Interruption of cenph Causes Mitotic Failure and Embryonic Death, and Its Haploinsufficiency Suppresses Cancer in Zebrafish*§

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Kinetochore proteins associate with centromeric DNA and spindle microtubules and play essential roles in chromosome segregation during mitosis. In this study, we uncovered a zebrafish mutant, stagnant and curly (stac), that carries the Tol2 transposon element inserted at the kinetochore protein H (cenph) locus. Mutant embryos exhibit discernible cell death as early as 20 hours postfertilization, extensive apoptosis, and upward curly tail during the pharyngula period and deform around 5 days postfertilization. The stac mutant phenotype can be rescued by cenph mRNA overexpression and mimicked by cenph knockdown with antisense morpholinos, suggesting the responsibility of cenph deficiency for stac mutants. We demonstrate that the intrinsic apoptosis pathway is hyperactivated in stac mutants and that p53 knockdown partially blocks excess apoptosis in stac mutants. Mitotic cells in stac mutants show chromosome missegregation and are usually arrested in G2/M phase. Furthermore, compared with wild type siblings, heterozygous stac fish develop invasive tumors at a dramatically reduced rate, suggesting a reduced cancer risk. Taken together, our findings uncover an essential role of cenph in mitosis and embryonic development and its association with tumor development.

Kinetochore is a structure formed at the outer surface of the centromere of a mitotic chromosome. In vertebrate cells, the kinetochore assembles as trilaminar ultrastructure on the centromere during prophase (1, 2) and acts as a bridge between the centromeric chromatin and spindle microtubules. The inner and outer kinetochore plates form the interface with the centromere and microtubules, respectively. Therefore, kinetochores play essential roles in sister chromatid adhesion and separation, connection of chromosome and microtubules, chromatid movement, and mitotic checkpoint control (3, 4).

In vertebrates, a kinetochore consists of a large number of proteins, many of which are highly conserved in invertebrates and plants (3). However, only a few of the vertebrate kinetochore proteins have been studied for their functions at the organism level. Cenpa, Cenpc, or Cenpe knock-out mice are embryonic lethal with severe chromosome instability and mitotic defects (5–8), whereas Cenph knock-out mice are viable (9), which suggest that different kinetochore proteins have different functions in development. Furthermore, altered levels of some kinetochore proteins have been shown to affect cancer susceptibility in animals (10, 11).

Kinetochore protein H (Cenph) was originally identified as a protein specifically and constitutively localized in kinetochores throughout the cell cycle in mouse cells (12). Subsequent studies indicate that Cenph is colocalized with Cenpa and Cenpc in the inner kinetochores and is required for recruiting Cenpc and Cenp−50 (13–15). Cenph overexpression in human HCT116 cells and mouse 3T3 cells induces aneuploidy due to chromosome missegregation that may result from abnormal location of ectopic Cenph (16). Depletion of Cenph in cultured chicken or human cells also causes chromosome missegregation and cell death (13, 17). These reports support the notion that Cenph is essential for normal chromosome segregation during mitosis. However, it remains unknown how Cenph deficiency leads to cell death and whether it plays a role in vertebrate development. Nevertheless, up-regulation of CENPH expression in several types of human tumors suggests the involvement of CENPH in tumorigenesis (16, 18–22).

In an attempt to identify developmentally important genes during zebrafish embryogenesis, we performed a mutagenesis using a Tol2 transposon-based gene trapping approach (23, 24). One of the mutant lines, stagnant and curly (stac) line, carries an insertion of the Tol2 transposon element at the cenph locus. Massive cell death occurs in stac mutant embryos and as a result leads to embryonic lethality, whereas heterozygous fish are viable. Compared with wild type sibling embryos, we found that stac mutants have more mitotic cells with aberrant spindles and chromosome missegregation with an arrest of the cell cycle in G2/M phase. In stac mutants, the intrinsic apoptotic pathway components, including tp53/p53, mdm2, and bbc3/puma genes as well as caspases 3, 7, and 9, are overexpressed or hyperactivated. Upon induction with the carcinogen MNNG, stac heterozygous fish develop malignant tumors at a significantly
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reduced rate than the wild type sibling fish. Thus, these findings provide novel insights into developmental roles and clinical significance of Cenph.

