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Links between Tumor Suppressors: p53 Is Required for TGF-β Gene Responses by Cooperating with Smads

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Summary

The p53 tumor suppressor belongs to a family of proteins that sense multiple cellular inputs to regulate cell proliferation, apoptosis, and differentiation. Whether and how these functions of p53 intersect with the activity of extracellular growth factors is not understood. Here, we report that key cellular responses to TGF-β signals rely on p53 family members. During *Xenopus* embryonic development, p53 promotes the activation of multiple TGF-β target genes. Moreover, mesoderm differentiation is inhibited in p53-depleted embryos. In mammalian cells, the full transcriptional activation of the CDK inhibitor *p21*^{WAF1} by TGF-β requires p53. p53-deficient cells display an impaired cytostatic response to TGF-β signals. Smad and p53 protein complexes converge on separate *cis* binding elements on a target promoter and synergistically activate TGF-β induced transcription. p53 can physically interact in vivo with Smad2 in a TGF-β-dependent fashion. The results unveil a previously unrecognized link between two primary tumor suppressor pathways in vertebrates.

Introduction

Members of the TGF-β growth factor family are prominent signals regulating cellular fates in a variety of physiological contexts, from embryonic development to adult tissue homeostasis (Massagué, 2000). The loss of this control leads to aberrant cell behaviors contributing to the development of cancer and inborn defects (Wakefield and Roberts, 2002).

In recent years, tremendous progress has been made in the elucidation of how cells sense and transduce TGF-β signals. TGF-β ligands bind to cognate serine/threonine kinase receptors leading, intracellularly, to phosphorylation and activation of the Smad family of signal transducers. Two different Smad signaling branches have been described: TGF-β-like signals, including TGF-βs, Activin, and Nodal, are transduced by Smad2 or Smad3, whereas BMPs are transduced by Smad1 (Massagué, 2000).

Once activated, the Smads translocate into the nucleus where they control gene expression in association with Smad4 and partner transcriptional regulators (Massagué, 2000). How the Smads recognize and properly activate a specific promoter is not fully understood. In part, specificity depends on the differential expression of distinct Smad partners in distinct cell types; however, several inputs can profoundly modify both the perception of the TGF-β signal and its biological output (Massagué, 2000). Understanding how this transcriptional plasticity is attained is central for embryonic development and cancer. For example, most carcinomas have selectively lost the growth arrest response and gained metastatic abilities in response to TGF-β (Wakefield and Roberts, 2002). Importantly, this change can occur without acquiring genetic defects in known components of the TGF-β pathway, indicating that alterations in other regulatory molecules can have a profound influence on the cellular responsivenss to TGF-β. Some of these regulators appear to act in parallel to the Smad signal transduction cascade, namely converging at the level of target gene expression (Lehmann et al., 2000).

To further understand the molecular mechanisms that control of TGF-β gene responses, we performed an unbiased screen for TGF-β modulators. Here, we report the unexpected identification of p53 as an in vivo relevant partner of Smad2 in the activation of multiple TGF-β target genes. p53 is a key tumor suppressor in mammals as it is mutated, or inactive, in the majority of human tumors (Vogelstein et al., 2000). p53 belongs to a family of proteins, including p63 and p73, that have evolved pleiotropic—and perhaps overlapping—cellular functions (Yang and McKeon, 2000).

We find that several TGF-β target genes are under joint control of p53 and Smads. p53 binds to Smads in vivo and strongly cooperates transcriptionally with the activated Smad complex. In *Xenopus* embryos and human cells, TGF-β requires the assistance of p53 to mediate the activation of key TGF-β target genes and to carry out some of its biological functions. Using the *Mix.2* promoter as a paradigm, we find that p53 adjusts TGF-β-induced transcription by interacting directly with a cognate binding site on promoter DNA. However, different from other Smad partners, this p53 binding element is located in a separate position from the Activin/TGF-β responsive element. We argue that these findings unveil a convergence of the p53 and the Smad signaling networks to regulate development and tissue homeostasis.

Results

Cloning of an Alternatively Spliced Isoform of p53 (p53AS) in a Screen for Activators of TGF-β Signaling

To identify molecules that modulate TGF-β/Activin/Nodal signaling during development, we performed an

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unbiased functional screen for genes whose expression promoted the differentiation of embryonic cells into endoderm and mesoderm, as this is the hallmark of TGF-β signaling in early vertebrate embryos (Whitman, 2001). We generated a mouse gastrula (embryonic day [E]6.5) cDNA library constructed in an RNA expression plasmid. Synthetic mRNA was prepared from pools of 100 bacterial colonies and injected into the animal hemisphere of 2-cell *Xenopus* embryos. At the blastula stage, the ectoderm was explanted and cultivated until siblings reached the gastrula stage. The injected animal caps were then assayed by RT-PCR to identify pools able to activate the expression of *Mixer* (endoderm) and *Xbra* (mesoderm). Of five positive pools, two of the active cDNAs isolated after sib selection corresponded to *Smad2* (Baker and Harland, 1996) and, unexpectedly, three corresponded to *p53AS*, a natural variant of p53 generated by alternative splicing at the C terminus (Wolkowicz et al., 1995). p53AS shares with commonly spliced p53 (p53R) the N-terminal transactivation domain, the central DNA binding and oligomerization domains, but lacks the most C-terminal 26 amino acids of p53R.

A wealth of data indicates that the TGF-β and p53 signaling networks operate independently as powerful tumor suppressors in mammalian cells; yet, the cloning of a p53 isoform in a TGF-β screen unveiled the possibility of a previously unrecognized partnership between these two types of molecules. We initially addressed this issue by characterizing the p53AS effects in more detail. Different doses of *p53AS* mRNA were injected in the animal pole of each blastomere at the 2-cell stage and tested for the induction of several tissue-specific markers in animal cap cells explanted from these embryos (Figure 1A). At lower doses of injected mRNA, *p53AS* induced first mesodermal (*Xbra*, *Eomes*) and then mesendodermal (*VegT*, *Mix.2*) genes; at a higher concentration, mainly endodermal markers were turned on (*Sox17*β, *Mixer*, *Xnr6*) (Figure 1A, lanes 1–5). This pattern of gene expression is typical of the ectopic activation of TGF-β/Activin/Nodal/Smad2 signaling in animal caps (Figure 1A, lanes 6 and 7) (Harland and Gerhart, 1997). However, other genes activated by Activin/Smad2 such as *goosecoid*, *XWnt8*, and *Xnr-1* were not induced in *p53AS*-injected cells (Figure 1A, bottom). This suggests that p53AS specifically activates a subset of TGF-β target genes.

We tested the biological activity of *p53* mRNA in the context of the whole embryo. When microinjected into a single ventral blastomere at the 4-cell stage, *p53AS* mRNA induced the formation of ectopic trunk-tail structures (n = 155, 77%), phenocopying the biological effects triggered by low doses of *Smad2* mRNA (Figure 1B) (Baker and Harland, 1996). Histological analysis showed that these secondary structures contained muscle, neural tissue, and in several cases an ectopic gut, but all lacked notochord (data not shown).

We conclude from these experiments that ectopic expression of p53 stimulates multiple gene responses and long-term phenotypic effects typically mediated by activation of the TGF-β signaling cascade in embryonic cells (Harland and Gerhart, 1997).

Alternative spliced p53AS represents up to 30% of total p53 in rodent cells but, curiously, a similar C terminally modified p53 isoform has not been described in human or frog cells that express regular p53 (p53R) (Wolkowicz et al., 1995). *Xenopus* and mammalian p53 proteins share similar functional properties and regulatory mechanisms (Cox et al., 1994). To assay for functional conservation, we compared the ability of mouse p53AS, human p53R, and *Xenopus* p53 (Xp53) to induce mesoendoderm differentiation in animal cap assays. Figure 1C shows that the inducing activities of *mp53AS* and *Xp53* are similar. *hp53R* can also stimulate expression of the same marker genes but at 5- to 10-fold lower efficiency than *mp53AS*, perhaps revealing a partial inhibitory role for p53 C terminus in these activation processes. Of note, injection of higher doses (above 400 pg for all mRNAs) was detrimental for survival (data not shown).

p53 may stimulate TGF-β gene responses acting in partnership with endogenous Smads or, alternatively, operating in an independent pathway. To discriminate between these two possibilities, we tested whether a blockade of Smad function had an effect on p53-mediated gene expression. As shown in Figure 1D, coinjection of *p53AS* and *dominant-negative Smad2* (Candia et al., 1997) mRNAs downregulated all the TGF-β-like inductions triggered by *p53AS*. We conclude that expression of p53 activates the transcription of TGF-β target genes in a Smad-dependent fashion.

To explore the possibility that p53 and Smad may jointly control the TGF-β output, we tested whether raising the levels of p53 may correspond to an enhanced responsiveness to TGF-β. Figure 1E shows that in animal caps explants, coinjection of suboptimal levels of *activin* and *p53AS* mRNAs cooperated in the induction of endodermal and mesodermal markers, whereas each component alone was weak or inactive. In contrast, the BMP4 target *Vent-1* was neither induced by *p53AS* alone nor in combination with BMP4 (see Supplemental Figure S1A online at http://www.cell.com/cgi/content/full/113/3/301/DC1).

