Mutation of DNA primase causes extensive apoptosis of retinal neurons through the activation of DNA damage checkpoint and tumor suppressor p53

Masahiro Yamaguchi1,2, Noriko Fujimori-Tonou2, Yukihiro Yoshimura1, Tsutomu Kishi3, Hitoshi Okamoto4 and Ichiro Masai1,2,*

Apoptosis is often observed in developing tissues. However, it remains unclear how the apoptotic pathway is regulated during development. To clarify this issue, we isolated zebrafish mutants that show extensive apoptosis of retinal cells during their development. pinball eye (piy) is one such mutant, in which retinal stem cells proliferate normally but almost all retinal neurons undergo apoptosis during differentiation. We found that a missense mutation occurred in the small subunit of DNA primase (Prim1) in the piy mutant. DNA primase is essential for DNA replication; however, this mutation does not affect cell proliferation but rather induces neuronal apoptosis. RNA synthesis catalyzed by Prim1 is important for the activation of the DNA damage response, which may activate Ataxia telangiectasia mutated (ATM), Checkpoint kinase 2 (Chk2) and the tumor suppressor p53. We found that the apoptosis induced by the prim1 mutation depends on the ATM-Chk2-p53 apoptotic pathway. These data suggest that the surveillance system of genome integrity strongly influences the cell fate decision between differentiation and apoptosis during retinal neurogenesis in zebrafish.

KEY WORDS: Apoptosis, Checkpoint, Chk, Prim1, p53, Retina, Zebrafish, Danio rerio

INTRODUCTION

In the vertebrate retina, six major classes of neurons and one class of glial cells differentiate to form the neural circuit responsible for phototransduction and visual processing (Dowling, 1987). In the developing zebrafish retina, neurogenesis is initiated in the cells adjacent to the optic stalk and progresses into the entire neural retina (Hu and Easter, 1999; Masai et al., 2000). Fibroblast growth factor (FGF) and Hedgehog (Hh) signaling pathways regulate the initiation and progression of retinal neurogenesis in zebrafish, respectively (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003; Masai et al., 2005; Martinez-Morales et al., 2005). In the zebrafish retina, Wnt and Notch signaling pathways promote cell proliferation and inhibit neurogenesis, respectively. Histone deacetylase 1 (Hdac1) antagonizes both Wnt and Notch signaling pathways to promote retinal neurogenesis in zebrafish (Yamaguchi et al., 2005). These studies suggest that at least five signaling pathways, namely FGF, Hh, Hdac1, Wnt and Notch, regulate retinal neurogenesis in zebrafish. However, the mechanisms regulating the later processes of neuronal differentiation in the zebrafish retina remain largely unknown.

Apoptosis is often observed in a developing tissue and is believed to remove abnormal cells such as cancer-predisposing cells. In some cases, apoptosis seems an indispensable event programmed in developmental processes. In mammalian retinas, retinal ganglion cells (RGCs) that project to inappropriate targets are removed by programmed cell death (Clarke and Cowan, 1976; Jeffery and Perry, 1982), maintaining the proper balance between the numbers of pre- and postsynaptic neurons (Oppenheim, 1991; Pettmann and Henderson, 1998). In the retinas of zebrafish, amphibians, birds and cats, apoptosis is observed initially in first-born neurons such as RGCs, followed by successive apoptosis in later-born neurons (Wong and Hughes, 1987; Cook et al., 1998; Marín-Teva et al., 1999; Glucksmann, 1940; Beazley et al., 1987; Bielmaier et al., 2001), suggesting a link between neuronal differentiation and apoptosis. However, it remains unclear how the apoptotic pathway is regulated during retinal neurogenesis.

