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Requirement of nuclear localization and transcriptional activity of p53 for its targeting to the yolk syncytial layer (YSL) nuclei in zebrafish embryo and its use for apoptosis assay

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Abstract

We expressed zebrafish p53 protein fused to GFP by a neuron-specific HuC promoter in zebrafish embryos. Instead of displaying neuronal expression patterns, p53-GFP was targeted to zebrafish YSL nuclei. This YSL targeting is p53 sequence-specific because GFP fusion proteins of p63 and p73 displayed neuronal-specific patterns. To dissect the underlying mechanisms, various constructs encoding a series of p53 mutant proteins under the control of different promoters were generated. Our results showed that expression of p53, in early zebrafish embryo, is preferentially targeted to the nuclei of YSL, which is mediated by importin. Similarly, this targeting is abrogated when p53 nuclear localization signal is disrupted. In addition, the transcriptional activity of p53 is required for this targeting. We further showed that fusion of pro-apoptotic BAD protein to p53-GFP led to apoptosis of YSL cells, and subsequent imperfect microtubule formation and abnormal blastomere movements.

Keywords: p53; Zebrafish; Yolk syncytial layer; Apoptosis; Nuclear localization; Neuron-specific promoter

Mutation of p53 is implicated in the pathogenic development of more than 50% of human cancers. Loss of p53 function is mainly due to missense mutations in its sequence-specific DNA-binding domain [1]. In cells under normal conditions, the wild-type p53 is rapidly turned over by ubiquitin-mediated proteosome degradation pathway. When cells are under stress and DNA damages take place, p53 is accumulated and induces cell-cycle arrest or apoptosis through transactivation of its downstream target genes [2]. Up to now, a number of p53-inducible target genes with apoptosis-inducing activities have been identified [3–5]. Transactivation of some p53 downstream genes, such as Bax [6], Noxa [7], and p53AIP1 [8], promotes the intrinsic mitochondrial apoptotic pathway, while the expression of others initiates the extrinsic apoptotic pathways, including death receptors Fas [9] and DR5 [10].

The fish p53 cDNA was initially cloned from rainbow trout [11] and later from other fish species including zebrafish [12,13]. Genes encoding p63 and p73, two other members of the p53 family, have also been described in the genomes of Fugu and zebrafish [14–18]. Recently, zebrafish lines with missense mutations in the p53 DNA-binding domain were established by target-selected mutagenesis

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and these mutant fishes develop tumors during their life time [19], providing important link between the loss of p53 function and tumorigenesis in zebrafish. Moreover, through genome-wide analysis, many genes involved in the p53-regulated apoptotic pathway were found in the Fugu genome with sequences highly homologous to their mammalian counterparts, suggesting that the p53 pathway is conserved from fish to mammals [16]. As an example, Perp (p53 apoptosis effector related to PMP-22) was recently identified as one of the p53 target genes based on its role involved in p53-mediated apoptosis in E1A-transformed mouse embryonic fibroblasts [20]. It was also demonstrated that overexpression of zebrafish Perp in early embryos also induces apoptosis [21].

The teleost yolk syncytial layer (YSL) was first described in *Fundulus* [22]. At the mid-blastula stage, the external YSL is formed and located between the anuclear yolk cytoplasmic layer (YCL) and the blastoderm rim, when marginal blastomeres collapse and release their nuclei into the cytoplasm of the YCL of zebrafish embryos [23,24]. Through vital staining and time-lapsed confocal imaging, several highly patterned movements of zebrafish YSL nuclei during gastrula stages have been described [25]. At the end of blastula stage, many YSL nuclei leave the marginal zone and move up toward the animal pole to form internal YSL. During mid-gastrulation, dorsal and lateral nuclei converge toward the dorsal midline, while the axial nuclei extend toward the animal pole to display the initial animal pole nuclei and push them toward ventral and lateral positions. The combination of YSL movements and radial intercalation of deep cells within the blastoderm initiates epiboly movement during gastrulation. Moreover, subsequent epiboly movement depends on the contraction of microtubules in the external YSL [26]. Therefore, the movements of YSL nuclei and deep cells of the blastoderm are dependent of each other [25].

In our initial attempt to examine the effect of p53-induced apoptosis in the neuronal development in zebrafish, we expressed the zebrafish p53 in fusion with GFP in the early embryos under the control of a neuron-specific HuC promoter. Interestingly, the expression of p53-GFP was not localized to the expected neuronal cells. Instead, it was targeted to the zebrafish YSL nuclei. The specific translocation of ectopically expressed p53-GFP into YSL cells has not been previously described in mammalian cells and vertebrate embryos. In this study, we further provide evidences to show that the targeting to YSL is dependent on the nuclear localization and transcriptional activity of p53. Alternatively, the expression of zebrafish p53 in the neuronal cells under the control of HuC promoter can be achieved when nuclear localization was impaired or transcriptional activity was lost.

Materials and methods

Materials. All restriction enzymes were purchased from Promega Biosciences (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). Chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (MO, USA).

Fish. Zebrafish (*Danio rerio*) were maintained at 28 °C on a 14 h-light/10 h-dark cycle. Embryos were incubated at 28 °C and different developmental stages were determined according to the description in Zebrafish Book [27].

Total RNA isolation and first-strand cDNA synthesis. Total RNA was isolated from the fertilized eggs (a pool of embryos at 12, 24, 36, 48, 72, 96, and 120 h post-fertilization, hpf), using the RNAzol reagent (Tel-Test, Friendswood, TX) according to the instructions of the manufacturer. After treatment with RQ1 RNase-Free DNaseI (Promega), 50–100 μg of total RNA was subjected to the first-strand cDNA synthesis in a 25 μl reaction mixture containing 10 pmol of oligo(dT) primer and 100 ng of random primer (Promega), 30 U of RNasin (Promega), 1 mM dNTP, 10 mM dithiothreitol, and 300 U of Superscript II RT (Invitrogen Life technologies, CA). The reaction mixture was incubated at 42 °C for 1 h. Two microliters of the cDNA products was used for subsequent PCR amplification.

Isolation of the full-length p53, ΔNp63, p73, and importin α1 cDNA from zebrafish. The cDNAs encoding the complete open-reading frame (ORF) of zebrafish *p53* family, i.e., p53, Δ*N*p63, p73, as well as importin α1 were obtained by PCR amplification using gene-specific primers according to the sequences from GenBank database with Accession Nos.: NM_131327, NM_152986, NM_183340 and NM_131851, respectively. PCR amplification was performed in a 50 μl reaction mixture containing 2 μl first-strand cDNA, 0.5 μg of forward primer (zp53F1, 5′-ATG GCG CAA AAC GAC AGC CAA GAG-3′; Δ*N*p63F1, 5′-ATG TTG TAC CTG GAG ACC AAT GCT CC-3′; zp73F1, 5′-ATG TCC CAG TCG TCC ACC GCT GAT GAG-3′; zImportin α1F, 5′-ATG TCT GCT GCA AAT GAG AAC ACC GCT-3′) and reverse primer (zp53R1, 5′-ATC AGA GTC GCT TCT TCC TTC GTC-3′; Δ*N*p63R1, 5′-TTC TCC TTC CTC CTT GAT GCG CTG C-3′; zp73R1, 5′-GTG AAC GTC GCC TTC GGC AAA CTC CTC-3′; zImportin α1R, 5′-GAA GTT GAA AGT GCT TTG GTT TTC GTT-3′), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U ExTaq (Takara Shuzo, Shiga, Japan). The samples were incubated in a thermal cycler (Hybaid MultiBlock System, Hybaid Limited, MA). PCR amplification was performed using the program of 96 °C for 2 min; 40 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s; and the final extension at 72 °C for 15 min. The PCR products were subcloned into the pGEM-T easy vector (Promega, WI) and the resulted clones were subjected to sequencing analysis.

