Sir protein–independent repair of dicentric chromosomes in *Saccharomyces cerevisiae*

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ABSTRACT Sir2 protein has been reported to be recruited to dicentric chromosomes under tension, and such chromosomes are reported to be especially vulnerable to breakage in *sir2Δ* mutants. We found that the loss of viability in such mutants was an indirect effect of the repression of nonhomologous end joining in Sir⁻ mutants and that the apparent recruitment of Sir2 protein to chromosomes under tension was likely due to methodological weakness in early chromatin immunoprecipitation studies.

INTRODUCTION

Sir proteins are necessary for the establishment and maintenance of heterochromatic gene silencing in the budding yeast *Saccharomyces cerevisiae* (reviewed in Rusche *et al.*, 2003; Kueng *et al.*, 2013). Sir2 is an NAD+-dependent protein deacetylase that primarily removes acetyl modifications from N-terminal tails of histones H3 and H4 (Imai *et al.*, 2000; Landry *et al.*, 2000a,b; Smith *et al.*, 2000). These deacetylated histone tails serve as high-affinity binding surfaces for Sir3 and Sir4 at telomeres and the silent mating loci (Hecht *et al.*, 1995). Sir-mediated heterochromatin formation represses transcription and recombination through the occlusion of factors necessary for these processes (Singh and Klar, 1992; Gottschling, 1992; Loo and Rine, 1994; Steakley and Rine, 2015). In many organisms, heterochromatin forms near and at the centromere, where it contributes to the fidelity of chromosome segregation (reviewed in Allshire and Karpen, 2008; Allshire and Ekwall, 2015).

The *SIR2*, *SIR3*, and *SIR4* genes encode proteins that assemble into a complex (Moazed *et al.*, 1997) that is essential for the proper regulation of cell type. Haploid yeast express a single mating-type locus allele, designated either *MATa* or *MATα*. Yeast also have additional copies of *MATa* and *MATα* genes at the *HMRa* and *HMLα* loci, respectively, which are transcriptionally silenced by the Sir proteins (Rine *et al.*, 1979; Rine and Herskowitz, 1987; Klar *et al.*, 1981).

Yeast strains with the *HO* allele can switch their mating type by undergoing a double-stranded DNA break at the *MAT* locus followed by gene conversion, with *MAT* being the recipient of genetic information from one of these silenced donor loci. *MATa* and *MATα* haploid cells can mate, producing an a/α diploid. Two proteins encoded by the *MAT* alleles, a1 and α2, form a heterodimer that represses haploid-specific genes, including some of those responsible for nonhomologous end joining, which is relevant to this study (Aström *et al.*, 1999; reviewed in Haber, 2012).

Unlike in *Schizosaccharomyces pombe* and most other eukaryotes, *Saccharomyces* centromeres have stood out as an exception due to their lack of heterochromatin. However, there is a report of a role for *S. cerevisiae* Sir2 in maintaining chromatin compaction when a chromosome is under artificial tension (Thrower and Bloom, 2001). This study used a strain with a second centromere whose function was made conditional by being under the control of the *GAL1* promoter (hereafter *GALCEN3* after Thrower and Bloom (2001)) 45 kb to the left of the endogenous centromere on chromosome III (Figure 1A). In cells grown in medium containing glucose, the promoter is inactive, and the conditional centromere is functional. In cells grown in medium containing galactose, the promoter is active, significantly inhibiting the function of this second centromere. The strain also contains a 10.1-kb *lacO* array between the centromeres and a *LacI-GFP* fusion gene to allow visualization of chromosome stretching during anaphase (Thrower and Bloom, 2001).

