Misregulation of Scm3p/HJURP Causes Chromosome Instability in Saccharomyces cerevisiae and Human Cells

Prashant K. Mishra¹, Wei-Chun Au¹, John S. Choy¹, P. Henning Kuich², Richard E. Baker³, Daniel R. Foltz², Munira A. Basrai¹*

¹ Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, United States of America, ² Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia, United States of America, ³ Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America

Abstract

The kinetochore (centromeric DNA and associated proteins) is a key determinant for high fidelity chromosome transmission. Evolutionarily conserved Scm3p is an essential component of centromeric chromatin and is required for assembly and function of kinetochores in humans, fission yeast, and budding yeast. Overexpression of HJURP, the mammalian homolog of budding yeast Scm3p, has been observed in lung and breast cancers and is associated with poor prognosis; however, the physiological relevance of these observations is not well understood. We overexpressed SCM3 and HJURP in Saccharomyces cerevisiae and HUJURP in human cells and defined domains within Scm3p that mediate its chromosome loss phenotype. Our results showed that the overexpression of SCM3 (GALSCM3) or HJURP (GALHJURP) caused chromosome loss in a wild-type yeast strain, and overexpression of HJURP led to mitotic defects in human cells. GALSCM3 resulted in reduced viability in kinetochore mutants, premature separation of sister chromatids, and reduction in Cse4p and histone H4 at centromeres. Overexpression of CSE4 or histone H4 suppressed chromosome loss and restored levels of Cse4p at centromeres in GALSCM3 strains. Using mutant alleles of scm3, we identified a domain in the N-terminus of Scm3p that mediates its interaction with CEN DNA and determined that the chromosome loss phenotype of GALSCM3 is due to centromeric association of Scm3p devoid of Cse4p/H4. Furthermore, we determined that similar to other systems the centromeric association of Scm3p is cell cycle regulated. Our results show that altered stoichiometry of Scm3p/HJURP, Cse4p, and histone H4 lead to defects in chromosome segregation. We conclude that stringent regulation of HJURP and SCM3 expression are critical for genome stability.

Citation: Mishra PK, Au W-C, Choy JS, Kuich PH, Baker RE, et al. (2011) Misregulation of Scm3p/HJURP Causes Chromosome Instability in Saccharomyces cerevisiae and Human Cells. PLoS Genet 7(9): e1002303. doi:10.1371/journal.pgen.1002303

Editor: Beth A. Sullivan, Duke University, United States of America

Received March 17, 2011; Accepted July 29, 2011; Published September 29, 2011

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: Support for this research was provided by the Intramural Research Program of the National Cancer Institute, National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: basraim@mail.nih.gov

Introduction

Proper chromosome segregation is essential for normal cell proliferation. Errors in this process lead to birth defects, developmental disorders, aneuploidy and possibly cancer [1]. The kinetochore (centromeric DNA and associated proteins), microtubules, spindle pole bodies, condensins, and telomeres, as well as regulatory components that establish checkpoints [2] are essential for faithful chromosome segregation. The centromere (CEN) is the cis-acting DNA locus that specifies the site of kinetochore assembly, participates in the attachment of chromosomes to the mitotic and meiotic spindle and maintains cohesion between sister chromatids [3,4]. CEN DNA sequences are highly variable among eukaryotes. Budding yeast contains “point” centromeres, whereas other eukaryotes, for example fission yeast, fruit fly, plants and mammals have “regional” centromeres. The centromeres, whereas other eukaryotes, for example fission yeast, fruit fly, plants and mammals have “regional” centromeres. The point centromeres are small (~125 bp in size) and consist of three conserved DNA elements (CDEI, CDEII, and CDEIII), whereas regional centromeres are relatively large in size (40–4000 kb) and contain species-specific arrays of repeated DNA [5–7]. Despite CEN DNA sequence variation, replacement of histone H3 in centromeric chromatin by the centromere-specific histone H3 variant CenH3 is universally conserved [6]. CenH3 homologs (Cse4p in budding yeast, Cnp1 in fission yeast, CID in fruit fly, HTR12 in Arabidopsis and CENP-A in humans) function as an epigenetic mark in all organisms and is essential for determining centromere identity and proper kinetochore function [8–14]. In budding yeast, kinetochore sub-complexes Ctf3p (Ctf3p, Mcm16p and Mcm22p) and COMA (Ame1p, Ctf19p, Mcm21p and Osk1p) exhibit genetic and physical interactions with Cse4p [15,16].

It has been shown that the point centromeres of Saccharomyces cerevisiae consist of a single Cse4p nucleosome [17], and a novel inner kinetochore protein Scm3p (Suppressor of Chromosome Missegregation) is required for the centromeric deposition of Cse4p [18–23]. Scm3p is evolutionarily conserved with homologs identified in a range of species including fission yeast (Sp-Scm3p), fungi, humans (HJURP), Holliday Junction Recognition Protein, and other vertebrates [24,25]. HJURP and Scm3p share a common ancestry and both proteins have an evolutionarily conserved N-terminal domain [24]. This evolutionary conserved domain of HJURP directly interacts with the centromere targeting domain (CATD) of CENP-A [25–27]. In budding yeast, depletion of Scm3p leads to cell cycle arrest and chromosome segregation.
Misregulation of SCM3 Causes Chromosome Instability

Author Summary

Proper chromosome segregation is essential for normal cell proliferation. Segregation errors lead to aneuploidy, a direct cause of birth defects and a hallmark of cancer. The kinetochore (centromeric DNA and associated proteins) is one of the key determinants for faithful chromosome transmission. Misregulation of kinetochore proteins such as HJURP has been observed in various cancers, however the biological relevance of this observation is not well understood. We determined that altered dosage of HJURP and its budding yeast homolog SCM3 leads to defects in chromosome segregation in yeast and human cells. We identified the centromeric DNA–interacting domain of Scm3p and determined that association of Scm3p devoid of Cse4p leads to chromosome segregation defects. Our findings suggest that stringent regulation of Scm3p/HJURP, Cse4p, and histone H4 is critical for maintenance of genome stability.

Results

Misregulation of SCM3 leads to defects in chromosome transmission fidelity (ctf) in wild-type strains and reduced levels of Cse4p at CEN DNA

To examine the physiological consequence of misregulation of SCM3, we assayed the chromosome loss phenotype of wild-type strains overexpressing SCM3 from a GAL1 promoter on a multi-copy plasmid. Western blot analysis confirmed the galactose-induced overexpression of GALSCM3HA (Figure 1A). The loss of a non-essential reporter chromosome fragment (CF) in this strain background results in red sectors in an otherwise white colony. We determined the chromosome loss rate by counting the number of colonies that were at least half red on galactose media reflecting the loss of CF in the first cell division (Figure 1B, see arrows). Overexpression of SCA2 results in a 10-fold higher chromosome loss rate compared to strains expressing vector alone (Figure 1C). The chromosome loss phenotype of the control vector alone strain is similar to that previously reported for wild-type strains on galactose media [35]. Strains expressing untagged GALSCM3 or GALSCM3HA integrated into the genome had chromosome loss rates similar to that of plasmid-borne GALSCM3SHA strains (Figure S1). We previously reported that overexpression of histone H3 (GALH3) alters the stoichiometry between Cse4p and histone H3 leading to defective kinetochores and a chromosome loss phenotype in wild type yeast strains [35]. The chromosome loss phenotype of strains co-expressing GALSCM3 and GALH3 is higher but statistically different than those expressing GALSCM3 or GALH3 alone (Figure 1C).

Scm3p directly binds and forms a stoichiometric complex with Cse4p and histone H4, and is required for their assembly into centromeric chromatin [18–20]. Similarly, HJURP interacts stoichiometrically with CENP-A/H4 [26,27]. We reasoned that the chromosome loss phenotype of GALSCM3 strains may be due to imbalanced stoichiometry between Scm3p and Cse4p/H4. Hence, we determined the effect of overexpression of CSE4 in GALSCM3 strains. Our results showed that GALCSE4 suppressed the chromosome loss phenotype of GALSCM3 strains (Figure 1C). Previous studies have shown that overexpression of histone H4 (GALH4) suppresses certain cse4 alleles [40]. Consistent with these observations, we found that GALH4 (GALHHF1) suppressed the chromosome loss phenotype of GALSCM3 strains (Figure 1C). We performed western blot analysis to determine if overexpression of Scm3p affects the levels of Cse4p, Histone H3 or Histone H4. We found that GALSCM3 strains show slightly higher levels of Cse4p, whereas levels of histone H3 or H4 are not affected in these strains (Figure 1A). We also observed higher levels of Cse4p in strains co-expressing GALSCM3 with either GALH4 or GALCSE4 when compared to strains expressing GALSCM3 or GALH4 alone (Figure 1D). The increased levels of Cse4p in GALSCM3 strains are consistent with a recent report describing a role for Scm3p in protecting Cse4p from ubiquitin-mediated degradation [41]. The increased levels of Cse4p in GALH4 strains can be explained by previous observations for genetic interactions between Cse4p and histone H4 [40].
Misregulation of SCM3 Causes Chromosome Instability

