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Coordinate Transcriptional and Translational Repression of p53 by TGF-β1 Impairs the Stress Response

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SUMMARY

Cellular stress results in profound changes in RNA and protein synthesis. How cells integrate this intrinsic, p53-centered program with extracellular signals is largely unknown. We demonstrate that TGF-β1 signaling interferes with the stress response through coordinate transcriptional and translational repression of p53 levels, which reduces p53-activated transcription, and apoptosis in precancerous cells. Mechanistically, E2F-4 binds constitutively to the *TP53* gene and induces transcription. TGF-β1-activated Smads are recruited to a composite Smad/E2F-4 element by an E2F-4/p107 complex that switches to a Smad corepressor, which represses *TP53* transcription. TGF-β1 also causes dissociation of ribosomal protein RPL26 and elongation factor eEF1A from p53 mRNA, thereby reducing p53 mRNA association with polyribosomes and p53 translation. TGF-β1 signaling is dominant over stress-induced transcription and translation of p53 and prevents stress-imposed downregulation of Smad proteins. Thus, crosstalk between the TGF-β and p53 pathways defines a major node of regulation in the cellular stress response, enhancing drug resistance.

INTRODUCTION

The cellular response to stress signals involves profound changes in RNA and protein synthesis whose net output directs specific cell-fate decisions (Spriggs et al., 2010). The tumor suppressor protein p53 is the main transcription factor that orchestrates the stress program, largely by inducing cell-cycle arrest or apoptosis. p53 activity is controlled predominantly by protein stability and posttranslational modifications and is a frequent target of mutations during tumorigenesis (Levine et al., 2006; Vousden and Prives, 2009). Much less is understood about regulation of p53 biosynthesis, although p53 mRNA deregulation has been observed in human cancers (Saldaña-Meyer and Recillas-Targa, 2011). Moreover, seminal studies have elegantly demonstrated that de novo p53 translation mediated by the 60S ribosomal protein RPL26 is required for efficient p53 accumulation to direct specific cell-fate outcomes (Chen and Kastan, 2010; Schumacher et al., 2005; Takagi et al., 2005).

Transforming growth factor β (TGF-β) has a dual role in cancer by acting as a tumor suppressor through cell-growth arrest and as a tumor facilitator at later stages (Massagué, 2008). Central to TGF-β1 signaling is phosphorylation of Smad 2/3 transcription factors by the TGF-βRI/TGF-βRII receptor complex. Phosphorylated Smads assemble into heterotrimeric and heterodimeric structures with Smad 4 and translocate into the nucleus as activated complexes. Interaction of Smad complexes with other DNA-binding proteins targets them to specific promoters where they activate or repress transcription (Massagué et al., 2005). TGF-β1 signaling controls cell growth, invasiveness, and the epithelial to mesenchymal transition (EMT) through both activation or repression of transcription and translation of its target genes (Massagué, 2008; Pardali and Moustakas, 2007; Chaudhury et al., 2010; Hussey et al., 2011; Lin et al., 2010). Yet the strategies used by TGF-β1 to switch from a tumor suppressor to a cancer enhancer and the stage in which it occurs are largely undefined.

In unstressed cells various p53 family members can cooperate with TGF-β/Smad signaling to facilitate *Xenopus* mesoderm differentiation. Also, in certain mammalian cells that lack p63 and p73, p53 can enhance TGF-β-mediated growth arrest (Cordenonsi et al., 2003). Smads also associate with mutant p53 to deregulate p63-mediated transcription and enhance metastasis (Adorno et al., 2009). Thus, the influence between p53 family members and TGF-β may be significant in tumor biology. But whether TGF-β signaling directly intersects with the p53-induced stress response to impact cell-fate decisions is poorly understood.

To address these issues, we examined the effects of TGF-β1 on the DNA damage response using nontumorigenic, spontaneously immortalized human mammary epithelial cells (HMECs). We found that TGF-β1-activated Smads attenuate the stress-induced p53 transcriptional program and protect damaged cells from apoptosis through coordinate transcriptional and translational repression of p53 protein levels. TGF-β1-mediated

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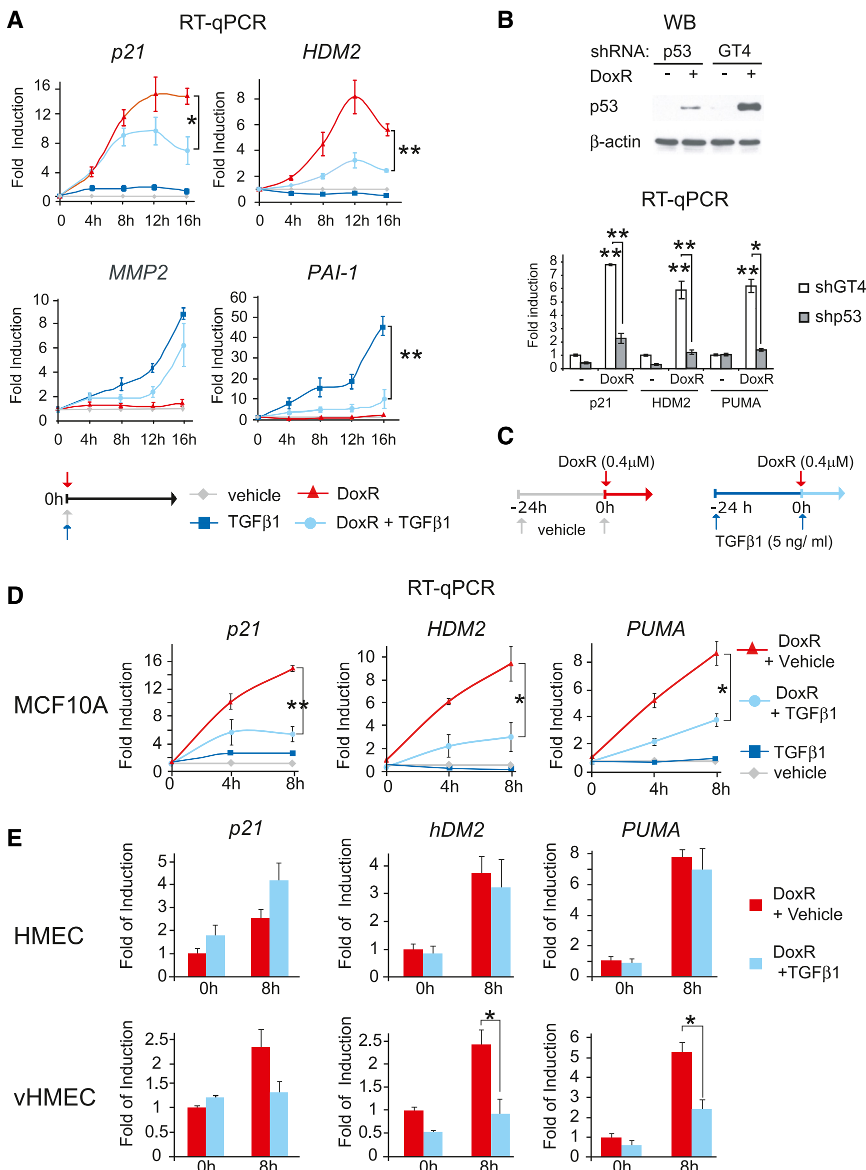

Figure 1. TGF-β1 Attenuates the p53-Mediated Stress Response in Precancerous but Not in Normal Human Mammary Epithelial Cells

(A) RT-qPCR analysis of gene expression for relevant p53 or TGF-β1-induced genes upon treatment with DoxR and/or TGF-β1, starting simultaneously with the color-coded arrows indicating the time at which compound was added. Fold of induction ± SEM was calculated after normalization with GAPDH mRNA.

(B) (Top) Western blot of p53 after 8 hr of DoxR treatment of MCF10A cells expressing the indicated shRNAs. (Bottom) Fold of induction ± SEM of p53 target genes measured by RT-qPCR as in (A).

(C) Protocol for eliciting both direct and indirect TGF-β1 effects on the DNA damage response. Arrows indicate the time of addition of each compound.

(D) Fold of induction ± SEM of p53 target genes in MCF10A cells after DoxR treatment following the protocol shown in (C) quantified as described in (A).

(E) RT-qPCR analysis of stress-responsive genes from patient-matched HMEC/vHMEC tissue samples treated as in (D). Red bars, DoxR treated; light blue bars, DoxR + TGF-β1. HMEC data are presented as mean ± SEM from six biological replicates from two patients (RM33, RM35). See also Figures S1 and S2.

downregulation of p53 occurs in precancerous and some breast and lung cancer cells, but not in patient-matched normal mammary cells, and confers apoptotic resistance to a variety of chemotherapeutic agents. Mechanistically, TGF-β signaling induces assembly of a Smad/E2F-4/p107 repressor complex on the *TP53* gene which downregulates transcription and disrupts interaction between the ribosomal protein RPL26 and the elongation factor eEF1A with p53 mRNA to attenuate p53 translation. Our findings demonstrate an unexpected dominance of TGF-β signaling over the cellular stress response by its ability to simultaneously affect two central nodes of regulation: transcription and translation. These results reveal a tumor-enhancing role for TGF-β in which it facilitates the survival of damaged precancerous and malignant cells by impairing the proapoptotic actions of p53.

