DNA Sequence-Specific Binding of CENP-B Enhances the Fidelity of Human Centromere Function

Graphical Abstract

Highlights
- CENP-B binding to alphoid DNA repeats stabilizes CENP-C and kinetochore nucleation
- Centromere function is enhanced by mutual dependencies of CENP-A, CENP-B, and CENP-C
- The CENP-B free Y and neocentromere chromosomes mis-segregate at elevated frequencies
- CENP-B binding to alphoid DNA enhances fidelity of epigenetically defined centromeres

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In Brief
Fachinetti et al. uncover the functional importance of CENP-B, the only human centromere protein known to bind in a DNA sequence-dependent manner. The authors show that by directly enhancing CENP-C recruitment and CENP-C-dependent nucleation of kinetochore assembly, CENP-B increases the fidelity of epigenetically defined human centromere function.
DNA Sequence-Specific Binding of CENP-B Enhances the Fidelity of Human Centromere Function

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http://dx.doi.org/10.1016/j.devcel.2015.03.020

SUMMARY

Human centromeres are specified by a stably inherited epigenetic mark that maintains centromere position and function through a two-step mechanism relying on self-templating centromeric chromatin assembled with the histone H3 variant CENP-A, followed by CENP-A-dependent nucleation of kinetochore assembly. Nevertheless, natural human centromeres are positioned within specific megabase chromosomal regions containing α-satellite DNA repeats, which contain binding sites for the DNA sequence-specific binding protein CENP-B. We now demonstrate that CENP-B directly binds both CENP-A’s amino-terminal tail and CENP-C, a key nucleator of kinetochore assembly. DNA sequence-dependent binding of CENP-B within α-satellite repeats is required to stabilize optimal centromeric levels of CENP-C. Chromosomes bearing centromeres without bound CENP-B, including the human Y chromosome, are shown to mis-segregate in cells at rates several-fold higher than chromosomes with CENP-B-containing centromeres. These data demonstrate a DNA sequence-specific enhancement by CENP-B of the fidelity of epigenetically defined human centromere function.

INTRODUCTION

The centromere is the fundamental unit for ensuring chromosomal inheritance. Correct centromere function is required for preventing errors in chromosome delivery that would lead to genomic instability and aneuploidy, both hallmarks of many human cancers. Although not conserved across species, centromere regions from fission yeast to man have arrays of repetitive sequences (Fukagawa and Earnshaw, 2014). Human centromeres carry extensive (1,500 to >30,000) copies of imperfectly repeated arrays of a 171 bp element, termed α-satellite DNA. In all but the centromere of the Y chromosome (Earnshaw et al., 1987, 1989; Miga et al., 2014), this array contains a 17-base pair (bp) consensus motif that is the binding site of CENP-B, the only known mammalian centromeric DNA sequence-specific binding protein (Verdaasdonk and Bloom, 2011).

Nevertheless, human centromere position is epigenetically specified (Ekwall, 2007; Karpen and Allshire, 1997). Among the strongest evidence for an epigenetically defined centromere was the discovery of neocentromeres in humans (Amor et al., 2004; du Sart et al., 1997; Ventura et al., 2004; Warburton, 2004) in which the initial functional centromere has moved from its previous location to a new site in formerly euchromatic DNA without α-satellite repeats or binding of CENP-B (Depinet et al., 1997; du Sart et al., 1997; Warburton et al., 1997). Often associated with chromosomal rearrangements and found in some types of cancer, each neocentromere is marked with chromatin stably assembled with CENP-A (Amor et al., 2004), the centromere-specific histone H3 variant (Earnshaw and Rothfield, 1985; Palmer et al., 1987).

CENP-A is essential for centromere identity (Black et al., 2007b). It marks and maintains centromere position (Black et al., 2004; Hori et al., 2013; Mendiburu et al., 2011) and recruits additional centromere and kinetochore components (Carroll et al., 2009, 2010; Foltz et al., 2006; Liu et al., 2006) to both normal and ectopic centromere locations (Barnhart et al., 2011; Guse et al., 2011; Hori et al., 2013; Mendiburu et al., 2011). Indeed, use of gene targeting in both human cells and fission yeast has demonstrated that CENP-A-containing chromatin is the primary epigenetic mark of centromere identity (Fachinetti et al., 2013).

Centromere identity and function is achieved through a two-step mechanism. First, new CENP-A assembly to centromeric chromatin is mediated by its CENP-A targeting domain (CATD) (Black et al., 2004, 2007a; Shelby et al., 1997) in conjunction with the CENP-A selective chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010) whose activity is tightly controlled across the cell cycle (Müller and Almouzni, 2014). In the second step, either the amino- or carboxy-terminal tail of CENP-A is required for nucleation of assembly of a kinetochore that mediates high fidelity chromosome segregation indefinitely (Fachinetti et al., 2013). Curiously, amino-terminal tail-dependent kinetochore nucleation requires the presence of CENP-B (Fachinetti et al., 2013). Starting from this suggestion of a CENP-B-dependent role in kinetochore function, we now use a combination of gene targeting and replacement in human and mouse cells, coupled with in vitro approaches, to identify a DNA sequence-dependent contribution to fidelity of human centromeric function that is mediated by CENP-B binding to centromeric α-satellite repeats.
CENP-A’s Amino-Terminal Tail Directly Binds the Alphoid DNA-Binding Protein CENP-B