If microtubules emanating from opposite spindle poles attach to two functional centromeres on a single chromosome during mitosis, the chromosome will usually break (Brock and Bloom, 1994). Dramatic stretching of the chromosome is observed by microscopy in *sir2Δ* and *ku70Δ/80Δ* mutants before a break occurs. The extent of stretching was reported as consistent with near-complete loss of higher-order chromosome structure. In addition to stretching, *sir2Δ* and *ku70Δ/80Δ* mutants also suffered a significant drop in viability
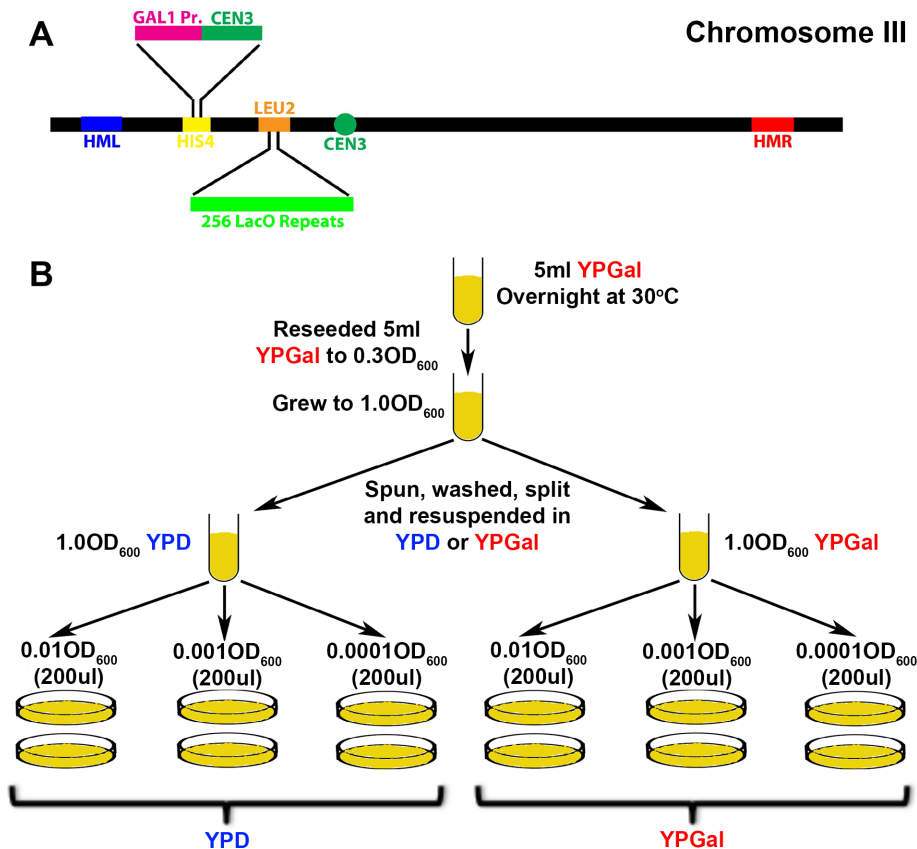
FIGURE 1: Experimental design. (A) Diagram of modified chromosome III. Conditional centromere downstream from *pGAL1* is functional in glucose and mostly nonfunctional in galactose medium. (B) Diagram of viability assay. Cells were grown to a density of 1.0 OD_{600} in YPGal medium. Cultures were split, washed, and resuspended in either YPD or YPGal medium. Serial dilutions of cultures were then plated on either YPGal or YPD medium. CFUs give a measure of viability on each medium.

when the second centromere was active. Chromatin immunoprecipitation (ChIP) with anti-Sir2 sera using primers complementary to the *lacO* array revealed enrichment of Sir2, but only under conditions in which the second centromere was active. No ChIP signal was observed when the second centromere was inactive, implying that Sir2 bound *lacO* chromatin only when it was under tension (Thrower and Bloom, 2001). This result was potentially of great interest, as it raised the possibility of condition-dependent assembly of Sir protein–based heterochromatin at repeated sequences and potentially at yeast centromeres when put under tension. While investigating this possibility further, we obtained results that offered a different explanation for key results from this study.

RESULTS

Sir 2/3/4 complex is necessary for preserving viability of dicentric cells

In addition to its role in heterochromatin gene silencing, Sir2 has a role independent of Sir3 and Sir4 in controlling the chromatin structure and recombination of rDNA. To determine whether Sir2 alone or the entire Sir2/3/4 complex was responsible for preserving viability of cells with dicentric chromosomes, we deleted *SIR3* and *SIR4* individually in a conditionally dicentric strain (KBY3615) and then measured the viability of these mutants alongside *sir2Δ* and *ku70Δ* mutants grown in both monocentric (galactose) and dicentric (glucose) conditions (Figure 1B). The viability of *sir3Δ* and *sir4Δ*

strains phenocopied that of the *sir2Δ and ku70Δ* mutants (Figure 2). Therefore the entire Sir2/3/4 complex was necessary to preserve viability of cells with a dicentric chromosome.

