The Tumor Suppressive Effects of HPP1 Are Mediated Through JAK-STAT-Interferon Signaling Pathways

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HPP1, a novel tumor suppressive epidermal growth factor (EGF)-like ligand, mediates its effects through signal transducer and activators of transcription (STAT) activation. We previously demonstrated the importance of STAT1 activation for HPP1 function; however the contribution of STAT2 remains unclear. We sought to delineate the components of JAK-STAT-interferon (IFN) signaling specifically associated with HPP1s biological effects. Using stable HPP1-HCT116 transfectants, expression analyses were performed by polymerase chain reaction (PCR)/western blotting while expression knockdowns were achieved using siRNA. Growth parameters evaluated included proliferation, cell cycle distribution, and anchorage-independent growth. STAT dimerization, translocation, and DNA binding were examined by reporter assays, fluorescent microscopy, and chromatin immunoprecipitation (ChIP), respectively. Forced expression of HPP1 in colon cancer cell lines results in the upregulation of total and activated levels of STAT2. We have also determined that JAK1 and JAK2 are activated in response to HPP1 overexpression, and are necessary for subsequent STAT activation. Overexpression of HPP1 was associated with significant increases in STAT1:STAT1 ($p=0.007$) and STAT1:STAT2 ($p=0.036$) dimer formation, as well as subsequent nuclear translocation. By ChIP, binding of activated STAT1 and STAT2 to the interferon-signaling regulatory element promoter sites of the selected genes, protein kinase RNA-activated (*PKR*), *IFI44*, and *OAS1* was demonstrated. STAT2 knockdown resulted in partial abrogation of HPP1s growth suppressive activity with increased proliferation ($p<0.0001$), reduced G1/G0 phase cell cycle fraction, and a restoration of growth potential in soft agar ($p<0.01$). Presumably as a consequence of upregulation of IFN signaling elements, HPP1 overexpression resulted in an acquisition of exogenous IFN sensitivity. Physiologic doses of IFN- α resulted in a significant reduction in proliferation ($p<0.001$) and increase in G1/G0 cell cycle arrest in HPP1 transfectants. STAT2 is necessary for HPP1-associated growth suppression, and mediates these effects through activation of IFN- α pathways. Given the interest in therapeutic targeting of oncogenic *erbB* proteins, further understanding of HPP1s role as a tumor suppressive EGF-like ligand is warranted.

Introduction

HPP1 WAS ORIGINALLY IDENTIFIED using a global methylation screening assay in colorectal neoplasms derived from patients with hyperplastic polyposis, suggesting a role in tumor suppression (Young *et al.*, 2001). *HPP1* was subsequently shown to be epigenetically downregulated in over 80% of colorectal cancer, 60% of adenomas, and the vast majority of colon cancer cell lines (Elahi *et al.*, 2008). *HPP1* is predicted to encode a transmembrane protein with a short cytoplasmic tail and an extracellular component containing an epidermal growth factor (EGF)-like domain (Young *et al.*, 2001). Through ectopic expression, we have previously demonstrated a dramatic reduction in prolifera-

tive and tumorigenic potential in both *in vitro* and *in vivo* models. HPP1s tumor-suppressive activity is mediated at least in part by upregulation and activation of signal transducer and activators of transcription-1 (STAT1). However, the upstream and downstream events underlying HPP1-associated STAT signaling pathway remain unclear.

STAT family proteins are involved in cytokine, hormone, and growth factor signal transduction and are capable of mediating a wide range of biological functions including cell growth, differentiation, apoptosis, transformation, inflammation, and immune responses (Ihle, 1996; Bromberg, 2001). Activation of STAT family members is known to occur through association with tyrosine kinases and, in particular, members of the Janus Kinase (JAK) family (Leonard

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and O'Shea, 1998). JAKs activate STAT proteins through phosphorylation following their association with activated cell surface receptors (Smithgall *et al.*, 2000). Importantly, differential engagement of specific JAK-STAT pathway components is capable of producing a specific response from each receptor complex (e.g., interferon [IFN] receptors) within the relevant cellular context (Liongue *et al.*, 2012).

