**dnmt1 function is required to maintain retinal stem cells within the ciliary marginal zone of the zebrafish eye**

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The ciliary marginal zone (CMZ) of the zebrafish retina contains a population of actively proliferating resident stem cells, which generate retinal neurons throughout life. The maintenance methyltransferase, *dnmt1*, is expressed within the CMZ. Loss of *dnmt1* function results in gene misregulation and cell death in a variety of developmental contexts, however, its role in retinal stem cell (RSC) maintenance is currently unknown. Here, we demonstrate that zebrafish *dnmt1*mutants possess severe defects in RSC maintenance within the CMZ. Using a combination of immunohistochemistry, in situ hybridization, and a transgenic reporter assay, our results demonstrate a requirement for *dnmt1* activity in the regulation of RSC proliferation, gene expression and in the repression of endogenous retroelements (REs). Ultimately, cell death is elevated in the *dnmt1*−/− CMZ, but in a p53-independent manner. Using a transgenic reporter for RE transposition activity, we demonstrate increased transposition in the *dnmt1*−/− CMZ. Taken together our data identify a critical role for *dnmt1* function in RSC maintenance in the vertebrate eye.

The distal region of the vertebrate retina, termed the ciliary marginal zone (CMZ), contains a population of resident retinal stem cells (RSCs). The CMZ remains proliferative throughout the life of fish, but it proliferates to a more limited extent during the lifetime of amphibians and birds¹⁻⁶. Whether an analogous structure exists in mammals is debated, but there are distinct, progenitor-like cells in the periphery of the retina that are active during embryogenesis⁷⁻⁹. Mammalian RSCs can also be isolated from the adult ciliary margin, cultured in vitro, and stimulated to produce retinal neurons¹⁰⁻¹³. However, this activity has not been demonstrated in the mature mammalian retina in vivo.

Studies of the CMZ have primarily focused on zebrafish and *Xenopus* models to determine genetic pathways required for RSC identity¹²⁻¹⁴ and to characterize the epigenetic networks which regulate RSC function¹⁷,¹⁸. By comparison, the mechanisms mediating RSC maintenance in vivo remain unknown. In studies of RSCs, the zebrafish has been advantageous given that it possesses a highly active RSC population and is tractable for genetic and pharmacological manipulations, transgenesis and in vivo imaging¹⁹,²⁰.

DNA methylation, a frequently studied epigenetic modification, is the process through which a methyl group is added to the fifth carbon of cytosine nucleotides and is commonly found at CpG dinucleotide sequences²¹. Members of the family of DNA methyltransferase (Dnmt) enzymes²²,²³ catalyze this epigenetic modification. Dnmt1 serves as a maintenance methyltransferase, copying the methylation pattern from parent to daughter strand during DNA replication and its function is required for cell cycle progression²⁴⁻²⁶. Loss of Dnmt1 function results in genomic hypomethylation²⁷⁻²⁹ and in developmental contexts and specific organ systems, this often compromises progenitor cell maintenance³⁰⁻³³ through numerous cellular mechanisms. These include: inducing cell cycle arrest¹⁴,³⁵, retroelement activation³⁶⁻³⁹, inflammatory responses³³,³⁷,⁴⁰, aberrant differentiation³⁸,³¹,⁴¹⁻⁴⁴ and/or p53-mediated apoptosis³⁴,³⁵.

Utilizing the *dnmt1*mutant zebrafish allele³⁰, we establish an in vivo requirement for *dnmt1* in RSCs. Through our analyses, we identify a decrease in overall RSC numbers, reduced RSC proliferation and aberrant gene expression patterns within the *dnmt1*-deficient CMZ. Additionally, we note increased retroelement

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expression and increased retrotransposition activity in \(dnmt1^{-/-}\) embryos. Remarkably, RSCs in \(dnmt1^{-/-}\) embryos are eliminated in a p53-independent manner, suggesting that \(dnmt1\) represses alternative, non-apoptotic cell death pathways in RSCs. Taken together, these data highlight a novel function for \(dnmt1\) in maintaining stem cell populations in the vertebrate retina.