We further tested whether p53-mediated effects are direct by assaying the biological activities of p53 and Smad2 in the presence of cycloheximide, a protein synthesis inhibitor. Transcription of *Xbra*, *Mix.2*, and *Eomes* is initiated as immediate response to Activin/Smad2 stimulation in *Xenopus* animal caps (Harland and Gerhart, 1997) and, as shown in Supplemental Figure S1B, p53 directly promotes transcription of the same genes in the absence of de novo protein synthesis. In keeping with this notion, injection of p53 alleles bearing inactivating mutations in the DNA binding (R273H) or transactivation domain (22-23) failed to induce any mesoendodermal marker (Figure 1F). This suggests that p53 relies entirely on its properties as sequence-specific transcription factor in these inductive events.

p53 is biochemically a latent transcriptional regulator that becomes active in response to a variety of stimuli (Vogelstein et al., 2000). Little is known on the activation status of Xp53 in early embryos. This can be visualized by monitoring p53-dependent transcription. To this end, we injected at the 1-cell stage a luciferase reporter for p53 signaling whose transcription is driven by multimerized p53 binding elements (PG13) (Kern et al., 1992). We compared PG13 transcription with MG13, in which the p53 binding elements are disrupted. Intriguingly, we

Figure 1. p53 Promotes Mesoderm and Endoderm Formation in *Xenopus* Embryos

(A) RT-PCR for mesoderm (Mes) and endoderm (Endo) markers activated in animal caps expressing the indicated mRNAs. Lane 1: *lacZ* mRNA (200 pg). Lanes 2–5: *p53AS* mRNA (3, 10, 30, 150 pg, respectively). Lane 6: *activin* mRNA (30 pg). Lane 7: *Smad2* mRNA (200 pg). Lane 8: *eFGF* (50 pg). Lanes 9 and 10: whole embryo total RNA without (−RT) and with reverse transcriptase.

(B) Injection of *p53AS* mRNA (50 pg; n = 155, 77%) mimicks the long-term effects of *Smad2* mRNA overexpression (100 pg; n = 12, 100%). Side view of stage 28 embryos. Dotted lines indicate the induced secondary trunk-tail structures.

(C) RT-PCR of animal caps expressing the following mRNAs: *hp53R* (20 pg and 200 pg), *mp53AS* (20 pg), and *Xp53* (30 pg).

(D) TGF-β-like activities promoted by *p53AS* mRNA (50 pg) are inhibited by *dominant-negative Smad2* (*DNS2*: 1 ng) mRNA.

(E) Lane 2: inductions by *activin* mRNA at a high dose (35 pg) but lower amounts of *p53AS* or *activin* mRNA (1 pg, each) were effective only in combination (Lanes 3–5). Lanes 6–8, synergism of *p53AS* with *activin*, both used at 5 pg of mRNA.

(F) RT-PCR on animal caps expressing the indicated mRNAs (200 pg each). Lane 2: *hp53* (WT). Lane 3: p53 L22E-W23S bears an inactive transactivation domain. Lane 4: p53 R273H is unable to bind DNA.

(G) Detection of p53 transcriptional activity in early embryos. *PG13-lux* and *MG13-lux* (40 pg per embryo) were injected at the 2-cell stage in each blastomere and luciferase activity was measured on extracts from gastrulae (n = 40 each).

found that *Xenopus* embryos have considerable endogenous p53 transcriptional activity (Figure 1G).

p53 Is Required for TGF-β/Activin/Nodal-Mediated Gene Responses in *Xenopus* Embryos

We sought to establish to what extent p53 is required for TGF-β/Activin/Nodal signaling in vivo. To this end, we reduced the endogenous p53 protein level with an anti-*p53* morpholino oligonucleotide (p53 MO) covering the initial codons of *Xp53* mRNA. As specificity control, we used a mutant morpholino oligonucleotide (control MO). p53 MO specifically blocked *Xp53* mRNA translation initiation in vivo, leading to effective knockdown of the endogenous levels of p53 protein, without affecting protein levels for β-catenin (Figure 2A) or actin (data not shown).

We examined the effect of p53 knockdown on mesoendoderm differentiation mediated by Activin protein in animal caps (Figure 2B). p53 MO and control MO were injected into the animal hemispheres at the 2-cell stage, animal caps were removed at blastula and treated in Activin-containing medium (50 pM). As shown in Figure 2B, Activin-mediated inductions of mesodermal (*Xbra* and *Eomes*) or endodermal (*Mix.2* and *Mixer*) markers were inhibited in p53-depleted caps (Figure 2B, compare lanes 3 and 4). Three evidences indicate that this interference is specific. First, the control MO has no effect on Activin signaling. Second, normal Activin-mediated gene responses can be restored by coinjec-

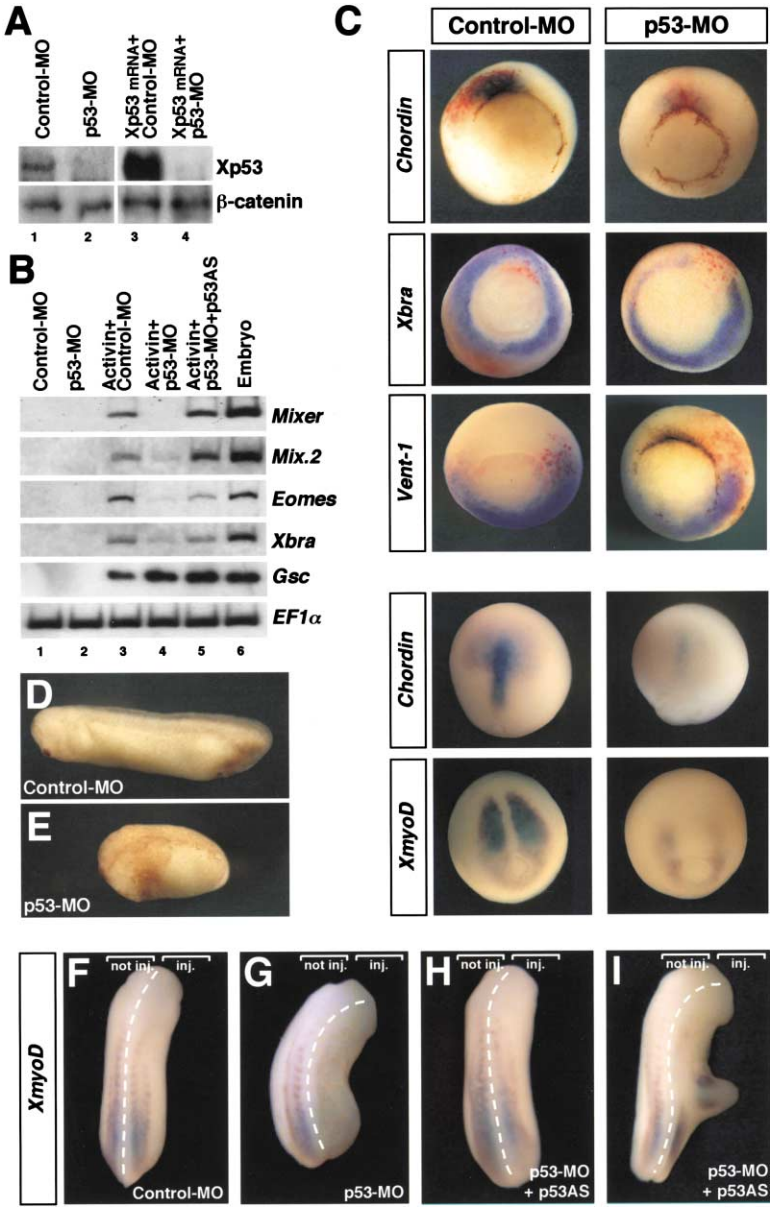

Figure 2. In Vivo Requirement of p53 in TGF-β Gene Responses

(A) p53 morpholino oligonucleotides (MO, 20 ng) specifically knockdown the translation of both endogenous (left) and overexpressed (right) *Xp53* mRNA (100 pg) in animal halves explanted at gastrula stage. β-catenin protein serves as specificity control.

(B) Animal caps, injected with the indicated MO, were explanted and treated with Activin protein (50 pM). Lane 4: p53 MO selectively blocks Activin inductions of *Mixer*, *Mix.2*, *Eomes*, and *Xbra*, but not of *goosecoid* (*Gsc*). Lane 5: *mp53AS* mRNA injection rescues Activin inductions in p53 MO injected animal caps.

(C) Whole-mount in situ hybridizations on control- and p53 morphant embryos showing severe phenotypes. Control MO or p53 MO (10 ng each), together with *lacZ* mRNA (200 pg), were injected at the 4-cell stage in a single blastomere. Embryos shown in the upper panels were collected at gastrula, stained for β-gal activity (red staining), and processed for in situ hybridization. Embryos shown in the lower panels were radially injected (20 ng per embryo) and collected at stage 13. Note that upon p53 knockdown, staining for *Chordin*, *Xbra*, and *XmyoD*, but not for *Vent-1*, is reduced.

(D and E) Phenotypes of p53-depleted embryos. Embryos were injected as in (C) and cultured until siblings reached the tailbud stage.

