BAC transgenic mice provide evidence that p53 expression is highly regulated in vivo

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p53 is an important tumor suppressor and stress response mediator. Proper control of p53 level and activity is tightly associated with its function. Posttranslational modifications and the interactions with Mdm2 and Mdm4 are major mechanisms controlling p53 activity and stability. As p53 protein is short-lived and hardly detectable in unstressed situations, less is known on its basal level expression and the corresponding controlling mechanisms in vivo. In addition, it also remains obscure how p53 expression might contribute to its functional regulation. In this study, we established bacterial artificial chromosome transgenic E.coli β-galactosidase Z gene reporter mice to monitor p53 expression in mouse tissues and identify important regulatory elements critical for the expression in vivo. We revealed preferentially high level of p53 reporter expressions in the proliferating, but not the differentiated compartments of the majority of tissues during development and tissue homeostasis. In addition, tumors as well as regenerating tissues in the p53 reporter mice also expressed high level of β-gal. Furthermore, both the enhancer box sequence (CANNTG) in the p53 promoter and the 3′ terminal untranslated region element were critical in mediating the high-level expression of the reporter. We also provided evidence that cellular myelocytomatosis oncogene was a critical player regulating p53 mRNA expression in proliferating cells and tissues. Finally, we found robust p53 activation preferentially in the proliferating compartment of mouse tissues upon DNA damage and the proliferating cells exhibited an enhanced p53 response as compared with cells in a quiescent state. Together, these results suggested a highly regulated expression pattern of p53 in the proliferating compartment controlled by both transcriptional and posttranscriptional mechanisms, and such regulated p53 expression may impose functional significance upon stress by setting up a precautionary mode in defense of cellular transformation and tumorigenesis.

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found high-level p53 mRNA expression during embryonic development and a striking p53 mRNA expression pattern in postnatal rat brain, with intensive signals in subventricular zone, rostral migratory stream and external granular layer (EGL), where new neurons were produced. In NIH3T3 cells, p53 mRNA level fluctuated with cell cycle progression. More works are needed to resulted in p53 stabilization preferentially in the proliferating compartments. These p53 mRNAs were at much lower levels in suprabasal layer of the skin, internal granular layer (IGL) of the cerebellum and villus of the small intestine (Figure 1a), which belong to the differentiated compartments.

High level of p53-LacZ reporter expression in the proliferating compartments of mouse tissues. To reveal p53 expression pattern and levels in mouse tissues, we performed X-gal staining in tissues of PZU mice at postnatal day 7–8, 2–3 months and 13–15 months of age, respectively. Intense β-gal staining was observed in multiple tissue compartments including germinal center of spleen, spermatogenic cells of seminiferous tubule in testis, dentate gyrus (DG) of hippocampus, islet of postnatal and adult pancreas, ependymocytes of choroid plexus, EGL of the postnatal cerebellum, renal tubule and glomerulus, and pulmonary
alveoli (Figure 2a). In contrast, β-gal was expressed at low or undetectable levels in the differentiated cells toward the center of the seminiferous tubule, mature neurons outside the DG region in adult hippocampus, acinous cells in adult pancreas and the IGL of cerebellum (Figure 2a), which were all non-proliferating in nature. Meanwhile, direct comparisons in the same tissues revealed that β-gal-staining intensities decreased when mice aged, especially in spleen, choroid plexus, kidney and lung (Figure 2a). Further, the whole mount X-gal staining on de-skinned mice also demonstrated attenuated β-gal expression in mouse muscle and skeleton with age (Supplementary Figure 2a). As few exceptions, the non-proliferating hepatocytes in the adult liver and purkinje cells of the cerebellum also demonstrated detectable levels of β-gal staining in the PZU mice (Figures 2a and d).

The preferential expressions of p53-LacZ reporter in proliferating compartment of tissues were further evaluated in the hair follicles of mouse skin which undergo step-wise growing and quiescent cycles. There were abundant strong β-gal-staining positive cells at the anagen stage of the hair cycle at P20, but only few at the quiescent telogen stage at P24 (Figure 2b). To study the link of proliferating or quiescent state with p53-LacZ expression, we performed X-gal staining in PZU MEFs either at proliferating or quiescent state. Upon