**Results**

**\(dnmt1\) mutants possess defects in the ciliary marginal zone.** Previously, we identified a requirement for \(dnmt1\) in maintaining lens epithelial cell viability using \(dnmt1^{s872}\) mutant zebrafish\(^2^7\). During these previous studies, we also detected photoreceptor layer abnormalities, similar to those documented in \(Dnmt1^{−/−}\) conditional knockout mice\(^4^5,4^6\), and an apparent defect in the CMZ. With an interest in the role that \(dnmt1\) plays in maintaining RSCs in vivo, here, we focused further on the CMZ phenotype. Using DAPI to label and count retinal nuclei, we confirmed a progressive degeneration of CMZ morphology beginning at 4 days post fertilization (dpf; Fig. 1A–F) and a significant decline in retinal cell numbers through 5dpf (Fig. 1G). The total number of cells present within central retina sections are equivalent between \(dnmt1^{−/−}\) and sibling larvae at 3dpf; however, numbers in \(dnmt1^{−/−}\) larvae diminish significantly between 4 and 5dpf (18.8% and 26.6% reduction respectively; \(p<0.0005\); Fig. 1G). Additionally, we compared the proportions of nuclei within the ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), and CMZ between \(dnmt1^{−/−}\) larvae and siblings from 3 to 5dpf (Fig. 1H). Interestingly, the proportions of cells in all three retinal laminae (GCL, INL, and ONL) remained equivalent over time in \(dnmt1^{−/−}\) larvae when compared to siblings, with only a slight increase in the ONL at 4dpf (Fig. 1H and Supplementary Figure 1A–C; \(p<0.005\)). In contrast, the CMZ proportion decreased significantly from 3 to 5dpf suggesting that \(dnmt1\) function in the retina is required within the CMZ to maintain the RSC population (Fig. 1H and Supplementary Figure 1D; \(p<0.0005\)).

**Cell death is elevated in the \(dnmt1^{−/−}\) CMZ in a p53-independent manner.** Previous publications have demonstrated increased p53 expression and TUNEL\(^{+}\) cells in \(Dnmt1\)-deficient tissues and cell types\(^3^0,3^4,3^5,4^7\) suggesting a p53-dependent apoptotic mechanism for cell loss. Based on these studies, we hypothesized that \(dnmt1^{−/−}\) RSCs would similarly undergo p53-dependent apoptosis. To test this hypothesis, we first assayed for the presence of DNA double-strand breaks in \(dnmt1^{−/−}\) and sibling retinae using TUNEL (Fig. 2A–F). \(dnmt1^{−/−}\) siblings displayed few TUNEL\(^{+}\) cells between 3 and 5dpf (Fig. 2L–N), whereas the \(dnmt1^{−/−}\) retina contained increased proportions of TUNEL\(^{+}\) cells at 3, 4, and 5dpf in the INL (+0.5–2.3%, \(p<0.05\)), ONL (+0.01–1.8%, \(p<0.05\)) and at 5dpf in the GCL (+1.3%, \(p<0.05\); Fig. 2H, I). Within the CMZ, we detected a 4.5% increase in TUNEL\(^{+}\) cells at 3dpf (\(p<0.005\), Fig. 2J) prior to the onset of CMZ disorganization. This proportion decreased to

**Figure 1.** Disruption of \(dnmt1\) function results in CMZ defects. A–F DAPI staining of nuclei (gray) within the CMZ (white dotted lines delineate CMZ boundaries) of siblings (A–C) and \(dnmt1^{−/−}\) (D–F) larvae from 3 to 5dpf. G Average number of all nuclei within the central retina of siblings and mutants. Each data point is the average of cell counts from three different 12 μm sections in one eye of a single larva. H Proportional changes of \(dnmt1^{−/−}\) retinal domains (GCL, INL, ONL, and CMZ) relative to siblings (set to 100%). Colors correspond with retinal domains in diagram. Scale bars = 30 μm. **\(p<0.005\), ***\(p<0.0005\), ****\(p<0.00005\). Dorsal is up in all images.
−/− CMZ. Previous reports have characterized the expression/distribution of the DNMT1 protein to be altered in the DNMT1−/− retinal layers (GCL, INL, ONL, and CMZ) labeled by TUNEL staining. GCL labeled with DAPI (gray; nuclei) and TUNEL (magenta; dsDNA breaks) from 3 to 5dpf.

Figure 2. Cell death is elevated in the dnmt1−/− CMZ. A–F dnmt1 sibling (A–C) and mutant (D–F) retinas labeled with DAPI (gray; nuclei) and TUNEL (magenta; dsDNA breaks) from 3 to 5dpf. G–J Proportion of retinal layers (GCL, INL, ONL, and CMZ) labeled by TUNEL staining. K Proportion of TUNEL+ cells within each layer from 3 to 5dpf. L–N Average number of TUNEL+ cells in each retinal layer of siblings and dnmt1−/− larvae from 3 to 5dpf. Yellow arrows in A–F indicate TUNEL+ nuclei. Scale bars = 30 μm. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005. Dorsal is up in all images.

