p53 isoform $\Delta 113p53$ is a p53 target gene that antagonizes p53 apoptotic activity via BclxL activation in zebrafish

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p53 is a well-known tumor suppressor and is also involved in processes of organismal aging and developmental control. A recent exciting development in the p53 field is the discovery of various p53 isoforms. One p53 isoform is human $\Delta 133p53$ and its zebrafish counterpart $\Delta 113p53$. These N-terminal-truncated p53 isoforms are initiated from an alternative p53 promoter, but their expression regulation and physiological significance at the organismal level are not well understood. We show here that zebrafish $\Delta 113p53$ is directly transactivated by full-length p53 in response to developmental and DNA-damaging signals. More importantly, we show that $\Delta 113p53$ functions to antagonize p53-induced apoptosis via activating *bcl2L* (closest to human *Bcl-x_L*), and knockdown of $\Delta 113p53$ enhances p53-mediated apoptosis under stress conditions. Thus, we demonstrate that the p53 genetic locus contains a new p53 response gene and that $\Delta 113p53$ does not act in a dominant-negative manner toward p53 but differentially modulates p53 target gene expression to antagonize p53 apoptotic activity at the physiological level in zebrafish. Our results establish a novel feedback pathway that modulates the p53 response and suggest that modulation of the p53 pathway by p53 isoforms might have an impact on p53 tumor suppressor activity.

[**Keywords:** p53 isoforms; $\Delta 113p53$; Bcl2; apoptosis; zebrafish]

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The tumor suppressor protein p53 is stabilized and activated in response to a wide variety of cellular stress (Laptenko and Prives 2006; Lavin and Gueven 2006). p53 is a transcription factor that regulates expression of genes involved in cell cycle arrest, DNA repair, and programmed cell death. Hence, p53 prevents genomic abnormalities being passed on to daughter cells, which could ultimately lead to cancer (Greenblatt et al. 1994; Vogelstein et al. 2000; Levine et al. 2004; Kops et al. 2005). The actual way in which p53 decides between cell cycle arrest and cell death in response to cellular stress remains, to an extent, unclear. In addition to its well-known function as a tumor suppressor, new data have shown that p53 is also involved in many other biological processes such as physiological responses to organism

aging, metabolism, and developmental control (Vousden and Lane 2007).

Until recently, the structure of the p53 gene was thought to be much simpler compared with *p63* and *p73*, two p53-homologous genes, which encode for several isoforms due to alternative promoter usage and alternative splicing. The N-terminal-truncated isoforms $\Delta Np63$ and $\Delta Np73$ are initiated from an alternative promoter in intron 3 in *p63* and *p73*, respectively (Yang et al. 1998; Grob et al. 2001). Studies on the *p73* alternative promoter showed that this promoter is subject to p53 regulation. In addition, both genetic and biochemical data have shown that $\Delta Np63$ and $\Delta Np73$ can function in a dominant-negative way to attenuate full-length TAp63 or TAp73 proteins (Yang et al. 1998; Grob et al. 2001).

We recently further assessed the structure of the p53 gene, using a PCR-based technique that specifically amplifies all capped mRNAs within normal human cells. This allowed us to identify several p53 mRNA variants encoding for nine different p53 protein isoforms (p53, p53 β , p53 γ , $\Delta 40p53$, $\Delta 40p53\beta$, $\Delta 40p53\gamma$, $\Delta 133p53$, $\Delta 133p53\beta$, and $\Delta 133p53\gamma$) due to alternative splicing,

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alternative promoters usage, and alternative initiation of translation (Bourdon et al. 2005). Moreover, we established that the *p53* gene family (*p53/p63/p73*) has a dual gene structure conserved in *Drosophila*, zebrafish, and man, suggesting that it has essential biological activities (Bourdon et al. 2005; Chen et al. 2005).

$\Delta 133p53$ in human and its orthologous $\Delta 113p53$ in zebrafish are both transcribed by the alternative *p53* promoter in intron 4 (Bourdon et al. 2005; Chen et al. 2005). Our analyses showed that a DNA fragment containing intron 4 of human *p53* could drive the expression of the reporter gene *luciferase* in H1299 cells, suggesting that this region is indeed transcriptionally active. The $\Delta 133p53/\Delta 113p53$ proteins are N-terminal-truncated forms of p53 with deletion of both the Mdm2-interacting motif and transcription activation domains together with partial deletion of the DNA-binding domain. However, the dimerization/tetramerization domain remains intact in $\Delta 133p53/\Delta 113p53$. We reported previously that, in the human cell line system, $\Delta 133p53$ might act as a dominant-negative regulator of p53 since cotransfection of full-length p53 with $\Delta 133p53$ impaired p53-induced cell apoptosis (Bourdon et al. 2005). Interestingly, $\Delta 113p53$ was found to be highly expressed in breast cancer and oral lichen planus (OLP) (Bourdon et al. 2005; Boldrup et al. 2007; Ebrahimi et al. 2008). Meanwhile, expression of $\Delta 113p53$ in zebrafish was specifically elevated in the digestive organs of the *def^{hi429}* mutant (Chen et al. 2005) and was induced by the injection of certain off-target morpholinos (Robu et al. 2007).

As the study of p53 isoforms is still at its infancy, these observations raise several questions. For example, how is $\Delta 113p53$ expression regulated? What is the biological function of $\Delta 113p53$, and what is the functional relationship between p53 and $\Delta 113p53$? Here we report that zebrafish $\Delta 113p53$ expression is directly transactivated by p53 upon DNA-damaging treatments and in the aberrant development of the *def^{hi429}* mutant fish. We show that p53-mediated induction of endogenous $\Delta 113p53$ inhibits p53-mediated apoptosis in response to DNA damage in zebrafish embryos. We also show that $\Delta 113p53$ does not work simply as dominant-negative toward p53 but rather differentially modulates gene expression to protect cells from apoptosis. Our results establish a negative feedback loop modulating p53 apoptotic activity that creates a new niche in the field and could have critical implications in cancer treatment and diagnosis.

Results

$\Delta 113p53$ expression is induced by DNA-damaging signals

The fact that the expression of $\Delta 113p53$ RNA was strikingly induced in the digestive organs by the loss of function of the *def* gene (*def* encodes for a novel digestive organ-enriched nuclear localized protein) (Chen et al. 2005) and by injection of certain morpholinos (Robu et al. 2007) suggests that the expression of $\Delta 113p53$ is

subjected to regulation by both developmental and external signals. To test if the expression of $\Delta 113p53$ is subjected to other regulation such as induction by DNA-damaging signals, we injected sheared herring sperm DNA into wild-type embryos. Northern blot hybridization using the *p53* probe I and RT-PCR using $\Delta 113p53$ -specific primers showed that the expression of $\Delta 113p53$ was obviously induced in the injected embryos at 24 h post-fertilization (hpf), while injection of baker's yeast total RNA or of buffer with phenol red dye failed to do so (Fig. 1A). Injection of linearized pGEMT plasmid DNA into one-cell-stage embryos also significantly induced the expression of $\Delta 113p53$ at 24 hpf when compared with the buffer-injected control embryos (Fig. 1B), possibly because the injected plasmid DNA mimicked genomic DNA with broken ends that triggered the cellular response. Such responses have been reported in mammalian tissue culture systems (Huang et al. 1996). We hypothesized that other means of causing DNA

