THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 7, Issue of February 13, pp. 5278–5287, 2004
Printed in U.S.A.

Identification of a *gadd45β* 3′ Enhancer That Mediates SMAD3- and SMAD4-dependent Transcriptional Induction by Transforming Growth Factor β*[S]

Received for publication, October 21, 2003, and in revised form, November 19, 2003
Published, JBC Papers in Press, November 20, 2003, DOI 10.1074/jbc.M311517200

Michael B. Major‡ and David A. Jones‡§¶

From the Departments of ‡Oncological Sciences and §Medicinal Chemistry, Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112

GADD45β regulates cell growth, differentiation, and cell death following cellular exposure to diverse stimuli, including DNA damage and transforming growth factor-β (TGFβ). We examined how cells transduce the TGFβ signal from the cell surface to the *gadd45β* genomic locus and describe how GADD45β contributes to TGFβ biology. Following an alignment of *gadd45β* genomic sequences from multiple organisms, we discovered a novel TGFβ-responsive enhancer encompassing the third intron of the *gadd45β* gene. Using three different experimental approaches, we found that SMAD3 and SMAD4, but not SMAD2, mediate transcription from this enhancer. Three lines of evidence support our conclusions. First, overexpression of SMAD3 and SMAD4 activated the transcriptional activity from this enhancer. Second, silencing of SMAD protein levels using short interfering RNAs revealed that TGFβ-induced activation of the endogenous *gadd45β* gene required SMAD3 and SMAD4 but not SMAD2. In contrast, we found that the regulation of plasminogen activator inhibitor type I depended upon all three SMAD proteins. Last, SMAD3 and SMAD4 reconstitution in SMAD-deficient cancer cells restored TGFβ induction of *gadd45β*. Finally, we assessed the function of GADD45β within the TGFβ response and found that GADD45β-deficient cells arrested in G_2 following TGFβ treatment. These data support a role for SMAD3 and SMAD4 in activating *gadd45β* through its third intron to facilitate G_2 progression following TGFβ treatment.

Normal epithelial cells are in constant communication with their surrounding environment, largely through the detection, interpretation, and response to extracellular signaling molecules. The TGFβ[1] superfamily of growth factors comprises 42 such signaling molecules in humans, many of which play fundamental roles in development and adult tissue homeostasis. The epithelial response to members of this family is highly varied and includes such diverse cellular processes as proliferation, movement, differentiation, and apoptosis. Indeed, cells harboring mutations within the signal transduction proteins or the TGFβ target genes either fail to respond or respond inappropriately to the TGFβ signal, often leading to developmental problems, oncogenesis, fibrotic disease, metastasis, and autoimmune disorders. Greater understanding of how cells interpret the TGFβ signal will facilitate the prevention, detection, and treatment of various human diseases.

The central elements of TGFβ signal transduction are now known (1, 2). TGFβ activates the serine/threonine kinase activity of a multimeric receptor complex. Activation of this complex initiates a cascade of intracellular events that culminate in altered gene expression. The SMAD proteins form the foundation of this signaling network, since they are the only proteins directly phosphorylated by the receptor complex. However, these transcription factors are by no means sufficient to impart a TGFβ response. To specifically target a gene for transcriptional regulation, the SMADs require assistance by accessory factors. Consequently, the presence and activity of these accessory factors is are important to the TGFβ transcriptional program as are the SMAD proteins. By designing the system in such a way, cell-specific responses to TGFβ can be achieved. Further, the logic of the TGFβ signaling network explains how the cell integrates multiple signals to generate highly specific phenotypic responses.

In an attempt to better understand how TGFβ regulates gene transcription and how those gene products contribute to TGFβ biology, we have partially defined the TGFβ transcriptional profile in normal human mammary epithelial cells (HMEC). cDNA microarray expression analysis of TGFβ-treated HMEC revealed a set of genes involved in cellular proliferation, differentiation, and apoptosis. One of these genes, *gadd45β/hMyD118*, is regulated by TGFβ in multiple cell types, thus suggesting that this gene is of central importance to the TGFβ response.

GADD45β and two similar small acidic nuclear proteins, GADD45α and GADD45γ, make up the GADD45 family (3). All three proteins regulate diverse cellular mechanisms including cell growth, DNA repair, differentiation, and apoptosis, four phenotypes that are also controlled by TGFβ signaling. Aside from sequence similarity, these genes share transcriptional regulation by DNA damage insult and growth factors. *gadd45β* is, however, the only member of this family regulated by TGFβ (4, 5). *gadd45β* was first discovered as a transcript rapidly induced by either TGFβ treatment or the onset of terminal differentiation in M1 murine myeloid cells (6, 7). Subsequent studies employing antisense-mediated silencing established

* This work was supported by a predoctoral fellowship from the Pharmaceutical Research and Manufacturers of America Foundation (to M. B. M.), a grant from the Huntsman Cancer Foundation, a grant from the Willard L. Eccles Foundation, and University of Utah Core Facility Technical Support Grant CA42104. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains an additional figure and table.

¶ To whom all correspondence should be addressed: Huntsman Cancer Institute, 2000 Circle of Hope, Salt Lake City, UT 84112-5550. Tel.: 801-585-6107; Fax: 801-585-0900; E-mail: david.jones@hci.utah.edu.

[1] The abbreviations used are: TGF, transforming growth factor; HMEC, human mammary epithelial cell(s); siRNA, short interfering RNA; siScr, scrambled siRNA; MES, 4-morpholineethanesulfonic acid; PAI1, plasminogen activator inhibitor-1.

GADD45β as an important regulator of the G_2/M checkpoint following genotoxic stress (8) and apoptosis during M1 myeloid cell terminal differentiation (6, 9). Human GADD45β, which was first identified in a complex containing the p38-activating kinase MTK1 (MEKK4), is now a well established regulator of p38 activity and consequently p38-regulated biology (5, 10, 11). TGFβ activates p38 kinase activity and induces apoptosis in normal murine hepatocytes, but not in hepatocytes derived from *gadd45β* knockout mice (11). An initial characterization of the molecular mechanism by which TGFβ induces *gadd45β* transcription has recently been reported. First, reconstitution of SMAD4 expression in SMAD4-null pancreatic cell lines restored *gadd45β* induction by TGFβ (5). The nature of the TGFβ-SMAD-*gadd45β* link appears to be direct; exogenously expressed SMAD2 and SMAD4 or SMAD3 and SMAD4 induce *gadd45β* proximal promoter activity 3–4-fold (11). However, the relative importance and function of each SMAD protein to the transcriptional activation of the endogenous *gadd45β* gene is not known.

Utilizing RNA interference and reconstitution of SMAD3 and SMAD4 protein expression in SMAD-deficient cell lines, we exclude SMAD2 and include SMAD3 and SMAD4 as transcription factors involved in the TGFβ induction of *gadd45β*. Additionally, through a genomics-based approach, we identified a SMAD-dependent TGFβ-responsive enhancer encompassing the third intron of *gadd45β*. The importance of this enhancer is indicated by a 3-fold greater transcriptional induction following TGFβ treatment than transcriptional effects mediated by 5′ promoter sequences. Finally, using a cell system that does not undergo TGFβ-induced apoptosis but does respond to TGFβ by *gadd45β* transcriptional induction, we establish an apoptosis-independent role for GADD45β as an important mediator of G_2/M progression following TGFβ treatment.

MATERIALS AND METHODS

Cell Culture and Drug Treatments—The following cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2.0 μM L-glutamine, 1.0 μM sodium pyruvate, penicillin, and streptomycin and split every third day or at 80% confluence: Mv1Lu (CCL64), HaCaT, HeLa, 293, and 10T1/2. HT29 adenocarcinoma colon cells were cultured in McCoy's medium supplemented with 10% fetal bovine serum. The HepG2 and JAR cell lines were cultured in minimal essential medium and RPMI supplemented with 10% fetal bovine serum, respectively. We obtained all of the cell lines from ATCC except for the HaCaT immortalized keratinocyte cell line, which was a kind gift from D. Grossman (University of Utah, Salt Lake City, UT), the Mv1Lu cells were a kind gift from D. Ayer (University of Utah), and the JAR cells were a kind gift from E. Adashi (University of Utah). Human mammary epithelial cells (HMEC) were obtained from BioWhittaker (Walkersville, MD) and cultured in complete mammary epithelial growth medium. HMEC were seeded at passage 7 or 8 and harvested at no greater than 80% confluence for all experiments. For treatments with TGFβ (isoform type 1; Peprotech, Rocky Hill, NJ), we found little to no difference with respect to gene transcription if the cells had been previously serum-starved. The vehicle control for TGFβ comprised 4 mM HCl, 1 mg/ml bovine serum albumin. Cyclohexamide and actinomycin D (Calbiochem) were used at 10 and 5 μg/ml, respectively, and treated as described in Fig. 2.