IFNs are important regulators of cancer cell growth and differentiation, affecting cellular communication and signal transduction pathways (Caraglia *et al.*, 2005). Although the exact basis for the IFN-dependent anticancer effect remains incompletely understood, likely pathways include the induction of apoptotic cell death (Panaretakis *et al.*, 2002; Thyrell *et al.*, 2002) and cell growth inhibition through transcriptional activation (Darnell, 1997). IFN receptor activation classically leads to the phosphorylation and activation of STAT family members and, not surprisingly, alterations in STAT expression have been shown to be an important mechanism of IFN resistance (Wong *et al.*, 1997; Brinckmann *et al.*, 2002). Type II IFNs (including IFN- γ) are known to elicit STAT1 homodimer formation, while type I IFNs (including IFN- α) are known to result in STAT1:STAT2 heterodimer formation. Interestingly, STAT2 is absolutely required for a fully effective response to IFN- α (Testoni *et al.*, 2011a). We have previously observed that STAT2 is upregulated and activated in response to HPP1 overexpression and consequently, we hypothesize that HPP1s tumor-suppressive effects are likely mediated by elements of the IFN- α pathway. We sought to delineate the components of JAK-STAT-interferon signaling specifically associated with HPP1s tumor suppressive effects.

Materials and Methods

Cell culture and transfection

The HCT-116 colorectal carcinoma cell line was obtained from American Type Culture Collection. The clonal derivatives of HCT-116 contain an empty vector (control cells) or full-length HPP1 cDNA, cloned into the pcDNA3 vector (Invitrogen) and selected in G418 (Elahi *et al.*, 2008). Three separate HPP1-overexpressing clones have been generated as previously described (Elahi *et al.*, 2008). All clonal transfectants were propagated in 1640 (Invitrogen/Gibco) growth media supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin (100 units/mL; Life Time Technologies, Inc.). Cell lines were authenticated by ATCC using 17 short tandem repeat loci and one gender loci.

Western blot

Protein was extracted using cell lysis buffer containing RIPA, sodium pyrophosphate, Na-orthovanadate, protease inhibitor, aprotinin, and leupeptin. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad). Fifty to one hundred micrograms of protein/sample was loaded and fractionated by electrophoresis in 8% and 10% SDS-PAGE gel. The protein was transferred on to a nitrocellulose membrane using Trans-Blot SD, Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat dry milk for 2 h and washed three times (5 min each) with TBST solution.

Primary antibodies (STAT1, pSTAT1, STAT2, pSTAT2, JAK1, pJAK1, JAK2, pJAK2, JAK3, pJAK3, TYK2, and

pTYK2) were purchased from Cell Signaling Technologies and diluted (1:1000) into buffer comprising 1 $\times$ TBS, 0.01% Tween-20 with either 5% bovine serum albumin (BSA) or 5% nonfat dry milk. The membranes were hybridized in a cold room with gentle shaking overnight. Membranes were then washed three times (5 min each) with 1 $\times$ TBST solution. Secondary antibodies (anti-rabbit or anti-mouse) were added to the hybridization solution and shaken gently for 1 h at room temperature. Membranes were then washed three times (5 min each) with TBST buffer. The target proteins (total and activated STAT2, JAK1, JAK2, JAK3, and TYK2) were detected by incubating membranes in ECL solution (Amersham) for 1 min at room temperature. Membranes were subsequently exposed to Kodak X-ray film and developed.

Cell cycle distribution assay

Analysis of cell cycle distribution in G0/G1 versus S versus G2/M phases was conducted by measuring cellular DNA content by propidium iodide methodology. One million cells per condition were washed in 1 $\times$ phosphate-buffered saline (PBS) and transferred into cold tubes containing 70% ethanol. Cells were kept overnight at -20°C and fixed in 70% ethanol and then rinsed with 1 $\times$ PBS. The cells were then suspended in 1 mL PI/Triton solution in the presence of RNase A and subsequently incubated at 37°C for 15 min. Cell fluorescence was then quantified by flow cytometry.

