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Interplay between the tumor suppressor p53 and TGFβ signaling shapes embryonic body axes in *Xenopus*

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SUMMARY

The transcription factor p53 has been shown to mediate cellular responses to diverse stresses such as DNA damage. However, the function of p53 in cellular differentiation in response to growth factor stimulations has remained obscure. We present evidence that p53 regulates cellular differentiation by modulating signaling of the TGFβ family of growth factors during early *Xenopus* embryogenesis. We show that p53 functionally and physically interacts with the activin and bone morphogenetic protein pathways to directly induce the expression of the homeobox genes *Xhox3* and *Mix.1/2*. Furthermore, functional knockdown of p53 in embryos by an antisense morpholino oligonucleotide reveals that p53 is required for the development of dorsal and ventral mesoderm. Our data illustrate a pivotal role of interplay between the p53 and TGFβ pathways in cell fate determination during early vertebrate embryogenesis.

Key words: Axis formation, TGFβ, p53, *Xenopus*, Embryogenesis

Supplemental data available online

INTRODUCTION

The TGFβ signaling pathway plays essential roles during embryogenesis (Massagué and Chen, 2000; Hill, 2001; Muñoz-Sanjuán and Hemmati-Brivanlou, 2001; Whitman, 2001). Members of the TGFβ family exert their biological functions by binding to two types of transmembrane receptors, type I and type II, that encode a serine/threonine kinase in the intracellular domain. Upon ligand binding, the type II receptor phosphorylates the type I receptor, which subsequently activates members of the Smad family of intracellular signal transducers in the cytosol. Ligands of the TGFβ family are subdivided into a few groups and each group activates different sets of the receptor-regulated Smad (R-Smad). Activin/TGFβ signals through Smad2 and Smad3, whereas bone morphogenetic proteins (BMPs) use Smad1, Smad5 and Smad8. Smad4, also known as the common-partner Smad (Co-Smad), forms a heteromeric complex with the R-Smads in response to ligand stimulation and is commonly used by both activin/TGFβ and BMPs. Upon ligand-induced complex formation, Smads translocate to the nucleus where they function as transcriptional activators or repressors to regulate gene expression. In the frog *Xenopus laevis*, activin directly activates the expression of the homeobox genes *goosecoid* and *Mix.1*, in addition to its close relative *Mix.2*, through binding of a complex containing Smad2, Smad4 and the transcription factor FAST-1 to an activin-responsive element (ARE) in their promoter (Chen et al., 1997; Labbé et al., 1998; Hill, 2001). During early stages of *Xenopus* development, activin-like signals emanating from the vegetal region of the egg are required for inducing mesodermal tissues in the overlaying ectoderm (Harland and Gerhart, 1997; Gurdon and Bourillot, 2001). BMP4 expressed in the ventral side at the gastrula stage has a ventro-posteriorizing activity that converts mesoderm induced by activin-like signals to form ventral and posterior mesoderm such as blood (Dale et al., 1992; Jones et al., 1992; Hemmati-Brivanlou and Thomsen, 1995).

Homeobox genes are not only induced by TGFβ ligands but also play a pivotal role in the regional specification of cell fates during development. The even-skipped-like homeobox gene *Xhox3*, which is responsive to both activin and BMPs, is expressed in ventral and posterior mesoderm during gastrulation and functions as a ventro-posteriorizing factor (Ruiz i Altaba and Melton, 1989a; Ruiz i Altaba and Melton, 1989b; Ruiz i Altaba et al., 1991; Dale et al., 1992; Jones et al., 1992). *Mix.1* was initially isolated as an immediate early response gene to activin, and its expression was detected in endoderm and mesoderm (Rosa, 1989). Moreover, Mix.1 has been proposed to function in the BMP pathway as BMP4 induces the expression of *Mix.1* and requires functional Mix.1 to cause ventro-posteriorization of embryos (Mead et al., 1996).

The *p53* gene is a tumor suppressor gene that is most frequently mutated or inactivated in a wide range of human tumors (Levine, 1997; Prives and Hall, 1999; Vogelstein et al., 2000). p53 protein functions as a sequence-specific transcription factor and its tumor suppressor function is attributed to its ability to regulate gene expression. Several p53 target genes mediating p53-induced responses have been reported, which include the cell-cycle inhibitor *p21/WAF* as well as the growth and differentiation factor inhibitors *IGF-BP3* and *Dkk1* (El-Deiry et al., 1993; Buckbinder et al., 1995; Wang et al., 2000). The transcriptional regulation of genes involved in growth factor signaling suggests that p53 has a role in cell differentiation processes. In fact, it has been shown that overexpression of dominant-negative forms of human p53 or the p53 negative regulator dm-2 in *Xenopus* embryos affects terminal differentiation of neural and mesodermal tissues (Wallingford et al., 1997). However, p53 appears to be largely dispensable for normal development during mouse embryogenesis (Donehower et al., 1992), although a small proportion of p53 null mice develop defects in neural tube closure (Armstrong et al., 1995; Sah et al., 1995). Therefore, the precise function of p53 during vertebrate development and the mechanisms by which p53 regulates cellular differentiation remain largely unknown.

In this paper, we describe a novel embryonic function for the transcription factor p53. We demonstrate that p53 functionally and physically interacts with the intracellular signaling of the TGFβ pathway to regulate the expression of homeobox genes *Mix.1/2* and *Xhox3* directly in *Xenopus* embryos. Furthermore, we show that in vivo function of p53 is required for the development of mesoderm.

MATERIALS AND METHODS

Embryo manipulations

Preparation and injection of *Xenopus laevis* embryos was carried out as previously described (Suzuki et al., 1997a). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Dexamethasone (DEX), cycloheximide (CHX) and activin treatments were performed as described (Suzuki and Hemmati-Brivanlou, 2000). Antisense morpholino oligonucleotides were obtained from Gene Tools (Philomath, USA) and the sequence is as follows: xp53-MO, 5′-GCC GGT CTC AGA GGA AGG TTC CAT T-3′; 5mis-MO, 5′-GCg GGa CTC AGA cGA AGc TTg CAT T-3′.

Expression library screening and RT-PCR analysis

Capped RNA was synthesized from a *Xenopus laevis* gastrula library (Weinstein et al., 1998; Suzuki and Hemmati-Brivanlou, 2000), and injected in combination with noggin mRNA (200 pg) in the animal pole of two-cell embryos. Animal caps were isolated from blastulae and subjected to RT-PCR analysis at neurula stages as described (Wilson and Hemmati-Brivanlou, 1995) except that PCR cycles were increased by two or three more cycles to allow the detection of amplified products by ethidium bromide staining. Primers used in the RT-PCR were described previously (Suzuki and Hemmati-Brivanlou, 2000). Other primer sequences are as follows: *xp53*, 5′-GGG TTC ACT GTA AGA TAT GG-3′ and 5′-GGC TGG AGG GCA CTA TTA CC-3′; *Sox17*, 5′-CAG AGC AGA TCA CAT CCA ACC G-3′ and 5′-GGA AAG GAC AGA AGA AAT GGG C-3′; *Mix.1*, 5′-AAT GTC TCA AGG CAG AGG TT-3′ and 5′-AGA TAC AGG TAT CTG AGG GC-3′. Nucleotide sequence of a positive single clone (pDH105-xp53) was determined and deposited with GenBank (Accession Number AY221266).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Suzuki et al., 1997a). For bleaching of wild-type embryos, the hybridized embryos were treated with bleaching solution (0.5×SSC with 1% hydrogen peroxide and 5% formamide) under a fluorescent light.

