Central Regulatory Role for SIN1 in Interferon γ (IFNγ) Signaling and Generation of Biological Responses[*]

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The precise signaling mechanisms by which type II IFN receptors control expression of unique genes to induce biological responses remain to be established. We provide evidence that Sin1, a known element of the mammalian target of rapamycin complex 2 (mTORC2), is required for IFNγ-induced phosphorylation and activation of AKT and that such activation mediates downstream regulation of mTORC1 and its effectors. These events play important roles in the assembly of the eukaryotic translation initiation factor 4F (eIF4F) and mRNA translation of IFN-stimulated genes. Interestingly, IFNγ-induced tyrosine phosphorylation of STAT1 is reduced in cells with targeted disruption of Sin1, leading to decreased transcription of several IFNγ-inducible genes in an mTORC2-independent manner. Additionally, our studies establish that Sin1 is essential for generation of type II IFN-dependent antiviral effects and antiproliferative responses in normal and malignant hematopoiesis. Together, our findings establish an important role for Sin1 in both transcription and translation of IFN-stimulated genes and type II IFN-mediated biological responses, involving both mTORC2-dependent and -independent functions.

Interferons (IFNs) are pleiotropic cytokines that possess antiviral, immunomodulatory, growth-inhibitory, and cyto-toxic properties (1–13). IFNs were among the earliest biologic therapeutic agents used to treat viral infections, certain solid tumors, hematologic cancers, and some autoimmune disorders (1–13). IFNγ, the only type II IFN, signals through a multimeric receptor complex, IFNGR, consisting of two different chains: the IFNγ receptor binding subunit (IFNGR1) and a transmembrane accessory factor (IFNGR2) (14, 15). Engagement of IFNγ with IFNGR leads to activation of the Janus kinases, JAK1 and JAK2, leading to tyrosine phosphorylation and activation of STAT1 (14–18). Upon activation, STAT1 homodimers bind DNA at IFNγ-activated site elements, leading to transcription of IFN-stimulated genes (ISGs)[6] (15–18).

There is evidence for different signaling pathways that are activated by IFNGR. These include protein kinase C (PKC), MAPK, Mnk kinase, PI3K/AKT, and mammalian target of rapamycin complex 1 (mTORC1) and mTORC2 signaling cascades (19–29). mTOR pathways promote mRNA translation in an mTOR/4E-BP1-dependent manner (25, 27–33). In previous studies, our group showed that AKT/mTOR pathways are engaged in IFNγ signaling and control initiation of mRNA translation of ISGs (25, 27, 28, 30). The 289-kDa mTOR kinase contains binding sites for multiple proteins regulating its activity or mediating its signals and consists of at least two independent multiprotein complexes: mTORC1 and mTORC2 (34–38). mTORC1 is formed by mTOR, mLST8/GβL (mammalian lethal with Sec13 protein 8/G-protein β-protein subunit-like), Raptor (rapamycin-sensitive companion of mTOR), Pras40 (Akt/PKB substrate 40 kDa), and Deptor (DEP domain-containing mTOR-interacting protein) and is sensitive to allosteric inhibitors, such as rapamycin, everolimus, temsirolimus, and other related agents (rapalogs) (34–36). mTORC2 includes mTOR, mLST8, Rictor (rapamycin-insensitive companion of mTOR), Sin1 (mammalian stress-activated protein kinase-in-
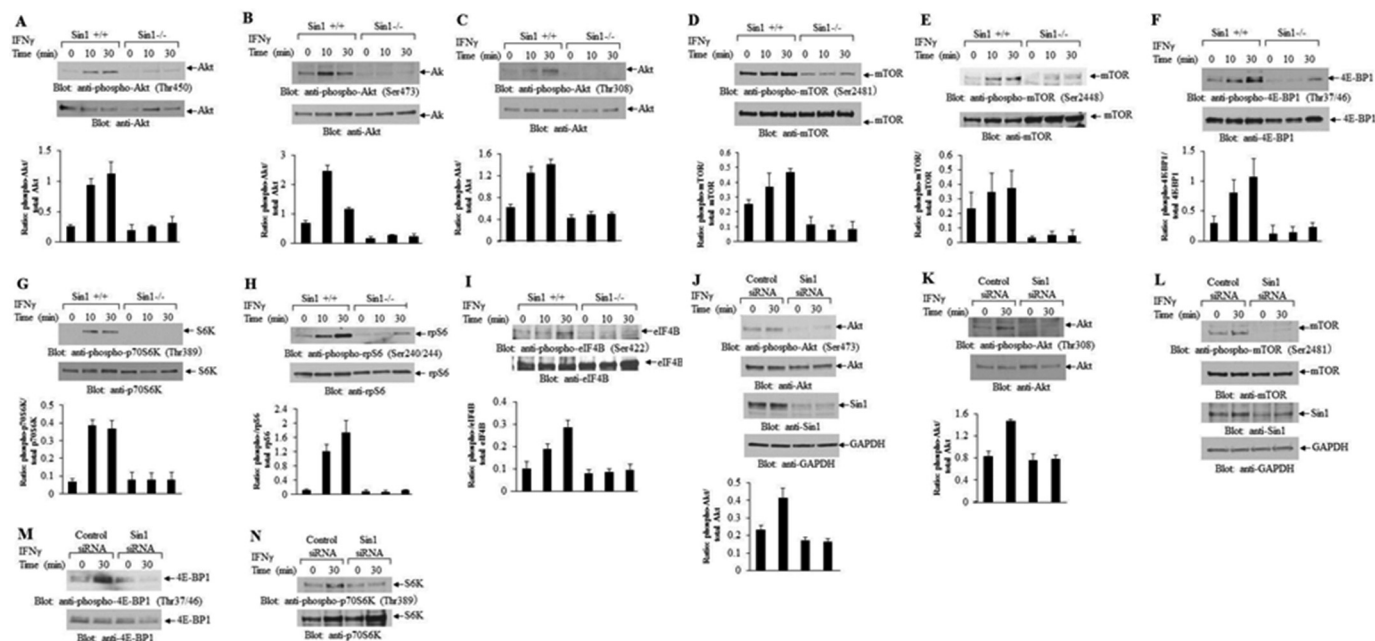
FIGURE 1. **IFNγ-dependent engagement of mTOR effectors is Sin1-dependent.** *A–I*, serum-starved Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were treated with mouse IFNγ (5×10^3 IU/ml) for the indicated times. *A–I* (*top panels*), cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE and then subjected to immunoblotting with the indicated specific anti-phosphoantibodies. The blots in the respective *top panels* were stripped and probed with anti-AKT (*A–C*), anti-mTOR (*D* and *E*), anti-4E-BP1 (*F*), anti-p70S6K (*G*), anti-rpS6 (*H*), and anti-eIF4B (*I*) antibodies. *J–N*, serum-starved U937 cells transfected with control siRNA or Sin1 siRNA were treated with human IFNγ (5×10^3 IU/ml) for the indicated times. Cell lysates were prepared, and proteins were resolved by SDS-PAGE and then processed for immunoblotting with anti- phospho-Ser-473 AKT, AKT, Sin1, and GAPDH antibodies (*J*), anti-phospho-Thr-308 AKT and anti-AKT antibodies (*K*), antibodies against phospho-Ser-2481 mTOR, mTOR, Sin1, or GAPDH (*L*), anti-phospho-Thr-37/46 4E-BP1 and anti-4E-BP1 antibodies (*M*), and anti-phospho-Thr-389 p70S6K and anti-p70S6K antibodies (*N*). *A–K* (*bottom panels*), bands from three independent experiments (including the blots shown) were quantified by densitometry. Data are expressed as ratios of phosphoprotein over respective total protein values, and bar graphs represent means ± S.E. (*error bars*) of three independent experiments for each experimental condition.

