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Topoisomerases solve the topological problems encountered by DNA throughout the lifetime of a cell. Topoisomerase IIα, which is highly conserved among eukaryotes, untangles replicated chromosomes during mitosis and is absolutely required for cell viability. A homozygous lethal mutant, can4, was identified in a screen to identify genes important for cell proliferation in zebrafish by utilizing an antibody against a mitosis-specific marker, phospho-histone H3. Mutant embryos have a decrease in the number of proliferating cells and display increases in DNA content and apoptosis, as well as mitotic spindle defects. Positional cloning revealed that the genetic defect underlying these phenotypes was the result of a mutation in the zebrafish topoisomerase IIα (top2a) gene. top2a was found to be required for decatenation but not for condensation in embryonic mitoses. In addition to being required for development, top2a was found to be a haploinsufficient regulator of adult liver regrowth in zebrafish. Regeneration analysis of other adult tissues, including fins, revealed no heterozygous phenotype. Our results confirm a conserved role for TOP2A in vertebrates as well as a dose-sensitive requirement for top2a in adults.

The accurate and complete replication and separation of chromosomes during mitosis is vital for the viability of cells. One potential complication encountered during cell division is the topological and tensional pressures put on DNA during these processes. Topoisomerases are responsible for enzymatically winding, unwinding, knotting, and unknotting reactions that are necessary for solving the topological problems of DNA. Genetic and biochemical studies in bacteria and yeast have revealed two classes of topoisomerases, type I and type II (38). Type I topoisomerases act on DNA by creating a temporary single-strand nick in DNA, passing the intact strand through the broken strand and then religating the nick. Type II topoisomerases, alternatively, function by binding to two double-stranded DNA molecules, generating a double-stranded break in one of the strands, passing the intact strand through the broken strand, and religating the broken strand.

During DNA replication, sister chromatids become tangled with each other as a by-product of their duplication. The primary cellular function of type II topoisomerases during mitosis is the decatenation (untangling) of sister chromatids. Consistent with this essential role, genetic studies Saccharomyces cerevisiae have revealed that temperature-sensitive topoisomerase II mutants (TOP2) are unviable at nonpermissive temperatures (10, 16, 36). These mutants arrest during M phase due to their incompletely decatenated chromatids.

Humans have two TOP2 homologues, topoisomerase II alpha (TOP2A) and topoisomerase II beta (TOP2B). Although they are both functionally redundant with the yeast homologue, TOP2A and TOP2B are genetically unique. As the products of different loci, TOP2A and TOP2B have different expression patterns. Specifically, TOP2A expression peaks during M phase, whereas TOP2B expression remains relatively low and constant throughout the cell cycle (15, 40). TOP2A is therefore thought to be the primary topoisomerase required for the decatenation of chromosomes during mitosis. This hypothesis is supported by the fact that, similar to yeast gene deletion studies, TOP2A knockout mice are not viable and arrest at the four- to eight-cell stage of development (1). Although these embryos do not have any clearly tangled chromosomes, they do have teardrop-shaped nuclei, a phenotype consistent with previous reports about yeast topoisomerase mutants (36). Additionally, mitotic human cells in which TOP2A has been depleted quickly develop tangled chromosomes, aberrant mitoses, aneuploidy, and cell cycle arrest (5).

In addition to its role in untangling chromosomes during mitosis, TOP2A activity has also been shown to be required for RNA polymerase II (Pol II)-dependent transcription of chromatin bound DNA (22, 23). During transcription, elongation along a DNA template results in a positively supercoiled topology ahead of Pol II. In the absence of TOP2A, this accumulation of positively supercoiled DNA prevents complete transcription. Despite the fact that there are many proteins known to be part of the Pol II holo enzyme, only TOP2A is

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necessary and sufficient to overcome this topological constraint.

Due to its vital role in untangling sister chromatids, the function of TOP2A during mitosis is monitored by a decatenation checkpoint (11). Similar to the G2/M DNA damage and spindle assembly checkpoints, the decatenation checkpoint monitors the cell’s progress through the cell cycle and can affect delay or arrest (14). Compounds such as ICRF-193, which inhibits TOP2A without generating DNA damage, have been used to elucidate the mechanism of this checkpoint and dissect any differences compared to the DNA damage checkpoint (25). The decatenation checkpoint becomes activated in G2 phase, and cells with persistent tangled chromosomes are unable to progress past metaphase (29).

