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ORIGINAL ARTICLE

Zebrafish *pten* genes have overlapping and non-redundant functions in tumorigenesis and embryonic development

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In human cancer, PTEN (Phosphatase and TENsin homolog on chromosome 10, also referred to as MMAC1 and TEP1) is a frequently mutated tumor suppressor gene. We have used the zebrafish as a model to investigate the role of Pten in embryonic development and tumorigenesis. The zebrafish genome encodes two *pten* genes, *ptena* and *ptenb*. Here, we report that both Pten gene products from zebrafish are functional. Target-selected inactivation of *ptena* and *ptenb* revealed that Ptena and Ptenb have redundant functions in embryonic development, in that *ptena*−/− and *ptenb*−/− mutants did not show embryonic phenotypes. Homozygous single mutants survived as adults and they were viable and fertile. Double homozygous *ptena*−/−*ptenb*−/− mutants died at 5 days post fertilization with pleiotropic defects. These defects were rescued by treatment with the phosphatidylinositol-3-kinase inhibitor, LY294002. Double homozygous embryos showed enhanced cellular proliferation. In addition, cell survival was dramatically enhanced in embryos that lack functional Pten upon γ-irradiation. Surprisingly, adult *ptenb*−/− zebrafish developed ocular tumors later in life, despite the expression of *ptena* in adult eyes. We conclude that whereas Ptena and Ptenb have redundant functions in embryonic development, they apparently do not have completely overlapping functions later in life. These *pten* mutant zebrafish represent a unique model to screen for genetic and/or chemical suppressors of Pten loss-of-function.

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Introduction

PTEN (Phosphatase and TENsin homolog on chromosome 10, also referred to as MMAC1 and TEP1) is a tumor suppressor gene that is mutated in the germline of patients with rare autosomal dominant cancer syndromes known as Cowden's disease, Bannayan–Zonana and Lhermitte–Duclose disease (Liaw *et al*., 1997; Marsh *et al*., 1997; Zhou *et al*., 2003). *PTEN* mutations are also detected in specific cancers in various tissues, including brain, prostate and ovary (Li and Sun, 1997; Li *et al*., 1997; Ali *et al*., 1999). Indeed, many studies have demonstrated a high frequency of *PTEN* mutations or deletions in various human cancers, making *PTEN* the second most frequently mutated tumor suppressor gene after *P53* (Stokoe, 2001).

PTEN belongs to the protein-tyrosine phosphatase superfamily and the *PTEN* gene product has lipid phosphatase activity (Maehama and Dixon, 1998; Myers *et al*., 1998). PTEN specifically dephosphorylates phosphatidylinositol(3,4,5)triphosphate (PI(3,4,5)P3) at the 3-position, making it the antagonist of phosphatidylinositol-3-kinase (PI-3 kinase), which is upstream of Akt/protein kinase B (Akt/PKB). Thus, PTEN is a negative regulator of the PI-3 kinase/Akt/PKB pathway, an important pathway involved in cell growth, proliferation and survival (Sun *et al*., 1999). Therefore, loss of PTEN leads to enhanced cell survival and ultimately results in tumor formation.

PTEN is also essential for normal development and tumor suppression in the mouse (Di Cristofano *et al*., 1998). Homozygous *Pten*−/− embryos die around day 8.5 of development, demonstrating that PTEN function is essential for early embryonic development. Hemizygous *Pten*+/− mice display hyperplastic–dysplastic changes in several organs and spontaneously develop germ cell, gonadostromal, thyroid and colon tumors. Furthermore, generation of conditional mutant mice using the Cre-loxP system showed that PTEN regulates cell size and growth in several organs (Kishimoto *et al*., 2003). For example, tissue-specific deletion of *Pten* in the brain induces an enlargement of this organ associated with an increase in soma size (Backman *et al*., 2001), and specific inactivation of *Pten* in cardiomyocytes causes hypertrophy and decrease of cardiac contractibility (Crackower *et al*., 2002). In *Drosophila*, dPten mutants die at early larval stages and mutations of dPten alter the size of cells and organs and affect cell survival, proliferation and migration (Goberdhan *et al*., 1999). It has also been shown that Daf-2, the *Caenorhabditis elegans* homolog of PTEN,

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regulates longevity and dauer development (Mihaylova *et al*., 1999). These data demonstrate that PTEN has fundamental roles in the development of many different organisms.

The zebrafish represents a powerful experimental model system to analyse gene function *in vivo* and to study vertebrate developmental mechanisms as well as human disease (Lieschke and Currie, 2007). Here, we have used the zebrafish to study the role of Pten in the development and to develop a vertebrate model that will allow us to screen for genetic and chemical suppressors of Pten loss-of-function. We identified two zebrafish *pten* genes, *ptena* and *ptenb* and demonstrated here that they both encode functional proteins. Mutants were identified in each gene by target-selected gene inactivation (TSGI) and we bred the mutants to homozygosity. *Ptena*−/− and *ptenb*−/− zebrafish are viable and fertile and show no detectable phenotypes during embryogenesis. Double homozygous embryos lack all Pten activity and die around 5 days post fertilization (dpf). Their phenotype is associated with enhanced proliferation and survival. Our results indicate that Pten is essential for early development and that Ptena and Ptenb have redundant functions. However, adult *ptenb*−/− zebrafish developed eye tumors around 7 months of age, indicating that Ptena did not rescue the lack of Ptenb in these tumors.