1% at 4dpf (p < 0.05) and increased again to 3.7% at 5dpf (p < 0.05; Fig. 2J), a time at which dnmt1−/− larvae begin to display severe systemic defects. During this 3–5dpf period, the majority of TUNEL+ cells in the CMZ were located within the retina proper, not within the CMZ (Fig. 2K and Supplemental Fig S2). In concordance with the TUNEL data, immunofluorescence of the pro-apoptotic marker, active-caspase3, displayed similar patterns to TUNEL (data not shown). Together, these data are consistent with those seen in previous studies; dnmt1 deficiency results in increased cell death30,35,47.

To identify if dnmt1 deficient RSCs are lost via p53-dependent apoptosis, we generated dnmt1−/−; p53 double mutants using the p53Δ5 allele, which is defective in p53-dependent apoptosis48,49. We hypothesized that p53-dependent apoptosis was the driving mechanism of RSC loss in dnmt1−/− mutants and therefore loss of p53 activity would rescue the CMZ phenotype. To test this hypothesis, we quantified nuclei in the DNMT1−/−; p53−/− larvae from 3 to 5dpf. Y ellow arrows in A indicate TUNEL+ nuclei. Scale bars = 30 μm. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005. Dorsal is up in all images.

To quantify these findings and further assess gene expression changes in dnmt1−/− larvae, we conducted quantitative PCR analysis of the expression of cell cycle, cell death, and immune response genes using whole larval samples (Fig. 4U). RNA was isolated from 4dpf sibling and dnmt1−/− larvae (n = 16 each) in three biological replicates, converted into cDNA, and analyzed for gene expression levels. Overall, the cell cycle progression...
genes (ccna2, ccnb1, ccnd1, ccne, cdk1, cdk2, and cdk4) displayed reduced expression levels while cell arrest genes (casp, caspb, mdm2, p53, and ripk1) showed equivalent or slightly increased expression in \( \text{dnmt1}^{-/-} \) larvae compared to sibling controls. Additionally, \( \text{dnmt1}^{-/-} \) larvae showed increased levels of immune response genes (tnfa and il-1β) consistent with previous reports. While these qPCR data correlate with in situ hybridization data for CMZ-specific expression, the changes were not statistically significant when assessed by 2-way ANOVA analysis. This is not surprising since whole larvae were used for qPCR and each of these genes is expressed in numerous larval regions outside of the CMZ; this non-ocular expression likely masks changes in the CMZ. Nonetheless, the trends are consistent with apparent loss or reduction of expression in the CMZ of \( \text{dnmt1}^{-/-} \) larvae detected by in situ hybridization. Taken together, these data suggest that RSCs are present at the onset of morphological defects in the \( \text{dnmt1}^{-/-} \) CMZ, but could be impaired in their ability to progress through the cell cycle and self-renew.

**Loss of \text{dnmt1} activity results in decreased RSC proliferation.** RSCs within the teleost CMZ remain proliferative throughout the lifespan of the animal and Dnmt1 is known to be required for cell cycle progression within stem cells of various tissue types. Based on the significant loss of RSCs in \( \text{dnmt1}^{-/-} \) larvae between 3 and 5dpf (Fig. 1) and the inability of \( \text{dnmt1}^{-/-} \) RSCs to maintain expression of cell cycle genes (Fig. 4), we hypothesized that \( \text{dnmt1}^{-/-} \) RSCs would be defective in their proliferative capacity. To test this hypothesis, larvae were incubated for 2 h in BrdU at 3, 4, and 5dpf, fixed immediately thereafter, and immunolabeled for BrdU and phosphohistone-H3-serine10 (pH3) to identify RSCs in late G2/M. \( \text{dnmt1} \) siblings maintained a constant proportion of BrdU+ cells within the CMZ between 3–5dpf (Fig. 5A–C, H). Notably, the proportion of BrdU+ \( \text{dnmt1}^{-/-} \) RSCs at 3dpf was comparable to sibling controls (compare images in Fig. 5A, D and nuclear proportions in Fig. 5G). However, beginning at 4dpf, the percentage of BrdU+ \( \text{dnmt1}^{-/-} \) RSCs is significantly reduced when compared to controls (Fig. 5B, E, H; \( p < 0.0001 \)), and this proportion continues to decrease through 5dpf (Fig. 5C, F, H; \( p < 0.0001 \)). Additionally, the proportion of cells in late G2/M phase (pH3+) was significantly reduced at 3 and 4dpf in the \( \text{dnmt1}^{-/-} \) CMZ when compared to siblings (Fig. 5G, I) indicating potential cell cycle defects in \( \text{dnmt1}^{-/-} \) RSCs that manifest as early as 3dpf.
**Figure 4.** *dnmt1* is required to maintain RSC gene expression. Gene expression shown in whole mount (A, C, E, G, I, K, M, O, Q, S) and transverse cryosections (B, D, F, H, J, L, N, P, R, T) between siblings and *dnmt1*−/− larvae. A–D *col15a1b* expression. E–H *ccnD1* expression. I–L *dnmt1* expression. M–P *cdkn1ca* expression. Q–T *atoh7* expression. Numbers in transverse cryosections designate the number of larvae that showed the displayed expression pattern versus the total number of larvae analyzed. Scale bars = 75 mm (whole mount) and 10 μm (transverse sections). Anterior is up in all whole-mounts and dorsal is up for all section images. U qPCR results showing relative gene expression levels of cell cycle genes (*ccna2*, *ccnb1*, *ccnd1*, *ccne*, *cdk1*, *cdk2*, and *cdk4*), cell arrest genes (*casp*, *caspb*, *mdm2*, *p53*, and *ripk1*), and inflammatory response genes (*tnfα* and *il-1β*) in whole 4dpf sibling (white bars) and *dnmt1*−/− (gray bars) larvae.