Figure 1. Overexpression of SCM3 causes chromosome instability and reduction in CEN-bound Cse4p and histone H4. (A) Western blot analysis was done using whole cell protein extracts prepared from wild type strains (RC154 or JG595) transformed with GALSCM3HA (pMB1306), or vector (pRS426 GAL1). Strains were grown to logarithmic phase of growth in synthetic media and gene expression induced in the presence of galactose (2%) at 30°C for 12 hours. Blots were probed with anti-HA for expression of GALSCM3HA, anti-FLAG for expression of FLAG tagged Scm3p expressed from its own promoter, anti-Myc for expression of Cse4p, anti-H3 (abcam # ab1791-100) for expression of histone H3, anti-H4 (Millipore # 62-141-13) for expression of histone H4 and α-Tub2p served as a loading control. (B) Wild type strains with reporter chromosome fragment (YPH1018) expressing SCM3 from GAL1 promoter (pMB1306) or vector (pRS426 GAL1) were plated on SC-URA with limiting adenine and galactose (2%). Representative images showing red sectors in white colonies represent the loss of the non-essential chromosome fragment (CF). Arrows indicate colonies that show a half red-half white phenotype which represent CF loss in the first cell division. (C) GALSCM3 induces chromosome missegregation, which is suppressed by GALCSE4 and GALH4. Quantification of chromosome loss by half-sector analysis. Reporter strains with chromosome fragment (YPH1018) transformed with vectors (pRS425 GAL1, pRS426 GAL1), GALSCM3HA (pMB1306), GALCSE4 (pMB1147), GALSCM3HA, GALH4 (pMB1158), GALSCM3HA, GALH4 (pMB1306, pMB1158), GALH3 (pMB1159), and GALSCM3HA, GALH3 (pMB1306, pMB1159) were assayed for chromosome loss. At least 3000 colonies were counted and values represent the average ± standard error for three independent transformants normalized to the value of 100. (D) Co-overexpression of histone H4 and SCM3 increases the levels of Cse4p. Western blot analysis was done using whole cell protein extracts prepared from a wild type strain (JG595) transformed with vector (pRS425 GAL1), GALSCM3 (pMB1193), GALCSE4 (pMB1193, pMB1158), GALSCM3, GALH4 (pMB1193, pMB1158), and GALSCM3, GALCSE4 (pMB1193, pMB1158). Strains were grown to logarithmic phase of growth in media selective for the plasmid and gene expression induced in the presence of galactose (2%) at 30°C for 12 hours. Blots were probed with anti-Myc for expression of Myc tagged Cse4p expressed from its own promoter and α-Tub2p served as a loading control. (E) GALSCM3 strains show reduced levels of CEN-associated Cse4p. ChIP experiments were done using wild type strain expressing Cse4p-Myc from its
Based on the suppression of GALSCM3-induced chromosome loss phenotype by \( \text{GALSCM3} \text{HA} \) and \( \text{GALH4} \), we hypothesized that excess Scm3p devoid of Cse4p/H4 may associate with CEN//DNA. Hence, we performed chromatin immunoprecipitation (ChIP) experiments to measure the levels of CEN-bound Cse4p in SCM3 overexpressing (GALSCM3) and vector strains. Consistent with our hypothesis, we observed that GALSCM3 strains showed about 3-fold reduction in the levels of Cse4p at both CEN1 (0.54% of input) and CEN3 (0.44%) compared to vector alone (1.50% at CEN1, and 1.48% at CEN3) (Figure 1E). CEN-bound H4 was also reduced in strains overexpressing SCM3. ChIP results showed a 2- to 3-fold reduction in CEN-bound histone H4 in GALSCM3 (2.30% at CEN1, and 2.57% at CEN3) than vector alone (5.61% at CEN1, and 4.22% at CEN3) strains (Figure 1F). We did not detect Scm3p enrichment at \( \text{ACT1} \) used as a background control (Figure 2C). These results show that centromeric association of Scm3p is cell cycle regulated. We validated these results in two additional experiments using cells arrested with: a) alpha factor and released into medium containing nocodazole (Figure S3A), or b) nocodazole and released into medium containing alpha factor (Figure S3B). Similar to results in Figure 2, we observed a cell cycle regulated pattern of CEN association of Scm3p with lower levels in cells released from alpha factor (15–30 min), maximal in S phase cells (45–60 min), and enriched in anaphase cells (105 min) (Figure 2). We did not detect Scm3p enrichment at \( \text{ACT1} \) used as a background control (Figure 2C). These results show that centromeric association of Scm3p is cell cycle regulated. We validated these results in two additional experiments using cells arrested with: a) alpha factor and released into medium containing nocodazole (Figure S3A), or b) nocodazole and released into medium containing alpha factor (Figure S3B). Similar to results in Figure 2, we observed a cell cycle regulated pattern of CEN association of Scm3p with lower levels in cells released from alpha factor (15–30 min, Figure S3A), maximal in S phase cells (45 min, Figure S3A), reduction in early mitotic cells (75 to 105 min, Figure S3A; and 0 min, Figure S3B) followed by enrichment in anaphase cells (20–30 min, Figure S3B). Western blot analysis showed that the expression of Scm3p is not cell cycle regulated (Figure S4). We conclude that the mitotic depletion of centromeric Scm3p in budding yeast is largely similar to that of fission yeast where Sp-Scm3p and to humans where \( \text{HJURP} \) show a transient depletion at the \( \text{CEN} \) during mitosis [26,30–32].

Next, we determined if overexpression of Scm3p affects its cell cycle regulated centromeric association pattern. Thus, we compared the centromeric association pattern of FLAG-Scm3p expressed from its native promoter (control strain SCM3, Figure 3A) to the HA-Scm3p expressed from \( \text{GAL1} \) promoter (GALSCM3, Figure 3B). Cultures were grown in synthetic media with 2% galactose, synchronized in G1 with alpha factor and released into pheromone free media. Centromeric enrichment pattern of Scm3p in S phase (90 min) and anaphase (340 min) is similar in strains expressing SCM3 either from its native (control, Figure 3A) or \( \text{GAL1} \) (GALSCM3, Figure 3B) promoter and the difference observed for these time points is not statistically significant (\( p \)-value >0.05, Figure 3C). However, we observed a statistically significant difference (\( p \)-value = 0.012, Figure 3C) in the level of centromeric Scm3p in early mitotic cells (300 minute,
Figure 2. Centromeric association of Scm3p is cell cycle regulated. A wild-type strain (RC154) with FLAG tagged Scm3p expressed from its own endogenous promoter was grown in YPD, synchronized in G1 with α-factor, washed, and released into pheromone-free YPD medium. Samples were taken at time points (min) after release from G1. (A) DNA content was determined by FACS. (B) Cell cycle stages were determined based on cell morphology and nuclear position by microscopic examination of 200 cells for each time point as described in Materials and Methods. (C) Enrichment levels of Scm3p at CEN3, CEN5 and ACT1 (background control). ChIP experiments were done using α-FLAG (Scm3p), and α-GST (mock) antibodies. The enrichment of Scm3p at CEN3, CEN5, and ACT1 was determined by qPCR and is shown as % input. Average from at least three independent experiments ± standard error is shown. *p value <0.05, **p value <0.01 derived from the Student’s t test.

doi:10.1371/journal.pgen.1002303.g002

Figure 3A, 3B) between the control (SCM3) and GALSCM3 strains. We confirmed the latter observations in an additional cell cycle experiment in which GALSCM3 strain was synchronized in G2/M with nocodazole and released into alpha factor (Figure S6). We did not observe a depletion of Scm3p in GALSCM3 strains at CENV DNA in G2/M cells (0 min, Figure S6). We conclude that the mitotic depletion of Scm3p at CEN is affected in GALSCM3 strains.