Plasmids

xp53ΔRD, xp53:GR, xp53Nmut:GR, Myc-tagged xp53 and Myc-tagged xp53ΔRD were made by a PCR-based strategy. The PCR fragments were cloned into expression vectors pDH105 (a gift from R. Harland), pDH105-GRHA (a vector constructed from the pSP64T-GRHA vector) (Tada et al., 1997) or Myc-pcDNA3 (Yagi et al., 1999). xp53Nmut:GR was designed to have conservative mutations, refractory to translational inhibition by xp53-MO, downstream of the initiation methionine. xp53 (R255T), Mix.2 (Smad mut), Mix.2 (FAST mut), Mix.2 (p53 mut) reporter mutants were made by a PCR-based method (Sawano and Miyawaki, 2000). p53 (X3), a p53 reporter gene was constructed by cloning annealed double strand oligonucleotides containing the p53-binding sites found in the human *PA26* gene (Velasco-Miguel et al., 1999) into the *Otx* minimal promoter vector pGL3-HpOtxE (–139~+180) (Kiyama et al., 1998). pXeX-RL was made by cloning a *Pst*I/*Xba*I fragment from pRL-CMV (Promega) into pXeX (Johnson and Krieg, 1994) downstream of the *EF-1α* promoter. A *Mix.2* reporter gene, pGL3-Mix.2 [–0.367], is a gift from M. Watanabe (Chen et al., 1997; Watanabe and Whitman, 1999; Yeo et al., 1999). For FLAG-tagged human p53, pDH105-hp53 was constructed by cloning a *Bam*HI/*Xba*I fragment of pcDNA3flag-hp53 (a gift from Y. Taya) into pDH105. Other plasmids used for mRNA synthesis are pSP64T-activinβB (Thomsen et al., 1990), pSP64TBX-CA-ALK2 (Suzuki et al., 1997b), pDH105-Smad1, pDH105-Smad2 (Lagna et al., 1996), pSP64T-xE2F(1-88):GR (Suzuki and Hemmati-Brivanlou, 2000) and pSP64T-noggin (Smith and Harland, 1992). In vitro translation of synthetic mRNA was carried out using Speed Read lysate kit (Novagen) and SDS-PAGE was performed using standard methods.

Electromobility-shift assay (EMSA) and oligonucleotides for EMSA

Whole-cell extract was prepared from early gastrula embryos injected animally with appropriate mRNA as described (Germain et al., 2000). Binding reactions were performed in 30 μl of buffer containing 1 μg Herring DNA, 3 mM DTT, 0.03% BSA, 20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP-40, protease inhibitor cocktail (Roche), 5 μl extract, the appropriate ^{32}P-labeled double-stranded oligonucleotides and monoclonal anti-p53 antibody, Pab421 (Oncogene). It has been shown that Pab421 recognizes human p53 and facilitates the binding of p53 to DNA in EMSA assay (Hupp et al., 1992). Thus, we used human p53, instead of xp53, for EMSA assay in the presence of Pab421. For supershift, anti-FLAG M2 monoclonal antibody (Sigma) was added to the binding reactions before electrophoresis. In the case of competition experiments, embryo extract were pre-incubated with competitor oligonucleotides before the binding reaction.

Oligonucleotides

5′-CCA CAT CCC AGA CAA GTT CAC ACT TCA GAG CT-3′ (*Mix.2*-upstream)
5′-CTG AAG TGT GAA CTT GTC TGG GAT GTG GAG CT-3′ (*Mix.2*-downstream)
5′-CCA CAT CCC ACA AAA CTG CAC ACT TCA GAG CT-3′ (*Mix.2* mut-upstream)
5′-CTG AAG TGT GCA GTT TTG TGG GAT GTG GAG CT-3′ (*Mix.2* mut-downstream)

Luciferase assay

Ten animal caps or four whole embryos injected with appropriate mRNA and reporter plasmid were homogenized in 100 μl of 50 mM

Tris-HCl, pH 7.4 and centrifuged for 5 minutes at 4°C. Supernatant of lysate (10 μl) was used to perform luminescence measurement using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol at half-scale. As an internal control for luciferase assay, we used pXeX-RL, which contains *Renilla luciferase* (RL) cDNA under the control of *EF-1α* promoter.

Chromatin immunoprecipitation (ChIP)

Twenty animal caps were isolated at stage 9 from embryos injected with appropriate mRNA, cultured until sibling embryos reached stage 11 and crosslinked with 1% formaldehyde at room temperature for 20 minutes. After rinse with ice-cold 0.5×MMR twice, animal caps were incubated in 100 mM Tris-HCl, pH 9.0, 10 mM DTT for 30 minutes at 30°C and followed by steps described by Shang et al. (Shang et al., 2000). Primer sequences used in PCR are as follows: *Mix.2* (upstream), 5′-GGT CTA TAG ATC TAT GGA GTG TGC C-3′ and *Mix.2* (downstream), 5′-AGT GCT GCT CAG TTG ACT CAA TGA C-3′; *goosecoid* (upstream), 5′-CGT TAA TGT CCC ATC ACG CTC AAT G-3′ and *goosecoid* (downstream), 5′-TGC AGA CTG CAG TCC TCT CCC ATC T-3′. Nucleotide sequence of the PCR products was confirmed by automated sequence.

Cell culture and immunoprecipitation

COS-7 cells were transiently transfected with the indicated plasmids using FuGene6 transfection reagent (Roche) following the manufacturer's instructions. Immunoprecipitation and immunoblotting were performed as previously described (Yagi et al., 1999).

RESULTS

Isolation of *Xenopus* p53 as a posteriorizing factor

To gain insights into molecular mechanisms involved in the establishment of the anteroposterior axis, we performed an expression screening in which the anterior neural inducer noggin was co-expressed in *Xenopus* ectodermal explants (animal caps) with pools of mRNA synthesized from a gastrula stage library (Fig. 1A). After sib-selections of a positive pool and reverse transcription-polymerase chain reaction (RT-PCR) analyses for marker gene expressions, we isolated *Xenopus p53* (*xp53*) as a gene that transforms anterior neural tissue induced by noggin to posterior neural tissue. The nucleotide sequence of *xp53* is 98% identical to that of previously reported *Xenopus p53* (Hoever et al., 1994). The temporal and spatial distribution of xp53 mRNA has been reported to be ubiquitous from cleavage to tailbud stages (Tchang et al., 1993; Hoever et al., 1994) and we have confirmed this by RT-PCR using RNA from staged whole embryos and dissected parts of gastrula stage embryos (not shown). Injection of xp53 mRNA with noggin mRNA induced the expression of posterior neural markers such as *En2* (mid-hindbrain boundary), *Krox20* (hindbrain) and *HoxB9* (spinal cord) in a dose-dependent manner, while explants from embryos injected with noggin mRNA alone induced the expression of the forebrain marker *Otx2* (Fig. 1B). The posteriorizing effect of xp53 could be direct because we observe little or no induction of the dorsal mesodermal marker *muscle actin*. However, we do not rule out the possibility of indirect posteriorization via mesoderm formation because we found that overexpression of xp53 alone in animal caps is capable of inducing several mesodermal markers (Fig. 2). For this reason, we focused our subsequent studies on the role of xp53 in mesoderm formation during embryogenesis.