Figure 1 Generation and validation of BAC transgenic reporter mice to monitor p53 expression. (a) In situ hybridization of p53 mRNA using sense and antisense probes on postnatal day 1 mouse skin, P7 cerebellum and 2-month-old mouse intestine. Boxed areas in left panel were shown in the middle panel. Scale bar = 100 μm. (b) Schematic representations of the p53 gene locus in the BAC and its modifications with the insertions of lacZ reporter in the ATG (exon 2) of full length p53 and EGFP reporter in the ATG (exon 5) of an isoform (Δ157) together with alterations in the regulatory elements in three BAC transgenic lines. ChIP primers used for detecting wild-type E-box-binding in tissues and cells (F1, R1 or F2, and WTR2); ChIP primers for detecting mutant E-box-binding in tissues and cells (F2, MutantR2). (c) β-gal staining of E10.5 mouse embryos from PZU, PMZU and PZS transgenic reporter lines with 1–4 BAC copies. Embryos were whole-mount stained in 1 mg/ml X-gal staining solution with gentle shaking for 24 h at 4 °C. Scale bar = 400 μm. (d) A comparison of p53 reporter expression in p7 skin, p7 cerebellum and 2-month-old mouse small intestine by β-gal staining with p53 mRNA in situ hybridization. Scale bar = 50 μm
Figure 2  Preferential p53 reporter expression in the proliferating compartments during mouse postnatal development, tissue homeostasis, regeneration and tumorigenesis. 

(a) β-gal staining of tissues including spleen, testis, dentate gyrus, pancreas, choroid plexus, cerebellum, kidney, lung and liver at P7, 2 months and 15 months of age respectively in PZU mice. Frozen slices of tissues were stained in 1 mg/ml X-gal staining solution at 37 °C for 10 h, except for 3 h in testis. Scale bar = 200 μm. (b) β-gal staining of skins at the anagen (p20) or telogen (p24) phase of the hair cycle in PZU mice. Frozen slices of skins were stained in 1 mg/ml X-gal staining solution at 37 °C for 10 h. Boxed areas were shown in the right panel. Scale bar = 200 μm. (c) Double immunofluorescence of β-gal and Ki67 in p8 cerebellum and adult small intestine of PZU mice. Scale bar = 200 μm. (d) β-gal staining of liver before and 48 h after hepatotectomy. Frozen slices of livers were stained in 1 mg/ml X-gal staining solution at 37 °C for 7 h in PZU mice. Boxed areas were shown in the right panel. Scale bar = 200 μm. (e) β-gal staining of small intestine in PZU mice and polyps in PZU, Apc

mice
withdraw of serum, proliferating MEFs gradually moved to a quiescent state. In parallel, PZU MEFs with high β-gal-staining intensity dropped from 46.2 to 2.6%, whereas cells with low-level β-gal increased from 18.7 to 68.3% (Supplementary Figure 2b). Next, we performed double immunofluorescence of ki-67 and β-gal in cerebellum and small intestine of the p7 PZU mice. β-gal positive cells were largely overlapped with those of Ki67 positive proliferating cells, establishing a direct link between high p53-LacZ expression and cellular proliferating state in multiple tissue compartments (Figure 2c).

Similar to the self-renewal of homeostatic tissues, tissue repair and regeneration also features cellular proliferation. β-gal expression was examined in the liver of PZU mice under hepatotectomy. In spite of the detectable basal level of β-gal staining, an enhanced β-gal expression was observed on the proliferation peak at 48 h (Figure 2d) as demonstrated by Ki67 staining (Supplementary Figure 2c), paralleling the level of endogenous p53 mRNA expression (Supplementary Figure 2d).

Tumorigenesis is marked by uncontrolled proliferation. To study p53 reporter expression during tumorigenesis, we crossed the PZU mice with Apcmin mice with a mutation in the Apc gene and studied β-gal expression in the adenomas developed in the small intestine of the PZU; Apcmin/+ mice. Most cells in these tumors expressed high levels of β-gal (Figure 2e), which paralleled with Ki67 immuno-staining (Supplementary Figure 2e), whereas high levels of β-gal was only confined to the crypts in normal intestine (Figure 2e).

Collectively, high level of β-gal expression was specifically observed in the proliferating cell compartments in a variety of homeostatic or pathological contexts in the PZU mice, suggesting p53 expression is highly regulated in the proliferating compartments.

p53 reporter expression is controlled by both the p53 promoter E-box element and 3’UTR. With the E-box-mutated PMZU mice and 3’UTR-deleted PZS mice, we addressed the respective roles of the conserved E-box and the p53 3’UTR sequence on the enhanced reporter expression in the proliferating cells during development and homeostasis. Significantly diminished β-gal staining was observed in the PMZU embryos as compared with the PZU embryos with the same BAC copy number (Figure 1c). At both P7-8 and 2–3 months of age, β-gal staining was all reduced in the PMZU mice as compared with the PZU mice in a panel of tissues examined, including spleen, testis, hippocampus, pancreas, choroid plexus, postnatal cerebellum, skin, lung and liver (Figures 3a and b). Immunohistochemistry (IHC) with β-gal antibody also revealed decreased β-gal expressions in the small intestine and bone marrow of the PMZU mice, whereas IHC of Ki67 remained the same between the PMZU and PZU mice (Figures 3c and d). Consistently, in the PMZU mice, the LacZ mRNA level normalized to that of endogenous p53 also dropped by 50–73% in embryos, postnatal and adult tissues as compared with those of the PZU mice (Figures 4a and b). These results indicated an important role of the E-box on p53-LacZ reporter expression in vivo.

