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

Masahiro Yamaguchi[1,2], Noriko Fujimori-Tonou[2], Yukihiro Yoshimura[1], Tsutomu Kishi[3], Hitoshi Okamoto[4] and Ichiro Masai[1,2,*]

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-Volhand, 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, amphibia, 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; Biehlmaier 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 phosphatidyl inositol 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.,

[1]Developmental Neurobiology Unit, Initial Research Project, Okinawa Institute of Science and Technology Promotion Corporation, 12-2 Suzaki, Uruma-shi, Okinawa 904-2234, Japan. [2]Initiative Research Program, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. [3]Kishi Initiative Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. [4]Laboratory for Developmental Gene Regulation, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.

*Author for correspondence (e-mail: masai@oist.jp)

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2006; Myers and Cortez, 2006). Replication fork stalling or UV treatment induces the ATR-dependent phosphorylation of ATM, which subsequently activates Chk2 (Stiff et al., 2006).

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*^{rw255} and *slow muscle omitted* (*smu*^{b577}) (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)^{rw021} (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 nucleic 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′-TTTCAGTCATAGCCTGGAAGGTGTA-3′ and reverse primer, 5′-TAAGACTGTCTCAATGACATGATTG-3′; Marker-piy (M) forward primer, 5′-AAGCTCAGTTTCTGTCTTCTATACT-3′ and reverse primer, 5′-TAGGCATGTGCAAAAAATTATTGTT-3′; (2) One single nucleotide polymorphic (SNP) marker: Marker-piy (R), 5′-CAGTAGTAGTTGG-ACTTCCTCTCATCCTTCCCTGCAATCCGCCTGAGGYCCTCCTAA-ACCAGACACTTACTGGCTGAACAGTAGTAAGTATAAGAGATT-3′ where Y=C (WIK) or T (Riken wild type).

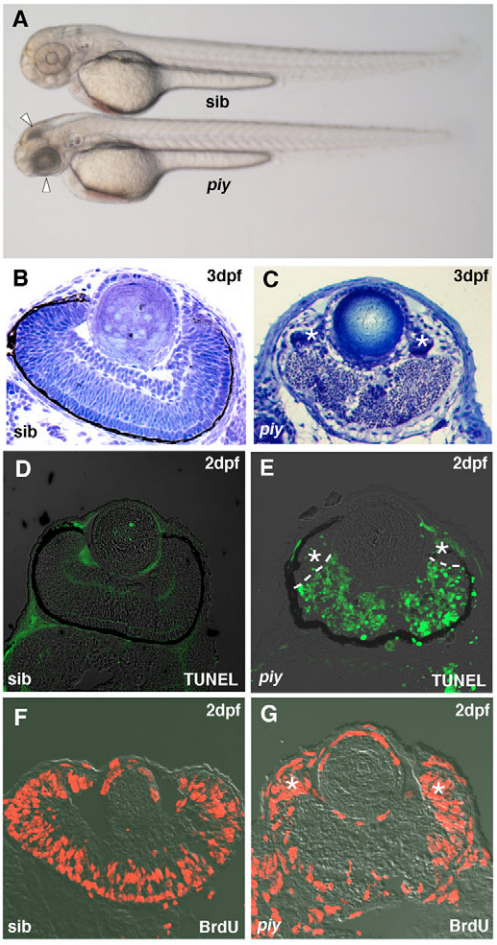

Fig. 1. Retinal neurons undergo extensive apoptosis in the *piy* mutant. (**A**) Morphology of wild-type and *piy* mutant zebrafish embryos at 2 dpf. Both embryos were treated with phenyltiourea to prevent pigmentation. Extensive cell death is observed in the retina and tectum of the *piy* mutant (arrowheads). (**B**,**C**) Plastic sections of wild-type (B) and *piy* mutant (C) retinas. In the *piy* mutant retinas, extensive cell death occurs in the central retina, where differentiated neurons are normally located. Retinal stem cells are retained in the CMZ of the *piy* mutant (asterisks). (**D**,**E**) TUNEL of wild-type (D) and *piy* mutant (E) retinas. In the *piy* mutant, most cells in the central retina are TUNEL-positive (green), whereas there are only a few TUNEL-positive cells in the CMZ (asterisks). (**F**,**G**) BrdU labeling of wild-type (F) and *piy* mutant (G) retinas. In the *piy* mutant, retinal stem cells in the CMZ incorporate BrdU (asterisks).