Eukaryotic cells have developed an elaborate network of checkpoints to ensure that damaged DNA is repaired. The central components of DNA damage checkpoints are two phosphatidylinositol 3-kinase-like kinase (PIKK) family proteins: Ataxia telangiectasia mutated (ATM) and ATM-and-Rad3-related (ATR) (Abraham, 2001; Shiloh, 2003). ATR is recruited to single-stranded DNA regions, which originate at stalled replication forks or in the processing of bulky lesions such as UV photoproducts, and activates the serine/threonine kinase Checkpoint kinase 1 (Chk1; also known as Chek1) (Andreassen et al., 2006). Chk1 prevents mitotic entry during DNA replication by inhibiting Cdc25 phosphatase activity. On the other hand, ATM is activated by DNA double-stranded breaks and phosphorylates several substrates, including Checkpoint kinase 2 (Chk2; also known as Chek2) (Shiloh, 2003; O’Driscoll and Jeggo, 2006). Chk2 has a function overlapping with that of Chk1, that is, it arrests cell-cycle progression by inhibiting Cdc25 activity. Furthermore, Chk2 facilitates DNA repair or induces apoptosis by activating the tumor suppressor p53 (Roos and Kaina, 2006; Helton and Chen, 2007). Although ATR- and ATM-dependent pathways were considered to function independently, recent studies have suggested a crosstalk between these two pathways. In response to double-stranded breaks during the S and G2 phases, ATM generates the single-stranded region of DNA, which subsequently activates ATR and Chk1 (Cuadrado et al., 2006; Jazayeri et al., 2007).
DNA primase catalyzes the synthesis of RNA primers in the lagging strands for DNA replication, and consists of two subunits: a small catalytic subunit and a large regulatory subunit (reviewed by Foiani et al., 1997; Arezi and Kuchta, 2000). DNA primase associates with DNA polymerase α, which extends RNA primers with about 20 bases of DNA. Following RNA-DNA hybrid nucleotide synthesis catalyzed by the DNA polymerase α-primase complex, DNA polymerase δ is thought to take over DNA synthesis against the lagging strand of the replication fork. DNA polymerases ε and δ catalyze the DNA synthesis against the leading strands of the replication fork (Johnson and O’Donnell, 2005). Thus, DNA primase is essential for DNA replication. Recently, it was reported that RNA primer synthesis catalyzed by DNA primase is required for the activation of the ATR-dependent DNA damage checkpoint pathway (Marini et al., 1997; Michael et al., 2000; MacDougall et al., 2007). DNA primase is also important for DNA damage checkpoints during replication.

In this study, we isolated a zebrafish mutant, pinball eye (piy), in which almost all retinal neurons undergo apoptosis during differentiation. We found that a missense mutation occurred in a small subunit of DNA primase (Prim1) in the piy mutant. Prim1 is essential for DNA replication. However, this missense mutation does not affect cell proliferation, suggesting that this amino acid substitution specifically induces neuronal apoptosis. Since DNA primase is important for the ATR-dependent DNA damage response, which may activate ATM and Chk2, we examined the relationship between piy mutation, DNA damage checkpoints, and their downstream target p53. We found that neuronal apoptosis in the piy mutant depends on ATM, Chk2 and p53. These data suggest that the surveillance of genomic integrity during DNA replication strongly determines whether zebrafish retinal cells will continue to differentiate normally or undergo apoptosis.

MATERIALS AND METHODS

Fish strains
RIKEN wild type and WIK were used as wild-type strains for mutagenesis and mapping, respectively. The piy

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and slow muscle omitted (smo) (Varga et al., 2001) alleles were used in this study. A transgenic strain carrying the green fluorescent protein (GFP) under the control of ath5, Tg(ath5:GFP)

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(Masai et al., 2005), was used.

Histological analysis, immunohistochemistry, whole-mount in situ hybridization and cell transplantation

Histological analysis, immunohistochemistry, whole-mount in situ hybridization and cell transplantation were performed as described previously (Masai et al., 2000). The antibodies used in this study were zpr1 (Oregon Monoclonal Bank, 1:100), zn5 (Oregon Monoclonal Bank, 1:50), anti-5-bromo-2′-deoxyuridine (BrdU) (Roche, 1:100), anti-gamma-amino butyric acid (GABA) (Sigma, 1:500) and anti-phosphorylated histone H3 (Upstate, 1:500). Sytox Green nuclear acid stain (Molecular Probes) was used at 1:50,000.

Labeling of apoptotic cells

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an In Situ Cell Death Detection Kit (Roche).

Mutagenesis, mapping and cloning of the piy gene

Mutagenesis, mapping and cloning were carried out as previously described (Masai et al., 2003). The sequences of the polymorphic markers used were as follows: (1) Two microsatellite markers: Marker-piy (L) forward primer, 5′-TTTCAGTCATAGCCTGAAGGTGTA-3′ and reverse primer, 5′-TAAGACTGTCTCAATGACATGATTG-3′; Marker-piy (M) forward primer, 5′-AAAGCTCATTTCTGCTCTATACT-3′ and reverse primer, 5′-TAGCCATGTCAAAAATATTGTGTT-3′; (2) One single nucleotide polymorphic (SNP) marker: Marker-piy (R), 5′-CAGTGATTGACTCTTCTCATCCTCTCCTGCAAATCCTGAGGGYCTCTTCAACCAGACACCTTACTGTGACAGTAGTGTTAAGAGATT-3′ where Y=C (WIK) or T (Riken wild type).

Trichostatin A (TSA) treatment

TSA stock solution (Sigma, 1 mg/ml in DMSO) was diluted to 1200 nM in water before use. Embryos were soaked in 1200 nM TSA from 18 hours post-fertilization (hpf) until 2 days post-fertilization (dpf).

