Depletion of minichromosome maintenance protein 5 in the zebrafish retina causes cell-cycle defect and apoptosis

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In multicellular organisms, the control of genome duplication and cell division must be tightly coordinated. Essential roles of the minichromosome maintenance (MCM) proteins for genome duplication have been well established. However, no genetic model has been available to address the function of MCM proteins in the context of vertebrate organogenesis. Here, we present positional cloning of a zebrafish *mcm5* mutation and characterization of its retina phenotype. In the retina, *mcm5* expression correlates closely with the pattern of cell proliferation. By the third day of development, *mcm5* is down-regulated in differentiated cells but is maintained in regions containing retinal stem cells. We demonstrate that a gradual depletion of maternally derived MCM5 protein leads to a prolonged S phase, cell-cycle-exit failure, apoptosis, and reduction in cell number in *mcm5*^{m850} mutant embryos. Interestingly, by the third day of development, increased apoptosis is detectable only in the retina, tectum, and hindbrain but not in other late-proliferating tissues, suggesting that different tissues may employ distinct cellular programs in responding to the depletion of MCM5.

stem cell | cell proliferation | ciliary marginal zone | embryogenesis | development

Proper duplication of the genome during the cell cycle is of paramount importance for the life of an organism. In eukaryotes, each replication origin is “licensed” to fire once per cell cycle through the cell-cycle-dependent formation and destruction of a prereplication complex (preRC) (1–3). A key component of this preRC is a family of six structurally related proteins, MCM2 through -7, which are evolutionarily conserved in all eukaryotes. The MCM proteins were originally identified as proteins required for minichromosome maintenance in *Saccharomyces cerevisiae* (4). MCM2 through -7 belong to a distinct subgroup of the large AAA+ ATPase family (5) and share a conserved central region of ≈200 amino acids (MCM box). Biochemical studies in *Xenopus* have established the role of MCM2 through -7 as replication-licensing factors (6–8). Subsequent studies illustrate that proper orchestration of the functional interactions among MCM2 through -7 proteins and other components of the preRC by cell-cycle-dependent protein kinases results in initiation of DNA synthesis once every cell cycle (9, 10). The MCM2 through -7 proteins appear to form heterohexamers and play important roles in initiation and elongation during DNA replication (4, 11, 12). Furthermore, recent evidence supports involvement of MCMs in many other chromosome transactions, including transcription, chromatin remodeling, and genome stability (13). However, *in vivo* analysis of MCM-protein functions in multicellular organism has been scarce. Here, we report the isolation, positional cloning, and in-depth characterization of a vertebrate mutant in an MCM-family protein. We demonstrate that the zebrafish *m850* allele harbors a mutation in the *mcm5* gene and exhibits developmental defects in late-proliferating tissues, including the retina and the brain. We focus on the analysis of retinal development because of its well described cell types and proliferation pattern and show that *mcm5* is expressed in the region where retinal stem cells and progenitor cells are found.

Furthermore, we find that the reduction in MCM5 level leads to S-phase prolongation, defects in cell-cycle progression, and a strong activation of apoptosis in the zebrafish retina. The activation of apoptosis is also observed in tectum and hindbrain but not in other proliferative tissues, indicating the existence of tissue-specific responses to the reduction of MCM5 level.

Materials and Methods

Fish Maintenance and Mutagenesis Screen. Zebrafish maintenance and breeding were carried out under standard conditions at 28.5°C (14). Embryos were staged and fixed at desired time points [hours or days postfertilization (hpf or dpf, respectively)] in 4% paraformaldehyde in PBS. To avoid formation of melanin pigments, embryos were incubated in 0.2 mM 1-phenyl-2 thiourea (Sigma). *mcm5*^{m850} was isolated during an ethyl-nitrosourea mutagenesis screen performed in our laboratory (15).

