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The DNase domain-containing protein TATDN1 plays an important role in chromosomal segregation and cell cycle progression during zebrafish eye development

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The DNase domain-containing protein TATDN1 is a conserved nuclease in both prokaryotes and eukaryotes. It was previously implicated to play a role in apoptotic DNA fragmentation in yeast and *C. elegans*. However, its biological function in higher organisms, such as vertebrates, is unknown. Here, we report that zebrafish TATDN1 (zTATDN1) possesses a novel endonuclease activity, which first makes a nick at the DNA duplex and subsequently converts the nick into a DNA double-strand break in vitro. This biochemical property allows zTATDN1 to catalyze decatenation of catenated kinetoplast DNA to produce separated linear DNA in vitro. We further determine that zTATDN1 is predominantly expressed in eye cells during embryonic development. Knockdown of TATDN1 in zebrafish embryos results in an abnormal cell cycle progression, formation of polyploidy and aberrant chromatin structures. Consequently, the TATDN1-deficient morphants have disordered eye cell layers and significantly smaller eyes compared with the WT control. Altogether, our current studies suggest that zTATDN1 plays an important role in chromosome segregation and eye development in zebrafish.

Introduction

TATD, known as a DNase domain-containing protein, is a conserved gene that is found in organisms across all kingdoms from archaebacteria to eukaryotes.[1] TATD was initially reported as a gene member of the operon of the *Escherichia coli* (*E. coli*) twin-arginine translocation (Tat) system that contains TATA, TATB, TATC and TATD.[2] The TATA, TATB and TATC genes in the Tat operon encode proteins that help the translocation of other fully folded proteins across the cytoplasmic membrane of most prokaryotes and the thylakoid membrane of plant plastids.[3] However, TATD in the operon is a cytoplasmic protein that not required for the export of Tat-targeted substrate.[4] Instead, TATD, which contains a putative DNase domain, was shown to have a DNase activity in the presence of Mg^{2+} ion.[4] Biochemical assays revealed that in *Saccharomyces cerevisiae*, TATD is both an endo- and 3' exonuclease.[1] It has been shown that in *Caenorhabditis elegans, S. cerevisiae* and Leishmania, TATD play a critical role in degradation of apoptotic DNA.[1,5-8] More recently, a study has also suggested that TATD is involved in programmed cell death in the protozoan parasite *Trypanosoma brucei*.[9]

It is unclear whether *TATD* nucleases in vertebrates also function in apoptotic DNA fragmentation. Unlike lower organisms, there are three *TATD* genes, *TATDN1, TATDN2* and *TATDN3*, that contain the *TATD* nuclease domain in vertebrates. Of these three genes, *TATDN1* is the most highly conserved, with TATD in lower organisms. To determine the role of TATDN1 in vertebrates, we cloned and purified TATDN1 from zebrafish and assayed its biochemical activities in vitro. We demonstrated that zebrafish TATDN1 (zTATDN1) is an endonuclease with distinct properties from its counterpart in yeast and *C. elegans*. Unlike yeast TATDN1, which degrades DNA duplex, zTATDN1 first makes a nick and subsequently converts the nick into a DNA double-strand break in DNA duplexes. This biochemical property allowed zTATDN1 to catalyze the decatenation of kDNA, which are circular DNA catenated to each other, producing linear DNA molecules. Furthermore, we demonstrate that knockdown of zTATDN1 in zebrafish fertilized eggs results in a delay