In the presence of catenated chromosomes, mitotic delay is dependent upon the presence of breast cancer gene 1 (BRCA1) and the ataxia-telangiectasia and rad3 related gene (ATR) (8). BRCA1, likely in response to activation by ATR, accumulates during S phase at sites of decatenation. In addition to their colocalization, TOP2A is polyubiquitinated in vivo by BRCA1, an activation event that enhances the decatenating activity of TOP2A (21). When the decatenation checkpoint is activated, ATR also affects cell cycle progression by inhibiting Polo-like kinase 1 (PLK1) (9). Since PLK1 activity is required for the activation of cyclin B/cyclin-dependent kinase 1, this inhibition of PLK1 by ATR prevents mitotic progression.

Due to the vital role of TOP2A during mitosis, its expression during the cell cycle is highly controlled. Prior to G2 phase, p21 and E2F1 bind to the cell cycle-dependent element in the TOP2A promoter, effectively suppressing expression (37, 41). As cells progress through G2 phase, this suppression is lifted by a collection of activating transcription factors, including CMYB, which promote the expression of TOP2A (3). Additionally, the cell cycle-regulated gene UHRF1 has been shown to bind to the TOP2A promoter and regulate its expression in response to protein kinase A signaling (17). In zebrafish, uhrf1 null mutants have been shown to have defects in embryonic liver growth, while adult uhrf1 heterozygotes are incapable of normal liver regeneration after partial hepatectomy (26).

In addition to its role in normal cell cycles, TOP2A was identified early as a reliable marker for abnormally proliferating and cancerous cells (39). Unsurprisingly, topoisomerase inhibitors have well-described mechanisms and are widely used as cancer chemotherapeutics (13). Here we describe the identification and characterization of can4, a novel zebrafish topoisomerase IIa mutant. can4 mutants display a variety of cell cycle defects that contribute to early lethality, including abnormal mitotic spindles and incompletely decatenated chromosomes during metaphase. In addition to embryonic phenotypes, we also show that adult heterozygous zebrafish have impaired liver regeneration after partial resection and that this defect is associated with decreased levels of top2a transcript. Finally, we demonstrate that the top2a-induced liver defect can be rescued with the protein kinase A stimulant forskolin.

MATERIALS AND METHODS

Cell proliferation screen. Screening procedures were done as described previously (27, 30). Adult male zebrafish of the AB strain were mutagenized with ethylnitrosourea and crossed to wild-type AB females in order to generate the F1 heterozygous generation. F2 haploid embryos were obtained by squeezing anesthetized F2 females and fertilizing the eggs in vitro with UV-inactivated sperm (6). At 36 h postfertilization (hpf) haploids were collected and used for anti-phosphorylated H3 (pH3) staining.

Genetic mapping. AB strain can4 heterozygous mutant zebrafish were outcrossed to wild-type wik in order to generate polymorphic mapping strains. Low-resolution mapping was done with 40 diploid mutant and 40 diploid wild-type embryos obtained from in-crossing mapping F2 fish. Microsatellite CA markers throughout the genome were used to scan for linkage. Linkage was initially established to chromosome 12 with marker z62244. Novel primer sets that scanned bacterial artificial chromosomes (BACs) in the region were designed and utilized to perform single-stranded conformational polymorphism analysis. Novel polymorphisms were identified, including flanking markers. An in silico chromosomal walk was then performed to order the sequence of BACs between flanking markers. Once complete, the critical interval was observed to contain only two genes, including the topoisomerase IIa genomic sequence.

pH3 staining. Embryos were fixed in 4% paraformaldehyde overnight at 4°C and stained as described previously (27).

Mitotic spindle/chromosome staining. At 26 to 28 hpf embryos were stained for alpha- and gamma-tubulin as previously described (27).