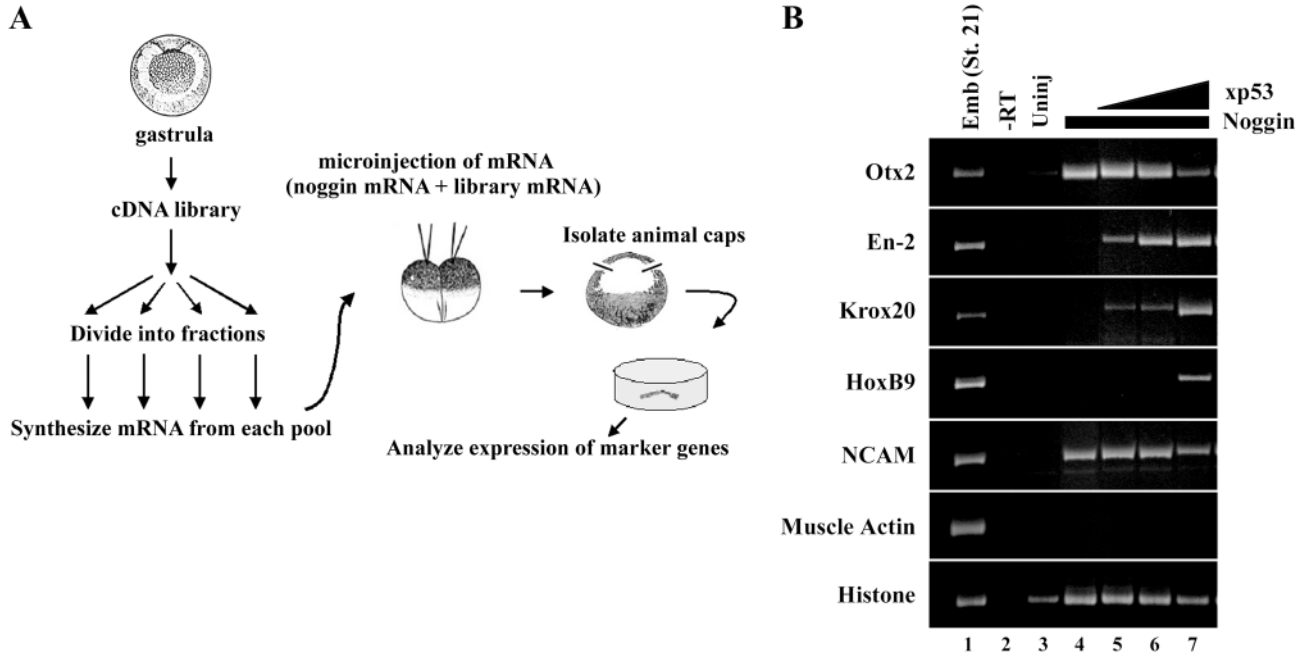

Fig. 1. Isolation of *Xenopus p53* gene as a posteriorizing factor. (A) Expression screening strategy. A gastrula expression library was divided into fractions and mRNA was synthesized from each pool. The synthetic library mRNA was injected in combination with noggin mRNA (200 pg), an anterior neural inducer, in the animal pole of two-cell stage embryos and ectodermal explants (animal caps) were isolated at blastula stages. The positive pools were identified by analyzing the expression of posterior neural marker genes by RT-PCR. (B) Animal caps injected with different amounts of xp53 mRNA (0-400 pg) and noggin (200 pg) mRNA were subjected to RT-PCR at neurula stages (stage 21). '-RT' indicates sibling control embryos processed without reverse transcriptase. 'Uninj' indicates uninjected caps. *Histone* was used as a loading control.

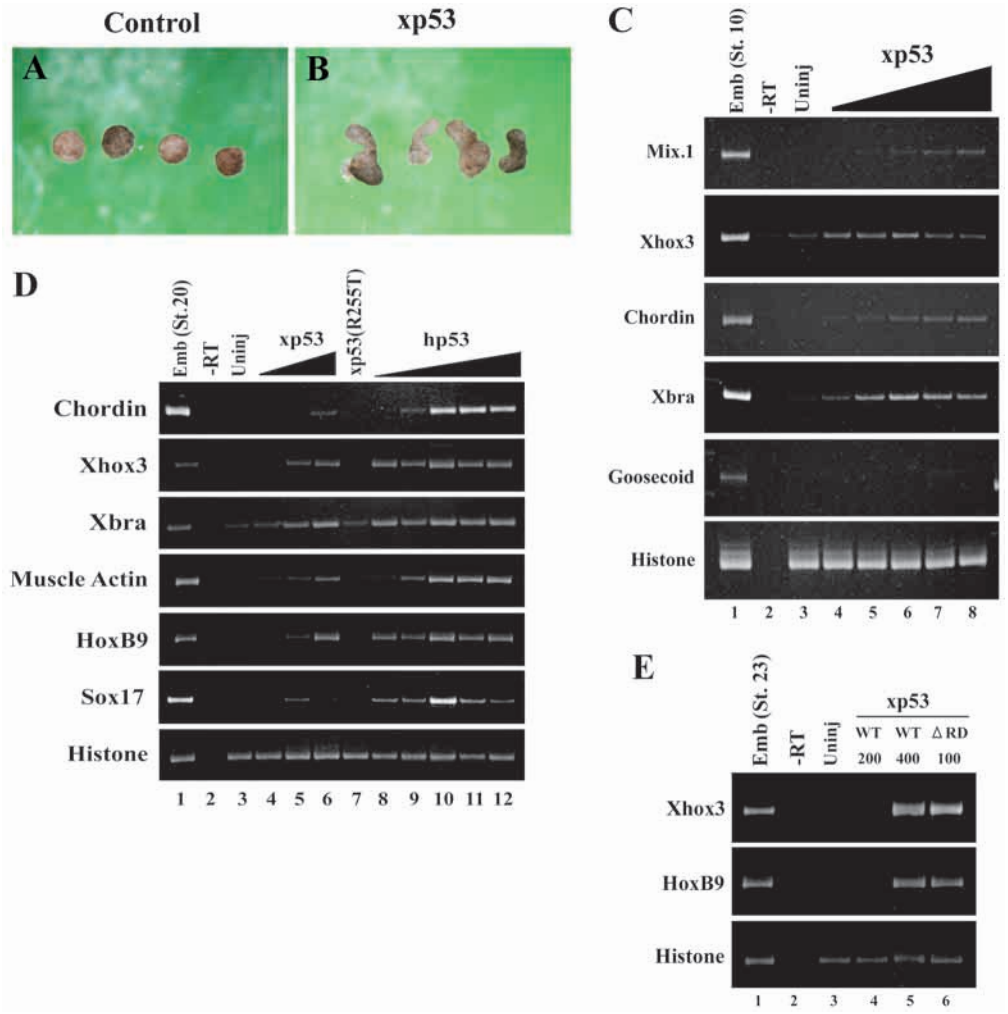

Fig. 2. Overexpression of xp53 induces mesodermal and endodermal markers. (A,B) Overexpression of xp53 causes elongation of animal caps. Uninjected (A) and xp53 mRNA (400 pg)-injected (B) animal caps were photographed at early neurula stage. (C,D) *Xenopus* and human p53 induce mesodermal and endodermal markers. Animal caps injected with different amounts of p53 mRNA were collected at early gastrula (stage 10; C) and neurula (stage 20; D) stages, and expression of marker genes was analyzed by RT-PCR. Injected mRNAs are 0.1-1.0 ng of xp53 (C; lanes 4-8), 0.2-0.8 ng of xp53 (D; lanes 4-6), 0.8 ng of xp53 (R255T) (D; lane 7) and 0.05-0.8 ng of human p53 (D; lanes 8-12). Although human p53 induced marker gene expression more efficiently than xp53, this is likely to be due to the more efficient translation of human p53, as revealed by in vitro translation (data not shown). (E) Deletion of the C-terminal regulatory domain (RD) enhances xp53-mediated transcription. The ability of wild type and Δ RD mutant of xp53 to induce marker genes was compared by animal cap assays. Note that 100 pg of xp53 Δ RD induces *Xhox3* and *HoxB9* genes to an extent similar to that obtained by 400 pg of wild-type xp53 (lanes 5 and 6).

