Type I interferon (IFN)-dependent activation of Mnk1 and its role in the generation of growth inhibitory responses

Sonali Joshi[a], Surinder Kaur[a], Amanda J. Redig[a], Katy Goldsborough[a], Kevin David[a], Takeshi Ueda[b], Rie Watanabe-Fukunaga[c], Darren P. Baker[d], Eleanor N. Fish[e], Rikiro Fukunaga[c], and Leonidas C. Platanias[a,1]

[a]Robert H. Lurie Comprehensive Cancer Center and Division of Hematology- Oncology, Northwestern University Medical School, and Jesse Brown Veterans Administration Medical Center, Chicago, IL 60611; [b]Campbell Family Institute for Breast Cancer Research, Princess Margaret Hospital, Toronto, Ontario, Canada M5G2M9; [c]Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto, Japan 606-8501; [d] Biogen Idec Inc, 14 Cambridge Center, Cambridge, MA 02142; and [e]Division of Cell and Molecular Biology, Toronto Research Institute, University Health Network and Department of Immunology, University of Toronto, Canada M5G2M1

Edited by George R. Stark, Cleveland Clinic Foundation, Cleveland, OH, and approved May 22, 2009 (received for review January 26, 2009)

We provide evidence for the existence of an IFN-regulated cellular pathway involving the mitogen-activated protein kinase (MAPK)-integrating kinase (Mnk) 1. Our data demonstrate that type I (α, β) IFNs induce phosphorylation/activation of Mnk1, which, in turn, regulates phosphorylation of the eukaryotic initiation factor 4E (eIF4E) on Ser-209. Such Mnk activation depends on upstream engagement of Jak1, and requires downstream activation of the Mek/Erk MAPK pathway. In studies using double Mnk1−/−Mnk2−/− knockout mouse embryonic fibroblasts (MEFs), we found that engagement of Mnk kinases is essential for mRNA translation of the *Isg15* and *Isg54* genes, suggesting an important role for this pathway in mRNA translation of IFN-stimulated genes (ISGs). Importantly, our data demonstrate that pharmacological inhibition of Mnk kinases or siRNA-mediated knockdown of Mnk1 and Mnk2 results in partial reversal of the suppressive effects of IFNα on normal and leukemic hematopoietic progenitors, establishing a key role for this pathway in the generation of the growth inhibitory effects of type I IFNs. Together, our findings establish that the Mnk/eIF4E kinase pathway is activated in an IFN-inducible manner and plays important roles in mRNA translation for ISGs and generation of IFN-inducible antiproliferative responses.

growth inhibition | interferon | signaling

Interferons (IFNs) are important cytokines that were originally defined by their ability to block cellular replication of different viruses (1–3). Subsequent work has established that beyond induction of antiviral effects, IFNs generate important growth inhibitory and proapoptotic effects and act as immune modulators to control a variety of innate immune responses (1–4). IFNs are classified into 2 major groups: type I IFNs that include multiple different subtypes (α, β, ω, τ, κ, ε, and δ) and IFNγ, which is the only known type II IFN (1–4). Over the last few years, a different class of IFNs, type III, has also been identified and defined. The type III IFN group includes the IFNλ proteins (λ1, λ2, and λ3) that also exhibit antiviral and antitumor properties (5).

The key roles of IFNs in antiviral host defense and immune surveillance against malignancies have provoked extensive studies that have resulted in a detailed understanding of the sequence of the signaling events that occur after binding of IFNs to their cellular receptors. Notably, the original discovery of Jak-Stat pathways resulted from work focused on IFN signaling (4, 6, 7) and provided important clues and insights on how IFNs and other cytokines regulate transcription of target genes. In the case of type I IFNs, the tyrosine kinases Tyk2 and Jak1 are constitutively associated with the IFN alpha receptor 1 (IFNAR1) and IFNAR2 subunits of the type I IFN receptor, respectively, and upon type I IFN engagement, they are activated and phosphorylate downstream targets, including Stat-proteins (1–4, 6, 7). However, the only type II IFN, IFNγ, generates signals via engagement of the kinases Jak1 and Jak2 that are associated with the IFN gamma receptor 1 (IFNGR1) and IFNGR2 subunits of the type II IFN receptor, respectively (1–4, 6, 7).

The engagement of Stat-proteins by activated IFN receptor-Jak kinase complexes plays critical and essential roles in the induction of IFN-responses, because it ultimately controls IFN-dependent transcriptional activation and generation of protein products that regulate the biological effects of IFNs (1–4, 6, 7). However, beyond the classic Jak-Stat pathways, there is evidence that IFNs activate several other signaling cascades, including the mTOR-regulated (8–12) and mitogen-activated protein kinase (MAPK) signaling cascades (13–15). Such non-Stat pathways play important roles in the induction of IFN-responses, via their ability to promote optimal IFN-dependent gene transcription (13) or mRNA translation of ISGs (12). Among the different MAPK pathways engaged by IFNs, signals generated by the p38 MAPK have been implicated in the generation of growth inhibitory responses by type I IFNs (14, 15).

Despite the well-established relevance of MAPK cascades in the control of IFN responses, the identity and functions of specific downstream effectors that mediate the effects of IFNs remains to be established. In this report, we provide evidence that type I IFNs activate MAPK-interacting kinase 1 (Mnk1), a MAPK-regulated kinase that phosphorylates the initiation factor eIF4E. Our studies establish that Mnk1 is phosphorylated in response to type I IFNs in an Erk/MAPK-dependent manner, and regulates downstream phosphorylation of eIF4E on Ser-209. Importantly, in experiments using double Mnk1/Mnk2 knockout MEFs, we found that Mnk activity is essential for regulation of IFNα-dependent mRNA translation of the *Isg15* gene and ISG15 protein expression. Moreover, siRNA-targeting of Mnk1/Mnk2 reversed the suppressive effects of type I IFNs on normal and leukemic hematopoietic progenitors, establishing key and essential roles for Mnk kinases in type I IFN signaling.

