Regulatory effects of SKAR in interferon α signaling and its role in the generation of type I IFN responses

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26). Notably, mTOR-induced signals are relevant for the mRNA and functional relevance in IFN signaling have been shown (23 verse cellular functions (22) and, in recent years, its activation
generation of IFN responses (20, 21).

Results

Significance

Type I interferons (IFNs) are cytokines with important biological effects, including antileukemic and antineoplastic properties. The biological responses of type I IFNs occur via up-transcriptional activation of specific genes, called interferon-stimulated genes (ISGs), ultimately resulting in generation of protein products that mediate their biological effects.

In the present study, we provide evidence that S6 kinase 1 (S6K1) Aly/REF-like target (SKAR) is an IFN-α-activated effector of either MAPK pathways downstream of p90 ribosomal protein S6 kinase (RSK1) or mTORC1 complexes downstream of S6K1. Our studies demonstrate that IFN-α-inducible phosphorylation of SKAR results in enhanced interactions of the protein with the eukaryotic initiation factor (eIF)4G and the formation of a unique IFN-inducible RSK1–eIF4G complex. Knockdown of SKAR results in defective mRNA translation of key ISGs and reverses generation of the antileukemic/antineoplastic effects of IFN-α on malignant hematopoietic progenitors from patients with chronic myeloid leukemia (CML) or polycythemia vera, establishing a key role for this protein in the generation of IFN-α responses.

Previous studies in other systems have identified SKAR as an mTOR/S6K1 effector recruiting S6K1 to cap-binding complex

Studies over several decades have firmly established that interferons (IFNs) exhibit important antiviral, immunomodulatory, and antineoplastic properties (1–6). Indeed, IFNs have shown important therapeutic properties and have been used extensively for the treatment of many human diseases, including infections, neurological disorders, and malignancies (6–10). Dysregulation of IFN gene expression and abnormally high IFN production contribute to the pathophysiology of certain diseases, and efforts are under way to limit IFN production or minimize their target effects to ameliorate disease (5, 11–13).

Type I IFNs generate biological responses by binding to specific cell-surface receptors and activating Jak-STAT signaling to mediate transcription of IFN-stimulated genes (ISGs) (14–16). In addition to Jak-Stat signaling, several other signaling cascades are activated following receptor activation and are essential for optimal transcriptional regulation and mRNA translation of ISGs (17). Among these are MAP kinase (MAPK) pathways and their effectors (18). In particular, the p38 MAPK pathway is of critical importance for type I IFN-dependent gene transcription and acts in an independent but complementary manner to Jak-Stat signaling (19). IFN-inducible MAPK-dependent signaling is critical for the control of IFN-induced mRNA translation and protein expression for key ISGs and, further downstream, Mnk kinases are essential for IFN-inducible protein expression and generation of IFN responses (20, 21).

The mTOR pathway plays a central role in the control of diverse cellular functions (22) and, in recent years, its activation and functional relevance in IFN signaling have been shown (23–26). Notably, mTOR-induced signals are relevant for the mRNA translation of genes whose transcription is regulated by Jak-Stat pathways, providing a link between Jak-Stat pathways and the signals needed for the ultimate expression of protein products of Stat-dependent genes (25, 26). Despite these advances in understanding the role of the mTOR pathway in IFN signaling, the precise contribution and unique roles of distinct mTOR effectors in cap-dependent mRNA translation of ISGs remain to be precisely defined.

In the present study, we provide evidence that S6 kinase 1 (S6K1) Aly/REF-like target (SKAR) is an IFN-α-activated effector of either MAPK pathways downstream of p90 ribosomal protein S6 kinase (RSK1) or mTORC1 complexes downstream of S6K1. Our studies define a requirement for SKAR in the generation of IFN-α-dependent inhibitory effects on malignant hematopoietic progenitors from patients with chronic myeloid leukemia or myelo-proliferative neoplasms. Taken altogether, these findings establish critical and essential roles for SKAR in the regulation of mRNA translation of IFN-sensitive genes and induction of IFN-α biological responses.