Results

Zebrafish Ptena and Ptenb display lipid phosphatase activities

The zebrafish genome encodes two *pten* genes *ptena* and *ptenb*, which are highly homologous to the human gene (Supplementary Figure 1). Due to chromosomal duplications in fish, it is not uncommon for zebrafish to have two homologous copies of a gene that are present in one copy in mammals. *In situ* hybridization (ISH) experiments showed that the two zebrafish *pten* genes were broadly expressed throughout embryonic development (Supplementary Figure 2; Croushore *et al*., 2005). Both *pten* genes were maternally expressed and later in development the expression was restricted to the more anterior region of the developing embryo. Whether the two Pten gene products are functional is not known. We cloned *ptena* and *ptenb* into vectors allowing the production of recombinant proteins. Next, we investigated the lipid phosphatase activity of the two recombinant zebrafish Ptens (Figure 1). Both Ptena and Ptenb displayed lipid phosphatase activity and dephosphorylated the D3 position of phosphatidylinositol(3,4)bisphosphate, phosphatidylinositol(3,5)bisphosphate and PI(3,4,5)P3, with PI(3,4,5)P3 as preferential substrate, similar to human PTEN (Taylor and Dixon, 2003). These results show that the zebrafish genome encodes two Pten proteins, which are both functional *in vitro* and, like human PTEN, are selective for the D3 position with a preference towards PI(3,4,5)P3.

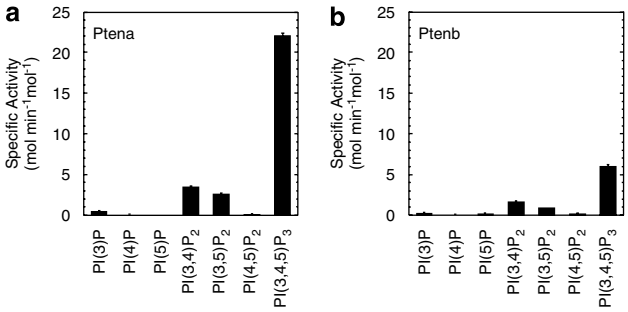


Figure 1 Zebrafish Ptena and Ptenb preferentially dephosphorylate the 3-position of phosphatidylinositol phosphates. The specific activities of bacterially expressed recombinant zebrafish Ptena (**a**) and Ptenb (**b**) towards a panel of synthetic di-C8-phosphoinositide substrates were determined using a malachite green-based assay for inorganic phosphate. Reactions were performed in triplicate and the specific activities are represented as mol phosphate released per min per mol of enzyme. The s.e. is indicated.

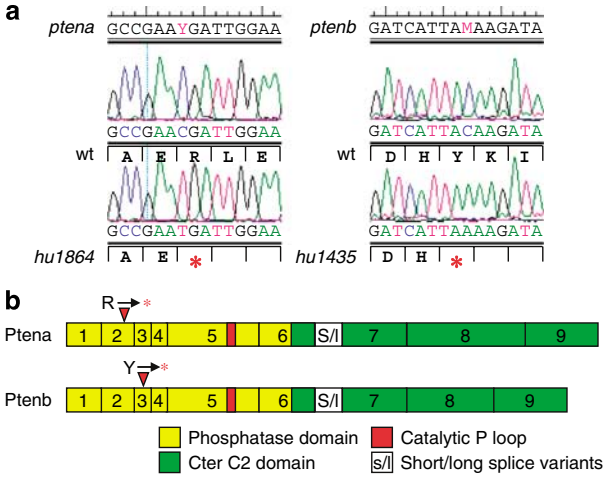


Figure 2 Nonsense mutations in zebrafish *ptena and ptenb*. (**a**) Sequence analysis of homozygous *ptena* and *ptenb* mutants. A C–T mutation in *ptena*^{hu1864} resulted in an Arg43 to STOP mutation (left) and a C–A mutation in *ptenb*^{hu1435} changed Tyr65 to STOP (right). (**b**) Schematic representation of the exon organization and structural domains of *ptena* and *ptenb*. Non-sense mutations upstream of the catalytic domain are represented by the red arrows in exons 2 and 3 for Ptena and Ptenb.

