Stat1 stimulates cap-independent mRNA translation to inhibit cell proliferation and promote survival in response to antitumor drugs

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The signal transducer and activator of transcription 1 (Stat1) functions as a tumor suppressor via immune regulatory and cell-autonomous pathways. Herein, we report a previously unidentified cell-autonomous Stat1 function, which is its ability to exhibit both antiproliferative and prosurvival properties by facilitating translation of mRNAs encoding for the cyclin-dependent kinase inhibitor p27Kip1 and antiapoptotic proteins X-linked inhibitor of apoptosis and B-cell lymphoma xL. Translation of the select mRNAs requires the transcriptional function of Stat1, resulting in the up-regulation of the p110γ subunit of phosphoinositide 3-kinase (PI3K) class IB and increased expression of the translational repressor translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1). Increased PI3Kγ signaling promotes the degradation of the eIF4A inhibitor programmed cell death protein 4, which favors the cap-independent translation of the select mRNAs under conditions of general inhibition of protein synthesis by up-regulated eIF4E-binding protein 1. As such, Stat1 inhibits cell proliferation but also renders cells increasingly resistant to antiproliferative effects of pharmacological inhibitors of PI3K and/or mammalian target of rapamycin. Stat1 also protects Ras-transformed cells from the genotoxic effects of doxorubicin in culture and immune-deficient mice. Our findings demonstrate an important role of mRNA translation in the cell-autonomous Stat1 functions, with implications in tumor growth and treatment with chemotherapeutic drugs.

Stat1 | phosphoinositide 3-kinase | programmed cell death protein 4 | eIF4E-binding protein 1 | mRNA translation

Stat1 is essential for innate immunity by protecting the host from infections with viruses and other pathogens (1). Stat1 mediates the transcriptional induction of IFN-inducible genes by acting downstream of type I (α/β) and II (γ) IFN receptors, resulting in the synthesis of proteins with antimicrobial and immune regulatory properties (1). Both types of IFNs induce Stat1 phosphorylation at tyrosine (Y) 701, which is mediated by receptor-associated Janus kinases (Jaks) and is required for homodimerization as well as heterodimerization with other Stat family members (1). Stat1 Y701 phosphorylation is crucial for DNA binding and transcriptional function, whereas serine (S) 727 phosphorylation promotes gene transcription in response to IFNs (1). Nevertheless, unphosphorylated Stat1 also possesses transcriptional functions in cells infected with viruses or exposed to DNA damage (1–3).

Genetic inactivation of mouse Stat1 has demonstrated that it functions as a tumor suppressor via two different but not mutually exclusive mechanisms (4, 5). One mechanism depends on the induction of antitumor immune responses (6) and the other on the suppression of oncogenic signaling in a cell-autonomous (tumor cell-specific) manner (7–11). The antitumor properties of Stat1 have been best documented in breast cancers in which Stat1 has been shown to inhibit cell proliferation or render cells increasingly resistant to antitumor drugs. Our work may result in the design of therapies that disarm the pro-survival function of Stat1 in tumors without compromising its ability to mount an effective antitumor immune response.

Significance

Stat1 functions as a tumor suppressor by inhibiting cell proliferation and mediating antitumor immune responses. Thus, Stat1 is thought to be a suitable target for the implementation of effective antitumor therapies. However, recent findings have shown that Stat1 can mediate resistance to antitumor drugs through mechanisms that are not well understood. Herein, we demonstrate the ability of Stat1 to induce phosphoinositide 3-kinase γ (PI3Kγ) signaling and inhibit general protein synthesis, which results in the translation of select mRNAs encoding for proteins that inhibit cell proliferation or render cells increasingly resistant to antitumor drugs. Our work may result in the design of therapies that disarm the pro-survival function of Stat1 in tumors without compromising its ability to mount an effective antitumor immune response.

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of the majority of capped mRNAs; eIF4F is negatively regulated by the eIF4E-binding proteins 1 and 2 (4E-BPs), which interact with eIF4E to prevent eIF4F formation (23). Activation of the PI3K pathway leads to mTORC1-mediated phosphorylation of 4E-BPs and their dissociation from eIF4E, resulting in an active eIF4F complex that stimulates cap-dependent translation (23). eIF4F activity is enhanced in tumor cells due to increased eIF4E expression and/or phosphorylation at S209 (24).

Cells respond to IFNs by increasing PI3K signaling, leading to increased phosphorylation of 4E-BPs and stimulation of cap-dependent translation of mRNAs transcriptionally induced by Jak-Stat activation (25–27). Herein, we demonstrate that Stat1 acts independent of IFNs to induce PI3K signaling by facilitating the expression of the p110γ catalytic subunit of PI3K class IB at the transcriptional level. We also show that the transcriptional properties of Stat1 are required to increase 4EBP1 expression, resulting in the inhibition of general protein synthesis and cell proliferation. Both pathways are mediated by unphosphorylated Stat1 and contribute to increased translation of select mRNAs encoding for the cyclin-dependent kinase (cdk) inhibitor p27kip1, X-linked inhibitor of apoptosis (XIAP), and B-cell lymphoma x1 (Bcl-x1) as a means to inhibit proliferation or promote survival in response to chemotherapeutic drugs.

