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REPORT

The homologous recombination component EEPD1 is required for genome stability in response to developmental stress of vertebrate embryogenesis

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ABSTRACT

Stressed replication forks can be conservatively repaired and restarted using homologous recombination (HR), initiated by nuclease cleavage of branched structures at stalled forks. We previously reported that the 5′ nuclease EEPD1 is recruited to stressed replication forks, where it plays critical early roles in HR initiation by promoting fork cleavage and end resection. HR repair of stressed replication forks prevents their repair by non-homologous end-joining (NHEJ), which would cause genome instability. Rapid cell division during vertebrate embryonic development generates enormous pressure to maintain replication speed and accuracy. To determine the role of EEPD1 in maintaining replication fork integrity and genome stability during rapid cell division in embryonic development, we assessed the role of EEPD1 during zebrafish embryogenesis. We show here that when EEPD1 is depleted, zebrafish embryos fail to develop normally and have a marked increase in death rate. Zebrafish embryos depleted of EEPD1 are far more sensitive to replication stress caused by nucleotide depletion. We hypothesized that the HR defect with EEPD1 depletion would shift repair of stressed replication forks to unopposed NHEJ, causing chromosome abnormalities. Consistent with this, EEPD1 depletion results in nuclear defects including anaphase bridges and micronuclei in stressed zebrafish embryos, similar to BRCA1 deficiency. These results demonstrate that the newly characterized HR protein EEPD1 maintains genome stability during embryonic replication stress. These data also imply that the rapid cell cycle transit seen during embryonic development produces replication stress that requires HR to resolve.

Introduction

Replication fork stalling and collapse is a major source of genome instability that can result in cell death and neoplasia.[1-3] Such stressed forks can be conservatively repaired and restarted using homologous recombination (HR), which can be initiated by cleaving the stressed replication fork junction.[3-5] While several candidate nucleases for replication fork cleavage have been proposed, including Mus81, Gen1, and Dna2,[5-9] most human stressed forks can be restarted without these nucleases, and the precise mechanisms of stalled fork cleavage remained undefined.[6-8] Replication fork cleavage permits extensive 5′ end resection that is required for HR, and prevents classical non-homologous end-joining (cNHEJ),[10-13] which can cause chromosomal fusions and thus genomic instability during replication stress.[14,15] This 5′ end resection is the major decision point in repair pathway choice during repair of stalled/collapsed replication forks.[10,12,13] Proper repair pathway choice at stalled forks is important for genome stability, because unopposed cNHEJ results in fusion of one-sided DNA ends at damaged replication forks, as in malignancies with inherited deficiencies in the HR proteins BRCA1 and BRCA2.[14,15] These chromosomal fusions cause unbalanced translocations and severe genome instability, resulting in catastrophic mitoses revealed as gross nuclear abnormalities including nuclear bridges and micronuclei.[14,15]

We recently reported that the previously uncharacterized 5′ endonuclease EEPD1 plays a critical role in initiating HR repair of stalled forks.[16] EEPD1 is recruited to stalled forks where it mediates fork cleavage, 5′ DNA end resection, and restart of stalled forks.[16] EEPD1-dependent fork cleavage and end resection is required for downstream damage signaling, including ATR and CHK1 phosphorylation, and formation of γ-H2AX foci, indicating that its promotion of replication fork cleavage and 5′ end resection were early events in accurate repair of stressed replication forks by HR.[16] EEPD1 is in an obligate complex with the 5′ end resection factors Exo1/BLM/RPA, within which EEPD1 is required for nucleolytic activity on stalled fork structures.[16] EEPD1 depletion caused nuclear and cytogenetic anomalies, especially after replication stress, highlighting the importance of accurate, HR-mediated fork repair to prevent both genome instability and mitotic

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catastrophe.[16,17] 53BP1 depletion rescues the nuclear and cytogenetic abnormalities seen with EEPD1 depletion,[16] as it does in BRCA1 deficient cells.[14,15] Thus, EEPD1 roles in 5′ cleavage of stalled replication forks and initiation of 5′ end resection appear to be critical in fork repair pathway choice.[16]

DNA replication and embryonic development are tightly linked.[18-20] While many features of the replication process can be specific to individual tissues, including replication initiation, rate, and extinction, all embryonic tissues require rapid proliferation to develop the cell numbers needed for organ differentiation.[21] During early embryogenesis, cells progress rapidly through the cell cycle with few gap phases, minimal control of cell cycle phase transitions, and a lack of coordination of cell cycle progression with DNA repair. Thus, there is little margin for error, and embryogenesis is exquisitely sensitive to perturbations in DNA replication.[18,19,21] We hypothesized that EEPD1 would be required to support the rapid proliferative rate during embryogenesis, especially during replication stress, such as effected by nucleotide depletion.