**dnmt1** is required for RSC differentiation and incorporation into the neural retina. Potential cell cycle progression defects coupled to the fact that the vast majority of *dnmt1*−/− RSCs elude *p53*-dependent apoptosis (Figs. 2, 3) led us to hypothesize that *dnmt1*−/− RSCs might instead be undergoing premature differentiation, as has been shown in vitro36. To test this hypothesis, we performed a BrdU birth-dating assay38. Our aim was to saturate RSCs with BrdU for a 12-h period (3–3.5dpf) and quantify the average starting number of proliferating cells at 3.5dpf and determine the final position of daughter cells at 5dpf, once they incorporated...
into the retina (Fig. 6A). Initial analysis of these samples revealed that most BrdU+ nuclei in both sibling and dnmt1−/− larvae were located within the CMZ after the 12 h incubation (Fig. 6C, E, G). However, there were a few BrdU+ cells that had incorporated into the neural retina (Fig. 6G). By comparing the number of BrdU+ nuclei of each retinal domain (CMZ, GCL, INL, ONL, Fig. 6B) to the total number of BrdU+ nuclei (Fig. 6H) at 3.5dpf, we noted a significant increase in the proportion of BrdU+ nuclei in the dnmt1−/− CMZ (79.5%, p < 0.05) compared to controls (71.7%, Fig. 6G; Supplemental Fig. S3A). Additionally, we found that the
proportion of BrdU+ cells in the \( \text{dnmt1}^{-/-} \) ONL (7.8%, \( p < 0.05 \)) was significantly reduced compared to siblings (10.9%, Fig. 6G; Supplemental Fig. 3A) at 3.5dpf. At 5dpf, all BrdU+ cells in the sibling controls had exited the cell cycle and incorporated into the neural retina (Fig. 6D, G), whereas \( \text{dnmt1}^{-/-} \) larvae retained 19.8% (\( p = 0.05 \)) of BrdU+ nuclei within the CMZ and had fewer BrdU+ cells overall within the retina (Fig. 6F, G). Additionally, there was a significant decrease in the proportion of BrdU+ nuclei in the GCL (9.8%, \( p < 0.0005 \)) (Fig. 6G; Supplemental Fig S3B) compared to controls (21.4%).

Surprisingly, among the cells that remained in the 5dpf \( \text{dnmt1}^{-/-} \) CMZ, there was an increase in the BrdU+ proportion when compared to siblings (19.76% vs. 0.9% respectively, \( p = 0.05 \); Fig. 6G; Supplemental Fig S3B) suggesting an inability for some RSCs to either successfully complete the cell cycle or to integrate into retinal laminae. These data also show that daughter cells produced from the \( \text{dnmt1}^{-/-} \) CMZ proportionally incorporate into the INL and ONL at similar levels to those detected in controls (Fig. 6G; Supplemental Fig S3B) supporting the notion that \( \text{dnmt1}^{-/-} \) RSCs are still capable of producing neurons that can successfully integrate into these two layers of the retina.