Sir 2/3/4 complex preservation of dicentric viability is an indirect effect of mating type

There were reports of Sir proteins having a role in DNA repair (e.g., Tsukamoto *et al.,* 1997), but these were proven to be indirect effects of mating type, since *sir* mutants have the properties of a/α diploids in which nonhomologous end joining is repressed (Aström *et al.,* 1999; Lee *et al.,* 1999). To test whether the vulnerability of dicentric chromosomes in *sir* mutants was also an indirect effect of mating type, we deleted *HML* in the *MATa sir3Δ* dicentric strain. This strain, despite being Sir⁻, expressed only a-specific cell-type genes. This deletion restored viability to wild-type levels (Figures 2 and 3). Sir2/3/4 repression of transcription at *HML* was therefore sufficient to prevent loss of viability in *MATa* cells with a dicentric chromosome.

Sir2 does not occupy chromatin between centromeres of a dicentric chromosome

We reevaluated the previously reported Sir2 enrichment at the *lacO* array during growth in glucose medium, when the dicentric chromosome would have the potential to create tension on the operator array, taking advantage of the enhanced sensitivity of

quantitative PCR (qPCR) over the endpoint-PCR analyses used previously (Thrower and Bloom, 2001). We fused a C-terminal 13x-myc tag to *SIR2* in the dicentric strain and used an anti-myc monoclonal antibody (Evan *et al.,* 1985), which we have used in multiple different studies to detect Sir2 with specificity and sensitivity, and in the context of the Sir2/3/4 complex, which our results indicated was relevant to these observations (Thurtle and Rine, 2014; Ellahi *et al.,* 2015). In addition to the lacO array, we measured enrichment of Sir2 at multiple other genomic positions that would experience tension in the dicentric state (Figure 4A). As a positive control, we observed 13x-myc-Sir2 enrichment at *HMLα1*, which was lost when cells were grown in 5 mM nicotinamide (NAM), an inhibitor of Sir2 (Figure 4B). Although Sir2 enrichment would not be expected for any experimental primer sets when cells were grown in galactose medium, enrichment would be expected in cells grown in glucose medium if Sir2 were recruited to chromatin under tension. Contrary to the results of the previous study, 13x-myc-Sir2 was not enriched at any of the regions tested in either galactose or glucose medium, with or without NAM (Figure 4, C–F). We observed inner kinetochore protein Mif2 enrichment at both centromeres but not at *HML*, demonstrating that our inability to detect 13x-myc-Sir2 at centromeres did not stem from a general inability to detect bound proteins in these regions by ChIP. The binding of Mif2 was unaffected by NAM treatment but increased upon activation of the conditional centromere *GALCEN3* by switching from galactose to dextrose medium
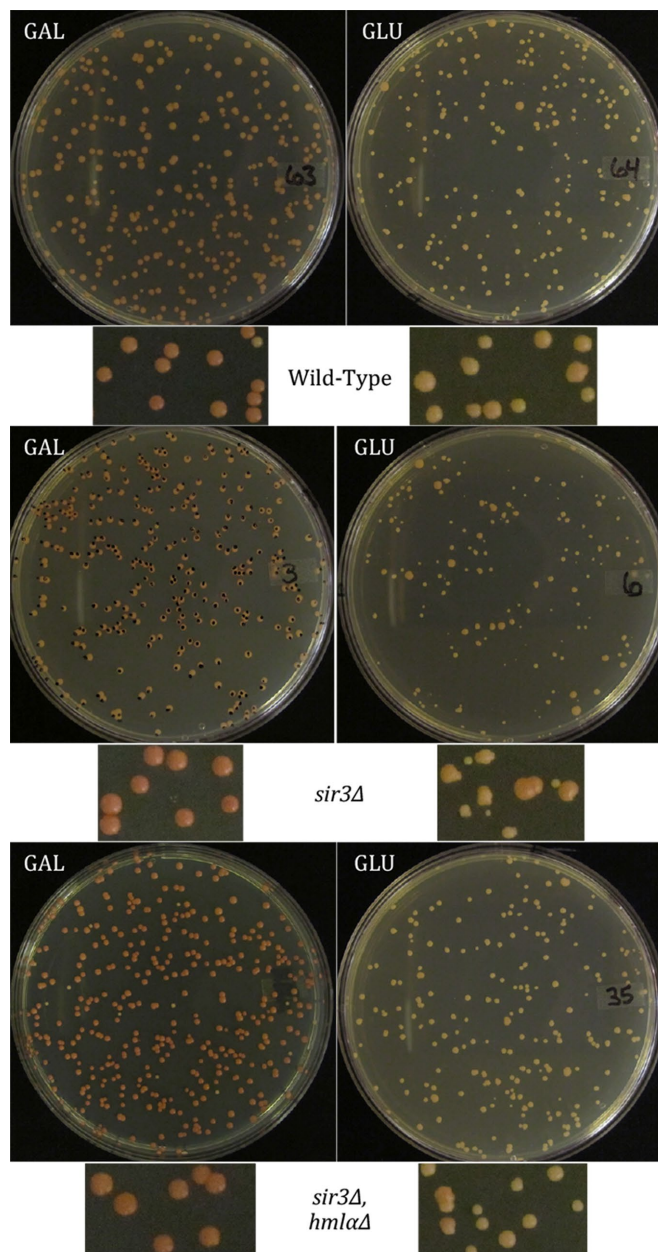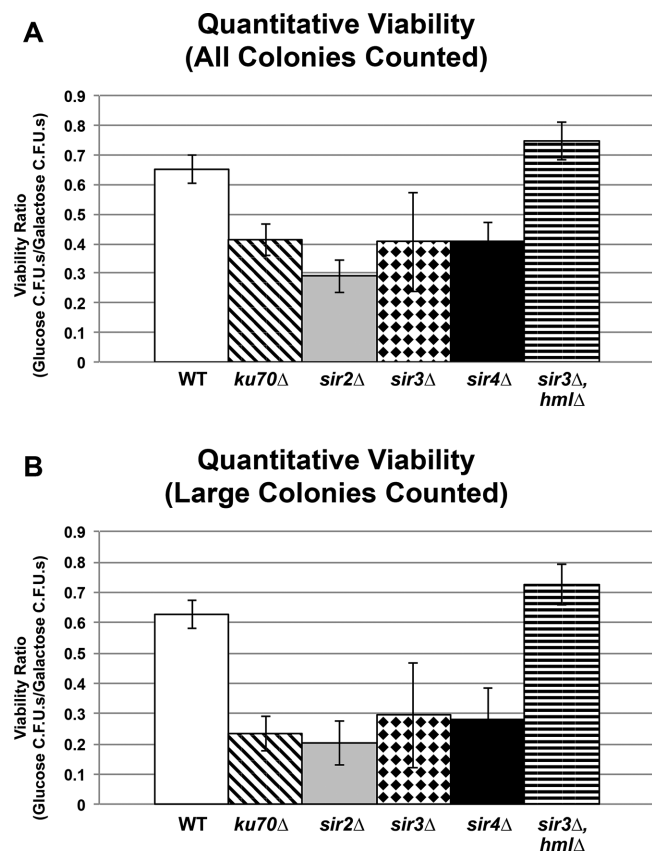
A