During development, vertebrate embryos produce serially repeated elements, the somites, on each side of the midline.[18,19] The somites generate the vertebral column, skeletal musculature, and dermis. Somites form sequentially, one pair at a time, from mesenchymal tissue near the tail. The embryo must control the number, size, and timing of somite formation, their subdivision into functional regions along three axes, regional identity (region-specific somite development), and interactions with neighboring tissues that coordinate somites with nearby structures.[18,19] Zebrafish somite development is a useful model to study the effects of cell cycle alterations on tissue specification.[18,19,21] Therefore, we asked whether depletion of EEPD1 would harm zebrafish somite development during replication stress. We found that EEPD1 is essential for proper somite development during rapid cell proliferation in zebrafish embryogenesis, especially when embryos were subjected to replication stress by nucleotide depletion. EEPD1-depleted somites had the canonical manifestations of fused chromosomes, such as nuclear bridges and micronuclei, that occur when stalled replication forks are repaired by unopposed NHEJ. These data demonstrate that EEPD1, by promoting HR and inhibiting NHEJ, maintains genome stability during embryonic replication stress.

Results

### EEPD1 is required for proper zebrafish embryonic development

Zebrafish (*Danio rerio*) *EEPD1* encodes a previously uncharacterized 2135 nt mRNA on chromosome 19 that encodes a 550 aa protein (mRNA: NM_205759.1, protein: NP_991322).[22] It has two N-terminal helix-hairpin-helix (HhH) DNA binding domains related to those *E. coli* RuvA, a component of the RuvABC Holliday junction resolvase that binds branched DNA structures,[23] and a C-terminal DNase I-like domain in the exonuclease-endonuclease-phosphatase (EEP) family, similar to the human protein.[16] EEPD1 is conserved from humans through fish to some insects, but it is not present in lower organisms. The zebrafish EEPD1 protein is 69.2% homologous

to human,[16] and it has two RuvA-like HhH DNA binding domains at aa36–100 and aa126–182, and a DNase-I-like domain at aa246–521 (Fig. 1A).

Proper replication stress responses are required to prevent gross chromosomal instability, which can be assessed by the formation of micronuclei and nuclear bridges.[14-16,19] EEPD1 depletion in human cell lines resulted in a defect in HR repair of stalled/collapsed replication forks, and nuclear defects such as micronuclei and nuclear bridges that reflect breakage and missegregation of fused chromosomes produced by unopposed NHEJ at stalled forks.[16] That report raised two questions that are addressed in this study. First, does this same phenomenon hold true at an organism level when EEPD1-mediated HR repair is defective, or is it only true in cell lines? Second, does rapid cell division during embryogenesis result in endogenous replication stress that requires EEPD1-mediated HR to maintain genome stability?

We assessed the role of EEPD1 in genome stability at an organism level, and whether delayed cell division due to defective HR-mediated fork repair would cause developmental defects, using zebrafish somite development as a model of rapid cell division.[18-20] We depleted EEPD1 in zebrafish zygotes by injection of an antisense morpholino (MO) [24] targeting the EEPD1 exon 3 splice site. This MO resulted in scission of the EEPD1 mRNA in zebrafish embryos (Fig. 1B). The exon 3 EEPD1 MO did not disrupt actin mRNA, an indication of its specificity (Fig. 1B). As further control for off-target effects, an MO targeting the EEPD1 ATG start codon was also tested, and this resulted in nearly identical phenotypes as the exon 3 MO (data not shown). A scrambled MO was used in control injections for comparison.

