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A naturally occurring 4-bp deletion in the intron 4 of *p53* creates a spectrum of novel p53 isoforms with anti-apoptosis function

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ABSTRACT

p53 functions as a tumor suppressor by transcriptionally regulating the expression of genes involved in controlling cell proliferation or apoptosis. p53 and its isoform Δ133p53/Δ113p53 form a negative regulation loop in that p53 activates the expression of Δ133p53/Δ113p53 while Δ133p53/Δ113p53 specifically antagonizes p53 apoptotic activity. This pathway is especially important to safeguard the process of embryogenesis because sudden activation of p53 by DNA damage signals or developmental stress is detrimental to a developing embryo. Here we report the identification of five novel p53 isoforms. p53β is generated due to alternative splicing of the intron 8 of p53 while the other four, namely, TA2p53, TA3p53, TA4p53 and TA5p53, result from the combination of alternative splicing of intron 1 (within intron 4 of the p53 gene) of the *Δ113p53* gene and a naturally occurring CATT 4 bp deletion within the alternative splicing product in zebrafish. The CATT 4 bp deletion creates four translation start codons which are in-frame to the open reading frame of Δ113p53. We also show that TAp53 shares the same promoter with Δ113p53 and functions to antagonize p53 apoptotic activity. The identification of Δ113p53/TA2/3/4/5p53 reveals a pro-survival mechanism which operates robustly during embryogenesis in response to the DNA-damage condition.

INTRODUCTION

p53 is a pan-transcription factor that regulates diverse biological and cellular processes (1,2). p53 plays its role by regulating the expression of hundreds of genes in response to different internal or external stimuli (3). The identi - cation of p53 isoforms marks a new era for the study of the p53 pathway and the dimension of the complexity of p53 function is further expanded by the involvement of p53 isoforms (4,5). Thirteen p53 human isoforms have been identi ed and these isoforms have been implicated to regulate p53 function in different ways (5,6). Human Δ133p53 and its zebra sh counterpart Δ113p53 belong to a special type of p53 isoforms whose expression is initiated by using intron 4 of the *53* gene as its promoter (7,8). Previous studies have shown that the transcriptional expression of *Δ113 53/Δ133 53* totally depends on the full-length p53 and the function of Δ113p53/Δ133p53 protein is to antagonize the p53 apoptotic activity selectively (9–11). Δ113p53/Δ133p53 functions at least in part through its interaction with p53 (9,12). Human Δ133p53 has been found to be highly expressed in certain cancer cells (6,13–15) while the expression of the zebra sh Δ113p53 is induced by morpholino injection (16) or by mutations in genes including ʃ (1) and *13* (17).

Digestive organ expansion factor Def is a nucleolar protein and loss-of-function of Def in the ʃ *429/ 429* mutant results in hypoplastic digestive organs in a cell autonomous manner (7,18). The p53 pathway is activated in ʃ *429/ 429* that in turn up-regulates the expression of *Δ113 53* (9). Recently, it has been shown that Def and Capn3 form a complex to mediate p53 degradation speci cally in the nucleoli, which explains why p53 protein is accumulated in the nucleoli of ʃ *429/ 429* mutant cells (1). Here we report the identi cation of ve novel p53 isoforms, namely, p53β, TA2p53, TA3p53, TA4p53 and TA5p53. p53β is generated due to alternative splicing of the intron 8 of p53 while TA2/3/4/5p53 are derived from a naturally occurring 4 bp genomic deletion in the intron 1 of the *Δ113 53* gene (part of the intron 4 of the *53* gene), which creates four new translation start codons in the product of alternative splicing of the intron 1 of the *Δ113 53* gene. We focused on studying the function of TAp53 isoforms and showed that these new isoforms

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function to antagonize the p53 apoptotic function in a way similar to that of Δ113p53.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Zebra sh were raised and maintained according to the standard procedure described in ZFIN (http://z n.org/zf_info/zfbook/zfbk.html). The ʃ 429 mutant line was provided by Prof Nancy Hopkins at Massachusetts Institute of Technology. Two pairs of primers derived from and ʃ were used to genotype the ʃ 429 mutant (7). The 53−/- mutant allele ◥ 53 214 line was provided by Prof Thomas Look at Harvard Medical School. (Δ113 53:ʃ) transgenic sh was as described (9). CATT genotypes were identi ed by sequencing, the primer pair 5′- GGCGAACATTTGGAGGG-3′ and 5′- AAAACACCCTAATGCGTCTTCAC-3′ were used for PCR and the primer 5′-CACAGAACAATAAACTAATAACAC-3′ was used for sequencing.

