RESEARCH COMMUNICATION

A mutation in separase causes genome instability and increased susceptibility to epithelial cancer

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Proper chromosome segregation is essential for maintenance of genomic integrity and instability resulting from failure of this process may contribute to cancer. Here, we demonstrate that a mutation in the mitotic regulator separase is responsible for the cell cycle defects seen in the zebrafish mutant, *cease&desist* (*cds*). Analysis of *cds* homozygous mutant embryos reveals high levels of polyploidy and aneuploidy, spindle defects, and a mitotic exit delay. Carcinogenesis studies demonstrated that *cds* heterozygous adults have a shift in tumor spectrum with an eightfold increase in the percentage of fish bearing epithelial tumors, indicating that *separase* is a tumor suppressor gene in vertebrates. These data strongly support a conserved cross-species role for mitotic checkpoint genes in genetic stability and epithelial carcinogenesis.

Supplemental material is available at <http://www.genesdev.org>.

Received July 18, 2006; revised version accepted November 8, 2006.

Results and Discussion

A previously described genetic screen in zebrafish for cell proliferation mutants identified *cease&desist* (*cds*), a homozygous lethal mutant with aberrant staining for the phosphorylated form of histone H3 (pH3), a mitotic marker (Shepard et al. 2005). pH3 staining of *cds* embryos 36 h post-fertilization (hpf) shows a slight decrease in the number of pH3-positive cells (Fig. 1A) and reveals that these pH3-positive foci are also larger than their wild-type sibling counterparts. Further characterization

of homozygous *cds* embryos shows that they also exhibit increased apoptosis as early as 24 hpf, as detected by TUNEL staining (Fig. 1B). 5-bromo-2-deoxyuridine (BrdU) incorporation also indicates that at 28 hpf, *cds* embryos have markedly decreased embryonic proliferation (Supplementary Fig. S1). To further characterize the larger foci phenotype and cell cycle defects exhibited by *cds* mutant embryos, DNA content analysis was performed on disaggregated 24-hpf embryos. This study demonstrated that *cds* embryos have a population of cells with 8N DNA content, indicating the presence of polyploid cells (Fig. 1C). Further analysis also demonstrated the presence of 16N cells (data not shown). These data suggest that *cds* mutants have a fundamental problem with control of the mitotic checkpoint.

To discover the gene responsible for the *cds* phenotype, a positional cloning project was undertaken and *cds* was mapped and localized to linkage group 6 in a 6.5-centimorgan (cM) interval between the microsatellite markers z5294 and z265 (Fig. 1D). Scanning of additional microsatellite makers narrowed the interval to 0.5 cM. A chromosomal walk was initiated from z11919, and led to the identification of a single BAC clone that contained the *cds* locus. Analysis of BAC sequence and human/zebrafish syntenic relationships led to the discovery that the zebrafish ortholog of the vertebrate gene *separase* was present in this critical interval. Sequence analysis of the *cds* mutant locus identified a C-to-A transversion introducing a nonsense mutation that is predicted to truncate the zebrafish *separase* gene after amino acid 597, which would cause loss of the catalytic domain and loss of half of the ARM repeats at the N terminus (Viadiu et al. 2005). Separase is a cysteine protease that, during mitosis, is responsible for cleaving the cohesin complex that binds sister chromatids together (Nasmyth 2001). Separase is tightly regulated by an anaphase-promoting complex target protein, securin, which has been shown to act as the oncogene pituitary tumor transforming gene (PTTG) in humans (Zou et al. 1999). Separase is also regulated by autocatalytic cleavage and inhibitory phosphorylation (Stemmann et al. 2001; Wai-zenegger et al. 2002; Zou et al. 2002). Additionally, in yeast, separase has been shown to act as part of the mitotic exit network (MEN) complex, suggesting that separase simultaneously triggers anaphase by cohesin cleavage and, independently of its protease function, initiates mitotic exit through activation of Cdc14 (Stegmeier et al. 2002). Zebrafish *separase* encodes a 2181-amino-acid protein that is 34% identical to human separase, and 13% identical to *Schizosaccharomyces pombe* Cut1 and *Saccharomyces cerevisiae* Esp1 (Supplementary Fig. S2). The catalytic residues that are present in all caspase-hemoglobinase fold family members are also conserved (Aravind and Koonin 2002). DNA content analysis of wild-type embryos injected with antisense morpholino-modified oligonucleotides (morpholinos) targeted to the *separase* exon 7 splice donor shows that ~30% of the cells are polyploid, which is similar to the level seen in *cds* homozygous mutants (Fig. 1E). These data strongly suggest it is a loss of function of *separase* that is responsible for the *cds* phenotype.