Results

In initial studies, we determined whether IFNα treatment of sensitive cell lines results in phosphorylation/activation of Mnk1. IFNα-sensitive U266 cells were treated with human IFNα for different times, and cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Mnk1 on Thr-197 and Thr-202 (corresponding to Thr-209 and

MEDICAL SCIENCES

Author contributions: E.N.F. and L.C.P. designed research; S.J., S.K., A.J.R., K.G., and K.D. performed research; T.U., R.W.-F., D.P.B., and R.F. contributed new reagents/analytic tools; S.J. and L.C.P. analyzed data; and S.J. and L.C.P. wrote the paper.

Conflict of interest statement: One of the authors, Darren P. Baker, is an employee of Biogen Idec Inc. and an owner of Biogen Idec stock.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: l-platanias@northwestern.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0900562106/DCSupplemental.

Thr-214 of human Mnk1, respectively). IFNα-treatment induced rapid phosphorylation of Mnk1 that was detectable within 15 min of treatment (Fig. 1*A*). Similarly, phosphorylation/activation of Mnk1 was clearly detectable when cells were treated with another type I IFN, IFNβ, suggesting that Mnk1 (Fig. 1*B*) is a common element in the signaling pathways of multiple type I IFNs. Similar results were obtained when the myelomonocytic cell line U937 was studied (Fig. 1*C*). It is well established that in response to stress-signals, Mnk kinases phosphorylate the eukaryotic initiation factor 4E (eIF4E) (16–18), a cap-binding protein that plays a critical role in the initiation of cap-dependent mRNA translation (19). In experiments in which cells were treated with human IFNα or IFNβ, we found strong induction of phosphorylation of eIF4E on Ser-209 (Fig. 1 *D–F*), strongly suggesting that activation of Mnk1 by the type I IFN receptor regulates downstream phosphorylation of eIF4E.

To definitively establish whether type I IFN-inducible eIF4E phosphorylation is Mnk-dependent, experiments were carried out using double Mnk1/Mnk2 knockout MEFs (20). Wild-type Mnk1+/+Mnk2+/+ MEFs and double knockout Mnk1−/−Mnk2−/− MEFs were treated with mouse IFNβ, and the phosphorylation of Mnk1 or eIF4E was assessed. As expected, IFNβ treatment induced phosphorylation of Mnk1 in parental MEFs, but no signal was detected in Mnk1−/−Mnk2−/− MEFs (Fig. 2*A*). In addition, IFNβ induced strong phosphorylation of eIF4E in parental MEFs but not in Mnk1−/−Mnk2−/− MEFs (Fig. 2*B*). Similarly, eIF4E phosphorylation was blocked by pretreatment of U937 cells with the Mnk inhibitor CGP57380 (Fig. 2*C*). As expected, this inhibitor did not block Mnk phosphorylation (Fig. 2*C*). In fact, the phosphorylation of Mnk1 increased after treatment of the cells with CGP57380 (Fig. 2*C*), likely reflecting a compensatory cellular response. Together, these data establish a requirement for Mnk kinase activity in the type I IFN-dependent phosphorylation of eIF4E.

There is extensive evidence that phosphorylation of Mnk1 in response to stress is regulated by upstream engagement of MAPKs (18, 21). As we have previously shown that types I IFNs activate the p38 MAPK, and that such activation is required for the generation of the growth suppressive effects of type I IFNs (13–15), we sought to determine whether activation of Mnk1, mediated via the type I IFN receptor, requires p38 activity. To address this question, we used immortalized MEFs from mouse embryos with targeted deletion of the p38α gene (22). p38α+/+ and p38α−/− MEFs were incubated in the absence or presence of mouse IFNβ, and cell lysates were resolved by SDS-PAGE and immunoblotted with the anti-phospho-Thr-197/Thr-202 Mnk1 antibody. IFN-dependent phosphorylation of Mnk1 was observed in both parental and p38α knockout MEFs (Fig. 2*D*), indicating that engagement of p38α is not required for such activation. Similarly, in experiments in which U937 cells were treated with the p38 inhibitor SB203580, we found that the inhibitor had no effects on the IFN-inducible phosphorylation of Mnk1 (supporting information Fig. S1*A*) or eIF4E (Fig. S1*B*), while it blocked the phosphorylation of the downstream p38 effector MAPKAPK-2 (Fig. S1*C*). Subsequently, we proceeded to determine whether Mek/Erk inhibition abrogates IFN-dependent Mnk activation and eIF4E phosphorylation. When cells were pretreated with the Mek/Erk inhibitor U0126, the IFN-inducible phosphorylation of Mnk1 and eIF4E were blocked (Fig. 2*E*). In addition, when Mek1 was knocked down