Morpholinos

Morpholinos were purchased from Gene Tools. ʃ-MO, Δ113 53-MO^{ATG} and the human β-globin antisense morpholino (st-MO) were used as described previously (7). A 53-MO^{spl} (5′-TTTAATCACACTTACATTCAAGCCT-3′) was designed to target the splice junction between exon 1 and intron 1 of the Δ113 53 transcript.

RNA and protein analysis

Total RNA was extracted from different samples using TRIzol reagent (Invitrogen). For real-time quantitative polymerase chain reaction (qPCR), total RNA was treated with DNase I prior to reverse transcription and puri ed with RNeasy mini kit (Qiagen). First strand cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). The qPCR was performed on CFX96TM Real-Time System (Bio-Rad) using SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. Primer pairs used for qPCR were listed in Supplementary Table S1.

The methods used for protein extraction from zebra sh embryos are as described previously (9). Protein electrophoresis and western blot were performed according to the instructions provided by the manufacturers (19).

TUNEL assay and embryo viability counting

◥ 53 214 mutant embryos injected with either p53 or Δ113p53 mRNA were harvested for Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays at 10 houes post injection (hpi). The survival rate for each treatment was counted at 24 hpi. CATT$^{+/+}$ or CATT$^{-/-}$ AB embryos were treated with γ-ray at 24 hpf at a dosage of 24 gray (Gy). Embryos at 8 h post-treatment were harvested for western and the survival rate for each treatment at 5 days post-treatment was counted.

Antibodies

The zf-p53 mouse monoclonal antibody A7-C10 was used as previously described (1). The zf-p53 N-terminal mouse monoclonal antibody 9.1 was purchased from abcam (ab77813). Rabbit monoclonal antibody (EPR1977Y) against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Epitomics (#2251–1), and rabbit polyclonal antibody against β-actin (#4967) was from Cell Signaling Technology.

RESULTS

Identification of a p53 alternative splicing isoform p53β

During the course of comparing the p53 and Δ113p53 protein levels in the ʃ 429/ 429 mutant using a monoclonal antibody (A7-C10) speci cally against the zebra sh p53, we noted that, in addition to the expected p53 and Δ113p53 bands, two extra bands, one with a molecular weight of ~35 kD and another ~45 kD, were detected (1). These two extra bands were also observed in the embryos treated with camptothecin (1,12). The higher extra band (45 kD) presumably corresponds to ΔN-p53 reported previously (20). To reveal the nature of the 35 kD band, we rst performed an reverse transcriptase-PCR (RT-PCR) to amplify the cDNA fragment between the exon 5 and exon 10 (this region corresponds to the production of human p53β and p53γ through alternative splicing) (8) (Figure 1A and Supplementary Table S1) and sequenced the PCR product to check whether there is an alternative splicing in the 3′-end of zebra sh 53 corresponding to the human p53β or p53γ (8). We obtained a PCR product (Figure 1B) which, when compared with the 53 full-length cDNA, contained an additional 85 bp originated from the intron 8 of the 53 gene due to alternative splicing (Figure 1C). This transcript is predicted to encode a peptide that retains the N-terminal 275 amino acids of p53 followed by the addition of 19 new amino acids (Figure 1C, Supplementary Figure S1). This new transcript is different from the GenBank sequence NC_007116 which contains an additional 12 bp originated from the intron 8 of p53 and has a predicted open reading frame (ORF) that is in-frame to the ORF of p53 (Figure 1C). To con rm that the new transcript is a genuine splicing product we performed an RT-PCR using a forward primer derived from exon 5 and a reverse primer from intron 8. A clear PCR band was obtained (Figure 1D). Sequencing of the PCR product revealed that it is identical to the new transcript (data not shown). The monoclonal antibody A7-C10 is known to recognize the C-terminus of the zebra sh p53, thus it was used to detect both the full-length p53 and Δ113p53 (1). In contrast, the monoclonal antibody 9.1 which recognizes the N-terminus of the zebra sh p53, was used to detect the full-length p53 but not Δ113p53 because Δ113p53 lacks its recognition motif (21). We cloned the PCR product into the expression vector and used the ◥ transcribed mRNA derived for injection. We found that this mRNA encoded for a product with much higher molecular weight which was detected by 9.1 but not by A7-C10 (Figure 1E). We designated this new p53 isoform as p53β. However, the expression of p53β protein is undetectable by the treatment of camptothecin (Figure 1E) or γ-ray (Supplementary Figure S2). Based on the above, we