We provide evidence that S6 kinase 1 (S6K1) Aly/REF-like target (SKAR) is engaged in IFN-α signaling and plays a key role in the generation of IFN responses. Our data demonstrate that IFN-α induces phosphorylation of SKAR, which is mediated by either the p90 ribosomal protein S6 kinase (RSK) or p70 S6 kinase (S6K1), in a cell type-specific manner. This type I IFN-inducible phosphorylation of SKAR results in enhanced interaction with the eukaryotic initiation factor (eIF)4G and recruitment of activated RSK1 to 5′ cap mRNA. Our studies also establish that SKAR is present in cap-binding CBP80 immune complexes and that this interaction is mediated by eIF4G. We demonstrate that inducible protein expression of key IFN-α-regulated protein products such as ISG15 and p21WAF1/CIP1 requires SKAR activity. Importantly, our studies define a requirement for SKAR in the generation of IFN-α-dependent inhibitory effects on malignant hematopoietic progenitors from patients with chronic myeloid leukemia or myeloproliferative neoplasms. Taken altogether, these findings establish critical and essential roles for SKAR in the regulation of mRNA translation of IFN-sensitive genes and induction of IFN-α biological responses.
(CBC) mRNA and enhancing translation efficiency (27, 28). As we have previously shown that the IFN-activated mTOR pathway has a critical role in mRNA translation of ISGs and generation of IFN responses (23–26), we sought to determine whether SKAR is an IFN-engaged mTOR effector and plays a role in type I IFN signaling. At the outset, we sought to determine whether SKAR is engaged in IFN signaling in malignant hematopoietic cells. Based on evidence that the consensus amino acid motif RXRXX*S/*T is present in SKAR (27), we examined whether there is IFN-inducible phosphorylation of SKAR on this motif. KT1 or U937 cells were treated for different times with IFN-α, and total cell lysates were immunoprecipitated with an anti-SKAR antibody. Phosphorylated SKAR was then identified by immunoblotting using the anti-phospho-RXRRXX*S/*T antibody. IFN-α treatment resulted in phosphorylation of SKAR both in KT1 (Fig. 1A) and U937 cells (Fig. S1), suggesting that this protein is engaged in IFN-α signaling.

Previous studies have established that SKAR can be found in CBP80-bound mRNA protein complexes (28). To determine whether IFN treatment results in formation of CBP80–SKAR complexes, communoprecipitation studies were performed. Serum-starved KT1 cells were treated with IFN-α, and then cell lysates were either left untreated, treated with RNase A, or immunoprecipitated with an anti-CBP80 antibody. IFN treatment increased the levels of SKAR protein present in CBP80 complexes, independent of treatment with RNase A. As expected (29–31), eIF4G was detected in association with CBP80 (Fig. 1B).

In subsequent studies, we sought to identify the IFN-dependent serine kinase that regulates phosphorylation of SKAR. Given our earlier evidence for differential, cell type-dependent phosphorylation of eIF4B and programmed cell death 4 (PDCD4) protein by either S6K1 or RSK1 (32, 33), we initially examined whether pharmacological inhibition of mTOR or RSK activity mediates inhibition of RSK1 expression partially blocked SKAR phosphorylation, experiments were carried out in which RSK1 was inhibited by combining the RSK inhibitor BI-D1870 and then treating cells with IFN-α. Controls included the RSK inhibitor, but it was blocked in cells treated with the RSK inhibitor. As expected, phosphorylation of SKAR after IFN-α treatment correlated with the amount of eIF4G detected in SKAR immunoprecipitates, suggesting that IFN treatment induces an association of the phosphorylated form of SKAR with eIF4G (Fig. 1C). Consistent with this, IFN-α–induced SKAR–eIF4G complex formation was not detected in cells pretreated with the RSK inhibitor (Fig. 1C).