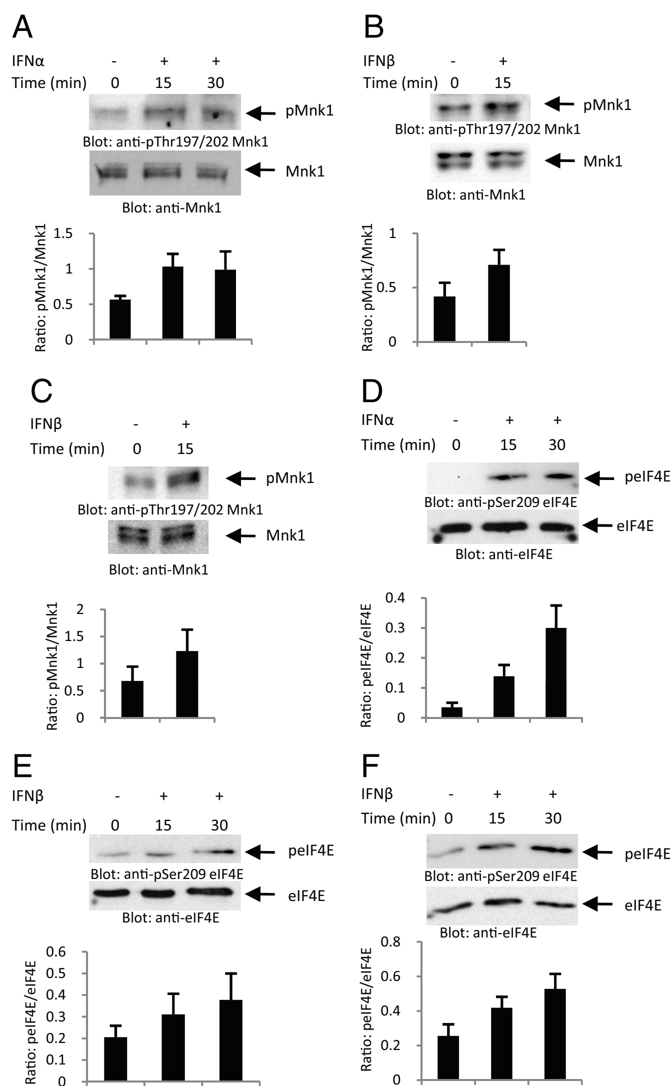

Fig. 1. Type I IFN-dependent phosphorylation/activation of Mnk1 and eIF4E. (*A*) U266 cells were treated with human IFNα for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) and Mnk1. The signals for pMnk1 and Mnk1 from 4 independent experiments (including the one shown) were quantified by densitometry. Data are expressed as the means of ratios of pMnk1/Mnk1 ± SE for each experimental condition. (*B*) U266 cells were treated with human IFNβ for 15 min. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) and Mnk1. The signals for pMnk1 and Mnk1 from 3 independent experiments (including the one shown) were quantified by densitometry. Data are expressed as the means of ratios of pMnk1/Mnk1 ± SE for each experimental condition. (*C*) U937 cells were treated with human IFNβ for 15 min. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) and Mnk1. The signals for pMnk1 and Mnk1 from 4 independent experiments (including the one shown) were quantified by densitometry. Data are expressed as the means of ratios of pMnk1/Mnk1 ± SE for each experimental condition. (*D*) U266 cells were treated with human IFNα for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated eIF4E (S209) and eIF4E. The signals for peIF4E and eIF4E from 3 independent experiments (including the one shown) were quantified by densitometry. Data are expressed as the means of ratios of peIF4E/eIF4E ± SE for each experimental condition. (*E*) U266 cells were treated with human IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated eIF4E (S209) and eIF4E. The signals for peIF4E and eIF4E from 3 independent experiments (including the one shown) were quantified by densitometry. Data are expressed as the means of ratios of peIF4E/eIF4E ± SE for each experimental condition. (*F*) U937 cells were treated with human IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated eIF4E (S209) and eIF4E. The signals for peIF4E and eIF4E from 3 independent experiments (including the one shown) were quantified by densitometry. Data are expressed as the means of ratios of peIF4E/eIF4E ± SE for each experimental condition.

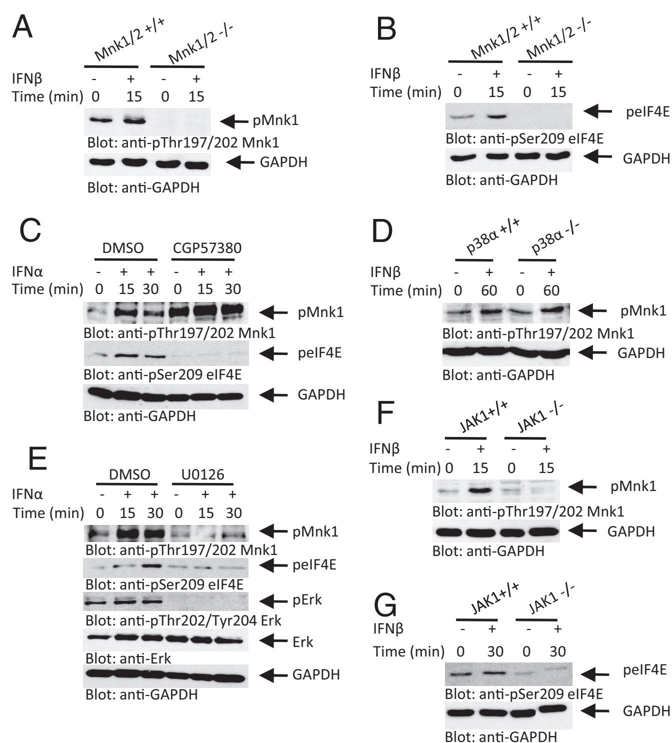

Fig. 2. Type I IFN-dependent phosphorylation of Mnk1 and eIF4E is p38α-independent, but Mek/Erk- and Jak1- dependent. (*A*) Mnk1/2 +/+ and Mnk1/2−/− MEFs were treated with mouse IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) and GAPDH. (*B*) Mnk1/2 +/+ and Mnk1/2−/− MEFs were treated with mouse IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated eIF4E (S209) and GAPDH. (*C*) U937 cells were pretreated with either DMSO or the Mnk1 inhibitor CGP57380 (10 μM) for 60 min, before treatment with human IFNα for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) or phosphorylated eIF4E (S209) or GAPDH, as indicated. (*D*) p38α +/+ and p38α−/− MEFs were treated with mouse IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) and GAPDH. (*E*) U937 cells were pretreated with either DMSO or the MEK1/2 inhibitor U0126 (10 μM) for 60 min, followed by treatment with human IFNα for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against phosphorylated Mnk1 (T197/202), phosphorylated eIF4E (S209), phosphorylated Erk1/2 (T202/Y204), Erk1/2 or GAPDH, as indicated. (*F*) Jak1 +/+ and Jak1−/− MEFs were treated with mouse IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Mnk1 (T197/202) or GAPDH. (*G*) Jak1 +/+ and Jak1 −/− MEFs were treated with mouse IFNβ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated eIF4E (S209) or GAPDH.