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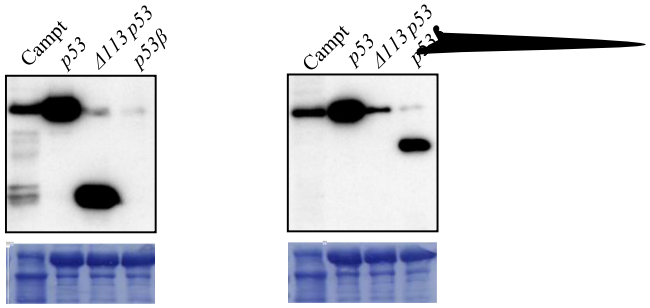
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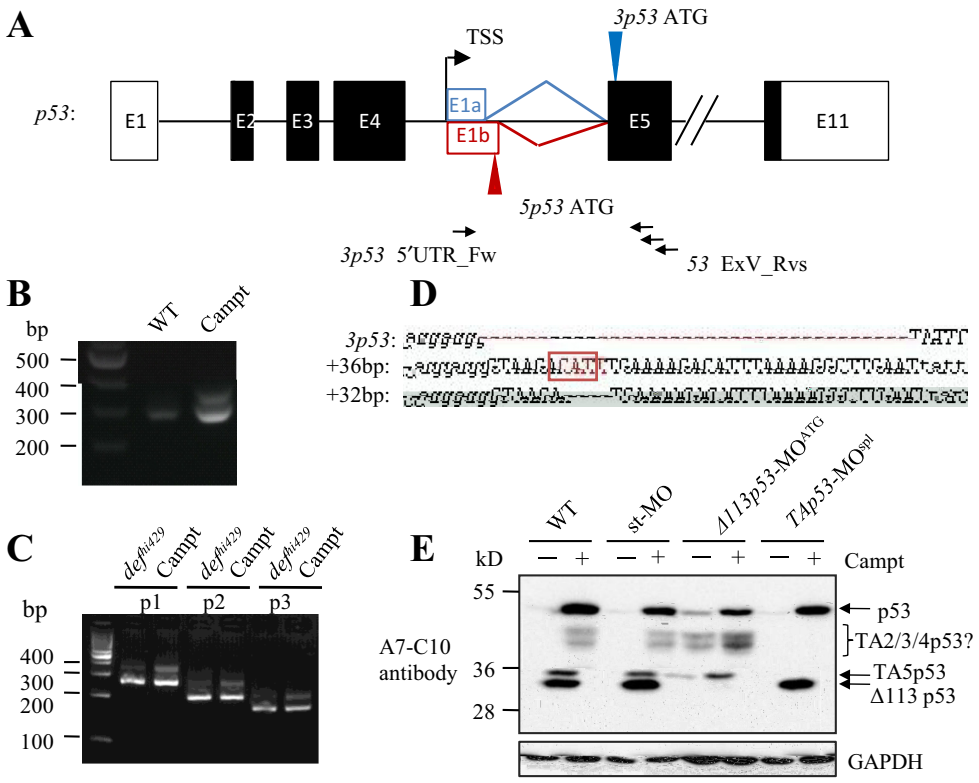
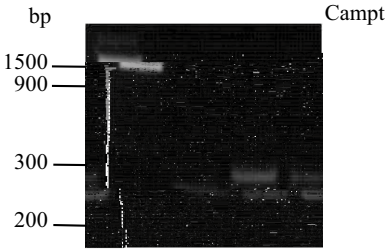