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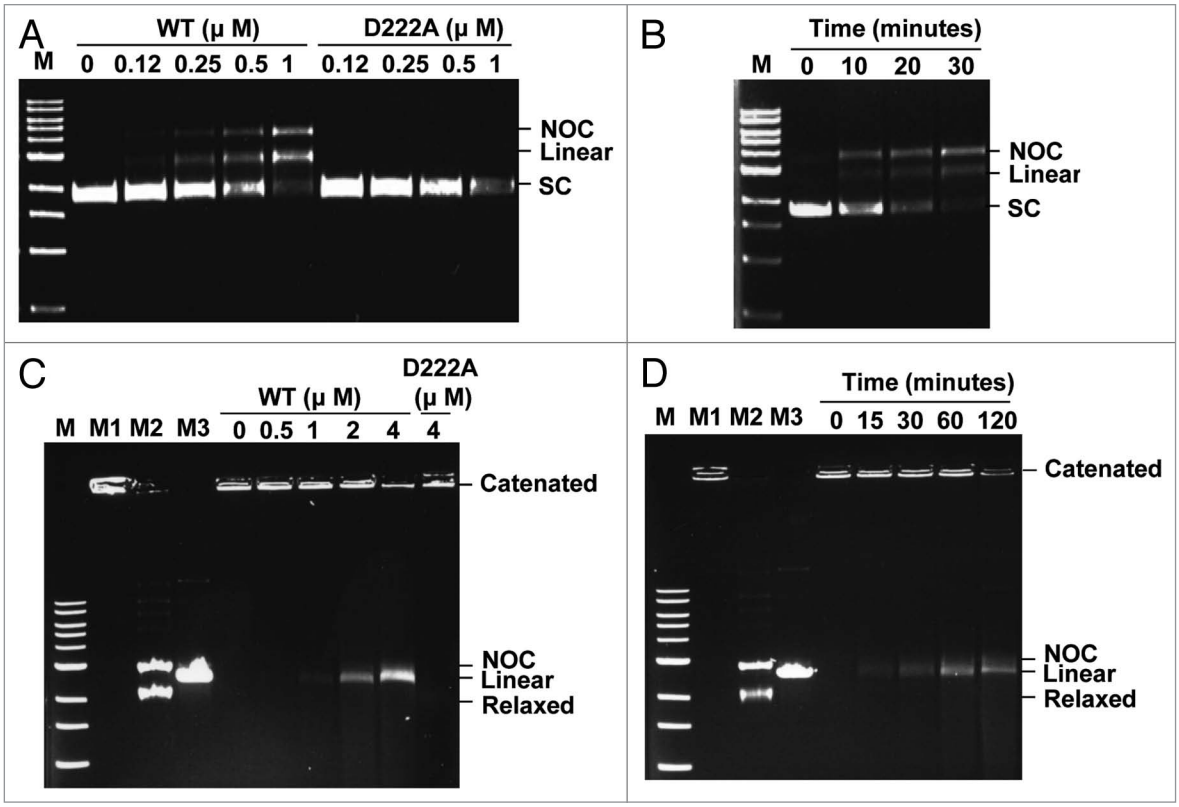

Figure 1. Purified recombinant zTATDN1 can cleave supercoiled pUC19 plasmid DNA and decatenated kDNA. (**A**) Purified recombinant TATDN1 cleaved supercoiled plasmid into relaxed, open circular and linearized DNA. The D222A point mutation in the active site of TATDN1 abolished most of this activity. (**B**) TATDN1 (1 μM) cleaved the supercoiled pUC19 plasmid in a time-dependent manner. (**C**) Purified TATDN1 decatenated kDNA into nicked open circular and linearized DNA; however, the D222A mutant TATDN1 could not. (**D**) TATDN1 (1 μM) cleaved kDNA in a time-dependent manner. Marker: 1 kb DNA ladder (NEB); M1, kDNA; M2, decatenated DNA (TOPOII treated); M3, X*ho*I treated kDNA; NOC, nicked open circular DNA; Linear, Linearized DNA; SC, supercoiled DNA; Relaxed, relaxed DNA.

in the cell cycle progression, formation of polyploidy and aberrant chromatin structures. Consequently, TATDN1 deficiency causes abnormal eye development in zebrafish.

Results

Zebrafish TATDN1 nuclease can catalyze DNA decatenation. Previously, we showed that yeast TATDN1 has both endonuclease and exonuclease activities. It cleaves plasmid DNA into nicked, relaxed DNA, and then degrades it into small DNA fragments, and it plays an important role in apoptotic DNA fragmentation in the yeast cell.[1] To determine if vertebrate TATDN1 can also mediate DNA fragmentation, we cloned, expressed and purified zebrafish TATDN1 (zTATDN1); the purity of recombinant TATDN1 proteins was obtained and confirmed by silver staining (**Fig. S1**). We assayed the nuclease activity of zTATDN1 on supercoiled plasmid DNA in vitro. Surprisingly, we found that unlike yeast TATDN1, zTATDN1 cleaved the supercoiled plasmid to produce a nicked, relaxed form and a linearized form of DNA (**Fig. 1A and B**). Only when plasmid DNA was incubated with excessive zTATDN1 (> 4 μM) was it degraded into small fragments (data not shown). Production of the nicked and the linearized DNA forms was abolished by a point mutation in the nuclease domain, D222A (**Fig. 1A**). These findings suggested that zTATDN1 was an incision nuclease, cleaving regular circular plasmid in the supercoiled form to produce DNA double-strand breaks. The cleavage pattern of supercoiled DNA by zTATDN1 was similar to that of METNASE, which has nuclease activity and is suggested to be involved in DNA decatenation and chromosome segregation.[10,11] Therefore, we hypothesized that zTATDN1 might also play a role in DNA decatenation. To assay the DNA decatenation activity of zTATDN1, we incubated zTATDN1 with catenated kDNA and generated linear DNA and a small amount of nicked open circular DNA (**Fig. 1C and D**). In contrast, the nuclease-deficient mutant D222A did not decatenate kDNA into linear DNA (**Fig. 1C**), suggesting that the endonuclease activity of zTATDN1 is critical for DNA decatenation in vitro.

