THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 50, pp. 42352–42360, December 7, 2012
Published in the U.S.A.

# Sprouty Proteins Are Negative Regulators of Interferon (IFN) Signaling and IFN-inducible Biological Responses*[S]

Received for publication, July 16, 2012, and in revised form, October 5, 2012 Published, JBC Papers in Press, October 16, 2012, DOI 10.1074/jbc.M112.400721

**Bhumika Sharma[‡], Sonali Joshi[‡], Antonella Sassano[‡], Beata Majchrzak[§], Surinder Kaur[‡], Priya Aggarwal[‡], Behnam Nabet[‡], Marinka Bulic[‡], Brady L. Stein[‡], Brandon McMahon[‡], Darren P. Baker[¶], Rikiro Fukunaga[‖], Jessica K. Altman[‡**], Jonathan D. Licht[‡], Eleanor N. Fish[§1], and Leonidas C. Platanias[‡**2]**

*From the [‡]Robert H. Lurie Comprehensive Cancer Center and Division of Hematology-Oncology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, the **Division of Hematology-Oncology, Department of Medicine, Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois 60612, the [§]Toronto General Research Institute, University Health Network and Department of Immunology, University of Toronto, Toronto, Ontario M5G 2M1, Canada, [¶]Biogen Idec Incorporated, Cambridge, Massachusetts 02142, and the [‖]Laboratory of Biochemistry, Osaka University of Pharmaceutical Sciences, Osaka 569-1094, Japan*

**Background:** The potential involvement of Spry proteins in IFN signaling is unknown.
**Results:** Type I IFN treatment results in up-regulation of Spry proteins, which negatively control generation of IFN responses.
**Conclusion:** Spry proteins play important regulatory roles in IFN signaling and the generation of the biological effects of IFNs.
**Significance:** This study provides evidence for the existence of a key signaling pathway that controls IFN responses.

**Interferons (IFNs) have important antiviral and antineoplastic properties, but the precise mechanisms required for generation of these responses remain to be defined. We provide evidence that during engagement of the Type I IFN receptor (IFNR), there is up-regulation of expression of Sprouty (Spry) proteins 1, 2, and 4. Our studies demonstrate that IFN-inducible up-regulation of Spry proteins is Mnk kinase-dependent and results in suppressive effects on the IFN-activated p38 MAP kinase (MAPK), the function of which is required for transcription of interferon-stimulated genes (ISGs). Our data establish that ISG15 mRNA expression and IFN-dependent antiviral responses are enhanced in Spry1,2,4 triple knock-out mouse embryonic fibroblasts, consistent with negative feedback regulatory roles for Spry proteins in IFN-mediated signaling. In other studies, we found that siRNA-mediated knockdown of Spry1, Spry2, or Spry4 promotes IFN-inducible antileukemic effects *in vitro* and results in enhanced suppressive effects on malignant hematopoietic progenitors from patients with polycythemia vera. Altogether, our findings demonstrate that Spry proteins are potent regulators of Type I IFN signaling and negatively control induction of Type I IFN-mediated biological responses.**

Due to the important biological effects of interferons (IFNs) (1–3) and the clinical efficacy of these cytokines in the treatment of various diseases (4), there has been a substantial interest in defining cellular pathways activated by IFN receptors and dissecting their contributions in the generation of the biological effects of IFNs. Although IFNs were originally discovered and described as agents that block replication of different viruses (1–3), subsequent work established that they also act as modulators of innate immune responses and exhibit important growth inhibitory and antineoplastic properties (1–3). Three major IFN groups exist, each of which includes different subgroups and members. These include Type I ($\alpha$, $\beta$, $\omega$, $\tau$, $\kappa$, $\epsilon$, and $\delta$); Type II ($\gamma$); and the more recently identified class of Type III IFNs ($\lambda$1, $\lambda$2, and $\lambda$3) (1–6). All different IFN classes utilize Jak-Stat pathways to activate elements in the promoters of IFN-stimulated genes (ISGs)[3] promoting transcriptional activation and induction of gene expression (7, 8). Common and distinct combinations of receptor-associated Jak kinases and Stat proteins are engaged by Type I, II, and III IFN receptors, and in each case, the coordinated functions of Jak-Stat pathways are essential for ultimate generation of ISG products and associated biological responses (1, 3, 7, 8). Importantly, there is also evidence that unphosphorylated Stats exhibit important functions as transcription factors, as well as modifiers of transcription factors and chromatin structure (9).

In addition to classical Jak-Stat pathways, other signaling cascades engaged by IFN receptors are essential for optimal transcriptional activation and mRNA translation of ISGs, and ultimately, the generation of IFN biological responses. The p38 MAP kinase (MAPK) pathway is activated in parallel to Jak-Stat pathways, and its function is essential for IFN-dependent gene transcription (10, 11), whereas the AKT/mTOR (mammalian target of rapamycin) signaling cascade is also engaged by IFN receptors and is required for mRNA translation of ISGs and ultimate production of ISG proteins (12–15). Other studies have shown that IFN-dependent engagement of the Erk MAP

* This work was supported, in whole or in part, by National Institutes of Health Grants CA155566, CA77816, and CA161796 (to L. C. P.). This work was also supported by a Merit Review grant from the Department of Veterans Affairs (to L. C. P.); by the Lynn Sage Foundation (to J. D. L.); and by Canadian Institutes of Health Research Grant 285791 (to E. N. F.).
[S] This article contains supplemental Fig. S1.
[1] A Canada Research Chair.
[2] To whom correspondence should be addressed: Robert H. Lurie Comprehensive Cancer Center, 303 East Superior St., Lurie 3-107, Chicago, IL 60611. Tel.: 312-503-4267; Fax: 312-908-1372; E-mail: l-platanias@northwestern.edu.

[3] The abbreviations used are: ISG, IFN-stimulated gene; Spry, Sprouty; mSpry, mouse Spry; MEF, mouse embryonic fibroblast; BFU-E, burst-forming unit, erythroid; CFU-L, colony-forming unit, lymphocyte; Mkk, Map kinase kinase; Mnk, MAPK-interacting kinase.

kinase pathway participates in ISG mRNA translation/protein expression via regulatory effects on the activation of Mnk kinases and eIF4E phosphorylation (16, 17).

In the present study, we provide the first evidence for engagement of Sprouty (Spry) proteins in IFN signaling. The family of Spry proteins includes four members (18–20), all of which are homologues of the *Drosophila melanogaster* Spry, which was originally identified as an inhibitor of FGF signaling (21). These proteins act as negative regulators of growth factor signaling pathways (18–20, 22), and their expression is deregulated in several malignancies (20). Our studies demonstrate that treatment of sensitive cells with IFN$\alpha$ or IFN$\beta$ results in stabilization/up-regulation of expression of Spry proteins in a Mnk kinase-dependent manner. IFN-activated Spry proteins act as negative feedback regulators and exhibit inhibitory effects on the p38 MAPK pathway and ISG expression. This leads to negative control of IFN-inducible antiviral effects and growth inhibitory responses. Importantly, inhibiting expression of the various Spry proteins results in enhanced IFN-dependent antileukemic effects and antiviral responses, suggesting that selective targeting of these proteins may provide an approach to enhance and optimize the therapeutic potential of IFNs.