EXPERIMENTAL PROCEDURES

Gene Trapping and Transposon Mutagenesis—The transposon-based gene trap vector T2BGS was modified from T2KSAG (23) by replacing the original splicing acceptor with a splicing acceptor in the first intron of the zebrafish bcl2 gene, which was a gift from Dr. Jian Zhang. Injection of transposon DNA and transposase mRNA, screening of transgenic fish, and identification of mutants were done essentially as described previously (24, 25).

Thermal Asymmetric Interlaced PCR, Genotyping, RT-PCR, and Real Time PCR—To identify the flanking sequences of the transposon insertion sites in stac<sup>aus55</sup> mutant line, genomic DNA was extracted from GFP-positive stac embryos, and thermal asymmetric interlaced PCR was performed as described previously (24).

Homoygotes, heterozygotes, and wild type siblings were easily separated based on GFP expression level around 24 hpf. For accurate genotyping, genomic DNA was extracted from single embryos, and the regions in the vicinity of the transposon insertion were amplified by PCR with the combination of three specific primers as follows: cenph<sub>5</sub> (5′-CCGCGATTCAGAGTAACGTTAATCC-3′), T3-1 (5′-CTCAAGTACAATTTTTTTGATGTAATCTT-3′), and cenph<sub>3</sub> (5′-CCGAGAACCACAACACCTAATTTACATC-3′). The amplification conditions were as follows: 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s, and extension at 72 °C for 45 s. An additional extension was executed at 72 °C for 7 min.

For RT-PCR, total RNA was isolated from individual embryos using the RNeasy mini kit (Qiagen), and the first-strand cDNA was synthesized by reverse transcription with Embryonic Lethality and Suppresses Cancer.
μl of propidium iodide solution (0.1% sodium citrate, 0.05 mg/ml propidium iodide (Sigma), 100 μg/ml RNase A, 0.0002% Triton X-100). Then the mixture was incubated at room temperature in the dark for at least 30 min. The DNA content was determined by flow cytometry analysis with the BD FACS AriaII flow cytometer.

Transmission Electron Microscopy—Zebrafish embryos at 30 hpf were fixed by 2.5% glutaraldehyde (pH 7.2) overnight at 4 °C and then incised with sharp blade to get the desired tissues (the brain from the optic tectum to the 4th ventricle combined with partial spinal cord). The tissues were washed three times, for 10 min each, with PBS (pH 7.2) and osmicated in 1% osmium tetroxide for 1.5 h, followed by washing three times with PBS. The tissues were dehydrated in graded cold ethanol from 50 to 100% at an interval of 15 min and further dehydrated in series acetone. Then the tissues were embedded in the Spur resin and polymerized at 60 °C for 1 or 2 days. Blocks were cut to produce 70-nm-thick sagittal sections with Leica EM UC6 ultracut microtome. Sections were stained with lead citrate and imaged.

RESULTS

stac Embryos Express GFP Ubiquitously, and Homozygotes Are Embryonic Lethal—We identified a mutant line stac, which was named for the stagnant and curly phenotype, by insertional mutagenesis using the Tol2 transposon-based gene trap vector T2BGS (23–25). In this line, the heterozygous females, when mated to wild type or heterozygous males, produced embryos all showing GFP expression at the one-cell stage (Fig. 1A), suggesting that the trapped gene is maternally expressed. Among embryos produced by pairwise heterozygous crosses, GFP expression (fluorescence) levels at or after 20 hpf could be categorized into the following three groups: weak, moderate, and strong (see Fig. 2D for examples); the difference should have resulted from zygotic expression of GFP, implying that the trapped gene is also zygotically expressed. We observed GFP expression in 1475 embryos at 30 hpf derived from 10 pairwise crosses of heterozygotes and wild type siblings, which were derived from crosses between heterozygotes and pre-sorted based on GFP expression at 36 hpf, were exposed to MNNG under dark conditions at a final concentration of 2 μg/ml for 24 h. Fish were sacrificed 6 months after exposure, and serial step 5-μm-thick sagittal sections were made for each fish and examined for the existence of tumors by hematoxylin and eosin staining.

