Co-Evolution of Transcriptional Silencing Proteins and the DNA Elements Specifying Their Assembly

Oliver A. Zill¹, Devin Scannell¹, Leonid Teytelman², Jasper Rine¹*

¹Department of Molecular and Cell Biology, and California Institute for Quantitative Biosciences, University of California–Berkeley, Berkeley, California, United States of America, ²Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, United States of America

Abstract

Co-evolution of transcriptional regulatory proteins and their sites of action has been often hypothesized but rarely demonstrated. Here we provide experimental evidence of such co-evolution in yeast silent chromatin, a finding that emerged from studies of hybrids formed between two closely related Saccharomyces species. A unidirectional silencing incompatibility between S. cerevisiae and S. bayanus led to a key discovery: asymmetrical complementation of divergent orthology of the silent chromatin component Sir4. In S. cerevisiae/S. bayanus interspecies hybrids, ChIP-Seq analysis revealed a restriction against S. cerevisiae Sir4 associating with most S. bayanus silenced regions; in contrast, S. bayanus Sir4 associated with S. cerevisiae silenced loci to an even greater degree than did S. cerevisiae’s own Sir4. Functional changes in silencer sequences paralleled changes in Sir4 sequence and a reduction in Sir1 family members in S. cerevisiae. Critically, species-specific silencing of the S. bayanus HMR locus could be reconstituted in S. cerevisiae by co-transfer of the S. bayanus Sir4 and Ko3 (the ancestral relative of Sir1) proteins. As Sir1/Ko3 and Sir4 bind conserved silencer-binding proteins, but not specific DNA sequences, these rapidly evolving proteins served to interpret differences in the two species’ silencers presumably involving emergent features created by the regulatory proteins that bind sequences within silencers. The results presented here, and in particular the high resolution ChIP-Seq localization of the Sir4 protein, provided unanticipated insights into the mechanism of silent chromatin assembly in yeast.

Introduction

Among all specialized chromatin structures, the difference between heterochromatin and euchromatin is perhaps the most fundamental, motivating intense study of the differences between these two structures. DNA sequences within heterochromatic regions evolve rapidly in animals [1,2], plants [3,4], and fungi [5], presenting a paradox of how the specification of heterochromatin structure persists despite rapid changes in the underlying sequence [6]. In Saccharomyces the biology of heterochromatin has proven eminently accessible to genetic studies through its role in gene silencing [7], and comparative studies of silencing now seem poised to illuminate key processes that underlie heterochromatin evolution.

Molecular co-evolution of transcriptional regulatory proteins with their sites of action has been proposed to maintain regulatory functions across species divergence [8,9]. In this context, “co-evolution” is typically understood as compensatory changes in a DNA sequence motif and the DNA-binding domain of the cognate transcription factor. Although it has been suggested that such co-evolution is prevalent in nature [8], in only a few instances has it been directly tested [10–12]. In Dipteran insects, for example, co-evolution of bicaud binding sites in the hunchback promoter and the bicaud homeodomain has been proposed to maintain hunchback-mediated developmental patterning along the anterior/posterior axis in Musca and Drosophila [13,14]. However, the large size and complexity of animal regulatory elements, and the difficulty of performing cross-species complementation tests in animals, have precluded clear distinction between regulatory divergence and bona fide co-evolution.

Transcriptional silencing by Sir (Silent Information Regulator) proteins is necessary for the specialized haploid mating-type system found in Saccharomyces [15,16]. DNA regulatory elements termed “silencers” contain binding sites for the Origin Recognition Complex (ORC), Rap1, and Abf1, which in turn direct the assembly of silent chromatin structures at the HML and HMR loci. The current model for the establishment of silencing holds that a Sir2/Sir3/Sir4 complex is brought to silencers by protein-protein interactions between ORC and Sir1, and between Rap1 and Sir4 [7]. Upon nucleation of these complexes, silent chromatin formation is catalyzed by the histone deacetylase activity of Sir2, and propagated, at least in part, through interactions between Sir3 and newly deacetylated histone tails [17–19]. Sir proteins are not thought to bind specific DNA sites; instead, efficient nucleation of silencing complexes at silencers requires interactions between Sir1 and Sir4, bridging the ORC-Sir1 and Rap1-Sir4 interactions [20]. Silencing also occurs at telomeres, which recruit Sir proteins primarily through arrays of Rap1 binding sites within the terminal
Author Summary

As eukaryotic species evolve, transcriptionally silent portions of their genomes—termed “heterochromatin”—mutate rapidly. To maintain the “off” state of certain genes in silenced regions, regulatory DNA sequences called silencers, which reside within a rapidly mutating region, must co-evolve with the regulatory proteins that bind these sequences to turn off transcription. Although hypothesized to occur widely in nature, such “molecular co-evolution” of genetic regulators has been demonstrated in only a few cases. Unlike previous examples of gene regulatory co-evolution, we found that the transcription factors that bind silencers in two budding yeast species are not, in fact, functionally interchangeable, even though the silencers are not. Surprisingly, the Sir1 and Sir4 silencing proteins, which are heterochromatin components that bind the transcription factors rather than the silencer DNA sequences per se, are the proteins engaged in rapid co-evolution with the silencers. Silencer sequences therefore contain additional, evolutionarily labile information directing the assembly of heterochromatin. As mutations in Sir1 and Sir4 over evolutionary time can compensate for changes in the silencers, this “extra information” likely involves cooperative assembly of the transcription factors with the Sir1 and Sir4 “adapter” proteins. The localization patterns of two species’ Sir4 proteins across both species’ genomes in interspecies yeast hybrids illuminate unexpected features of heterochromatin structure and assembly.

Results

An Incompatibility between S. cerevisiae Sir4 and S. bayanus HMR Revealed by Genetic Analysis of Interspecies Hybrids

In the course of a genetic screen for S. bayanus silencing mutants, we discovered that S. cerevisiae Sir4 failed to complement S. bayanus sir4A mutants for silencing of both HML and HMR, but S. cerevisiae Sir2 and Sir3 complemented mutations in S. bayanus orthologs (Figure S1; Zill et al. in preparation). This result was unanticipated as there are many cases of human proteins that can replace their yeast counterparts, even for proteins that function in large complexes and have considerably more sequence divergence than that seen between S. cerevisiae and S. bayanus proteins [31–34]. The incompatibility was unidirectional as S. bayanus Sir2, Sir3, and Sir4 complemented S. cerevisiae sir2A, sir3A, and sir4A, respectively.

Importantly, Sir4 functional divergence was due to one or more coding changes, as the level of expression of the two Sir4 orthologs, measured at either the RNA or protein level, was equivalent (Figure S2). To assay the function of both species’ silencing machineries in the same cellular milieu, we developed a highly sensitive transcriptional reporter assay in S. cerevisiae/S. bayanus interspecies hybrid diploids that allowed us to monitor silencing of each species’ HMR locus (hereafter referred to as Sc-HMR or Sb-HMR). The reporter consisted of the K. lactis URA3 open reading frame placed under the control of the endogenous HMR1 promoter of each species, in two separate, but otherwise isogenic, hybrid strains (Figure 2A).

In these hybrids the S. cerevisiae Sir4 (Sc-Sir4) allele could not, on its own, silence Sb-HMR (Figure 2A, row 2). Reduced dosage of Sir4 per se did not cause loss of silencing at Sb-HMR, as S. bayanus diploids with only one copy of Sb-Sir4 showed no silencing defect (Figure 2B, row 2), nor did S. cerevisiae diploids with only one copy of Sc-Sir4 (unpublished data). Furthermore, a hybrid diploid containing two copies of Sc-Sir4 (the Sb-Sir4 gene was replaced by Sc-Sir4) also failed to silence Sb-HMR (Figure 2A, row 5). In contrast, one Sc-Sir4 gene was able to silence Sc-HMR in all hybrid strains tested (Figure 2A, bottom panel; Figure 3A). Thus, the hybrid cellular environment did not interfere with Sc-Sir4 function, and within a species, Sir4 was not haplo-insufficient. It appeared that Sc-Sir4 was either inhibited at Sb-HMR by something encoded by the S. bayanus genome specifically or somehow failed to interact with proteins that promoted Sb-HMR silencing.

Transcription analysis of a critical set of the hybrid strains showed good correspondence between expression of the HMR::URA3 reporter and growth patterns observed on FOA and CSM/-Ura media (Figure 3B).

