DISEASES AND DISORDERS

Tdp1 protects from topoisomerase 1–mediated chromosomal breaks in adult zebrafish but is dispensable during larval development

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Deficiency in the DNA end-processing enzyme, tyrosyl-DNA phosphodiesterase 1 (TDP1), causes progressive neurodegeneration in humans. Here, we generated a tdp1 knockout zebrafish and confirmed the lack of TDP1 activity. In adulthood, homozygotes exhibit hypersensitivity to topoisomerase 1 (Top1) poisons and a very mild locomotion defect. Unexpectedly, embryonic tdp1−/− zebrafish were not hypersensitive to Top1 poisons and did not exhibit increased Top1-DNA breaks. This is in contrast to the hypersensitivity of Tdp1-deficient vertebrate models reported to date. Tdp1 is dispensable in the zebrafish embryo with transcript levels down-regulated in response to Top1-DNA damage. In contrast, apex2 and ercc4 (xpf) transcripts were up-regulated. These findings identify the tdp1−/− zebrafish embryo as the first vertebrate model that does not require Tdp1 to protect from Top1-DNA damage and identify apex2 and ercc4 (xpf) as putative players fulfilling this role. It highlights the requirement of distinct DNA repair factors across the life span of vertebrates.

INTRODUCTION

Defects in DNA repair are linked to a variety of human disorders (1, 2). DNA repair protects from cancer, neurological disorders, immunodeficiency, and premature aging. One example is spinocerebellar ataxia with axonal neuropathy 1 (SCAN1), which is caused by an autosomal recessive mutation in tyrosyl-DNA phosphodiesterase 1 (TDP1), primarily causing progressive cerebellar atrophy, neuropathy, and distal muscle weakness (3). This leads to the development of an ataxic gait, areflexia, dysarthria, loss of vibration sensation, and confinement to a wheelchair by early adulthood. TDP1 repairs a variety of damaged 3′ termini, namely 3′-phosphoglycolate, 3′-deoxyribose phosphate, 3′-histidine, and, importantly, 3′-topoisomerase 1 cleavage complexes (Top1-CCs) (4–7). Studies in yeast and chicke, as well as studies using recombinant human TDP1, have shown that TDP1 also plays a role in the repair of 5′-phosphotyrosyl lesions, caused by Top2-CCs (8, 9). Top1-CCs occur because of the abortive activity of topoisomerase 1 (Top1).

TOP1 relieves torsional stresses of DNA by transiently nicking one DNA strand and creating a phosphodiester bond between its active site tyrosine and the DNA, allowing controlled rotation of the nicked strand (10). The topoisomerase-DNA intermediate is called a TOP1-CC and can turn into a persistent single-strand break or double-strand break (DSB) because of collision with replication, transcription machinery, or the presence of proximal oxidative or bulky lesions (11–15). Out of all the lesions that TDP1 repairs, TOP1-CCs are the preferred substrates and have received the most interest because of their antireplicative properties, which can be used in cancer therapy by employing agents that stabilize TOP1-CCs, such as camptothecin (CPT) and its water-soluble analog topotecan (TPT) (16).

TDP1 cleaves the phosphodiester bond between the DNA and topoisomerase 1 by first generating a TDP1-DNA intermediate, which is then hydrolyzed using the histidine-493 active site (6, 17). The H493R mutation in SCAN1 not only reduces TDP1 activity by 25-fold but also causes the accumulation of TDP1-DNA complexes; thus, it may have neomorphic properties (18, 19). It is unclear whether the SCAN1 phenotype arises because of a reduction in TDP1 activity and, thus, elevated TOP1-CC levels, accumulation of TDP1-DNA complexes, or both.

Tdp1−/− mouse models were generated by three separate groups with some evidence of mild cerebellar degeneration (19–21). Notably, Atm−/− mice also do not adequately recapitulate the most notable clinical phenotype in ataxia telangiectasia, which is neurodegeneration, raising the question whether mouse is the ideal organism to model human neurological disorders (22). In an attempt to study the physiological function of TDP1 at the whole organismal level, we used CRISPR-Cas9 to generate a zebrafish tdp1−/− model. Zebrafish are vertebrates with high genetic similarity to humans that offer external development, high fecundity, and larval transparency and reach sexual maturity quickly, allowing large-scale studies that were not previously possible in mammalian models. Here, we describe the zebrafish tdp1−/− model and characterize its phenotype. We show that adult, but not embryonic, tdp1−/− zebrafish exhibit hypersensitivity to TOP1 poisons and a very mild behavioral defect. Our findings indicate that in the embryos, tdp1 is down-regulated and, instead, apex2 and ercc4 (xpf) are up-regulated in response to Top1 inhibition. This model will be a valuable tool for the generation of a humanized SCAN1 zebrafish and will form the basis for genetic and chemical modifier screens to unravel new physiologically relevant TOP1-mediated repair mechanisms and adjuvants for TOP1-targeting chemotherapeutics.

RESULTS

Generation and validation of tdp1−/− zebrafish

Similar to mammals, zebrasfish contain a single tdp1 ortholog. The zebrafish tdp1 gene encodes a protein of 615 amino acids with a high degree of similarity to its human counterpart (Fig. 1, A and B).
Two deletion alleles, SH475 and SH476, were generated in exon 2 of the zebrafish tdpl gene using the CRISPR-Cas9 system (Fig. 2, A to C). SH475 and SH476 contained a 4– and 5–base pair (bp) deletion, respectively, which both cause a frameshift and result in a putative early stop codon 21 and 6 amino acids downstream of the deletion, respectively (Fig. 2C). The founders carrying these alleles were out-crossed to a wild-type strain to create tdpl+/+ zebrafish (tdpl<sup>SH475+/</sup> and tdpl<sup>SH476+/</sup>), which were then crossed to produce tdpl<sup>SH475/SH476</sup> and tdpl<sup>SH475/SH475</sup> (hereafter referred to as tdpl<sup>−/−</sup>) fish. Tdpl<sup>−/−</sup> animals were born at expected Mendelian ratios, had normal longevity, and appeared to be in good health (Fig. 2D). To confirm the loss of Tdpl protein in the mutants, we used a Tdpl biochemical activity assay. In this assay, a labeled oligonucleotide harboring a 3′ phosphotyrosyl moiety (3′-PY) is incubated with whole embryo lysate. If active Tdpl1 is present, then a band shift on a DNA sequencing gel is observed (Fig. 2E). The band shift denotes the cleavage of the phosphodiester bond between the phosphate and tyrosine, which mimics the bond linking the tyrosine residue in TOP1 and the 3′ terminus of DNA. We incubated lysates from 4-dpf (days post-fertilization) embryonic and adult fish with such an oligonucleotide and did not observe a band shift in tdpl<sup>−/−</sup> samples, confirming that Tdpl1 activity has been abolished by the mutation at 4 dpf (Fig. 2, F and G).