Figure 1. $\Delta 113p53$ is responsive to DNA-damaging signals. (A) Induction of $\Delta 113p53$ expression by sheared herring sperm DNA injection. Northern analysis using the *p53* probe I (detecting both p53 and $\Delta 113p53$) and RT-qPCR using $\Delta 113p53$ -specific primers showed that injection of 50 pg of sheared herring sperm DNA (lane 4) induced the expression of $\Delta 113p53$ in wild-type (WT) embryos, whereas injection of 50 pg of baker's yeast total RNA (lane 3) or buffer containing phenol red dye (lane 2) failed to do so when compared with the uninjected wild-type control (lane 1). (B, top panel) Northern analysis of p53 and $\Delta 113p53$ transcripts using the *p53* probe I. (Third panel) RT-qPCR analysis of $\Delta 113p53$ using $\Delta 113p53$ -specific primers. (Lane 1) Uninjected wild type. (Lane 2) Buffer-injected wild type. (Lane 3) pGEMT plasmid DNA-injected wild type. (C) Wild-type and the *tp53^{M214K}* mutant embryos were treated with γ -ray, camptothecin (campt), or roscovitine (roscov). Northern analysis was performed using the *p53* probe I (top panel), a $\Delta 113p53$ -specific probe (second panel) and a *p53*-specific probe (third panel). Control: Untreated wild-type (lanes 1,5) and *tp53^{M214K}* (lane 8) embryos.

damage, such as γ -ray irradiation and drug treatment, would be expected to trigger the expression of $\Delta 113p53$ as well. Wild-type fish embryos were treated with γ -ray (Lu and Lane 1993), camptothecin, and roscovitine (Langheinrich et al. 2002), respectively, and embryos 18 h post-treatment were harvested and assayed for $\Delta 113p53$ RNA levels using either the *p53* probe I, or a $\Delta 113p53$ -specific probe or a *p53*-specific probe. The results showed that all treatments dramatically induced $\Delta 113p53$ RNA expression in the treated embryos (Fig. 1C). Interestingly, while γ -ray treatment also increased full-length *p53* expression, camptothecin and roscovitine treatments did not show obvious effect on full-length *p53* RNA levels (Fig. 1C).

The Tg($\Delta 113p53:gfp$) reporter system faithfully recapitulates the response of endogenous $\Delta 113p53$ to both developmental and DNA-damaging signals

As there are no antibodies recognizing specifically zebrafish $\Delta 113p53$ protein, to follow in vivo $\Delta 113p53$ expression in response to irradiation or developmental defect, we generated *Tg($\Delta 113p53:gfp$)* transgenic fish expressing Gfp protein under the control of the zebrafish *p53* internal promoter. A 4.113-kb DNA fragment immediately upstream of the start codon ATG of $\Delta 113p53$ (Fig. 2A,B) was amplified using primers P0-F and P0-R (Supplemental Table S1) and then cloned to the pEGFP vector upstream of the *gfp* reporter gene (designated as $\Delta 113p53:gfp$).

Because P0-F (starts from +26) and P0-R (ends at +477) are derived from the *p53* mRNA (accession no. AF365873), this DNA fragment excludes the *p53* promoter region. Because the endogenous $\Delta 113p53$ expression is induced by plasmid DNA injection (Fig. 1B), it is expected that if the 4.113-kb DNA fragment contains the regulatory elements for the expression of $\Delta 113p53$, then injection of the $\Delta 113p53:gfp$ plasmid would induce *gfp* expression. Indeed, when the $\Delta 113p53:gfp$ plasmid was injected into the single-cell-stage embryos, Gfp was strongly expressed in the injected embryos (Fig. 2C; Supplemental Fig. 1), suggesting that the cloned $\Delta 113p53$ promoter is a functional promoter. We then generated *Tg($\Delta 113p53:gfp$)* transgenic fish and crossed the *Tg($\Delta 113p53:gfp$)* fish with the *def^{hi429}* heterozygous mutant fish. The F2 progeny of the heterozygous F1 sibling crosses were checked for Gfp fluorescence. At 3 d post-fertilization (dpf), Gfp was found to be weakly expressed in the wild-type and *def^{hi429}* heterozygous siblings (Fig. 2D, highlighted with red arrow) but was highly enriched in the head region and digestive organs in the *def^{hi429}* homozygous mutants (Fig. 2D; Supplemental Fig. 2A,B, highlighted with yellow arrow), displaying a pattern resembling the endogenous $\Delta 113p53$ RNA expression as reported previously (Chen et al. 2005). We then treated the *Tg($\Delta 113p53:gfp$)* transgenic embryos with γ -ray, camptothecin, and roscovitine and assayed for both Gfp fluorescence and *gfp* transcripts in the treated embryos.

Figure 2. *Tg($\Delta 113p53:gfp$)* reporter fish faithfully recapitulate the response of $\Delta 113p53$ to both developmental and stress signals. (A)

Diagram showing the genomic structure of the zebrafish *p53* gene and the relative position of the 4.113-kb genomic DNA fragment cloned for the $\Delta 113p53$ promoter activity analyses. The zebrafish *p53* gene (derived from BAC clone CH211-190-H10) has 10 exons (light-blue box) and nine introns (black lines linking exons). The start codon ATG of *p53* is located in the second exon. The lengths of introns 1–4 of *p53* are 637, 240, 92, and 2692 bp, respectively, as shown in the diagram. Transcription of $\Delta 113p53$ starts in intron 4 (dark-blue box ES), and the mature $\Delta 113p53$ transcript contains a 155-bp intron 4 sequence joined to exon 5 of *p53* after splicing an intron of length 842 bp (intron 1 for $\Delta 113p53$). The start codon ATG of $\Delta 113p53$ is located in exon 5 of *p53*. The 4.113-kb genomic DNA fragment cloned is immediately upstream of the $\Delta 113p53$ start codon ATG and ends in exon 1 of *p53*. The 4.113-kb DNA fragment was cloned into the pEGFP-1 vector to generate the $\Delta 113p53:gfp$ plasmid. (B) Diagram showing the main domains of *p53* and $\Delta 113p53$. Each domain is represented by different bars with descriptions above or under the bar. (C) $\Delta 113p53:gfp$ plasmid-injected wild-type embryos at 24 hpf. (D) Gfp fluorescence in a heterozygous *def^{hi429}* sibling (red arrow) and a *def^{hi429}* homozygote (yellow arrow) in the *Tg($\Delta 113p53:gfp$)* background at 3 dpf. (in) Intestine; (lv) liver; (ex) exocrine pancreas. (E,F) *gfp* transcripts (E) and Gfp fluorescence (F) in *Tg($\Delta 113p53:gfp$)* embryos untreated or treated with γ -ray, camptothecin, or roscovitine.

The result showed that both levels of *gfp* transcripts and Gfp fluorescence were induced by these treatments (Fig. 2E,F) and that the γ -ray treatment showed a much stronger effect than the two drugs on induction of *gfp* expression (Fig. 2E). The amplified 4.113-kb fragment is known to contain the 5'-untranslated region (5'-UTR) of $\Delta 113p53$; therefore, transcription of *gfp* in *Tg*($\Delta 113p53:gfp$) transgenic fish is expected to be initiated from the cloned

$\Delta 113p53$ transcription initiation start site (TSS) (Fig. 3A). Indeed, sequencing the 5'-RACE product obtained by using a *gfp*-specific primer revealed that the *gfp* transcript contained the expected $\Delta 113p53$ 5'-UTR region (data not shown). The results demonstrate that the 4.113-kb DNA fragment faithfully recapitulates the regulation of endogenous $\Delta 113p53$ expression in response both to DNA-damaging and developmental signals.