Pten mutants display severe late phenotype due to disturbed PI-3,4,5-P3 homeostasis

We used the TSGI strategy that was developed in house at the Hubrecht Laboratory (Wienholds *et al*., 2002) to identify germline mutations in the *pten* genes. Mutant alleles *ptena*^{hu1864} and *ptenb*^{hu1435} contain nonsense mutations in *ptena* and *ptenb* in exons 2 and 3, respectively (Figures 2a and b). We refer to these mutant alleles as *ptena*− and *ptenb*−, because the premature stops are well upstream of the phosphatase catalytic site and no functional Ptena or Ptenb will be produced. We generated homozygous *ptena*−/− and *ptenb*−/− fish both of which were viable and fertile. Homozygous fish lines were generated by incrossing. Embryos derived

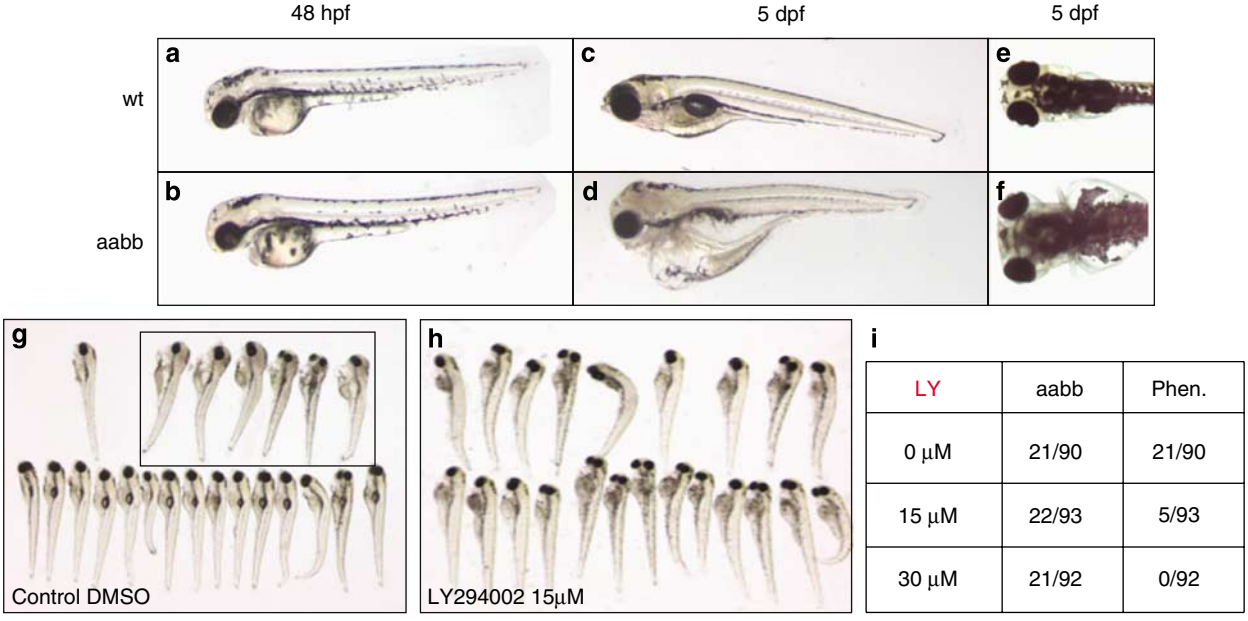
from fifth generation incrosses of *ptena−/−* or *ptenb−/−* zebrafish did not show any specific phenotype, demonstrating that either *pten* gene was dispensable for normal zebrafish development.

We then intercrossed these lines to investigate zebrafish development in the absence of any functional Pten. Double heterozygotes as well as fish with only one single *pten* allele were viable and fertile and did not display any defects. Double mutant (*ptena−/−ptenb−/−*) embryos were indistinguishable from wild-type embryos up to 48 h post-fertilization (hpf) (Figures 3a–b). However, none of these survived past 5 dpf. Indeed, at 4 dpf, double homozygous embryos displayed significant hyperplastic–dysplastic changes (Figures 3c–f). The phenotype was characterized by reduced body length, smaller eyes, enlarged head and heart edema formation. Furthermore, although the heart was still beating, there was no blood circulation and the embryos were unable to move, even following the stimulation. Notably, the eyes were set wider apart and anterior structures had not extended normally (Figures 3e–f). We tried to confirm the absence of Pten proteins in those mutants by immunoblotting and immunohistochemistry (data not shown). Unfortunately, none of the anti-zebrafish Pten specific antibodies that we generated, nor the anti-human PTEN antibodies we tested recognized the zebrafish Ptens. Our results demonstrate that the lack of Pten induced severe defects leading to lethality at 5 dpf, indicating that Pten was essential for zebrafish development.

At the molecular level, deletion of Pten leads to defects in PI-3,4,5-P3 homeostasis, resulting in hyperactivation of Akt/PKB and further downstream signalling. We surmised that re-equilibration of the PI-3 kinase/Pten balance by inhibition of PI-3 kinase might shift the balance back to normal. Indeed, exposure of embryos from incrossed *ptena+/−ptenb−/−* fish to the PI-3 kinase inhibitor, LY294002, showed a dose-dependent decrease in the number of embryos with severe phenotypes (Figures 3g–h). Subsequent genotyping confirmed that in all clutches, approximately 25% of the embryos were double homozygous mutants as expected (Figure 3i). These results demonstrate that the observed phenotypes are due to perturbed PI(3,4,5)P3 homeostasis and are, therefore, directly attributable to the lack of Pten.