Loss of *separase* in yeast, human, and mouse cells has

[Keywords: Cancer; chromosome segregation; mitotic checkpoint; zebrafish]

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1470407>.

Figure 1. *cds* has mitotic cells with increased size of pH3 foci and increased apoptosis and polyploidy, and is caused by a mutation in the zebrafish separase gene. (A) High-power views of a 36-hpf *cds* mutant show that pH3-positive foci in *cds* are larger in size than in wild-type siblings. (B) TUNEL staining of 24-hpf *cds* embryos shows increased apoptosis in the head and dorsal part of the tail. (C) DNA content analysis of *cds* homozygous 36-hpf embryos and wild-type siblings showing the presence of polyploid cells in the mutant. (D) Positional cloning of the *cds* gene. The *cds* locus is depicted by a black bar between the flanking markers z11919 and z7248 identified by analysis of ~3000 meioses from diploid mutants. Below is an enlarged view of the *cds* locus that depicts the BAC genomic clones identified in a chromosomal walk. The number of recombination events identified from the distal side (squares) and proximal side (circles) are indicated. (E) Phenocopy of the *cds* polyploid phenotype by injection of morpholinos targeted to the *separase* exon 14 splice donor.

been shown to cause mitotic spindle defects such as multiple spindle poles (Baum et al. 1988; Uzawa et al. 1990; Waizenegger et al. 2002; Chestukhin et al. 2003; Kumada et al. 2006; Wirth et al. 2006). Examination of the mitotic spindle in 32-hpf *cds* mutants demonstrated that ~50% of the visible spindles were abnormal (Fig. 2A–I; data not shown). Analysis of *cds* cells for pH3 and mitotic spindles showed that these abnormal mitotic figures do exhibit multipolar spindles some with three to four visible centrosomes (Fig. 2B,D–H). Monopolar spindles are also visible (Fig. 2C). Also present are multipolar spindles that appear to have lobed or distinct nuclei with spindles that may nucleate from shared centrosomes (Fig. 2G,H). These may represent failed mitoses resulting from fused cells or midbody retraction. Another defect commonly seen in *cds* embryos is lagging chromosomes (Fig. 2I). This phenotype has also been seen in *separase* knockdowns in human cells (Chestukhin et al. 2003). Our findings indicate that separase is required for normal chromosome segregation in zebrafish, and supports a conserved role in regulating mitosis in eukaryotic cells.

Studies have shown that the presence of lagging chromosomes slows the spindle elongation rate indicating that cells may detect the presence of such chromosomes and slow anaphase in order to allow the chromosomes

extra time to reach the pole (Pidoux et al. 2000). Our studies have demonstrated that such a mitotic exit defect can be detected. To examine the cell cycle timing of *cds* cells from S to M phase and determine if a mitotic entry or exit defect existed, we performed a BrdU pulse/chase experiment and double stained the embryos with anti-BrdU antibody and pH3 antibody. Examining the timing of when BrdU-positive cells become pH3 positive allows calculation of the length of time between the S phase and mitotic entry and exit in *cds* as compared with wild types (Fig. 2J). The S-phase cell population in wild-type embryos begins to enter G2/M 2 h after BrdU labeling and begins to exit from mitosis at 6 h, with exit completed by 8 h. Cells in *cds* embryos enter M phase at the same time as wild types, and also begin to exit at 6 h post-incorporation. There are still a significant proportion of *cds* cells in mitosis at the 8-h time point, indicating slower progression through mitosis, perhaps due to mitotic complications, or a potential mitotic exit defect. Separase has been shown to act as part of a complex that promotes mitotic exit in yeast (Stegmeier et al. 2002), so this phenotype is consistent with a similar role in vertebrates. However, recent studies in mouse embryonic fibroblasts lacking Separase have not uncovered any mitotic exit defects (Kumada et al. 2006). This discrepancy between zebrafish embryos and mouse embryonic fibroblasts (MEFs) may be attributed to studying this phenomenon in a whole organism versus in a cell culture environment; the defect may only be visible in the former.