To test the effect that complete loss of the CENP-A amino-terminal tail has on centromere-bound CENP-B and on overall cell viability, we stably expressed (by retroviral integration) a full-length CENP-A or a CENP-A variant lacking its amino-terminal tail (\(^{\text{NH}_{2}}\)-CENP-A) in human cells containing one disrupted endogenous CENP-A allele and one floxed allele (CENP-A \(^{\text{F}}\)) (Figure 1A). After Cre-recombinase mediated inactivation of the floxed allele and subsequent loss of endogenous CENP-A protein (Figures S1A and S1B), long-term cell viability was rescued by \(^{\text{NH}_{2}}\)-CENP-A (Figure 1B), albeit with a 4-fold increase in chromosome mis-segregation and micronuclei formation (scored by live cell imaging in cells stably expressing H2B-mRFP to label chromosomes) (Figures 1A and 1C). Furthermore, loss of the CENP-A amino-terminal tail was accompanied by reduced CENP-B binding at centromeres (Figures 1A and 1D), as measured by quantifying centromeric CENP-B intensity by immunofluorescence.

To determine if this CENP-A-dependent binding of CENP-B at centromeres could result from a direct interaction, recombinant CENP-B was incubated with GST or GST-tagged CENP-A fragments and GST-containing proteins were affinity purified on glutathione-immobilized beads (Figures 1E and 1F). CENP-B bound directly to the amino-terminal tail of CENP-A (GST–CENP-A\(^{1–44}\)) but not to GST alone or to a CENP-A mutant lacking its amino-terminal tail (GST–CENP-A\(^{1–44}\)) (Figure 1F). The first 29 amino acids of the CENP-A tail were sufficient for this interaction (Figure S1C), in agreement with the observation that the first 29 amino acids of CENP-A’s amino terminal tail stabilize CENP-B binding at centromeres (Fachinetti et al., 2013).

CENP-B Supports CENP-C Maintenance at Centromeres

Deletion of the CENP-A amino-terminal tail not only affected CENP-B binding, but also reduced by half centromere-bound CENP-C (Figure 1D), a major centromere component required for kinetochore assembly (Fukagawa et al., 1999). In vitro (Carroll et al., 2010; Guse et al., 2011) and in vivo (Fachinetti et al., 2013) findings have reported that the small (six amino acid) carboxy-terminal tail of CENP-A is one element for CENP-C recruitment to centromeres. Complete loss of the CENP-A carboxy-terminal tail did not, however, abolish centromeric CENP-C binding (Fachinetti et al., 2013), indicating the existence of another pathway for its recruitment. Because the CENP-A amino-terminal tail binds to CENP-B and its deletion reduced both CENP-B and CENP-C bound to centromeres (Figure 1), we tested if CENP-B was required for the maintenance of a fraction of centromeric CENP-C. Long-term dependency of centromere recruitment of CENP-C on CENP-B was tested by disrupting both CENP-B alleles in human diploid RPE-1 cells using a CRISPR/Cas9 nuclease (Figure 2A and Figure S2A). Complete loss of CENP-B (Figures 2B–2D) resulted in a 50% reduction of CENP-C at centromeres but not of its total cellular levels (Figures 2B and 2D), with only a slight decrease of centromeric CENP-A levels (Figure 2D), a reduction insufficient to explain the observed CENP-C reduction (CENP-A must be depleted >75% to produce 2-fold decrease of centromere-bound CENP-C [Fachinetti et al., 2013]).

To determine whether the CENP-B influence on the level of centromere-bound CENP-C in cells could be mediated by physical interaction between CENP-B and CENP-C, we tested direct binding of the proteins to each other in vitro (Figure 3A). After co-incubation of GST-CENP-C and His-CENP-B, either protein was affinity purified (Figure S2E), followed by immunoblot. GST pull-down for CENP-C co-purified a portion of CENP-B and vice versa (Figure 3B), indicative of direct binding and in agreement with a previous two-hybrid study (Suzuki et al., 2004).

CENP-B Is Not Required for Initial Loading of New CENP-C at Centromeres

We then tested if CENP-B was also responsible for CENP-C deposition at centromeres besides its role in CENP-C stabilization. To test the initial loading of CENP-C at centromeres and its dependency on CENP-B, a tetracycline-inducible CENP-C tagged with EYFP and AID was stably integrated at a specific chromosomal locus. After culturing in IAA to induce degradation of the accumulated tagged CENP-C assembled at centromeres, IAA was withdrawn and immunofluorescence was used to monitor CENP-C\(^{\text{AID–EYFP}}\) re-accumulation and its assembly at centromeres (Figures S3A and S3B). Interestingly, siRNA-mediated reduction in CENP-B by more than 90% (Figure S3C) did not significantly change the percentage of cells that efficiently loaded new CENP-C\(^{\text{AID–EYFP}}\) (Figure S3D), but did reduce the intensity of centromeric CENP-C\(^{\text{AID–EYFP}}\) (Figure S3E). This result indicates that CENP-B is not required for CENP-C deposition at centromeres, but only for its retention.
Figure 1. CENP-A Amino-Terminal Tail Interacts with CENP-B

(A) Schematic representing the different CENP-A rescue constructs amino terminally tagged with EYFP (enhanced yellow fluorescent protein), the CENP-A−/− cell line, and the experiments described in (B)-(D).