**Adult, but not embryonic, tdpl<sup>−/−</sup> zebrafish exhibit a very mild locomotion defect**

To assess potential progressive neurodegeneration in tdpl<sup>−/−</sup> zebrafish, behavioural analysis using a camera system was performed in tdpl<sup>−/−</sup>-fish and tdpl<sup>WT</sup> siblings every 2 months from 14 to 24 months of age (Fig. 3 and fig. S1). Parameters measured were the number of times each of the three speeds (low, medium, and high) were initiated (speed count), the length of time spent swimming at each speed (speed duration), total distance, and average speed. We defined low speed as <30 mm/s, medium speed as 30 to 60 mm/s, and high speed as 60 mm/s or above. Data analysis revealed a modest but significant reduction in two of six time points for low speed count, medium speed duration, and count in the tdpl<sup>−/−</sup>-fish in comparison to wild types (Fig. 3). Although an overall trend of reduced locomotion was observed across all parameters, the difference between tdpl<sup>−/−</sup>-fish and their wild-type siblings in low speed count, medium speed, high speed duration and count, total distance traveled, and average speed did not reach statistical significance (Fig. 3 and fig. S1, A and B). These data suggest a trend of mildly impaired locomotion across all parameters tested.

The fish were also subjected to swim tunnel analysis at 19 months of age (fig. S1, C to F). In this assay, the fish were placed in a tube filled with water and an increasing current was applied against them. Endurance of the fish can be determined by calculating the critical swimming speed, $U_{\text{crit}}$, as described in Materials and Methods. Critical swimming speed is dependent on the percentage of fish still swimming at each flow rate, which was not affected in tdpl<sup>−/−</sup>-fish (fig. S1D). Since the weight and length of the fish could affect the result, these parameters were measured among the fish tested and found to be equal (fig. S1, E and F). Recovery after vestibular disorientation was also examined using the “drop test” (fig. S1, G to I). Fish were dropped into a deep tank from 10 cm above the water, and time spent at the bottom part of the tank, freezing duration, and number of transitions between the top and the bottom of the tank were recorded. Wild-type fish tend to first freeze at the bottom, then swim at the bottom of the tank, and later explore the top region (23). Any deviation from this typical response could indicate vestibular defects. However, no significant differences between wild-type and tdpl<sup>−/−</sup>-fish were found in the duration of time spent in the bottom area of the tank (fig. S1G), number of transitions between top and bottom of the tank (fig. S1H), or freezing duration (fig. S1I).
To assess whether any ataxic phenotypes were present during development, we monitored the photomotor response of 4- and 5-dpf embryos with and without exposure to the TOP1 inhibitor, CPT. On the basis of the known function of tdp1 and observations in mice, CPT treatment was expected to exacerbate otherwise mild phenotypes (19, 21). Tdp1−/− females were crossed with tdp1+/− males to avoid any maternal contribution of tdp1 mRNA in the progeny. The embryos were then treated with 500 nM CPT overnight and subjected to 5-min light/5-min dark cycles (Fig. 3, G to J), which have been shown to produce a highly robust pattern of larval movement (24–28). Measurement of total distance traveled by the larvae at each 5-min cycle (Fig. 3, G and I) and in all light and dark cycles combined (Fig. 3, H and J) revealed no significant differences between tdp1−/− and tdp1+/− siblings at 4 and 5 dpf.

