THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 37, pp. 25051–25064, September 11, 2009
Printed in the U.S.A.

Role of Schlafen 2 (SLFN2) in the Generation of Interferon α-induced Growth Inhibitory Responses*

Received for publication, June 5, 2009, and in revised form, July 9, 2009 Published, JBC Papers in Press, July 10, 2009, DOI 10.1074/jbc.M109.030445

Efstratios Katsoulidis[‡], Nathalie Carayol[‡], Jennifer Woodard[‡], Iwona Konieczna[‡], Beata Majchrzak-Kita[§], Alison Jordan[‡], Antonella Sassano[‡], Elizabeth A. Eklund[‡], Eleanor N. Fish[§], and Leonidas C. Platanias[‡1]

From the [‡]Robert H. Lurie Comprehensive Cancer Center and Division of Hematology-Oncology, Northwestern University Medical School and Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois 60611 and the [§]Division of Cell and Molecular Biology, Toronto Research Institute, University Health Network and Department of Immunology, University of Toronto, Toronto, Ontario M5G 2M1, Canada

The precise STAT-regulated gene targets that inhibit cell growth and generate the antitumor effects of Type I interferons (IFNs) remain unknown. We provide evidence that Type I IFNs regulate expression of Schlafens (SLFNs), a group of genes involved in the control of cell cycle progression and growth inhibitory responses. Using cells with targeted disruption of different STAT proteins and/or the p38 MAP kinase, we demonstrate that the IFN-dependent expression of distinct Schlafen genes is differentially regulated by STAT complexes and the p38 MAP kinase pathway. We also provide evidence for a key functional role of a member of the SLFN family, SLFN2, in the induction of the growth-suppressive effects of IFNs. This is shown in studies demonstrating that knockdown of SLFN2 enhances hematopoietic progenitor colony formation and reverses the growth-suppressive effects of IFNα on normal hematopoietic progenitors. Importantly, NIH3T3 or L929 cells with stable knockdown of SLFN2 form more colonies in soft agar, implicating this protein in the regulation of anchorage-independent growth. Altogether, our data implicate SLFN2 as a negative regulator of the metastatic and growth potential of malignant cells and strongly suggest a role for the SLFN family of proteins in the generation of the antiproliferative effects of Type I IFNs.

Type I interferons (IFNs)[2] are potent inhibitors of cell growth of both normal and malignant cells *in vitro* and *in vivo* and play critical roles in the immune surveillance against cancer (1–4). The potent antitumor properties of Type I IFNs have prompted extensive efforts over the years to understand the mechanisms by which these cytokines generate signals and induce biological responses. Key events elicited during engagement of the Type I IFN-receptor have been identified, and major signaling cascades that are activated in an IFN-dependent manner have been defined. The Jak-STAT pathway is the most important pathway in the regulation of IFN-inducible gene transcription and probably the best studied and characterized IFNα-regulated signaling pathway to date (reviewed in Refs. 2 and 5–7). Beyond the Jak-STAT pathway, other highly relevant cellular cascades in IFN signaling are MAP kinase pathways (8–13) that control auxiliary signals for optimal gene transcription and Akt/mTOR pathways that promote mRNA translation of IFN-stimulated genes (ISGs) (14–18). An emerging model for the production of Type I IFN-inducible gene products involves transcriptional regulation of ISGs by Jak-STAT pathways, immediately followed by mRNA translation of such transcripts in an mTOR/4EBP1-dependent manner (17, 18).

The identification and definition of Type I IFN receptor-generated signals that promote transcription and mRNA translation of target genes has provided critical information of how early signals at the receptor level ultimately translate to Type I IFN responses. A remaining challenge in the IFN signaling field is the identification of specific genes or groups of genes that specifically account for the induction of the diverse biological responses of IFNs. Various proteins that are involved in the generation of the antiviral effects of IFNs have been identified over the years (19). However, very little is known on ISG products that participate in the generation of IFN-dependent antiproliferative responses. In fact, the key IFN-inducible gene products that mediate growth inhibitory responses in different cell types remain largely unknown.

The Schlafen (SLFN) (from the German word *schlafen* or sleeping) family of proteins includes several members that have previously been shown to control cell cycle progression and growth arrest (20–26). These proteins contain a common N-terminal (AAA) domain that is involved in GTP/ATP binding (20, 22), whereas a subgroup of these proteins, the long SLFNs, have motifs found in members of Superfamily I of DNA/RNA helicases (21). There is evidence that Schlafen proteins promote growth inhibitory responses (20) and modulate cell cycle progression by inhibiting cyclin D1 (22). Although limited studies have been conducted on the roles of distinct Schlafen group members on the regulation of cellular functions, there is emerging evidence indicating a potentially important role for these proteins in the control of cell cycle progression. Regardless, very little is known on the potential involvement of SLFN genes and their products in the induction

* This work was supported, in whole or in part, by National Institutes of Health Grants CA100579, CA77816, and CA121192. This work was also supported by a Merit Review Grant from the Department of Veterans Affairs and by Canadian Institutes of Health Research Grant MOP 15094.

[1] To whom correspondence should be addressed: Robert H. Lurie Comprehensive Cancer Center, Northwestern University Medical School, 710 North Fairbanks St., Olson 8250, Chicago, IL 60611. Tel.: 312-503-4267; Fax: 312-908-1372; E-mail: l-platanias @northwestern.edu.

[2] The abbreviations used are: IFN, interferon; STAT, signal transducers and activators of transcription; MAP, mitogen-activated protein; ISG, IFN-stimulated gene; MEF, mouse embryonic fibroblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; Ctrl, control; shRNA, small hairpin RNA; MAPK, MAP kinase; RT, reverse transcription; SLFN, Schlafen.

of antiproliferative responses induced by IFNs or other growth-suppressive cytokines.

In the present study we examined the induction of expression of various mouse SLFN family members during treatment of sensitive cells with IFNα. Our data demonstrate that *SLFN1* and *SLFN2* (group I), *SLFN3* (group II), as well as *SLFN5* and *SLFN8* (group III) are all genes inducible by treatment of sensitive cells with mouse IFNα. Using defined knock-out cells for different STAT proteins and/or the p38 MAP kinase, we provide evidence for differential regulation of distinct SLFN members by different STAT complexes and the p38 MAP kinase. In other studies we provide evidence that knockdown of SLFN2 enhances murine hematopoietic progenitor colony formation and reverses the growth-suppressive effects of IFNα and IFNγ on normal hematopoiesis. In addition, our data show that NIH3T3 and L929 fibroblast cells with stable knockdown of SLFN2 form more colonies in soft agar compared with control cells, implicating this member of the SLFN family of proteins in the regulation of anchorage-independent growth. Altogether, our results indicate that SLFN2 acts as a negative regulator of the metastatic and growth potential of malignant cells, and it is an effector element in the generation of Type I IFN-induced antiproliferative responses.

MATERIALS AND METHODS

Cells Lines and Antibodies—NIH3T3 and L929 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and antibiotics. Immortalized mouse embryonic fibroblasts (MEFs) from p38α knock-out mice (27) were kindly provided from Dr. Angel Nebreda (CNIO (Spanish National Cancer Center), Madrid, Spain). Immortalized STAT1 knock-out (28) and STAT3 knock-out (29) MEFs were generously provided by Dr. David Levy (New York University, New York, NY). In the figures, *STAT3 WT* refers to $STAT3^{flox/-}$ MEFs (29), whereas *STAT3 KO MEFs* refers to MEFs resulting from deletion of exons 16–21 of STAT3 by infection with a retrovirus encoding Cre recombinase (29). The different MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. A custom-made polyclonal antibody against the N-terminal region (amino acids 1–14) of mouse SLFN2 was produced and purified via New England Peptide LLC (Gardner, MA). Antibodies against Cyclin D1, Cyclin D3, CDK 4, CDK 6, p15 INK, and p27 KIP were obtained from Cell Signaling Technology (Danvers, MA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Chemicon International (Temecula, CA). An antibody against Lamin A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lysis, Isolation of Nuclear and Cytosolic Fractions, Immunoprecipitations, and Immunoblotting—The cells were lysed in phosphorylation lysis buffer as described in our previous studies (12, 30). For the detection of IFN-dependent SLFN2 translocation, the cells were treated with 10^4 IU/ml IFNα for the indicated times or were left untreated. Nuclear and cytosolic fractions were isolated using the Pierce NE-PER kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). For the detection of SLFN2 protein expression, cells were treated with 1.5×10^3 IU/ml IFNα for the indicated times or were left untreated. Immunoprecipitations and immunoblotting using an ECL method were performed as previously described (12, 30).

Antiviral Assays—The antiviral effects of IFNα were determined using standard methodologies as in previous work (13), using encephalomyocarditis virus as the challenge virus.

Mobility Shift Assays—Actively growing cells were treated with 10^4 IU/ml mouse IFNα for 15 min. Equal amounts of nuclear extracts from untreated or IFNα-treated cells were analyzed using electrophoretic mobility shift assays with oligonucleotides to detect SIF or ISGF3 complexes, as in our previous studies (33, 34).

siRNA Transfection and Generation of Stable SLFN2 Knockdown Cells—Transient knockdown of SLFN2 was performed using either SLFN2 ON-TARGETplus SMARTpool siRNA (SLFN2 siRNA1) and nontargeting control pool siRNA (Ctrl siRNA1) (Thermo Fisher Scientific, Waltham, MA) or a Silencer select SLFN2 siRNA pool (SLFN2 siRNA) and a Silencer select control nontargeting siRNA (Ctrl siRNA2) (Applied Biosystems, Foster City, CA). The siRNA transfection reagent TransIT-TKO was used according to the manufacturer's instructions (Mirus Bio Corporation, Madison, WI). For the generation of stable SLFN2 knockdown NIH3T3 and L929 cells, a commercially available system from Clontech was used. Briefly, SLFN2 ON-TARGETplus SMARTpool siRNA and control scrambled sequences were used as templates in the Clontech shRNA sequence designer tool for Clontech pSIREN vectors. Plasmids were sequenced to verify the presence of siRNA encoding insert and then used for retroviral infection of NIH3T3 and L929 cells. Infected pSIREN-shRNA expressing cells were green fluorescent and were selected by flow cytometry.

Cell Proliferation Assays—Cell proliferation assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide method were performed as in our previous studies (35, 36).