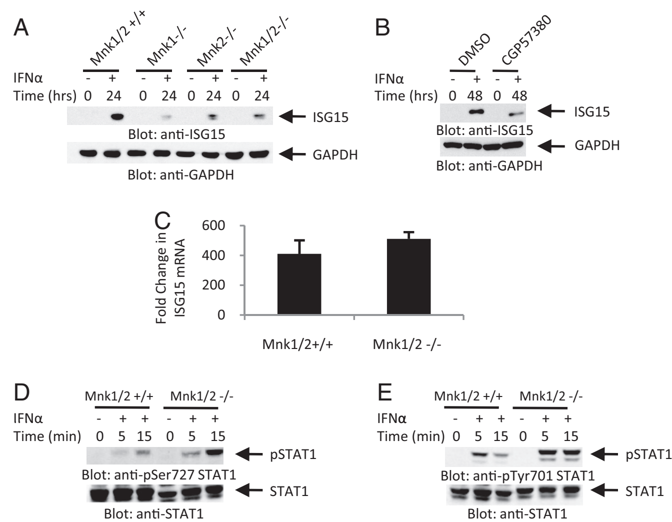

Fig. 3. ISG15 protein expression is Mnk kinase-dependent. (*A*) Mnk1/2 +/+, Mnk1 −/−, Mnk2 −/−, or Mnk1/2 −/− MEFs were treated with mouse IFNα, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against mouse ISG15 or GAPDH. (*B*) U266 cells were pretreated with either DMSO or CGP57380 (10 μM), followed by treatment with human IFNα for 48 h. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against human ISG15 and GAPDH. (*C*) Mnk1/2 +/+, Mnk1/2 −/− MEFs were treated with mouse IFNα. The expression of ISG15 mRNA was assessed by quantitative RT-PCR, using GAPDH as a control. The data are expressed as the fold induction over corresponding untreated samples and represents means ± SE of 3 independent experiments. (*D* and *E*) Mnk1/2 +/+ and Mnk1/2 −/− MEFs were treated for the indicated times with mouse IFNα. Equal amounts of protein were resolved by SDS-PAGE and sequentially immunoblotted with antibodies against phosphorylated Stat1 (S727) and Stat1 (*D*) or with antibodies against phosphorylated Stat1 (Y701) and Stat1 (*E*).

using specific siRNA, there was a decrease in the IFN-dependent phosphorylation of Mnk1 (Fig. S2*A*) and eIF4E phopshorylation (Fig. S2*B*). Together, these studies establish that in the type I IFN system, phosphorylation/activation of Mnk1 is regulated by the Mek/Erk MAPK cascade, but not the p38 MAPK signaling pathway. Notably, IFN-inducible phosphorylation/activation of Mnk1 and eIF4E were also blocked in cells with targeted disruption of the Jak1 gene (Fig. 2 *F* and *G*), consistent with a requirement for IFN-dependent activation of Jak kinases in the process.

In subsequent studies, we sought to determine the functional relevance of Mnk engagement in type I IFN signaling. As our data demonstrated that Mnk-activity is required for IFN-inducible eIF4E phosphorylation, we examined whether engagement of Mnk mediates signals important for IFN-dependent mRNA translation of target genes and IFN-dependent protein expression. Initially, we examined the effects of targeted disruption of the Mnk1 and/or Mnk2 genes on ISG15 expression. ISG15 is an IFN-inducible protein, whose expression is enhanced in 4E-BP1 knockout cells (11) and is known to play important roles in the generation of IFN-dependent biological responses via regulation of ISG15 conjugation to cellular proteins (ISGylation) (23, 24). Mnk1, Mnk2, and double Mnk1/Mnk2 knockout MEFs were treated with mouse IFNα, and the expression of ISG15 protein was compared with parental wild-type MEFs. There was a decrease in the induction of expression of ISG15 in both the single Mnk1 and Mnk2 knockout cells (Fig. 3*A*), indicating that the functions of Mnk1 and Mnk2 are essential for ISG15 induction. There was also defective ISG15 expression in the double knockout cells (Fig. 3*A*). Consistent with this finding, when U266 cells were treated with human IFNα, in the absence or presence of the Mnk inhibitor CGP57380, induction of ISG15 protein was substantially decreased as compared with control, DMSO-treated, cells (Fig. 3*B*). Such inhibition was partial, consistent with the involvement of additional pathways in the regulation of ISG15 expression.

To understand the mechanisms that may result in defective ISG15 expression in Mnk-deficient cells, experiments were carried out to determine the effects of Mnk1/Mnk2 knockout on IFN-inducible transcriptional activation of the *Isg15* gene using quantitative RT-PCR. Induction of *Isg15* mRNA expression was intact in the absence of Mnk1 and Mnk2 (Fig. 3*C*), suggesting that Mnk1 and Mnk2 are not involved in the regulation of IFN-dependent gene transcription. Consistent with this finding, the Type I IFN-inducible phosphorylation of Stat1 on serine 727 or tyrosine-701 was intact in Mnk1/Mnk2 knockout cells (Fig. 3 *D* and *E*), indicating that