**Adult tdp1−/− zebrafish are hypersensitive to Top1 poisons**

TDp1-deficient human, avian, and murine cells, as well as Tdp1−/− mice, are hypersensitive to topoisomerase-linked breaks induced by CPT and its clinical derivatives, TPT and irinotecan (8, 19, 21, 29). To ascertain the role of Tdp1 in zebrafish following exposure to topoisomerase 1 poisons, 27-month-old animals were subjected to intraperitoneal injections of TPT. The fish received one daily injection of 22.5 mg/kg for two consecutive days, and their locomotion was monitored after each injection and then 24, 48, and 72 hours after the second injection (Fig. 4 and fig. S2). Locomotion analysis revealed a significant reduction in total distance traveled over 6 hours in TPT-treated tdp1−/− fish in comparison to dimethyl sulfoxide (DMSO)-injected tdp1−/− fish at 24 (Fig. 4A) and 48 hours (Fig. 4B) after the second injection. Likewise, a corresponding decrease of average speed was found in TPT-treated tdp1−/− fish in comparison to dimethyl sulfoxide (DMSO)-injected tdp1−/− fish at 24 (Fig. 4A) and 48 hours (Fig. 4B) after the second injection. TPT treatment did not significantly affect low speed count at 24 hours (Fig. 4E) but markedly reduced it at 48 hours in TPT-treated tdp1−/− fish, in relation to control tdp1−/− fish (Fig. 4F). On the other hand, low speed duration was not altered because of the treatment (Fig. 4, G and H). Medium speed count was not affected at 24 hours (Fig. 4I) but strongly reduced at 48 hours in the TPT-treated tdp1−/− fish in comparison to DMSO-injected tdp1−/− fish (Fig. 4I). Likewise, differences in medium speed duration between TPT-treated and DMSO-treated tdp1−/− fish were
**Fig. 3.** Tdp1<sup>−/−</sup> fish have a mild locomotion defect in adulthood but not at 4 to 5 dpf. (A to F) Adult zebrafish movement was recorded with a camera system for 6 hours. Time spent swimming at low (<30 mm/s) (A), medium (30 to 60 mm/s) (C), and high (E) speeds (60 mm/s or above) and count of times low (B), medium (D), and high (F) swimming speeds were plotted. Each of the 18 fish from either genotype was recorded twice (n = 36) and shown as average ± SEM. P values were calculated by two-tailed Student’s t test with Holm adjustment for multiple comparisons. (G to J) Four-dpf (G and H) and 5-dpf (I and J) embryos from a single female tdp1<sup>−/−</sup> and male tdp1<sup>−/+</sup> incross were subjected to 3 cycles of 5-min darkness and 5-min light using the photomotor response assay. (G and I) Total distance traveled in each cycle was plotted in each data point as average ± SEM. P value, two-tailed Student’s t test. (H and J) Total distance traveled in all light or dark cycles was measured. Lines indicate average ± SEM. (I) Total distance traveled each cycle was plotted as average ± SEM. P values were calculated using two-tailed Student’s t test. DMSO, dimethyl sulfoxide. *P < 0.05; **P < 0.01.
**Fig. 4. Adult tdp1−/− zebrafish are hypersensitive to TPT.** (A to P) Zebrafish (27-month-old) were intraperitoneally injected with TPT (22.5 mg/kg) or DMSO (22.5 mg/kg) on two consecutive days for a final concentration of 45 mg/kg and monitored for 1.5 hours using a camera system 24 and 48 hours after the second injection. *P* values, two-tailed Student’s *t* test with Holm post hoc analysis for multiple comparisons. Total distance traveled (A and B), average speed (C and D), low speed count (E and F), low speed duration (G and H), medium speed count (I and J), medium speed duration (K and L), high speed count (M and N), and high speed duration (O and P) were quantified after the second injection. (Q) Tissues were dissected from 29-month-old fish and treated with 14 µM CPT for 2 hours and then examined for Top1-CC accumulation using CsCl fractionation and immunoblotting, as described in Materials and Methods. (R) Quantification of (Q); two biologically independent experiments for heart and gut and four for brain; for each independent repeat, either four hearts or four guts were pooled. One brain was used for each independent repeat; ±SEM. *P* values, two-tailed Student’s *t* test. n.s., not significant. *P* < 0.05; **P** < 0.01.
not significant until 48 hours after the second injection (Fig. 4, K and L). Furthermore, no obvious differences were observed in high speed count at 24 hours after treatment (Fig. 4M), while at 48 hours, high speed count was reduced in TPT-treated tdp1−/− fish, in relation to control tdp1+/− fish (Fig. 4N). High speed duration was significantly lower at both the 24- and 48-hour time points in the TPT-treated tdp1−/− fish in comparison to DMSO-injected tdp1−/− fish (Fig. 4, O and P). All locomotion parameters were also quantified 72 hours after the second injection, but no significant differences were found (fig. S2). To summarize, TPT treatment significantly decreased low speed count, medium speed duration, and high speed duration and count in tdp1−/− animals at 48 hours after the second injection. High speed duration was also lower in TPT-treated tdp1−/− fish than in DMSO-treated tdp1−/− fish at 24 hours after the second injection. Notably, none of the locomotion parameters were significantly different in wild-type siblings before and after treatment at this dose, showing that adult tdp1−/− zebrafish are hypersensitive to increased Top1-CCs.

To confirm TPT hypersensitivity in adult tdp1−/− zebrafish, we dissected the gut, brain, and heart from 29-month-old fish and treated the tissues with CPT (Fig. 4, Q and R). Tissue lysates were subjected to cesium chloride fractionation to purify Top1-linked DNA breaks. CPT treatment caused a significant increase in Top1-CCs in the gut of tdp1−/− zebrafish in comparison to their wild-type siblings, but there were no significant differences in the brain or heart tissues. Increased Top1-CC level in the gut of CPT-treated adult tdp1−/− zebrafish is consistent with the observed reduced mobility phenotype after TPT treatment.

**Embryonic tdp1−/− zebrafish are not hypersensitive to Top1 poisons**

We next wondered whether embryonic tdp1−/− zebrafish would also be hypersensitive to Top1 poisons. Tdp1−/− fish were incrossed, and the progeny was treated with various concentrations of CPT at 4 dpf for 16 hours. As each concentration produced a range of phenotypes at 5 dpf, the fish exhibiting the most severe phenotypes, such as body curvature, brain necrosis, and lack of swim bladder were blindly selected and genotyped (Fig. 5A). From previous studies, we predicted that the most severely affected embryos should be positive for tdp1 mutation (19, 21, 30). In notable contrast to our prediction, genotyping revealed a Mendelian distribution of genotypes across the selected embryos (Fig. 5B). The lack of hypersensitivity of tdp1−/− embryos could be a result of maternal contribution of tdp1 mRNA. To address this possibility, we repeated this experiment by crossing female tdp1−/− fish to male tdp1−/− fish. Unexpectedly, the genotypes blindly selected this way were also consistent with Mendelian ratios from such a cross (Fig. 5C). To ascertain whether CPT was indeed inhibiting topoisomerase 1 in zebrafish, Top1-CC induction in 3-dpf tdp1−/− embryos was measured by cesium chloride fractionation of lysates into free protein, DNA-protein complexes and free DNA after a 2-hour CPT treatment (Fig. 5, D and E). Immunoblotting of fractions with a Top1-CC antibody revealed that CPT did induce Top1-CCs in equal levels in tdp1−/− and tdp1WT fish. Together, these results suggest that zebrafish embryos, but not adults, can tolerate Top1-induced DNA breaks despite Tdp1 loss.

**Embryonic tdp1−/− fish do not exhibit increased CPT-induced DNA double-stranded breaks**

Top1-CCs could be converted into DNA DSBs during replication. Therefore, we measured overall and local phosphorylated H2A histone family member X (γH2AX) levels in tdp1−/− embryos after CPT and ionizing radiation (IR) treatment. IR primarily causes DNA strand breaks and base modifications, both of which can trap Top1 on DNA (31, 32). First, 4-dpf embryos were treated with CPT overnight and then harvested for immunoblotting for γH2AX at 5 dpf (fig. S3, A and B). An induction of γH2AX was observed in both genotypes, without a significant increase in tdp1−/− embryos. The embryos were also treated with irradiation at 24 hpf, and overall γH2AX levels were analyzed by immunoblotting; however, similar levels of γH2AX were observed (fig. S3, C and D). As the cerebellum is selectively vulnerable in SCAN1 patients and mice, local induction of γH2AX foci in the 24-hpf cerebellum and optic tectum was also quantified. Embryos (24 hpf) were irradiated with 22 gray and fixed either immediately or after a 30-min recovery for immunofluorescence analysis (fig. S3E). Quantification of γH2AX foci revealed no significant differences in IR-induced DSBs between genotypes in both tissues (fig. S3F).