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).

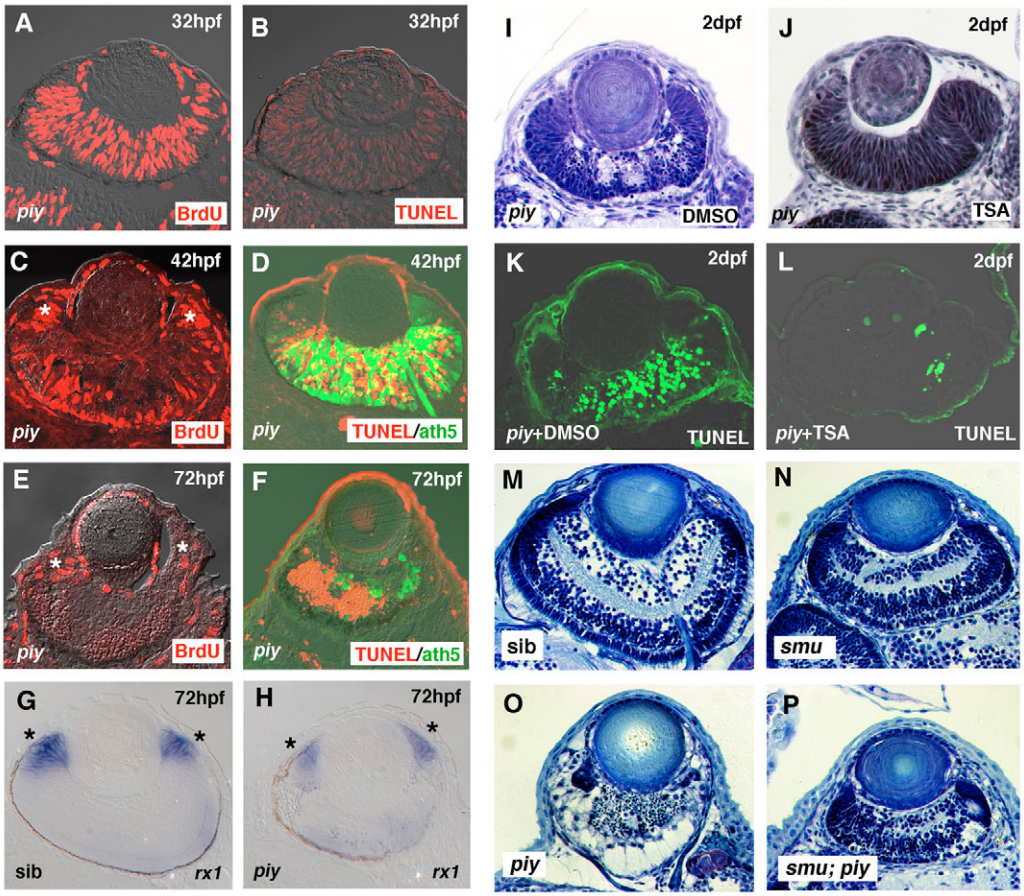

Fig. 2. Apoptosis occurs in differentiating neurons in the *piy* mutant retina. (**A-F**) BrdU labeling (A,C,E) and TUNEL (B,D,F) of *piy* mutant zebrafish retinas. At 32 hpf, many retinal cells incorporate BrdU (A, red) and there are no TUNEL-positive cells (B, red). At 42 hpf, many neurons are produced in the central retina and these express the neuronal marker *ath5* (D, green). Apoptotic cells are observed in the *ath5*-positive central retina, but are rare in the CMZ (D, red). At 72 hpf, dense dead cells are observed in the central retina (F, red) and ath5-positive neurons markedly decrease in number (F, green), suggesting that differentiating neurons undergo apoptosis in the *piy* mutant. Cells in the CMZ incorporate BrdU (C,E, asterisks), suggesting that retinal stem cells continue to proliferate in the *piy* mutant. (**G**,**H**) In situ hybridization of wild-type (G) and *piy* mutant (H) retinas with *rx1* RNA probe (asterisks). (**I**,**J**) Plastic sections of *piy* mutant retinas treated with DMSO (I) or TSA (J). (**K**,**L**) TUNEL (green) of *piy* mutant retinas treated with DMSO (K) or TSA (L). (**M-P**) Plastic sections of wild-type (M), *smu* (N), *piy* (O) and *smu; piy* (P) mutant retinas. Although retinal neurogenesis is delayed in the *smu* mutant, retinal lamination occurs until 3 dpf (N). In the *smu; piy* double mutant, retinal cell apoptosis is suppressed (P).

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 *piy* 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 *piy* 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 *Not*I and used for the synthesis of capped mRNA with SP6 RNA polymerase (Ambion). *piy* homozygous embryos were generated by crossing *piy* heterozygous parent fish carrying WIK-derived wild-type allelic chromosome 23 and identified by genotyping with the polymorphic microsatellite