RNA Interference—siRNAs were designed to specifically target either *smad2*, *smad3*, or *smad4* in accordance with the guidelines developed by Tuschl *et al.* (12). Because the sequence of mink *smad2* and *smad3* cDNAs is unknown, siRNAs were designed against the human sequences. The human-designed *smad2* and *smad3* siRNAs efficiently and specifically silenced mink SMAD2 and SMAD3 protein expression, thus indicating that these sequences are conserved in mink. We designed the *smad4* siRNA-A and siRNA-B against the mink sequence, and consequently they do not silence human *smad4* (data not shown). The control siRNA (scrambled siRNA; siScr) specifically recognizes human *smad4* and thus does not affect mink SMAD2, SMAD3, or SMAD4 expression. The sequences of the chemically synthesized and high pressure liquid chromatography-purified RNA oligomers are as follows (sense strand shown): Smad2 5′-UCUUUGUGCAGAGCCCCA-Att; Smad3 5′-ACCUAUCCCCGAAUCCGAUtt; Smad4-A 5′-GGACGA-AUAUGUUCAUGACtt; Smad4-B 5′-UUGGAUUCUUUAAUAACAGtt; siScr 5′-GGAUGAAUAUGUGCAUGACtt. To silence *gadd45β* expression, three siRNAs were designed (sense strand shown): siGadd45β-A (5′-GUU GAU GAA UGU GGA CCC Att), siGadd45β-B (AUC CAC UUC ACG CUC AUC Ctt), and siGadd45β-C (CUU GGU UGG UCC UUG UCU Gtt). Of these three siRNAs, siGadd45β-A was the most efficacious and was used to generate the data seen in Fig. 8. All RNA oligomers were reconstituted and annealed following the protocol of Tuschl *et al.* (12). Mv1Lu cells were plated 24 h prior to transfection and transfected at 70% confluence. All siRNAs were transfected using 18 μl of LipofectAMINE 2000/10-cm^2 plate according to manufacturer's guidelines (Invitrogen). For the SMAD silencing experiments, total RNA or protein was isolated 40–48 h after transfection. In time course experiments, we found that maximal silencing occurred 36 h after transfection for all three SMAD proteins (data not shown). For Gadd45β silencing, 3 h after the start of Gadd45β siRNA transfection, cells were treated with vehicle or TGFβ for an additional 2 h prior to RNA isolation or 12 h prior to flow cytometry.

Plasmids and Genomic Alignments—We electronically cloned the human, murine, and rat *gadd45β* genomic loci from publicly available sequence databases. Approximately 8 kb of the genomic loci, starting at 5000 kb upstream of the transcriptional start site, were aligned using the MAVID alignment algorithm (13, 14). The portion of this piece of genomic DNA showing conservation among all three species is shown in Fig. 7*A*. The G45β-1 (−1470 bp, +362 bp), G45β-2 (−972 bp, +362 bp), G45β-3 (−476 bp, +362 bp), G45β-A (−1535 bp, −1042 bp), G45β-B (−572 bp, −79 bp), and G45β-C (+941 bp, +1428 bp) reporter constructs were created as follows. The indicated region of the human *gadd45β* genomic locus was PCR-amplified from HMEC genomic DNA and cloned into the pCR2.1-TOPO vector (Invitrogen). These DNAs were then subcloned into pGL3basic, sequence-verified, and utilized in subsequent dual luciferase assays. J. Massague generously provided the 3TPLux reporter construct (Memorial Sloan-Kettering Cancer Center, New York). The murine Smad7 cDNA (generously provided by R. Derynk, University of California, San Francisco, CA) was subcloned into pcDNA3.1. Similarly, the FLAG-tagged Smad expression vectors used in the reporter experiments were created by subcloning the cDNAs from constructs provided by D. Satterwhite into pCMV2-FLAG (University of Utah, Salt Lake City, UT). For luciferase assays, all reporters were co-transfected with an SV40-*Renilla* luciferase reporter plasmid that was used to normalize transfection efficiencies. For retroviral infections, we PCR-amplified the *smad3* or *smad4* open reading frames from HMEC cDNA and then cloned them into the pBabe retroviral vector. D. Ayer generously provided the GFP-pBabe vector (University of Utah, Salt Lake City, UT).

Luciferase Assays—Fugene 6 (Roche Applied Science) was used to transfect HaCaT cells as instructed by the manufacturer. We seeded cells at a density of 80,000 cells/well in 24-well plates and transfected them the next day. Transfections were performed using 0.6 μg of DNA (including either 0.1 μg of normalization vector and 0.5 μg of reporter vector or 0.1 μg of normalization vector, 0.2 μg of reporter vector, and 0.3 μg of expression vector) and harvested 20 h after the start of transfection. For TGFβ treatment, medium containing either TGFβ (200 pM) or an equal volume of vehicle was added to cells 3 h after the start of transfection. Luciferase values were analyzed using a dual luciferase assay system (Promega). Dividing the firefly luciferase activity from each well by the *Renilla* luciferase activity from the same well normalized transfection efficiencies. Data in each experiment are presented as the mean ± S.D. of triplicates from a representative experiment. All experiments were performed at least three times, producing qualitatively similar results.

Retroviral Transduction—Expression of the GFP, SMAD3, or SMAD4 retroviral constructs was verified by Western blot in a transient assay prior to virus production. To produce the retrovirus, Phoenix helper cells were seeded in 60-mm^2 plates 24 h prior to transfection with LipofectAMINE 2000. 24 h after transfection began, we split the cells 1:3 to 10-cm^2 plates. 48 h after the cells had been split, virus-containing medium was removed from the Phoenix cells, filtered (0.22 μM; low protein binding filter), and added to a 6-well plate containing the HT29 or JAR target cells (at 60% confluence). We added Polybrene (4 μg/ml) to the virus immediately before transduction of the target cells to facilitate infection. 24 h after infection, the target cells were split to 10-cm^2 plates and placed under selection with 750 ng/ml puromycin for 10 days.

Quantitative Reverse Transcription-PCR—Trizol (Invitrogen) was used to isolate the total RNA from the HT29 and JAR retroviral poly-

clonal stables according to the manufacturer's guidelines. cDNA was synthesized from 2 μg of total RNA using Superscript III (Invitrogen). Real time PCR was performed using the Roche Light Cycler instrument and software, version 3.5 (Roche Applied Science). Intron-spanning primers (Gadd45β, forward (5′-CGGTGGAGGAGCTTTTGGTG-3′) and reverse (5′-CACCCGCACGATGTTGATGT-3′); 18 S rRNA, forward (5′-GGTGAAATTCTTGGACCGGC-3′) and reverse (5′-GACTTTGGTTTC-CCGGAAGC-3′)) were designed to amplify 200-bp products in order to minimize contamination from genomic DNA.

PCR was performed in duplicate (or triplicate for 18 S rRNA) with a master mix consisting of cDNA template, buffer (500 mM Tris, pH 8.3, 2.5 mg/ml bovine serum albumin, 30 mM $MgCl_2$), dNTPs (2 mM), TaqStart antibody (Clontech), Biolase DNA polymerase (Bioline), gene-specific forward and reverse primers (10 μM), and SYBR Green I (Molecular Probes, Inc., Eugene, OR). The PCR conditions are as follows: 35 cycles of amplification with 1-s denaturation at 95 °C and 5-s annealing at 57 °C for Gadd45β and 53 °C for 18 S rRNA. A template-free negative control was included in each experiment. We determined the copy number by comparing gene amplification with the amplification of standard samples that contained 10^3 to 10^7 copies of the gene or 10^5 to 10^9 for 18 S rRNA. The relative expression level of each gene was calculated by averaging the replicates and then dividing the average copy number of Gadd45β by the average copy number of 18 S rRNA. S.E. of the ratios was calculated using a confidence interval.

Northern and Western Blotting—Total RNA was isolated using Trizol following the manufacturer's protocol (Invitrogen). Where indicated, total RNA isolation was followed by poly(A) RNA selection using a PolyATtract™ mRNA Isolation kit (Promega). Total RNA or poly(A) RNA was fractionated through formaldehyde-containing agarose gels and transferred onto N+Hybond nylon membranes (Amersham Biosciences). Labeled probes were generated using the Rediprime II random prime labeling system (Amersham Biosciences) supplemented with [^{32}P]dCTP (ICN). To generate Northern blot probes, we PCR-amplified gene-specific sequences from human, mink, or murine cDNA. Mink *gadd45β* was PCR-amplified using the following degenerate primers: 5′-CTGCARATYCACTTCACSCT and 3′-GGRAYCCAYTGGTTDTTGC. Hybridizations with ^{32}P-labeled probes were carried out using ULTRAhyb buffer (Ambion) as recommended by the manufacturer. For Western blotting, protein lysates were harvested in a buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$. 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1 mg/ml pepstatin A, and 1 mg/ml phenanthroline. The resulting whole cell lysates were centrifuged at 20,800 × *g* for 10 min at 4 °C. Following protein quantitation using the DC Protein Assay (Bio-Rad), equal amounts of protein were fractionated through Tris-glycine 4–12% gradient NuPAGE gels using the MES buffer system (Invitrogen). The following antibodies were used to detect the SMAD proteins in both Mv1Lu and human cell lines: SMAD3 (catalog no. 51-1500; Zymed Laboratories Inc.), SMAD2 (catalog no. S66220; Transduction Laboratories), SMAD4 (catalog no. sc-7966; Santa Cruz Biotechnology), and β-catenin (catalog no. 610153; Transduction Laboratories). Immune complexes were visualized using a secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) and Western Lighting chemiluminescence reagent (PerkinElmer Life Sciences).