(F–I) Embryos were injected on the right side at the 2-cell stage with 15 ng of control MO (F) or p53 MO either alone (G) or in combination with *mp53AS* mRNA ([H], 30 pg; [I], 100 pg). Embryos were collected at stage 26 and processed for in situ hybridization with the *XmyoD* probe.

tion of mouse *p53AS*, whose translation is not antagonized by the p53 MO (Figure 2B, lane 5). Third, Activin-mediated induction of *goosecoid* is p53 independent (Figure 2B, lane 4). Collectively, these findings indicate a critical role of p53 for the activation of a subset of TGF-β/Activin targets.

We next asked whether p53 function was also required in the whole embryo. Several findings have demonstrated that Nodals and Derriere, a group of TGF-β-related ligands, induce and pattern the mesoderm in vertebrates (Whitman, 2001; Sun et al., 1999). If p53 plays a role in endogenous TGF-β signaling, p53 knockdown should attenuate these inductions in embryos. We injected p53 MO in a single dorsal or ventral blastomere at the 4-cell stage and assayed the resulting phenotype using molecular markers by in situ hybridization. As shown in Figure 2C, p53 MO, but not control MO, attenuated the expression of the organizer marker *Chordin* and of the pan-mesodermal marker *Xbra* in early gastrulae; in contrast, expression of *Vent-1* was not affected (see closer images in Supplemental Figure S2 online at http://www.cell.com/cgi/content/full/113/3/301/DC1). In embryos injected in all blastomeres with p53 MO, the development of dorsal mesoderm derivatives was assayed molecularly using probes for *Chordin* and *XmyoD*, whose expression at the neurula stage marks axial mesoderm and prospective skeletal muscle, respectively. As shown in Figure 2C, p53 MO, but not control MO, severely downregulated *Chordin* and *XmyoD* expression. In monolateral injection experiments (right side in Figures 2F–2I), coinjection of p53 MO with increasing levels of *p53AS* mRNA rescues this phenotype. At the tailbud stage, major developmental changes occurred in p53-depleted embryos compared to control MO injected embryos (Figures 2D and 2E). Part of the p53 morphants (23%) failed to gastrulate properly due

to defective blastopore formation. Among the p53 morphants that survived after gastrulation and neurulation, several (n = 76, 54%) lacked tail structures and developed a reduced trunk, indicative of defective mesoderm formation. These phenotypes recapitulate aspects of embryonic TGF-β deficiencies, such as embryos injected with low doses of *Cerberus-short*, a secreted antagonist of Nodal, or with an inhibitory construct for *derriere*, both leading to defective trunk development (Piccolo et al., 1999; Sun et al., 1999).

Together, these loss-of-function experiments suggest that depletion of p53 leads to impaired edogenous TGF-β/Activin/Nodal gene responses resulting in defective embryonic development.

p53 Is Required for Full TGF-β Gene Responses and TGF-β-Mediated Growth Arrest in Mammalian Cells

Having established the biological activities of p53 in the frog embryo, we wished to determine to what extent p53 is required for TGF-β-mediated gene responses in human cells. We monitored the expression of a group of genes playing key roles in different aspects of the cellular response to TGF-β signaling in mammals, such as *p21^{WAF1}*, *plasminogen activator inhibitor-1* (*PAI-1*), *matrix metalloprotease-2* (*MMP2*), and *TGF-β inducible early gene* (*TIEG*). p21^{WAF1} is a Cyclin-dependent kinase (CDK) inhibitor and a central mediator of the cellular growth arrest program (Deng et al., 1995). PAI-1 and MMP2 are secreted proteins required for extracellular matrix remodeling and epithelial-mesenchymal transformation, whereas TIEG is thought to be important in TGF-β-mediated apoptosis.

We analyzed the relevance of p53 in the activation of these TGF-β targets. For this purpose, we reduced the endogenous levels of p53 using the small interfering RNA (siRNA) technique in the HepG2 model system (Figure 3A), as this hepatoma cell line is highly TGF-β/Activin responsive and expresses wild-type p53, but not p63 or p73. Figure 3B shows RT-PCR analyses in which all the markers were clearly upregulated in HepG2 cells as an early response to Activin treatment. In the presence of anti-p53 siRNA, these inductions were reduced for *p21^{WAF1}*, *MMP2*, and *PAI-1*. Notably, induction of *TIEG* was p53 independent, implicating that p53 knockdown has no effect, per se, on the overall TGF-β responsiveness. This further suggests that knockdown of p53 affects a significant proportion, but not all the TGF-β target genes, as previously noted in the TGF-β differentiation program of the frog embryo.

The CDK inhibitor p21^{WAF1} is a critical determinant of growth arrest in response to a variety of stimuli; these activate p21^{WAF1} expression p53 dependently, such as DNA damage, or p53 independently, such as terminal differentiation and senescence (Macleod et al., 1995). As our data argued that Activin/TGF-β signaling and p53 elevate in concert *p21^{WAF1}* expression in HepG2 cells, we examined in more detail the effect of p53 depletion on the induction of p21^{WAF1} by Activin at the protein level. Mock and anti-p53 siRNA transfected HepG2 cells were stimulated for different times with Activin and then analyzed by Western blotting. As shown in Figure 3C, the elevation of *p21^{WAF1}* as response to Activin requires p53 activity, although a weaker, p53-independent *p21^{WAF1}* induction can be observed after hours of Activin signaling.

Of note, activation of *p21^{WAF1}* by TGF-β has been shown to be p53 independent in some tumorigenic or immortalized epithelial cell types (Datto et al., 1995). This indicates, perhaps not surprisingly, that different genetic programs may be at work in distinct cells (Massagué, 2000); additionally, it also leaves open the possibility that p63 of p73 may be able to compensate for p53 loss-of-function in some contexts. HaCaT cells are a point in case, as this cell line of immortalized keratinocytes is p53 mutant, but highly TGF-β responsive (Datto et al., 1995). However, these cells express high levels of *p63* (Hall et al., 2000), whose reduction by anti-p63 siRNA led to a concomitant reduction in of *p21^{WAF1}* inducibility by TGF-β1 (see Supplemental Figure S3 online at http://www.cell.com/cgi/content/full/113/3/301/DC1). These results suggest that p53 and p63 may have partially overlapping roles in modulating the expression of at least one key gene in the TGF-β cytostatic program in keratinocytes.

Given the gene expression changes observed in the response to Activin after ablation of p53 in HepG2 cells, we assayed for their biological importance in the context of the TGF-β cytostatic program. For this purpose, bromodeoxyuridine (BrdU) incorporation was analyzed as an indication of DNA synthesis and S phase entry of control and p53-depleted cells (Figure 3D). As expected, Activin inhibited cell-cycle progression of control HepG2 cells. Strikingly however, Activin had limited effect on the BrdU incorporation after siRNA-mediated ablation of p53, indicating that p53 knockdown is sufficient to overcome the growth arrest imposed by TGF-β signaling.

Lack of sensitivity to TGF-β growth-suppressing effects is a landmark of most cancers. Given that a high proportion of human cancers carry mutations in p53, our data at least suggest that inactivation of p53 may be one of the possible mechanisms for the selective loss of TGF-β tumor-suppressing effects in cancer cells. We explored this hypothesis trying to restore the antiproliferative effects of TGF-β in cancer cell lines lacking p53. SAOS-2 is a *p53* null osteosarcoma cell line, not expressing p63 or p73, that is insensitive to growth arrest mediated by TGF-β treatment or overexpressed Smad2 (Figure 3E, lane 2, and data not shown) (Prunier et al., 2001). Strikingly, however, coexpression of Smad2 and low amounts of p53AS, by themselves unable to trigger any effect, resulted in a marked inhibition of cell growth as measured by BrdU incorporation (compare lane 5 and 6). Thus, reintroduction of p53 activity in p53-deficient cancer cells may restore part of the TGF-β control over the cell cycle.

p53 Is Required for TGF-β1-Mediated Growth Arrest in Mouse Embryonic Fibroblasts and Hematopoietic Progenitors

Since transient siRNA ablation of p53 had a strong impact on the TGF-β response of HepG2 cells, we next asked whether the genetic inactivation of p53 was also relevant for some biological responses to TGF-β in normal cell types. One complicating issue in the interpreta-

