p53 deficiency rescues apoptosis and differentiation of multiple cell types in zebrafish *flathead* mutants deficient for zygotic DNA polymerase δ 1

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Abstract

Cell culture work has identified the tumor suppressor p53 as a component of the S-phase checkpoint control system, while *in vivo* studies of this role of p53 in whole-vertebrate systems were limited. Here, we describe zebrafish mutants in the DNA polymerase delta catalytic subunit 1, based on the positional cloning of the *flathead* (*fla*) gene. *fla* mutants display specific defects in late proliferative zones, such as eyes, brain and cartilaginous elements of the visceral head skeleton, where cells display compromised DNA replication, followed by apoptosis, and partial or complete loss of affected tissues. Antisense-mediated knockdown of p53 in *fla* mutants leads to a striking rescue of all phenotypic traits, including completion of replication, survival of cells, and normal differentiation and tissue formation. This indicates that under replication-compromised conditions, the p53 branch of the S-phase checkpoint is responsible for eliminating stalled cells that, given more time, would have otherwise finished their normal developmental program.

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Abbreviations: AP, anterior–posterior; ATR, Ataxia telangiectasia mutated (ATM) and Rad3 related; BrdU, 5' Bromo-2'-desoxyuridine; Chk1, checkpoint kinase 1; DAPI, 4',6-Diamidino-2-phenylindole; dpf, days postfertilization; ef1 α , elongation factor 1 alpha; FACS, fluorescence-activated cell sorting; *fla*, flathead mutant; hpf, hours postfertilization; MO, morpholino oligonucleotide; pcna, proliferating-cell nuclear antigen; pol δ 1, DNA polymerase delta1; RT-PCR, polymerase chain reaction after reverse transcription of RNA; SSCP, simple sequence length polymorphism; TGF β , transforming growth factor beta; TUNEL,

terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling; 4mm, four mismatch control

Introduction

During S phase of every cell cycle, the entire genome must be replicated in an error-free manner. This not only requires DNA-replicating proteins with very high fidelity but also the ability of the cell to check for damage caused by endogenous and environmental forces. To date, 19 different DNA polymerases have been identified. Among the 'classical' polymerases (α , β , γ , δ , ϵ), Pol α , Pol δ , and Pol ϵ are believed to be responsible for the majority of nuclear DNA synthesis, each with specific and unique roles, while Pol γ largely acts during mitochondrial replication, and Pol β during DNA repair (reviewed in Hindges and Hübscher,¹ and Hübscher *et al.*²). The Pol δ holoenzyme consists of several subunits, the catalytic subunit (Pol δ 1; p125), which is highly conserved among eukaryotes (50–60% between mammals and yeast), and two to four more divergent smaller subunits with structural and regulatory functions.² Pol δ plays a role in both leading and lagging strand synthesis after taking over for Pol α in replication initiation, as demonstrated in the *in vitro* SV40 replication system.³ Disruptions of Pol δ 1 in various organisms support its proposed role in DNA replication. In fission yeast, temperature-sensitive mutations of Pol δ lead to cell cycle arrest in S phase.⁴ Furthermore, studies with *Xenopus* egg extracts have shown that disruption of Pol δ via immunodepletion leads to an accumulation of single-stranded DNA gaps, highlighting Pol δ 's role in lagging strand synthesis, and the inability of Pol ϵ to functionally replace Pol δ .⁵ In addition, Pol δ 1 displays 3'–5' exonuclease activity and can function in DNA repair.^{1,2} The importance of this 3'–5' exonuclease activity has been highlighted by genetic analyses in mouse, showing that a point mutation specifically disrupting the 3'–5' exonuclease proofreading activity of Pol δ 1 causes cancer susceptibility.^{6,7} The phenotype of mouse mutants lacking entire Pol δ 1 function, however, has not been reported.

In the event of dysfunctions at the replication fork during S phase, a replication checkpoint is activated. The function of this checkpoint is four-fold. First, it blocks additional origin firing in the event of DNA damage. Second, elongation is slowed to allow for the assembly and function of repair machinery. Third, the structure of the stalled replication forks is maintained to prohibit additional damage to the DNA, and finally, the affected cells are prohibited from undergoing mitosis (for reviews see Nyberg *et al.*⁸ and Bartek *et al.*⁹). If replication forks are not repaired and/or stalled for too long, cells either proceed to G2/M arrest^{10,11} or undergo apoptosis by the instructions of the tumor suppressor and transcription factor p53 (reviewed in Haupt *et al.*¹²). Phosphorylation, stabilization, and activation of p53 are accomplished at least

in part by members of the replication checkpoint, namely the PI3K-like protein kinase ATR (Ataxia telangiectasia mutated (ATM) and Rad3 related)^{13,14} and its substrate checkpoint kinase 1 (Chk1),¹⁵ which have been shown to play a major role during the elimination of cultured cells in response to DNA damage and replication blocks.^{16–20} Studies with *Xenopus* egg extracts have further demonstrated that immunodepletion of Pol δ leads to an increase in the phosphorylation of Chk1.⁵ Together, this suggests that disruption of Pol δ might lead to the activation of the replication checkpoint and downstream effectors like p53 via the function of ATR. However, *in vivo* studies of the interplay of Pol δ 1 and p53 during replication, cell cycle progression, and apoptosis in a whole-vertebrate system had been missing thus far.

Here, we describe combined Pol δ 1 and p53 loss-of-function studies in zebrafish larvae. Large-scale genetic screens led to the isolation of hundreds of zebrafish mutants, providing an excellent resource for traditional forward genetic approaches in studying gene function.^{21,22} Via positional cloning, we identified the *flathead* (*fla*) gene, initially defined according to the craniofacial defects of its mutants,²³ as zebrafish Pol δ 1. Most likely due to early compensation by maternally supplied *pol δ 1* gene products, zygotic zebrafish *pol δ 1* null mutants display rather late phenotypes, characterized by compromised DNA replication and subsequent apoptosis of cells in late proliferative zones, such as the chondrocyte precursors forming the branchial arches. Furthermore, applying morpholino antisense technology, we studied the role of p53 under *pol δ 1*-deficient conditions, demonstrating that loss of p53 function leads to a rescue of all phenotypic traits of *pol δ 1* mutants, including apoptosis and cell differentiation, indicating that in this particular case, the p53 control system might be too 'impatient', and that cells slowed down in replication can give rise to normal tissues when their p53-dependent elimination is blocked.