Loss of \( \text{dnmt1} \) activity leads to altered Long Terminal Repeat retroelement expression within the CMZ. Half of the zebrafish genome is comprised of endogenous viral elements known as transposons\(^{59,60} \), and \( \text{dnmt1} \) is required for repressing the retroelement (RE) lineage of transposons\(^{37,61–63} \). Though many REs have lost their ability to “jump” throughout evolution, some still retain this ability\(^{64,65} \). These studies led us to hypothesize that aberrant DNA methylation resulting from loss of \( \text{dnmt1} \) activity in RSCs would result in upregulation of RE expression within the \( \text{dnmt1}^{-/-} \) CMZ. To identify RE expression within the CMZ, we performed in situ hybridizations targeting several REs that belong to the Long Terminal Repeat (LTR) class of retrotransposons, specifically Bel20, ERV1, ERV1-N5, ERV4, and Gypsy10 LTRs. We noted endogenous expression of Bel20, ERV4, and Gypsy10 REs within the CMZ but not the neural retina of control larvae at 4dpf (Fig. 7A, D, E). This result was unexpected since REs can be deleterious to cellular function\(^{66–68} \). However, not all of the LTR REs were detected within control CMZs; ERV1 and ERV1-N5 expression was not detected in the CMZ of siblings (Fig. 7B, C), but rather ERV1-N5 seemed to be expressed within the ONL of some control larvae (Supplemental Fig. S4O). Remarkably, \( \text{dnmt1}^{-/-} \) larvae displayed patches of ERV1-N5 expression in the CMZ and within the overlying retinal pigmented epithelium (Fig. 7H). The distributions of Bel20 and ERV4 were also expanded beyond the CMZ into the neural retina of \( \text{dnmt1}^{-/-} \) larvae (Fig. 7F, I) when compared to controls. Of note, we also identified several non-ocular tissues that displayed altered RE expression between \( \text{dnmt1}^{-/-} \) and sibling control larvae (Supplemental Fig S4). Interestingly, these LTR RE expression patterns were larvae-dependent, suggesting that not all RSCs respond uniformly to loss of \( \text{dnmt1} \) function.

**A L1RE3-EGFP transgene reports increased LINE1 retrotransposition activity in \( \text{dnmt1}^{-/-} \) CMZ.** To expand our analysis of RE expression in \( \text{dnmt1}^{-/-} \) RSCs, and more specifically, visualize retrotransposition activity in vivo, we generated a non-LTR, LINE1 element transgenic reporter line by modifying the pLRE3-EGFP plasmid\(^{69,70} \) (referred to as L1RE3-EGFP for the remainder of this study). The L1RE3-EGFP

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**Figure 7.** Loss of \( \text{dnmt1} \) function results in misregulation of retroelement expression. A–J Transverse cryosections of sibling (A–E) and \( \text{dnmt1}^{-/-} \) (F–J) larvae at 4dpf. A, F Expression of Bel20 LTR. B, G Expression of ERV1 LTR. C, H Expression of ERV1-N5 LTR. D, I Expression of ERV4 LTR. E, J Expression of Gypsy10 LTR. Dotted lines: domains of retroelement expression. Scale bars = 10 μm. Dorsal is up in all images.
construct contains a human-derived LINE1 RE sequence that requires retrotransposition for EGFP to be expressed and translated into a functional protein. p53 is known to repress REs and when used transiently in p53−/− zebrafish, L1RE3-EGFP was shown to have increased transposition activity and EGFP expression. We validated the stability and effectiveness of the L1RE3-EGFP transgenic using again p53 mutants and immunolabeling for EGFP (Supplemental Fig S5). When L1RE3-EGFP was incorporated into the dnmt1 genetic background, ectopic EGFP expression could be seen within the dnmt1−/− eye when compared to control siblings (Supplemental Fig S5B,C). Notably, we were able to detect ectopic EGFP expression within the dnmt1−/− CMZ at both 3dpf (Fig. 8C) and 4dpf (Fig. 8D) timepoints when compared to controls (Fig. 8A, B). However, similar to RE expression patterns, clonal EGFP expression patterns were variable, both within and between sibling controls and dnmt1−/− larvae, again suggesting that the effects of dnmt1 loss is variable from cell to cell and larva to larva.

Discussion

The zebrafish, with its lifelong, actively cycling RSCs within the CMZ, is a powerful model through which we can address how epigenetic regulators function to maintain these stem/progenitor cell populations in vivo. This study focused on the role of the DNA maintenance methyltransferase, dnmt1, within the CMZ, with the goal of determining how dnmt1 activity facilitates RSC maintenance. Previous work has shown that loss of dnmt1 function results in ocular defects, but no studies have yet analyzed RSC populations and determined whether dnmt1 activity modulates their behavior.