Carcinogenesis—MNNG was freshly dissolved in 100% ethanol at a concentration of 2 mg/ml. Twenty one-day-old stac heterozygous fry and wild type siblings, which were derived from crosses between heterozygotes and pre-sorted based on GFP expression at 36 hpf, were exposed to MNNG under dark conditions at a final concentration of 2 μg/ml for 24 h. The tissues were dehydrated in graded cold ethanol from 50 to 100% at an interval of 15 min and further dehydrated in series acetone. Then the tissues were embedded in the Spur resin and polymerized at 60 °C for 1 or 2 days. Blocks were cut to produce 70-nm-thick sagittal sections with Leica EM UC6 ultracut microtome. Sections were stained with lead citrate and imaged under a HITACHI H-7650 electron microscope.

FIGURE 1. GFP expression pattern and phenotype of stac transgenic embryos. A, fluorescent images of stac embryos showing GFP expression at indicated stages. The genotypes of pictured embryos at early stages were not determined, whereas pictured embryos at 24 – 48 hpf were heterozygotes. B, morphology of stac mutant and wild type (WT) sibling embryos at indicated stages. The boxed trunk area was enlarged in the corner, and the head was enlarged in the right panel. Note that mutant embryos had a darker head, rough skin, and curly posterior trunk with stage-dependent degrees.

cenph Locus in the stac Line Is Interrupted by a Transposon Insertion—To identify the transposon insertion position in the genome of the stac line, we performed thermal asymmetric interlaced PCR. The result revealed that the transposon element has been inserted into the second exon of the gene NP_001107081.1 in Ensembl on the 8th linkage group, which was originally named cenph-h like gene. This gene has an open reading frame of 702 bp, which encodes a putative peptide of...
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The zebrafish cenph locus consists of 10 exons and 9 introns. The insertion of the transposon element into the 2nd exon in the 5' UTR was expected to allow the transcription of GFP expression cassette under the control of the cenph promoter without interrupting the cenph coding region (Fig. 2A). The correlation between GFP expression levels and the interrupted cenph locus was tested by PCR-based genotyping using specific primers. Results showed that strong GFP embryos and moderate GFP embryos carry homozygous and heterozygous transposon insertion at the cenph locus, respectively, although embryos with weak GFP have no insertion (Fig. 2B). Thus, GFP expression in the stac line is driven by the cenph promoter.

Next, we asked whether the transposon insertion would affect the expression level of cenph in stac embryos. RT-PCR analysis disclosed that, compared with the wild type siblings (weak GFP), the heterozygous siblings at 24 or 30 hpf express cenph at a reduced level, whereas homozygotes express cenph at almost undetectable levels (Fig. 2C). Analysis by real time PCR indicated that heterozygous and homozygous embryos at 30 hpf retained ~50 and 3.8% of cenph mRNA amount in the wild type embryos, respectively (Fig. 2D). It is clear that the transposon insertion in the stac line inhibits the expression of wild type cenph mRNA.

Interruption of the cenph Locus Accounts for the stac Mutant Phenotype—To further verify the relationship between the stac mutant phenotype and cenph disruption, we first performed a rescue experiment using in vitro synthesized cenph mRNA. Injection of 233 amino acids. The putative peptide shares an overall sequence similarity of 59 and 58% to the human and mouse centromere protein Cenph, respectively. Importantly, several genes located adjacent to the CENPH locus on human chromosome 5 have their counterparts in the vicinity of the zebrafish cenph like locus on chromosome 8 (data not shown). This information suggests that zebrafish Cenph-like protein is in fact an orthologue of mammalian Cenph and should be renamed Cenph (GenBank accession number GU977276).

cenph mRNA into wild type embryos did not produce visible abnormalities (data not shown). We then injected stac mutant embryos at the one-cell stage with different doses of cenph mRNA, and we observed morphology at several stages with focuses on cell death in the head (visible at 30 hpf) and curly posterior trunk (visible at 36 hpf). As shown in Fig. 3A, the mutant embryos injected with 50 pg of cenph mRNA had reduced degrees of cell death at 30 hpf and straight trunk before or at 36 hpf, which resembled wild type embryos, but the
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![Image](https://example.com/image)