Results

fla mutants are characterized by defects in various tissues derived from late proliferative zones

Zebrafish *flathead* (*fla*) mutants were originally isolated during the Tuebingen 1996 large-scale ethylnitrosourea (ENU) mutagenesis screen, based on their severe craniofacial defects.^{21,23} Specifically, the cartilage of the neurocranium and of pharyngeal arches 1–3 was reduced, while arches 4–7 were completely absent in *fla* mutants at 120 hpf (Figure 1g, h). Cartilage of the pharyngeal arches is largely derived from neural crest.⁴² However, according to *in situ* hybridization analyses with *dlx2*, marking prechondrogenic neural crest cells,²⁹ there was no difference in the amount of neural crest populating the pharyngeal arches of mutants and wild-type siblings at 36 hpf (data not shown), while at 48 hpf, the number of *dlx2*-positive cells in the *fla* mutants was moderately reduced (Figure 1c, d). Similarly, the endodermal pouches, which play an important role in patterning and survival of the pharyngeal arches,⁴³ were formed normally in *fla* mutants, as indicated by the normal staining intensity and pattern of Zn5 immunostainings before 48 hpf (data not shown). However, craniofacial defects became more apparent during the next

Figure 1 *fla* mutants display defects in craniofacial, brain, and eye development. All panels show *fla* mutants (b, d, f, h) in comparison to wild-type siblings (WT; a, c, e, g). (a, b) Live larvae at 120 hpf; lateral view over entire length of larvae. (c–f) Whole mount *in situ* hybridization with probes staining cartilage-forming neural crest cells at different stages of development. (c, d) 48 hpf, *dlx2*; (e, f) 72 hpf, *sox9*. Arrowheads point to crest cells forming the first two pharyngeal arches, mandibular and hyoid, arrows to more posterior crest cells forming pharyngeal arches 3–7 (gill arches). Comparison of (e) and (f) reveals that *sox9* expression in arches 1 and 2 is reduced in *fla* mutants, while staining in the gill arches is completely absent. Similarly, *sox9* expression in the chondrocytes of the pectoral fin is absent in *fla* mutants (indicated with asterisks). (g, h) Alcian blue stainings of cartilaginous elements of the head skeleton at 120 hpf. 'ep' indicates the ethmoid plate of the neurocranium; numbers 1–7 indicate the pharyngeal arches (1 = mandibular; 2 = hyoid; both are jaw-forming arches; 3–7 = gill/branchial arches). Abbreviations: WT, wild type; h, hours post fertilization (hpf)

day of development, when the arch-populating crest cells undergo increased cell proliferation, as revealed by *in situ* hybridizations for the replication marker *pcna* (proliferating-cell-nuclear-antigen; see below; Figure 3j), and by BrdU incorporation analyses (see below; Figure 3k). Thus, at 72 hpf, expression of *sox9*,³⁰ a marker for differentiating neural crest-derived chondrocytes, was particularly compromised in the gill arches (pharyngeal arches 3–7) of *fla* mutants (Figure 1e, f), suggesting that cells either fail to specify, or that they have become severely reduced in number due to decreased proliferation and/or enhanced apoptosis. Consistent with the latter notion, *fla* mutants also showed defects in other tissues known to be highly proliferative during late developmental stages, such as the eyes and the brain, which

were of normal size during the first 40 h of development, but much smaller than in wild-type siblings during later stages of development (see Figure 1a, b for 120 hpf). In contrast, other regions and tissues of *fla* mutant larvae appeared morphologically normal.

***fla* mutations disrupt zebrafish polδ1 catalytic subunit**

During the Tuebingen screen, five different alleles of *fla* were isolated, all of which yield morphologically indistinguishable phenotypes.²³ We genetically mapped one of them, *fla*^{ta53c}, to linkage group 3 between markers z8208 and z14228. For fine-mapping, additional markers were designed, based on genomic sequences in the vicinity of z14228 (Figure 2a; see also Materials and Methods and Supplementary Data). Ultimately, this led to the identification of Ensembl genomic contig ctg24833.1, which contains four putative open reading frames (ORFs), and which is flanked on either side by a north or a south marker, respectively, each with a distance of approximately 0.05 cM to the *fla* locus (NP3, NP4; one recombination in 1912 meioses) (Figure 2a). One of the putative ORFs was predicted to encode Polδ1 catalytic subunit, consistent with the phenotype of *fla* mutants in proliferative zones (see above). 5' and 3' RACE primers were designed to the putative predicted *polδ1* transcript sequence. Sequencing of the RACE products revealed that this transcript encodes a protein of 1105 amino-acid residues (GenBank Accession Number AY883095), with 70.9% amino-acid residue identity to both human and mouse Polδ1 (calculated by Jotun Hein method, DNASTAR software). We determined the genomic structure of *polδ1* and amplified all 27 coding exons from genomic DNA of homozygous wild-type and mutant larvae for three different alleles, *fla*^{ta53c}, *fla*^{th5b}, and *fla*^{ty76a}. For the *fla*^{ta53c} allele, we identified a G to A transition in the splice acceptor site preceding coding exon 24 (989–1026), while the *fla*^{ty76a} allele contains a T to A transversion causing a nonsense mutation in exon 12 (C531*), and the *fla*^{th5b} allele a nonsense A to T transversion in exon 16 (L696*) (Figure 2b). C531* results in a truncation of the protein eliminating five of the six polymerase domains, all three conserved C-terminal (CT) domains, and both zinc-finger domains of Polδ1, while L696* mutant Polδ1 lacks three polymerase domains, all three CT domains, and both zinc-finger domains (Figure 2c). In order to examine the effects of the aberrant splice acceptor site, we PCR-amplified and sequenced the 3' end (corresponding to coding exons 19–27) of *polδ1* from wild type and mutant cDNA. We detected two isoforms of mutant transcript, resulting from the usage of alternative splice acceptor sites (Figure 2b). In one case, the splice acceptor preceding exon 25 is used, resulting in an in-frame deletion of exon 24. Exon 24 encodes the first half of the first zinc-finger domain of Polδ1. In the second case, the coding AG sequence at the beginning of exon 24 is used as an alternative splice site, producing a frame-shift and a premature termination of the polymerase removing both zinc-finger domains of Polδ1. The zinc-finger domains have been suggested to be important for internal stability of the protein.¹ Furthermore, in the case of mouse Polz1⁴⁴ and yeast Polδ⁴⁵ and Polε,⁴⁶ these domains

have been shown to be required for binding of the catalytic subunits to the B subunits (Cdc1/Polδ2) of the holo-enzymes.^{44–46} Accordingly, mutational analyses in budding yeast revealed that mutations within the zinc-finger domains of Polδ and Polε abolish polymerase function *in vivo*.^{46,47}

In sum, given the importance of the domains lost in the different mutant versions of zebrafish Polδ1, together with the indistinguishable phenotypic strengths of the three alleles, we suspect that they all represent *polδ1* null mutations.

***polδ1* is restricted to areas with high cell proliferation**

Given the rather late and restricted defects caused by loss of Polδ1 function, we examined *polδ1* expression by RT-PCR analyses and whole mount *in situ* hybridization during normal zebrafish development up to 72 hpf. *polδ1* mRNA could be detected throughout all investigated stages, including earliest stages before midblastula transition (MBT),⁴⁸ indicating that it is both maternally and zygotically supplied (data not shown). During cleavage, blastula, gastrula, and early segmentation stages, *polδ1* transcripts were ubiquitously and uniformly distributed throughout the entire embryo (Figure 3a; and data not shown). However, during mid-segmentation stages, *polδ1* expression started to become restricted. At 24 hpf, expression had largely ceased in the posterior half of the embryo, while it was prominent in the brain, the eye vesicles, and the neural crest (Figure 3b, c). At 32 hpf (data not shown), 48 hpf (Figure 3d–f), and 57 hpf (Figure 3g, h), *polδ1* expression had become further restricted, and was largely confined to a ventral patch of the retina and the ciliary marginal zone of the eyes, the midbrain–hindbrain boundary, neural crest cells of the branchial arches, the pectoral fin buds, and the endoderm of the gastrointestinal system. This expression pattern was very similar to that of the replication marker *pcna*,³¹ which also showed prominent and restricted expression in the ciliary marginal zone, the midbrain–hindbrain boundary, the tectum, and the neural crest cells of the branchial arches at 57 hpf (Figure 3j). Furthermore, it resembled the pattern obtained in BrdU incorporations studies to label proliferating cells at 48 hpf (Figure 3k, l). At 72 hpf, *polδ1* expression had ceased in most tissues except the digestive organs (Figure 3i). In sum, these data indicate that during zebrafish development, *polδ1* expression is restricted to proliferating cells, outlining proliferative zones during later stages of development, whereas expression is switched off in differentiating cells.