Overexpression of SCM3 (GALSCM3) reduces viability in a subset of kinetochore mutants and leads to premature separation of sister chromatids

The chromosome loss phenotype of GALSCM3 strains may be due to compromised kinetochore function and hence, we examined genetic interactions between GALSCM3 and genes encoding other kinetochore components, specifically subunits of the COMA and Cdt3p complexes. These proteins interact with Cse4p and are important for maintenance of kinetochore integrity [15,16]. GALSCM3 showed reduced viability in a subset of the kinetochore mutants, with the most severe phenotype in the mcm21A strain (Figure 4A). Deletion of MCM21 is known to cause defects in pericentromeric cohesion, leading to precocious separation of sister chromatids and premature initiation of anaphase [44]. Since GALSCM3 showed the most severe phenotype in mcm21A strains, we examined if GALSCM3 exacerbated the sister chromatid cohesion defect of mcm21A strains. We monitored the segregation of sister chromatids in metaphase cells by assaying the binding of GFP-LacI to operator sequences inserted 12-kb from the centromere (pericentromere) on Chromosome IV [44]. Wild-type strains arrested in metaphase show a predominance of cells with a single GFP-LacI focus marking two closely associated sister chromatids, whereas premature separation of sister chromatids results in the appearance of two GFP-LacI foci [44]. Consistent with previous findings, we observed a low incidence of two GFP-LacI foci in wild-type cells (5%) compared to that in mcm21A cells (26%) (Figure 4B, 4C). Strains overexpressing SCM3 showed a higher incidence of two GFP-LacI foci in wild-type (16%) as well as mcm21A strains (39%) (Figure 4B, 4C) suggesting that overexpression of SCM3 enhances premature separation of sister chromatids. The higher incidence of two GFP-LacI foci was observed in mitotic cells but not in G1-arrested cells (Figure 4B), mcm21A cells depend on the IPL1 orientation checkpoint for viability [44]. Similarly, we found that GALSCM3 leads to growth defects in the qpl5-321 strain but not in wild-type strains (Figure 4D). Together, these results show that overexpression of SCM3 enhances premature separation of sister chromatids.

Scm3p can associate with CEN DNA independently of Cse4p

Our observation that GALSCM3 causes reduction in CEN-bound Cse4p led us to postulate that the chromosome loss phenotype of GALSCM3 may be due to centromeric association of Scm3p devoid of Cse4p. Hence, we determined if CEN-association of Scm3p requires bound Cse4p. Previous studies of Scm3p have defined two essential domains, a nuclear export signal (NES) that presumably mediates export of Scm3p from the nucleus, and a coiled-coil heptad repeat (HR) domain that mediates interaction with Cse4p [19,20]. Since deletion of NES and HR domains from endogenous SCM3 causes lethality [20], we used galactose inducible forms of mutant scm3 alleles for our analysis. We constructed plasmids expressing scm3nes (amino acid residues 13–24 deleted) and scm3hr (amino acids 101–139 deleted) from GALI promoter (Figure 5A). Our results showed that GALscm3nesA and GALscm3hrA strains exhibit a 6- and 8-fold higher chromosome loss than GALSCM3 strains (Figure 5B). We reasoned that the chromosome loss phenotype of strains overexpressing scm3nesA, which can interact with Cse4p, but not scm3hrA, which lacks the Cse4p interacting domain, would be suppressed by GALCSE4 [20]. Indeed, GALCSE4 suppresses the chromosome loss phenotype of GALscm3nesA strains but not GALscm3hrA strains (Figure 5B). ChIP results showed that both GALscm3nesA and GALscm3hrA can associate
with CEN DNA (Figure 5C). Co-immunoprecipitation experiments verified previous observations that a deletion of the HR domain of Scm3p but not the NES abolishes interaction with Cse4p (Figure 5D) [20]. Since GALscm3hr does not interact with Cse4p and can still bind CEN DNA, we conclude that Scm3p can associate with CEN DNA independent of its interaction with Cse4p.

Figure 3. Overexpression of SCM3 alters its cell cycle-regulated centromeric association pattern. (A) Wild-type strain expressing FLAG-tagged Scm3p (SCM3) from its own endogenous promoter (RC154) were grown in minimal media with galactose (2%) to logarithmic phase, treated with α-factor for G1 arrest, washed, and released into pheromone-free media. Samples were taken at time points (min) after release from G1 arrest. DNA content (FACS), cell cycle stages based on cell shape and nuclear position (cell morphology), and levels of Scm3p at CEN DNA (ChIP) were determined as described in Figure 2. (B) Wild-type strain (RC100) overexpressing HA-tagged Scm3p from GAL1 promoter (GALSCM3) was grown in minimal media with galactose (2%), synchronized in G1 with α-factor, washed, and released into pheromone-free media. Samples were taken at time points (min) after release from G1. DNA content (FACS), cell cycle stages (cell morphology) and the levels of Scm3p at CEN DNA (ChIP) were determined as described in (A) above. (C) Statistical comparisons of CEN-bound Scm3p levels at different cell cycle stages between strains expressing Scm3p from endogenous (panel A above) or GAL1 promoter (panel B above). Significance was determined using Student’s t-test. The t-statistic and p-values are shown: significant (s), not significant (ns).

doi:10.1371/journal.pgen.1002303.g003
The N-terminus of Scm3p is required for centromeric association of Scm3p

If the GALSCM3-induced chromosome loss phenotype is due to centromeric association of Scm3p devoid of Cse4p, overexpression of scm3 alleles that do not associate with CEN DNA should not affect chromosome segregation. To identify the putative DNA interacting domain of Scm3p, we used computational analysis using BindN-RF [45] and MEME [46] software. These analyses suggested that the first 100 amino acids of the N-terminus of Scm3p exhibit properties of DNA binding sequences (Figure S7); therefore, we constructed Scm3p variants with a deletion of the putative CEN DNA interacting motif (amino acid residues 1–103).
Figure 5. Scm3p can associate with CEN DNA independently of Cse4p, and the N-terminus of Scm3p mediates its centromeric association. (A) Schematic of full-length Scm3p and its allelic mutant proteins constructed using gene deletion approach. NES: nuclear export signal; HR: heptad repeat; and D/E: C-terminus acidic domain. Results from chromosome loss (B), CEN-binding (C) and Cse4p interactions (D) are summarized in the table. (B) Overexpression of N-terminal mutants (scm3-D103HA, scm3-D140HA) does not result in increased chromosome loss. Reporter strains with chromosome fragment (YPH1018) transformed with vector (pRS426 GAL1), GALSCM3HA (pMB1306), GALscm3nes.DHA (pMB1393), GALscm3hr.DHA (pMB1455), GALscm3-D103HA (pMB1520), GALscm3-D140HA (pMB1521), GALCSE4 (pMB1147), GALscm3nes.DHA, GALKCE4 (pMB1393, pMB1147), GALscm3hr.DHA, GALCSE4 (pMB1455, pMB1147), GALscm3-D103HA, GALCSE4 (pMB1520, pMB976), GALscm3-D140HA, GALCSE4 (pMB1521, pMB976) were assayed for chromosome loss as described in Figure 1 (C). (C) N-terminal mutants of Scm3 (scm3-D103HA, scm3-D140HA) do not bind CEN DNA. ChIP experiments were done using wild type strain (YMB6398 or JG595) with GALSCM3HA (pMB1306), GALscm3nes.DHA, GALCSE4 (pMB1393, pMB1147), GALscm3hr.DHA, GALCSE4 (pMB1455, pMB1147), GALscm3-D103HA, GALCSE4 (pMB1520, pMB976), GALscm3-D140HA, GALCSE4 (pMB1521, pMB976) grown in minimal media with galactose (2%) for 12 hours at 30°C and immunoprecipitated with α-HA, and α-GST (mock) antibodies. Enrichment of Scm3p and its allelic mutant forms at CEN1, and ACT1 (internal control) is determined by PCR. Lanes: IN (input), IP (DNA from chromatin immunoprecipitation using α-HA antibodies), and M (DNA from chromatin immunoprecipitation using α-GST antibodies). (D) Western blots of proteins copurifying with Scm3p or its allelic mutant forms. Extracts from cells coexpressing Myc-tagged Cse4p from its native promoter (Cse4-12Myc) and HA-tagged Scm3p or scm3 mutant proteins expressed from GAL1 promoter were used in immunoprecipitation (IP) experiments using anti-HA conjugated agarose beads. Eluted proteins were analyzed by western blotting with anti-HA and anti-Myc antibodies. Ten-fold more protein was loaded for IP samples. (E) Overexpression of Scm3p and its mutant alleles (GALscm3nes.DHA and GALscm3hr.DHA) cause reduction in Cse4p at centromeres. ChIP experiments were done using wild type strain...
either alone or in combination with the HR domain (amino acid residues 1–140) (Figure 5A). Consistent with our hypothesis, GALScm3-A103 or GALScm3-A140 strains do not exhibit a chromosome loss phenotype (Figure 5B) nor do the mutant proteins associate with CEN DNA (Figure 5C). As expected, results of co-immunoprecipitation showed that Scm3-A103p interacts with Cse4p, while Scm3-A140p does not (Figure 5D). Consistent with the lack of chromosome loss phenotype, overexpression of scm3-A103 and scm3-A140 alleles do not affect the level of Cse4p at CEN DNA, whereas the overexpressed scm3nesA, scm3hrA alleles showed about 3- to 5-fold reduction in the levels of Cse4p at SCM3 and CEN3 relative to that of the vector control strain (Figure 5E). Our results show that the N-terminus of Scm3p mediates its association with CEN DNA and overexpression of N-terminal scm3 mutants (scm3-A103 and scm3-A140) that fail to associate with centromeric chromatin do not result in increased chromosome loss.