To further define the involvement of RSK1 in SKAR phosphorylation, experiments were carried out in which RSK1 was knocked down in KT1 cells. As shown in Fig. 1D, siRNA–mediated inhibition of RSK1 expression partially blocked SKAR phosphorylation, indicating that in these cells RSK1 is the dominant kinase controlling IFN-dependent SKAR phosphorylation (Fig. 1D). Consistent with this, when immune complex kinase assays were performed with anti-RSK1 immunoprecipitates from IFN-treated KT1 cells using GST-SKAR as a substrate, we found strong SKAR phosphorylation by the IFN-α–activated RSK1 (Fig. 1E). Similarly, RSK inhibition blocked IFN-induced phosphorylation of exogenously overexpressed SKAR protein in KT1 cells (Fig. S2), further establishing a role for RSK1 in IFN-inducible SKAR phosphorylation on the RXRXX*S/*T motif. Altogether, these studies definitively establish that SKAR is a substrate for RSK1 in cells of hematopoietic origin.

To determine whether S6K1 may also have a role in IFN-α–inducible SKAR phosphorylation, we conducted a series of experiments in immortalized mouse embryonic fibroblasts (MEFs) with targeted disruption of either the S6K1 or S6K2 gene or both. Serum-starved S6K1−/−, S6K1+/−, S6K2−/−, and double S6K1/S6K2 knockout MEFs were treated with mouse IFN-α, total lysates were immunoprecipitated with a SKAR antibody, and phosphorylated SKAR was examined by anti-phospho-RXRRXX*S/*T

**Fig. 1.** IFN-α induces phosphorylation of SKAR. (A) Serum-starved KT1 cells were treated with IFN-α for the indicated times. Total cell lysates were subjected to immunoprecipitation (IP) using an anti-SKAR antibody conjugated to biotin and pulled down by streptavidin agarose. Phosphorylated SKAR was detected by immunoblotting with an anti–phospho-RXRRXX*S/*T motif antibody. Immunoblotting with an anti-SKAR antibody is also shown. (B) Serum-starved KT1 cells were transfected with siRNA targeted against RSK1 or control siRNA and treated for 30 min with IFN-α. Total cell lysates were subsequently immunoprecipitated with anti-CBP80 antibody. Immunoprecipitates were resolved by SDS/PAGE and immunoblotted with the indicated antibodies. (C) Serum-starved KT1 cells were transfected for 60 min with SI-027 or BI-D1870 and then treated with IFN-α for 30 min. Total cell lysates were subjected to IP using an anti-SKAR antibody conjugated to biotin and pulled down by streptavidin agarose. The immunoprecipitates were subsequently resolved by SDS/PAGE and immunoblotted with antibodies against the anti–phospho-RXRRXX*S/*T motif, SKAR, or eIF4G, as indicated (Top). Total cell lysates used for IP were analyzed separately by SDS/PAGE and immunoblotted with the indicated antibodies (Bottom). Signals were quantified by densitometry and used to calculate the intensity of phosphorylated SKAR relative to that of total SKAR. Data are expressed as the ratio of phospho-SKAR to SKAR for each experimental condition and represent means ± SD of the results of four experiments, including the one shown (Top). (D) KT1 cells were transfected with either control siRNA or siRNA specifically targeting RSK1 and treated with IFN-α, as indicated. Equal amounts of total cell lysates were subjected to IP using anti-SKAR antibodies conjugated to biotin and pulled down by streptavidin agarose. The immunoprecipitates were resolved by SDS/PAGE and immunoblotted with antibodies against the anti–phospho-RXRRXX*S/*T motif, SKAR, or eIF4G, as indicated (Top). Total cell lysates used for IP were analyzed separately by SDS/PAGE and immunoblotted with the indicated antibodies (Bottom). Data were quantified by densitometry and used to calculate the intensity of phosphorylated SKAR relative to that of total SKAR. The experiment was performed at least three times, and data are representative of three experiments.
found in the 5′ cap complexes, further supporting that eIF4G is the mediator of SKAR binding to the complex (Fig. 2C).