To further characterize the polyploidy seen in *cds* embryos and to look for further evidence of chromosomal instability as indicated by the presence of grossly abnormal mitoses, interphase fluorescent in situ hybridization (FISH) analysis was performed using probes specific to two randomly chosen linkage groups. This analysis shows that *cds* embryos contain 48% polyploid and 34% aneuploid cells versus 2% and 7%, respectively, in wild types (Fig. 3A,B). Further analysis of metaphase spreads demonstrated the presence of cells in *cds* mutant embryos with 16N DNA content (Fig. 3C). These cells also contain diplochromosomes (chromosomes that have undergone DNA replication but have not segregated) that appear to remain attached at the centromeres. Diplochromosomes have been previously observed in *Drosophila*, and more recently in mouse, *separase* mutants (Jager et al. 2001; Kumada et al. 2006). This confirms the role of vertebrate Separase in cleavage of the bonds between sister chromatids during mitosis.

Given Securin's role as an oncogene (Zou et al. 1999) and that mutation of *separase* affects chromosome segregation and chromosomal stability, we next asked whether the *separase* mutation affected cancer susceptibility in heterozygous adults. Twenty-eight-day-old zebrafish (heterozygotes and wild-type siblings) were subjected to a single dose of the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). At 3, 6, or 12 mo after exposure, the 663 treated fish were sacrificed, and serial step sections of these fish were examined for the presence of tumors. Each fish was genotyped using a restriction fragment length polymorphism (RFLP) specific

Figure 2. Abnormal mitoses and a mitotic exit defect are present in *cds*. (A–D) Mitotic spindles (α -tubulin, red), centrosomes (γ -tubulin, green), and DNA (DAPI, blue) seen in *cds* embryos. (E–I) Mitotic figures (pH3 staining, green) and spindles (α -tubulin, red) seen in *cds* embryos. A shows a normal bipolar mitotic spindle. (J) Time course of *cds* and wild-type movement from S phase into/out of G2/M as shown by BrdU incorporation followed by double pH3/BrdU staining at varying time points post-incorporation.

to the *separase* mutant allele. *cds* heterozygotes have a 2.5-fold increase in cancer susceptibility relative to wild-type siblings after MNNG exposure (Fig. 4A; $p = 0.005$). These studies demonstrate that *separase* mutations increased cancer susceptibility in zebrafish, and this is the first demonstration of *separase* as a tumor suppressor gene.

Work in other organisms has shown that certain genetic mutations may cause an increased incidence of a particular tumor type. To address this issue in the *cds* heterozygotes, analysis of the tumor spectrum in *cds* heterozygotes and wild types after MNNG exposure was performed. This investigation shows that treated *cds* heterozygotes have an approximately eightfold increase in the percentage of *cds* heterozygous fish with epithelial tumors as compared with treated wild types (Fig. 4B, $p = 0.001$). The level of epithelial tumors seen in *cds* heterozygotes is also higher than that seen in MNNG-treated fish in previous studies (Spitsbergen et al. 2000; Shepard et al. 2005). These epithelial tumors consist of, in part, intestinal adenomas (Fig. 4C,D) and cholangiocarcinomas (Fig. 4E,F). Epithelial neoplasms, including adenomas and carcinomas, are common tumors in human adults, and the rates of such cancers have been shown to increase with age (Chang et al. 2001; American Cancer Society 2004). Another feature of epithelial tumors in humans is their aberrant cytogenetic profiles with visible aneuploidy and chromosomal amplification, deletions, and translocations (Atkin 1986, 1989). Genomic instability caused by the heterozygous loss of *separase* in zebrafish may be the causative factor in the shift in the tumor spectrum toward epithelial neoplasms. Interestingly, loss of *separase* in *Drosophila* has been shown to cause defects in epithelial organization and integrity (Pandey et al. 2005). A specific role for *separase* in epithelial cells may also provide an explanation for this shift in tumor spectrum. Consistent with the findings presented here, Sotillo et al. (2006) have shown recently that Mad2 overexpression, which leads to Securin stabilization and Separase inhibition, results in spontaneous tumor initiation in mice with a tumor spectrum similar to that seen in *separase* heterozygous zebrafish.