In Situ Hybridization, Immunohistochemistry, Cartilage Staining, and Apoptosis Assay. Whole-mount *in situ* hybridization was performed as described in ref. 16. Digoxigenin- or fluorescein-labeled antisense RNA probes were prepared by using RNA-labeling reagents (Roche Biochemicals). We prepared RNA probes for the following genes: *th* (17), *ath5* (18), and rhodopsin (a gift of K. Dürre, University of Freiburg). To generate an antisense probe for *mcm5*, the N-terminal 725-bp fragment of the *mcm5* gene was PCR-amplified. Immunohistochemistry was performed as described in ref. 15. Anti-Zn5 (19), anti-BrdUrd (Becton Dickinson), and anti-phospho histone H3 (Upstate Biotechnology, Lake Placid, NY) were used at 1:1,000 dilution. Whole-mount stained embryos were cut into 15-μm sections by using a vibratome. Unstained 3-dpf embryos were embedded in plastic (Technovit, Heraeus), cut into 6-μm sections, and stained for a few minutes with methylene blue solution. For S-phase analysis, 48-hpf embryos were incubated for 15 min at room temperature in 10 mM BrdUrd solution (containing 15% DMSO in egg water), fixed immediately in 4% paraformaldehyde in PBS, and embedded in paraffin. Transverse sections of 6-μm thickness were subjected to antigen heat retrieval. A biotin-avidin horseradish peroxidase system (Vectastain ABC-kit, Vector Laboratories) was used for detection. TUNEL assay was performed by using the Apoptag peroxidase *in situ* apoptosis-detection kit (Intergen). Fixed and permeabilized embryos were incubated in ethanol/acetic acid mixture for 15 min and incubated in equilibration buffer, followed by terminal transferase enzyme solution. digoxigenin-labeled DNA fragments were detected by using anti-digoxigenin antibody conjugated to alkaline phosphatase. Anti-active caspase-3 immunohistochemistry and detection of MCM5

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Abbreviations: CMZ, ciliary marginal zone; dpf, days postfertilization; hpf, hours postfertilization; MCM, minichromosome maintenance.

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protein are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Mapping and Cloning of *mcm5^{m850}*. We mapped *mcm5^{m850}* using bulked-segregant analysis (20) with pooled DNA from F₂ homozygous mutants and F₂ wild-type siblings from a *mcm5^{m850}* AB × India map cross. We linked *mcm5^{m850}* to linkage group 3 between simple-sequence-polymorphism markers z11015 and z59609. We generated additional polymorphic markers based on sequences from EST fa94d12 and the netrin gene. z59609 was used to identify bacterial artificial chromosome (BAC) zC100E2 from the Wellcome Trust Sanger Center BAC fingerprinting project (www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish). An end-sequence of BAC zC100E2 was used to identify contig z06s019176 from the Wellcome Trust Sanger zebrafish genome project assembly (www.ensembl.org/Danio_rerio). The *mcm5* sequence was identified within this contig and represented the sequence identical to EST BC044460. *mcm5* isolated from cDNA generated from seven dpf *m850* mutants and their sibling embryos was sequenced, revealing the lack of exon 2 in transcripts derived from *mcm5^{m850}* mutant embryos. An ≈500-bp region surrounding exon 2 was amplified from genomic DNA isolated from individual *mcm5^{m850}* mutants and their sibling embryos. Sequencing of these PCR products revealed a T-to-C base change within the splice donor site at the end of the second exon in the mutant embryos.

RNA Injection. To generate *mcm5* mRNA, full-length *mcm5* was PCR-amplified from cDNA from wild-type embryos and cloned into pCS2+ vector (Clontech). Synthetic capped mRNA was generated by using the mMessage mMachine kit (Ambion), and 200 pg of mRNA was injected into single one-cell-stage embryos. The morpholino-injection procedure can be found in *Supporting Materials and Methods*.

DNA-Content Analysis. Retinae were dissected from anesthetized 48- to 50-hpf mutant and wild-type embryos. Single-cell suspension was achieved after room-temperature incubation for 1 hour in 20 units/ml papain in L15 tissue culture media (Sigma) and repeated trituration using fire-polished glass pipettes. Cells were resuspended in 4 mM citrate buffer, pH 6.5, containing 0.1 mg/ml propidium iodide (Sigma), 200 μg/ml RNase, and 0.1% Triton X-100 and were stored in the dark at 4°C until analysis. Data acquisition was performed by using a Becton Dickinson FACS-Calibur machine and analyzed by using the program CELLQUEST.

Results

m850 Mutant Embryos Display Reduction in Cell Number in the Retina.