teracting protein 1), Protor 1/2 (protein observed with rictor 1/2), and Deptor and is generally insensitive to rapalogs (34–45). Because mTORC2 is a critical regulator of AKT, a kinase that is often activated in cancer cells and mediates survival signals, this mTOR complex has become a target for cancer therapy and has led to efforts to develop catalytic mTOR inhibitors that block the mTOR kinase (24, 34, 44).

In efforts to map and define the roles of different mTORC2-driven elements in the induction of IFNγ responses, we evaluated the role of Sin1 in the IFNγ system. Our studies demonstrate that Sin1 is required for IFNγ-induced phosphorylation of AKT and that Sin1-mediated AKT activation mediates downstream regulation of mTORC1 effectors to control mRNA translation of ISGs. Remarkably, IFNγ-induced STAT1 activation and subsequent transcription of several ISGs is diminished in cells with targeted disruption of Sin1, demonstrating the existence of an mTORC2-independent function of Sin1 as a regulator of Jak-Stat pathways.

Results

In initial studies, we compared type II IFN-dependent mTORC2 phosphorylation of AKT in wild type (Sin1$^{+/+}$) *versus* Sin1 knock-out (Sin1$^{-/-}$) mouse embryonic fibroblasts (MEFs). IFNγ treatment induced phosphorylation of AKT on Thr-450, Ser-473, and Thr-308 in Sin1$^{+/+}$ MEFs but not in Sin1$^{-/-}$ MEFs (Fig. 1, *A–C*, respectively), thus establishing a requirement for Sin1 in induction of AKT activity. This result is consistent with previous findings demonstrating that mTORC2 activation is required for phosphorylation of the turn motif in

AKT on Thr-450 and of the hydrophobic motif on Ser-473 and that this phosphorylation is required for enabling PDK1 to phosphorylate AKT at Thr-308 (45–49). Similarly, the absence of Sin1 resulted in decreased IFN-inducible phosphorylation of mTOR at Ser-2481 (Fig. 1*D*) and Ser-2448 (Fig. 1*E*), possibly reflecting reduced mTORC2 and mTORC1 catalytic activity, respectively (50). The defective type II IFN-dependent phosphorylation of AKT and mTOR at Ser-2448, taken together with our previous findings implicating AKT upstream of mTORC1 in IFNγ signaling, led us to examine mTORC1 effectors in the type II IFN system. IFNγ-induced phosphorylation of 4E-BP1 on Thr-37/46 (Fig. 1*F*) and S6K on Thr-389 (Fig. 1*G*) was reduced in Sin1$^{-/-}$ MEFs compared with Sin1$^{+/+}$ MEFs. As expected, IFNγ treatment resulted in induction of phosphorylation of the downstream effectors of S6K, rpS6, and eukaryotic translation initiation factor 4B (eIF4B) (Fig. 1, *H* and *I*), but these phosphorylation levels were decreased in Sin1$^{-/-}$ MEFs. We also examined whether transient knockdown of Sin1 by specific siRNA-mediated targeting in malignant hematopoietic cells also results in reduced type II IFN-dependent mTOR activity. IFNγ-induced phosphorylation of AKT, mTOR, 4E-BP1, and S6K were impaired in U937 cells where Sin1 was selectively knocked down (Fig. 1, *J–N*), consistent with the findings in Sin1$^{-/-}$ MEFs.

We subsequently sought to determine whether engagement of Sin1 in IFNγ signaling regulates expression of IFNγ-inducible proteins. For this purpose, Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were incubated in the presence or absence of mouse IFNγ, and cell
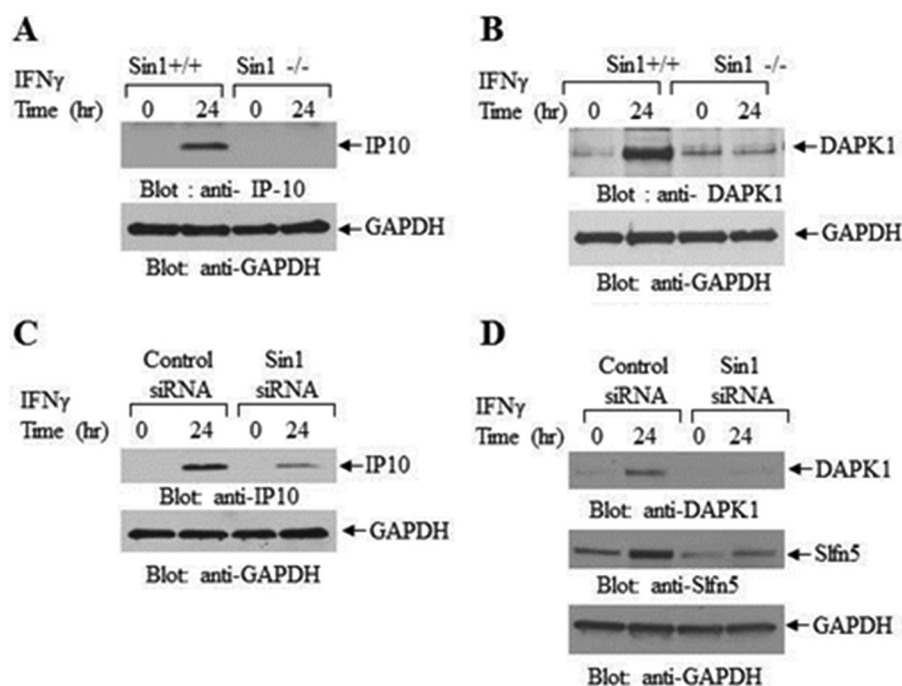
FIGURE 2. **Requirement of Sin1 for type II IFN-induced ISG protein expression.** *A* and *B*, serum-starved Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were treated with mouse IFNγ (1.5×10^3 IU/ml) as indicated. Cell lysates were prepared, and proteins were resolved by SDS-PAGE and immunoblotted with anti-IP10 (*A*) or anti-DAPK1 (*B*). *C* and *D*, serum-starved U937 cells transfected with control siRNA or Sin1 siRNA were treated with human IFNγ (1.5×10^3 IU/ml) for the indicated times. Cell lysates were prepared, and proteins were resolved by SDS-PAGE and then processed for immunoblotting with anti-IP10 (*C*) and anti-DAPK1 and anti-SLFN5 (*D*) antibodies. *A–D*, anti-GAPDH antibody was used for loading control.