(B) Images of representative crystal violet-stained colonies from the colony formation assay in (A).

(C) Frequency of mitotic errors in the indicated cell lines. Bars represent the mean of >50 cells per condition. Error bars represent the SEM of three independent experiments.

(D) Bar graph showing centromere intensities of EYFP-rescue constructs, CENP-B and CENP-C for the indicated cell lines. Values represent the mean of six independent experiments (>30 cells per experiment, average of 30 centromeres per cell). Error bars represent the SEM. Unpaired t test: ***p < 0.0001.

(E) Schematic of the experiment performed in (F).

(F) GST pull down and subsequent immunoblot for GST-tagged CENP-A co-incubated with His-tagged CENP-B.

See also Figure S1.
Figure 2. CENP-B Is Required for Full CENP-C Maintenance at Centromeres

(A) Schematic of the experiments described in (B)–(D) with the use of CRISPR/Cas9-mediated gene deletion.

(B) Immunoblot for accumulated CENP-B and CENP-C after identification of cells with both CENP-B alleles disrupted by action of the CRISPR/Cas9; α-tubulin was used as a loading control.

(C) Representative immunofluorescence images of centromere-bound CENP-B following CRISPR-mediated disruption of both CENP-B alleles in RPE1 cells. ACA was used to mark centromere positions. Scale bar represents 5 μm.

(D) Bar graphs of centromere intensities for CENP-A (red), CENP-B (blue), and CENP-C (green) in the indicated cell lines quantified with specific antibodies as described in (A). Bars represent the mean of three independent experiments (>30 cells per experiment). Error bars represent the SEM. Unpaired *p < 0.006.

(E) Schematic of the experiments described in (F)–(H) with the use of TALEN-mediated gene targeting.

(F) CENP-C genotypes validated in the indicated cell lines using PCR to distinguish normal (+/+) and double tagged (EYFP/EYFP) alleles.

(G) Immunoblot for CENP-C to distinguish non-tagged, single, or double allele-tagged CENP-C with EYFP.

(H) Bar graphs represent CENP-C-EYFP centromere intensity in the indicated cell lines measured by live cell imaging with or without siRNA treatment of CENP-B. Bars represent the mean of three independent experiments (>30 cells per experiment, average of 30 centromeres for cell). Error bars represent the SEM. Unpaired t test: **p < 0.0001.

See also Figure S2.
CENP-B Acts to Enable Highest Fidelity of Chromosome Segregation

To determine the consequence of CENP-B loss on the fidelity of centromere function, we measured chromosome mis-segregation rates using live cell imaging (Figure 3C) in CENP-A−/− cells deleted in both endogenous CENP-A alleles and with centromere function rescued by stable expression of full-length CENP-A or a CENP-A variant (CENP-AH3-C) in which its CENP-C binding site (CENP-A's carboxy terminus) was replaced with the corresponding region of histone H3 (Figures 3C and 3E). In CENP-A−/− cells rescued with full-length CENP-A, reduction in CENP-B lowered centromeric CENP-C by 50% and produced a 2-fold increase in mitotic errors relative to the siRNA control (Figures 3C–3F). In CENP-A−/− cells rescued by CENP-AH3-C, almost all centromere-bound CENP-C was lost following siRNA-mediated depletion of CENP-B (Figures 3C–3E). This CENP-B-dependent loss of CENP-C at centromeres was accompanied by more than half of mitoses with misaligned chromosomes and micronuclei found within 60% of interphase cells (Figures 3C and 3F).

The KMN network (comprised of the Mis12, Knl1, and Ndc80 complexes) is essential for connection between the centromere and spindle microtubules (Hori and Fukagawa, 2012). Recognizing that CENP-C has been proposed to be required for kinetochore function through recruitment of the Mis12 complex (Przewloka et al., 2011; Screpanti et al., 2011), we measured the centromeric levels of Dns1 and Hec1, subunits of the Mis12 and Ndc80 complexes, respectively, on metaphase centromeres in cells with normal or reduced centromere-bound CENP-C (Figures S3F and S3G). Reduction of centromeric CENP-C in CENP-B−/− cells or in CENP-A−/− cells rescued by ΔH2CENP-A lead to correspondingly reduced levels of Dns1 and slightly reduced Hec1, similar to what was observed in CENP-A−/− cells rescued by CENP-AH3-C (Figures S3H and S3I) (Fachinetti et al., 2013). Thus, diminution of centromeric CENP-C drives chromosome mis-segregation at least in part from reduced recruitment of the Mis12 complex.