Hematopoietic Progenitor Cell Assays and Soft Agar Assays—Mouse hematopoietic progenitor colony formation was assessed as previously described (12, 37). Colony formation assays were performed using Sca1+ cells isolated from mouse bone marrow stem cells according to the manufacturer's instructions (MACS kit, Miltenyi Biotec Inc., CA). The cells were plated in methocult methylcellulose media (Stemcell Technologies, Seattle, WA) in the presence or absence of 10^3 IU/ml IFNα, and colony formation was assessed after 7 days of culture. Anchorage-independent growth was assessed in soft agar assays in duplicate, carried out essentially as previously described (38). Briefly, the cells were suspended in 0.3% top agar over a bottom layer of 0.5% agar in 6-well plates. The solidified soft agar was overlaid with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. The medium was changed every 4–5 days. The colonies were scored after 11 days (NIH3T3 cells) or 8 days (L929 cells) of culture.

mRNA Isolation and Real Time PCR Probes and Primers—Cells were treated with 5×10^3 IU/ml of IFNα for the indicated times. Isolation, purification of mRNA, and conversion into cDNA was performed using the respective kits and oligo(dT)s

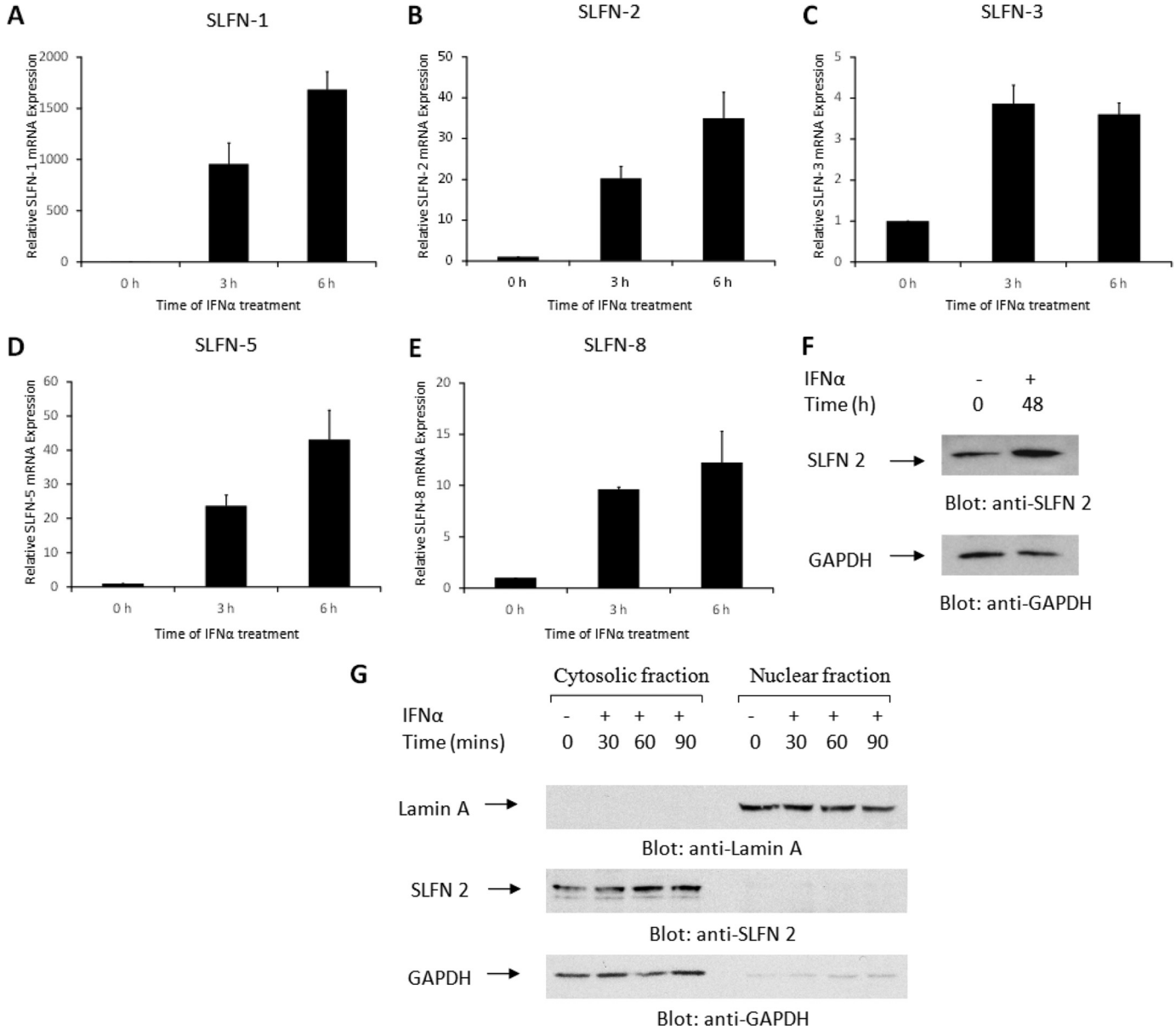

FIGURE 1. **IFNα-inducible expression of *SLFN* family members.** *A–E*, NIH3T3 cells were treated with IFNα for 3 or 6 h or left untreated as indicated. Total RNA was subsequently isolated, and the expression of *SLFN1* (*A*), *SLFN2* (*B*), *SLFN3* (*C*), *SLFN5* (*D*), and *SLFN8* (*E*) was analyzed by real time RT-PCR, using specific primers and *GAPDH* as an internal control. The data are expressed as fold increase over control untreated samples and represent the means ± S.E. of several experiments for *SLFN1* ($n = 3$), *SLFN2* ($n = 7$), *SLFN3* ($n = 6$), *SLFN5* ($n = 4$), and *SLFN8* ($n = 5$). *F*, NIH3T3 cells were treated with IFNα for 48 h, and after cell lysis, the proteins were resolved by SDS-PAGE and immunoblotted with an anti-SLFN2 antibody. *G*, NIH3T3 cells were treated with mouse IFNα, as indicated. Cytosolic and nuclear fractions were obtained, and the proteins were resolved by SDS-PAGE and immunoblotted with an anti-SLFN2 antibody. Immunoblotting with antibodies against Lamin A and GAPDH was also performed to control for successful separation of nuclear and cytosolic fractions.

from Qiagen according to the manufacturer's instructions. Validated, inventoried probes and primers for real time PCR and TaqMan PCR master mix were purchased from Applied Biosystems (Foster City, CA). The probes and primers were: *SLFN1*, Mm00488306_m1; *SLFN2*, Mm 00488307_m1; *SLFN3*, Mm00488309_g1; *SLFN5*, Mm00806095_m1; *SLFN8*, Mm00824405_m1; and *ISG15*, Mm01705338_s1. *GAPDH* (Mm99999915_g1) was used as an internal control.

RESULTS

In initial studies we determined whether treatment of cells with IFNα induces expression of different *SLFN* genes. NIH3T3 cells were treated with mouse IFNα for different times, and the induction of mRNA expression for key members of the *SLFN* gene family was determined. As shown in Fig. 1, mRNA expression for different *SLFN* genes was inducible at various degrees in response to IFNα treatment. The most pronounced induction was for *SLFN1* (Fig. 1*A*), followed by *SLFN5*, *SLFN2*, and *SLFN8* (Fig. 1, *B*, *D*, and *E*). *SLFN3* was induced clearly to a lesser degree than other *SLFN*s (~4-fold), but its induction was consistently seen (Fig. 1*C*).

To better understand the regulation of SLFN proteins during engagement of the Type I IFN receptor, we generated and used an anti-SLFN2 antibody to directly examine the expression of SLFN2 protein after IFNα treatment of cells. This antibody was custom-generated via a commercial vendor against a conserved

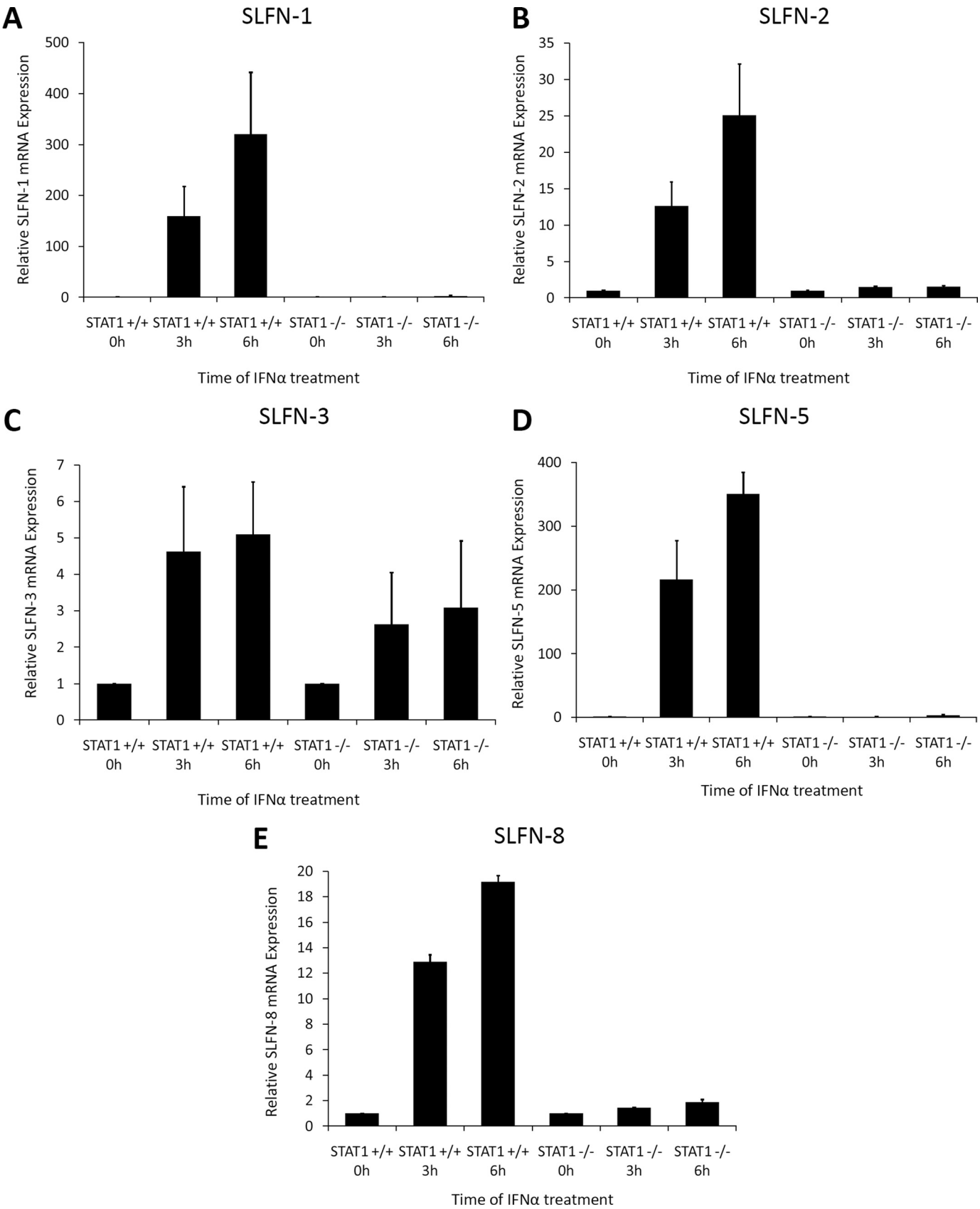

FIGURE 2. **Differential requirement for STAT1 in IFNα-inducible expression of distinct *SLFN* mRNAs.** STAT1$^{+/+}$ and STAT1$^{-/-}$ MEFs were treated with IFNα for the indicated times. Total RNA was isolated and the expression of *SLFN1* (*A*), *SLFN2* (*B*), *SLFN3* (*C*), *SLFN5* (*D*), and *SLFN8* (*E*) mRNAs was determined by real time RT-PCR, after normalization for *GAPDH* expression. The data are expressed as fold increases over control untreated samples and represent the means $\pm$ S.E. of two independent experiments for *SLFN3* and *SLFN5*, three for *SLFN8*, four for *SLFN1*, and seven for *SLFN2*.