Overexpression of HJURP causes chromosome loss in budding yeast and mitotic defects in human cell lines

Overexpression of HJURP has been observed in mammalian cancer cell lines, such as lung and breast cancers [38,39]. Based on our results with GALSCM3, we tested if overexpression of HJURP leads to mitotic defects in yeast. We cloned HJURP into a yeast expression vector allowing regulated expression of the gene from a GALI promoter. A wild-type reporter strain overexpressing HJURP exhibits a chromosome loss phenotype (7.3±0.3%) similar to that observed for GALSCM3 (8.9±0.8%) (Figure 6A). GALCSE4 suppresses the chromosome loss phenotype of the GALHJURP strain to the same extent as that observed for strains co-expressing GALSCM3 and GALCSE4 (Figure 6A). We confirmed galactose-induced expression of GALHJURP by western blotting (Figure 6B).

The chromosome loss phenotypes observed upon Scm3p and HJURP overexpression in S. cerevisiae led us to ask if increased levels of HJURP in human cells would also result in chromosome segregation errors. HeLa cells were transfected with a construct to express GFP-tagged HJURP. The population was enriched for chromosome loss with overexpression of both CENP-A and HJURP are consistent with observations in breast cancer where there is a correlation between increased levels of HJURP and CENP-A [39]. Overexpression of both CENP-A and HJURP may result in stabilization of HJURP (Figure 7C). Alternatively, overexpression of CENP-A and HJURP may independently lead to a phenotype that is more susceptible to acquiring a tumorigenic potential.

Discussion

Here we report that misregulation of Scm3p/HJURP causes defects in kinetochore function and results in chromosome instability in yeast and human cells. We used budding yeast to show that overexpression of SCM3 exhibits reduced viability in kinetochore mutants, premature separation of sister chromatids and lower levels of Cse4p and histone H4 at centromeres. To understand the molecular basis of the chromosome loss phenotype of GALSCM3 strains, we created strains overexpressing mutant alleles of SCM3, and these studies have shown that: a) Scm3p can associate with centromeres independently of its association with Cse4p, b) the N-terminal domain of Scm3p mediates its association with centromeric chromatin, and c) centromeric association of Scm3p devoid of Cse4p is the likely cause of the same cell, the incidence of micronuclei (32±18%) and lagging chromosomes (30±17%) was increased relative to CENP-A or HJURP overexpression alone (Figure 7B). Increased rates of chromosome loss with overexpression of both CENP-A and HJURP may result in stabilization of HJURP (Figure 7C).

![Figure 6. Overexpression of HJURP causes chromosome mis-segregation in budding yeast. (A) Overexpression of HJURP leads to chromosome loss in budding yeast. Reporter strains with chromosome fragment (YPH1018) transformed with a vector (pRS426 GALI), GALHJURP (pMB1490), GALCSE4 (pMB1147), GALHJURP, GALCSE4 (pMB1490, pMB1147), and GALSCM3HA (pMB1306) were assayed for chromosome loss as described in Figure 1 (C). (B) Western blot analysis showing galactose induced expression of HJURP. Whole cell protein extracts prepared from wild type strains (YPH1018) transformed with GALHJURP (pMB1490), or vector (pRS426 GALI). Strains were grown to logarithmic phase of growth in synthetic media and gene expression induced in the presence of galactose (2%) at 30°C for 12 hours. Blots were probed with anti-HJURP. A non-specific band served as a loading control.

doi:10.1371/journal.pgen.1002303.g006
Our results establish that balanced stoichiometry of Scm3p and its human homolog *HJURP* are critical for maintenance of genome stability. Since mutations affecting the HR, NES, and N terminus of Scm3p are lethal, limiting their usefulness, we used overexpression alleles to gain insight into Scm3p function. Overexpression studies have been conducted in other systems to understand the molecular mechanisms of chromosome segregation and kinetochore assembly. For example, overexpression of wild-type or mutant forms of *CENP-A* or its homologs in different organisms such as flies, budding and fission yeasts, leads to chromosome segregation defects [34–36]. In flies, excess *CENP-A* promotes the formation of ectopic kinetochores [36]. In budding yeast, excess Cse4p is degraded by ubiquitin mediated proteolysis [41,47], however a mutant form of Cse4p (Cse4K16R) can be stably overexpressed [48], and such overexpression results in Cse4p mis-localization and chromosome loss [35]. Our results showed that the increased chromosome loss of *GALSCM3* strains does not appear to be due to spreading of Scm3p to non-centromeric regions, as we found no evidence for mis-localization of overexpressed Scm3p to non-CEN regions.

In *in vitro* studies in budding yeast have shown that Scm3p directly binds and forms a stoichiometric complex with Cse4p and histone H4 [19]. Similarly, *HJURP* in humans also interacts at a stoichiometric ratio with CENP-A/H4 tetramers [27]. Our results here have shown that overexpression of *SCM3* adversely affects the incorporation of Cse4p/H4 into centromeric chromatin as is evident from the reduction in the levels of Cse4p and H4 at the CEN DNA and suppression of *GALSCM3*-induced chromosome loss phenotype by *GALCSE4* or *GALH4* (Figure 1C, 1E, 1F). We propose that *GALSCM3* leads to defects in kinetochore integrity and that this contributes to reduced viability in kinetochore mutants such as *mcm21Δ*. Previous reports have shown that *mcm21* genetically interacts with *cse4* and *ipl1* mutants and *mcm21Δ* strains exhibit precociously separation of sister chromatids in metaphase [44]. It is possible that the lower levels...
of centromeric Cse4p in \textit{GALSCM3} strains contribute to altered pericentromeric cohesion, which in turn results in premature separation of sister chromatids in the \textit{GALSCM3} strains. Defect in pericentromeric cohesion has been previously reported for \textit{cse4} mutants [49].

We propose a model whereby Scm3p overexpression leads to increased levels of unbound Scm3p, which localizes to \textit{CEN} DNA and interferes with the productive association of Cse4p/H4-Scm3p complexes, resulting in decreased Cse4p incorporation, defects in kinetochore function, and, ultimately, chromosome loss (Figure 8).

\textbf{Figure 8. Schematic model for \textit{GALSCM3}-induced chromosome instability in budding yeast.} (A) In wild type cells, Scm3p forms a stoichiometric complex with Cse4p/H4 tetramers and mediates the assembly of Cse4p at the \textit{CEN} DNA resulting in faithful chromosome segregation. (B) Centromeric association of Scm3p devoid of Cse4p contributes to the chromosome loss phenotype of \textit{GALSCM3} strains. In \textit{GALSCM3} expressing cells, there is an excess of Scm3p compared to the available pools of Cse4p and H4. Centromeric association of Scm3p devoid of Cse4/H4 leads to chromosome loss. Support for this is based on chromosome loss, ChIP experiments and suppression analysis from Figure 1. (C) Scm3p devoid of Cse4p can associate with centromeric DNA. \textit{GALscm3hr\Delta} that cannot interact with Cse4p can bind \textit{CEN} DNA and association of Scm3p devoid of Cse4p leads to the chromosome loss phenotype of \textit{GALscm3hr\Delta} strains (Figure 5). (D) The N-terminus of Scm3p defines the centromeric association domain of Scm3p. Overexpression of \textit{scm3} mutants (\textit{GALscm3-\Delta103}) that can interact with Cse4p but do not associate with \textit{CEN} DNA do not exhibit increased chromosome loss (Figure 5). Thick arrows represent excess Scm3p or its allelic mutant forms. Taken together our data support the model that centromeric association of Scm3p devoid of Cse4p contributes to the chromosome loss phenotype of \textit{GALSCM3} strains.

doi:10.1371/journal.pgen.1002303.g008
The model posits that Scm3p devoid of Cse4p can associate with CEN DNA, and it correctly predicts that mutant alleles of scm3 that fail to associate with CEN DNA should not affect chromosome segregation (Figure 3B). Mutant alleles of scm3 deleted of the N terminus (GALscm3m-A140) either alone or in combination with the HR domain (GALscm3m-A140) do not associate with CEN DNA (Figure 5C). Our results are consistent with in vitro studies demonstrating a DNA-binding domain located within the N-terminal 113 of Scm3p (Carl Wu, personal communication). That GALscm3m-A140, which fails to bind Cse4p (Figure 5D), is still found associated with CEN DNA (Figure 5C) indicates that CEN DNA association of Scm3p is not dependent on Cse4p interaction even though centromeric localization of Cse4p is dependent on Scm3p [18-20,29]. The higher chromosome loss phenotype of GALscm3m-A140, and GALscm3mnes strains compared to that of GALSCM3 strains suggests that these mutant alleles exhibit a dominant interfering phenotype and this may be due to additional roles of the HR and NES domains. For example, Shivaraju et al. have reported that expression of scm3m-A140 from its native promoter and simultaneous depletion of Scm3p results in checkpoint activation, and G2/M arrest [29]. Future studies should help us better understand the additional roles of different Scm3p domains.