lysates were resolved by SDS-PAGE and immunoblotted with anti-IP10 and DAPK1 antibodies. The expression of IP10 (CXCL10), an IFN-induced chemokine that has diverse roles in infectious diseases and induces apoptosis and cell growth inhibition (51), was defective in Sin1$^{-/-}$ MEFs (Fig. 2*A*). We also examined whether Sin1 is required for the expression of DAPK1, a tumor suppressor protein (52, 53). As anticipated, IFNγ treatment induced the expression of DAPK1 in Sin1$^{+/+}$, but this did not occur in Sin1$^{-/-}$ MEFs (Fig. 2*B*). Consistent with these findings, IFNγ-induced expression of IP10, DAPK1, and SLFN5 (54) was also reduced in U937 cells in which Sin1 was knocked down using specific siRNAs (Fig. 2, *C* and *D*).

Next, using the 5′-cap binding assay, we undertook studies to evaluate the effects of Sin1 targeting the assembly of the translation initiation complex during IFNγ signaling (55). As expected, IFNγ treatment resulted in dissociation of 4E-BP1 from eIF4E and subsequently enhanced binding of eIF4G and eIF4A to the 5′-cap analog in Sin1$^{+/+}$ cells. This was not the case in Sin1$^{-/-}$ MEFs (Fig. 3*A*). To determine whether impaired assembly of the eIF4F complex, which consists of eIF4E, eIF4G, and eIF4A, in Sin1$^{-/-}$ MEFs accounts for defective mRNA translation of type II IFN-dependent ISGs, we next evaluated the mRNA levels for *Ip10*, *Dapk1*, and *Slfn2* (56) in polysome fractions. Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were left untreated or were treated with IFNγ, polysomal mRNA was fractionated (Fig. 3*B*), and quantitative real-time RT-PCR was used to measure mRNA levels of ISGs in the polysome fractions. *Ip10*, *Dapk1*, and *Slfn2* mRNA levels were decreased in Sin$^{-/-}$ MEFs compared with Sin1$^{+/+}$ MEFs (Fig. 3, *C–E*), indicating a reduction in translation of those mRNAs when Sin1 is absent. Although these results are consistent with the impaired activa-

tion of mTOR effectors, whose functions are required for initiation of mRNA translation, observed in Sin1$^{-/-}$ cells, these studies do not address the effects of Sin1 on the transcription of ISGs. Accordingly, we next examined the mRNA levels of *Dapk1*, *Ip10*, and *Slfn2* in IFNγ-treated Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs by quantitative real-time RT-PCR. Interestingly, *Dapk1* mRNA expression was not defective in Sin1$^{-/-}$ cells (Fig. 4*A*), but both *Ip10* and *Slfn2* mRNA levels were reduced in the absence of Sin1 (Fig. 4, *B* and *C*). Together, these results suggest that Sin1 expression is required for mRNA translation of ISGs, but also for IFNγ-induced transcription of selective ISGs.