The truncated forms of zebrafish p53, ΔCp53, were derived from the full-length cDNA described above by PCR cloning method. Primers used were as follows: ΔCp53, forward 5′-GGA TCC ATG GCG CAA AAC GAC AGC CAA GAG-3′, reverse 5′-GAA TTC TTG GTC TTT CTT GAA GTT GCT-3′. Similarly, the N-terminal region of importin α1 (aa 1–158) was derived from its full-length sequence by PCR using forward primer, 5′-ATG TCT GCT GCA AAT GAG AAC ACC GCT-3′ and reverse primer, 5′-TGT TCC AGA TGC AAT GTT GGT CAG-3′.

DNA sequence analysis. DNA sequence analysis was performed by using PRISM Ready Reaction Big-Dye Termination Cycle sequencing Kit (Applied Biosystems, CA) on an Applied Biosystems 310 automated DNA sequencer.

Construction of expression plasmids. Complementary DNA encoding each of p53, p73, ΔNp63, ΔCp53, and importin α1(1–158) was re-amplified by PCR using primers with built-in restriction sites followed by subcloning of the PCR products into pHA-YUN-GFP or pHA-YUN at appropriate sites to generate pCMV-p53-GFP, pCMV-p73-GFP, pCMV-ΔNp63-GFP, pCMV-ΔCp53-GFP, pCMV-HA-p53, and pCMV-HA-Impα1(1–158), respectively. The plasmid pHA-YUN-GFP was constructed by inserting the cDNA corresponding to the green fluorescence protein (GFP) coding region into pHA-YUN, a gift from Dr. H.J. Kung (University of California at Davis Cancer Center, Sacramento, CA). pCMV-Bad-p53-GFP and pCMV-Bad-p53-NLSmut-GFP were generated by restriction digestion of the individual coding regions of Bad from pcDNA-BAD-HA [28] followed by subcloning into the corresponding sites of pCMV-p53-GFP or pCMV-p53-NLSmut-GFP plasmid.

To express GFP fusion proteins in the neuron, each DNA fragment encoding GFP-fusion protein from pCMV-p53-GFP, pCMV-HA-p53, pCMV-p73-GFP, pCMV-ΔCp53-GFP, pCMV-p53NLSmut-GFP, and pCMV-Impα1(1–158), respectively, was released by the *Bam*HI and *Xho*I sites, and inserted into the corresponding sites of pHuC-GFP plasmid to replace GFP coding region and to generate pHuC-p53-GFP, pHuC-HA-p53, pHuC-p73-GFP, pHuC-ΔCp53-GFP, and pHuC-Impα1(1–158), respectively. The control plasmid pHuC-GFP or pHuC-RFP [29] was previously described and GFP or DsRed genes were driven by a zebrafish neuron-specific HuC promoter [30]. Similarly, the DNA fragment encoding GFP-fusion protein from pCMV-Δ Np63-GFP, pCMV-Bad-p53-GFP, and pCMV-Bad-p53NLSmut-GFP was released by *Hin*dIII, followed by Klenow fill-in, and *Xho*I digestion, and subcloned into the pHuC-GFP at blunt-ended *Bam*HI and *Xho*I sites to generate pHuC-Δ Np63-GFP, pHuC-Bad-p53-GFP, and pHuC-Bad-p53NLSmut-GFP.

Site-directed mutagenesis of DNA-binding domain or nuclear localization signal (NLS) in zebrafish p53. Site-directed mutagenesis was performed to generate plasmids encoding p53 NLS mutants (K292A and K293A) and others (R143H and R241H) using pHuC-p53-GFP plasmid as template by the Quick Change Site-Directed Mutagensis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The corresponding oligonucleotides used were as follows with the altered bases underlined [31]: NLS-F: 5′-CCT GAG GGG AGC GCA GCG GCC AAG GGC-3′, NLS-R: 5′-GCC CTT GGC CGC TGC GCT CCC CTC AGG-3′; R143H-F: 5′-GAA GTG GTC CGC CAT TGC CCC CAT CAT-3′, R143H-R: 5′-ATG ATG GGG GCA ATG GCG GAC CAC TTC-3′; R241H-F: 5′-TCT TTT GAG GTG TGC GTG TGT GCA TGT-3′, R241H-R: 5′-ACA TGC ACA CAC GCACAC CTC AAA AGA-3′. The sequences of the resultant plasmids were verified using DNA sequencing.

Microinjection of expression plasmid into zebrafish embryos. The expression plasmid was linearized by digestion with suitable restriction enzymes and purified with PCR Gel extraction kit (Qiagen, GmbH, Germany). DNA was adjusted to a final concentration of 100 μg/ml in 1× Danieau solution (5 mM Hepes, pH 7.6, 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, and 0.6 mM $(NO_3)_2$) containing 0.5% phenol red and injected into the zebrafish embryo at one-cell stage using Narishige IM 300 microinjector system (Narishigi Scientific Instrument Lab., Tokyo, Japan). Embryos at 24, 48, and 120 hpf were observed under an Olympus IX70-FLA inverted fluorescence microscope. Images were taken by using the SPOT system (Diagnostic Instruments, Sterling Heights, MI) and assembled by PhotoShop program (Adobe System, CA).

Morpholino injection. Antisense morpholinos (MOs) against importin β and α1 were obtained from Gene Tools (Philomath, OR) with the sequences as: β, 5′-TGC AGT CGG TCG GTA AAG TCG GTC G-3′ and α1, 5′-CGG TGT TCT CAT TTG CAG CAG ACA T-3′. The 4-mismatch control MO of importin α1 was generated with sequences as 5′-CGG TcT TCT CtT TTG CtG CAG tCA T-3′. Each MO was dissolved in 1× Danieau solution containing 0.5% phenol red to a final concentration of 0.3 mM and 1.5 nl (6 pg) was co-injected with pHuC-p53-GFP (3 pg) into each embryo at the 1–2 cell stage.

TUNEL assay. For detection of apoptotic cells, the embryos were fixed overnight in 4% paraformaldehyde followed by staining. TUNEL analysis was performed using the In situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany).