Enhanced proliferation and reduced cell survival in ptena−/− ptenb−/− *mutants*

Because the double mutant fish display hyperplastic–dysplastic changes in several organs at 4 dpf, we decided to investigate the proliferation and apoptotic responses. First, we compared proliferation rates between wild-type and double mutant embryos by whole-mount immunohistochemistry using an anti-phosphohistone H3 antibody. The nuclei of proliferating cells stained positive in the developing retina (Figures 4a and b), which is known to be a proliferative tissue during the late developmental stages and even in the adult fish



LY	aabb	Phen.
0 μM	21/90	21/90
15 μM	22/93	5/93
30 μM	21/92	0/92

Figure 3 Severe late phenotype in Pten double mutants due to disturbed PI(3,4,5)P3 homeostasis. (**a**–**b**) Morphology of 48 hpf and (**c**–**f**) 4 dpf old zebrafish. Representative wild-type (wt, top) and ptena−/−ptenb−/− double homozygous mutant (aabb, bottom) are depicted. (**g**–**h**) Inhibition of PI-3 kinase rescued the Pten knockout phenotype. Clutches of embryos from ptena+/−ptenb−/− zebrafish were split and mock-treated with DMSO (**g**) or treated with 15 μM LY294002 (**h**) from 2 dpf onwards. The embryos were sorted based on their morphology at 4 dpf. Six of 22 embryos displayed the characteristic ptena−/−ptenb−/− phenotype under control conditions (box), whereas none of the LY-treated embryos showed this phenotype. (**i**) Correlation of genotype/phenotype under control conditions or after exposure to 15 or 30 μM of LY294002. aabb, number of Pten double knockout genotype over total number of embryos. Phen., number of embryos showing characteristic *ptena−/−ptenb−/−* phenotype at 4 dpf.

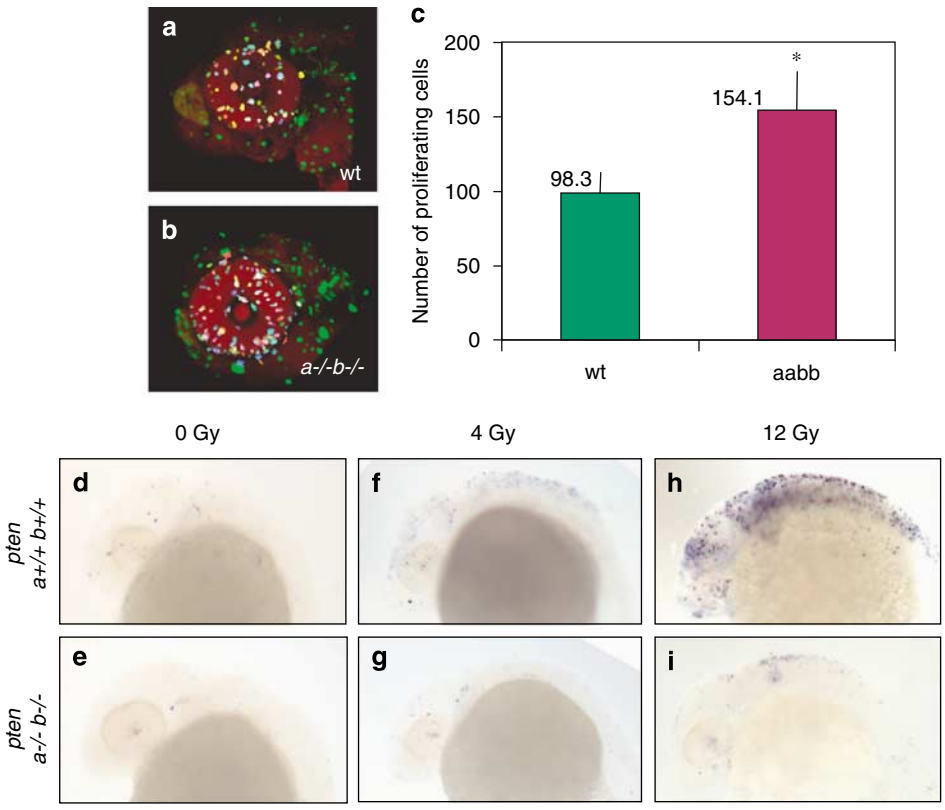


Figure 4 Enhanced cell proliferation and reduced apoptosis in *ptena−/−ptenb−/−* embryos. (**a**–**c**) Anti-phosphohistone H3 immunostaining was performed on 72-hpf-old embryos to detect proliferating cells. Lateral views of the head of a wild-type embryo (**a**) and of a double mutant embryo (**b**). All nuclei are stained with propidium iodide (red) and nuclei of proliferating, phosphohistone H3-positive cells (green) in the eye were counted using the Volocity program after confocal imaging. (**c**) Quantification of proliferating cells. The bars represent the average number of phosphohistone H3-positive nuclei per eye of wild-type (wt, $n=10$) and double mutant (aabb, $n=10$) embryos. The error bars represent the s.e. of the mean. The asterisk indicates a statistically significant increase in the number of proliferating cells ($P< 0.000193$) as calculated by a Student's *t*-test. (**d**–**i**) Embryos (24 hpf old) were left untreated (**d**, **e**) or γ-irradiated with 4 Gy (**f**, **g**) or 12 Gy (**h**, **i**). At 30 hpf, the embryos were fixed and whole-mount TUNEL assays were performed to label apoptotic cells. Lateral views of representative sibling (**d**, **f**, **h**) and double mutant embryos (**e**, **g**, **i**) are depicted.