Previously, we demonstrated that mTORC2 elements regulate transcription of type I ISGs by controlling activation of STAT-driven pathways, through regulation of STAT2 phosphorylation on Tyr-689 and STAT1 phosphorylation on Ser-727 (29). In addition, prior data showed that the transcription of *Ip10* and *Slfn2* is STAT1-dependent, and transcription of DAPK1 is STAT1-independent (51, 52). This led us to consider the possibility that defective transcription of IFNγ-regulated genes in the absence of Sin1 could reflect impaired STAT1 activation. Previous studies have established that phosphorylation of STAT1 on Tyr-701 is required for STAT1 dimerization (15, 16), whereas phosphorylation on Ser-727 is essential for full transcriptional function of the protein (14–16). We performed studies to examine the effects of targeted disruption of the *Sin1* gene on phosphorylation of STAT1. As shown in Fig. 5 (*A* and *B*), IFNγ treatment led to greater phosphorylation of STAT1 on Tyr-701 and on Ser-727 in Sin1$^{+/+}$ cells compared with Sin1$^{-/-}$ MEFs, suggesting that Sin1 is essential for optimal STAT1 transcriptional activity. In different settings, Sin1 has been shown to interact with IFNAR2 and TGF receptors (57, 58), which led us
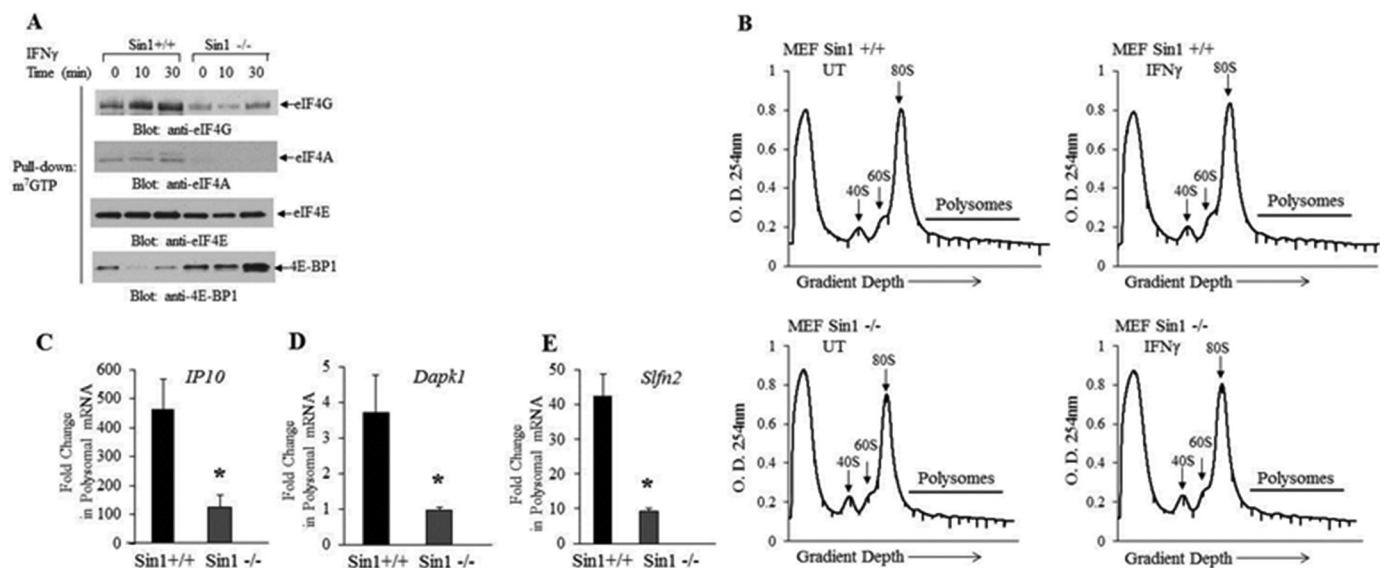
FIGURE 3. **Essential role for Sin1 in mRNA translation of type II ISGs.** *A*, serum-starved Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were either left untreated or treated with mouse IFNγ (5×10^3 IU/ml) for the indicated times. Cell lysates were bound to the cap analog m^{7}GTP biotin-labeled and streptavidin beads (39). After extensive washing, bound proteins were resolved by SDS-PAGE and immunoblotted with antibodies against eIF4G, eIF4A, eIF4E, and 4E-BP1. The cell lysates used for this experiment are from the same experiment shown in Fig. 1*F*. *B*, Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were either left untreated or treated with mouse IFNγ (1.5×10^3 IU/ml) in DMEM containing 0.5% FBS for 24 h. Cell lysates were layered on 5–50% sucrose gradients and subjected to density gradient centrifugation, and fractions were collected by continuous monitoring of optical density (*O.D.*) at 254 nm. Optical density at 254 nm is shown as a function of gradient depth and the 40S, 60S, and 80S peaks, and polysomal fractions are indicated. *C–E*, polysomal fractions were pooled, and RNA was isolated. Subsequently, quantitative real-time PCR was carried out to determine *Ip10* (*C*) *Dapk1* (*D*), and *Slfn2* (*E*) mRNA expression in the polysomal fractions, using *Gapdh* for normalization. Data are expressed as -fold change over control untreated cells, and bar graphs represent means ± S.E. (*error bars*) of three independent experiments, including the ones shown in *B*. Statistical analyses were performed using Student's *t* test (*, $p < 0.05$).
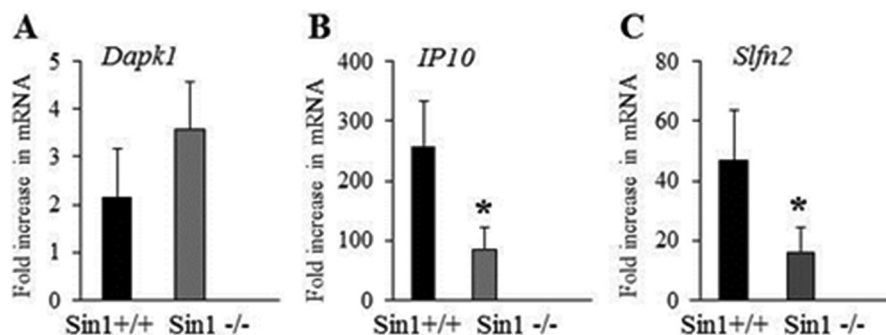

FIGURE 4. **Essential role of Sin1 in transcription of Type II ISGs.** Serum-starved Sin1$^{+/+}$ or Sin1$^{-/-}$ MEFs were either left untreated or were treated with mouse IFNγ (2.5×10^3 IU/ml) for 6 h, and total RNA was isolated as described under "Experimental Procedures." mRNA expression of *Dapk1* (*A*), *Ip10* (*B*), and *Slfn2* (*C*) was evaluated by quantitative real-time PCR, and *Gapdh* was used for normalization. Data are expressed as -fold change over control untreated cells, and bar graphs represent means ± S.E. (*error bars*) of three independent experiments. Statistical analyses were performed using Student's *t* test (*, $p < 0.05$).

to examine whether Sin1 binds IFNGR1. As shown in Fig. 5*C*, Sin1 co-immunoprecipitates with IFNGR1, suggesting that Sin1 may be required for transmission of IFNGR downstream signaling events. Notably, STAT1 was found to bind IFNGR1 only in the presence of Sin1, suggesting that IFNGR1 requires Sin1 to create a docking site for the STAT1 protein. Binding of IFNγ to IFNGR2 and IFNGR1 induces phosphorylation of JAKs (14, 15). Active JAK kinases phosphorylate the cytoplasmic domain of IFNGR1, which allows for the recruitment of STAT1 and its phosphorylation at Tyr-701 (14, 15). In subsequent studies, we sought to determine whether Sin1 knock-out affects IFNγ-dependent JAK1 and JAK2 phosphorylation. IFNγ treatment induced phosphorylation of JAK1 and JAK2 in Sin1$^{+/+}$ but not in Sin$^{-/-}$ MEFs (Fig. 5, *D* and *E*), suggesting defective IFNγ-dependent activation of JAKs in the absence of Sin1. To further examine the regulation of IFNγ-dependent STAT1

activation, we immunoprecipitated IFNGR1 from mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs and evaluated binding to STAT1 and Jak1 (Fig. 5*F*). Importantly, the interaction between Sin1, JAK1, STAT1, and IFNGR1 is independent of mLST8 expression (Fig. 5*F*), a key component of both mTORC1 and mTORC2, suggesting that Sin1 controls IFNγ-mediated STAT1 activation independently of its role in mTORC2. In agreement, mLST8 expression is not required for IFNγ-induced phosphorylation of STAT1 on Tyr-701 (Fig. 5*G*).