***fla* mutants display a transient subtle increase in the number of cells in S phase**

Different approaches were taken to study the proliferation and cell cycle state of cells before and during the appearance of morphological defects in *fla* mutants. At 48 hpf, the staining intensity obtained via BrdU incorporations was strongly reduced in *fla* mutants (Figure 4b) compared to wild-type siblings (Figure 4a), suggesting that DNA replication was compromised. Consistent results were obtained in FACS analyses determining the DNA content of dissociated cells from heads of single *fla* mutants and wild-type siblings,

Figure 2 Phenotype of *fla* mutants is caused by potential null mutations in zebrafish DNA polymerase delta1. (a) Positional cloning of *fla* allele *ta53c*. Meiotic segregation linkage analyses placed *fla* into an interval on linkage group 3, defined by two flanking polymorphic markers (NP3, NP4), both of which display a genetic distance of 0.05 centimorgan (cM) to the *fla* mutation. Numbers in middle diagram indicate found recombinations for investigated markers in 1912 meioses. Physically, the NP3/NP4 interval corresponds to 130 kb of DNA containing four predicted open reading frames (ORF), one of which is *polδ1*. (b) Sequencing of three independent *fla* alleles revealed a mutation in the splice acceptor site upstream of exon 24 in the case of the *ta53c* allele (left panel), and nonsense mutations in exon 12 (*ty76*; middle panel) or exon 16 (*th5b*; right panel), respectively. First amino-acid residues lost in the resulting truncated proteins are indicated with an asterisk. (c) Cartoon demonstrating the pattern of false splicing leading to the identified mutant *polδ1* transcripts (A, B) of the *ta53c* allele, and cartoons demonstrating the domain structures of wild type and the various mutant Pol δ 1 proteins encoded by the three *fla* alleles *ta53c*, *ty76*, and *th5b*. The region deleted in Pol δ 1 encoded by transcript A of *fla*^{ta53c} is indicated in black

Figure 3 *polδ1* is uniformly expressed early, whereas expression becomes restricted to zones of high proliferation during later stages of zebrafish development. All panels show wild-type embryos or larvae of ages indicated in lower right corner (in h = hours post fertilization/hpf). (a–j) Whole mount *in situ* hybridizations for *polδ1* (a–i) or the replication marker *pcna* (j), respectively. (k, l) BrdU incorporation patterns. (a, b, d, f, g, i–k) Lateral views on entire embryo (a) or on the head region (others), and (c, e, h, l) dorsal views on head region. Arrows in (b,c) point to trunk region that has become devoid of *polδ1* transcripts. Abbreviations: cmz, ciliary marginal zone of eyes; ey, eye; gi, gastrointestinal system; gt, gut; liv, liver; mbh, midbrain–hindbrain boundary; nc, neural crest cells; pcr, pancreas; pfb, pectoral fin buds; tec, tectum; vr, ventral retina; vz, ventricular zone of brain

accompanied by genotyping of the embryos with genomic DNA extracted from the corresponding trunk and tail region. At 36 hpf, before morphological defects become apparent, *fla* mutants displayed a subtle, but consistent increase in the fraction of cells with a DNA content higher than 2N ($17.5 \pm 0.86\%$; $n=5$) (Figure 4d), compared to wild-type siblings ($14.5 \pm 1.18\%$; $n=14$) (Figure 4c). The difference is statistically significant ($P=0.0002$). Given that at these stages, most cells have initiated terminal differentiation (DNA content = 2N), while only a rather small subset of cells is proliferative (see *pcna* and *polδ1* *in situ* hybridizations and BrdU incorporation studies; Figure 3), these results suggest that within this population of proliferating cells, loss of *polδ1* leads to a slower progression through S phase.

In contrast, no such increase in the relative ratio of cells in S phase was observed in *fla* mutants at a later stage, when the defects have become morphologically visible. Rather, at 52 hpf, the number of cells with a DNA content higher than 2N in *fla* mutants ($11.3 \pm 0.46\%$; $n=15$; three pools with five embryos each; Figure 4f) was similar to that of wild-type siblings ($13.1 \pm 1.7\%$; $n=15$; Figure 4e; difference statistically nonsignificant; $P=0.2\%$). Instead, *fla* mutants at 52 hpf

displayed a significant increase in the fraction of cells with a DNA content lower than 2N, compared both to wild-type siblings of the same stage ($18.7 \pm 1.68\%$ in *fla* versus $4.3 \pm 1.54\%$ in wild type; $P=0.0006$) (Figure 4e, f), and to *fla* mutants at 36 hpf ($3.8 \pm 1.03\%$; $n=5$; compare Figure 4f with Figure 4d; $P=0.0009$). This <2N fraction is commonly regarded to represent apoptotic cells, suggesting that at these later stages, cells arrested or slowed down in S phase might undergo programmed cell death.

fla mutants display increased apoptosis in late proliferative zones

To directly examine apoptosis in *fla* mutants, we carried out TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; Figure 5a–d) and acridine orange stainings (Figure 5e–p), both of which yielded very similar results. No increased apoptosis rates were found in *fla* mutants prior to 36 hpf. At 38 hpf, TUNEL stainings revealed higher numbers of apoptotic cells particularly in the tectum of *fla* mutants (Figure 5a–d). At 50 hpf, *fla* mutants displayed high numbers of acridine orange-positive cells in all late proliferative zones, such as the midbrain–hindbrain boundary (Figure 5f, j), the eye vesicles (Figure 5n), and the branchial arch region (Figure 5j), while wild-type embryos only showed dispersed acridine orange-positive cells (Figure 5e, i, m). Actually, the pattern of acridine orange staining in *fla* mutant embryos resembled very much the pattern obtained via BrdU incorporation studies in wild-type embryos (compare Figure 5f with Figure 3l). In sum, this suggests that in *fla* mutant embryos, cells slowed down or arrested in S phase due to the loss of Polδ1 activity, are eliminated by apoptosis.

Disruption of Polδ1 leads to an upregulation of genes involved in the p53 pathway

Studies in cell culture systems have indicated that the tumor suppressor protein p53 is part of the S-phase checkpoint control systems, eliminating replication-arrested cells by inducing programmed cell death. In order to examine if the p53 pathway is activated in response to disruption of Polδ1 function, we examined wild type and *fla*^{ta53c} mutant single embryos for p53 transcript levels. Quantitative real-time RT-PCR analysis showed that p53 transcript levels were elevated approximately two-fold in mutants (Figure 6a). Additionally, p53 protein activity appeared to be increased, as indicated by the two-fold increased mRNA levels of the direct p53 target gene *p21*.^{36,49} p21 encodes a CDK inhibitor that mediates DNA damage-induced cell cycle arrest both during G1/S and G2/M transition.⁵⁰ In contrast, the reported p53 target gene *bax*,^{39,51} which is involved in mediating p53-dependent apoptosis via the mitochondrial pathway,^{12,52} showed unaltered mRNA levels in *fla* mutants (Figure 6a). These *in vivo* results support previous data obtained in cell culture studies showing a role of p53 for G2/M arrest after replication blockage,^{10,11} while its role to mediate apoptosis remains elusive.