Figure 3. $\Delta 113p53$ is a p53-target gene. (A) Diagram showing the details of each $\Delta 113p53$ promoter deletion construct. The $\Delta 113p53:gfp$ plasmid is designated as P0 and the TSS of $\Delta 113p53$ as the position +1. The start positions of intron 4 (−1692) and exon 5 (+995) of *p53* are also highlighted. The nucleotide position +1054 is immediately 5' upstream of the start codon ATG of $\Delta 113p53$. (Red line) Internal deletions. Three putative p53-binding sites (site I: −1125 to −1106 bp, AGACATGAATGGGCATGTTTC; site II: −243 to −219 bp, TGACATGTTA-TATTTATCAAGTCC; site III: −103 to −84 bp, GAACATGTCTGAACCTTGTC; underlined bases denote mismatches) are marked with red lined white boxes. (B) Gfp fluorescence in P0 to P9 plasmid-injected wild-type embryos at 24 hpf. (C, first and second panels) Northern analysis of *gfp* transcripts and 18S rRNA control in wild-type embryos (24 hpf) injected with P0 to P9 plasmids to assay their transcriptional activity. The longer transcripts in P0-, P1-, P2-, and P6-injected samples represent the unspliced product, and the lower band represents the mature mRNA in each case. (Third and fourth panels) RT-qPCR analysis of the endogenous $\Delta 113p53$ and *elongation factor a* gene (*el1a*) (control). (ctl) Uninjected wild-type control. (D) The *p53*-probe I (top panel) and the $\Delta 113p53$ -specific probe (middle panel) were used in Northern analysis. (Lane 1) *tp53*^{M214K} *def*^{+/+} or *def*^{hi429/+} siblings. (Lane 2) *def*^{+/+} *tp53*^{M214K} double mutant. (Lane 3) *def*^{+/+} or *def*^{hi429/+} siblings. (Lane 4) *def*^{hi429} single mutant. (E) Detection of $\Delta 113p53$ transcripts in *p53*-MO^{ATG}-injected γ -ray-treated embryos, in *def*-MO or *p53*-MO^{ATG} and *def*-MO-coinjected wild-type embryos. Morpholinos were injected at the one-cell stage, and the injected embryos were treated with γ -ray at 24 hpf and harvested for total RNA extraction 6 h after the γ -ray treatment. (F, top panel) *gfp* transcripts in the *tp53*^{M214K} embryos injected with P0, P5, or P9 plasmids alone or together with *p53* mRNA. (Third panel) The endogenous $\Delta 113p53$ expression was examined via RT-qPCR. Uninjected wild type (lane 1) and P0-injected wild type (lane 2) were used as the negative and positive controls, respectively. (G) ChIP down the HA-p53-DNA complex using an anti-HA antibody (α -HA Co-IP) from the input. Control: Uninjected embryos. (H) HA-p53-DNA complex obtained by ChIP and respective inputs (input

panel) were used as the templates for PCRs using a pair of primers from exon 10 (negative control) (lanes 1,2), a pair for region −112 to +98 (*p53*-binding site III) (lanes 3,4), and a pair for the region −1086 to −1300 (*p53*-binding site I) (lanes 5,6).

Identification of two cis-regulatory regions essential for $\Delta 113p53$ expression

To characterize the $\Delta 113p53$ promoter in detail, nine deletion constructs were generated from the $\Delta 113p53:gfp$ plasmid (P0 construct) (Fig. 3A) and injected into wild-type embryos to assay their transcriptional ability in response to DNA-damaging signals (i.e., plasmid DNA injection). Both the Gfp fluorescence (Fig. 3B) and *gfp* transcripts (Fig. 3C) together with the endogenous $\Delta 113p53$ transcripts (Fig. 3C) were examined in each case. Deletion from –1992 to –3059 from the 5'-end (construct P1 and P2) (Fig. 3A) did not alter the promoter activity in driving the expression of the *gfp* reporter gene (Fig. 3B,C). However, deletion from –1544 to –3059 from the 5'-end (construct P3) (Fig. 3A) significantly reduced the promoter activity in driving the expression of the *gfp* reporter gene (Fig. 3B,C). Extension of the deletion from –1544- to –1039-base-pair (bp) (construct P4) (Fig. 3A) further reduced the promoter activity, but this reduction was not enhanced by further deletion up to –506 bp (construct P5) (Fig. 3A–C). These data suggest that there are cis-regulatory elements between –1041 and –1991 bp. The internal deletion from –506 and –1059 bp in P6 seems to activate the promoter because the induction of *gfp* transcription by P6 plasmid injection is stronger than that by P0 plasmid injection, suggesting that there might be a repressive element in this region (construct P6) (Fig. 3A–C). Deletion +564 to +1054 bp from the 3'-end (construct P8) (Fig. 3A) did not obviously alter the promoter activity because the *gfp* transcript levels were similar to that observed for the control (P0 construct) (Fig. 3C). However, the Gfp fluorescence level was much weaker in the P8 injected embryos than that observed in the P0 injected embryos (Fig. 3B, P0 and P8 panels), and the likely explanation is that the P8 construct probably lacks a suitable Kozak sequence for efficient translation (Fig. 3A). On the other hand, deletion –278 to +1054 bp from the 3'-end (construct P7) (Fig. 3A) completely abolished the promoter activity (Fig. 3B,C), most likely because the –278- to +1054-bp region contains (a) crucial regulatory element(s) together with the TSS (position +1) (Fig. 3A). We further deleted the internal region between –1 and –239 bp upstream of the TSS (construct P9) (Fig. 3A) and found that this internal deletion also completely abolished the expression of *gfp* (Fig. 3B,C). In conclusion, deletion analyses have identified two regions, namely, –1041 to –1991 bp and –1 to –239 bp to contain crucial cis-elements for the $\Delta 113p53$ expression. RT-PCR results showed that, in all of the above cases, endogenous $\Delta 113p53$ expression was increased due to plasmid DNA injection (Fig. 3C), demonstrating that the two regions identified in our promoter analysis are likely genuinely used to regulate $\Delta 113p53$ expression in vivo.

Loss of function of full-length p53 abolishes the response of $\Delta 113p53$ to either developmental or DNA-damaging inductive signals

Sequence analysis revealed three putative p53-binding sites in the $\Delta 113p53$ promoter (Fig. 3A, highlighted in