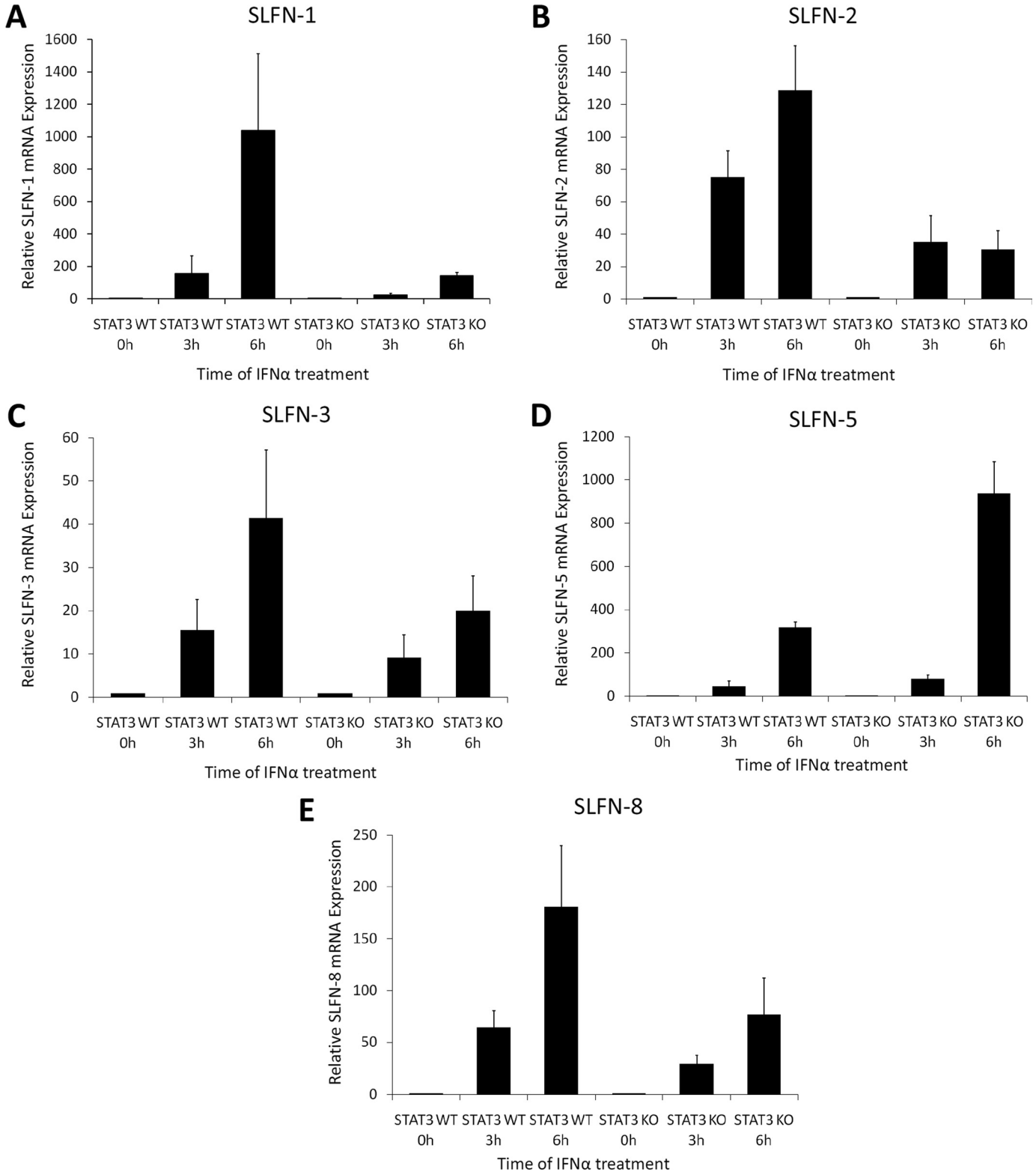

FIGURE 3. **Differential requirement for STAT3 in IFNα-dependent expression of distinct *SLFN* mRNAs.** The indicated MEFs were treated with IFNα for the indicated times. Total RNA was isolated and the expression of *SLFN1* (*A*), *SLFN2* (*B*), *SLFN3* (*C*), *SLFN5* (*D*), and *SLFN8* (*E*) mRNAs was determined by real time RT-PCR, after normalization for *GAPDH* expression. The data are expressed as fold increases over control untreated samples and represent the means ± S.E. of three independent experiments for *SLFN1*, *SLFN2*, and *SLFN5* and six for *SLFN8*.

region in the N terminus of the protein and detects a single band at ~44 kDa, which is consistent with the predicted molecular mass of SLFN2. As shown in Fig. 1*F*, base-line expression of SLFN2 in NIH3T3 cells was clearly detectable, but treatment of cells with IFNα resulted in up-regulation of the expression of the protein (Fig. 1*F*). We also examined the subcellular localization of the protein. In a previous study, it was shown that overexpressed FLAG-tagged SLFN2 in HEK-293T cells is

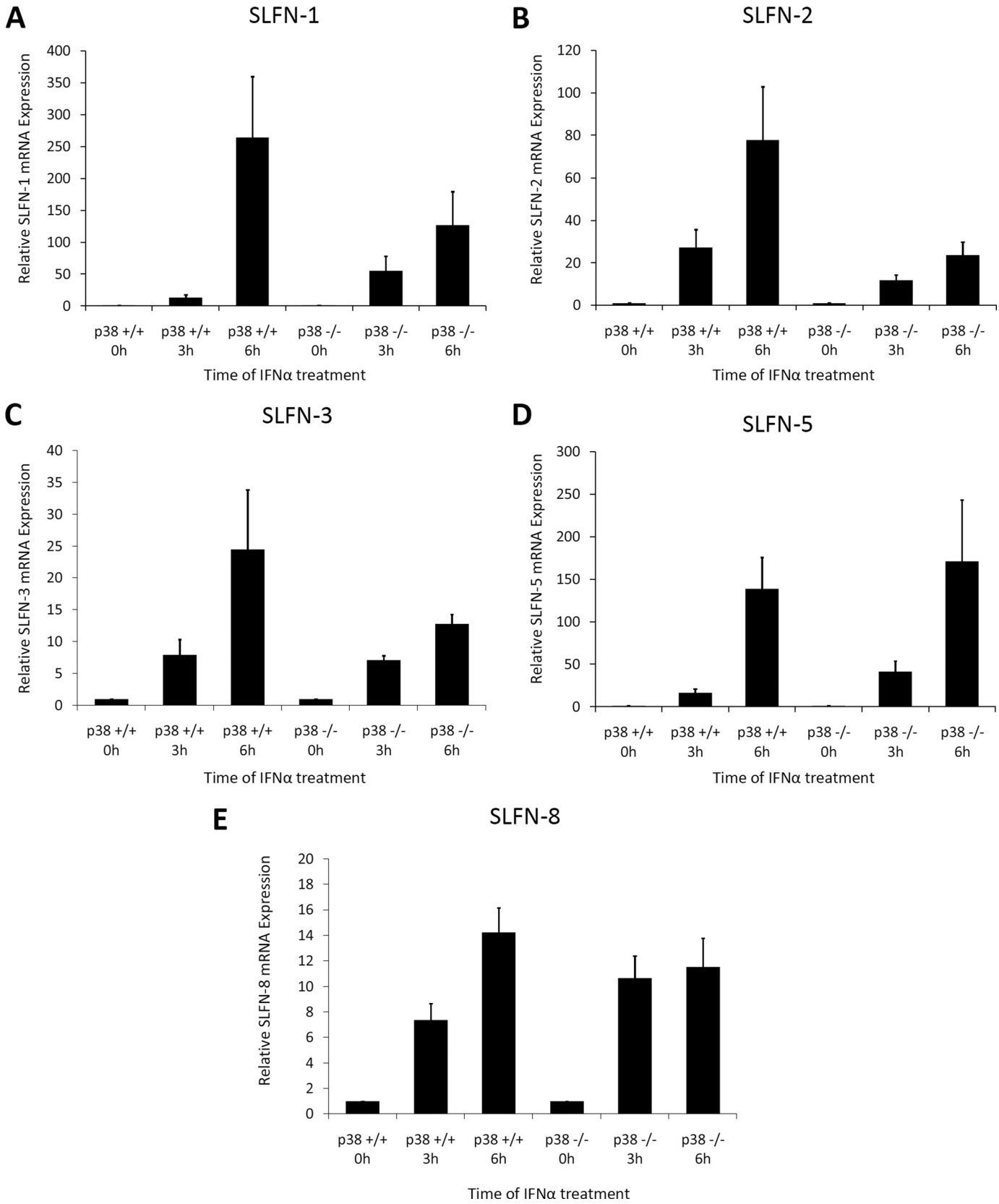

FIGURE 4. **Role of p38α MAPK in the regulation of expression of *SLFN* genes.** p38$\alpha^{+/+}$ and p38$\alpha^{-/-}$ MEFs were treated with IFNα for the indicated times. Total RNA was isolated and the expression of *SLFN1* (*A*), *SLFN2* (*B*), *SLFN3* (*C*), *SLFN5* (*D*), and *SLFN8* (*E*) mRNAs was determined by real time RT-PCR, after normalization for *GAPDH* expression. The data are expressed as fold increases over control untreated samples and represent the means ± S.E. of three independent experiments for *SLFN2*, four for *SLFN1* and *SLFN3*, and five for *SLFN5* and *SLFN8*.