marker, marker-piy (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′-GCGCCATTGCTTTGCAAGAATTG-3′ (Langheinrich et al., 2002); MO-Chk1 (0.25 mM), 5′-TTAACAAAAGGCACAGCCATTATGC-3′; MO-Chk2 (0.25 mM), 5′-CAGACATGATGCTTTTATTCTGGAC-3′; MO-ATM (0.1 mM), 5′-GAAAACGGCACCACCTGGTAAAAAC-3′; and MO-ATR (0.25 mM), 5′-TGACATTTCTAGTCCTTGCTCCATC-3′ (Stern et al., 2005). To confirm the genotype of morphants, *piy* homozygous embryos were identified using the polymorphic marker, marker-piy (M). Oligos with five mismatches (indicated in lowercase) for MO-Chk1 (5mis-MO-Chk1, 5′-TTAAgAAAAcGCAgAGCgATTAaGC-3′) and MO-Chk2 (5mis-MO-Chk2, 5′-CAcACATcATcCTTTTATaCTGcAC-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 *ef1α* 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′-GCGATGAGGAGAT-CTTTACCC-3′ and reverse primer, 5′-ACAAAGGTCCCAGTGGAGTG-3′; *ef1α* forward primer, 5′-TGGGCACTCTACTTAAGGAC-3′ and reverse primer, 5′-TGTGGCAACAGGTGCAGTTC-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 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*, in which retinal lamination 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).

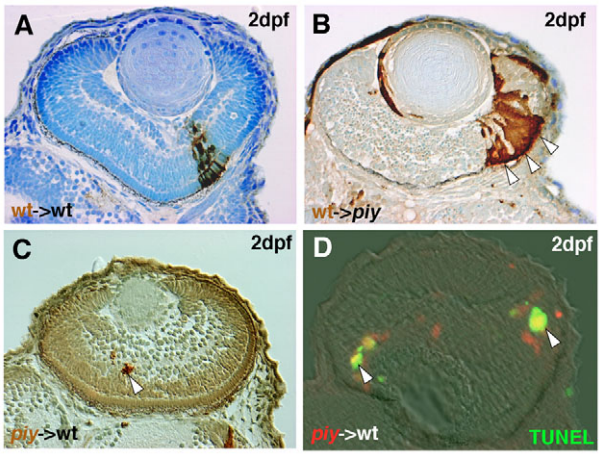

Fig. 3. *piy* mutation behaves in a cell-autonomous manner. (**A**) Transplantation of wild-type zebrafish cells into wild-type recipient retinas. Wild-type donor cells (brown) form retinal columns. (**B**) Transplantation of wild-type cells into *piy* mutant recipient retinas. Although most of the recipient cells undergo apoptosis, wild-type donor cells (brown) survive and form retinal columns (arrowheads). (**C**) Transplantation of *piy* mutant cells into wild-type recipient retinas. Transplanted mutant cells fail to form retinal columns and instead remain as a very small cell mass (arrowhead). (**D**) Transplantation of *piy* mutant cells into wild-type recipient retinas. *piy* donor mutant cells (red) are TUNEL-positive (green, arrowheads) in the wild-type recipient retina.