RESULTS

gadd45β Is a Primary TGFβ-responsive Gene in Normal Human Mammary Epithelial Cells—TGFβ induces a G_1 cell cycle arrest and epithelial to mesenchymal transition, but not apoptosis, in primary normal human mammary epithelial cells grown in culture (15) (data not shown). To understand the mechanisms behind these TGFβ-induced phenotypes, we partially defined the TGFβ transcriptome in normal human mammary epithelial cells (HMEC). Specifically, we used cDNA microarray expression analysis to determine the relative expression of 7000 genes at 2 and 12 h after TGFβ treatment in HMEC. Data analysis revealed 54 up-regulated and 10 down-regulated TGFβ-regulated genes. Genes included in this list had a -fold change of greater than 1.3 or less 0.7 at both time points and a *p* value of less than 0.05 at both time points (Supplemental Table I and methods therein). Next, we identified genes within this data set that were in common to TGFβ-regulated genes identified through transcriptional profiling in other TGFβ-responsive cell systems. We surmised that because genes in this subgroup were regulated by TGFβ irrespective of cell origin or transformation status, they would be of central importance to the TGFβ cytostatic program. Plasminogen activator inhibitor-1 (PAI1) is a well established TGFβ-induced gene and was induced 8-fold 2 h after TGFβ treatment in HMEC (Supplemental Table I). Consequently, PAI1 served as an important positive control in the microarray, in Northern blots, and in subsequent experiments (Fig. 1*A*). A second common TGFβ target gene identified in our expression analysis was *gadd45β/hMyD118*. In addition to our studies in primary normal HMEC, previous findings indicate that *gadd45β* is a TGFβ-induced gene in transformed cell lines derived from my-

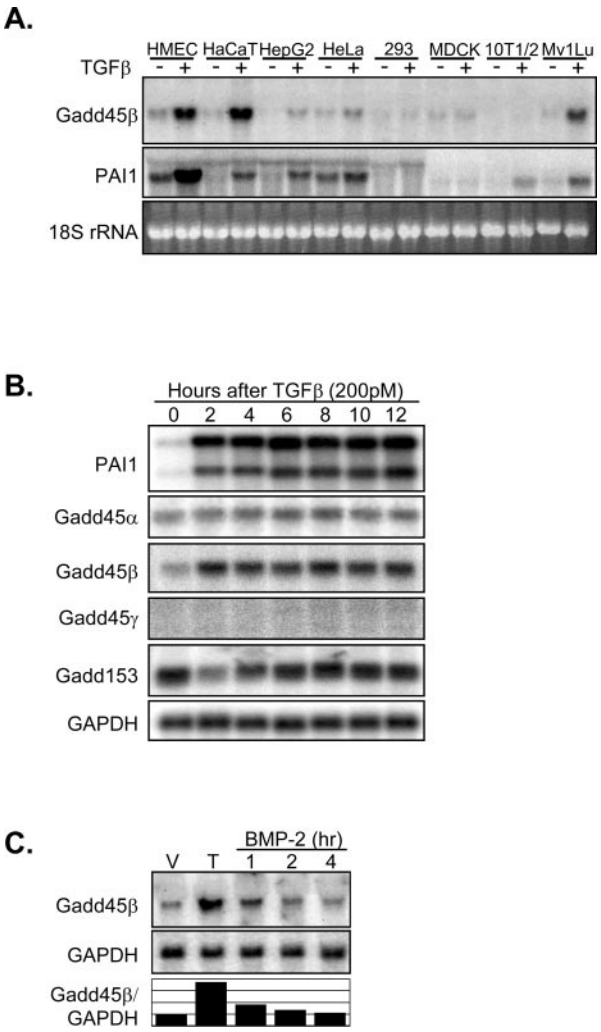

FIG. 1. ***gadd45β* is a TGFβ-inducible gene.** *A*, various established cell lines that have been previously reported to be TGFβ-sensitive by some measure were treated with TGFβ for 1 h prior to total RNA isolation and Northern blotting for Gadd45β and PAI1. The PAI1 3.2-kb transcript is shown; we could not detect the 2.2-kb PAI1 transcript in Mv1Lu or 10T1/2 using human, mink, or murine PAI1 probe. Extended exposure of the Northern blots and additional experiments not shown verified that the human PAI1 and *gadd45β* Northern probes are capable of recognizing their respective orthologues. The 18 S ribosomal band was visualized in the ethidium bromide-stained gel prior to Northern blotting and serves as the loading control. *B*, randomly cycling HMEC were treated with either TGFβ (200 pM) or an equal volume of vehicle. At the indicated time, total RNA was isolated from the cells. Following mRNA purification, Northern blot analysis was performed to visualize the relative transcript abundance of the indicated genes. Both the 3.2- and 2.2-kb alternatively spliced forms of the mature PAI1 mRNA are shown. *C*, HMEC were treated with TGFβ (200 pM for 2 h) or BMP-2 (4 nM) for the indicated times before RNA isolation and Northern blot analysis for the *gadd45β* transcript. GAPDH serves as a loading control. The *gadd45β* and GAPDH signals were quantitated using a PhosphorImager, and the resulting *gadd45β*/GAPDH ratio was plotted *below* the Northern blots.

eloid, breast, skin, breast, pancreas, and bone (4, 5, 11, 16). Because of its frequent presence in the TGFβ transcriptional response and because of its previously described role in growth arrest, differentiation, and apoptosis, we chose to characterize the upstream signal transduction pathway necessary for *gadd45β* transcriptional induction and, second, to examine the role of GADD45β in the TGFβ response.

We first determined the scope of *gadd45β* transcriptional activation. Specifically, we monitored its induction by TGFβ in several cell lines and by other members of the TGFβ superfamily in HMEC. To determine whether other TGFβ-responsive cell lines responded similarly to HMEC with respect to *gadd45β* transcription, several cell lines were treated with TGFβ or vehicle for 1 h. The *gadd45β* and PAI1 transcripts were induced by TGFβ in the following cell lines: HMEC, HaCaT, Mv1Lu, PANC-1, primary breast organoid outgrowths, and to a lesser extent in HepG2 and HeLa cells (data not shown) (Fig. 1*A*). The Madin Darby canine kidney and 293 cell lines did not respond to TGFβ stimulation by inducing either *gadd45β* or PAI1. TGFβ treatment of 10T1/2 murine fibroblasts caused a moderate increase in PAI1 transcription but did not affect *gadd45β* mRNA levels. We also asked whether other members of the TGFβ superfamily of growth factors could regulate *gadd45β* transcription. Fig. 1*C* illustrates that both TGFβ and BMP2 induced *gadd45β* transcription. However, the kinetics of *gadd45β* induction as well as the strength of induction differed between the two ligands. Finally, of the three genes that comprise the *gadd45* family, only *gadd45β* was found to be TGFβ-inducible in HMEC; *gadd45α* was not affected by TGFβ treatment, and Gadd545γ was not detected (Fig. 1*B*). Gadd153/Chop10, a GADD family member by virtue of its induction by cellular stress, was transiently repressed by TGFβ.

To distinguish whether TGFβ treatment resulted in increased *gadd45β* transcription or increased *gadd45β* mRNA stability, we measured the *gadd45β* mRNA half-life before and after TGFβ treatment. HMEC were treated with TGFβ for 1 h before the addition of the transcription inhibitor actinomycin D for various periods of time. Quantitative analysis of the Northern blot revealed that TGFβ failed to stabilize the *gadd45β* mRNA (Fig. 2, *A* and *B*). The accumulation of *gadd45β* mRNA within 2 h of TGFβ treatment suggested that it is an immediate early TGFβ-induced target gene. To test this idea, we pretreated HMEC with the protein translation inhibitor cyclohexamide 15 min before a 3-h combined TGFβ/cyclohexamide treatment. We found that the levels of *gadd45β* increased in a TGFβ-dependent manner irrespective of cyclohexamide pretreatment, indicating that new protein synthesis is not required for TGFβ induction of *gadd45β* (Fig. 2*C*). These data indicate that *gadd45β* is a direct TGFβ transcriptional target.

gadd45β Is Partly Dependent upon SMAD3 and Independent of SMAD2 in Its Regulation by TGFβ—We first sought to determine whether specific inhibition of SMAD2, SMAD3, and SMAD4 abrogated *gadd45β* responsiveness to TGFβ. To approach this, we employed siRNA-mediated silencing of the SMAD2, SMAD3, and SMAD4 proteins. Because we were unable to achieve silencing greater than 60% of wild-type levels in HMEC, we chose to use Mv1Lu cells for our siRNA studies. Transfection of Mv1Lu cells with siRNAs specific to SMAD2 or SMAD3 reduced the respective protein expression to nearly undetectable levels (Fig. 3*A*). Loss of SMAD2 caused a 70% decrease in the induction of PAI1 by TGFβ. In contrast, siRNA silencing of SMAD2 had no significant effect on *gadd45β* induction following TGFβ treatment (Fig. 3*A*). SMAD3-deficient cells, however, responded to TGFβ stimulation with reduced levels of induction for both *gadd45β* and PAI1. Although the

