Unstable Kinetochore-Microtubule Capture and Chromosomal Instability Following Deletion of CENP-E

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Summary

A selective disruption of the mouse CENP-E gene was generated to test how this kinetochore-associated, kinesin-like protein contributes to chromosome segregation. The removal of CENP-E in primary cells produced spindles in which some metaphase chromosomes lay juxtaposed to a spindle pole, despite the absence of microtubules stably bound to their kinetochores. Most CENP-E-free chromosomes moved to the spindle equator, but their kinetochores bound only half the normal number of microtubules. Deletion of CENP-E in embryos led to early developmental arrest. Selective deletion of CENP-E in liver revealed that tissue regeneration after chemical damage was accompanied by aberrant mitoses marked by chromosome missegregation. CENP-E is thus essential for the maintenance of chromosomal stability through efficient stabilization of microtubule capture at kinetochores.

Introduction

Each successful eukaryotic cell cycle culminates in the accurate segregation of duplicated chromosomes to daughter cells. This is accomplished by the mitotic spindle, whose dynamic microtubules are essential for the complex movements of chromosomes during mitosis. Attachments between spindle microtubules and chromosomes are mediated by kinetochores, which are specialized, multilayered structures located at the surfaces of each centromere. After nuclear envelope breakdown, some of the microtubules emanating from the centrosome (or spindle pole) attach to kinetochores. The initial linkage is often lateral, allowing rapid poleward movement of the chromosome along a microtubule’s surface, apparently powered by kinetochore-associated cytoplasmic dynein (Rieder and Alexander, 1990). The subsequent attachment of sister kinetochores to microtubules growing from sister poles yields bipolar chromosome orientation, which is usually accompanied by interactions between the kinetochores and the microtubule ends. The formation of this arrangement initiates chromosome “congression”, a motion often characterized by discontinuous movements toward the spindle equator and then oscillatory movements about that plane (Cassimeris et al., 1994; Skibbens et al., 1993). With the onset of anaphase, sister chromatids separate and the individual chromosomes exhibit mostly poleward movements, with the centromeres leading as the kinetochore-associated microtubules depolymerize.

Kinetochores not only link chromosomes to spindle microtubules, but they also actively participate in chromosome movement through the action of microtubule motors that are bound to them (Hyman and Mitchison, 1991; Nicklas, 1989). These motors include dynein (Pfarr et al., 1990; Steuer et al., 1990), CENP-E (centromere-associated protein E) (Yen et al., 1992), and members of the MCAK/XKCM1 family (Walczak et al., 1996; Wodemann and Mitchison, 1995). Kinetochore motors have been proposed not only to power the movement of chromosomes along spindle microtubules, but also to act as a tether between chromosomes and dynamic microtubule assembly. CENP-E, which is localized to kinetochores from prometaphase through anaphase A (Brown et al., 1996), is appropriately positioned to participate in all phases of chromosome movement. Disruptions of CENP-E function by its removal from Xenopus extracts (Wood et al., 1997) or by genetic mutation in Drosophila (Yucel et al., 2000) have demonstrated that CENP-E is required in those contexts for chromosome alignment at metaphase.

Additionally, despite a requirement for CENP-E in mitotic checkpoint activation in Xenopus extracts (Abrieu et al., 2000), antisense oligonucleotide-mediated suppression of synthesis (Yao et al., 2000) and antibody microinjection (McEwen et al., 2001; Schaar et al., 1997) in immortalized mammalian cells have resulted in preanaphase chromosome misalignment with chronic checkpoint activation, albeit there are discrepancies among the results from the latter three approaches. In one case, antibody injection was interpreted to lead to bioriented sister chromatids that oscillate normally, but which disjoin at the centromeres, despite an arrest in prometaphase (Schaar et al., 1997). In a subsequent study, the injection of a similar antibody was reported to cause no changes in the types and velocities of movements of bioriented chromosomes, despite a mild reduction in the number of microtubules per kinetochore and the absence of tension across bioriented centromeres (McEwen et al., 2001).

These findings not notwithstanding, it remains untested whether, and, if so, why, CENP-E is essential for mammalian development or for tissue regeneration in the adult. We have therefore disrupted the CENP-E gene using the Cre/loxP system, creating a complete, systemic gene disruption and a selectively deletable allele in parallel. Analysis of the systemic deletion revealed the requirement for CENP-E function at a very early stage in whole-animal development, accompanied by mitotic chromosome misalignment. Selective deletion...
of CENP-E within adult animals has allowed us to document mitotic errors indicative of chromosome instability during tissue regeneration that result from a requirement for CENP-E in the stabilization of kinetochore-microtubule attachments and for sustained mitotic checkpoint activation.

**Results**

**Targeting the Mouse CENP-E Locus**

A portion of the murine CENP-E gene was isolated from a 129Sv genomic DNA library with a segment of the human CENP-E cDNA as a probe. Exon/intron boundaries were mapped by reference to sequences derived from mouse CENP-E cDNA clones isolated from an embryonic library and from the EST database. The nucleotide binding consensus sequence, or “P loop” sequence (Endow, 1991; Yang et al., 1989), was encoded by a 120-nucleotide exon, hereafter referred to as “exon P”. The sequence of this and the flanking exons (numbered P − 1 and P + 1) revealed that the deletion of exon P would introduce a frameshift mutation in the resultant mRNA, creating a premature stop codon at amino acid 82 of the 2474 amino acids that comprise the full-length mouse CENP-E sequence; B. Weaver, F.R.P., and D.W.C., unpublished data. The predicted truncation should thus produce a null allele, removing all domains required for nucleotide and microtubule binding as well as kinetochore targeting.

Using a combination of PCR and subcloned fragments of the CENP-E gene, we assembled a targeting construct in which neomycin resistance and thymidine kinase genes flanked by lox recombination sites were placed within intron P − 1 of the CENP-E gene and an additional lox site was placed in intron P (Figure 1A). These were flanked by two portions of the CENP-E genomic sequence, a 1.8 kb portion of sequence 5’ to exon P and a 6.8 kb portion of sequence 3’ to exon P.