We isolated the *m850* mutant allele in a genetic screen for mutations affecting the formation of the catecholaminergic system, based on the severe reduction in the number of tyrosine hydroxylase (*th*)-expressing cells in the CNS of mutant embryos. Furthermore, *m850* mutant embryos develop morphological defects during dpf 3, including a smaller retina and head compared with wild-type embryos (Fig. 1 *A* and *B*). The jaw and branchial arches remain small and do not develop properly in mutant embryos (see Fig. 6, which is published as supporting information on the PNAS web site). In contrast, the overall body size is similar in mutant and wild-type embryos, and early-forming tissues, including somites and notochord, are largely normal during the first 4 days of development. To better understand the defects in *m850* mutants, we focused our phenotypic analysis on the retina, because it contains several well characterized cell types, and its cell-proliferation pattern has been well described (21). The zebrafish retina, as is typical for vertebrates, consists of three major cell-body layers. The innermost ganglion cell layer consists of ganglion cells whose processes form the optic nerve. The amacrine, bipolar, and horizontal cells are found in the intermediate inner nuclear layer, and photoreceptor cells are found in the outermost photoreceptor layer (Fig. 1*C*).

Fig. 1. *m850* mutant embryos display reduction in cell number in the retina. Live wild-type (*A*) and *m850* mutant (*B*) embryos at 3 dpf. A smaller head and smaller eyes characterize the *m850* mutant. (*C* and *D*) Transverse sections through the eye at 3 dpf, stained with methylene blue. (*C*) The wild-type retina shows characteristic stratification in three nuclear layers (GCL, INL, and PR) and two plexiform layers. (*D*) In *m850* mutant embryos, the stratification of retinal layers occurs, although the number of cells across all three retinal nuclear layers is severely reduced. (*E* and *F*) Transverse sections of 3-dpf embryos stained with anti-Zn5 (α -Zn5) antibody exhibit the presence of optic nerves in wild-type (*E*) and *m850* mutant (*F*) embryos, albeit they are with reduced intensity. (*G* and *H*) Transverse sections through the retinae of wild-type embryos (*G*) display dopaminergic amacrine cells, as detected by the expression of tyrosine hydroxylase (*th*) at 3 dpf. Dopaminergic amacrine cells can also be detected in mutant embryos (*H*). (*I* and *J*) Transverse sections through the retinae of 3-dpf embryos showing rod photoreceptors, as detected by the expression of rhodopsin (*rhod.*). The presence of differentiated rod photoreceptors is clearly visible in mutants (*I*). (*A* and *B*) Lateral views, anterior left, dorsal up; (*C*–*J*) transversal sections, dorsal up. (Scale bars, 100 μm.) Black arrows indicate the optic nerve (*F*), a dopaminergic amacrine cell (*H*), or a photoreceptor cell (*I*). GCL, ganglion cell layer; INL, inner nuclear layer; PR, photoreceptor layer.

In *m850* mutant retinae, at dpf 3, the relative positioning of the individual layers appears to be preserved in the central retina; however, the number of cells in each layer is severely reduced (Fig. 1*D*).

To elucidate whether differentiation and cell-type specification are affected in *m850* mutant retinae, we used markers for differentiated cell types present in each of the three retinal layers. In

Fig. 2. The zebrafish *mcm5* gene is disrupted in *m850*. (A) Schematic representation of the genetic map of linkage group 3 (LG 3) and genomic organization of the *mcm5* locus showing the position of the mutation. Some of the closest simple-sequence-polymorphism and SNP markers are listed, and their genetic distances relative to the mutation are indicated as the number of recombinants in a total of 1,786 meioses. (B) RT-PCR from wild-type and mutant RNA using primers from exons 1 and 3 reveals that the mutant transcript is shorter. M, 100-bp DNA ladder. (C) Chromatogram of genomic sequences from the 3' end of exon 2 from wild-type, heterozygous, and mutant embryos. The T-to-C base change in the mutant embryo affects the first of the two core bases, highly conserved in eukaryotic splice donor sites. (D) Skipping exon 2 produces a frame shift, which introduces a stop codon at amino acid residue 74 in mutant embryos. (E–G) RNA rescue of the *m850* mutant phenotype. The mutant phenotype was judged by the eye and head size and the presence of dopaminergic amacrine cells, as detected by *th* whole-mount *in situ* hybridization at 3 dpf. All pictures show lateral views, dorsal up. (E) Wild-type retina. (F) *m850* mutant retina. (G) Injection of 200 pg of *mcm5* RNA into *m850* mutant embryos rescued the mutant phenotype ($n = 19$ of 26).

zebrafish, the Zn5 monoclonal antibody recognizes a surface adhesion molecule of the Ig superfamily neurolin/DM-GRASP (22) and marks retinal ganglion cells and their projections. Albeit reduced in its intensity, the projection of retinal ganglion cells is clearly visible in the mutant retinae (Fig. 1 E and F). To test whether the differentiation of cells from other retinal layers is affected, we used *th* expression to mark the dopaminergic subset of amacrine cells within the inner nuclear layer (17) and rhodopsin expression to label photoreceptors. We detected both of these neuronal types in mutant retinae, although their cell number was severely reduced (Fig. 1 G–J). These results suggest that patterning and cell-type specification occur in *m850* mutant embryos similar to wild-type embryos and that the primary defect in the *m850* mutant retina may involve cell proliferation or cell survival.