Figure 4 *fla* mutants display compromised replication and a transient accumulation of cells with a DNA content higher than 2N, followed by an accumulation of cells with a DNA content smaller than 2N, which can be rescued by p53 deficiency. (a,b) BrdU incorporation in wild-type (a) and *fla* mutant (b) at 48 hpf, revealing strongly reduced staining intensity in the proliferative zones of the mutant. (c–h) Representative histograms from FACS analyses after DAPI staining of dissociated cells from single heads of embryos at 36 hpf (c, d) or 50 hpf (g, h), and from pooled heads of five embryos at 52 hpf (e, f). At 52 hpf, mutants (f) and wild-type siblings (e) were distinguished by their morphological phenotype, while at 36 hpf (c, d), and at 50 hpf after injection of *p53* MO (g, h), when the morphological defects of mutants are very subtle, the genotype was determined via PCR of the corresponding posterior part of the specimen, as described in Materials and Methods. Numbers show the percentages of cells with DNA contents lower (<) or higher (>) than 2N in the area indicated by bars

Figure 5 *fla* mutants display increased apoptosis in late proliferative zones, which can be completely rescued by *p53* deficiency. (a–d) TUNEL stainings at 38 hpf, all other panels show acridine orange stainings at 50 hpf. The genotype of larvae (wild type or *fla*), and the injected morpholino antisense oligonucleotides (*p53* MO or four mismatch control morpholino *p53* 4mm MO) are shown in lower left corner. (a, b, i–l) Lateral view, (c, d) frontal view, (e–h) dorsal view on head region; (m–p) magnified lateral view on eye. In (j, k) cells forming the cartilage of the gill arches (arrowheads) and of the pectoral fin buds (arrows) are indicated (compare with Figure 1e, f). Abbreviations: mbh, midbrain–hindbrain boundary; tc, tectum; vz, ventricular zone of tectum

p53 deficiency rescues both apoptotic and morphological traits of the *fla* phenotype

To investigate whether despite the normal *bax* mRNA levels in *fla* mutants, the observed elimination of replication-compromised cells is p53-dependent, we generated *polδ1-p53* double deficient zebrafish. Previous studies have demonstrated that p53 protein levels can be efficiently decreased and p53-induced apoptosis can be inhibited by the injection of *p53* antisense MOs (*p53*-MO). *p53* morphant embryos themselves show no developmental defects (data not shown; Langheinrich *et al.*³⁶), similar to p53-deficient mice.⁵³ This is in contrast to p53-deficient *Xenopus* embryos, which display compromised cell differentiation during early stages of development.^{54–56} Here, p53 is required for proper transforming growth factor beta (TGFβ) signaling and mesoderm formation, consistent with a physical interaction of p53 with Smad proteins.^{55,56} The reasons for the different requirements of p53 during early mammalian, fish and amphibian development are unclear, but might be due to the presence of other p53 family members (p63, p73) in mammals and zebrafish, whereas they appear to be absent during early stages of *Xenopus* development.⁵⁶

Two different *p53* MOs were injected into clutches of *fla*^{ta53c} or *fla*^{th5b} mutant embryos at the one-cell stage. Consistent

with a role of p53 as a proapoptotic factor in replication-compromised cells, we found a significant rescue of apoptosis in *fla* mutants injected with *p53* MOs. Acridine orange stainings revealed that apoptosis rates in all investigated proliferative zones (tectum, midbrain–hindbrain boundary, eye vesicles, craniofacial domain) of *p53*-deficient *fla* mutants (Figure 5g, k, o) were indistinguishable from those in wild-type siblings (Figure 5e, i, m) during days 2 and 3 of development, and strongly reduced compared to regular *fla* mutants (Figure 5f, j, n) or mutants injected with a 4-mismatch control MO (Figure 5h, l, p).

We also investigated cell proliferation behavior of *polδ1*-deficient cells in the absence of p53, carrying out DNA content-dependent flow cytometric experiments with heads of single genotyped embryos at 50 hpf, as described above. Consistent with the aforementioned results obtained via acridine orange stainings, the ratio of cells with a DNA content smaller than 2N, representing apoptotic cells, was significantly reduced in *p53*-deficient *fla* mutants ($5.8 \pm 0.37\%$; $n=6$) compared to noninjected *fla* mutants ($18.7 \pm 1.46\%$) (compare Figure 4h with Figure 4f; $P=0.002$), and was similar to that of wild-type embryos injected with *p53* MO ($4.6 \pm 0.71\%$; $n=8$) (compare Figure 4h with Figure 4g). However, the ratio of cells with a DNA-content higher than 2N was not increased accordingly. Rather, it was only

Figure 6 *fla* mutants display increased expression of *p53* and *p53* target genes, while morphological structures can be completely rescued by *p53* deficiency. **(a)** Quantitative real time RT-PCR of *fla* and wild-type sibling mutants at 46 hpf, showing increased transcript levels of *p53* and its target gene *p21* in *fla* mutants, while *bax* transcript levels are normal. Mean relative expression ratios compared to *ef1α* (fold), standard deviations (Stdev), and *P*-values for comparison of wild type and *fla* numbers (*P*, statistically significant difference when < 0.05) are listed on the right side. **(b–m)** Injection of *p53* MO, but not the four mismatch control MO, leads to a rescue of *fla* morphology. The genotype of larvae, and the injected morpholino antisense oligonucleotides are shown in upper right corner. **(b–e)** Dorsal views on the head region of live larvae at 120 hpf; *fla* mutants injected with *p53* MO display a significant normalization of eye size. **(f–m)** Ventral view on heads stained with alcian blue at 72 hpf, **(f–i)** or 120 hpf **(j–m)**. Numbers indicate pharyngeal arches (compare with Figure 1). At 120 hpf *fla* mutant injected with *p53* MO **(l)** has regained pharyngeal arches 4–7 absent in regular or control-injected *fla* mutants **(k, m)**. At 72 hpf, the differentiation of rescued chondrocytes in pharyngeal arches 4–7 of the *fla* mutant injected with *p53* MO **(h)** is slightly delayed compared to the non-injected wild-type sibling **(f)** and to the wild-type sibling injected with *p53* MO **(i)**

moderately higher ($11.8 \pm 0.93\%$; Figure 4h) than that of the *p53* MO-injected wild-type controls ($10.2 \pm 0.87\%$; Figure 4g; difference statistically significant; $P = 0.008$). This suggests that in the absence of *p53*, replication-compromised *fla* mutant cells neither die nor irreversibly arrest, but can progress through S phase, possibly with slightly reduced speed.

Such surviving *p53*-deficient *fla* mutant cells also appear to be able to finish their normal developmental program, as indicated by the rescue of all morphological traits of *fla* mutants upon *p53* MO injection. Noninjected *fla* mutants or mutants injected with a four mismatch *p53* control MO could easily be detected at 48 hpf by their characteristic small head/small eyes phenotype, which is even more apparent at 72 hpf (data not shown) and at 120 hpf (Figure 6c; and data not shown). However, *p53* MO-injected clutches displayed no mutant phenotypes at 48 hpf, while at 72 and at 120 hpf, the

size of the eyes and the head of *p53*-deficient *fla* mutants was much less reduced than in noninjected *fla* mutants (compare Figure 6d with Figure 6c). In addition, alcian blue stainings at 120 hpf revealed that the craniofacial cartilage defects of *fla* mutants were rescued to almost wild-type condition upon loss of *p53* activity. Specifically, gill arches 4–7, while absent in noninjected *fla* mutants at 72 and 120 hpf (Figure 6g, k), and not fully developed in *p53* MO-injected *fla* mutants at 72 hpf (Figure 6h), were present at normal sizes and shapes in injected mutants at 120 hpf (Figure 6l). These regained alcian blue-positive cartilaginous elements mostly consist of post-mitotic, differentiated chondrocytes, indicating that in the absence of *p53*, *polδ1*-deficient cells rescued from apoptosis can undergo terminal differentiation. However, rescued *fla* mutants were not viable. Although they developed swim bladders and swam normally, they died around 12 dpf, while non-injected *fla* mutants lived until 8 or 9 dpf.