The final 15.5 kb vector was linearized and introduced by electroporation into mouse embryonic stem (ES) cells from a 129Sv background. Because the targeting vector introduces an additional BamHI site, DNA from neomycin-resistant clones was analyzed for targeted integration after restriction digestion with this enzyme (Figure 1C). Of the ES cell clones screened, 22% were homologously recombined at the CENP-E locus. Two of the correctly targeted clones were chosen for expansion and transfection with genes encoding the Cre recombinase. ES cells that had undergone an excision event leading to the loss of the thymidine kinase gene were selected by the addition of gancyclovir. ES cells harboring either the complete or conditional disruption were identified with PCR primers that would yield 1150-, 950-, 48 hr postinfection (Figure 2D), whereas similar figures selected by the addition of gancyclovir. ES cells harboring misaligned and/or centrophilic chromosomes increased in number. Chimeric mice were identified and bred to obtain primary mouse fibroblasts for culture. Since previous work has shown that, in such cultured cells, CENP-E is quantitatively degraded at the end of mitosis, just like the mitotic cyclins (Brown et al., 1994), disruption of the single functional CENP-E allele in these cells would, by the first subsequent mitosis, produce an effective CENP-E null, so long as the disruption took place sufficiently early in the cell cycle. Gene disruption in G2 would not be expected to affect the subsequent mitosis, since CENP-E levels would already be maximal, but the second mitosis would take place in the absence of CENP-E.

In control cells that properly align their chromosomes at a mitotic metaphase position, kinetochore-bound CENP-E was always present (Figure 2A). Visualization of DNA by DAPI staining and indirect immunofluorescent detection of microtubules revealed that cells lacking CENP-E showed frequent mitotic abnormalities. While a subset of CENP-E-deficient cells established an apparently normal metaphase plate, the remaining preanaphase cells lacking detectable CENP-E kinetochore staining displayed obvious defects in chromosome alignment. In some cases, one or two chromosomes were displaced from the metaphase plate (Figure 2B, arrow). In other examples, many chromosomes clearly revealed the characteristic double-dot pattern of centromere antigens on centrophilic chromosomes deficient in CENP-E (Figure 2E). Thus, these misaligned chromosomes result from the failure of chromosomes to congress, not from premature sister chromatid separation.

CENP-E Null Primary Fibroblasts Display Abnormalities in Chromosome Alignment

To examine the phenotype of normal, untransformed cells that are deficient for CENP-E, we mated mice heterozygous for the complete and the conditional disruptions of CENP-E to each other and harvested day 14 embryos. The embryos were individually genotyped by PCR, and those of the desired genotype were dissected to obtain primary mouse fibroblasts for culture. Since previous work has shown that, in such cultured cells, CENP-E is quantitatively degraded at the end of mitosis, just like the mitotic cyclins (Brown et al., 1994), disruption of the single functional CENP-E allele in these cells would, by the first subsequent mitosis, produce an effective CENP-E null, so long as the disruption took place sufficiently early in the cell cycle. Gene disruption in G2 would not be expected to affect the subsequent mitosis, since CENP-E levels would already be maximal, but the second mitosis would take place in the absence of CENP-E.

Rapid, efficient, and uniform introduction of the Cre recombinase into these fibroblasts was achieved by infecting them with a replication-incompetent adenovirus (Ad-Cre) encoding Cre under control of a human cytomegalovirus immediate early promoter (Anton and Graham, 1995). Cells grown on coverslips were infected with Ad-Cre and fixed 1 or 2 days after infection. The use of PCR to follow Cre excision at the CENP-E locus revealed that, by 48 hr postinfection, the conditional disruption was converted to a complete disruption in greater than 75% of the cell population. Immunofluorescence with CENP-E antibodies (Figures 2B and 2C) revealed that, within 48 hr of the addition of Ad-Cre, as many as 90% of the preanaphase cells lacked detectable CENP-E.

In control cells that properly align their chromosomes at a mitotic metaphase position, kinetochore-bound CENP-E was always present (Figure 2A). Visualization of DNA by DAPI staining and indirect immunofluorescent detection of microtubules revealed that cells lacking CENP-E showed frequent mitotic abnormalities. While a subset of CENP-E-deficient cells established an apparently normal metaphase plate, the remaining preanaphase cells lacking detectable CENP-E kinetochore staining displayed obvious defects in chromosome alignment. In some cases, one or two chromosomes were displaced from the metaphase plate (Figure 2B, arrow). In other examples, many chromosomes clearly failed to align (Figure 2C, yellow arrows) or were clustered near the poles in a centrophilic position (Figure 2C, white arrows). The percentage of mitotic cells with misaligned and/or centrophilic chromosomes increased over time, from 28% at 24 hr postinfection to 43% at 48 hr postinfection (Figure 2D), whereas similar figures were never seen in more than 4.4% of the control samples.

An anti-centromere antibody (ACA), which was used to identify centromeres after infection with Ad-Cre, clearly revealed the characteristic double-dot pattern of centromere antigens on centrophilic chromosomes deficient in CENP-E (Figure 2E). Thus, these misaligned chromosomes result from the failure of chromosomes to congress, not from premature sister chromatid separation.
**Unstable Microtubule Capture without CENP-E**

**Figure 1. Strategy for Modification of the CENP-E Locus and Identification of ES Cell Clones in which the CENP-E Locus Is Modified**

(A) A schematic of the mouse CENP-E locus. Three Cre recombinase recognition sites, light blue triangles; P loop exon, dark blue. Nucleotide numbers corresponding to a full-length mouse CENP-E cDNA are listed above the exons (light blue). Amino acids of the mouse CENP-E protein are listed below the exons.

(B) Schematic diagrams of conditional and complete CENP-E gene disruptions after excision by the Cre recombinase. After removal of exon P, the splicing of exon P/11002 to P/11001 introduces a premature stop codon at amino acid 82, removing all of the sequence encoding-identified functional domains.