RESULTS

TGF-β1 Signaling Attenuates the p53-Mediated Transcriptional Response to Stress

To examine the impact of TGF-β signaling on the p53-mediated DNA damage response, we used immortalized, nontumorigenic human breast epithelial MCF10A cells. DNA damage in these cells by the anticancer drug Doxorubicin (DoxR) resulted in p53-dependent expression of *p21*, *HDM2*, and the proapoptotic gene *PUMA* (Figures 1A and 1B) and induced p53-dependent apoptosis (see Figure S1 online). TGF-β1 also efficiently activated the Smad pathway in MCF10A cells (Figures 1A, 1D, and 2E; Figure 2C; Figure S6; and data not shown). We then examined the effects of crosstalk between TGF-β and p53 signaling by simultaneously inducing both pathways with TGF-β1 and DoxR. Unexpectedly, we found that activation of p53 target genes *p21* and *HDM2* was reduced in the presence of TGF-β1 (Figure 1A). Since either DNA damage or TGF-β signaling can activate the *p21* gene, the observed interference between these two pathways on *p21* expression was surprising. We further investigated the effects of TGF-β signaling on the p53-mediated damage response by priming MCF10A cells with TGF-β1 24 hr before the addition of DoxR (Figure 1C). This protocol allows TGF-β1 signaling to exert both direct and indirect effects and may approximate a more physiological condition, since sustained

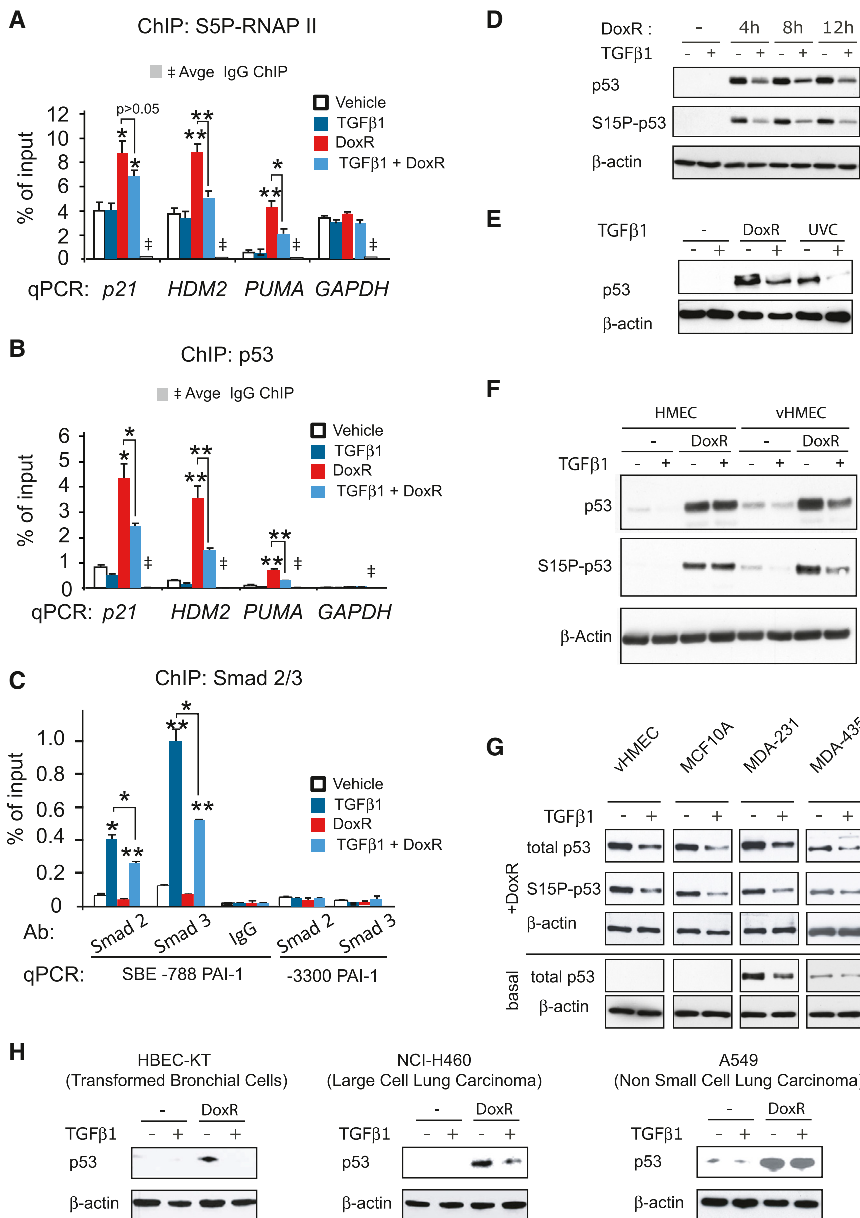

Figure 2. TGF-β1 Reduces p53 Accumulation in Precancerous and Transformed Cells

(A–C) ChIP analyses from MCF10A cells treated with DoxR in the presence of TGF-β1 or vehicle with the indicated antibodies. Relevant sequences were quantified by real-time PCR. Sequence and antibody specificity (average from all treatments or individually) controls are included. Data are presented as percentage of input DNA ± SEM.

(D) Western blot analysis after DNA damage, according to the protocol shown in Figure 1C in the presence of activated TGF-β1 signaling in MCF10A cells.

(E) p53 accumulation in MCF10A cells after 8 hr of DoxR or UVC (25J/cm2) in the presence of activated TGF-β1 signaling in MCF10A cells.

(F) Western analysis of p53 levels from patient-matched normal (HMEC)/precancerous (vHMEC) tissue samples treated as indicated above. Data presented corresponds to cells from patient RM35 and are representative of two matched pairs.

(G) Total and S15P-p53 accumulation before or after 8 hr of DoxR treatment ± TGF-β1 in vHMEC RM45, MCF10A, MDA-231, and MDA-435 breast cancer cells.

(H) Western blot analysis of p53 levels in WT p53-expressing human lung cells.

See also Figure S2.

TGF-β activation of Smads is observed in both normal and neoplastic breast tissues, which we corroborated (Figures S2 and S3). This approach also resulted in attenuation of the p53-mediated response (Figure 1D). Therefore, the priming protocol was chosen for all subsequent studies since it more closely recapitulated the in vivo microenvironment.

We next analyzed primary patient-matched sets of normal HMECs and its rare variant vHMEC subpopulation, which displays precancerous properties (Crawford et al., 2004; Romanov et al., 2001), obtained from cancer-free individuals undergoing reduction mammoplasty. Remarkably, we found that TGF-β1 downregulated the p53 pathway in the precancerous, extended life span vHMECs, but not in normal HMECs (Figure 1E). Interestingly, DNA damage of MCF10A cells impaired TGF-β1-induced expression of *PAI-1* and *MMP2* (Figure 1A) and downregulated cellular levels of Smads 2-3 and 4, which could be reversed by TGF-β1 stimulation (Figure S1). Together, these data support the notion of reciprocal interference between the stress response and TGF-β pathways and suggest that TGF-β1 can overcome the general stress-mediated shutdown of gene expression.

Stress-Induced p53 Protein Accumulation and p53 Interaction with Its Target Promoters Are Decreased by TGF-β1 Activation

Chromatin immunoprecipitation studies revealed reduced association of the active form of RNA polymerase II (S5P-RNAPII) with p53 target promoters in TGF-β1-treated cells (Figure 2A). Correspondingly, a specific significant reduction of DNA damage-induced association of p53 with the p21, HDM2, and PUMA promoters in TGF-β1-treated cells was also detected (Figure 2B). Conversely, TGF-β1 strongly induced interaction of Smads 2 and 3 with the PAI-1 promoter, which is reciprocally reduced by DoxR (Figure 2C). Thus, TGF-β1 signaling results in insufficient recruitment of p53 and RNAP II complexes to target promoters, causing a downregulation of p53-responsive genes upon stress.