Here, we demonstrate that dnmt1 is essential for RSC homeostasis by maintaining CMZ-specific gene expression (Fig. 4), facilitating cell cycle progression (Fig. 5), and incorporation of CMZ-derived cells into the retina (Fig. 6). These data are consistent with Dnmt1 functions described in other in vivo progenitor models such as the lens, hippocampus, kidney, pancreas and intestine. RSCs in S- and G2/M-phases of the cell cycle were detected in reduced proportions in the dnmt1−/− CMZ and this correlated with a reduction in CMZ expression of genes encoding proteins that function in cell cycle progression, namely ccnD1 (Fig. 4G, H) and

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**Figure 8.** RSCs require dnmt1 function to repress L1RE3-EGFP transposition. A–D Transverse sections of Tg(CMV:Hsa.L1RE3, EGFP, myl7:EGFP; dnmt1+/+) (A, B) and Tg(CMV:Hsa.L1RE3, EGFP, myl7:EGFP; dnmt1−/−) (C, D) larvae at 3dpf (A, A′, C, C′) and 4dpf (B, D). Nuclei labeled with DAPI (gray). Endogenous EGFP expression activated after L1RE3-EGFP transposition labeled in green. Arrows delineate EGFP + cells. Scale bars: 30 μm. Dorsal is up in all images.
Defects in cell cycle progression may also contribute to aberrant daughter cell integration into retinal laminae detected in *dnmt1*−/− larvae (Fig. 6).

It is critical to note that while the RSCs are more affected by loss of *dnmt1* function than fully differentiated neurons within the GCL, INL, and ONL, we cannot rule out the possibility that any of the surrounding tissues could be contributing to the CMZ phenotype. Indeed, it is known *dnmt1* loss can influence cells and tissues through both autonomous73,74 and non-autonomous65 mechanisms. There are multiple tissues surrounding the CMZ that influence RSC identity16,72 and these include differentiated neurons in the retina, lens, RPE and vasculature; loss of *dnmt1* function in any of these could non-autonomously result in CMZ defects. Future work focused on tissue and/or cell type-specific loss of *dnmt1* function will be critical for defining its autonomous and non-autonomous roles in RSC maintenance.

While loss of *p53* function in the *dnmt1*−/− background significantly rescued cell death within the laminated retina, validating that the *p53* allele is in fact inhibiting *p53*-driven apoptosis, loss of *p53* in the *dnmt1*−/− CMZ had no effect on CMZ cell numbers suggesting a *p53*-independent cell death pathway is likely modulated by *dnmt1* in the CMZ23. Recent reports have demonstrated an upregulation of an innate inflammatory response in *dnmt1*−/− larvae75. Necroptosis, a programmed cell death pathway tightly linked to a cell's innate viral detection system and inflammatory response, also results in DNA fragmentation and, in its later stages, is detected by TUNEL73. Indeed, we noted upregulation of the inflammatory genes, *tra5* and *il-1β*, and some cell death pathway markers, *p53* and *ripk1* (Fig. 4U); however, these data were obtained from whole larvae qPCR and thus are compounded by systemic expression changes. Accordingly, we considered the possibility that *dnmt1*−/− RSCs were instead lost via necroptosis. We tested this hypothesis using several chemical inhibitors of necroptosis, some of which have been reported to function in the zebrafish77,78,79; however, we were unable to replicate necrotic inhibition nor validate drug efficacy. None the less, we predict that either necroptosis or pyroptosis (a programmed cell death pathway triggered by intracellular bacterial infections76,77) are the most likely mechanisms of cell death in *dnmt1*-deficient RSCs, but this will require the development of new tools to enable further analysis.

Alterations in RE expression activity the *dnmt1*−/− CMZ (Figs. 7, 8) are exciting given Dnmt1's known roles in repressing RE activity36-39. RE expression was aberrant in most *dnmt1*−/− CMZs examined (Fig. 7); however, expression changes and levels were variable between larvae, suggesting that the location and extent of genomic hypomethylation resulting from loss of *dnmt1* function is inherently variable between cells of each larva. Previous reports demonstrated innate RE activity within somatic neural tissue64,65,78-80. Indeed, we detected retrotransposition activity within the larval zebrafish brain (Supplemental Fig S5D–I) of both siblings and *dnmt1*−/− larvae from 2 to 4dpf, similar to activity detected in human hippocampal neurons64,65,80. However, RE retrotransposition is highly variable between larvae. Further studies will be required to determine what cellular processes might sensitize a cell- or tissue-type to upregulate REs and whether these REs have a mechanistic purpose within the cell.