In situ hybridization. A 2.6-kb portion of the zTOP2A open reading frame was amplified with forward and reverse primers, 5'-AGTTGTCCTTCGTGTGATG-3' and 5'-CAACTACAGATGACTATTTCGGATG-3', from RNA prepared from wild-type 24-hpf embryos and cloned into the pCR-Blunt II vector (Invitrogen). The resulting plasmid was digested with EcoRI and Hpal to remove 1.6 kb of the partial open reading frame, yielding a 1-kb fragment that was used to generate RNA probes. Antisense RNA probe was generated using SP6 RNA polymerase linearized with EcoRI. Sense control RNA probes were generated using T7 polymerase on Kpdl-digested plasmids. Probes were purified by size exclusion chromatography. RNA in situ hybridization was performed as described previously (34).

Genotyping. can4 genotyping was performed with forward and reverse primers, 5'-CTCAGAAACCCCTGTGTAAG-3' and 5'-AGGGGATTGACCTCTCCTGGTG-3', followed by sequencing utilizing the same primers. hi3635 mutants were genotyped as described previously (2).

Cytogenetics. Metaphase chromosomes were prepared as described elsewhere and visualized with 4',6-diamidino-2-phenylindole (DAPI) (27).

DNA content analysis. Cell cycle analysis was performed on disaggregated 24-hpf embryos fixed in ethanol and stained with propidium iodide and analyzed by flow cytometry (20).

Apoptosis assays. At 36 hpf embryos were used to generate protein lysates using standard biochemical techniques. Caspase-3 activity in the lysates was measured with the Caspase-Glo kit (Promega), according to the manufacturer’s protocol. Luminescence was measured on a luminometer. Embryos were also stained with acridine orange, as previously described (24).

Partial hepatectomy. Visicsection of anesthetized adult zebrafish was performed as described previously to remove the ventral lobe of the liver (12, 26). At least five fish of each genotype were used per experiment. Animals were allowed to recover for 3 days, at which point they were sacrificed and liver size analyzed. Forskolin-treated fish were soaked in 0.5 μM forskolin in fish water overnight, rinsed, and allowed to recover as normal.

qRT-PCR. Regenerated liver was dissected from euthanized fish and RNA was isolated utilizing Trizol (Invitrogen) reagent, according to the manufacturer’s protocol. Luminescence was measured on a luminometer. Primers against the top2a and the control, β-actin, were used as described previously (26, 32).

Morpholinos. An ATG morpholino against the top2a transcript with the following sequence was obtained from Gene Tools: TCAGAGCTCTTCAGGTCACAGCCAT. Nuclei were visualized with DAPI.

RESULTS

can4 is a zebrafish cell cycle mutant. In order to identify genes important for embryonic cell division, we performed an F2 ethylnitrosourea mutagenesis screen in zebrafish for homozygous recessive mutations. Mutagenized F0 male zebrafish were outcrossed in order to generate heterozygous F1 adults. Haploid F2 offspring were probed at 36 hpf with an antibody against the mitotic marker phosphorylated serine-10 of histone H3 (pH3). One of the mutants recovered, can4, displayed a...
nied by a corresponding increase in cell death, we assayed the embryos die at 4 to 5 dpf.

ture at 24 hpf (Fig. 1B). These phenotypes persist until the high levels of brain necrosis and develop abnormal tail curvature compared to wild type. (C and D) Assays for caspase-3 activity in embryo protein extracts (C) and acridine orange staining (D) indicate increased apoptosis in can4 mutants.

slight decrease in pH3 staining compared to wild-type siblings (Fig. 1A). This phenotype was observable as early 12 hpf. Quantification of pH3-positive cells from 12-hpf embryos revealed that wild-type embryos contain 192 (±9) cells while can4 mutant embryos contain only 161 (±12) cells per embryo (P = 0.038). Additionally, homozygous mutant embryos have high levels of brain necrosis and develop abnormal tail curvature at 24 hpf (Fig. 1B). These phenotypes persist until the embryos die at 4 to 5 dpf.