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)^{rw021}, 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.

In zebrafish, retinal neurogenesis is markedly inhibited in the absence of Hdac1 activity (Yamaguchi et al., 2005) or delayed in the Hh pathway mutant *smu* (also known as *smo* – ZFIN) (Masai et al., 2005). To elucidate whether *piy*-mediated apoptosis correlates with neurogenesis, we examined the retinal phenotype of the *piy* mutant treated with TSA, which is a potent inhibitor of Hdac. Compared with the untreated *piy* mutant, neuronal apoptosis was significantly suppressed in the retina of the *piy* mutant treated with TSA (Fig. 2I-L). Next, we examined 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).

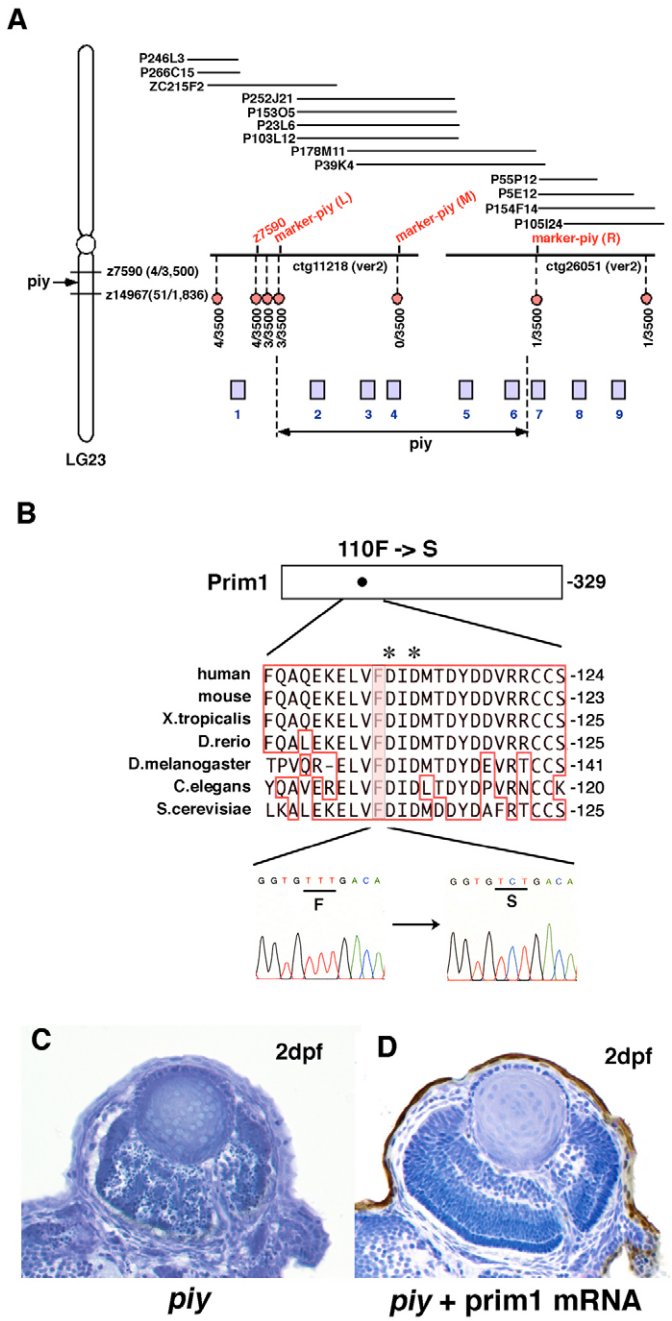

Fig. 4. Missense mutation occurs in Prim1 in the *piy* mutant. (**A**) Schematic of zebrafish LG23. The *piy* mutation maps to the genomic region between the SSLP markers z7590 (recombination 4/3500) and z14967 (recombination 51/1836). z7590 maps to the genomic fragment ctg11218, which was shown in the zebrafish genomic database (version 2) released by the Wellcome Trust Sanger Institute. ctg11218 is connected to another genomic fragment, ctg26051, through six overlapping PAC clones. According to the most current version of the zebrafish genomic database, the following nine genes are annotated within this genomic region: 1, *zgc:111964* (bHLH transcription factor neuroB); 2, *rnd1* (Rho family GTPase 1); 3, *prim1* (DNA primase small subunit); 4, *zgc:86644* (Tuftelin interacting protein 11); 5, *naca* (Nascent polypeptide-associated complex alpha); 6, 559664 (EntrezGene) (intracellular membrane-associated calcium-independent phospholipase A2 gamma); 7, 55959 (EntrezGene) (Neurofascin); 8, *zgc:65788* (Chitinase); and 9, *zgc:123113* (Peptidase). Red circles indicate polymorphic markers that we examined; their recombination rates are shown beneath the red circles. We identified three polymorphic markers, marker-piy (L) (3/3500), marker-piy (M) (0/3500) and marker-piy (R) (1/3500), two of which flanked the *piy* mutation. 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.