We next tested if a role of CENP-B in supporting centromere function via maintenance of centromeric CENP-C in human cells was conserved in an additional mammalian species. We tested if chromosome mis-segregation rates and centromeric CENP-C loading were affected in immortalized mouse embryonic fibroblasts (MEFs) in which both CENP-B alleles had been inactivated...
Figure 4. CENP-B Is Required for Faithful Chromosome Segregation in Mouse Cells by Supporting CENP-C Association with Centromeres

(A) Quantifications of CENP-A and CENP-C protein levels at centromeres in MEFs with or without CENP-B. Bars represent the mean of three independent experiments (>30 cells per experiment). Error bars represent the SEM. Unpaired t test: *p < 0.04.

(B) Schematic of the experimental design to test for frequency of micronuclei formation in CENP-B+/+ or CENP-B−/− MEFs before and after recovery from nocodazole-induced mitotic arrest or by live cell imaging.

(C) CENP-B deletion increases chances of chromosome mis-segregation leading to micronuclei formation. Representative images of nuclei in CENP-B-containing and CENP-B-depleted MEFs quantified in (D). Scale bar represents 5 μm. An arrow marks a micronucleus in the CENP-B−/− cell.

(D) Quantification of micronuclei frequency in CENP-B+/+ and CENP-B−/− MEFs measured as in (B). Bars represent the mean of >100 cells per condition. Error bars represent the SEM of three independent experiments. Unpaired t test: *p < 0.04.

(E) Bar graphs quantifying lagging chromosomes in mitosis or micronuclei in interphase in the indicated cell lines. Bars represent the mean of >50 cells per condition scored by live cell imaging. Error bars represent the SEM of three independent experiments. Unpaired t test: *p < 0.04.

(Kapoor et al., 1998; Okada et al., 2007). As in the human RPE-1 or DLD-1 cells following CENP-B reduction, CENP-B null MEFs displayed a 50% reduction in centromeric CENP-C, relative to wild-type MEFs, despite unchanged levels of CENP-A (Figure 4A). This was accompanied by an increased mitotic error frequency (measured by imaging of fixed cells or by live imaging of cells expressing H2B-mRFP), with accumulation of micronuclei at more than twice the rate seen in CENP-B wild-type cells (Figures 4B–4E). Using release from nocodazole arrest to enrich for mitotic errors (Thompson and Compton, 2011), higher error frequencies were again observed in CENP-B-deleted MEFs relative to wild-type MEFs (Figures 4B–4D).

Elevated Mis-segregation of a Neocentromere without Bound CENP-B

We then tested whether it was the presence of CENP-B per se or the sequence-dependent CENP-B binding at centromeres that was required to reinforce both CENP-C binding at centromeres and fidelity of chromosome segregation. To address this point, we examined a patient-derived cell line (PD-NC4) containing an alphoid DNA-free neocentromere on chromosome 4 (hereafter Neo4) (Amor et al., 2004) (Figure 5A). The intensities of CENP-A/B/C were measured by immunofluorescence on chromosome spreads. The neocentromere was identified by scoring for a chromosome in which CENP-A (at the Neo4 centromere) and CENP-B (at the inactive, original chromosome 4 centromere DNA locus containing CENP-B boxes in its alphoid DNA repeats) did not colocalize (Figure 5A). CENP-A level at the Neo4 centromere was nearly normal (83% of the average level at the other centromeres; Figure 5B, top), in agreement with previous reports (Bassett et al., 2010; Bodor et al., 2014). In contrast, CENP-C intensity at the CENP-B-free Neo4 was half of that of CENP-B-containing centromeres (49% reduction; Figure 5B, bottom). Also, in agreement with a role of CENP-A in stabilizing CENP-B via its amino tail, CENP-B level at the inactive original centromere on the neocentromere-containing chromosome 4 was also 57% compared to other centromeres (Figure 5B, middle).

We next tested the influence of the presence or absence of cellular CENP-B on CENP-C binding at the Neo4 centromere and the fidelity of segregation of the Neo4 chromosome. To do this, we measured the frequency of Neo4 incorporation into micronuclei by scoring for (1) centromeric CENP-A (or CENP-C) without CENP-B (representing the functional Neo4 centromere) and (2) an additional CENP-B spot (representing the inactive original centromere 4). In cells with normal CENP-B levels, Neo4 was found to be encapsulated in a micronucleus with a 3-fold higher frequency than other chromosomes. Neo4 was present in 6% of observed micronuclei, a rate three times higher than the 2.1% level expected for mis-segregation by chance, assuming that each chromosome has an equal chance to mis-segregate (Figures 5C–5E).

Because Neo4 centromere function relies only on CENP-A, but not on CENP-B, for CENP-C recruitment and consequently kinetochore nucleation, we then tested whether reduction of CENP-A preferentially increased mis-segregation of the Neo4 chromosome. We decreased centromere-assembled CENP-A levels (by simultaneous siRNA-mediated reduction of CENP-A and its chaperone HJURP—Figures 5C and 5F). Remarkably, this CENP-A reduction selectively and significantly enhanced mis-segregation frequency of the Neo4 containing chromosome, yielding a 5- to 6-fold (by release from nocodazole arrest) increased error frequency compared to all other chromosomes (Figure 5E).
Elevated Mis-segregation of the CENP-B-free, Alphoid DNA-Containing Y Chromosome