Previously, AKT was found to be required for Sin1 phosphorylation at Thr-86 (59). Thus, in subsequent studies, we sought to determine whether IFNγ-induced Sin1 phosphorylation at this site affects IFNγ-induced STAT1 phosphorylation. For this, we assessed the effects of rapamycin, an mTORC1 inhibitor, and MK-2206, an AKT inhibitor, in MEFs (Fig. 6). IFNγ-induced phosphorylation of AKT and Sin1 was blocked by co-
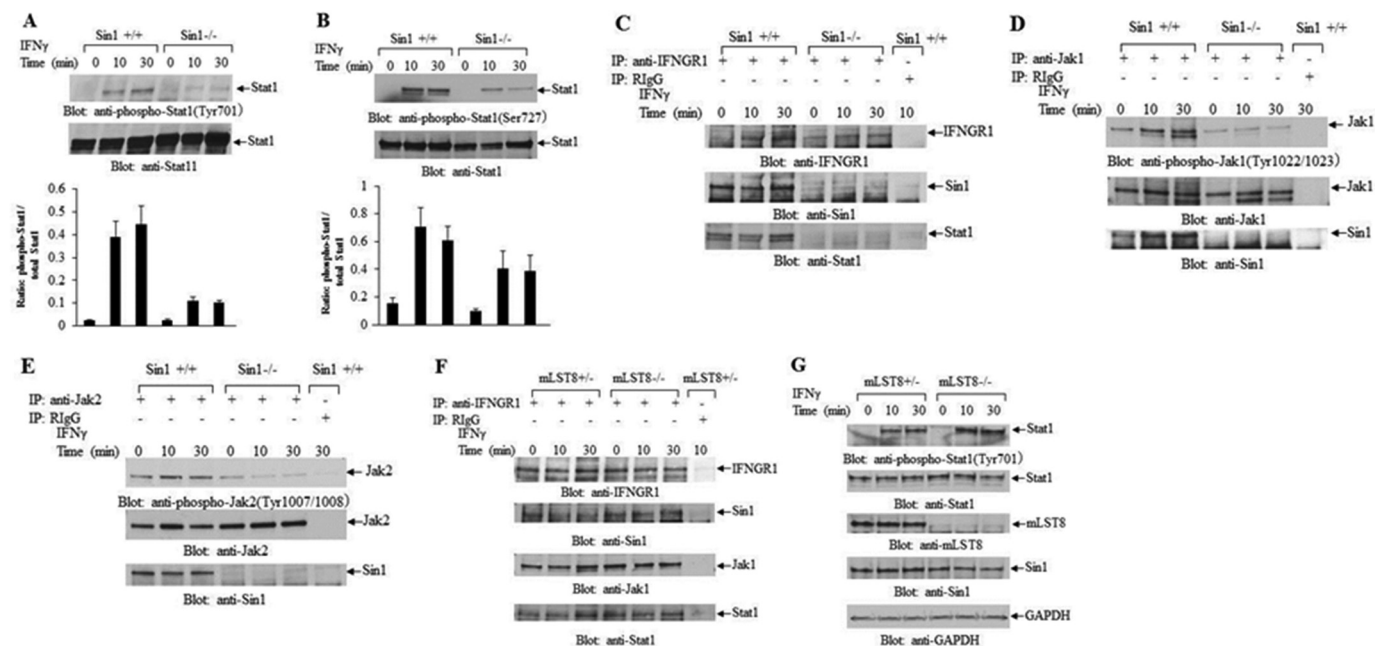
FIGURE 5. **IFNγ-induced tyrosine phosphorylation of STAT1 is Sin1-dependent.** *A* and *B*, serum-starved Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were treated with mouse IFNγ (5×10^3 IU/ml) for the indicated times. Cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE and then subjected to immunoblotting analyses with the indicated antibodies. *A* and *B* (*bottom panels*), bands from three independent experiments (including the blots shown) were quantified by densitometry. Data are expressed as ratios of phospho-Stat1 over total Stat1, and bar graphs represent means ± S.E. (*error bars*) of three independent experiments for each experimental condition. *C–E*, serum-starved Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were treated with mouse IFNγ (5×10^3 IU/ml) for the indicated times. *C*, equal amounts of protein were processed for immunoprecipitation (*IP*) with anti-IFNGR1 antibody or control IgG, as indicated. The immunoprecipitated proteins were resolved by SDS-PAGE and then subjected to immunoblot analyses with anti-IFNGR1, anti-Sin1, and anti-Stat1 antibodies. *D* and *E*, equal amounts of protein were processed for immunoprecipitation with anti-Jak1 (*D*) or anti-Jak2 (*E*) and control IgG, as indicated. The immunoprecipitated proteins were resolved by SDS-PAGE and then subjected to immunoblot analyses with anti-phospho-Tyr-1022/1023 Jak1, Jak1, and Sin1 antibodies (D) or with anti-phospho-Tyr-1007/1008 Jak2, Jak2, and Sin1 antibodies (E). *F*, serum-starved mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were treated with mouse IFNγ (5×10^3 IU/ml) for the indicated times. Equal amounts of protein were processed for immunoprecipitation with anti-IFNGR1 antibody or control IgG as indicated. The immunoprecipitated proteins were resolved by SDS-PAGE and then subjected to immunoblot analyses with anti-IFNGR1, Sin1, Jak1, and Stat1 antibodies. *G*, the same cell lysates used in *H* were resolved by SDS-PAGE and then subjected to immunoblot analyses with anti-phospho-Tyr-701 Stat1, Stat1, mLST8, Sin1, and GAPDH antibodies.

treatment with MK-2206 (Fig. 6, *A* and *B*). However, the AKT inhibitor did not affect STAT1 phosphorylation at Tyr-701 and Ser-727 (Fig. 6*C*). As expected, rapamycin treatment had no effect on IFNγ-mediated phosphorylation of Sin1, AKT, and STAT1 (Fig. 6, *A–C*). These results confirm that IFNγ-mediated Sin1 phosphorylation at Thr-86 is AKT-dependent. However, Sin1 phosphorylation at this site does not affect IFNγ-dependent activation of JAK-STAT signaling, further supporting the requirement of Sin1 activity, independently of both mTORC1 and mTORC2 activation.