We then measured total and stress-stabilized serine 15 phosphorylated (S15P) p53 accumulation over time in DoxR-treated MCF10A cells in the presence and absence of TGF-β1. Surprisingly, we found that levels of both phosphorylated and total p53

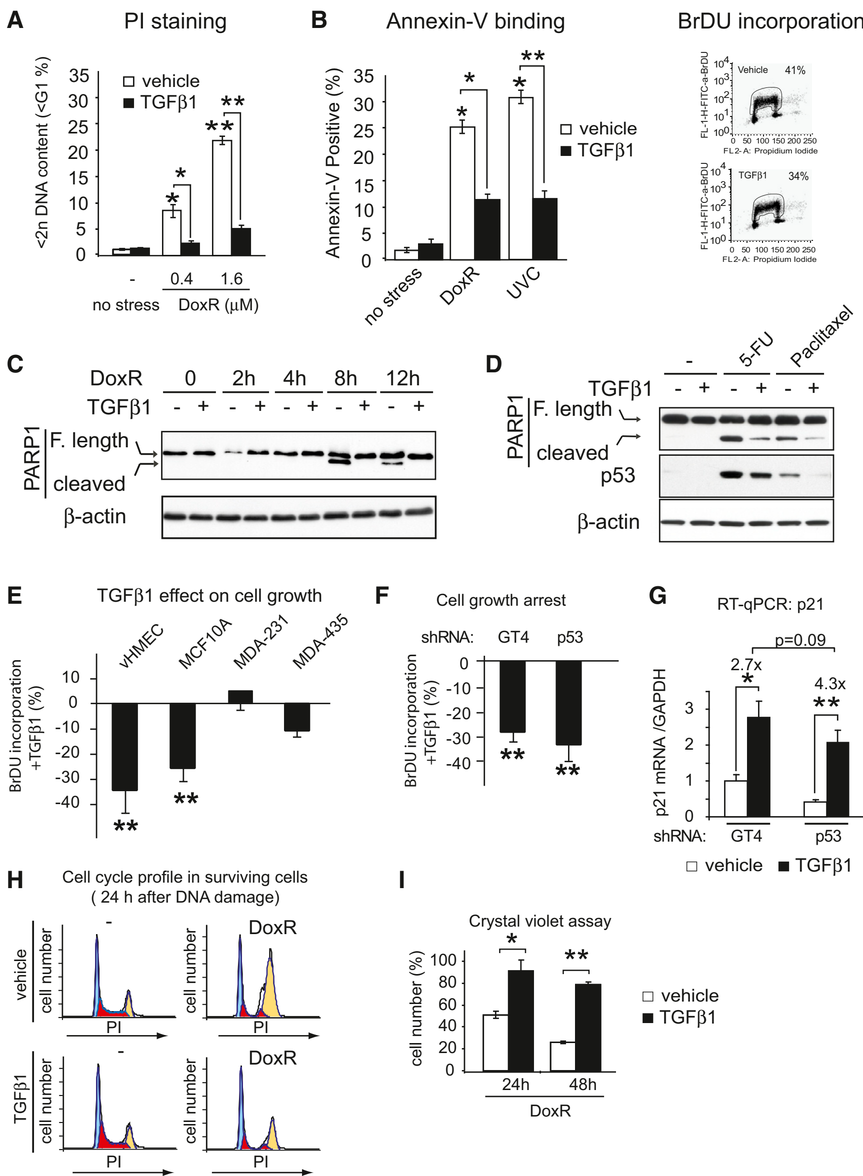

Figure 3. TGF-β1 Prevents DNA Damage-Induced Apoptosis

(A) DNA content loss (DNA < 2N) analysis. Cells treated as described were harvested after 8 hr of stress, fixed, stained with propidium iodide, and analyzed by FACS. Data are presented as the percentage of cells ± SEM in the subG1 population.

(B) (Left) Annexin V binding-based apoptotic indexes ± SEM on MCF10A cells after 8 hr of exposure to DoxR (1.6 μM) or UVC irradiation (25 J/m2) in the presence or absence of TGF-β1. (Right) Proliferation rate in TGF-β1- or vehicle-treated cells at the time of DNA damage induction was measured by BrDU incorporation.

(C and D) Western blot analysis of PARP-1 cleavage in the presence or absence of TGF-β1 in cells treated with Doxorubicin (C), 5-Florouracil, or Paclitaxel (D).

(E and F) TGF-β1 growth suppression in the absence of DoxR in various mammary cells (E) and in p53-depleted MCF10A cells described in Figure 1B (F). Cells were treated with TGF-β1 for 48 hr, and the proliferative rate ± SEM was determined by BrDU incorporation.

(G) p21 mRNA fold of induction ± SEM by TGF-β1 after 4 hr in p53-depleted cells was determined as described above.

(H) Cell-cycle profiles of viable cells 1 day after DNA damage, determined by FACS as in (A) G1 (blue), S (red), and G2/M (yellow) phases of the cell cycle, are shown from one representative experiment.

(I) Number of viable cells ± SEM determined by crystal violet staining after 24 or 48 hr of DNA damage. Unstressed control cells are set as 100%.

were significantly reduced in cells exposed to TGF-β1, indicating a decrease in cellular concentrations of p53 (Figure 2D). A similar effect was obtained when MCF10A cells were stressed by UV irradiation (Figure 2E). Strikingly, TGF-β1 also decreased DNA damage-induced wild-type p53 levels in vHMECs, but not in patient-matched normal HMECs (Figure 2F), suggesting that this phenotype is acquired at a very early stage in tumorigenic transformation.

This effect was found in mutant p53-expressing breast cancer cell lines MDA-MB-231 and MDA-MB-435 as well as in transformed (immortalized) bronchial epithelial cells and some wild-type p53-expressing lung cancer cells (Figures 2G and 2H). Thus, TGF-β1-mediated downregulation of stress-induced p53 protein levels is independent of wild-type p53 activity and is not restricted to breast tissues. These results suggest that TGF-β1 might attenuate both the wild-type and mutant DNA damage-induced p53 pathways in precancerous and tumorigenic human cells by reducing levels of bulk cellular and promoter-bound p53 protein, downregulating critical p53 target genes, and potentially compromising p53-directed cell-fate choices.

Additionally, immunohistochemistry analyses of normal human breast tissues and breast carcinomas indicated that coactivation of TGF-β and p53 (either WT or mutant) pathways does not always exist. Moreover, western analyses of freshly frozen lysates from another tumor set showed that activated Smad 2 might inversely correlate with both wild-type and mutant p53 levels. This suggests that cooperation between TGF-β and apoptotic (p53-dependent or -independent) pathways might represent a potential node of deregulation in specific tumor contexts (Figures S2 and S3).

TGF-β1 Protects Cells from DNA Damage-Induced Apoptosis

We then addressed whether TGF-β1 can modify cell-fate decisions upon DNA damage. Indeed, in the presence of TGF-β1, DoxR-induced cell death was significantly reduced as measured by propidium iodide DNA content analysis (Figure 3A). Consistently, TGF-β1 decreased both DoxR- and UVC-induced