The Y chromosome is known to have repetitive alphoid DNA sequences but lacks functional CENP-B boxes (Earnshaw et al., 1987, 1989; Miga et al., 2014). To determine whether it is binding of CENP-B at each centromere rather than the presence of repetitive alphoid sequences that acts to maintain CENP-C level at centromeres and thereby support fidelity of chromosome segregation, we measured the level of centromeric CENP-C on mitotic chromosomes from a male human cell line (DLD-1) (Figures 6A and 6B). CENP-A level on the Y was only slightly reduced relative to other centromeres (82% of the average level on all other centromeres; Figure 6C, left), in agreement with a previous report (Bodor et al., 2014). However, as was observed for the Neo4 centromere, the intensity of centromeric CENP-C on the CENP-B-free Y centromere (Figures 6B and 6C, middle) was only about half (60%) of that of the other chromosomes (Figures 6B and 6C, right).

We next measured the rate of chromosome mis-segregation of the Y chromosome compared to the X chromosome or chromosome 4, the latter two both having alphoid DNA repeats with functional CENP-B boxes. To do this, we used dual fluorescence in situ hybridization (FISH) with centromere-specific probes to concomitantly depict two chromosomes. We then counted the number of micronuclei containing chromosome 4, X or Y relative to the total number of micronuclei (Figures 6A, 6D, and 6E). Remarkably, the CENP-B-free Y chromosome accumulated into micronuclei with 4-fold higher frequency (8.3%) compared to what would be expected from random mis-segregation of haploid chromosomes into micronuclei (2.1%) or relative to the measured X or 4 mis-segregation rate (2.8% and 4.8%, respectively). In agreement with the dependency on CENP-A (but not CENP-B) for chromosome segregation observed for the Neo4 neocentromere-containing chromosome (Figure 5E), siRNA-mediated reduction of CENP-A by as little as four-fold (Figure 6F) selectively increased the frequency of Y-positive micronuclei to...
18%, while only slightly increasing mis-segregation of the chromosome X or 4 (Figure 6E). In agreement with what was observed earlier (Figures 3 and 4), an influence of CENP-B on fidelity of chromosome segregation was seen by more than a 2-fold elevation in the overall rate of micronuclei formation (Figure 6G) following CENP-B depletion by siRNA (Figure 6F). Despite this, reduction in CENP-B did not significantly affect the rate of mis-segregation of the CENP-B-free Y chromosome (Figure 6E).

CENP-B Contributes to Cell-Cycle-Dependent CENP-A Loading at Centromeres

With our evidence that CENP-B together with CENP-A is required to maintain a normal CENP-C level at each centromere, we tested whether there is a contribution to CENP-A deposition provided by CENP-B. For this test (see schematic in Figure 7A), we took advantage of the SNAP-tag technique (Jansen et al., 2007) which permits in vivo covalent marking of proteins linked to a 20 kD suicide enzyme (named SNAP). siRNA was used to deplete CENP-B (Figure S4A) from CENP-A−/− cells in which centromere function was rescued with a stably integrated full-length, SNAP-tagged CENP-A. CENP-A loading at centromeric chromatid was only slightly affected by loss of CENP-B (Figures 7A and 7C and Figures S4A and S4B). As expected, HJURP depletion, on the other hand, eliminated new CENP-A deposition (Figure 7C and Figures S4A and S4B) (Dunleavy et al., 2009; Foltz et al., 2009). Remarkably, CENP-A−/− cells rescued with a histone H3 variant carrying the CATD and amino-terminal tail of CENP-A, but not its carboxy-terminal tail (SNAP, NH₄H₃CATD) showed a marked reduction in new NH₄H₃CATD deposition following CENP-B depletion (Figures 7A–7C and Figure S4B). Accordingly, total centromere-bound CENP-A levels decreased in cells supported by a variant that lacks the CENP-A/CENP-C interaction domain, but not in cells rescued with full-length CENP-A (Figure S4C).

CENP-B depletion also reduced M18BP1 (a subunit of the Mis18 complex) binding at centromeres assembled with a CENP-C binding-deficient CENP-A variant (Figure S4D), in agreement with a previously reported CENP-C/Mis18 interaction (Dambacher et al., 2012; Moree et al., 2011) and consistent with the CENP-B-dependent portion of CENP-A loading at centromeres mediated in part through the Mis18 complex.

DISCUSSION

CENP-A-containing chromatid has previously been demonstrated to be the epigenetic mark that is sufficient for long-term maintenance of human centromere positioning and identity and for nucleation of a functional kinetochore (Fachinetti et al., 2013). Adding to this, our current evidence has identified a parallel DNA sequence-specific role for direct stabilization of CENP-C and recruitment of CENP-A that is provided by CENP-B, the sole sequence-specific mammalian centromeric DNA binding protein identified so far (Muro et al., 1992; Stimpson and Sullivan, 2011). This discovery offers an explanation for the existence and retention of CENP-B boxes and the high degree of CENP-B conservation in divergent mammals (Sullivan and Glass, 1991).