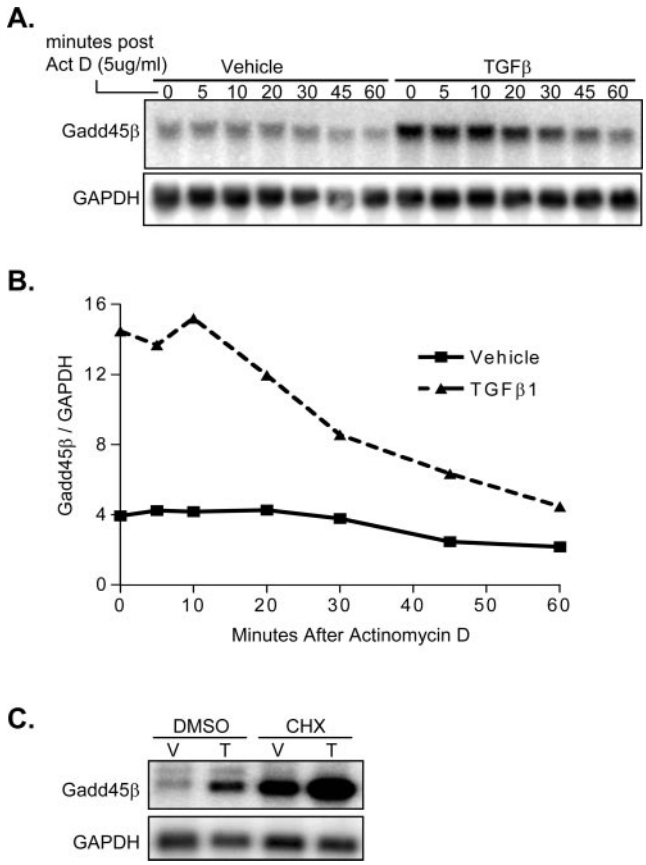

FIG. 2. **Transcriptional activation of *gadd45β* by TGFβ.** *A*, following a 2-h treatment with TGFβ (200 pM) or an equal volume of vehicle, HMEC were treated with 5 μg/ml actinomycin D for the indicated times. RNA was subsequently harvested and analyzed for the *gadd45β* and GAPDH transcripts. *B*, plotting the GAPDH-normalized *gadd45β* mRNA levels from Fig. 2*A* demonstrates that TGFβ does not stabilize the *gadd45β* mRNA. *C*, HMEC were treated with 10 μg/ml cyclohexamide (*CX*) for 15 min prior to a 3-h combined TGFβ (200 pM)-cyclohexamide treatment. RNA was then isolated and analyzed for the *gadd45β* and GAPDH transcripts. *DMSO*, Me_2SO. *ActD*, actinomycin D.

decrease in PAI1 induction by TGFβ observed in the SMAD2 and the SMAD3 single-knockout cells was enhanced in the double-knockout cells, the SMAD2/SMAD3 double-knockout cells behaved similarly to SMAD3-deficient cells with respect to *gadd45β* induction (Fig. 3*A*). Dose-response curves with the Smad3 siRNA (IC_{50} ~1 nM) further demonstrated that TGFβ activates PAI1 and *gadd45β* through a mechanism that is partly dependent upon SMAD3 (Fig. 3*B*).

SMAD4 Silencing Prevents gadd45β and PAI1 Induction by TGFβ—Of the many proteins involved in mediating the different facets of TGFβ signal transduction, SMAD4 is considered central to many of the responses. Two different siRNAs were designed against mink *smad4*, and the efficacy of their silencing was tested in Mv1Lu cells by Western blot (Fig. 4*A*). Consistent with the central role of SMAD4 in TGFβ signaling, siRNA silencing of SMAD4 resulted in a dramatic loss of *gadd45β* transcriptional induction following TGFβ treatment (Fig. 4*B*). As a confirmation of specificity, a human-specific SMAD4 siRNA, which contains mismatches at two positions relative to the mink sequence, did not affect SMAD4 protein expression or TGFβ-regulated transcription of *gadd45β* or PAI1. siSmad4-A and siSmad4-B both robustly silenced SMAD4 protein expression and did not interfere with SMAD3 protein expression (Fig. 4*A*). Examination of the *gadd45β* and PAI1 transcript levels in these SMAD4-deficient cells revealed a clear necessity for SMAD4 in targeting these genes for tran-

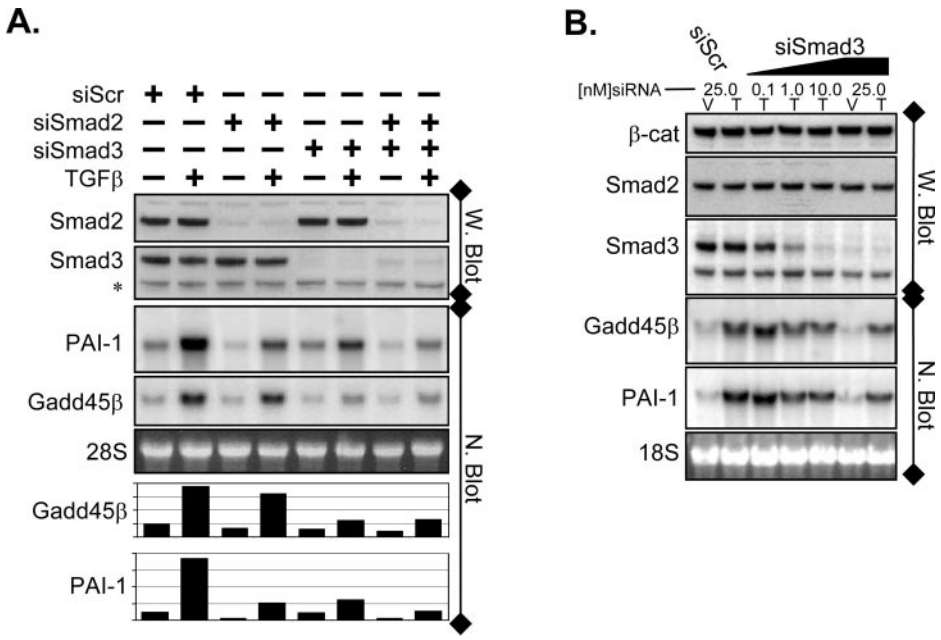

FIG. 3. ***gadd45β* induction by TGF*β* is independent of SMAD2 and dependent upon SMAD3 expression.** *A*, total cellular protein and total RNA was isolated from Mv1Lu cells transfected with the indicated siRNAs (15 nM). 1 h prior to RNA and protein harvest, the designated plates received 200 pM of TGF*β*. The protein lysates and RNA were then examined by Western blot and Northern blot for the SMAD2 or SMAD3 proteins and for the *gadd45β* and PAI1 transcripts, respectively. A graphical representation of the quantitated *gadd45β* and PAI1 Northern blots is shown *below*. For both *bar graphs*, all values were normalized to the transcript expression level in the siScr and vehicle-treated sample. The nonspecific band detected by the SMAD3 polyclonal antibody (indicated by an *asterisk*) and the ribosomal 28 S RNA serve as loading controls. *B*, Mv1Lu cells were transfected with the Smad3 siRNA at the indicate concentrations for 40 h prior to a 1-h treatment with vehicle (*V*) or TGF*β* (*T*). Total cellular protein and total RNA were then analyzed for the expression of the SMAD proteins and the *gadd45β* and PAI1 transcripts. *β*-Catenin and the 18 S ribosomal band serve as loading controls for the Western and Northern blots, respectively.

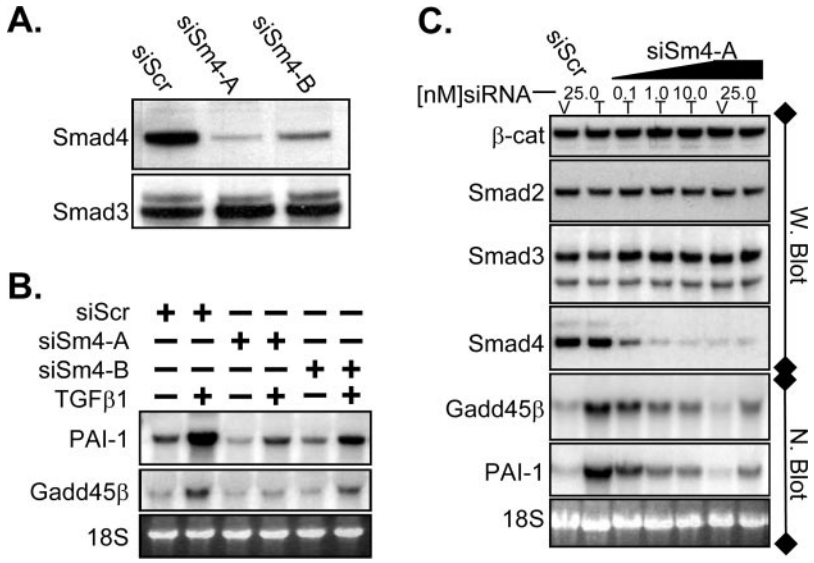

FIG. 4. **siRNA-mediated silencing of SMAD4 prevents *gadd45β* and PAI1 induction by TGF*β*.** *A*, two different siRNAs, siSmad4-A and siSmad4-B, were transfected into Mv1Lu cells at 15 nM for 40 h. Western blot analysis of total cellular protein for the SMAD4 and SMAD3 proteins demonstrates the efficacious and specific silencing of SMAD4. *B*, total RNA isolated from vehicle or TGF*β*-treated SMAD4-silenced Mv1Lu cells was analyzed for the presence of the *gadd45β* and PAI1 transcripts by Northern blot. These RNA samples were prepared in parallel to the protein extracts shown in Fig. 4*A*. *C*, dose-response relationship between *gadd45β* induction by TGF*β* and SMAD4 protein levels in siRNA-transfected Mv1Lu cells. Mv1Lu cells were transfected with siSmad4-A at the indicated concentration for 40 h prior to a 1-h TGF*β* (200 pM) treatment. Protein and total RNA were harvested in parallel. *β*-Catenin and the 18 S ribosomal band serve as loading controls for the Western and Northern blots, respectively.