TATDN1 deficiency delays cell cycle progression and induces polyploidy of zebrafish eye cells. To investigate the in vivo role of zTATDN1, we first analyzed the expression of TATDN1 during different zebrafish developmental stages. At the one- or two-cell stage, we observed considerable TATDN1 mRNA, which is likely to be the maternal mRNA (**Fig. 2**). Between 50%-epiboly and the 13-somite stage, there was no obvious TATDN1 expression pattern detected. From 14-somite

to Prim-5 stage, TATDN1 was predominantly expressed in zebrafish retina and tectum, and after that, expression spread to the dorsal telencephalon (**Fig. 2**). The expression of TATDN1 in optic vesicles suggested that the nuclease may play a critical role in the development of the zebrafish eye.

During DNA replication, it is thought that newly synthesized DNA strands may be catenated and the topologically entangled DNA strands must be decatenated to allow proper chromosome segregation.[12,13] Because TATDN1 decatenated kDNA in vitro, we thought that it may play a role in DNA decatenation during chromosome replication and segregation. To test this hypothesis, we injected the fertilized zebrafish egg with morpholino oligos, which targeted either the splice site (O3-MO) or the sequence flanking the ATG start codon (ATG-MO) of zebrafish TATDN1, to inhibit its expression throughout zebrafish development. At 48, 72 and 96 h post-morpholinos injections, TATDN1 protein levels in the embryos injected with O3-MO or ATG-MO decreased by 90% compared with embryos without injection (control) or those injected with standard morpholinos (STD-MO) (**Fig. 3**). We then examined DNA replication and progression of the cell cycle during embryo development. First, we injected EdU into the morphant embryos at different development stages and analyzed the number of EdU-incorporated cells at 1 h post-EdU labeling. On day 1, the number of eye cells in the ATG-MO and O3-MO embryos that are EdU-positive are similar compared with STD-MO embryos (**Fig. 4A**). This is likely due to the fact that TATDN1 is a maternal protein and morpholino knockdown of TATDN1 does not significantly decrease its protein level until day 2; therefore, at this time point, morphants were not significantly affected. At day 3, the number of EdU-positive eye cells in STD-MO embryos decreased approximate 80%, most likely because most of the cells had finished DNA synthesis, and only cells in and near the ciliary marginal zone (CMZ) were still proliferating (**Fig. 4A**). The CMZ, a stem cell niche, has long been known as a source of postembryonic neuronal production in the retinas of fish.[14] However, the level of EdU-positive cells remained at 32% (ATG-MO) or 30% (O3-MO) at day 3 in the morphant embryos, indicating a delay in the cell cycle progression (**Fig. 4A**). To analyze the impact TATDN1 knockdown had on the delay in cell cycle progression, we isolated eye cells from embryos of different stages and analyzed the cell cycle stages by FACS. **Figure 4B** shows that the percentage of cells in S phase decreased rapidly from day 1 to day 5 in control and STD-MO, but smoothly in ATG-MO and O3-MO. **Table 1** contains one representative data selected from four individual experiments. Consistent with the EdU-staining result, we found that on day 1, the proportion of S phase eye cells from control and STD-MO embryos were 41 and 42% (**Table 1**). At day 5, the proportion of S phase eye cells decreased to approximately 7% for both control and STD-MO embryos (**Table 1**), indicating that over 80% of the cells had completed DNA replication and cell division and had likely become differentiated cells. In contrast, on day 1, the proportion of S phase cells from the O3-MO and ATG-MO embryos were 34 and 30% (**Table 1**). On day 5, the level of S phase cells from the ATG-MO and O3-MO morphant embryos were 15 and 23%, respectively (**Table 1**), suggesting that the knockdown of TATDN1 resulted in a delay of cell cycle progression.