red), and this observation fits well with the general observation that most of the p53 target genes have at least several p53-binding sites in their promoters (Bourdon et al. 1997; Zhao et al. 2000; Hoh et al. 2002; Inga et al. 2002; Laptenko and Prives 2006), indicating that the expression of $\Delta 113p53$ might be regulated by p53. To test this hypothesis, we took advantage of the $p53^{-/-}$ mutant that carries the $tp53^{M214K}$ mutation converting the M²¹⁴ to K²¹⁴ in the p53 DNA-binding domain (Berghmans et al. 2005), inactivating p53 transcriptional activity (Lee et al. 2008). We constructed the $def^{hi429} tp53^{M214K}$ double mutant and examined the levels of $\Delta 113p53$ transcripts in the double mutant. Consistent with our previous report (Chen et al. 2005), $tp53^{M214K}$ mutation fully or partially rescued the hypoplastic digestive organ phenotype conferred by the def^{hi429} mutation (data not shown). We reasoned that if $\Delta 113p53$ expression is regulated by p53 and if $tp53^{M214K}$ is epistatic to def^{hi429} , the up-regulated $\Delta 113p53$ expression in the def^{hi429} mutant (Chen et al. 2005) would be compromised or abolished in the $tp53^{M214K}$ background. Indeed, while $\Delta 113p53$ was highly expressed in the def^{hi429} single mutant, its expression was almost undetectable in the $def^{hi429} tp53^{M214K}$ double mutant (Fig. 3D). This observation suggests that the $\Delta 113p53$ response to developmental signals is p53-dependent. To test if the $\Delta 113p53$ response to DNA-damaging signals is also p53-dependent, we treated the $tp53^{M214K}$ embryos with γ -ray, camptothecin, and roscovitine. The results showed that these treatments failed to induce $\Delta 113p53$ expression (Fig. 1C). Furthermore, knockdown of endogenous p53 by the p53-MO^{ATG} morpholino that specifically targets the start codon ATG of the full-length p53 (Langheinrich et al. 2002) greatly reduced the induction of $\Delta 113p53$ expression in wild type by γ -ray or in the *def*-MO morphants (3 dpf) (Fig. 3E; Chen et al. 2005). To further test the necessity and sufficiency of p53 for $\Delta 113p53$ expression, we injected the P0 plasmid ($\Delta 113p53:gfp$) (Fig. 3A) into the $tp53^{M214K}$ mutant and wild-type control embryos. Both Gfp fluorescence and *gfp* transcripts together with endogenous $\Delta 113p53$ transcripts were examined. The results showed that the *gfp* reporter gene (Fig. 3F) and Gfp fluorescence (Supplemental Fig. 3) were highly expressed in the injected wild-type embryos but were barely detectable in the injected $tp53^{M214K}$ embryos. More importantly, coinjecting p53 mRNA with the P0 plasmid rescued the *gfp* expression (Fig. 3F) and Gfp fluorescence (Supplemental Fig. 3) in the injected $tp53^{M214K}$ embryos, while coinjecting the plasmid with $\Delta 113p53$ mRNA failed to do so (Fig. 3F; Supplemental Fig. 3). RT-PCR showed that the expression of the endogenous $\Delta 113p53$ phenocopied the *gfp* expression pattern of P0 in each treatment (Fig. 3F). Therefore, induction of the $\Delta 113p53$ expression by either developmental or DNA-damaging signals is totally p53-dependent.

$\Delta 113p53$ expression is directly regulated by p53

Next, we asked if the predicted p53-binding sites in the $\Delta 113p53$ promoter are used by p53 to regulate $\Delta 113p53$ expression. First, we injected the P5 and P9 plasmids [P5 is deleted of the predicted p53-binding site I, and P9 is

deleted of sites II and III) into the $tp53^{M214K}$ mutant embryos, and as expected, no Gfp and *gfp* expression was detected (Fig. 3F; Supplemental Fig. 3). Coinjecting P5 and P9, respectively, with $p53$ mRNA into $tp53^{M214K}$ embryos showed that p53 could rescue the weak expression of Gfp and *gfp* in P5-injected embryos but not in P9-injected embryos, while RT-PCR results showed that the endogenous $\Delta 113p53$ was well induced in both cases (Fig. 3F; Supplemental Fig. S3). This observation is fully consistent with our previous observation that P5 still retains certain promoter activity, while P9 is abolished of such activity (Fig. 3B,C). We went further to test if p53 binds to the predicted putative p53-binding sites in the $\Delta 113p53$ promoter to regulate $\Delta 113p53$ expression directly. mRNA encoding HA-tagged $p53$ was injected into single-cell-stage embryos, and the embryos at 5 h post-injection were harvested and used for a chromatin coimmunoprecipitation (ChIP) assay using anti-HA antibody to pull down the HA-p53-DNA complex (Fig. 3G). Two pairs of primers (one pair to amplify the -1086 to -1300 fragment containing the putative p53-binding site I and the other pair to amplify the -112 to +98 fragment containing the putative p53-binding site III) (Supplemental Table S1) were designed and used to perform PCR using the HA-p53-DNA complex as the template. The results showed that both the -112 to +98 fragment and -1086 to -1300 fragments were specifically coimmunoprecipitated together with HA-p53 but not in the uninjected controls (Fig. 3H). On the other hand, PCR using a pair of primers derived from exon 10 of $p53$ failed to yield any products using the HA-p53-DNA complex as the template (Fig. 3H). Furthermore, deletion of 10 bp from the p53-binding site III abolished the promoter activity, whereas the endogenous $\Delta 113p53$ was induced by plasmid injection as expected (Supplemental Fig. 4). Therefore, p53 directly regulates $\Delta 113p53$ expression by binding to response elements in the promoter.

$\Delta 113p53$ antagonizes p53's apoptotic activity

In the above, we showed that $\Delta 113p53$ expression is induced by DNA-damaging signals via a p53-dependent pathway. What, then, is the biological significance of such a dramatic increase in $\Delta 113p53$ expression for an embryo in response to DNA-damaging signals? $\Delta 133p53$ has been implicated to act as a dominant-negative regulator of p53 since cotransfection of p53 with $\Delta 133p53$ impaired p53-induced cell apoptosis in cultured cells (Bourdon et al. 2005). To study if $\Delta 113p53$ can modulate p53 activity at the organismal level, we first injected 0.01, 0.05, and 0.1 ng of $p53$ mRNA (in same volume) into the $tp53^{M214K}$ mutant embryos and found that the viability in these injected embryos was ~69%, 39%, and 13%, respectively; together these embryos displayed a p53 dosage-dependent abnormal morphology (deformed tail, trunk, and head), whereas injection of the $tp53^{M214K}$ mutant mRNA did not cause abnormal development and high mortality (Fig. 4A,B; Supplemental Figs. 5, 6). On the other hand, injection of $\Delta 113p53$ mRNA up to a concentration of 1 ng into the

$tp53^{M214K}$ embryos did not cause obvious morphological change and increase in mortality when compared with the control injection (Fig. 4A-C; Supplemental Fig. 6). Interestingly, coinjection of 0.1, 0.5, or 1 ng of $\Delta 113p53$ mRNA, but not the *gfp* mRNA, with 0.1 ng of $p53$ not only reduced the high mortality but also rescued the abnormal morphology caused by 0.1 ng of $p53$ mRNA in a $\Delta 113p53$ mRNA dosage-dependent manner (Fig. 4A,C; Supplemental Fig. 5). TUNEL assay showed that, compared with the $tp53^{M214K}$ control embryos, cell apoptosis was strongly increased in the $p53$ mRNA-injected $tp53^{M214K}$ embryos. Coinjection of $\Delta 113p53$ mRNA with $p53$ mRNA greatly compromised the p53-induced apoptotic activity (Fig. 4D).

$\Delta 113p53$ knockdown in *def*-MO morphants by $\Delta 113p53$ -MO enhances cell apoptosis in the digestive organs specifically

After showing that overexpression of $\Delta 113p53$ can inhibit p53-induced apoptosis, we then investigated whether knockdown of endogenous $\Delta 113p53$ expression can promote p53-mediated apoptosis in conditions known to induce p53-dependent cell cycle arrest under physiological conditions. We designed a translation-blocking morpholino ($\Delta 113p53$ -MO) targeting the 5'-UTR of $\Delta 113p53$ that is included and transcribed together with *gfp* in the $\Delta 113p53:gfp$ construct. Coinjecting $\Delta 113p53$ -MO with the $\Delta 113p53:gfp$ plasmid into wild-type embryos (Supplemental Fig. 7A,B) or injecting $\Delta 113p53$ -MO into *Tg*($\Delta 113p53:gfp$) embryos (Fig. 5A,B) effectively blocked the translation of Gfp protein and Gfp fluorescence. We showed previously that the hypoplastic digestive organs in the *def^{hi429}* mutant are due to p53-dependent cell cycle arrest with specific induction of p53 target genes involved in cell cycle arrest concomitantly with increased expression of $\Delta 113p53$. *def*-MO morphants mimicked the *def^{hi429}* mutant phenotype together with elevated $\Delta 113p53$ transcript levels (Chen et al. 2005). When $\Delta 113p53$ -MO was coinjected with the *def*-MO (Supplemental Fig. 8), we found that the coinjected embryos exhibited unusual cell apoptosis specifically in the digestive organs (Fig. 5C-K; Supplemental Fig. 7C), and consequently, the coinjected embryos (Fig. 5F-H) exhibited more severe hypoplastic digestive organs than the *def^{hi429}* mutant (Chen et al. 2005). Therefore, the highly elevated levels of $\Delta 113p53$ in the *def^{hi429}* embryos prevent mutant cells from undergoing apoptosis under normal physiological conditions.