**FIGURE 3.** *stac* mutants are rescued by *cenph* overexpression and phenocopied by *cenph* knockdown. A, overexpression of *cenph* mRNA rescued the mutant phenotype. One-cell embryos from *stac* heterozygote intercrosses were injected with *cenph* mRNA. The homozygous mutant embryos were sorted out at 24 hpf by their strong GFP expression, and their morphology was observed at indicated stages. B, spatiotemporal expression pattern of *cenph* in zebrafish wild type embryos was investigated by in situ hybridization at indicated stages. C, knockdown of *cenph* in wild type embryos mimicked the *stac* phenotype. One-cell wild type embryos were injected with *cenph* morpholinos alone or in combination with *cenph* mRNA. The number of affected embryos and the total number of injected embryos are indicated.

injected mutant embryos still showed curly trunk at 48 hpf and later stages. Increasing the amount of *cenph* mRNA led to a better rescue effect as observed at 48 and 72 hpf. Nevertheless, these results support the idea that the deficiency of Cenph accounts for the *stac* mutant phenotype.

We inspected spatiotemporal expression of *cenph* in wild type embryos by whole-mount in situ hybridization. The *cenph* transcripts were detected in one-cell embryos and distributed evenly until midsegmentation (Fig. 3B). From the 20-somite stage to 30 hpf, *cenph* expression occurred at higher levels in the head and spinal cord, but at 36 hpf or later stages, its expression was retained in the head region only. The similarity between *cenph* expression pattern in wild type embryos and GFP expression pattern in *stac* embryos is another indication that the *stac* mutant phenotype arises from the interruption of the *cenph* locus.

We took another strategy to confirm the relatedness of the Cenph deficiency to the *stac* mutant phenotype by inhibiting *cenph* expression in wild type embryos with antisense morpholinos. Two morpholinos, *cenph*-MO1 and *cenph*-MO2, were found to effectively block the expression of the reporter p*cenph*-5′UTR-GFP in which GFP was fused to 5′UTR and a 5′UTR of *cenph* mRNA led to a widespread apoptosis.

**Extensive Apoptosis Occurs in *stac* Mutant Embryos**—To determine whether excess cell death in *stac* mutants belongs to apoptosis, we performed TUNEL assay that detects DNA fragmentation. We found that *stac* mutant embryos had many more TUNEL-positive cells, mainly in the brain and spinal cord, than in the wild type siblings at or after 24 hpf (Fig. 4A). Staining with acridine orange, a vital dye for detecting apoptotic cells with hypercondensed chromatin and apoptotic bodies, also detected more dying cells in *stac* mutants than in the wild type siblings (data not shown). By confocal microscopy of DAPI (diamidino-2-phenylindole)-stained embryonic nuclei, we found that, compared with the wild type siblings, *stac* mutants had more nuclei with superfluous apoptotic bodies and chromosomal condensation/fragmentation in the spinal cord and optic tectum of *stac* mutants (Fig. 4B). Electronic microscopy revealed that many cells in *stac* mutants displayed nuclear chromatin compaction and segregation, nuclear budding, and fragmentation (Fig. 4C), which are ultrastructural manifestations of apoptosis. Taken together, we conclude that the deficiency of Cenph in *stac* mutants induces widespread apoptosis.