Results

Stat1 Stimulates PI3K Signaling in Response to Mitogenic Treatment.

Much is known about Stat1’s role in cytokine signaling (1), but its function in mitogenic responses is less understood. We observed that serum stimulation of primary mouse embryonic fibroblasts (MEFs) resulted in the induction of Akt phosphorylation at T308 and S473, which was higher in Stat1+/+ than Stat1−/− MEFs (Fig. 1A). Serum stimulation induced the phosphorylation of the 70 kDa isoform of the ribosomal S6 kinase 1 (S6K1) at T398, which is mediated by mTORC1, at higher levels in primary Stat1+/+ than Stat1−/− MEFs (Fig. 1A). In line with increased S6K activity, ribosomal S6 protein phosphorylation at S235/S236 was more highly increased in primary Stat1+/+ than Stat1−/− MEFs (Fig. 1A). The ability of Stat1 to increase Akt phosphorylation and mTORC1 activity was also detected in spontaneously immortalized MEFs (Fig. S1A). It is noteworthy that Akt phosphorylation was equally induced in IFN-γ-treated immortalized Stat1+/+ and Stat1−/− cells, suggesting that stimulation of PI3K signaling by IFNs may not be regulated by Stat1 (Fig. S1B).

We next examined whether the effects of Stat1 on the PI3K pathway were affected by cell transformation. We used Stat1+/+ and Stat1−/− MEFs transformed by Ha-Ras G12V based on studies showing the induction of PI3K signaling by activated Ras and the ability of Stat1 to suppress Ras-mediated tumorigenesis (7, 28). We found that Akt S473 phosphorylation as well as phosphorylation of the p70 and p85 isoforms of S6K1 and 2, respectively, were more pronounced in Ras-transformed Stat1+/+ than Stat1−/− cells after serum stimulation (Fig. S1C). Reconstitution of Ras-transformed Stat1−/− cells with either wild-type Stat1 or Stat1 Y701F and/or S727A mutants resulted in the induction of Akt and S6K1/2 phosphorylation at comparable levels, indicating that increased PI3K signaling is mediated by unphosphorylated Stat1 (Fig. S1B).

To determine how Stat1 increases PI3K signaling, we examined the levels of the regulatory and catalytic subunits of the PI3K class I family by immunoblotting. We observed that expression of the catalytic p110γ subunit of PI3K class IB was decreased in immortalized as well as Ras-transformed Stat1−/− cells compared with Stat1+/+ cells, as opposed to expression of the p110α catalytic subunit of class IA, which was not affected by Stat1 (Fig. 1B). We further noticed that expression of the p101 and p87 regulatory subunits of PI3K class IB as well as phosphorylation and expression of the p85α/β regulatory subunit of class IA PI3K were not controlled by Stat1 (Fig. 1B). Loss of Stat1 was associated with a decreased expression of PI3CG mRNA encoding for the p110γ subunit in immortalized or Ras-transformed Stat1−/− cells compared with Stat1+/+ cells, supporting a transcriptional role of Stat1 (Fig. 1C and D).

Stat1 Inhibits Protein Synthesis at the Level of mRNA Translation Initiation.

Activation of mTORC1 by increased PI3K signaling results, directly or indirectly, in the phosphorylation of several factors, with key roles in mRNA translation (21). Thus, we looked at the phosphorylation of eIF4B at S422, which facilitates its interaction with eIF4A, leading to increased RNA helicase activity (29, 30). Also, we examined the phosphorylation of eIF4G1 at S1108, which stabilizes its interaction with eIF3 and the subsequent loading of ribosomal small subunits (31, 32). We found that phosphorylation of either eIF4B or eIF4G1 was more highly increased in primary, immortalized, or Ras-transformed Stat1−/− than Stat1+/+ cells after serum treatment (Fig. S3). Also, immortalized and Ras-transformed Stat1−/− cells expressed increased levels of eIF4G2, an initiation factor involved in the stimulation of mRNA translation under stress (33) (Fig. S3). Furthermore, serum-deprived Stat1−/− cells contained decreased levels of the translational suppressor programmed cell death protein 4 (PDCD4) compared with Stat1+/+ cells, an effect that was more pronounced in Ras-transformed than immortalized cells (Fig. S3).

Despite the positive effects of Stat1 on eIF4G and eIF4B, we noticed that Stat1 inhibited general protein synthesis in immortalized as well as Ras-transformed cells (Fig. 2A and Fig. S4A). In addition, primary, immortalized, as well as Ras-transformed Stat1−/− cells contained higher levels of 4EBP1 than the corresponding Stat1+/+ cells (Fig. 2B and Fig. S4 B and C). Furthermore, serum stimulation increased 4EBP1 phosphorylation at
higher levels in immortalized or Ras-transformed Stat1+/+ than Stat1−/− cells as a result of PI3K stimulation (Fig. 2B and Fig. S4C). Because detection of the total amount of 4EBP1 is hindered by the delayed mobility of the phosphorylated protein in polyacrylamide gels, the total levels of unphosphorylated 4EBP1 were assessed after treatment with the PI3K/mTOR inhibitor BEZ235 (34). We found that 4EBP1 but not 4EBP2 was more highly expressed in immortalized Stat1+/+ than Stat1−/− cells, a difference that was more evident by Ras transformation (Fig. S4E). 4EBP1 up-regulation was a transcriptional effect because a higher amount of 4EBP1 mRNA was expressed in immortalized or Ras-transformed Stat1+/+ than Stat1−/− cells (Fig. 2C and Fig. S4D).