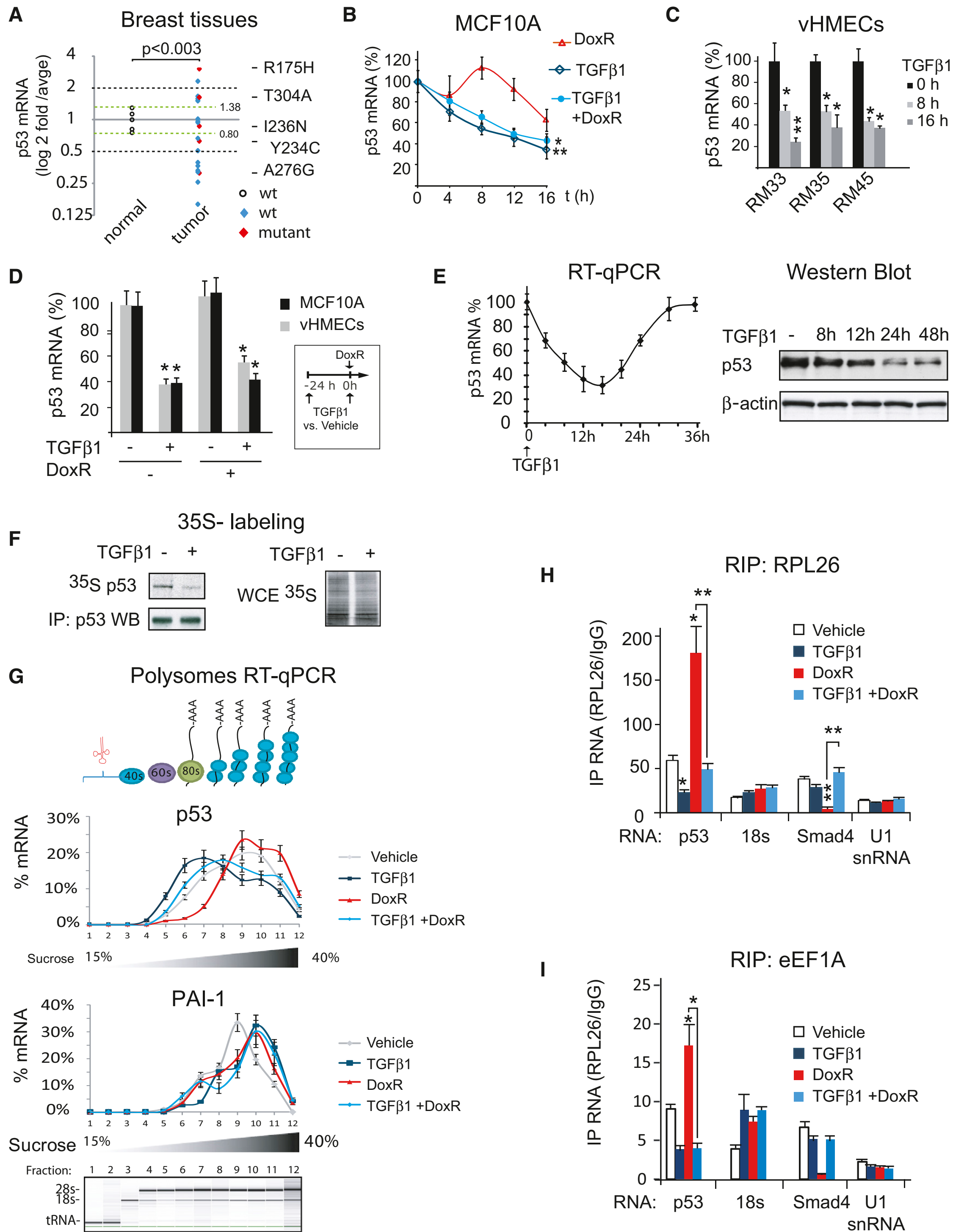

Figure 4. TGF-β1 Signaling Represses p53 Transcription and Translation

(A) p53 mRNA quantification by RT-qPCR in human breast tissues. Normalized p53 mRNA expression is presented as fold relative to the mean value for each group. p53 transcripts were sequenced, and all p53 mutants (5/21 = 24%) are indicated; p = 0.0027.

(B) MCF10A cells were treated as in Figure 1A, and p53 mRNA was quantified at the indicated times by RT-qPCR and normalized to GAPDH and untreated controls. Values are expressed as the percentage ± SEM of TGF-β1-treated cells relative to untreated cells.

(C) RM-derived vHMECs from three donors were incubated with TGF-β1 or a vehicle buffer, and p53 mRNA level ± SEM was determined as described in (B).

(D) p53 mRNA level ± SEM was determined in MCF10A and vHMECs (averaged from donors RM33, RM35, and RM45) following the protocol indicated in Figure 1C.

(legend continued on next page)

apoptosis (Figure 3B, left panel). Notably, the proliferative rates at the times of these measurements were similar for both TGF-β1- and vehicle-treated cells, as assessed by BrDU incorporation (Figure 3B, right panel). This suggested that impaired cell death mediated by TGF-β1 was independent of cell-proliferative rates. Strikingly, TGF-β1 markedly abrogated cleavage of poly(ADP-ribose) polymerase (PARP)-1 into its inactive 89 kDa catalytic fragment when induced by three types of conventional chemotherapeutic drugs: Doxorubicin, 5-Fluorouracil, and Paclitaxel (Figures 3C and 3D and data not shown). PARP-1 participates in the TGF-β-induced transcriptional cycle by interacting with Smad complexes at TGF-β-regulated promoters (Lönn et al., 2010). Thus, our results support the existence of a positive feedback in which TGF-β1 may protect PARP-1 from cleavage to ensure homeostasis of the TGF-β1 pathway in damaged cells.

Interestingly, TGF-β1 treatment of vHMECs and MCF10A cells induces cell-cycle arrest at G1 after prolonged incubation (48 hr) as determined by BrDU incorporation and cell-cycle profiles (Figure 3D and data not shown). Conversely, MDA-231 and MDA-435 cells were refractory to growth arrest. Therefore, cell-cycle arrest and protection from apoptosis resulting from TGF-β1 signaling appear as two independent events. These observations prompted us to test whether p53 is required for TGF-β1-mediated cell-cycle arrest in MCF10A cells. We found that p53 knockdown in MCF10A cells, which express similar levels of p63 and p73 as control cells (Figure S1B), did not impede TGF-β1-induced growth arrest or p21 or PAI-1 mRNA activation (Figures 3F and 3G; data not shown).

TGF-β1 also reduces DNA damage-induced G2/M cell-cycle arrest at the expense of an increased number of cells arrested in G1, indicating that they can overcome the G2/M-imposed arrest (Figure 3H). In addition, TGF-β1 increased the number of surviving cells after 48 hr exposure to DNA damage from 20% to 75%, suggesting that TGF-β1 signaling initiates a cell-survival pathway (Figure 3I). Together, our results show that TGF-β1 protects precancerous, immortalized mammary epithelial cells from apoptosis and promotes a shift from the G2/M arrest toward a G1 cell-cycle arrest in surviving cells, in part by impairing p53 function.

TGF-β1 Signaling Represses *TP53* Gene Transcription

TGF-β1 repressed p53 levels over a wide range of physiological and pathological concentrations (Figures S4A and S4B) but cannot repress exogenously expressed p53 (either wild-type or HA-tagged) from a constitutively active promoter (Figures S4C and S4D). This argued against the possibility that TGF-β1 destabilizes p53 by posttranslational modulation. Moreover, both basal and DoxR-induced levels of HDM2 are also downregulated upon TGF-β1 treatment of MCF10A cells and vHMEC primary cultures (Figure S4E). Thus, the negative effect of TGF-β1 on p53 levels is independent of p53 protein degradation through increased HDM2 expression. Curiously, a previous report showed that TGF-β can activate the *HDM2* gene in colon carcinoma HCT116 cells which were genetically modified to re-express the TGF-β receptor (Araki et al., 2010).

Interestingly, an analysis of p53 mRNA levels in human breast tumors revealed that the expression range of both WT and mutant p53 is far greater than that found in normal breast tissues (Figure 4A, Table S2). Decreased abundance of p53 mRNA has been correlated with poor prognosis in aggressive breast carcinomas that express WT p53 (Miller et al., 2005). We hypothesized that TGF-β1 might attenuate the p53-mediated DNA damage response by restricting p53 mRNA abundance. p53 mRNA levels were reduced (3- to 4-fold) by TGF-β1 in MCF10A cells and in primary cultures of vHMECs from three different patients in the presence or absence of DNA damage (Figures 4B–4D). In addition, mRNA stability experiments indicated that TGF-β1 does not promote p53 mRNA decay but slightly increases its stability (Figure S4F). The delay in TGF-β-mediated repression of p53 mRNA (Figure 4B) is consistent with the p53 mRNA half-life extending over 9 hr. Together, these results reveal that TGF-β1 potentially acts by repressing *TP53* RNA synthesis rather than by facilitating mRNA or protein turnover, which was further confirmed by RT-qPCR using intron-specific primers on vHMEC primary cells (Figure S4G).

TGF-β1 Represses RPL26-Mediated p53 mRNA Translation

Interestingly, repression of p53 protein lasted much longer than p53 mRNA repression after a single addition of initial TGF-β1 (Figure 4E), and we speculated that TGF-β1 might downregulate p53 mRNA translation. A short pulse metabolic labeling experiment with ^{35}S-methionine showed reduced levels of newly synthesized p53 in TGF-β1-treated cells (Figure 4F). Furthermore, ribosome analysis indicated that TGF-β1 treatment specifically displaced p53 mRNA from heavier to lighter polysomes/monosomes (Figure 4G, Figure S4L, and data not shown). TGF-β1 also prevented the redistribution to lighter poly/monosomes of RPL26 induced by DoxR (Figure S5A).

In addition, RNA immunoprecipitation (RIP) experiments revealed that TGF-β1 specifically inhibits interaction of p53 mRNA with both the 60S ribosomal protein RPL26 and the eukaryotic elongation factor 1A (eEF1A) in the presence or absence of DoxR (Figures 4H and 4I, Figures S5B–S5E). RPL26 interaction is essential for efficient translation of p53 mRNA in a CAP- and poly(A)-independent manner through specific pairing of both the 5′UTR and 3′UTR (Chen and Kastan, 2010), while eEF1A delivers aminoacyl-tRNAs to the A site of the ribosomes,

(E) Levels of p53 mRNA ± SEM and protein in MCF10A cells treated once (t = 0 hr) with TGF-β1.

(F) Downregulation of p53 translation by TGF-β1. MCF10A cells were treated with TGF-β1 for 24 hr, and equal amounts of p53 were immunoprecipitated from (S^{35})-methionine-labeled, TGF-β1-treated, or control cells incubated with MG132 (20 μM). IP proteins were analyzed by autoradiography (top left) or western blot (bottom left). Autoradiography of whole-cell extracts (WCE) shows equal amount of S^{35}-methionine incorporation.