**Tdp1 is dispensable for Top1-CC repair in the zebrafish embryo**

The hypersensitivity to Top1 poisons in adult zebrafish, but not embryos, suggests the presence of a compensatory pathway during the embryonic stages, which could be either overwhelmed or inaccessible during adulthood. It has been shown that Tdp2 can repair Top1-CCs in the absence of Tdp1 (33). To investigate the possibility of compensation by Tdp2, we treated 4-dpf embryos with 500 nM CPT overnight and harvested the embryos for protein and RNA analyses. We chose a low dose of CPT (500 nM) for a longer time (16 hours) over a shorter pulse with a high dose to observe the long-term effects on transcription. Embryo lysates were incubated with a labeled oligonucleotide with a 5′ phosphothioatyrosyl moiety, mimicking Top2-CCs, and then run on a DNA sequencing gel, where a band shift indicates Tdp2 activity. No overt differences were observed between wild-type and tdp1−/− embryos (Fig. 6A). The RNA was transcribed into cDNA and used for quantitative polymerase chain reaction (qPCR) analysis of mRNA levels. Contrary to expectations, tdp2b mRNA was significantly reduced in DMSO-treated tdp1−/− embryos (Fig. 6B). This difference, however, seemed to be abrogated by CPT whereby tdp2b mRNA exhibited similar levels in tdp1−/− and tdp1WT embryos. Thus, tdp2b is not the responsible compensatory factor. We then tested the next most likely compensation candidates mre11a, mus81, ercc1-ercc4, apex2, and rbbp8. These endonucleases have been shown to have the ability for Top1-CC repair in mouse, chicken, and yeast cells (34–39). We treated 4-dpf embryos with 500 nM CPT overnight for 16 hours and harvested RNA at 5 dpf. The RNA was transcribed into cDNA, which was used as a template in qPCR (Fig. 6C). Two different primer pairs were used for apex2: one targeting both transcript 201 (ENSEDART00000021514.7) and 202 (ENSEDART000000189272.1), denoted apex2, and another one targeting only one transcript 201, denoted apex2_201. qPCR results showed no significant difference in the expression of mre11a, mus81, ercc1, ercc4, apex2, and rbbp8 between tdp1−/− and tdp1WT embryos both in DMSO- and CPT-treated conditions. However, we did note a remarkable increase in apex2 and ercc4 mRNA after CPT treatment in both genotypes. There was a significant increase in expression of apexx2 using both primer pairs; however, transcript 201 showed an even higher increase than transcripts 201 and 202 together. We also observed that CPT treatment resulted in a significant decrease in ercc1 and mus81.

To examine the mechanism/s that enable zebrafish embryos to tolerate Tdp1 loss, we conducted microarray analyses comparing gene...
expression profiles of CPT-treated tdp1−/− and tdp1WT embryos (fig. S4). RNA was extracted from 5-dpf embryos after an overnight 500 nM CPT treatment and processed for microarray investigations. Data analysis revealed 1720 transcript clusters that were differentially expressed between tdp1−/− and tdp1 WT embryos (fig. S4A, B). A total of 1021 of these transcript clusters were up-regulated, and 699 were down-regulated. As expected, tdp1 was significantly down-regulated in tdp1−/− fish (fig. S4C). The top three hits with the lowest P value were zinc finger protein 644 (znf644b), si:dkeyp-53e4.4 (Ensembl accession: ENSDARG00000114935), and CABZ01069162.1 (Ensembl accession: ENSDARG00000115161). Znf644b and si:dkeyp-53e4.4 were up-regulated by 4.88- and 10.13-fold, respectively, while CABZ01069162.1 was down-regulated by 6.54-fold. Znf644b is one of two genes encoding for the Znf644 transcription factor in zebrafish (40). It contains five C2H2 and one atypical zinc finger motif. Znf644 regulates H3K9-mediated gene silencing during neurogenesis and has been linked to autosomal dominant high-grade myopia (40–42). Si:dkeyp-53e4.4 and CABZ01069162.1 are uncharacterized fish-specific genes. CABZ01069162.1 belongs to the TF613015 PiggyBac transposable element-derived family. The expression of sprtn and neil1 was significantly increased in tdp1−/− embryos (fig. S4C). Sprtn is a DNA-dependent metalloprotease that plays a role in the resolution of DNA-protein cross-links, including Top1-CCs, while Neil1 is a glycosylase that participates in the first step of base excision repair (43–46). As sprtn and neil1 were the most likely compensation candidates from the microarray screen, we carried out qPCR to confirm these hits, as described previously for tdp2b (fig. S4D). qPCR results showed a decrease in sprtn and neil1 in tdp1−/− embryos.
in comparison to wild types, both after DMSO and CPT treatment. This decrease was only significant for *neil1*. qPCR also showed a significant decrease in *neil1* expression after CPT treatment.