Next, we examined the effects of Stat1 on the eIF4F preinitiation complex. We observed that eIF4E S209 phosphorylation was lower in immortalized or Ras-transformed Stat1+/+ than Stat1−/− cells and less sensitive to pharmacological inhibition of MEK1 by PD325901 in the former than latter cells (Fig. S5). eIF4E S209 phosphorylation is mediated by the mitogen-activated protein (MAP) kinase-interacting kinase 1 and 2 (Mnk1/2) and takes place within the eIF4F complex (35). As such, increased eIF4E S209 phosphorylation was indicative of a better eIF4F complex formation in Stat1+/+ than in Stat1−/− cells. Consistent with this interpretation, cap-analog binding assays showed increased binding of eIF4G1 and decreased binding of 4EBP1 to eIF4E by the loss of Stat1 in either immortalized or Ras-transformed cells (Fig. 2D and Fig. S4F). Collectively, the data were consistent with an inhibitory effect of Stat1 on the eIF4F preinitiation complex as a result of 4EBP1 up-regulation.

**Stat1 Stimulates Cap-Independent mRNA Translation.** We reasoned that the ability of Stat1 to stimulate PI3K signaling and at the same time inhibit general protein synthesis may have had implications in the expression of proteins acting in the PI3K pathway. Several effectors of the PI3K pathway were shown to be under translational control, among which the XIAP and Bcl-xl proteins play prominent roles (20). Also, the PI3K pathway impacts the expression of the cdk inhibitor p27Kip1 (20), which is an important mediator for the antiproliferative and tumor-suppressive effects of Stat1 on Ras-transformed cells (7, 9). The 5′ untranslated region (UTR) of p27Kip1, XIAP, or Bcl-xl mRNAs contains regulatory features known as internal ribosome entry sites (IRESes) that are highly conserved in mouse and human genes with a specific role in stimulation of cap-independent translation (36–38).

We observed an up-regulation of p27Kip1, XIAP, and Bcl-xl proteins in immortalized or Ras-transformed Stat1+/+ cells compared with Stat1−/− cells under serum starvation (Fig. 3A and B). Serum stimulation caused the down-regulation of p27Kip1 and XIAP proteins in both Stat1+/+ and Stat1−/− cells, although expression of each protein was maintained at a higher level in the former than latter cells (Fig. 3A). Bcl-xl protein was not affected by serum treatment and was maintained at elevated levels in immortalized as well as Ras-transformed Stat1+/+ cells compared with Stat1−/− cells (Fig. 3B). Increased p27Kip1 protein was in line with a transcriptional effect of Stat1 on CDKN1B expression, as previously reported (7). Increased XIAP and Bcl-xl expression was not a transcriptional effect of Stat1 inasmuch as their mRNA levels did not significantly differ between Stat1+/+ and Stat1−/− cells. XIAP mRNA was expressed in immortalized or Ras-transformed Stat1−/− cells (Fig. 2C) and XIAP and Bcl-xl proteins in both Stat1+/+ and Stat1−/− cells, although expression of each protein was maintained at a higher level in the former than latter cells (Fig. 3A). Bcl-xl protein was not affected by serum treatment and was maintained at elevated levels in immortalized as well as Ras-transformed Stat1+/+ cells compared with Stat1−/− cells (Fig. 3B). Increased p27Kip1 protein was in line with a transcriptional effect of Stat1 on CDKN1B expression, as previously reported (7).

Fig. 2. Stat1 increases the expression and phosphorylation of 4EBP1. (A) Ha-Ras G12V-transformed Stat1+/+ and Stat1−/− MEFs were subjected to [35S]methionine labeling in the presence of 10% (vol/vol) dialyzed calf serum for the indicated time (h). (B) Serum-starved Ha-Ras G12V-transformed Stat1+/+ and Stat1−/− MEFs were stimulated with 10% (vol/vol) calf serum for the indicated time (h). (C) Detection of 4EBP1 and PKR mRNAs in Ha-Ras G12Vtransformed cells by qPCR. (D) Detection of eIF4E interaction with eIF4G1 and 4EBP1 by 7-methyl-GTP Sepharose 4B affinity chromatography in whole-cell extracts (WCEs) from Ha-Ras G12V-transformed cells maintained in the absence or presence of 10% (vol/vol) calf serum for 6 h. (B and D) Cell extracts (50 μg of protein) were subjected to immunoblot analyses for the indicated proteins.