Because our studies suggested that RSK1 is required for SKAR phosphorylation in cells of hematopoietic origin, we next examined whether RSK1 associates with SKAR during IFN-α treatment of cells. As shown in Fig. 3A, the IFN-inducible phosphorylated form of RSK1 was detected in SKAR immunoprecipitates (Fig. 3A), in support of a physical interaction between SKAR and RSK1. To confirm this finding, protein-binding assays were used. Equal amounts of phosphorylated/activated His-tagged-RSK1 (His-RSK1) and the dephosphorylated form of His-RSK1 (as a result of PP2A treatment) (Fig. S4) were mixed with GST-SKAR (Fig. 3B). Immunoblot analysis of eluted fractions from a His60 Ni Superflow resin column demonstrated the presence of GST-SKAR together with activated His-RSK1, whereas the dephosphorylated form of His-RSK1 did not bind to GST-SKAR (Fig. 3C), providing further evidence that only the activated form of RSK1 binds to SKAR.

To examine the role of SKAR in eIF4G binding to the 5′ cap complex, we conducted a series of experiments using lysates from cells transfected with control siRNA or siRNA specifically targeting SKAR. As anticipated (Fig. 3D), we found that IFN treatment resulted in enhanced recruitment/binding of eIF4G to motif immunoblotting. IFN-α–inducible SKAR phosphorylation was defective in S6k1 knockout cells and double S6k1/S6k2 knockout cells but not in single S6k2 knockout cells, demonstrating a cell type–specific, selective requirement for S6k1 but not S6k2 in the process (Fig. S3).

Earlier studies in other systems have identified SKAR as an S6k1-interacting protein recruiting S6k1 to CBC mRNA (28). SKAR and eIF4G are found in CBP80 immune complexes, but the specific protein that interacts directly with SKAR has not been identified (28). The nuclear CBC involves a CBP20 and CBP80 heterodimer that interacts with the 5′ cap structure of mRNA (34–36) and functions analogously to the cap-bound eIF4E as a translation initiation factor (37). Using 7-methylguanosine cap complexes (Fig. 2C), we undertook studies to identify which factors are important for the recruitment of SKAR to the complex. As shown in Fig. 2A, time–dependent binding of eIF4G and eIF4A to the 5′ cap structure, and knockdown of eIF4G (Fig. 2A) decreased not only the amount of eIF4A but also of SKAR (Fig. 2A). In lysates from cells transfected with eIF4G-specific siRNA, we found less SKAR in CBP80 complexes compared with lysates from cells transfected with control siRNA (Fig. 2B), suggesting that eIF4G is the necessary protein for binding of SKAR to the complex. To confirm this, we used lysates from cells transfected with control siRNA or siRNA specifically targeting CBP80. As expected, CBP80 knockdown resulted in decreased levels in the amount of CBP20 in 5′ cap complexes (Fig. 2C). Notably, siRNA-mediated CBP80 knockdown did not affect the amount of SKAR.
the 5′ cap complex, which was clearly detectable in lysates from cells in which SKAR was knocked down, but knockdown of SKAR dramatically decreased the levels of phosphorylated RSK1 compared with lysates from cells transfected with control siRNA (Fig. 3D).

We have previously shown that elf4B and PDCD4 are phosphorylated by RSK1 during IFN-α treatment of hematopoietic cells and that their phosphorylation is important for regulation of mRNA translation of ISGs (32, 33). Accordingly, we hypothesized that the SKAR interaction with elf4G may result in positioning phosphory-RSK1 in close proximity to the translational machinery. To test this, we examined phosphorylation of elf4B and PDCD4 in lysates from cells transfected with either control siRNA or siRNA targeting SKAR and treated with IFN-α. Phosphorylation of elf4B and PDCD4 was diminished in lysates from cells transfected with siRNA targeting SKAR and IFN-treated (Fig. 3E and Figs. S5 A and B). Moreover, PDCD4 levels were higher in SKAR siRNA-transfected cells compared with control siRNA-transfected cells, consistent with the observation that nonphosphorylated PDCD4 is resistant to proteosomal degradation (33). On the other hand, IFN-α-inducible phosphorylation of RSK1 and the S6 ribosomal protein (rpS6) was not affected by SKAR knockdown.