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

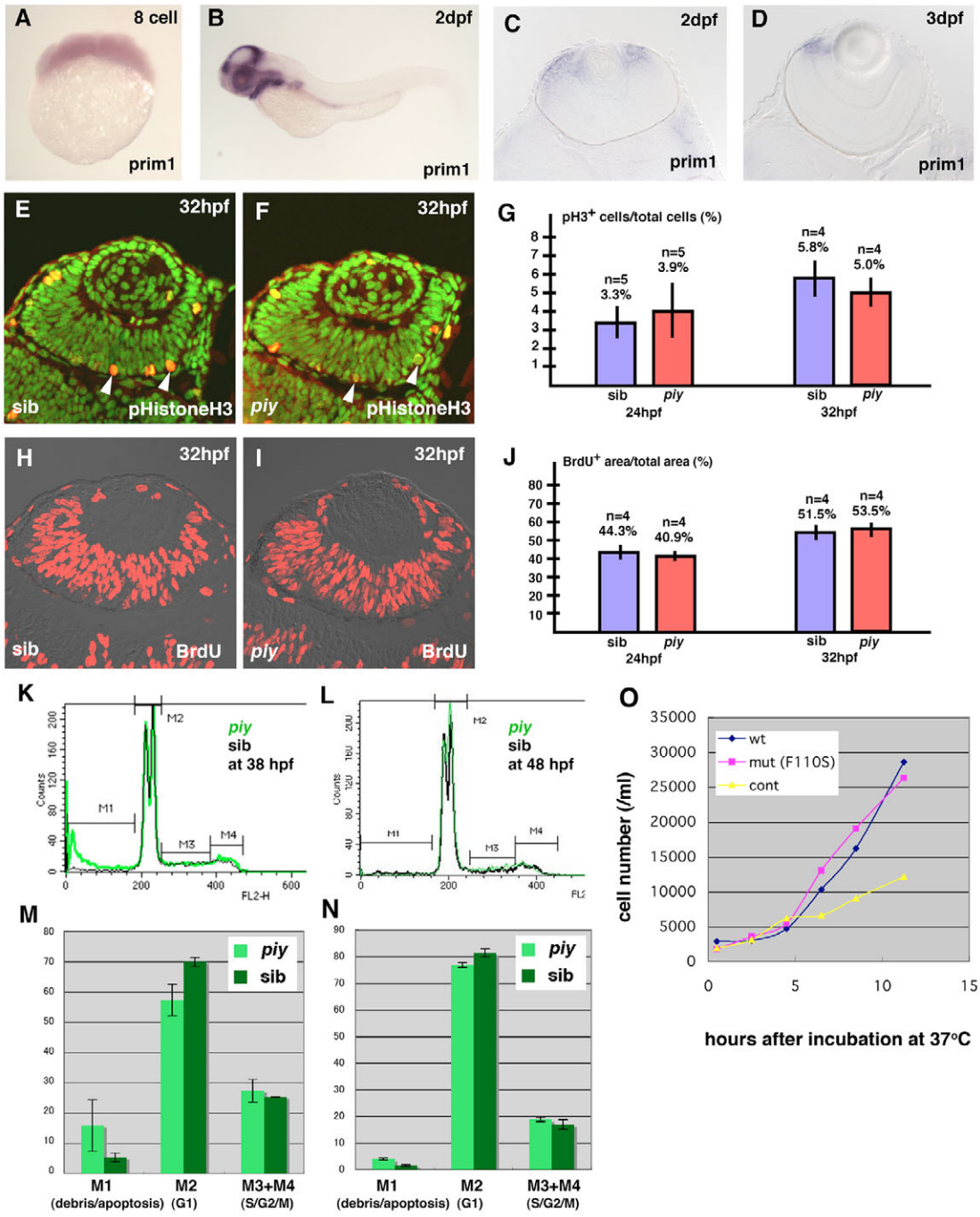

Fig. 5. The missense mutation F110S does not affect cell proliferation. (**A**,**B**) Expression of *prim1* mRNA in wild-type zebrafish embryos at the eight cell stage (A) and 2 dpf (B). The maternal expression of *prim1* is observed (A). The *prim1* gene is predominantly expressed in regions with high 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 propiduim isodide staining of dissociated cells from wild-type (black line) and *piy* mutant (green line) heads at 38 hpf (K) and 48 hpf (L). The traces are divided into four regions, M1, M2, M3 and M4, which represent cells with <2N, 2N, 2N-4N and 4N DNA, respectively. (**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.