(G) TGF-β1 alters the polysome distribution of p53 mRNA. Cytosolic extracts of MCF10A cells were fractionated by sucrose gradient centrifugation and p53 and PAI-1 mRNA levels ± SEM were determined by RT-qPCR on purified RNA. Bioanalyzer results show the ribosomal rRNA species distribution (1, lightest fraction; 12, heaviest fraction).

(H and I) RIP analysis of RPL26 and eEF1A association with p53 mRNA in MCF10A cells treated as in (G). Data are shown as percentage of input RNA ± SEM. See also Figures S4 and S5.

and its transient association with mRNAs also indicates an efficient translational elongation process.

Importantly, neither cellular levels of RPL26 and eEF1A nor their specific distribution across cytosolic fractions were affected by TGF-β1 (Figure S5A). Moreover, the distribution between cytoplasmic (ribosome-bound) and nuclear (ribosome-free) pools of RPL26 was also unaffected by DoxR (Figures S5E and S5F), in agreement with results by Kastan and colleagues (Chen and Kastan, 2010), or by TGF-β1. TGF-β1 prevented DoxR-induced accumulation of RPL26 in the 60S/80S ribosomes, indicating its dominance over the DNA damage response (Figure S5A). We consistently found that association of RPL26 and eEF1A with Smad 4 mRNA was dampened by DoxR-induced general translational repression (Spriggs et al., 2010) but restored in the presence of TGF-β1 (Figures 4H and 4I). Mechanistically, our data suggest that TGF-β1 represses p53 translation at the elongation stage, consistent with reports showing that TGF-β1 affects this step in translation (Lin et al., 2010).

Altogether, these results demonstrate that TGF-β1 abrogates the DNA damage response at the level of protein translation in at least two ways: by directly repressing p53 mRNA translation through interference with RPL26 and eEF1A binding and by relieving DoxR-induced repression of Smad mRNA translation.

Identification of a Functional Smad Binding Element within the *TP53* Gene that Directs Transcriptional Repression by TGF-β1

Our findings also suggested that activated TGF-β1 signaling might repress *TP53* gene expression through Smad proteins. We identified three potential Smad binding regions (SR1–3) in the *TP53* gene containing shortly spaced SBEs (Figure 5A). The SR2 localizes in the regulatory region of the *TP53* gene and contains an E2F-4 binding site that overlaps an imperfect SBE. Depletion of Smad 4 protein from MCF10A cells resulted in both increased p53 protein levels (Figure 5B) and abrogation of TGF-β1-induced *TP53* gene repression (Figure 5C). Thus, TGF-β1 represses *TP53* through the canonical Smad-dependent pathway.

ChIP assays showed that TGF-β1 induced a clear recruitment of phosphorylated P-Smad 2 to the *TP53* gene (Figure 5D), with highest association near the SR2 region. P-Smad 2 remained bound for up to 6 hr with maximal occupancy at 2 hr (Figure S6A). In addition, Smad 3 was also recruited to similar locations with faster but weaker association than P-Smad 2 (Figure 5D, Figure S6A). These results support the hypothesis that Smads contribute directly to transcriptional repression of the *TP53* gene.

Additional ChIP experiments indicated that both Smad 2 and Smad 3 associate with the SR2 region upon TGF-β1 stimulation in unstressed cells and, importantly, in the presence of DoxR (Figure 5E), indicating that TGF-β1-induced repression of *TP53* is stable in the context of damaged cells and predominates during the stress response. Furthermore, association of Smad 2/3 proteins with the SR2 region of *TP53* was accompanied by a simultaneous decrease in promoter recruitment of the initiating form of RNA polymerase II (S5P-RNAP II) and abundance of the elongating form of RNAP II (S2P-RNAP II) throughout the coding region of the gene (Figure 5F). Moreover, TGF-β1-induced decrease of RNAP II interaction with the p53 promoter was also observed in the presence of DoxR. These results indicate that upon TGF-β1 signaling in both unstressed and stressed cells, Smad 2/3 complexes are recruited to the *TP53* gene and repress transcription by reducing the assembly of transcriptional initiation complexes on the promoter. TGF-β1 also decreased association of RNAP II with the p53 promoter in MDA-MB-231 and HaCaT cells, which express mutant p53 proteins (Figures S6D and S6E).

Using high-resolution IP analysis with purified mononucleosomes (MnIP), we confirmed P-Smad 2 binding specifically within the SR2 sequence of the *TP53* gene (Figure 6A). Next, we performed cotransfection assays with reporter plasmids containing fragments from the p53 promoter fused to a luciferase reporter gene (Figure 6B). As expected, TGF-β/Smad 4 cotransfection efficiently transactivated the PAI-1-derived SBE multimeric, activatable pBV-SBE4-luc control reporter (Figure 6C). By contrast, both p53 promoter constructs were downregulated by TGF-β/Smad 4 (Figure 6D). Deletion or mutation of the SR2 region in the p53 promoter (+80 to +150) abrogated TGF-β/Smad-mediated repression (Figure 6E, Figure S6), indicating that the SR2 sequences are required for repression. Together, these results demonstrate that *TP53* transcription is directly repressed by TGF-β1/Smads through the SR2 sequence within the p53 promoter.

Sequential Assembly of an E2F-4/Smad Corepressor Complex at the p53 Promoter Underlies Attenuation of the p53 Pathway by TGF-β1

The TGF-β-inhibitory element in the c-MYC promoter contains a composite sequence formed by an E2F-4 site and an imperfect SBE (GGCT) similar to SR2 in the *TP53* gene. Smads 2/3 and 4 repress *c-MYC* transcription by associating with E2F-4 or -5 and retinoblastoma-like (RBL)-1/p107 (thereafter p107) to form a complex in the cytoplasm that is recruited to the c-MYC promoter upon TGF-β stimulation (Chen et al., 2002). Indeed, endogenous E2F-4 and p107 specifically associate with the SR2 element in the p53 promoter before and after TGF-β1 stimulation (Figure 7A). This suggested that a conditional corepressor complex was preassembled on the p53 promoter and responsive to subsequent TGF-β1 signaling. Next we depleted E2F-4 protein from MCF10A cells by shRNA expression (Figure 7B and Figure S7A). Quantitative E2F-4 depletion (shRNAs A6, A10) not only significantly impaired TGF-β1-imposed repression of the p53 promoter but also downregulated basal p53 transcription in the absence of TGF-β1 (Figure 7C, Figures S7B and S7C). Thus, E2F-4 normally functions as an activator of basal *TP53* transcription, but upon TGF-β1 signaling it switches to a Smad corepressor to negatively regulate the p53 promoter.

We then analyzed interaction of Smad-containing complexes with the SR2 sequence in Smad 4- or E2F-4-depleted cells by ChIP. Loss of Smad 4 had no effect on E2F-4 binding to the p53 promoter (Figure 7D); however, both Smad 4 and E2F-4 were necessary to recruit P-Smad 2 to the SR2 region upon TGF-β1 induction (Figure 7E). Furthermore, E2F-4 was specifically required for recruitment of P-Smad 2 to the *TP53* gene but not to the *PAI-1* locus, whereas Smad 4 was necessary for