## EXPERIMENTAL PROCEDURES

*Cells and Reagents*—U937 cells were grown in RPMI supplemented with 10% (v/v) fetal bovine serum and antibiotics. Immortalized Sprouty1,2,4 knock-out MEFs (23) were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and antibiotics. Recombinant human IFN$\alpha$ was obtained from Hoffmann-La Roche. Recombinant human and mouse IFN$\beta$ were from Biogen Idec. Antibodies against human Spry1 and Spry2 were purchased from Abcam (Cambridge, MA). An antibody against human Spry4 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against mouse Spry1 and Spry2 have been previously described (23). Antibodies against the phosphorylated forms of STAT1, STAT3, ERK1/2 (Thr-202/Tyr-204), and p38 MAPK (Thr-180/Tyr-182), as well as antibodies against ERK1/2 and p38 MAPK, were obtained from Cell Signaling Technology (Danvers, MA). An antibody against GAPDH was obtained from Millipore. siRNAs targeting human Sprouty1, Sprouty2, and Sprouty4 as well nontargeting siRNAs were obtained from Santa Cruz Biotechnology.

*Cell Lysis and Immunoblotting*—Cells were treated with $5 \times 10^3$ or $1 \times 10^4$ IU/ml of IFN$\alpha$ and IFN$\beta$ for the indicated times. For the experiments in which ISG15 expression was assessed, the concentration of IFN used was $2.5 \times 10^3$ IU/ml. Cells were then collected and lysed in phosphorylation lysis buffer as in our previous studies (12). Immunoblotting was performed using an enhanced chemiluminescence (ECL) method were performed as in previous studies (12).

*mRNA Expression Assays*—Cells were treated with $5 \times 10^3$ units/ml of IFN$\alpha$ or IFN$\beta$ for 6 h, and quantitative RT-PCR was carried out as described previously (12). Real-time RT-PCR to determine expression of ISG15 mRNA was carried out by using commercially available 6-carboxyfluorescein-labeled probes and primers (Applied Biosystems). For knockdown experiments, cells were nucleofected with siRNA, and Spry1,2,4 mRNA expression was assessed using real-time RT-PCR. GAPDH was used for normalization.

*Overexpression of Murine Spry1 and Murine Spry2 in Spry1,2,4$^{-/-}$ MEFs*—For these studies, virus supernatants were generated from a Phoenix packaging cell line that had been transfected with empty vector (pBabe-puro), pBabe-puro murine Spry1 (FLAG/HA), or pBabe-puro murine Spry2 (FLAG/HA) plasmids using FuGENE (Roche Diagnostics). Spry1,2,4$^{-/-}$ MEFs were infected in the presence of Polybrene (8 $\mu$g/ml) with retroviruses and selected for 4 days in puromycin (1 $\mu$g/ml), and protein expression was verified by immunoblotting, as indicated.

*Antiviral Assays*—The antiviral effects of mouse IFN$\alpha$ in Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs were determined in assays using encephalomyocarditis virus as the challenge virus, as in our previous studies (12).

*Hematopoietic Cell Progenitor Assays*—Peripheral blood from polycythemia vera patients was collected after obtaining consent approved by the Institutional Review Board of Northwestern University. Hematopoietic progenitor colony formation for late erythroid progenitors (BFU-E) was determined in clonogenic assays in methylcellulose, as in our previous studies (16).

## RESULTS

In initial studies, we examined the effects of Type I IFNs (IFN$\alpha$ and IFN$\beta$) on protein expression of different Spry family members. U937 cells were treated with IFN$\alpha$ or IFN$\beta$ for different times, and Spry protein levels were assessed by immunoblotting. Type I IFN treatment resulted in the up-regulation of expression of Spry2 that was detectable within 60 min of treatment and persisted for 4–5 h (Fig. 1, *A* and *B*). Similarly, IFN treatment resulted in sustained up-regulation of Spry4 (Fig. 1, *C* and 1*D*) and Spry1 (Fig. 1, *E* and *F*).

Evidence from previous work shows that phosphorylation of Spry2 on serines 112 and 121 by Mnk1 stabilizes the protein and antagonizes c-Cbl binding and polyubiquitination that would otherwise lead to its degradation (24). As we have shown that Mnk1 is engaged in Type I IFN signaling and plays critical roles in the generation of IFN responses (16), we examined whether Mnk kinase activity is required for stabilization/up-regulation of Spry protein expression by the Type I IFN receptor. Immortalized MEFs from mice with targeted disruption of both Mnk1 and Mnk2 (25) or control wild-type (WT) MEFs were treated with mouse IFN$\beta$ for increasing times, and protein levels of Spry1 and Spry2 were analyzed in parallel. Treatment of WT MEFs with mouse IFN$\beta$ resulted in rapid up-regulation of Spry1 levels, consistent with stabilization of protein expression, but this up-regulation was not seen in Mnk1/2 double knockout MEFs (Fig. 2*A*). Similarly, up-regulation of expression of Spry2 was Mnk kinase-dependent (Fig. 2*B*), suggesting that IFN-dependent, Mnk-mediated phosphorylation of Spry proteins stabilizes them and promotes their expression (24).

Spry expression is stimulated by growth factors and plays key regulatory roles in growth factor signaling (18–22, 24). By contrast to growth factors, IFNs are cytokines that generally inhibit cell proliferation, and our finding that Spry protein levels are regulated by IFNs raised the possibility that Spry proteins might