Cell cycle exit, reentry and progression can be manipulated in cell culture systems. p53 mRNA was elevated 8 h after serum stimulation and reached its peak at 16 h, paralleling cell cycle progression13,14 (Supplementary Figure 3). LacZ mRNA from PZU MEFs also exhibited a similar elevation upon serum stimulation, whereas LacZ mRNA level from the PMZU mice remained unchanged (Figure 4c), again suggesting a role of E-box in coordinating cell cycle progression and p53 expression.

In contrast, upstream stimulatory factor-1 (USF-1), another bHLH transcription factor, could bind the E-box in endogenous p53 promoter but not the mutant E-box in transgenic reporter. As expected, c-Myc could bind wild-type E-box in the endogenous p53 promoter, but not the mutant E-box in transgenic p53 promoter in the proliferating tissues of the PMZU mice (Figures 4d and e). However, little c-Myc binding was observed on endogenous p53 promoter in liver (Figure 4e).

Together, these results indicated a significant role of c-Myc in upregulating p53 reporter expression through the E-box element, suggesting that c-Myc may be one of the major transcriptional factors driving high level of p53 mRNA expression in proliferating cells.

Compared with PZU mice, PZS mice lacking p53 3’UTR exhibited no or very low-level β-gal expression visible only in limb bud of the E10.5 embryos with 1–4 BAC copies (Figure 1c). In addition, no or very weak β-gal staining was detected in multiple tissue compartments of the PZS mice with 2 or 4 BAC copies at both P7 (Figure 5a) and 2 months of age (Figure 5b). Only the PZS mice with 4 BAC copies exhibited weak β-gal staining in the spermatogenic cells of seminiferous tubules in testis and dentate gyrus of hippocampus (Figures 5a and b). These results indicated an essential role of p53 3’UTR in supporting high level of β-gal expressions in the proliferating tissue compartments. Interestingly, in both embryos and a variety of adult tissues examined, LacZ mRNA levels of the PZS mice were not significantly different from those of the PZU mice.
indicating that p53 3’ UTR does not significantly influence LacZ mRNA levels.

Finally, we directly compared the β-gal protein level in MEFs from the PZU, PMZU and PZS mice with two BAC copies. Western blot analysis showed that β-gal level dropped by >50% in PMZU MEF, and was almost undetectable in PZS MEFs (Figure 5c), consistent with the β-gal staining results in multiple tissues. These results further supported that p53 E-box and 3’UTR were critical in sustaining high-level expression of the reporter gene.