An initial suggestion of a functional role at centromeres for CENP-B emerged from Masumoto and colleagues’ demonstration that CENP-B strongly enhanced de novo centromere formation in artificial chromosomes (Ikeno et al., 1998; Ohzeki et al., 2002; Okada et al., 2007; Okamoto et al., 2007), possibly by altering modifications to centromere-associated histone H3 (Okada et al., 2007), thereby affecting HJURP and CENP-A recognition/deposition (Bergmann et al., 2011). To these earlier proposals, our effort has established that repetitive alphoid centromeric DNA sequences to which CENP-B binds do provide a contribution for binding of centromeric CENP-A and CENP-C on native centromeres. Indeed, we have now shown that a CENP-A variant without its CENP-C binding domain (AH3CATD), which fully supports long-term centromere function despite inability to directly recruit CENP-C (Fachinetti et al., 2013), supports incorporation or stabilization of new CENP-A into centromeric chromatin in a CENP-B-dependent manner (Figure 7C). This CENP-B-dependent loading/stabilization of CENP-A occurs via the recruitment of CENP-C to centromeres in a parallel pathway to direct CENP-C recruitment by the CENP-A carboxy-terminal tail (Carroll et al., 2010; Guse et al., 2011; Fachinetti et al., 2013; Kato et al., 2013). Our findings on this mutual dependency of CENP-A and CENP-C are in agreement with the demonstration that targeting of CENP-C to an ectopic chromosomal site is sufficient for CENP-A recruitment following removal of the endogenous centromere on that chromosome (Hori et al., 2013) and with the CENP-C-dependent recruitment of the methyl-transferase DNMT3B (Gopalakrishnan et al., 2009; Kim et al., 2012) or the Mis18 complex (Dambacher et al., 2012; Hori et al., 2013; Moree et al., 2011), which in turn is recognized by the CENP-A chaperone HJURP (Barnhart et al., 2011).

It is important to note that this CENP-B-dependent stabilization of centromeric CENP-A and CENP-C requires sequence-dependent DNA binding, as it is found only on centromeres carrying CENP-B boxes and indeed is absent from the Y centromere and neocentromeres. In addition, our findings that CENP-B does not play a role in initial CENP-C recruitment (Figure S3), but rather stabilizes it once centromere bound, reinforces the importance of a crucial epigenetically defined contribution to centromere function. CENP-A-containing chromatid is essential for the mutual reinforcement of CENP-A/B/C binding at centromeres. Without it, the silenced centromere on the Neod chromosome cannot stabilize CENP-C despite a direct affinity for CENP-B bound to the CENP-B boxes in its α-satellite repeats.

The idea that CENP-B might play an important role in centromere function had previously been dismissed by demonstration that CENP-B null mice are viable (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998). In addition, the existence of human neocentromeres and the Y chromosome lacking CENP-B boxes had established that CENP-B is dispensable for human centromere function.

Nevertheless, we have shown here in both mouse and human cells that depletion of CENP-B yields a higher rate of chromosome mis-segregation for chromosomes with centromeres to which CENP-B had initially been bound (Figures 3 and 4). This observation offers at least a partial explanation for the reproductive and developmental dysfunctions reported in CENP-B null mice due to disruption in the normal morphogenesis of the highly mitotically active uterine epithelial tissue (Fowler et al., 2000; 2004). When combined with evidence that widespread somatic
Figure 6. The CENP-B-Free Alphoid DNA-Containing Y Chromosome Has Reduced Level of CENP-C and Increased Rate of Chromosome Mis-segregation

(A) Schematic of the experiments shown in (B)–(F).

(B) Representative image of Y chromosome in mitosis (yellow arrow) stained with CENP-C (green) and CENP-B (red) antibodies.

(C) Box-and-whisker plots of CENP-A, CENP-B, or CENP-C intensities at the centromere of the Y chromosome compared to all other centromeres measured on metaphase spreads (see Experimental Procedures for details). Error bars represent the SEM. Bar in the box represents the median; the whiskers represent points distribution. Unpaired t test: ***p < 0.0001.

(D) (Left) Representative immunofluorescence image of a FISH experiment on a metaphase spread using centromeric probes for the Y (red) and X (green) chromosomes. (Right) Representative immunofluorescence image of a FISH experiment on interphase cells using centromeric probes for the Y chromosome (red) or chromosome 4 (green). Yellow arrow indicates a nucleus in a cell that has undergone Y chromosome mis-segregation to accumulate two Y chromosomes. White arrow indicates a nucleus in which mis-segregation has led to loss of the Y chromosome.

(E) Chromosome mis-segregation rates of chromosomes 4, Y, and X determined by FISH. (Left) Representative immunofluorescence image of a micronucleus-containing cell after siRNA treatment to lower CENP-A levels. Scale bar represents 5 μm. (Right) Bars represent the frequency of micronuclei formation.

(F) Immunoblot for CENP-A and CENP-B after HJURP/CENP-A or CENP-B siRNA transfection, followed by Co-FISH of chr X and Y.

(G) Total number of micronuclei (%).