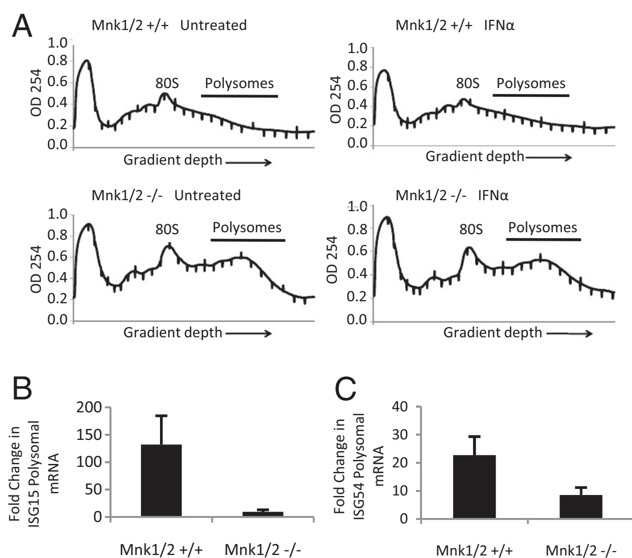

Fig. 4. Essential role of Mnk kinases in IFN-dependent mRNA translation. (*A*) Mnk1/2 +/+ and Mnk1/2 −/− MEFs were either left untreated or treated with mouse IFNα. The cells were subjected to hypotonic lysis followed by separation on a 10%–50% sucrose gradient and the OD at 254 nm was recorded. The OD254 is shown as a function of gradient depth for each treatment. (*B* and *C*) *Isg15* (*B*) or *Isg54* (*C*) mRNA expression in the polysomal fractions was determined by quantitative RT-PCR, using GAPDH for normalization. Data are expressed as fold increase in the IFNα-treated samples over untreated samples and represent means ± SE of 5 independent experiments.

engagement of the Stat-pathway by the type I IFN receptor does not require Mnk kinase activities.

Together, our studies with the Mnk knockout cells indicated that although induction of ISG15 protein by type I IFNs is defective in the absence of Mnk1 and Mnk2, targeted disruption of the genes for these kinases does not alter transcriptional activation of the *Isg15* gene, suggesting that IFN-dependent engagement of Mnk kinases regulates mRNA translation of *Isg15* and, possibly, other regulated genes. In subsequent studies, we directly examined the role of Mnk kinases in type I IFN-dependent mRNA translation of the *Isg15* gene. Mnk1/2 +/+ and Mnk1/2−/− cells were treated with IFNα, polysomal mRNA was isolated (Fig. 4*A*), and the induction of *Isg15* mRNA was assessed directly in the polysomal fractions. IFNα-inducible polysomal *Isg15* mRNA expression was clearly defective in Mnk1/Mnk2 double knockout cells (Fig. 4*B*), indicating an essential role for Mnk kinases on IFN-dependent mRNA translation for this gene. Similarly, IFNα-inducible mRNA translation of another IFN-stimulated gene, *Isg54*, was also defective (Fig. 4*C*), strongly suggesting a critical role for the Mnk pathway in mRNA translation of ISGs.

Subsequently, experiments aimed at determining the effects of Mnk inhibition on the generation of the growth suppressive effects of IFNα on normal and leukemic hematopoiesis were carried out. Concomitant treatment with the Mnk-inhibitor CGP57380 reversed the suppressive effects of IFNα on leukemic progenitor (CFU-L) colony formation derived from U937 cells (Fig. 5*A*), implicating Mnk1 in the control of the suppressive effects of IFNα on primitive leukemic progenitors. To definitively establish the roles Mnk kinases play in the generation of the growth inhibitory effects of IFNα, experiments were carried out in which the expression of Mnk1/Mnk2 was blocked using siRNAs, and the effects of Mnk1/Mnk2 knockdown on the generation of the suppressive effects of IFNα were examined. As shown in Fig. 5*B*, Mnk1 or Mnk2 siRNAs effectively inhibited the expression of the corresponding kinase mRNA expression, and had a partial, but significant, inhibition on the other isoform. The combination of both Mnk1 and