In subsequent studies, we examined the functional relevance of SKAR in mRNA translation of ISGs and the generation of IFN responses. In experiments to directly establish whether SKAR engagement in IFN signaling is important for ISG expression, we found that SKAR knockdown results in defective IFN-dependent induction of ISG15 and p21WAF1/CIP1 proteins (Fig. 4A). The inhibitory effects on ISG15 and p21WAF1/CIP1 protein expression in cells in which SKAR was knocked down were not the result of suppression of IFN-α-dependent transcription of these genes (Fig. 4 B and C), suggesting effects on mRNA translation. In parallel studies in which SKAR was overexpressed in KT1 cells, IFN-α-dependent ISG protein expression, including ISG15 and p21WAF1/CIP1, was increased (Fig. 4D). To determine whether the regulatory effects of SKAR on ISG protein expression reflect effects on mRNA translation of key ISGs, we examined the effects of siRNA-mediated knockdown of SKAR on ISG15 and p21WAF1/CIP1 mRNA expression in polysomal fractions from KT1 cells (Fig. 4 E–G). We found a significant decrease of IFN-α-induced ISG15 and p21WAF1/CIP1 polysomal mRNA expression in cells in which SKAR was knocked down (Fig. 4 F and G), establishing an essential role for SKAR in mRNA translation of these key ISGs.

In subsequent studies, we examined the potential involvement of SKAR in the generation of the antileukemic effects of IFN-α. We assessed the effects of SKAR knockdown on the generation of the inhibitory effects of IFN-α on leukemic progenitor colony formation. IFN treatment suppressed CFU-blast (CFU-L) colony formation of KT1 cells transfected with control siRNA, but this inhibition was ablated by SKAR knockdown (Fig. 5A). Similarly, siRNA knockdown of SKAR in primary leukemic CFU-GM progenitors from CML patients (Fig. 5B), or in primary malignant early erythroid progenitors (BFU-E) from patients with polycythemia vera (Fig. 5C), reversed the inhibitory effects of IFN-α on colony formation, strongly suggesting that SKAR plays an important role in the generation of the antileukemic and antineoplastic effects of IFN-α.

To determine whether SKAR has a role in the generation of IFN-α responses in nonhematopoietic malignant cells, we assessed the effects of SKAR knockdown on the suppressive effects of IFN-α on anchorage-independent growth of malignant colon adenocarcinoma HT29 cells. IFN-α treatment resulted in inhibition of anchorage-independent growth of HT29 cells (Fig. S6 A and B), but this inhibition was reversed by SKAR knockdown (Fig. S6 A and B). In other experiments, we examined the effects of overexpression of SKAR and the ability of IFN-α to suppress anchorage-independent HT29 cell growth. Ectopic expression of SKARα did not affect baseline anchorage-independent growth but significantly enhanced the inhibitory effects of IFN-α (Fig. S6 C and D), further establishing an important role for SKAR in the generation of the antineoplastic effects of IFN-α.

![Figure 4: Requirement of SKAR for IFN-α-dependent ISG15 and p21WAF1/CIP1 expression.](image-url)
Discussion
In recent years, there has been accumulating evidence that the mTOR and MEK/ERK pathways are essential elements of sig-
naling following activation of IFN receptors and play key roles in
controlling initiation of mRNA translation for several ISGs and
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generation of specific IFN-functional responses (20, 23–26, 32,
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tiple signaling substrates. mTORC1 interacts with eIF3 and
phosphorylates S6K at Thr389, and this leads to release of S6K
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lization of several elements in pathways involved in mRNA trans-
lation (32, 33, 38). However, the precise molecular mechanisms by which IFN-activated RSK interacts with vari-
ous effector substrates are not well-understood.