In conclusion, our results demonstrate that *dnmt1* functions to maintain RSC proliferation, gene expression, and integration of RSC daughters into the retina. Additionally, some REs are innately expressed within RSCs, however *dnmt1* function is required to maintain tight control of these viral elements. Without *dnmt1* activity, LTR expression remains active within the retina and *LIRE3-EGFP* retrotransposition activity is increased. Interestingly, RE activity within RSCs does not result in *p53*-mediated apoptosis, supporting a model in which *dnmt1*−/− RSCs are lost through another mechanism of cell death. As discussed above, we predict that this increase in RE activity most likely activates necrotic or pyroptotic cell death pathways, which are both known to result from intracellular responses to invading pathogens37,56,77. Regarding the innate LTR expression within *dnmt1*−/− RSCs, in conjunction with previous reports of inherent RE activity within human neural tissue, it is worth considering how RE activity may contribute to neural stem cell biology. It is well known that dysregulation of REs is a hallmark of many human neurodegenerative diseases67,81-84. Future evaluations regarding the innate cost-to-benefit ratio of RE activity could provide crucial evidence for the development of neurodegenerative therapies.

Methods
Zebrafish maintenance. Zebrafish (*Danio rerio*) were maintained at 28.5 °C on a 14 h light/10 h dark cycle. All protocols used within this study were approved by the Institutional Animal Care and Use Committee of The University of Pittsburgh School of Medicine, and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mutant alleles used in this study were *dnmt1*−/− and *p53*−/−, *dnmt1*−/− and *tp53*−/− zebrafish were genotyped using BioRad's CFX Manager 3.1 and Precision Melt Analysis software (v4.05.20602). All genotyping primers are listed in Supplemental Table 1. Transgenic Tg(CMV:Has.L1RE3, EGFP, myl7:EGFP)fo279 zebrafish were generated as described85 using constructs generously provided by Kristen Kwan and Chi-Bin Chien (University of Utah, Salt Lake City).

BrdU labeling. To assess cellular proliferation, larvae were incubated in 10 mM BrdU for either 2 or 12 h, after which the BrdU was washed out and larvae were either collected or used for BrdU pulse-chase experiments.

Immunohistochemistry and fluorescent labeling. Immunohistochemistry performed as described previously86. The following antibodies and dilutions were used: anti-BrdU antibody (Abcam, ab6326, 1:250), anti-phospho-histone H3 (Ser10) (EMD Millipore, 06-570, 1:250), anti-GFP (Thermo Fisher Scientific, A-11122, 1:50), goat anti-rat Cy3 secondary (Jackson Immuno Research, 112-165-003, 1:500), goat anti-rabbit Cy3 secondary (Jackson Immuno Research, 111-165-144, 1:500), and goat anti-rabbit Cy5 secondary (Jackson Immuno Research, 711-035-152, 1:500). Nuclei were counterstained with DAPI using Vectashield with DAPI (Vector Laboratories, H-1200). F-actin was labeled using AlexaFluor 633 Phalloidin (Thermo Fisher Scientific, A-11122, 1:50), goat anti-rat Cy3 secondary (Jackson Immuno Research, 112-165-003, 1:500), goat anti-rabbit Cy3 secondary (Jackson Immuno Research, 111-165-144, 1:500), and goat anti-rabbit Cy5 secondary (Jackson Immuno Research, 711-035-152, 1:500).
Cloning and probe synthesis. CMZ-specific probes have been published previously\(^{14,27}\). Retroelement probes were generated using reverse transcription-polymerase chain reaction (RT-PCR) on Trizol-isolated RNA from 4dpf and 5dpf embryos. Primer sequences were kindly provided by Dr. Kirsten Sadler (NYU Abu Dhabi) and PCR products were ligated into pGEM-T Easy vector (Promega Cat# PR-A1360) and verified by Sanger sequencing. Plasmids containing the correct clones were linearized and used as templates to in vitro transcribe digoxigenin-labeled RNA probes (Roche).

In situ hybridization. Hybridizations using digoxigenin labeled antisense RNA probes were performed essentially as described\(^{88}\), except that they were pre-incubated with 1 mg/mL Collagenase type 1A (Sigma, C9891) to allow probe diffusion throughout the tissue. All probe primer sequences are listed in Supplemental Table S1.