(C) Genomic DNA blotting from neomycin-resistant ES cell clones after digestion with BamHI and hybridization with the 5' probe listed in (A). HR, homologously recombined; WT, wild-type.

(D) PCR with primers A and B of cell clones after the transient transfection of targeted ES cells with pMC-Cre or pIC-Cre. Lane 1, conditional/disruption; lane 2, conditional/wild-type; lane 3, homozygous wild-type; lane 4, wild-type/disruption.

**CENP-E Is Required for the Stability of Kinetochore-Microtubule Interactions**

To examine, at higher resolution, why the absence of CENP-E affected chromosome behavior, we used conventional electron microscopy of serial thin sections and electron tomographic reconstruction of thick sections to examine kinetochore structure and kinetochore-microtubule associations in CENP-E-containing and CENP-E-deficient cells. Primary fibroblasts were grown on coverslips and rapidly fixed with a prewarmed solution of glutaraldehyde (2%) and then prepared for electron microscopy (see Experimental Procedures). Serial thin sections revealed that aligned kinetochores in wild-type fibroblasts were associated with the ends of between five and seven microtubules (Figure 3A). Some aldehyde-fixed samples were rapidly frozen in a high-pressure freezer and then prepared for thick (approximately 300 nm) sectioning. Tomographic reconstruction...
Figure 2. Chromosome Misalignment in Primary Fibroblasts that Lack CENP-E

(A) Metaphase in a control primary fibroblast 48 hr after infection with Ad-Cre. DNA, blue; tubulin, green; CENP-E, red.

(B and C) Conditional/disruption cells infected with Ad-Cre.

(B) An example of one cell with one CENP-E-depleted chromosome near a spindle pole.

(C) A second example with at least six misaligned chromosomes. White arrows in (B) and (C) point to chromosomes clustered near a spindle pole; yellow arrows point to misaligned chromosomes farther from a pole.

(D) Frequency of metaphase figures with misaligned chromosomes determined at 24 and 48 hr post-Ad-Cre infection in conditional/disruption cells (CENP-E null after infection) or conditional/wild-type cells (CENP-E heterozygote after infection). The presence (blue columns) or absence (burgundy columns) of kinetochore-bound CENP-E is indicated.

(E) The determination of whether misaligned chromosomes are paired sister chromatids. Following Ad-Cre infection, cells were stained for DNA (blue), tubulin (green), and centromere proteins (red) (with ACA). Left panel, all three images; right panel, DNA and ACA alone. The inset shows one misaligned pair of sister chromatids.

of thick sections allowed the generation of graphic models for two wild-type kinetochores, confirming the number of microtubules seen in thin sections (Figure 3C; see also tomogram and 3D reconstructed model in Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/3/17039/DC1). Overall, combining data
from both methods, there were $5.6 \pm 0.9$ (mean ± SEM, $n = 7$ kinetochores counted) microtubules associated with each kinetochore. Similar analyses of cells with a CENP-E disruption, identified in the Ad-Cre-infected populations by the presence of centrophilic chromosomes, revealed a reduced number of kinetochore-associated microtubules (Figure 3B). Only $2.4 \pm 0.5$ microtubules per kinetochore were seen (five kinetochores counted). For example, tomographic reconstruction of an equatorial chromosome from one CENP-E-deficient cell (Figure 3D; see also tomogram and 3D reconstructed model in Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/3/DC1) identified only two microtubules at that kinetochore.

The kinetochores and their associated microtubules were also examined on the centrophilic chromosomes commonly found in CENP-E-deficient cells. These chromosomes were almost always V shaped, with the tip of the V pointing directly toward the nearby pole and the arms trailing away. Such geometry is characteristic of normal early prometaphase, when poleward chromosome movement is mediated through the attachment of astral microtubules to kinetochore-associated motors that provide a pole-directed force at the kinetochores (Rieder and Alexander, 1990). Examination of three such chromosomes by serial sectioning revealed that their centromeres were positioned very close to the pericentriolar material of the adjacent pole (Figure 4A), so close that the centriole was within $430 \text{ nm} \pm 65 \text{ nm}$ ($n = 3$).
Figure 4. Centrophilic Chromosomes in CENP-E-Depleted Cells Lack Kinetochore Microtubules
Electron micrographs of a CENP-E-depleted cell with a centrophilic chromosome in close proximity to the centriole, but no microtubules end in the vicinity of the kinetochore. The kinetochore (arrowhead) and centriole (boxed region in left panel) are shown enlarged in a series of serial sections in the four right panels.
(A) A low-magnification view.
(B) Four serial sections of an enlarged view of this centrophilic chromosome. Bar, 0.5 μm.

Despite this and the abundance of microtubules found in that vicinity, no microtubules were attached to either of the sister kinetochores of any of the examples sectioned (Figure 4B). This observation, combined with the presence of Mad2 on centrophilic chromosomes (see below; Figure 5), reveals that centrophilic, CENP-E-deficient chromosomes lack stable kinetochore-microtubule interactions.

Microtubule Attachment, Tension, and Mitotic Checkpoint Signaling
To further test how absence of CENP-E affects functional kinetochore attachment, we examined the status of the spindle assembly checkpoint in CENP-E-depleted cells. In the current checkpoint model, the presence of Mad2 at unattached kinetochores reflects the combination of an active recruitment of it and its rapid conversion to an activated form that is rapidly released. This produces a “wait anaphase” signal that delays the metaphase to anaphase transition (reviewed in Shah and Cleveland, 2000). In CENP-E-containing control cells, Mad2 was present at the kinetochores of prophase and nonaligned prometaphase cells (data not shown), as previously described (Howell et al., 2000; Li and Ben ezra, 1996; Waters et al., 1998). In cells depleted of CENP-E by gene disruption, both kinetochores of centrophilic chromosomes were found to contain easily detectable Mad2, consistent with active checkpoint signaling (Figures 5A and 5B, white arrows).