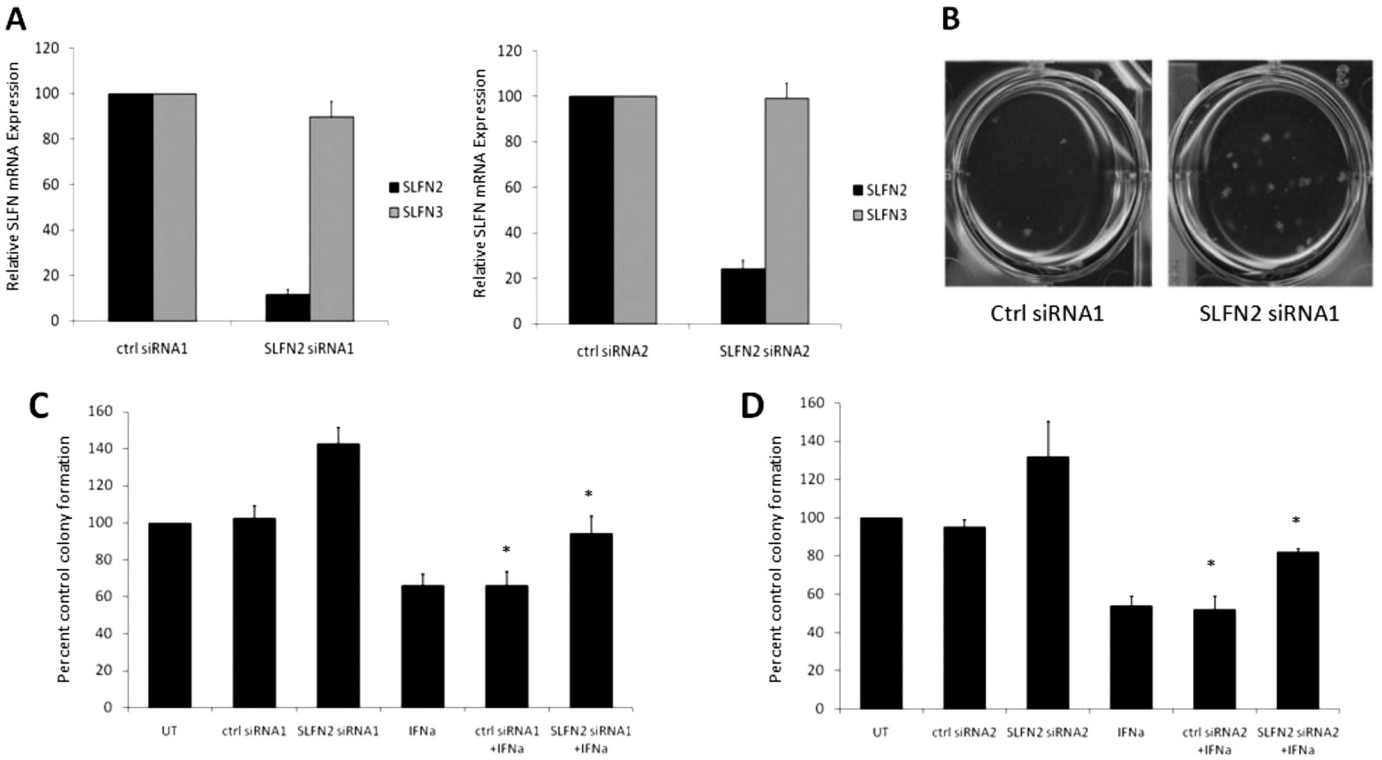

FIGURE 5. **SLFN2 controls hematopoietic progenitor colony formation and promotes the growth-suppressive effects of IFNs on primitive hematopoietic precursors.** *A*, to test whether the available siRNAs were efficient and selective for the knockdown of SLFN2 in murine cells, NIH3T3 cells were transfected with control siRNAs or siRNAs selectively targeting *SLFN2*, and expression of *SLFN2* or *SLFN3* mRNAs was subsequently examined by real time RT-PCR. Two different pools of SLFN2 siRNA (siRNA1 and siRNA2) and control siRNA (Ctrl siRNA1 and Ctrl siRNA2) were used. The data are presented as percentages of expression in control siRNA transfected cells and represent the means ± S.E. of three experiments. *B*, Sca1+ derived, murine hematopoietic progenitor cells were transfected with control siRNA or *SLFN2*-siRNA, and hematopoietic colony progenitor was assessed in clonogenic assays in methylcellulose. Representative plates are shown. *C–D*. Sca1+ stem cells were isolated from murine bone marrows and plated in methylcellulose in the presence or absence of IFNα (*C* and *D*). The cells were either not transfected or were transfected with the control nontargeting siRNAs or SLFN2-targeting siRNAs shown in *A*. Colony formation (colony forming units) of primitive hematopoietic precursors was assessed at day 7 of culture. The data are expressed as percentages of control untransfected cells colony formation and represent means ± S.E. of five (*C*) or four (*D*) independent experiments. Paired *t* test analysis demonstrated a *p* value of 0.0004 for IFNα-treated Ctrl siRNA1 *versus* SLFN2 siRNA1 transfected cells (*C*) and a *p* value of 0.007 for IFNα-treated Ctrl siRNA2 *versus* SLFN2 siRNA2 transfected cells (*D*).

exclusively expressed in the cytoplasm (25). However, FLAG tagging could theoretically interfere with the structural properties and localization of the protein, and the potential translocation of endogenous SLFN2 in response to cytokine treatment has not been known. In studies in which the localization of the endogenous protein was directly determined using the newly generated anti-SLFN2 antibody, we found that endogenous SLFN2 is exclusively expressed in the cytoplasm, and IFNα treatment does not induce its translocation to the nucleus (Fig. 1*G*).

IFNα binding to the type I IFN receptor results in activation of STAT1, STAT2, and STAT3 transcription factors, which form various homo- and/or heterodimers that can bind specific sequences in the promoters of IFN inducible genes (2–7). In addition, IFNα-mediated gene transcription is regulated by auxiliary pathways, such as the p38 MAPK pathway (7–13). To define the roles of distinct STAT proteins and the p38 MAPK in *SLFN* gene expression, experiments were performed using cells with targeted disruption of *STAT1*, *STAT3*, or p38α genes. In initial studies, STAT1$^{-/-}$ MEFs and STAT1$^{+/+}$ parental MEFs were treated with mouse IFNα, and mRNA expression for *SLFN1*, *SLFN2*, *SLFN3*, *SLFN5*, and *SLFN8* was determined. IFNα-dependent expression of all *SLFN* genes was decreased in STAT1 knock-out MEFs compared with parental MEFs, and the effect ranged from a partial impairment (*SLFN3*) to completely defective transcription (*SLFN1*, *2*, *5*, and *8*) (Fig. 2). Similarly, IFNα-inducible *SLFN* expression was examined in STAT3 knock-out MEF cells. The induction of expression of *SLFN1*, *SLFN2*, *SLFN3*, and *SLFN8* genes was decreased in STAT3 knock-out cells although not abrogated (Fig. 3, *A–C*). The expression of *SLFN5* was completely STAT3-independent, and in fact, *SLFN5* expression was enhanced in STAT3 knock-out cells (Fig. 3*D*).

p38 MAPK-activated signaling cascades play important roles in Type I IFN-dependent transcriptional regulation, acting as auxiliaries to STAT pathways, and their function is essential for full transcriptional activation of ISGs (reviewed in Refs. 7 and 39). To determine the role of p38α MAPK-mediated signals in *SLFN* gene expression, we used MEF cells with targeted disruption of the p38α gene (27) in which we have previously shown that IFNα-inducible transcription via ISRE or GAS elements is defective (33). IFNα-dependent mRNA expression for *SLFN1*, *SLFN2*, and, to a lesser degree, *SLFN3* was suppressed in the absence of p38α MAPK (Fig. 4, *A–C*). On the other hand, the group III *schlafen* genes, *SLFN5* and *SLFN8*, were induced by IFNα in a p38α MAPK-independent manner (Fig. 4, *D* and *E*), suggesting that p38 activity is essential for IFN-dependent expression of group I and II but not group III Schlafen genes.