Studies in other systems suggest that many of the protein
elements involved in steady-state translation participate in the
first round of mRNA translation (41). As mentioned, the nuclear
CBC involves a CBP20 and CBP80 heterodimer that interacts
with the 5′ cap structure of mRNA (34–36) and functions anal-
gously to the cap-bound eIF4E as a translation initiation factor
(37). The CBC, as well as eIF4E, interacts directly with eIF4G,
the protein that binds eIF3, and subsequently recruits the 40S
ribosomal subunit-containing preinitiation complex to the mRNA
(29, 31). CBP80 also interacts with the components of the exon-
junction complex (EJC) (42, 43), a group of proteins localized to
20–24 nt upstream of exon–exon junctions. Before the steady-state
translation initiation complex is formed, mammalian mRNA
undergoes quality control (44). Studies show that if translation
termination is premature, then the mRNA is subject to nonsense-
mediated decay and that this process requires the splicing-
dependent deposition of EJC proteins (43, 44), necessary for the
splicing-dependent increase in gene expression by a mechanism
involving association of spliced mRNA with polyosomes (44–46).

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A

B

C

Fig. 5. Requirement of SKAR for the generation of the antineoplastic
effects of IFN-α. (A) KT1 cells were transfected with either control siRNA or
siRNA specifically targeting SKAR and subsequently cultured in methyl-
cellulose in the presence or absence of IFN-α, as indicated. Data are expressed
as % control colony formation of untreated control siRNA-transfected cells and
represent means ± SD of three experiments. Two-tailed paired t test analysis,
\( P = 0.0001 \), for the combination of control siRNA and IFN-α versus
the combination of SKAR siRNA and IFN-α. (B) Mononuclear cells derived
from the peripheral blood of CML patients were transfected with either control
siRNA or siRNA targeting SKAR and subjected to clonogenic assays in
methylcellulose to assess for myeloid leukemic progenitor colony formation.
Data are expressed as % control colony formation of untreated control siRNA-
transfected cells and represent means ± SD of three experiments. Two-tailed paired t test analysis,
\( P = 0.0035 \), for the combina-
tion of control siRNA and IFN-α versus the combination of SKAR siRNA and
IFN-α. (C) Mononuclear cells derived from peripheral blood of patients with
polycythemia vera were transfected with either control siRNA or siRNA
targeting SKAR and then plated in methylcellulose in the absence or pres-
ence of human IFN-α, as indicated, and BFU-E colony formation was assessed.
Data are expressed as % control colony formation of untreated control
siRNA-transfected cells and represent means ± SD of five experiments.
Two-tailed paired t test analysis, \( P = 0.0135 \), for the combination of control
siRNA and IFN-α versus the combination of SKAR siRNA and IFN-α.

Materials and Methods

Cells and Reagents. KT1, U937, and HT29 cells were grown in RPMI medium
1640 with 10% FBS and antibiotics. OSI-027 (ChemieTek) and Bl-D1870

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Immunoprecipitation and Immunoblotting. Cells were lysed and processed for immunoblotting as previously described (32).

In Vitro Kinase Assays. Assays to detect the phosphorylation of SKAR were performed as in our previous studies (33).

Isolation of Polysomal RNA. Polysomal fractionation was performed according to previously described methods (26).

Cap-Binding Assays. Cell lysates were incubated overnight with 7-methyl-GTP-Sepharose, and retained proteins bound were eluted by boiling the resin-bound complex in SDS/PAGE sample buffer, resolved by SDS/PAGE, and probed with the indicated antibodies (33).

Hematopoietic Progenitor Assays. Peripheral blood from CML or polycythemia vera patients was collected after obtaining informed consent approved by the Institutional Review Board of Northwestern University. Hematopoietic progenitor colony formation was determined in clonogenic assays in methylcellulose, as in our previous studies (49).

Additional information is available in SI Materials and Methods.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants CA77816, CA15566, and CA161796; Merit Review Grant ID1C00001601 from the Department of Veterans Affairs; and funds made available to E.N.F. as a Tier 1 Canada Research Chair.