We first assessed whether EEPD1 was required for normal zebrafish developmental progression. This would shed light on whether normal embryogenesis requires HR to appropriately deal with endogenous replication stress associated with rapid cell proliferation.[18,25] We analyzed whether MO repression of
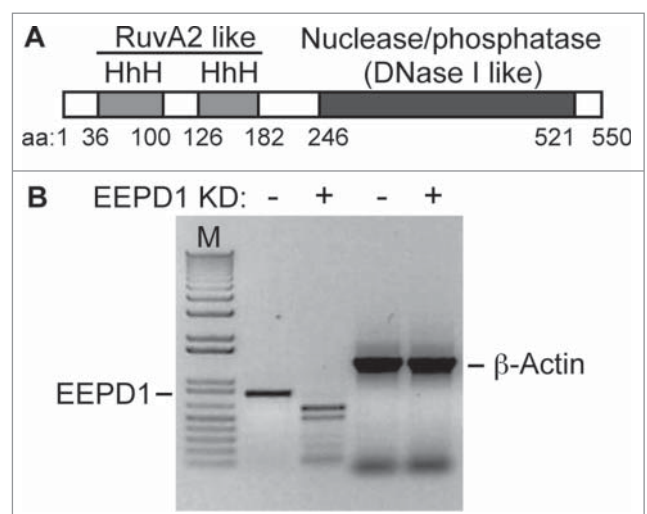
Figure 1. Homologous recombination nuclease EEPD1 domain structure and mRNA depletion by antisense morpholino (MO). (A) *Denio rerio* zebrafish EEPD1 protein has tandem DNA binding Helix-hairpin-Helix (HhH) domains and a nuclease domain in the DNase-I superfamily. (B) Zebrafish zygotes were injected with EEPD1 or scrambled MOs, mRNA was prepared, and RT-PCR was used to amplify EEPD1 mRNA, and β-actin mRNA as control to demonstrate specificity. No EEPD1 mRNA scission was observed with a scrambled control MO injection.

EEPD1 in zygotes produced developmental delays or embryonic death in the absence of exogenous replication stress, such as that induced by hydroxyurea (HU). There were no developmental delays among embryos injected with the scrambled MO control population, while 27% of EEPD1 MO-injected embryos showed developmental delay (Fig. 2A; P < 0.0001). Embryonic death was rare among control scrambled MO injected embryos (4%), but death increased to >25% (6.3-fold) among EEPD1 MO-injected embryos (Fig. 2A; P < 0.0001). Morphological examination of the EEPD1 MO-injected dead embryos demonstrated clear somite region abnormalities (Fig. 2B).[18,25]

EEPD1 is critical for replication stress signaling during zebrafish embryogenesis

We next analyzed whether EEPD1 depletion prevented replication stress signaling during embryogenesis. When a replication fork stalls, the ATR/Chk1 kinases arrest DNA synthesis and activate the repair of the stalled replication fork.[26-28]. An important downstream target of the ATR kinase is H2Ax, the phosphorylated form of which (γ-H2Ax) serves as a marker of active repair of stressed replication forks.[26-28] We previously demonstrated in human cells that EEPD1 functions upstream of ATR/Chk1 activation, mediating 5′ end resection to create single-stranded DNA that is bound by RPA and necessary for ATR activation.[16] We therefore monitored γ-H2Ax using immunofluorescence microscopy in untreated and HU treated embryos 24 h post fertilization with and without EEPD1 depletion. Representative images are shown in Figure 3A, and quantitation of the results is shown in Fig. 3B. EEPD1 depletion greatly diminished HU-induced γ-H2Ax in embryos, consistent with results in cultured cells.[16] Without exogenous replication stress, γ-H2Ax positive cells were extremely rare, and EEPD1 knockdown did not cause a significant increase (P = 0.34). In embryos treated with 5 mM HU for 4 hr, embryos injected with control scrambled MO, nearly 90% of cells had > 5 γ-H2Ax foci, while EEPD1 knockdown caused a marked reduction to only 1.7%, which is not significantly different than that of untreated EEPD1 knockdown embryos (P = 0.18). Thus, EEPD1 plays a critical role in replication stress signaling during zebrafish embryonic development. In addition, if EEPD1 deficiency alone causes replication stress and/or DSBs,

signaling of such stress to γ-H2Ax is blocked, most likely because EEPD1 has a key role in end resection.[16]