We note that in the interspecies hybrids with both species’ Sir4 alleles (Sc-Sir4/Sb-Sir4), Sb-HMR silencing appeared weakly defective relative to the complete silencing of Sc-HMR in S. bayanus diploids by both the reporter assay and direct RNA measurement (compare Figure 2A, row 1 with Figure 2B, row 1; Figure 3B). In addition, Sb-HMR silencing was further weakened in hybrids lacking Sc-Sir4 (Figure 2A, compare row 3 with row 1). This result was paradoxical because Sc-Sir4 appeared to have very little ability to silence Sb-HMR in hybrids lacking Sc-Sir4. As explained below, these weak Sb-HMR silencing defects were likely due to an emergent property of the hybrids, resulting from unusually strong interactions between Sc-Sir4 and S. cerevisiae silent loci that effectively reduced Sb-Sir4 associations with Sb-HMR. The presence of Sc-Sir4 limited the competition for Sb-Sir4.

repeats [TG₅₋₆] [21]. However, more complex regulatory elements reside near the terminal repeats, and at some telomeres these may also serve to recruit Sir proteins [22,23].

We have recently shown that silencer elements are among the most rapidly evolving regulatory sequences in Saccharomyces genomes [5]; however, the regulatory proteins that directly bind silencers are highly conserved, essential proteins. Intriguingly, the Sir1 and Sir4 proteins parallel the silencers in their rapid evolution, but these proteins show distinct patterns of evolution. Sir1-related genes have undergone multiple duplication and loss events: for example, S. bayanus has four functional paralogs of the single S. cerevisiae Sir1 gene, including the ancestral Kos3 (Kin Of Sir1) paralog, which S. cerevisiae has lost along with two other paralogs (Figure 1A) [24]. In contrast, the Sir4 protein is among the 40 most diverged proteins between S. cerevisiae and S. bayanus (Figure 1B), with 45% identity between its orthologs relative to a genome-wide average of 83% identity [25,26]. Although Sir2 and Sir3 each has a paralog resulting from the whole-genome duplication (Sir2 has three additional, more ancient paralogs), neither gene has experienced subsequent duplication or loss events [27,28].

S. cerevisiae and S. bayanus are post-zygotically isolated—haploids of these two species can mate to form mitotically stable hybrid diploids, but meiotic spores derived from these diploids are usually inviable [29,30]. The rapid evolution of the silencers, the Sir4 protein sequence, and the elaboration of Sir1 paralogs make these two species an excellent phylogenetic context for comparative studies of silencing. Here, we describe functional studies in S. cerevisiae/S. bayanus interspecies hybrids that demonstrated how co-evolution among two heterochromatin proteins, Sir1 and Sir4, and multiple silencer DNA elements allowed two divergent lineages to maintain robust silencing despite these rapid genetic changes. This example of regulatory co-evolution is of particular interest because the co-evolving proteins are not the agents that directly bind to the divergent regulatory DNA sites.
Figure 1. Comparative analysis of Sir proteins in *S. cerevisiae* and *S. bayanus*. (A) Comparison of the Sir protein complements of *S. cerevisiae* and *S. bayanus*. Percent identity (ID) and similarity (sim) for each orthologous pair of proteins, as determined by BLASTP alignments, is indicated above each *S. bayanus* ortholog. Protein lengths in numbers of amino acids (a.a.) are given to the right of each schematic. Black boxes indicate known domains within each protein (to approximate scale), with domain names indicated below the *S. cerevisiae* orthologs. OIR, ORC-Interacting Region; BAH, Bromo-Adjacent Homology domain; PAD, Partitioning and Anchoring Domain; CC, Coiled-Coil. (B) Percent identities of orthologous *S. cerevisiae* and *S. bayanus* proteins as reported by BLASTP. Histogram shows percent identities of orthologous *S. cerevisiae* and *S. bayanus* proteins based on
Conditional Association of Sc-Sir4 with *S. bayanus* HML and HMR

The inability of Sc-Sir4 to function at *Sb-HML* and *Sb-HMR* could have been manifested either during its recruitment or after its assembly into chromatin [35]. To determine where in the assembly of *S. bayanus* silenced chromatin Sc-Sir4 protein was blocked, we compared the ability of Sc-Sir4 and Sb-Sir4 proteins to associate with all silent loci of both species at high resolution using chromatin-immunoprecipitation followed by deep-sequencing of the precipitate (ChIP-Seq). Sir4 ChIP-Seq was performed using hybrid diploids expressing Sc-Sir4 only, Sb-Sir4 only, or both Sc-Sir4 and Sb-Sir4. Because of the sequence divergence between HML and HMR of the two species, the occupancy of each species' HML and HMR loci could be evaluated simultaneously. In each strain, only one SIR4 allele carried a 13xMyc epitope tag [36]. In hybrids expressing Sc-Sir4 only, robust enrichment of Sc-HML and Sc-HMR silencers was observed as expected, with very weak enrichment of Sb-HML and Sb-HMR silencers (Figure 4A, Table 1). Strikingly, Sc-Sir4 association with an internal region of Sb-HMR was indistinguishable from non-silenced regions. In contrast, as predicted from the genetic results, Sb-Sir4 associated robustly with HML and HMR loci from both species, and did so most robustly at *S. cerevisiae* silencers (Figure 4A, Table 1). The ChIP-Seq results were validated at Sc-HMR, Sb-HMR, and control loci using standard ChIP-qPCR analysis (Figure 5A). Thus, Sc-Sir4 showed strongly reduced association with Sb-HML and Sb-HMR silencers and no detectable association with their internal regions. The relative absence of Sc-Sir4 from these normally silenced regions of the *S. bayanus* genome was consistent with two possibilities. Perhaps Sc-Sir4...
Sir4 could not interact properly with Rap1, ORC, or the S. bayanus Sir1 paralogs assembling on their silencers, or perhaps an S. bayanus protein was preventing stable association between Sc-Sir4 and S. bayanus silencers. The comparative Sir4 ChIP-Seq data provided a surprising insight into the mechanism of Sir4 incorporation into silent chromatin. Although Sc-Sir4 binding to Sb-HML and Sb-HMR loci was barely detectable in hybrids expressing Sc-Sir4 only (Figure 4A, center and right panels; Table 1), in hybrids expressing both Sc-Sir4 and Sb-Sir4, Sc-Sir4 binding increased substantially at Sb-HML and Sb-HMR silencers and internal regions (Figure 4A; Table 1). Thus, despite the poor ability of Sc-Sir4 to associate with Sb-HML and Sb-HMR on its own, Sb-Sir4 somehow provided Sc-Sir4 access to them. It appeared that Sir4 association with S. bayanus HML and HMR involved two distinguishable modes of interaction, but Sc-Sir4 was capable of only one (Sb-Sir4 dependent). Moreover, the divergent mode was apparently critical only for the initial association of Sir4 with a silencer, and not for subsequent associations with the silenced region. The ability of Sc-Sir4 to form dimers suggested one straightforward explanation for the Sb-Sir4-dependent chromatin association: inter-specific Sc-Sir4/Sb-Sir4 dimerization through a conserved coiled-coil domain [37,38].

Sb-Sir4-assisted incorporation of Sc-Sir4 into Sb-HML and Sb-HMR was consistent with Sc-Sir4 contributing to silencing at these loci, as suggested by the decreased Sb-HMR silencing in hybrids lacking Sc-Sir4 (Figure 2A, row 3). However, this hypothesis per se could not explain the sensitivity of Sb-HMR silencing to reduced Sb-SIR4 dosage that was observed in interspecies hybrids, but not in S. bayanus diploids (compare Figure 2A, rows 1 and 3, with Figure 2B, row 2; Figure 3B). Further analysis of Sir4 localization on the S. cerevisiae/S. bayanus hybrid genome by ChIP-Seq provided an explanation of this hybrid-specific Sb-SIR4 dosage sensitivity, as described next.

Figure 3. Further characterization of the silencing incompatibility. (A) Sc-SIR4 was unable to silence Sb-HMR in S. bayanus. Top row: Silencing of Sb-HMR::URA3 in an S. bayanus haploid strain bearing Sc-SIR4 integrated in place of Sb-SIR4. Bottom row: Control showing that the Sb-sir4D::Sc-SIR4 replacement allele could supply silencing function to Sc-HMR in an S. cerevisiae/S. bayanus hybrid. (B) RNA analysis of HMR::URA3 reporters in S. cerevisiae/S. bayanus hybrids and S. bayanus diploids. URA3 amplification values were normalized to those of actin (ACT1) for each strain. Error bars show standard deviations (n = 3).