Our results for the cell cycle regulated enrichment of Scm3p in S phase cells correlates with a similar enrichment of Cse4p in S phase cells and is consistent with the role of Scm3p as a Cse4p assembly factor [18-20,29-31]. Studies with budding yeast have shown that replication of CEN DNA occurs in early S phase [50], and the centromeres transiently detach from the microtubules in order for the replication machinery to pass through the centromeric region [51]. The S phase co-enrichment of Scm3p and Cse4p could likely reflect the CEN DNA replication dependent centromeric assembly of Cse4p. Scm3p homologs in fission yeast also exhibits cell cycle dependent centromeric localization pattern and an enrichment in S phase that correlates with the centromeric assembly of Cnp1 [30,31]. Our results for depletion of Scm3p in early mitotic cells and enrichment in anaphase cells is similar to findings from fission yeast, wherein Scm3p dissociates from CEN in metaphase cells and reappears in anaphase [30,31]. Despite the difference in centromere structure of fission and budding yeast, the overall cell cycle regulated centromeric localization pattern of Scm3p seems to be evolutionarily conserved in these two systems. However, in humans no HJURP signals were detected at centromeres in anaphase and telophase cells [26]. The mechanism and physiological relevance of the mitotic depletion of Scm3p or its homologs has not been investigated in fission yeast or human cells. It is possible that the lack of mitotic depletion of Scm3p in GALSCM3 strains (300 min, Figure 3B) may contribute to the chromosome loss phenotype. Future studies using mutant alleles of SCM3, which fail to show mitotic depletion should give us insights into the role of the cell cycle regulated centromeric association of Scm3p.

We have shown that overexpression of SCM3 or HJURP causes chromosome loss in a wild-type yeast strain, and overexpression of HJURP leads to mitotic defects in human cells. Unlike our observations in budding yeast where overexpression of CSE4 suppresses GALSCM3 induced chromosome loss, overexpression of CENP-A does not suppress, but exacerbates the chromosome loss phenotype of human cells overexpressing HJURP. We propose that these differences may be due to the composition of centromeric chromatin, timing of incorporation of CenH3 or additional roles of HJURP in humans. For example, in contrast to the point centromeres of budding yeast, human centromeres contain a large number of CENP-A nucleosomes that are stably propagated through S phase [52], whereas the single budding yeast Cse4p nucleosome is replaced during S phase [43]. Furthermore, HJURP occupies the centromere during a limited time in the cell cycle [26]. Therefore, overexpression of HJURP would not be expected to deplete stable CENP-A nucleosomes. Recently, HJURP mediated deposition of CENP-A has been shown to be sufficient to recruit centromeric proteins to an ectopic site within the chromatin [53]. Co-overexpression of CENP-A and HJURP in human cells may result in the increased incorporation of CENP-A at non-centromeric loci. As a result, limiting amounts of critical centromere proteins may be titrated away from endogenous centromeres under these conditions leading to chromosome missegregation. Alternatively, the non-centromeric roles of HJURP may contribute to chromosome missegregation [38] and this effect may be increased when CENP-A is present because of the increased stability of HJURP (Figure 7C) [39]. Unlike HJURP overexpression, which causes its mislocalization to different genomic regions [38], overexpression of Scm3p does not result in mislocalization to non-centromeric regions (Figure S2A).

Overall, our results show that proper regulation of SCM3 and HJURP contribute to genome stability. The importance of our results with SCM3/HJURP is further reinforced by the fact that HJURP overexpression has been observed in human cancer cells and is associated with chromosomal aberrations and aneuploidy [38,39]. Also, altered dosage and mis-localization of kinetochore proteins, such as CENP-A, CENP-H, Aurora-B and INCENP has been observed in various types of tumor cells [37–39,54]. The elucidation of mechanisms underlying regulation of SCM3/HJURP in humans may help us identify and develop therapeutic targets for cancer therapy.

Materials and Methods

Media, strains, plasmids, and growth conditions
All yeast strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. Yeast growth media, and protocols are as described previously [35]. Plasmid pMB1193 (2µ LEU2 GAL1/10-SCM3) was derived by inserting a PCR-amplified SCM3 fragment between the BamHI and XhoI sites of pRS425 GALI. The NES domain of SCM3 was deleted from pMB1306 using QuickChange Site Directed Mutagenesis Kit (Stratagene) resulting in pMB1393 (2µ URA3 GAL1/10-scm3nes-HA). pMB1457 (2µ LEU2 GAL1/10-scm3nes-HA) was constructed by cloning the PCR-amplified SCM3nes-HA fragment from pRB923 between SpeI and XhoI sites of pRS425 GALI, whereas pMB1490 (2µ URA3 GAL1/10-HJURP) was derived from subcloning the EcoRI -XhoI fragment of HJURP (clone id 2920741, Open Biosystem), into pRS426 GALI. Plasmids pMB1520 (2µ LEU2 GAL1/10-scm3-A105HA) and pMB1521 (2µ URA3 GAL1/10-scm3-A140HA) were constructed by cloning the PCR-amplified SCM3-A105HA, and SCM3-A140HA fragments from pMB1306 between SpeI and XhoI sites of pRS425 GALI. Strains were grown inYPD (1% yeast extract, 2% Bactopeptone, 2% glucose) or in synthetic medium containing either 2% glucose or 2% galactose and supplements to allow selection of plasmids being tested.

Chromosome transmission fidelity (ctf) and viability assays
We used an assay developed previously [55] to measure the loss of a non-essential reporter chromosome fragment (CF). Reporter strains were grown to logarithmic phase in synthetic media to maintain the CF and plasmids to be assayed. Cultures were then diluted and plated on synthetic medium to maintain plasmid
Table 1. *S. cerevisiae* strains used in this study.

| Strain     | Genotype                                                                 | Reference |
|------------|---------------------------------------------------------------------------|-----------|
| YPH1676    | MATa ura3-52 lys2-801 ade2-101 trp1-1 his3-1200 leu2-11 CFII (CEN3;YPH278) HIS3 SUP1 | [60]      |
| YPH1678    | MATa okp1-5:TRP1                                                         | [61]      |
| YPH1712    | MATa mfa1::MFA1pr-LEU2 can1-1::MFA1pr-HIS3 ura3-30 leu2-10 his3-11 ly2-1 ctf3-1::natR | [16]      |
| YPH1713    | MATa mfa1::MFA1pr-LEU2 can1-1::MFA1pr-HIS3 ura3-30 leu2-10 his3-11 ly2-1 ctf19-1::natR | [16]      |
| YPH1714    | MATa mfa1::MFA1pr-LEU2 can1-1::MFA1pr-HIS3 ura3-30 leu2-10 his3-11 ly2-10 mcm16-1::natR | [16]      |
| YPH1715    | MATa mfa1::MFA1pr-LEU2 can1-1::MFA1pr-HIS3 ura3-30 leu2-10 his3-11 ly2-10 mcm21-1::natR | [16]      |
| YPH1716    | MATa mfa1::MFA1pr-LEU2 can1-1::MFA1pr-HIS3 ura3-30 leu2-10 his3-11 ly2-10 mcm21-1::natR | [16]      |
| R421       | MATa trp1-1 ura3-52 leu2-2::PET56 ade2-2 SCm3deg::NAT CSE4GFP::TRP1 | [20]      |

Table 2. List of plasmids used in this study.

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pRS424-GAL1 | 2 μm TRP1 GAL1 | [61] |
| pRS425-GAL1 | 2 μm LEU2 GAL1 | [61] |
| pRS426-GAL1 | 2 μm URA3 GAL1 | [61] |
| pMB976 | 2 μm TRP1 GAL1/10-CSE4-13Myc | [35] |
| pMB1147 | 2 μm LEU2 GAL1/10-CSE4-13Myc | [35] |
| pMB1158 | 2 μm TRP1 GAL1/10-HHF1 | [35] |
| pMB1159 | 2 μm TRP1 GAL1/10-H3 | [35] |
| pMB1193 | 2 μm LEU2 GAL1/10-SCM3 | This study |
| pMB1306 | 2 μm URA3 GAL1/10-SCM3-HA | [62] |
| pMB1393 | 2 μm URA3 GAL1/10-SCM3nes-4-HA | This study |
| pMB1455 | 2 μm LEU2 GAL1/10-SCM3nes-4-HA | This study |
| pMB1490 | 2 μm URA3 GAL1/10-HUUP | This study |
| pMB1520 | 2 μm LEU2 GAL1/10-SCM3-Δ100HA | This study |
| pMB1521 | 2 μm LEU2 GAL1/10-SCM3-Δ140HA | This study |
| pOC52 | URA3 PDS1-HA | Orna Cohen-Fix |
| pRB835-2 | TRP1 SCM3-HA | Richard Baker |
| pRB914 | TRP1 SCM3nes-4 | [20] |
| pRS314 | TRP1 CEN Vector | [61] |
| pRB923 | TRP1 SCM3-ΗΗΗΗΗΗΗΗΗΗΗΑ | [20] |

doi:10.1371/journal.pgen.1002303.t001

doi:10.1371/journal.pgen.1002303.t002

selection with limiting adenine. The loss of non-essential CF leads to red sectors in an otherwise white colony. Chromosome loss was measured by counting the percentage of colonies that were at least half red, which represents the loss of reporter chromosome during the first cell division. A minimum of 3000 colonies were assayed from three individual transformants for each strain.