Quantification of the rate of proliferation of retinal cells

To estimate the rate of cell proliferation, we examined the ratio of BrdU-labeled cells and mitotic cells to the total number of retinal cells, as described in our previous study (Yamaguchi et al., 2005).
Yeast experiments

The budding yeast Saccharomyces cerevisiae was used. The yeast prim1 (also known as pri1) gene was amplified from the yeast genome by PCR. The mutated yeast prim1 sequence, in which phenylalanine is substituted by serine as in the zebrafish pity mutant (yeast F110S prim1), was amplified using the megaprimer method (Barik, 1993). The amplified DNA was ligated into the shuttle vector YCplac33 (Gietz and Sugino, 1988). These constructs were transformed into the yeast temperature-sensitive prim1 mutant strain pri1-1 (Francesconi et al., 1991) by a method using lithium acetate (Sherman et al., 1986). The transformed yeast cells were cultured in a complete minimal (CM) dropout medium (CM-ura) (Ausubel et al., 1987) at 30°C. After culture for 12 hours, the cultures were diluted with the medium 30 times and recultured at 37°C. Cell number was counted every 2 hours using a hemocytometer.

Rescue experiment of pity mutant

The full-length prim1 cDNA fragment was amplified by PCR and subcloned in frame into the Myc-tagged pCS2 expression vector (Rupp et al., 1994). This plasmid was cut with NotI and used for the synthesis of capped mRNA with SP6 RNA polymerase (Ambion). pity homozygous embryos were generated by crossing pity heterozygous parent fish carrying WIK-derived wild-type allelic chromosome 23 and identified by genotyping with the polymorphic microsatellite marker, marker-pity (M) (see Fig. 4A), which showed no recombination to the prim1 mutation in 3500 meioses. These embryos were injected with the synthesized prim1 RNA (1.26 mg/ml) at the one-cell stage, fixed in 4% paraformaldehyde (PFA) at 2 dpf and sectioned using a microtome.

Inhibition of ATR, ATM, Chk1, Chk2 and p53 by injection of morpholino antisense oligos

The following morpholino oligos (Gene Tools) were injected into embryos at the one-cell stage: MO-p53 (1 mM), 5’-GGGCCATAGGTGGCA-AGAATTG-3’ (Langheinrich et al., 2002); MO-Chk1 (0.25 mM), 5’-TTAA-CAAAAGGCACAGCCATTATGC-3’; MO-Chk2 (0.25 mM), 5’-CAGATCTGTGCTTTTTATTTATTGAC-3’; MO-ATM (0.1 mM), 5’-GAAAACGGCACCACCTGGTAAAAAC-3’; and MO-ATR (0.25 mM), 5’-TGACATTCTAGTTCCTGCCATTGAC-3’ (Stern et al., 2005). To confirm the genotype of morphants, pity homozygous embryos were identified using the polymorphic marker, marker-pity (M). Oligos with five mismatches (indicated in lowercase) for MO-Chk1 (5mis-MO-Chk1, 5’-TTAAgaA- AAacGCaAGCgATTaGC-3’) and MO-Chk2 (5mis-MO-Chk2, 5’-CAc-ACATcATcCTTTTTATAC-3’) were used as negative controls. We also...
confirmed the efficiency and specificity of MO-Chk1 and MO-Chk2 by examining whether morpholinos specifically suppress the translation from the hybrid RNAs that encode GFP following the 5’ untranslated region and initial 20-bp coding region of target genes (see Fig. S1 in the supplementary material).

**Quantification of p53 mRNA in wild-type and piy mutant embryos**

Total RNA were prepared from 20 wild-type and 20 piy mutant embryos using the Ultraspec RNA Isolation System (Biotecx Laboratories) and cDNA was generated using an RNA LA PCR Kit (AMV) version 1.1 (Takara). p53 and ef1a cDNA fragments were amplified from the same amount of wild-type and piy mutant cDNA in a series of PCR amplification cycles using primers: p53 forward primer, 5’-GGATGAGGAGAT-CTTACC-3’ and reverse primer, 5’-TGGGCACCTCTCAAGGAC-3’ and reverse primer, 5’-GGAGATGCAGTGCAGTGCAGTTCC-3’. The amount of PCR product was compared between wild-type and piy mutant embryos following electrophoresis.

**Optokinetic response (OKR) assay**

OKR was measured for 5 dpf embryos as described (Brokerhoff, 2006). After OKR assay, embryos were fixed with 4% PFA. Genomic DNA was extracted from the posterior part of each embryo, and the heads of single embryos were used for the labeling with anti-GABA antibody. piy homozygous embryos were identified by genotyping using marker-piy (M). The percentage of piy homozygous embryos that were OKR-positive was determined.

**DNA content analysis**

Heads were dissected from wild-type sibling and piy mutant embryos at 38 and 48 hpf. FACS analysis was carried out using dissociated cells from pooled heads of three embryos at 38 hpf and five embryos at 48 hpf, respectively, as previously described (Plaster et al., 2006). To determine the ratio of cells undergoing apoptosis and G1 phase/postmitotic, S phase and G2–M phases, the areas of histograms with <2N, 2N, 2N-4N and 4N were measured and compared with the total area. Standard deviation was determined using Student’s t-test (38 hpf piy mutant, n=5; 38 hpf wild-type sibling, n=3; 48 hpf piy mutant, n=3; 48 hpf wild-type sibling, n=3).