(expected frequency for a diploid chr. (4))

2 / 46 = 4.3%

(expected frequency for a haploid chr. (X, Y))

1 / 46 = 2.1%
aneuploidy is surprisingly well tolerated in mice (e.g., in mice with reduced levels of the centromeric motor protein CENP-E [Weaver et al., 2007], the mitotic checkpoint protein Mad2 [Michel et al., 2001], or mitotic checkpoint kinase Bub1 [Jeganathan et al., 2007]), we predict that mice deficient in CENP-B develop similar somatic aneuploidy. While this was not tested by any of the three groups that had produced CENP-B null mice (none of which have been maintained) (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998), we demonstrated that fibroblasts from those mice (Kapoor et al., 1998) are chromosomally unstable with a chronically elevated rate of mis-segregation (Figure 4).

We also demonstrated that two human chromosomes that lack DNA-containing CENP-B binding sites (the Y chromosome and the neocentromere) mis-segregate at higher frequency than do chromosomes with the alphoid DNA-containing, CENP-B-bound centromeres (Figures 5 and 6). (Note that an additional contributor for neocentromere mis-segregation may be inefficient Aurora B function, as previously proposed [Bassett et al., 2010]). The reduced segregation fidelity, which had previously been reported for the Y chromosome to be associated with shorter survival and high risk of blood cancer in an age- and smoke-dependent manner (Dumanski et al., 2015; Forsberg et al., 2014; Nath et al., 1995), correlates with reduced CENP-C binding, which we argue is the probable cause of reduced centromere function. Whereas our evidence has only tested CENP-B influence on mitotic chromosome segregation, if a similar contribution is also present in meiosis the selective pressure for maintaining males will act to maintain the Y chromosome despite increased mis-segregation frequency. Therefore, our findings strongly implicate CENP-B as a key contributor to centromere strength and the rate of faithful chromosome segregation through stabilization of binding of other centromere/kinetochore proteins.
Finally, our evidence for a direct contribution of CENP-B to CENP-C stabilization at centromeres together with the discovery of a direct interaction between CENP-A and CENP-B (via the CENP-A amino-terminal tail) demonstrates a redundant, mutual dependency among CENP-A, CENP-B, and CENP-C for centromere association and function. Unresolved is whether individual CENP-C dimers bind simultaneously to both CENP-A and CENP-B. Thus, whereas human centromeres are specified primarily by an epigenetic mechanism mediated through CENP-A-containing chromatin, we identified a sequence-dependent component to centromere function provided by the centromeric DNA binding protein CENP-B. We now propose that CENP-B does this by supporting a parallel pathway for CENP-A stabilization and kinetochore formation via CENP-C recruitment. This CENP-B-mediated enhancement in CENP-C binding at centromeres acts as a backup mechanism to ensure chromosome segregation when CENP-A levels are compromised (Figure 7D). This might explain the observation that centromere function is still partially retained even in cells with as little as 1% of the original CENP-A level (Fachinetti et al., 2013).

Although other natural metazoan centromeres had been identified to be without repetitive sequences including horses (Wade et al., 2009), chickens (Shang et al., 2010), and orangutans (Locke et al., 2011), the overall evidence implies that repetitive alphoid sequences can provide increased fidelity to human centromere function, consistent with the suggestion that the observed human neocentromeres are likely to be centromeres “in progress” (Marshall et al., 2008). Incorporation of repetitive sequences during evolution is therefore likely to have enhanced full centromere maturation and fixation, as proposed in primates (Ventura et al., 2007). Additionally, it is tantalizing to speculate that α-satellites and CENP-B play a role in the meiotic drive for egg specification (the preferential segregation of one chromosome over another) (Dawe and Henikoff, 2006; Henikoff et al., 2001), not only by supporting CENP-A binding (Chmátil et al., 2014; Marshall and Choo, 2012), but also by actively reinforcing centromere function.

**EXPERIMENTAL PROCEDURES**

**siRNA, SNAP-Tagging, Clonogenic Colony Assay, and Adeno-Cre Treatment**

siRNAs were introduced using Lipofectamine RNAiMax (Invitrogen). A pool of four siRNAs directed against CENP-C, CENP-B, HJURP, GAPDH (Fachinetti et al., 2013), and CENP-A (GAGCACACACCCUCUGUAU, UUAUACUGAGG CCGAAGUA, AGAAGGGUUGCGCAAAGG, UAAUUCACUGCGUGUGUS) were purchased from Dharmacon. SNAP labeling was conducted as described previously (Jansen et al., 2007). Clonogenic colony assays and Adeno-Cre treatment were done as described (Fachinetti et al., 2013).

**CENP-C Gene Deletion by the CRISPR/Cas9 System**

An expression vector (Addgene plasmid 42239) expressing cas9 and sgRNA (Cong et al., 2013) was digested with BbsI and the linearized vector was gel purified. A pair of complementary oligos to the targeting site (5′ GAAGAA CAAGCGCGCCATCC 3′) were annealed and ligated into the vector. RPE-1 CENP-A<sup>−</sup> cells were co-transfected with the sgRNA expression vector and a GFP-expressing vector (to identify transfected cells) by nucleofection (Lonza) using program U-017 and nucleofector solution V (Lonza). Forty-eight hours after transfection, high GFP-expressing cells were single cell sorted into 96-well plates using a fluorescence in situ hybridization (FACS)/Aria II. Genomic DNA sequencing, immunostaining, and immunoblotting were used to identify cells with disruption of both targeted alleles.