Viability assays for Ctf3p and COMA complex strains containing GALSCM3HA (pMB1306) or vector (pRS426 GAL1) were carried out by plating equal numbers of cells from three independent transformants for each strain on synthetic media with either glucose (2%) or galactose (2%) and grown at 30°C for 5–7 days. At least 2500 colonies were counted for each strain. Percent viability is expressed as the ratio of the number of colonies obtained on galactose media over the number of colonies obtained on glucose plates and normalized to the value of 100.

Chromatin immunoprecipitation (ChIP) and quantitative PCR

All ChIP experiments were carried out (three biological replicates) as described [35] with minor modifications. Cultures were cross-linked with formaldehyde (1% final concentration) at room temperature for 15 min and excess formaldehyde was quenched with 125 mM glycine for 5 min. Cell pellets were collected by centrifugation and spheroplasts prepared using Zymolyase 100T, followed by sequential washes with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4), and FA buffer (50 mM Na-Hepes pH 7.6, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 0.1% Na-deoxycholate). Spheroplasts were resuspended in FA buffer with protease inhibitors (1 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotonin) and sonicated on ice at setting 3, 100% duty cycle, for four 12 sec
Immunoprecipitation and Western blot analysis

Immunoprecipitation experiments were performed as described previously [18]. Protein samples for western blot analysis were prepared using the TCA procedure as described previously [57], and protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, CA). Equal amounts of protein for each sample were size separated on 4–12% gradient polyacrylamide gels and transferred to nitrocellulose membrane. Primary antibodies used were anti-NA (clone 12G5, Roche), anti-Myc (Z-5, sc-789, Santa Cruz Inc), anti-histone H3 (ab1791-100, Abcam), anti-histone H4 (clone 62-141-13, Millipore), anti-Flag (A-6457, Molecular Probes) or anti-HJURP [26]. Secondary antibodies used were HRP-conjugated sheep anti-mouse IgG (NA931V–Amersham) and HRP-conjugated sheep anti-rabbit IgG (NA934V–Amersham). Rabbit polyclonal antibodies against Tub2p were custom made by Covance, Inc.

Cell cycle arrest and release experiments

For determining the CEN enrichment of Scm3p through the cell cycle, wild-type strain (RC154) with FLAG-tagged Scm3p expressed from its endogenous promoter was used. A wild type strain (JG595) with Myc-tagged Cse4p expressed from its endogenous promoter was used to determine the CEN enrichment of Cse4p through the cell cycle. Cells were grown in YPD to logarithmic (LOG) phase at 30°C, treated with 3 μM α-factor (T-6901, Sigma, St. Louis) for 90 minutes to arrest cells in G1, washed, and released into pheromone-free YPD medium. Samples were taken at 15 min time points after release from G1 arrest and used for FACS, protein and ChIP analysis. Additional synchronizations were carried out to confirm the results: cells were arrested in G1 with α-factor as described above and released into YPD medium containing 15 μg/ml nocodazole (M1404, Sigma); cells were arrested in G2/M with nocodazole for 2 hours and released into YPD medium containing 3 μM α-factor (T-6901, Sigma).

To examine the CEN enrichment of overexpressed Scm3p (GALSCM3) through the cell cycle, strain RC100 with HA tagged Scm3p integrated at its endogenous locus (only copy in the genome) and expressed from GAL1 promoter was used. Cells were grown in SC media with 2% galactose to logarithmic (LOG) phase at 30°C, treated with 3 μM α-factor (T-6901, Sigma, St. Louis) for 90 minutes for G1 arrest, washed, and released into pheromone-free SC media with 2% galactose medium. Samples were taken at different time points after release from G1 arrest and were used in FACS and ChIP analysis.

Table 3. List of primers used in this study.

| Locus     | Forward primer (5’ to 3’)                       | Reverse primer (5’ to 3’)                   |
|-----------|------------------------------------------------|---------------------------------------------|
| CEN1      | CTGCATTTCGATAAAGTGCGCC                          | GTGCTTAAAGAGTGCTGACCA                      |
| CEN3      | GAATCCGCGCACAACATAATGG                         | AACTCCACGAGTAACACGTC                      |
| CEN5      | AAGACTATGATACATAATGCGCC                         | CTGCCAATACAGCAATTTCATATAGTTTAG            |
| ACT1      | ACAACAGTATGAGGCTCGGAC                         | AATTGCTTAAAGAGTGCGCGCC                      |
| IR1       | TACTGCGGACGACGGAGGC                            | AATCCACAGTGACCCATAC                       |
| IR2       | ATGAAATAGCAAGATCTCAC                         | CTGCTTAAACACGCTTCCC                       |
| IR3       | TGCGCTCAGAGTCATGGAGTGT                         | GTAGCTTAAATTCATGCTATACCC                  |
| IR4       | GTCACCAGGATTAATACCTGACTG                      | TTGTGTTATTGTTATAGTCGAG                     |
| AGP1      | CCATGAAATGCTCGGAGA                            | CAATCGTTCGAGACACTTAC                      |
| CWH4      | AAAAAAGAAAAACCGCCTGCT                         | GAAGCGGTTCAAGCAGACAG                      |
| YGL036W   | GGGGGGCAAAATAAGTAAAAAC                       | CAAGAAGCAGAACATTACCAAC                    |

doi:10.1371/journal.pgen.1002303.t003
FACS and nuclear morphology analyses

FACS assays was performed to confirm the cell cycle arrest and release. Cells were fixed in 70% ethanol, washed in 0.2 M Tris buffer, treated with RNase A, Proteinase K, stained with propidium iodide, and analyzed using a Becton-Dickinson FACSort flow cytometer and Cell Quest software (BD Biosciences, Boston, MA). Cell cycle stages were determined by examining cell morphology and nuclear position in propidium iodide-stained cells under the Zeiss Axioskop 2 microscope (Carl Zeiss Inc., Thornwood, USA) as described previously [42]. Cell cycle stages were defined as follows: G1, single cells with undivided nuclei; S-phase, cells showing a small bud with undivided nuclei; metaphase, large budded cells with nucleus at the neck; anaphase, large budded cells with elongated/separated nuclei; and telophase, large budded cells with nuclei separated between mother and daughter cells (see Figure 2).

Human cell culture

HeLa cells were plated 24 hours prior to transfection to poly-lysine coated 12mm square coverslips in a 6-well plate. Cells were incubated with DNA and Effectene transfection reagent (Qiagen) according the manufacturer instructions in Optimem (Invitrogen) for 8 hours and at which time the media was replaced with normal growth media (DMEM, 10% FBS, supplemented with Penicillin and Streptomycin). Beginning 24 hours after transfection, cells were incubated with 2mM thymidine for 20 hours, then released from thymidine by washing cells in PBS and incubating cells in normal growth media supplemented with 20mM deoxyctydine for 12 hours prior to fixation. Cells were fixed after 4% formaldehyde and blocked in PBS with 2% FBS, 2% BSA and 0.1% TritonX-100. Antibody incubates were conducted for 1 hour at room temperature in blocking buffer. Centromeres were detected using anti-centromere auto sera (Antibodies incorporated) and Cy5 conjugated goat-anti-human secondary antibodies (Jackson Labs). DNA was stained using 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and cells were mounted in anti-fade (Invitrogen). Z-stacked images were collected on a Deltavision microscope (Applied Precision Instruments) using a 60X objective. Images were deconvolved and are presented as maximum projections.

Supporting Information

Figure S1 Overexpression of SCMi causes chromosome instability in wild-type strains. Quantification of chromosome loss by half-secton analysis. Reporter strains with chromosome fragment (YPH1018) transformed with vector (pRS426 GAL1), GALSCMiHA (pMB1306), GALSCMi (pMB1193), or GALSCMiHA (pMB1306) integrated at URA3 locus in the genome were plated on SC-URA with limiting adenine and galactose (2%). At least 300 colonies were counted. Values represent the average and standard error of chromosome loss for three independent transformants and were normalized to the value of 100.