**Intrinsic Apoptotic Pathway Is Triggered in *stac* Mutants**—Like in human, both intrinsic and extrinsic apoptotic signaling pathways are involved in apoptosis in zebrafish (29, 30). We asked which apoptotic pathways are stimulated in *stac* mutants. We then did a microarray analysis using the Affymetrix GeneChip® Zebrafish Genome Array (NCBI GEO accession number GSE20707) and total RNA extracted from 24- or 30-hpf *stac* mutants or the wild type siblings. This analysis identified 49 up-regulated and 55 down-regulated genes in *stac* mutants (supplemental Tables S1 and S2 for known genes). We chose those involving apoptosis and cell cycle regulation, including *tp53/p53*, *mdm2*, *fadd*, *bbc3/puma*, *gadd45al*, *folistatin (fist)*, *caspase 8* (*casp8*), and *cdn1c/p57*, for further validation by quantitative RT-PCR analysis and whole-mount in...
situ hybridization. Two other mitosis-related genes, cyclin B1 (ccnb1) and cyclin E2 (ccne2), were also analyzed, although they were not included in the array. The quantitative RT-PCR results indicated that in stac mutants bbc3, tp53, casp8, and mdm2 were significantly up-regulated at and after the 20-somite stage. The up-regulation of fst became apparent from 24 hpf onward; gadd45al was also up-regulated at 36 hpf. In contrast, ccne and cdkn1c were down-regulated from 24 hpf onward; however, the expression of fadd and ccnb1 appeared unchanged at examined stages (Fig. 5, A–C). The changes in the expression levels of these markers, as detected by in situ hybridization, showed a general tendency similar to the quantitative RT-PCR results (supplemental Figs. S2–S4). These data imply that both the intrinsic (mitochondria-dependent caspase 9 pathway) and extrinsic (death receptor-mediated caspase 8 pathway) apoptotic pathways might have been induced in stac mutant embryos and that the progression of the cell cycle of mutant cells may have been affected by down-regulation of genes such as ccne2 and cdkn1c.

As the activation of caspases is a critical step of the extrinsic and intrinsic apoptotic pathways, we went on to measure the activities of caspase 3/7, caspase 8, and caspase 9 in embryonic cells at 30 hpf using the luminescent method (31). In stac mutant cells, the activities of caspase 3/7 and caspase 9 were increased significantly (Fig. 5D), compared with embryonic cells derived from the wild type siblings. The increase of the caspase 8 activity was less, but still statistically significant, if the incubation time was longer (3 h) (Fig. 5D). Thus, we hypothesize that the disruption of cenph expression in stac mutants primarily triggers the intrinsic but not extrinsic apoptotic pathway.

Depletion of p53 Can Partially Rescue stac Mutants—Tp53/p53 is a key player of the intrinsic apoptotic pathway (32, 33). As the expression of p53 and several other components of the pathway is up-regulated in stac mutant embryos as demonstrated above, we expected that knockdown of p53 in stac mutants could inhibit massive apoptosis. To verify this, we injected different doses (5 or 10 ng) of p53 antisense morpholino (p53-MO) (27) into stac mutant embryos at the one-cell stage, followed by morphological observation and TUNEL assay at 30 hpf of development. As reported before (27), injection of p53-MO
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FIGURE 5. Apoptosis signaling pathway components are highly activated in stac mutants. A–C, quantification of apoptosis and cell cycle regulator expression levels by real-time RT-PCR at different stages. The tested genes were selected from cDNA microarray assay. D, bioluminescent assay of caspase 8, 9, and 3/7 activities. Twenty stac and wild type (WT) sibling embryos at 30 hpf were treated with corresponding Caspase-Glo reagents (Promega), and luminescence was measured 2 and 3 h after treatment. The p value was 0.0847, 0.0273, or 0.0014 for 2-h treated caspase 8, 9, or 3/7 activity, respectively, and 0.0445, 0.0165, or 0.0004 for 3-h treatments. Significance of differences is as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001 (by Student’s t test). Error bars indicate S.D. E, Immunostaining of activated caspase 3 using an antibody against active caspase 3. F, excessive apoptosis in stac mutant embryos was inhibited by injection of p53 morpholino (p53-MO). One-cell embryos derived from stac heterozygote intercrosses were injected. The mutants were sorted based on GFP fluorescence confocal microscopy, microtubules/mitosis spindle and chromosomes of mitotic cells in embryos that were stained using α-tubulin antibody and DAPI. From the data together, we conclude that the mitotic cells in stac embryos can proceed through S and G0/G1 phases but are arrested in G2/M phase.

into wild type embryos did not cause any observable developmental defects (data not shown). In contrast, the cell death and the curly posterior trunk phenotype of stac mutant were partially inhibited by knockdown of p53 in a dose-dependent manner (Fig. 5F); this rescue effect was declined later, and as a result, the injected mutant embryos still developed the curly posterior trunk phenotype at 48 hpf. The TUNEL assay also detected varying degrees of decrease of signal for DNA fragmentation in the p53-MO-injected stac mutant embryos, which was dependent on the p53-MO doses (Fig. 5F). These results confirm that the p53 apoptosis pathway plays a role in cell death of stac mutant embryos. But the p53-independent apoptotic pathway may also contribute to the development of the stac mutant phenotype. On the other hand, the partial rescue effect by knocking down p53 could be ascribed to continuing dilution of the morpholino concentration during embryonic development.