scription following TGF*β* stimulation (Fig. 4*B*). The small difference between siSmad4-A and siSmad4-B in silencing SMAD4 protein expression directly reflected the levels of *gadd45β* and PAI1 induction by TGF*β*. The siSmad4-A silences SMAD4 protein expression with an IC_{50} of less than 1 nM, which is consistent with the IC_{50} of silencing imparted by siSmad3 (compare Fig. 4*C* with Fig. 3*B*). The induction of *gadd45β* and PAI1 transcripts by TGF*β* in these cells showed close correlation with each other and with the levels of SMAD4 protein (Fig. 4*C*).

Finally, we asked whether Mv1Lu cells lacking SMAD2, SMAD3, and SMAD4 responded differently to TGF*β* with respect to *gadd45β* transcriptional induction than cells deficient in only one or two of the SMADs. Mv1Lu cells were transfected with siRNAs directed against each of the SMADs alone and in all combinations thereof (Fig. 5*A*). Northern blot analysis of

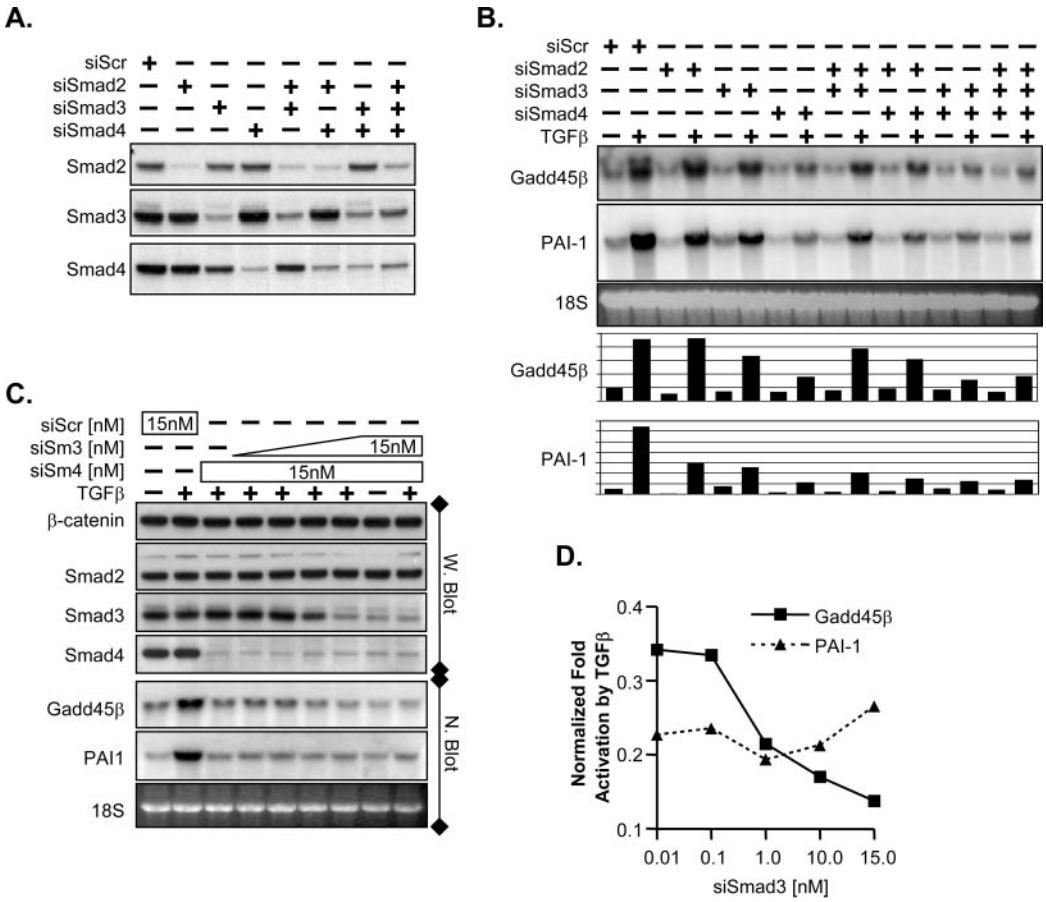

FIG. 5. **SMAD3 silencing in SMAD4-deficient cells represses *gadd45β* transcriptional induction by TGFβ.** *A*, Mv1Lu cells were transfected with the indicated siRNA at 15 nM for 40 h. The siSmad4-A siRNA was used to silence Smad4 expression. Total cellular protein was harvested and analyzed by Western blot for the indicated proteins. *B*, RNA was harvested from either vehicle or TGFβ treated Mv1Lu cells transfected with the indicated siRNAs (15 nM) in parallel to the protein seen in Fig. 5*A*. The *gadd45β* and PAI1 transcripts were detected by Northern blot. A graphical representation of the quantitated *gadd45β* and PAI1 Northern blots is shown *below*. For both bar graphs, all values were normalized to the transcript expression level in the siScr- and vehicle-treated sample. The 18 S ribosomal band serves as a loading control. *C*, Mv1Lu cells were transfected with either the scrambled siRNA (15 nM) alone or the Smad4-A siRNA (15 nM) in the presence of increasing concentrations of Smad3 siRNA (at 0.01, 0.1, 1.0, 10.0, and 15.0 nM) for 40 h prior to TGFβ treatment for 1 h. As before, the RNA and protein were harvested in parallel for Northern and Western blot analysis, respectively. *D*, the PAI1 and *gadd45β* Northern blots seen in Fig. 5*C* were quantitated by PhosphorImager analysis and normalized to the -fold induction observed in Mv1Lu cells transfected with a scrambled siRNA (-fold induction in *lane 1 versus lane 2*).

gadd45β again demonstrated a SMAD3 and SMAD4 dependence for TGFβ-induced transcription. Loss of SMAD2 in these SMAD3/SMAD4-deficient cells had no further effect on *gadd45β* induction. Interestingly, although PAI1 depends partly upon SMAD2 for TGFβ-induced transcription (Fig. 3*A*), loss of SMAD2 did not affect PAI1 induction in cells lacking SMAD3 and SMAD4 (Fig. 5*B*).

Our data generated with siRNA-mediated silencing have revealed no differences between *gadd45β* and PAI1 with respect to their regulation by SMAD3 and SMAD4 (Figs. 3 and 4). To examine the role of SMAD3 and SMAD4 more closely, we asked whether loss of SMAD3 in a SMAD4-reduced background would further inhibit *gadd45β* and PAI1 induction by TGFβ. Mv1Lu cells were transfected with a constant amount of siSmad4-A (15 nM) in the presence of an increasing concentration of siSmad3 (Fig. 5*C*). siRNA-mediated silencing of SMAD3 in a SMAD4-reduced background had no effect on PAI1 induction (Fig. 5, *C* and *D*). SMAD3 silencing in these SMAD4-deficient cells did, however, further repress the transcriptional induction of *gadd45β* following TGFβ treatment. These data support a transcriptional model that distinguishes Gadd45β from PAI1 in their regulation by SMAD3 and SMAD4.