Figure 2. Identi cation of *Δ113 53* splicing transcripts. (**A**) Schematic diagram showing the genomic structure of the p53 gene. White/black boxes, exons (E1–E11); E1a box, exon 1 of *Δ113 53*; E1b, exon 1 of +36/+32 bp transcript; solid line, introns; double slashes, omitted genomic region; black arrow, position and direction of primers; blue and red arrow heads, indicating the translation start codon for Δ113p53 and TA5p53, respectively. TSS, *Δ113 53* transcription start site. (**B** and **C**) RT-PCR analysis of transcripts in WT and camptothecin-treated embryos using the *Δ113 53*_5′UTR_Fw and *53*_ExV_Rv413 primers pair (**B**) and in *defhi429* and camptothecin-treated embryos using *Δ113 53*_5′UTR_Fw and *53*_ExV_Rv413 (p1), *Δ113 53*_5′UTR_Fw and *53*_ExV_Rv351 (p2) and *Δ113 53*_5′UTR_Fw and *53*_ExV_Rv318 (p3) three primers pairs (**C**). (**D**) Alignment of *Δ113 53*, +36 bp and +32 bp cDNA sequences. CATT 4 bp (boxed and lettered in red) is deleted in the +32 bp transcript. (**E**) Western blot analysis of p53 and its isoforms in 36 hpf WT and camptothecin-treated embryos that were injected with either control morpholino (st-MO), *Δ113 53*-MO^{ATG} or *A 53*-MO^{spl}. Morpholinos were injected into one-cell stage zebra sh embryos. WT and injected embryos were treated with camptothecin at 24 hpf for 12 h. A7-C10, zebra sh p53 monoclonal antibody. TA2/3/4p53?, the identity of each of these bands in correspondence to TA2p53, TA3p53 and TA4p53 has not been determined. GAPDH was used as a loading control.

sponds to the *Δ113 53* transcript. Surprisingly, sequencing of the higher PCR product yielded two DNA sequences that differed from a genuine *Δ113 53* sequence by addition of either 32 bp (+32 bp transcript) or 36 bp (+36bp transcript) originated from the intron 1 of the *Δ113 53* gene (Figure 2D). Sequence alignment of +36 bp and +32 bp two transcripts revealed that both transcripts are likely to be derived from the same alternative splicing product of *Δ113 53* and the difference between the +36 bp and +32 bp two transcripts is that the +32 bp transcript lacks CATT 4 bp (Figure 2D and Supplementary Figure S3).

To con rm whether +36/+32 bp transcripts resulted from alternative splicing, we designed a morpholino (*A 53*-MO^{spl}) that speci cally targets the presumed splicing donor site (Supplementary Figure S3). The injection of *A 53*-MO^{spl} abolished the expression of the 35 kD product together with other unidenti ed bands induced by camptothecin but did not affect on the production of Δ113p53 (Figure 2E). In contrast, the injection of *Δ113 53*-MO^{ATG}, which has been shown to knock down the expression of Δ113p53 effectively (9), only blocked the expression of Δ113p53 but not the 35 kD product and other unidenti ed products induced by camptothecin (Figure 2E).

B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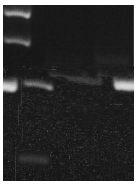

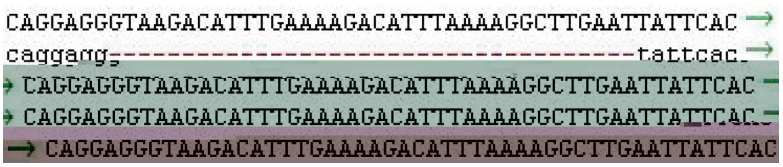
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The result showed that CATT$^{+/+}$ sh produced *Δ113 53* and +36 bp transcripts, whereas CATT$^{-/-}$ sh produced the *Δ113 53* and +32 bp transcripts as expected (Figure 3B). In addition, we con rmed that the alternative splicing of intron 8 also occurs in +32 bp transcript by RT-PCR using a forward primer derived from the exon 1 of *Δ113 53* and a reverse primer from intron 8 of *53* (data not shown).