Discussion

Zygotic Pol δ 1 is essential for proper development of multiple tissues derived from late proliferative zones

Thus far, genetic analyses addressing the *in vivo* role of DNA Pol δ 1 in higher eukaryotic systems had been limited. Here, we identified zygotic *pol δ 1* null mutants in zebrafish, based on the positional cloning of the gene causing the *fla* phenotype in three independent alleles.²³ During development, *pol δ 1* is expressed ubiquitously early, while expression becomes restricted to proliferative zones later (Figure 3). Consistently, defects of *pol δ 1/fla* mutants are restricted to tissues derived from these zones, such as eyes, brain, and cartilaginous elements of the head skeleton, all of which are strongly reduced in size. The gill arches are even completely missing, although their progenitor cells were present in normal numbers during earlier stages of development (Figure 1). In FACS analyses determining the DNA content of cells from dissociated zebrafish heads, we found only a subtle accumulation of cells in S phase, suggesting that replication is not completely arrested, but only slowed down (Figure 4). Later, these cells are eliminated by apoptosis, as indicated by the high numbers of acridine orange-positive cells in proliferative zones of *fla* mutants (Figure 5), and by the accumulation of signals with a DNA content lower than 2N in the FACS studies, while the fraction of cells in S-phase drops (Figure 4). In sum, these data indicate that zygotic Pol δ 1 is required in multiple proliferative zones of zebrafish larvae to ensure proper DNA replication, and the survival and subsequent differentiation of late proliferating cells.

Maternally supplied Pol δ 1 protein might be sufficient to drive DNA replication during early stages of zebrafish development

DNA replication is required during all stages of development. Thus, treatment of blastula stage zebrafish embryos with drugs blocking DNA replication leads to an arrest of early development.⁵⁷ Given the essential role of Pol δ 1 during DNA replication revealed in lower eukaryotes (e.g. fission yeast⁴) and in higher *in vivo*-like systems (*Xenopus* egg extracts⁵), the rather late appearance of defects in zebrafish *pol δ 1* zygotic null mutants seems surprising. We can only speculate about possible reasons. There could be a second *pol δ 1* gene, resulting from the genome duplication during teleost evolution.⁵⁸ However, we thus far failed to identify such a second gene in genomic and EST database searches. Also, functional redundancy with its closest relative Pol ϵ seems quite unlikely in light of results obtained via immunodepletions in *Xenopus* egg extracts, demonstrating that the two polymerases cannot replace each other.^{5,59}

In this light, we favor a model in which zygotic Pol δ 1 might be dispensable for early DNA replication because of functional compensation by maternally supplied *pol δ 1* gene products. mRNAs and proteins of many so-called house-keeping genes have been shown to be synthesized during oogenesis and deposited in the egg. Indeed, we could detect high levels of *pol δ 1* mRNA in zebrafish embryos before the onset of MBT,

when zygotic transcription of genes is initiated (4 hpf).⁴⁸ However, maternal mRNAs have been shown to be usually degraded during the first 10–15 hpf,⁶⁰ whereas the phenotype of *fla* mutants only becomes apparent during day 2 of development. Accordingly, knocking down both maternal and zygotic *pol δ 1* mRNAs with antisense MOs targeting the translational start codon generated phenotypes not much stronger and earlier than those of *fla* mutants (NP and MH, unpublished observations).

Thus, it appears more likely that it is maternally provided Pol δ 1 protein, rather than maternal *pol δ 1* mRNA, that compensates for zygotic Pol δ 1 during early stages of zebrafish development. We do not know how much maternally supplied Pol δ 1 protein is present in early stage zebrafish embryos, since none of several tested antibodies against mammalian Pol δ 1 crossreacted with the zebrafish protein. However, in amphibian oocytes, DNA polymerases have been shown to be over 100 000-fold enriched compared to somatic cells, and we suspect that the same might be true for zebrafish. Maternal proteins can be much more stable than maternal mRNAs, as for instance revealed for Mcm5, another component of the replication complex, which is detectable in zygotic *mcm5* mutants until day 2 of development (Soojin Ryu and Wolfgang Driever, personal communication). A similar slow and gradual loss of maternal Pol δ 1 protein would not only provide an excellent explanation for the late appearance of the *fla* mutant phenotype but also for the observed normalization of the *fla* phenotype upon p53 deficiency.

Zygotic Pol δ 1 becomes more dispensable under p53-deficient conditions

Thus far, implications of p53 deficiency on the rescue of apoptosis during vertebrate development have been mainly studied in the context of DNA repair, such as in the case of Pol β -deficient mice⁶¹ and of Fancd-morphant zebrafish.³⁹ In the context of DNA replication stress, similar studies have been largely restricted to cell culture systems. Thus, it was shown that blockage of DNA replication through application of aphidicolin or hydroxyurea in mouse or human fibroblast cultures leads to an increase in p53 protein level and to an S-phase arrest, which is released in p53-deficient fibroblasts.¹⁰ Similarly, S-phase arrest and subsequent apoptosis of mouse ES cells deficient for the protein kinase Cdc7 are accompanied by an increase in *p53* mRNA, protein and activity levels, while apoptosis can be repressed upon chemical inactivation of p53.⁶²

Here, we show that *pol δ 1* zebrafish mutants display an increase in *p53* transcription and p53 activity (Figure 6), while loss of p53 allows completion of replication (Figure 4), as well as survival (Figure 5) and differentiation of replication-compromised cells (Figure 6). For the same reasons as described above, we think that completion of replication in rescued *pol δ 1-p53* double-deficient cells is driven by maternally supplied Pol δ 1 protein. Assuming that in zygotic *pol δ 1* mutants, such maternal protein only runs out slowly, p53-dependent apoptosis of replication-compromised cells might occur when maternal Pol δ 1 has dropped below a critical threshold, for instance when not all replication forks initiated

under the participation of Pol α can be directly taken over by Pol δ . These transiently stalled replication forks, if present for too long, would then lead to the activation of p53 to eliminate the corresponding cells. However, there might still be enough maternal Pol δ 1 present to complete DNA replication in the absence of p53, when cells are given more time. This notion is supported by our findings that in *polδ1-p53* double-deficient zebrafish, chondrocytes of the rescued gill arches differentiate slightly later than in regular *p53* morphants (Figure 6). In this light, the p53 branch of the S-phase control checkpoint might be too tight and 'impatient', eliminating cells that would otherwise have caught up. However, *polδ1-p53* double-deficient zebrafish are not perfectly rescued; death is just postponed for 3–4 days (compare with Kim *et al.*⁶²). This could be due to the progressive reappearance of p53; MO-based gene knockdown normally starts to be attenuated after 3 dpf. In addition, it could mean that zygotic Pol δ 1 eventually becomes absolutely essential even under p53-deficient conditions, for instance when maternal protein has been completely lost.