**Treatment with KU55933 and CGK733**

Embryos were soaked in water containing KU55933 (Calbiochem) at 2 ppm and CGK733 (Calbiochem) at 15 μM, or CGK733 (Calbiochem) at 15 μM, from 24 to 52 hpf.

**RESULTS**

**Differentiating retinal neurons undergo apoptosis in piy mutant retina**

To elucidate the mechanism regulating apoptosis during retinal neurogenesis, we screened zebrafish mutants by visualizing their retinal layers with an anti-acetylated α-tubulin antibody (Masai et al., 2003). In this screening, we identified a recessive lethal mutant which we named piy, which retinal laminar was not apparent (data not shown). In the piy mutant, extensive cell death was observed in the retina and tectum at 2 dpf (Fig. 1A). Plastic sections and TUNEL of the piy mutant retina revealed that apoptosis occurred in the central region of the neural retina, where differentiated neurons are normally located (Fig. 1B-E). Retinal stem cells are normally located in the most peripheral region of the neural retina called the ciliary marginal zone (CMZ) (Perron and Harris, 2000). In the piy mutant, retinal stem cells maintained their morphology (Fig. 1C) and incorporated BrdU (Fig. 1G). These data suggest that retinal stem cells appear intact, but almost all differentiating neurons undergo apoptosis in the piy mutant retina. In contrast to the neural retina, there was no gross defect observed in the retinal pigment epithelium in the piy mutant (data not shown).

To determine whether apoptosis occurs in differentiating neurons or mitotic progenitor cells in the piy mutant retina, we examined the relationship between apoptotic cells, the neuronal marker ath5 (also known as atoh7 – ZFIN) (Masai et al., 2000) and BrdU incorporation in the piy mutant retina. At 32 hpf, many retinal cells incorporated BrdU (Fig. 2A), but there were almost no apoptotic cells in the piy mutant retina (Fig. 2B). At 42 hpf, BrdU-positive cells were localized in the CMZ and the outer part of the central retina (Fig. 2C). In the zebrafish transgenic line Tg(ath5:GFP)w02, retinal neurons express GFP under the control of the ath5 promoter (ath5:GFP) (Masai et al., 2005). Apoptosis occurred in the central retina of the piy mutant where ath5:GFP expression was observed (Fig. 2D). At 72 hpf, massive apoptotic cells were observed in the central retina of the piy mutant, and ath5:GFP-positive neurons decreased in number (Fig. 2F). The CMZ cells incorporated BrdU (Fig. 2E) and expressed a molecular marker of retinal stem cells, rx1 (Fig. 2H), suggesting that retinal stem cells are maintained in the piy mutant. These data suggest that apoptosis occurs in differentiating neurons and not in mitotic progenitor cells in the piy mutant retina.

To determine whether apoptosis occurs in differentiating neurons, we examined the expression of upregulated genes in the piy mutant retina. The gene expression of p53, which is a potent inhibitor of Hdc, was significantly upregulated in the retina of the piy mutant treated with TSA (Fig. 2I-L). Next, we measured whether the smu mutation suppresses piy-mediated retinal apoptosis. We found that retinal apoptosis was significantly inhibited in the double mutant smu; piy (Fig. 2M-P).
These data suggest that the blockade of neurogenesis suppresses the apoptosis of retinal cells in the piy mutant. Thus, it is very likely that apoptosis occurs in differentiating neurons in the piy mutant retina.

**piy mutation behaves in a cell-autonomous manner**

To elucidate whether the piy mutation behaves in a cell-autonomous manner, we carried out transplantation experiments. When wild-type cells were transplanted into the piy mutant retina, wild-type donor cells survived to form retinal columns (Fig. 3B). When piy mutant cells were transplanted into the wild-type retina, the mutant donor cells failed to form retinal columns and instead remained as a small aggregated cell mass (Fig. 3C) that was TUNEL-positive (Fig. 3D), suggesting that the piy mutant cells undergo apoptosis in a wild-type environment. These data suggest that the piy mutation behaves in a cell-autonomous manner.