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Differential Association of the Two Sir4 Proteins with Native Telomeric Regions: Sb-Sir4 Sequestration by S. cerevisiae Subtelomeres

Given the differential association of Sc-Sir4 and Sb-Sir4 with the two species’ HML and HMR loci, we asked if any other loci,
genome-wide, also showed a dramatic discrepancy. In *S. cerevisiae*, silencing by Sir proteins occurs at telomeres and subtelomeres, in addition to *HML* and *HMR* [18,39,40]. A comparison of the interspecies hybrids expressing Sc-Sir4 only versus Sb-Sir4 only showed that all *S. cerevisiae* TG1–3 terminal repeats (which contain embedded Rap1 binding sites), including those present on the centromere-proximal side of some Yₚ₉ elements, were comparably occupied by both species’ Sir4 proteins (Figure 4B). (Yₚ₉ elements are helicase-encoding repetitive sequences of unknown origin and function that are found in some subtelomeric regions immediately adjacent to the terminal repeats [22].) This result was not surprising as the telomerase-replicated repeated sequence, templated by the *TLC1* RNA, is identical in the two species (our unpublished observations). Thus, it appeared that Sir4 association with the *S. cerevisiae* genome, as promoted by Rap1, was not substantially different between Sc-Sir4 and Sb-Sir4. Indeed, the C-terminal residues of Sc-Sir4 critical for its interaction with Rap1 are conserved in Sb-Sir4 (our unpublished observations). We note that the smaller ChIP-Seq peaks observed in these regions in the “No tag” control strain (Figure 4B, yellow shading) are likely due to non-specific DNA binding to the anti-myc beads.

Unexpectedly, *S. cerevisiae* subtelomeres had two types of regions notably more enriched by Sb-Sir4 ChIP than by Sc-Sir4 ChIP. These regions corresponded to X elements, which are regulatory sequences near telomere ends that contain ORC and Abf1 binding sites [22], and the ORFs within Yₚ₉ elements. For X elements, ChIP-Seq of Sc-Sir4 showed an average of 7-fold enrichment, whereas Sb-Sir4 showed an average of 14-fold enrichment, with even greater disparity often evident immediately adjacent to X elements (Figure 4B). Therefore, Sb-Sir4 either associated more robustly with factors bound to X elements than did Sc-Sir4 or conceivably was excluded less effectively. X element core

Figure 4. Sc-Sir4 versus Sb-Sir4 ChIP-Seq analysis in *S. cerevisiae*/S. bayanus hybrids. (A) Left: Sir4 IP/Input ratios, normalized to control regions within each experiment, for the Sc-HMR-E silencer. Center: Normalized IP/Input ratios for the Sb-HMR-E silencer. Right: Normalized IP/Input ratios for the Sb-HMR::URA3 ORF. “Sc-Sir4” or “Sb-Sir4” labels indicate which species’ Sir4 protein was examined by ChIP. Species’ identities of both SIR4 alleles in each strain are given in parentheses, with the allele bearing the 13x-Myc tag indicated in red: (Sc/Sc), JRY9062; (Sb/Sb), JRY9063; (Sc/Sb), JRY9064 (see Table S1 for complete strain genotypes). Dashed lines indicated IP/Input ratio of non-silenced control regions. Error bars indicate the standard error of the mean of all 100 bp windows covering a region. See Table 1 for non-normalized IP/Input ratios and Methods for a description of data processing. (B) ChIP-Seq profiles of Sc-Sir4 (JRY9062), Sb-Sir4 (JRY9063), and the “No tag” control (JRY9054) at two *S. cerevisiae* telomere regions. The ratio of IP/Input read counts for each base of a telomeric region is plotted. Diagrams indicate salient genetic features of two telomeres (see key at left) with X elements (yellow boxes), Yₚ₉ elements, and terminal repeats (TR) containing Rap1 binding sites, labeled above. TELXV-L (left panel) has an X-element-only end, whereas TELVIII-R (right panel) has an X-Yₚ₉ end. The TELVIII-R Yₚ₉ element spans nucleotide positions 556986–562456, with two helicase-encoding ORFs located between positions 558014 and 562047 (www.yeastgenome.org). For the ORFs within this Yₚ₉ element, Sc-Sir4 had a mean IP/Input ratio of 1.2, and the “No tag” control had a mean IP/Input ratio of 0.9 (the mean IP/Input ratio for all non-silenced regions, genome wide, was approximately 0.7 for both Sc-Sir4 and Sb-Sir4 ChIPs).

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Table 1. Average IP/Input ChIP-Seq values for selected regions of the *S. cerevisiae/S. bayanus* hybrid genome.

| Region       | Sc-Sir4 (Sc*/Sc) | Sb-Sir4 (Sb*/Sb) | Sc-Sir4 (Sc*/Sb) | Sb-Sir4 (Sc/Sb*) |
|--------------|------------------|------------------|------------------|------------------|
| Sc-HML-E     | 12.49            | 17.99            | 8.59             | 10.07            |
| Sb-HML-E     | 2.75             | 13.78            | 6.11             | 8.28             |
| Sc-HMR-E     | 18.58            | 45.98            | 12.44            | 14.51            |
| Sb-HMR-E     | 1.04             | 7.33             | 2.85             | 4.83             |
| Sc-HMRa1     | 1.76             | 3.33             | 1.16             | 1.39             |
| Sb-HMR::URA3 | 0.44             | 1.86             | 1.04             | 1.11             |
| Sc-Control   | 0.54             | 0.68             | 0.47             | 0.40             |
| Sb-Control   | 0.56             | 0.38             | 0.22             | 0.21             |

Each genetic element indicated at left represents a 600 bp region containing a silencer, an ORF inside an HMR locus, or a non-silenced control region. These control regions were located 3 kb to the left of HMR-E in either species’ genome and correspond to syntenic regions of both species’ YCR095C genes. Asterisks in column headers indicate which SIR4 allele bears the 13x-myc tag. Strains used in this analysis, from left to right: JRY9062, JRY9063, JRY9064, JRY9065.

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Figure 5. Additional comparative Sir4 ChIP and expression analyses. (A) ChIP-qPCR analysis of Sc-Sir4 versus Sb-Sir4. For each primer set, the IP/Input ratios for Sc-Sir4 (JRY9062), Sb-Sir4 (JRY9063), and the “No-tag” control (JRY9054) are shown. Error bars show standard deviations (n = 3). (B) ChIP-Seq analysis of Sc-Sir4 versus Sb-Sir4 association on an *S. bayanus* contig containing subtelomeric sequence (GenBank accession number AACG02000166). Hybrid strains used in this analysis were identical to those used in Figures 4B and 5A: Sc-Sir4, JRY9062; Sb-Sir4, JRY9063; No tag, JRY9054. Per-base IP/Input ratios, determined as in Figure 4B, are plotted versus contig position. We note that the terminal TG1-3 repeats are not present in the current *S. bayanus* genome assembly [77]. (C) RNA expression analysis of putative *S. bayanus* subtelomeric genes. Quantitative RT-PCR was performed on RNA isolated from *S. bayanus* wild-type (Sb-SIR4), Sb-sir4::Sb-SIR4 (Sc-SIR4), or sir4A strains. The actin gene (ACT1) served as a euchromatic control gene. Error bars show standard deviations (n = 3).

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sequences (containing the ORC and Abf1 binding sites) are bordered on the telomere-proximal side by X-element combinatorial repeats (formerly known as subtelomeric repeats or STRs; [22]) and the terminal repeats (see http://www.yeastgenome.org/images/yeastendfigure.html for schematics depicting X-only and X-Y telomere ends). The differential pattern of Sir4 association with X elements was consistent with Sb-Sir4 associating more robustly than Sc-Sir4 with sequences at, and immediately adjacent to, the ORC binding sites, presumably via ORC-mediated interactions (Figure 4B). Other S. bayanus proteins produced in the hybrids, such as the Sir1 paralogs, may contribute to the enhanced association of Sb-Sir4 with X elements, as discussed below.

We observed weak Sc-Sir4 association with Y’ elements despite its strong association with neighboring terminal repeats (Figure 4B, right panel), consistent with earlier observations using ChIP-chip and transcription reporter analyses [23,41]. Surprisingly, Sb-Sir4 associated considerably better than Sc-Sir4 with all Y’ elements, showing an average of 5-fold enrichment across their coding regions by Sb-Sir4 ChIP versus 1.2-fold enrichment by Sc-Sir4 ChIP. We note that the S. bayanus genome lacks Y’ elements, and thus S. bayanus subtelomeres may have reduced Sir4 recruitment potential relative to S. cerevisiae subtelomeres [42,43]. Thus, the enhanced associations of the Sb-Sir4 protein with X and Y’ elements suggested that, in the hybrid strains, S. cerevisiae telomeres might have competed with Sb-Sir4 association with Sb-HML and Sb-HMR, leading to the somewhat weakened Sb-HMR silencing observed in hybrids with only one copy of Sb-Sir4 (Figure 2A, rows 1 and 3; Figure 3B). Sb-Sir4 association was indeed reduced at Sb-HMR and Sb-HML silencers in a hybrid expressing only one copy of Sb-SIR4, relative to a hybrid with two copies of Sb-SIR4 (Table 1, compare columns 2 and 4). Thus, Sc-Sir4 may have, in effect, protected Sb-HMR silencing in hybrids when Sc-Sir4 was present (Figure 2A, compare rows 1 and 3) by occupying sites at S. cerevisiae telomeres that would otherwise have been bound by Sb-Sir4. (Although the S. cerevisiae Y’ elements are bound by Sb-Sir4 and not by Sc-Sir4 in cells with only a single species’ Sir4, in the Sb-SIR4/Sb-SIR4 hybrids, Sc-Sir4 and Sb-Sir4 both occupy Y’ elements (unpublished data). However, the extent of occupancy by Sb-Sir4 is less than in cells with Sb-Sir4 only, consistent with Sc-Sir4’s ability to spare Sb-Sir4 binding to Y’ elements in the hybrids.)