RNA isolation and cDNA synthesis. Total RNA was extracted from three biological replicates of whole 4dpf zebrafish sibling and \(dnmt1^{−/−}\) larvae (n = 16–18 per replicate) using Trizol Reagent (Thermo Fisher Scientific, 15–596-018) according to the manufacturer’s instructions. RNA concentrations and absorbance ratios \((A_{260/280} \text{ and } A_{260/230})\) were measured using a Nanodrop spectrophotometer. RNA from each sample was reverse-transcribed using iScript cDNA Synthesis Kit (BioRad, 1708891).

Quantitative PCR. qPCR was performed using a BioRad CFX384 Real-Time PCR machine. All reactions were carried out in triplicate using iTaq Universal SYBR Green Supermix (BioRad, 1725121), following the manufacturer’s instructions. Each reaction was performed in the final volume of 10 µL. The thermocycler program consisted of an initial hot start cycle at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Product specificity and melt curve analysis was performed after each amplification (65–95 °C in 0.5 °C increments; 5 sec per step). Three controls were used for expression normalization: \(c\text{fla}, \text{gapdh, and } \beta\text{-actin.}\) Primer efficiencies were determined using cDNA serial dilution tests and melt curve analysis. All qPCR primer sequences are listed in Supplemental Table S1.

qPCR statistical analyses. \(C_q\) values were transformed to linear scale and the normalization factor was calculated as the geometric mean of candidate reference genes included in the dataset as described\(^{88}\). Variance analyses between siblings and \(dnmt1^{−/−}\) larvae were performed using 2-way ANOVA test followed by a post-hoc Bonferroni test with significance set to \(p < 0.05\). Graph (Fig. 4U) depicts average relative fold expression levels with 95% confidence intervals of \(dnmt1^{−/−}\) larvae relative to sibling controls.

Microscopy and image processing. For sectioned embryos, imaging was performed with an Olympus FV1200 confocal microscope. Confocal Z-stacks were collected in 1 µm optical sections. Z-stacks were max-projected using ImageJ (version 1.52r) software (National Institutes of Health) and quantification was conducted using the “Cell Counter” plugin. Figures were prepared using Adobe Illustrator CS6 (Adobe Systems). In situ cryosections were imaged utilizing a Leica DM2500 with a 100X oil immersion objective (NA: 1.25).

Cell counting and quantification. Each data point was collected from an individual larva. Each larva was analyzed using three consecutive 12 µm sections of the central retina using the optic nerve and lens morphology as retinal landmarks. The CMZ domain was defined as the region of cells posterior to the RPE and anterior to the IPL and OPL, using both nuclear and Phalloidin staining as markers. Nuclear morphology was taken into consideration when determining layer-specific cellular locations where CMZ nuclei display an elongated, or oval, shape in comparison to the spherical nuclei seen in the GCL and INL. Photoreceptor nuclei were defined by elongated morphology and with peripheral phalloidin staining of outer segments. The average of the three consecutive sections was used as a single data point (n ≥ 4 for all datasets). Proportions of retinal domains were calculated by dividing the number of DAPI-labeled nuclei in each domain over the total number of retinal nuclei.

Statistics. For all statistical analysis, data were imported into GraphPad Prism 8 software. Quantification of nuclei and immunolabeled cells was statistically assessed using Student’s two-tailed unpaired \(t\) test with \(p < 0.05\) as a significance threshold.

Generation of Tg(CMV:Hsa.L1RE3, EGFP, myl7:EGFP)\(^{p701}\). pLRE-mEGFPi plasmid was generously donated by Dr. John V. Moran (The University of Michigan School of Medicine)\(^{69}\). The Hsa.L1RE3-EGFP sequence was isolated from the pCEP4 backbone using NotI and Sall restriction enzymes and then inserted into pME-MCS plasmid from the Tol2 Gateway Kit. LR Clonase II Plus was used to carry out all Multisite Gateway assembly reactions\(^{85}\) using p5E-MCS (19 ng), pME-Hsa.L1RE3-EGFP (77 ng), p3E-polyA (19 ng), and pDestTol2CG2 (103 ng) plasmids. Capped Tol2 mRNA was synthesized using iScript cDNA Synthesis Kit (BioRad, 1708891). 4dpf zebrafish sibling and \(dnmt1^{−/−}\) larvae relative to sibling controls.
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Author contributions
J.M.G and K.M.A. designed and conceived the study; K.M.A. collected all samples and performed the experiments and analyses; J.M.G and K.M.A. interpreted the results, wrote and reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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