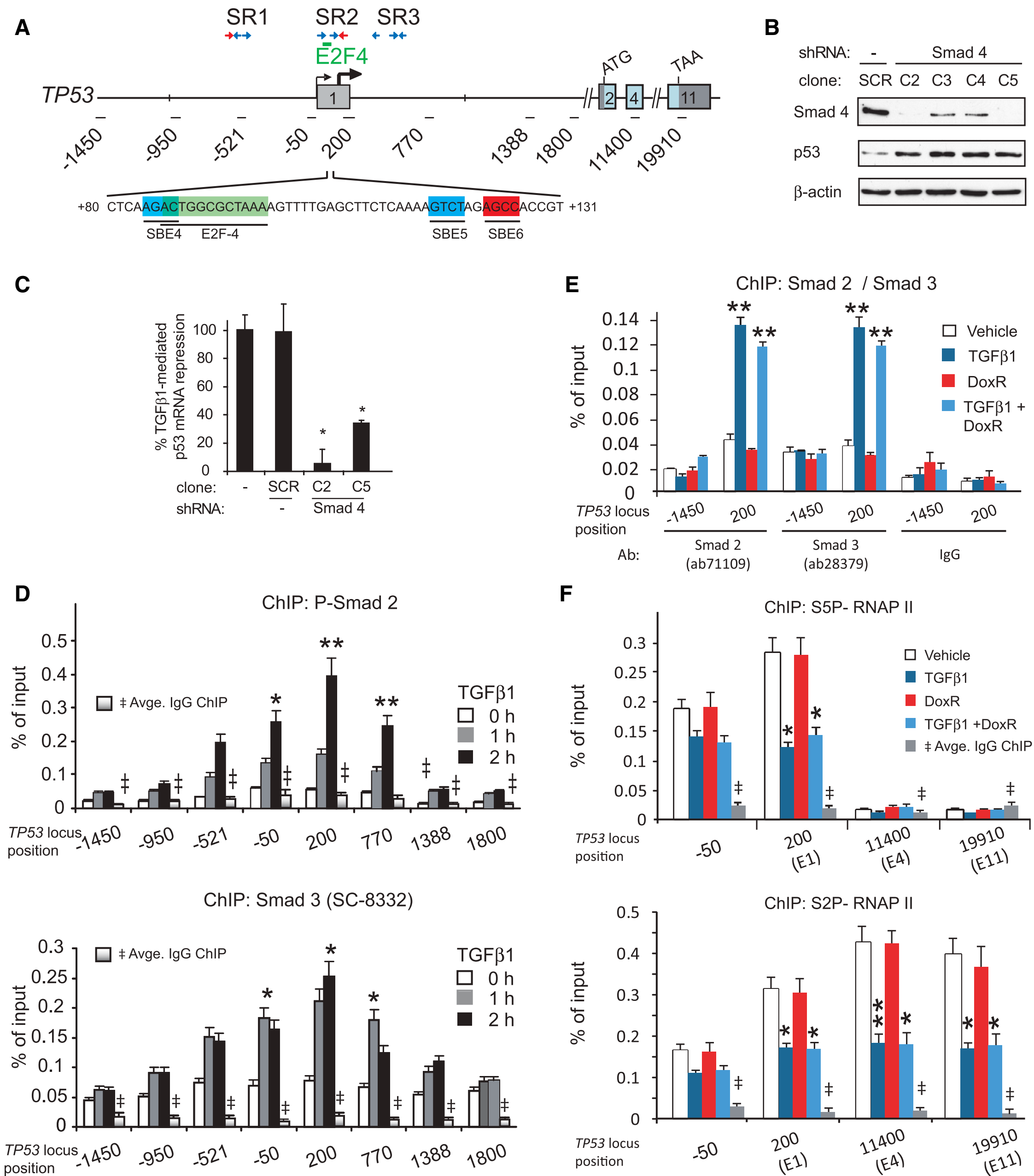

Figure 5. Smad 2/3-4 Complexes Associate with the p53 Promoter and Mediate TGF-β1 Repression

(A) Diagram of the human *TP53* locus (gray, 5′UTR; blue, coding sequence) showing the first annotated transcription start site (arrow). Putative Smad binding regions (SR1–3) containing canonical (blue arrows) or imperfect (red arrows) SBEs are indicated. The E2F-4 binding sequence that overlaps an SBE in SR2 is shown in green. Positions of ChIP amplicons within the *TP53* locus are also specified.

(B) Western blot analysis of Smad 4 and p53 levels in MCF10A cells transduced with lentiviruses expressing shRNAs directed to Smad 4 mRNA or a scramble (SCR) sequence, after selection with puromycin.

(C) p53 mRNA repression by TGF-β1 (12 hr) in Smad 4-deficient cells (MCF10A-C2) compared to WT cells was determined by RT-qPCR. Data are presented as percentage of repression ± SEM.

(D–F) ChIP assays from MCF10A cells between 0 and 2 hr (D) or after 2 hr (E and F) of treatment using the indicated antibodies specific for the following: (D and E) phosphorylated Smad 2 or Smad 3, (F) phospho (P)-Ser5 RNAPII, P-Ser2 RNAPII. Normal nonimmune IgG controls were included, and individual or averaged results for the different treatments are shown. Results are shown as percentage of input DNA ± SEM of four IPs from two independent experiments. See also Figure S6.

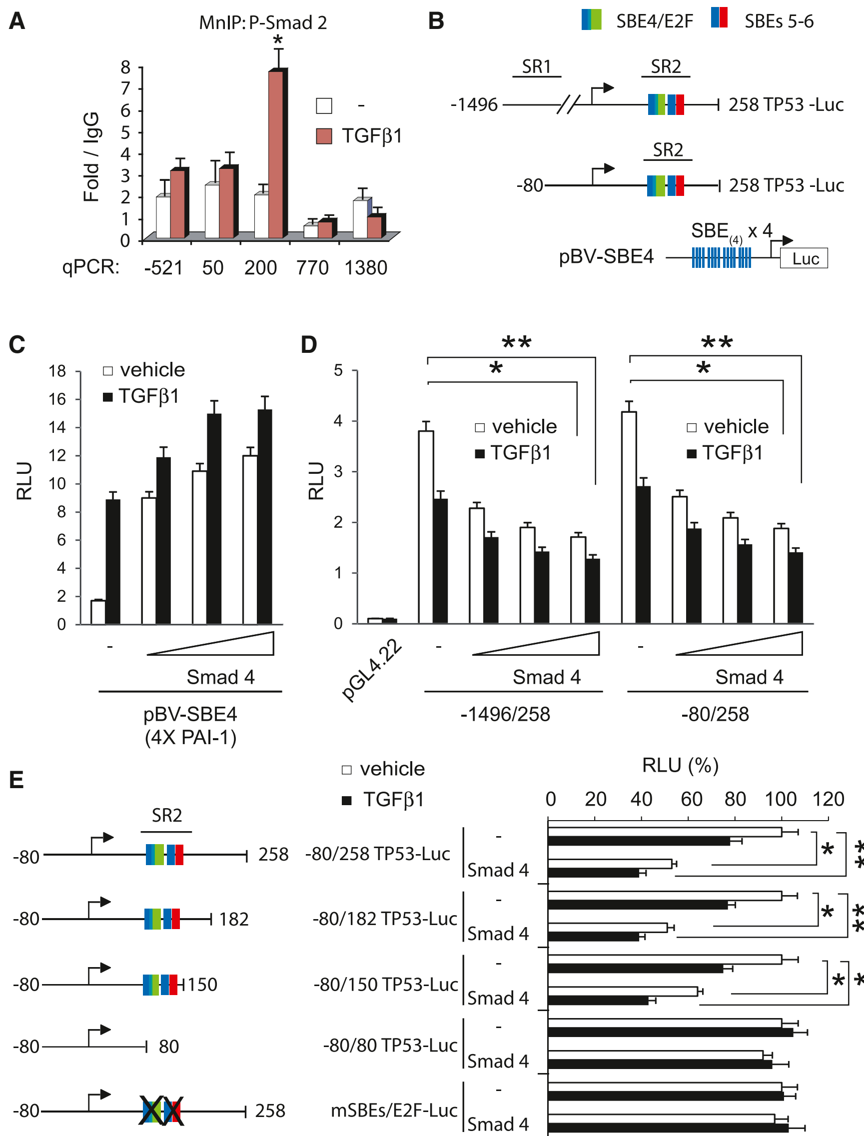

Figure 6. TGF-β1/Smad Signaling Directly Represses *TP53* Gene Transcription through the SR2 Region

(A) MnIP assay. Mononucleosomes prepared from MCF10A cells treated with TGF-β1 or vehicle were immunoprecipitated with an anti-P-Smad 2 antibody. The amount of indicated IP-enriched DNA sequences is expressed as fold of percentage enrichment ± SEM relative to the control IP with normal IgGs.

(B) Diagram of the long/short p53 promoter-luciferase reporter plasmids. The control plasmid, pBV-SBE4-Luc, contains four tandem repeats of a 30 bp concatamer of four SBEs from the PAI-1 promoter.

(C and D) Gene reporter assays. MCF10A cells were cotransfected with the indicated reporter plasmids plus identical increasing amounts of an expressing Smad 4 plasmid and treated with TGF-β1 for 6–8 hr. Normalized luciferase activity ± SEM in cell extracts is indicated as relative light units.

(E) Extended deletion/mutation analysis of the p53 promoter in gene reporter assays was performed as described with deletions from +258 to +80 or mutations in the SBEs and the E2F binding site. Normalized relative light units are indicated as the percentage activity ± SEM relative to the WT short p53-luciferase reporter.

See also Figure S6.

P-Smad 2 binding to *PAI-1* (Figure 7E and Figure S7D). Thus, association of E2F-4 with the p53 promoter precedes Smad activation by TGF-β1 and does not require the presence of nuclear activated Smad complexes. These results indicate that E2F-4 is part of a pre-existing, transcription-engaged, Smad-independent complex with a dual role as either an activator or a TGF-β1-induced corepressor and serves as an interface between Smads and *TP53* gene activity.

Given that TGF-β1 impairs the stress response at both the transcriptional and translational levels, we examined whether this would result in enhanced survival of MCF10A cells treated with either DoxR or Paclitaxel. A comparison of the drug concentration required to inhibit cell growth by 50% (IC_{50}) revealed that exposure of cells to TGF-β1 increased resistance to both DoxR (ΔIC_{50} = 2-fold) and Paclitaxel (ΔIC_{50} = 8-fold), resulting in a greater number of surviving damaged cells (Figure 7F, upper panels). Importantly, depletion of Smad 4 in MCF10A-C2 cells abolished the enhanced drug resistance and cell survival to both drugs, indicating that Smad-mediated transcriptional downregulation of the stress response plays a central role in the ability of TGF-β1 to interfere with DNA damage-induced cell death. Together, our results indicate that TGF-β1/Smad signaling has a protective role against elimination of damaged cells, which is achieved, at least in part, through transcriptional and translational repression of the *TP53* gene (Figure 7F, lower panels).