It remains unclear how p53 mediates apoptosis of replication-compromised cells. Interestingly, we found *bax* mRNA levels to be normal in *fla* mutant embryos, suggesting that the mitochondrial death pathway is not involved. This is consistent with data obtained in mouse, showing that although DNA damage fails to induce apoptosis in p53-deficient cells, it can do so in cells deficient for Bax or death receptors (reviewed in Rich *et al.*⁶³)

p53 is only one of the effectors of the S-phase checkpoint control system

Cell culture work has identified the kinase ATR as the central component of the S-phase checkpoint control, which upon replication stress activates other proteins in parallel to p53 (see Introduction and Nyberg *et al.*⁸ and Bartek *et al.*⁹ for reviews). We have preliminary data that in contrast to p53 itself, the ATR checkpoint control system in its entirety is required for normal cell cycle progression in replication-compromised zebrafish cells. Thus, when knocking down zebrafish ATR with antisense MOs, we observed a significant worsening of the *fla* mutant defects, with a loss of pharyngeal arches 2 and 3 in addition to arches 4–7 absent in regular *fla* mutants. In contrast, ATR deficiency in wild-type embryos had no visible effects (NP and MH, unpublished data). This suggests that cells of arches 2 and 3 of *fla* mutants also display (more moderately) compromised replication, but can complete S phase before they are caught by p53, whereas in the absence of ATR, they undergo precocious entry into mitosis, ultimately leading to their elimination. This is in clear opposition to the situation observed in *p53/polδ1*-double deficient animals, in which the absence of p53 appears to allow all replication-compromised cells of arches 2–7 to survive, while the presence of ATR enables them to enter mitosis only after they have finished replication. This implies that in developing zebrafish, other ATR target proteins of the S-phase checkpoint system regulate entry into mitosis, whereas p53 itself is dispensable for this process, in contrast to its reported role in cultured cells.¹⁰

Craniofacial development under Polδ1-compromised conditions

Within the pharyngeal arches, *fla* mutants display a deletion of arches 4–7, while arches 1–3 develop with rather normal size, which initially had prompted us to believe that *fla* might be involved in anterior–posterior (AP) patterning of the visceral head skeleton. What might account for these AP effects under replication-compromised conditions? Specification of cartilage-forming cephalic neural crest cells occurs in an AP-wave, with cells from anterior arches differentiating before cells from more posterior arches.⁶⁴ In this light, chondrocytes forming arches 1–3 might already have completed their final round of replication before Pol δ 1 levels drop below the critical threshold for p53 activation, whereas chondrocytes forming arches 4–7 are caught by the checkpoint control system and eliminated. Along the same lines, the differential responses among arches 1–3 to ATR deficiency could be explained (see above). In any case, our data suggest that the defects of other craniofacial zebrafish mutants with *fla*-like phenotypes^{23,65} might be caused by defects in genes involved in DNA replication and/or S-phase checkpoint control.

Materials and Methods

Maintenance and identification of *fla*^{ta53c}, *fla*^{ty76}, and *fla*^{th5b}

Zebrafish were bred and maintained, and embryos were treated using standard conditions.²⁴ *fla*^{ta53c}, *ty76*, *th5b* mutant lines²⁵ were obtained from the Tuebingen stock center and were maintained in the Tuebingen background. *fla*^{ta53c} was outcrossed to the WIK line for genetic mapping.²⁶ Mutant larvae were identified live at 3–5 days postfertilization (dpf) by their obvious craniofacial morphology and eye size reduction.

Genetic and physical mapping of the *fla* locus

The *fla*^{ta53c} mutation was mapped to linkage group 3 via PCR analysis of simple sequence length polymorphism (SSLP) markers (Tuebingen version 4) on mutant (25 larvae) and wild-type (75 larvae) genomic DNA pools.²⁷ Genomic sequence from the *fla* locus was identified by sequence similarity searches of markers previously mapped near z14228 (<http://wwwmap.tuebingen.mpg.de/>) against the Ensembl Zv2 genome database (<http://www.ensembl.org/>). New polymorphic markers were designed from this sequence for fine mapping of single mutant larvae (see Supplementary Data). Contig ctg24833.1 was identified by a sequence similarity search with the marker fi11h05.x1 against the Ensembl genomic database. All PCR fragments were amplified with a 55°C annealing temperature using standard protocols.

Cloning of zebrafish *polδ1* and identification of mutations were carried out as described in Supplementary data.

Tissue labeling procedures

Whole mount *in situ* hybridization was carried out as previously described.²⁸ For *polδ1* *in situ* probe synthesis, plasmid *pCR11-zfpolδ3'* was linearized with *NotI* and transcribed with SP6 RNA polymerase. In addition, riboprobes of the following cDNAs were used as described: *dlx2*,²⁹ *sox9*,³⁰ *pcna*.³¹

Whole mount immunostainings were carried out as previously described,³² using the Vectastain Elite ABC kit (Vector Laboratories), and the primary antibody Zn5 (Zebrafish International Resource Center; University of Oregon, Eugene) at 1:500. To visualize craniofacial cartilage, 120 hpf (hours postfertilization) embryos were stained with alcian blue (Sigma) as described.²³

For detection of apoptotic cells, TUNEL (terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling) stainings were performed using the *In Situ* Cell Death Detection Kit (Roche), essentially as described by Cole and Ross.³³ Acridine orange stainings of apoptotic cells were performed as previously described.³⁴

For labeling of proliferating cells via incorporation of 5-Bromo-2'-deoxyuridine (BrdU), BrdU was delivered by incubating live zebrafish embryos for 30 min in BrdU solution (10 mM BrdU; 15% DMSO in E3 medium) on ice, followed by three washes in E3 medium and further incubation for 1 h at 28.5°C. Afterwards, embryos were fixed in 4% PFA/PBS for 2 h at room temperature or over night at 4°C. After several washes in PBS, 0.1% Tween-20 (PBST), and one brief wash in distilled water, embryos were incubated in acetone at -20°C for 10 min. For further permeabilization, embryos were digested for 6 min at room temperature in 0.25% trypsin in PBS without Mg²⁺ and Ca²⁺, and refixed in 4% PFA for 20 min. Embryos were then rinsed three times in 0.1% Tween-20 and two times with 2 N HCl, followed by incubation in 2 N HCl for 1 h. Afterwards, embryos were rinsed three times in PBST and blocked for 10 min in immunostaining blocking solution,³² followed by subsequent incubation with the primary anti-BrdU antibody (Boehringer 1170376; 1:100 in blocking solution) and the secondary AlexaFluor647-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR, USA; 1:200 in blocking solution), using standard conditions.

Morpholino injections

Antisense morpholino oligonucleotides (MOs) were injected at the 1–4 cell stage and at a concentration of 0.1 mM, as previously described.³⁵ The sequences of used MOs were: AGA ATT GAT TTT GCC GAC CTC CTC T (p53-MO1); GCG CCA TTG CTT TGC AAG AAT TG (p53-MO2³⁶); the mismatch control MO, with mismatched nucleotides denoted in lowercase, was GCa CCA TcG CTT gGC AAG cAT TG (p53-4mm³⁶).