The ChIP-Seq data allowed us to determine whether the species restriction to Sc-Sir4 association, evident at Sb-HML and HMR, also applied to S. bayanus telomeres. Although subtelomeric regions of the S. bayanus genome are presently incompletely assembled and annotated (see Saccharomyces Genome Database, www.yeastgenome.org), we identified several candidate subtelomeric contigs based on homology to S. cerevisiae subtelomeric genes and X elements. Contigs from the S. bayanus genome assembly that contained regions bound by both Sc-Sir4 and Sb-Sir4 (as determined by peak-calling software, see Methods) and putative subtelomeric sequence were further examined for Sir4 ChIP enrichment (an example is shown in Figure 5B). Sb-Sir4 associated with one end of each of these contigs and usually with an internal region as well, typically within 10 kb of the contig end. Interestingly, in the Sc-Sir4-only hybrid, Sc-Sir4 association was observed at the contigs’ ends, but not at the internal regions that bound Sb-Sir4. This result suggested that Sc-Sir4, even in the absence of Sb-Sir4, was capable of associating with S. bayanus telomere ends, presumably via the conserved Rap1 protein, but could not make some additional contacts necessary to associate with internal sequences.

To test whether the Sc-Sir4 molecules bound to S. bayanus telomeres were capable of silencing S. bayanus subtelomeric genes, we measured the transcription of candidate subtelomeric ORFs in S. bayanus wild-type, Sb-sir4A::Sc-SIR4, and Sb-sir4A strains. Importantly, the expression of all three putative subtelomeric genes increased in Sb-sir4A cells (Figure 3C). Although Sc-Sir4 was capable of silencing Sb-TIR039c and an ORF located on Contig_626, it could not repress the transcription of an ORF on Contig_511 located almost 9 kb from the main peak of Sc-Sir4 ChIP. Thus, Sc-Sir4 could bind to and silence at least a subset of S. bayanus telomeric regions. It was possible that S. bayanus had subtelomeric regulatory elements that promoted silencing, in addition to the Rap1-binding terminal repeats. Depending on the sequence of a particular element, or its proximity to the telomere end, Sc-Sir4 may or may not have been capable of binding and silencing.

The Sb-HMR Silencers Mediated the Species Restriction of Sc-Sir4

The cross-species complementation and ChIP analyses suggested that the incompatibility between Sc-SIR4 and Sb-HML and HMR was caused by the failure of one or more physical interactions occurring at S. bayanus silencers. In principle, the lack of productive Sc-Sir4 association with Sb-HML and Sb-HMR could have resulted either from an S. bayanus-specific inhibitor of silencing that Sc-Sir4 could not overcome or an S. bayanus-specific positive regulator of silencing (e.g., Sb-Rap1 or Sb-Sir1) with which Sb-Sir4 could not interact. To distinguish between these models, in an S. cerevisiae strain, we replaced the Sc-HMR locus with Sb-HMR containing the URA3 reporter, including the flanking silencer elements (Figure 6A). If S. bayanus encoded an inhibitor of silencing that Sc-Sir4 could not overcome, Sb-HMR should be silenced in S. cerevisiae, given the strong conservation of ORC, Rap1, and Abf1 proteins and the Rap1 and Abf1-binding sites in the HMR-E silencer [5]. If, however, Sc-Sir4 failed to be recruited to S. bayanus silencers, we would expect little or no silencing of Sb-HMR in S. cerevisiae.

Upon transfer into S. cerevisiae, Sb-HMR was silenced extremely poorly (Figure 6B, row 1). However, the transplanted Sb-HMR locus could still be silenced in the context of the S. cerevisiae chromosome in hybrids made by mating the S. cerevisiae Sb-HMR strain to wild-type S. bayanus. The transplanted Sb-HMR locus was silenced to approximately the same degree as the native Sb-HMR locus in hybrids (Figure 6B, row 2, compare with 6C rows 1 and 2). The slightly incomplete silencing of the transplanted Sb-HMR was largely due to the Sb-SIR4 dosage sensitivity observed in the original set of hybrids (Figure 2A, row 3), as silencing was strengthened in Sb-SIR4/Sb-SIR4 hybrids (Figure 6B, row 3). Thus, the lack of silencing of Sb-HMR in hybrids expressing only Sc-Sir4 (Figure 2A, rows 2 and 5) was not due to an inhibitor of silencing encoded elsewhere in the S. bayanus genome. Rather, the incompatibility was encoded in the Sb-HMR locus itself, requiring S. bayanus-specific silencing proteins to interpret Sb-HMR-specific sequence information. These “interpreter” proteins potentially included DNA-binding proteins such as ORC, Rap1, and Abf1 or proteins indirectly associated with silencers, such as Sir1, Sir4, or both.

Alignments of Sc-HMR and Sb-HMR suggested that their functional divergence was due to changes in the silencer sequences between the two species (Figure 7). The HMRα1 gene was 83% identical between S. cerevisiae and S. bayanus (the promoter was 93% identical), well above the genome-wide average of 62% identity for all intergenic regions, and the mating-type cassette-homology sequences (shared with MAT and HML) approached 100%
identity (Figures 6A and 7). Notably, the silencer sequences share well below the genome-wide average identity for intergenic regions and are difficult to align outside of the conserved Rap1 and Abf1 sites [5].

Reconstitution of \textit{S. bayanus} Silencing in \textit{S. cerevisiae} with \textit{Sb-SIR4} and \textit{Sb-KOS3}

The simplest model consistent with the results so far was that the silencing incompatibility was limited to Sir4, with \textit{Sc-Sir4} having a more restricted range of interactions than \textit{Sb-Sir4}. To test this possibility, we replaced \textit{Sc-SIR4} with \textit{Sb-SIR4} in the \textit{S. cerevisiae} strain bearing \textit{Sb-HMR}. If the incompatibility involved only \textit{SIR4} and silencers, \textit{Sb-SIR4} should restore silencing to \textit{Sb-HMR}. Indeed, the \textit{S. cerevisiae} strain with \textit{Sb-SIR4} and \textit{Sb-HMR} indeed showed a modest increase in silencing relative to the \textit{Sc-SIR4} \textit{Sb-HMR} strain, confirming that changes in Sir4 itself contributed to the silencing incompatibility. However, this silencing increase—a 5-fold change—was detectable only as an increase in FOA resistance, and was still at least 100-fold below the level of \textit{HMR} silencing seen in the hybrids (Figure 8A, row 2; compare with Figure 6B, row 2).

Thus, although a portion of the incompatibility could be explained by \textit{SIR4} and silencer co-evolution, one or more additional \textit{S. bayanus} proteins were likely required to recruit \textit{Sb-Sir4} efficiently or to stabilize its association with \textit{S. bayanus} silencers.

Interestingly, \textit{Sc-Sir4}'s very weak ability to silence the transplanted \textit{Sb-HMR} locus resulted in the low-frequency appearance of FOA-resistant colonies (occurring at an approximate frequency of $5 \times 10^{-7}$; Figure 8A, row 1). Within these
colonies, which grew at nearly the rate expected of Ura- strains, the cells were able to grow under conditions that killed the majority of cells that did not form colonies. Hence this silencing occurred at low frequency, but was nonetheless heritable. Indeed, Sb-HMR silencing by either Sb-Sir4 or Sc-Sir4 was fully dependent on S. cerevisiae Sir1 (Figure 8A), whose role is to promote the establishment of heritable silencing. That Sb-HMR could be silenced at all in S. cerevisiae suggested that a critical subset of Sc-ORC, Rap1, and Abf1 bound productively to Sb-HMR silencers. It was therefore possible that providing additional S. bayanus silencing proteins could stabilize interactions between the S. cerevisiae DNA-binding proteins and S. bayanus silencers. Likely candidates to provide this presumptive function were the S. bayanus Sir1 paralogs, Kos1, Kos2, and Kos3, with Kos3 being the most structurally distinct from Sir1, yet the most similar to the ancestral member of the Sir1 family [24]. Interestingly, Sb-KOS3 enhanced Sb-HMR silencing synergistically with Sb-SIR4, but not with Sc-SIR4 (Figure 8B; compare rows 1, 5, and 10). None of the other Sir1 paralogs of S. bayanus provided a dramatic enhancement of Sb-HMR silencing. The Sb-HMR Sc-SIR4 + Sb-KOS3 strain showed 100-fold better silencing than the Sb-HMR Sc-SIR4 strain (Figure 8B, compare rows 1 and 10). This result was particularly interesting because Sir4 interacts weakly and non-specifically with DNA [44], and Kos3 is not thought to bind DNA at all. Thus, the “interpretation” of differences between the Sb-HMR and Sc-HMR silencers by Sb-Kos3 and Sb-Sir4 presumably required some sort of HMR-allele-specific collaboration with silencer-binding proteins that could be interpreted by Sb-Kos3 and Sb-Sir4 in a species-specific way.

