type I IFN–dependent antiproliferative responses. In summary, our study supports a model in which SIRT2 regulates acetylation/deacetylation of CDK9 on Lys-48, which in turn is required for full STAT1 phosphorylation on Ser-727 and consequent optimal transcription of type I IFN–induced genes and biological responses. It remains to be determined whether SIRT2 regulates activation of other type I IFN–dependent signaling pathways.
SIRT2 in type I IFN responses

Viewed together with previous studies, our results provide further support for the concept that not only phosphorylation, but other post-translational modifications, such as acetylation, glycosylation, and ubiquitination, are required for optimal type I IFN signaling (6, 12) and we now provide evidence for the involvement of SIRT2. By establishing the first association between sirtuins and the IFN system, our data also raise the possibility that targeting sirtuins might provide a unique approach for the development of therapies for immune-related diseases.

Experimental procedures

Cells and reagents

KT-1 (33), U937 (CRL-1593.2; ATCC), and HEL (TIB-180; ATCC) cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), and SET-2 (ACC 608; DSMZ) were grown in RPMI 1640 medium supplemented with 10% FBS, and antibiotics. U937 cells were transduced with lentiviral control shRNA or lentiviral SIRT2 shRNA (43). Transduction was carried out by spinoculation and stably transduced cells were selected using puromycin (2 μg/ml). The empty vector, CDK9 WT, CDK9-K44R, and CDK9-K48R plasmids have been previously described (29). Control siRNA-B and human SIRT2 and SIRT1 siRNA were from Santa Cruz Biotechnology. Control siRNA and human CDK9 siRNA were purchased from Dharmacon. Recombinant human IFNα was from Hoffman-La Roche Inc. and recombinant human and mouse IFNβ were from Biogen Idec. Antibody against SIRT2 was from Proteintech. Antibodies against phospho-STAT1 (Ser-727), phospho-STAT1 (Tyr-701), and CDK9 were from Abcam. The antibody against SIRT2 was from Proteintech. Antibodies against phospho-STAT1 (Ser-727), phospho-STAT1 (Tyr-701), and CDK9 were from Abcam. The antibody against SIRT2 was from Proteintech.

Immunoblotting, immunoprecipitation, and in vitro kinase assays

Cells were either left untreated or were treated with the indicated type I IFN (10^4 IU/ml for short term treatments and 10^3 IU/ml for 24-h treatments, as indicated). For immunoblotting analyses, cells were lysed in phosphorylation lysis buffer (1 M HEPES, pH 7.3, 5 m NaCl, 1 M MgCl2, 0.5 M EDTA, pH 8.0, 100 μM sodium fluoride, 100 μM sodium pyrophosphate, 0.5% Triton X-100, and 10% glycerol) supplemented with protease and phosphatase inhibitors. Equal amounts of total cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P polyvinylidine difluoride membrane (Millipore). Membranes were then probed with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and antibody binding was detected by enhanced chemiluminescence using Amersham Biosciences ECL Prime Western blotting detection reagent (GE Healthcare Life Sciences). For kinase assays, KT-1 and Sirt2+/+ and Sirt2−/− MEFs were treated with human IFNβ and mouse IFNγ, respectively, for 30 and 60 min, and lysed in Nonidet P-40 buffer (20 mM HEPES, pH 7.4, 180 mM KCl, 0.2 mM EGTA, 10% glycerol, 0.1% Nonidet P-40) supplemented with protease and phosphatase inhibitors. 300 μg of protein (total cells lysates) from each sample were used for immunoprecipitation of CDK9 using anti-CDK9 rabbit polyclonal antibody (1:100), followed by incubation with protein G-Sepharose 4 Fast Flow beads (GE Healthcare Life Sciences). As control, the same procedure was followed, but using nonimmune rabbit IgG antibody, instead of anti-CDK9 antibody. The beads were washed three time with Nonidet P-40 buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 10 mM magnesium acetate, and 1 mM DTT) prior to the kinase reaction. In vitro kinase reactions to detect CDK9 kinase activity were performed for 30 min at 30°C as in previous studies (45). STAT1 recombinant human inactive protein (5 μg) (Thermo Fisher Scientific) was used as an exogenous substrate. [γ-32P]ATP was from PerkinElmer Life Science.