Cell proliferation

Determination of alterations in cell proliferation by MTT assay (Sigma-Aldrich) in response to various experimental conditions was carried out as previously described (Elahi *et al.*, 2008).

Growth in soft agar

A 3% bacto-agar (BD Biosciences) stock was made in sterile water and autoclaved. For constitution of the bottom layer, 0.6% gel was prepared in growth medium with 3% bacto-agar stock and kept at 48°C . Two milliliter per well was plated into a 12-well tissue culture plate and solidified at room temperature. Cells were counted using Trypan blue dye and a hemacytometer. Separate suspensions of 3×10^3 cells were prepared and mixed with agar medium. The cell-agar mixture (1.5 mL) was dispensed on top of the base layer with subsequent incubation for 2–3 weeks. One milligram per milliliter of MTT was dissolved in the growth medium and 100 μL of MTT dye was added in each well and incubated overnight. Colonies were counted and photographed.

Apoptosis

Determination of programmed cell death was carried out as previously described following 48-h treatment with physiologic doses of IFN- α (100 IU) added to the growth media (Elahi *et al.*, 2008).

STAT activation

Following assessment of transfection efficiency with pCIS-CK plasmid according to the manufacturer's protocol (Stratagene), we transfected HPP1-overexpressing transfectants

and control cells in triplicate with pGAS and pISRE plasmids. The pGAS-Luc plasmids and pISRE-Luc plasmids (Stratagene) contained four interferon-gamma activated sequence or five interferon-signaling regulatory element (ISRE) sequences, respectively, in a promoter upstream of a TATA box and luciferase gene. Luminescence was determined using a luminometer.

1×10^3 Cells were seeded onto coverslips in six-well plates and incubated overnight in appropriate growth media. Cells were washed with PBS and PIPES buffer. Cells were then fixed in methanol at -20°C for 6 min. Nuclei were made permeable with 0.5% Nonidet P-40/PIPER buffer for 13 min at room temperature. Coverslips were washed with PBS and cells blocked in 1% BSA/PBS for 1 h followed by incubation for 50 min with $1 \mu\text{g/mL}$ of anti-pSTAT1 antibody or anti-pSTAT2 antibody (Cell Signaling). Cells were then washed in PBS and incubated with fluorescein-conjugated secondary antibody (1:1000) for 1 h followed by three additional washings. Coverslips were then mounted onto glass slides and examined by fluorescent microscopy.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was carried out as described previously. HPP1-overexpressing cells and control cells were harvested. ChIP was carried out using 6×10^6 cells and $1 \mu\text{g}$ of specific antibody per immunoprecipitation. The antibodies used were anti-phospho-STAT1 and anti-phospho-STAT2 antibodies (Cell Signaling Technology). The immunoprecipitated DNA was treated with RNase (Ambion) at 37°C and proteinase K (Roche) for 1 h at 45°C . The DNA was purified with Qiagen PCR spin columns. Purified DNA was analyzed by quantitative polymerase chain reaction (PCR) using specific primers.

RNA interference assay

Using commercially available siRNAs (Dharmacon), transient knockdown was performed following the manufacturer's recommended protocol with concentrations of 40–100 nM and with confirmation by western blot analysis.

Results

HPP1 overexpression induces activation, dimerization, and nuclear translocation of STAT1 and STAT2