Heterozygous mutations in tumor suppressor genes often contribute to tumor formation through a mechanism of loss of heterozygosity. To determine if this mechanism was applicable to the epithelial tumors arising in

the *separase* heterozygotes, we performed FISH on 10 epithelial tumors from *cds* heterozygotes using a probe that contained the *separase* locus and a centromeric control probe from the same linkage group. In two large and advanced epithelial tumors we found a single copy of *separase* and two copies of the control centromeric probe (in ~70% of the cells in each tumor, $n = 200$ cells) (Fig. 4G; Supplementary Fig. S4), indicating loss of one allele of the *separase* gene. Sequence analysis of laser-capture microdissected tissue from the tumor depicted in Figure 4G indicated that the wild-type allele was lost (Supplementary Fig. S5). Areas outside the tumor margins had two copies of each probe (Fig. 4H; Supplementary Fig. S4). It is also possible that loss of function of *separase* occurs in some of the tumors by a mechanism other than deletion, such as point mutation or silencing, although sequencing of the conserved protease domain in three epithelial tumors did not reveal any mutations (data not shown). There is no apparent polyploidy in the tumor cells with *separase* loss of heterozygosity (LOH) (Fig. 4G; Supplementary Fig. S4; data not shown). The zebrafish system currently does not have a platform technology to comprehensively assess global chromosomal instability. Until a comprehensive approach is possible, we cannot be certain whether instability exists in these tumors. Our data clearly demonstrates that heterozygous loss of *separase* contributes to initiation and progression of epithelial tumors. While haploinsufficiency of the *separase* gene appears to be the mechanism for the increased tumor incidence in the majority of cases, loss of heterozygosity of the *separase* gene appears to play a role in tumor progression in some tumors. Tumor formation may be achieved by similar mechanisms in both instances. In many environments, complete loss of *separase* function would likely have a negative effect on cell viability because of gross chromosomal missegregation. It is possible that only after additional mutations have occurred in the tumor that affect the cell cycle and mitotic control would loss of the functional allele of *separase* give a selective advantage to the tumor cells and advance tumor progression. Therefore, in more advanced tumors, complete loss of function could provide a competitive advantage for the tumor cells, enough to see LOH at some frequency. The absence of polyploidy in these tumors suggests that the cells have developed a

Figure 3. *cds* mutant embryos demonstrate polyplody and aneuploidy. (A) Percentage of polyplod and aneuploid cells in interphase nuclei of *cds* (black bars) and wild-type embryos (white bars). (B) Chromosome copy number enumeration for linkage group 2 (red) and linkage group 16 (green). The aneuploid cell shown in B exhibits tetrasomy for linkage group 2 and disomy for linkage group 16. (C) Metaphase spreads from *cds* and wild-type embryos.

way to partially compensate for complete loss of *separase*. This supports our notion that genetic lesions in the advanced tumor leads to a selective advantage for complete *separase* loss. While LOH is observed in some tumors, our data demonstrates that loss of only one copy of *separase* is sufficient in most cases to promote tumorigenesis and shift tumor spectrum toward epithelial neoplasms.

These data represent the first link between *separase* and increased cancer susceptibility and the first evidence that *separase* acts as a tumor suppressor. The presence of genomic instability and severe mitotic abnormalities in *cds* homozygous mutant embryos also demonstrates that zebrafish *separase* functions in a similar capacity to its mammalian and yeast counterparts. It is possible that *separase* may also act as a tumor suppressor in humans, although initial sequencing of the *separase* locus in 82 human tumor cell lines representing a myriad of tumor types did not reveal any obvious mutations (data not shown). The identification of Securin as a human oncogene does support a role for this pathway in human tumor formation. A more focused sequencing approach concentrating on tumors of epithelial origin may have to be undertaken in order to reveal such a role in humans.

This work also suggests interesting implications for the study of the interactions between carcinogens and genetic mutations. In zebrafish, carrying *separase* mutations causes a clear susceptibility to cancers outside the

normal tumor spectrum when exposed to the carcinogen, MNNG. Examples of this type of specific genetic susceptibility to carcinogens have already been shown to exist in certain cases (Bennett et al. 1999; Tan et al. 2000). Therefore, in addition to providing the first evidence of *separase* tumor suppressor function, our studies demonstrate that zebrafish can serve as an excellent model system for the study of epithelial cancers, and could provide insight into the interactions between carcinogens and genetic predispositions.

Material and methods

Whole-mount immunohistochemistry

pH3 staining, TUNEL staining, BrdU incorporation, DNA content analysis, and mitotic spindle/centromere staining were done as previously described (Shepard et al. 2005).