Quantitative Viability (All Colonies Counted)

B

Quantitative Viability (Large Colonies Counted)

FIGURE 2: Quantitative viability decreases in Sir⁻ mutants rescued by deletion of *HMLα*. Data represent CFUs in glucose medium, in which the second centromere is functional, normalized to CFUs in galactose medium, in which the second centromere is mostly nonfunctional. (A) All colonies, regardless of size, were counted. (B) Only large colonies were counted.

(Supplemental Figure S1). The dependence of Mif2 binding at *GALCEN3* on growth in dextrose was consistent with data showing that Mif2 is mostly evicted from a conditional centromere that has been inactivated by galactose-induced transcription (Collins *et al.*, 2005).

DISCUSSION

Double-strand DNA breaks can be repaired by either homologous recombination with a sister chromatid or nonhomologous end joining. Typically, homologous recombination is the preferred mechanism except for breaks that occur in haploids in G1, where there is no sister chromatid or homologous chromosome to guide repair (Aström *et al.*, 1999; Lee *et al.*, 1999). The repair of a dicentric chromosome broken by the forces of chromosome segregation is imperfectly amenable to homologous repair because the force that breaks a chromosome also moves the sister chromatid away from one of the two broken fragments. In addition, at some frequency, both of the sister dicentric chromatids would be broken in the same cell cycle. Hence, nonhomologous end joining would be expected to play a significant role in the repair of double-stranded DNA breaks created by missegregation of dicentric chromosomes.