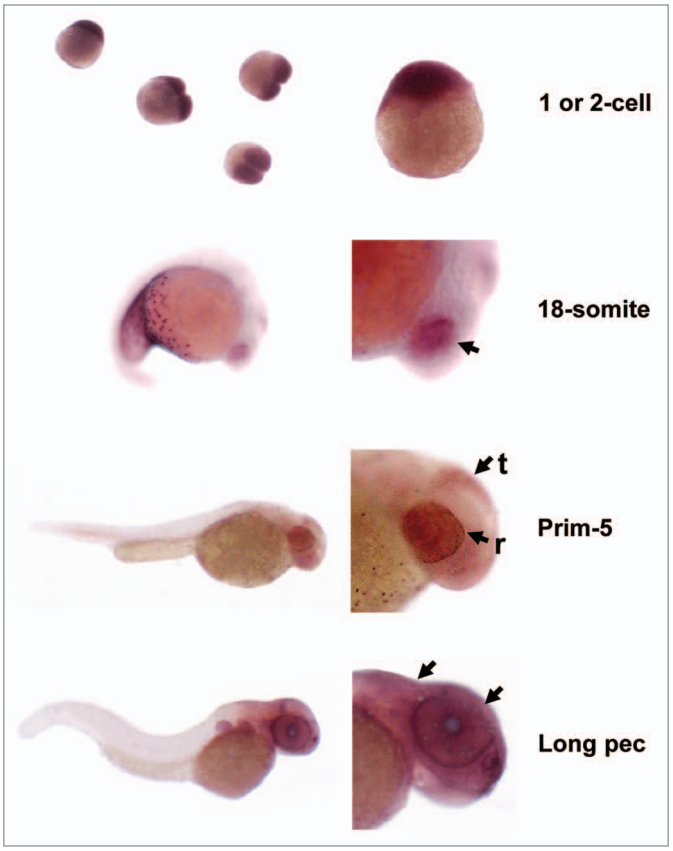

Figure 2. Zebrafish TATDN1 is predominantly expressed in eye cells at certain developmental stages. The pCS2-*TATDN1* plasmid was the template for synthesis of a digoxygenin-labeled antisense RNA probe and the WISH assay was performed to analyze the expression pattern of *TATDN1* at different developmental stages. TATDN1 was obviously expressed in the optic vesicle from 14-somite until Prim-5 stage and showed a basal level of expression in the dorsal telencephalon after Prim-15 stage. t, tectum; r, retina. Arrows mark the TATDN1 expression.

To determine if the cell cycle delay resulted in chromosomal instability in morphant embryos, metaphase spreads were performed and analyzed. zTATDN1 morphant embryos contained 67.21% polyploid cells compared with 15.75% in STD-MO embryos, representing more than a 4-fold increase (**Fig. 4C**). Taken together with the previous results, indicating that zTATDN1 can decatenate DNA in vitro, the cell cycle delay in zTATDN1-knockdown cells was probably due to TATDN1 deficiency-induced defects in DNA decatenation. Meanwhile, it might also result in chromosome segregation errors or failure, leading to polyploidy formation and aberrant chromatin structure in zebrafish.

Knockdown of zTATDN1 results in developmental defects on zebrafish eye development. In addition to the cellular defects described above, the O3-MO and ATG-MO morphant embryos displayed a series of abnormal phenotypes, including delayed embryonic development, smaller eyes, incomplete yolk

Table 1. Percentage of eye cells in difference cell cycle stages with or without TATDN1 knockdown

	Control			STD-MO			ATG-MO			O3-MO		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Day 1	53.79	41.02	5.19	54.21	42.01	3.78	61.76	34.34	3.89	65.15	29.86	5.0
Day 2	67.54	29.03	3.43	73.08	25.42	1.5	60.65	33.67	5.68	71.87	25.19	2.94
Day 3	86.45	9.61	3.95	83.03	9.46	7.5	58.0	39.25	2.75	58.63	37.81	3.56
Day 4	89.97	6.39	3.64	88.76	8.23	3.01	75.74	17.84	6.42	65.74	25.86	8.4
Day 5	85.86	7.33	6.8	88.77	6.96	4.27	81.46	15.22	3.32	72.53	22.73	4.73

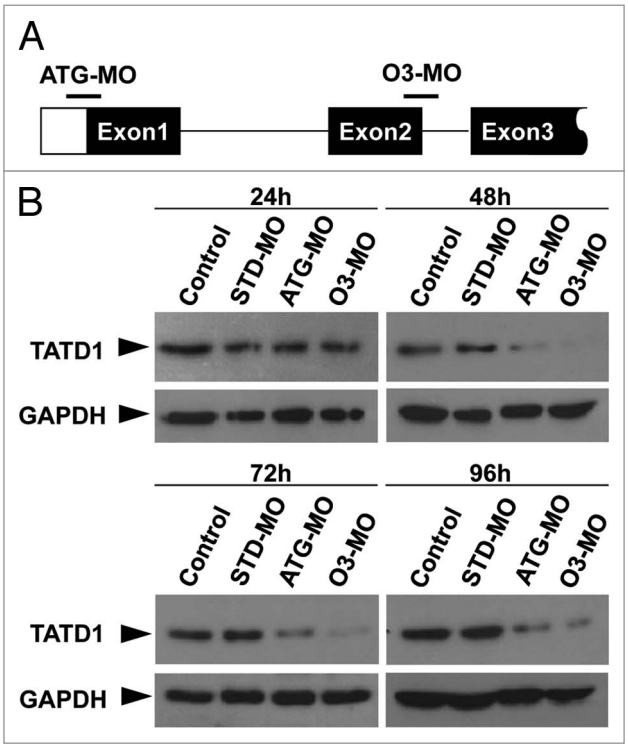

Figure 3. Decrease of the TATDN1 protein level after knockdown. (**A**) Schematic of the morpholinos target sites. (**B**) The eggs with or without morpholino injection were collected at different time points. The whole-embryo proteins were extracted and detected with western blot using anti-TATDN1 antibody. STD-MO, standard human β-globin antisense morpholino.