Whole-mount antibody staining. Embryos were collected at appropriate stage, fixed in 4% paraformaldehyde (W/V) (overnight, 4 °C), rinsed with PBS, and dehydrated using 100% methanol following standard dehydration procedures. After rehydaration, embryos were incubated with the anti-tubulin primary antibody, DM1A (ICN Immunobiologicals, Costa Mesa, CA), or anti-HA primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for overnight. After washing with PBST, embryos were incubated with biotin-conjugated secondary antibody at 4 °C for overnight. Detection of the antigen was carried out using the Vectastain Avidin/Biotin/Horseradish peroxidase ABC Elite System (Vector Laboratories, CA) according to standard procedures [32]. Embryos stained were kept in 100% glycerol.

Cell culture, transfection, and luciferase reporter assay. All of the plasmids used for transfection were purified by Nucleospin Plasmid kit (Clontech Laboratories, CA). Approximately 80% confluent COS-1 cells were transiently transfected by using Lipofectamine/Plus kit (Invitrogen Life technologies, CA) at 37 °C for 6 h with 1.5 μg of designated plasmid in a 12-well dish. For luciferase reporter assay, 100 ng of p53CON-Frag.A-luciferase plasmid [33], which contains both double-stranded p53 consensus-binding site (5′-GGACATGCCCGGGCAT GTCC-3′) and fragment A (nucleotides 106–138 fragment of human genomic clone 772CBE) [34], was cotransfected with 1 μg of p53 effector plasmid as indicated. Cells were harvested 48 h after transfection, and luciferase activity was measured. Luciferase activity was normalized to the protein tested and expressed as a fold change as compared to that derived from control cells. Each transfection was performed in duplicate and data were obtained from three individual experiments.

Results

Expression of p53-GFP driven by neuron-specific promoter in the nuclei of yolk syncytial layer in zebrafish embryos

As an attempt to express p53 protein in neuronal cells, zebrafish cDNA encoding p53 was expressed in fusion to the GFP protein under the control of the neuron-specific HuC promoter [30] in zebrafish embryos. *HuC* gene is known as a useful early marker for neurons in zebrafish and the upstream 3.4 kb-long promoter fragment has been demonstrated to be sufficient to confer its downstream target gene a neuron-specific expression pattern closely resembling that of endogenous *HuC* gene [30]. As shown in Fig. 1, the HuC promoter-driven GFP was expressed in trigerminal ganglion, axon as well as interneurons at 48 hpf (panels A and B). However, p53-GFP was expressed as small polka dots in yolk ball and yolk extension (panels G and H), and these fluorescent spots sustained for 5 days post-fertilization (dpf) (panels I and J). Such an expression pattern has been previously reported in the Sytox Green-injected zebrafish embryos (panels K and L), that Sytox Green can vitally stain the yolk syncytial layer (YSL) nuclei of zebrafish embryos and those cells do not contribute to the formation of liver, pancreas, and gut [25]. Similarly, Sytox Green signal in YSL nuclei also sustained and remained visible for 7 dpf until the yolk has been completely absorbed. Moreover, free fluorescent spots were observed at 4 hpf (panels N and O), suggesting that the targeting to YSL is corresponding to YSL development. In addition, the HA-tagged p53 protein was also governed by HuC promoter to be expressed in the yolk ball (panel P), with similar expression pattern to p53-GFP. These data demonstrate that HuC promoter-directed p53 protein was targeted to YSL nuclei, but not to neuronal cells, in zebrafish embryos. In contrast, GFP fusion proteins of other members of the p53 family, ΔNp63 and p73, were expressed only in the nuclei of neuronal cells and no signal in axon (panels C–F). Based on these findings, we propose that targeting to zebrafish YSL nuclei is p53-specific.