Fig. 3. Stat1 promotes cap-independent translation of p27Kip1, XIAP, and Bcl-xl. (A and B) Control (immortalized) and Ras G12V-transformed Stat1+/+ and Stat1−/− MEFs were serum starved for 18 h followed by stimulation with 10% (vol/vol) calf serum for the indicated time points (h). Protein extracts (50 μg) were subjected to immunoblot analyses for the indicated proteins. The ratio of p27Kip1, XIAP, Bcl-xl, and Mcl-1 to actin for each lane is indicated. (C–E) Ha-Ras G12V-transformed Stat1+/+ and Stat1−/− MEFs were transfected with plasmids containing the 5′ UTR of p27Kip1 (C), GAPDH (C), Bcl-xl (D), or XIAP mRNA (E) between either the renilla and firefly luciferase genes (C), or the CAT and β-galactosidase (β-gal) genes (D and E). The ratio of firefly to renilla luciferase (C) or CAT to β-gal activity (D and E) was determined 36 h after transfection.
Moreover, PDCD4 down-regulation by siRNAs increased the rate of degradation of p27Kip1, XIAP, and Bcl-xl mRNAs in polyribosomes as assessed by sucrose gradient centrifugation to distinguish between efficiently translated mRNAs associated with polyribosomes from poorly translated mRNAs bound to monosomes (i.e., 4SOS) or disomes (i.e., 8SOS). We observed that the overall amount of monosomes and disomes was higher in Ras-transformed Stat1+/+ cells than in Stat1−/− cells, whereas the amount of polyribosomes was substantially decreased in the former cells compared with latter cells (Fig. S7). This result was in line with the interpretation that general mRNA translation is less efficient in Stat1+/+ than in Stat1−/− cells. Nevertheless, we observed that a higher amount of p27Kip1, XIAP, and Bcl-xl mRNAs was associated with polyribosomes in Stat1−/− cells than in Stat1+/+ cells as opposed to Mcl-1 mRNAs, which were less efficiently bound to polyribosomes of the former than latter cells (Fig. S7). The association of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA with polyribosomes was more efficient in Stat1−/− than Stat1+/+ cells, consistent with the interpretation that loss of Stat1 facilitates general mRNA translation (Fig. S7). To further substantiate the findings, we determined the effects of Stat1 on the IRES activity in the 5′ UTR of each mRNA in transient transfection assays with dicistronic reporter gene constructs. We observed that the 5′ UTR of p27Kip1 mRNA displayed a stimulatory effect on reporter gene expression in Stat1−/− cells compared with Stat1+/+ cells as opposed to the 5′ UTR of GAPDH, which lacked such an effect (Fig. 3C). Also, transient transfection assays of dicistronic constructs bearing the 5′ UTR of either Bcl-xl or XIAP mRNA between reporter genes provided further evidence that the IRES activity of each mRNA is increased in Stat1−/− cells compared with Stat1+/+ cells. Collectively, the data concluded that Stat1 stimulates the cap-independent translation of p27Kip1, XIAP, and Bcl-xl mRNAs.

**PDCD4 Degradation Is an Important Determinant of the Translational Effects of Stat1.** PDCD4 acts as suppressor of mRNA translation through its ability to interact with and impair the RNA helicase activity of eIF4A (40, 41) (Fig. S8A). We observed that PDCD4 protein was down-regulated by Stat1 in immortalized as well as Ras-transformed cells (Figs. S3 and S8B). Increased PI3K signaling leads to Akt- and S6K-mediated phosphorylation and proteasomal degradation of PDCD4 (42, 43), which can also be mediated by IFN treatment (44). Treatment with the proteasome inhibitor MG132 caused the stabilization of PDCD4 and decreased the differences in PDCD4 expression between Stat1+/+ and Stat1−/− cells, suggesting a role of Stat1 in PDCD4 degradation (Fig. S8B). Because PDCD4 exhibits a negative effect on the IRES-mediated translation of XIAP and Bcl-xl mRNAs (45), we examined the expression of p27Kip1, XIAP, and Bcl-xl proteins in PDCD4−/− MEFs. Loss of PDCD4 caused an increase in p27Kip1, XIAP, and Bcl-xl proteins in primary MEFs without a significant effect on their mRNA levels (Fig. 4A and Fig. S8C). Moreover, PDCD4 down-regulation by siRNAs increased p27Kip1, XIAP, and Bcl-xl proteins in Ras-transformed Stat1−/− cells to equal levels in Stat1+/+ cells treated with scrambled siRNAs (Fig. 4B). These data suggested that PDCD4 down-regulation by Stat1 contributes to increased expression of p27Kip1, XIAP, and Bcl-xl proteins at the translational level.