To assess the biological relevance and functional significance of Sin1 in the type II IFN system, we next examined the role of Sin1 in the generation of IFNγ-dependent anti-leukemic activity. The inhibitory effects of IFNγ on primitive leukemic progenitors (CFU-L) were partially reversed in U937 cells transfected with Sin1 siRNA (Fig. 7*A*), suggesting that Sin1 mediates IFNγ-induced anti-leukemic responses. Studies were also carried out to determine the potential role of Sin1 as a mediator of the suppressive effects of IFNγ on normal hematopoiesis. Normal human bone marrow-derived CD34$^+$ cells transduced with control siRNA or Sin1-specific siRNA were treated with IFNγ, and normal myeloid (CFU-GM) or early erythroid (BFU-E) colony formation was determined by clonogenic assays in methylcellulose. As expected, treatment with IFNγ resulted in suppression of hematopoietic progenitor colony formation, but

these effects were partially reversed by Sin1 knockdown (Fig. 7*B*), directly establishing a role for Sin1 in the process.

Because our data demonstrated defective IFNγ signaling events in Sin1$^{-/-}$ cells, we compared induction of IFNγ-dependent antiviral responses in Sin1$^{+/+}$ and Sin1$^{-/-}$ cells. Notably, Sin1$^{-/-}$ cells are more sensitive to encephalomyocaditis virus (EMCV) infection (data not shown). We found that Sin1$^{+/+}$ MEFs were responsive to the antiviral effects of IFNγ, as reflected by protection from the cytopathic effects (CPE) of EMCV (Fig. 7*C*). Using the same infective dose, we found that identical IFNγ doses did not provide protection in the Sin1$^{-/-}$ MEFs, suggesting a role for Sin1 in the generation of IFNγ-induced antiviral effects.

Discussion

Recent advances in cancer immunotherapy underscore the critical relevance of the type II IFN system in immune surveillance against cancer (2, 6, 7, 13). The emerging understanding of the central role of IFNγ in the network of immune regulatory mechanisms that control malignant cell survival underscores the importance of precisely defining type II IFN signaling events. Recently, there has been an increased interest in identifying the mechanisms by which IFNs regulate transcription and mRNA translation of ISGs to develop unique approaches that could modulate the IFN response and lead to ways to enhance
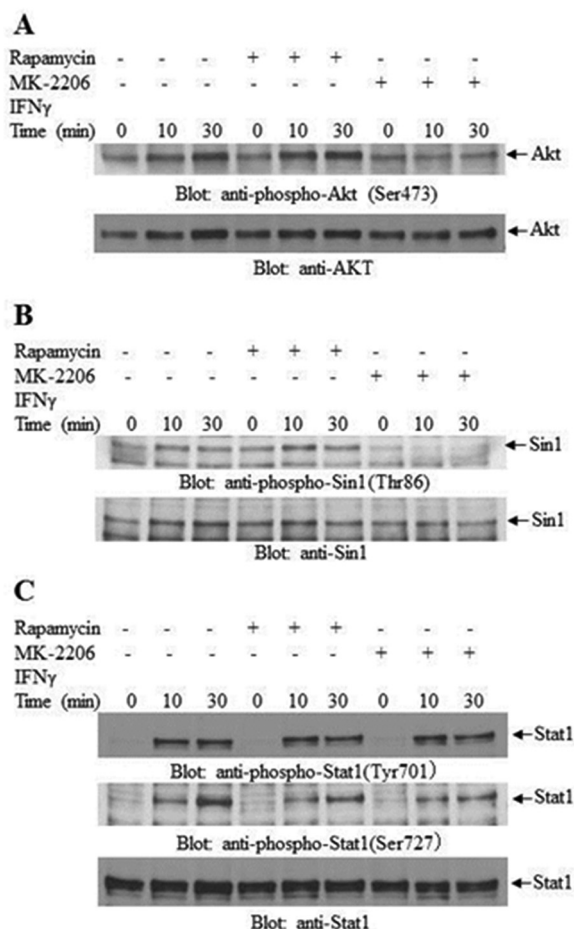
FIGURE 6. **IFNγ-induced phosphorylation of Sin1 at Thr-86 is AKT-dependent but not required for STAT1 phosphorylation.** *A–C*, serum-starved Sin1$^{+/+}$ MEFs were treated with rapamycin (20 nM) or MK-2206 (10 μM) for 30 min, followed by co-treatment with mouse IFNγ (5 × 10^3 IU/ml) for the indicated times. Cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE and then subjected to immunoblotting with the indicated specific anti-phosphoantibodies. These blots were stripped and probed with the respective anti-total antibodies.

the generation of antiviral or antiproliferative responses by these cytokines. In earlier studies, we provided evidence implicating mTOR complexes in the expression of type I and II ISG products (27–33). Other earlier work from our group raised the possibility that mTORC2 is engaged in a unique way in IFN signaling, as reflected by IFN-specific effects on the AKT/mTORC1 axis (28, 30). Notably, in a recent study, we established that Rictor and Sin1 are essential for type I IFN-mediated activation of STAT-driven signaling pathways required for optimal type I ISG transcription (29), suggesting cross-talk between mTOR and STAT signaling cascades upon engagement of the type I IFN receptor (60). Overall, the emerging evidence has been consistent, with a key role for mTOR pathways in the generation of IFN responses, but the elements of the mTOR signaling machinery that mediate interactions with other IFN-activated pathways remain to be identified, and their roles have yet to be precisely defined.

In the current study, we sought to determine the role of Sin1 in the generation of IFNγ responses. We used cells with targeted disruption of the *Sin1* gene to define the role of Sin1 in IFNγ signaling and the control of IFNγ responses. IFNγ-in-

duced phosphorylation of AKT was defective in Sin1$^{-/-}$ cells, establishing that intact mTORC2 is required for induction of AKT activity during its engagement by the type II IFN receptor. Moreover, we found decreased or defective IFNγ-induced phosphorylation of downstream mTORC1 effectors in the absence of Sin1, indicating Sin1-dependent sequential activation of mTORC2 and mTORC1. Our data also demonstrate that Sin1 expression is required for protein expression of ISGs, such as IP10 and DAPK1. This could be explained, in part, by the fact that in the absence of Sin1, there appears to be defective mTORC1-dependent formation of eIF4F and consequent decreased mRNA translation (56).