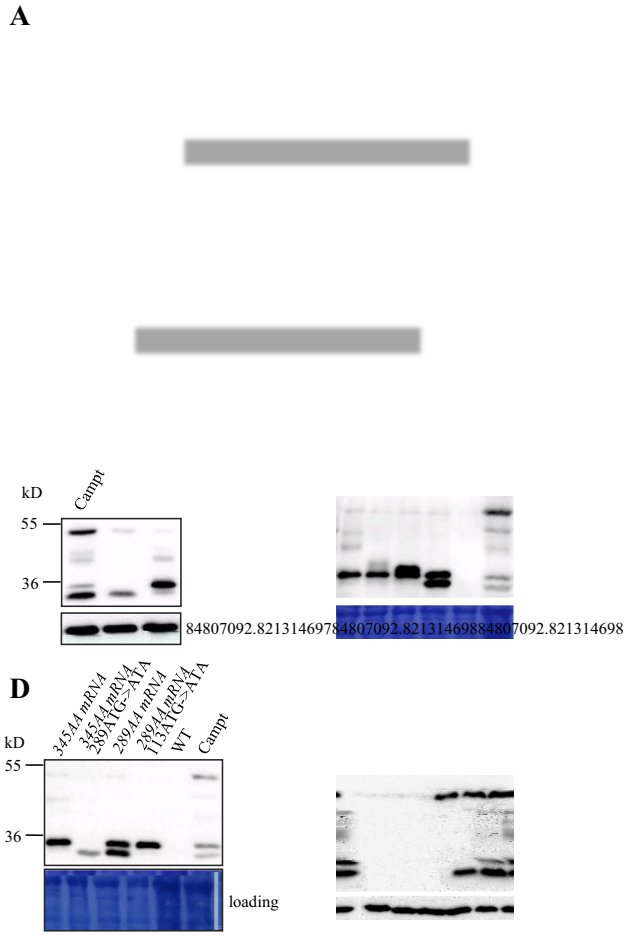
Δ113p53 and +36/+32 bp transcripts share the same promoter

We previously reported that the *(Δ113 53:)* transgenic sh which harbors the reporter gene under-driven by the *Δ113 53* promoter can faithfully recapitulate the response of *Δ113 53* to the developmental and DNA-damaging signals (9). Knowing that there are two genotypes for the intron 1 of *Δ113 53*, namely, CATT^{+} and CATT^{-}, we rst determined the genotype of the intron 1 of *Δ113 53* fused with the reporter and found that the transgene belongs to CATT^{+}. We surmised that, if the transcription of the +36/+32 bp transcripts shares the same promoter with that of *Δ113 53*, both *Δ113 53* and the +36 bp transcript must be transcribed from the *Δ113 53:* transgene in the *(Δ113 53:)* sh under the DNA damage stress. To test this hypothesis, we used a forward primer in the 5′-upstream sequence of *Δ113 53* (*Δ113 53*_5′UTR_Fw) and a reverse primer in the gene sequence (_Rv22) (Figure 3C and Supplementary Table S1) for PCR using the cDNA templates obtained from *(Δ113 53:)* embryos. Clearly, two discrete PCR products were obtained in the camptothecin-treated samples, whereas only one weak band was observed in the control embryos (Figure 3D). We recovered the two PCR products by gel extraction for subsequent cloning and sequencing. The result showed that these two products represent the splicing variants of the *Δ113 53:* transgene that exactly matches the endogenous *Δ113 53* and +36 bp transcripts (Figure 3E).

We performed 5′-RACE to determine the transcription start site for the endogenous +36/+32 bp transcripts, however, due to the fact that p53 has many isoforms it was hard to identify and obtain the corresponding 5′-RACE products for the the +36/+32 bp transcripts. As an alternative, we performed 5′-RACE using the cDNA template obtained from camptothecin-treated *(Δ113 53:)* embryos with a primer derived from the gene (Figure 3C, Supplementary Table S1). We cloned the 5′-RACE products for sequencing. Detailed analysis of the DNA sequence revealed that, the transcription start site for the +36 transcript is nearly identical to that of Δ113p53 (Figure 3F) (7). Thus, the transcription of the +36/+32 bp transcripts and *Δ113 53* was driven by the same promoter.