Most kinetochores of equatorially positioned chromosomes lacked Mad2 (≈36 of the 40 chromosome pairs in a diploid cell), indicating that checkpoint signaling is silenced (or fails to activate) when kinetochores are attached to the reduced microtubule number characteristic of loss of CENP-E. However, in those same cells, intense Mad2 staining was found at both kinetochores of some nearly equatorial chromatid pairs (Figure 5B, yellow arrows). The positioning and orientation of these chromatid pairs along the spindle axis suggest prior bioriented attachment. Moreover, kinetochores of other equatorial chromatid pairs exhibited striking asymmetry in Mad2 abundance at sister kinetochores (Figure 5B, blue boxes and insets). One kinetochore of each pair in two examples in Figure 5B (boxed and inset) was weakly Mad2 reactive (Figure 5B, green arrowhead in inset), while the other displayed much higher Mad2 intensity (Figure 5B, blue arrowhead in inset), suggesting occupancy of microtubule attachment sites at only one of the sister kinetochores.

Tension developed between paired sister chromatids has previously been estimated by the stretching of the sister kinetochores (Waters et al., 1996). To test the influence of CENP-E on tension development, we measured the positions of Mad2 or anticentromere antigens (ACA) on centrophilic and equatorial kinetochore pairs in the presence or absence of CENP-E. Kinetochore spacing on fully aligned chromosomes in CENP-E-deficient cells (n = 44 kinetochores from four cells) was
Unstable Microtubule Capture without CENP-E

Figure 5. Unstable Microtubule Attachment in the Absence of CENP-E Revealed by Continued Recruitment of the Spindle Assembly Checkpoint Protein Mad2 to Kinetochores

(A and B) CENP-E-depleted cells (48 hr after Ad-Cre infection of conditional/disruption MEFs). DNA, blue; tubulin, red; Mad2, green; merge, an overlay of all 3 images. Centrophilic chromosomes, white arrows; nearly aligned chromosomes with high levels of Mad2, yellow arrows. Asymmetric Mad2 staining at equatorial chromatid pairs, one kinetochore (blue arrowheads) with a staining intensity similar to that for centrophilic kinetochores and the other kinetochore (green arrowheads) with barely detectable Mad2.

(C) Interkinetochrome distances of centrophilic and equatorial chromatid pairs in wild-type (CENP-E-containing), CENP-E-depleted, and colcemid-treated cells. KT-bound CENP-E indicates a control cell (+), whereas KT-bound CENP-E indicates a CENP-E null cell (−). A “+” or “−” indicates pooled data from a population of CENP-E-containing and CENP-E-depleted cells.

indistinguishable from that found in CENP-E-containing cells, whereas kinetochores of centrophilic chromosomes had spacings about half that of the aligned ones (n = 6 kinetochores from two cells) (Figure 5C). As expected, most aligned kinetochore pairs had lost Mad2, and all centrophilic chromosomes were Mad2 positive (n = 10 kinetochores from five cells). Unexpectedly, we found that some equatorially positioned kinetochore pairs (n = 4 kinetochores from two cells) exhibited Mad2 staining and reduced sister stretching (e.g., Figure 5B, yellow arrows). The kinetochore spacings for centrophilic and Mad2-positive chromosomes were similar to those for kinetochores under no tension (as measured in the absence of microtubules induced by colcemid, n = 26 kinetochores from two cells).

The presence of Mad2 at centrophilic, as well as at some equatorial, chromatid pairs and the absence of tension generated across sister kinetochores of these
Table 1. Results of Crossing CENP-E Disruption Heterozygotes

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<th>Wild-Type</th>
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<td>Observed</td>
<td>22 (25%)</td>
<td>32 (46%)</td>
<td>17 (24%)</td>
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chromosomes provide additional measures for the loss of microtubule occupancy and stability at these CENP-E-depleted kinetochores.

Abnormal Development and Mitoses in Early CENP-E Null Embryos

To test whether homozygous disruption of CENP-E would affect mouse development and viability, we intercrossed mice heterozygous for the disruption and analyzed the genotypes of the resulting offspring. Of the first 150 mice genotyped, there were no mice homozygous for the CENP-E disruption (Table 1). Recovery of embryos at 7.5 or 8.5 days revealed that homozygous CENP-E disruptants were also absent at these ages, indicating that embryos lacking a functional CENP-E gene are unable to implant and/or develop past implantation.

A culture of CENP-E null embryos was performed at even earlier ages to examine why these embryos were dying in utero. Embryos from CENP-E heterozygote intercrosses were collected at 3.5 days of age, just prior to the normal time of implantation. Nested PCR was performed to genotype the embryos (see Experimental Procedures). This revealed that, from the 71 embryos collected at embryonic day 3.5, 24% were homozygous for the CENP-E disruption (Table 1), a number consistent with that expected from a heterozygous intercross.

These embryos were then cultured in vitro in glass-bottomed chamber slides for 2–4 days (Glasser et al., 1981). To identify mitotic cells with confidence, we fixed embryos at varying times of culture and used indirect immunofluorescence with tubulin antibodies to detect spindle assembly and with an antibody specific for phosphorylated histone H3, a mitotic-specific chromatin modification (Gurley et al., 1978). Images were collected by deconvolution light microscopy, and the genotype of each embryo was determined retrospectively by solubilizing it from the slide, transferring it to a lysis buffer, and performing nested PCR with primers whose products identified the wild-type or disrupted CENP-E alleles. Normal embryos were found to hatch from the zona pellucida and efficiently attach to the culture chamber (100%, n = 28), a behavior that is analogous to in vivo implantation. The trophoblast cells of the embryo spread onto the chamber slide, and the inner cell mass maintains a ball-shaped structure atop the trophoblast cell layer.