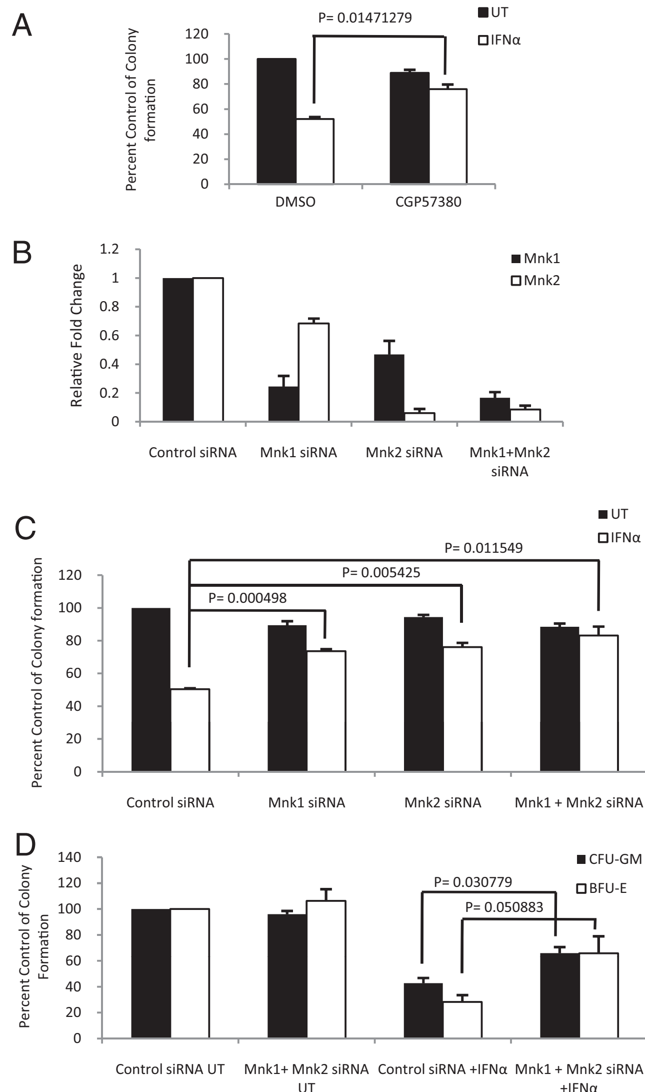

Fig. 5. Mnk1 and Mnk2 mediate the antiproliferative effects of IFNα. (*A*) U937 cells were incubated in clonogenic assays in methylcellulose with or without human IFNα, in the presence of DMSO or CGP57380, as indicated. Leukemic CFU-L colonies were scored and data are expressed as percentage of control DMSO treated colonies and represent means ± SE of 3 independent experiments. Paired *t* test analysis showed $P = 0.01471279$ for the combination of DMSO and IFNα versus the combination of CGP57380 and IFNα. (*B*) U937 cells were transfected with control siRNA or a mixture of Mnk1 and/or Mnk2 specific siRNAs, as indicated. Expression of mRNAs for Mnk1 and Mnk2 genes was evaluated by quantitative RT-PCR using GAPDH for normalization. Data are expressed as fold increase over control samples and represent means ± SE of 3 independent experiments. (*C*) U937 cells were transfected with the indicated siRNAs and plated in a methylcellulose assay system in the absence or presence of human IFNα. Data are expressed as percentage of control siRNA transfected cells-derived colony formation and represent means ± SE of 3 independent experiments. Paired *t* test analysis showed $P = 0.000498$ for the combination of control siRNA and IFNα versus the combination of Mnk1 specific siRNA and IFNα; $P = 0.005425$ for the combination of control siRNA and IFNα versus the combination of Mnk2 specific siRNA and IFNα; and $P = 0.011549$ for the combination of control siRNA and IFNα versus the combination of Mnk1 and Mnk2 specific siRNAs and IFNα. (*D*) CD34+ cells derived from normal bone marrow were transfected with the indicated siRNAs and were then plated in a methylcellulose assay system, in the absence or presence of human IFNα, as indicated. CFU-GM and BFU-E progenitor colonies were scored after 14 days in culture. Data are expressed as percentage control colony formation from control siRNA trasfected cells and represent means ± SE of 4 independent experiments. Paired *t* test analysis showed $P = 0.030779$ for the combination of control siRNA and IFNα versus the combination of Mnk1 and Mnk2 siRNAs and IFNα for CFU-GM colonies; and $P = 0.050883$ for the combination of control siRNAs and IFNα versus the combination of Mnk1 and Mnk2 siRNAs and IFNα for BFU-E colonies.

Mnk2 siRNAs almost completely silenced mRNA expression for both Mnk isoforms (Fig. 5*B*). Treatment with IFNα resulted in inhibition of CFU-L colony formation from U937 cells (Fig. 5*C*). However, such inhibition was partially reversed when cells were transfected with the various Mnk siRNAs (Fig. 5*C*), establishing a requirement for Mnks in the generation of the suppressive effects of IFNα on leukemic progenitors.

It is well-established that type I IFNs are potent regulators of normal hematopoiesis and exhibit potent inhibitory effects on primitive human hematopoietic precursors including erythroid (BFU-E) and myeloid (CFU-GM) bone marrow-derived progenitors (14). Interestingly, siRNA-mediated knockdown of both Mnk1 and Mnk2 resulted in partial reversal of the suppressive effects of IFNα on eythroid (BFU-E) and myeloid (CFU-GM) progenitors (Fig. 5*D*), strongly suggesting a critical role for IFN-induced Mnk activation in IFNα-dependent regulation of normal hematopoiesis.

Discussion

Despite the dramatic advances in the field of IFN-signaling, a number of questions and issues remain to be answered and elucidated to complete our understanding of how IFNs generate signals to induce their diverse biological effects. A challenging task that remains is the identification of mechanisms that account for signaling specificity for different IFNs and the clarification of the mechanisms of generation of signals for distinct biological responses. Although the mechanisms of type I IFN-induced transcriptional regulation have been extensively studied over the years, very little is known on the mechanisms by which mRNAs for different ISGs are translated, leading to the generation of specific IFN-inducible protein products. In previous studies, we provided evidence that type I IFNs induce activation of mTOR and its effector p70 S6 kinase (8). In addition, we have shown previously that type I IFN treatment results in phosphorylation/de-activation of the translational repressor 4E-BP1, leading to its dissociation from the eukaryotic initiation factor eIF4E (8), thus enabling initiation of cap-dependent translation.

We have also established the relevance of this pathway in the generation of IFN-antiviral responses and identified Tsc2 and 4E-BP1 as key and essential elements in the control of generation of the biological effects of IFNs (11). Work from others has also implicated mTOR in the regulation of IFN-induced apoptosis (10), while in recent studies using cells with targeted deletion of both the *Akt1* and *Akt2* genes, we established Akt as a critical element of the IFN-signaling pathway required for engagement of mTOR and for mRNA translation of IFN-regulated genes (12, 25). Together, the emerging evidence points toward an essential role for mTOR and its effectors in the generation of the biological effects of IFNs. However, the precise signals downstream of mTOR that account for specific IFN-responses and the cross-talk between the IFN-activated Akt/mTOR pathway and other IFN-signaling cascades remains to be determined.

An issue of particular interest to us has been to define whether there is cross-talk and synergistic interactions between the IFN-regulated mTOR pathway and IFN-activated MAPK cascades that have been shown previously to play key roles in IFN-mediated signaling (13–15). In the present study we provide evidence that Mnk1, a serine kinase known to act as a downstream effector of MAPK pathways (16, 17), is rapidly phosphorylated following type I IFN-treatment of sensitive cell lines. In addition, we provide evidence that eIF4E is phosphorylated on Ser-209 in a type I IFN-dependent manner, and establish that such phosphorylation is mediated by Mnk kinase activity downstream of Jaks. It also appears that as in the case of the Akt/mTOR pathway, the Mnk/eIF4E pathway is shared by the type II IFN receptor, because IFNγ also induces phosphorylation of Mnk1 and eIF4E (S. Joshi and L. C. Platanias, unpublished observations). Our studies also implicate the Mek/Erk MAPK pathway as the key MAPK cascade regulating Mnk kinase activity in the IFN system. These data suggest that Mnk kinases mediate signals for the generation of IFN-responses downstream of MAPKs, and led us to examine further the role of Mnk kinases in the control of IFN cellular responses.