xp53 activates mesodermal and endodermal gene expression

In order to determine the function of xp53, we expressed xp53 in animal caps and analyzed the expression of tissue-specific marker genes. As shown in Fig. 2, xp53 overexpression caused the explants to elongate (Fig. 2B) and to express a variety of endodermal and mesodermal genes at both early gastrula and neurula stages (Fig. 2C,D, respectively), which include *Mix.1* (mesoderm and endoderm), *Xhox3* (ventro-posterior mesoderm and posterior ectoderm), *chordin* (dorsal mesoderm), *Xbra* (pan mesoderm), *muscle actin* (paraxial mesoderm), *HoxB9* (lateral plate mesoderm) and *Sox17* (endoderm). The marker genes induced range from dorsal to ventral types for mesoderm with the exception that the dorsal mesodermal marker *goosecoid* is not induced. The induction of markers are specific to functional xp53 because its inducing ability was dependent on the intact DNA-binding domain, as indicated by the failure of marker gene activation by xp53 (R255T), which carries an amino acid substitution from arginine to threonine at position 255 within the DNA-binding domain (Fig. 2D, lane 7). A human p53 mutation corresponding to the xp53 (R255T) [hp53 (R280T)] has been reported in human cancer and is proposed to function as a dominant-negative mutant (Sun et al., 1992). The gene expression profile exhibited by human p53 is similar to that observed for xp53, suggesting the presence of a conserved function of the *p53* gene during evolution to regulate early embryonic development. Moreover, a xp53 mutant lacking the regulatory domain in the C terminus (xp53 Δ RD, Fig. 3A) showed an elevated activity compared to that obtained by wild type (Fig. 2E, lane 6). The level of protein expression of xp53 Δ RD examined by in vitro translation was comparable with wild-type xp53 (not shown). It has been proposed that the deletion of the regulatory domain mimics an in vivo process of human p53 activation through post-translational modifications at the C terminus (Hupp et al., 1992; Hupp et al., 1995). Therefore, these results suggest that xp53 is able to activate mesodermal and endodermal gene expressions via mechanisms that are similar, at least in part, to those of mammalian p53 during early development.

In order to identify direct target genes for xp53, we searched for genes induced by xp53 without the need for de novo protein synthesis. For this purpose, we made use of a xp53:GR fusion protein that can be activated by dexamethasone (DEX) (Fig. 3A). We confirmed that overexpression of xp53:GR in animal caps followed by DEX treatment led to activation of marker genes similar to those observed for wild-type xp53 (Fig. 3B, lane 8). At the early gastrula stage, we found that the homeobox genes *Xhox3* and *Mix.1* as well as *chordin* were induced by xp53:GR even in the presence of cycloheximide

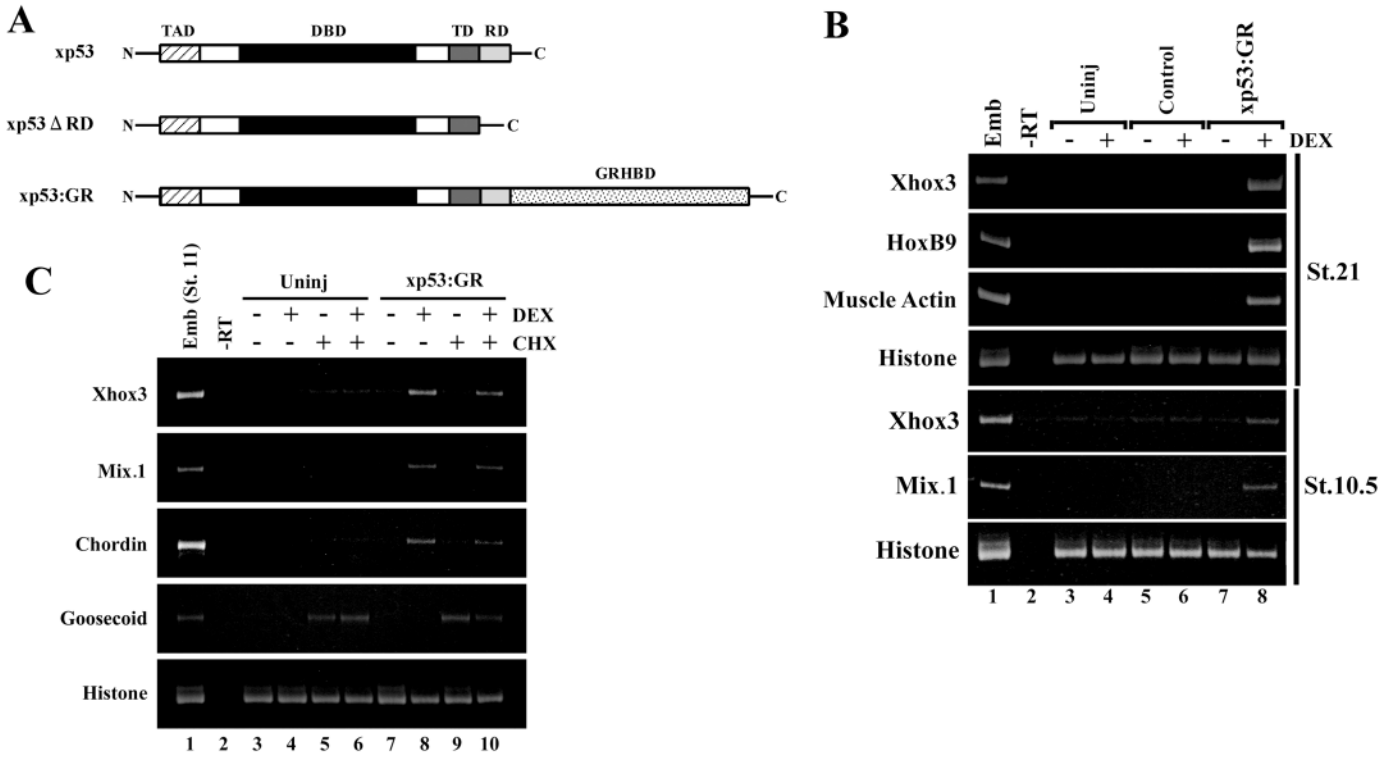

Fig. 3. xp53 does not require de novo protein synthesis to induce *Xhox3* and *Mix.1*. (A) Schematic representation of xp53 constructs used in this study. xp53 contains an N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a tetramerization domain (TD) and a C-terminal regulatory domain (RD). xp53:GR contains the hormone binding domain of the glucocorticoid hormone receptor (GRHBD) at the C terminus. (B) Conditional activation of xp53:GR by dexamethasone (DEX). Animal caps expressing xp53:GR were prepared at blastula stages (stage 9) and cultured in the absence or presence of 20 μM DEX. The expression of molecular markers was detected by RT-PCR at the stages indicated. Animal caps expressing xE2F(1-88):GR are used as a control for expression of GRHBD (Suzuki and Hemmati-Brivanlou, 2000). (C) xp53 does not require de novo protein synthesis to induce target gene expression. Animal caps expressing xp53:GR were treated with cycloheximide (CHX) for 30 minutes, and then transferred into medium containing both CHX and DEX to activate xp53:GR. The expression of marker genes was detected by RT-PCR after 3 hours of DEX treatment (equivalent to stage 11).