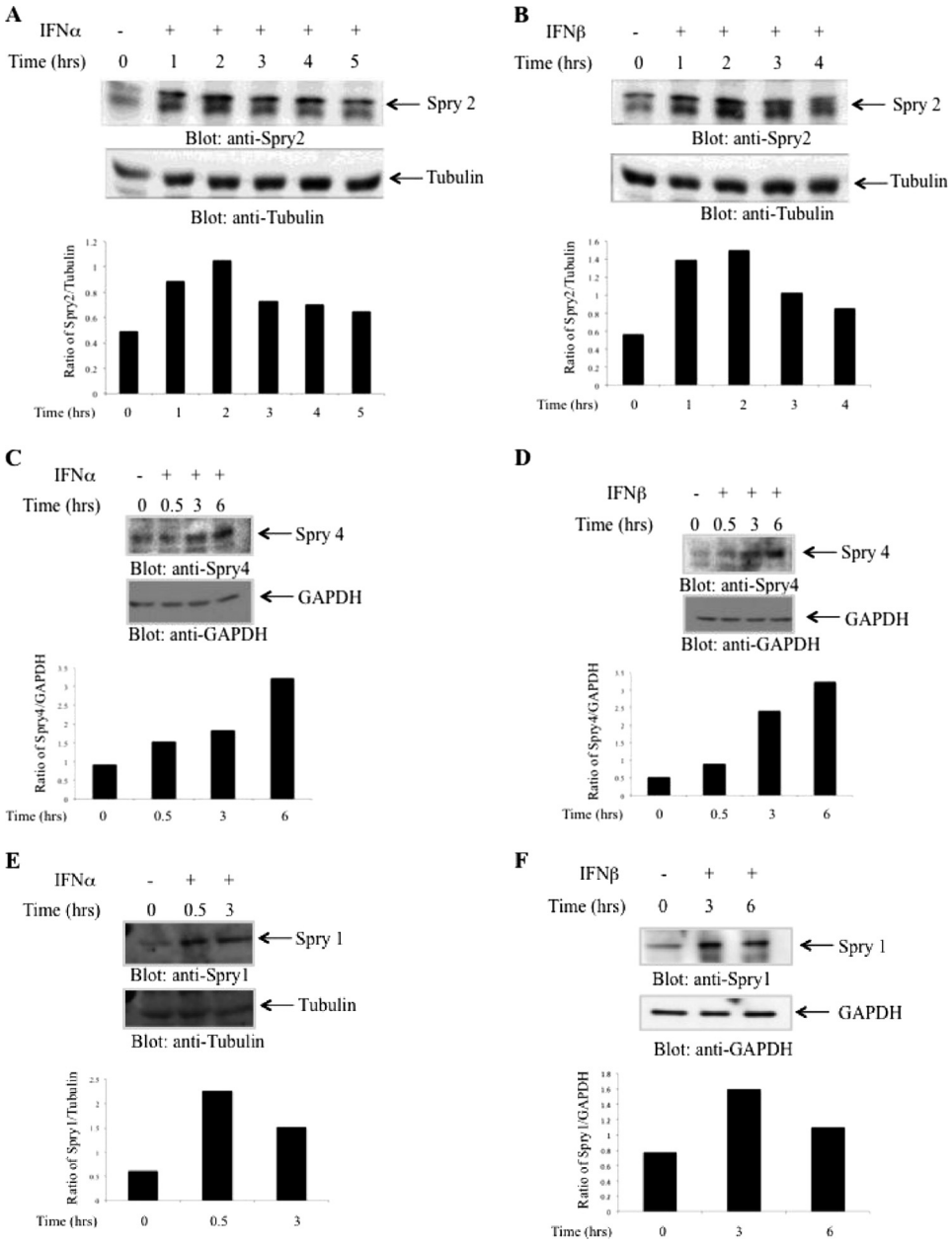


FIGURE 1. **Effects of Type I IFNs on Spry protein levels.** *A* and *B*, U937 cells were treated with human IFN$\alpha$ or IFN$\beta$ for the indicated times. Proteins in lysates were resolved by SDS-PAGE and immunoblotted with an antibody against Spry2. The same blots were probed with an antibody against tubulin. The signals for Spry2 and tubulin from the experiments shown in *A* and *B* were quantitated by densitometry, and the intensities of Spry2 relative to tubulin expression were calculated. *C* and *D*, U937 cells were treated with human IFN$\alpha$ or IFN$\beta$ for the indicated times. Proteins in lysates were resolved by SDS-PAGE and immunoblotted with an antibody against Spry4. The same blots were probed with an antibody against GAPDH. The signals for Spry4 and GAPDH from the experiment shown in *C* and *D* were quantitated by densitometry, and the intensities of Spry4 relative to GAPDH expression were calculated. *E* and *F*, U937 cells were treated with human IFN$\alpha$ or IFN$\beta$ for the indicated times. Proteins in lysates were resolved by SDS-PAGE and immunoblotted with an antibody against Spry1. The same blots were probed with an antibody against GAPDH. The signals for Spry1 and tubulin (*E*) or GAPDH (*F*) were quantitated by densitometry, and the intensities of Spry1 relative to tubulin or GAPDH expression were calculated.

modulate IFN-mediated growth inhibition. To examine the effects of Spry proteins on Type I IFN-induced signaling, we utilized MEFs created by Cre-mediated excision of lox P-linked alleles deficient for all three Spry proteins up-regulated in response to Type I IFNs (Spry1,2,4$^{-/-}$) (23). Serum-starved parental Spry1,2,4$^{flox/flox}$ or Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\beta$, and phosphorylation/activation of Stat proteins and MAP kinases was determined. As expected, Type I IFN treatment resulted in strong phosphorylation of Stat1 on Tyr-701 (Fig. 3*A*) and Ser-727 (Fig. 3*B*) in Spry1,2,4$^{flox/flox}$ MEFs. This IFN-inducible phosphorylation was intact and similar in Spry1,2,4$^{-/-}$ cells (Fig. 3, *A* and *B*), indicating that Spry proteins do not modulate phosphorylation or protein expression of Stats. In a similar manner, IFN-inducible phosphorylation of Stat3 on Tyr-705 or Ser-727 was similar in Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs (Fig. 3, *C* and *D*), indicating that this phosphorylation is independent of Spry activity. Furthermore, in Spry1,2,4$^{-/-}$cells, IFN-dependent Erk1/2 phosphorylation was enhanced (Fig. 3*E*), suggesting that, as in the case of growth factor signaling (18–20), Spry proteins act as