(Marcus *et al*., 1999). Proliferative cells were visualized as described in the experimental procedures and all of the proliferative cells were counted in 10 eyes from each genotype. At 48 hpf, no differences were observed in the numbers of proliferating cells between wild-type and the double mutant fish (data not shown). However, at 72 hpf, the number of proliferating cells in the double mutant fish increased by approximately 50% compared to the wild-type fish (Figure 4c).

Pten is known to have a role in cell survival at least in tissue culture cells growing *in vitro* and we studied the effect of the lack of Pten on the apoptotic response. Under control conditions, only a few apoptotic, TUNEL-positive cells were detected in 30 hpf double mutant embryos and their siblings. Embryos were γ-irradiated at 24 hpf and TUNEL assays were performed at 30 hpf to detect apoptotic cells (Figures 4d–i). Sibling embryos showed a massive increase in TUNEL-positive apoptotic cells in a dose-dependent manner, whereas Pten-mutant embryos displayed a much reduced response to γ-irradiation. Thus, cell survival was enhanced in γ-irradiated zebrafish embryos that lack functional Pten. Taken together, these results show that the *ptena−/−ptenb−/−* phenotype observed in the 4 dpf mutants could be linked to enhanced proliferation and survival of cells in developing zebrafish embryos.

Eye tumors in ptenb*−/− mutants display hyperphosphorylation of Akt/PKB*

Unlike the double mutants, none of the homozygous *ptena−/−* or *ptenb−/−* mutants showed defects during early developmental stages. Nevertheless, at around 7 months of age, *ptenb−/−* fish developed severe eye tumors (Figure 5a). It is noteworthy that these eye tumors developed spontaneously, at a relatively high incidence (16 of 49 homozygous *ptenb−/−* fish, that is, the tumor incidence was 33% by 18 months). Spontaneous tumor formation is extremely rare in zebrafish and we have never observed similar eye tumors in wild-type fish, nor in *ptena−/−* fish. Interestingly, while most of the affected fish displayed unilateral tumors, some of them also developed bilateral tumors that appeared to arise independently. Ocular sections indicated that the eyes were strongly dilated and filled with neurogenic tissue with cells that were organized in rosette-like structures (Figures 5b–d). The overall architecture of the eye of *ptenb−/−* fish was strikingly different from the

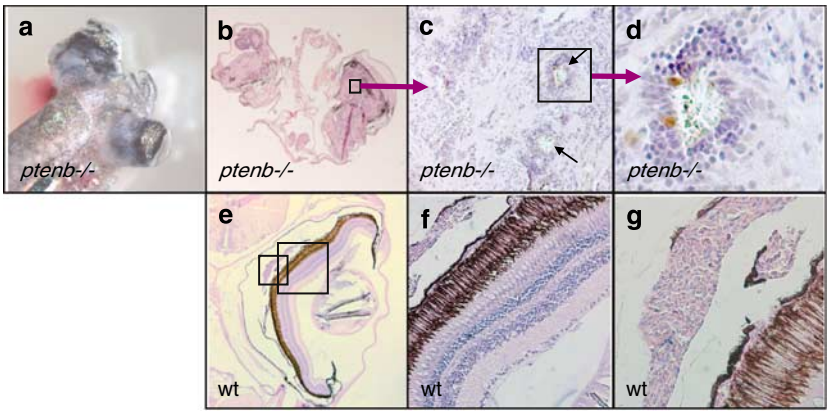


Figure 5 Ocular tumors in adult *ptenb*−/− mutants. (**a**) Dorsal view of a 7-month-old *ptenb*−/− fish head. (**b**–**d**) Transversal section of *ptenb*−/− fish head, including the tumor area (**b**) and higher magnification of the indicated areas (black boxes) (**c**, **d**). Arrows in (**c**) point to disorganized tumor tissue with cells that are organized in rosette-like structures (**d**). (**e**) Transversal section of wild-type adult zebrafish eye with magnifications of the indicated boxes showing the layered structure of the retina (**f**) and cellular morphology just outside the eye (**g**).