absorption and swimming defects when compared with the control or STD-MO embryos (**Fig. 5A**). These defects became more pronounced as embryonic development progressed from 2 d post-fertilization to 5 d post-fertilization, and the O3-MO or ATG-MO zebrafish could not survive longer than 10 d. The most striking phenotype was that O3-MO ant ATG-MO zebrafish had small eyes. The eye area in the mutant zebrafish was significantly smaller than that of the control embryos (**Fig. 5A and B**), in parallel with the cell cycle delay. To eliminate the possibility that the phenotypes were due to the morpholino off-target effects,[15] we did the same experiment in $p53^{-/-}$ embryos that potentially suppress off-target effects.[15] Similar phenotypes were observed in the $p53^{-/-}$ embryo injected with O3-MO and ATG-MO morpholinos (data not shown), suggesting that the developmental defects we observed were specific to the knockdown of zTATDN1 in the zebrafish embryos. To further analyze the eye development defect, we stained the nuclei of developing embryos. The morphant embryos had obviously fewer eye cells than the control or the STD-MO embryos (**Fig. 5C**), and the nuclei of the eye cells in the O3-MO or ATG-MO embryos generally displayed a longer shape compared with eye cells in the control embryos. The ordered layers of the eye structure were also missing or disrupted (**Fig. 5C**). Due to the cell cycle progression problem, cell proliferation becomes abnormal and many of the cells undergo apoptosis (**Fig. S2**). As a consequence, the distribution of proliferating cells became irregular, and the level of mature cells decreased in the eye.

Discussion

We have revealed an unexpected biochemical activity of zTATDN1, which can decatenate DNA in vitro. Previously, DNA topoiserases were suggested to be the major enzymes that carry out the DNA decatenation. Top1 and Top2 were previously shown to be important for DNA decatenation during DNA replication.[12,16] Recently, Top3 was found to complex with single-stranded DNA-binding protein RPA, RecQ helicase Sgs1 and Rmi 1 to dismantle the topologically linked DNA.[17] A nuclease, METNASE, was observed to catalyze DNA decatenation in vitro.[17] Our current studies indicate that TATDN1 nuclease in zebrafish possesses similar activity to METNASE, supporting that the nuclease may be important in resolution of entangling DNA structures.

DNA decatenation has been suggested as a critical process during DNA replication and chromosome segregation.[12] It was shown that duplication of double-stranded DNA molecules produces topological stress, which could result in catenated daughter DNA molecules.[12,18,19] The cantenated DNA molecules, if not properly decatenated, could delay DNA replication and chromosome segregation.[12,18,19] A previous study showed that depletion of Topo II in *S. cerevisiae* prevented DNA decatenation, leading to DNA damage, and delayed S-phase progression and cytokinesis failure.[18] DNA damage-induced cell cycle arrest and/or cytokinesis failure have been shown to cause polyploidy.[20,21] BRCA1 deficiency in human cells impaired DNA decatenation and induced polyploidy.[13] Consistently, TATDN1 deficiency, which may lead to defects in DNA decatenation, causes S-phase arrest and leads to chromosome segregation errors or failure. This, in turn, results in polyploidy and aberrant chromatin structures and impairs eye development in the morphant embryos, as the enzyme is dominantly expressed in eyes.