### EEPD1 prevents genome instability during embryogenesis

Defects in HR proteins such as BRCA1, BRCA2, RAD51-paralogs, and H2Ax are known to confer genome instability,[2,15,29-32] revealed as micronuclei (aberrantly retained chromosomes after mitosis).[17] Because EEPD1 deficiency confers both HR defects and genome instability in human cells,[16] we assessed whether EEPD1 depletion in zebrafish zygotes resulted in genome instability in developing embryos. We measured micronuclei formation in control MO-injected and EEPD1 MO-injected embryos, which is an indication of chromosomal mis-segregation at mitosis from chromosomal fusions.[17] We found that EEPD1 MO-injection greatly increased micronuclear abnormalities in embryos scored 24 hr post-fertilization in embryos without exogenous replication stress (Fig. 4A). In the EEPD1 MO-injected embryos, a mean of 6.4% of cells had micronuclei, compared to a control MO embryo mean of 0.04% (P < 0.0001, t test) (Fig. 4B). Replication stress induced by depletion of nucleotides with HU increased micronuclei in control embryos several fold (P = 0.0001), but this treatment did not appreciably change the fraction of cells with micronuclei in the EEPD1 MO-injected embryos (P = 0.72) (Fig. 4B). These results suggest that HR deficiency during normal zebrafish embryogenesis results in gross chromosomal abnormalities, represented by the marked increase in micronuclei. Exogenous replication stress induced by HU does not further exacerbate genome instability in EEPD1-deficient embryos, rather disrupting HR during somite development is sufficient to generate genome instability.

Discussion

We show here that MO depletion of the recently discovered HR protein EEPD1 during zebrafish somitogenesis results in developmental delay and embryonic death, likely caused by genome instability, since EEPD1 depletion markedly increases micronuclei formation (Figs. 2 and 4). This genomic instability indicates that rapid cell proliferation during vertebrate somite development induces replication stress, requiring HR to maintain the
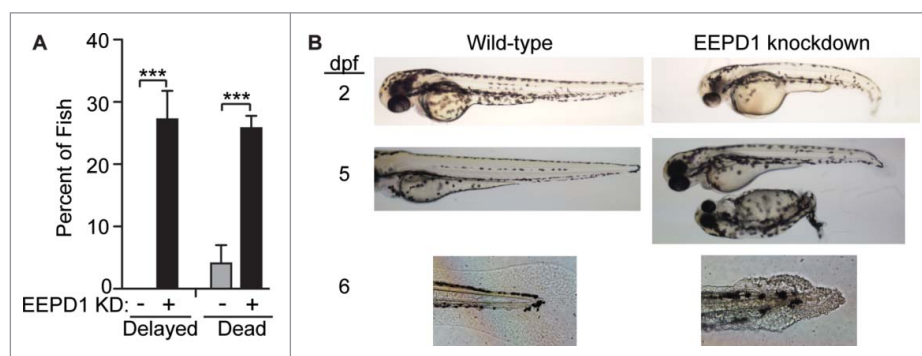
Figure 2. EEPD1 deletion causes developmental delay and death during Zebrafish embryogenesis. (A) Depletion of the HR nuclease EEPD1 by MO injection into zygotes results in delayed embryonic development or death. Averages ±SD for two determinations are plotted. A total of 115–240 embryos were scored per condition. *** indicates P < 0.0001 by Fisher exact test for the combined data from the two determinations. (B) Morphologic abnormalities in developing zebrafish depleted of EEPD1, especially in the somite region; representative embryos are shown at 2, 5, and 6 days post-fertilization (dpf).
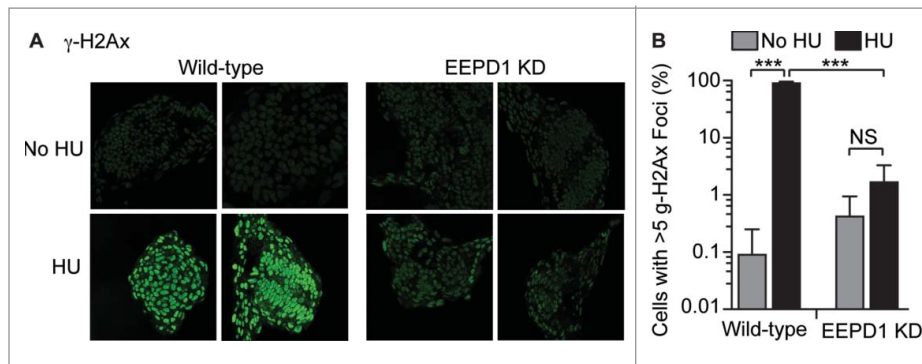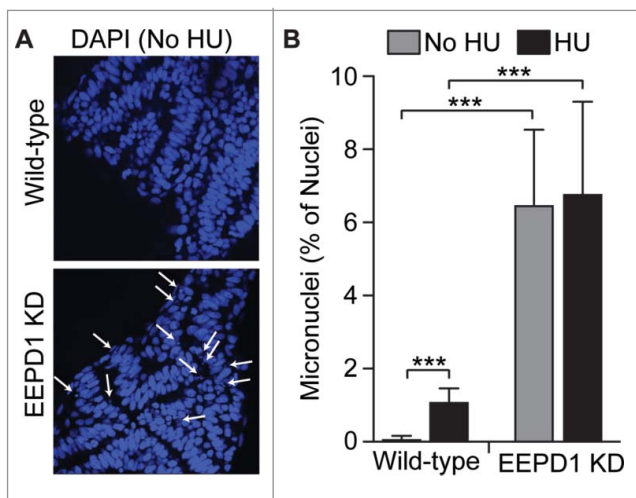
Figure 3. EEPD1 depletion prevents replication stress signaling. (A) Representative confocal immunofluorescent photomicrographs of the somite region of zebrafish embryos stained for γ-H2Ax. (B) Percentage of somite nuclei >5 γ-H2Ax foci. Values are averages ($\pm$SD) for 3–6 embryos per condition, 56–375 nuclei scored per embryo. *** indicates P < 0.0001, t-tests.