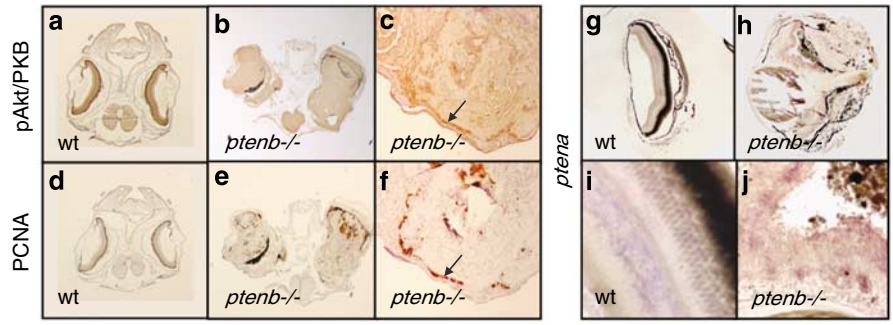


Figure 6 Co-localized pAkt/protein kinase B (pAkt/PKB) and proliferating cell nuclear antigen (PCNA) staining in ocular tumor of *ptenb*−/− fish. (**a**–**f**) Immunostaining on transversal section of *ptenb*−/− fish head. Anti-P-Thr308-Akt (pAkt/PKB) staining shows levels of phosphorylated Akt/PKB (**a**–**c**) and anti-PCNA staining (**d**–**f**) reveals proliferating cells in wild-type (**a**, **d**) and *ptenb* mutant fish (**b**, **c**, **e**, **f**). Arrows indicate overlapping staining of PCNA and phosphoAkt/PKB (**c**, **f**). (**g**–**j**) *In situ* hybridization (ISH) of eye sections from wild-type (**g**, **i**) and *ptenb*−/− (**h**, **j**) fish with a *ptena*-specific probe.

highly regular, organized structure of wild-type adult eyes (Figures 5e–g). Moreover, it appears that the observed tumors in *ptenb*−/− fish eyes grew locally outside the eye, suggesting that they were malignant. To determine if the lack of Pten could induce an over-activation of Akt/PKB leading to these tumors, we investigated Akt/PKB phosphorylation as phosphorylation correlates directly with Akt/PKB activation (Alessi *et al*., 1996). We performed immunostaining with an antibody raised against phosphorylated Akt/PKB (anti-pThr308-Akt) on sections of wild-type and mutant fish (Figures 6a–c). In the tumor region of the mutant fish, we detected areas where the level of phospho-Akt/PKB was enhanced (Figure 6c). We then performed an anti-proliferating cell nuclear antigen (PCNA) staining to detect proliferating cells (Figures 6d–f). Unlike the wild-type fish, the *ptenb*−/− mutant show high-proliferating areas in the expanded tissues (Figure 6f). Moreover, high levels of PCNA were observed in the cells with high phospho-Akt/PKB levels (Figures 6a–f). These observations indicate that hyper-activation of the PI-3 kinase/Akt/PKB pathway due to the lack of Ptenb leads to overproliferation resulting in eye tumors.

Interestingly, it seems that the lack of Ptenb is sufficient to induce tumor formation. We sequenced all exons of *ptena* and *ptenb* after laser micro-capture of tumor material and DNA extraction from the tumor section. We confirmed that the *ptenb* nonsense mutation was present and no mutations were found in the *ptena* gene (data not shown). Moreover, we performed ISH on eye sections using a *ptena*-specific probe (Figures 6g–j). *Ptena* mRNA was expressed in the wild-type as well as in the Ptenb mutant eye. These results demonstrate that Ptena is expressed in adult zebrafish eyes and does not compensate for the lack of Ptenb in tumors of *ptenb*−/− fish.

Discussion

The zebrafish genome encodes two Pten genes *ptena* and *ptenb*. Both zebrafish *pten* gene products display similar

lipid phosphatase activity when compared to human PTEN, with PI(3,4,5)P3 being the preferential substrate. Using TSGI, we identified nonsense mutations in *ptena* and *ptenb* that abolished Pten catalytic activity and function. The homozygous single mutant fish lacking Ptena or Ptenb were viable, fertile and lack any developmental defects. In the light of the early embryonic lethality of *Pten*−/− mouse embryos (Di Cristofano *et al*., 1998; Suzuki *et al*., 1998), our results suggest that Ptena and Ptenb have redundant functions in zebrafish embryogenesis. Surprisingly, it has previously been shown that morpholino (MO)-mediated knockdown of either Ptena or Ptenb induced exclusive defects during embryogenesis, suggesting that each Pten gene has a distinct role in zebrafish development (Croushore *et al*., 2005). However, our single homozygous mutants developed normally. Maternal expression of Ptena or Ptenb might account for the lack of phenotypes in our single knockout zebrafish. However, offspring of fifth generation homozygous fish do not express maternal Ptena or Ptenb, respectively. Yet, these embryos did not exhibit developmental defects either, excluding maternal contribution. We cannot exclude the possibility that truncated Pten proteins are expressed in our mutants. However, the positions of the stop mutations in ptena and ptenb, well upstream of the catalytic site (Figure 2), indicate that it is unlikely that these proteins will be functional. Therefore, our results indicate that the observed MO-induced defects (Croushore *et al*., 2005) are not due to the loss of either Pten protein.