SMAD3 and SMAD4 Expression in SMAD3- and SMAD4-null Cancer Cells Reconstitutes TGFβ-mediated Induction of gadd45β—The second approach we utilized to study the transcriptional regulation of *gadd45β* by TGFβ relied upon the preponderance of inactivating mutations within the SMAD proteins in human cancer cell lines. HT29 colon adenocarcinoma cells do not express SMAD4 protein because of a nonsense mutation that renders the transcript unstable (17). JAR cells, on the other hand, do not express SMAD3 (18). TGFβ treatment of these cell lines results in the phosphorylation of SMAD2, indicating that both cell lines express functional TGFβ receptor complexes and that SMAD2 phosphorylation is not dependent upon SMAD3 or SMAD4 (Fig. 6*A*). Retroviral transduction followed by polyclonal selection of these cells with either a GFP-encoding retrovirus or a SMAD3- or SMAD4-encoding virus provided an experimental approach to further examine the role of the SMADs in *gadd45β* transcription. Two weeks after the transduced cells were placed under selection, expression of the transduced genes was verified by fluorescence microscopy (for GFP expression; data not shown) and Western blot (Fig. 6*B*). RNA harvested in parallel to the protein samples analyzed in Fig. 6*B* was reverse transcribed and used in real time quantitative PCR to measure the *gadd45β* transcript levels. TGFβ treatment of JAR-SMAD3 cells revealed a small but statistically significant increase in *gadd45β* message levels (Fig. 6*C*). The SMAD4-HT29 cells responded to TGFβ through

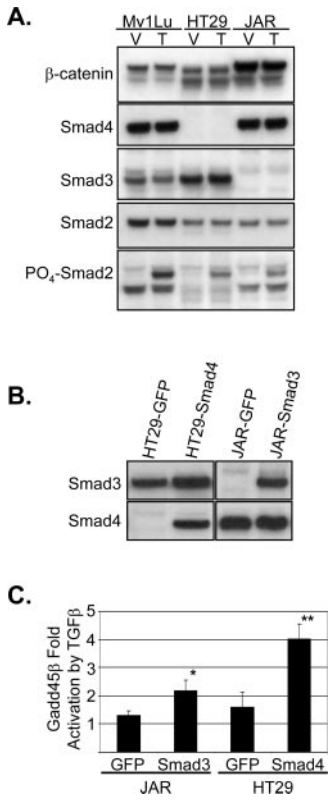

FIG. 6. **Reconstitution of Smad3 and Smad4 expression in SMAD-null cancer cells restores TGFβ signaling to *gadd45β*.** *A*, the Mv1Lu, HT29, and JAR cell lines were treated with TGFβ (200 pM) for 1 h prior to protein isolation and Western blot analysis for the indicated proteins. A Ponceau S-stained membrane (data not shown) and β-catenin verified that equal protein was loaded into each lane. *B*, HT29 cells and JAR cells were infected with the indicated pBabe retrovirus and selected for 2 weeks in puromycin. Total cellular protein harvested from the polyclonal stables was analyzed by SDS-PAGE for the SMAD4 and SMAD3 proteins. GFP expression was visualized by fluorescence microscopy (data not shown). *C*, the indicated polyclonal stable cell line was treated with either vehicle or TGFβ (200 pM) for 1 h before the total cellular RNA was harvested. Following reverse transcription, real time quantitative PCR for the *gadd45β* transcript was performed. The *gadd45β* transcript values were normalized to copies per 100,000 copies of 18 S ribosomal RNA. The values plotted are the average of two independent experiments, and the *error bars* represent the confidence interval of the ratios between the experiments. *, $p < 0.02$; **, $p < 0.001$.

a robust induction of *gadd45β* (Fig. 6*C*). Northern blot analysis of these RNAs confirmed the quantitative PCR results (data not shown).

gadd45β Contains a TGFβ-responsive Enhancer That Encompasses the Third Intron—Next, we analyzed the *gadd45β* genomic locus for transcriptional responsiveness to TGFβ. First, 1500 bp of the proximal promoter of *gadd45β* was cloned upstream of firefly luciferase for use in reporter assays (G45β-1) (Fig. 7*A*). TGFβ stimulation of HaCaT or Mv1Lu cells increased the transcriptional activity of G45β-1, G45β-2, and G45β-3 ~2-fold (Fig. 7*B*) (Mv1Lu data not shown). In contrast, the endogenous *gadd45β* transcript levels increased 8–15-fold in responsive cell lines following TGFβ treatment (Fig. 1*A*). We were unable to see increased reporter activity when other portions of this 5′-flanking region were analyzed or in numerous other cell lines or when the cells were treated for different lengths of time with TGFβ (Fig. 7*A*) (data not shown). We reasoned that because the *gadd45β* coding sequence is highly conserved between human, mouse, rat, and zebrafish, the region of the genomic locus mediating TGFβ responsiveness might also be conserved. To address this possibility, we aligned the human, mouse, and rat *gadd45β* genomic sequences and plotted the degree of conservation utilizing the MAVID algorithm (13, 14). In addition to the coding regions, three domains of the *gadd45β* genomic locus demonstrate high conservation between species. Each of these regions was cloned upstream of Firefly luciferase and used in reporter experiments (Fig. 7*A*). Remarkably, TGFβ robustly activated transcription from the G45β-C enhancer, which contains part of the third exon, the complete third intron, and part of the fourth exon, but not from G45β-A or G45β-B (Fig. 7*B*, Supplemental Fig. 1) (Mv1Lu data not shown). We took two approaches to test if SMAD proteins were mediating TGFβ-dependent transcriptional induction off of G45β-C. First, the inhibitory SMAD7 protein was overexpressed to block SMAD activation. SMAD7 overexpression inhibited TGFβ-induced activation of the 3TPLux reporter, which contains the PAI1 promoter, and the G45β-C reporter, it but did not affect an SV40-driven luciferase construct (Fig. 7*C*). Second, overexpression of SMAD3 and SMAD4 greatly enhanced G45β-C reporter activity in HaCaT cells (Fig. 7*C*) and in HeLa cells (data not shown). In contrast, SMAD2 expression did not affect the transcriptional activity. Interestingly, the increase in reporter activity was dependent upon both SMAD3 and SMAD4, because neither one alone significantly affected the G45β-C transcriptional activity. These data support a role for SMAD3 and SMAD4 in regulating Gadd45β transcription through a 3′ enhancer that contains the third intron. Indeed, sequence analysis of G45β-C revealed four conserved putative SMAD binding elements (SBEs) (Supplementary Fig. 1).

GADD45β Regulates G_2 Progression following TGFβ Stimulation—To examine the contribution of GADD45β to the TGFβ phenotype, a siRNA was designed to silence *gadd45β* expression. Dose-response analysis revealed potent (IC_{50} ~1 nM) and specific knockout of TGFβ-induced *gadd45β* expression (Fig. 8*A*). TGFβ rapidly induces a G_1 cell cycle arrest, but not apoptosis, in Mv1Lu cells. We asked whether Mv1Lu cells deficient in GADD45β would undergo a G_1 cell cycle arrest. Introduction of a scrambled siRNA had no detectable effect on TGFβ-induced cell cycle arrest (Fig. 8*B*). However, cells containing reduced levels of *gadd45β* demonstrated a slight reduction in G_1 accumulation and failed to progress through G_2 following TGFβ treatment (Fig. 8*B*). Loss of the *gadd45β* transcript did not affect cell cycle progression in the absence of TGFβ treatment. Dose-response analysis further verified this finding; 0.01 and 0.1 nM siRNA did not significantly affect *gadd45β* transcript levels or cell cycle progression following TGFβ stimulation. These findings indicate that GADD45β is an important regulator of cell cycle progression following TGFβ treatment.

DISCUSSION

The intracellular domain of a ligand-bound TGFβ receptor complex ignites an intertwined cascade of signaling events that induces one of many possible phenotypic responses (1, 2). Consequently, the mechanism by which a cell decides how to respond to TGFβ is fundamental to many aspects of eukaryotic life. One approach to decipher the cellular interpretation of the TGFβ signal and how that interpretation might be altered in a diseased tissue is to define and utilize the TGFβ target genes as a starting point in a retrograde molecular characterization of the upstream transcriptional program. Concurrent studies would assess the gene function as it contributes to the phenotypic response. We have employed this approach to the *gadd45β* gene. We found that *gadd45β* transcriptional induction by TGFβ was dependent upon SMAD4 and to a lesser extent on SMAD3 but independent of SMAD2. Further, SMAD3 and SMAD4 mediated the transcriptional induction of *gadd45β* through an enhancer that encompasses the third intron of the *gadd45β* gene. Finally, TGFβ stimulation of