Interestingly, for the first time, we uncovered a unique role for Sin1 in IFNγ signaling that appears to be independent of its function as a component of mTORC2. Upon binding of IFNγ to IFNGR1, these two chains associate with the two IFNGR2 chains, and JAK1 and JAK2 are activated by phosphorylation (61). This interaction leads to JAK1 and JAK2 dimerization, activation, and ultimately phosphorylation of Tyr-457 of IFNGR1, which comprises the receptor docking site for STAT1 (61). Subsequently, STAT1 binds to IFNGR1 at this site and is phosphorylated at Tyr-701 by JAK1 and JAK2 (14–17, 61–63). Our data revealed that Sin1 binds IFNGR1 and that this interaction is essential for the IFNγ-induced association of JAK1 and subsequent docking of STAT1 to the IFNGR. IFNγ-mediated tyrosine phosphorylation of JAK1, JAK2, and STAT1 was impaired in Sin1$^{-/-}$ cells, leading to defective transcription of STAT-driven ISGs. Taken in context, these studies provide the first evidence implicating Sin1 in the functional activation of IFNGR-mediated signaling. Moreover, we found that AKT-dependent phosphorylation of Sin1 at Thr-86 is not necessary for STAT1 phosphorylation. We infer that Sin1 may act as a scaffold protein between JAK1 and IFNGR. Overall, our studies establish that Sin1-dependent type II IFN signaling events are required for the IFNγ suppressive effects on normal hematopoiesis and the generation of antiproliferative and antiviral responses, via control of both transcription and translation of specific type II ISGs. Because of the key role of the type II IFN system in the pathophysiology of different diseases, a better understanding of the involvement of Sin1 in the functional activation of IFNGR may contribute to the design of novel and improved therapeutic approaches toward several pathologies.

Experimental Procedures

Cell Lines and Reagents—Immortalized Sin1$^{+/+}$, Sin1$^{-/-}$ (28, 45), mLST8$^{+/-}$, and mLST8$^{-/-}$ MEFs provided by David Sabatini (Whitehead Institute, Cambridge, MA) (28, 46) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Bone marrow-derived CD34$^+$ cells were from Stemcell Technologies (Vancouver, Canada). U937 cells were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Phospho-specific antibodies against mTOR, AKT, Sin1, p70S6K, rpS6, 4E-BP1, eIF4B, JAK1, JAK2, and Stat1 and antibodies against mTOR, AKT, p70S6K, rpS6, 4E-BP1, eIF4B, JAK1, JAK2, mLST8, eIF4G, eIF4A, and eIF4E were from Cell Signaling (Boston, MA). The antibody against CXCL10 (IP10) was purchased from Abcam (Cambridge, MA). Antibodies against Sin1 and GAPDH were from Millipore (Bil-
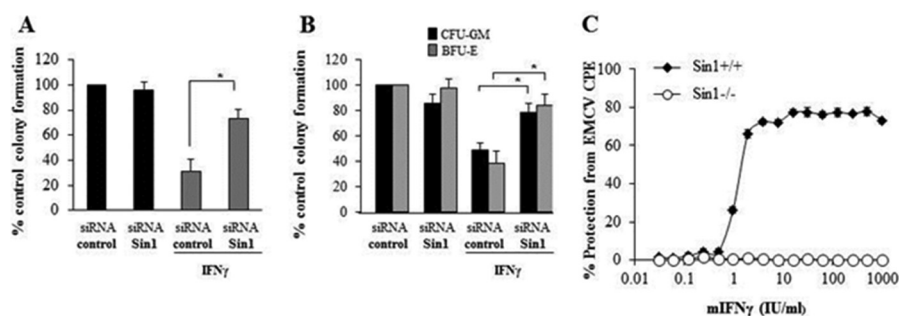
FIGURE 7. **Requirement of Sin1 for generation of IFNγ biological effects.** *A*, U937 cells were transfected with either control siRNA or siRNA specifically targeting Sin1, as indicated. The cells were subsequently plated in methylcellulose, in the absence or presence of human IFNγ, and leukemic CFU-L colony formation was assessed. Data are expressed as percentage of colony formation of untreated control siRNA-transfected cells, and bar graphs represent means ± S.E. (*error bars*) of three independent experiments. Statistical significance was calculated using one-way ANOVA followed by Tukey's test: *, $p < 0.05$. *B*, normal human CD34$^+$ bone marrow-derived cells were transfected with control siRNA or Sin1 siRNA and incubated in methylcellulose in the presence or absence of human IFNγ, as indicated. CFU-GM and BFU-E progenitor colonies were scored after 14 days in culture. Data are expressed as percentage of colony formation of untreated control siRNA-transfected cells, and bar graphs represent means ± S.E. of three independent experiments. Statistical significance was calculated using one-way ANOVA followed by Tukey's test: *, $p < 0.05$. *C*, Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were seeded in wells of a 96-well plate in DMEM. 24 h later, the cells were either left untreated or treated with the indicated doses of mouse IFNγ for 16 h and subsequently challenged with EMCV (0.02 MOI). Virus-induced CPE were quantified 24 h later, as described under "Experimental Procedures." Data are expressed as percentage protection from EMCV CPE and are representative of three independent experiments. Values are means ± S.E. of quadruplicate assays.

lerica, MA). Anti-DAPK1 and anti-IFNGR1 antibodies were from Proteintech Group, Inc. (Chicago, IL). Antibody against STAT1 was from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-SLFN5 was from Sigma-Aldrich (St. Louis, MO). Recombinant human and mouse IFNγ were from Life Technologies, Inc. (Waltham, MA). Rapamycin was obtained from Calbiochem (Billerica, MA). The Akt inhibitor MK-2206 was purchased from Santa Cruz Biotechnology.

Cap Binding Assays—These studies were performed as described previously (30). Briefly, Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were incubated overnight in serum-free medium and then treated with mouse IFNγ (5 × 10^3 IU/ml) for the indicated times. Cell lysates were incubated for 24 h with a dinucleotide mRNA 5′-cap analog (m7GpppG) labeled with biotin attached to ribose of the second dinucleotide (64, 65) and subsequently with streptavidin beads for 4 h. After extensive washing with lysis buffer, the retained proteins were eluted from the beads, boiled and resolved by SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), and probed with the indicated antibodies.