$\Delta 113p53$ knockdown by $\Delta 113p53$ -MO sensitizes the zebrafish embryos to ionizing radiation treatment

We then investigated the role of $\Delta 113p53$ in wild-type embryos in response to ionizing radiation. When the $\Delta 113p53$ -MO-injected wild-type embryos at 1 dpf were treated with γ -ray (24 Gy), they exhibited 100% mortality (448 embryos examined) at 5 d post-treatment (Fig. 6A; Supplemental Fig. 9). In contrast, both buffer and standard control morpholino (*st*-MO) injected wild-type embryos exhibited stronger tolerance against the γ -ray treatments

Figure 4. $\Delta 113p53$ antagonizes p53's apoptotic activities. (A, panels 2–4) Injection of p53 mRNA into the $tp53^{M214K}$ mutant embryos caused death to the injected embryos in a dosage-dependent manner. (Panels 6–9) Coinjecting $\Delta 113p53$ mRNA with p53 mRNA antagonized p53's apoptotic activity and reversed the viability of the injected embryos in a dosage-dependent manner. Injection of 1 ng of $\Delta 113p53$ (panel 5) did not cause an overt effect on injected $tp53^{M214K}$ embryos when compared with the buffer-injected $tp53^{M214K}$ embryos (panel 1). The embryos shown were at 24 hpf. (B,C) Viability counting corresponding to panels in A. Viability of the $tp53^{M214K}$ embryos injected with buffer (panel 1), 0.01, 0.05, and 0.1 ng of p53 mRNA (panels 2–4), 1 ng of $\Delta 113p53$ mRNA alone (panels 5,5'), or coinjected with 0.1 ng of p53 mRNA with 0, 0.1, 0.5, and 1 ng of $\Delta 113p53$ mRNA (panels 6–9). Each test was repeated three times, and ~100–200 embryos were counted in each test. Dead embryos are characterized by the presence of large, dark debris in the embryo. The high death rate in p53 mRNA-injected embryos was not caused by possible toxins released from dead embryos because when such egg water was reused to culture new embryos, the embryos grew normally without an abnormal death toll (data not shown). (D) Apoptosis TUNEL assay (red spots) in $tp53^{M214K}$ embryos at 10 hpf injected with p53 mRNA alone or coinjected with p53 and $\Delta 113p53$ mRNA. Controls: Uninjected or buffer-injected $tp53^{M214K}$ embryos.

and had 67% and 52% survival rates, respectively (>200 embryos examined in each case) (Fig. 6A; Supplemental Fig. 9). We also treated the $\Delta 113p53$ -MO- or st-MO-injected $tp53^{M214K}$ embryos with γ -ray, and no obvious differences between the two samples were observed regarding viability (70% and 71%, respectively) and abnormal development (Fig. 6A; Supplemental Fig. 9). TUNEL assay revealed that mortality was due to massive apoptosis in γ -ray-treated $\Delta 113p53$ -MO-injected wild-type embryos (Fig. 6B; Supplemental Fig. 10). To prove the specificity of the $\Delta 113p53$ -MO effect, we coinjected the embryos with $\Delta 113p53$ mRNA and $\Delta 113p53$ -MO and found that $\Delta 113p53$ mRNA prevented the $\Delta 113p53$ -MO morphants from the γ -ray-induced apoptosis (Fig. 6B; Supplemental Fig. 10). Furthermore, knockdown of p53 using a morpholino specifically targeting the p53 start codon ATG (Langheinrich et al. 2002) blocked the synergistic effect of $\Delta 113p53$ -MO and γ -ray on apoptosis in the treated embryos (Fig. 6C; Supplemental Figs. 11, 12). Therefore, endogenous $\Delta 113p53$ expression protects wild-type embryos from p53-mediated apoptosis in response to ionizing radiation or DNA-damaging environment.

$\Delta 113p53$ alters full-length p53 transcriptional selectivity on p53 response genes

To study the molecular mechanism of $\Delta 113p53$ anti-apoptotic activity, we examined the expression of p53 response genes *p21*, *bcl2L* (*bcl2*-like, closest to human *Bcl-x_L*), *bax*, and *mdm2* in $tp53^{M214K}$ embryos, $tp53^{M214K}$ embryos injected with p53 mRNA alone or $\Delta 113p53$ mRNA alone, or coinjected with p53 and $\Delta 113p53$ mRNAs. As expected, *p21*, *bax*, and *mdm2* were all significantly up-regulated, whereas *bcl2L* was obviously down-regulated upon p53 mRNA injection (Fig. 7A, lane 3; Supplemental Fig. 13). We also examined *bcl2* expression (Kratz et al. 2006) and did not observe any obvious differences at the transcript level among these samples (data not shown). Notably, *p21*, *bax*, and *mdm2* were unresponsive to $\Delta 113p53$ mRNA injection, suggesting that $\Delta 113p53$ alone has little or subtle transcriptional activity on these three genes, whereas *bcl2L* was slightly up-regulated in $tp53^{M214K}$ embryos injected with $\Delta 113p53$ mRNA (Fig. 7A, lane 2; Supplemental Fig. 13). Interestingly, coinjection of $\Delta 113p53$ with p53 caused an

Figure 5. Knockdown $\Delta 113p53$ enhances cell apoptosis specifically in the digestive organs in the *def* morphants. (A,B) Analysis of Gfp fluorescence (A) and Gfp protein (B) in Tg($\Delta 113p53:gfp$) embryos injected with st-MO or $\Delta 113p53$ -MO and treated with or without 50 nM camptothecin as indicated (>100 embryos were examined in each case). Lanes 1–3 in B correspond to samples 1–3 in A. (C–K) A *def*-MO morphant (C–E), a *def*-MO and $\Delta 113p53$ -MO double morphant (F–H), and a $\Delta 113p53$ -MO morphant (I–K). (C,F,I) TUNEL staining. (D,G,H) DAPI staining. (E,H,K) Superimposition of corresponding TUNEL and DAPI staining. (in) Intestine; (pa) pancreas. For the $\Delta 113p53$ -MO and *def*-MO double morphants, out of 1965 cells counted (total 30 sections from four fish) in the liver, pancreas, and intestine, 66 apoptotic cells were identified. For *def*-MO morphants, out of 3019 cells counted (total 23 sections from three fish) in the liver, pancreas, and intestine, no apoptotic cell was found. For $\Delta 113p53$ -MO morphants, out of 2195 cells counted (total 25 sections from three fish) in the liver, pancreas, and intestine, no apoptotic cell was found.

obvious up-regulation of *p21* and *mdm2* and twofold to threefold up-regulation of *bcl2L*, whereas it slightly down-regulated *bax* when compared with their respective expression in the *p53* mRNA-alone injected embryos (Fig. 7A, lane 4; Supplemental Fig. 13). We showed previously that the *def^{hi429}* mutant has elevated levels of $\Delta 113p53$, *p21*, and *mdm2* (Chen et al. 2005). In fact, *bcl2L* is also up-regulated in *def^{hi429}* (Fig. 7B). Treating *def^{hi429}* mutant embryos with $\Delta 113p53$ -MO down-regulated *bcl2L* (Fig. 7B). Similarly, $\Delta 113p53$ -MO also down-regulated *bcl2L* in the wild-type embryos treated with γ -ray (Fig. 7C). Therefore, $\Delta 113p53$ does not work simply as dominant-negative toward p53, but rather, it modulates p53 response by differentially altering the expression profiles of p53-responsive genes.