We have previously demonstrated that STAT1 plays an integral role in mediating the tumor suppressive effects of HPP1 (Elahi *et al.*, 2008). By gene expression profiling, STAT2 is also upregulated, although less dramatically, in HPP1-overexpressing transfectants as compared to empty vector control cells (Table 1). In Figure 1A, expression validation of both total and activated STAT2 is demonstrated in the setting of HPP1 overexpression. These findings suggest that HPP1-mediated signal transduction may potentially occur through STAT1:STAT1 homodimerization (IFN- γ pathway) and/or STAT1:STAT2 heterodimerization (IFN- α pathway). To distinguish between IFN pathways we exploited the fact that activated STAT1 is able to stimulate transcription of target genes that contain GAS sites (by STAT1:STAT1 homodimer binding) or ISRE sites (by STAT1:STAT2 heterodimer binding) within their promoter regions. A statistically significant increase in signal intensity was observed for both pGAS and pISRE plasmid transfection suggesting an increase in STAT1:STAT1 homodimer and STAT1:STAT2 heterodimer formation in HPP1-overexpressing cells as compared to control cells (Fig. 1B). To affect cellular transcription, the formation of STAT dimers must be followed by translocation to the nucleus. We demonstrated a significant increase in cytoplasmic to nuclear localization with both pSTAT1 and pSTAT2 (Fig. 1C), indicating that there may be at the very least, functional STAT1:STAT2 heterodimer signaling and possibly STAT1:STAT1 homodimer activity.

HPP1's tumor suppressive activities are predominantly mediated by type I IFN signaling pathways

From a functional standpoint, by gene expression profiling, the differential upregulation of STAT1 and STAT2 in HPP1-overexpressing transfectants was associated with a concomitant dramatic upregulation of IFN-stimulated genes [18 of 42 upregulated genes (Elahi *et al.*, 2008)]. Virtually all of these genes were associated with IFN- α inducibility

TABLE 1. SELECTED GENES UPREGULATED WITH HPP1 OVEREXPRESSION AS COMPARED TO CONTROL CELLS

Accession No.	Gene name	Gene symbol	Fold change
NM_005532	Interferon, alpha-inducible protein 27	IFI27	40.31
NM_002038	Interferon, alpha-inducible protein (clone IFI-6–16)	G1P3	23.15
NM_001548	Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	21.6
NM_007315	Signal transducer and activator of transcription 1	STAT1	7.63
NM_002534	2',5'-Oligoadenylate synthetase 1, 40/46 kDa	OAS1	7.29
NM_003641	Interferon-induced transmembrane protein 1 (9–27)	IFITM1	6.2
NM_006417	Interferon-induced protein 44	IFI44	6.2
NM_001572	Interferon regulatory factor 7	IRF7	5.4
NM_002534	2',5'-Oligoadenylate synthetase 1, 40/46 kDa	OAS1	4.59
NM_006084	Interferon-stimulated transcription factor 3, gamma 48 kDa	ISGF3G	4
NM_001547	Interferon-induced protein with tetratricopeptide repeats 2	IFIT2	3.81
NM_001549	Interferon-induced protein with tetratricopeptide repeats 4	IFIT4	3.81
NM_016192	Transmembrane protein with EGF-like and two follistatin-like domains 2	HPP1	3.56
NM_011905	Signal transducer and activator of transcription 2	STAT2	1.5

EGF, epidermal growth factor; STAT, signal transducer and activators of transcription.

FIG. 1. Signal transducer and activators of transcription (STAT) signaling with HPP1 overexpression. **(A)** Total and activated STAT2 protein levels are upregulated with HPP1 overexpression as compared to control cells as measured by western blot. **(B)** Using pGAS-luc and pISRE-luc plasmids, we demonstrated significant increase of STAT1 homodimer ($p=0.007$) and STAT1:STAT2 heterodimer ($p=0.036$) formation, respectively, as measured by luminescence. **(C)** Fluorescein-conjugated antibodies were used to demonstrate pSTAT1 (top panels) and pSTAT2 (bottom panels) accumulation in the nucleus of HPP1 overexpressing cells. **(D)** Chromatin immunoprecipitation assay demonstrated increased pSTAT1 (left) and pSTAT2 (right) binding to interferon-signaling regulatory element sequences in the promoters of PKR, IFI44, and OAS1 in HPP1 cells as compared to control cells. GAS, gamma activated sequence; PKR, protein kinase RNA-activated.