**Missense mutation in the small subunit of DNA primase in the piy mutant**

To elucidate the mechanism underlying the apoptosis of retinal neurons in the piy mutant, we cloned the piy mutant gene. The piy locus was mapped to chromosome 23 (Fig. 4A). We searched the polymorphic markers and found one marker, marker-piy (M), which showed no recombination in 3500 meioses, and two markers, marker-piy (L) (3/3500) and marker-piy (R) (1/3500), which flanked the piy mutation. Using the zebrafish genomic database, we found five candidate genes, rnd1, prim1, zgc:86644 (tfip11), naca and 559664 are located in this flanking region. (B) Missense mutation occurred in the **prim1** gene in the piy mutant. Amino acid comparison of Prim1 among human (Stadlbauer et al., 1994), mouse (Prussak et al., 1989), Xenopus tropicalis (GenBank accession BC067914), Danio rerio (GenBank accession NM_201448), Drosophila melanogaster (Bakkenist and Cotterill, 1994), Caenorhabditis elegans (GenBank accession NM_06488) and Saccharomyces cerevisiae (Plevani et al., 1987) is shown. Phenylalanine (F) 110 is conserved among these organisms (highlighted). Aspartic acids (D) marked by asterisks indicate the putative active site residues of Prim1 (Augustin et al., 2001). Sequences of the prim1 cDNA in the wild type (left) and piy mutant (right) are shown beneath. The transversion from T to C replaces phenylalanine (F) 110 with serine (S) in the piy mutant. (C, D) piy mutant retinas injected without (C) and with (D) **prim1** mRNA.
Fig. 5. The missense mutation F110S does not affect cell proliferation. (A, B) Expression of prim1 mRNA in wild-type zebrafish embryos at the eight cell stage (A) and 2 dpf (B). The maternal expression of prim1 is observed (A). The prim1 gene is predominantly expressed in regions with high cell proliferation rate, such as the eyes and tectum at 2 dpf (B). (C, D) Plastic sections of wild-type retinas labeled with prim1 RNA probe at 2 dpf (C) and 3 dpf (D). The prim1 gene is expressed in the CMZ of the neural retina, suggesting that this gene is expressed in the proliferating cells of the retina. (E, F) Labeling of wild-type (E) and piy mutant (F) retinas with anti-phosphorylated histone H3 antibody (red, arrowheads). All nuclei are counterstained with Sytox Green (green). (G) Percentage of retinal cells that are dividing in wild-type (blue bar) and piy mutant (red bar) retinas at 24 hpf and 32 hpf. (H, I) BrdU labeling (red) of wild-type (H) and piy mutant (I) retinas. (J) Percentage of retinal area that is BrdU-positive in wild-type (blue bar) and piy mutant (red bar) retinas at 24 hpf and 32 hpf. (K, L) Representative traces from FACS analyses after propidium isodide staining of dissociated cells from wild-type (black line) and piy mutant (green line) embryos at 38 hpf and 48 hpf. (M, N) Bar graph showing summary of FACS results from wild-type sibling and piy homozygous mutant dissociated cell pools at 38 hpf (M) and 48 hpf (N). The piy mutant embryos (light green) contain fewer G1/postmitotic cells and more apoptotic cells than wild type. However, there is no significant difference in the ratio of S/G2/M cells between wild-type and piy mutant embryos. (O) Proliferation rate of yeast pri1-1 mutant cells transformed with wild-type prim1, F110S mutated prim1 and control vector at 37°C. The proliferation rate of the yeast pri1-1 mutant cells transformed with the F110S mutant prim1 (pink) is not significantly different from that of the yeast pri1-1 mutant cells transformed with the wild-type prim1 (blue), whereas the proliferation rate of the yeast pri1-1 mutant cells transformed with the control vector (yellow) is low.
five candidate genes in this flanking genomic region. The sequencing of all of these cDNAs prepared from the piy mutant embryos revealed that a missense mutation occurred in the DNA primase small subunit (prim1) gene. Phenylalanine 110 is highly conserved among eukaryote Prim1 proteins, but is substituted by serine in the piy mutant genome (Fig. 4B). Furthermore, the injection of wild-type prim1 mRNA significantly rescued the piy-mediated phenotypic defect (Fig. 4D). Taken together, these data suggest that this missense mutation in the prim1 gene causes severe apoptosis of retinal neurons in the piy mutant.

The piy mutation does not affect cell proliferation
Prim1 is essential for DNA replication. The prim1 gene was ubiquitously expressed until the tailbud stage (Fig. 5A) and prominently in the brain at 24 hpf (Fig. 6A). At 2 dpf, prim1 expression was restricted to the CMZ of the neural retina and the peripheral rim of the tectum (Fig. 5B-D). This expression profile suggests that the prim1 gene is expressed in proliferating retinal cells. However, retinal stem cells in the CMZ seem normal in the piy mutant at 3 dpf (Fig. 2E,H), suggesting that this missense mutation does not affect cell proliferation. To examine whether cell proliferation is affected in the piy mutant retina, we carried out three sets of experiments.

First, we compared the rate of cell proliferation in piy mutant and wild-type retinas. In zebrafish, retinal neurogenesis is initiated at ~24 hpf; however, we did not observe apoptosis in the piy mutant until at least 32 hpf (Fig. 2A,B). Therefore, we examined the rate of cell proliferation at 24 and 32 hpf. The rate of cell proliferation correlates with the ratio of the number of BrdU-labeled cells or mitotic cells to the total number of cells for a given period. We found that the ratio of mitotic cells to the total number of retinal cells was not significantly different between the wild-type and piy mutant retinas at both 24 and 32 hpf (Fig. 5H-J).