**Stat1 Protects Cells From the Antiproliferative Effects of Antitumor Drugs.** Next, we examined Stat1 function in cells treated with chemotherapeutic drugs. We found that Stat1 mediated a twofold increase in the viability of immortalized cells to the antiproliferative effects of the PI3K/mTOR inhibitor BEZ235 (34) or mTOR catalytic inhibitor KL0063794 (46) (Fig. S9A and B). Also, Stat1 increased the resistance of immortalized or Ras-transformed cells to the antiproliferative effects of doxorubicin by fivefold and protected them from the killing effects of the drug by 50% (Fig. 5A and B and Fig. S9 C and D). To further support the findings, we examined the response of Ras-transformed cells to doxorubicin treatment in xenograft tumor assays in severe combined immunodeficient (SCID) mice. Stat1 inhibited the growth of Ras tumors in SCID mice, consistent with our previous findings demonstrating the antitumor function of Stat1 in Ras-mediated transformation (7) (Fig. 5C). When mice were treated with doxorubicin, tumor growth inhibition was more evident for Stat1−/− than Stat1+/+ cells, which was in line with a prosurvival role of the protein in response to genotoxic stress (Fig. 5C). At the end point of the experiment, doxorubicin treatment caused a ∼threefold reduction of Ras-Stat1−/− tumor mass, as opposed to Ras-Stat1+/+ tumor mass, which was marginally affected by the drug (Fig. 5D). These data strongly supported a prosurvival function of Stat1 in response to doxorubicin.

**Discussion**
Unphosphorylated Stat1 acts at the transcriptional level to increase the expression of the p110γ catalytic subunit of PI3K class IB, which associates with either the p84/87 or p101 regulatory subunit to promote PI3Kγ signaling and cell survival (47, 48) (see model in Fig. 6). PI3Kγ plays a central role in G protein-coupled receptor (GPCR) signaling via direct interactions with Gi/Go heterodimers but can also function through direct and transient interactions with other regulators including Ras (47, 48). In regard to cancer, p110γ overexpression can transform chicken embryo fibroblasts in culture via a Ras-dependent mechanism (49). Also, increased PI3Kγ signaling is implicated in the development of breast, pancreatic, and brain tumors or Kaposis sarcoma by promoting tumor inflammation and metastasis (50–54). Despite the tumorigenic properties of PI3Kγ, Stat1 inhibits cell proliferation and tumorigenesis, suggesting the presence of control pathways mediated by Stat1 to counteract PI3Kγ-mediated tumorigenesis. Consistent with this interpretation, Stat1 increases 4EBP1 as a means to impair general protein synthesis.
to regulate important biological functions including the transcriptional induction of immune regulatory genes as well as inhibition of the oncogenic ErbB2/HER2 signaling and tumorigenesis (10, 61).

An emerging salient property of Stat1 is its ability to increase resistance of cells to chemotherapeutic drugs. Previous studies showed that Stat1 mediates the expression of a set of IFN-inducible genes known as IFN-related DNA damage-resistance signature, which renders different tumor types increasingly resistant to chemotherapy and radiation therapies (62–65). More recent work demonstrated that unphosphorylated Stat1 mediates the induction of genes that protect cells from virus infection or DNA damage in response to prolonged treatment with IFN-γ (2). Our data are in agreement with those findings and suggest that the transcriptional function of unphosphorylated Stat1 impacts on the translational induction of select mRNAs. Although Bcl-xL and XIAP are bona fide antiprotective proteins, p27Kip1 may also contribute to survival by inhibiting cell cycle progression and blocking the effects of chemotherapeutic drugs that are effective in proliferating cells (66). Some studies, however, indicated that Stat1 can act as a checkpoint in DNA-damaging chemotherapies and synergize with either p53 or IFNα to induce cell death in response to doxorubicin treatment (67–69). Stat1 function in cell survival as well as cell death may be explained by its ability to respond to drugs via pathways that are controlled by its phosphorylation in a temporal fashion. For example, phosphorylated Stat1 may be initially required to increase cell sensitivity to drugs, whereas de/unphosphorylated Stat1 acts at a later stage to contribute to survival and development of drug resistance (2, 62).

Stat1 functions as a double-edged sword by inhibiting proliferation and establishing an environment that helps cells survive under genotoxic stress (Fig. 6). Apart from acting in a cell-autonomous manner, Stat1 may increase PI3Kγ to regulate immune and inhibit cell proliferation. Translational repression by 4E-BPs is selectively exerted on mRNAs containing a terminal oligopyrimidine (TOP) or a TOP-like motif at the 5′ UTRs (55–57), which encode proteins of the translational machinery, including ribosomal proteins and translation factors (58). Inhibition of the elf4F complex is thought to have detrimental effects on translation of mRNAs with increased secondary structure in the 5′ UTRs (59). Considering that several mRNAs with increased secondary structure in their 5′ UTRs encode proteins with crucial roles in cell proliferation (24), translational inhibition of such mRNAs may contribute to the antiproliferative and antitumor effects of Stat1 (Fig. 6). It is noteworthy that Stat1 may use additional mechanisms to inhibit protein synthesis, such as the increased expression of the double-stranded RNA-activated protein kinase PKR and increased eIF2α S51 phosphorylation (Fig. 1 C and D and Fig. S1A), which displays antiproliferative and tumor suppressor functions (60).