Transfection, hematopoietic progenitor assays, and cell viability assays

Sirt2−/− MEFs were transfected with empty vector, CDK9 WT, CDK9-K44R, CDK9-K48R (29) plasmids using Amaxa Biosystems MEF2 Nucleofector Kit (Lonza) per manufacturer’s instructions. The next day, cells were left untreated or were treated with IFNβ as indicated and processed for immunoblotting analysis. SET-2 megakaryoblastic cells were transfected with either control siRNA, CDK9, or SIRT2 siRNA and HEL cells were transfected with either control siRNA or SIRT2 siRNA using Amaxa Biosystems Nucleofector Kit V (Lonza) per the manufacturer’s instructions. Cellular proliferation of transfected SET-2 cells in the absence or presence of human IFNα (10^4 IU/ml) was assessed using WST-1 assay (Hoffman-La Roche Inc.). Leukemic colony formation of transfected HEL cells was assessed in clonogenic assays in methylcellulose in the presence or absence of human IFNα (10^3 IU/ml). KT-1 cells were transfected with control siRNA or siRNA targeting SIRT1 and SIRT2 using TKO transfection reagent (Mirus) per the manufacturer’s instructions. Clonogenic assays in methylcellulose (StemCell Technologies) in the absence or presence of human IFNα (10^3 IU/ml) were then performed. Human normal bone marrow CD34+ cells (Stem Cell Technologies) were transfected with either control or SIRT1 and SIRT2 siRNA using TKO transfection reagent (Mirus) per the manufacturer’s instructions. Clonogenic assays in methylcellulose (StemCell Technologies) in the absence or presence of human IFNα (10^3 IU/ml) were then performed and erythroid (BFU-E) or myeloid (CFU-GM) colonies were scored. Cellular viability was assessed by plating Sirt2+/+, Sirt2−/− MEFs, SET-2, and HEL cells in 96-well plates in triplicates. Sirt2+/+ and Sirt2−/− MEFs were incubated with the indicated doses of mouse IFNα for 5 days. SET-2 and HEL were treated with human IFNα for 7 days at the
indicated doses. Cell viability and proliferation were quantified using WST-1 Reagent (Hoffman-La Roche Inc.) according to the manufacturer’s protocol. IC_{50} values were calculated using GraphPad Prism 6.0 for PC.

**Microarray analysis**

Sirt2^{+/+} and Sirt2^{-/-} MEFs were plated and were then left untreated or were treated for 4 h with 2.5 × 10^3 IU/ml of mouse IFNβ (3 independent replicates). Total RNA was isolated using the RNeasy Mini Kit (Qiagen) per the manufacturer’s instructions. The expression analysis was performed at the Genomics Core Facility at Northwestern University. From the total RNA, cRNA was synthesized and labeled using Illumina TotalPrep RNA Amplification Kit (Thermo Fisher). Mouse WG-6 v2.0 Expression BeadChips were hybridized with the labeled cRNA. After staining, the chips were scanned with Illumina iScan (Illumina) according to the manufacturer’s protocol. Raw data were imported to GenomewStudio (Illumina), quantile normalized, and the average signal intensities were analyzed in Partek Genomic Suite v.6.6 (Partek, Inc.) after log_{2} transformation. Qualitative principal component analysis (PCA) did not reveal any outlier samples or artifacts on the microarray. Differential expression between the experimental groups, Sirt2^{-/-} versus Sirt2^{+/+} + IFNβ, and Sirt2^{+/+} versus Sirt2^{+/+} + IFNβ, was assessed by using two-way analysis of variance (ANOVA) and Method of Moments together with a false-discovery rate correction of the p-value below 0.05 (and with −1.5 > fold-change > 1.5) (46). Fisher’s least significant difference was used as the contrast method to compare the experimental groups.

**Gene set enrichment analysis**

The list of differentially expressed genes identified only in IFNβ-treated Sirt2^{+/+} cells was submitted to the Metascape database, a gene annotation and analysis resource (http://metascape.org/) (48), for pathway and process enrichment analysis. For each given gene list, Metascape carries pathway and process enrichment analysis using the following ontology sources: GO Biological Processes, KEGG Pathway, and Reactome Gene Sets. All genes in the genome were used as the enrichment background. Terms with p value <0.01, minimum count 3, and enrichment factor >1.5 are collected and grouped into clusters based on their membership similarities. More specifically, p values are calculated based on accumulative hypergeometric distribution, q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testing. Kafka scores were used as the similarity metric when performing hierarchical clustering on the enriched terms and then subtrees with similarity >0.3 are considered a cluster. The most statistically significant term within a cluster is chosen as the one representing the cluster.

**Quantitative RT-PCR analysis**

Sirt2^{+/+} and Sirt2^{-/-} MEFs were plated and then either left untreated or treated for 6 h with 5 × 10^3 IU/ml of mouse IFNβ. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) per the manufacturer’s instructions. 2 μg of total cellular mRNA was reverse-transcribed into cDNA using the OmniScript RT kit (Qiagen) and oligo(dT)_{12–18} primers (Life Technologies). Quantitative RT-PCR was carried out using an ABI7500 sequence detection system (Applied Biosystems) using commercially available FAM-labeled probes and primers (Applied Biosystems) to determine mouse Oas1l2, Cxcl10, Isg15, and Isg54 mRNA expression. Mouse Gapdh was used for normalization. The mRNA amplification was calculated as described previously (47).

**Statistical analyses**

All statistical analyses were performed using Prism GraphPad 6.0. Unpaired two-tailed t test with Welch’s correction was used for comparison of one observation between two groups and one-way ANOVA was used to compare more than two groups followed by Tukey’s multiple comparisons test. Differences were considered statistically significant when p values were less than 0.05.

**Author contributions**—E. M. K. formal analysis; E. M. K., S. M., D. S., B. K., B. M.-K., D. G., E. N. F., A. V., and L. C. P. validation; E. M. K., S. M., C. D., A. R., A. T., T. L., and L. C. P. investigation; E. M. K., E. M. K., E. N. F., and L. C. P. review and editing; P. L. visualization; L. C. P. supervision.

**Acknowledgment**—We thank Dr. Chu-Xia Deng (NIDDK, National Institutes of Health) for kindly providing the Sirt1^{+/+}, Sirt1^{-/-}, Sirt6^{+/+}, and Sirt6^{-/-} MEFs.

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SIRT2 in type I IFN responses