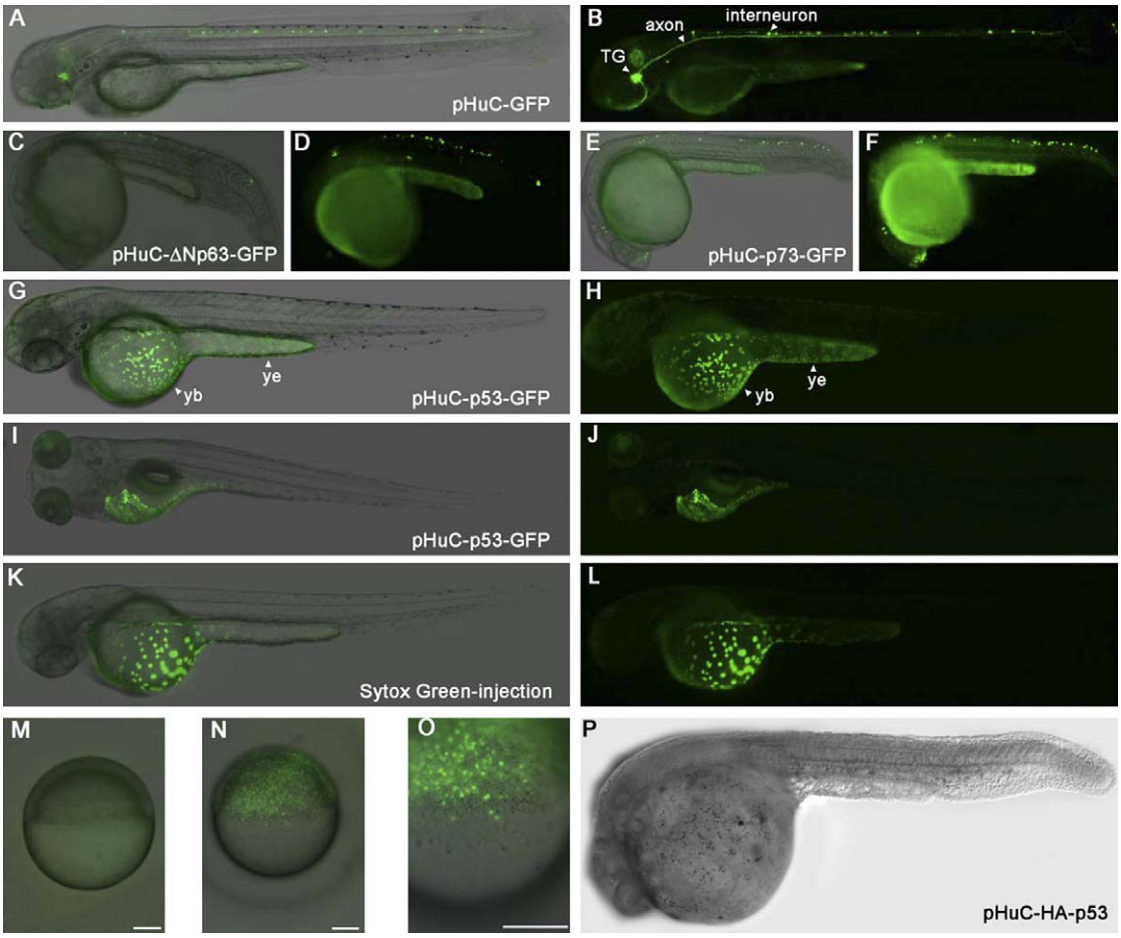

Fig. 1. Expression of p53, ΔNp63, and p73 driven by a neuron-specific HuC promoter in zebrafish embryos. To express the p53 family proteins under the control of the neuron-specific HuC promoter, plasmids pHuC-p53-GFP, pHuC-ΔNp63-GFP, and pHuC-p73-GFP were individually injected into zebrafish embryos at one-cell stage, with pHuC-GFP as a control. Merged images of bright-field and fluorescence are shown in (A, C, E, G, I, K, M, N, and O), while fluorescence images only in (B, D, F, H, J, and L). At 48 hpf, pHuC-GFP-injected embryos displayed GFP expression in trigerminal ganglion (TG), axon as well as interneurons (A,B; 76%, $n = 115$). Similarly, HuC-ΔNp63-GFP and HuC-p73-GFP were expressed in the nuclei of neurons and interneurons, but not axon at 24 hpf (C–F; 85%, $n = 132$ and 84%, $n = 143$, respectively). In contrast, pHuC-p53-GFP-injected embryos displayed fluorescence spots in yolk ball (yb) and yolk extension (ye) at 48 hpf (G,H; 40%, $n = 148$). Those GFP spots sustained for 5 days post-fertilization (dpf) (I,J) and disappeared at 7 dpf. Sytox Green-injected zebrafish embryos also showed GFP signals in yolk ball and yolk extension at 48 hpf (K,L; 80%, $n = 56$). Fluorescence signals were observed in pHuC-p53-GFP-injected embryos at 4 hpf (N) and some free fluorescent spots moved toward the yolk cell with high magnification (O). Control embryo at 4 hpf without GFP (M). At 48 hpf, pHuC-HA-p53-injected embryos were fixed and stained with anti-HA monoclonal antibody (P). Bars, 100 μm. (A–L) Lateral views with anterior to the left and dorsal to the top.

Zebrafish p53-GFP can be expressed in neuronal cells when it is localized in the cytosol

The zebrafish p53 is composed of 373 amino acids which shares 48% identity to its human counterpart [31]. Analogous to p53 in other species, the zebrafish p53 also contains the five highly conserved domains (Fig. 2A), of which domains II–V are involved in DNA binding, while N-terminal domain I is involved in interaction with other proteins including Mdm2. In addition, the carboxyl terminal region of the zebrafish p53 contains a putative nuclear localization signal (NLS) and the oligomerization domain [35]. To study the structural domains of p53 that are involved in targeting to YSL in zebrafish embryos, C-terminal truncated form of zebrafish p53, from which the C-terminal region (261–373 amino acid residues) was removed, was generated as shown in Fig. 2A. It is noted that expression of ΔCp53-GFP under the control of HuC promoter was targeted to neuronal cells (panels B and C).

The human p53 contains two nuclear localization signals (NLSs) that have been demonstrated to interact with importin α and mediate the translocation of p53 to nucleus [36]. To test whether the putative NLS signal (aa 287–295 RPEGSKKAK) located in the C-terminal region of zebrafish p53 determines whether p53 can express in neuronal cells, site-directed mutagenesis was performed to generate a mutated NLS (RPEGSAAAK). As shown, the p53-GFP with mutated NLS signal also displayed neuron-specific expression (panels D–G) as that of ΔCp53-GFP, and it was not targeted to YSL nuclei.