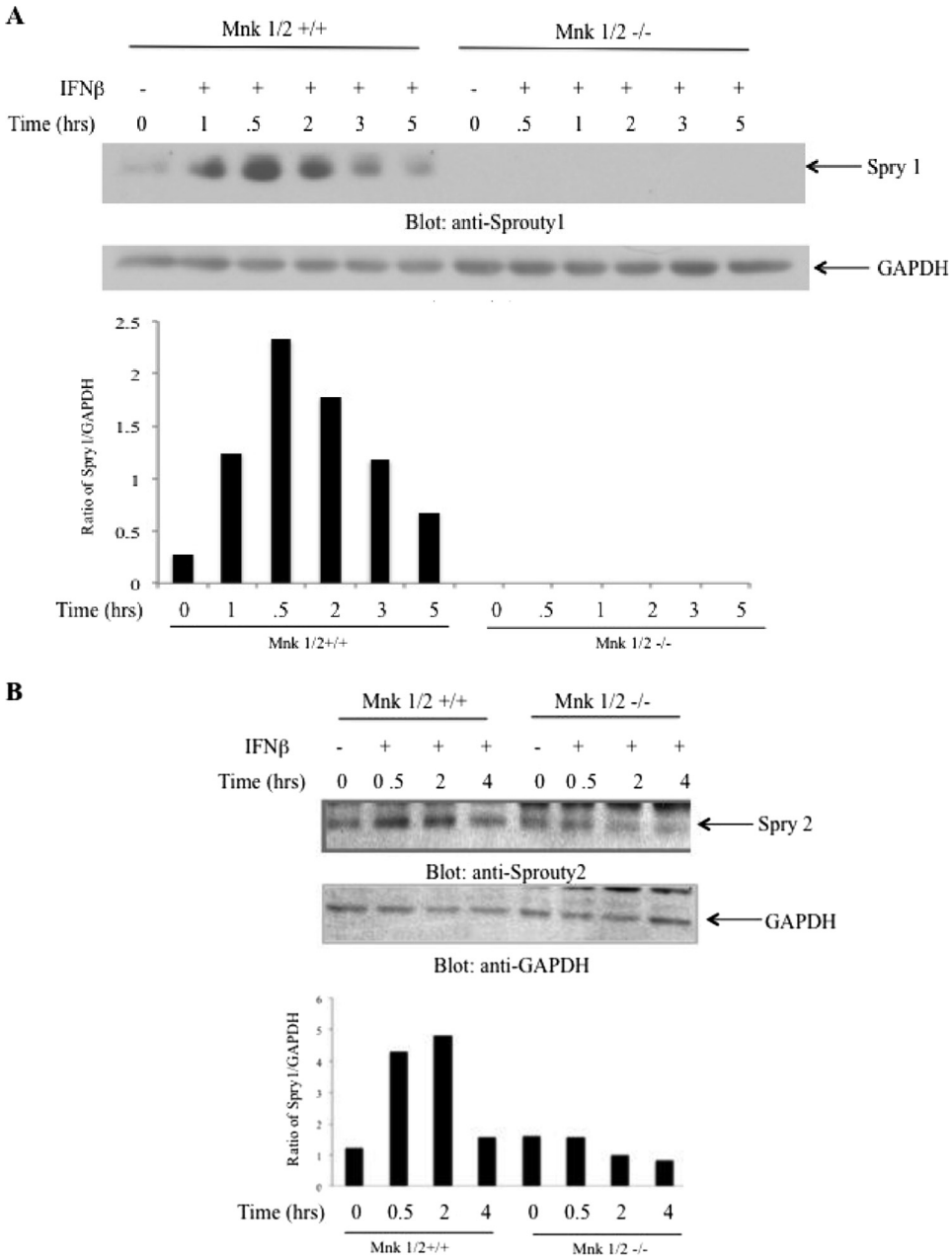


FIGURE 2. **Type I IFN-dependent up-regulation of Spry proteins is Mnk kinase dependent.** *A*, Mnk1/2$^{+/+}$ and Mnk1/2$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for the indicated times, and proteins in cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against Spry1. The same blot was probed with an antibody against GAPDH. The signals for Spry1 and GAPDH were quantitated by densitometry, and the intensity of Spry1 relative to GAPDH expression was calculated. *B*, Mnk1/2$^{+/+}$ and Mnk1/2$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for the indicated times, and proteins in cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against Spry2. The same blot was probed with an antibody against GAPDH. The signals for Spry2 and GAPDH were quantitated by densitometry, and the intensity of Spry2 relative to GAPDH expression was calculated.

negative feedback regulators of the MEK/Erk pathway. Importantly, targeted disruption of the Spry1, Spry2, and Spry4 genes also resulted in enhancement of phosphorylation/activation of p38 MAPK (Fig. 3*F*), suggesting unique regulatory effects of Spry proteins in IFN signaling.

Previous work established that the p38 MAPK pathway complements the function of Stat pathways and is required for optimal transcription of ISGs with interferon-sensitive response elements in their promoters (26, 27). As our data demonstrated regulatory effects of Spry proteins on p38 MAPK activity, we determined the effects of the targeted disruption of Spry genes on mRNA expression of the ISG15 gene, the protein product of which accounts for IFN-dependent ISGylation and plays an important role in the control of IFN responses (28). Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs were treated with murine IFN$\alpha$ or IFN$\beta$, and ISG15 mRNA expression was determined by quantitative real-time RT-PCR. As shown in Fig. 4*A*, there was strong induction of ISG15 mRNA expression in response to either IFN$\alpha$ or IFN$\beta$ in Spry1,2,4$^{flox/flox}$ MEFs, but this expression increased further in Spry1,2,4$^{-/-}$ cells (Fig. 4*A*). Consistent with this, IFN-dependent ISG15 protein expression was strongly enhanced in Spry1,2,4$^{-/-}$cells (Fig. 4, *B–C*). To

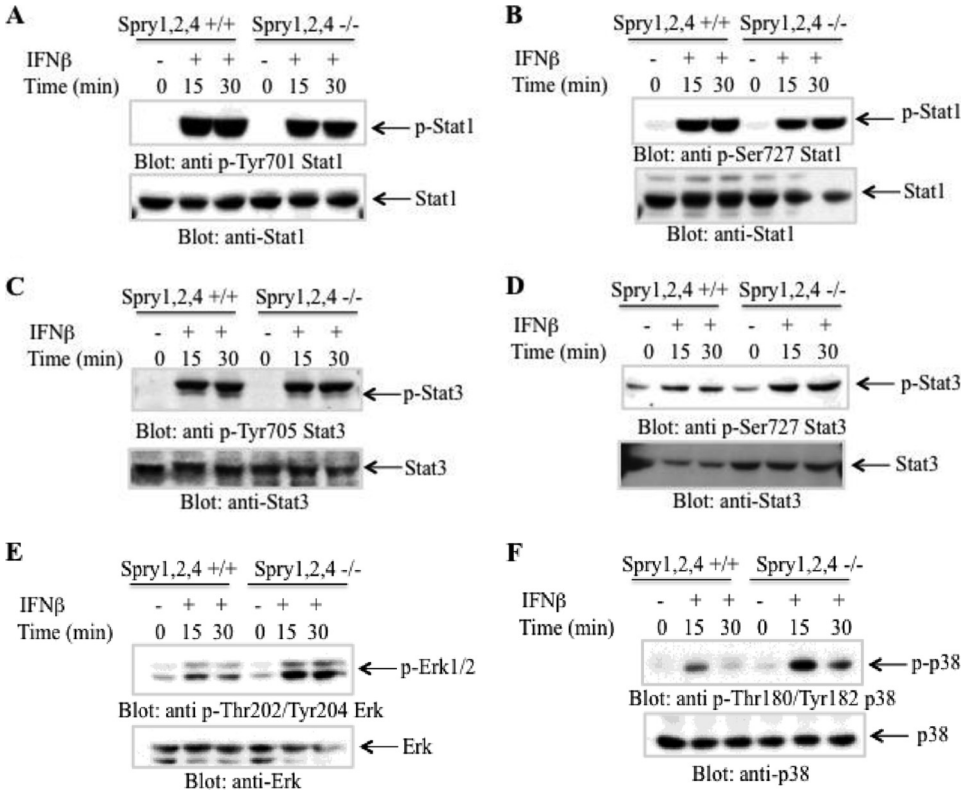


FIGURE 3. **Regulatory effects of Spry proteins on elements of Type I IFN signaling.** *A* and *B*, Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and then immunoblotted with antibodies against phosphorylated STAT1 (Tyr-701) (*anti p-Tyr701 Stat1*) (*A*) or STAT1 (Ser-727) (*anti p-Ser727 Stat1*) (*B*). The blots were then stripped and reprobed with an antibody against STAT1. *C* and *D*, Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and then immunoblotted with antibody against phosphorylated STAT3 (Tyr-705) (*anti p-Tyr705 Stat1*) (*C*) or STAT3 (Ser-727) (*anti p-Ser727 Stat1*) (*D*). The blots were then stripped and reprobed with an antibody against STAT3. *E*, Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and then immunoblotted with an antibody against phosphorylated Erk1/2 (Thr-202/Tyr-204) (*anti p-Thr202/Tyr204 Erk*). The blot was then stripped and reprobed with an antibody against Erk1/2. *F*, Spry1,2,4$^{flox/flox}$ and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and then immunoblotted with an antibody against phosphorylated p38 MAPK (Thr-180/Tyr-182) (*anti p-Thr180/Tyr182 p38*). The blot was then stripped and reprobed with an antibody against p38 MAPK.

further establish the role that Spry proteins play in regulation of ISG15 expression, we ectopically expressed Spry1 (Fig. 4*D*) or Spry2 (Fig. 4*E*) in Spry1,2,4$^{-/-}$cells and examined the effects of this complementation on ISG15 protein expression. As shown in Fig. 4*F*, ectopic expression of either Spry1 or Spry2 substantially decreased the levels of IFN-dependent expression of ISG15 protein (Fig. 4*F*).

To evaluate the functional relevance of Spry proteins in the biological effects of IFNs, we compared the antiviral properties of mouse IFN$\alpha$ against encephalomyocarditis virus infection in Spry1,2,4$^{-/-}$ and Spry1,2,4$^{flox/flox}$ MEFs. Spry1,2,4 triple knock-out MEFs showed enhanced protection from viral infection in response to IFN$\alpha$ when compared with Spry1,2,4$^{flox/flox}$ MEFs (Fig. 5*A*), a finding consistent with the regulatory effects that Spry proteins exhibit on ISG expression.