Differential ORC Utilization by S. bayanus Silencers

By sequence conservation, Rap1 and Abf1 binding sites can be detected in the Sb-HMR-E silencer, but the ORC binding site is not readily identified (Figure 7) [5]. Given Sb-Sir4’s dependence on Sir1 and Kos3, and their dependence on ORC [20,43,46], our results suggested two likely explanations for why Sb-HMR was not silenced in S. cerevisiae: either Sc-ORC bound S. bayanus silencers less well than S. cerevisiae silencers, or Sc-ORC bound equivalently but failed to promote silencing because it was in a suboptimal conformation or context with respect to other silencer binding proteins. In either case, the subsequent interactions with Sc-Sir1 and Sc-Sir4 might suffer. To test whether Sc-ORC indeed bound to S. bayanus silencers, we performed ChIP analysis with HA-tagged Sc-Orc5 in S. cerevisiae bearing Sb-HMR. Sc-Orc5 associated with the Sb-HMR-E silencer, albeit at a level several-fold below its association with Sc-HMR-E (Figure 9A, left panel; note log scale on y-axis). A parallel analysis with Sc-Abf1 ChIP showed robust association of this protein with both Sc-HMR-E and Sb-HMR-E silencers (Figure 9A, right panel). We note that both Sc-Orc5 and Sc-Abf1 associations with Sb-HMR-E showed small alterations in the Sb-SIR4 strain relative to the Sc-SIR4 strain. However, these changes did not correlate with Sb-HMR silencing levels (Figure 8A, rows 1 and 2). These ChIP data were consistent with Sb-HMR silencers having conserved functional binding sites for ORC and Abf1.

To test whether Sc-ORC, Rap1, and Abf1 indeed participated in S. bayanus silencing, we monitored silencing of Sb-HMR in hybrids lacking either species’ complement of each of these proteins (out of the six ORC subunits, we focused on Orc1 because it directly interacts with Sir1). Because Rap1, Abf1, and ORC1 are essential, we assayed silencing in hybrids heterozygous for each gene. S. cerevisiae diploids sensitized to detect silencing defects at HMR show strong silencing defects if either Sir1 or Sir4 dosage is also reduced [47]. Similarly, Sb-HMR silencing was weakly compromised in hybrids whereas Sc-HMR was not (Figure 2A), potentially providing a sensitized background to uncover similar types of genetic interactions. For this reason, any such silencing defects in heterozygous hybrids were expected to affect silencing of Sb-HMR but not Sc-HMR. Indeed, Sb-HMR, but not Sc-HMR, was further derepressed in hybrids lacking either Sc-RAP1 or Sc-RAPI (Figure 9B, top panel; Figure S3). Note that Sb-HMR was fully silenced in S. bayanus rap1 disaploid; therefore, reduced rap1 dosage per se did not cause the loss of silencing observed in the hybrid (Figure 9B, bottom panel). Thus, Sc-Rap1 participated in Sb-HMR silencing in hybrids, likely by direct binding to S. bayanus silencers. In contrast to the analysis with rap1, Sb-HMR was derepressed to a greater extent in hybrids lacking Sc-ORC1 but not in hybrids lacking Sb-ORC1 (Figure 9B, top panel). Again, Sb-HMR was fully silenced in S. bayanus ORC1/αc1 rap1 disaploid (Figure 9B, bottom panel), ruling out simple dosage explanations. Hence, Sb-Orc1 was more important for Sb-HMR silencing in hybrids than Sc-Orc1, suggesting that a partial species restriction existed with respect to ORC binding or activity at Sb-HMR silencers. Heterozygosity of Abf1 had no effect on either Sb-HMR or Sc-HMR silencing (Figure 9B, top panel; Figure S3).
Evidence for Positive Selection on SIR4

The rapid sequence and functional divergence of SIR4 between closely related species suggested that an interesting evolutionary force may have contributed to the functional divergence of this gene. To test whether a specific function of the Sir4 protein had been under positive selection within the sensu stricto clade, we aligned SIR4 coding sequence from all five species and computed the ratio of nonsynonymous to synonymous divergence (henceforth \( v \), also known as dN/dS) across the whole gene. The value of \( v \) for SIR4 was 0.44, substantially higher than the genomic average of 0.10. Only 16 of 4,894 loci we analyzed had a higher \( v \), indicating that SIR4 was indeed one of the most rapidly evolving genes in the budding yeast genome.

A value of \( v \) significantly greater than 1 is evidence of positive selection [48]. Therefore, a value of 0.44 might suggest that the SIR4 coding region did not evolve under positive selection. However, because Sir4 is a large protein we investigated whether sub-regions or individual codons might have \( v > 1 \). To determine whether rapidly evolving Sir4 residues might lie within known functional regions of the protein, we computed \( v \) in 102 bp (34-codon) windows throughout the SIR4 open reading frame (Figure 10A). Consistent with our previous whole-gene estimate, the median \( v \) value for all windows in SIR4 was 0.43 (Figure 10A, solid horizontal line) with a range from 0.02 to 1.87. Because \( v \) estimates calculated in short windows are subject to stochastic noise, we compared the results of this analysis to 1,500, 102 bp windows drawn from other S. cerevisiae coding regions. The median of these \( v \) values was 0.05, and 95% of windows lie between 0.0001 and 0.42 (Figure 10A, dashed lines). These comparisons supported two conclusions. First, because the median \( v \) for SIR4 was comparable to the most extreme values in other genes, the unusual molecular evolution of this gene extended over a large fraction of its length. Second, the non-random distribution of windows with high \( v \) suggested that the rapid evolution of certain residues was connected

Figure 8. Partial reconstitution of Sb-HMR silencing in S. cerevisiae by transfer of S. bayanus Sir4 and Kos3 proteins. (A) Top panel: Silencing of the Sc::(Sb-HMR::URA3) replacement allele in S. cerevisiae MATα haploids bearing either the endogenous Sc-SIR4 gene or an integrated Sb-SIR4 gene (top panel). Bottom panel: Silencing of the Sc::(Sb-HMR::URA3) replacement allele in the absence of Sc-SIR1. (B) S. cerevisiae strains bearing the Sc::(Sb-HMR::URA3) replacement allele, and either the endogenous Sc-SIR4 gene or an integrated Sb-SIR4 gene, were transformed with plasmids encoding individual S. bayanus Sir1 paralogs and assayed for silencing function (FOA/-His, CSM/-His-Ura, or CSM/-His indicate silencing reporter media also selective for maintenance of plasmids bearing the HIS3 marker). Quantification of relative silencing function, based on growth on FOA/-His, is indicated at right. Fold-change comparisons were made relative to the Sc::(Sb-HMR::URA3) Sc-SIR4 strain bearing an empty vector (row 1). We note that the CEN/ARS plasmid itself appeared to enhance Sb-HMR silencing relative to the untransformed strains (compare Figure 8B, “empty vector” rows, to Figure 8A, rows 1 and 2). However, relative comparisons among transformed strains were still possible.

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to functional changes within specific regions of the Sir4 protein. In support of this suggestion, simulations of *SIR4* evolution indicated that 1.3% of 102 bp windows are expected to have v = 1 by chance compared to 6.7% observed in *SIR4* sensu stricto (unpublished data). The high-v windows in *SIR4* were therefore unlikely to reflect noise and instead indicated that the most rapidly evolving codons are concentrated in particular regions of *SIR4*. Indeed, although the Rap1- and Sir3-binding coiled-coil domain was largely protected from the rapid evolution of *SIR4*, residues within the PAD (Partitioning and Anchoring of plasmids) and the putative N-terminal regulatory domains [37] showed striking signatures of rapid evolution (Figure 10A).