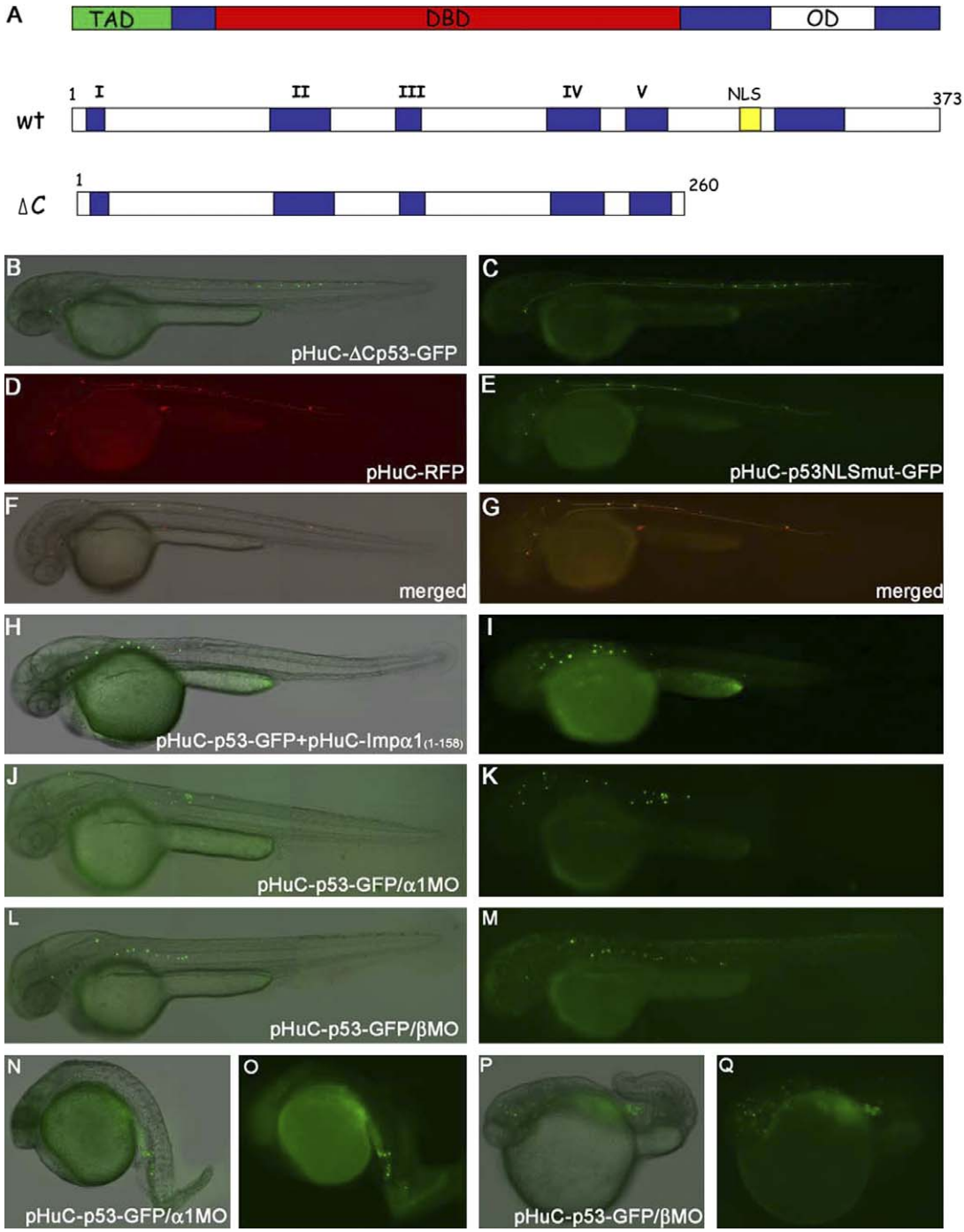

Fig. 2. Expression of p53-GFP in neuronal cells when it is localized to the cytosol. Schematic representations of functional domains in the full-length and C-terminal deleted p53 are shown in (A). Zebrafish p53 protein has the N-terminal transactivation domain (TAD), DNA-binding domain (DBD), and the C-terminal oligomerization domain (OD). A deleted form of C-terminal region, which contained a putative nuclear localization signal (NLS), of zebrafish p53 was generated. At 48 hpf, embryos injected with pHuC-ΔCp53-GFP displayed GFP signal in neuronal cells (B,C; 49%, $n = 132$). Embryos coinjected with pHuC-p53NLSmut-GFP and pHuC-RFP showed both green and red fluorescence signals in neuronal cells (D–G; 50%, $n = 70$). pHuC-Impα1(1–158) was coinjected with pHuC-p53-GFP into zebrafish embryos at one-cell stage, and embryos displayed GFP expression in neuronal cells, not in YSL at 48 hpf (H,I; 35%, $n = 72$). Morpholino oligonucleotides specifically targeted at zebrafish importin α1 and β, designated as α1-MO and β-MO, were synthesized and individually coinjected with pHuC-p53-GFP into zebrafish embryos at one-cell stage. At 48 hpf, embryos injected with pHuC-p53-GFP/α1MO (J,K,N,O; 18% and 25%, respectively, $n = 142$) and pHuC-p53-GFP/βMO (L,M,P,Q; 10% and 38%, respectively, $n = 138$) displayed GFP signal in neuronal cells, not in YSL cells. Merged images of bright-field and fluorescence are shown in (B, F, H, J, L, N, and P), while fluorescence images only in (C, D, E, G, I, K, M, O, and Q). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

We next investigated whether other factors including the importin family proteins that control the subcellular localization of p53 are involved in the expression of p53-GFP in neuronal cells. A vector expressing a truncated importin α1 (aa 1–158) was constructed and co-injected with pHuC-p53-GFP into zebrafish embryos at one-cell

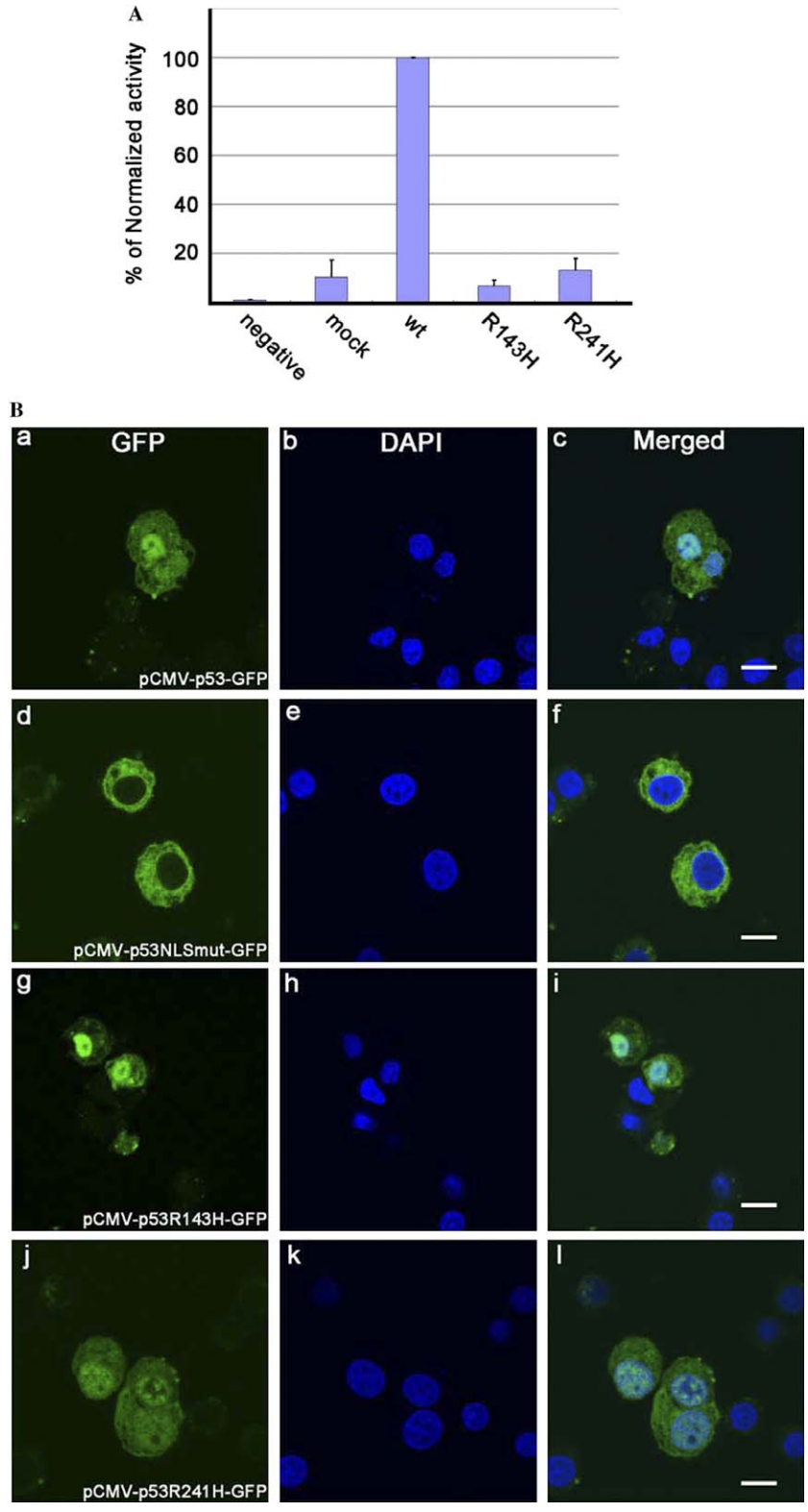

Fig. 3. Transcriptional activity and cellular localization of p53 mutants in COS-1 cells and their expression in neuronal cells of zebrafish embryos. (A) Effect of p53 mutants on transcriptional activity. Analysis of transcriptional activity of wild-type p53 and mutants p53 in COS-1 cells toward the p53RE consensus sequence reporter. Error bars indicate standard deviations. (B) Cellular localization of p53 mutants in COS-1 cells. After 16 h post-transfection, cells transfected with pcDNA-p53-GFP or p53 mutants were observed under fluorescence microscope. Cellular nucleus was stained with DAPI (blue). Bars, 10 μm. (C) Expression of p53 mutants in zebrafish embryos. Individual expression construct was coinjected with pHuC-RFP into zebrafish embryos at one-cell stage. At 48 hpf, embryos injected with pHuC-RFP and pHuC-p53R143H-GFP (a,b,c; 24%, $n = 62$) and pHuC-p53R241H-GFP (d,e,f; 27%, $n = 65$) displayed colocolized RFP and GFP signal in neuronal cells, but not YSL. Merged images of bright-field and fluorescence are shown in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