Second, we performed FACS analysis of dissociated cells from wild-type and piy mutant heads to measure DNA content per cell. A typical DNA content distribution is shown for piy mutant and wild-type sibling heads at 38 hpf (Fig. 5K) and 48 hpf (Fig. 5L). At both stages, the percentage of the number of cells with <2N DNA to the total number of dissociated cells was significantly higher in piy mutant than in wild-type sibling heads (Fig. 5M,N), suggesting that the percentage of apoptotic cells increases in the piy mutant. Furthermore, the percentage of cells with 2N DNA was lower in the piy mutant than in the wild-type sibling (Fig. 5M,N). Since a majority of retinal cells become postmitotic in these stages, postmitotic neurons seem to be selectively eliminated by apoptosis in the piy mutant. By contrast, the percentage of cells with >2N DNA was not significantly different between the piy mutant and the wild-type sibling (Fig. 5M,N), suggesting that cell cycle progression proceeds normally in the piy mutant embryos until at least 48 hpf.

Third, we utilized a yeast temperature-sensitive prim1 mutant, pri1-1, in which cell proliferation is normal at 25°C but delayed at 37°C (Francesconi et al., 1991). We transformed the yeast prim1 gene carrying the same missense mutation as that of the piy mutant (F110S) into the yeast pri1-1 mutant, and examined its proliferation profile at 37°C. The rates of cell proliferation showed no significant difference between transformation with the wild-type prim1 gene and with the F110S mutant prim1 gene (Fig. 5O), indicating that the F110S mutant form of Prim1 rescued the pri1-1-mediated proliferation defect. Taken together, these data suggest that this missense mutation does not affect cell proliferation.

Apoptosis in the piy mutant retina depends on ATM, Chk2 and p53
DNA primase is crucial not only for DNA replication but also for the ATR-dependent DNA damage checkpoint, which may activate the ATM-Chk2 pathway. This led us to examine the possibility that Chk2 and its downstream target p53 are aberrantly activated in piy mutant retinas. First, we examined the expression of the zebrafish...
chk2 (GenBank accession AF265346; zgc:55865 – ZFIN) and p53 (Tp53 – ZFIN) genes during development. We found that the expression pattern of these genes is similar to that of the prim1 gene (Fig. 6), suggesting that the Chk2-p53 apoptotic pathway correlates with DNA replication in zebrafish. Next, we compared the expression of the p53 gene in the piy mutant with that in wild type, and found that the expression level of p53 mRNA is at least fourfold higher in the piy mutant than in wild-type embryos (Fig. 7A). This suggests that the p53-dependent apoptotic pathway is activated in the piy mutant retina.

To elucidate whether neuronal apoptosis in the piy mutant retina depends on DNA damage checkpoints and p53, we injected morpholinos MO-ATR, MO-ATM (ATM, GenBank accession AB191208), MO-Chk1 (Chk1, zgc:56093 – ZFIN), MO-Chk2 and MO-p53 into the piy mutant embryos. Neuronal apoptosis was effectively suppressed in the piy mutant embryos injected with MO-Chk2 (82.4%, Table 1; Fig. 7C) or MO-p53 (100%, Table 1; Fig. 7E) at 2 dpf. By contrast, introduction of MO-Chk1 did not suppress piy-mediated apoptosis (0%, Table 1). We also examined the retinal phenotype of piy mutant embryos injected with MO-ATR and MO-ATM, but these embryos showed severe developmental arrest before retinal neurogenesis occurred (data not shown). KU55933 and CGK733 are inhibitors of ATM and ATR/ATM, respectively (Hickson et al., 2004; Won et al., 2006). We used these inhibitors to determine whether ATM is involved in piy-mediated apoptosis, and found that the treatment with KU55933 or CGK733 significantly suppressed piy-mediated apoptosis (Fig. 7F–H; Table 1). Although it is still unclear whether...
ATR activation is indispensable for retinal apoptosis in the piy mutant, these data suggest that neuronal apoptosis in the piy mutant retina depends on the ATM-Chk2-p53 signaling pathway.

Next, we examined whether piy mutant retinal cells differentiate normally into mature retinal neurons and form functional neural circuits when Chk2 or p53 is inhibited. We examined neuronal differentiation using antibodies, zn5 and zpr1, which label RGCs (Fig. 7I) and double-cone photoreceptors (Fig. 7L), respectively. Both types of retinal neurons differentiated normally in the piy mutant retinas injected with MO-p53 (Fig. 7K,N). Furthermore, we found that some of embryos injected with MO-p53 or MO-Chk2 displayed a normal visual response, OKR, at 6 dpf (Table 2). The expression of a neurotransmitter, GABA, was also recovered in these OKR-positive piy mutant embryos (Fig. 7O-Q). Although the percentage of OKR-rescued embryos (Table 2) was lower than that of apoptosis-rescued embryos (Table 1), probably owing to the reduction in the effect of morpholino antisense oligos with stage progression, we conclude that functional neural circuits are formed normally in the piy mutant embryos injected with MO-p53 and MO-Chk2. When the Chk2-p53-mediated apoptotic pathway is blocked, the piy mutant retinal cells can differentiate normally to form functional neural circuits responsible for visual transduction.