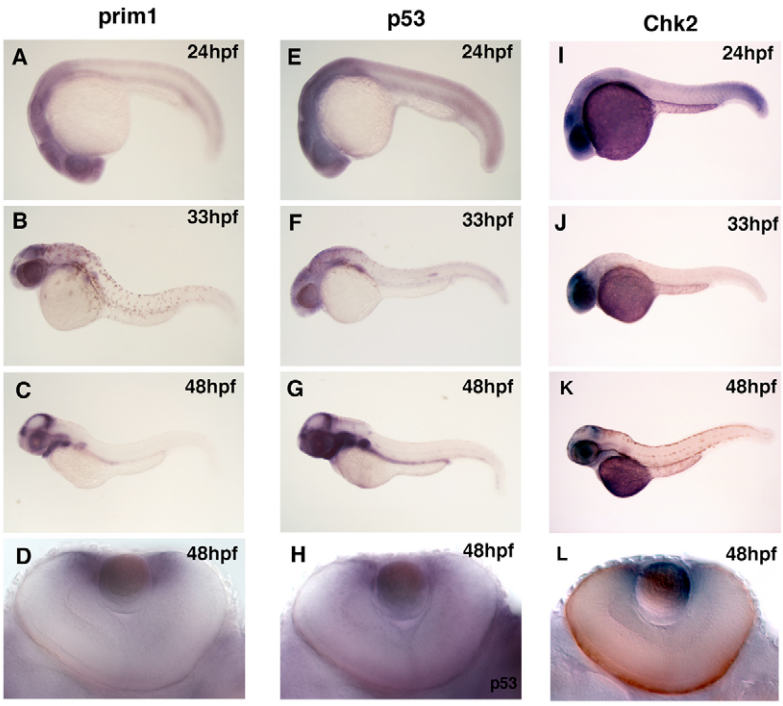

Fig. 6. Expression of zebrafish *prim1*, *p53* and *chk2* genes. (**A-D**) Expression of *prim1*. Lateral views of embryos (A-C) and sectioned retina (D). The expression of the *prim1* gene is observed in the entire head at 24 hpf (A) and is prominent in the eyes and tectum at 33 hpf (B). At 48 hpf, *piy* expression is observed mainly in the proliferative zones of the eye and tectum (C-D). (**E-H**) The expression pattern of *p53* is similar to that of *prim1*. (**I-L**) The expression pattern of the zebrafish *chk2* gene is similar to that of *prim1*.