To determine if this decrease in proliferation was accompanied by a corresponding increase in cell death, we assayed the relative levels of apoptosis in our fish. Protein extracts from can4 mutant and wild-type embryos were harvested and tested for activated caspase activity. In this assay, can4 mutant extracts showed a sevenfold increase in caspase-3 activity compared to their wild-type siblings (Fig. 1C) (P = 0.039). Acridine orange staining of 24-hpf embryos revealed that the majority of this apoptosis was occurring in the brain and along the neural tube (Fig. 1D).

DNA content analysis revealed that can4 mutants have increased polyploidy and aneuploidy (Fig. 2A). This defect indicates a required role of the can4 gene in maintaining genome stability. To further probe the cause of aneuploidy and polyploidy seen by fluorescence-activated cell sorting, we visualized mitotic spindles in 24-hpf mutant embryos by costaining for alpha-tubulin and gamma-tubulin (Fig. 2B and C). In a wild-type tail, spindles were bipolar and normally formed (n = 50). By contrast, 23% (11/48) of spindles observed in can4 mutants were multipolar (P = 0.0002). Taken together, these results suggest that can4 mutants have a problem accurately segregating their chromosomes as well as regulating mitotic spindle formation.

Topoisomerase Ila is the can4 gene. We utilized positional cloning to discover the genetic defect responsible for the observed embryonic phenotypes. The can4 mutation was mapped to a 1.5-cM region of chromosome 12 (Fig. 3A). The assembly of BACs across the region and analysis of this critical interval led to the identification of the candidate gene, top2a. Sequence analysis of mutant cDNA revealed a polymorphism that was unique when compared to the wild-type allele at amino acid 685 of the predicted protein sequence. The polymorphism consists of 11 additional nucleotides in the cDNA which causes a frameshift and premature translational stop. Sequencing the genomic DNA revealed that the can4 polymorphism is an intrinsic mutation that creates a cryptic splice donor in intron 14, which allows for the inclusion of 11 intronic nucleotides in the can4 cDNA (Fig. 3B and C). The can4 mutation occurs nearly halfway through the expected polypeptide, well before the catalytic Tyr-839 (Fig. 3D).

top2a was verified as the gene responsible for the can4 phenotypes by crossing can4 heterozygotes to the previously identified top2a mutant hi6535 (2). The hi635 allele is thought to be null (Fig. 3D). As expected, the hi635 allele failed to complement the can4 mutation and resulted in a clutch of embryos with the predicted Mendelian ratio of 92/404 (24%) of the progeny exhibiting morphological phenotypes similar to both can4 and hi635.

In order to determine the endogenous expression pattern of top2a during development, RNA in situ hybridization was performed (Fig. 4). A fragment, 5′ in relation to the can4 mutation of the zebrafish top2a open reading frame was cloned and used for in situ hybridization (Fig. 3D). At 36 hpf, wild-type embryos express top2a throughout the head and hindbrain proliferative zone (Fig. 4A). Conversely, homozygous can4 mutant embryos have very little signal, suggesting nonsense-mediated decay of abnormal can4 transcripts and extinction of any maternally deposited top2a mRNA (Fig. 4B). In wild-type embryos, top2a is expressed early and ubiquitously throughout early embryogenesis (Fig. 4D to F). The presence of top2a
Early embryonic development underscores the importance of cell cycle genes like \textit{top2a} to early cell division and strongly suggests that these early divisions are dependent on maternally derived transcripts. At 24 hpf, \textit{top2a} expression becomes restricted to proliferating tissues, and by 3 dpf neural expression is limited to the tectum, posterior midbrain, and hindbrain proliferative zone (Fig. 4G) (33). Expression in 3-dpf embryos was also observed in the branchial arches, retina, fin buds, liver, pancreas, and gut (Fig. 4G to I).