with ISRE sites within their promoters, suggesting that STAT1:STAT2 pairing may represent the more biologically relevant STAT dimer (and thus type I IFN signaling, the more important pathway) in the setting of HPP1 activity. By PCR, we have previously confirmed increased expression of three of the upregulated IFN-stimulated genes, *OAS1*, *PKR*, and *IFI44* in the HPP1 transfectants (Elahi *et al.*, 2008). For additional physiological confirmation of STAT-dependent downstream activation of IFN-inducible genes, we performed ChIP for the three selected genes (*OAS1*, *PKR*, and *IFI44*) (Table 1). Using anti-phospho-STAT1 and anti-phospho-STAT2 antibodies, we have confirmed direct interaction of activated STAT1 and STAT2 with the ISRE sequences in the promoter regions of *OAS1*, *PKR*, and *IFI44* (Fig. 1D).

Abrogation of STAT2 signaling in HPP1-overexpressing transfectants results in partial restoration of proliferation and anchorage-independent growth

We have previously demonstrated that abrogation of STAT1 expression in HPP1-overexpressing transfectants results in an augmentation of proliferative capacity and restoration of anchorage-independent growth. To further confirm the contribution of STAT2 and thus STAT1:STAT2 heterodimers to HPP1 function, we have used siRNA-mediated knockdown in HPP1 transfectants. Maximum reduction in STAT2 expression was achieved after 72-h treatment with siRNA (Fig. 2A).

With STAT2 knockdown, HPP1-overexpressing transfectants demonstrated a twofold augmentation in proliferative capacity (Fig. 2B), a reduction in G1/G0 phase cell cycle distribution (Fig. 2C), and an increased capacity for anchorage-independent growth ($p=0.006$) as compared to the same HPP1-overexpressing transfectants treated with nontargeting control siRNA (Fig. 2D).

JAK1 and JAK2 are upregulated with HPP1 overexpression and are responsible for the activation of STAT1 and STAT2

Janus Kinases (JAK) are one of several mammalian nonreceptor tyrosine kinase families that are essential mediators of cellular signaling through cytokine receptors (Aringer *et al.*, 1999). There are four members of the JAK family: JAK1, JAK2, JAK3, and TYK2. Receptor-associated JAKs are able to autophosphorylate as well as to phosphorylate the receptor, which in turn can recruit inactive STAT monomers (Pinkas-Kramarski *et al.*, 1996). JAK-mediated tyrosine phosphorylation then results in STAT dimerization and consequent activation (Pinkas-Kramarski *et al.*, 1996). Although we have demonstrated activation of STAT1 and STAT2 with HPP1 overexpression, the responsible upstream kinase(s) remain unknown. The baseline expression and activation levels of the four JAK family members (JAK1, JAK2, JAK3, and TYK2) were determined by western blot analysis in HPP1-overexpressing and control transfectants.

FIG. 2. Abrogation of STAT2 counteracts HPP1's tumor suppressive effects. (A) Quantification of STAT2 protein expression by western blot following 72-h transfection with STAT2 siRNA at various concentrations. Of note, 40 nM of STAT2 siRNA was used in all subsequent experiments. (B) Proliferation significantly increased in HPP1 cells treated with STAT2 siRNA as compared to HPP1 cells treated with nontargeting siRNA ($p < 0.0001$), although still significantly less than control cells ($p = 0.02$). (C) Alterations in cell cycle distribution for HPP1 cells treated with STAT2 siRNA and nontargeting siRNA, as well as control cells. (D) STAT2 siRNA treatment resulted in a significant increase in soft agar colony formation as compared to nontargeting siRNA treatment in HPP1 cells ($p = 0.0061$).