The a/α diploid yeast and haploid Sir⁻ mutants that express both *HMLα* and *HMRa* repress nonhomologous end joining at least 20-fold (Aström *et al.*, 1999; Lee *et al.*, 1999), favoring repair via homologous recombination (Shrivastav *et al.*, 2008). As shown here and reported earlier (Thrower and Bloom, 2001), the viability of a

FIGURE 3: Loss of viability and colony phenotypes in Sir⁻ mutants rescued by deletion of *HMLα*. Left, strains grown on galactose medium, in which the second centromere should be mostly inactive. Right, strains grown on glucose medium, in which the second centromere should be active. *sir3Δ* strain (middle) is less viable than wild type (top), and colony size and shape change when grown on glucose medium, consistent with increased DNA damage. Deletion of *HMLα* in a *sir3Δ* background (bottom) restores these phenotypes to wild type. Close-up views of representative colonies are shown below.

strain with a dicentric chromosome was lost in cells lacking Sir protein function. However, rather than reflecting a role of Sir2 in controlling the probability that a chromosome would break under tension, our work established that preservation of viability by Sir protein function was mediated indirectly through the role of the Sir 2/3/4 complex in maintaining cell-type identity via repression of the mating-type genes at *HML* and *HMR*. This drop in viability was likely a result of repression of the nonhomologous end-joining repair pathway by the a1/α2 repressor in Sir⁻ mutants. Restoration of haploid
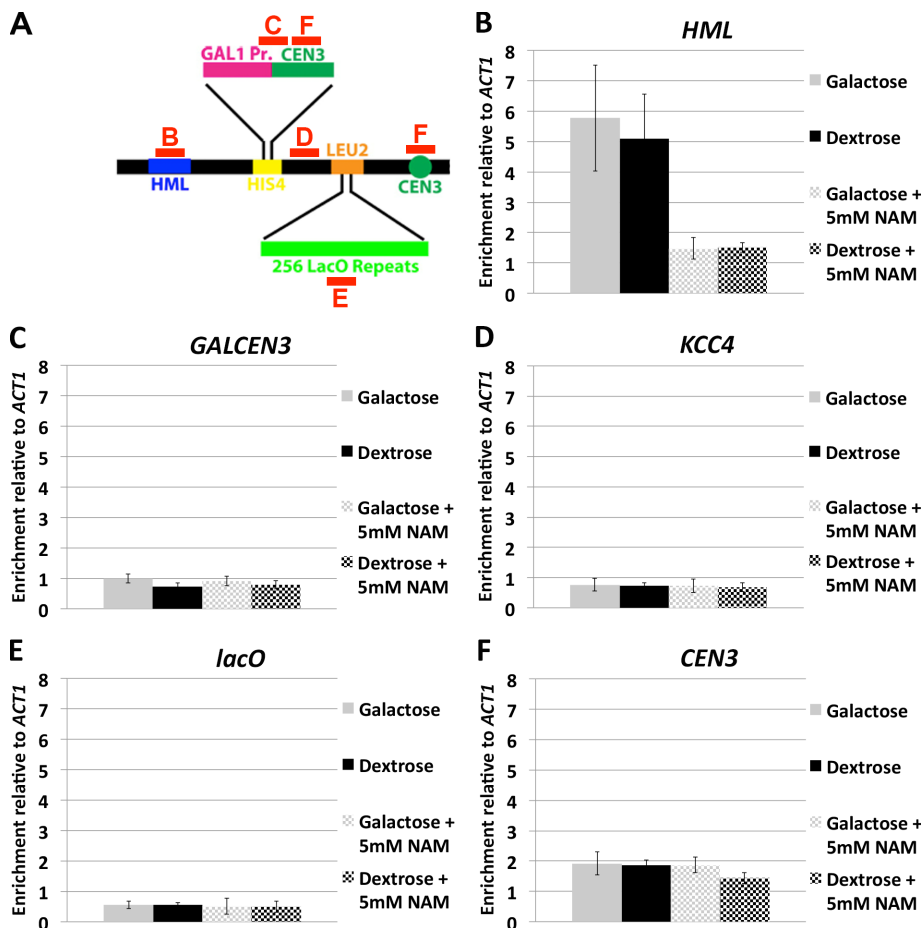
FIGURE 4: Sir2 did not occupy chromatin between centromeres of a dicentric chromosome. (A) Regions of chromosome III homologous to qPCR primers used in this figure. (B–F) 13x-myc-Sir2 enrichment using primer sets diagrammed in A was performed in glucose medium (second centromere functional) and galactose medium (second centromere nonfunctional) with or without 5 mM NAM. All enrichment values are normalized to *ACT1* primer set enrichment. Sir2 enrichment is not observed anywhere, regardless of the status of the conditional centromere. The appearance of an approximately twofold 13x-myc-Sir2 enrichment at *CEN3* in all samples is due to the *CEN3* primers amplifying both centromeres on chromosome III, not to Sir2 occupancy.

enrichment. The earlier work used primers that flanked a *lacO* sequence that is repeated 32 times in the 10.1-kb *lacO* array. In addition, the primers used in the earlier study were insufficiently specific, possibly as a result of the repetitive nature of the array. When we performed qPCR using these primers, the dissociation curve revealed their suboptimum performance, with multiple products of various sizes being amplified. Although it is not clear how many PCR cycles were used in the previous study, the presence of 32 *lacO* octamer repeats means that it might have been the equivalent of five cycles (2^5) too many.