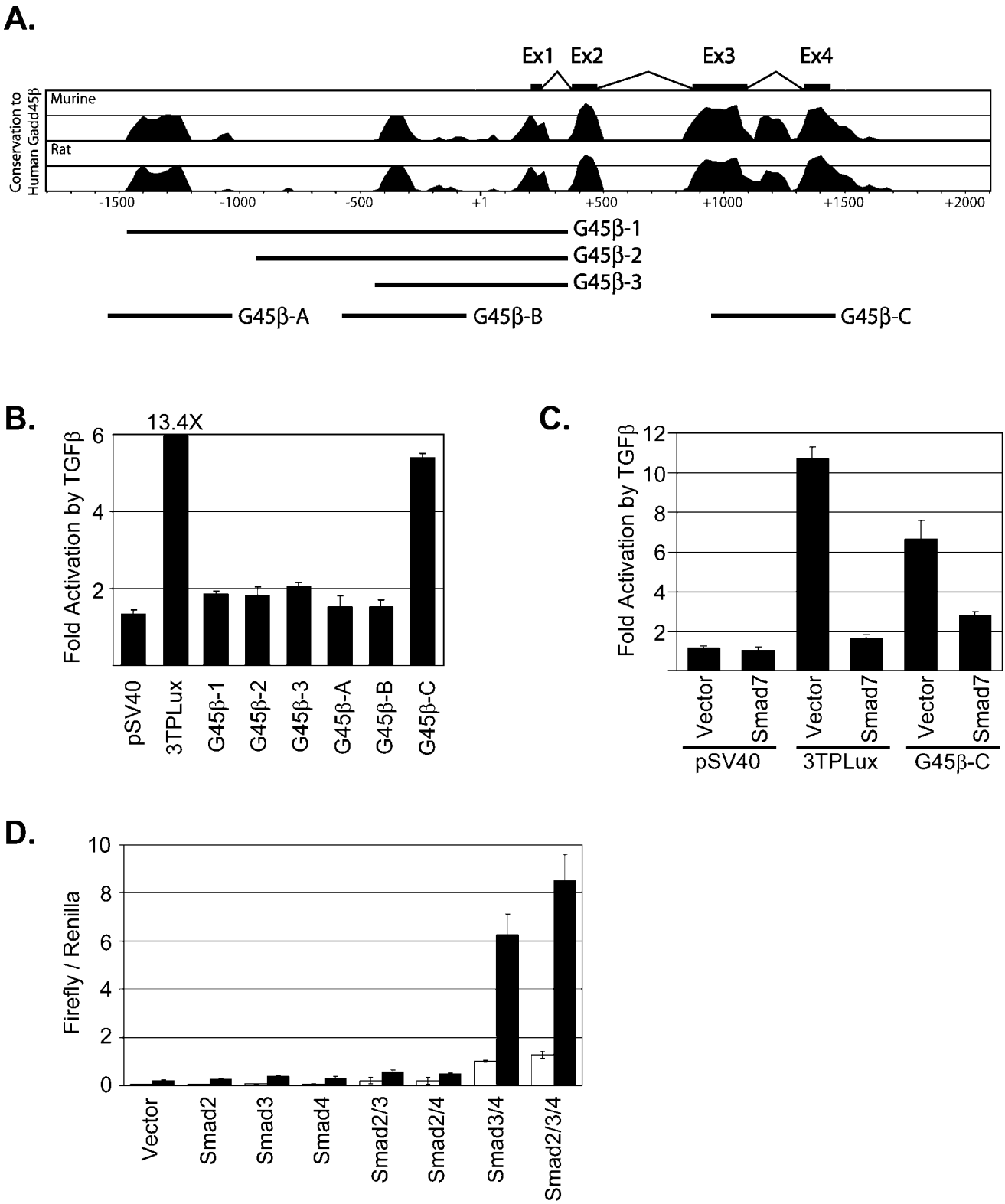

FIG. 7. ***gadd45β* is activated by TGFβ through a 3′ enhancer.** *A*, schematic representation of the *gadd45β* genomic locus. The relative position of the exons and introns are indicated *above* the *graph*. The MAVID algorithm was used to determine the relative degree of sequence conservation between human *gadd45β* (*x* axis) and rat *gadd45β* (*bottom half* of *plot*; *y* axis) and murine *gadd45β* (*top half* of *plot*; *y* axis). The degree of genomic conservation is indicated by the *height* of the *black curve*. *Below* the *graph* is a schematic diagram of six pieces of the *gadd45β* genomic locus that were cloned upstream of firefly luciferase for use in subsequent reporter assays. Of note, G45β-C contains 93 bp of exon 3, the complete third intron (237 bp), and 98 bp of exon 4 of *gadd45β* (Supplementary Fig. 1). *B*, HaCaT cells were transfected with the indicated reporter construct for 24 h in the presence or absence of TGFβ (200 pM) before firefly luciferase values were quantitated, normalized, and plotted as -fold induction by TGFβ. Qualitatively similar results were obtained with Mv1Lu cells (data not shown). *C*, reporter assay in HaCaT cells that were transiently co-transfected with either an empty vector (*Vector*) or a SMAD7 expression vector and the indicated luciferase reporter construct. TGFβ or vehicle control was added 4 h after the transfection began. Luciferase values were read 24 h after the TGFβ was administered. *D*, HaCaT cells co-transfected with G45β-C and the indicated SMAD expression construct in the presence (*black bars*) or absence (*open bars*) of TGFβ. All firefly luciferase values were normalized to *Renilla* luciferase before data analysis. The *error bars* represent the S.D. values from three independent experiments. All luciferase reporter experiments were repeated a minimum of five times (each time in triplicate) and produced qualitatively identical results.

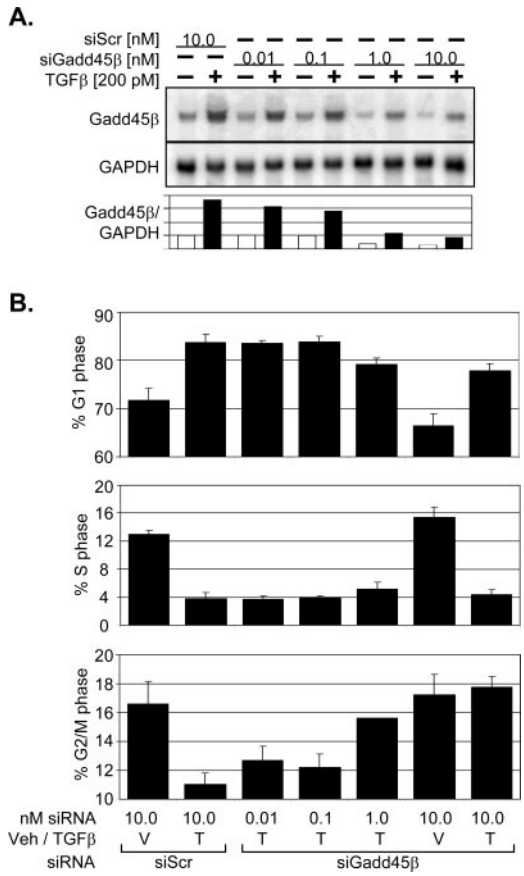

FIG. 8. ***gadd45β* regulates cell cycle progression following TGFβ treatment.** *A*, Mv1Lu cells were transfected with a siScr or a *gadd45β*-specific siRNA (*siGadd45β*) at the indicated concentration. Three hours after the start of transfection, the cells received an equal volume of vehicle or TGFβ (200 pM). TGFβ treatment lasted 2 h, at which point RNA was extracted and analyzed for the *gadd45β* or GAPDH transcript by Northern blot. Quantitative representation of the GAPDH-normalized *gadd45β* Northern blot is plotted below. *B*, Mv1Lu cells were transfected with *gadd45β* siRNA at the indicated concentration for 3 h prior to vehicle or TGFβ (200 pM) treatment for an additional 12 h. Samples were then harvested for flow cytometry analysis. The *error bars* represent the S.D. across three independent experiments.

gadd45β-deficient cells, but not of *gadd45β*-expressing cells, resulted in the activation of a G_2/M checkpoint.

We used RNA interference as a tool to probe the upstream signal transduction components necessary for *gadd45β* and PAI1 transcriptional induction following TGFβ stimulation. We chose Mv1Lu cells as a cell system for these studies rather than a human cell line such as HMEC or HaCaTs, because we found that in these cells our siRNAs were more efficacious as compared with a panel of TGFβ-responsive human cells. In fact, silencing SMAD protein expression in HaCaT cells to 30% of wild type levels resulted in no detectable effect on PAI1 or *gadd45β* transcription following TGFβ treatment.[2] These results and our findings presented in Fig. 4 argue that with respect to the transcriptional activation of *gadd45β* and PAI1, the SMAD proteins are expressed in excess. Coupled to the immediate early transcriptional induction of *gadd45β* and PAI1 by TGFβ, these data suggest that the *gadd45β* and PAI1 promoters share a relatively high affinity for the SMAD proteins. Further, this provides a possible molecular mechanism explaining how *gadd45β* and PAI1 are regulated by TGFβ irrespective of tissue type. Analogous findings have recently been discovered in *Caenorhabditis elegans* where the FoxA protein, PHA4, achieves transcriptional discrimination among target genes through a differential affinity to gene promoter sequences (19). Consequently, high affinity PHA4 promoters are responsive to relatively low levels of PHA4 protein expression. Further studies are in progress to classify TGFβ transcriptional targets by their sensitivity to changes in SMAD protein expression.

With the exception of a few genes, such as p15 (20) and MMP2 (21), most well characterized immediate early TGFβ-regulated genes appear to depend upon SMAD3 and SMAD4, but not SMAD2, for TGFβ transcriptional regulation. Our work places *gadd45β* within this SMAD2-independent, SMAD3/SMAD4-dependent class of TGFβ-responsive genes. Our conclusion that *gadd45β* is a SMAD4-dependent TGFβ target gene agrees with the findings of Yoo *et al.* (11) and Takekewa *et al.* (5), who have also reported SMAD4 dependence in *gadd45β* regulation, although through different experimental approaches. Conversely, our findings that TGFβ regulated *gadd45β* independently of SMAD2 contradict previous findings. Yoo *et al.* (11) recently reported that overexpression of SMAD2 and SMAD4 together, but not separately, induced a *gadd45β* reporter construct in a TGFβ-dependent fashion. Although additional work is necessary to reconcile these outcomes, they could result from cell type-specific responses to TGFβ (hepatocytes *versus* keratinocytes and fibroblasts). It is important to note, however, that SMAD2-deficient fibroblasts show *gadd45β* transcriptional induction following TGFβ with kinetics and efficacy similar to that of wild-type cells, an observation that is consistent with a SMAD2-independent model of *gadd45β* regulation (22).