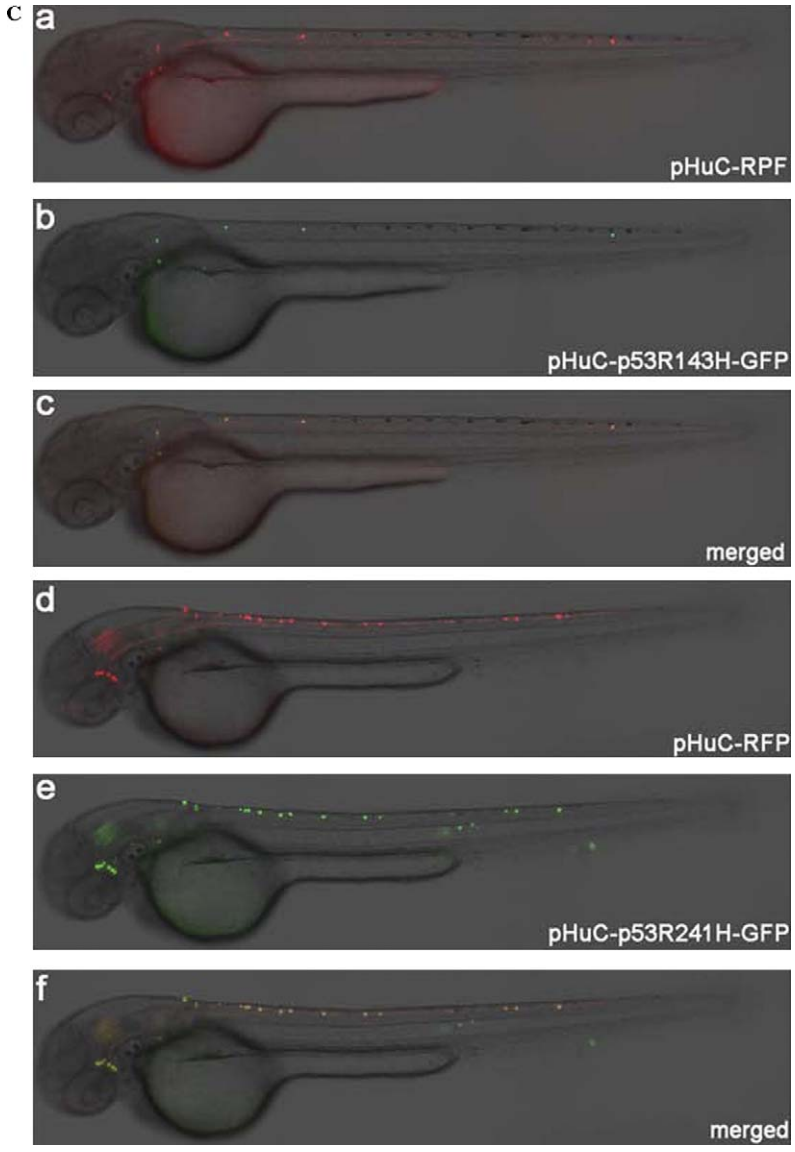

Fig 3. (*continued*)

stage. Upon overexpression of the non-functional importin α1, p53-GFP was targeted to zebrafish neuronal cells (panels H and I). These data also demonstrated the cytosolic distribution of wild-type p53-GFP in neurons when co-expressed with the truncated importin α1 which acts in a dominant-negative fashion to interact with importin β and prevents importin β from binding to p53 NLS [37]. Thus, p53-GFP remained in the cytosol and targeted to the neuronal cells. We further test whether elimination of the expression of importin α1 and β will facilitate the expression of p53-GFP in neuronal cells. Morpholino-modified oligonucleotides specifically targeted against zebrafish importin α1 and β, designated as 1-MO and β-MO, were synthesized and co-injected with pHuC-p53-GFP into zebrafish embryos. Our results showed that α1-MO (panels J and K) and β-MO (panels L and M) yielded similar results as that of truncated importin α1 (panels H and I). These data suggest that targeting of p53-GFP to neuronal cells is dependent on its cytosolic distribution. In addition, importin α1-MO and β-MO morphants showed significant abnormality in zebrafish embryo development (panels N–Q), suggesting that importin α1 and β may be also involved in the nuclear translocation of other transcription factors required for embryo development.

Expression of zebrafish p53-GFP in YSL nuclei depends on its transcriptional activity

We further determined the factors that may affect the targeting of HuC promoter-directed p53-GFP fusion protein to YSL nuclei in zebrafish embryos. We first examined whether the transcriptional activity of p53 is required for this targeting. A series of zebrafish p53 mutants were generated based on the conserved sequences in the DNA-binding domain (DBD) between zebrafish and human p53 proteins by site-directed mutagenesis.