A functional link between cellular proliferating state and p53 response. Our findings that p53 reporter expression was preferentially upregulated in proliferating cells suggested the possibility of a more robust p53 response upon stresses in proliferating cells compared with quiescent and differentiated cells. To directly explore the possible regulatory difference of p53 in vivo, B6 mice was either untreated or treated with 6 Gray of X-irradiation and immunostained for p53 protein in multiple tissue compartments. Consistent with previous results, p53 protein was undetectable in normal tissues from untreated mice (Figure 6a). In contrast, upon irradiation, p53 positive cells were readily detected in EGL of cerebellum in the postnatal mice, and in the basal cells of tongue and intestine crypt of both postnatal and adult mice (Figure 6a). In contrast, there was weak or no p53 staining in the more differentiated cell compartments of these tissues, such as IGL of the adult cerebellum, villi of small intestines and differentiated cells in the tongue epithelium (Figure 6a). Double staining with p53 and 5-Bromo-2-Deoxy Uridine (Brdu) antibodies further
Figure 4  Disrupting c-Myc binding on the conserved E-box in p53 promoter downregulated p53 expression. (a) RT-PCR results of LacZ mRNA expression as normalized to p53 mRNA in embryos at 10.5 day from PZU and PMZU mice. (b) RT-PCR results of LacZ mRNA expression as normalized to p53 mRNA in small intestine, skin and kidney from PZU and PMZU mice. (c) RT-PCR results of lacZ mRNA expression normalized to 18 s RNA in a time course upon serum stimulation in MEFs from PZU and PMZU mice with the same BAC copy number. (d) ChIP analysis on c-Myc binding on the endogenous p53 promoter (wild-type E-box) or p53 promoter in transgenic BAC (mutant E-box) in the E11.5 embryo of the PMZU mice. (e) ChIP analysis on c-Myc and USF binding on the endogenous p53 promoter (wild type E-box) or p53 promoter in transgenic BAC (mutant E-box) in small intestine and liver of 2-month-old PMZU mice. (f) ChIP analysis on c-Myc binding on the endogenous p53 promoter (wild-type E-box) or p53 promoter in transgenic BAC (mutant E-box) in the MEFs from the PMZU mice under the treatment of Myc inhibitor 10058-F4 for 6 h (upper panel) and ChIP analysis on c-Myc binding on the p53 promoter (wild-type E-box) in the MEFs from the PZU mice under the treatment of Myc inhibitor 10058-F4 for 6 h (lower panel). (g) RT-PCR results of LacZ and p53 mRNA expressions normalized to β-actin in MEFs from PZU and PMZU mice under the treatment of 10058-F4 for 8 h. (h) β-gal staining of MEFs from the PZU and PMZU mice under the treatment of 10058-F4 for 10 h. MEFs were stained in 1 mg/ml X-gal staining solution at 37 °C for 10 h. Scale bar = 200 μm. Values are means ± S.E.M.s. *P<0.05; **P<0.01; ***P<0.001 (t-test)
p53 3' UTR was essential in supporting high level of p53 reporter expression. (a) β-gal staining of multiple tissues including spleen, testis, dentate gyrus, pancreas, choroid plexus, cerebellum, skin, kidney, lung and liver in P7 PZU and PZS mice. Slices of frozen tissues were stained in 1 mg/ml X-gal staining solution at 37 °C for 10 h, except for 3 h for testis. Scale bar = 200 μm. (b) β-gal staining of multiple tissues including spleen, testis, dentate gyrus, pancreas, choroid plexus, cerebellum, skin, kidney, lung and liver in 2-month-old PZU and PZS mice. Slices of frozen tissues were stained in 1 mg/ml X-gal staining solution at 37 °C for 10 h, except for 3 h for testis. Scale bar = 200 μm. (c) Western blot analysis of β-gal expression in MEFs from PZU, PMZU and PZS mice with the two copies of transgenic BAC.
Figure 6 A direct link between cellular proliferation and p53-mediated stress response. (a) IHC of p53 in small intestine, cerebellum and tongue of p7 and 2-month-old C57BL/6J control mice or mice treated with X-irradiation for 4 h. Arrows depicted positive stained cells. Scale bar = 200 μm. (b) Double immunostaining of p53 and BrdU in intestine and testis of 2-month-old C57BL/6J mice treated with X-irradiation for 4 h. (c) Western blot analysis of p53 and p21 expression on quiescent and proliferating 3T3 cells treated with Doxorubicin. (d) Western blot analysis of p53 and p21 expression on quiescent and proliferating MEFs treated with Cisplatin. (e) Immunofluorescence of p53 or p21 in quiescent and proliferating MEFs treated with Cisplatin or Doxorubicin. Scale bar = 200 μm.
revealed that p53-positive-staining largely overlapped with that of Brdu in proliferating compartment of fast turn-over tissues including small intestine and testis (Figure 6b). Therefore, p53 protein was preferentially expressed in the proliferating tissue compartment upon induction, essentially recapitulating its gene expression pattern.

To directly analyze p53 functional differences in cells at different proliferating state, quiescent and proliferating cells were treated with either Doxorubicin or Cisplatin. p53 protein was undetectable regardless of the cell cycle status in untreated controls (Figures 6c and d). However, upon treatment, p53 as well as its transcriptional target gene p21 was much more greatly elevated in the cycling cells (Figures 6c and d). Immunofluorescence on MEFs treated with Cisplatin also demonstrated much more elevated level of p53 activation and response in the cycling cells (Figure 6e). Thus, the concordant elevation in p53 expression may provide one possible functional basis for robust p53 responses selectively in the proliferating compartments upon stress.

Discussion

The regulatory and functional mechanisms of p53 have been under intensive investigations at molecular, cellular and organismal levels to better understand its roles in tumor suppression and stress responses. Aiming to study p53 expression pattern and regulatory mechanisms both under physiological contexts and in a comprehensive manner, we established an in vivo reporter system in BAC transgenic mice that were able to recapitulate endogenous p53 mRNA expression and identified a highly selective expression pattern for the reporter across diverse tissue compartments in both physiological and pathological conditions. We provided further evidence suggesting that the preferential expression of the reporter in proliferating compartments is critically dependent on both p53 promoter and 3’UTR elements through transcriptional or posttranscriptional mechanisms. In addition, this study also revealed a distinctive functional difference of p53 in proliferating compartments versus their differentiated or quiescent counterparts upon stress.