In wild-type embryos and those homozygous for the CENP-E disruption, all stages of mitosis were represented, including prophase, prometaphase, metaphase, and anaphase (Figure 6A, yellow, blue, white, and green arrows, respectively). The cells of the inner cell mass continued to divide for at least 5 days in culture. CENP-E null embryos, on the other hand, were much less successful in attaching to the chamber slide and much less robust: only 12% survived in vitro, compared with 97% for wild-type or CENP-E heterozygotes. While these gross abnormalities precluded assessment of intracellular defects in the most abnormal null embryos, the least-affected CENP-E depleted embryos (the ones that did attach to the culture chambers) exhibited obviously retarded growth of the inner cell mass, with less than one-fifth as many cells as wild-type embryos (compare right panels of Figures 6A and 6B). Growth in some embryos was so severely abnormal that the inner cell mass detached from the trophoblast layer.

The CENP-E null embryos that were sufficiently healthy to remain attached to the culture chamber throughout fixation and staining contained multiple abnormal mitoses (Figure 6B). These included chromosomes displaced from an otherwise normal metaphase plate (Figure 6B, orange arrows in inset). By projecting a 3D reconstruction of this spindle in an appropriate orientation, we saw that two misaligned chromosomes were closely associated with one spindle pole (whose position can be inferred from the convergence of the spindle microtubules). Similar figures were never seen (~100 mitoses examined) in normal embryos. A more aberrant mitotic cell in this embryo (Figure 6B, white arrows in inset) also had chromosomes clustered around each pole, as well as an imperfectly aligned metaphase. (Images of two other mitotic cells in this embryo were consistent with prophase (yellow arrow) or early prometaphase (blue arrow), although they could also represent even more-extreme phenotypes of failure of chromosome attachment.) Nested PCR of DNA from this embryo demonstrated that it was indeed a CENP-E null embryo (Figure 6C).

The high frequency of aberrant mitoses as prominent
Figure 6. Culture of CENP-E Null Embryos Reveals Growth Defects and Chromosome Misalignment

(A and B) Innercell mass growth in culture for a wild-type embryo (A) and a CENP-E null embryo (B). Embryos were fixed, and mitotic cells were identified by indirect immunofluorescence with an antibody to phosphorylated histone H3 (left), tubulin (middle), and DAPI to identify DNA (right). Colored arrows in (A) point to cells in prophase (yellow), prometaphase (blue), metaphase (white), or anaphase (green). The insets in (B) are higher magnifications of the marked areas. The white arrows in the inset point to chromosomes located near spindle poles in a prometaphase-like cell; the orange arrows point to two chromosomes located at one spindle pole in a cell otherwise at metaphase; the blue arrow points to cells in prometaphase or with many misaligned chromosomes.

(C) Nested PCR analysis to genotype embryos. Lane 1, the wild-type embryo (950 bp product); lane 2, a CENP-E null embryo (600 bp product).

Chromosome Instability during Liver Regeneration without CENP-E

To examine the contribution of CENP-E to regeneration of a mature, fully developed tissue, we chose to study liver regeneration in mice after Cre-mediated disruption of the conditional CENP-E gene. Adenovirus administered through tail vein injection is readily absorbed by the liver; thus, the Cre recombinase can be effectively delivered to this tissue. Additionally, liver cells readily proliferate in response to toxic injury, a feature that provides a source of mitotic cells in a mature tissue. Ad-Cre was administered to adult mice by tail vein injection, and, 5–11 days later, the toxin carbon tetrachloride was
injected to induce liver damage (see Figures 7A and 7B). Because of differences in oxygen concentration and expression of cytochrome p450 isozymes, carbon tetrachloride-induced damage preferentially occurs in the hepatocytes surrounding the central vein (Figure 7B, marked “CV”) (Lindros et al., 1990; Morrison et al., 1965). In contrast, hepatocytes in the region surrounding the portal tract (PT) respond to the toxic injury by dividing and replacing the damaged hepatocytes (Figure 7B) (Morrison et al., 1965). This induces a wave of DNA synthesis that peaks between 40 and 44 hr after damage (Melvin, 1968); this is followed by one or two divisions of each active hepatocyte (reviewed in Fausto, 2000). Regeneration is normally completed within 7 days of the toxic injury.

Livers were examined at 3 days, during the regeneration phase, or at 7 days, when regeneration was nearing completion, in wild-type animals. We analyzed genomic DNA extracted from liver tissue by quantitative PCR to determine the efficiency of loxP-CENP-E excision by the Cre recombinase. Earlier work has shown an efficiency of Cre-mediated excision of loxP-flanked regions in liver as high as 99% (Wang et al., 1996); in our hands, excision ranged from 34%–95% (see Experimental Procedures for quantitation protocol), and, in eight animals, it averaged approximately 70%. The extent and localization of carbon tetrachloride-induced damage was assessed in hematoxylin- and eosin-stained sections of formalin-fixed, paraffin-embedded livers from both CENP-E-containing and CENP-E-deficient livers. Three days after injection, both wild-type and CENP-E-deficient livers exhibited tissue damage characteristic of that induced by carbon tetrachloride. The area around the central vein was necrotic, whereas the portal tract region displayed normal histology (Figure 7C). When livers were examined 7 days after carbon tetrachloride injection, liver regeneration and recovery were apparent (Figure 7C), with fewer mitoses present than at earlier times after injury.

To investigate CENP-E function in regeneration, we counted mitotic figures and examined them for abnormalities. CENP-E-deficient cells were easily identified in the livers from which the CENP-E gene had been deleted (Figure 7D). Both CENP-E-positive and CENP-E-negative hepatocytes were found, sometimes adjacent to one another. Mitotic figures were scored for the presence of bipolar spindles with and without misaligned chromosomes (Figure 7E). The frequency of mitotic cells was indistinguishable in CENP-E gene-deleted and wild-type livers, both 3 and 7 days after carbon tetrachloride injection. However, more than 90% (100 fields of 7 mm² analyzed from four animals) of the CENP-E-depleted mitotic figures with bipolar spindles included misaligned chromosomes (examples in Figures 7D and 7E; quantified in Figure 7G). Some chromosomes were displaced from the metaphase plate (yellow arrows), some were clustered near a pole (blue arrows), and some were completely separated from the spindle (white arrows). This condition was characteristic of all four livers with ~70% efficiency of CENP-E gene deletion. The frequency of preanaphase chromosomal misalignments was four times that in wild-type livers, a comparison that was highly statistically significant (p = 0.03). Moreover, >50% of all mitotic figures from CENP-E-depleted livers had misaligned chromosomes, a number nine times that seen in wild-type cells (p = 0.02).