**CENP-C Gene Targeting with TALENs**

TALENs were assembled using the Golden Gate cloning strategy and library as described previously (Cermak et al., 2011) and cloned into a modified version of pcDNA3.1 (Invitrogen) also containing the Fok I endonuclease domain as previously described (Miller et al., 2011). TALENs were designed to the C-terminal region of CENP-C gene: GAGGAAAGTGTCTTC and GGTGTAGCTTT CATC. DLD-1 cells were co-transfected with the TALEN expression vectors and the donor cassette (containing the two homology arms for CENP-C C-terminal region and the AID and EF1A gene) by nucleofection (Lonza) using program T-020, and positive clones were selected with FACS.

**Protein Purification**

GST tagged human CENP-A (1–29 or 1–44 amino acids) and 6xHis tagged CENP-B variants were expressed in Rosetta E. coli after induction with IPTG. GST-CENP-C variants were overexpressed in Sf9/High-Five insect cells using the Bac-to-Bac expression system (Invitrogen). Briefly, genes encoding GST tagged human CENP-C variants were cloned into pFastBac1. Bacmids were obtained from DH10Bac E. coli after transformation with the pFastBac1 containing CENP-C gene and used to transfect Sf9 cells (72 hr) to produce baculovirus. Hi-Five insect cells were infected by CENP-C baculovirus (1:10 dilution) for 48 hr, collected, and frozen until required. Cells were resuspended in PBS containing protease inhibitors and lysed by sonication. Soluble lysate was recovered after centrifugation and proteins were affinity purified over nickel-nitrilotriacetic acid sepharose beads (QiAGEN) or glutathione sepharose beads (GE Healthcare Life Sciences).

**In Vitro Binding Assay**

In vitro binding assays were conducted in 50 mM Tris-HCl (pH 7.7), 100 mM KCl, 0.1% Triton X-100, and 10% glycerol. For the GST pull-down assay, glutathione sepharose-bound GST-tagged protein and other recombinant proteins were combined and incubated at room temperature for 1 hr. Bound protein complexes were washed four times with 20 volumes of the binding buffer, eluted from the beads in 15 mM glutathione buffer. For the His pull-down assay, nickel sepharose-bound 6xHis-tagged protein and other recombinant proteins were combined and incubated in the presence of 20 mM imidazole at 4°C for 1 hr. Bound protein complexes were washed four times with 20 volumes of the binding buffer supplemented with 20 mM imidazole, and eluted from the beads in 200 mM imidazole buffer. Eluted proteins were analyzed by immunoblotting.

**FISH Experiment**

Cells were fixed in Carnoy’s Fixative (Meth-Acetic Acid 3:1) for 15 min at room temperature, rinsed in 80% ethanol, and air-dried for 5 min. Probe mixtures (MetaSystems) were applied and sealed with a coverslip. Slides were denatured at 75°C for 2 min and incubated at 37°C overnight in a humidified chamber. Slides were washed with 0.4×SSC at 72°C for 2 min, 2×SSC, 0.05% Tween-20 at room temperature for 1 min, and rinsed with ddH2O. Slides were incubated with DAPI solution for 10 min before mounting in anti-fade reagent.

For information regarding DNA constructs, cell culture conditions, generation of stable cell lines, the surveyor nuclease assay, antibodies and experimental conditions for immunoblotting and immunofluorescence, live-cell microscopy and procedures for centromere quantifications, see the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.03.020.

**AUTHOR CONTRIBUTIONS**

J.S.H. performed in vitro protein purification. D.F. and J.S.H. performed the binding assay. D.F. and M.A.M. performed gene targeting of CENP-B and CENP-C. D.F. and P.L. performed FISH experiments. D.F., A.A., and A.J.W. performed all the remaining experiments. D.F. and D.W.C. conceived the experimental design, analyzed the data, and wrote the manuscript.
ACKNOWLEDGMENTS

The authors thank William C. Earnshaw (Wellcome Trust Centre for Cell Biology, University of Edinburgh), P. Kaltiös (Murdoch Childrens Research Institute Victoria, Australia), A. Desai, R. Khalilulin, Neil Hattersley, and C. Bartocci (Ludwig, La Jolla, California), F. Dibazar, Y. Arbely, B. Vitre, and others members of the Cleveland lab for helpful suggestions. We thank P. Maddox (University of North Carolina), I. Cheeseman (MIT, Boston), A. Desai (Ludwig, La Jolla, California), P. Kaltiös (Murdoch Children’s Research Institute Victoria, Australia), H. Masumoto (Kazusa DNA Research Institute, Japan), and B.E. Black (University of Pennsylvania, Philadelphia) for providing reagents. We also thank the Neuroscience Microscopy Shared Facility (P30 NS047101, University of California, San Diego) and the FACS facility in the Sanford Consortium for Regenerative Medicine, La Jolla, California. This work was supported by a grant from the NIH (R01-GM 074150 to D.W.C.). D.W.C. receives salary support from the Ludwig Institute for Cancer Research.

Received: December 21, 2014
Revised: February 9, 2015
Accepted: March 25, 2015
Published: May 4, 2015

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