In summary, the enhanced loss of viability of dicentric chromosomes in Sir⁻ mutants can be attributed to an indirect effect on the repression of nonhomologous end joining, and we found no evidence of recruitment of Sir2 to DNA under tension between two centromeres. As a final yet important point, we have no reason to doubt any of the microscopy data from the earlier work.

MATERIALS AND METHODS
Yeast strains and media

a-cell identity through deletion of *HMLα* restored viability in a Sir⁻ dicentric strain. In addition, loss of nonhomologous end joining in the *ku70Δ* mutant phenocopied the reduced viability of a *sir2Δ*, *sir3Δ*, or *sir4Δ* mutant. Taken together, the similarity of Sir⁻ and Ku⁻ phenotypes, the rescue of *sir3Δ* viability loss by deletion of *HML*, and the previously known repression of nonhomologous end joining by the a1/α2 heterodimer (Frank-Vaillant and Marcand, 2001) indicate that aberrant repression of nonhomologous end joining in Sir⁻ mutants was responsible for loss of viability.

The discrepancy between our finding that Sir2 could not be detected at regions of chromatin that were under tension and the earlier work is most likely due to methodological improvements over the intervening years. Our ChIP experiments used a more sensitive method than used previously yet failed to reveal enrichment of Sir2 between the potentially tensioned pair of centromeres in anaphase-enriched cultures under any experimental condition tested. Moreover, we failed to detect Sir2 enrichment at additional regions under tension, as well as at the *lacO* array, yet did detect robust Sir2 enrichment at *HMLα*, as expected. We suggest that the repetitive nature of the *lacO* array was responsible for the artifactual signal of Sir2

Quantitative viability of strains under dicentric conditions
Viability of dicentric strains was determined by plating equal numbers of galactose-grown cells (as measured by OD_{600} cell density) on both YPGal and YPD and counting colony-forming units (CFUs) after 4 d of growth. CFU count on YPD was normalized to CFU count on YPGal for each strain to measure relative viability under dicentric conditions.

ChIP and qPCR
Cells for ChIP experiments were grown and treated as previously described (Thrower and Bloom, 2001), taking care to collect cells enriched for anaphase after release from 20 μg/ml nocodazole. Anaphase enrichment was confirmed by microscopy, defined as >80% of cells with large buds and bilobed nuclei. The cells were fixed with 1% formaldehyde for 20 min and processed for ChIP analysis as previously described (Steakley and Rine, 2015) using EZview Red

Anti-c-Myc Affinity Gel (E6654-1ML; Sigma-Aldrich, St. Louis, MO) for pull down of 13x-c-myc-Sir2. Cultures containing 5 mM NAM were included as a negative control for Sir2 function (Landry *et al.*, 2000a,b). For pull down of kinetochore protein Mif2, chromatin was precleared with 45 µl of 50% (vol/vol) Protein A Sepharose CL-4B beads (17-0780-01; GM Healthcare, Uppsala, Sweden) for 1 h before being incubated overnight with polyclonal anti-Mif2 antibody (1:2000), generously provided by Arshad Desai (Ludwig Institute for Cancer Research, San Diego, CA; Akiyoshi *et al.* 2009). Chromatin was then incubated with 45 µl of 50% (vol/vol) Protein A Sepharose CL-4B beads for 3 h before being washed and treated as previously described (Steakley and Rine, 2015). qPCR of input and immunoprecipitated (IP) DNA was performed using Thermo Scientific DyNAmo HS SYBR Green PCR Kits (F-410L; Thermo Scientific, Waltham, MA) and a Stratagene Mx3000P qPCR system (Agilent Technologies, Waldbronn, Germany). Primers that detect *HMLα1* were used as a positive control for Sir2 binding. Primers that detect *KCC4*, *GAL-CEN3*, *CEN3*, and the *LEU2/lacO* junction were also used. Sequences of qPCR oligonucleotides are included in Supplemental Table S2. IP enrichment values were normalized to input enrichment values, as well as to relative enrichment using primers that detect *ACT1*, for which Sir2 has been shown to not bind [(IP(primer)/IN(primer)]/[IP(control)/IN(control)].

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