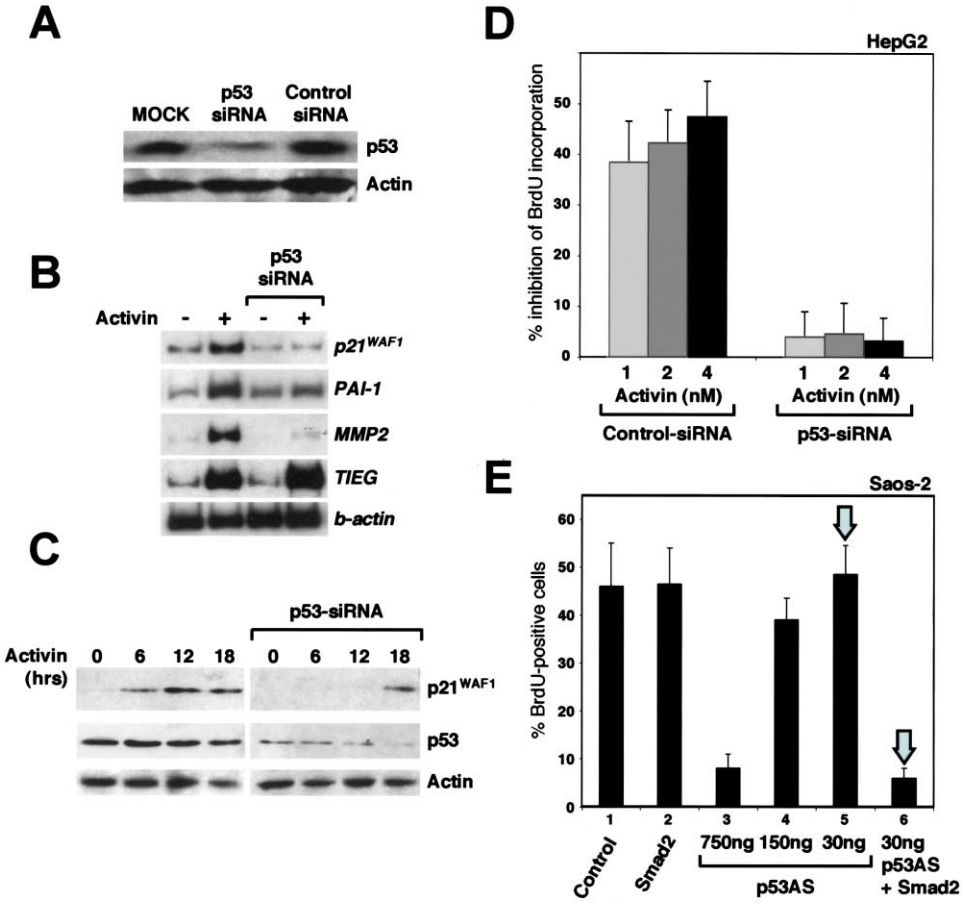

Figure 3. p53 Activity Is Required for Full TGF-β Responsiveness in Human Cells

(A) Effectiveness of siRNA-mediated depletion of p53 in HepG2 cells controlled by Western blotting.

(B) Control and p53-depleted HepG2 cells were treated with Activin (1 nM) for 2 hr and subjected to RT-PCR analyses for endogenous targets of Activin/TGF-β.

(C) Time course of $p21^{WAF1}$ induction by Activin (1 nM) in mock and p53-depleted HepG2 cells.

(D) p53 is required for Activin-mediated growth inhibition in HepG2 cells treated with increasing doses of Activin for 24 hr. Columns show the effects of Activin expressed as percentage of inhibition of BrdU incorporation relative to unstimulated cultures.

(E) p53 rescues Smad2 growth suppressing activity in *p53* null SAOS-2 cells. Cells were transfected with the indicated plasmids and then plated onto coverslips for BrdU incorporation assay. Transfection of *p53AS* expression vector at high doses (lane 3, 750 ng) blocked cell growth, but less than 10-fold lower doses of *p53AS* had no effect (lane 4, 150 ng; lane 5, 30 ng). Cotransfection of of *p53AS* and *Smad2* (lane 6, 30 ng and 1 μg, respectively) inhibited BrdU incorporation (up to 87% inhibition). Arrows indicate the comparable amounts of p53AS.

tion of genetic analyses is that the three p53 family members are coexpressed in most tissues in vivo (Yang and McKeon, 2000). Nonetheless, recent data by Flores et al. (2002) unveiled that induction of $p21^{WAF1}$ by DNA damage relies on p53 and is independent from p63 and p73 in mouse embryonic fibroblasts (MEFs). Thus, p53 may be the main member of its family regulating p53-dependent growth arrest in this cell type. This simplifying finding provided us with a window of opportunity to test a genetic requirement of p53 for TGF-β mediated growth suppression. Primary wild-type and $p53^{-/-}$ MEFs were seeded at low density and treated with different doses of TGF-β1; the distribution of the cell population in the G1 and S phases of the cell cycle was analyzed by flow cytometry after TGF-β1 treatment and the percentage of cells in active DNA synthesis was assayed by BrdU incorporation. As shown in Figures 4A–4D, wild-type MEFs were efficiently growth arrested by TGF-β1, as judged by the decreased number of cells in S phase, the concomitant increase in the G1 phase and blockade of BrdU incorporation. In contrast, $p53^{-/-}$ MEFs were largerly insensitive to TGF-β1 antiproliferative properties. As a control, we verified that the TGF-β signaling cascade itself was effective in p53-deficient cells. For this purpose, we monitored the TGF-β1-dependent transcription of two synthetic reporters for Smad activation, *ARE3-lux* and $CAGA_{12}$-*lux* (Dennler et al., 1998; Huang et al., 1995) and found that their inducibility was indistinguishable in wild-type and mutant cells (Figure 4E). We conclude that p53 is a significant player in the antiproliferative effects mediated by TGF-β in embryonic fibroblasts.

We next aimed to extend these findings to an additional experimental system reflecting a role of TGF-β in vivo. TGF-β signaling has been shown to restrain the proliferative potential of hematopoietic progenitors

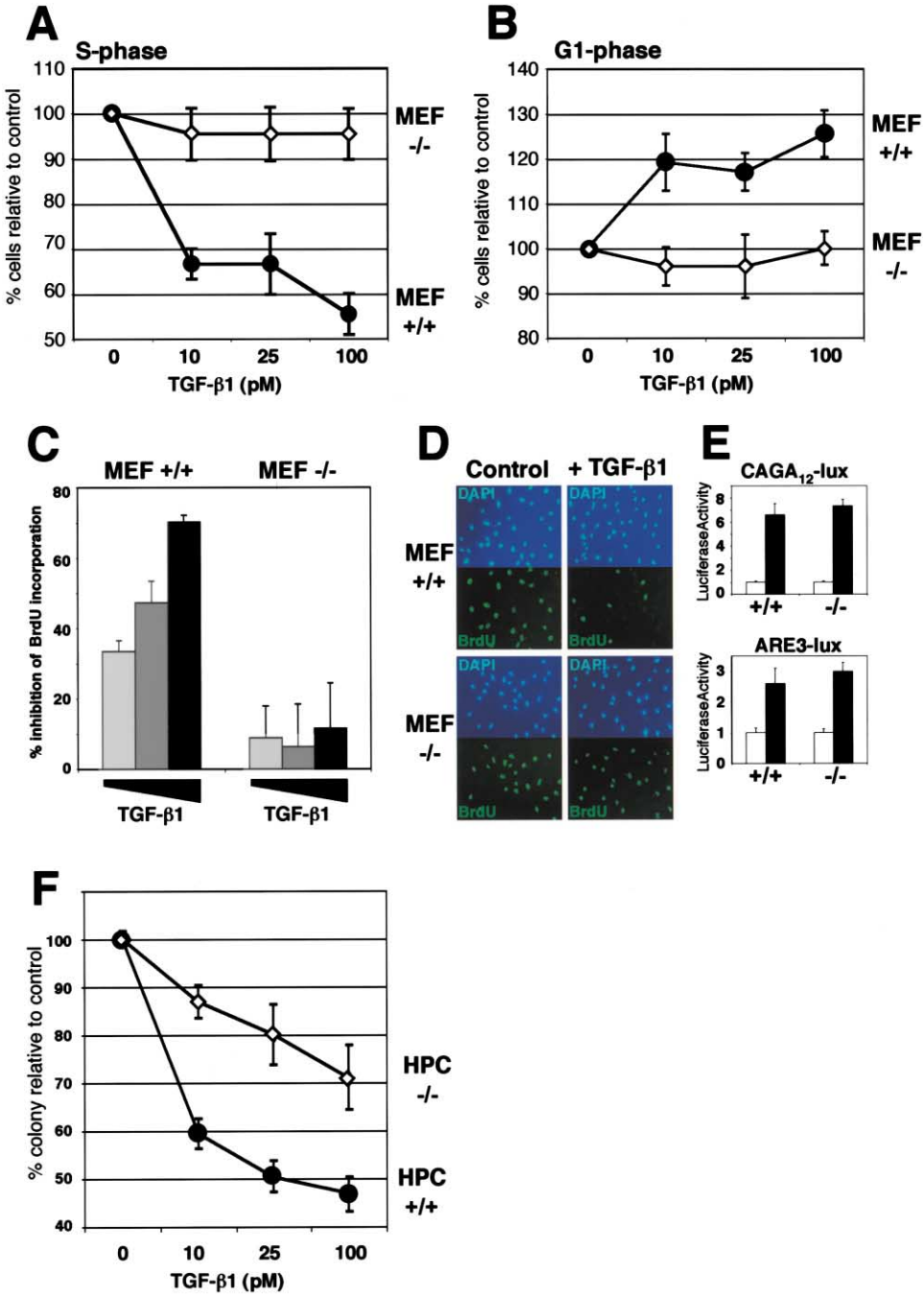

Figure 4. Growth Arrest Induced by TGF-β1 Requires p53 in Mammalian Cells

(A and B) Cell cycle analysis by flow cytometry of wild-type (+/+) and *p53* knockout (−/−) MEFs treated with increasing doses of TGF-β1. The number of cells in each phase for unstimulated cultures was given an arbitrary value of 100%, and all other values are depicted relative to this. $p53^{-/-}$ MEFs are insensitive to TGF-β1 stimulation.

(C and D) Effects of TGF-β1 on +/+ and −/− MEFs proliferation as measured by BrdU incorporation. (C) Columns show the inhibitory effects of increasing doses of TGF-β1 (10, 25, and 100 pM) relative to unstimulated cultures. (D) Pictures show representative fields of cells.