Double homozygous embryos, however, died during embryogenesis, indicating that Pten is essential for zebrafish embryonic development. This is consistent with the observation that PTEN knockout mice die around day E 8.5. However, there appears to be a difference in the developmental stage at which the lack of PTEN is lethal between mice and zebrafish. This may be explained by maternally contributed Pten in zebrafish eggs. Indeed, ISH experiments demonstrated that *ptena* and *ptenb* are maternally expressed (Supplementary Figure 2). Apparently, the heterozygous zebrafish mother provides the eggs with sufficient Pten mRNA and/or protein to survive until 5 dpf. There are many examples of maternally contributed essential mRNAs and proteins that rescue very early lethality in zebrafish. Whereas Pten function is essential for zebrafish development, the single Ptena and Ptenb mutants show no developmental phenotypes, indicating that Ptena and Ptenb have redundant functions.

At 4 dpf, the double homozygous Pten mutant zebrafish embryos displayed major hyperplastic–dysplastic changes in several organs including the brain, the eyes and the heart. It appears that enhanced cell proliferation and survival are responsible for the observed phenotypes. Loss of function studies in mice and reconstitution experiments in mammalian cells have shown that PTEN is a key regulator of mammalian cell cycle progression. PTEN-mutant mouse embryos display regions of increased cellular proliferation (Stambolic *et al*., 1998). Loss of PTEN function and Akt/PKB activation *in vitro* result in enhanced cell survival (Li *et al*., 1998; Stambolic *et al*., 1998; Wu *et al*., 2007). For example, *pten*−/− mouse embryo fibroblasts are impaired in their response to apoptotic stimuli and *pten*+/− mutant mice develop a lymph-node hyperplasia syndrome resulting from defects in FAS-mediated apoptosis (Li *et al*., 1998; Di Cristofano *et al*., 1999). Groszer *et al*. (2001) have also shown significant increases in the number of TUNEL positive cells in the telencephalon of conditional Pten knockout Nestin-CrePten$^{flox/flox}$ mice. Nevertheless, due to the constraints of the mouse model, it is difficult to study the effect of loss of PTEN on apoptosis thoroughly. We provide a model where the effects of loss of Pten can be detected by the inhibition of apoptosis in the whole organism. The p53 tumor suppressor protein is known to be the key regulator of apoptosis induced by DNA damage. However, it seems that the presence of p53 in our Pten mutants is not sufficient to trigger apoptosis in response to γ-irradiation-induced DNA double-strand breaks. This is consistent with the previous studies showing that PTEN protects p53 against survival signals, by inhibiting the activation of the p53-inhibitor Mdm2 via the PI 3 kinase/Akt/PKB pathway (Mayo *et al*., 2002). Further studies of the signalling pathways involved in this phenomenon could help us to better understand the role of Pten during vertebrate development and how distinct tumor suppressor proteins cooperate.

Importantly, we have found that *ptenb*−/− fish developed tumors at around 7 months of age. Akt/PKB was hyperphosphorylated in the tumors in areas that proliferate. It is noteworthy that we also isolated a Ptenb splice donor mutant by TSGI, *ptenb*^{hu863} (splice-site mutation at the exons 3–4 boundary). Homozygous *ptenb*$^{hu863/hu863}$ mutants also developed eye tumors associated with hyperphosphorylated Akt/PKB (data not shown). Despite the apparent redundancy between the two zebrafish Pten genes during an early development, Ptena cannot compensate for the lack of Ptenb during tumor formation in adult zebrafish eyes. Loss of Ptena activity and/or expression in the eyes would explain this phenomenon. However, we demonstrated that *ptena* was not mutated and was expressed in adult zebrafish eyes and in the tumors. Perhaps, subtle differences in Ptena and Ptenb function account for the inability of Ptena to block tumor formation in the absence of Ptenb.

We have generated Pten mutant zebrafish, which show defects that are consistent with enhanced cell proliferation and survival. We demonstrated that the two *pten* genes in zebrafish have redundant functions during embryonic development and at the same time, Ptena did not protect against tumor formation later in life. These pten mutant zebrafish provide a unique model to screen for suppressors of pten loss-of-function. Using the PI-3 kinase inhibitor, LY294002, we have already provided proof-of-principle for a chemical screen, as the administration of this compound rescued the embryonic phenotype resulting from the lack of both zebrafish Ptens. In conclusion, the zebrafish pten model provides a powerful system to unravel the

role of Pten in vertebrate development and tumor formation.

Materials and methods

Fish care and generation of zebrafish Pten mutant lines
Zebrafish maintenance, breeding and staging were performed as described (Westerfield, 1995). ENU mutagenesis was performed on TL males (Wienholds *et al*., 2002) and exons 1–4 of the zebrafish *ptena* and *ptenb* genes were sequenced by using DNA from F1 generation zebrafish. Each mutation was confirmed by resequencing. Genomic DNA was extracted from adult fish or embryo tail clip obtained from anesthetization of fixed fish. The genotyping assay for the *pten* mutations was performed by nested PCR with primer sets: *ptena*, 5′-GCGCTAGTTTCTTGTTTAGATTG, 5′-CAGACTATTA TTTCCCCAAAC; nested *ptena* 5′-TGTTAACCTGGTGTA CAGTGC, 5′-TGGGCAAAATTAAAGAGACC; *ptenb*, 5′-AAAGAACAGAAATCCAGTTCCA, 5′-TGCTTAGAACT TTGCACCAA and nested *ptenb* 5′-TGTTGAGCTTTTG TTGGATGA, 5′-TGCCAAAACCAACAGAACAA, followed by sequencing to detect the mutations.