Moreover, 95% of the anaphases in the CENP-E-depleted livers had abnormal chromosome positioning (Figure 7H). This included lagging chromosomes (Figure 7F, green arrows) that were found in 27% of anaphases and polar chromosomes (Figure 7F, blue arrows; quantified in Figure 7I) in 73% of anaphases. Chromosomes that were not obviously spindle associated (Figure 7F, white arrows) were found in 56% of anaphases. Thus, stable attachment of spindle microtubules to kinetochores is strongly compromised during regeneration in the absence of CENP-E, but, at least for the one or two mitotic cycles needed for regeneration after a single toxic insult, this did not provoke sustained mitotic checkpoint activation, unlike what has been reported in immortalized cell cultures (Schaar et al., 1997; Yao et al., 2000; McEwen et al., 2001).

Despite these mitotic abnormalities, serum alanine aminotransferase levels, which were elevated in mice with a damaged or diseased liver, returned to normal at approximately normal rates in the CENP-E mice. The levels were elevated in serum of all mice 3 days after carbon tetrachloride injection and returned to the normal range in all genotypes of mice allowed to recover for 7 days. Albumin levels (measured only at 7 days) were within the normal range, consistent with functional recovery from liver damage. Moreover, hepatocyte differentiation appeared to proceed normally. Therefore, despite the chromosomal instability observed after CENP-E depletion and a single toxic injury, the liver appears to recover functionally.

Discussion

Our evidence demonstrates that CENP-E is required in vivo and in vitro to establish and maintain stable attachments between kinetochores and spindle microtubules and thus to prevent chromosome loss during anaphase (chromosomal instability). Evidence for this conclusion includes the following: (1) chronic centrophilic chromosomes in primary cells after CENP-E gene excision; (2) the absence of stable microtubule capture by either kinetochore of such centrophilic chromatid pairs; (3) 50% fewer microtubules bound to kinetochores of bioriented, aligned chromosomes; (4) despite apparently normal congression, a subset of the kinetochores that lack CENP-E is displaced from full metaphase alignment, with each of these sister kinetochores recruiting the same amount of Mad2 as is found on microtubule-free kinetochores; (5) other chromatid pairs appear fully aligned, yet have highly asymmetric levels of kinetochore Mad2, suggesting that, even after initial bipolar attachment and chromosome alignment, kinetochore attachment is unusually labile in the absence of CENP-E; (6) early developmental arrest in mouse embryos accompanied by aberrant mitoses with centrophilic chromosomes that do not make timely bioriented attachments; (7) a 9-fold increase in the number of mitoses with misaligned, centrophilic chromosomes that fail to efficiently achieve bioriented attachment and congression during regeneration of a fully differentiated tissue; (8) 95% of anaphases with aberrantly positioned
Figure 7. Mitotic Errors during Liver Regeneration in the Absence of CENP-E

(A) A schematic of experiment to follow CENP-E function in liver regeneration after injection of Ad-Cre and carbon tetrachloride in mice with one conditional and one disrupted CENP-E allele.

(B) A schematic of liver damage and regeneration. Blood enters the liver in the portal region and exits in the central region. Due to low oxygen levels and expression of the appropriate cytochrome p450 isozymes, damage from carbon tetrachloride occurs preferentially in the central region (light pink cells) and is followed by regeneration in the portal region (dark pink cells).

(C) Hemotoxylin- and eosin-stained sections of formalin-fixed, paraffin-embedded liver tissue. Livers from animals 3 days (left) or 7 days (right) after carbon tetrachloride exposure. At 3 days, damaged areas (encircled black areas) are near the central vein (CV). PV, portal vein. By 7 days, regeneration is nearly complete. Wild-type (top panels) and CENP-E-deficient (bottom panels) livers. Bar, 100 μm.

(D) Immunofluorescent detection of CENP-E in paraformaldehyde-fixed cryosections of liver tissue. All images were found in liver from an animal harboring the conditional CENP-E allele that received adenCre. The cells in the top two panels, one with and one without detectable CENP-E, are hepatocytes found adjacent to one another. CENP-E-negative cells (bottom two panels) display misaligned chromosomes (yellow arrows). Left images, CENP-E; right images, DAPI. Bar, 5 μm.

(E) Bipolar spindles in hemotoxylin- and eosin-stained livers. Left, normal bipolar spindle from CENP-E-containing liver; right, bipolar spindle with chromosome alignment defects from a CENP-E-disrupted liver. Yellow arrows, slightly misaligned chromosomes; blue arrows, chromosomes clustered near the pole; white arrows, chromosomes completely displaced from spindle; white asterisks, pole regions. All images are of livers 7 days after carbon tetrachloride-induced damage. Bar, 5 μm.

(F) Anaphase figures in hemotoxylin- and eosin-stained livers. Left, normal bipolar spindle from CENP-E-containing liver; right, anaphase
chromosomes, demonstrating lack of chronic checkpoint activation and generation of aneuploidy from chromosome missegregation. For this last point, given that CENP-E gene excision is incomplete, it is likely that the 5% normal anaphases arise from CENP-E-containing cells and, thus, that essentially all of the CENP-E-depleted anaphases are aberrant.

In all of these contexts, CENP-E-depleted kinetochores do interact transiently with spindle pole-nucleate microtubules: the centromere and chromosome arms of centrophilic chromosomes are juxtaposed to one pole in a fashion fully consistent with microtubule-mediated movement of these centrophilic chromosomes to, and retention near, that pole by kinetochore-associated, minus end-directed microtubule motor(s).