(E) The Smad/TGF-β pathway is functional in $p53^{-/-}$ MEFs. +/+ and −/− MEFs were transfected with synthetic TGF-β luciferase reporters $CAGA_{12}$-*lux* and *ARE3-lux* (200 ng each). After transfection, cells were left unstimulated (white bars) or stimulated with 75 pM TGF-β1 (black bars).

(F) Reduced sensitivity of $p53^{-/-}$ hematopoietic progenitor cells (HPC) to TGF-β1 growth inhibition. Data are mean ± SD from two to four replicates.

(HPC) (Fortunel et al., 2000). Thus, we asked whether p53 is modulating this process. Bone marrow progenitors from wild-type and $p53^{-/-}$ mice were purified and plated in semisolid stem-cell media containing different doses of TGF-β1 and assayed for colony formation. By comparing the dose-response curves (Figure 4F), TGF-

β1 reduced the number of HPC colonies from wild-type animals, but it was less effective toward *p53* null cultures. These data parallel with a similar requirement of Smad5 in the inhibition of hematopoietic stem cells by TGF-β1 (Liu et al., 2003).

We conclude from these experiments that p53 family members and TGF-β/Smad jointly control cell growth, at least in specific cellular contexts.

p53 Family Members Cooperate with TGF-β-Induced Transcription in Human Cells but Require Promoter Sequences Separate from Smad-Responsive Element

We showed above that p53 modulates TGF-β-induced transcription of only a subset, albeit large, of TGF-β target genes. How can p53 operate in such gene-specific manner? To answer this question we investigated the molecular mechanism by which p53 family members impinge on TGF-β-induced transcription. For this purpose, we investigated the regulation of the *Mix.2* promoter because this is a paradigm for Smad-mediated transcription and an example of gene transcription under joint-control of Activin/TGF-β signaling and of p53 (Figures 1E and 2B). Smads are recruited to the *Mix.2* promoter by FAST-1, whose expression is an obligatory requirement for activation of the natural *Mix.2* promoter by TGF-β signals (Chen et al., 1996). As shown in Figure 5A, transfection of *p53AS* led to a discrete increase of the basal level of *Mix.2* promoter transcription (Figure 5A, lanes 1 and 2). This activation was insensitive either to *FAST-1* expression (Figure 5A, lanes 3 and 4) or to stimulation by TGF-β signaling alone (Figure 5A, lanes 5 and 6). More importantly, however, in the presence of both *FAST-1 and* TGF-β signaling, *p53AS* promoted a robust increase in the absolute level of activation as well as in the fold induction of the promoter by TGF-β (Figure 5A, lane 8).

Ablation of p53 by siRNA in HepG2 cells reduced inducibility by TGF-β, but had no effect on the unstimulated promoter basal state (Figure 5B). Similar results were obtained with the promoter of *PAI-1*, another gene under dual control of p53 and TGF-β in mammalian cells (see Supplemental Figure S4 online at http://www.cell.com/cgi/content/full/113/3/301/DC1). We conclude from these experiments that p53 enhances Smad-dependent transcription in vivo.

Mechanistically, p53 may act at any level upstream of the Smad pathway (for example, inhibiting Smad degradation); if so, its partnership with TGF-β should be observed not only on the natural promoter fragments used so far, but also on synthetic reporters of Smad activity. Importantly, as shown in Figure 5C, we found no transcriptional cooperation between TGF-β and *p53AS* on *ARE3-lux*, a synthetic sensor of Activin/TGF-β signaling derived from multimerization of the *activin responsive element* (*ARE*) of the *Mix.2* promoter (Huang et al., 1995). We conclude from these experiments that the partnership between TGF-β signaling and p53 is specific and must operate through separate enhancer elements; thus, p53 appears as an independent input in TGF-β-mediated transcription, rather that a general component of the activated Smad complex.

As p53 regulates TGF-β gene responses, we verified whether the converse was true, that is whether p53 may be activated by the TGF-β cascade. We exclude that this possibility may be generally applicable, as TGF-β stimulation did not activate neither the basal nor the p53 activated transcriptional response of a p53 sensor (PG13-lux, Figure 5D).

A parallel set of experiments to those described for p53AS were carried out to test how overexpression of p53R, p63, and p73 can modulate *Mix.2* promoter transactivation. p63 and p73 are expressed in several isoforms either containing or lacking an N-terminal transactivation domain similar to p53 (TA and ΔN, respectively); other isoforms are generated by alternative splicing at the C terminus (α, β, and γ). The table in Figure 5E summarizes the effect of several p53-related molecules on the inducibility of the *Mix.2* promoter by TGF-β signaling. In agreement with the *Xenopus* animal caps data, p53R was slightly less efficient that p53AS in cooperating with TGF-β-induced transcription. Interestingly, all the TA-p63 and ΔN-p63 isoforms, as well as TA-p73α, were capable of cooperating with the Smad-dependent activation of the promoter, albeit with significant differences. As judged by Western blotting, these functional disparities did not reflect variations in protein expression (data not shown). Thus, p53 family members can cooperate efficiently with TGF-β in mediating Smad-dependent transcriptional responses.

A p53 Responsive Element on the *Mix.2* Promoter Is Required for Endogenous Expression In Vivo and Full TGF-β Responsiveness

Next, we attempted to identify the regulatory regions conferring p53 responsiveness to the *Mix.2* promoter. We found a putative p53 binding consensus (*p53BE*) located between bases −88 and −69 relative to the transcription start site, in a separate position from the *ARE* (−215, −166) (Figure 6A). To test whether *p53BE* can recruit endogenous p53, we carried out DNA affinity precipitation experiments (DNAP) with a *p53BE* double-stranded biotinylated oligonucleotide using nuclear extracts from control and Activin-treated HepG2 cells. Endogenous p53 bound to *p53BE*, but not to a mutant *p53BE* probe, irrespectively from Activin stimulation (Figure 6B). In gel shift assays, recombinant purified GST-p53AS, p53R, and p73 specifically bound the *Mix.2 p53BE* (Figure 6C), indicating the direct contact of p53 with its consensus site.

To investigate whether the interaction of p53 on its target DNA is instrumental for cooperation with TGF-β in *Mix.2* transactivation, we generated a mutant luciferase reporter construct in which the p53 site was deleted (Δ*p53*). In HepG2 cells, deletion of *p53BE* completely blocked the TGF-β/p53 transcriptional synergism (Figure 6D).

We then examined the relative importance of the Activin- and p53 responsive elements in the transcriptional response to Activin in animal cap assays. Two constructs were generated bearing point mutations in the p53 consensus (*M-p53BE*) and in the FAST-1 binding site (*M-ARE*). In animal cap assays, *M-p53BE* was activated less efficiently than wild-type *Mix.2* at all concentrations of *activin* mRNA tested (Figure 6E). As expected *M-ARE* showed no activity (Huang et al., 1995). Thus, binding of endogenous Xp53 to its cognate site is re-