To provide an independent, statistically robust analysis of *SIR4* evolution in this clade, we used a likelihood-ratio test to compare nested models of sequence evolution that either allowed or did not allow a subset of codons to have a value of v = 1. The model allowing v = 1 (M8) fit the data significantly better than the alternative model (M7; p = 5 × 10^-4), indicating that some codons were likely to be evolving under positive selection (Figure 10B). The posterior probability of v = 1 exceeded 0.75 for 11 codons (Figure 10A and C; v = 1.5 in all cases), however for no single codon did the posterior probability exceed the nominal significance level of 0.95. Inclusion of *SIR4* sequences from species outside the *sensu stricto* was not possible because of poor alignment quality. In summary, although we were not able to identify specific codons that were unambiguously under positive selection, these data suggested that multiple codons within *SIR4*, including some within the PAD and N-terminal regulatory domains, exhibit signatures of extremely rapid sequence evolution in the *Saccharomyces sensu stricto* clade.

To examine whether the rapid sequence evolution of *SIR4* showed a phylogenetic correlation with the functional divergence we observed, we fit models that allowed different branches of the *SIR4* tree to have different values of Ω. If such a correlation were

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**Figure 9. ChIP and genetic interaction analysis of ORC, Rap1, and Abf1 silencing functions.** (A) ChIP analysis of Sc-Orc5 and Sc-Abf1 in *S. cerevisiae* at Sc-HMR-E versus Sb-HMR-E. Relative enrichment of silencer sequences was verified by comparison to amplification values for a positive control region, the ARS1 replication origin, and a negative control region in the *SEN1* gene (unpublished data). Note log scale on y-axis. Error bars show standard deviations (n = 3). (B) Top panel: Silencing of the Sb-HMR::URA3 reporter gene in *S. cerevisiae/S. bayanus* hybrids each lacking a single allele of the *RAP1*, *ORC1*, or *ABF1* genes. Bottom panel: Silencing of the Sb-HMR::URA3 reporter gene in *S. bayanus* diploids lacking one allele of *RAP1* or *ORC1*. Note that to properly document the silencing differences shown in (B), the CSM/-Ura plates were photographed after 5 d, whereas in Figure 2 CSM/-Ura plates were photographed after 3 d.

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observed (i.e., more rapid evolution of \textit{SIR4} along the \textit{S. cerevisiae} lineage than along the \textit{S. bayanus} lineage), then positive selection on a specific silencing function of \textit{SIR4} during the evolution of \textit{S. cerevisiae} would be likely. Although increased estimates of $v$ were obtained for some branches (notably the shared \textit{S. cerevisiae}/\textit{S. paradoxus} branch; $v = 0.55$), none were statistically supported, suggesting that there have been no dramatic shifts in the selection pressures operating on \textit{SIR4} since the divergence of the \textit{sensu stricto} (Figure S4). We note that a change in selection pressure that affected only a subset of codons could easily have gone undetected.

\section*{Discussion}

Using interspecies hybrids, we have shown by three functional criteria—cross-species complementation assays, cross-species \textit{cis-trans} tests, and genome-wide localization by ChIP-Seq—that the functions of both the Sir4 protein and multiple silencer elements have strikingly diverged over the short divergence time between closely related yeast species. Cross-species complementation assays revealed an incompatibility between Sc-Sir4 and \textit{Sb}-HML and \textit{Sb}-HMR (Figure 2A). The inability of Sc-Sir4 to silence \textit{Sb}-HML and \textit{Sb}-HMR was due to a difference in the protein sequence of Sir4 between the two species rather than a difference in expression level (Figure 10, Figure S2). This incompatibility likely resulted from the coordinated divergence of multiple heterochromatin determinants: Sir1, Sir4, and silencers. Two pieces of evidence implicated \textit{cis}-acting changes in silencer sequences as being key to the incompatibility. First, comparative ChIP-Seq analysis of Sir4 pinpointed an inability of Sc-Sir4 to associate stably with \textit{S. bayanus} silencers (Figure 4A, Table 1). Second, and more definitively, transfer of the \textit{Sb}-HMR locus into

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{Evolutionary analyses of \textit{SIR4} in the \textit{sensu stricto} clade. (A) Ratios of nonsynonymous to synonymous divergence ($dN/dS$, or $\omega$) computed in 102 bp windows every 3 bp along alignments of the \textit{SIR4} gene from five \textit{sensu stricto} species (\textit{S. cerevisiae}, \textit{S. paradoxus}, \textit{S. mikatae}, \textit{S. kudriavzevi}, and \textit{S. bayanus}). Horizontal lines show the value of $\omega$ for the median \textit{SIR4} window (red solid line), the median window of all genes (blue solid line), and the limits within which 95\% of all $~1,500$ windows, sampled from across coding regions of the genome, fall (dashed lines). Diamonds indicate codons having a posterior probability of $(\omega>1) \geq 0.75$ (corresponding to positions given in panel C). Each of these 11 rapidly evolving codons is labeled with the inferred ancestral amino acid at that position, the amino acid number, and the amino acid present in \textit{S. cerevisiae} Sir4. Labeled boxes at top indicate the locations of functional domains. (B) Table summarizing statistics of PAML's M7 versus M8 evolutionary models for \textit{SIR2}, \textit{SIR3}, and \textit{SIR4}. For each gene, the starting nucleotide alignments were generated using sequences from the five \textit{sensu stricto} species used in panel A (see Methods for further description of PAML analysis). NS, not significant. (C) Table summarizing a subset of the Bayes Empirical Bayes (BEB) analysis for model M8 of PAML. The identities of the 11 sites with posterior probability of $(\omega>1) \geq 0.75$ are shown. Nucleotide (nt) and amino acid (a.a.) positions from \textit{S. cerevisiae} \textit{SIR4} are given.}
\end{figure}
S. cerevisiae demonstrated that this locus was inherently unrecognizable to Sc-Sir4 (Figure 6B). This result established that S. bayanus did not produce an inhibitor of Sc-Sir4 function, and mapped the locus of the Sir4 species specificity to the Sb-HMR silencers. As silencing of the transplanted Sb-HMR locus was largely restored in an S. cerevisiae/S. bayanus hybrid (Figure 6B), S. bayanus-specific proteins were required to assemble silent chromatin at Sb-HMR in the manner dictated by the Sb-HMR silencers, with Sb-Sir4 and the Sb-Sir1 paralogs being the most likely candidates for species-specific ‘‘interpreter’’ proteins.

Co-Evolution of Silencer Elements and Heterochromatin Proteins in Budding Yeast

The Sir4 protein and silencers diverged rapidly in concert, a process that was accompanied by loss of three Sir1 paralogs in the S. cerevisiae lineage [24]. As silencing was robustly maintained in each species, it was likely that these factors had co-evolved such that coding changes in Sir4 and a reduction in Sir1 family members led to compensatory changes in silencers, or vice versa. The asymmetrical complementation of SIR4 alleles (Figure 2A), and the enhanced ability of Sb-Sir4 to bind S. cerevisiae silent loci compared to its own silent loci (Figure 4B), suggested that S. cerevisiae silent elements had become stronger than those of S. bayanus, while S. cerevisiae Sir1 and Sir4 proteins had become weaker (operationally defined) than S. bayanus Sir4 and its four Sir1 paralogs. The intra-species combinations of Sir1 and Sir4 proteins and silencers allowed efficient nucleation of silencing complexes at HML and HMR in each species.

Broadly speaking, we imagine two possible evolutionary paths for this co-evolution, with variations on either path possible. In an ‘‘adaptive’’ model, hypothetical selective pressure(s) induced coding changes in Sir4 and reduction in Sir1 family members (Zill et al. in preparation), which then required ‘‘strengthening’’ mutations (for example, a change that increased the affinity of ORC for a silenter) in the silencers to maintain robust silencing. In a ‘‘constructive neutral’’ model [49], strengthening mutations accumulated in silencers at random, thus relaxing the selective constraints to maintain Sir1 paralogs and certain Sir4 residues. Once Sir1 paralogs were lost, the ‘‘stronger’’ silencers would need to be maintained by purifying selection. Our evolutionary analyses using the PAML software supported a role for positive selection in each species (Figure S4). However, the SIR4 gene was highly divergent from other SIR4 orthologs (Figure 8B), indicating that the SIR4 gene from K. lactis, an outgroup to the Saccharomyces clade, was able to complement silencing function in S. bayanus sir4A mutants (Zill et al. in preparation). That a Sir4 protein from a species outside of Saccharomyces is compatible with S. bayanus silencers suggests that these elements did not ‘‘gain’’ a restrictive property in the S. bayanus lineage. The more likely scenario is that Sir4 changed in the S. cerevisiae lineage such that its range of interactions with other species’ silencers has become restricted, consistent with earlier observations of cross-species function of Sir4 [50]. It will therefore be of interest to understand in detail the mechanism of silencing in S. bayanus and to determine what forces caused the dramatic shift in Sir1 and Sir4 functionality in the S. cerevisiae lineage. We measured the rates of SIR4 evolution (Table 1) along all branches in the sensu stricto clade but did not observe a notable asymmetry in these rates (Figure S4). Thus, the functional asymmetry between Sc-Sir4 and Sb-Sir4 was probably localized to a few sites and may not be related to the broad evolutionary forces that have acted on SIR4 across all five species in this clade.