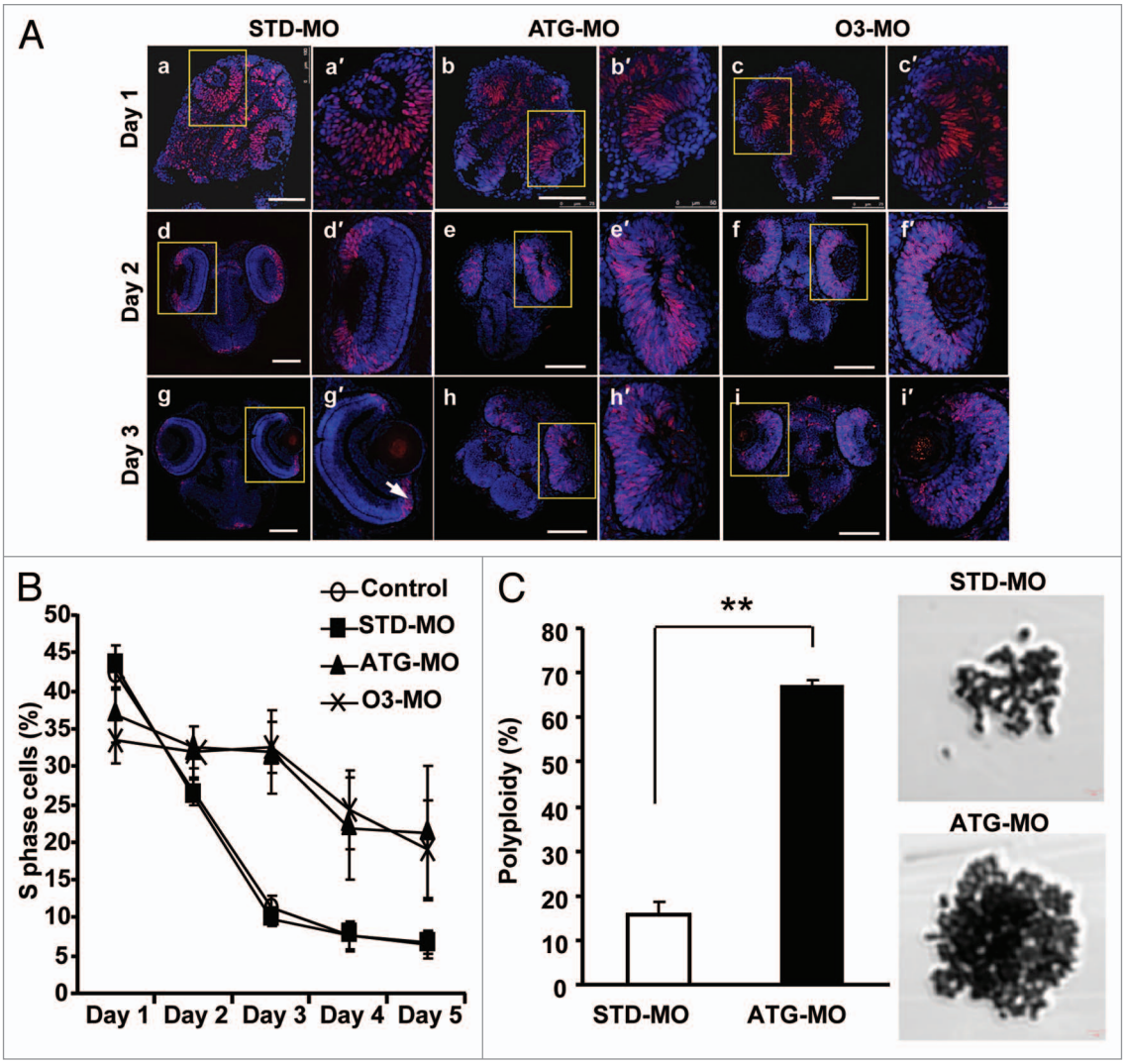

Figure 4. Knockdown of TATDN1 results in abnormal DNA replication, cell cycle delay and polyploidy in eyes. (**A**) The morpholino-injected embryos were treated with 10 μM EdU for 1 h on days 2 and 3. After fixation, the embryos were sectioned, processed for EdU detection and stained with DAPI, then examined by confocal fluorescence microscopy. Merged images show the nuclei stained with DAPI (blue) and the proliferating cells stained with EdU (red). Arrow, CMZ. Scale bars: 100 μm. (**B**) The eye regions of the morphant embryos were isolated from different developmental stages and the cells were processed to analyze DNA content by PI staining and flow cytometry. The percentage of S-phase cells for each sample was calculated using a modified peak reflection method. Values are the mean ± s.e.m. (standard error of the mean) from four individual experiments. (**C**) On 4 d post-fertilization, the eye cells were cultured and processed for metaphase spread. The percentage of polyploid eye cells was calculated from slides of metaphase spreads. The representative metaphase spread images were shown. **p < 0.01, two-tailed Student's t-test.

Materials and Methods

Ethics statement. This study has been performed in full accordance to the requirements by "Governing Regulation for the Use of Experimental Animals in Zhejiang Province" (Zhejiang Provincial Government Order No 263). According to the Chapter for Biosafety and Animal Ethics (Chapter 4), ethical approval is not stated to be required for scientific research using adult or embryonic zebrafish by the Regulation. Every effort was made to minimize any suffering of the animals used in this study. Tricane solution was used to euthanize zebrafish before tissue collection. Zebrafish were raised and maintained in the standard Zebrafish Unit (produced by Aisheng Zebrafish Facility Manufacturer Company).