Topoisomerase IIa is required for chromosome decatenation during mitosis. TOP2A has been shown to be absolutely required for mammalian development as well as for human somatic cell cycles (1, 2, 5). Although other topoisomerases are capable of condensing replicated chromatin, TOP2A alone is required for decatenating mitotic chromosomes. In order to assess whether \textit{top2a} is required for chromosome decatenation in zebrafish, metaphase chromosome spreads were analyzed (Fig. 5A to C). Consistent with other systems, \textit{top2a} deficiency in zebrafish results in incompletely decatenated metaphase chromosomes. Figure 5A shows the normal spectrum of condensed chromosomes. Tangled chromosomes are characterized by poor chromosome spreading and poor resolution of individual chromosomes (Fig. 5B and C). Unresolved linkages between sister chromosome telomeres were observed, as were aneuploid and polyploid cells. Interestingly, chromosomes from both \textit{hi3635} (not shown) and \textit{can4} mutant embryos had no observable defect in chromosome condensation. These results suggest that Top2a is necessary for chromosome decatenation but does not have a required role in chromosome condensation in zebrafish.

In order to further probe the role of maternally derived transcripts, an ATG morpholino targeted against the \textit{top2a} mRNA was injected into the yolk of one-cell embryos. Consistent with high levels of maternally deposited \textit{top2a} protein and transcript, morpholino concentrations at and below 100
were not observed in these animals, a small number of bridged nuclei were found in these animals. This phenotype was previously described in the *top2a* knockout mouse and is thought to be the result of cytokinesis in the presence of incompletely segregated chromosomes (Fig. 5D) (1). Arguing that the effect of this *top2a* morpholino is specific, animals injected with similar concentrations of a p53 morpholino control were not observed to contain bridged nuclei.

**Topoisomerase IIα is haploinsufficient for liver regeneration.**

Due to its required and transcriptionally regulated role in cell division, *TOP2A* has long been used as a marker of proliferation for both normal and cancerous tissues. Uhrf1 is a known positive regulator of *TOP2A* activity. Recent studies in zebrafish have implicated Uhrf1 as an important regulator of liver growth and regeneration (26). Arguing for a tissue-specific role, heterozygous *uhrf1* mutant fish are unable to regenerate their livers after partial hepatectomy but have normal fin regeneration. However, even though these studies show a concurrent decrease in *top2a* transcript in *uhrf1* heterozygotes, it is not possible to determine if this observation is due to a direct effect of Uhrf1 on *top2a* transcription or if decreased *top2a* levels are a secondary effect merely reflecting the decreased mitotic index of mutant liver regrowth. We hypothesized that Top2a activity in adult liver regulates liver regrowth after resection. To test this hypothesis we analyzed the effect of *top2a* heterozygosity on liver regrowth by performing partial hepatectomies on *can4* and *hi3635* heterozygous fish. Shown are representative animals 3 days after resection for both wild-type sibling and *top2a*+/−/− fish (Fig. 6A and B). Liver regrowth was quantified and found to be significantly decreased in both *can4* and *hi3635* heterozygotes following partial resection (Fig. 6C).

In order to determine if *top2a* heterozygosity causes organism-wide regenerative defects, we assessed fin regrowth in a partial amputation assay. In agreement with the liver-specific sensitivity to *uhrf1* heterozygosity, *top2a* heterozygosity had no observable effect on fin regeneration at 10 days postamputation (Fig. 6E). One possible explanation for this tissue specificity is reduced basal transcription of the *top2a* locus in *hi3635* heterozygous liver. In order to test this possibility, we analyzed homeostatic liver and fin from *hi3635* heterozygous and sibling fish for baseline levels of *top2a* transcript. qRT-PCR analysis revealed no difference in *top2a* transcript levels between heterozygous mutants and siblings in either tissue (Fig. 6F). However, in an effort to confirm that the defect in liver regeneration was associated with the decreased level of *top2a* transcript in *hi3635* heterozygous liver compared to wild-type siblings, we performed qRT-PCR on regenerating liver 1 day postresection. Unlike homeostatic liver, our analysis of 10 *hi3635* heterozygotes and 10 sibling wild types revealed that *top2a* transcripts were depressed in regenerating liver from *hi3635* heterozygotes compared to their wild-type siblings (*P* = 0.0035) (Fig. 6F).