Phosphatase activities
We generated zebrafish *ptena* and *ptenb* cDNA constructs and cloned them in a pET-21 vector, allowing production and purification of recombinant Pten proteins, fused to six histidine residues in their C-termini. Ptena and Ptenb enzymatic activities were assayed exactly as previously described (Taylor and Dixon, 2003). Reactions were performed in a volume of 20 μl for various times at 37°C, then terminated by the addition of 20 μl of 0.1 M *N*-ethylmaleimide and 50 μl of malachite green reagent. The absorbance at 620 nm was measured and phosphate release was quantified by comparison to a standard curve of inorganic phosphate.

LY294002 treatment
Clutches of embryos from *ptena*+/− *ptenb*−/− zebrafish were split and mock-treated with DMSO or treated with 15 or 30 μM LY294002 (Calbiochem, San Diego, CA, USA) added to the embryo medium from 2 dpf onwards. The embryos were sorted based on their morphology at 4 dpf. The presence of mutants was confirmed by genotyping of the analysed embryos.

Whole-mount anti-phosphohistone H3 antibody staining
Embryos (72 hpf) were dechorionated and fixed overnight in 4% paraformaldehyde. Embryos were rinsed in PBS, dehydrated in PBS/0.1% Tween (PBST)/methanol series (25, 50, 75 and 100%, 5 min each at room temperature), kept in methanol 100% for at least 4 h and then brought back to PBST. Permeabilization was performed by incubating the embryos for 5 min on ice in trypsin 2.5 mg/ml (Worthington Biochemical Corporation, Lakewood, NJ, USA) and washed three times in PBST (5 min each). Embryos were blocked for 30 min with 0.1% BSA, 2% lamb serum, 0.1% DMSO, 1% Triton X-100 and incubated with a polyclonal anti-phosphohistone H3 antibody 5 μg/ml (Upstate, Charlottesville, VA, USA) in blocking buffer. Embryos were washed four times in PBST (5 min each) before the addition of anti-rabbit Alexa Fluor 488 secondary antibody 1:250 (Invitrogen, Carlsbad, CA, USA) in blocking buffer overnight at 4°C. Embryos were rinsed 10 times in PBST (10 min each) and treated with RNAse 200 μg/ml for 1 h at 37°C before incubation with propidium iodide (Molecular Probes, Leiden, The Netherlands) 2 μg/ml for 30 min at 37°C. Finally, embryos were rinsed three times in PBST (5 min each) and mounted on a slide with 1% agarose and confocal analysis was performed on a Zeiss LSM510. Stained nuclei were counted using the Volocity program (Improvision, Coventry, UK). The presence of mutants was confirmed by genotyping the analysed embryos.

Apoptosis assay
Embryos were raised at 28°C and γ-irradiated at 24 hpf (4 or 12 Gray). At 30 hpf, the embryos were fixed overnight in 4% paraformaldehyde and dehydrated in PBST/methanol series (25, 50, 75 and 100%, 5 min each at room temperature), kept in methanol 100% for at least 4 h and then brought back to PBST. TUNEL assays were performed as described (Cole and Ross, 2001). The presence of mutants was confirmed by genotyping the analysed embryos.

Paraffin embedding and sectioning
Adult fish were killed and placed in 4% paraformaldehyde at 4°C for 4 days and then transferred to 0.25 M EDTA (pH 8.0) for no less than 2 days. The fish were then dehydrated in alcohol, cleared in xylene and infiltrated with paraffin. Tissue sections (6 μm thick) from paraffin-embedded tissue blocks were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions and stained with hematoxylin–eosin.

PCNA and phospho-Akt/PKB immunostaining on sections
Paraffin-embedded fish were transversally sectioned, placed on charged slides and deparaffinized. Peroxidases were blocked by incubating the slides 15 min at room temperature in 83.2 g/l citric acid, 215.2 g/l di-sodium-hydrogen-phosphate-2-hydrate, 20 g/l sodium azide and 1.5% peroxide. For antigen retrieval, the sections were boiled 20 min in 5.88 g/l tri-sodium-citrate-2-hydrate, pH 6. Sections were blocked 20 min in 1% BSA–PBS and incubated with either rabbit anti-phospho-Thr308-Akt 1:100 (Cell Signaling, Boston, MA, USA) or mouse anti-PCNA 1:100 (Euro Diagnostica, Arnhem, The Netherlands) in blocking buffer at 4°C overnight. Slides were washed three times in PBS (5 min each) and incubated at least 20 min in 0.005% BSA–PBS and then 30 min with biotinylated secondary respectively anti-rabbit or anti-mouse antibodies. Sections were rinsed four times in PBS (10 min each) and developed in diaminobenzidine solution for 5 min.

In situ *hybridization on sections*
In situ hybridization on sections were performed as described previously (Gregorieff *et al*., 2004) using a probe specific for *ptena* (gb|AAR04345 from NCBI Protein database).

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