Previous studies on p53 mRNA expression and promoter analyses provided a number of relatively separated clues on p53 expression regulation in general and a few of them were implicated in proliferating cells. However, to our knowledge, there is no commonly recognized theme for p53 expression pattern in vivo and the importance and relevance remained to be further established for many of the cis-elements and transacting factors identified in regulating p53 expression. Here we took a transgenic approach in integrating different lines of studies and shed new insights on p53 expression and its regulatory mechanisms in a diverse set of conditions in vivo. Our results corroborated the existing results and argued strongly for a unified and distinctive expression pattern for p53 in vivo.

It becomes increasingly apparent that the studies on the regulatory mechanisms of gene expression can be better oriented and more meaningful with a clear understanding of the expression pattern of the gene. As an immediate early gene, c-Myc is induced earlier than p53 mRNA accumulation and its mRNA level parallels with p53 mRNA level in cell lines.\textsuperscript{14,28} Ectopic c-Myc or N-Myc was able to trans-activate reporters driven by an E-box from the p53 promoter in 3T3 and neuroblastoma cells\textsuperscript{17,29} in an effort to decipher the requirements of p53 expression in vivo, we found the E-box element in the p53 promoter and its binding by c-Myc contributed significantly to high-level expression of p53 reporters in the proliferating compartments. Notably, as regulators of cell cycle or proliferation, E2F1,\textsuperscript{20} C/EBP$\beta$,\textsuperscript{21} EGR-1\textsuperscript{22} and Ets-1/2\textsuperscript{23} were also implicated in regulating p53 expression. In PM2U mice with high BAC copy numbers, weak β-gal staining was still detected in the proliferating compartments of tissues. In addition, the variations in the degree of reduction of lacZ expression in the PM2U mice of different ages also suggested a differential dependence on the E-box regulation. Thus, the strength of Myc activity, together with the contribution of other factors may help to coordinate p53 expression levels with the cellular proliferation state in multiple tissue compartments.

In spite of the dominant p53 reporter expression pattern identified in the vast majority of tissues examined, there were a few exceptions including the purkinje cells and hepatocytes with detectable levels of β-gal staining while being largely quiescent. Interestingly, the E-box mutation also abrogated the reporter expression in liver. Our ChIP analysis suggested that the moderate reporter expression in liver may be regulated by USF-1, which is known to be expressed in liver.\textsuperscript{30,31} When the hepatocytes reentered the cell cycle, a strong increase in β-gal expression suggested that these cells were still subjected to the regulatory mechanisms distinguishing proliferating and quiescent states.

Replacement of p53 3’UTR with a commonly used SV40pA greatly reduced p53 reporter expression in the proliferating compartments, suggesting a general role of the 3’UTR as a positive regulatory elements in supporting p53 expression. RNA-binding factors such as HuR,\textsuperscript{32} Wig1,\textsuperscript{33} CPEB,\textsuperscript{34} and certain miRNAs were reported to regulate mRNA stability and/or translational efficiency by directly binding on cis-elements in p53 3’UTR.\textsuperscript{25,26,35} Although HuR and Wig1 could stabilize reporter mRNA and/or strengthen its translational efficiency upon DNA damage stress, CPEB did not significantly influence p53 mRNA level, but instead regulated the p53 mRNA polyA length and protein level through translation.\textsuperscript{34} In our study, reporter mRNA levels were not significantly altered in the PZS mice, thus pointing to the possible translational control in mediating the high-level expression mediated by the 3’UTR sequences.

Our results suggested concerted effects of both transcriptional and posttranscriptional regulations in supporting the selective p53 expression pattern in proliferating compartments. Known to be anti-proliferative, p53 protein is kept to undetectable levels in normal cells. However, this could also obscure many possible links between p53 expression and its function. For example, proliferative tissues are often more radiosensitive,\textsuperscript{36,37} and while complex mechanisms may underlie such phenomenon, heightened p53 basal expression may have a role. Through genetic deletion of Mdm2 in a p53 hypomorphic background, we previously discovered that p53 protein stabilization and accumulation only appeared in the proliferating compartments of mice,\textsuperscript{6} a pattern fully recapitulated in the p53 reporter mice in this study. Here we
demonstrated that DNA damage elicited a greater p53 response in the proliferating cells. Therefore, the posttranslational controls seem to act possibly in sequence with the transcriptional and posttranscriptional mechanisms in fine-tuning p53 activity in a variety of cell types depending on their proliferative and differentiation status.