(CHX) (Fig. 3C, lane 10). CHX treatment alone was sufficient to induce the expression of *goosecoid* as previously reported (Cho et al., 1991), ensuring the efficacy of the CHX treatment (Fig. 3C, lanes 5, 6, 9 and 10). These results indicate that the induction of *Xhox3*, *Mix.1* and *chordin* by xp53 does not require de novo protein synthesis, thus identifying these genes as potential direct targets for xp53.

xp53 interacts functionally and physically with the TGFβ pathway for the regulation of homeobox gene expression

Our analysis and previous reports (see Fig. S1 at http://dev.biologists.org/supplemental/) (Vize, 1996) have shown that BMPs and activin-like molecules are able to induce directly the expression of *Xhox3* and *Mix.1* genes that are identified as potential direct targets for p53 (Fig. 3C). Therefore, we analyzed if xp53 requires signals mediated by TGFβ ligands to activate *Xhox3* and *Mix.1* gene expression in animal cap assays (Fig. 4A). In order to inhibit TGFβ ligand-dependent signals, we used a dominant-negative activin type II receptor (Δ ActR) that has been shown to inhibit both activin and BMPs at the plasma membrane (Hemmati-Brivanlou and Thomsen, 1995; Wilson and Hemmati-Brivanlou, 1995; Yamashita et al., 1995; Macias-Silva et al., 1998). We found that the expression of Δ ActR prior to activation of xp53:GR had no effect on the ability of xp53:GR to activate *Xhox3* and *Mix.1* genes (lane 5). In addition, in the presence of ΔActR, xp53:GR induced a reporter plasmid for the *Mix.2* gene, the transcriptional regulation of which is similar to that of *Mix.1* (Fig. 4B). Thus, xp53 regulates these homeobox genes either downstream of TGFβ ligand-induced receptor activation or independently of TGFβ ligands. To distinguish these two possibilities, we tested if endogenous xp53 is required for activin or BMP-mediated induction of *Xhox3* and *Mix.1* gene expression. We established that an antisense morpholino oligonucleotide designed around the initiation methionine of xp53 (xp53-MO) is able to inhibit translation of xp53 mRNA in vitro, while a control morpholino oligonucleotide containing five mismatched sequence (5mis-MO) had no effect (Fig. 4C). In addition, the xp53-MO suppressed endogenous p53 activity as monitored by a p53 reporter gene [p53 (X3)] (Fig. 4D). The effect of p53-MO is specific because the inhibition of endogenous p53 activity is restored by expression of xp53Nmut:GR, a transcript that is refractory to the p53-MO inhibition because of conservative mutations in the p53-MO target region (Fig. 4C). As shown in Fig. 4E, injection of the

intracellular signal transducers Smad2 or Smad1 mRNA, which transmit activin and BMP signals, respectively, induced the expression of both *Xhox3* and *Mix.1* genes, while co-injection of xp53-MO partially suppressed these responses (lane 6). Furthermore, the inhibition of marker gene expression is restored by p53Nmut:GR, indicating the effect of p53-MO is specific (lanes 7 and 8). In summary, these results suggest that xp53 functions downstream of the receptor activation and may act together with a transcriptional machinery involving Smads to regulate homeobox gene expression.

In order to further support the notion that xp53 and TGFβ pathways functionally interact on the expression of homeobox genes, we analyzed regulatory sequences of the *Mix.2* gene. *Mix.1* and *Mix.2* are thought to be derived from the tetraploidy

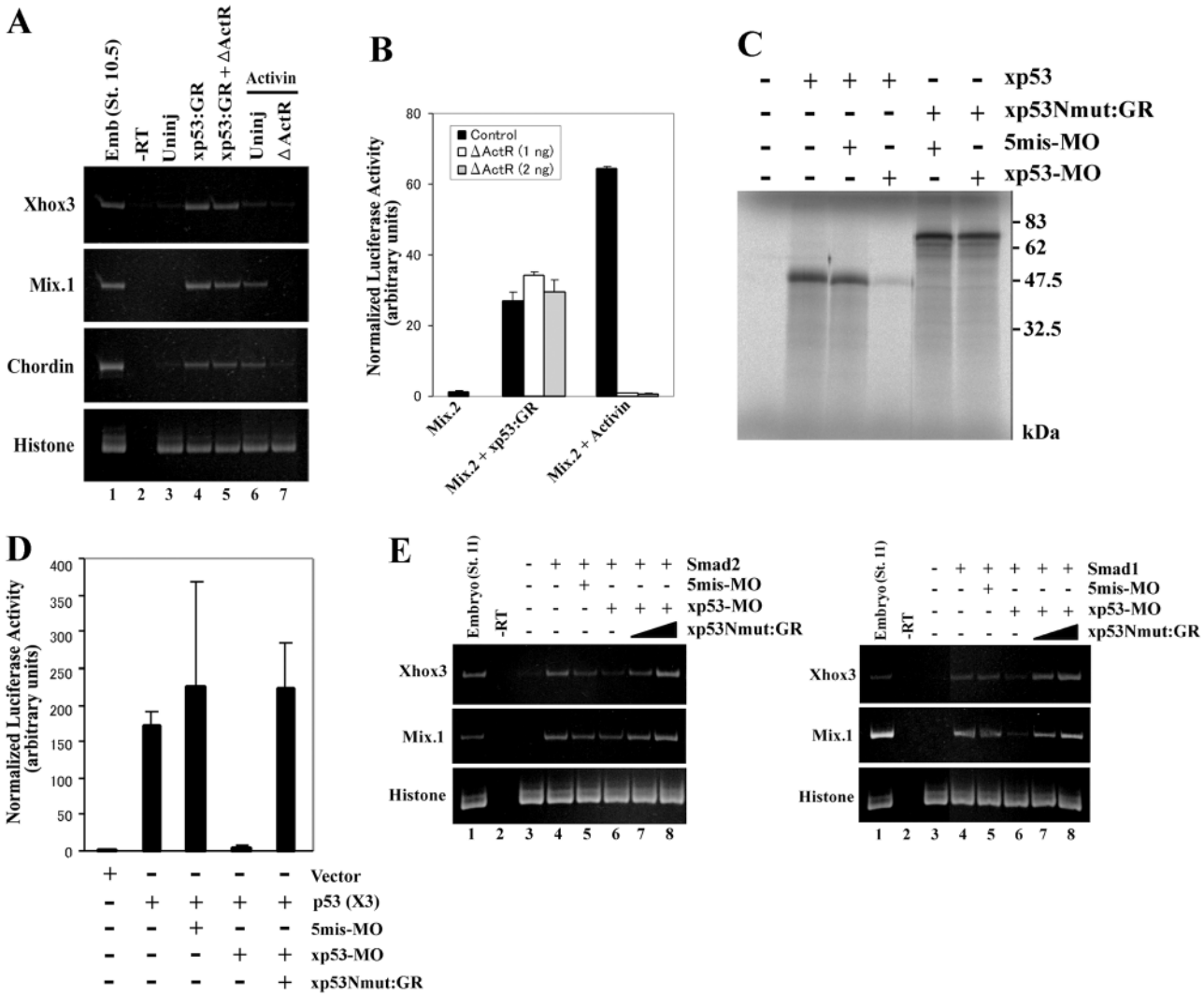

Fig. 4. xp53 interacts with activin and BMP pathways downstream of the receptor activation. (A) A dominant-negative activin type II receptor (Δ ActR) does not interfere with the marker gene induction by xp53:GR. Animal caps injected with xp53:GR (1 ng) alone or together with Δ ActR (2 ng) were treated with DEX to activate xp53:GR. The expression of marker genes was determined at the gastrula stage (stage 10.5) by RT-PCR. Δ ActR is able to inhibit marker gene expression induced by activin protein (lanes 6 and 7). (B) Δ ActR does not interfere with transcription from a *Mix.2* reporter gene induced by xp53:GR. Animal caps injected with a *Mix.2* reporter gene in combination with indicated RNA [xp53:GR (1 ng), activin (15 pg) and Δ ActR mRNA (1 ng or 2 ng)] were treated with DEX and harvested at mid-gastrula stage for luciferase assay. The columns indicate the averages of duplicate assays and the error bars indicate the ranges. (C) An antisense morpholino oligonucleotide against xp53 inhibits translation of xp53 mRNA in vitro. In vitro translation of xp53 or xp53Nmut:GR was performed in the presence or absence of antisense xp53 morpholino oligonucleotide (xp53-MO) or five mismatched control-MO (5mis-MO). The [^{35}S]-labeled translation products were analyzed on a SDS-PAGE gel. (D) xp53-MO inhibits endogenous p53 transcriptional-activating activity. Whole embryos were injected in the animal pole with xp53-MO (170 ng) or 5mis-MO (170 ng) at the four-cell stage and followed by injection of a p53 reporter plasmid, p53 (X3), alone or together with xp53Nmut:GR at the eight-cell stage. The injected embryos were subjected to the dual luciferase assay at the gastrula stage. 'Vector' indicates a reporter vector lacking p53-responsive elements. The columns indicate the averages of duplicate assays and the error bars indicate the ranges. (E) Knockdown of xp53 partially inhibits target gene expression induced by activin and BMP pathways. xp53-MO (170 ng) or 5mis-MO (170 ng) were injected into two animal blastomeres at the two-cell stage and followed by injection of either Smad2 (1 ng) or Smad1 (2 ng) mRNA into four animal blastomeres of four-cell embryos. xp53Nmut:GR mRNA was injected with Smad mRNA to rescue the effect of xp53-MO (lanes 7 and 8). The injected embryos were treated with DEX to activate xp53:GR from stage 7, and animal caps isolated at stage 9 were subjected to RT-PCR at stage 11.