We next examined the effects of Spry proteins on the ability of Type I IFNs to suppress leukemic progenitor colony formation. Spry1, Spry2, or Spry4 expression was targeted in U937 cells using specific siRNAs (supplemental Fig. S1), and the effects of Spry knockdowns on the generation of the suppressive effects of IFN$\alpha$ or IFN$\beta$ on leukemic progenitor (CFU-L) colony formation were examined. Treatment with either IFN$\alpha$ or IFN$\beta$ resulted in partial inhibition of CFU-L colony formation (Fig. 5, *B* and *C*), which was significantly enhanced in cells depleted of Spry1 or Spry2 expression (Fig. 5*B*). A similar trend was seen in cells in which Spry4 was knocked down (Fig. 5*C*). Thus, Spry proteins oppose both the antiviral and the antiproliferative/antileukemic effects of Type I IFNs.

To ascertain the clinical importance of this latter effect, we determined the function of Spry proteins on the action of IFN$\alpha$ on primary malignant hematopoietic progenitors from patients with polycythemia vera. As shown in Fig. 5*D*, selective targeting of Spry1, Spry2, or Spry4 resulted in enhanced IFN-inducible suppressive effects on malignant early erythroid progenitor (BFU-E) colony formation (Fig. 5*D*), further implicating these proteins as negative regulators of IFN responses, specifically in primary malignant progenitors.

## DISCUSSION

The first member of the Sprouty family (dSpry) was originally discovered in *Drosophila* as a novel cysteine-rich protein functioning as an FGF antagonist (21). In that original study, which ultimately defined the existence of the Spry family of proteins, it was demonstrated that the FGF pathway is overactive in *Sprouty* mutant embryos and is associated with ectopic branches, as compared with wild-type embryos (21). Subsequent work identified four mammalian homologs, *Spry1*, *Spry2*, *Spry3*, and *Spry4* (29). Expression of Spry1, Spry2, and Spry4

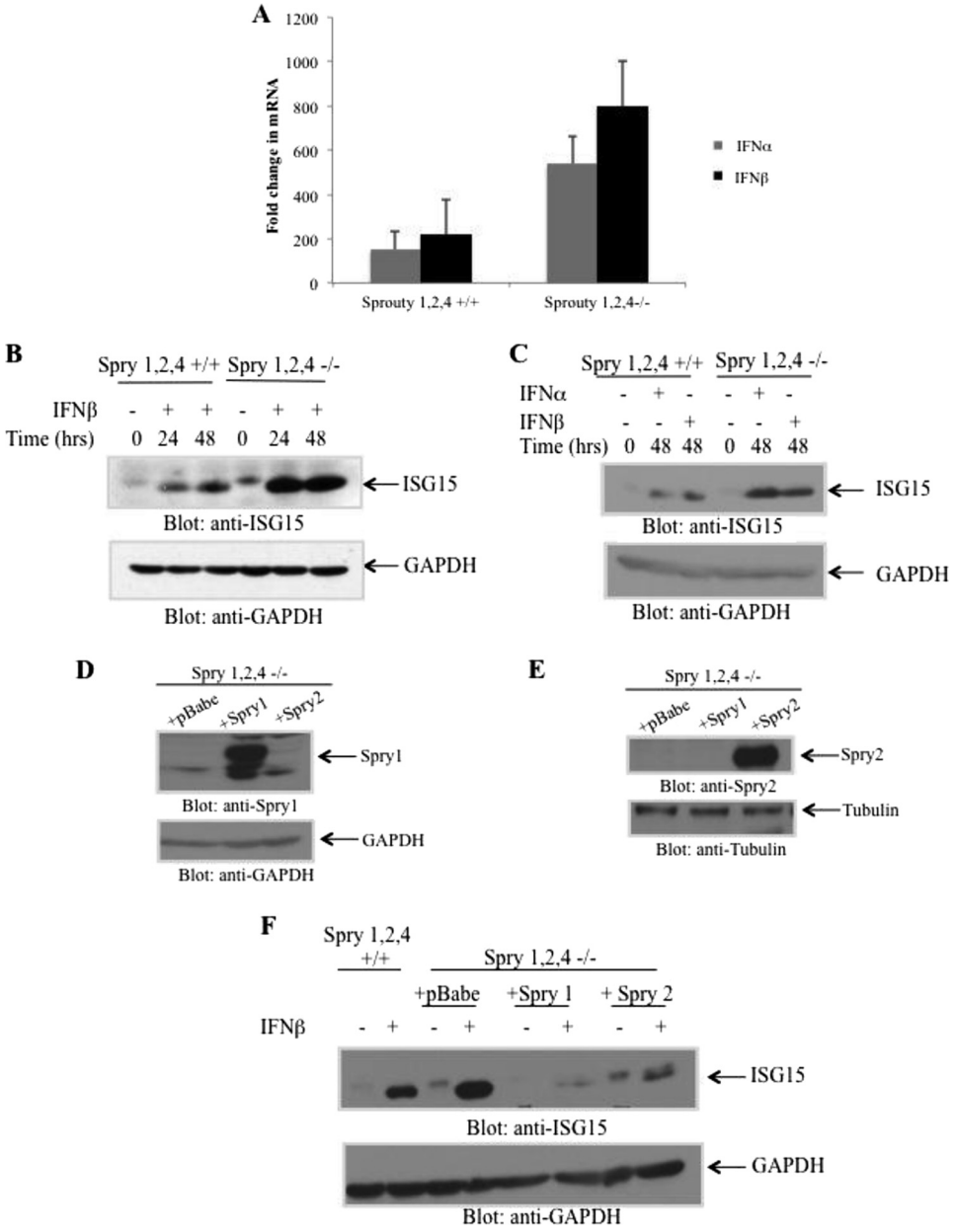


FIGURE 4. **Regulatory effects of Spry proteins on ISG expression.** *A*, Spry1,2,4$^{flox/flox}$ MEFs and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\alpha$ or IFN$\beta$ for 6 h, as indicated. The expression of ISG15 mRNA was assessed by quantitative RT-PCR, normalized for GAPDH expression. Data are expressed as -fold induction over corresponding untreated samples and represent means ± S.E. of three independent experiments. *B*, Spry1,2,4$^{flox/flox}$ MEFs and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\beta$ for 24 and 48 h, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibody against mouse ISG15. The same blot was probed with an antibody against GAPDH. *C*, Spry1,2,4$^{flox/flox}$ MEFs and Spry1,2,4$^{-/-}$ MEFs were treated with mouse IFN$\alpha$ or IFN$\beta$ for 48 h, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibody against mouse ISG15. The same blot was probed with an antibody against GAPDH. *D*, Spry null MEFs complemented with empty vector or vectors containing cDNA for mSpry1 or mSpry2 were lysed, and equal amounts of protein were resolved by SDS-PAGE and immunoblotted with an antibody against mSpry1. The same blot was probed with an antibody against GAPDH. *E*, Spry null MEFs complemented with empty vector or cDNA for mSpry1 or mSpry2 vector were lysed, and equal amounts of protein were resolved by SDS-PAGE and immunoblotted with an antibody against mSpry2. The same blot was probed with an antibody against tubulin. *F*, Spry1,2,4$^{flox/flox}$ MEFs and Spry1,2, 4$^{-/-}$ MEFs in which mSpry1 or mSpry2 were ectopically re-expressed, as indicated, were treated with mouse IFN$\beta$ 24 h, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibody against mouse ISG15. The same blot was probed with an antibody against GAPDH.

proteins is widespread in embryonic and adult tissues, whereas expression of Spry3 is restricted to brain and testes in adult tissues (30, 31). Spry proteins play critical roles in normal cells, and there is accumulating evidence for important regulatory effects during embryogenesis and organ development, including expansion of the organ of Corti (auditory sensory epithelium) (32), diastema tooth development (33), and morphogenesis of the ureteric epithelium in kidney development (34). These important functions of Spry proteins during development are to a large extent reflections of their properties as modifiers of intracellular pathways and growth factor responses during development.