high rate and accuracy of cell division.[18,19,21] The genome instability expressed as micronuclei in EEPD1 depleted zebrafish embryos parallels the nuclear defects in EEPD1 depleted human cells, including mitotic bridges and micronuclei indicative of severe genome instability.[16] Such nuclear abnormalities are generated by fused chromosomes that fail to segregate properly at mitosis. The fused chromosomes contain either two centromeres, or none, which prevents their proper movement to either cell pole in anaphase, forming micronuclei distinct from either daughter nucleus.[17] The zebrafish embryo defects here completely mimic the human cell defects, implying that the MO depletion used here indeed targeted EEPD1, and the phenotype observed was from EEPD1 depletion.

Chromosomes can fuse when unopposed cNHEJ causes mis-repair of DSBs arising at stressed replication forks. We previously discovered that EEPD1 promotes HR and suppresses cNHEJ, and thus prevents chromosome fusions and other forms of genome instability.[16] During vertebrate embryonic development, cell proliferation is very rapid and this in itself induces replication stress that requires constant vigilance by HR to maintain genome integrity. Our results indicate that EEPD1's role in HR repair of stalled/collapsed replications forks is not limited to cultured cells, but is an important factor in maintaining genome integrity during normal embryogenesis. The endogenous replication stress associated with rapid proliferation of embryogenesis is reminiscent of "oncogene stress" observed in rapidly dividing tumor cells.[33] The idea that HR is required to manage replication stress associated with rapid proliferation during embryogenesis is supported by evidence that defects in the HR proteins RAD51, BRCA1 and BRCA2 confer embryonic lethality in mice,[34-37] and that conditional inactivation of RAD51 causes rapid cell death in chicken DT40 cells.[38] The present results extend the concept that HR is required for rapid cell division during embryogenesis to zebrafish, and it is likely that this concept applies to all vertebrates.

Our previous study demonstrated that the EEPD1 nuclease plays a key role in repairing stressed replication forks via HR.[16] We found that EEPD1 initiated stalled replication fork repair and restart by cleavage of fork junctions to allow 5' end resection, the commitment step for HR. Such end resection is required for HR, but it also creates RPA-bound single-stranded DNA which activates replication stress signaling via ATR.[26-28] EEPD1 depletion abrogates γ-H2Ax formation in human cells [16] and zebrafish embryos (Fig. 3), indicating resection promoted by EEPD1 precedes phosphorylation of H2Ax during replication fork repair. Thus, the end resection defect in EEPD1-depleted cells prevents downstream ATR signaling and this is revealed as a defect in γ-H2Ax formation. The present study provides new insight into the mechanisms of faithful DNA replication during embryogenesis. Our results demonstrate that without EEPD1, there is essentially no γ-H2Ax formation, suggesting little ATR activation. This implies that during embryogenesis, replication stress signaling requires EEPD1-dependent 5' end resection to generate single-stranded DNA that is critical for ATR activation, and further implies that single-stranded DNA arising by de-coupling MCM unwinding from DNA polymerization has a lesser role.[26-28] Thus, fork cleavage and end resection are not only crucial for fork repair, but also cell cycle arrest in response to replication stress, and hence genome stability.