(TIF)

Figure S2 Scm3p and Cse4p are not mislocalization to non-centromeric DNA regions in strains overexpressing SCMi. (A) A wild-type strain (RC154) with FLAG-tagged Scm3p expressed from its own endogenous promoter was transformed with vector (pRS426 GAL1), or GALSCMiHA (pMB1306) and grown in minimal media with galactose (2%) for 12 hours at 30°C. Chromatin immunoprecipitation were done with α-HA (Scm3p expressed from GAL1 promoter), α-FLAG (Scm3p expressed from its endogenous promater), and α-GST (mock) antibodies. Enrichment of Scm3p in these strains were assayed by qPCR using primers representing CEN3, intergenic (IR1, IR2, IR3, IR4), and transcribed (AGP1, CHW43, ACT1, and YGL036W) regions. Chromosomal coordinates of these DNA regions were derived from yeast genome database (www.yeastgenome.org) and are as follows: CEN3 (Chromosome III, 114385-114501), IR1 (Chromosome III, 163689-163699), IR2 (Chromosome III, 277629-277969), IR3 (Chromosome VI, 224045-224329), IR4 (Chromosome XVI, 520851-521150), AGP1 (Chromosome III, 76166-76400), CHW43 (Chromosome III, 146651-146886), ACT1 (Chromosome VI, 54093-53886), and YGL036W (Chromosome VII, 431253-431610). Average from at least three independent experiments ± standard error is shown as % input. *p value <0.05, **p value <0.01. Student’s t test. (B) GALSCMi strains show reduced levels of CEN-asso ciated Cse4p. ChIP experiments were done using wild type strain expressing Cse4p-Myc from its endogenous promoter (YMB6094) with vector (pRS426 GAL1), or GALSCMiHA (pMB1306) grown in minimal media with galactose (2%) for 12 hours at 30°C and immunoprecipitation were done with α-Myc, and α-GST (mock) antibodies. Enrichment of Cse4p was determined using primers for DNA regions described in (A) above.

(TIF)

Figure S3 Centromeric enrichment of Scm3p is cell cycle regulated. A wild-type strain (RC154) with FLAG-tagged Scm3p expressed from its own endogenous promoter was used. Cells were grown in YPD to logarithmic (LOG) phase, synchronized with α-factor in G1, and released into YPD containing nocodazole (A), or synchronized with nocodazole in G2/M, and released into YPD containing α-factor (B). Samples were taken at time points (min) after release. (FACS): DNA content was determined by FACS analysis. (cell morphology): cell cycle stages were determined based on cell shape and nuclear position by microscopic examination of at least 200 cells for each time point. Numbers represent the percentage of cells in each of the categories (G1, S, M, A, T) as described in Materials and Methods. (ChIP): enrichment of Scm3p at CEN DNA was examined by chromatin immunoprecipitation using α-FLAG (Scm3p), and α-GST (mock) antibodies. The immunoprecipitated DNA fragments were purified and used as templates for traditional PCR using primers specific for CEN1 DNA. To determine the enrichment of Scm3p to CEN1 DNA, signals obtained from the immunoprecipitated DNA were divided by signals of the corresponding input DNA and normalized to the values from ACT1 (bottom DNA band in the gel images). The average enrichment from at least three independent experiments, with standard errors is shown. *p value <0.05, **p value <0.01, Student’s t test. Lanes: IN (input), IP (chromatin immunoprecipitated DNA with α-FLAG antibodies), and M (chromatin immunoprecipitated DNA with α-GST antibodies).

(TIF)

Figure S4 Expression of Scm3p is not cell cycle regulated. Western blot analysis was done on whole cell protein extracts prepared using samples used for Figure 2, which were taken at time points after release from G1. Western blots were probed with α-FLAG (Scm3p), α-HA (Pds1p), and α-Tub2p (loading control) antibodies.

(TIF)

Figure S5 Centromeric association of Cse4p through the cell cycle. A wild-type strain (JG595) with Myc tagged Cse4p expressed from its own endogenous promoter was grown in YPD, synchronized in G1 with α-factor, washed, and released into pheromone-free YPD medium. Samples were taken at time points (min) after release from G1. (A) DNA content was determined by FACS. (B) Cell cycle stages were determined based on cell
morphology and nuclear position by microscopic examination of 200 cells for each time point. (C) Enrichment levels of Scm3p at nuclear position by microscopic examination of 200 cells for each time point. (C) Enrichment levels of Cse4p at nuclear position by microscopic examination of 200 cells for each time point.

**Figure S6** Centromeric enrichment pattern of Scm3p expressed from a Gal1 promoter (Gal1SCM3) through the cell cycle. Wild-type strain expressing HA tagged SCM3 from a Gal1 promoter (RC100) was grown in minimal media with galactose (2%) at 30°C, treated with nocodazole for 2 hours to synchronize cells in G2/M, washed, and released into minimal media containing α-factor. Samples were taken at time points (min) after release from G2/M arrest. (A) DNA content was determined by FACS. (B) Cell cycle stages were determined based on cell morphology and nuclear position by microscopic examination of 200 cells for each time point. (C) Enrichment levels of SCM3 at CEN3. ChIP experiments were done using α-HA (Scm3p), and α-GST (mock) antibodies. The enrichment of Scm3p at CEN3 was determined by qPCR and is shown as % input. Average from at least three independent experiments ± standard error is shown. No significant differences in enrichment were observed among time points.

**Figure S7** Identification of DNA binding sequences of Scm3p. Predicted DNA binding sequences identified by computational analysis using BindN-RF and MEME software are shown in red and marked with an arrow (amino acids 1-103). Amino acids residues predicted to interact with DNA with high affinity are shown as brown color letters. Symbols, NES = nuclear export signal; HR = Cse4p interacting heptad repeat domain; acidic D/E region, and BR1 and BR2 are basic regions 1 and 2, respectively.

**Acknowledgments**

We are highly thankful to Carl Wu for sharing unpublished data on DNA-binding domain of Scm3p. We thank Jennifer Gerton, Sue Biggins, Vivian Measday, Orna Cohen-Fix, and Mitch Smith for strains and plasmids and Agnes Sahat for technical assistance. We are grateful to Michael Lichten and the members of the Barslab for useful discussions and comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: PKM REB DRF MAB. Performed the experiments: PKM W-CJ JSC PHK. Analyzed the data: PKM PHK DRF MAB. Contributed reagents/materials/analysis tools: REB. Wrote the paper: PKM REB DRF MAB.

**References**

1. Lengauer C, Kinzler KW, Vogelstein B (1998) Genetic instabilities in human cancers. Nature 396: 645–648.

2. Skibbens RV, Hieter P (1998) Kinetochores and the checkpoint mechanism that monitor defects in the chromosome segregation machinery. Annu Rev Genet 32: 307–337.

3. Elsworth K (2007) Epigenetic control of centromere behavior. Annu Rev Genet 41: 63–80.

4. Buscaino A, Allshire R, Pidoux A (2010) Building centromeres: home sweet home or a nomadic existence? Curr Opin Genet Dev 20: 118–126.

5. Cheeseman IM, Drubin DG, Barnes G (2002) Simple centromere, complex kinetochore: finding the spindle microtubules and centromeric DNA in budding yeast. J Cell Biol 157: 199–203.

6. Cleveland DW, Mao Y, Sullivan KF (2003) Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell 112: 407–421.

7. Hyman AA, Sorger PK (1995) Structure and function of kinetochores in budding yeast. Annu Rev Cell Dev Biol 11: 471–495.

8. Blower MD, Karpen GH (2001) The role of Drosophila Cid in kinetochore formation, cell-cycle progression and heterochromatin interactions. Nat Cell Biol 3: 730–739.

9. Buchwitz BJ, Ahmad K, Moore LL, Roth MB, Henikoff S (1999) A putative protein complex consisting of Ctf19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. Genes Dev 13: 1140–1155.

10. Chen ES, Saitoh S, Yanagida M, Takahashi K (2003) A cell cycle-regulated novel histone H4 mutant defective in nuclear division and mitotic chromosome transmission. Mol Cell Biol 23: 1175–1187.

11. Meluh PB, Yang P, Glowczewski L, Koshland D, Smith MM (1998) Cse4p is a subkinetochore component of the core centromere of Saccharomyces cerevisiae. Cell 94: 607–613.

12. Smith MM, Yang P, Santisteban MS, Boone PW, Goldstein AT, et al. (1996) A centromere-specific heptad repeat domain; acidic D/E region, and BR1 and BR2 are basic regions 1 and 2, respectively.

13. Ortiz J, Stemmann O, Rank S, Lechner J (1999) A putative protein complex consisting of Cid19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. Genes Dev 13: 1140–1155.

14. Meadav Y, Hailey DW, Pot I, Givan SA, Hyland KM, et al. (2002) Cid1p, the Mi6 budding yeast homolog, interacts with Mcm22p and Mcm16p at the yeast outer kinetochore. Genes Dev 16: 101–113.

15. Furuyama S, Biggins S (2007) Centromere identity is specified by a single centromeric nucleosome in budding yeast. Proc Natl Acad Sci U S A 104: 14706–14711.