The *m850* Mutation Disrupts the Zebrafish *mcm5* Gene. To elucidate the molecular basis of the *m850* mutation, we identified the affected gene by using a positional cloning strategy. Using the simple-sequence-polymorphism-marker-based map (23) and EST-derived markers, we defined a critical interval of 0.45 cM flanked by markers z59609 and EST fa94d12 (Fig. 2A). Using z59609 se-

quences, we isolated BAC zC110E2, whose end sequence overlapped with the contig z06s019176 from the Wellcome Trust Sanger zebrafish genomic sequence database (www.emsembl.org/Danio_rerio). A polymorphic marker generated from this contig sequence had no recombination event in 1,786 meioses, suggesting close proximity to the mutation. The contig z06s019176 contained two ORFs: an ORF with 80% identity to human MCM5 at the protein level and the heme oxygenase 1 (*hmox1*) gene. In vertebrates, Hmox1 catabolizes cellular heme to biliverdin, carbon monoxide, and free iron (24) and, in zebrafish, is expressed in the extraembryonic yolk syncytial layer, lens, and a small population of blood cells (Thisse *et al.*, 2004 at www.zfin.org). Because *hmox1* is not expressed in the tissues where we observe strongest mutant phenotypes, including retina and the brain, we decided to focus on the *mcm5* gene for our mutation search. Sequencing of the genomic *mcm5* locus from the mutant embryos revealed a T-to-C base change within the highly conserved splice donor site at the end of the second exon (Fig. 2C). RT-PCR using mRNA from mutants and wild-type embryos revealed the presence of a shorter *mcm5* transcript in mutant embryos that lacks the entire second exon (Fig. 2B). The mutant transcript contains a frame shift and a premature stop codon at amino acid 74, resulting in a protein that lacks $\approx 90\%$ of the wild-type sequence, including the highly conserved MCM box, and, thus, is likely to be a null allele (Fig. 2D). To provide further evidence that *mcm5* is the gene disrupted in the *m850* allele, we rescued the retinal phenotypes by *mcm5* mRNA injection into mutant embryos and phenocopied *m850* by morpholino-knockdown experiments (Figs. 2 E–G; and see Figs. 7 and 8, which are published as supporting information on the PNAS web site). Taken together, our results demonstrate that *m850* is an amorphic mutant allele at the zebrafish *mcm5* locus. Thus, we will refer to the *m850* mutation as *mcm5*^{*m850*} from here onward.

Although *mcm5*^{*m850*} is likely a null allele, the presence of maternally provided *mcm5* mRNA and MCM5 protein may partially substitute for zygotic loss of *mcm5* during early embryonic development. By generating antibody against MCM5 protein, we demonstrated the existence of maternally derived MCM5 protein, which persists beyond 3 dpf in zebrafish embryos (see Fig. 9, which is published as supporting information on the PNAS web site).

***mcm5* Expression Correlates with the Pattern of Cell Proliferation.** To learn more about the function of *mcm5* in the zebrafish retina, we analyzed its expression pattern during development by using whole-mount *in situ* hybridization. Until ≈ 28 hpf, zebrafish retinae consist of an apparently uniform population of rapidly proliferating neuroepithelial cells (25, 26). The onset of terminal mitoses begins ≈ 28 hpf and continues in three major bursts separated by two 10-hour intervals (27). Most retinal cell proliferation has slowed down by the end of this period (58 hpf); however, it continues in the ciliary marginal zone (CMZ), where retinal stem cells are maintained, even in adult fish (28, 29). The pattern of *mcm5* expression follows closely this pattern of proliferation in the zebrafish retina. Early on, *mcm5* is ubiquitously expressed in the entire retina (data not shown). However, by 38 hpf, *mcm5* expression has begun to decrease where differentiation occurs in the retina but is maintained in a region that includes the CMZ (Fig. 3A). By 58 hpf, *mcm5* expression remains detectable only in the CMZ (Fig. 3C).