RNA extraction and quantitative real time RT-PCR analysis

Total RNA was isolated from 46 hpf whole wild-type or mutant embryos using 750 µl of Trizol reagent (Gibco/BRL). First strand cDNA synthesis was performed on 2 µg of total RNA using oligo dT primer and Superscript III reverse transcriptase (Invitrogen). Quantitative real time RT-PCR (QRT-PCR) was performed with the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon-Continuous Fluorescence Detection system (MJ Research), using the following primer sets: elongation factor 1α (*ef1α*)³⁷ (control): sense, TCA CCC TGG GAG TGA AAC AGC; antisense, ACT TGC AGG CGA TGT CAG CAG; *p53*:³⁸ sense, GCG ATG AGG AGA TCT TTA CCC; antisense, ACA AAG GTC CCA GTG GAG TG; *p21*:^{39,40} sense, AGC TGC ATT CGT CTC GTA GC; antisense, TGA GAA CTT ACT GGC AGC TTC A; *bax*:^{39,40} sense, GCA GTG GCA ATG ACC AGA TA; antisense, GGA AAA CTC CGA CTG TCT GC. All samples were quantified by the comparative cycle threshold (Ct) method for relative quantification of gene expression, normalized to *ef1α*.⁴¹ Differences between two groups were analyzed using a one-tailed Student's *t*-test assuming unequal variances.

Cell dissociation, genotyping, and fluorescence-activated cell sorting (FACS) analysis

Embryos were anaesthetized with triacine,²⁴ and heads were cut immediately posterior to the eyes. Genomic DNA was isolated from the posterior part of each embryo, PCR-amplified with exon24 primers giving a 498 bp fragment, and digested overnight with *Hpy188I*. Digests were run on a 2% agarose gel and scored for their genotype. As the *fla*^{ta53c} mutation results in the disruption of an *Hpy188I* site, the digest of wild-type DNA yields bands of 257, 174, and 67 bp, while mutant DNA results in fragments of 324 and 174 bp. The heads of single embryos were incubated at room temperature in 100 µl papain solution (2 mg/ml papain (Sigma), 0.3 mg/ml cysteine (Sigma) in magnesium/calcium-free PBS) for 30 min. An additional 100 µl of papain solution was added to the suspension for 30 min, cells were dissociated by trituration, and kept on ice. Triton X-100 was added to dissociated cells at a final concentration of 0.1%, followed by addition of 4',6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR; Product number D-3571) at a final concentration of 3 µM. Cells were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Data evaluation was carried out using FlowJo (Tree Star, Ashland, OR, USA). To avoid a loss of apoptotic cells by gating onto the forward/sideward scatter population, ungated histograms were used for the measurement of DNA content. To determine the ratio of cells with DNA contents smaller or higher than 2N, the area of the histograms with 10–80% (<2N) or 125–250% (>2N) of the peak channel were measured in comparison to the total area. Standard deviations (±) and probabilities associated with Student's *t*-test (*P*; Tail 2; Type 3) were calculated using Microsoft Excel software.

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References

- Hindges R and Hübscher U (1997) DNA polymerase delta, an essential enzyme for DNA transactions. *Biol. Chem.* 378: 345–362
- Hübscher U, Maga G and Spadari S (2002) Eukaryotic DNA polymerases. *Annu. Rev. Biochem.* 71: 33–63
- Waga S and Stillman B (1994) Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. *Nature* 369: 207–212
- Francesconi S, Park H and Wang TS (1993) Fission yeast with DNA polymerase delta temperature-sensitive alleles exhibits cell division cycle phenotype. *Nucleic Acids Res.* 21: 3821–3828
- Fukui T, Yamauchi K, Muroya T, Akiyama M, Maki H, Sugino A and Waga S (2004) Distinct roles of DNA polymerases delta and epsilon at the replication fork in *Xenopus* egg extracts. *Genes Cells* 9: 179–191
- Goldsby AE, Lawrence NA, Hays LE, Olmsted EA, Chen X, Singh M and Preston BD (2001) Defective DNA polymerase-δ proofreading causes cancer susceptibility in mice. *Nat. Med.* 7: 638–639
- Goldsby AE, Hays LE, Chen X, Olmsted EA, Slayton WB, Spangrude GJ and Preston BD (2002) High incidence of epithelial cancers in mice deficient for DNA polymerase δ proofreading. *Proc. Natl. Acad. Sci. USA* 99: 15560–15565
- Nyberg KA, Michelson RJ, Putnam CW and Weinert TA (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* 36: 617–656