Supporting Information

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SI Materials and Methods

Cells and Reagents. Immortalized mouse embryonic fibroblasts (MEFs) from S6 kinase (S6k)/S6k2−/− mice were cultured in DMEM with 10% FBS and antibiotics. Recombinant human IFN-α was provided by Hoffmann-La Roche. Antibodies against the p-RXRXXpS/T motif, S6 ribosomal protein (rpS6), S6K, phospho-eF4B, eukaryotic initiation factor (eIF)4B, anti-p70S6k, phospho-T389-p70S6k, phospho-Ser240/244-rpS6, rpS6, eIF4A, eIF4G, p90 ribosomal protein S6 kinase (RSK1), His, and GST were obtained from Cell Signaling Technology, and the anti-S6K1 Aly/REF-like target (SKAR) antibody was from Bioss. A monoclonal antibody recognizing human ISG15 was kindly provided by Ernest Borden (Tauscing Cancer Center, Cleveland, OH). An antibody against programmed cell death 4 (PDCD4) protein was purchased from Rockland. An antibody against S6K2 was from Abgent. Antibodies against p21WAF1/CIP1, phospho-Ser67-PDCD4, and phospho-Ser221-RSK1 were purchased from Abcam. Recombinant GST-SKAR protein was purchased from Abnova. Activated His-tagged-RSK1 (His-RSK1) recombinant protein was from Life Technologies. 7-Methyl-GTP-Sepharose was from GE Healthcare. siRNA specific for SKAR was from Bioroll Biotechnology, and transfections were performed using the siRNA transfection reagent TransIT-TKO from Mirus Bio. Nucleofector solution was from Lonza Cologne. Protein phosphatase 2A (PP2A) and anti-GAPDH were from Millipore. Anti-GFP and anti-Myc epitope tag were from Pierce Biotechnology. CleanBlot IP (immunoprecipitation) Detection Reagent was from Thermo Scientific. The His60 Ni Gravity Kit was purchased from Clontech. Anti-chicken IgY agarose and chicken anti-mouse were from Aves Labs. [r-32P]ATP was from PerkinElmer Life Sciences.

Immuno precipitation and Immunoblotting. Cells were treated with 1,000 IU/mL IFN-α for the indicated times shown in the figures and lysed in phosphorylation lysis buffer (PLB) (2). Cell lysates were incubated overnight with anti-SKAR antibody and biotin-conjugated. Streptavidin agarose was added to the anti-SKAR mixture and incubated with mixing for 2 h at 4 °C. After extensive washing with PLB + 1% Nonidet P-40, the bound immunoprecipitates were eluted by boiling the resin-bound complex in SDS/PAGE sample buffer and processed for immunoblotting as previously described (2).

In Vitro Phosphatase Assays. These assays were performed according to previously described methodologies (3). Briefly, 1 μg of active His-RSK1 was incubated at 37 °C for 30 min in PP2A buffer (50 mM Tris, 1 mM DTT, 1 mM MnCl2, pH 8.0) containing 0.1 U of PP2A and used for protein-binding assays.

Protein Binding. Recombinant His-RSK1 (0.5 μg) active or not active proteins were annealed with 0.5 μg of GST-SKAR in 100 μl of 50 mM Tris, 100 mM sodium chloride (pH 8.0) for 1 h in 4 °C before they were bound to the His affinity column as described (3).}

Isolation of Polysomal RNA. KT1 cells were transfected with control siRNA or siRNA targeting SKAR, and after 24 h of starvation were treated with 2,500 IU/mL IFN-α for 24 h. Polysomal fractionation was performed using slight modifications to previously described methods (4). The polysomal fractions were pooled, and total RNA was isolated using the RNA AllPrep Kit (Qiagen).

Quantitative RT-PCR. Cells were treated with 5,000 IU/mL IFN-α for 6 h, and total RNA was isolated using the RNeasy Kit (Qiagen). Real-time RT-PCR (TaqMan) for the Isg15 and p21WAF1/CIP1 genes was performed as previously described (4, 5).