DISCUSSION

We show that cellular signaling by the pleiotropic cytokine TGF-β1 interferes with the stress response through coordinate transcriptional and translational repression of p53, which impacts cell-fate decisions upon stress (Figure 7G). We propose that TGF-β/Smad signaling can attenuate many downstream events in the p53-mediated DNA damage response in both precancerous and tumorigenic cells that retain WT p53.

Crosstalk between the DNA Damage Response and TGF-β Signaling in Cancer Cells

Crosstalk between TGF-β and p53 plays a pivotal, albeit complex, role in tumorigenesis and tumor progression. We focused

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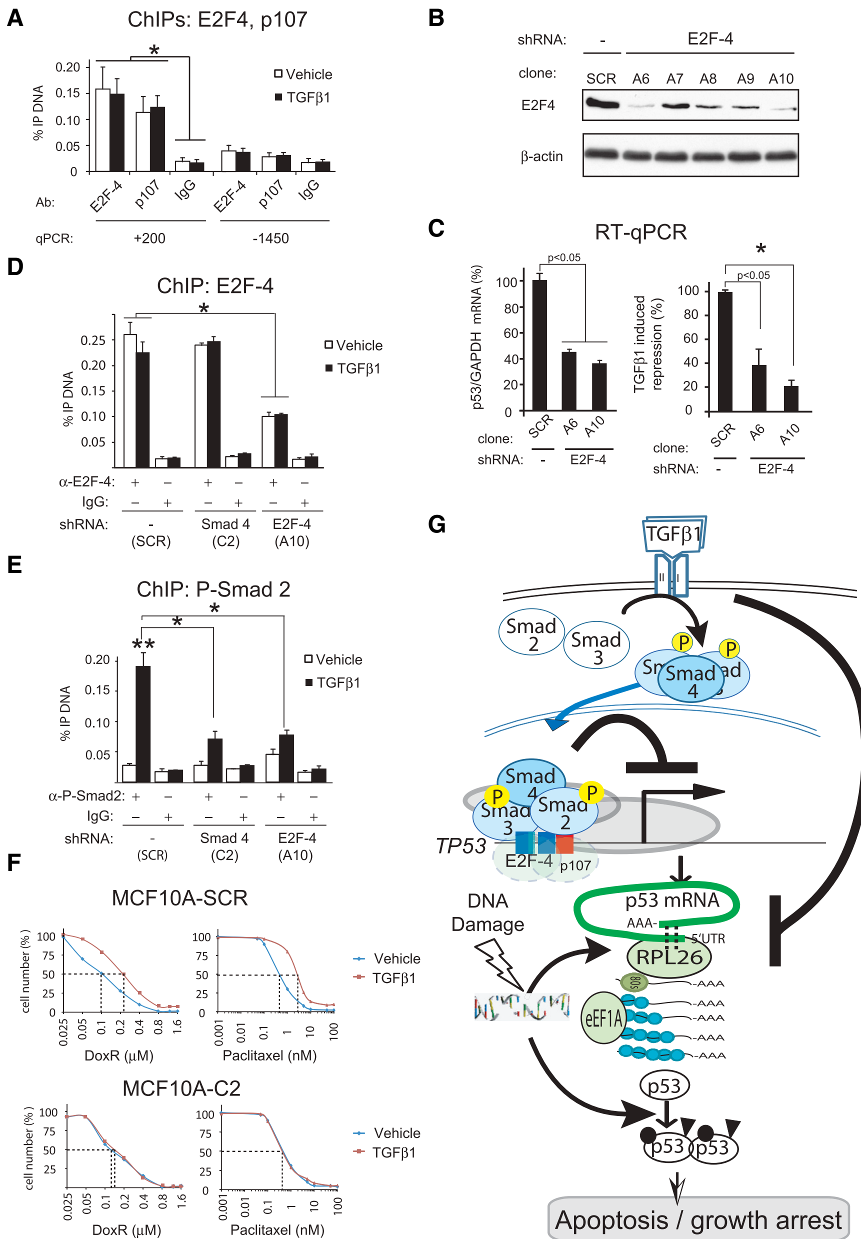

Figure 7. TGF-β1-Activated Smads Are Recruited by Chromatin-Bound, Preassembled E2F-4/p107 Complexes to Repress the *TP53* Gene and Impair Cell Death

(A) ChIP assays were performed on MCF10A cells after treatment with TGF-β1 or vehicle for 2 hr using antibodies specific to E2F-4 and p107 or normal IgGs. Enrichment of the SR2 region (centered at +200) of the p53 promoter or a control sequence (at −1450) is shown as percentage of input DNA ± SEM.

(B) Western blot analysis of E2F-4 depletion of MCF10A cells. Cells were transduced with lentiviruses expressing E2F-4-specific shRNAs or a scramble (SCR) sequence and selected for 2 days with puromycin.

(C) RT-qPCR analysis in E2F-4-deficient cells of p53 mRNA (left) and its repression by TGF-β1 compared to that seen in WT cells (right). Data are shown as the means ± SEM.

(D and E) Association of E2F-4 (D) and P-Smad 2 (E) with the SR2 region of the *TP53* gene in cells depleted of Smad 4 (MCF10A-C2) or E2F-4 (MCFA10A-A10) was analyzed by ChIP as described in (A). Data are presented as percentage of input DNA ± SEM.

(F) The protective effect of TGF-β against drug toxicity 4 days after addition of DoxR or Paclitaxel was evaluated in control or Smad 4-depleted cells. Cell number percentage ± SEM was determined by crystal violet staining. IC_{50} under each condition is indicated by dashed lines.

(G) Schematic representation of how TGF-β1/Smads attenuate the DNA damage response by downregulating p53 transcription and translation. This survival mechanism may facilitate progression of transformed cells through distinct stages of cancer.

See also Figure S7.

on the intersection between TGF-β and p53 specifically during DNA damage when p53 has a critical function in directing the stress response. In human precancerous and tumorigenic mammary and lung cells, but not normal primary HMECs, we revealed a direct antagonistic crosstalk in which TGF-β protects cells from the p53-mediated DNA damage response. By repressing *TP53* transcription and translation, TGF-β1 very effectively lowers cellular levels of p53 protein before and after stress, a process conserved in various precancerous and malignant (wild-type and mutant p53) human cells.

Certainly, the disparities that exist among published reports may reflect the complex nature of TGF-β and p53 pathway interactions that are influenced not only by tissue- and cell-stage specificity but also by the experimental paradigm used in each study. We found that TGF-β-p53 cooperation is absent in various cancer cell lines, which corresponded with an inverse correlation between TGF-β signaling and expression of p53 and the apoptotic marker PUMA in subsets of human breast tumors.

Coordinate Transcriptional and Translational Control of p53 by TGF-β

Long-term functional abrogation of p53 is a hallmark of many cancers and mainly occurs by mutations in the coding sequence or degradation rather than by decreasing p53 protein synthesis. In contrast to HDM2/HDMX-mediated posttranslational deregulation of p53 levels, transcriptional or translational control of p53 gene expression as a mechanism to evade WT p53 signaling in cancer is poorly understood. Several studies have reported the existence of potential regulatory elements in the p53 promoter.

Yet only a few have shown that transcriptional downregulation of p53 mRNA synthesis impedes p53-mediated responses in tumorigenesis (Saldaña-Meyer and Recillas-Targa, 2011).

Elegant studies by Kastan and colleagues demonstrated the significance of RPL26 in mediating p53 mRNA translational induction in stressed cells by interacting with the 5′UTRs and 3′UTRs of p53 mRNA (Chen and Kastan, 2010; Takagi et al., 2005). Translational induction of p53 is CAP and poly(A) independent, which suggests that it may occur at the level of elongation. This raised the possibility that control of p53 translation may be a potential target for malignant transformation, which had not been previously demonstrated. TGF-β1 is known to regulate mRNA translation during tumorigenesis, generally by repressing elongation while stimulating translation of select EMT-related genes (Chaudhury et al., 2010; Hussey et al., 2011; Lin et al., 2010). Indeed, our results reveal opposite effects of TGF-β1 on p53 and Smad4 interaction with RPL26 and the elongation factor eEF1A as well as accumulation of p53 mRNA in monosomes.

The ability of TGF-β1 to coordinately deregulate p53 by directly targeting both transcription and translation was unexpected. Importantly, TGF-β1-signaling is dominant over stress-activated transcription and translation of p53 (Spriggs et al., 2010; Takagi et al., 2005) while simultaneously preventing stress-imposed downregulation of Smad levels and PARP-1 cleavage.