The fact that none of the key Top1-CC repair enzymes were up-regulated in the *tdp1*−/− embryos led us to consider the possibility that Tdp1 is not required for Top1-CC repair at this stage. We thus investigated the expression of *tdp1* mRNA after incubation with CPT. We treated 4-dpf embryos with 500 nM CPT overnight for 16 hours and harvested RNA at 5 dpf. As expected, *tdp1* mRNA was reduced by more than 50% in DMSO-treated *tdp1*−/− embryos in comparison to wild-type siblings (Fig. 6D). To our surprise, however, *tdp1* expression was significantly reduced after incubation with CPT, suggesting that *tdp1* may not be required for Top1-CC repair in zebrafish embryos. In contrast, the expression of *ercc4* and
apex2 was increased following CPT treatment (Fig. 6C). We next generated apex2 crisps whereby mosaic mutations are introduced in the target gene using CRISPR-Cas9 to obtain a stable knockdown of the protein. A pool of four guide RNAs (gRNAs) designed across the apex2 gene was injected before or at the one-cell stage. PCR assessment of gRNA efficiency revealed an average 42% of detectable mutations (fig. S5, A and B). Treatment of the 4-dpf apex2 crisps treated embryos with CPT revealed no overt morphological differences when compared to controls (fig. S5, C and D). In contrast, recording embryo movement with a camera followed by quantification of the total distance traveled revealed a putative role for apex2 in response to Top1-CC. Whereas apex2 gRNA injections did not affect the movement of DMSO-treated embryos, it significantly reduced the total distance traveled in CPT-treated apex2 crisps in comparison to CPT-treated controls (fig. S6E). These data suggest a role for apex2 in repairing Top1-CCs in zebrafish embryos, which is consistent with recently reported in vitro biochemical assays and studies in human cells (38, 39), and lay the foundation for further validations in follow-up studies.

**DISCUSSION**

SCAN1 is caused by an active site mutation in TDPI that leads to progressive neurodegeneration. It is thought that neurodegeneration is a combined result of the accumulation of protein-linked DNA breaks, a lack of alternative repair pathways, high oxidative stress, and transcription levels in postmitotic neurons (47). However, studies show that TDPI is also required in replicating cells under genotoxic stress (30), show that TDP1 is also required in replicating cells under genotoxic breaks, a lack of alternative repair pathways, high oxidative stress, and transcription levels in postmitotic neurons (30). Next, we assessed the sensitivity to CPT in embryonic tdpi−/− zebrafish and, notably, found that, in contrast to studies in mice, human, and avian cells, tdpi−/− embryos were not hypersensitized to CPT. We note that our results here are reminiscent of a recent report in plants showing that Arabidopsis Tdp1 knockout is also not hypersensitive to TOP1-induced DNA damage (53). At the molecular level, there were no differences in Top1-CC and γH2AX levels between tdpi−/− and wild-type embryos either before or after CPT or IR treatment, confirming that loss of Tdp1 had no measurable effect in embryos, even under challenging conditions. We have found the lack of CPT sensitivity to be corroborated by an unexpected down-regulation of tdpi mRNA after CPT treatment, suggesting that tdpi is not required to repair Top1-CCs in zebrafish embryos. Instead, we show that apex2 and ercc4 are up-regulated. While apex2 crisps did not show overt morphological differences following CPT treatment, they exhibited a significant reduction in total distance traveled. This adds weight to the notion that apex2 is responsible for Top1-CC repair in zebrafish embryos. Although these findings need further experimental proof in follow-up studies, they are consistent with recent reports showing a role for apex2 in TOP1-CC repair. For a while, it has been known that the yeast apex2 ortholog apo2 has phosphodiesterase activity (53–55). Only recently, it was shown that the vertebrate Ape2 has the unique ability to process phosphotyrosine-DNA conjugates into readily ligatable DNA ends, which our data corroborate (38, 39). Ercc4 would also be an intriguing candidate for Top1-CC repair; however, we found ercc1, an essential part of the ercc1-ercc4 heterodimer, to be slightly down-regulated after CPT treatment. It is likely that zebrafish Ercc4 does not require Ercc1 for DNA binding and activity, which could explain this conundrum. It is reminiscent of Ercc4 in archaea, which do not have Ercc1 at all (56). Mus81 and neil1 were significantly down-regulated as well. This result indicates that, as well as tdpi, these factors are likely not required for Top1-CC repair in the zebrafish embryo. Tdp2b, mre11a, and rbbp8 (citi) did not show significant expression changes in response to increased Top1-CCs, and tdp2 did not show a measurable increase in activity. Despite the lack of a phenotype or CPT hypersensitivity in tdpi−/− embryos, microarray analysis identified gross transcriptional changes in CPT-treated tdpi−/− embryos in comparison to wild types. This included a slight ~1.3-fold increased expression of sprtn and neil1 in tdpi−/− embryos. However, qPCR validation showed a down-regulation in neil1, both before and after CPT treatment, and a nonsignificant trend of sprtn down-regulation in tdpi−/− embryos.

To summarize, we have generated and extensively characterized a tdpi−/− zebrafish mutant, which is hypersensitive to Top1 poisons and has a very mild locomotion defect in adulthood. We found that Tdp1 in zebrafish embryos does not appear to play a role in Top1-CC repair, which is corroborated by the lack of hypersensitivity to Top1 poisons at this stage. We show that apex2 and ercc4 are up-regulated in response of CPT treatment and are, thus, the factors that are most likely repairing this type of damage in the zebrafish embryo. Our findings are exciting because the lack of Tdp1 requirement to cope with Top1 poisons has only been observed in Arabidopsis thaliana.
(57), thereby identifying the zebrafish embryo as the first vertebrate model that does not require Tdp1 to protect from TOP1-mediated DNA damage. We propose apex2 and ercc4 (xp) as primary players protecting from TOP1-induced damage in zebrafish embryos and suggest the utility of their inhibition as adjuvants to Top1-targeting chemotherapeutics.