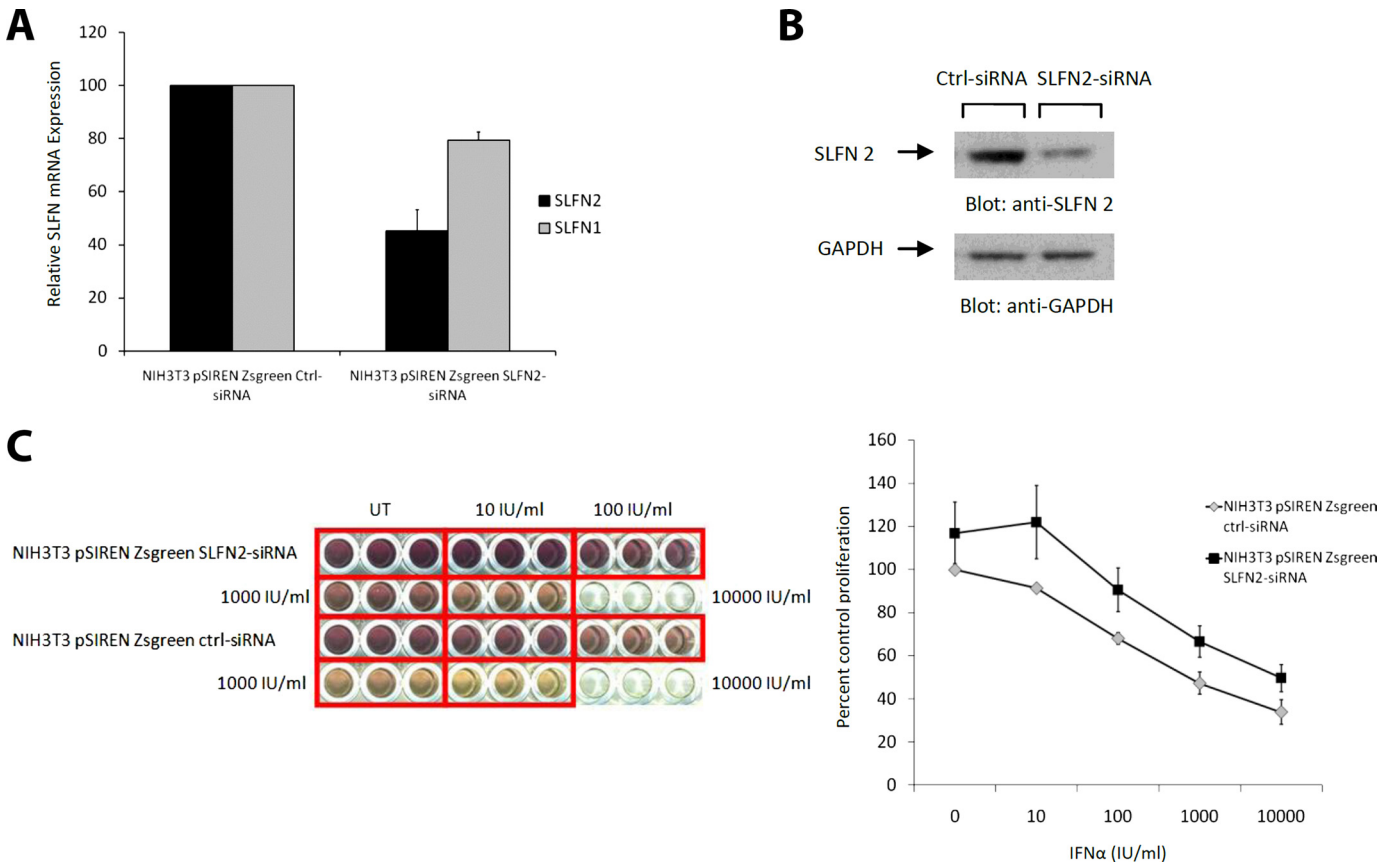

FIGURE 6. **Stable knockdown of SLFN2 enhances cell proliferation and impairs IFNα-dependent growth inhibitory responses but has no effects on the generation of antiviral responses.** *A*, expression of *SLFN2* or *SLFN1* mRNAs in pSIREN Zsgreen-SLFN2 siRNA and pSIREN Zsgreen control-siRNA NIH3T3 cells was determined by real time RT-PCR using specific primers and *GAPDH* as an internal control. The data are presented as percentages of expression in pSIREN Zsgreen control-siRNA cells and represent the means ± S.E. of three experiments. *B*, total cell lysates from pSIREN Zsgreen SLFN2-siRNA or pSIREN Zsgreen control-siRNA NIH3T3 cells were resolved by SDS-PAGE and immunoblotted sequentially with anti-SLFN2 or anti-GAPDH antibodies. *C*, equal numbers of pSIREN Zsgreen SLFN2-siRNA or pSIREN Zsgreen control-siRNA NIH3T3 cells were plated and were left untreated or were treated with the indicated doses of mouse IFNα. After 5–7 days cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assays. A representative experiment is shown in the *left panel*. The means ± S.E. of three experiments, including the one shown in the picture on the *left*, are shown on the *right panel*. *D* and *E*, pSIREN Zsgreen SLFN2 siRNA or pSIREN Zsgreen control-siRNA NIH3T3 cells were treated with IFNα for 15 min, as indicated. Nuclear extracts were reacted with 40,000 cpm of ^{32}P-labeled ISRE (*D*) or SIE (*E*) oligonucleotides, and complexes were resolved by native gel electrophoresis and visualized by autoradiography. The migration of the different STAT complexes is indicated by *arrows*. *F*, wild-type NIH3T3 cells, pSIREN Zsgreen SLFN2 siRNA NIH3T3 cells, or pSIREN Zsgreen control-siRNA NIH3T3 cells were treated with IFNα for the indicated times. Expression of *Isg15* mRNA was determined by real time RT-PCR using *GAPDH* as an internal control. The data are expressed as the means ± S.E. of three experiments. *G*, wild-type NIH3T3 cells, pSIREN Zsgreen SLFN2-siRNA NIH3T3 cells, or pSIREN Zsgreen control-siRNA NIH3T3 cells were incubated in triplicate, in the presence or absence of the indicated concentrations of IFNα. The cells were subsequently challenged with encephalomyocarditis virus (*EMCV*), and cytopathic effects were quantified 24 h later. The data are expressed as percentages of protection from the cytopathic effects of encephalomyocarditis virus. A representative of three independent experiments is shown.

It is well known that Type I and II IFNs are potent regulators of normal and leukemic hematopoiesis and inhibit the growth of primitive hematopoietic precursors *in vitro* and *in vivo* (4, 39, 40). It has been also established that activation of the p38 MAP kinase pathway is required for the generation of the myelosuppressive effects of IFNs on both normal and leukemic progenitors (12, 13). Because SLFN2 is induced by IFNs and its expression is regulated via both STAT and p38 MAPK pathways, we examined whether this protein plays a role in the generation of the myelosuppressive effects of IFNα. In initial experiments, two different specific siRNAs (Fig. 5*A*) were used to knock down SLFN2 expression in murine bone marrow-derived Sca1+ stem cells. Primitive progenitor colony formation was subsequently assessed in clonogenic assays in methylcellulose. Knockdown of SLFN2 in normal hematopoietic progenitors resulted in increased hematopoietic colony formation (Fig. 5, *B–D*), suggesting that this protein plays a critical role in the control of normal hematopoietic progenitor cell growth. Also, as expected, treatment of cells with IFNα (Fig. 5, *C* and *D*) or IFNγ (data not shown) resulted in suppression of hematopoietic progenitor colony formation compared with untreated cells, although the suppressive effects of IFNα were much less noticeable in cells in which SLFN2 was knocked down (Fig. 5, *C* and *D*). Thus, SLFN2 participates in the control of normal hematopoiesis and the generation of the myelossuppressive effects of IFNs, suggesting that this protein may be an effector in the regulation of p38-mediated hematopoietic suppression.

To further analyze the functional relevance of SLFN2 in cell growth regulation and its role in the generation of IFN responses in other cell types, we generated stable SLFN2 knockdown NIH3T3 cells via expression of shRNA-targeting SLFN2 using the pSIREN Zsgreen retroviral system. SLFN2 expression was selectively knocked down in NIH3T3 cells (Fig. 6, *A* and *B*). Cells in which SLFN2 was knocked down exhibited enhanced proliferation compared with their control counterparts (Fig. 6*C*). IFNα treatment resulted in dose-dependent growth sup-

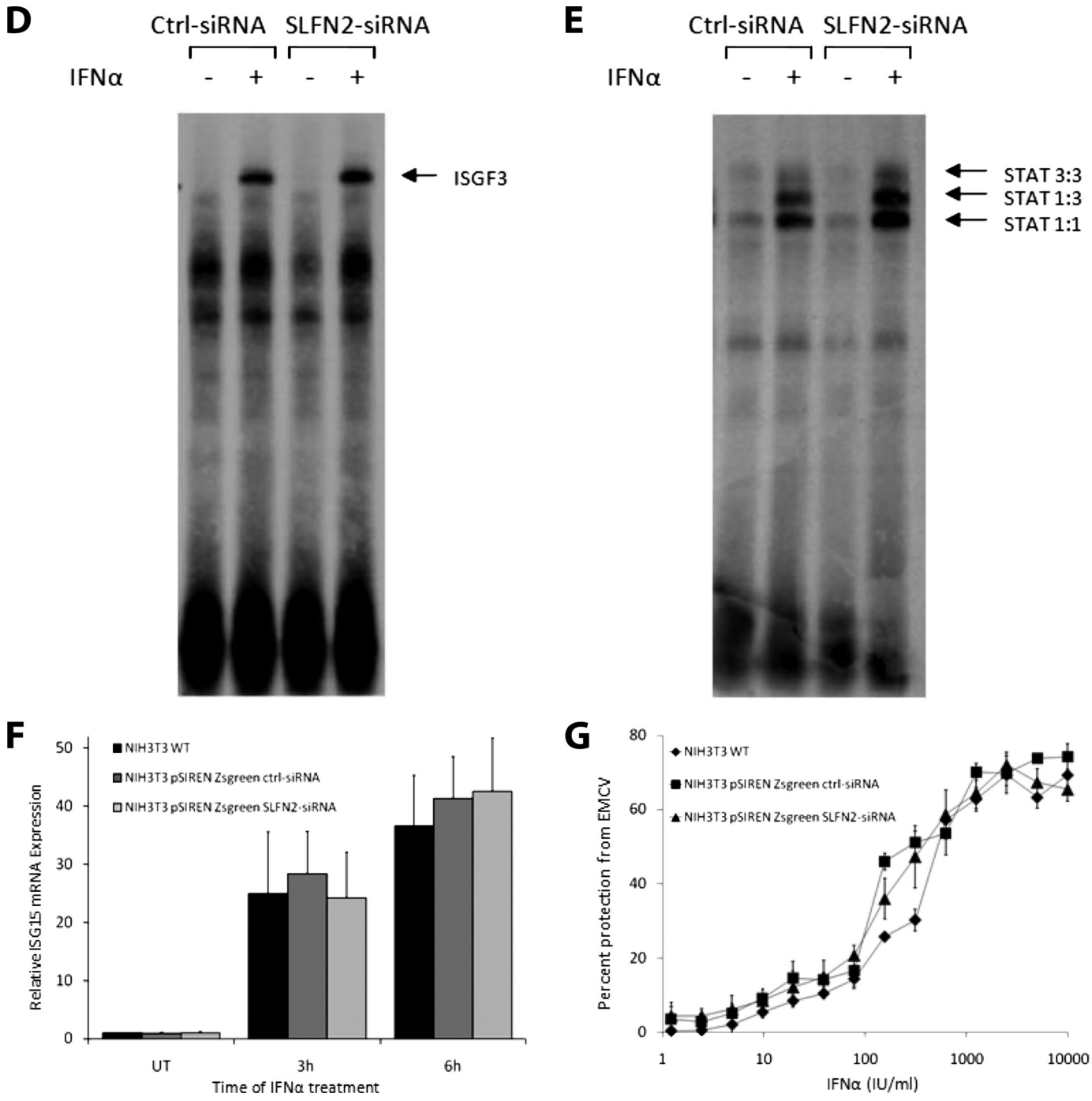

FIGURE 6—*continued*

pression in both NIH3T3 pSIREN Zsgreen Ctrl siRNA and NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells (Fig. 6*C*). However, in NIH3T3 cells in which SLFN2 was knocked down, IFNα-induced antiproliferative responses were clearly decreased compared with cells expressing SLFN2 (Fig. 6*C*), indicating that SLFN2 participates in the generation of the growth inhibitory effects of IFNα. On the other hand, SLFN2 knockdown had no effect on IFNα-dependent formation of STAT-containing DNA-binding complexes (Fig. 6, *D* and *E*). Similarly, IFNα-dependent *Isg15* gene transcription (Fig. 6*F*) or generation of IFNα-induced antiviral responses (Fig. 6*G*) were not affected by SLFN2 knockdown. Thus, targeting SLFN2 appears to be impairing IFNα-dependent cell cycle arrest but not IFN-inducible gene transcription or generation of antiviral responses.