of the *Xenopus laevis* genome and reported to be under essentially the same transcriptional control (Rosa, 1989; Vize, 1996; Chen et al., 1997). We found a potential p53-binding site (AGACAAGTTC) 64 bp upstream of the reported transcription start site using the TFBIND program (http://tfbind.ims.u-tokyo.ac.jp/; p53 consensus RRRCWWGYYY) (Fig. 5A). Previous studies have shown that the *Mix.2* promoter contains an activin-responsive element (ARE) consisting of FAST-1 and Smad-binding sites (Vize, 1996; Chen et al., 1997; Yeo et al., 1999). Interestingly, we observed a weak, but significant, induction of the *Mix.2* reporter gene by BMP signaling augmented by CA-ALK2 mRNA injection (Fig. 5C), indicating that this reporter is also responsive to BMP signaling. Moreover, xp53:GR appears to activate the *Mix.2* reporter (Fig. 5D). By introducing a mutation in the p53-binding site of the *Mix.2* promoter, we found that a *Mix.2* promoter containing a mutation in the putative p53-binding site [Mix.2 (p53 mut)] is less active than the wild type when induced by activin or CA-ALK2 (Fig. 5B,C), indicating the importance of the p53-binding site in the TGFβ signal-dependent *Mix.2* gene transcription. Furthermore, constructs bearing mutations in either FAST-1 or Smad-binding sites [Mix.2 (FAST mut) and Mix.2 (Smad mut)] responded poorly to xp53 activation (Fig. 5D). These results suggest that FAST-1 and Smads are in fact involved in the xp53-mediated *Mix.2* expression, and that FAST-1, Smads and xp53 function together at the level of *Mix.2* gene transcription.

To confirm directly the intracellular convergence of TGFβ

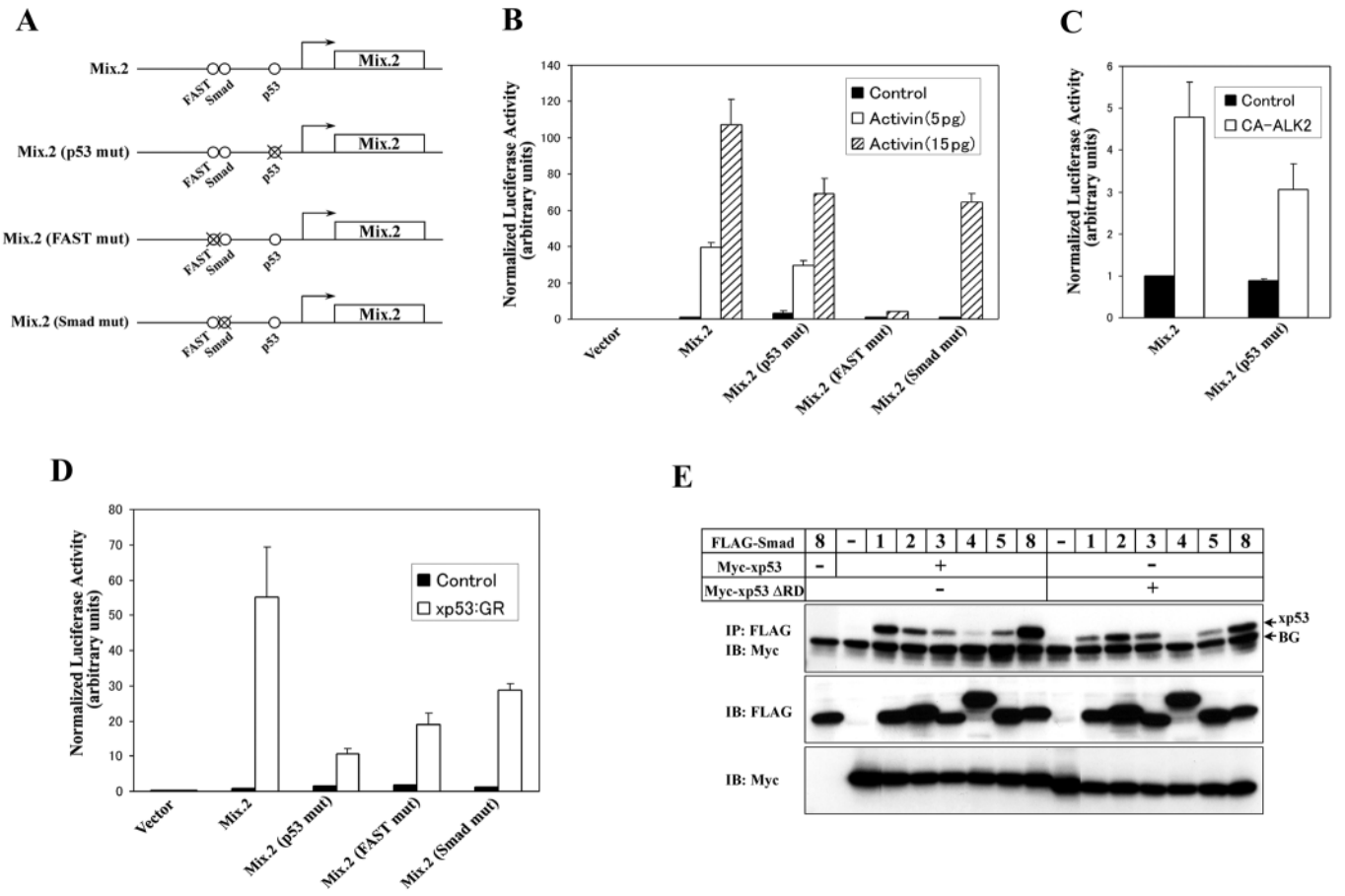

Fig. 5. xp53 interacts with activin and BMP pathways to regulate the expression of *Mix.2* and binds to Smads. (A) Schematic representation of *Mix.2* reporter constructs. Open circles indicate putative binding sites for FAST-1, Smad and p53. Arrows indicate the putative transcription start site of the *Mix.2* gene. (B) The putative p53-binding site is important for *Mix.2* transcription induced by activin. A wild-type or mutant *Mix.2* reporter gene was injected with or without activinβB mRNA (5 pg or 15 pg) in the animal pole of four-cell embryos. Animal caps were isolated at blastula stages and harvested at mid-gastrula stage (about 3 hours after isolation) for luciferase assay. 'Vector' indicates a negative control vector, pGL3-Basic (Promega). The columns indicate the averages of duplicate assays and the error bars indicate the ranges. Four other independent experiments gave similar results. (C) The putative p53-binding site is important for *Mix.2* transcription induced by the BMP signal. A wild-type or mutant *Mix.2* reporter was injected with or without CA-ALK2 mRNA (150 pg) in the animal pole of four-cell embryos. The luciferase assay was performed and presented as described in B. Six other independent experiments gave similar results. (D) FAST-1 and Smad-binding sites are important for p53-mediated induction of *Mix.2* reporter. A wild-type or mutant *Mix.2* reporter was injected with or without xp53:GR mRNA (1 ng) in the animal pole of four-cell embryos. Animal caps were isolated at blastula stages and treated with DEX for 3 hours before determination of luciferase activity. The luciferase assay was performed and presented as described in B. Three other independent experiments gave similar results. (E) xp53 binds to R-Smads. Myc-tagged xp53 (Myc-xp53) or xp53 Δ RD (Myc-xp53 Δ RD) was co-precipitated with FLAG-tagged R-Smads (Smad1, 2, 3, 5 and 8), but not with FLAG-tagged Co-Smad (Smad4) when expressed in COS-7 cells. 'BG' indicates nonspecific background signals. IP, immunoprecipitation; IB, immunoblotting.