It is now well established that Spry proteins inhibit signaling from various growth factor receptors, including epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth fac-

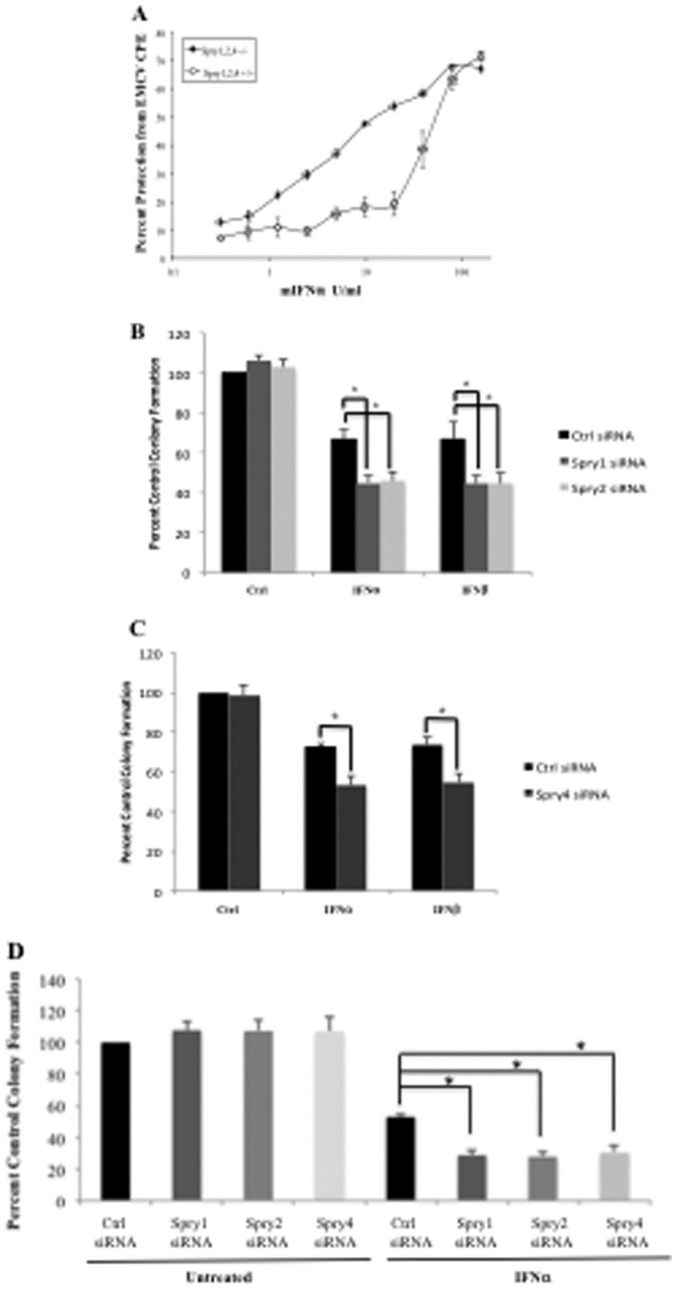


FIGURE 5. **Negative regulatory effects of Spry proteins in induction of IFN-dependent antiviral and antileukemic effects.** *A*, Spry1,2,4$^{flox/flox}$ MEFs and Spry1,2,4$^{-/-}$ MEFs were incubated with the indicated doses of mouse IFN$\alpha$. The cells were subsequently challenged with encephalomyocarditis virus (*EMCV*), and the viral cytopathic effects (*CPE*) were quantified 4 days later. Data are expressed as the percentage of protection from viral cytopathic effects of encephalomyocarditis virus. *B* and *C*, U937 cells were transfected with the indicated siRNAs and plated in a methylcellulose assay system in the absence or presence of human IFN$\alpha$ and IFN$\beta$. Data are expressed as a percentage of the control (*Ctrl*) siRNA-transfected cell-derived colony formation and represent means $\pm$ S.E. of four independent experiments. For *panel B*, paired *t* test analysis showed $p = 0.001$ for the combination of control siRNA and IFN$\alpha$ *versus* the combination of Spry1 siRNA and IFN$\alpha$; $p = 0.00009$ for the combination of control siRNA and IFN$\alpha$ *versus* the combination of Spry2 siRNA and IFN$\alpha$; $p = 0.019$ for the combination of control siRNA and IFN$\beta$ *versus* the combination of Spry1 siRNA and IFN$\beta$ for CFU-L; and $p = 0.035$ for the combination of control siRNA and IFN$\beta$ *versus* the combination of Spry2 siRNA and IFN$\beta$ for CFU-L colonies. For *panel C*, paired *t* test analysis showed $p = 0.02$ for the combination of control siRNA and IFN$\alpha$ *versus* the combination of Spry4 siRNA and IFN$\alpha$; and $p = 0.008$ for the combination of control siRNA and IFN$\beta$ *versus* the combination of Spry4 siRNA and IFN$\beta$ for CFU-L. *D*, mononuclear cells derived from peripheral blood of patients with polycythemia vera were transfected with the indicated siRNAs and were then plated in a methylcellulose assay system, in the absence or presence of human IFN$\alpha$. BFU-E progenitor colonies were scored after 14 days in culture. Data are expressed as the percentage of control colony formation of the control untreated siRNA-transfected cells and represent means $\pm$ S.E. of five independent experiments. For *panel D*, paired *t* test analysis showed $p = 0.0008$ for the combination of control siRNA and IFN$\alpha$ *versus* the combination of Spry1 siRNA and IFN$\alpha$; $p = 0.0017$ for the combination of control siRNA and IFN$\alpha$ *versus* the combination of Spry2 siRNA and IFN$\alpha$; and $p = 0.0074$ for the combination of control siRNA and IFN$\alpha$ *versus* the combination of Spry4 siRNA and IFN$\alpha$.

tor (PDGF), and glial cell-derived neurotrophic factor (GDNF) (19–22, 35). A key mechanism by which Spry proteins modulate cell proliferation and survival is by their ability to inhibit the Ras/MEK/Erk pathway (35). Several mechanisms for these responses have been delineated and involve direct interactions with known regulators or effector molecules of this signaling cascade, such as Grb2 (36), SHP2 (37), Raf1 (38), and Gap1 (39). There is also recent evidence that Spry proteins modulate other cellular signals and pathways, such as the phosphatidylinositol-specific phospholipase C-$\gamma$, which accounts for regulatory effects on calcium-mediated signaling and T cell proliferation (23). Notably, the effects of Spry proteins on growth factor-activated MAP kinase pathways appear to be limited to the MEK/Erk pathway (20–22), and there have been no reports on effects on other MAP kinase cascades.