Mitotic Cells in stac Mutants Are Arrested in G2/M Phase—Considering the important roles of Cenph and other centromere proteins on chromosome segregation and cell division (3, 4, 34, 35), we hypothesize that the deficiency of Cenph in the zebrafish stac mutants would lead to spindle conformation damage and mitotic arrest. To confirm this, we performed FACS-based cell cycle analysis of the DNA content of cells derived from stac mutant or wild type sibling embryos from 24 to 36 hpf. Results showed that the percentage (48.7%) of cells in G0/G1 phase in 24-hpf stac mutants was already lower than that (59.1%) in the wild type siblings, and the decrease became more apparent at 30 and 36 hpf (Fig. 6A). A much bigger difference between stac mutants and the wild type siblings was found in the proportion of the G2/M cells; the stac mutants at 36 hpf showed 3.43-fold increase compared with the wild type siblings, which suggests the accumulation of the G2/M cells in stac mutants. In contrast, the percentage of the S phase cells appeared unchanged between two groups of samples. Immunostaining for Ser-10-phosphorylated histone H3 (pH3), the mitosis marker, detected a dramatic increase of signals, particularly in the head and spinal cord, in stac mutants embryos at 24–36 hpf compared with the wild type siblings (Fig. 6B), which would suggest normal chromosome condensation but failure of chromosome segregation in the stac mutant cells. Taking these data together, we conclude that the mitotic cells in stac embryos can proceed through S and G0/G1 phases but are arrested in G2/M phase.

Chromosome Missegregation Occurs in Mitotic Cells of stac Mutants—Our next question is why mitotic cells in stac mutant embryos are arrested in G2/M phase. Considering that Cenph is a component of the kinetochores (36), we would expect a failure of chromosome segregation due to the insufficiency of Cenph in stac mutant cells. To test this idea, we observed, by immunofluorescent confocal microscopy, microtubules/mitosis spindles and chromosomes of mitotic cells in embryos that were stained using α-tubulin antibody and DAPI. From the 20-somite stage to 36 hpf, we consistently identified a much higher proportion of abnormal mitotic cells in stac mutants than in the wild type siblings; and the difference became more dramatic at later stages (Fig. 6, C and D). Typically, the abnormal mitotic cells at the metaphase exhibited hypercondensation of chromosomes with failure to congregate in the metaphase plate, and the distorted (deformed, disordered and curved) organization of mitotic spindles; at anaphase, the abnormal cells showed aggregation or random dispersion of the chromosomes as well as disorganized and asymmetrical spindles (Fig. 6C). In a few extreme cases, we found mitotic cells with multipolar spindles (see an example in Fig. 6C). These results indicate that the deficiency of Cenph in stac mutants causes abnormal mitosis by affecting chromosome segregation and stability.
stac Heterozygous Adults Have Reduced Cancer Susceptibility in Response to Carcinogen Induction—Zebrafish has been successfully used as a cancer model system (37, 38). Because some kinetochore proteins have been shown to be involved in cancer development (10), we extended our study to assess the effects of stac/cenph heterozygosity on the development of cancer.