To investigate the biological significance of the elevated *bcl2L* mentioned above, we coinjected the wild-type *bcl2L* mRNA or the *bcl2L^m* mutant (a C-to-T substitution converted the sixth codon CGA for Arg to the stop codon TGA) mRNA with *p53* mRNA into *tp53^{M214K}* and found that *bcl2L*, but not *bcl2L^m*, could inhibit p53-mediated high mortality and apoptosis as effectively as $\Delta 113p53$ (Fig. 7D,E; Supplemental Figs. 14A,B, 15A,C). Conversely, coinjecting 0.06 pmol of *bcl2L*-specific morpholino (*bcl2L*-MO) (Supplemental Fig. 14A–D) but not the control st-MO (Supplemental Fig. 15A) with *p53* and $\Delta 113p53$ mRNAs blocked $\Delta 113p53$ anti-apoptotic activity and induced high mortality in the injected *tp53^{M214K}* embryos (Fig. 7D; Supplemental Fig. 15B) due to massive cell apoptosis (Fig. 7E; Supplemental Fig. 15C). Therefore, considering the fact that *bcl2L* expression was down-regulated by $\Delta 113p53$ -MO in the *def^{hi429}* mutant or γ -ray-treated wild-type embryos (Fig. 7B,C), it is reasonable to

argue that the $\Delta 113p53$ anti-apoptotic activity is at least partly mediated by induction of *bcl2L* expression under physiological conditions.

Discussion

It was reported that $\Delta 133p53$ was overexpressed in human breast tumors and in human samples from chronic inflammatory disease OLP (Bourdon et al. 2005; Boldrup et al. 2007; Ebrahimi et al. 2008). In zebrafish, the level of $\Delta 113p53$ transcripts was specifically induced in the abnormal digestive organs of the *def^{hi429}* mutant (Chen et al. 2005), suggesting that $\Delta 133p53/\Delta 113p53$ play important roles during carcinogenesis and zebrafish embryos development. Moreover, it has been shown that injections of “off-target” morpholino in zebrafish embryos induce $\Delta 113p53$ expression, suggesting that it is exquisitely sensitive (Robu et al. 2007). However, the biological activities and regulation of expression of $\Delta 133p53/\Delta 113p53$ are still unknown. In this report, we specifically focused our studies on $\Delta 113p53$ in zebrafish. Our data showed that $\Delta 113p53$ expression is induced by various DNA-damaging treatments such as γ -ray, camptothecin, and roscovitine chemicals. We further showed that a 4.113-kb genomic DNA fragment immediately upstream of the start codon ATG of $\Delta 113p53$ is a functional promoter and carries the regulatory elements that can faithfully recapitulate the regulation of endogenous $\Delta 113p53$ expression in response to both developmental signals and DNA-damaging signals. Promoter deletion analysis showed that the 4.113-kb $\Delta 113p53$ promoter region contains two regulatory regions (–1041 to –1991 bp and –1 to –239 bp) important for the expression of $\Delta 113p53$.

Figure 6. Knockdown $\Delta 113p53$ sensitizes the zebrafish embryos to ionizing radiation treatment. (A) Photos showing $\Delta 113p53$ -*MO*- or *st*-*MO*-injected wild-type or $tp53^{M214K}$ embryos 5 d after being treated or untreated with γ -ray as indicated. All surviving embryos after γ -ray treatments showed abnormal phenotype (body curving). (B) Wild-type embryos were injected with different reagents as indicated and treated with γ -ray at 6 hpf and were harvested 6 h after treatment for TUNEL assay. The embryos shown were at 12 hpf. (C) The γ -ray-induced apoptosis is p53-dependent. Embryos injected with *st*-*MO*, $\Delta 113p53$ -*MO*, $p53$ -*MO*^{ATG}, or $\Delta 113p53$ -*MO* and $p53$ -*MO*^{ATG} were treated with γ -ray at 24 hpf and harvested 6 h later for TUNEL assay. The embryos shown were at 30 hpf.

Sequence analysis revealed that both regions contain putative p53-binding sites, suggesting that $\Delta 113p53$ expression is probably regulated by p53. Indeed, the elevated expression of $\Delta 113p53$ induced by the def^{hi429} mutation is abolished in the $def^{hi429} tp53^{M214K}$ double mutant, and DNA-damaging signals are no longer able to induce the expression of $\Delta 113p53$ in the $tp53^{M214K}$ background. More importantly, $p53$ mRNA could rescue both expression of the *gfp* reporter gene under the $\Delta 113p53$ promoter and endogenous $\Delta 113p53$ in the $tp53^{M214K}$ mutant embryos. ChIP assay showed that p53 could directly bind to the two promoter regions containing putative p53-binding sites. Therefore, the expression of $\Delta 113p53$ is p53-dependent and is directly controlled by p53.

The fact that the $\Delta 113p53$ protein retains the p53 dimerization/tetramerization domain raises the possibility that $\Delta 113p53$ might interact with p53 to form a hetero-oligomer. Our coimmunoprecipitation result showed

that $\Delta 113p53$ and p53 can indeed form a complex (Supplemental Fig. 16). The formation of the $\Delta 113p53$ -p53 complex opens an avenue to speculate that $\Delta 113p53$ might act as a dominant-negative regulator of p53 activity since $\Delta 113p53$ lacks the p53 activation domain and Mdm2-interacting motif and is partially deleted of the DNA-binding domain. This speculation seems to be true based on the observations that $\Delta 113p53$ antagonized p53's apoptotic activity on p53-injected $tp53^{M214K}$ embryos, while knockdown of endogenous $\Delta 113p53$ in γ -ray-treated embryos enhanced p53's apoptotic activity and resulted in 100% mortality. However, further molecular analysis showed that the interplay between $\Delta 113p53$ and p53 is more complex than a dominant-negative mechanism. Coinjection of $\Delta 113p53$ and p53 altered differentially the expression patterns of p53 response genes in a gene-dependent manner. For example, it was observed that coinjection of $\Delta 113p53$ and p53 greatly enhanced the expression of *p21* and *mdm2*, while it only

Figure 7. Bcl2L mediates the $\Delta 113p53$ anti-apoptotic activity. (A) Semiquantitative RT-PCR analysis of *bcl2L* and Northern analysis of *p21*, *bax*, and *mdm2* in the uninjected *tp53^{M214K}* control (lane 1), 1 ng of $\Delta 113p53$ mRNA alone injected (lane 2), 0.1 ng of *p53* mRNA alone injected (lane 3), and $\Delta 113p53$ and *p53* mRNA coinjected (lane 4) *tp53^{M214K}* embryos. (B) *def^{hi429}* homozygous mutant (*def^{-/-}*) and its *def^{+/+}* or *def^{hi429/+}* siblings (*def^{+/+}*) were injected with buffer (as control) or $\Delta 113p53$ -MO and were then genotyped (*def* panel) and harvested (pool of at least 20 embryos for each sample) for assessing *bcl2L* and $\Delta 113p53$ expression at 3 dpf. The *el1a* gene was used as the normalization control. Expression fold changes for *bcl2L* and $\Delta 113p53$ are shown below their corresponding panel. (C) The st-MO- or $\Delta 113p53$ -MO-injected embryos were treated with γ -ray at 6 hpf and then harvested (pool of more than 100 embryos for each sample) 6 h later for total RNA extraction. Transcripts of *bcl2L* and $\Delta 113p53$ were examined via semiquantitative RT-PCR, and 18S RNA was used as the normalization control. Expression fold changes against the st-MO-injected embryos (set as 1) for *bcl2L* and $\Delta 113p53$ are shown under their corresponding panel. (D, E) Photos showing *tp53^{M214K}* embryos at 24 hpf injected with various reagents as indicated. (D) *bcl2L*-MO (0.06 pmol) was injected per embryo. (E) TUNEL assay of embryos at 12 hpf treated as in D (marked with number).