and p53 signals, we analyzed the physical interaction between xp53 and Smads. In this experiment, COS-7 cells were transiently transfected with expression plasmids for Myc-tagged xp53 and FLAG-tagged R-Smads (Smad1, Smad2, Smad3, Smad5 or Smad8) or FLAG-tagged Co-Smad (Smad4). The total cell lysates prepared from the transfected cells were precipitated by anti-FLAG antibody and subsequently analyzed for levels of co-precipitated xp53 by the immunoblot analysis with anti-Myc antibody. As illustrated in Fig. 5E, all the Smads, except for Smad4, were co-precipitated with xp53, suggesting that xp53 associates with R-Smads but not with Co-Smad. Similar results are also obtained when xp53ΔRD was used instead of wild type. We did not observe a significant change in this association even in the presence of activin or BMP signals (data not shown), suggesting that xp53 constitutively associates with Smads, at least in overexpression experiments using COS-7 cells. Collectively, these results strongly indicate that xp53 in concert with Smads regulates the expression of target genes to pattern the embryo.

p53 binds directly to *Mix.2* gene

We next examined if p53 binds to the putative p53-binding sites found in the regulatory sequence of *Mix.2* in vitro by using electromobility-shift assays (EMSA). We identified binding complexes with a 26 bp labeled probe containing a p53-binding site from *Mix.2* in cell extracts from embryos injected with FLAG-tagged human p53 mRNA (Fig. 6A, lane 2). The formation of these complexes was diminished by addition of an excess amount of the non-labeled probe, but not by probe bearing a mutation in the consensus p53-binding site (lanes 3-6). Furthermore, addition of a monoclonal antibody recognizing FLAG tag caused a large shift in the electrophoretic mobility of the complexes (lane 7). To examine whether xp53 binds to this homeobox gene in vivo, we performed a chromatin immunoprecipitation assay in which Myc-tagged xp53 was expressed in embryos in the absence or presence of TGFβ signals and followed by precipitation of chromatin bound to xp53 with an anti-Myc antibody (Fig. 6B). PCR amplification using specific primer sets flanking p53-binding sites of *Mix.2* gene revealed the in vivo association of xp53 with the proximity of these genes in response to activin and BMP signals (lanes 4 and 5), given that the size of the genomic DNA fragment produced by sonication is 300-1000 bp (not shown). In the absence of TGFβ signals, no significant binding of xp53 to *Mix.2* gene was detected. This may be due to the detection limit of this assay, because xp53 alone was able to activate the target gene expression in the animal cap assay (Fig. 2C). The *goosecoid* gene was not precipitated with xp53 even in the presence of TGFβ signals, showing the specificity of xp53. Overall, these results, in conjunction with the in vitro EMSA data described above, strongly suggest that xp53 binds to p53-binding sites in the *Mix.2* gene in vivo, and that TGFβ ligand stimulation can enhance the binding of p53 to its target genes.

Requirement for xp53 function in the formation of mesoderm

To determine whether inhibition of endogenous xp53 might affect mesoderm development, which is known to be regulated by TGFβ signals, we injected xp53-MO into the marginal zone at the two-cell stage. Embryos injected with xp53-MO (170 ng/embryo), but not 5mis-MO, exhibited truncation of trunk and posterior regions with relatively small head structures (57%, n=86; Fig. 7A). Whole-mount in situ hybridization analysis of the xp53-MO injected embryos revealed that functional knockdown of xp53 caused reduction of both dorsal and ventral mesodermal markers, *muscle actin* and α-globin, respectively (Fig. 7F,G). In addition, xp53-MO appears to slightly reduce the expression of the early mesodermal marker Xbra, indicating partial inhibition of mesoderm at early gastrula stages (Fig. 7E). These results suggest that perturbation of endogenous xp53 causes partial loss of both dorsal and ventral mesoderm.

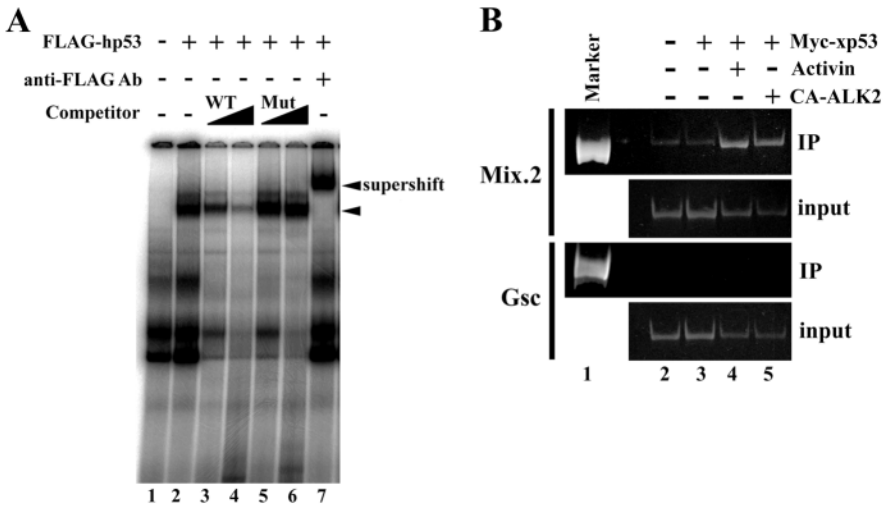

Fig. 6. p53 binds to *Mix.2* gene in vitro and in vivo. (A) p53 binds to the putative p53-binding sites from *Mix.2* gene in vitro. Cell extracts from embryos injected with FLAG-tagged human p53 mRNA (lanes 2-7) or uninjected embryos (lane 1) were incubated with a labeled double-stranded oligonucleotide. Unlabeled wild-type (WT) or mutant (Mut) oligonucleotides were added in eightfold (lanes 3 and 5) or 40-fold (lanes 4 and 6) molar excess over labeled oligonucleotide. Arrowheads indicate protein-DNA complex or supershifted complex (supershift). (B) xp53 binds to the proximity of *Mix.2* gene in vivo in response to activin and BMP signals. Soluble chromatin was prepared from embryos injected with mRNA as indicated and immunoprecipitated (IP) with antibody against Myc tag. The final DNA extractions were amplified using pairs of primers that cover the regions of *Mix.2* and *goosecoid* (Gsc) genes (see Materials and Methods). 'input' represents a portion of the sonicated chromatin prior to immunoprecipitation.