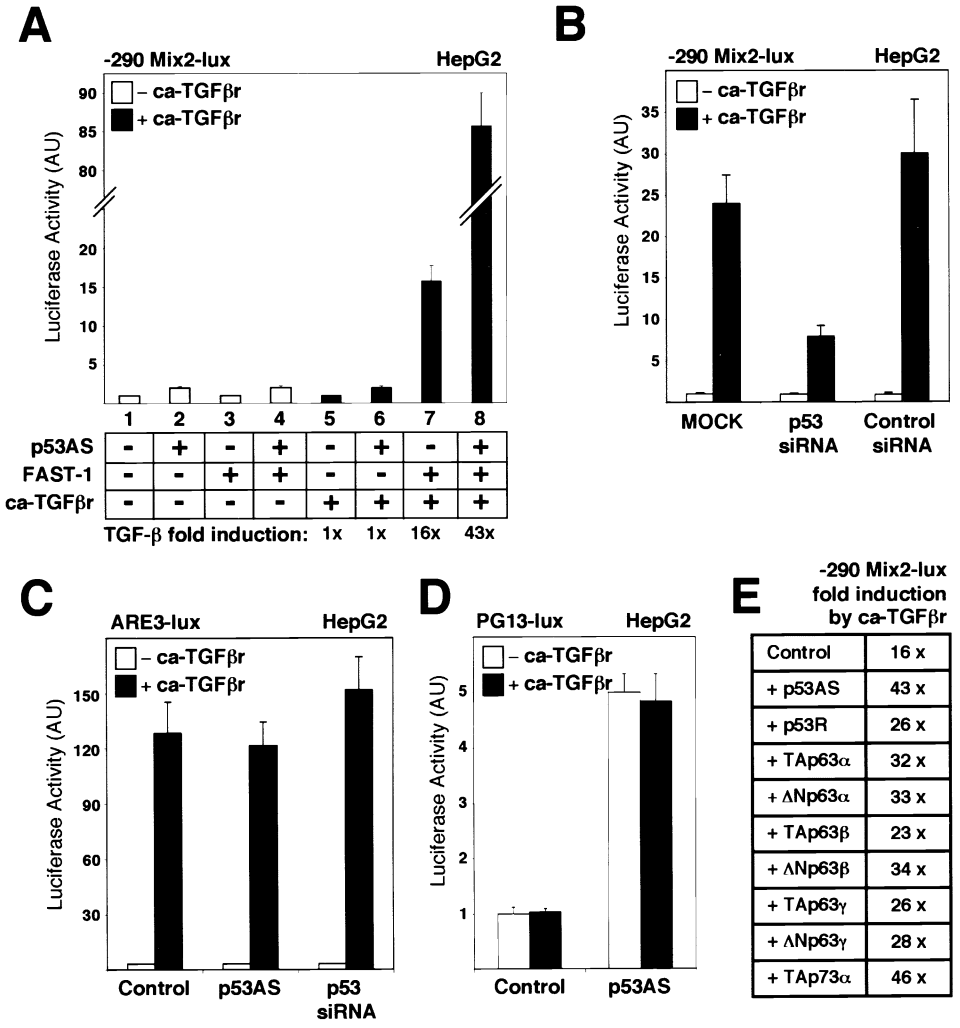

Figure 5. Transcriptional Partnership between p53 and TGF-β

(A) p53 enhances TGF-β-mediated transcription in HepG2 cells. −290 *Mix.2* reporter (200 ng) was transfected in HepG2 cells alone or with combinations of *FAST-1* (100 ng) and *mp53AS* (100 ng) expression vectors as indicated. TGF-β stimulation (black bars) was provided by transfected constitutive-active type I TGF-β receptor (*ca-TGFβr*, 100 ng).

(B) p53 is required for full activation of *Mix.2* reporter by TGF-β. After 24 hr from siRNA transfection (as described in Figure 3), −290 *Mix.2* reporter (200 ng) was transfected with FAST-1 (100 ng) expression vector, without or with *ca-TGFβr*.

(C) p53 cooperation with TGF-β requires sequences separated from the *ARE* of the *Mix.2* promoter. The synthetic *ARE3-lux* reporter (200 ng) was transfected in HepG2 cells in the presence of FAST-1, with or without p53AS. Where indicated, transfections were performed on cells deprived of p53 by siRNA treatment.

(D) TGF-β signaling does not promote p53 transcriptional activation. *PG13-lux* reporter construct (200 ng) was transfected in HepG2 cells alone or in combination with *p53AS* expression plasmid as indicated.

(E) The table summarizes the effects of p53 family members on TGF-β transcriptional activation, measured in transfection assays with −290 *Mix.2* reporter in HepG2 cells. ca-TGFβr fold induction indicates the ratio between stimulated and basal (without FAST-1) promoter activities, in the presence of empty vector (control) or expression vectors for *p53AS* (100 ng), *p53R* (100 ng), and 20 ng for each indicated p63/p73 isoform.

quired for complete promoter responsiveness to overexpressed *activin* mRNA.

We then assayed the requirement of the p53 binding site for the activation in vivo of the *Mix.2* promoter mediated by endogenous TGF-β ligands. Wild-type, *M-p53BE*, and *M-ARE* reporter constructs were microinjected at the 8-cell stage in the vegetal pole, as these blastomeres generate the cells expressing the endogenous *Mix.2* gene. Intriguingly, we found that the integrity of the p53 binding element has almost similar importance than *ARE* inactivation (Figure 6F). These data suggest that endogenous levels of TGF-β ligands are insufficient or partially sufficient for target gene activation. By contrast, TGF-β target gene expression is under the combinatorial control of Smad and p53 signaling, each converging to independent *cis*-regulatory elements.

p53 Physically Interacts with Smads

We then investigated whether the transcriptional cooperation between p53 and TGF-β signaling connected with a physical interaction between p53 and Smads in vivo and in vitro. In a first set of experiments, total cell

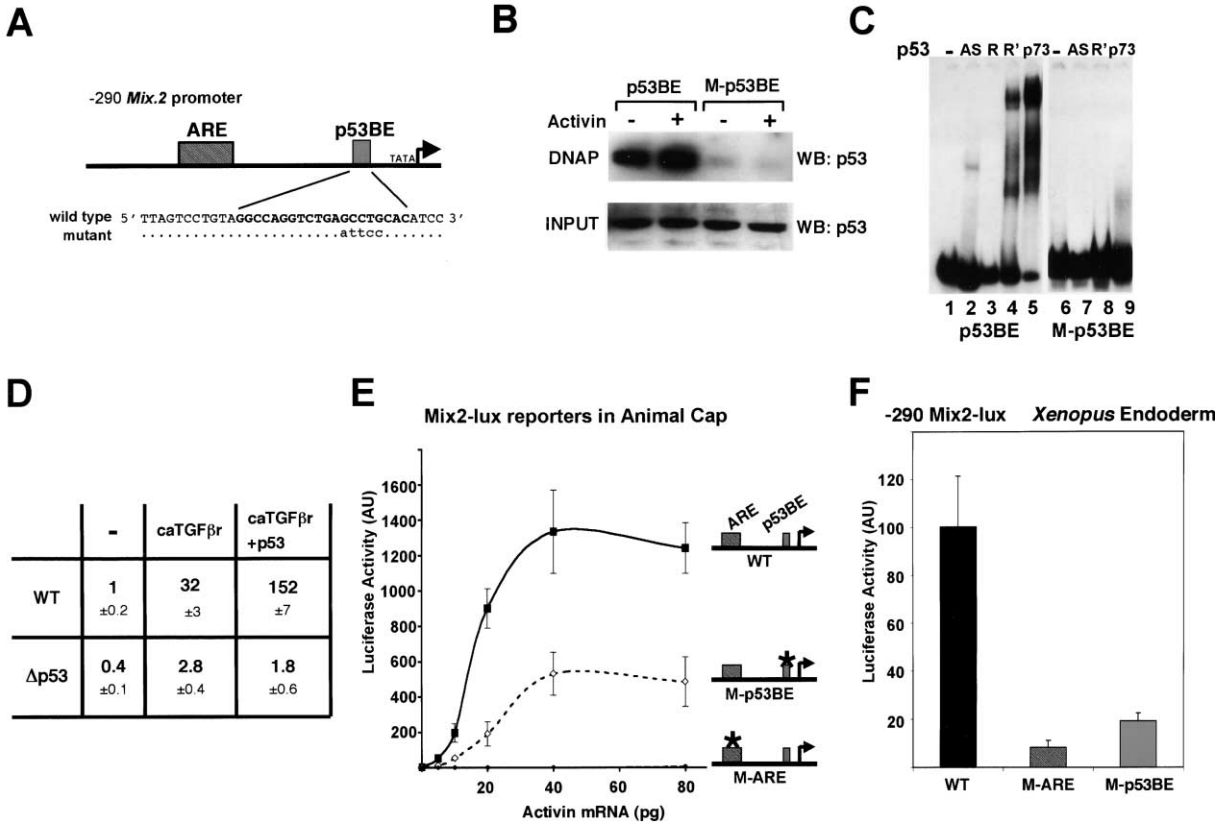

Figure 6. Identification of a Functional p53 Binding Site on the *Mix.2* Promoter that Is Required for Endogenous Expression and Full TGF-β Responsiveness

(A) Schematic representation of the natural −290 *Mix.2* promoter. A putative p53 consensus is located 105 bp downstream of the FAST-1 binding site in the *ARE*. The nucleotide sequence of the p53 consensus (*p53BE*) is highlighted in bold. Point mutations are in lowercase.

(B) DNAP showing Activin-independent binding of endogenous p53 protein to *p53BE*.

(C) Direct binding of recombinant GST-p53 and p73 to the *p53BE* in gel shift experiments. mp53AS (lanes 2 and 7), hp53R (lanes 3, 4, and 8) and hp73β (lanes 5 and 9) were incubated with wild-type or mutated *p53BE* radioactive probes as indicated. p53R binds its consensus only in the presence of PAb421 antibody (R', lane 4).

(D) The synergism between p53 and TGF-β on *Mix.2* promoter requires *p53BE*. The table summarizes the results obtained using the wild-type (WT) and *p53BE*-deleted (Δ*p53*) *Mix.2* reporter constructs. HepG2 cells were transfected with WT or Δ*p53* reporters together with *caTGFβr*, alone or in combination with *p53*. Values are relative to basal expression (−) of the WT promoter.

(E) *p53BE* is required for complete *Mix.2* promoter activation by Activin in *Xenopus* animal caps. The schematized reporter DNAs (40 pg each) were injected, alone or with different doses of *activin* mRNA, in the animal pole of 8-cell stage embryos and harvested at gastrula for luciferase detection. Induction of reporters was calculated as the ratio between *activin*-injected and uninjected embryos.

(F) *Mix.2* activation by endogenous TGF-β signals depends on the presence of a functional *p53BE*. 8-cell stage embryos were injected in the vegetal blastomeres with wild-type or mutated reporter constructs as indicated (40 pg per embryo). Inductions of the reporter constructs are relative to that of WT *Mix2-lux* (100%).

lysates from 293T cells transfected with *Smad2* and *Smad4* were incubated with immobilized GST, GST-p53, or GST-p73. As shown in Figure 7A, Smad2, but not Smad4, bound to immobilized p53 and p73 in a TGF-β manner. The interaction between p53AS and Smad2 was confirmed in vivo with coimmunoprecipitation experiments with overexpressed proteins (Figure 7B).