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. 5E-G). Furthermore, the ratio of BrdU incorporation 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

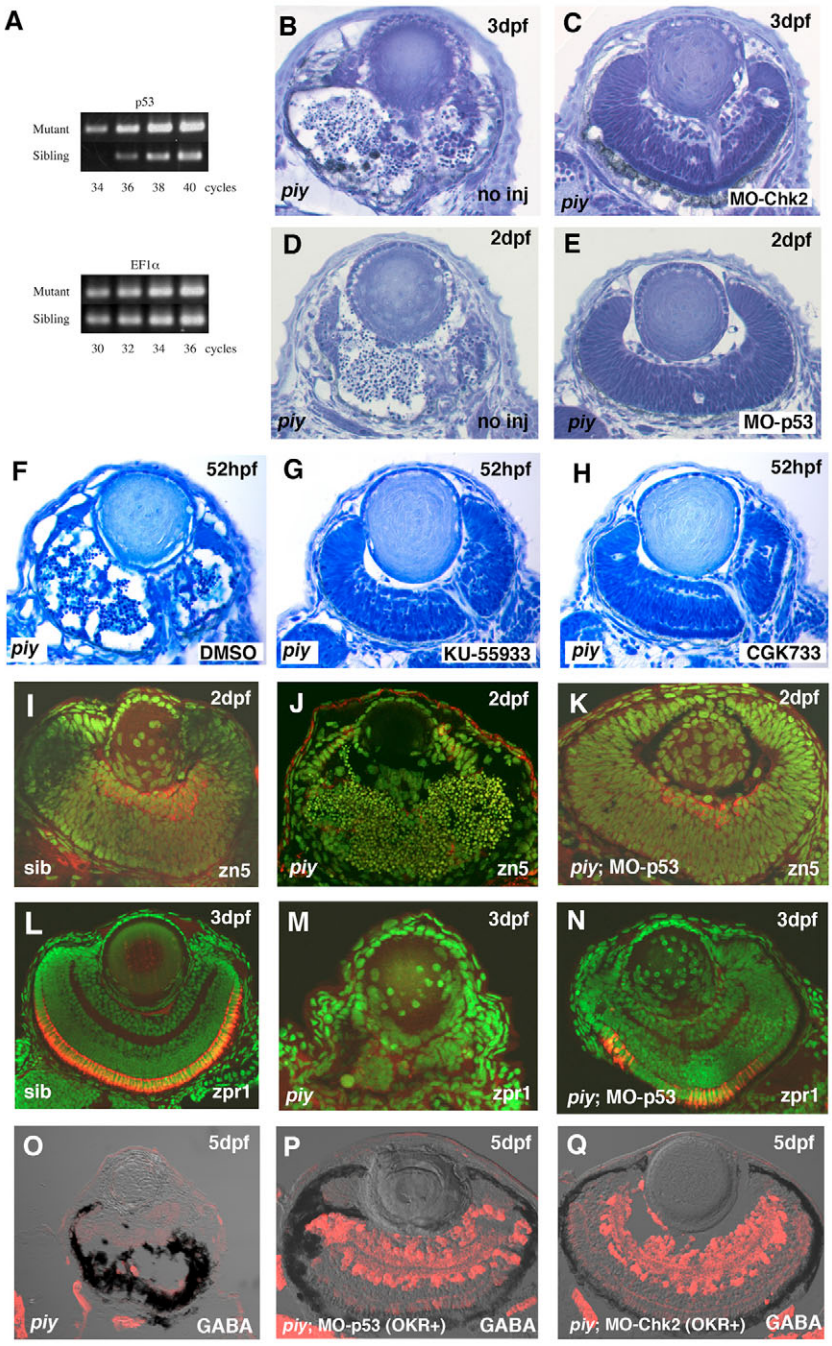

Fig. 7. *piy*-mediated apoptosis depends on the ATM-Chk2-p53 pathway. (**A**) Quantitative PCR analysis of *p53* mRNA in wild-type and *piy* mutant zebrafish embryos. There is no difference in the amplification of a control gene, *ef1α* (lower panel), from wild-type and the *piy* mutant cDNA pools. By contrast, the *p53* gene is amplified from the *piy* mutant cDNA pool by a smaller number of PCR cycles than from the wild-type cDNA pool (upper panel), suggesting that *p53* mRNA is more abundant in the *piy* mutant embryos than in wild-type embryos. (**B**,**C**) Plastic sections of *piy* mutant retinas injected without (B) and with (C) MO-Chk2. (**D**,**E**) Plastic sections of *piy* mutant retinas injected without (D) and with (E) MO-p53. (**F-H**) Treatment of the *piy* mutant embryos with DMSO (F), KU55933 (G) or CGK733 (H). (**I-K**) Labeling of wild-type sibling (I), *piy* mutant (J) and MO-p53-injected *piy* mutant (K) retinas with the zn5 antibody, which labels RGCs (red). All nuclei are counterstained with Sytox Green (green). (**L-N**) Labeling of wild-type sibling (L), *piy* mutant (M) and MO-p53 injected *piy* mutant (N) retinas with the zpr1 antibody, which labels double-cone type photoreceptors (red). (**O-Q**) Labeling of retinas of *piy* mutant (O), OKR-restored MO-p53-injected *piy* mutant (P) and OKR-restored MO-Chk2-injected *piy* mutant (Q) with anti-GABA antibody (red). GABA expression is normal in RGCs and amacrine cells in both MO-injected retinas.

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.

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

Genotype	Total number examined	Number of rescued embryos	Percentage
Wild-type sib	20	20	100
piy$^{-/-}$	22	0	0