In summary, this study took a genetic approach to address the long existing question of p53 expression and revealed novel insights suggesting a general intrinsic mechanism for upregulating p53 in proliferating cells and tissues. Linking to p53 function, the potential advantage and significance for such regulation at the expression level can be several folds: first, higher basal level of p53 would allow a fast and robust protein stabilization/activation and stress response upon stress; on the other hand, a retarded or weak p53 response may protect the cells from apoptosis in the terminally differentiated cells and reduce tissue damage; finally, the switches of cell proliferative state or fate constantly occur for stem cells and progenitors during homeostasis. The coordinated control of p53 expression may allow the uninterrupted monitoring of proliferation to fulfill its tumor suppressor functions.

As one of the most fundamental cellular processes, cellular proliferation and its regulation are an integral part of development, tissue homeostasis, tissue regeneration and tumorigenesis. Close monitoring of this process by a tumor suppressor controller at multiple levels may set up a precautionary mode in the cells without causing much disturbance.

Materials and Methods

Mice. Mice were bred and maintained under specific pathogen-free conditions and experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the animal facility of Model Animal Research Center of Nanjing University, China. p53PZ(S) (P2U, p53PMZS (PM2U) and p53PZS(PZS) mice were established on C57BL/6J and CBA mixed background and were backcrossed to C57BL/6J background for three generations. PZU mice were crossed to C57BL/6J-ApCΔNk mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) to establish P2U, ApCΔNk mice.

In situ hybridization. Tissues were quickly immersed into cold 4% PFA solution for 1–2 h, dehydrated with 30% sucrose in PBS (pH 7.4) at 4 °C overnight, embedded in OCT and cut into 12 μm in microinjection buffer, and 1 pl DNA was injected into embryos. For NIH3T3 cells: to make cells in a proliferating state, 2.2 × 10^6 cells were cultured in 10-cm dish with 10% FBS in DMEM for 12 h. To make cells in a quiescent state, 2.2 × 10^5 NIH3T3 cells were cultured in 10-cm dish with 10% FBS in DMEM until cells reached 80–90% confluency; then cells were cultured with 0.05% FBS in DMEM for next 72 h. Cells were collected, sonicated and ChIP analysis was performed according to manufacturer's instructions (Upstate) with Rabbit polyclonal antibody against β-gal (Abcam, Cambridge, MA, USA; ab9361, 1:200) and rat monoclonal antibody against β-gal (Jackson, 1:500) for an hour at room temperature. Signals were imaged by either a bio- or confocal microscope (OLYMPUS).

Generation of BAC transgenic mice. p53 BAC (No.bMQ-441J16, Research Genetics, USA) was modified by homologous recombination to insert a LacZ reporter gene (from placeZtet<sup>β</sup>) together with various alterations of p53 cis-regulatory elements to establish p53PMZS, p53PMZU and p53PMZS reporter constructs. The lox and lox511 sequences in the BACe3.6 backbone were then replaced with loxp and loxp511 sequences in the BAC transgenic mice. Close monitoring of this process by a tumor suppressor controller at multiple levels may set up a precautionary mode in the cells without causing much disturbance.

X-gal staining. Embryos or tissues were fixed with cold 4% PFA for 1.5 h on ice. Embryos were stained in 1 mg/ml X-gal staining solution with gentle shaking for 24 h at 4 °C. Tissues were dehydrated, embedded in OCT and cut into 10μm slices, which were stained in 1 mg/ml X-gal staining solution for 3–10 h at either 25 or 37 °C (for comparing β-gal expression levels, staining conditions and time should be consistent between samples). X-gal staining for cells was similar as that for tissues except the fixation step: after washed with cold 1× PBS, cells were fixed with 0.25% glutaraldehyde in 1× PBS for 5 min as described.