CENP-E thus appears to require for efficient conversion of these labile interactions into stable associations. Previous experiments have demonstrated that CENP-E antibodies, but not antibodies to cytoplasmic dynein, disrupt microtubule disassembly-driven, ATP-independent, chromosome movement along microtubules in vitro (Lombillo et al., 1995). These experiments offered the initial evidence that CENP-E is important for microtubule assembly/attachment to kinetochores, suggesting that CENP-E could mediate continued attachment of kinetochores to disassembling microtubule ends. Taken together with the evidence here that the absence of CENP-E leads to a failure of stable, efficient chromosome biorientation and fewer kinetochore-associated microtubules on those that are attached, we conclude that one essential role for CENP-E is in stable kinetochore-microtubule association and/or in conversion of initial attachments into stable ones.

CENP-E is essential for mammalian development, yet CENP-E-deficient primary cultured cells and hepatocytes can proceed through a few mitotic divisions. We attribute this apparent discrepancy to the likelihood that strict diploidy is essential for the complex events of early mammalian development. The observed lethality during early embryogenesis probably arises from the accumulation of missegregated chromosomes. It is known, however, that many mature fully differentiated hepatocytes are not diploid in wild-type mice and that some have chromosome numbers consistent with tetraploidy or octaploidy (Sargent et al., 1996). One might expect, however, that the multiple rounds of cell division that accomplish tissue regeneration would lead, in the absence of CENP-E, to higher degrees of aneuploidy. This is now being tested, along with its corollary that such genetic instability might uncover recessive alleles that alter growth control.

Despite the presence of aberrant mitotic figures after CENP-E disruption both in vitro and in vivo, we find no in vivo evidence for this condition provoking a significant long-term mitotic arrest. Although mouse cells recruit high levels of Mad2 to some misaligned chromosomes (Figures 5A and 5B), thus apparently initiating a “wait anaphase” signal, there is no apparent elevation in the mitotic index of CENP-E-null embryos or regenerating hepatocytes, as one might expect if the mitotic checkpoint were chronically activated. Likewise, liver regeneration is not delayed after efficient removal of CENP-E. Indeed, the striking increase in anaphase figures with polar and/or lagging chromosomes demonstrates that hepatocytes lacking CENP-E are not able to sustain mitotic arrest, despite the presence of unattached/unstably attached kinetochores, consistent with earlier biochemical evidence in vitro using Xenopus extracts (Abrieu et al., 2000). As yet unexplored is how immortalized HeLa cells have acquired the ability to override a CENP-E requirement for sustained checkpoint signaling after suppression of CENP-E function with antibodies or antisense oligonucleotides (McEwen et al., 2001; Yao et al., 2000). One possibility is that CENP-E may be necessary for the maximal strength of mitotic checkpoint signal per kinetochore. Only with CENP-E present can one or a few unattached kinetochores generate a sufficiently robust checkpoint signal for a long-term block of advance to anaphase. This model is especially appealing because CENP-E has been identified as a binding partner for the kinetochore-associated checkpoint kinase BubR1 (Chan et al., 1998; Yao et al., 2000). If BubR1 function were modulated by binding to CENP-E, deletion of one or both CENP-E alleles from individual adult mouse cell types should provoke higher rates of aneuploidy during the multiple regenerative events that occur during the lifetime of an animal. With the further discovery that mice heterozygous for the checkpoint component Mad2 develop late onset lung tumors (presumably through a weakened mitotic checkpoint) (Michel et al., 2000), testing for whether loss of CENP-E may also exacerbate tumor initiation or progression (as well as aneuploidy) will now be of central interest in identifying how partial disruption of such components may promote tumorigenesis.

Experimental Procedures

Library Screening and Targeting Vector Construction

A female 129Sv mouse kidney genomic DNA library was screened with a mouse EST AA008158 (Research Genetics). One of four clones found was restriction mapped and sequenced. One cDNA clone from the EST database (I.M.A.G.E. #1108008; Research Genetics) and one cDNA clone found in a screen of a mouse embryonic cDNA library were also sequenced. Exon P was amplified by PCR with a 5’ oligonucleotide, which added a terminal BamHI site, and a 3’ oligonucleotide, which added a terminal BglII site. The PCR product, after restriction endonuclease digestion with BamHI and BglII, was inserted into the BamHI site of the pfx vector (a gift from Jamey Marth, University of California, San Diego). A 6.8 kb HindIII-Xhol piece of the genomic clone 3’ to exon P was inserted between the HindIII and Xhol sites of pfxo. A 1.8 kb HindIII-ClaI piece of the genomic clone 5’ to exon P was shuttled through pBluescript (Stratagene) to add required restriction sites and was subsequently inserted between the SalI and XbaI sites in the pfx vector.
Unstable Microtubule Capture without CENP-E

ES Cell Culture and Transfection
RI embryonic stem cells (Nagy et al., 1993; a gift from Andras Nagy, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto) from a 129Sv strain were cultured in DMEM (Gibco) supplemented with 15% FBS (HyClone), 50 $\mu$g/ml pen/strep (Gibco), 100 mM glucose (Gibco), 1 mM sodium pyruvate (Gibco), nonessential amino acids (Gibco), 0.1 mM $\beta$-mercaptoethanol (Cell and Molecular Technologies), and 1000 units/ml murine leukemia inhibitory factor (Chemicon). Except when grown for DNA, ES cells were always grown on a layer of neomycin-resistant mouse embryonic fibroblasts (supplied by the Burnham Institute Transgenic Core Facility), mitotically inactivated by treatment with 100 $\mu$g/ml Mitomycin-C (Roche) for 2 hr. When approaching confluence, cells were passages with 0.05% trypsin (Difco) and replated at 1:5 for standard expansions or 1:2 for expansion of selection-resistant clones. ES cells were frozen in 25% FBS (HyClone) with 10% DMSO (Sigma). Transfections were done using a Bio-Rad Gene Pulser set at 240 V and 500 $\mu$F. For positive selection after transfection with 40 $\mu$g of the linearized targeting vector, G418 sulfate (Gibco) was used at 175 $\mu$g/ml. For negative selection after transfection with pIC-Cre (Sauer and Henderson, 1998; Stemberg and Hamilton, 1991) or pMC-Cre (Chui et al., 1997), gancyclovir was used at 2 $\mu$g/ml.