Recombinant protein expression and purification. The plasmid pET28b-*TATDN1* was constructed for the expression of 6His-tagged zebrafish TATDN1 by inserting a DNA fragment encoding zebrafish TATDN1 into the pET28b (+) vector. The pET28b-$TATDN1_{D222A}$ mutant was created using a one-step site-directed mutagenesis protocol[22] with primers 5'-GAA ACA GCC GCT CCG TGG TGC GGC ATT AAA AAC AC-3' and 5'-CGG AGC GGC TGT TTC GAT CAT CAG TCT GTC AGA CG-3'. The 6His-tagged TATDN1 proteins were expressed in *E. coli* C41 and purified by affinity chromatography using a

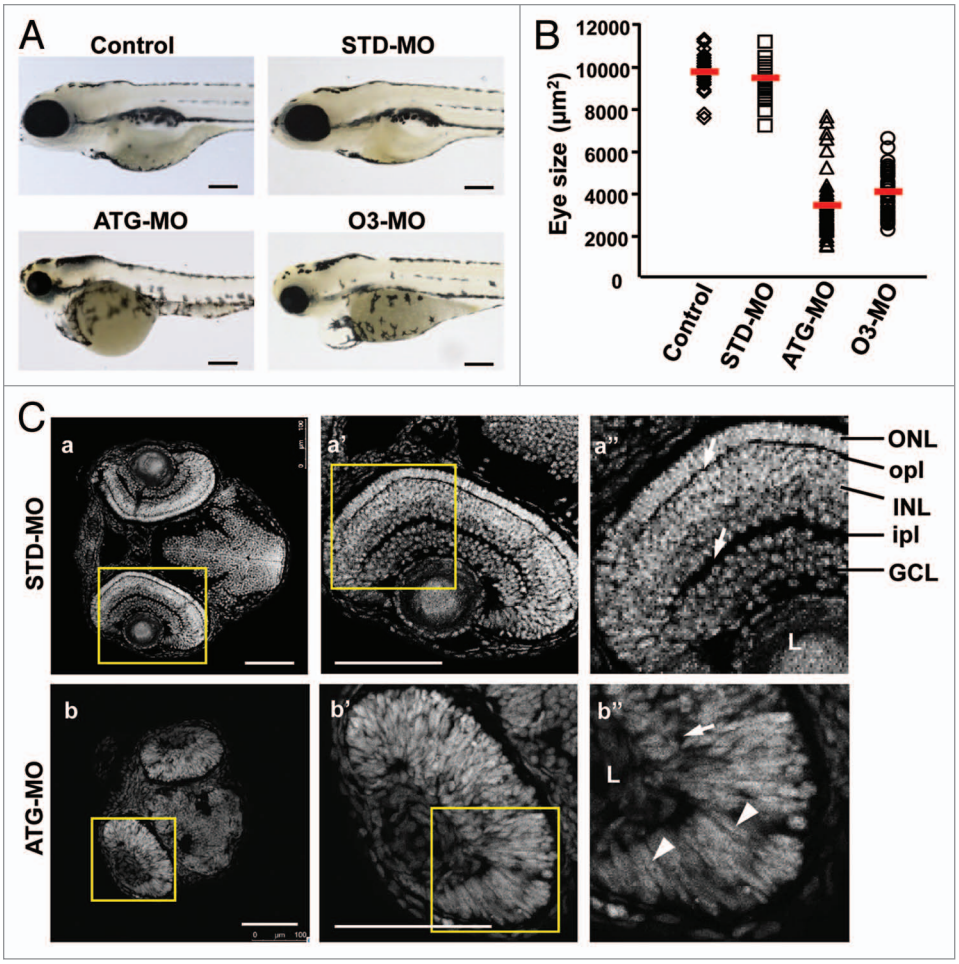

Figure 5. Knockdown of TATDN1 results in disordered eye cell layers and small eyes in zebrafish. (**A**) Compared with the control zebrafish embryos, the morphants showed a series of abnormal phenotypes, including delayed development, smaller eyes, incomplete yolk absorption and swimming defects. The images of zebrafish with or without microinjection were recorded on day 3 after fertilization. (**B**) The sizes of zebrafish eyes were measured using a microscope system, Nikon DIGITAL SIGHT DS-Ril 150943 and software, NIS-Elements D 3.0 and calculated with the formula Π (diameter/2).[2] Each eye measurement represents an individual zebrafish taken at 72 h post-fertilization. For each group, the sizes of 50 individual fish are shown in the chart and the red line indicates the average value. (**C**) Day 3 morpholino-injected embryos were fixed and processed into frozen sections. After permeabilization, they were stained with DAPI and imaged using a fluorescence confocal microscope. Arrows (a'') indicate the regular cell layer observed in a standard control but not in the morphant, and arrowheads (b'') indicate the shape alteration in morphant. GCL, ganglion cell layer; INL, inner nuclear layer; ipl, inner plexiform layer; L, lens; ONL, outer nuclear layer; opl, outer plexiform layer. Scale bars: 100 μm.