IHC and immunofluorescence. Tissues were dissected and fixed in cold 4% PFA over night at 4 °C, dehydrated by gradient alcohol (from 50 to 100%), rendered transparent in xylol and embedded in paraffin, then cut in a microtome to slices of 6 μm in thickness and affixed onto the APES coated slides. After deparaffinized and rehydrated, slices were performed with heat-induced epitope retrieval in sodium citrate (pH 6.0), blocked with normal goat serum block solution (Boster, Wuhan, China) for 1 h at room temperature. For IHC, slices were incubated with rabbit polyclonal antibody against β-gal (Invitrogen, Shanghai, China; A11131, 1:200), rat monoclonal antibody against K67 (Dako, Glostrup, Denmark; 1:100) or rabbit polyclonal antibody against p53 (Vector, Burlingame, CA, USA; C561:1:500) for 18–22 h at 4 °C. β-gal or p53 was detected using Ultra Sensitive S-P (rabbit) kit (MaxInBio, Fuzhou, China); K67 was incubated with the biotin-tagged goat anti-rat secondary antibody (Jackson, West Grove, PA, USA; 1:300) for 1 h at RT, then incubated with avidin-tagged horseradish peroxidase. All signals were developed with DAB agents (MaxInBio) and then stained with hematoxylin. For immunofluorescence analysis, slices were incubated with chicken polyclonal antibody against β-gal (Abcam, Cambridge, MA, USA; ab9361, 1:200) and rat monoclonal antibody against K67 (Dako, 1:100) for 18–22 h at 4 °C, and then were incubated with the Cy3-tagged goat anti-chicken (Jackson, 1:500), FITC-tagged goat anti-rat antibodies (Jackson, 1:500) and the nucleus was stained with Hoechst (Sigma, St. Louis, MO, USA) for an hour at room temperature. Signals were imaged by either a bio- or confocal microscope (OLYMPUS).

NIFHST3 cells: to make cells in a proliferating state, 2.2 × 10<sup>6</sup> NIH3T3 cells were cultured in 10-cm dish with 20% fetal bovine serum (FBS) in DMEM for 12 h. To make cells in a quiescent state, 2.2 × 10<sup>5</sup> NIH3T3 cells were cultured in 10-cm dish with 10% FBS in DMEM until cells reached 80–90% confluency; then cells were cultured with 0.05% FBS in DMEM for next 72 h. Cells were collected, sonicated and ChIP analysis was performed according to manufacturer's instructions (Upstate) with Rabbit polyclonal antibody against c-Myc (Santa Cruz, Santa Cruz, CA, USA; sc-764-x) or Rabbit IgG (Santa Cruz, Sc2763). Primer sequences used: 5ʹ-CAGCTTTTGTGCCAGGAGTCT-3ʹ (F1), 5ʹ-TAAGTGAGCTCCTACTC-3ʹ (R1). For embryo and tissues: CHIP was performed as described with modifications on sonication: for 1 s and pause for 2 s with the total time being 1 min for small intestine, 6 min for liver, and 3.8 min for embryos. For MEFs: CHIP was performed similarly as 3T3 cells with a few differences: early passage MEFs from P2U or PM2U mice were plated on 10-cm dish at 1.5 × 10<sup>6</sup> density and cultured in 0.1% FBS in DMEM for 7–8 h, then cultured with 20% FBS in DMEM for 6 h. 10058-F4 (Sigma) was added to the medium at a final concentration of 180 μM, and MEFs were cultured for another 6 h and was sonicated for 4.8 min with sonication for 1 s, pause for 2 s.

Primer used for embryo, tissues and MEFs: wild type E-box, 5ʹ-ACTTTCAGACGGCTTCT-3ʹ (F2); mutant 5ʹ-ACTTTCACAGACGGCTTCT-3ʹ (F2). Time PCR with SYBR Green reagents (Invitrogen) and were reverse transcribed to CDNA using M-MV Reverse Transcription kit (Invitrogen) following manufacturer's instructions. Diluted cDNAs were used for Real Time PCR with SYBR Green reagents (Invitrogen) on an ABI Prism Step-One bio-analyzer (Foster City, CA, USA). Sequences of primers are available upon request. Expression data were normalized to β-actin mRNA. 18 s rRNA or endogenous p53 mRNA expression. Expression changes were calculated using the ΔΔCt method and expressed as fold change over control. Experiments were repeated three times with similar results.
Western blotting. MEFs and 3T3 cells were lysed in RIPA buffer supplemented with complete Protease Inhibitor Cocktail Tablets (Roche). The membrane was incubated with anti-p53 (Vector, CM5, 1: 200), anti-p21 (Santa Cruz, M19, 1 : 200), and appropriate secondary antibody (Pierce, Rockford, IL, USA; 1: 2000) sequentially. Protein detection was performed using the ECL substrate (Thermo, Rockford, IL, USA) before exposure to film.

X-ray treatment. Mice were exposed to X-ray irradiation at 6 Gy dosage at a rate of 1.2 Gy per minute using Biological X-ray irradiator (RS2000, Rad Source, Brentwood, TN, USA). Tissues were collected 4 h after X-ray irradiation.