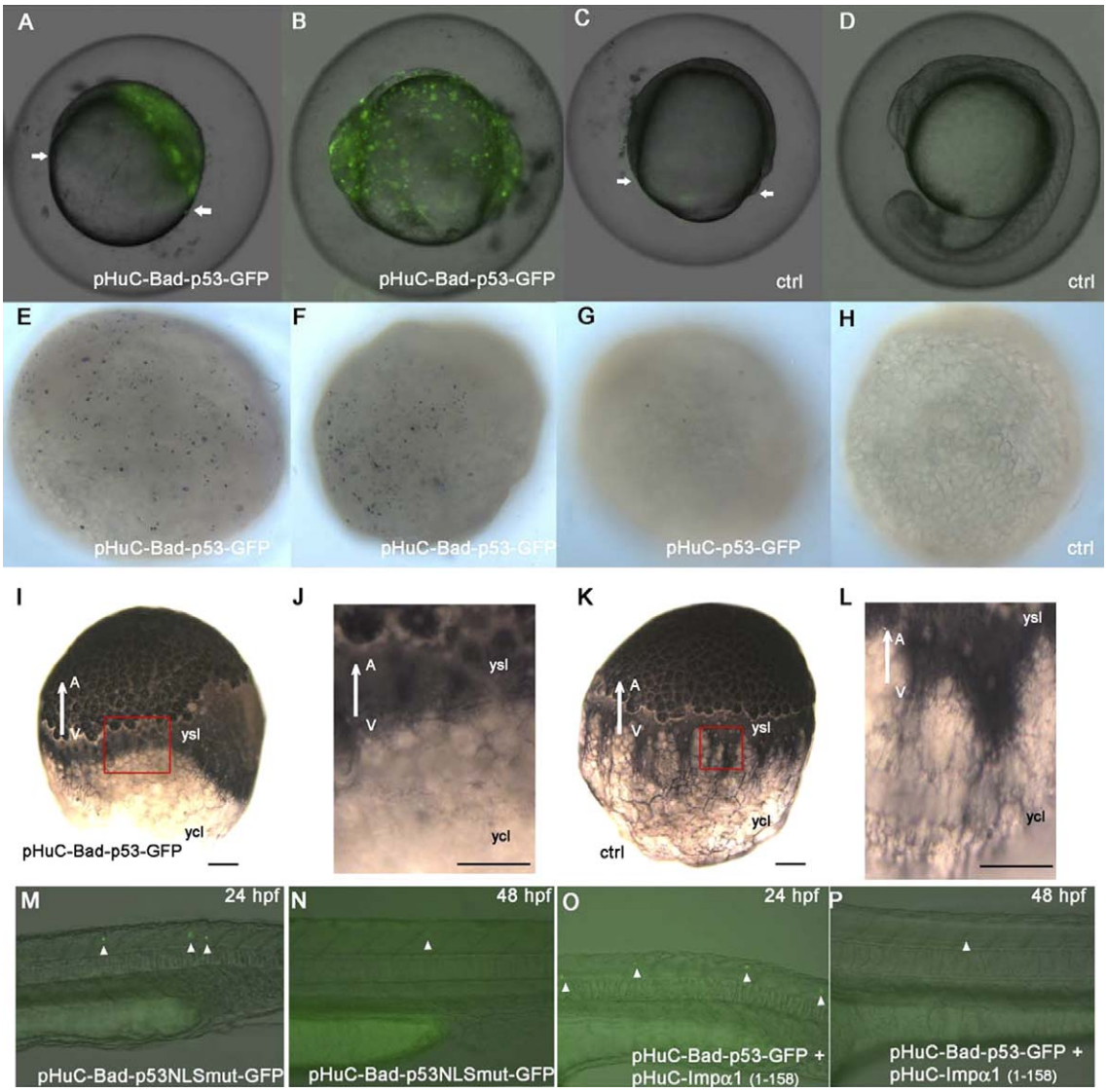

Fig. 4. Expression of Bad-p53-GFP in zebrafish embryos induces apoptosis of YSL cells while expression of Bad-NLSmt-p53-GFP results in neuronal cell death. pHuC-Bad-p53-GFP was injected into zebrafish embryos at one-cell stage. At 8 hpf, the pHuC-Bad-p53-GFP-injected embryos remained at 50% epiboly (A), while wild-type embryos were at 75% epiboly (C). At 20 hpf, pHuC-Bad-p53-GFP-injected embryos showed abnormal phenotype with GFP expression in the yolk ball (B) whereas the wild-type embryos developed normally (D). Significant TUNEL-positive cells were observed in pHuC-Bad-p53-GFP-injected embryos at 10 hpf (E,F), while very few or no TUNEL-positive cell was observed in the pHuC-p53-GFP-injected (G) or wild-type embryo (H). Microtubule arrays in the yolk cells are stained by anti-tubule antibody and visible in the yolk syncytial layer (ysl) and in the yolk cytoplasmic layer (ycl) of the wild-type embryos at 6 hpf (K,L). Very few microtubules were observed in the YSL of the pHuC-Bad-p53-GFP-injected embryos (I,J). High magnification of microtubules marked by red boxes in (I,K) is shown in (J,L). On the other hand, zebrafish embryos were injected with pHuC-Bad-p53 NLSmut-GFP (M,N; 31%, $n = 65$) or coinjected with pHuC-Bad-p53-GFP and pHuC-Impα1 (1–158) (O,P; 30%, $n = 70$) at one-cell stage, respectively. At 24 hpf, all injected embryos displayed GFP signal in neuronal cells (M,O) and these fluorescence signals in the same fish decreased at 48 hpf (N,P). The vegetal–animal (V–A) axis is indicated by the white arrow. Bars, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

They belong to two classes of p53 DBD mutants, conformational mutant (R175H [38]) and contact site mutant (R273H [39]). These mutants have been described as hot-spot mutants that are frequently found in human cancer [40]. Although the overall amino acid identity between zebrafish and human p53 is only 48.4%, the four domains conserved within the DNA-binding core of zebrafish p53 and the human orthologue share 92%, 100%, 100%, and 94% sequence identity, respectively. In zebrafish, the equivalent corresponding mutants are R143H and R241H due to the lack of 32 amino acid residues in the N-terminal region [12,31]. Upon overexpressing in COS-1 cells, these zebrafish p53 mutant proteins were expressed and mainly localized in the nucleus, and as analogous to their human p53 mutants, they were completely inactive toward transactivation of p53 consensus-binding site (Figs. 3A and B). When the zebrafish p53 mutant proteins were expressed under the control of HuC promoter in zebrafish embryos, unlike the wild-type p53 being targeted to YSL nuclei, they were

expressed in neuronal cells (Fig. 3C). These data suggested that the transcriptional activity of p53 is directly or indirectly required for the targeting of p53-GFP to YSL nuclei.

Injection of pHuC-Bad-p53-GFP into zebrafish embryos induces apoptosis of YSL cells while injection of pHuC-Bad-p53NLSmut-GFP results in neuronal cell death

Because that p53 expression is localized to YSL nuclei in the early embryo development, we then tested whether p53 can be utilized as a tool to deliver target proteins of specific functions to YSL in zebrafish embryos. The gene encoding proapoptotic protein Bad was cloned preceding that of p53 in the pHuC-p53-GFP or pHuC-p53NLSmut-GFP, and the resultant plasmid pHuC-Bad-p53-GFP or pHuC-Bad-p53NLSmut-GFP was injected into zebrafish embryos at one-cell stage. In comparison to the control embryos that displayed 75% epiboly at 8 hpf (panel C) and developed normally at 20 hpf (panel D), approximately 50% of the pHuC-Bad-p53-GFP-injected embryos ($n = 150$) showed delayed development with 50% epiboly at 8 hpf (Fig. 4A) and abnormal phenotype was visible at 20 hpf, with fluorescence signals observed in the yolk ball (panel B). In embryos injected to express Bad-p53-GFP, significant apoptosis was observed at 10 hpf as detected by TUNEL assay (panels E and F), while very few or no TUNEL-positive cell was detected in the pHuC-p53-GFP-injected (panel G) and the control embryos (panel H). In consistence, microtubule arrays in the yolk cell are visible in the wild-type embryo at 6 hpf (panels K and L), which are the structural prerequisite for YSL and blastomere movements [26]. In contrast, relatively sparse distribution of microtubules can be seen in the YSL of embryos injected with pHuC-Bad-p53-GFP (panels I and J). These data indicate that Bad-p53-GFP-induced apoptosis results in the loss of YSL cells that impairs the normal formation of microtubule arrays in the yolk cell, thus leading to abnormal YSL and blastomere movements. In contrast, zebrafish embryos injected with pHuC-Bad-p53NLSmut -GFP displayed a neuronal expression pattern (panel M). The GFP signals were quickly lost (panel N), suggesting that activation of caspases may occur to cleave the fusion protein. Similar phenomena were also observed in zebrafish embryos that were coinjected with pHuC-Bad-p53-GFP and pHuC-Impα1(1–158) (panels O and P).