In contrast to growth factors, IFNs are cytokines with important antiproliferative properties and tumor suppressive effects on malignant cells (1–3). In addition to the classical Jak-Stat pathways that regulate transcription of IFN-stimulated genes and their products (1, 3–8), IFNs activate several other cellular cascades, the functions of which complement the activities of Jak-Stat pathways in the generation of IFN responses. In recent studies, we demonstrated that activation of the Mnk/eIF4E pathway plays important roles in the generation of IFN-induced biological effects (16, 17). As there is evidence in other systems that phosphorylation of Spry2 on Ser-112 and Ser-121 by Mnk kinase activity stabilizes the protein and antagonizes c-Cbl binding and polyubiquitination (24), we examined the effects of Type I IFN treatment on expression of different members of the Spry family of proteins. Our data established that IFN treatment leads to increased levels of Spry proteins, whereas such expression is defective in Mnk1/2 double knockout MEFs, establishing that Mnk activity is required for the process. In experiments using Spry1/Spry2/Spry4 triple knockout MEFs, we found that IFN-dependent phosphorylation/activation of the MEK/Erk pathway is augmented, in a manner similar to what others have reported previously for growth factor signaling (35). However, the p38 MAPK was also strongly enhanced in the absence of expression of these Spry proteins, establishing regulatory effects of Spry proteins on the p38 MAPK pathway. In parallel studies, we found that induction of *ISG15* gene transcription and ISG15 protein expression are Spry-regulated and that targeted disruption of all three widely expressed Spry genes (*Spry1*, *Spry2*, and *Spry4*) results in enhanced ISG15 expression. Such effects do not reflect effects of Spry proteins on IFN-activated Jak-Stat pathways as IFN-dependent phosphorylation of Stat1 and Stat3 is intact in Spry1,2,4 triple knock-out cells. However, they do correlate

with enhanced p38 MAP kinase activity and are in agreement with previous work that has established that the p38 MAPK pathway plays an essential role in ISG transcription without modulating tyrosine or serine phosphorylation of Stat proteins (26, 27). Such Spry-dependent regulatory effects on the IFN-activated p38 MAPK pathway have important functional consequences as our data demonstrate that knockdown of Spry1, Spry2, or Spry4 potentiates the inhibitory effects of IFN$\alpha$ or IFN$\beta$ on U937-derived leukemic precursors or primary malignant erythroid hematopoietic progenitors from patients with polycythemia vera. It is of interest that although selective knock-out of distinct Spry proteins only partially enhances the suppressive effects of Type I IFNs on malignant hematopoiesis, different Spry proteins do not appear to compensate for each other in that context. It should also be noted that previous work demonstrated that the p38 MAP kinase pathway is essential for the generation of the suppressive effects of IFN$\alpha$ on normal (40) and leukemic hematopoietic progenitors (41), and recent work has shown that p38 MAPK is required for the inhibitory effects of IFN$\alpha$ on malignant erythroid progenitors from patients with polycythemia vera (42). Taken together with the findings of the current study, the data suggest that the enhancing effects of Spry knockdown on IFN-mediated suppression of malignant hematopoiesis may result from regulatory effects on the p38 MAPK pathway.

The precise protein target(s) of Spry proteins among elements of the IFN-activated p38 MAPK pathway remain to be determined. Putative candidates include various kinases in the p38 MAPK cascade, such as MAP kinase kinase (Mkk) 3, Mkk6, or Mkk4 (10, 11), or various upstream MAP kinase kinase kinases (MAPKKK) that are known to control MKK/p38 MAPK cascades (10, 11). Alternatively, effects on early upstream G-proteins that regulate engagement of the IFN-activated pathway, such as Rac1 (27), may be involved, but this remains to be directly addressed in future studies. Independent of the precise mechanisms involved, the results of this study may prove to have important translational implications in the future use of IFNs in clinical medicine. IFN$\alpha/\beta$ is used in various clinical settings, such as the treatment of various viral infections, multiple sclerosis, and certain malignancies (4). Importantly, it has major clinical activity, and it is one of the most effective agents in the treatment of Philadelphia negative ($Ph^-$) myeloproliferative neoplasms. In recent years, there has been a dramatic emergence of IFN$\alpha$ as an agent with major activity in the treatment of polycythemia vera and essential thrombocytosis (43–45). Nevertheless, not all patients respond or achieve long-term remissions, and efforts to further improve the clinical activity of IFN$\alpha$ should have a substantial impact in the management of patients with myeloproliferative neoplasms. Our studies suggest that Spry proteins are important negative feedback regulators of IFN responses and raise the potential of approaches to block their expression or inhibit their function to enhance and promote the antineoplastic effects of IFNs *in vitro* and *in vivo*.

*Acknowledgment—We thank Elisabeth Stecki for expert secretarial assistance.*

**REFERENCES**


1. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) How cells respond to interferons. *Annu. Rev. Biochem.* **67,** 227–264
2. Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. (1987) Interferons and their actions. *Annu. Rev. Biochem.* **56,** 727–777
3. Platanias, L. C. (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* **5,** 375–386
4. George, P. M., Badiger, R., Alazawi, W., Foster, G. R., and Mitchell, J. A. (2012) Pharmacology and therapeutic potential of interferons. *Pharmacol. Ther.* **135,** 44–53
5. Donnelly, R. P., and Kotenko, S. V. (2010) Interferon-λ: a new addition to an old family. *J. Interferon Cytokine Res.* **30,** 555–564
6. Lasfar, A., Abushahba, W., Balan, M., and Cohen-Solal, K. A. (2011) Interferon λ: a new sword in cancer immunotherapy. *Clin. Dev. Immunol.* **2011,** 349575
7. Stark, G. R. (2007) How cells respond to interferons revisited: From early history to current complexity. *Cytokine Growth Factor Rev.* **18,** 419–423
8. Stark, G. R., and Darnell, J. E. (2012) The JAK-STAT pathway at twenty. *Immunity* **36,** 503–514
9. Cheon, H., Yang, J., and Stark, G. R. (2011) The functions of signal transducers and activators of transcriptions 1 and 3 as cytokine-inducible proteins. *J. Interferon Cytokine Res.* **31,** 33–40
10. Platanias, L. C. (2003) The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol. Ther.* **98,** 129–142
11. Platanias, L. C. (2003) Map kinase signaling pathways and hematologic malignancies. *Blood* **101,** 4667–4679
12. 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) Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. *J. Biol. Chem.* **282,** 1757–1768
13. Kaur, S., Sassano, A., Dolniak, B., Joshi, S., Majchrzak-Kita, B., Baker, D. P., Hay, N., Fish, E. N., and Platanias, L. C. (2008) Role of the Akt pathway in mRNA translation of interferon-stimulated genes. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 4808–4813
14. Kroczynska, B., Kaur, S., Katsoulidis, E., Majchrzak-Kita, B., Sassano, A., Kozma, S. C., Fish, E. N., and Platanias, L. C. (2009) Interferon-dependent engagement of eukaryotic initiation factor 4B via S6 kinase (S6K)- and ribosomal protein S6K-mediated signals. *Mol. Cell. Biol.* **29,** 2865–2875
15. Kaur, S., Sassano, A., Majchrzak-Kita, B., Baker, D. P., Su, B., Fish, E. N., and Platanias, L. C. (2012) Regulatory effects of mTORC2 complexes in type I IFN signaling and in the generation of IFN responses. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 7723–7728
16. Joshi, S., Kaur, S., Redig, A. J., Goldsborough, K., David, K., Ueda, T., Watanabe-Fukunaga, R., Baker, D. P., Fish, E. N., Fukunaga, R., and Platanias, L. C. (2009) Type I interferon (IFN)-dependent activation of Mnk1 and its role in the generation of growth inhibitory responses. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12097–12102
17. Joshi, S., Sharma, B., Kaur, S., Majchrzak, B., Ueda, T., Fukunaga, R., Verma, A. K., Fish, E. N., and Platanias, L. C. (2011) Essential role for Mnk kinases in type II interferon (IFNγ) signaling and its suppressive effects on normal hematopoiesis. *J. Biol. Chem.* **286,** 6017–6026
18. Kim, H. J., and Bar-Sagi, D. (2004) Modulation of signalling by Sprouty: a developing story. *Nat. Rev. Mol. Cell Biol.* **5,** 441–450
19. Mason, J. M., Morrison, D. J., Basson, M. A., and Licht, J. D. (2006) Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol.* **16,** 45–54
20. Edwin, F., Anderson, K., Ying, C., and Patel, T. B. (2009) Intermolecular interactions of Sprouty proteins and their implications in development and disease. *Mol. Pharmacol.* **76,** 679–691
21. Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., and Krasnow, M. A. (1998) Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell* **92,** 253–263
22. Egan, J. E., Hall, A. B., Yatsula, B. A., and Bar-Sagi, D. (2002) The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 6041–6046