Up-regulation of PI3Kγ signaling by Stat1 depends, at least in part, on PDCD4 down-regulation to promote translation of select mRNAs under condition of general protein synthesis inhibition by increased 4EBP1 (Fig. 6). As such, Stat1 favors the IRES-mediated translation of p27Kip1, which plays an important role in the suppression of cell proliferation and Ras-mediated cell transformation (7, 9). Our previous work demonstrated that Stat1 promotes p27Kip1 expression by inducing CDKN1B transcription and increasing protein stabilization through the transcriptional repression of the E3 ubiquitin ligase Skp2 (7, 9). Control of p27Kip1 at distinct levels may underscore the importance of the cdk inhibitor in the antiproliferative and antitumor functions of Stat1. It is of interest that p27Kip1 expression is controlled by Stat1 through phosphorylation-dependent as well as -independent mechanisms. That is, although transcriptional and posttranscriptional control of p27Kip1 depends on Stat1 Y701 phosphorylation (7, 9), p27Kip1 mRNA translation is mediated by unphosphorylated Stat1. Our data further support previous findings showing the ability of unphosphorylated Stat1

Fig. 5. Stat1 protects cells from doxorubicin treatment. (A) Ha-Ras G12V-transformed Stat1−/− and Stat1+/− MEFs were subjected to SRB cytotoxicity assays after treatment with doxorubicin. (B) Ha-Ras G12V-transformed Stat1−/− and Stat1+/− MEFs were treated with 100 nM of doxorubicin for 24 h followed by evaluation of death by FACS analysis (sub-G1 population). (C) and (D) Ha-Ras G12V-transformed Stat1−/− and Stat1+/− MEFs were injected s.c. in the flanks of 10 female nude mice. Each mouse received two s.c. injections (1 × 106 cells per site). Five mice from each group (n = 2 × 5 = 10 tumors) were treated with doxorubicin (4 mg/kg) and the rest with phosphate buffer saline (PBS). Tumor growth was monitored for up to 4 wk (C), and tumor mass was measured at the end point of the experiment (D).

Fig. 6. Schematic model of the translational effects of Stat1. Stat1 increases p110γ and 4EBP1 expression at the transcriptional level, which contributes to stimulation of the PI3K pathway and inhibition of cap-dependent translation, respectively. Down-regulation of PDCD4 by increased PI3K signaling stimulates elf4Fα activity, which in conjunction with increased levels of 4EBP1 establishes conditions that favor the cap-independent translation of mRNAs encoding for the prosurvival proteins Bcl-xL and XIAP. Stat1 also promotes the cap-independent translation of p27Kip1 mRNA, which results in the inhibition of cell proliferation. The antiproliferative effects of Stat1 may be further facilitated by the general inhibition of cap-dependent mRNA translation as a result of 4EBP1 up-regulation.
responses given that both proteins are involved in the activation of natural killer (NK) and CD8 T cells (70–73). Considering the implications in cancer treatment, Stat1’s response to chemotherapeutic drugs is likely to be controlled by both cell-autonomous and immune regulatory mechanisms. This notion is supported by a recent study showing that efficient treatment of ErbB2/HER2 mouse mammary tumors with the RTK inhibitor lapatinib and doxorubicin requires the immune regulatory function of Stat1 (14). Nevertheless, Stat1’s function in drug-sensitive cells may be different from its function in drug-resistant tumor cells. That is, Stat1 may rely on its immune regulatory properties to promote the antitumor effects of drugs at initial stages of treatment. However, tumor escape from immune surveillance may depend on Stat1 for survival and resistance to antitumor treatments. Stat1 may also be implicated in therapies inducing immunogenic tumor death, which requires the activation of select immune effector cells by the dying tumors under conditions of immunosuppression caused by chemotherapeutic drugs (74). Thus, understanding the mechanisms of Stat1 function in antitumor therapies may be helpful for the design of strategies that distinguish the cell-autonomous from the immune regulatory effects of Stat1 as a means to combat cancer.

Materials and Methods

Cell Culture and Treatments. Primary MEFs were isolated from Stat1−/− mice and PDCD4−/− mice together with isogenic control MEFs and were used for experiments in early passages (75, 76). Spontaneously immortalized Stat1−/− and Stat1−/− MEFs as well as immortalized MEFs infected with either pBABE insert-less retroviruses (control) or pBABE retroviruses expressing Ha-Ras G12V were previously described (7). Reconstitution of Ha-Ras G12V Stat1−/− cells with either wild type or phosphorylation mutants of Stat1 was previously described (7). Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) (Wisent) supplemented with 10% (vol/vol) FCS (Wisent) and 100 U/ml penicillin-streptomycin (Wisent). Mouse IFN-γ was obtained from Biosource (Invitrogen), whereas BEZ235 and KU0063794 as well as doxorubicin were purchased from Selleckchem and Sigma, respectively.