negligibly suppressed the expression of *bax*. In contrast, while *p53* down-regulated the *bcl2L* expression, coinjection of $\Delta 113p53$ restored the expression of the anti-apoptotic *bcl2L* gene. Our results demonstrate that $\Delta 113p53$ does not act simply as dominant-negative toward *p53*; instead, $\Delta 113p53$ differentially modulates gene expression in a promoter-dependent manner and in a stress-dependent manner. We noticed that $\Delta 113p53$ mRNA increased *bcl2L* expression by twofold to threefold at the transcription level. Since *bcl2L* is normally expressed at a very low level and can only be detected by quantitative RT-PCR (qRT-PCR) (this is reflected by the fact that merely 0.125 pmol of *bcl2L*-MO injection can cause a severe phenotype, and we used 0.06 pmol of *bcl2L*-MO in our experiments), we speculate that this twofold to

threefold change in *bcl2L* expression might be enough to produce the effect observed. Certainly, it is also possible that *bcl2L*-MO might have a more profound effect on the production of Bcl2L protein. Further studies will be required to fully understand how the selectivity is achieved.

p53 is usually kept at a low level in normal cells (Montes de Oca et al. 1995; Langheinrich et al. 2002). Mdm2-mediated *p53* degradation plays a key role in maintaining the low level of *p53* in a cell (Momand et al. 1992). In addition to the negative feedback loop between Mdm2-*p53*, *p53* is also regulated at multiple levels via protein stabilization and activation (Lavin and Gueven 2006). We show here that *p53* and $\Delta 113p53$ form another feedback loop in zebrafish: *p53* activates $\Delta 113p53$ expression in response to developmental or DNA-damaging

signals that in turn inhibits p53-mediated apoptosis via activation of the Bcl2-related anti-apoptotic gene (Fig. 8). Therefore, while p53 eliminates abnormal cells caused by DNA-damaging treatments, the cells have a mechanism to mobilize $\Delta 113p53$ that acts as a buffer that demands a threshold of p53 signaling to fire or to modulate its output under particular circumstances, sometimes even at a cost of abnormal development (Chen et al. 2005; Robu et al. 2007). Our findings may have profound significance to extend our understanding of the mechanisms of p53 tumor suppressor activity and/or other biological functions (Fig. 8). They demonstrate a new feedback loop in which p53-induced $\Delta 113p53$ acts to alter the p53 response to favor growth arrest rather than an apoptotic phenotype. These results also imply that the p53 response has complex kinetics. Since the $\Delta 113p53$ promoter and the promoter for full-length p53 are distinct, they potentially allow the ratio between these two isoforms to be modulated independently in different tissues and organs and in response to different stresses. Research in the human cell line system showed that human $\Delta 133p53$ is regulated and functions in similar ways (J.-C. Bourdon and D.P. Lane, unpubl.) as that we found for $\Delta 113p53$ in zebrafish.

Last but not the least, because the *Tg*($\Delta 113p53:gfp$) transgenic fish model can faithfully recapitulate the expression regulation of the endogenous $\Delta 113p53$, it can be used in drugs screening for cancer treatment. Meanwhile, the *Tg*($\Delta 113p53:gfp$) transgenic fish can be used as an effective and cheap reporter to detect polluted environments, especially to detect DNA-damaging agents

Figure 8. A new feedback loop for p53 pathway. p53 and Mdm2 form a negative feedback regulation loop in that p53 activation induces the Mdm2 expression and the latter then triggers the degradation of p53 to remove p53. Here we show that p53 and $\Delta 113p53$ also form a negative feedback loop in that activation of p53 induces the expression of $\Delta 113p53$ that in turn acts specifically to antagonize p53's apoptotic activity without inactivating p53-promoted cell cycle arrest. We propose that $\Delta 113p53$ switches the p53 pathway to favor cell cycle arrest in response to developmental or stress signals.

Materials and methods

Fish lines and fish maintenance

Zebrafish were raised and maintained according to standard procedures described in ZFIN (http://zfin.org/zf_info/zfbook/zfbk.html). Zebrafish mutant *def^{hi429}* was kindly provided by Professor Nancy Hopkins at Massachusetts Institute of Technology (Golling et al. 2002; Chen et al. 2005). The primer pairs used to genotype the *def^{hi429}* mutant were as described (Chen et al. 2005). The *p53*^{−/−} mutant allele *tp53^{M214K}* was kindly provided by Professor Thomas Look at Harvard Medical School (Berghmans et al. 2005).

$\Delta 113p53:gfp$ plasmid and *Tg*($\Delta 113p53:gfp$) transgenic fish

A 4.113-kb DNA fragment immediately upstream of the start codon ATG of $\Delta 113p53$ (Fig. 2A,B) was amplified from genomic DNA (AB strain wild-type zebrafish) with primer pair P0-F and P0-R (Supplemental Table S1) using the Expand Long Template Kit (Roche). The PCR product was first cloned into a pGEMTeasy vector (Promega), then digested with BamHI and EcoRI enzymes for subcloning into the pEGFP-1 vector (Clontech) to generate the $\Delta 113p53:gfp$ plasmid. The $\Delta 113p53:gfp$ plasmid was linearized with EcoRI prior to injection. Approximately 25 pg of linearized $\Delta 113p53:gfp$ plasmid DNA was injected into one-cell-stage embryos to generate T0 fish. *Tg*($\Delta 113p53:gfp$)-positive fish were screened via PCR using the *gfp*-specific primers (Supplemental Table S1). *Tg*($\Delta 113p53:gfp$) transgenic fish were crossed with *def^{hi429}* heterozygous fish to study the effect of *def^{hi429}* mutation on the $\Delta 113p53:gfp$ reporter. The *lacZ* and *def* gene-specific primers (Chen et al. 2005) were used to identify the *def^{hi429}* mutant in the transgenic fish population. Gfp fluorescence was visualized under a Leica DMIRE2 fluorescence microscope.

Deletion analysis of the $\Delta 113p53$ promoter

Each truncated promoter (Fig. 3A) was amplified from $\Delta 113p53:gfp$ (P0) using iProof enzyme (Bio-Rad) and cloned into a pEGFP-1 vector using the Infusion kit (Clontech) according to the manufacturer's protocol. The corresponding primer pairs used are listed in Supplemental Table S1. To make the P6 constructs, primer pairs (−U3059)–(−L1060) and (−U505)–(L1054) were used to amplify the parts flanking the left and right side of the deletion, respectively. PCR products from these two pairs of primers were mixed and denatured together to allow annealing of the sticky ends to join the two parts, and this mixture was then used as the template for the second-round PCR using primers (−U3059) and (L1054) to get the deleted product for P6. Similarly, for P9, primer pairs (−U3059)–(−L240) and (U1)–(L1054) were used to amplify the two parts flanking each side of the deletion, and the PCR products were mixed together and used as the template for the second-round PCR using primers (−U3059) and (L1054) to get the deletion product for P9. All fragments cloned were sequenced and confirmed to be identical to $\Delta 113p53:gfp$ except the deleted regions. Ten picograms of each plasmid DNA were injected into AB fish embryos at the one-cell stage for promoter activity assay.