We demonstrated an increase in baseline (protein and RNA) and activated JAK1 and JAK2 expression in response to HPP1 overexpression (Fig. 3A, B), suggesting that one or both kinases may play a role in mediating STAT activation with HPP1 overexpression. We found no correlation between JAK3 or TYK2 activation and HPP1 overexpression (data not shown). To confirm the importance of JAK1 and JAK2 in mediating STAT activation, we utilized validated oligonucleotide siRNA sequences against both kinases. We identified maximal reduction in protein expression with 72-h treatment using 50 nM concentration of siRNA (Fig. 3C). With both JAK1 and JAK2 knockdown, we demonstrated a significant decrease in phosphoSTAT1 and phosphoSTAT2 expressions (Fig. 3D). We also demonstrated an increase in proliferative capacity with knockdown of JAK1 and JAK2 ($p = 0.01$) (Fig. 3E), although interestingly, the impact on proliferation did not differ significantly with concomitant knockdown of both JAK1 and JAK2 as compared to either individually.

IFN- α attenuates proliferative capacity without impacting cell survival in HPP1-overexpressing transfectants

IFNs initiate signaling by binding to their receptors resulting in transphosphorylation and activation of JAK1/JAK2, which in turn, results in the phosphorylation of STAT1 and STAT2, which are important mediators of IFN target gene expression (Darnell *et al.*, 1994). IFNs are well known to regulate cell cycle progression, to induce cell differentiation, and to modulate immune response. The majority of cancer cell lines and primary tumors including colon cancer are resistant to IFNs (Kaplan *et al.*, 1998). This

resistance is reportedly acquired by a number of mechanisms, including defects in the expression of IFN-associated transcription factors such as STATs. The HCT-116 colon cancer cell line, which has low levels of STAT1 and STAT2, is resistant to IFN treatment despite substantial IFN receptor expression. Of note, HPP1 expression had no impact upon IFN receptor expression (Fig. 4A) suggesting that receptor upregulation was not responsible for increased JAK-STAT activation. With the introduction of HPP1 in HCT-116 cells, we have demonstrated an upregulation of the components associated with IFN- α stimulation. We, therefore, sought to determine if HPP1 overexpression also confers sensitivity to IFN- α treatment. We first utilized dose-escalation experiments to determine maximal STAT activation. We determined that maximal STAT activation occurs at 100 IU/mL, which is physiologically relevant (Fig. 4B). Next, we demonstrated that IFN- α treatment limits proliferative capacity in HPP1-overexpressing transfectants as compared to control cells (twofold reduction) (Fig. 4C), and also results in alterations in cell cycle distribution consistent with G1/G0 arrest (Fig. 4D). Interestingly however, IFN- α treatment was not sufficient to induce apoptotic cell death in HPP1-overexpressing transfectants or control cells (Fig. 4E). Of note, IFN- γ failed to illicit any demonstrable phenotypic changes in HPP1-overexpressing transfectants or control cells.

Discussion

HPP1 is a novel epigenetically silenced tumor suppressor gene that appears to be a suppressive EGF-like ligand (Uchida *et al.*, 1999) and is downregulated in the majority of adenomas and colorectal cancers. It has gained emerging

FIG. 3. JAK1 mediates STAT activation in HPP1-overexpressing cells. (A) Baseline and activated JAK1 and JAK2 protein levels are upregulated with HPP1 overexpression as compared to control cells as measured by western blot. (B) JAK1 and JAK2 RNA levels at baseline with HPP1 overexpression as compared to control, as measured by PCR. (C) Optimal JAK1 and JAK2 protein knockdown in HPP1 cells was achieved with 50 nM concentration of siRNA for 72-h duration. (D) Western blot demonstrating a significant reduction in pSTAT1 and pSTAT2 expression with JAK1 and JAK2 knockdown. (E) Proliferation significantly increased in HPP1 cells treated with JAK1 ($p=0.01$), JAK2 ($p=0.001$), and concomitant JAK1/JAK2 ($p=0.008$) siRNA as compared to HPP1 cells treated with nontargeting siRNA. PCR, polymerase chain reaction.

interest as a potential serum and stool-based methylated biomarker in colorectal cancer (Belshaw *et al.*, 2004). However, there is very little known about the mechanisms by which HPP1 inhibits tumorigenicity. Herein, we have demonstrated that HPP1s growth suppressive activity is mediated by components of the Type 1 IFN pathway through the predominant activation of JAK1 and JAK2, and STAT1/STAT2. We have confirmed that the antitumorigenic effects of HPP1 are associated primarily with STAT1: STAT2 activation, heterodimerization, nuclear translocation, and activation of downstream genes such as *OAS1*, *IFI44*, and *PKR*, that harbor ISRE promoter sites. We have also shown that HPP1 overexpression re-establishes the biological infrastructure to restore sensitivity to IFN- α *in vitro*.