Perhaps the most striking finding of this study was that the heterochromatin proteins that showed the most dramatic evidence of co-evolution with silencers, Sir1 and Sir4, were not the ones that bind specific DNA sites. Rather, these proteins associate with DNA indirectly via the conserved regulatory proteins Rap1, Abf1, and ORC (Figure 11). The key evidence demonstrating functional co-evolution between Sir4 and the Sir1 family and silencers came from attempts to reconstitute Sb-HMR silencing in S. cerevisiae. The changes in Sir4 sequence were not sufficient to explain the inability of Sc-Sir4 to function at S. bayanus silencers: expression of Sb-Sir4 in an S. cerevisiae strain was only modestly effective in silencing an Sb-HMR locus transplanted into that strain (Figure 8A). The Sir1-dependence of the rare, but heritable, silencing events mediated by Sb-Sir4 at Sb-HMR in S. cerevisiae suggested that the limitation involved proteins dedicated to establishing silencing. Indeed, adding Sb-Kos3, the ancestral member of the Sir1 family, together with Sb-Sir4 enhanced silencing of Sb-HMR in S. cerevisiae by 100-fold (Figure 8B), although not completely. It was possible that the site-specific DNA-binding proteins ORC, Rap1, and Abf1 had also co-evolved with silencer sequences. If this were the case, we would expect hybrids lacking the Sb-ORC, Sb-Rap1, or Sb-Abf1 proteins to have shown defective Sb-HMR silencing. However, only Sb-Orc1 inactivation (and by inference, inactivation of the entire Sb-ORC complex) showed the expected S. bayanus allele-specific effect on Sb-HMR silencing (Figure 9B). This effect of Sb-ORC1 deletion on Sb-HMR silencing was relatively modest, and the addition of Sb-ORC1 (together with Sb-SIR4) had no effect on Sb-HMR silencing in S. cerevisiae reconstitution experiments (unpublished data). Because Sc-Orc1, Sc-Rap1, and Sc-Abf1 were capable of supporting Sb-HMR silencing in hybrids (Figure 9B) and in S. cerevisiae (Figure 9B), their DNA-binding domains’ interactions with silencers were largely conserved across species and hence were not engaged in notable co-evolution with silencers or with Sir4. Indeed, we were able to ChIP Sc-Orc3 and Sc-Abf1 on the Sb-HMR-F silencer in S. cerevisiae (Figure 9A). Together, these results suggested that the co-evolving differences between the two species’ silencers were interpreted largely indirectly, via interactions between ORC, Sir1/Kos3, and Sir4, with a somewhat lesser contribution of differences in ORC-silencer DNA interactions.

Asymmetrical Interactions of Heterochromatin Determinants in Interspecies Hybrids Yielded Insights into the Silencing Mechanism

Why did Sc-Sir4 not bind efficiently to S. bayanus silencers? Simple explanations such as sequence divergence between S.
Silencing proteins, as shown by reconstitution experiments in S. bayanus silencers. cis-regulatory information in the S. bayanus silencer was specifically tuned to the Sb-Sir4 and Sb-Kos3 silencing proteins, as shown by reconstitution experiments in S. cerevisiae (Figure 8). Loss of Kos3 and changes in Sir4 in the S. cerevisiae lineage were compensated by changes in the silencers, thereby maintaining robust silencing.

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Figure 11. A new twist on co-evolution of transcriptional regulatory proteins and DNA target sites. Although the site-specific DNA binding proteins, ORC, Rap1, and Abf1 were largely interchangeable between S. cerevisiae and S. bayanus, the Sc-Sir4 protein showed a striking inability to function on S. bayanus silencers. cis-regulatory information in the S. bayanus silencer was specifically tuned to the Sb-Sir4 and Sb-Kos3 silencing proteins, as shown by reconstitution experiments in S. cerevisiae (Figure 8). Loss of Kos3 and changes in Sir4 in the S. cerevisiae lineage were compensated by changes in the silencers, thereby maintaining robust silencing.

cerevisiae and S. bayanus silencers precluding sequence-specific contacts with Sir4 are unlikely because biochemical data on Sir4 point to a lack of sequence-specific binding to DNA [44]. Instead, Sir4 is recruited to silencers predominantly via protein-protein interactions [20,37,51]. It is unlikely that different proteins bind the silencers in the two species as the preponderance of evidence points to ORC, Rap1, and Abf1 as the critical silencer-binding proteins in both species (Figures 5, 7, and 9). Further, the residues mediating Sc-Orc1 interaction with Sc-Sir1 [52,53] are conserved in Sb-Orcl (our unpublished observations). Hence we are forced to consider models in which something special about how ORC, Rap1, and Abf1 bind S. bayanus silencers prevents Sc-Sir4 from interacting with Rap1 or creates a requirement for specific interactions with the Sir1 paralogs that can only be made by Sb-Sir4. Perhaps the precise juxtaposition or conformation of these site-specific DNA-binding proteins allow or restrict interactions with a particular species of Sir4. Alternatively, perhaps a reduced affinity of S. bayanus silencers for ORC or Rap1, or the ensemble of nucleation proteins, is compensated by binding energy provided by Sh-Kos3 [and possibly additional Sir1 paralogs] and Sh-Sir4, but not by Sc-Sir4. Indeed, complete silencing of both Sb-HML and Sb-HMR requires Sh-Sir1, Sh-Kos1, and Sh-Kos2 [24]. Therefore, it is possible that a relatively weak binding site (such as for Rap1) in the Sh-HMR-E silencer could be compensated by increased binding energy provided to the nucleation complex in trans by the combination of Sb-Sir4 and the Sir1 paralogs. Additionally, we note that a requirement for multivalent interactions may help explain why Sir4 fails to interact stably with Rap1 at the many Rap1 binding sites throughout the genome.

An unexpected finding of the Sir4 comparative ChIP-Seq experiment provided insight into the mechanism of silent chromatin assembly. The Sh-Sir4-assisted Sc-Sir4 incorporation into Sh-HML and HMR (Figure 4A) suggested two distinct types of interactions made by Sir4 proteins at these loci: only Sb-Sir4 was capable of making stable contacts either with the Sir1 paralogs, or perhaps with Rap1. However, as Sc-Sir4 was capable of mediating telomeric silencing at some S. bayanus telomeres (Figure 5C), it appeared that Sc-Sir4 could interact productively with the Sb-Rap1 protein. In addition, there was a second and qualitatively distinct mode of Sir4 protein association that was species-independent but occurred only if the species-specific interaction occurred. Three types of interactions might account for the secondary mode of Sc-Sir4 association with Sh-HML and Sh-HMR: direct Sc-Sir4-Sh-Sir4 interaction via a conserved dimerization surface [38], Sc-Sir4 binding to Rap1 via the conserved C-terminal coiled-coil domain, or Sc-Sir4 interaction with deacetylated histone tails [18]. We note that Sc-Sir4 association with the Sh-HMR-E silencer increased in the presence of Sb-Sir4 at least as much as did its association with internal regions of Sh-HMR (Figure 4A). Thus, this secondary mode of Sir4 interaction did not appear to be restricted to regions of Sh-HMR where the deacetylated histones reside (silencers are nucleosome-free regions). Further studies will resolve whether Sb-Sir4-assisted Sc-Sir4 incorporation involves contacts with multiple silencing proteins versus simple Sir4-Sir4 dimerization and whether it requires Sir2 catalytic activity.

Additionally, the enhanced interaction of Sh-Sir4 across Y' elements at S. cerevisiae telomeres (Figure 4B) suggested that novel or changed interactions in the hybrids somehow led to enhanced Sir4 occupancy of these regions. This differential long-range occupancy by Sir complexes presents an opportunity to ask whether Sir1 and Sir4-mediated interactions during Sir complex nucleation regulate the “strength” of silent chromatin over a distance. Alternatively, Sh-Sir4 (and potentially other S. bayanus silencing proteins) may have been less sensitive to factors that exclude Sc-Sir4 from the Y' elements. The species-specific Sir4 distributions occurring in these interspecies hybrids should be

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further dissected to understand the determinants limiting silent chromatin formation across subtelomeric regions.