In experiments using double knockout MEFs for both Mnk1 and the related Mnk2 kinase, we found defective mRNA translation for the *Isg15* and *Isg54* genes in the absence of Mnk1/Mnk2, as assessed directly in polysomal mRNA fractions. This finding is of particular interest, because it suggests that 2 key signaling events are essential for IFN dependent mRNA translation of *Isg15*, *Isg54*, and possibly other ISGs. One event apparently involves IFN-dependent hierarchical phosphorylation of the translational repressor 4E-BP1 that results in its dissociation from eIF4E (9, 11, 12); while a second event involves Mnk-dependent phosphorylation of eIF4E on serine 209. Notably, in previous studies we have shown an important regulatory role for the PI 3′K/mTOR pathway in the regulation of *Isg15* mRNA translation (11, 12, 26), consistent with the hypothesis that coordinated function of both MAPK pathways regulating eIF4E phosphorylation and mTOR pathways are required for optimal mRNA translation of ISGs. However, it is also possible that additional kinases beyond Mnk regulate IFN-dependent eIF4E phosphorylation in different cell types, and that only a selected group of ISGs is regulated by this pathway. It should be noted that a very recent study (27) that attempted to define the characteristics of eIF4E-regulated/targeted mRNAs demonstrated that accrual of distinct mRNAs to polyribosomal complexes in response to ectopic overexpression of eIF4E correlates with mRNA G+C content. However, a negative correlation of such mRNA accrual with total and 3′UTR mRNA length was also established (27). Other studies have also shown that Mnks phosphorylate hnRNPA1 and PSF (the polypyrimidine tract-binding protein-associated splicing factor) that bind to RNAs that contain AU-rich elements (AREs) (28, 29), providing additional putative characteristics for potential ISG mRNAs whose translation could be regulated by this pathway. Future studies, involving microarray analyses, to identify groups of ISGs whose translation requires Mnk kinases and compare them to genes whose mRNA translation requires elements of the mTOR pathway should provide valuable information on the coordination of IFN-dependent signaling cascades to regulate mRNA translation of ISGs.

Beyond providing evidence for an important role for Mnk1/2 in IFN signaling, our findings may have implications as regards the overall perception of the function of Mnk kinases in mRNA translation. It has been difficult to define the precise roles of Mnk kinases in the regulation of mRNA translation in different systems. Previous work has suggested that Mnk-inducible phosphorylation of eIF4E is important in the control of cap-dependent translation and protein synthesis in other cellular systems (30–32), and there is evidence that such phosphorylation promotes HSV-1 mRNA translation and replication (33), and cytokine (TNFα) biosynthesis (28, 29). However, it appears that under certain conditions, Mnk kinases exhibit negative effects on protein translation and synthesis (34, 35), while there is evidence for a unique Mnk-regulatory mechanism that involves conversion of the activation segment into an autoinhibitory module (36). It is possible that such an auto-inhibitory mechanism may explain the diversity of responses seen in response to Mnk kinases in different systems, but this remains to be directly established in future studies.

Our finding, that IFN-induced mRNA translation is defective in cells with targeted disruption of the *Mnk1* and *Mnk2* genes, is consistent with a positive role for Mnk kinases in IFN-dependent signaling and the regulation of IFN responses. Consistent with this finding, in experiments aimed at determining the functional relevance of the Mnk pathway in the growth inhibitory effects of IFNα, we found that siRNA-mediated Mnk1/Mnk2 knockdown reverses generation of type I IFN-dependent suppressive responses in primitive leukemic hematopoietic progenitors. Importantly, our studies demonstrate a requirement for Mnk activity in the suppres-

sive effects of IFNα on normal hematopoietic erythroid (BFU-E) and myeloid (CFU-GM) progenitors derived from normal bone marrow. Although the precise mechanisms by which such growth suppression is mediated by Mnk kinases remains to be defined, it is possible that engagement of eIF4E downstream of Mnk kinases by the IFN-pathway sequesters away this key element of the mRNA translation machinery from mitogenic/cell proliferative pathways and redirects it toward the regulation of ISG mRNA expression. Moreover, it is likely that the translation of other ISGs, beyond *Isg15* and *Isg54*, is regulated by this mechanism, but the identity of such genes that mediate growth suppressive effects on normal hematopoietic progenitors remains to be established in future studies. Studies involving comparative microarray analyses between Mnk1/2+/+ and Mnk1/2−/− cells may lead to the identification of genes accounting for such inhibitory effects, and future studies in that direction are warranted. Nevertheless, independent of the specific genes and protein products involved in such regulation, our findings may have important future clinical-translational implications. For instance, it is well-known that when administered to humans, type I IFNs may induce cytopenias. As such cytopenias result from suppression of normal hematopoietic progenitor growth (37) and the function of Mnk kinases is critical for such effects, it is possible that selective pharmacological or molecular targeting of Mnk activity in hematopoietic cells may provide an approach to diminish such IFN toxicities. Based on our findings, future work to precisely define the mechanisms by which Mnk kinases and eIF4E-generated signals participate in the induction of IFN responses is warranted, and may ultimately provide the basis for the design of approaches to more effectively use IFNs as therapeutics.

Materials and Methods

Cells and Reagents. Immortalized MEFs from Mnk1/Mnk2 double-knockout mice (20) were grown in DMEM with 10% FBS and antibiotics. Immortalized MEFs from p38α knockout mice (22) were kindly provided by Dr. Angel Nebreda [CNIO (Spanish National Cancer Center), Madrid, Spain]. Immortalized MEFs from Jak1 (38) knockout mice were kindly provided by Dr. Robert Schreiber (Washington University School of Medicine, Saint Louis, MO). Recombinant human IFNα was obtained from Hoffman LaRoche. Recombinant human and mouse IFNβ were obtained from Biogen Idec.