**DISCUSSION**

**Mutations in the DNA replication system induce p53-dependent retinal apoptosis**

In this study, we identified a zebrafish piy mutant that shows apoptosis of retinal neurons. Our cloning of the mutant gene revealed that a missense mutation occurred in the prim1 gene in the piy mutant. We also found that neuronal apoptosis in the piy mutant retina depends on the ATM-Chk2-p53 apoptotic pathway. This finding suggests that a dysfunction of DNA primase induces ATM-, Chk2- and p53-dependent apoptosis in the zebrafish retina. Recently, it has been reported that extensive apoptosis occurs in the retina of the zebrafish minichromosome maintenance deficient 5 (mcm5) mutant (Ryu et al., 2005) and the zebrafish DNA polymerase delta 1 (pold1) mutant, flathead (fla) (Plaster et al., 2006). Mcm5 and DNA polymerase δ regulate the unwinding of the DNA replication fork and DNA synthesis of the replication fork, respectively. The fla mutant displays defects in cartilaginous elements of the head skeleton as well as in the eye and tectum. We observed a similar defect in cartilage formation in the piy mutant embryos (see Fig. S2 in the supplementary material). Taken together, these observations suggest that a common signaling pathway causes similar defects in these mutants, and that the defects in the DNA replication system induce p53-dependent apoptosis in the zebrafish retina.

How do the defects in the DNA replication system induce p53-dependent apoptosis in the zebrafish retina? Missense mutation occurred in phenylalanine (F) 110 of Prim1 in the piy mutant. This residue is located in the most highly conserved motif, 107-ELVFID-113, in which three negatively charged residues (E107, D111 and D113) are essential for primase activity (Augustin et al., 2001). It has been reported that cell cycle progression is delayed or arrested in the mcm5 and fla mutants (Plaster et al., 2006; Ryu et al., 2005). Thus, it is possible that defective DNA synthesis in the replication fork induces retinal apoptosis. However, we found that the missense mutation of the prim1 gene does not compromise cell proliferation. Although we cannot exclude the possibility that the maternal Prim1 partly rescues some defects in DNA replication of the piy mutant, it seems unlikely that this amino acid substitution affects the efficiency of DNA replication. One possibility is that the F110S-mutant Prim1 activates the DNA damage response without any defects in DNA replication. In this case, the mechanism that activates p53-dependent apoptosis in the piy mutant might be different from that in the mcm5 and fla mutants.

It has been reported that functional uncoupling between MCM helicases and DNA polymerase activities activates the ATR-dependent checkpoint (Byun et al., 2005). It is possible that missense mutation occurring in the piy mutant compromises the balance between DNA unwinding by Mcm5, DNA synthesis against the lagging strand by the DNA polymerase α-Prim1 complex, and DNA synthesis against the leading strand by DNA polymerase δ. Although it seems unlikely that

**Table 1. Efficiency of rescue of piy-mediated apoptosis by inhibition of DNA damage checkpoint and p53**

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<th>Genotype</th>
<th>Total number examined</th>
<th>Number of rescued embryos</th>
<th>Percentage</th>
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<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; MO-Chk1</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; Smis-MO-Chk1</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; MO-Chk2</td>
<td>17</td>
<td>14</td>
<td>82.4</td>
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<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; Smis-MO-Chk2</td>
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<td>0</td>
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<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; KU55933</td>
<td>7</td>
<td>5</td>
<td>71.4</td>
</tr>
<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; CGK733</td>
<td>5</td>
<td>4</td>
<td>80</td>
</tr>
</tbody>
</table>

**Table 2. Injection of MO-53 and MO-Chk2 rescues visual response OKR in the piy mutant**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total number examined</th>
<th>Number of OKR&lt;sup&gt;+&lt;/sup&gt; embryos</th>
<th>Percentage</th>
</tr>
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<tr>
<td>Wild-type sib</td>
<td>15</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;</td>
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<td>0</td>
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<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; MO-p53</td>
<td>6</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>piy&lt;sup&gt;–/–&lt;/sup&gt;; MO-Chk2</td>
<td>12</td>
<td>6</td>
<td>50</td>
</tr>
</tbody>
</table>

**Fig. 8. Model of ATM-Chk2-p53 pathway in zebrafish retinal neurogenesis.** In the zebrafish retina, mitotic progenitor cells start to generate neurons at 24 hpf. After 24 hpf, the cell cycle duration of retinal progenitor cells shortens and the cell proliferation rate increases. The increase in cell proliferation rate may increase the number of DNA replication errors during retinal neurogenesis. One possible model is that the ATM-Chk2-p53 pathway repairs the replication errors or induces apoptosis to remove retinal neurons with unrepaired DNA damage. This pathway might prevent the accumulation of abnormally differentiated neurons, which possibly compromise the subsequent formation of neural circuits, and might also remove cancer-predisposing cells.
the piy mutation affects the efficiency of DNA replication, a slight imbalance between MCM, DNA polymerase α, and DNA polymerase δ, might be sufficient to activate the DNA damage response. If this is the case, retinal apoptosis in piy, fla and mcm5 mutants is triggered by a common molecular mechanism. It will be interesting and important to examine whether retinal apoptosis in fla and mcm5 mutants depends on ATM or Chk2.