**FIGURE 6.** **A**, cell cycle analysis by flow cytometry. Cells from stac mutant or wild type (WT) sibling embryos at indicated stages were labeled with propidium iodide (PI), and DNA content was counted. The percentages of cells in different phases of the cell cycle are shown in bar graphs. Significance of differences is as follows: ***, p < 0.001 (by Student’s t test). Error bars indicate S.D. B, mitotic cells labeled with anti-pH3 antibody in stac mutant and wild type sibling embryos at indicated stages. The boxed area is enlarged at the right corner of each panel. Note that more mitotic cells are found in stac mutants. C and D, mitotic spindles and chromosome aggregation and segregation at metaphase and anaphase of mitotic cells in stac mutant and wild type sibling embryos. Immunohistochemistry was performed with anti-α-tubulin for spindles (red) and DAPI for chromosomes (blue) (C). Cells were picked up from the spinal cord. An abnormal mitotic cell in the anaphase from a mutant embryo was shown to have multipolar spindles (on the right lower corner of panel). Scale bar, 5 μm. The percentage of mitotic cells with aberrant spindles is shown in D.

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...tumors. The stac heterozygous fish and wild type siblings at day 21, which were pre-sorted based on the GFP expression at 36 hpf, were treated with 2 μg/ml MNNG, a carcinogen that can induce different types of tumors (39). Six months later, fish were sacrificed, and serial cross-sections were made to examine the existence of tumors. Among 138 treated wild type sibling fish, we identified 9 fish carrying malignant tumors in the brain, esophagus, liver, spleen, retina, or lens (Fig. 7A), which showed hyperproliferation and in most cases invasion into the adjacent tissues/organs. The carcinogenesis rate (6.52%) in wild type sibling fish was comparable with those reported previously by other researchers (40, 41). In contrast, only 0.97% (1:103) of the treated stac heterozygous fish had retina hyperplasia. These data suggest that reduced levels of cenph due to loss of one allele suppress the development of invasive tumors. The reduction of cancer susceptibility in stac heterozygous fish may be related to the activation of the apoptotic pathways as seen in stac mutants (Fig. 5).

We also paid attention to noninvasive neoplasms or aberrant hyperplasia in the treated fish. We found that comparable proportions of stac heterozygous fish and wild type sibling fish developed intestinal adenomas (Fig. 7, B and C). The intestinal adenomas manifested large outgrowth of polyps, loss of goblet cells, and pseudostratification of nuclei (Fig. 7B). It appears that stac haploinsufficiency in zebrafish has little impact on noninvasive neoplasms.

DISCUSSION

Cenph is a constitutive component of the centromeric inner kinetochores. Previous in vitro studies have disclosed that Cenph is required for normal chromosome segregation during mitosis. However, the importance of Cenph at the organism level has not been demonstrated. In this study, we identified a cenph-deficient zebrafish mutant, stac, in which the cenph locus is interrupted by the insertion of the Tol2 transposon element, and as a result, cenph expression is blocked. stac mutants are embryonic lethal due to widespread cell death via hyperactivation of the intrinsic apoptosis pathway. In stac mutants, mitotic cells show aberrant mitotic spindles and chromosome missegregation, resulting in the arrest of the cell cycle in G2/M phase. We also demonstrate that stac heterozygous adults are less susceptible to induction of the carcinogen MNNG for malignant tumor development. Thus, our study uncovers an essential role of Cenph in vertebrate development and its implications in tumorigenesis.

More apoptotic cells in stac mutant embryos than in wild type embryos are not obvious until about 20 hpf of development, being much later than the detectable time point (6 hpf during early gastrulation) of apoptosis in zebrafish embryos (42, 43). This phenomenon may be ascribed to the presence of maternal cenph transcripts and/or Cenph protein. Although stac mutant embryos injected with cenph mRNA survive a couple of days longer, they can never grow to adulthood, which suggests that constant supply of Cenph protein is necessary for life. Thus, it is not practical to obtain stac homozygous adult females by mRNA rescue or germ cell transfer. To study the requirement of maternal Cenph for the cleavage of blastomeres, new approaches should be taken to deplete maternal Cenph. Another interesting phenomenon is that more serious apoptosis occurs in the brain and spinal cord in stac mutants. It is likely that highly proliferating tissues require a large amount of Cenph.