Statistical analysis. Data are expressed as mean ± S.E.M. from triplicates. Statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) and Hist analysis was performed between two groups. All P-values < 0.05 were considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

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1. MacCallum DE, Hupp TR, Midgley CA, Stuart D, Campbell SJ, Harper A et al. The p53 response to ionising radiation in adult and developing murine tissues. Oncogene 1996; 16: 2575.
2. Franch S, Froment P, Bogaerts S, De Clercq S, Maetens D, Doumont G et al. Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. Proc Natl Acad Sci USA 2006; 103: 3232–3237.
3. Cheng Q, Chen J. Mechanism of p53 stabilization by ATM after DNA damage. Cell Cycle 2009; 8: 472.
4. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci USA 1992; 89: 4495.
5. Garcia-Cao I, Garcia-Cao M, Martin-Caballero J, Criado LM, Klatt P, Flores JM et al. Super p53/Mice exhibit enhanced DNA damage response, are tumor resistant and age normally. EMBO J 2002; 21: 6225–6235.
6. Liu G, Terzian T, Xiong S, Van Pett CS, Audiffred A, Box NF et al. The p53-Mdm2 network in progenitor cell expansion during mouse postnatal development. J Pathol 2007; 209: 360–366.
7. Garcia D, Wain MR, Martins CP, Brown Swigar L, Passeggi E, Evan GI. Validation of Mdmx as a therapeutic target for reactivating p53 in tumors. Genes Dev 2011; 25: 1746.
8. Kang JH, Kim SJ, Nho DY, Park IA, Choe KJ, Yoo OJ et al. Methylation in the p53 promoter is a supplementary route to breast carcinogenesis: correlation between CpG methylation in the p53 promoter and the mutation of the p53 gene in the progression from ductal carcinoma in situ to invasive ductal carcinoma. Lab Invest 2001; 81: 573–579.
9. Raman V, Martensen SA, Reisman D, Evron E, Odenwald WF, Jaffee E et al. The p53 promoter and the mutation of the p53 gene in the progression from ductal carcinoma in situ to invasive ductal carcinoma. J Natl Cancer Inst 1999; 91: 1140.
10. Rogel A, Popliker M, Webb C, Oren M. p53 cellular tumor antigen: analysis of mRNA levels in transformed human cells. Proc Natl Acad Sci USA 1983; 80: 5815–5819.
11. Schmid P, Lorenz A, Hameister H, Montenarh M. Expression of p53 during mouse postnatal development. Proc Natl Acad Sci USA 1986; 83: 2574–2577.
12. Lookeren Campagne MV, Gill R. Tumour suppressor p53 is involved in cell cycle progression in mouse microglioma. Proc Natl Acad Sci USA 2003; 100: 1730–1735.
13. Tian S, Huang S, Wu S, Guo W, Li J, He X. MicroRNA-125B inhibits the expression of p53 by directly targeting its 3' untranslated region. Biochem Biophys Res Commun 2010; 396: 435–439.
14. Swarbrick A, Woods SL, Shaw A, Balakrishnan A, Phyu Y, Nguyen A et al. miR-380-3p represses p53 to control cellular survival and is associated with poor outcome in MYCN amplified neuroblastoma. Nat Med 2010; 16: 1134–1140.
15. Van der Kogel F, van der Veer J, ten Doesschate J, van den Akker J, Reuten P et al. Post-transcriptional control of myc and p53 expression during mouse postnatal development. Genes Dev 2001; 15: 863.
16. Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM, Chase TN et al. Nuclear factor-kappaB-dependent regulation of p53 gene expression induced by daunomycin genotoxic drug. Oncogene 1998; 17: 1187.
17. Choi M, Lee H, Rho HM. E2F1 activates the human p53 promoter and overcomes the repressive effect of hepatocyte B viral X protein (Hbx) on the p53 promoter. JUNB/Lile 2002; 53: 309–317.
18. Boggi K, Reisman D, CEBPβ participates in regulating transcription of the p53 gene in response to mitogen stimulation. J Biol Chem 2007; 282: 7982.
19. Liu J, Groogan L, Nau MM, Allegra CJ, Chu E, Wright JJ et al. Physical interaction between p53 and primary response gene Egr-1. J Int J Oncol 2001; 18: 863.
20. Venanzoni MC, Robinson LR, Hodgkiss DR, Kola I, Seth A. ETS1 and ETS2 in p53 regulation: spatial separation of ETS binding sites (EBS) modulate protein: DNA interaction. Oncogene 1996; 12: 1199.
21. Chen J, Kastan MB. S–3′ UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes Dev 2010; 24: 2146–2156.
22. Tian S, Huang S, Wu S, Guo W, Li J, He X. MicroRNA-125B inhibits the expression of p53 by directly targeting its 3′ untranslated region. Biochem Biophys Res Commun 2010; 396: 435–439.