In contrast to *gadd45β*, we found that SMAD2, SMAD3, and SMAD4 all contributed to TGFβ regulation of PAI1, although to varying degrees (Figs. 3–5). Extensive research on PAI1 has not implicated SMAD2 (23–25) in its regulation with the notable exception that fibroblasts derived from *smad2* knockout mice failed to induce PAI1 following TGFβ treatment (21). The ability of *smad2* siRNAs to phenocopy the Smad2 knockout fibroblasts in this respect strongly supports the use of siRNA-mediated gene silencing in future TGFβ transcriptional studies. Clearly, genome-wide analysis of TGFβ responsiveness in SMAD-silenced or SMAD knockout cells will be of great importance.

Yoo *et al.* (11) have recently shown that 220 bp of the *gadd45β* proximal promoter is activated by TGFβ and that this activation is enhanced by overexpression of SMAD2, SMAD3, and SMAD4, but not dominant negative forms of SMAD2 or SMAD3. Our data support their results in that we have also found the 5′ promoter sequence to be TGFβ-responsive (Fig. 7*B*). However, through a genomics-based alignment strategy, we identified a second TGFβ-responsive domain encompassing the highly conserved third intron of the *gadd45β* gene. In contrast to the 2-fold activation we observed with 5′ promoter sequences, the 3′ enhancer is activated 5–7-fold following TGFβ treatment. It will be important to determine which of the conserved transcription factor binding sites within this enhancer account for induction by TGFβ. Notably, we identified four conserved SMAD binding elements, three of which are located in exonic sequence (Supplementary Fig. 1). The endogenous *gadd45β* gene may likely respond to TGFβ through a concerted action of the 3′ enhancer and 5′ promoter sequences. A similar transcriptional model has been reported for the *gadd45α* gene where highly conserved sequences within the third intron or fourth exon facilitate transcriptional induction following genotoxic stress (26, 27) and vitamin D3 (28), respectively. Thus, in addition to primary sequence and genomic organization, *gadd45α*, *gadd45β*, and *gadd45γ* might also

[2] M. B. Major and D. A. Jones, unpublished results.

share an intronic/exonic enhancer as an important transcriptional regulatory element.

Last, utilizing the power of siRNA mediated gene silencing, we discovered that Mv1Lu cells made deficient for *gadd45β* arrested at the G_2/M checkpoint following TGFβ treatment. Previous research has established GADD45β as a negative regulator of cell cycle progression, and several molecular mechanisms behind this inhibition have been put forth (3). Following genotoxic stress, GADD45β acts to inhibit Cdc2/cyclin B1 kinase to induce a G_2/M cell cycle checkpoint in RKO lung carcinoma cells (8). In contrast, normal fibroblasts microinjected with a GADD45β expression vector fail to undergo a G_2/M arrest, although GADD45β was found to associate with Cdc2 in these cells (29). Our findings support these previous data in that we also see a GADD45β-dependent effect on the G_2/M cell cycle checkpoint. However, we show that GADD45β acts to promote G_2/M progression following TGFβ treatment in Mv1Lu cells (Fig. 8). This finding supports the notion that GADD45β does not act to modulate cell cycle progression in isolation, but rather the presence of other proteins might ultimately determine how cells respond to increases in GADD45β protein levels (3). Indeed, GADD45β associates with many nuclear proteins involved in cell cycle progression, including proliferating cell nuclear antigen, p21, GADD45α, and Cdc2/cyclin B2 (8, 30, 31).

Perhaps the most well understood function of the GADD45 family of proteins is their ability to regulate apoptosis through the activation of MTK1 (MEKK4) (32) and subsequently p38 kinase (5, 10). Although *gadd45β* is rapidly induced by TGFβ in Mv1Lu and HMEC, we have not detected an apoptotic response following TGFβ treatment in these cells.[2] An apoptosis-independent cellular response to GADD45β induction was recently shown, where tumor necrosis factor α signaling through NF-κB induced *gadd45β* transcription to prevent c-Jun N-terminal kinase activation and cell death (33, 34). Further, several research laboratories have been successful in generating *gadd45β* overexpression systems and have not observed cell death (35). Future studies utilizing siRNA silencing of *gadd45β* following transcriptional agonists other than TGFβ will be invaluable in determining the functional consequences of GADD45β expression.

REFERENCES

1. Massague, J. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 169–178
2. Shi, Y., and Massague, J. (2003) *Cell* **113,** 685–700
3. Liebermann, D. A., and Hoffman, B. (2002) *Leukemia* **16,** 527–541
4. Zhang, W., Bae, I., Krishnaraju, K., Azam, N., Fan, W., Smith, K., Hoffman, B., and Liebermann, D. A. (1999) *Oncogene* **18,** 4899–4907
5. Takekawa, M., Tatebayashi, K., Itoh, F., Adachi, M., Imai, K., and Saito, H. (2002) *EMBO J.* **21,** 6473–6482
6. Selvakumaran, M., Lin, H. K., Sjin, R. T., Reed, J. C., Liebermann, D. A., and Hoffman, B. (1994) *Mol. Cell. Biol.* **14,** 2352–2360
7. Abdollahi, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991) *Oncogene* **6,** 165–167
8. Vairapandi, M., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2002) *J. Cell. Physiol.* **192,** 327–338
9. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Fornace, A. J., Jr. (1994) *Mol. Cell. Biol.* **14,** 2361–2371
10. Takekawa, M., and Saito, H. (1998) *Cell* **95,** 521–530
11. Yoo, J., Ghiassi, M., Jirmanova, L., Balliet, A. G., Hoffman, B., Fornace, A. J., Jr., Liebermann, D. A., Bottinger, E. P., and Roberts, A. B. (2003) *J. Biol. Chem.* **278,** 43001–43007
12. Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) *Methods* **26,** 199–213
13. Bray, N., and Pachter, L. (2003) *Nucleic Acids Res.* **31,** 3525–3526
14. Bray, N., Dubchak, I., and Pachter, L. (2003) *Genome Res.* **13,** 97–102
15. Hosobuchi, M., and Stampfer, M. R. (1989) *In Vitro Cell Dev. Biol.* **25,** 705–713
16. Zawel, L., Yu, J., Torrance, C. J., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Zhou, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 2848–2853
17. Woodford-Richens, K. L., Rowan, A. J., Gorman, P., Halford, S., Bicknell, D. C., Wasan, H. S., Roylance, R. R., Bodmer, W. F., and Tomlinson, I. P. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9719–9723
18. Xu, G., Chakraborty, C., and Lala, P. K. (2002) *Biochem. Biophys. Res. Commun.* **294,** 1079–1086
19. Gaudet, J., and Mango, S. E. (2002) *Science* **295,** 821–825
20. Feng, X. H., Lin, X., and Derynck, R. (2000) *EMBO J.* **19,** 5178–5193
21. Piek, E., Ju, W. J., Heyer, J., Escalante-Alcalde, D., Stewart, C. L., Weinstein, M., Deng, C., Kucherlapati, R., Bottinger, E. P., and Roberts, A. B. (2001) *J. Biol. Chem.* **276,** 19945–19953
22. Yang, Y. C., Piek, E., Zavadil, J., Liang, D., Xie, D., Heyer, J., Pavlidis, P., Kucherlapati, R., Roberts, A. B., and Bottinger, E. P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10269–10274
23. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) *EMBO J.* **17,** 3091–3100
24. Song, C. Z., Siok, T. E., and Gelehrter, T. D. (1998) *J. Biol. Chem.* **273,** 29287–29290
25. Stroschein, S. L., Wang, W., and Luo, K. (1999) *J. Biol. Chem.* **274,** 9431–9441
26. Hollander, M. C., Alamo, I., Jackman, J., Wang, M. G., McBride, O. W., and Fornace, A. J., Jr. (1993) *J. Biol. Chem.* **268,** 24385–24393
27. Chin, P. L., Momand, J., and Pfeifer, G. P. (1997) *Oncogene* **15,** 87–99
28. Jiang, F., Li, P., Fornace, A. J., Jr., Nicosia, S. V., and Bai, W. (2003) *J. Biol. Chem.* **278,** 48030–48040
29. Yang, Q., Manicone, A., Coursen, J. D., Linke, S. P., Nagashima, M., Forgues, M., and Wang, X. W. (2000) *J. Biol. Chem.* **275,** 36892–36898
30. Kovalsky, O., Lung, F. D., Roller, P. P., and Fornace, A. J., Jr. (2001) *J. Biol. Chem.* **276,** 39330–39339
31. Vairapandi, M., Azam, N., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2000) *J. Biol. Chem.* **275,** 16810–16819
32. Mita, H., Tsutsui, J., Takekawa, M., Witten, E. A., and Saito, H. (2002) *Mol. Cell. Biol.* **22,** 4544–4555
33. Jin, R., De Smaele, E., Zazzeroni, F., Nguyen, D. U., Papa, S., Jones, J., Cox, C., Gelinas, C., and Franzoso, G. (2002) *DNA Cell Biol.* **21,** 491–503
34. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) *Nature* **414,** 308–313
35. Yang, J., Zhu, H., Murphy, T. L., Ouyang, W., and Murphy, K. M. (2001) *Nat. Immunol.* **2,** 157–164
36. Karpf, A. R., Peterson, P. W., Rawlins, J. T., Dalley, B. K., Yang, Q., Albertsen, H., and Jones, D. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14007–14012
37. Miller, G. S., and Fuchs, R. (1997) *Comput. Appl. Biosci.* **13,** 81–87