To examine whether SLFN2 plays a role in the control of anchorage-independent growth, we assayed transduced NIH3T3 cells for colony formation in soft agar (41). Colony formation was clearly increased in NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells as compared with NIH3T3 pSIREN Zsgreen Ctrl siRNA cells (Fig. 7, *A* and *B*). Notably, the colonies from NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells were consistently larger (Fig. 7*A*), and the numbers of colonies were increased (Fig. 7*B*) as compared with NIH3T3 pSIREN Zsgreen

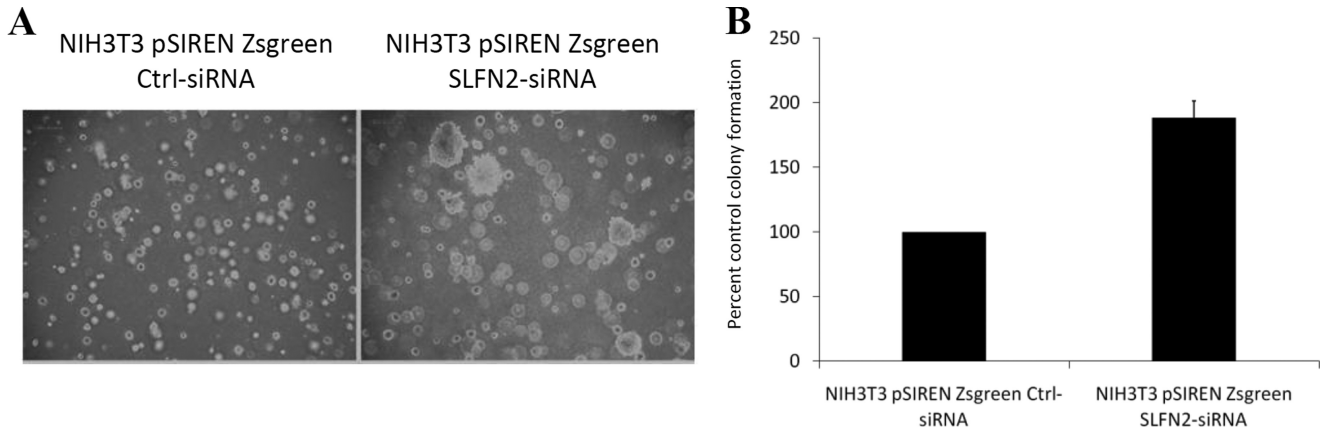

FIGURE 7. **Effects of SLFN2 knockdown on anchorage-independent growth.** *A*, equal numbers of NIH3T3 pSIREN Zsgreen control-siRNA and NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells were plated in a soft agar assay system. Colony formation was analyzed after 11 days of culture. Representative areas of the soft agar plates for NIH3T3 pSIREN Zsgreen control-siRNA and NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells are shown. *B*, colonies were counted, and the results were expressed as percentages of control of NIH3T3 pSIREN Zsgreen control-siRNA-derived colonies. The data shown represent the means ± S.E. of three independent experiments, including the one shown in *A*. Paired *t* test analysis showed a *p* value of 0.01.

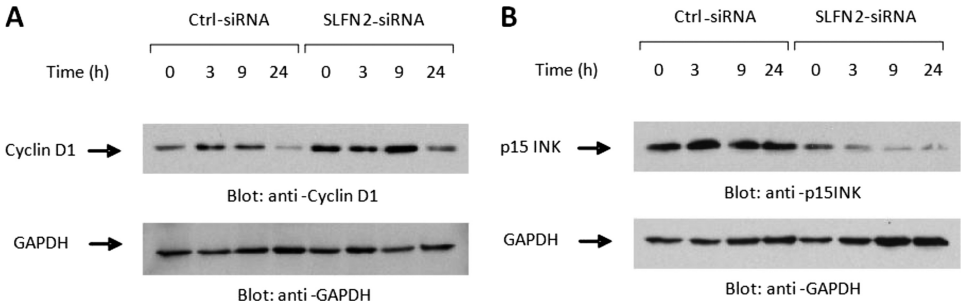

FIGURE 8. **SLFN2 knockdown in NIH3T3 cells modulates expression of Cyclin D1 and p15 INK cell cycle regulators.** NIH3T3 pSIREN Zsgreen control-siRNA and NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells were synchronized via serum starvation. After re-entry of cells into the cell cycle by the addition of serum, the cells were collected at the indicated time points. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against Cyclin D1 (*A*) or p15 INK (*B*), as indicated.

Ctrl siRNA cells. Taken altogether, these data for the first time implicate SLFN2 in the regulation of anchorage-independent growth.

In subsequent studies, we sought to obtain information on the mechanisms by which SLFN2 regulates anchorage-independent cell growth and blocks cell proliferation. Initially, we examined the effects of SLFN2 knockdown on the expression of various key cell cycle regulators. We compared the levels of expression of Cyclin D1, Cyclin D3, CDK4, CDK6, and the CDK inhibitors p27 KIP1 and p15 INK in serum-starved and cycling NIH3T3 control cells or NIH3T3 cells in which SLFN2 was knocked down. As shown in Fig. 8*A*, stable SLFN2 knockdown in NIH3T3 cells resulted in higher basal Cyclin D1 levels of expression than control cells, whereas Cyclin D1 levels were also consistently higher in cycling SLFN2 knockdown cells compared with control cells (Fig. 8*A*). On the other hand, Cyclin D3, as well as CDK4 and CDK6, levels were not significantly altered in cells in which SLFN2 was knocked down across the time points analyzed (data not shown). When the levels of expression of the CDK inhibitors p27 KIP1 and p15 INK were assessed, we noticed that unlike p27 expression, which was not consistently altered (data not shown), p15 INK levels were clearly lower in resting and cycling NIH3T3 pSIREN Zsgreen SLFN2-siRNA transfected cells, compared with NIH3T3 pSIREN Zsgreen Ctrl siRNA transfected cells (Fig. 8*B*). Thus, although additional mechanisms may be involved, these findings suggest that SLFN2 inhibits cell growth and colony formation in part via suppression of cyclin D1 and up-regulation of the CDK inhibitor p15 INK.

To definitively establish the role of SLFN2 in the generation of IFN responses and anchorage-independent growth in nonhematopoietic cells, we stably knocked down SLFN2 in another murine fibroblast cell line, L929. Initially, we examined the IFN-inducible expression of SLFN2 in L929 cells. The cells were treated with mouse IFNα for different times, and the induction of mRNA and protein expression was analyzed. As expected, both SLFN2 mRNA (Fig. 9*A*) and protein (Fig. 9*B*) expression were up-regulated in response to IFNα treatment. We then generated stable SLFN2 knockdown L929 cells via expression of shRNA-targeting SLFN2 using the same pSIREN Zsgreen retroviral system we utilized before to knock down SLFN2 in NIH3T3 cells. Green fluorescent L929 pSIREN Zsgreen cells were selected after retroviral transfection and analyzed for SLFN2 expression. As shown in Fig. 9 (*C* and *D*), stable SLFN2 expression was selectively knocked down in L929 pSIREN Zsgreen SLFN2-siRNA cells compared with L929 pSIREN Zsgreen Ctrl siRNA cells. We next analyzed IFNα-dependent *Isg15* gene transcription (Fig. 9*E*) in SLFN2 stable knockdown L929 cells, as well as the effects of stable SLFN2 knockdown on IFNα-induced antiproliferative responses (Fig. 9*F*). Consistent with the results obtained with NIH3T3 cells, L929 cells with stable SLFN2 knockdown showed enhanced proliferation and were less sensitive to the suppressive effects of IFNα compared with their control counterparts (Fig. 9*F*), whereas IFNα-dependent *Isg15* gene transcription was unaltered (Fig. 9*E*).

We also determined whether SLFN2 knockdown in L929 cells enhances anchorage-independent growth. L929 pSI-