ChIP in zebrafish

Approximately 200 pg of *HA-p53* mRNA were injected into one-cell-stage embryos. At 5 hpf, ~500 embryos were deyolked in PBS with 1× Complete (Complete Protease Inhibitor Cocktail Tablets; Roche). The supernatant was removed after 300g centrifugation. The cell pellet was homogenized in 1 mL of NIM

buffer of 0.25 mM sucrose, 25 mM KCl, 10 mM Tris-Cl (pH 7.4), 5 mM MgCl₂, and 1× Complete. Pelleted nuclei were then treated with formaldehyde (final concentration 1%) for 15 min at room temperature. The reaction was quenched with glycine (final concentration 125 mM). The nuclei were pelleted at 800g at 4°C. The nuclei were washed with NIM buffer three times, then resuspended in 500 μ L of SDS lysis buffer (this buffer is provided in the ChIP assay kit; Upstate Biotechnologies). The nuclei lysate was subjected to 40 sets of 5-sec pulses using Misonix 3000 equipped with a 2-mm tip, and the energy output was set to 2. The lysate was left for 2 min on ice between each pulse. The chromatin was sheared into 200- to 1000-bp fragments. After sonication, 50 μ L of lysate were taken out as the template for positive control PCRs and 25 μ L of lysate for Western blot. The rest of the lysate was spun at 14,000 rpm, and the supernatant was diluted 10-fold with ChIP dilution buffer. One hundred microliters of HA antibody matrix (Roche) were added into the diluted solution and incubated overnight at 4°C. The HA antibody-matrix was washed and eluted as described in the protocol recommended by the manufacturer. The histone-DNA cross-links were reversed according to the manufacturer's protocol. DNA was recovered by phenol/chloroform extraction and precipitated by ethanol. The pellet was resuspended in water and used as template for the PCR reaction.

Morpholinos

Morpholinos were purchased from Gene Tools. The $\Delta 113p53$ -MO (5'-GCAAGTTTTTCCAGCTGACAGAAG-3') and the *bcl2L*-MO (5'-AATTCAGGTTGTTGCTCGTTCTCCG-3') were designed to specifically target against the 5'-UTR regions of $\Delta 113p53$ and *bcl2L*, respectively. The human β -globin antisense morpholino (5'-CCTCTTACCTCAGTTACAATTATATA-3') was used as the standard control (st-MO). One nanoliter of 1 mM def-MO (Chen et al. 2005), 0.4 mM p53-MO^{ATG}, 0.25 or 0.4 mM $\Delta 113p53$ -MO, 0.25 or 0.4 mM st-MO, and 0.06 mM *bcl2L*-MO were injected into the embryos at the one-cell stage as specified in the text.

RNA and protein analysis, RT-PCR

Total RNA was extracted from different samples using TRIzol reagent (GIBCO-BRL). Northern analysis using the *p53* probe I, $\Delta 113p53$ -specific probe, *p53*-specific probe, and *p21* and *bax* probes was as described previously (Chen et al. 2005). The *gfp* probe was amplified from the *gfp* gene using primer pair EGFP-U322 and EGFP-L742 (Supplemental Table S1). Digoxigenin (DIG)-labeled probes were used in Northern blot hybridization as described (Cheng et al. 2006). Primer pairs used in RT-qPCR for detecting $\Delta 113p53$ and *el1a* were as described previously (Chen et al. 2005). RNA was treated with DNAase I before being used in RT. For semiquantitative RT-PCR analysis of *bcl2L* transcripts, the RT-PCR products were amplified and removed at linear amplification cycles and were then subjected to 1% agarose gel electrophoresis and blotted onto Hybond N⁺ membranes and subjected to Southern blot hybridizations using DIG-labeled DNA probes.

Plasmid injection, γ -ray irradiation, and drug treatment

Fifty picograms of linearized pGEMT plasmid DNA were injected into AB fish embryos at the one-cell stage. Embryos at 6 hpf or 24 hpf were treated with γ -ray irradiation (with a dosage of 16 or 24 Gy) or 500 nM camptothecin or 50 μ M roscovitine, respectively. At 6 or 18 h post-treatment, the embryos were harvested for RNA extraction, and the extracted RNA was used for Northern blot analysis of *p53* and $\Delta 113p53$ expression. To assay the response of the $\Delta 113p53$ promoter, *Tg*($\Delta 113p53$:*gfp*)

transgenic embryos were treated with γ -ray and the two drugs, respectively. RNA was extracted from the treated embryos and used for the analysis of *gfp* transcripts.

bcl2L, *bcl2L^m*, and the *cmv:5'utr-bcl2L-gfp* construct

The *bcl2L* full-length coding region (accession no. 114401) was amplified using primer pair Bcl2l-ATG-EcoRI and Bcl2l-TGA-XhoI, and the mutant form of *bcl2L* (*bcl2L^m*) was amplified using primer pair Bcl2l mutant-ATG-EcoRI and Bcl2l-TGA-XhoI (Supplemental Table S1). After being digested with EcoRI and XhoI, the PCR products were cloned into a pCS2+ vector, respectively. For *bcl2L^m*, the +16-nucleotide (nt) C (the "A" of the start codon ATG of *bcl2L* is designated as +1) was changed to T such that the sixth codon CGA (Arg) became TGA (stop codon). One nanoliter of 0.5 μ g/ μ L *bcl2L* or *bcl2L^m* mRNA was injected into the embryos at the one-cell stage.

To examine the efficiency of *bcl2L*-MO in blocking the translation, we constructed the *cmv:5'utr-bcl2L-gfp* plasmid as the reporter system. Firstly, the Bcl2l-U(-87)-EcoRI and Bcl2l-L556-XhoI primer pair was used to amplify a cDNA fragment of *bcl2l* containing the +1 to +556 nt coding region and -1 to -87 nt 5'-UTR region (the "A" of the start codon ATG of *bcl2L* is designated as +1). This PCR fragment was digested with EcoRI and XhoI and then cloned in frame and N-terminal to the *gfp* reporter, which was cloned previously into the pCS2+ vector.

TUNEL assay and embryo viability counting

One nanoliter of 0.25 mM $\Delta 113p53$ -MO, 0.4 mM p53-MO^{ATG}, or human β -globin antisense morpholino (st-MO) or phenol red dye was, respectively, injected with or without 1 ng of $\Delta 113p53$ mRNA into one-cell-stage embryos. At 6 hpf or 24 hpf, embryos were γ -ray-irradiated with a dosage of 16 Gy. Embryos 6 h post- γ -ray-irradiation were fixed with 4% PFA overnight and then subjected to the TUNEL assay using the In Situ Cell Death Detection Kit, TMR red (Roche) (Chen et al. 2005). For *p53* or $\Delta 113p53$ mRNA injection into the *tp53^{M214K}* mutant, embryos at 12 hpf were harvested for TUNEL assays. For def-MO and $\Delta 113p53$ -MO double morphants, 1 nL of 1.0 mM def-MO and 0.4 mM $\Delta 113p53$ -MO was coinjected into wild-type embryos at the one-cell stage. At 3 dpf, embryos were fixed with 4% PFA and cryo-sectioned for TUNEL assay (Chen et al. 2005). For viability counting, 1 nL of 0.4 mM $\Delta 113p53$ -MO or 0.4 mM st-MO was, respectively, injected into wild-type or *tp53^{M214K}* mutant embryos, and then the injected embryos were treated with γ -ray at 24 hpf at a dosage of 24 Gy. The mortality for each treatment was counted at 5 d post- γ -ray-irradiation.

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