A Transcription-Competent, p53 Promoter-Bound E2F-4/p107 Scaffold Switches to a Smad-Dependent Corepressor upon TGF-β Signaling

The E2F-4 binding site within the *TP53* gene, which is also recognized by E2F1 and E2F6 (data not shown), constitutes a functionally composite E2F/Smad recognition sequence. Curiously, repression of the c-MYC promoter by TGF-β1 was shown to be mediated by a composite E2F/SBE sequence and to require the interaction of Smads 3/4 with E2F-4/5 and p107/DP1 complexes in the cytoplasm. These complexes only associate with the c-MYC promoter after TGF-β1 induction (Chen et al., 2002). Thus, our study has an intriguing difference in the mechanism of Smad-mediated repression of the *TP53* gene compared to *c-MYC*. Although Smad recruitment to each promoter is dependent upon TGF-β signaling, the E2F-4/p107 complex is already assembled on the p53 promoter before stimulation, unlike the situation with c-MYC. While E2F-4 is considered to be a transcriptional repressor, it does contain a transactivation domain, as does E2F1-5 (Rowland and Bernards, 2006). We hypothesize that E2F-4/p107 proteins form a transcriptionally permissive scaffold at the p53 promoter whose activity can be switched after associating with Smads 2/3 upon TGF-β1 signaling. This mature corepressor complex then triggers repression of *TP53* transcription and subsequently impairs downstream p53-mediated events (Figure 7).

Cooperation between p53/p63 Family Members and Smads

p53 family members, including ΔN /TA p63 isoforms, interact with Smads 2/3 and bind to a p53/p63-recognition sequence overlapping the Smad-binding element of the Activin-inducible Mix.2 promoter in mesoderm *Xenopus* cells. TGF-β/Smads can also activate their transcription program and cell-cycle arrest in p53 null/mutated epithelial cells (Chen et al., 2002; Datto et al., 1995). Importantly, Cordenonsi and colleagues showed that p53 was only required for Activin or TGF-β to induce cell-cycle arrest and gene expression if cells lacked p63 and p73, either naturally or through siRNAs. Notably, p63 is selectively expressed in basal epithelia in breast, prostate, and bronchia and most stratified epithelial cells (Di Como et al., 2002). This might explain why TGF-β1 reduces proliferation of basal-like MCF10A cells which express both p63 and p73 even after p53 depletion (Figure S1) but fails to induce growth arrest in MEFs and hematopoietic progenitors from $p53^{-/-}$ mice (Cordenonsi et al., 2003).

Mutant p53 proteins disrupt the balance between Smads and ΔNp63/TAp63-mediated transcription to enhance the metastatic potential of TGF-β (Adorno et al., 2009). In studies using murine and *Xenopus* embryos, the only p53 isoform identified as a Smad 2 mesoderm coinducer was the p53 splicing variant p53AS. Paradoxically, p53AS lacks the C-terminal domain that is rapidly acetylated by CBP/p300 upon DNA damage in human cells (Vousden and Prives, 2009). Altogether, these data suggest that in human epithelial cells it is more likely that p53 family members such as p63, rather than p53 itself, normally cooperate with TGF-β signaling. It is possible that in mammary epithelial cells TGF-β might repress p53 while still cooperating with p63 to induce TGF-β-related phenotypes.

A Molecular Link between TGF-β1, Cell Survival, Cancer Progression, and Drug Resistance

In breast cancers, analyses of a large cohort of tumors have shown that low p53 mRNA levels correlate with decreased p53 signaling, reduced therapeutic response, and poor prognoses (Miller et al., 2005). Our work also demonstrates that *TP53* gene expression is deregulated in primary breast tumors. Mechanistically, we observed that TGF-β1/Smad signaling represses p53 mRNA levels in precancerous and metastatic human cell lines in both unstressed and DNA damaged cells. p53 repression by TGF-β1 also occurs in immortalized (nontumorigenic) bronchial epithelial cells, highly metastatic NCI-H460 lung cancer cells (Figure 2H), and keratinocyte-derived HaCaT cells (Figure S6), which extends the implications of our findings beyond breast tumors. Interestingly, TGF-β1 signaling represses expression of both WT and mutant *TP53* genes (Figure 2, Figure S6). Since mutant p53 proteins often acquire a gain of function (Goh et al., 2011), TGF-β might influence the spectrum of p53-dependent activities, both positive and negative, in tumors that contain such mutants.

Studies with genetically modified mice have shown that TGF-β1 signaling suppresses apoptosis while increasing the invasiveness and metastatic potential of ErbB2-expressing primary tumors (Muraoka-Cook et al., 2006; Muraoka et al., 2002). Moreover, blocking TGF-β signaling improved the effectiveness of chemotherapeutic drugs in cancer cells and murine models of breast cancer (Barcellos-Hoff and Akhurst, 2009; Biswas et al., 2007). Our data support these observations and provide a mechanistic basis by which to understand the inhibitory interaction between TGF-β and DNA damage in breast cancer cells both in vivo and in vitro (reviewed by Barcellos-Hoff and Akhurst,

2009). In agreement with these reports, we found that TGF-β protects cells not only from DoxR but also from 5-Fluorouracil and Paclitaxel-induced cell death specifically though Smad 4-mediated complexes (Figure 3 and Figure 7). During the course of our studies, TGF-β1 signaling was shown to mediate MED12-induced resistance to cisplatin and various tyrosine kinase receptor inhibitors in several cancer cell types (Huang et al., 2012). Although the mechanism was not elucidated in this report, we anticipate that our findings may be generally applicable to other biological contexts that display TGF-β1-mediated drug resistance.

Interestingly, although in certain cell types TGF-β can induce apoptosis, in most epithelial cells, including mammary cells, TGF-β induces cell-cycle arrest and EMT rather than apoptosis (Massagué, 2008; Pardali and Moustakas, 2007). Our study provides mechanistic insights to explain how cancer progression may be facilitated by the ability of TGF-β1 signaling to promote survival of damaged cells. Moreover, our data indicate that TGF-β can become a tumor promoter by impeding the p53 pathway very early in tumorigenesis (vHMECs, MCF10A), but not in normal cells (HMECs). On this basis, one may predict that breast tissue containing both normal HMECs and slightly abnormal vHMECs will undergo very different cell-fate outcomes when exposed to stress in a TGF-β-rich microenvironment, with facilitated elimination of damaged normal cells but enhanced survival of abnormal cells by the mechanisms that we have deciphered. In this scenario, tissue heterogeneity can be understood in part by the distinct programmed stress responses of normal and abnormal cells in a TGF-β-rich or -poor microenvironment.

EXPERIMENTAL PROCEDURES

Human Tissues, Cells, Lentiviral Transductions, and Drug and TGF-β1 Treatments

Human tissues were obtained from the UCSF Cancer Center, from the Cooperative Human Tissue Network Western Division (Nashville, Tennessee), and from the Innsbruck Medical University, Austria, in compliance with approved protocols by Institutional Review Boards. Primary cultures of HMECs and vHMECs were derived from healthy human donors by reduction mammoplasty. shRNA-expressing cells were generated by lentiviral transduction and selected with puromycin or GFP-based cell sorting. Puromycin was removed from the media 16–24 hr before treatment with TGF-β1 (Peprotech or in-house purified) or vehicle buffer.

Luciferase Reporter Assays

MCF10A cells were transfected using Fugene HD reagent (Roche) with luciferase reporter plasmids containing the p53 promoter, different amounts of the pLV-Smad-HA plasmid, and a Renilla-Luciferase reporter. After 16 hr, cells were treated with TGF-β1 (5 ng/ml) for an additional 6–8 hr, and luciferase activity was determined with the dual-luciferase system (Promega).

ChIPs, MnIP, and RIPs

ChIPs were performed essentially as described with minor modifications (Gomes et al., 2006). Mononucleosomes were obtained using the ChIP-IT-enzymatic kit (Active Motif) with minor modifications, and IPs were performed as for ChIPs. For RIPs, extracts from 5×10^7 MCF10A cells were prepared and incubated with specific antibodies (Table S3) using the Magna RIP kit (Millipore).

Analyses of Cell-Cycle Distribution and Apoptosis

Cell-cycle distribution assays of MCF10A cells were performed using the Watson distribution model as described (Gomes et al., 2006). Apoptotic index assays were performed as in Gomes et al. (2006) using Alexa Fluor 350-labeled Annexin V (Molecular Probes). Cells were sorted in a Becton Dickinson LSR instrument and analyzed by FlowJo software.

Statistical Analysis

Unless specified otherwise, all data are presented as the mean values ± SEM from at least three independent experiments; two-sided t tests assuming unequal variance were used to test the relationships between the means of data sets, and p values indicate the probability of the means compared being equal with $*p < 0.01$ and $**p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2013.04.029.

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