DISCUSSION

Regulation of homeobox genes by p53

Our analysis provides evidence that the homeobox genes *Mix.1/2* and *Xhox3* are under the regulation of xp53. As the spatiotemporally regulated expression of homeobox genes is central to the determination of cell fates by extracellular stimulations, our finding points to xp53 as

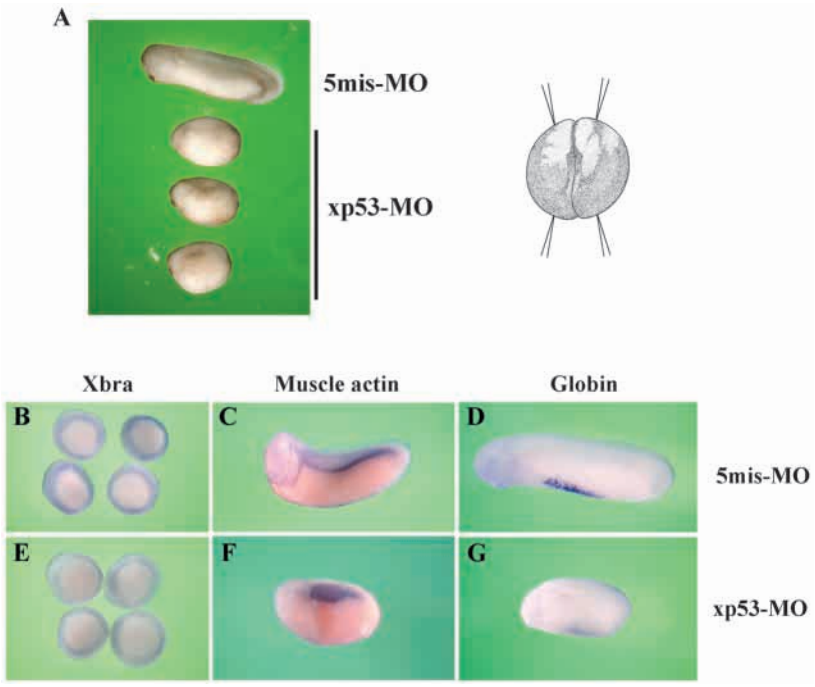

Fig. 7. Functional knockdown of xp53 affects mesoderm formation in embryos. (A) xp53-MO or 5mis-MO (170 ng each) was injected into the ventral and dorsal sides of two-cell stage embryos as shown on the right (animal view). Embryos injected with xp53-MO showed truncation of trunk and posterior structures. (B-G) Whole-mount in situ hybridization for *Xbra* (B,E), *muscle actin* (C,F) and α-*globin* (D,G) genes in embryos injected with 5mis-MO (B-D) or xp53-MO (E-G). Embryos injected with xp53-MO demonstrate slightly reduced level of expression of these marker genes.

an important regulator of cell fate specification during early development. In fact, loss-of-function studies of xp53 indicate that xp53 is not only important for the expression of target homeobox genes but also essential for the formation of mesoderm. Recently, a genome-wide screening for p53 target genes was employed by using a DNA chip technology with p53-stimulated cultured cells (Zhao et al., 2000). This genome-wide analysis and previous reports have shown that, in addition to genes involved in apoptosis and cell cycle regulation, the expression of growth factors, growth inhibitors and receptors is also regulated by p53 in mammalian cells. For example, two TGFβ family members (*BMP4* and *PTGFB*), *IGF-BP3* (an IGF inhibitor), *EGF receptor* and *Dkk-1* (a Wnt inhibitor) are expressed upon p53 activation (Deb et al., 1994; Buckbinder et al., 1995; Tan et al., 2000; Wang et al., 2000; Zhao et al., 2000). We found a bona fide p53-binding site on *Xenopus Mix.2* gene and this p53-binding site appears to support the induction of the *Mix.2* gene by activin and BMP signaling (Fig. 5B,C). The supportive, but not essential, role of p53 in the TGFβ-mediated gene expression may indicate that the p53 pathway is used to achieve a maximal induction of these homeobox genes by TGFβ signaling during embryogenesis. Alternatively, p53 could function in the maintenance of homeobox gene expression to ensure the determination of cell fates in the early and/or late phases of cell fate decision. It is also possible that endogenous p53 may provide the cells with a competence to respond to TGFβ signals emanating from the vegetal hemisphere. In support of these possibilities, we observed a higher endogenous p53 activity in the vegetal and marginal regions of the gastrula embryo, where mesoderm and endoderm form and overlap with the expression domains of *Mix.1/2* and *Xhox3*, than in the animal region (see supplemental Fig. S2 at http://dev.biologists.org/supplemental/). Delineation of the above possibilities as well as the temporal and spatial regulation of p53 activity in relation to the regulation of TGFβ-mediated homeobox gene expression awaits further studies.

Interplay between the TGFβ and p53 pathways

Although we show that p53 overexpression induces a variety of genes involved in the establishment of mesoderm and endoderm, we found that *goosecoid*, an organizer-specific homeobox gene, is not induced by xp53. As the *goosecoid* promoter contains an activin-responsive element that has been shown to bind a transcription complex involving Smads (Watabe et al., 1995; Labbé et al., 1998), this result indicates that not all genes that respond to the Smad-mediated activin pathway are regulated by p53. The extent to which p53 interacts with the TGFβ pathway as well as the selection of the TGFβ pathways to be connected to p53 could be a context dependent and may involve other factors that are differentially expressed in different cell or tissue types. For example, *p21/WAF1*, a p53 target gene, is known to be directly regulated by TGFβ signaling in several types of cells, but at least in the cultured mouse B-cell hybridoma cells, inactivation of endogenous p53 does not affect the TGFβ ligand-mediated induction of *p21* gene expression (Yamato et al., 2001). However, using bioinformatic and microarray approaches for the human genome, Wang et al. have found that the majority of TGFβ1-induced genes they characterized contain p53-binding sites (Wang et al., 2001). Based on our analysis, we propose that the interplay between TGFβ and p53 pathways at the level of transcription is crucial for mesoderm formation in *Xenopus* embryos. However, the interplay may be limited to genes involved in early development. It will be interesting to address if the interplay is also subject to downstream genes important for other aspects of p53 function such as apoptosis and cell cycle arrest in physiological contexts including early mammalian embryogenesis and primary cell culture from tumor tissues.

In addition to the importance of the p53-binding site in the *Mix.2* promoter, we observed that both Smad and FAST-1-binding sites also are important for p53-mediated *Mix.2* expression (Fig. 5D). The mutual requirement for p53 and Smad-binding sites for *Mix.2* expression may result from the physical interaction between Smads and p53 (Fig. 5E). The identification of signals and mechanisms regulating the physical interactions in embryos may provide a clue to understanding the dynamics of interplay between p53 and TGFβ signaling during embryogenesis.

Developmental functions for p53

Despite evidence that p53 appears to be largely dispensable for normal development during mouse embryogenesis (Donehower et al., 1992), we have demonstrated that *Xenopus* p53 plays an important role in the formation of mesoderm. This result is consistent with the previous observation, by Wallingford et al. (Wallingford et al., 1997), that the blockade of p53 activity results in inhibition of terminal differentiation of mesoderm and neural tissues. In addition, several lines of evidence have already implied the developmental functions of p53 during early mammalian development (Hall and Lane, 1997). For example, it has been shown that the overexpression of p53 in transgenic mice results in altered differentiation of the ureteric bud without causing cell cycle arrest and apoptosis (Godley et al., 1996). Mice homozygous for *p53* are viable, but a significant proportion of $p53^{-/-}$ mice die during embryogenesis due to a spectrum of abnormalities including defects in neural tube closure and craniofacial malformations (Armstrong et al., 1995; Sah et al., 1995). Mice embryos homozygous for *mdm2*, a negative regulator of p53, die between implantation and days E6.5, but the phenotype is rescued by the absence of *p53*, suggesting that the embryonic lethality of the *mdm2* null mutation is caused mainly by activation of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995).

The fundamental question is why defects in mesoderm are observed in *Xenopus* embryos, but not in the mouse, upon knockdown of p53? We expect that the severity of the phenotype may depend on the extent of redundancy among members of the p53 family (p53, p63 and p73) in a given species. At least in *Xenopus*, major expression of p63 begins in the ectoderm, not the mesoderm, at neurula stages, following the establishment of early mesoderm formation (Lu et al., 2001). This could explain our observation that p53 knockdown affects mesoderm development in this species. In mammals, however, the expression of p63 and p73 genes during gastrulation has not been examined and the determination of the involvement of p63 and p73 in mesoderm formation awaits further studies. We also do not rule out the possibility that mammalian embryos contain a system that bypasses the loss of p53 function, rather than the use of redundant functions among p53 family members. A detailed analysis of the expression profile as well as the determination of functional redundancy between p53 family members will be required to understand precisely the developmental functions of this family during early vertebrate embryogenesis.

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