**MATERIALS AND METHODS**

**Zebrafish husbandry**

Zebrafish (*Danio rerio*) were housed in the Bateson Centre aquaria and fed *Artemia* or dry food (Gemma Micro, SKRETTING). Zebrafish were kept at a constant temperature of 28°C and 14-hour on/10-hour off light cycle. All work were performed in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986 under personal license I023015BA held by R.Z. and project licenses PB2866EDO and PC39B259E held by F.V.E.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was carried out as previously described (58). A probe of 1030 bp was amplified with KOD Hot Start DNA polymerase (Merck, 71086) from zebrafish cDNA using primers listed in Table 1, according to the manufacturer’s instructions. The thermocycling conditions were as follows: 95°C for 3 min, followed by 30 cycles of 95°C for 20 s, 53°C for 10 s, and 70°C for 45 s, finished with 5 min at 70°C. The products were run on a 2% agarose gel, and required bands were extracted using the QIAquick Gel Extraction Kit (QIAGEN, 28704). To increase the DNA concentration, a second round of PCR was carried out. A 20-μl transcription reaction was set up using 2 μl of the final PCR product, 1× DIG-UTP (digoxigenin-uridine triphosphate) labeling mix (Roche, 0000001127703910), 10 U of T7 or T3 RNA polymerase (Promega, P207B, P208C), 1× polymerase buffer, and 40 μl of RNaseOUT RNase inhibitor (Invitrogen, 10777019). The reaction was incubated at 37°C for 2 hours. The sample was treated with 2 μl of TURBO DNase (Life Technologies, AM1340), while sgRNA was transcribed with the MEGAshortscript kit (Life Technologies, AM1340), and hygG (pH 6) in PBSTw at least 1 hour at room temperature with gentle shaking. The reaction was terminated by replacing the BM Purple solution with BCL3 developing buffer and then fixing in 4% PFA in PBS at least overnight at 4°C. For imaging, samples were washed in a series of 5-min washes: three times in PBSTw, once in 50% glycerol (Invitrogen, 15514-011) in 2× SSC, 50% glycerol in MABTw, and 100% MABTw. The embryos were blocked in 2% Blocking Reagent (Roche, 11096176001) in MABTw for at least 1 hour at room temperature with gentle rocking. Following this, the sample was rocked for 1 hour at room temperature to complete the antibody reaction. The sample was then washed eight times for 15 min in MABTw with gentle rocking. Embryos were equilibrated in BCL3 developing buffer [100 mM tris (pH 9.5), 100 mM NaCl, 50 mM MgCl2, and 0.1% Tween 20] three times for 5 min at room temperature. The developing buffer was then aspirated and replaced with 50% BM Purple (Roche, 11442074001) in BCL3. The staining was developed by gently rocking the embryos at room temperature in tubes wrapped in foil until desired levels of staining were achieved. The reaction was terminated by replacing the BM Purple solution with BCL3 buffer and then fixing in 4% PFA in PBS at least overnight at 4°C. For imaging, samples were washed in a series of 5-min washes: three times in PBSTw, once in 25% glycerol (Invitrogen, 15514-011) in PBSTw, once in 50% glycerol in PBSTw, and once in 70% glycerol in PBSTw. The embryos were imaged on the Leica M165 FC dissecting microscope with the Leica Application Suite version 4.3.0 program.

**Generation of tdp1−/− zebrafish**

CRISPR-Cas9 was performed as described by Hruscha et al. (59). Single-cell stage embryos were injected with 2.4-μg Cas9 mRNA and single gRNA (sgRNA; 0.4 μg/μl), targeting the first coding exon of zebrafish tdp1 (Table 3), and raised. Cas9 mRNA was in vitro transcribed from 1 to 2 μg of NotI-linearized pCS2-nCas9 plasmid using the mMESSAGE mMACHINE kit (Life Technologies, AM1340), while sgRNA was transcribed with the MEGASHortscript T7 Transcription Kit (Life Technologies, AM1354), according to the manufacturer’s instructions. Once the injected animals reached sexual maturity, they were outcrossed to wild-type fish, and the

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligo (upper case, gene-specific sequence)</th>
<th>F/R</th>
<th>Tm (°C)</th>
<th>Template</th>
<th>Product size (bp)</th>
</tr>
</thead>
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<td>taatacgactcactatagggAGCACGATATCGCCAGAATT</td>
<td>F</td>
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<td>cDNA</td>
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<td>R</td>
<td>64.9</td>
<td></td>
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</tbody>
</table>
progeny were sequenced to identify founders carrying the desired mutations. The selected founders were outcrossed to a wild-type strain to generate \( tdp1^{-/-} \) zebrafish, which were later incrossed to give rise to \( tdp1^{+/+} \) zebrafish. The line was maintained in a mixed London wild-type/nacre background.

**TDP1 activity assay**

TDP1 activity assay was performed similarly to the protocol described by Meisenberg et al. (60). Embryos (4 dpf) were anesthetized and deyolked in ice-cold PBS by pipetting up and down with a 200-\( \mu \)l pipette tip. The embryos were then washed twice in PBS, homogenized with a micropestle, and lysed in lysis buffer [200 mM Hepes, 40 mM NaCl, 2 mM MgCl\(_2\), 0.5% Triton X-100, and 1X protease inhibitor cocktail (Roche, 4693159001)] for 30 min on ice. The fin clips were collected, snap-frozen, homogenized, and lysed in lysis buffer. The tissue debris was pelleted at 13,300 rpm for 15 min at 4°C, and the supernatant was collected. Ten to 600 ng of total protein were combined with 1X loading buffer (44% deionized formamide, 2.25 mM tris-borate, 0.05 mM EDTA, 0.01% xylene cyanol, and 1% bromophenol blue) and boiling at 90°C for 10 min. The sample was then loaded onto a prerun 20% Urea SequaGel (Fisher Scientific, EC-833-1) and subjected to 150-V electrophoresis for approximately 1 hour. The bands were imaged using the ChemiDoc MP imaging system (Bio-Rad, 1708280).

**Locomotion analysis**

All zebrafish locomotion analysis was carried out in the Sheffield Zebrafish Screening Unit. During the photomotor response analysis one 5-dpf embryo was added per well of a 24-well plate and acclimatized to the room for 30 min before habituation in 10% light in the ZebraBox Viewpoint system. The larval movement was then recorded during 3 cycles of 5-min darkness (0% light) and 5-min light (10% light) using ZebraLab version 3.20.5.3 software. Adult zebrafish locomotion of up to 10 animals at a time was measured in the ZebraCube (Viewpoint). The data from the first 30 min (TPT/DMSO intraperitoneal injections) or 1 hour (untreated fish movement) were removed to account for acclimatization and were analyzed using ZebraLab software version 3.22.3.9.
Swim tunnel analysis was adapted from the work of Plaut (61). Adult zebrafish were habituated in the experiment room for 1 hour before being placed into a transparent water tunnel. The water flow rate in the tunnel was then gradually increased to 6.58 cm/s, maintained for 5 min, and increased in increments of 6.58 cm/s for 5 min at a time until the animals got tired and fell into a mesh at the end of the tunnel. The fish were allowed a second attempt at swimming at a time until the animals got tired and fell into a mesh at the end of the tunnel. The fish were then recorded when they fell into the mesh.