Discussion

In this study, we demonstrated that the expression of zebrafish p53 under the control of a neuron-specific HuC promoter was not localized to the neuronal cells as expected. Instead, it was targeted to the zebrafish YSL nuclei (Fig. 1). The expression pattern of p53-GFP in YSL resembled the staining feature previously reported for Sytox Green [25]. We further provided evidences to show that the targeting to YSL is p53 sequence-specific and dependent on the nuclear localization and transcriptional activity of wild-type p53 (Figs. 2 and 3). When p53 was retained in the cytosol, the zebrafish p53 was expressed in the neuronal cells under the control of HuC promoter (Fig. 2). We also showed that p53 mutants that harbor mutations in DNA-binding domain (DBD) were also expressed in the neuronal cells under the control of HuC promoter (Fig. 3). This is the first report to demonstrate that targeting to YSL nuclei or tissue-specific expression of zebrafish p53 in developing embryos is determined by its subcellular localization.

Nuclear translocation is a prerequisite for proper functionality of transcription factors. Translocation of large proteins into the nucleus is facilitated by the signal motif through its interaction with importin α/β heterodimer. The NLS-containing proteins interact through its NLS motif with importin α, which in turn associates with importin β, the subunit that mediates the docking of the complex with the nuclear pore complex (NPC) and facilitates the import into nucleus [41–43]. The characterized NLS consists of a stretch of amino acids, enriched in arginines and lysines [44,45]. Currently, there are six members of the importin α family, i.e., α1 and α3–α7, and each forms heterodimer with the only β subunit [46,47]. In human, the nuclear import of p53 is mediated by importin α1 and the introduction of an importin α1 mutant with N-terminal region truncated inhibits nuclear translocation of p53, thus leading to the accumulation of p53 in the cytoplasm [37].

In analogy to the monopartite NLS sequences (^{316}PQPKKKP^{322}) located in human p53 which has been demonstrated to be responsible for its nuclear import [48], a putative monopartite NLS was identified in the C-terminal region of zebrafish p53 with the amino acid sequences of RPEGSKKAK (aa 287–295). Through deletional analysis, we showed that the C-terminal region of zebrafish p53 is responsible for its targeting to YSL. By site-directed mutagenesis, we further provided evidence to demonstrate that the NLS within this region plays an essential role in targeting zebrafish p53 to the YSL or expression in neuronal cells (Fig. 2). The absolute requirement of the NLS sequences for YSL targeting was further substantiated by our findings that the targeting to YSL is mediated through importin heterodimers. By morpholino-mediated knockdown approach, we showed that injection of specific morpholino oligonucleotides to knock down the expression of zebrafish importin β and α1 leads to the expression of p53-GFP in neuronal cells, not targeting to zebrafish YSL. Moreover, if we coinjected pHuC-p53-GFP with pHuC-Impα1(1–158) into zebrafish embryos, we could demonstrate the expression of p53-GFP in neuronal cells (Fig. 2, panels H and I). It has been reported that the importin α1 mutant (1–158) with N-terminal region truncated inhibits nuclear translocation of p53, thus leading to the accumulation of p53 in the cytoplasm [37]. These data suggest that cytosolic or nuclear localization of wild-type p53 determines its expression in neuronal cells or targeting to YSL nuclei.

Apparently, not all NLS sequences confer the targeting to zebrafish YSL nuclei, as zebrafish p53 does. It is noted that the amino acid sequences of zebrafish ΔNp63 and p73 are 74% and 70% identical to those of human counterparts, respectively [15,18]. In addition, a putative NLS was identified in the C-terminal region of zebrafish ΔNp63 with the amino acid sequences of LNSIKKRRST (aa 288–297), and p73 contains a stretch of amino acid residues of SINIKKRRHG (aa 348–357). However, the GFP fusion proteins of ΔNp63-GFP and p73-GFP were not targeted to zebrafish YSL when expressed under the control of HuC promoter (Fig. 1). Therefore, only p53, not ΔNp63 nor p73, in the p53 family confers the targeting to zebrafish YSL nuclei.

During zebrafish development, the external YSL was formed due to the collapse of marginal blastomeres and the release of their nuclei into the cytoplasm of the YCL of zebrafish embryos [23,24]. In this study, it is noted that HuC promoter-directed p53-GFP fusion protein is targeted to YSL nuclei and not localized to the neuronal cells in zebrafish embryos as expected. To test whether the transcriptional activity of zebrafish p53 is required for this targeting, we generated several p53 mutants based on the conserved sequences in the DNA-binding domain (DBD) between zebrafish and human p53 protein by site-directed mutagenesis. As expected, these mutants had lost their transcriptional activity (Fig. 3A), but retained the ability to localize in the nucleus when overexpressed in COS-1 cells (Fig. 3B). We then microinjected and expressed these mutant p53 proteins under the control of HuC promoter in zebrafish embryos. Our results showed that they were expressed in neuronal cells of zebrafish embryos and not targeted to YSL nuclei (Fig. 3C). These data demonstrate that the transcriptional activity of wild-type p53 protein is required for the targeting of p53-GFP to YSL nuclei of zebrafish embryos, suggesting the possibility that p53 may transactivate one or more target genes and the expression of these genes may affect or change the initial neural cell fate that is determined by HuC promoter.

Because of the similarity between the movements of YSL nuclei and deep cells of the blastoderm, the dependence of each other on their specific movements has been addressed. Although it is difficult to tackle, several lines of evidence have demonstrated that the epiboly of the blastoderm is partially dependent on the normal epiboly of the YSL [24,49]. In this study, for the first time, we showed that zebrafish p53 could be utilized as a tool to specifically target the proapoptotic BAD protein to YSL nuclei and induce apoptosis in the YSL cells as shown by TUNEL assay (Fig. 4). We further showed that loss of YSL during zebrafish early development results in imperfect formation of microtubules (panels I and J), thus leading to delayed movement of the blastoderm (panel A). As a consequence, severely abnormal embryos were observed (panel B), supporting the notion that movements of YSL cells are related to movements of deep cells of the blastoderm. On the other hand, pHuC-Bad-p53NLSmut-GFP-injected zebrafish embryos only displayed a quick loss of GFP signal in neuronal cells (panels M and N), suggesting possible activation of caspases. Similarly, zebrafish embryos co-injected with pHuC-Bad-p53-GFP and pHuC-Impα1(1–158) also showed neuronal cell death (panels O and P) without death of YSL cells.

In conclusion, we showed that expression of p53 in zebrafish early embryo is preferentially targeted to YSL nuclei, and the targeting to YSL is dependent on the nuclear localization and transcriptional activity of p53.

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