Immunoprecipitation, Immunoblotting Analysis, and siRNA Knockdown—Immortalized MEFs were starved overnight in DMEM containing 0.5% FBS and were then treated with mouse IFNγ at the indicated doses in DMEM containing 0.5% FBS for the indicated times. U937 cells were nucleofected with control siRNA or a pool of four different synthetic Sin1 siRNA (Dharmacon) using the Amaxa nucleofector kit C, following the manufacturer's protocol (Lonza). Transfected cells were starved overnight in RPMI containing 0.5% FBS and then treated with human IFNγ at the indicated doses in RPMI containing 0.5% FBS for the indicated times. Following treatment, cells were washed with PBS and lysed in 40 mM HEPES, pH 7.5, containing 0.1% Nonidet P-40, 120 mM sodium chloride, 1 mM EDTA, 10 mM sodium pyrophosphate, 50 mM NaF, 10 mM B-glycerophosphate, and protease and phosphatase inhibitors (Calbiochem). For immunoblotting analyses, lysates were resolved by SDS-PAGE gradient gels (Bio-Rad), transferred to Immobilon-P PVDF membranes (Millipore), which were probed with pri-

mary and secondary antibodies, and then detected by enhanced chemiluminescence as in previous studies (25, 30). For co-immunoprecipitation analyses, total cell lysates were incubated overnight at 4 °C with rotation with rabbit antibody against either IFNGR1, JAK1, or JAK2 (Cell Signaling), as indicated, followed by incubation at 4 °C with protein G-Sepharose 4 Fast Flow beads (GE Healthcare). As a control, the same procedure was followed, but using Rabbit IgG. After immunoprecipitation, the beads were washed with Nonidet P-40 buffer. Protein complexes were eluted from the beads, and eluates were resolved by SDS-PAGE and processed for immunoblotting analyses.

Polysomal Isolation—Immortalized Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were either left untreated or were treated with 1.5 × 10^3 IU/ml of mouse IFNγ for 24 h in DMEM supplemented with 0.5% FBS. Cells were washed twice with Dulbecco's PBS with 100 μg/ml cycloheximide and then lysed in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 5 mM Tris, pH 7.5, 2.5 mM MgCl₂, 1.5 mM KCl, 100 μg/ml cycloheximide, 2 mM DTT, protease inhibitor, and 1 unit/μl RNase inhibitor). Lysates were then centrifuged at 12,000 × g for 5 min at 4 °C, and supernatants were collected and snap-frozen in liquid nitrogen. Equal amounts of cell lysates of each sample were layered on a sucrose gradient of 5–50% prepared by using the BioComp Gradient Master 108 (Biocomp Instruments, Fredericton, Canada), according to the manufacturer's instructions. Samples were centrifuged at 4 °C for 110 min at 35,000 rpm using a Beckman SW41-Ti rotor. After centrifugation, samples were loaded on the Density Gradient Fractionation System (Brandel), and absorbance was measured continuously at 254 nm with the following settings: pump speed, 0.80 ml/min; 10 drops/fraction; chart speed, 300 cm/h; sensitivity, 1; peak separator, off; noise filter, 0.5 s. Assignments of the 40S, 60S, and 80S peaks and polysomes were determined based on the absorbance profile. RNA from polysomal fractions was isolated using an RNA all-prep kit from Qiagen. 1 μg of polysomal RNA was reverse transcribed using oligo(dT) primers (Life Technologies) and the Omniscript RT kit (Qiagen), as described previously (30).

Sin1 in IFNγ Responses

Hematopoietic Progenitor Assays—Malignant leukemic progenitor (CFU-L) colony formation in methylcellulose from U937 cells transfected with control siRNA or Sin1 siRNA was assessed as described previously (29). Cells were plated in methylcellulose, in the absence or presence of human IFNγ (5×10^2 IU/ml), and leukemic CFU-L colony formation was assessed as in our previous studies (29). Normal hematopoietic progenitor colony formation for late erythroid progenitors (BFU-E), or myeloid progenitors (CFU-GM) from normal $CD34^+$ bone marrow-derived cells transfected with control siRNA or Sin1 siRNA was determined in clonogenic assays in methylcellulose, in the absence or presence of human IFNγ (50–100 IU/ml), as in our previous studies (30). The transfections were performed using the siRNA transfection reagent TransIT-TKO (Mirus).

Quantitative RT-PCR—$Sin1^{+/+}$ and $Sin1^{-/-}$ MEFs were starved overnight in DMEM containing 0.5% FBS and then treated for 6 h with 2.5×10^3 IU/ml mouse IFNγ. Total RNA was isolated using the RNeasy minikit (Qiagen) per the manufacturer's instructions. 1 μg of total cellular mRNA was reverse transcribed into cDNA using the Omniscript RT kit (Qiagen) and oligo(dT)$_{12-18}$ primers (Life Technologies). Real-time PCR was carried out using an ABI7500 sequence detection system (Applied Biosystems) using commercially available FAM-labeled probes and primers (Applied Biosystems) to determine mRNA expression of *Ip10*, *Dapk1*, *Slfn2*, and *Gapdh*. *Gapdh* was used for normalization as described in our previous studies (28, 30).

Antiviral Assays—$Sin1^{+/+}$ and $Sin1^{-/-}$ MEFs were seeded in individual wells of 96-well plates. 24 h later, the cells were either left untreated or treated with mouse IFNγ at the indicated doses for 16 h, before infection with EMCV. The infective dose of EMCV was predetermined as the minimum viral dilution that leads to 100% CPE in the $Sin1^{+/+}$ cells at 24 h (0.02 MOI). Virus-induced CPE was quantified 24 h postinfection using a colorimetric assay as described earlier (28, 30). Data are expressed as percentage protection from EMCV CPE and are representative of three independent experiments. Values are means ± S.E. of quadruplicate assays.

Statistical Analyses—Statistical significance was analyzed using Student's *t* test or one-way ANOVA followed by Tukey's test as indicated. Differences were considered statistically significant when *p* values were <0.05.

Author Contributions—B. K., G. T. B., R. L. R., L. X., E. M. K., B. M.-K., D. S., S. M., and G. T. B. performed research and analyzed data; B. S. and J. J. developed, produced and provided key experimental reagents for the study; B. K., E. N. F., J. K. A., E. A. E., and L. C. P. conceived and analyzed data; D. S., L. X., G. T. B., R. L. F., and E. N. F., contributed to manuscript drafting. B. K. and L. C. P. designed research and wrote the manuscript.

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