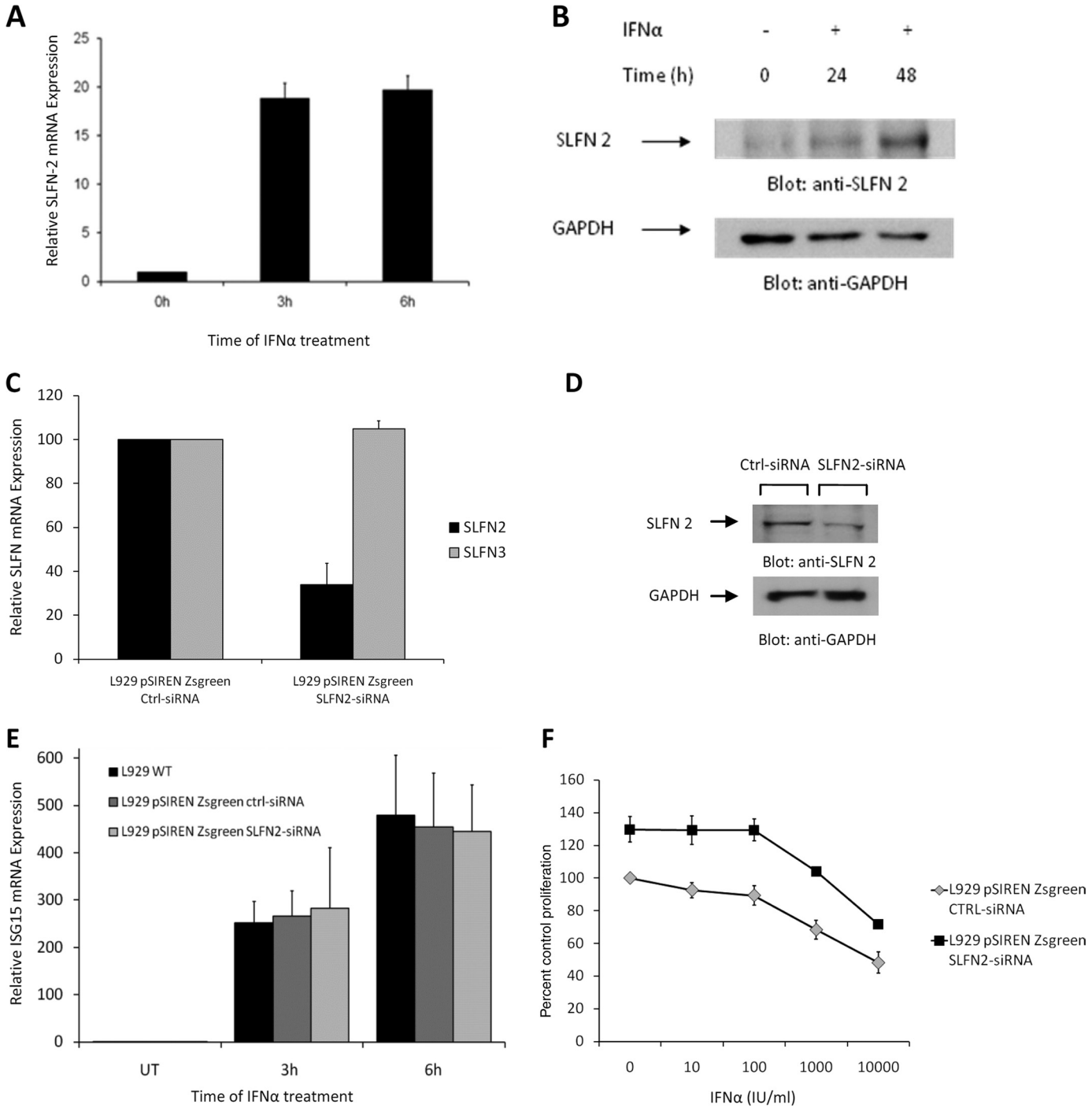

FIGURE 9. **Stable knockdown of SLFN2 enhances cell proliferation and impairs IFNα-dependent growth inhibitory responses.** *A*, L929 cells were treated with IFNα for 3 or 6 h or left untreated as indicated. Total RNA was subsequently isolated, and the expression of *SLFN2* mRNA was analyzed by real time RT-PCR, using specific primers for *SLFN2* and *GAPDH* as an internal control. The data are expressed as fold increases over untreated samples and represent the means ± S.E. of four independent experiments. *B*, L929 cells were either left untreated or were treated with IFNα for 24 or 48 h, as indicated. After cell lysis, the proteins were resolved by SDS-PAGE and immunoblotted with anti-SLFN2 or anti-GAPDH antibodies, as indicated. *C*, expression of *SLFN2* or *SLFN3* mRNAs in L929 Zsgreen-Ctrl siRNA and pSIREN Zsgreen SLFN2-siRNA in L929 cells was determined by real time RT-PCR using specific primers and *GAPDH* as an internal control. The data are presented as percentages of expression in pSIREN Zsgreen control-siRNA cells and represent means ± S.E. of five experiments. *D*, total cell lysates from pSIREN Zsgreen SLFN2-siRNA or pSIREN Zsgreen control-siRNA L929 cells were resolved by SDS-PAGE and immunoblotted sequentially with anti-SLFN2 or anti-GAPDH antibodies. *E*, wild-type L929 cells, pSIREN Zsgreen SLFN2 siRNA L929 cells or pSIREN Zsgreen control-siRNA L929 cells were treated with IFNα for the indicated times. Expression of *Isg15* mRNA was determined by real time RT-PCR using *GAPDH* as an internal control. The data are expressed as fold increases over control untreated cells and represent the means ± S.E. of four experiments. *F*, equal numbers of L929-pSIREN Zsgreen SLFN2-siRNA or L929-pSIREN Zsgreen control-siRNA cells were either left untreated or treated with the indicated doses of mouse IFNα for 5 days, and cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assays. The means ± S.E. of three experiments are shown.

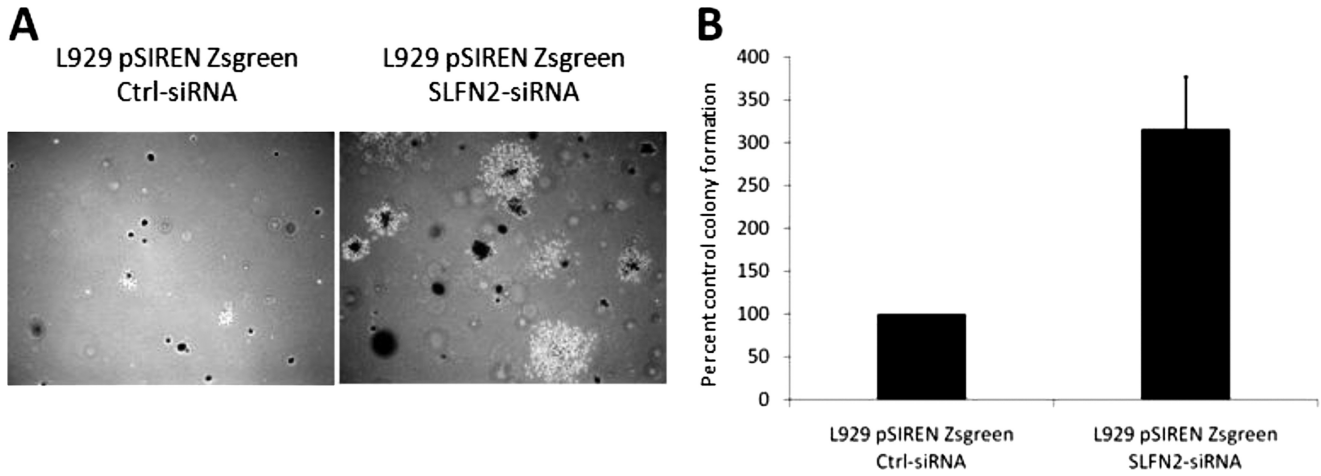

FIGURE 10. **Effects of SLFN2 knockdown on anchorage-independent growth of L929 cells.** *A*, equal numbers of L929 pSIREN Zsgreen control-siRNA and L929 pSIREN Zsgreen SLFN2-siRNA cells were plated in a soft agar assay system. Colony formation was analyzed after 8 days of culture. Representative areas of the soft agar plates for L929 pSIREN Zsgreen control-siRNA and L929 pSIREN Zsgreen SLFN2-siRNA cells are shown. *B*, colonies were counted, and the results were expressed as percentages of control of L929 pSIREN Zsgreen control-siRNA-derived colonies. The data shown represent the means ± S.E. of four independent experiments, including the one shown in *A*. Paired *t* test analysis showed a *p* value of 0.04.

REN Zsgreen SLFN2-siRNA and L929 pSIREN Zsgreen Ctrl siRNA cells were plated, and colony formation was determined after 8 days of culture in soft agar. As depicted in Fig. 10, L929 cells with stable SLFN2 knockdown showed consistently larger colonies (Fig. 10*A*), and there were increased numbers of colonies compared with L929 pSIREN Zsgreen Ctrl siRNA cells (Fig. 10*B*).

DISCUSSION

The family of Schlafen genes was originally identified during screening for growth regulatory genes that are differentially expressed during lymphocyte development (20). Originally, *SLFN* family members 1, 2, 3, and 4 were identified and studied (20). Initial studies had suggested that *SLFN* genes suppress growth and participate in the maintenance of the quiescent state of naive T lymphocytes, as shown by experiments involving ectopic expression of *SLFN1*, demonstrating disruption of thymic development (20). Subsequently, and based on sequence homology, Geserick *et al.* (21) identified additional *SLFN* genes (*SLFN5*, *SLFN8*, *SLFN9*, and *SLFN10*) forming a cluster on mouse chromosome 11 where the *SLFN1–4* genes are also located.

The different members of the *SLFN* family of proteins can be classified into three subgroups (20, 21). The first group includes *SLFN1* and *SLFN2*, which encode for the smallest two SLFN proteins, with predicted molecular masses of 37 and 42 kDa, respectively (20). They contain an AAA domain, found in ATPases (42), and an adjacent "SLFN box," which is common to all SLFN proteins (21, 25). Overexpression of *SLFN1* results in potent growth suppression by inducing G_1 cell cycle arrest (20) through inhibition of cyclin D1 expression (22). In addition, it appears that accumulation of SLFN1 protein to the nucleus correlates with induction of its growth-suppressive effects (43).

The second group of SLFN proteins includes SLFN3 and SLFN4, which have predicted molecular masses of 58 and 68 kDa, respectively. These proteins have in their structures a small sequence motif (SWA(L/V)DL) (21, 25), also shared by the third group. This third group of SLFN proteins contains a Superfamily I DNA/RNA helicase motif not found in group I/II SLFNs, whereas the members of this group are significantly larger proteins with molecular masses ranging between 100 (SLFN5) and 104 kDa (SLFN8) (21). Although the roles of members of this SLFN group remains to be established, studies with *SLFN8* transgenic mice have suggested an important regulatory role for this *SLFN gene* in T cell development and differentiation (21). Notably, different SLFN family members have been shown to be induced in response to a wide variety of stimuli, including CpG-DNA (24), the bacterial pathogens *Brucella* and *Listeria* (44), and terminal differentiation of myeloid cells (21), suggesting that signals from divergent stimuli converge on SLFN family members to control cell cycle progression.

Despite the fact that studies on the functional relevance and biochemical activities of SLFN proteins have been very limited so far, the emerging evidence suggests key regulatory roles for these proteins on cell cycle progression and growth arrest. Yet very little is known on their potential involvement in the generation of the suppressive effects of growth inhibitory cytokines. Type I IFNs are probably the most prominent cytokines that generate growth inhibitory and antitumor effects; and these properties have over the years led to their introduction in the treatment of various leukemias and solid tumors (3). Importantly, although it is well established that IFNs regulate cell cycle progression and induce G_0/G_1 cell cycle arrest, very little is known about the IFN-inducible proteins that mediate such responses. In the present study, we provide the first evidence that IFNs regulate expression of members of the *SLFN* family of genes and proteins. Our data demonstrate that IFNα is a potent inducer of different *SLFN* family members, including members of Group I (*SLFN1* and *SLFN2*), Group II (*SLFN3*), and Group III (*SLFN5* and *SLFN8*). Moreover, in work aimed to define the regulation of expression of these proteins by IFNs, we established the differential involvement of distinct IFN-activated STAT proteins and the p38 MAP kinase in their regulation.