Smad proteins consist of an N-terminal MH1 domain, a linker, and a C-terminal MH2 domain. Using purified, recombinant proteins, we found that p53 and p73 associate directly with Smad2 and with the closely related Smad3, but not Smad4 (Figure 7C). Moreover, p53 recognizes specifically the MH1 domain of Smad2 and Smad3 (see Supplemental Figures S5A and S5B online at http://www.cell.com/cgi/content/full/113/3/301/DC1), without affecting with the ability of recombinant Smad3-MH1 to contact DNA (Supplemental Figure S5C).

To determine whether the interaction of p53 and Smad2/3 occurred with physiological levels of these proteins, we precipitated endogenous p53 from HepG2 cells either untreated or stimulated with Activin for 1 hr. To select for the pool of active cellular p53 able to bind to DNA, we carried out DNA affinity precipitation using a biotinylated oligonucleotide sequence from the high-affinity p53 binding site of the *GADD45-apha* gene (Qian et al., 2002; Vairapandi et al., 1996), which is unable to interact with Smads in any assay (data not shown). The purified complexes were analyzed by Western blotting with anti-p53 and anti-Smad2/3 antibodies. As shown in Figure 7D (lanes 1 and 2), p53 was precipitated equally from untreated and Activin-treated HepG2 cells; importantly, we could clearly detect the association of Smad2 and Smad3 with p53 upon Activin stimulation. Notably, the combined treatment of the cells with Activin and with

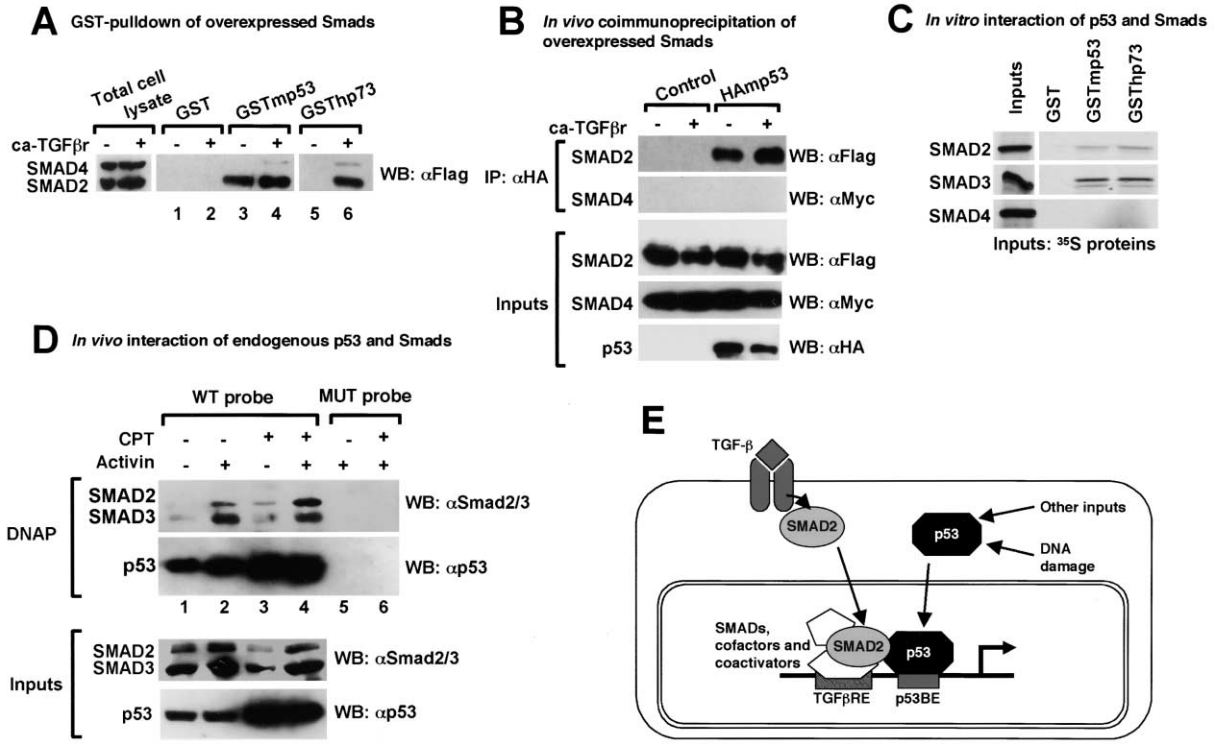

Figure 7. p53 Directly Interacts with Smad2 and Smad3

(A) Anti-Flag Western blot of GST pull-down experiments using total cell lysates from HEK 293T cells transiently expressing Flag-Smad2 and Flag-Smad4. Immobilized mp53AS (lanes 3 and 4) and hp73β (lanes 5 and 6) formed a complex with Smad2. Note the small amount of Smad4 coprecipitating in conjunction with Smad2.

(B) p53 interacts in vivo with Smad2. Immunoprecipitation (anti-HA antibody) of 293T cell lysates transfected with control, *HA-mp53AS*, *Flag-Smad2*, *Myc-Smad4* plasmids, with or without *ca-TGFβr*.

(C) GST pull-down of in vitro translated ^{35}S-labeled Smad proteins by immobilized GST-mp53AS and GST-hp73β.

(D) In vivo interaction between endogenous p53 and Smads. The panels show Western blotting for endogenous Smad2/3 and p53. Lanes 1 and 2: protein complexes from HepG2 cells, untreated or treated with Activin (1 nM), were precipitated using biotinylated oligonucleotides containing the *GADD45*-α p53 consensus (WT probe). Note the Activin-dependent coprecipitation of Smad2 and Smad3 with endogenous p53. Lanes 3 and 4: HepG2 cells were pretreated for 3 hr with DNA-damaging agent camptothecin (CPT, 0.5 μM), and then treated as above. Percentage of Smad2 bound to p53: lane 1, 0.9%; lane 2, 4.8%; lane 3, 11.3%; and lane 4, 14.4%. Percentage of endogenous Smad3 bound to p53: lane 1, 1.7%; lane 2, 4.9%; lane 3, 2.8%; and lane 4, 7.0%. As a control of the DNA precipitation procedure, Activin-treated samples, − or + CPT, were precipitated with a mutated *GADD45*-α oligo (MUT probe). Lower panels indicate the inputs for each corresponding sample.

(E) A model depicting the convergence of p53 and Smads in TGF-β-mediated gene transcription. Upon TGF-β ligand stimulation, Smad2 moves to the nucleus, where it associates with specific DNA binding cofactors. p53 and the TGF-β-activated Smad complex bind to each other and to their cognate sites on the promoter DNA of specific genes, leading to their synergistic transcriptional activation. Abbreviations: TGFβRE, TGF-β responsive element; p53BE, p53 binding element. Smad4 and other components of the TGF-β signaling cascade were omitted for simplicity.

the DNA-damaging drug camptothecin (CPT) increased p53 stability and further enhanced Smad2/p53 interaction (Figure 7D, lanes 3 and 4). As a specificity control, no interaction was observed in experiments performed with a *GADD45*-α oligonucleotide point mutated in the p53 consensus (Figure 7D, lanes 5 and 6). These biochemical results suggest that the functional cooperativity between p53 and TGF-β signaling is paralleled by the formation of a p53/Smad specific interaction.

Discussion

In the present work, we provide evidences that p53 family members are critical determinants for key TGF-β gene responses in different cellular and developmental settings. We showed that p53 associates with Smad2 and Smad3 in vivo in a TGF-β-dependent manner and that p53 family members can strongly cooperate with the activated Smad complex. Several TGF-β target genes in mammalian cells and *Xenopus* embryos are under such joint control of p53 and Smad.

Using a combination of loss-of-function approaches, we provided evidence of the biological importance of such cooperation. In frog cells, specific depletion of p53 leads to diminished responsiveness to Activin signaling and, in the context of the whole embryo, to severe developmental phenotypes recapitulating aspects of Nodal/Derriere deficiencies. In mammalian cells, the biological relevance of the p53/Smad cooperation was investigated in the context of TGF-β growth arrest program. Transient depletion of p53 or its genetic ablation impaired the antiproliferative response to Activin/TGF-β1 signaling. Finally, in p53 null cancer cells that do not respond to TGF-β signaling, reintroduction of p53 activ-

ity leads to the rescue of Smad-dependent growth inhibition.