To elucidate the cell types in which *mcm5* is expressed, we compared the expression pattern of *mcm5* with that of proliferating cell nuclear antigen (*pcna*), *ath5*, and *elavl3* at 48 hpf (Fig. 3 D–H). Whereas *pcna* is expressed in noncommitted proliferating cells, the expression of the bHLH transcription factor *ath5* serves as one of the first markers of neurogenesis in the zebrafish retina. *ath5* is first expressed in a small group of cells in the ventronasal retina at 25 hpf, and its expression spreads in a manner preceding differentiation (18). By 48 hpf, *ath5* is expressed in retinoblast cells and, possibly, postmitotic neurons before full differentiation but not in differentiated cells in the central retina. In contrast, the zebrafish *elavl3* gene

Fig. 3. In the retina, *mcm5* expression is down-regulated in regions containing differentiated cells but is maintained in the region containing retinal stem cells. (A–C) Transverse sections of the retina of wild-type embryos after whole-mount *in situ* hybridization for *mcm5*. (A) At 38 hpf, whereas *mcm5* expression in the retina is broad, it is stronger in the marginal zone. At 48 (B) and 58 (C) hpf, strong *mcm5* expression is maintained in the ciliary marginal zone (cmz) but is down-regulated in other areas of the retina. (D–F) Expression of *pcna* (blue) (E), marking noncommitted proliferating cells, overlaps exactly with that of *mcm5* (red) (D), resulting in brown staining (F). (G) *ath5* (blue), marking neurons before full differentiation, overlaps with the lateral extent of *mcm5* (red). (H) *elavl3* (blue), marking fully differentiated neurons, shows no overlap with *mcm5* (red). Anterior is up.

encodes an RNA-binding protein homologous to *Drosophila elav* that serves as an early marker for differentiated neurons (30). In vertebrate retinae, *elavl3* is expressed in ganglion cells and the majority of amacrine cells in the inner nuclear layer (31). Our analysis shows that *mcm5* and *pcna* are expressed in identical populations of proliferating cells (Fig. 3F). *mcm5* and *elavl3* are expressed in a complimentary manner with virtually no overlap, whereas *mcm5*- and *ath5*-expression domains partially overlap (Fig. 3 G and H), revealing that *mcm5* expression is maintained in proliferative retinal cells in the CMZ region but is down-regulated in differentiated cells in the central retina.

Activation of Apoptosis in CNS Tissues in *mcm5^{m850}* Mutant Embryos.

mcm5m850 mutant embryos die as larvae between 5 and 10 dpf with small head and eyes. To test whether this is because of the lack of cell proliferation or whether activation of programmed cell death is involved, we performed TUNEL assays in *mcm5m850* mutants at 48 hpf, after the maternally derived MCM5 levels have significantly decreased. We observed a strong activation of apoptosis in the retina, tectum, and hindbrain (Fig. 4). This activation was most dramatic at 2 dpf and continued beyond 3 dpf (Fig. 4 and data not shown). The same pattern of apoptosis was detected by immunohistochemistry using anti-active caspase-3 (see Fig. 10, which is published as supporting information on the PNAS web site). Caspase-3 is a key protease that is activated during the early stages of apoptosis and is, therefore, a sensitive and specific marker for cells undergoing apoptosis (32). To-

Fig. 4. Apoptosis is activated in CNS tissues in *m850* mutant embryos. (A–J) TUNEL assay for apoptosis in whole-mount wild-type and *mcm5^{m850}* mutant embryos at 2 dpf. (A and B) Apoptosis is activated in retina, tectum, and hindbrain in the mutant embryos. (C–H) *mcm5^{m850}* mutant embryos show more apoptotic cells in the retina (C and D), the tectum (E and F), and the hindbrain (G and H) but not in the branchial arches and the brain ventricular zone (I and J), compared with wild-type embryos. (A–D) Lateral view; (E–H) dorsal views; (I and J) ventral views. Anterior toward the left. (Scale bars, 100 μ m.) ba, branchial arches; hb, hindbrain; t, tectum; vz, ventricular zone.

gether, these results suggest that activation of programmed cell death might be the prominent cause of reduction in head and eye size in *mcm5^{m850}* mutant embryos. Interestingly, retina and tectum are only some of the proliferative tissues in the zebrafish embryo at 2–3 dpf. Strong *mcm5* expression is detected in highly proliferative tissues such as forebrain ventricular zones, the branchial arches, and endoderm (Thisse *et al.*, 2004 at www.zfin.org; S.R. and W.D., unpublished data), which do not show activation of apoptosis at 2 dpf (Fig. 4*A* and *J*) or at 3 dpf (data not shown). This result suggests that different proliferative tissues may employ distinct cellular programs in responding to the reduction in MCM5 level.