23. Akbulut, S., Reddi, A. L., Aggarwal, P., Ambardekar, C., Canciani, B., Kim, M. K., Hix, L., Vilimas, T., Mason, J., Basson, M. A., Lovatt, M., Powell, J., Collins, S., Quatela, S., Phillips, M., and Licht, J. D. (2010) Sprouty proteins inhibit receptor-mediated activation of phosphatidylinositol-specific phospholipase C. *Mol. Cell. Biol.* **21,** 3487–3496
24. DaSilva, J., Xu, L., Kim, H. J., Miller, W. T., and Bar-Sagi, D. (2006) Regulation of Sprouty stability by Mnk1-dependent phosphorylation. *Mol. Cell. Biol.* **26,** 1898–1907
25. Ueda, T., Watanabe-Fukunaga, R., Fukuyama, H., Nagata, S., and Fukunaga, R. (2004) Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. *Mol. Cell. Biol.* **24,** 6539–6549
26. Uddin, S., Majchrzak, B., Woodson, J., Arunkumar, P., Alsayed, Y., Pine, R., Young, P. R., Fish, E. N., and Platanias, L. C. (1999) Activation of the p38 mitogen-activated protein kinase by type I interferons. *J. Biol. Chem.* **274,** 30127–30131
27. Uddin, S., Lekmine, F., Sharma, N., Majchrzak, B., Mayer, I., Young, P. R., Bokoch, G. M., Fish, E. N., and Platanias, L. C. (2000) The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon $\alpha$-dependent transcriptional activation but not serine phosphorylation of Stat proteins. *J. Biol. Chem.* **275,** 27634–27640
28. Ritchie, K. J., and Zhang, D. E. (2004) ISG15: the immunological kin of ubiquitin. *Semin. Cell Dev. Biol.* **15,** 237–246
29. Li, X., Wheldon, L., and Heath, J. K. (2003) Sprouty: a controversial role in receptor tyrosine kinase signalling pathways. *Biochem. Soc. Trans.* **31,** 1445–1446
30. Minowada, G., Jarvis, L. A., Chi, C. L., Neubüser, A., Sun, X., Hacohen, N., Krasnow, M. A., and Martin, G. R. (1999) Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126,** 4465–4475
31. Leeksma, O. C., Van Achterberg, T. A., Tsumura, Y., Toshima, J., Eldering, E., Kroes, W. G., Mellink, C., Spaargaren, M., Mizuno, K., Pannekoek, H., and de Vries, C. J. (2002) Human Sprouty 4, a new ras antagonist on 5q31, interacts with the dual specificity kinase TESK1. *Eur. J. Biochem.* **269,** 2546–2556
32. Shim, K., Minowada, G., Coling, D. E., and Martin, G. R. (2005) Sprouty2, a mouse deafness gene, regulates cell fate decisions in the auditory sensory epithelium by antagonizing FGF signaling. *Dev. Cell* **8,** 553–564
33. Klein, O. D., Minowada, G., Peterkova, R., Kangas, A., Yu, B. D., Lesot, H., Peterka, M., Jernvall, J., and Martin, G. R. (2006) Sprouty genes control diastema tooth development via bidirectional antagonism of epithelial-mesenchymal FGF signaling. *Dev. Cell* **11,** 181–190
34. Basson, M. A., Watson-Johnson, J., Shakya, R., Akbulut, S., Hyink, D., Costantini, F. D., Wilson, P. D., Mason, I. J., and Licht, J. D. (2006) Branching morphogenesis of the ureteric epithelium during kidney development is coordinated by the opposing functions of GDNF and Sprouty1. *Dev. Biol.* **299,** 466–477
35. Cabrita, M. A., and Christofori, G. (2008) Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* **11,** 53–62
36. Tefft, D., Lee, M., Smith, S., Crowe, D. L., Bellusci, S., and Warburton, D. (2002) mSprouty2 inhibits FGF10-activated MAP kinase by differentially binding to upstream target proteins. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283,** L700–L706
37. Hanafusa, H., Torii, S., Yasunaga, T., and Nishida, E. (2002) Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat. Cell Biol.* **4,** 850–858
38. Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M., and Yoshimura, A. (2003) Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat. Cell Biol.* **5,** 427–432
39. Casci, T., Vinós, J., and Freeman, M. (1999) Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96,** 655–665
40. Verma, A., Deb, D. K., Sassano, A., Uddin, S., Varga, J., Wickrema, A., and Platanias, L. C. (2002) Activation of the p38 mitogen-activated protein kinase mediates the suppressive effects of type I interferons and transforming growth factor-$\beta$ on normal hematopoiesis. *J. Biol. Chem.* **277,** 7726–7735
41. 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) The p38 MAPK pathway mediates the growth inhibitory effects of interferon-$\alpha$ in BCR-ABL-expressing cells. *J. Biol. Chem.* **276,** 28570–28577
42. Lu, M., Zhang, W., Li, Y., Berenzon, D., Wang, X., Wang, J., Mascarenhas, J., Xu, M., and Hoffman, R. (2010) Interferon-$\alpha$ targets JAK2V617F-positive hematopoietic progenitor cells and acts through the p38 MAPK pathway. *Exp. Hematol.* **38,** 472–480
43. Bracarda, S., Eggermont, A. M., and Samuelsson, J. (2010) Redefining the role of interferon in the treatment of malignant diseases. *Eur. J. Cancer.* **46,** 284–297
44. Kiladjian, J. J., Cassinat, B., Chevret, S., Turlure, P., Cambier, N., Roussel, M., Bellucci, S., Grandchamp, B., Chomienne, C., and Fenaux, P. (2008) Pegylated interferon-$\alpha$-2a induces complete hematologic and molecular responses with low toxicity in polycythemia vera. *Blood* **112,** 3065–3072
45. Quintás-Cardama, A., Kantarjian, H., Manshouri, T., Luthra, R., Estrov, Z., Pierce, S., Richie, M. A., Borthakur, G., Konopleva, M., Cortes, J., and Verstovsek, S. (2009) Pegylated interferon $\alpha$-2a yields high rates of hematologic and molecular response in patients with advanced essential thrombocythemia and polycythemia vera. *J. Clin. Oncol.* **27,** 5418–5424