We have demonstrated HPP1-mediated nuclear translocation of activated STAT1 and STAT2 and promoter binding to IFN-inducible genes such as *OAS1*, *IFI-44*, and *PKR*. In this regard, our results are similar to those demonstrated in IFN- α -treated hepatoma cells, including pSTAT2 recruitment and resultant expression of OAS-1 and IFI-44 (Testoni *et al.*, 2011a). Interestingly, in a fibrosarcoma cell

line, the functional activity of STAT2 is augmented with serine phosphorylation in an IFN-dependent manner (Steen *et al.*, 2012). This post-translational event is important for signaling and results in enhanced antiproliferative and antiviral response to IFN- α . This finding suggests the presence of a regulatory serine residue in STAT2 that affects the response to IFN treatment (Steen *et al.*, 2012). We did not investigate the specific nature of STAT2 phosphorylation that occurs in response to HPP1 overexpression with or without IFN- α treatment in our colon cancer model.

Most intriguingly, pSTAT2 has been shown to be involved in target gene repression. In response to IFN- α , STAT2 mediates repression of DNp73 (a transrepressor of p53) resulting in apoptotic death in hepatoma cells (Testoni *et al.*, 2011b). Similarly, about one-quarter of the IFN-inducible genes found to be repressed following IFN- α treatment recruited both STAT2 and pSTAT2 to the promoter regions. Moreover, half of the genes repressed by IFN- α treatment demonstrated STAT2 binding before treatment and pSTAT2 binding afterward (Testoni *et al.*, 2011a). We focused our investigation of HPP1-mediated tumor suppression on three typical

FIG. 4. Interferon (IFN)- α attenuates proliferative capacity without impacting cell survival in HPP1 cells. (A) PCR demonstrating equivalent mRNA expression of IFN- α receptor (*top*) and IFN- γ receptor (*bottom*) in HPP1 and control cells. (B) Treatment of HPP1 cells with escalating doses of IFN- α resulting in maximal pSTAT2 expression at 100 IU/mL. (C) Treatment of HPP1 cells with 100 IU/mL of IFN- α resulted in an increase in the percentage of cells in G1/G0 arrest and a decrease in the percentage of cells in G2/S phase. (D) Proliferation significantly decreased in HPP1 cells treated with 100 IU/mL of IFN- α ($p=0.0001$). (E) Treatment of HPP1 cells with 100 IU/mL of IFN- α failed to impact apoptotic cell death ($p=0.8779$).

members of IFN-inducible gene family. However, our initial microarray data revealed both upregulated and downregulated candidate HPP1-sensitive genes.

STAT phosphorylation is mediated by kinases of the Janus Family (Schindler and Darnell, 1995). We have demonstrated increased activation of two of the Janus Family kinases, JAK1 and JAK2, with HPP1 overexpression, which in turn phosphorylate STAT1 and STAT2. We have demonstrated that abrogation of JAK1 or JAK2 activation results in partial alleviation of HPP1-mediated tumor suppression. Interestingly, JAK inhibitors have recently entered the clinical arena for patients with Philadelphia chromosome-negative myeloproliferative neoplasms after the identification of constitutive JAK2 activation (through a single base-pair substitution) in the majority of cases (Quintas-Cardama *et al.*, 2010). Two randomized controlled trials have demonstrated the clinical and survival benefits of ruxolitinib, a selective inhibitor of JAK1 and JAK2, in patients with myelofibrosis (Harrison *et al.*, 2012; Verstovsek *et al.*, 2012). Interestingly, JAKs have been demonstrated to have functional activity in the nucleus in leukemic cell lines, independent of its association with STATs. Specifically, JAK-mediated histone 3 phosphorylation prevents transcription factor binding, which would otherwise lead to increased mitotic recombination and aneuploidy (Quintas-Cardama *et al.*, 2011). Clearly, the results of JAK activation are contextually dependent, although direct chromosomal effects of JAK activation in HPP1 overexpression is a possibility.