Polysome Profile Analysis, RT-PCR, and Real-Time PCR. Polysome profile analysis was performed as previously described (77). Total RNA or RNA from polysomal fractions was isolated by TRIzol (Invitrogen), and 1 μg of it was subjected to reverse transcription (RT) with 100 μM oligo(dt) primer using the SuperScript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Quantitative (q) real-time PCR was performed with SYBR Green PCR reverse transcription (RT) with 100 μM of it was subjected to reverse transcription (RT) with 100 μM oligo(dt) primer using the SuperScript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Quantitative (q) real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems by Life Technologies) according to the manufacturer’s instructions, following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines by including both mouse GAPDH and actin mRNAs as internal controls (78). The synthesized cDNAs from the polysome fractions were amplified with 2.5 μg of Taq DNA polymerase (GenScript) by PCR as follows: 94 °C 1 min, 57 °C 1 min, and 70 °C 1 min × 28 cycles. The sequences of the primers are shown in Table 51.

35S-Methionine Cell Labeling. Metabolic labeling of cells with [35S]methionine was performed as described (79).

Transfections and Luciferase Reporter Gene Assays. DNA transfections were performed as previously reported (7). The reporter gene constructs containing the p27Kip1, XIAP or Bcl-xL 5′UTR were previously described (80, 81). Quantification of firefly and renilla luciferase activity was performed with the Dual-Luciferase Reporter Assay System (Promega). Quantification of β-galactosidase and chloramphenicol acetyltransferase (CAT) activity was performed as described (81).

siRNA Transfection. Cells (2.5 × 106/100 mm plate) were transfected with 200 pmol siRNA, for mouse PDCD4 or control siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Cells were refreshed with 10% FBS DMEM media and incubated at 37 °C for 48 h before further analysis.

Immunoblot Analysis. Protein extraction and immunoblot analysis were performed as described (7). Anti-Stat1 α p91 (C-111), α-Myc (9E10), Stat3 (C-20), and Stat1 (M-23) antibodies were purchased from Santa Cruz Biotechnology; pY701-Stat1, pS277-Stat1 and phospho-p44/42 MAPK (Th202/Yr204), MAPK, pT308-AKT, pS473-AKT, AKT, pT389 S6K, S6, pS324/235 S6, S6, pSer1109 elF4GI, elF4GI, elF4G1, pS221 elF4α, pSer450 elF4α, elF4α, PDCD4, elF4α, elF2p551, pY545 p85, p85, p110el, p110y, p101, and p87 antibodies were purchased from Cell Signaling Technology; anti-actin (C4) was purchased from Biosource International; anti-p212α antibody was purchased from BD Transduction Laboratories; and horseradish peroxidase-conjugated anti-mouse IgG antibody and HRP anti-rabbit IgG antibody were purchased from Amersham Pharmacia Biotech. Quantification of bands in linear range of exposure was performed by densitometry using Scion image software.

IC50 Evaluation of Drug Treatments. Cell viability assays and calculation of IC50 were performed using the sulforhodamine B (SRB) cytotoxicity assay as previously described (82).

Cell Death Analysis. Detection of cell death by propidium iodide and flow cytometry analysis was performed as previously described (83).

Xenograft Tumor Assays. Injection of 1 × 106 cells per site in 8-wk-old female athymic nude mice (Charles River Inc.) and tumor monitoring were performed as described (83). Mice were treated with 4 mg/kg doxorubicin delivered by i.p. injections twice per week based on established protocol (83). The animal studies were performed in accordance with approved protocols and regulations by the Animal Welfare Committee of McGill University (protocol 5754).

Statistical Analysis. Experiments were performed in triplicate, and data represent the average of three independent experiments. Error bars represent SE, as indicated, and significance in differences between arrays of data tested was determined using two-tailed Student t test. * P < 0.05, ** P < 0.01.

Acknowledgments. We thank Drs. Y. Chen for PDCD4−/− mice, M. Holic for XIAP and Bcl-xL IRES dicistronic constructs and anti-XIAP antibodies, and L. Kobor for p27(1−/−) and GAPDH IRES dicistronic constructs. The work was supported by funds from the Cancer Research Society Inc., the Marjorie Sheridan Innovation grant of the Canadian Cancer Society (CCSR 701631), and the Quebec Breast Cancer Foundation (to A.E.K.). C.P. was an awardee of a studentship from the McGill Integrated Cancer Research Training Program.

Supporting Information

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Fig. S1. Stat1 increases PI3K signaling in immortalized or Ras-transformed cells independent of IFN-γ. (A and B) Serum-starved spontaneously immortalized MEFs were subjected to either 10% (vol/vol) calf serum stimulation (A) or 100 IU/mL of mouse IFN-γ (B) for the indicated time points (h). (C) Control (immortalized MEFs infected with pBABE insert-less viruses) and Ha-Ras G12V-transformed MEFs were serum deprived for 18 h followed by 10% (vol/vol) calf serum stimulation for the indicated time (h). (A–C) Cell extracts (50 μg of protein) were subjected to immunoblot analyses for the indicated proteins.