9. Bartek J, Lukas C and Lukas J (2004) Checking on DNA damage in S phase. *Nat. Rev. Mol. Cell Biol.* 5: 792–804
10. Taylor WR, Agarwal ML, Agarwal A, Stacey DW and Stark GR (1999) p53 inhibits entry into mitosis when DNA synthesis is blocked. *Oncogene* 18: 283–295
11. Taylor WR and Stark GR (2001) Regulation of the G2/M transition by p53. *Oncogene* 20: 1803–1815
12. Haupt S, Berger M, Goldberg Z and Haupt Y (2003) Apoptosis – the p53 network. *J. Cell Sci.* 116: 4077–4085
13. Tibbets RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh S-Y, Taya Y, Prives C and Abraham RT (1999) A role of ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev.* 13: 152–157
14. Lakin ND, Hann BC and Jackson SP (1999) The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53. *Oncogene* 18: 3989–3995
15. Shieh SY, Ahn J, Tamai K, Taya Y and Prives C (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev.* 14: 289–300
16. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnicka-Worms H and Elledge SJ (1997) Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* 277: 1497–1501
17. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA and Elledge SJ (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev.* 14: 1448–1459
18. Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K and Nakanishi M (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1(–/–) mice. *Genes Dev.* 14: 1439–1447
19. Guo Z, Kumagai A, Wang SX and Dunphy WG (2000) Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts. *Genes Dev.* 14: 2745–2756
20. Feijoo C, Hall-Jackson C, Wu R, Jenkins D, Leitch J, Gilbert DM and Smythe C (2001) Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. *J. Cell Biol.* 154: 913–923
21. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJM, Jiang Y-J, Heisenberg C-P, Kelsh RN, Furutani-Seiki M, Warga RM, Vogelsang E, Beuchle D, Schach U, Fabian C and Nüsslein-Volhard C (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* 123: 1–36
22. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SCF, Malicki J, Stemple DL, Stanier D, Zwartkruis F, Abdelah S, Rangini Z, Belak J and Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123: 37–46
23. Schilling TF, Piotrowski T, Grandel H, Brand M, Heisenberg CP, Jiang YJ, Beuchle D, Hammerschmidt M, Kane DA, Mullins MC, van Eeden FJ, Kelsh RN, Furutani-Seiki M, Granato M, Haffter P, Odenthal J, Warga RM, Trowe T and Nüsslein-Volhard C (1996) Jaw and branchial arch mutants in zebrafish I: branchial arches. *Development* 123: 329–344
24. Westerfield M (1994) *The Zebrafish Book: A Guide for the Laboratory use of Zebrafish* (Eugene, Oregon: University of Oregon Press)
25. Schilling TF (1997) Genetic analysis of craniofacial development in the vertebrate embryo. *BioEssays* 19: 459–468
26. Rauch G-J, Granato M and Haffter P (1997) A polymorphic zebrafish line for genetic mapping using SSLPs on high-percentage agarose gels Technical Tips Online T01208
27. Geisler R (2002) In *Zebrafish: A Practical Approach*, Nüsslein-Volhard C, Dahm R, eds (Oxford: Oxford University Press) pp. 175–212
28. Hammerschmidt M, Pelegri F, Mullins MC, Kane DA, van Eeden FJM, Granato M, Brand M, Furutani-Seiki M, Haffter P, Heisenberg C-P, Jiang Y-J, Kelsh RN, Odenthal J, Warga RM and Nüsslein-Volhard C (1996) *dino* and *mercedes*, two genes regulating dorsal development in the zebrafish embryo. *Development* 123: 95–102
29. Akimenko M-A, Ekker M, Wegner J, Lin W and Westerfield M (1994) Combinatorial expression of three zebrafish genes related to *distal-less*: part of a homeobox gene code for the head. *J. Neurosci.* 16: 3475–3486
30. Chiang EF-L, Pai CI, Wyatt M, Yan Y-L, Postlethwait J and Chung B-C (2001) Two Sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. *Dev. Biol.* 231: 149–163
31. Lee J-S and Gye MC (1999) Zebrafish *Danio rerio* proliferating cell nuclear antigen (PCNA) cloning and characterization. *Fish. Sci.* 65: 955–958
32. Schulte-Merker S, Ho RK, Herrmann BG and Nüsslein-Volhard C (1992) The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* 116: 1021–1032
33. Cole LK and Ross LS (2001) Apoptosis in the developing zebrafish embryo. *Dev. Biol.* 240: 123–142
34. Herzog W, Sonntag C, von der Hardt S, Roehl HH, Varga ZM and Hammerschmidt M (2004) Fgf3 signaling from the ventral diencephalon is required for early specification and subsequent survival of the zebrafish adenohipophysis. *Development* 131: 3681–3692
35. Nasevicius A and Ekker SC (2000) Effective targeted gene ‘knockdown’ in zebrafish. *Nat. Genet.* 26: 216–220
36. Langheinrich U, Hennen E, Stott G and Vacun G (2002) Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. *Curr. Biol.* 12: 2023–2028
37. Nordness S, Krauss S and Johansen T (1994) cDNA sequence of zebrafish (*Brachydanio rerio*) translation elongation factor-1 alpha: molecular phylogeny of eukaryotes based on elongation factor-1 alpha protein sequences. *Biochim. Biophys. Acta* 1219: 529–532
38. Cheng R, Ford BL, O’Neal PE, Mathews CZ, Bradford CS, Thongtan T, Barnes DW, Hendricks JD and Bailey GS (1997) Zebrafish (*Danio rerio*) p53 tumor suppressor gene: cDNA sequence and expression during embryogenesis. *Mol. Mar. Biol. Biotechnol.* 6: 88–97
39. Liu TX, Howlett NG, Deng M, Langenau DM, Hsu K, Rhodes J, Kanki JP, D’Andrea AD and Look AT (2003) Knockdown of zebrafish *Fancd2* causes developmental abnormalities via p53-dependent apoptosis. *Dev. Cell* 5: 903–914
40. Nowak M, Köster C and Hammerschmidt M (2005) Perp is required for tissue-specific cell survival during zebrafish development. *Cell Death. Diff.* 12: 52–64
41. Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402–408
42. Schilling T, Walker C and Kimmel CK (1996) The *chinless* mutation and neural crest cell interactions in zebrafish jaw development. *Development* 122: 1417–1426
43. David NB, Saint-Etienne L, Tsang M, Schilling TF and Rosa FM (2002) Requirement for endoderm and FGF3 in ventral skeleton formation. *Development* 129: 4457–4468
44. Mizuno T, Yamagishi K, Miyazawa H and Hanaoka F (1999) Molecular architecture of the mouse DNA polymerase α -primase complex. *Mol. Cell. Biol.* 19: 7886–7896
45. Sanchez Garcia J, Cuifo LF, Kearsey SE and MacNeill SA (2004) The C-terminal zinc finger of the catalytic subunit of DNA polymerase δ is responsible for direct interaction with the B-subunit. *Nucl. Acids Res.* 32: 3005–3016
46. Dua R, Levy DL and Campbell JL (1999) Analysis of essential functions of the C-terminal protein/protein interaction domain of *Saccharomyces cerevisiae* pol ϵ and its unexpected ability to support growth in the absence of the DNA polymerase domain. *J. Biol. Chem.* 274: 22283–22288
47. Simon M, Giot L and Faye G (1993) A random mutagenesis procedure – application to the *pol3* gene of *Saccharomyces cerevisiae*. *Gene* 127: 139–144
48. Kane DA and Kimmel CB (1993) The zebrafish midblastula transition. *Development* 119: 447–456
49. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817–825
50. Niculescu III AB, Chen X, Smeets M, Hengst L, Prives C and Reed SI (1998) Effects of p21 (Cip1/Waf1) at both the G1/S and the G2/M cell cycle transition: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol. Cell. Biol.* 18: 629–643
51. Mayashita T and Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human BAX gene. *Cell* 80: 293–299
52. Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407: 770–776

53. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery Jr CA, Butel JS and Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356: 215–221
54. Wallingford JB, Seufert DW, Virta VC and Vize PD (1997) p53 activity is essential for normal development in *Xenopus*. *Curr. Biol.* 7: 747–757
55. Takebayashi-Suzuki K, Funami J, Tokumori D, Saito A, Watabe T, Miyazono K, Kanda A and Suzuki A (2003) Interplay between the tumor suppressor p53 and TGF β signaling shapes embryonic body axes in *Xenopus*. *Development* 130: 3929–3939
56. Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C and Piccolo S (2003) Links between tumor suppressors: p53 is required for TGF- β gene responses by cooperating with Smads. *Cell* 113: 301–314
57. Ikegami R, Rivera-Bennetts AK, Brooker DL and Yager TD (1997) Effects of inhibitors of DNA replication on early zebrafish embryos. Evidence for coordinate activation of multiple intrinsic checkpoints at midblastula transition. *Zygote* 5: 153–175
58. Postlethwait JH, Yan Y-L, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelik P, Beier D, Joly J-S, Larhammar D, Rosa F, Westerfield M, Zon LI, Johnson SL and Talbot WS (1998) Vertebrate genome evolution and the zebrafish gene map. *Nat. Genet.* 18: 345–349
59. Waga S, Masuda T, Takisawa H and Sugino A (2001) DNA polymerase ϵ is required for coordinated and efficient chromosomal DNA replication in *Xenopus* egg extracts. *Proc. Natl. Acad. Sci. USA* 98: 4978–4983
60. Hammerschmidt M, Blader P and Strähle U (1999) In *Methods in Cell Biology*, Vol. 59, Detrich HW, Westerfield M, Zon LI (eds) (San Diego: Academic Press) pp. 87–115
61. Sugo N, Niimi N, Aratani Y, Takiguchi-Hayashi K and Koyama H (2004) p53 deficiency rescues neuronal apoptosis but not differentiation in DNA polymerase β -deficient mice. *Mol. Cell. Biol.* 24: 9470–9477
62. Kim JM, Nakao K, Nakamura K, Saito I, Katsuki M, Arai K-i and Masai H (2002) Inactivation of Cdc kinase in mouse ES cells results in S-phase arrest and p53-dependent cell death. *EMBO J.* 21: 2168–2179
63. Rich T, Allen RL and Wyllie AH (2000) Defying death after DNA damage. *Nature* 407: 777–783
64. Schilling TF and Kimmel CB (1997) Musculoskeletal patterning in the pharyngeal segments of the zebrafish. *Development* 124: 2945–2960
65. Neuhauss SCF, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki DL, Abdelilah S, Stainier DFR and Driever W (1996) Mutations affecting craniofacial development in zebrafish. *Development* 123: 357–367

Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)