16. Camahort R, Li B, Flores R, Swanson SK, Washburn MP, et al. (2007) Scm3p is essential to recruit the histone h3 variant cse4 to centromeres and to maintain a functional kinetochore. Mol Cell 26: 853–865.

17. Mizuguchi G, Xiao H, Wiusinski J, Smith MM, Wu C (2007) Nonhistone Scm3 and histones CenH3/H4 assemble the core of centromere-specific nucleosomes. Cell 129: 1153–1164.

18. Stoler S, Rogers K, Weitze S, Morey L, Fitzgerald-Hayes M, et al. (2007) Scm3, 14706–14711.

19. Mizuguchi G, Xiao H, Wiusinski J, Smith MM, Wu C (2007) Nonhistone Scm3 and histones CenH3/H4 assemble the core of centromere-specific nucleosomes. Cell 129: 1153–1164.

20. Zhang W, Mellen BG, Karpen GH (2007) A specialized nucleosome has a "point" to make. Cell 129: 1047–1049.

21. Zhou Z, Feng H, Zhou BR, Ghirlendo R, Hu K, et al. (2011) Structural basis for recognition of centromere histone variant CenH3 by the chaperone Scm3. Nature 474: 232–237.

22. Cho US, Harrison SC (2011) Recognition of the centromere-specific histone Cse4 by the chaperone Scm3. Proc Natl Acad Sci U S A.

23. Sauze-Paludo L, Pidoux A, Ponting CP, Allshire RC (2009) Common ancestry of the CENP-A chaperones Scm3p and HJURP. Cell 137: 1173–1174.

24. Hu H, Liu Y, Wang M, Fang J, Huang H, et al. (2011) Structure of a CENP-A-histone H4 heterodimer in complex with chaperone HJURP. Genes Dev 25: 502–516.

25. Foltz DR, Jansen LE, Bailey AO, Yates JR, 3rd, Bassett EA, et al. (2009) Centromere-specific assembly of CENP-A nucleosomes is mediated by HJURP. Cell 137: 472–484.

26. Shibaib M, Ouazarhni K, Dimitrov S, Hamiche A (2010) HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. Proc Natl Acad Sci U S A 107: 1349–1354.

27. Shibaib M, Ouazarhni K, Dimitrov S, Hamiche A (2010) HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. Proc Natl Acad Sci U S A 107: 1349–1354.

28. Camahort R, Shivaraju M, Mattingly M, Li B, Nakamichi S, et al. (2009) Cse4p is part of an octameric nucleosome in budding yeast. Mol Cell 35: 793–805.

29. Shivaraju M, Camahort R, Mattingly M, Gerton JLR (2011) Scm3p is a centromeric nucleosome assembly factor. J Biol Chem 286: 12016–12023.

30. Pidoux AL, Choi ES, Abbott JK, Liu X, Kagayusu B, et al. (2009) Fission yeast Scm3p is a CENP-A receptor required for integrity of subkinetochore chromatin. Mol Cell 33: 299–311.

31. Williams JS, Hayashi T, Yanagida M, Russell P (2009) Fission yeast Scm3p mediates stable attachment of Cnp1/CENP-A into centromeric chromatin. Mol Cell 33: 287–298.
32. Dunleavy EM, Roche D, Tagami H, Lacoste N, Ray-Gallet D, et al. (2009) HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. Cell 137: 485–497.

33. Bernad R, Sanchez P, Rivero T, Rodriguez-Cosino M, Boyarchuk E, et al. (2011) Xenopus HJURP and condensin II are required for CENP-A assembly. J Cell Biol 192: 569–582.

34. Castillo AG, Mellone BG, Partridge JF, Richardson W, Hamilton GL, et al. (2007) Plasticity of fusion yeast CENP-A chromatin driven by relative levels of histone H3 and H4. PLoS Genet 3: e121. doi:10.1371/journal.pgen.0030121.

35. Au WC, Crip MJ, DeLuca SZ, Rando OJ, Basrai MA (2008) Altered dosage and mislocalization of histone H3 and Cse4p lead to chromosome loss in Saccharomyces cerevisiae. Genetics 179: 263–275.

36. Heun P, Erhardt S, Blower MD, Weiss S, Skora AD, et al. (2006) Mislocalization of the Drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores. Dev Cell 10: 303–315.

37. Tomonaga T, Matsushita K, Yamaguchi S, Ohashi T, Shimada H, et al. (2003) Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. Cancer Res 63: 3511–3516.

38. Kato T, Sato N, Hayama S, Yamakuki T, Ito T, et al. (2007) Activation of Holliday junction recognizing protein involved in the chromosomal stability and immortality of cancer cells. Cancer Res 67: 8544–8553.

39. Hu Z, Huang G, Sadanandam A, Huang JH, Suen AL, et al. (2010) The expression level of HJURP has an independent prognostic impact and predicts the sensitivity to radiotherapy in breast cancer. Breast Cancer Res 12: R18.

40. Glowczewski L, Yang P, Kalashnikova T, Santisteban MS, Smith MM (2000) Histone-histone interactions and centromere function. Mol Cell Biol 20: 5700–5711.

41. Hesawazam G, Shrirajini M, Mattingly M, Venkatesh S, Martin-Brown S, et al. (2010) Phl1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. Mol Cell 40: 444–454.

42. Calvert ME, Lannigan J (2010) Yeast cell cycle analysis: combining DNA staining with cell and nuclear morphology.Curr Protoc Cytom Chapter 9: Unit 9.32 31–16.

43. Pearson CG, Yeh E, Gardiner M, Oddie D, Salmon ED, et al. (2004) Stable kinetochore-microtubule attachment constrains centromere positioning in metaphase. Curr Biol 14: 1968–1972.

44. Wang L, Yang MQ, Yang JY (2009) Prediction of DNA-binding residues from protein sequence information using random forests. BMC Genomics 10(Suppl 1): S1.

45. Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization. Biochemical and genetic analysis of the yeast Cse4p/CENP-A histone H3 variant. J Cell Biol 168: 971–972.

46. Eckert CA, Gravdahl DJ, Mege P (2007) The enhancement of pericentromeric cohesion association by conserved kinetochore components promotes high-fidelity chromosome segregation and is sensitive to microtubule-based tension. Genes Dev 21: 278–291.

47. McCarroll RM, Fangman WL (1988) Time of replication of yeast centromeres and telomeres. Cell 54: 505–513.

48. Collins KA, Furuyama S, Biggin S (2004) Proteolysis contributes to the exclusive centromere localization of the yeast Cse4p/CENP-A histone H3 variant. Curr Biol 14: 1968–1972.

49. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

50. Tong AH, Boone C (2006) Synthetic genetic array analysis in Saccharomyces cerevisiae. Methods Mol Biol 313: 171–237.

51. Jansen LE, Black BE, Foul EA, Cleveland DW (2007) Propagation of centromeric chromatin requires exit from mitosis. J Cell Biol 176: 785–795.

52. Barnhart MC, Kiss PH, Stellfox ME, Ward JA, Bassett EA, et al. (2011) HJURP is a CENP-A chromatin assembly factor sufficient to form a functional de novo kinetochore. J Cell Biol 194: 229–243.

53. Amato A, Schillaci T, Lentini I, Di Leonardo A (2009) CENPA overexpression promotes genome instability in pRb-depleted human cells. Mol Cancer 8: 119.

54. Spencer F, Gerring SL, Connelly C, Hieter P (1990) Mitotic chromosome transmission fidelity mutants in Saccharomyces cerevisiae. Genetics 124: 237–249.

55. Levk RJ, Schmitting TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

56. Tong AH, Boone C (2006) Synthetic genetic array analysis in Saccharomyces cerevisiae. Methods Mol Biol 313: 171–192.

57. Pot I, Knoock J, Aneliunas V, Nguyen T, Ah-Kye S, et al. (2005) Spindle checkpoint maintenance requires Ame1 and Okp1. Cell Cycle 4: 1448–1456.

58. Collins KA, Camahort R, Seidel C, Gerton JL, Biggin S (2007) The overexpression of a Saccharomyces cerevisiae centromeric histone H3 variant protein leads to a defect in kinetochore biorientation. Genetics 175: 513–525.

59. Tong AH, Boone C (2006) Synthetic genetic array analysis in Saccharomyces cerevisiae. Methods Mol Biol 313: 171–192.

60. Pot I, Knoock J, Aneliunas V, Nguyen T, Ah-Kye S, et al. (2005) Spindle checkpoint maintenance requires Ame1 and Okp1. Cell Cycle 4: 1448–1456.

61. Mumberg D, Muller R, Funk M (1994) Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res 22: 5767–5769.

62. Saltz LP, White MA, Wilkinson ML, Kon Y, Kung LA, et al. (2005) Transcriptional activity and their use for heterologous expression. Nucleic Acids Res 22: 5767–5769.