Fig. S2. Induction of PI3K signaling by Stat1 phosphorylation mutants. (A) Ha-Ras G12V-transformed Stat1−/− MEFs were reconstituted with either Stat1 WT or the indicated Stat1 phosphorylation mutants. (B) Cells were serum-starved for 18 h followed by 10% (vol/vol) calf serum stimulation for the indicated time (h). (A and B) Cell extracts (50 μg of protein) were subjected to immunoblot analyses for the indicated proteins.
Fig. S3. Stat1 controls expression and phosphorylation of translation initiation factors. (A) Control (immortalized cells infected with pBABE insert-less retroviruses) and Ha-Ras G12V-transformed Stat1<sup>+/+</sup> and Stat1<sup>−/−</sup> MEFs were serum starved for 18 h followed by stimulation with 10% (vol/vol) calf serum for the indicated time points (h). (B) Serum-starved primary Stat1<sup>+/+</sup> and Stat1<sup>−/−</sup> MEFs were treated with 10% (vol/vol) calf serum for the indicated time (h). (A and B) Cell extracts (50 μg of protein) were subjected to immunoblot analyses for the indicated proteins.
Fig. S4. Translational effects of Stat1 in primary and immortalized MEFs. (A) Spontaneously immortalized Stat1+/+ and Stat1−/− MEFs were subjected to [35S]methionine labeling in response to 10% (vol/vol) stimulation with dialyzed calf serum for the indicated time (h). (B and C) Serum-starved primary Stat1+/+ and Stat1−/− MEFs (B, passage 2) or spontaneously immortalized Stat1+/+ and Stat1−/− MEFs (C) were subjected to 10% calf serum stimulation for the indicated time (h). (D) Detection of 4EBP1 and PKR mRNA expression in immortalized MEFs by qPCR. (E) Control (immortalized) and Ha-Ras G12V-transformed Stat1+/+ and Stat1−/− MEFs maintained in complete medium in the absence or presence of 100 nM BEZ235 for 6 h. (F) Detection of elf4E interaction with either elf4G or 4EBP1 in WCEs from immortalized cells grown in the absence or presence of serum for 6 h using 7-methyl-GTP Sepharose 4B affinity chromatography. (B, C, E, and F) Protein extracts (50 μg) or proteins were subjected to immunoblot analyses for the indicated proteins.
**Fig. S5.** Inhibition of eIF4E S209 phosphorylation by Stat1. Serum-starved immortalized (A) or Ha-Ras G12V-transformed Stat1\textsuperscript{+/+} and Stat1\textsuperscript{-/-} MEFs (B) were left untreated (−) or treated with 50 nM of PD0325901 for 30 min (+) followed by stimulation with 10% (vol/vol) calf serum for the indicated time (h). Protein extracts (50 μg) or proteins were subjected to immunoblot analyses for the indicated proteins.

**Fig. S6.** Detection of Bcl-xl and XIAP mRNA expression by qPCR. Immortalized (A) or Ha-Ras G12V-transformed Stat1\textsuperscript{+/+} and Stat1\textsuperscript{-/-} MEFs (B) were analyzed for Bcl-xl and XIAP mRNAs by qPCR. PKR mRNA was used as a positive control of Stat1 transcriptional function.
Fig. S7. Polysome profile analysis of Ras-transformed Stat1^+/+ and Stat1^-/- MEFs. The distribution of p27^Kip1, Bcl-xL, XIAP, Mcl-1, and GAPDH mRNAs across the different polysome fractions from cells maintained in medium with 10% (vol/vol) calf serum was determined by semiquantitative RT-PCR. The data represent one of three independent experiments.
Fig. S8. Control of PDCD4 degradation by Stat1. (A) Schematic representation of the inhibitory effect of PDCD4 on eIF4A RNA helicase activity in eIF4F preinitiation complex (40S, small ribosomal subunit; AUG, initiator codon; PABP, polyA-BP). (B) Control (immortalized) as well as Ha-Ras G12V-transformed Stat1\(^{+/+}\) and Stat1\(^{-/-}\) MEFs were serum starved for 18 h followed by treatment with 10% (vol/vol) calf serum in the absence or presence of 10 \(\mu\)M MG132 for the indicated time (h). Protein extracts (50 \(\mu\)g) or proteins were subjected to immunoblot analyses for the indicated proteins. (C) Primary PDCD4\(^{+/+}\) and PDCD4\(^{-/-}\) MEFs were analyzed for p27\(^{Kip1}\), Bcl-xL, and XIAP mRNAs by qPCR.
Fig. S9. Stat1 protects immortalized MEFs from the effects of antiproliferative drugs. (A–C) Immortalized MEFs were subjected to SRB cytotoxicity assays after treatment with the mTOR inhibitor KU-0063794 (A), PI3K/mTOR inhibitor NVP-BEZ235 (B), or doxorubicin (C). (D) Immortalized MEFs were treated with 100 nM doxorubicin for 24 h followed by evaluation of death by FACS analysis (sub-G1 population).

Table S1. Sequences of primers used for PCR

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<tr>
<th>Primer</th>
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<td>Forward 5′-GATGTCAACACGTGAGAGTGCTCTAAC-3′</td>
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<td>Reverse 5′-GCCACTCGTATCTGGCTCCAGGGG-3′</td>
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