TALON Metal Affinity Resin column (Clontech). The purified recombinant TATDN1 was examined by SDS-PAGE and confirmed by western blotting using an anti-TATDN1 antibody (Genescript).

Nuclease activity and DNA decatenation assays. To assay the nuclease activity and DNA decatenation activity of TATDN1, 200 ng of pUC19 plasmid or kDNA (catenated, Topogen) was incubated with various concentrations of recombinant WT TATDN1 or TATDN1 $_{D222A}$ at 37°C for specified times in reaction buffer (50 mM Tris–HCl, pH 7.4, 1 mM DTT, 20 mM $MgCl_2$). DNA substrates and products were analyzed on 1.2% agarose gels. For kDNA cleavage, kDNA from the DNA Gyrase Assay kit (Topogen) was used as a positive control for kDNA decatenation.

Zebrafish whole-mount in situ hybridization (WISH). Wild-type AB stocks of *Danio rerio* and mutant lines were maintained according to standard procedures described in ZFIN (www.zfin.org/zf_info/zfbook/zfbk.html). Embryos were raised in egg water containing 0.2 mM N-phenylthiourea at 28°C to the desired stage.[23] To prevent the pigment formation during post-gastrulation stages, the embryos were treated with 0.0045% 1-Phenyl-2-thiourea (PTU, Sigma) after 24 h. After removal of chorions, the embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C and dehydrated in 100% methanol for 15 min at room temperature followed by at least 2 h at -20°C before use. Whole-mount RNA in situ hybridizations were performed essentially as previously described.[24,25] Probes of *TATDN1* were labeled with digoxigenin (DIG, Roche Diagnostics) as previously described.[24]

Morpholinos and microinjection. Morpholinos were purchased from Gene Tools. The ATG-MO (5'-TCA TGG CGA GTT TAG CAG AAA CAG A-3') and the splice-MO named O3-MO (5'-CAG CGA GGA GTT TTC ATT ACC TTC A-3') were designed to specifically target *TATDN1* against the 5'-UTR translation start site and the splice site of Exon 2, respectively. The human β-globin antisense morpholino (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') was used as the standard control (STD-MO). One nanoliter of 0.5 mM morpholinos were injected into the embryos at the one-cell stage after fertilization. The efficacy of the *TATDN1* exon 2 splice site-targeting morpholino was tested by reverse transcription (RT)-PCR.

EdU injection and immunofluorescence imaging. To examine cells undergoing DNA replication, live dechorionated morphant embryos were injected with 1 nl 10 mM 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen) into heart at desired age (54-h and 76-h) and incubated for 1 h at 28°C. The embryos were then collected and fixed for frozen sectioning and immunostaining. PFA-fixed embryos (4%) were washed three times

with PBS and embedded in 1.5% agarose and 30% sucrose in PBS. After trimming, the blocks were dehydrated in 30% sucrose overnight at 4°C and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc.) for frozen section. Transverse sections of 12 μm thickness were cut using a Cryotome FE. After permeabilization with 0.5% Triton X-100, EdU was detected first with an Alexa Fluor® azide 594 reaction cocktail from the EdU Imaging Kit according to the kit protocol (Invitrogen). Nuclei were labeled with 4', 6-diamidino-2-phenylindole (DAPI). Images were obtained using a Leica TCS SP5 confocal microscope.

FACS and cytogenetics. The eyes portion of the morphant embryos were isolated at different stages and trypsinized to generate single-cell suspensions. After filtration through cell strainers, they were fixed in cold 70% ethanol and processed for DNA content by staining with propidium iodide (PI) (Sigma), using 50 μg/ml PI, 0.05% Triton X-100, 0.1 mg/ml RNase A in PBS. Cells were analyzed using BD FACS Calibur flow cytometer.

Metaphase chromosome spreads were performed on 4-day-post-fertilization embryos following standard cytogenetic procedures.[21] Cells were arrested at metaphase with colcemid (100 ng/ml) at 37°C for 4 h, then transferred to a hypotonic solution of 75 mM KCl at room temperature for 10 min and fixed onto glass slides with 3:1 methanol-acetic acid. The chromosomes were visualized by Giemsa staining (Sigma).

Zebrafish eye size measurements. At 72 h post-fertilization, fish were anesthetized with 0.05% Tricaine and imaged at a fixed magnification from a lateral perspective using a Nikon DIGITAL SIGHT DS-Ril 150943 microscope system. The eye diameters were measured using NIS-Elements D 3.0 software and the sizes of the eye areas were calculated with the formula (diameter/2).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/22886/

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