Top1-CCs, according to Chiang (62). Thirty to 40 3-dpf zebrafish embryos or tissues were homogenized and lysed in 1.1-ml lysis buffer (8 M guanidine hydrochloride, 30 mM tris-HCl (pH 7.5), 10 mM EDTA, and 1% sarkosyl (pH 7.5)) for 15 min at 65°C. Embryos or tissues were then centrifuged at 16,000 × g for 10 min; then, 1 ml of the supernatant was carefully layered on top of each other to form a gradient (from bottom to top: 1.45, 1.5, 1.72, and 1.82 g/ml) in a 5-ml polyallomer centrifuge tube (Beckman, 326819). The lysate was centrifuged at 30,000 rpm in a Beckman Ultima LE-80K ultracentrifuge with a swinging rotor (2/2) 40 This manuscript.

Measurement of Top1-CCs by fractionation

In vivo complex of enzyme assay was used to purify and quantify Top1-CCs, according to Chiang et al. (62). Thirty to 40 3-dpf zebrafish embryos or tissues were homogenized and lysed in 1.1 ml lysis buffer [8 M guanidine hydrochloride, 30 mM tris-HCl (pH 7.5), 10 mM EDTA, and 1% sarkosyl (pH 7.5)] for 15 min at 65°C. One-milliliter aliquots of cesium chloride (CsCl) in a range of densities were gently layered on top of each other to form a gradient (from bottom to top: 1.45, 1.5, 1.72, and 1.82 g/ml) in a 5-ml polyallomer centrifuge tube (Beckman, 326819). The lysate was centrifuged at 16,000 g for 10 min; then, 1 ml of the supernatant was carefully layered on top of the CsCl gradient. The layered sample was centrifuged at 30,000 rpm in a Beckman Ultima LE-80K ultracentrifuge with a swinging rotor for 24 hours at 25°C and stopped gradually without a brake. In the meantime, 10 μl of the remaining lysate was made up to 100 μl in 1× TE buffer [10 mM tris and 1 mM EDTA (pH 8)] and incubated with chloride (Sigma-Aldrich) or 30% DMSO in sterile Hank’s buffered solution (Gibco, 11530476). A daily dose of TPT hydrochloride (22.5 mg/kg) was injected for two consecutive days for a final concentration of 45 mg/kg in a total volume of 10 μl.

### Table 5. RT-qPCR probes.

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<th>Name</th>
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<th>F/R</th>
<th>Product size (bp)</th>
<th>Target exon(s)</th>
<th>Targeted splice variants</th>
<th>No. of qPCR cycles</th>
<th>Source</th>
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<td>Same as target gene</td>
<td>Bower et al. (66)</td>
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<tr>
<td></td>
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<td>tdp2b</td>
<td>TTGAAGCCGCAATGCTGAAA F</td>
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<td>2, 3</td>
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<td>45 This manuscript</td>
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<tr>
<td></td>
<td>AGCTTTGCTTCCATCTTCGAC R</td>
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<td></td>
<td>CTGTCCTCCCCTTTCTGAC R</td>
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<td>TCTCATGACGCCAGCATCTTG R</td>
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<td></td>
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<td>tdp1</td>
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<td>12, 13</td>
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<tr>
<td></td>
<td>ATGTTCCAGATTCGGAAGCGG R</td>
<td></td>
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</table>
RNase A (ribonuclease A; 0.5 µg/ml) at 37°C overnight. Fifty microliters of the RNase-digested sample or 1× TE buffer was mixed with an equal volume of 1× TE buffer with a 1:200 dilution of PicoGreen (Invitrogen, P7581). λ DNA standard (5 µg/ml) was also mixed with 1× TE buffer with PicoGreen, and a serial dilution was carried out to obtain a range of DNA standards (125 ng to 5 µg). DNA concentration was quantified in a FLUOSsar Omega microplate reader (BMG) with fluorescence at EX485-12/EM520. Fractionated lysates were collected by piercing the bottom of the tube with a 19-gauge syringe needle (positioned at 45° with the bevel upward), connected to a Pharmacia Biotech P-1 peristaltic pump with a silicone tube. Each sample was collected in 10 fractions of 0.5 ml. Fractions with equal double-stranded DNA amounts between samples (maximum, 200 µl) were subjected to slot blotting onto a PBS-wetted 0.45-µm nitrocellulose membrane (GE Healthcare, 106000002). The membrane was air-dried and subjected to immunoblotting with a 1:2000 dilution of a Top1-CC antibody (Table 2).

Western blotting
Zebrafish embryos were deyolked by trituration in PBS, then homogenized, and lysed in 1 to 1.5 µl of lysis buffer [200 mM Hepes, 40 mM NaCl, 2 mM MgCl2, 0.5% Triton X-100, and 1× protease inhibitor cocktail (Roche)] per embryo. Total protein concentration was determined using Bradford reagent (Bio-Rad); then, 100 µg per lane were run on a 15% SDS–polyacrylamide gel electrophoresis gel and transferred onto a 0.45-µm nitrocellulose membrane (Bio-Rad, 170-4271) using the Trans-Blot Turbo Transfer System (Bio-Rad, 17001915), according to the manufacturer’s instructions. The nitrocellulose membrane was blocked in blocking buffer [5% milk, 200 mM tris, 140 mM NaCl, and 0.1% Tween 20 (pH 7.4)] for 1 hour at room temperature and then incubated at 4°C overnight with the primary antibody in blocking buffer. The membrane was then washed three times for 5 min in 1× TBST buffer [200 mM tris, 140 mM NaCl, and 0.1% Tween 20 (pH 7.4)] and incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody in blocking buffer at room temperature. The three washes were repeated before adding the Clarity Western ECL blotting substrate (Bio-Rad, 1705060) onto the membrane. Bands were visualized in the ChemiDoc MP imaging system (Bio-Rad, 1708280) and quantified using Image Lab version 4.1 (Bio-Rad) software. Details of antibodies are provided in Table 2.