Our finding that members of the *SLFN* family of proteins are engaged by the Type I IFN receptor in a STAT- and/or p38

MAPK-dependent manner provided a direct link between IFN-activated Jak-STAT pathways and cellular elements controlling cell cycle progression. Such a link led us to further studies aimed to define the functional relevance of the SLFN pathway in the generation of IFN responses. We focused our efforts on SLFN2, a member of group I SLFNs, whose expression was greatly induced by activation of the Type I IFN receptor. Selective knockdown of this protein resulted in enhanced bone marrow-derived hematopoietic progenitor cell growth, whereas IFN-dependent suppression of normal hematopoietic progenitor colony formation was less noticeable in such cells. Nevertheless, it is possible that some functional redundancy among different SLFN members may account for residual IFN-dependent hematopoietic suppression in the absence of SLFN2. Stable knockdown of SLFN2 also substantially diminished the ability of IFNα to generate antiproliferative responses in non-hematopoietic cells, strongly implicating this protein in the generation of the growth-suppressive effects of IFNs. Our data also suggest for the first time an important role for SLFN2 in the control of anchorage-independent cell growth, whereas we did not find any requirement for this protein in the generation of IFN-dependent antiviral effects. There was also no requirement for SLFN2 in Type I IFN-dependent formation of STAT-binding complexes or IFN-inducible transcription of *Isg15*. Thus, it appears that this member of the SLFN family of proteins specifically mediates signals that participate in the induction of the growth-suppressive effects of IFNs but not their antiviral effects.

Our studies establish that beyond engagement of SLFN2, IFNα up-regulates expression of several other members of the SLFN family. The functional differences among distinct *SLFN* groups and individual *SLFN* members in the generation of IFN responses remain to be established. Nevertheless, the Type I IFN-dependent induction of expression of several members of this family raises the possibility that beyond SLFN2, other members participate in the generation of IFN-inducible responses, but this remains to be directly determined in future studies. Interestingly, the *SLFN* gene cluster was recently linked to development of rheumatoid arthritis by combining microarray analyses of two independent rheumatoid arthritis mouse models (45). Because overproduction of various cytokines is linked to the pathogenesis of rheumatoid arthritis (31), these data raise the possibility that beyond IFNs, *SLFN* genes and their products may be involved in cellular pathways activated by several other cytokines, and this needs to be examined in future work.

The involvement of *SLFN2*, and possibly other Schlafens, in the control of IFNα-antiproliferative effects may ultimately prove to be of clinical-translational therapeutic relevance. IFNα exhibits potent antineoplastic properties *in vitro* and *in vivo*, and it has substantial clinical activity in the treatment of various malignancies. However, a limiting factor in the administration of higher, more effective, doses of IFNs has been the various side effects that reflect the diversity of responses elicited by these pleiotropic cytokines. It is possible that *SLFN* genes and their products selectively mediate the antiproliferative effects of IFNs, because different groups of genes are mediators of IFN-regulated antiviral effects (32). Moreover, it is conceivable that development of methodologies to selectively induce *SLFN* gene expression may specifically promote the antitumor effects of IFNs in the absence of engagement of other pathways associated with various IFN-inducible adverse effects. Although the validity of such a hypothesis remains to be determined, further work in this direction is warranted and may provide interesting new information and help in attempts to optimize the antitumor effects of IFNα.

REFERENCES

1. Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. (1987) *Annu. Rev. Biochem.* **56,** 727–777
2. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) *Annu. Rev. Biochem.* **67,** 227–264
3. Parmar, S., and Platanias, L. C. (2003) *Curr. Opin. Oncol.* **15,** 431–439
4. Platanias, L. C., and Fish, E. N. (1999) *Exp. Hematol.* **27,** 1583–1592
5. van Boxel-Dezaire, A. H., Rani, M. R., and Stark, G. R. (2006) *Immunity* **25,** 361–372
6. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) *Science* **264,** 1415–1421
7. Platanias, L. C. (2005) *Nat. Rev. Immunol.* **5,** 375–386
8. Katsoulidis, E., Li, Y., Mears, H., and Platanias, L. C. (2005) *J. Interferon Cytokine Res.* **25,** 749–756
9. David, M., Petricoin, E., 3rd, Benjamin, C., Pine, R., Weber, M. J., and Larner, A. C. (1995) *Science* **269,** 1721–1723
10. Uddin, S., Majchrzak, B., Woodson, J., Arunkumar, P., Alsayed, Y., Pine, R., Young, P. R., Fish, E. N., and Platanias, L. C. (1999) *J. Biol. Chem.* **274,** 30127–30131
11. Uddin, S., Lekmine, F., Sharma, N., Majchrzak, B., Mayer, I., Young, P. R., Bokoch, G. M., Fish, E. N., and Platanias, L. C. (2000) *J. Biol. Chem.* **275,** 27634–27640
12. Verma, A., Deb, D. K., Sassano, A., Uddin, S., Varga, J., Wickrema, A., and Platanias, L. C. (2002) *J. Biol. Chem.* **277,** 7726–7735
13. Mayer, I. A., Verma, A., Grumbach, I. M., Uddin, S., Lekmine, F., Ravandi, F., Majchrzak, B., Fujita, S., Fish, E. N., and Platanias, L. C. (2001) *J. Biol. Chem.* **276,** 28570–28577
14. Lekmine, F., Uddin, S., Sassano, A., Parmar, S., Brachmann, S. M., Majchrzak, B., Sonenberg, N., Hay, N., Fish, E. N., and Platanias, L. C. (2003) *J. Biol. Chem.* **278,** 27772–27780
15. Lekmine, F., Sassano, A., Uddin, S., Smith, J., Majchrzak, B., Brachmann, S. M., Hay, N., Fish, E. N., and Platanias, L. C. (2004) *Exp. Cell Res.* **295,** 173–182
16. Thyrell, L., Hjortsberg, L., Arulampalam, V., Panaretakis, T., Uhles, S., Dagnell, M., Zhivotovsky, B., Leibiger, I., Grander, D., and Pokrovskaja, K. (2004) *J. Biol. Chem.* **279,** 24152–24162
17. Kaur, S., Lal, L., Sassano, A., Majchrzak-Kita, B., Srikanth, M., Baker, D. P., Petroulakis, E., Hay, N., Sonenberg, N., Fish, E. N., and Platanias, L. C. (2007) *J. Biol. Chem.* **282,** 1757–1768
18. Kaur, S., Sassano, A., Dolniak, B., Joshi, S., Majchrzak-Kita, B., Baker, D. P., Hay, N., Fish, E. N., and Platanias, L. C. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 4808–4813
19. Sadler, A. J., and Williams, B. R. (2008) *Nat. Rev. Immunol.* **8,** 559–568
20. Schwarz, D. A., Katayama, C. D., and Hedrick, S. M. (1998) *Immunity.* **9,** 657–668
21. Geserick, P., Kaiser, F., Klemm, U., Kaufmann, S. H., and Zerrahn, J. (2004) *Int. Immunol.* **16,** 1535–1548
22. Brady, G., Boggan, L., Bowie, A., and O'Neill, L. A. (2005) *J. Biol. Chem.* **280,** 30723–30734
23. Bell, T. A., de la Casa-Esperón, E., Doherty, H. E., Ideraabdullah, F., Kim, K., Wang, Y., Lange, L. A., Wilhemsen, K., Lange, E. M., Sapienza, C., and de Villena, F. P. (2006) *Genetics* **172,** 411–423
24. Sohn, W. J., Kim, D., Lee, K. W., Kim, M. S., Kwon, S., Lee, Y., Kim, D. S., and Kwon, H. J. (2007) *Mol. Immunol.* **44,** 3273–3282
25. Neumann, B., Zhao, L., Murphy, K., and Gonda, T. J. (2008) *Biochem. Biophys. Res. Commun.* **370,** 62–66
26. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) *Mol. Cell. Biol.* **17,** 338–344

27. Adams, R. H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R., and Nebreda, A. R. (2000) *Mol. Cell* **6,** 109–116
28. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) *Cell* **84,** 443–450
29. Schlessinger, K., and Levy, D. E. (2005) *Cancer Res.* **65,** 5828–5834
30. Uddin, S., Fish, E. N., Sher, D. A., Gardziola, C., White, M. F., and Platanias, L. C. (1997) *J. Immunol.* **158,** 2390–2397
31. Brennan, F. M., and McInnes, I. B. (2008) *J. Clin. Invest.* **118,** 3537–3545
32. Sen, G. C. (2001) *Annu. Rev. Microbiol.* **55,** 255–281
33. Fish, E. N., Uddin, S., Korkmaz, M., Majchrzak, B., Druker, B. J., and Platanias, L. C. (1999) *J. Biol. Chem.* **274,** 571–573
34. Li, Y., Sassano, A., Majchrzak, B., Deb, D. K., Levy, D. E., Gaestel, M., Nebreda, A. R., Fish, E. N., and Platanias, L. C. (2004) *J. Biol. Chem.* **279,** 970–999
35. Kannan-Thulasiraman, P., Katsoulidis, E., Tallman, M. S., Arthur, J. S., and Platanias, L. C. (2006) *J. Biol. Chem.* **281,** 22446–22452
36. Giafis, N., Katsoulidis, E., Sassano, A., Tallman, M. S., Higgins, L. S., Nebreda, A. R., Davis, R. J., and Platanias, L. C. (2006) *Cancer Res.* **66,** 6763–6771
37. Deonarain, R., Verma, A., Porter, A. C., Gewert, D. R., Platanias, L. C., and Fish, E. N. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 13453–13458
38. Hamburger, A. W., and Salmon, S. E. (1977) *Science* **197,** 461–463
39. Platanias, L. C. (2003) *Pharmacol. Ther.* **98,** 129–142
40. Borden, E. C., Sen, G. C., Uze, G., Silverman, R. H., Ransohoff, R. M., Foster, G. R., and Stark, G. R. (2007) *Nat. Rev. Drug Discov.* **6,** 975–990
41. Reddig, P. J., and Juliano, R. L. (2005) *Cancer Metastasis Rev.* **24,** 425–439
42. Hanson, P. I., and Whiteheart, S. W. (2005) *Nat. Rev. Mol. Cell Biol.* **6,** 519–529
43. Zhang, Y., Yang, Z., Cao, Y., Zhang, S., Li, H., Huang, Y., Ding, Y. Q., and Liu, X. (2008) *Biochem. J.* **413,** 239–250
44. Eskra, L., Mathison, A., and Splitter, G. (2003) *Infect. Immun.* **71,** 1125–1133
45. Fujikado, N., Saijo, S., and Iwakura, Y. (2006) *Arthritis Res. Ther.* **8,** R100