TAp53 proteins arise from the CATT 4 bp deletion in the *Δ113p53* alternative splicing transcript

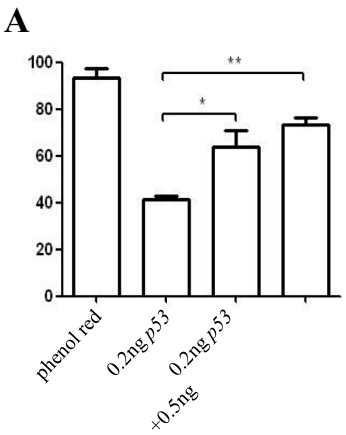
The +36-bp transcript was predicted to encode a product that is identical to that encoded by the *Δ113 53* transcript, however, the +32-bp transcript adds four new ATG codons which are in frame to the ORF of *Δ113 53* due to the CATT 4 bp deletion, leading to predicted peptide products of 345, 325, 298 and 289 AA, respectively (Figure 4A and Supple-



mentary Figure S3). We cloned the +32-bp transcript containing coding sequence for 345 AA product (Supplementary Table S1) and obtained its mRNA by transcription. The +32-bp mRNA was injected into embryos and total proteins were extracted and subjected to western blot analysis. We found that the +32-bp transcript produced a major protein product of the size of 35 kD and also other protein products identical to that observed in the $^{429/429}$ or camptothecin-treated embryos (Figure 4B). Thereafter, we named the new p53 isoforms derived from +32 bp mRNA as TA2p53 (corresponding to 345 AA product), TA3p53 (corresponding to 325 AA product), TA4p53 (corresponding to 298 AA product) and TA5p53 (corresponding to 289 AA product), collectively called TAp53 isoforms.

To determine whether these four new ATG codons in +32 bp transcript are genuinely used in the translation of corresponding TAp53 isoforms, we generated four constructs, namely, *345*-, *325*-, *298*- and *289*- *A 53*, respectively, corresponding to the four new ATG codons in the +32-bp transcripts (Supplementary Table S1), for transcription. The mRNA corresponding to each of these four constructs was injected and total protein was extracted and subjected to western blot analysis. The result showed that the *289*- *A 53* mRNA yielded two protein products identical to those observed in $^{429/429}$ and camptothecin-treated embryos (Figure 4C). Injection of *298*- *A 53* mRNA produced two products, one corresponding to TAp53 plus a new higher band (Figure 4C). The predominant product by *325*- *A 53* mRNA injection produced TA5p53, meanwhile two faint higher bands, one corresponding to the higher band observed by *298*- *A 53* mRNA injection and another being a new band (Figure 4C). Injection of *345*- *A 53* mRNA also produced TA5p53 as the predominant product and a few faint higher bands (Figure 4C). Apparently, protein translation can be initiated from all four ATG codons. However, because all four mRNAs produced TA5p53 as the major product and the *289*- *A 53* is the shortest one, we concluded that TA5p53 is translated by using the ATG for the 289 AA product and ATG^{289} is a preferred translation start codon.

To unequivocally prove that TA5p53 is translated from the ATG in *289*- *A 53* unequivocally we mutated the 289 ATG to ATA in the *345*- *A 53* transcript (Supplementary Table S1). This mutant mRNA failed to produce TA5p53 and but produced Δ113p53. When the ATG for Δ113p53 translation initiation was mutated to ATA in the *289*- *A 53* transcript, the resultant mutant mRNA was no longer able to produce Δ113p53 but only produced TA5p53 (Figure 4D). These results demonstrate that the 289 ATG is responsible for the translation of TA5p53 (Supplementary Figure S4).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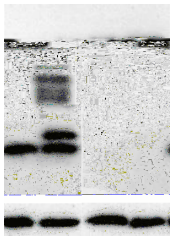
0.2ng p53
+0.5ng



ported by the fact that CATT$^{-/-}$ sh (producing both the Δ113p53 and TAp53 isoforms) is more tolerant to ionizing radiation treatment than the CATT$^{+/+}$ sh (producing only the Δ113p53 isoform). In this regard, the CATT 4 bp deletion is a bene cial mutation. While we showed that the CATT 4 bp deletion is bene cial to embryos upon irradiation treatment, the individual sh we genotyped was randomly picked from the population raised in our facility. We reckon that sh growing in our sh facility are under less selection pressure, together with the consideration that CATT 4 bp deletion is probably a late event in the sh population, which might explain the ratio of CATT$^{-/-}$ observed in the population. It is not surprising that nature normally maximizes the use of the existing system to protect itself.

ACCESSION NUMBER

p53 intron IV genomic DNA containing CATT 4bp deletion: KM981740; +32 bp transcript cDNA sequence: KM981741; +36 bp transcript cDNA sequence: KM981742; p53β cDNA sequence: KM981743.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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