piy$^{-/-}$; MO-p53	14	14	100
piy$^{-/-}$; MO-Chk1	6	0	0
piy$^{-/-}$; 5mis-MO-Chk1	5	0	0
piy$^{-/-}$; MO-Chk2	17	14	82.4
piy$^{-/-}$; 5mis-MO-Chk2	4	0	0
piy$^{-/-}$; KU55933	7	5	71.4
piy$^{-/-}$; CGK733	5	4	80

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

Genotype	Total number examined	Number of OKR^{+} embryos	Percentage
Wild-type sib	15	15	100
piy$^{-/-}$	15	0	0
piy$^{-/-}$; MO-p53	6	2	33
piy$^{-/-}$; MO-Chk2	12	6	50

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-ELVFDID-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.

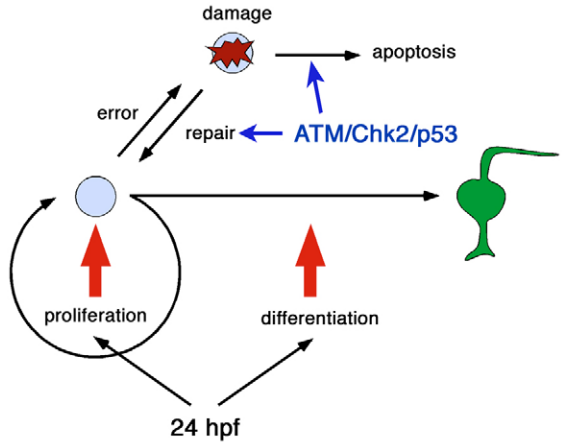

Fig. 8. Model of ATM-Chk2-p53 pathway in zebrafish retinal neurogenesis. In the zebrafish retina, mitotic progenitor cells start to generate neurons at 24 hpf. After 24 hpf, the cell cycle duration of retinal progenitor cells shortens and the cell proliferation rate increases. The increase in cell proliferation 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.

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

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, at 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 does not contribute to p53-mediated tumor suppression (Efeyan 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 *piy* 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 *piy* mutant retina, differentiating neurons undergo apoptosis; however, retinal stem cells seem to proliferate normally. It seems likely that retinal stem cells are protected from *piy*-mediated apoptosis. How do retinal stem cells survive in the *piy* 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 (Rfwd2 – 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 (Laurie 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 *piy* 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.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/7/1247/DC1

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