Regulation of TGF-β-Mediated Gene Responses by p53 Family Members, Key Sensors of Multiple Cellular Inputs

The combinatorial control of gene expression by p53 and Smad establishes a new tier in the regulation of TGF-β gene responses. Our data indicate that p53 neither serves as a DNA binding platform for the Smads, nor can it adjust the general magnitude of gene responses to TGF-β. Depletion of p53 leaves the Smad response fully operational on artificial promoters containing only the Activin/TGF-β responsive element and on some endogenous TGF-β targets, such as *goosecoid* or *TIEG*. Instead, p53 appears as an independent input that is integrated on specific target promoters to modulate TGF-β induced transcription. Multiple cellular inputs converge on p53 (Vogelstein et al., 2000) and it is tempting to speculate that specific posttranslational modifications of p53 may further tune its crosstalk with Smads. In Figure 7E, we propose a model in which p53 and the activated Smad complex are recruited at distinct *cis*-regulatory elements on a common target promoter, leading to synergistic activation of transcription. We demonstrated this model for the *Mix.2* promoter, a paradigm of TGF-β-induced transcription (Huang et al., 1995). A point mutation in the p53 binding element of the *Mix.2* promoter caused a reduced Activin responsiveness in human cells and in the frog embryo, suggesting that p53 activity is required on DNA for full TGF-β transactivation. Of note, we find a correlation between other genes that in our assays are under joined control of p53 and Smad, and the presence of a functional p53 binding element in their promoters. This is the case for $p21^{WAF1}$, *PAI-1*, and *MMP2* (Qian et al., 2002). Oppositely, we could not identify putative p53 elements in the known regulatory sequences of *goosecoid* or *TIEG*, two genes not aided by p53.

p53/TGF-β Cooperation: Implications for Tumorigenesis

The TGF-β cascade plays both positive and negative roles on tumorigenesis (Wakefield and Roberts, 2002). Several mammalian cell systems studied here appear exquisitely sensitive to the level of p53 during TGF-β-mediated growth suppression; however, in different cell types, the contribution of p53 may be balanced by several factors, including redundant/compensatory mechanisms involving p63 and p73, differences in the cells' genetic make up, or other signaling inputs. For instance, it is worthwhile noting that the requirement of p53 for TGF-β1 growth control in hematopoietic progenitors is not as marked as it appears in embryo fibroblasts or in HepG2 cells.

Yet, our data suggest that inactivation of p53 may contribute to the lack of TGF-β antiproliferative effects in some cancer cell types. In several tumors, p53 is found as a mutant protein that is unable to bind DNA specifically; these altered p53 proteins are stable and dominantly inhibit the activity of wild-type p53, and of p63/p73 as well (Gaiddon et al., 2001). In other cancers, TGF-β/p53 dependent growth arrest may be overcome by epigenetic mechanisms. Nonetheless, it is well established that several TGF-β responses are fully operational in cancer cells and contribute positively to tumor invasiveness (Wakefield and Roberts, 2002). As p53 appears to impact on a significant subset of TGF-β target genes, it remains possible that p53/p63/p73 may also regulate new aspects of the TGF-β response in cancer cells.

A Developmental Role for p53 in Embryonic TGF-β Signaling

Specific depletion of p53 in *Xenopus* embryos reveals a developmental role for p53 in embryonic TGF-β signaling. This finding is challenged by the observation that *p53* knockout mice do not display developmental phenotypes (Donehower et al., 1992). It is possible that amphibian and mammalian embryonic cells may have different requirements. Additionally, functional redundancy or compensation between family members may account for the discrepancy. We think the latter is a likely explanation given that p53R, p53AS, p63, and p73 are all coexpressed, at significant levels, in gastrula-stage mouse early embryos (E6.25–E6.5) (see Supplemental Figures S6A–S6F online at http://www.cell.com/cgi/content/full/113/3/301/DC1). These findings, complemented by our data on the efficient interaction between TGF-β signaling and p63/p73, suggest a widespread possibility for Smad/p53 cooperation that may not be revealed by individual inactivation of p53 family/Smad cooperation during early embryogenesis.

At difference with mammals, we find little potential for redundancy in frog embryos, which express only p53 during early development (Supplemental Figure S6G). Indeed, p73 is not found in the lower vertebrates, and p63 is only expressed at later stages during organogenesis (Supplemental Figure S6G and Lu et al., 2001). It is therefore not surprising that a developmental role for p53 may be revealed in the *Xenopus* system.

p53 knockdown attenuates the induction of developmentally relevant genes controlled by endogenous Nodal/Derriere signaling (Figure 2). These data indicate that endogenous Nodal signaling is insufficient, by itself, to induce the expression of several target genes but requires assistance from p53. TGF-βs operate as morphogens in embryonic tissues (Gurdon and Bourillot, 2001). Should a promoter be under dual control of p53 and Smad, this would represent at least one mechanism to enhance—or sharpen—the otherwise subtle differences in TGF-β signaling between neighboring cells.

In sum, we have provided a mechanism encasing the sensory capabilities of p53 within the TGF-β gene expression program. Enrolling information on growth, stress, and cellular signaling in TGF-β-induced transcription would greatly expand, yet in a promoter-selective manner, the range and avenues for modulation of the cellular responses to this pleiotropic family of cytokines.

Experimental Procedures

cDNA Library Construction, Functional Screen, and Biological Assays in *Xenopus*

A cDNA library (2.1 kb average size) containing 80,000 primary transformants was prepared from gastrula-stage mouse embryos (from E6.0–E6.5 dpc). A total of 20,000 clones were assayed as described in the text.

Xenopus embryos manipulations, in situ hybridization, and capped mRNA preparation were as in Piccolo et al., 1999.

The morpholino antisense oligonucleotide (GeneTools) directed against Xp53 (p53 MO) was CCATGCCGGTCTCAGAGGAAGGTTC, whereas in the negative control morpholino (control MO), this sequence was mutated in six residues.

Plasmids and RT-PCR Primers

For a list of plasmids and the sequences of the RT-PCR primers used in this study, please refer to the Supplemental Data.

BrdU Analysis and Flow Cytometry

SAOS-2 cells were plated onto coverslips 24 hr after transfection with the indicated plasmids. HepG2 cells and MEFs were plated onto coverslips the day before adding Activin or TGFβ1 (R&D). Cells were incubated without or with added growth factors as follows: HepG2, 24 hr; MEFs, 48 hr; and SAOS-2, 48 hr. For BrdU incorporation, cells were labeled with 10 μM BrdU (Roche) and processed according to BrdU labeling and detection kit I (Roche) instructions. Cells were counted with DAPI staining and then scored for BrdU staining. Experiments were performed at least twice, each time in duplicate.

For flow cytometry, MEFs were plated at low density in 10 cm plates 1 day before adding TGF-β1. After 48 hr of TGF-β1 treatment, cells were harvested by trypsinization, fixed in 70% ethanol, and then treated with 50 μg/ml of propidium iodide and 100 U/ml of RNase. In each assay, 10,000 cells were scored using a FACScalibur flow cytometer and analyzed with the CellQuest program (Becton Dickinson). Data are mean of three independent experiments.

Clonogenic Assay for Hematopoietic Progenitor Cells (HPC)

HPC were purified from the bone marrow as indicated (Minucci et al., 2002). Single cell suspensions were plated (4000 cells/ml) in methylcellulose stem cell medium. HPC were cultured for 10 days, and large colonies were scored under a dissecting microscope ($n > 120$ in untreated samples).

Cell Culture and Transcription Assays in Mammalian Cells

HepG2, SAOS-2, H1299, and HEK293 were purchased from ATCC and were cultured as indicated by provider. HaCaT and early passage wild-type and *p53* null MEFs were a gift from G. Del Sal and were cultured as described (Flores et al., 2002; Datto et al., 1995).

For transcriptional response assays, *lacZ* constitutive expression vector (150 ng) was used to normalize for transfection efficiency. After overnight transfection (Lipofectamine 2000), HepG2 cells were placed in medium with 0.1% serum for 16 hr.

For siRNA treatment, 75 ng/cm^2 of dsRNA oligos were transfected using Oligofectamine reagent (Gibco). Control siRNA was anti-GFP (Xeragon). Anti-p53 siRNA sense sequence: CCGCGCCAUGGC CAUCUACAAG.

Immunoprecipitations and GST Pulldowns

Total cell lysates were prepared from transfected HEK293T cells by sonication in 25 mM Hepes (pH 7.9), 0.4 M KCl, 0.4% NP40, 5 mM EDTA, 1 mM DTT, 10% glycerol with protease, and phosphatase and deacetylase inhibitors. For immunoprecipitation, HA-tagged p53 protein was collected from extracts (300 μl) using anti-HA affinity resin (SantaCruz) in 600 μl of 0.25× lysis buffer/10 mM $MgCl_2$. For GST pulldowns, beads with purified proteins were incubated with total cell lysates (80 μl) or ^{35}S-methionine labeled in-vitro-translated Smad proteins as above for 3 hr. After four washes, copurified proteins were analyzed by Western blotting.

DNA Affinity Precipitation and EMSA

Nuclear extracts were prepared from control and Activin-treated HepG2 cells. Extracts were incubated with 600 ng of biotinylated double-stranded oligonucleotides and 5 μg of poly(dI-dC) at 4°C for 16 hr in DB Buffer (60 mM NaCl, 20 mM HEPES (pH 7.8), 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 1 mM DTT, 2% BSA, and 0.1% of NP-40). DNA bound proteins were collected with streptavidin-agarose beads (Pierce) for 1 hr, washed extensively with DB, separated on a SDS-polyacrylamide gel, and identified by Western blotting. The sequence for *p53BE* probe was TTTAGTCCTGTAGGC CAGGTCTGAGCCTGCACATCCCAGACAAG, *M-p53BE* was mutated as in Figure 6A. WT and MUT *GADD45* probes are AATTCT CGAGCAGAACATGTCTAAGCATGCTGGGCTCGAGCC and AATTC TCGAGCAGAATCGCTCTAAGCATGCTGGGCTCGAGCC, respectively.

Electrophoretic mobility shift assays (EMSA) were done as described in Supplemental Figure S5 Legend.

Antibodies and Western Blotting

The list of antibodies and the Western blotting protocol used in this study are available as Supplemental Data.

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