Cell Lysis and Immunoblotting. Cell lysis and immunoblotting were performed as described (14, 15).

Human Hematopoietic Progenitor Cell Assays. Bone marrow aspirates from healthy donors were collected after obtaining informed consent approved by the Institutional Review Board of Northwestern University. Hematopoietic progenitor assays in methylcellulose were performed as in previous studies (14, 15).

Isolation of Polysomal RNA. Polysomal fractionation was performed as in our previous studies (12).

SI. Further information is available in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants CA100579, CA77816, and CA121192; Department of Veterans Affairs Grant (L.C.P.); Canadian Institutes of Health Research Grant MOP15094 (to E.N.F.); Malkin Scholars Award (to S.J.); and Predoctoral National Research Service Award F30ES015668 (to A.J.R.)

1. Pestka S, Langer JA, Zoon KC, Samuel CE (1987) Interferons and their actions. *Annu Rev Biochem* 56:727–777.
2. van Boxel-Dezaire AH, Rani MR, Stark GR (2006) Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 25:361–372.
3. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD (1998) How cells respond to interferons. *Annu Rev Biochem* 67:227–264.
4. Platanias LC (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 5:375–386.
5. Kotenko SV, et al. (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4:69–77.
6. Darnell JE, Jr., Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415–1421.
7. Stark GR (2007) How cells respond to interferons revisited: From early history to current complexity. *Cytokine Growth Factor Rev* 18:419–423.
8. Lekmine F, et al. (2003) Activation of the p70 S6 kinase and phosphorylation of the 4E-BP1 repressor of mRNA translation by type I interferons. *J Biol Chem* 278:27772–27780.
9. Lekmine F, et al. (2004) Interferon-gamma engages the p70 S6 kinase to regulate phosphorylation of the 40S S6 ribosomal protein. *Exp Cell Res* 295:173–182.
10. Thyrell L, et al. (2004) Interferon alpha-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway. *J Biol Chem* 279:24152–24162.
11. Kaur S, et al. (2007) Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. *J Biol Chem* 282:1757–1768.
12. Kaur S, et al. (2008) Role of the Akt pathway in mRNA translation of interferon-stimulated genes. *Proc Natl Acad Sci USA* 105:4808–4813.
13. Uddin S, et al. (1999) Activation of the p38 mitogen-activated protein kinase by type I interferons. *J Biol Chem* 274:30127–30131.
14. Verma A, et al. (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.
15. Mayer IA, et al. (2001) The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells. *J Biol Chem* 276:28570–28577.
16. Scheper GC, Morrice NA, Kleijn M, Proud CG (2001) The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. *Mol Cell Biol* 21:743–754.
17. Waskiewicz AJ, et al. (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. *Mol Cell Biol* 19:1871–1880.
18. Wang X, et al. (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J Biol Chem* 273:9373–9377.
19. Pestova TV, et al. (2001) Molecular mechanisms of translation initiation in eukaryotes. *Proc Natl Acad Sci USA* 98:7029–7036.
20. Ueda T, Watanabe-Fukunaga R, Fukuyama H, Nagata S, 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.
21. Waskiewicz AJ, Flynn A, Proud CG, Cooper JA (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J* 16:1909–1920.
22. Adams RH, et al. (2000) Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* 6:109–116.
23. Ritchie KJ, et al. (2004) Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. *Nat Med* 10:1374–1378.
24. Ritchie KJ, Zhang DE (2004) ISG15: The immunological kin of ubiquitin. *Semin Cell Dev Biol* 15:237–246.
25. Kaur S, Katsoulidis E, Platanias LC (2008) Akt and mRNA translation by interferons. *Cell Cycle* 7:2112–2116.
26. Kaur S, et al. (2008) Dual regulatory roles of phosphatidylinositol 3-kinase in IFN signaling. *J Immunol* 181:7316–7323.
27. Santhanam AN, et al. (2009) Role of 3′UTRs in the translation of mRNAs regulated by oncogenic eIF4E–a computational inference. *PLoS ONE* 4:e4868.
28. Buxade M, et al. (2005) The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. *Immunity* 23:177–189.
29. Buxadé M, Morrice N, Krebs DL, Proud CG (2008) The PSF.p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha. *J Biol Chem* 283:57–65.
30. Beretta L, et al. (1998) Differential regulation of translation and eIF4E phosphorylation during human thymocyte maturation. *J Immunol* 160:3269–3273.
31. Pyronnet S, Dostie J, Sonenberg N (2001) Suppression of cap-dependent translation in mitosis. *Genes Dev* 15:2083–2093.
32. Takei N, Kawamura M, Hara K, Yonezawa K, Nawa H (2001) Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes: Comparison with the effects of insulin. *J Biol Chem* 276:42818–42825.
33. Walsh D, Mohr I (2004) Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. *Genes Dev* 18:660–672.
34. Knauf U, Tschopp C, Gram H (2001) Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. *Mol Cell Biol* 21:5500–5511.
35. Shenberger JS, et al. (2007) Roles of mitogen-activated protein kinase signal-integrating kinases 1 and 2 in oxidant-mediated eIF4E phosphorylation. *Int J Biochem Cell Biol* 39:1828–1842.
36. Jauch R, et al. (2006) Mitogen-activated protein kinases interacting kinases are autoinhibited by a reprogrammed activation segment. *EMBO J* 25:4020–4032.
37. Raefsky EL, Platanias LC, Zoumbos NC, Young NS (1985) Studies of interferon as a regulator of hematopoietic cell proliferation. *J Immunol* 135:2507–2512.
38. Rodig SJ, et al. (1998) Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell* 93:373–383.