**Role of the Chk2-p53 pathway in retinal development**

In this study, we showed that activation of the ATM-Chk2-p53 pathway induces extensive apoptosis of retinal neurons in zebrafish. What is the significance of the ATM-Chk2-p53 pathway in zebrafish retinal development? The first possibility is that the ATM-Chk2-p53 pathway monitors the integrity of a genome and protects it from DNA damage during retinal neurogenesis. In the zebrafish developing retina, the cell cycle duration is initially long, about 30 hours; however, it abruptly decreases to about 10 hours after the initiation of retinal neurogenesis, leading to a higher proliferation rate of retinal progenitor cells (Li et al., 2000). Although the mechanism underlying this increase in the proliferation rate of progenitor cells coupled with retinal neurogenesis is as yet unclear, it might be important to prevent the depletion of retinal progenitor cells during neurogenesis. However, it is possible that the increase in cell proliferation rate increases the frequency of DNA replication errors, which might be detrimental to retinal neurogenesis. One possible model is that the DNA damage checkpoint pathway might repair such replication errors or induce apoptosis to remove unrepaired differentiating neurons (Fig. 8). Because the accumulation of DNA replication errors potentially affects neuronal differentiation, the DNA damage checkpoint pathway might function as one of the systems that ensure retinal neurogenesis.

The second possibility is that the Chk2-p53 pathway removes genomic lesions, which potentially cause retinal tumors. It was reported that 28% of zebrafish p53 homozygous mutant fish developed tumors by 16.5 months, and that more than 50% of these tumors occurred in the eyes (Berghmans et al., 2005). Because it is possible that such retinal tumors are derived from retinal cells in which DNA damage fails to be repaired, the ATM-Chk2-p53 pathway might suppress tumor formation in the late stages of retinal development. In the early stage of tumorigenesis, human cells activate the ATM/ATR-dependent DNA damage response pathway, which may delay or prevent cancer development (Bartkova et al., 2005; Gorgoulis et al., 2005). In contrast to these reports, it has been reported that the DNA damage checkpoint pathway does not contribute to p53-mediated tumor suppression (Eleyan et al., 2006; Christophorou et al., 2006). The piy mutant will provide a useful model for studies on whether p53 activation by the DNA damage response pathway suppresses tumor formation in zebrafish.

**Retinal stem cells survive in the p53 mutant**

In humans, syndromes associated with a defective DNA damage response include neurological symptoms as a primary feature of their phenotypes (Rolig and McKinnon, 2000; Shiloh, 2003; O’Driscoll and Jeggo, 2006). Apoptosis occurs in differentiating neurons but not in stem cells in mice knocked out for DNA damage repair signaling enzymes (Barnes et al., 1998; Frank et al., 1998; Gao et al., 1998; Deans et al., 2000; Gu et al., 2000; Sugo et al., 2000). However, it remains unclear why the disruption of a process fundamental to proliferating cells as the DNA damage response network is selectively lethal to postmitotic neurons. In the p53 mutant retina, differentiating neurons undergo apoptosis; however, retinal stem cells seem to proliferate normally. It seems likely that retinal stem cells are protected from p53-mediated apoptosis. How do retinal stem cells survive in the p53 mutant retina? The most likely possibility is that p53 activity is normally suppressed in retinal stem cells in zebrafish. The protein levels and transcriptional activity of p53 are tightly regulated in response to DNA damage caused by post-translational modifications (Brooks and Gu, 2006; Ronai, 2006; Toledo and Wahl, 2006). Under unstressed conditions, p53 is degraded by several E3 ubiquitin ligases, such as mouse double minute 2 (Mdm2), Mdm4, Cop1 (Rifw2 – Mouse Genome Informatics) and Pirh2 (Rchy1). It has been reported that Mdm2 antagonizes p53 in zebrafish (Langheinrich et al., 2002). The p53 pathway is inactivated by the increased expression level of MdmX (Mdm4) in Retinoblastoma 1-deficient retinal cells (Lauret et al., 2006). It is important to examine whether retinal stem cells have high activities of p53 inhibitors, such as Mdm2/4, that prevent p53-dependent apoptosis in zebrafish. Future research on the p53 mutant phenotypes will reveal the mechanism underlying the protection against apoptosis in retinal stem cells and hopefully increase our understanding of neurological symptoms associated with DNA damage response defects in humans.