Protein kinase A directly phosphorylates Uhrf1 in response to forskolin-induced cyclic AMP (cAMP) signaling (35). In order to determine if this activation is sufficient to overcome the regeneration defect in *top2a* heterozygotes, we challenged *hi3635* heterozygotes with the cAMP-activating drug forskolin. After liver resection, fish were either treated with 0.5 μM forskolin overnight or with 1% dimethyl sulfoxide as a control. While forskolin had a minimal effect on liver

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**FIG. 5.** Zebrafish embryos deficient in *Topoisomerase IIα* fail to deccatenate their chromosomes during mitosis. Shown are representative metaphase spreads from wild-type (A) and *can4* mutant (B and C) embryos. Wild-type chromosomes are condensed, discrete, and spread evenly. Although *can4* mutant metaphase display condensed chromo-

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μM had no effect on injected embryos. At doses in excess of 1 mM, general toxicity as early as the 64-cell stage was visible. However, the absence of tangled chromosomes suggests this phenotype was nonspecific and the result of morpholino-induced toxicity. Embryos injected with morpholino at the intermediate concentration of 500 μM developed normally until 24 hpf, at which point they developed brain necrosis and tail curvature similar to the phenotypes of *can4* homozygous mutant animals. Although catenated metaphase chromosomes
In the present study we describe the identification of can4, a novel zebrafish topoisomerase IIα mutant. can4 was identified in a forward genetic screen for genes required for vertebrate cell cycles. The ability to identify cell cycle mutants in zebrafish epitomizes the unique utility of the system in studying cell cycle genetics. The requirement of top2a during zebrafish development is consistent with the well-known role of TOP2A in chromosome decatenation. Additionally, results in zebrafish embryos lacking functional top2a alleles are in agreement with the observed requirement of TOP2A in mouse development, yeast division/fission, and cell cycle progression in human cells. However, our observations of catenated chromosomes, aneuploidy, and polyploidy in can4 mutants indicate a defective decatenation checkpoint during early zebrafish development. The deficiencies in decatenation checkpoint signaling in zebrafish appear similar to the observed defects in embryonic stem (ES) cells. Indeed, ES cells cultured in vitro are poorly able to sense catenated chromosomes and become better able to sense chromosome entanglement as they are forced to differentiate. Like ES cells, zebrafish embryos lacking Top2a activity accumulate aberrant mitoses and aneuploidy instead of succumbing to checkpoint arrest.

This similarity between zebrafish embryos and ES cells may reflect the inherent nature of developmental cell cycles. The deemphasis of G2 checkpoints during development, including the decatenation checkpoint, may simply reflect the rapid nature of development. Because early zebrafish embryos have a yolk that contains the protein and mRNA complement re-
quired for rapid development, early cell divisions are more similar to the cyclic progression of S and M phases without the benefit of normal checkpoints. The fact that mouse Top2a-deficient embryos, but not ES cells, arrest almost immediately suggests that mammalian systems may have developed alternative mechanisms for sensing decatenation deficiencies in vivo. The abundance of top2a transcript in early embryos further underscores the importance of this transcript and implicates maternal transcript as a primary source of top2a mRNA and protein in early zebrafish embryos.

The accumulation of multipolar spindles as the primary mitotic defect in our can4 embryos is consistent with the role of TOP2A in the resolution of sister centromeres during mitosis. Sumoylation of TOP2A protein during the metaphase-anaphase transition by RanBP2, a SUMO E3 ligase, is required for proper centromeric localization (7). The lack of RanBP2 results in the mislocalization of several centromeric proteins that are required for normal kinetochore-microtubule interactions. Similar to top2a-deficient zebrafish embryos, the primary spindle defect in these cells is multipolar spindles (19). Although it is unknown whether the polyploidy is the primary defect or a residual consequence from an earlier aberrant mitotic event, it seems clear that the localization of TOP2A to the centromeres is required for both complete chromosome decatenation as well as proper kinetochore-microtubule dynamics.

It is interesting that adult regenerating liver is particularly sensitive to top2a heterozygosity. This observation is consistent with the hypothesis that liver is particularly sensitive to certain genes, like UHRF1, that directly regulate DNA topoisomerase IIa genes in zebrafish embryos may provide a useful platform for dissecting the genetic pathways of chemosensitivity.

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