Mouse Embryonic Fibroblast Preparation and Culture
MEFs were cultured in DMEM (Gibco) containing 10% fetal bovine serum, 100 U/ml penicillin/streptomycin (Gibco), and 1 mM sodium pyruvate (Gibco). Mouse embryonic fibroblasts were prepared according to a protocol supplied by the ES Core Facility at Washington University (St. Louis, MO). Briefly, a pregnant female was sacrificed, the embryos were removed from the uterus and amniotic sac, the head, liver, and tail were removed, and the embryo was transferred to a dish containing 0.05% trypsin. The embryo was dissected into small pieces, and, after 15 min at 37°C, the trypsin and digested embryo were transferred to a conical tube containing MEM media. After allowing the large tissue pieces to settle for 2 min, the supernatant was transferred to a new tube and spun at 1000 rpm in a clinical centrifuge, and the pellet was resuspended in 5 ml of media and plated on a 6 cm dish. Genomic DNA was prepared from embryonic tails and analyzed by PCR as described for mouse tail DNA. For DNA blotting, ES cell DNA was digested with BamHI and run overnight on a 1% agarose gel to Hybond N+ membranes (Amer sham) using standard methods. Membranes were hybridized with $^{32}$P-dATP-labeled DNA fragments and exposed to X-ray film. Mouse cells were genotyped by PCR with 0.2 $\mu$L each of primers MCE10 (5'-CCGCGCTACAGGCTCACTA-3') and MCE exon Bgl (5'-GATCTC CAGTGAAGAGATTG-3'). For DNA blotting, ES cell DNA was digested with BamHI and run overnight on a 1% agarose gel to Hybond N+ membranes (Amer sham) using standard methods. Membranes were hybridized with $^{32}$P-dATP-labeled DNA fragments and exposed to X-ray film. Mouse cells were genotyped by PCR with 0.2 $\mu$L each of primers MCE10 (5'-CCGCGCTACAGGCTCACTA-3') and MCE exon Bgl (5'-GATCTC CAGTGAAGAGATTG-3'). For DNA blotting, ES cell DNA was digested with BamHI and run overnight on a 1% agarose gel to Hybond N+ membranes (Amer sham) using standard methods. 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Electron Microscopy

pMEFs were grown either on 12 mm glass coverslips or on small chips (0.5 mm square) of Thermofax plastic (NaIgenc Nunc Interna-
tional). For thin serial sections, cells on glass coverslips were fixed as previously described (McDonald, 1984). Cells used for thick sec-
tional). For thin serial sections, cells on glass coverslips were fixed and embedded in EPOX-Araldite. Three hundred nanometer sections were col-
collected, stained as above, and imaged on a JEM-1000 high-voltage electron microscope operating at 750 kV. Procedures for image acquisition and dual-axis tomography were as described previously (Ladinsky et al., 1999; Mastronarde, 1997). The tomographic recon-
construction was modeled with Silicon Graphics computers running the IMOD software (Kremer et al., 1996).

CENP-E Disruption in Liver

A total of 1 x 10^6 purified adenovirus particles, encoding either Cen recombinase or β-galactosidase (β-gal) (kindly supplied by Frank J. Giordano, Yale University, New Haven, CT), were injected into the tail vein of adult male mice (2–3 months old). To induce liver damage, we intraperitoneally injected 1000 μl per 10 g body weight of a 20% solution of carbon tetrachloride (Sigma) dissolved in olive oil (Sigma) into mice. Mice were sacrificed, and livers were dissected for fixation and DNA preparation. For paraffin sectioning, livers were fixed in 10% formalin for at least 4 hr at 4°C, dehydrated in a graded series of ethanol, and embedded in paraffin wax. Embedding, sectioning, and hematoxylin/eosin staining was performed by San Diego Pathol-
ogical. For frozen sectioning, livers were incubated in a solution contain-
ing 2% paraformaldehyde, 0.01 M sodium-periodate, and 0.075 M L-lysine-HCl in 0.037 M phosphate buffer (pH 7.4) for 4 hr at 4°C and then subjected to successive 4–8 hr incubations in PBS containing 10%, 15%, and 20% sucrose and embedding in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA). Ten microm-
eter sections were cut on a Jung 2800 Frigocut-N cryostat (Leica Microsystems, Bannockburn, IL). Immunofluorescent detection of CENP-E in frozen sections was performed as described for MEFS after permeabilization in PBS containing 0.5% Triton X-100.

Quantitative PCR

DNA from liver tissue was prepared by digestion in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, and 0.5% SDS with 0.4 μg/μl proteinase K (Roche) overnight at 55°C. DNA was extracted first with phenol:chloroform (1:1) and next with chloroform:isoamyl alco-
hol (24:1) and then precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate. DNA was resuspended in 10 mM Tris and 1 mM EDTA (pH 8.0). Primer express software (Applied Biosystems) was used to design forward (5’-TGGACAAACTGGAT CAGGAAAAG-3’) and reverse (5’-TGAATTGGCGCTTGTAAACTC), and fluorescein dye-tagged (5’-FAM-CAACACAGATGATGGGTCT GAAAGTTGTG[BHQ1-FAM]-3’) oligonucleotides (Operon) for use in real-time PCR. The loss of the conditional CENP-E allele was measured with 0.3 μM each of the forward and reverse primers, 0.25 μM of the fluorescein dye-tagged oligonucleotide, and TaqMan Universal PCR Master Mix (Roche). Reaction conditions were: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The degree of excision was calculated by comparison of CENP-E-intact DNA relative to an unexcised gene, in this case, c-jun.

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