IFN- α has emerged as an important regulator of cancer cell growth and differentiation by affecting signal transduction pathways (Pestka, 2000). However, colon cancer cell lines, including HCT-116, are resistant to IFN treat-

ment. We have demonstrated that overexpression of HPP1 leads to an upregulation and activation of JAK1, STAT1, and STAT2, which are also the components of IFN- α signaling (Caraglia *et al.*, 2005). We, therefore, hypothesized that HPP1 overexpression would restore IFN- α sensitivity. Although we were able to demonstrate decreased proliferation and cell cycle arrest with IFN- α in HPP1-overexpressing cells, we observed no increase in apoptotic cell death. IFN- α -mediated apoptosis involves ordered activation of caspases and the mitochondrial pathway (Thyrell *et al.*, 2002). More specifically, IFN- α activates the proapoptotic bcl-2 related proteins Bak and Bax (Panaretakis *et al.*, 2003). Interestingly, HCT-116 cells have an intact mitochondrial-dependent apoptosis pathway (Lim do and Park, 2009). Moreover, we have demonstrated HPP1-dependent upregulation of *PKR*, which is also activated by IFN- α , and induces the specific expression of selected proteins (including Bax) that trigger cell death by engaging with the caspase pathway (Caraglia *et al.*, 1999). However, HCT-116 cells have mutant K-ras, which may abrogate any proapoptotic IFN- α effects, given the recent evidence indicating that IFN-induced upregulation of epidermal growth factor receptor is an important reactive mechanism of resistance for tumor cells. This inducible survival pathway involves activation of ras/raf/mek/erk signaling (Caraglia *et al.*, 2005). The constitutive activity of ras in HCT-116 may, therefore, abrogate IFN- α -induced cell death, and explain our observed failure of IFN to promote apoptosis. Given that we have previously demonstrated that HPP1 was sufficient to induce apoptotic cell death (fourfold) as compared to control cells, failure of IFN- α to induce apoptotic cell death may also simply be the result of pathway saturation.

We approached our investigation of STAT signaling from the classic point of view that STAT family proteins are latent transcription factors, requiring phosphorylation for activation (Williams, 2000). However, recent evidence suggests that unphosphorylated STAT1 and STAT2 are able to shuttle in and out of the nucleus (Banninger and Reich, 2004; Cheon and Stark, 2009). Moreover, unphosphorylated STAT1 and STAT2 have been shown to play a role in regulating the expression of IFN-inducible genes (Cheon and Stark, 2009; Testoni *et al.*, 2011a), including some typical IFN-inducible genes such as *RIG-G* (Lou *et al.*, 2009). Interestingly, Testoni *et al.* (2011a) also demonstrated recruitment of unphosphorylated STAT2 to the promoter region of *OAS1* with IFN- α treatment. We did not evaluate the effect of HPP1 on unphosphorylated STAT recruitment, although this potential mechanism is intriguing and deserves further investigation.

Conclusions

HPP1 is capable of mediating tumor inhibition, contrary to the well-recognized tumorigenic signaling cascades mediated by erbB family members in response to the binding of other homologous ligands. These effects are mediated through canonical JAK-STAT signaling involving STAT1 and STAT2, as well as JAK1 and JAK2, and confer sensitivity to exogenous IFN- α . Given the substantial interest in the therapeutic targeting of oncogenic erbB family members, further understanding of HPP1's role and mechanisms of action as a tumor suppressive EGF-like ligand is of substantial interest.

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Disclosure Statement

No competing financial interests exist.

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