Genetic mapping, genotyping, and library screens

cds *wik* strains were outcrossed to AB to create polymorphic mapping strains. Low-resolution mapping was done using pools of 40 diploid mutant and 40 diploid wild-type embryos and scanning for linked polymor-

Figure 4. *cds* adult heterozygotes have a higher susceptibility to cancer and a distinct tumor spectrum as compared with wild types. (A) After treatment with MNNG, tumors were detected in 9.7% of *cds* heterozygotes and 4% of sibling wild types. A two-sided Fisher's Exact test gives this finding a *p* value of 0.005. (B) Percentage of MNNG-treated *cds* and wild-type fish bearing either epithelial, mesenchymal, or germ cell tumors. (C,D) Intestinal adenoma (P) arising as a polyp in otherwise normal intestine (I). Mitotic figures are abundant (arrowheads), and there is nuclear crowding with stratification. (E,F) Ductal adenocarcinoma arising from intestine or pancreas. Bars: C,F, 100 μ m; D,G, 50 μ m. (G,H) Fluorescent in situ hybridization on paraffin sections seen in F and G using a BAC probe containing the *separase* gene (orange) and a control LG6 centromeric probe (green). DAPI is in blue. G shows an area within the tumor. H shows an area in the normal intestine.

phic markers using a range of CA microsatellite markers on each linkage group. After linkage was established to LG 6, CA microsatellites on this linkage group were tested for polymorphism in mapping families and then tested on a medium resolution panel of 88 mutants and eight wild types. Close flanking markers z5294 and z265 were identified, and then the entire mapping panel consisting of ~1500 diploid *cds* embryos was tested with both markers. Recombinants were identified for both markers. ESTs in that region on the RH panel map and additional microsatellite markers were scanned for polymorphisms and close flanking markers of z11919 and z7248 were identified. A chromosomal walk was initiated from z11919. Overgo oligos were designed from BAC end sequence and hybridized to CHORI and DanioKey BAC library filters. Polymorphic markers were identified on BAC ends by SSCP analysis. BAC end sequence was used to identify a BAC in the critical interval that contained the *cds* locus and the entire *separase* gene.

cds RFLP genotyping

Genomic DNA was extracted from embryos or adult tailclips and amplified with nested PCR primers flanking the *cds* mutation (forward primer1, 5'-GACACACGCCTGAGGTAAC-3'; reverse primer1, 5'-CAT AACCCTTAAACATTTTCCTG-3'; forward primer2, 5'-CGGGTTAG GAGAATCCTTAG-3'; reverse primer2, 5'-TGTATGCAAGTCTCGA GTGG-3'). The initial PCR conditions were as follows: 20 sec at 94°C, 30 sec at 65°C (decrease 0.5°C per cycle), 1 min at 72°C for 30 cycles and then an additional 10 cycles of 20 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C. The second nested PCR conditions were 20 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C for 30 cycles. The PCR products were digested for 2.5 h at 37°C with the enzyme Hpy188I (New England Biolabs), which cuts the mutant but not the wild-type allele. Digests were run on a 2.5% agarose gel and the presence of digestion fragments indicated the mutant allele.

Embryo injection experiments

Morpholinos were designed to the exon 14 splice donor site (5'-ACTG GCTGAGTGTCTGCAGTTTGGT-3') (GeneTools). One-cell stage embryos were injected into the yolk sac with 1 nL of a 500-μM solution of the splice morpholino (or control vehicle). Embryos were examined at 24 hpf for the characteristic morphology of *cds* as well as undergoing DNA content analysis.

Cytogenetics

Metaphase chromosome spreads and interphase nuclei were obtained from 24-hpf embryos using standard cytogenetic procedures. Cells were arrested at metaphase with colchicine, subjected to hypotonic treatments, and fixed onto glass slides with 3:1 methanol:acetic acid. Fluorescence in situ hybridization was performed using standard protocols. Fluorescently labeled probes included a rhodamine-labeled zC039P05 BAC DNA probe for linkage group 2 and a FITC-labeled zC132M17 BAC DNA probe for linkage group 16. For tumor sections, the *separase* locus probe was zC233B16 and the centromere probe was zK11J4.

Carcinogenesis

Twenty-eight-day-old fry from backcrosses of mutant heterozygotes were exposed to MNNG (Spitsbergen et al. 2000) and analyzed as previously described (Shepard et al. 2005).

Statistical methods

A Fisher exact test was used to assess whether tumor susceptibility differed between *cds* heterozygotes and wild-type zebrafish. All time points were combined for this calculation. All *p* values are two sided.

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Genes Dev. 2007, **21**:

Access the most recent version at doi:[10.1101/gad.1470407](https://doi.org/10.1101/gad.1470407)

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