Figure 4. EEPD1 depletion in zebrafish embryos causes genome instability with or exogenous replication stress induced by 4 hr treatment with 5 mM HU. (A) Representative photomicrographs of somite nuclei in zebrafish embryos stained with DAPI. Arrows indicate micronuclei. (B) Percentage of somite nuclei displaying micronuclei. Values are averages ($\pm$SD) for 6–16 embryos per condition, 115–421 nuclei scored per embryo. *** indicates P ≤ 0.0001, t-tests.

DNA replication is not a smooth, continuous process, but one of stalls and restarts, with stress arising from many endogenous sources, such as nutrient depletion, DNA methylation, or metabolically-generated oxygen free radicals.[1-4] The more rapid the cell cycle, the more important mechanisms of replication fork stability and accurate fork repair become, as there is less margin of error for repairing damaged forks resulting from endogenous replication stress. Thus, the data presented here also imply that rapid proliferation during embryonic development commonly results in stressed replication forks that if not accurately repaired result in genome instability. Our results indicate that EEPD1 plays a critical role in the response to endogenous replication stress, but there appears to be no additional requirement for EEPD1 when embryos are exposed to exogenous replication stress by HU (Fig. 4B). Finally, it is well established that proper somite development during embryogenesis is highly dependent on maintaining the rapid timing of cell division.[18,19,21] Here we further establish that HR is required to maintain genome stability during rapid rate of cell cycle progression during embryogenesis.

Materials and methods

Zebrafish development

Zebrafish embryonic development was analyzed as we described.[39,40] Zebrafish were grown and maintained at 28.5°C. Mating was routinely carried out at 28.5°C and embryos were staged according to established protocols. The TuAB zebrafish strain was used in this study. All zebrafish studies were performed according to the University of Florida animal protocol guidelines under protocol #AUA320, approved by the University of Florida IACUC committee.

Morpholino depletion of EEPD1

Gene Tools, Inc. designed ATG and exon 3 splice MOs targeting the ATG start codon or the exon 3-intron 3 splice junction of zebrafish EEPD1 gene (NM 205759) as we described.[39,40] The two targeting MOs were used in distinct experiments to control for off-target effects. The phenotypes were identical, and data is shown for the exon 3 splice MO. Down-regulation of target mRNA was analyzed by RT-PCR performed with primers located in exons adjacent to target sequences. The RT-PCR sequences used in this study were: Forward primer CC473: 5′-TCCAGTGAGAAGGCGAACAACC, reverse primer CC475: 5′-AGTGAAGACGGACGGTGCGAGG. The MO sequences were: ATG MO: 5′-GCACCCGAGATTCC-CACCCATGTGT; exon 3 splice MO: 5′-GCTAGAAGAACA-TAAACTCACGCTA. Both gene-specific and control MOs were reconstituted to a final concentration of 1 mM in deionized distilled water. Microinjection was performed with 2 nanoliters (nL) injected into each embryo at the 1–2 cell stage as we described.[39,40] Doses of MO, empirically determined, were 8 ng each for control MO, EEPD1 ATG MO, and EEPD1 exon 3 splice MO. Each injection experiment was repeated three independent times with approximately 100 embryos per condition. Embryonic delay and death were analyzed as described.[25]

Confocal immunofluorescence microscopy

Developing embryos were stained with DAPI and examined by confocal microscopy for micronuclei as we described.[16] For immune-staining, embryos were fixed at 24 hpf and frozen in tissue freezing medium (TFM). A Leica Cryocut1800 was used to generate 10 μm cryo-sections embedded in TFM, which were mounted onto glass slides. Immunohistochemistry was performed with rabbit anti-zebrafish γ-H2AX (Gene Tex, GTX127342) as primary, and anti-rabbit IgG-Alexa 488 (Invitrogen, A-21204) as secondary antibodies. Briefly, the section slides were washed 3 times with PBST, incubated 2 h in blocking solution (1% BSA in PBST, 2 h at RT) and then incubated with primary antibody (1:2000) overnight at 4°C. Slides were washed 3 times with PBST and then incubated with secondary antibody for 3 h at room temperature. The slides were washed thoroughly in PBST, mounted using VectorMount (Vector Laboratory, H-5501) and allowed to dry overnight in 4°C in the dark. The embryos were imaged the next day using a Leica TCS-SP5 confocal immunofluorescent microscope. Confocal parameters and magnification were identical for each embryo in each condition to normalize for immunofluorescence.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

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