loss of the MCM complex, prompting the notion that the presence of the replication-licensing system defines the proliferative state of a cell (39). Similarly, we find that *mcm5* expression correlates remarkably closely with *pcna* expression and with the pattern of cell proliferation. Furthermore, we compared the *mcm5*-expression pattern with those of early stage (*ath5*) and later stage (*elavl3*) markers of neurogenesis. *mcm5* expression overlaps partially with that of *ath5* but does not overlap with that of *elavl3*, suggesting that *mcm5* is rapidly down-regulated as differentiation proceeds. Interestingly, at 48 hpf, most *mcm5* expression is in the marginal zone of the retina, although BrdUrd and phosphorylated histone H3 immunoreactivity indicate the presence of cycling cells in the periphery, representing cells that will soon undergo cell-cycle exit and differentiation. The retinæ of many fish and amphibians grow throughout life, and new retinal cells are continually added at the CMZ. Furthermore, retinal regeneration in these animals occurs through retinal stem cells present in the CMZ (40). In contrast, the other areas within the retina are largely composed of fully differentiated cells with no further potential for proliferation. Thus, our expression analysis in the retina suggests that *mcm5* expression may provide a sensitive marker to define tissues that possess the capacity for further proliferation. *mcm5* is expressed in cells that can continue to proliferate. However, it appears to be down-regulated, ahead of the actual cell cycle exit, in those cells that will soon differentiate.

To assay the effect of reduced MCM5 at the cellular level, we assayed DNA content by FACS analysis at 48–50 hpf, when the maternal MCM5 level is significantly reduced. Using dissociated retinal cells from *mcm5^{m850}* mutant and wild-type embryos, we observed an increased fraction of cells with >2C DNA content in the mutant retinæ. This increase could indicate either an increase in the number of cells entering S phase or a prolongation of S phase in *mcm5^{m850}* mutant embryos. Our data supports the latter explanation, because we observe no increase in cells positive for BrdUrd incorporation in *mcm5^{m850}* mutant embryos at this stage (see Fig. 11, which is published as supporting information on the PNAS web site). The prolongation of S phase is most likely caused by a defect in DNA replication, because MCM2 through -7 have been suggested to be important for both initiation and elongation during DNA replication. With the reduced level of functional MCM complex in *mcm5^{m850}* mutants, fewer replication forks would likely be initiated, and the elongation of existing replication forks might slow, perhaps resulting in delayed progression through S phase. Interestingly, many *mcm5^{m850}* mutant cells accumulate near 4C

peak and may have finished replicating the bulk of their DNA, reminiscent of the situation in budding yeast *mcm* mutants, which exhibit a cell-division-cycle arrest in S phase, with a nearly doubled DNA content (41, 42).

In both *Arabidopsis* and *Drosophila*, null mutations in MCM proteins are lethal, and the lethality has been largely attributed to defects in cell proliferation (34, 35). However, in these mutants, whether an active cell-death process is involved has not yet been described. Our data suggest that, in *mcm5^{m850}* mutant embryos, some tissues may respond to the reduced level of MCM5 by activating programmed cell death, whereas others do not. Activation of apoptosis may be a direct consequence of the replication defect which could activate the S phase checkpoint system responsible for coordinating cell-cycle progression and cellular responses to DNA damage (43, 44). For example, induction of rereplication through perturbation of prereplication-complex components activates ATM/ATR-dependent checkpoint pathways that lead to an arrest of the cell cycle and/or apoptosis (45–47). Alternatively, MCM5 may have a role in the checkpoint system independent of its replication function. MCM proteins are expressed far in excess of what is required to support normal levels of DNA replication (4). Recently, human MCM7 was shown to interact directly with ATRIP, a protein that binds and activates ATR kinase. Interestingly, MCM7 was shown to regulate S-phase checkpoint response, even under the conditions where DNA replication is not perturbed (48). It remains to be tested whether MCM5 could also regulate S-phase checkpoint response in a similar manner.

In conclusion, our study reveals the crucial role of MCM5 in ensuring efficient genomic duplication and cell-cycle progression during vertebrate development. Furthermore, we show that MCM5 is a sensitive marker to identify cells with proliferative capacity. Finally, our data point to the existence of an added level of complexity in multicellular organisms in responding to the reduction in MCM5 by activation of a tissue-specific apoptosis program.

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