TDP2 activity assay
Cy5.5-labeled substrate oligomer (100 pmol) was combined with 100 pmol of a 20-bp complementary oligonucleotide with a 5’ overhang in a total volume of 33.3 µl (Table 4). The sample was denatured at 95°C for 5 min and reannealed by dropping the temperature by 2°C/ls for 5 s and then by 0.1°C/s for 600 s. Once annealed, 3 µM double-stranded substrate oligomer with a 5’ overhang was generated. We hypothesized that such a substrate should prevent ligation of product by zebrafish RNA ligases and, thus, undesirable full repair. Zebrafish were deyolked and lysed as described for the TDP1 activity assay. A total of 0.5, 1, and 5 µg of the lysate were combined with 1× TDP2 activity assay buffer [5 mM tris (pH 7.5), 5 mM KCl, 0.1 mM DTT, bovine serum albumin (10 µg/ml), and 0.1 mM MgCl2], 60 nM Cy5.5-labeled substrate oligomer, and 2 µM competitor oligo. The bands were imaged using the ChemiDoc MP imaging system (Bio-Rad, 1708280).

Reverse transcription qPCR
Thirty-five to 45 zebrafish embryos were homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted, according to the manufacturer’s instructions. The NanoDrop (Thermo Fisher Scientific) was used to quantify RNA; then, 1 µg of RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA from all conditions was pooled, serially diluted to 20, 4, 0.8, and 0.16%, and run alongside unknown samples to serve as a standard curve for extrapolating the sample concentration. Two microliters of a 1:16 dilution of individual cDNA was used in a 20-µl qPCR reaction with SensiMix SYBR no-ROX master mix (Bioline) in a Rotor-Gene 6000 real-time thermocycler (Corbett Research) under the following conditions: 95°C for 10 min, followed by 40 to 45 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s (Table 5). All samples were run in duplicate and normalized to rps29. Quantification was performed using the Rotor-Gene Q version 2.3.5 software. Primer sequences and other details are listed in Table 5.

Microarray
Zebrafish embryos were homogenized in TRIzol reagent (Invitrogen) to extract RNA, according to the manufacturer’s protocol. RNA was then sent to the Affymetrix Microarray Core Facility at the University of Sheffield. There, samples were processed using the Affymetrix WT plus protocol. Total RNA (200 ng) was taken forward to biotin labeling. Samples were hybridized using the Affymetrix standard protocol and the hybridization, wash, and stain solutions. Hybridization was carried out overnight at 45°C for 16 hours in a rotating hybridization platform. Post-hybridization stringency washing was performed using the Affymetrix fluidics station, following the protocol outlined in the Affymetrix instructions. The samples were scanned on an Affymetrix GeneChip Scanner 3000, according to the manufacturer’s protocols. Raw data were processed and Robust Multi-array Average (RMA) normalized using the Transcriptome Analysis Console version 4.0 (Applied Biosystems).

Apex2 crisprants
Wild-type embryos at one-cell stage and under were injected with a pool of four gRNAs against genomic apex2 (Table 3). Each embryo was injected with 1 nl of a solution containing 2.5 fmol each gRNA (total, 10 fmol), 10 fmol of Cas9 protein (NEB, M0386), and 10 fmol of trans-activating CRISPR RNA (tracrRNA). gRNA efficiency was determined by PCR on DNA extracted from single embryos. DNA extraction was carried out using the HotSHOT DNA isolation method. A total of 25 µl of 1× base solution (1.25 M KOH crystals and 10 mM EDTA) was added to each embryo and incubated for 30 min at 95°C. Tubes were vortexed, and 25 µl of 1× neutralization solution (2 M tris-HCl in

Table 6. apex2 genotyping primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>F/R</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apex2</td>
<td>ACACACCTCATGGGCCCATAC</td>
<td>F</td>
<td>IDT</td>
</tr>
<tr>
<td></td>
<td>TGGTACCATAGCAACCAATA</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>

Milli-Q water) was added before vortexing again; the DNA was kept on ice. The solution was centrifuged for 2 min at 4200 rpm. PCR was carried out using primers listed in Table 6. Embryo images were taken using the Leica M165 FC dissecting microscope with Leica Application Suite version 4.3.0; total distance traveled was quantified using ImageJ, as described by Meijering et al. (63), after correcting for camera movement.

Statistical analyses

For adult behavioral analysis, n = 36 corresponds to each of the 18 fish recorded twice. P values were calculated using two-tailed Student’s t test with Holm adjustment for multiple comparisons. For embryonic behavioral analysis, P values were calculated between tdp1 WT and tdp1 Δ/Δ pairs from n = 39 (4 dpf) or n = 14 (5 dpf) for tdp1 WT DMSO, n = 49 (4 dpf) or n = 25 (5 dpf) for tdp1 Δ/Δ DMSO, n = 43 (4 dpf) or n = 15 (5 dpf) for tdp1 WT CPT, and n = 45 (4 dpf) or n = 22 (5 dpf) for tdp1 Δ/Δ CPT using a two-tailed Student’s t test with Holm adjustment for multiple comparisons. In the swim tunnel test, n = 6 and the P value for the “survival” curve was calculated using the Mantel-Cox test, while for the tunnel test, a 2-sided Student’s t test with Holm post hoc analysis for multiple comparisons; in the drop test (n = 39 (4 dpf) or n = 14 (5 dpf)) for IR0 H2AX, n = 8 for IR30 H2AX, and n = 10 for IR0 H2AX, while n = 10 for IR30 H2AX. Embryo images were plotted ±SEM. P values were calculated using two-way ANOVA with Sidak post hoc analysis. All values were plotted ±SEM. P values are indicated as follows: not significant, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/5/eabc4165/DC1

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES


38. C. TDP1. reversed by the


zebrafish mutant and performed all experiments. R.Z., F.v.E., and S.F.E.-K. analyzed and interpreted the data. R.C.T. performed and analyzed the apex2 crispant experiments. R.Z. and S.F.E.-K. wrote the manuscript. All authors edited the manuscript. S.F.E.-K. conceived the study. S.F.E.-K. and F.v.E. supervised and managed the project. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Ringaille Zakauskaite, Ruth C Thomas, Freek van Eeden and Sherif F. El-Khamisy

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