FIGURE 7. stac heterozygous adults have a decreased susceptibility of carcinogenesis. stac heterozygous and wild type (WT) sibling fish at 21 days postfertilization were treated with the carcinogen MNNG and sectioned 6 months post-treatment. All pictures were taken from wild type embryos. A, malignant tumors were found in various tissues of wild type embryos. The percentage of fish with tumors was statistically insignificant (p = 0.046, Fisher’s exact test), although the difference in intestine adenoma rate between two groups was statistically significant (p = 0.046, Fisher’s exact test), although the difference in intestine adenoma rate was statistically insignificant (p = 1).
Among known Cenp genes, Cenpa, Cenpb, Cenpc, or Cenpe null mice have been generated by site-specific targeting (5–9, 44). Homozygous mutants for Cenpa, Cenpb, or Cenpe all display mitotic defects and are embryonic lethal (5, 7, 8), indicating that these kinetochore genes are essential for embryonic development. In this study, we demonstrate for the first time an essential role of Cenph in vertebrate embryonic development using the zebrafish model. In contrast, Cenph null mice develop normally; the mutant adults have reduced body weight and show age-dependent reproductive deterioration (9, 44). These reports suggest that different kinetochore genes/proteins have distinct roles in development, which may depend on their dynamic requirement for centromere architecture and function (4, 45). Each kinetochore in vertebrate cells consists of more than 80 proteins, most of which have not been studied for their function in development (3). Future effort should be made to address their developmental roles at the whole organism level.

In zebrafish stac mutants, many mitotic cells display disorganized spindles, chromosome hypercondensation, and missegregation, resembling those seen in chicken DT40 or human HEP-2 cells depleted of CENPH (13, 17), which suggests a conserved function of Cenph in mitosis. Compared with wild type sibling embryos, stac mutant embryos have a significantly higher fraction of mitotic cells in G2/M phase, suggesting mitotic arrest in mutants. The same phenomenon has also been observed in chicken DT40 cells depleted of CENPH (13). The mitotic arrest in mutants. The same phenomenon has also been observed in chicken DT40 cells depleted of CENPH (13). The observation that the cell cycle progresses normally in human HEP-2 cells depleted of CENPH by RNAi knockdown (17) may result from inefficient depletion of CENPH.

Previous studies have shown that the deficiency of kinetochore proteins usually leads to cell death following defective mitosis in cultured cells or organisms of vertebrates (for examples see Refs. 5, 7, 8, 13, 17, 46–48); however, the mechanisms underlying cell death are poorly understood. In this study, we demonstrate that in stac mutants several components of the intrinsic apoptotic pathway, including tp53/p53, mdm2, and bbc3/puma genes as well as caspases 3, 7, and 9, are expressed at higher levels or hyperactivated. Knockdown of p53 gene expression in stac mutants rescues the mutant phenotype only partially and temporarily. This suggests that p53-independent and even caspase-independent apoptosis pathways are involved in extensive cell death in zebrafish stac mutants. Another unsolved question is how the deficiency of kinetochore proteins or abnormal kinetochores triggers apoptosis. It is likely that microtubule/spindle damage due to abnormal kinetochores/centromeres causes activation of microtubule-associated apoptosis inducers or inactivation of apoptosis inhibitors. Another possibility is that mitotic arrest due to abnormal kinetochores will lead to DNA damage, and the latter activates related apoptosis pathways. Unfortunately, technical bottlenecks in fish have hindered our further studies.

The genome instability caused by depletion of kinetochore or spindle checkpoint proteins could raise cancer susceptibility in most cases and inhibit the tumorigenesis in other contexts (10, 11). We demonstrate that haploinsufficiency of Cenph in stac heterozygous fish suppresses development of malignant tumors in several tissues upon chemical induction. This finding is similar to the previous report that Cenpe heterozygous mice have reduced tumor susceptibility to carcinogen stimulation (49). This possibly could be explained by the following: a higher level of apoptosis in the organisms triggered by dysfunctional kinetochores, which is supported by higher levels of casp8, tp53, bbc3, and mdm2 in stac heterozygous fish than in wild type fish (supplemental Fig. S5), may help destroy hyperproliferating cells. Interestingly, up-regulation of human CENPH expression has been reported in different types of cancers, including colorectal cancer (16), oral squamous cell carcinomas (18), esophageal (20), nasopharyngeal carcinomas (19), tongue cancer (22), and lung cancer (21). These findings suggest a potential significance of Cenph in tumor prognosis and therapy.

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