Soft Agar Assays. Anchorage-independent cell growth was determined in soft agar assays as previously described (6). Equal numbers of cells were plated in soft agar and allowed to form colonies for 14 d.

**Fig. S1.** IFN-α–dependent phosphorylation of SKAR in U937 cells. Serum-starved U937 cells were treated with IFN-α for the indicated times. Total cell lysates were immunoprecipitated using anti-SKAR antibody conjugated to biotin and pulled down by streptavidin agarose. Phosphorylated SKAR was detected by immunoblotting with an anti–phospho-RXRXX*S/*T motif antibody. Protein levels of SKAR in immunoprecipitates and lysates are shown.

**Fig. S2.** Effects of different kinase inhibitors on IFN-α–dependent phosphorylation of SKAR. KT1 cells were transfected with pCMV-Myc-SKARα and treated with IFN-α for 30 min in the presence or absence of OSI-027 or BI-D1870. Equal amounts of cell lysates were immunoprecipitated with chicken anti-myc antibody and purified by anti-chicken IgY agarose. The immunoprecipitates were subsequently resolved by SDS/PAGE and immunoblotted with antibodies against the phospho-RXRXX*S/*T motif or anti-myc, as indicated.
**Fig. S3.** IFN-α induces phosphorylation of SKAR in MEF cells in an S6K1-dependent manner. Serum-starved S6k1+/+ S6k2+/+ (wild type), S6k1−/−, S6k2−/−, and S6k1,2−/− MEFs were incubated with IFN-α for 30 min. Equal amounts of total cell lysates were immunoprecipitated with an anti-SKAR antibody, resolved by SDS/PAGE, and immunoblotted with the anti–phospho-RXRXS*/T* motif antibody or anti-SKAR, as indicated. Immunoblotting of total cell lysates from the same experiment with the indicated antibodies is also shown.

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**Fig. S4.** Equal amounts of active His-RSK1 (His-RSK1-A) or active His-RSK1 subjected to an in vitro phosphatase (PP2A) assay (His-RSK1-NA) were resolved by SDS/PAGE, immunoblotted with anti-His antibody, and, after stripping the membranes, reprobed with p-Ser221-RSK1 antibody.

A

**Fig. S5.** Requirement of SKAR for IFN-α–dependent phosphorylation of PDCD4 (A) and eIF4B (B). U937 cells were transfected with either control siRNA or siRNA specifically targeting SKAR and treated with IFN-α, as indicated. Equal amounts of total cell lysates were resolved by SDS/PAGE and immunoblotted with the indicated antibodies.

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Fig. S6. Requirement of SKAR for the generation of the antineoplastic effects of IFN-α. (A) HT29 cells were transfected with control siRNA or SKAR siRNA. Equal numbers of cells were plated in soft agar in the presence or absence of IFN-α, and anchorage-independent growth was assessed. Representative images of the soft agar wells for the indicated conditions are shown. UT, untreated. (B) Quantitation of colony numbers from five independent experiments including the one shown in A. Data are expressed as % untreated control siRNA-transfected cells and represent means ± SD of five experiments. Two-tailed paired t test analysis showed $P = 0.0002$ for the combination of control siRNA and IFN-α versus the combination of SKAR siRNA and IFN-α. (C) HT29 cells were transfected with either the vector pCMV-Myc or pCMV-Myc-SKARα, as indicated. Equal numbers of cells were subsequently plated in soft agar in the presence or absence of IFN-α and allowed to form colonies for 14 d. Representative images of the soft agar wells are shown. (D) Quantitation of colony numbers from four independent experiments including the one shown in C. Data are expressed as % control siRNA-transfected cells and represent means ± SD of four experiments. Two-tailed paired t test analysis revealed $P = 0.0051$ for the combination of control pCMV-Myc and IFN-α versus the combination of pCMV-Myc-SKARα and IFN-α.