Another unusual property of the interspecies hybrids led to a weak silencing defect affecting Sb-HMR but not Sc-HMR (Figure 2A, row 1; Figure 3B). In hybrids lacking Sc-Sir4 this defect was more evident (Figure 2A, row 3), which paradoxically suggested that Sc-Sir4 protected Sb-HMR silencing in the presence of Sb-Sir4, despite having no ability to silence Sc-HMR on its own. How might Sc-Sir4 have “enhanced” Sb-Sir4 function at S. bayanus silent loci in hybrids? Strong evidence compatible with Sc-Sir4 protecting Sb-Sir4 from being titrated by Sc-specific sequences was the ability of both Sb-Sir4 and Sc-Sir4 to bind extensively to S. cerevisiae telomeres, as described in the Results. Hence, the hybrid state may result in a dosage sensitivity to Sc-Sir4 not evident in S. bayanus SIR4/sir4A intra-species diploids due to additional binding sites provided by the Sc-X and Y' elements, and potentially other elements. We note the resemblance of this “Sb-Sir4 sequestration” model to the “Circe effect” proposed to explain Sc-Sir4-mediated clustering of S. cerevisiae telomeres [34].

On the Special Properties of Interspecies Hybrids with Regard to Heterochromatin

Gregor Mendel’s studies were motivated by a desire to understand the emergent properties of interspecies hybrids, such as hybrid vigor, that were of great practical significance at the time. Although he became famously distracted by discovering two fundamental laws of genetics, his original interest in the processes by which hybrid species are not necessarily the “average” of the two parental species remains as interesting today as it was practically important in Mendel’s day. Indeed, the striking asymmetry in the ability of Sb-Sir4 to silence S. bayanus HMR, but inability of Sc-Sir4 to silence S. bayanus SIR4, observed in the seminal observation that inspired this study. By and large, however, in interspecies hybrids of S. cerevisiae and S. bayanus, a protein from either species was fully capable of providing all of that protein’s function to hybrids. Although this result could be anticipated from the ability to “clone by complementation” genes of one species by their function in another, this study established that symmetry of complementation is an important general consideration. For example, the essential proteins Rap1 and Abf1 from either species had all the functions necessary to support viability of the hybrids, and we established that Sir2 and Sir3 of both species were fully interchangeable (Figure S1), despite being members of a complex in which another member of that complex, Sir4, has extraordinary divergence. By extrapolation, asymmetrical deviations from a general expectation of cross-species compatibility, such as in the case of Sir4, may signal situations of uncommon interest.

The studies presented here capitalized on the extraordinary genetic properties of interspecies hybrids to tease out important dimensions to the evolution and structure of silent chromatin in yeast. Although silencing behavior in these yeast hybrids was rather unusual, some type of defect might have been anticipated from recent studies of hybrid sterility or lethality genes in Drosophila, which have implicated rapidly evolving heterochromatin proteins as key factors contributing to interspecies genetic incompatibility [55,56]. There is presently no reason to believe that Sir1 or Sir4 play roles in the post-zygotic genetic incompatibility between budding yeast species. It is notable, however, that in budding yeast multiple regulatory sites mediating silencing have rapidly evolved in a phylogenetically asymmetrical fashion along with a set of divergent silencing proteins, paralleling observations of rapid evolution in Drosophila heterochromatin [55,57,58]. It will be of great interest to determine whether the similar patterns of heterochromatin evolution in these distant taxa reflect similar underlying evolutionary processes.

Implications for the Mechanism of Heterochromatin Formation

The unprecedented resolution of Sir4 distribution provided by ChIP-Seq methods calls into question earlier models for silenced chromatin assembly, and in particular the so-called mechanism of spreading [reviewed in [7,59]]. In the common view, Sir protein recruitment to the silencers or to telomeres allows the deacetylation of H4K16-Ac on adjacent histones, creating new binding sites for additional Sir protein complexes, with sequential cycles of deacetylation and binding leading to spreading of Sir-protein complexes across all nucleosomes in silenced chromatin. The strikingly uneven distribution of Sir4 at HML and HMR (Figure 4A, note y-axes), and at the telomeres (Figure 4B), as shown here and in E. lactis [60], is not entirely inconsistent with the common view of heterochromatin spreading, but is in no way anticipated by it. Clearly, high-resolution characterization of all Sir proteins by ChIP-Seq has the potential to force substantial revision or replacement of the current view.

Materials and Methods

Yeast Strain Construction and Genetic Manipulations

All S. cerevisiae strains were of the W303 background. Generation of marked S. bayanus strains from type strain CBS 7001 has been described [61]. All yeast strains were cultured at 25°C in standard yeast media. One-step gene replacement and C-terminal 13xMyc tag integration have been described previously [36,62], and these genetic manipulations were performed identically for S. bayanus, S. cerevisiae, and S. cerevisiae/S. bayanus hybrids. The HMR::URA3 reporter strains were constructed independently in S. cerevisiae sir4A and S. bayanus sir4A haploid strains, wherein the HMRa1 ORF was replaced with the K. lactis URA3 ORF by PCR-based gene targeting, leaving the HMRa1 promoter intact. For most experiments, interspecies hybrids were made by crossing S. bayanus MATa HMR::URA3 strains (wild-type, sir4A, or SIR4-13xMyc) to S. cerevisiae MATa strains (wild-type, sir4A, or SIR4-13xMyc). For ORC1, RAP1, and ABF1 heterozygote analysis, gene targeting was performed directly in hybrid diploids or S. bayanus diploids. Three independent transformants were analyzed in all cases. Sc-SIR4-13xMyc and Sb-SIR4-13xMyc allelic series were shown to be functional by two independent silencing assays in each case: by mating ability in S. cerevisiae SIR4-13xMyc and S. bayanus SIR4-13xMyc haploid strains and by FOA resistance in hybrid diploids bearing the appropriate HMR::URA3 reporter (unpublished data).

The Sc::(Sb-HMR::URA3) replacement allele (Figure 6A) was generated in two steps. The Sb-HMR::URA3 cassette plus 1 kb of leftward-flanking sequence was PCR-amplified out of the S. bayanus genome, and the PCR product was used to replace the syntenic portion of Sc-HMR (including the E silencer) in S. cerevisiae sir4A strains. A HygMX marker was then targeted into the S. bayanus genome 3 kb to the right of Sb-HMR. The entire rightward-flanking 3 kb region plus the HygMX marker was PCR-amplified out of the S. bayanus genome, and the PCR product was used to replace the syntenic portion of Sc-HMR in the S. cerevisiae genome (including the I silencer). The Sc::(Sb-HMR::URA3) replacement allele therefore included a total of 5.5 kb of Sb-HMR sequence, plus the 1.7 kb HygMX marker.

To construct the Sir4 replacement alleles, the Sc-SIR4 and Sb-SIR4 genes were separately cloned into the yeast plasmid pRS315 [63] such that the LEU2 marker was 5' of, and in opposite orientation to, each SIR4 gene. Each SIR4 gene plus the LEU2
marker was PCR-amplified from each plasmid. The LEU2-Sc-
SIR4 PCR product was used to replace the URA3 marker at the
6S-SIR4 locus in an S. bayanus sir4::URA3 leu2 strain; likewise, the
LEU2-Sb-SIR4 PCR product was targeted into the Sc-SIR4 locus in an
S. cerevisiae sir4::URA3 leu2 strain. The integrated Sc-SIR4 gene
was shown to silence Sc-HMR::URA3 in hybrids (Figure 3A), and the
integrated Sb-SIR4 gene was shown to silence Sb-HMR::URA3 in
hybrids (Figure 6C) and Sc-HML and Sc-HMR in S. cerevisiae
strains (Zill et al. in preparation). The expression level of each SIR4
replacement allele was determined by quantitative RT-PCR
(Figure S2A).

Silencing Reporter Assays
Assays of yeast strain growth on FOA and CSM/-Ura media
were performed using standard “frogging” techniques. Briefly, for
each strain, a 10-fold dilution series of yeast cells at an
approximate density of 4 × 10⁶/mL was spotted onto each plate.
For Figures 2, 3A, 6, and S8A plates were photographed after 2 d
for YPD, and after 3 d for FOA and CSM/-Ura. For Figure 6B,
plates were photographed after 3 d for all media. For Figure 9B,
plates were photographed after 3 d for FOA and YPD, and after
5 d for CSM/-Ura. We note that some changes in silencing could
be seen only on FOA and not on CSM/-Ura. Incomplete silencing of
the HMR1 promoter likely led to heterogeneous expression
states within the population of cells, with some remaining silent
while others were expressed [64].

RNA and Protein Analysis
RNA isolation was performed using the hot-phenol method
[65]. Total RNA was digested with Amplification grade DNase I
(Invitrogen) and purified using the RNeasy MiniElute kit (Qiagen).
cDNA was synthesized using the SuperScript III First-Strand
Synthesis System for RT-PCR and oligo(dT) primer (Invitrogen).
Quantitative PCR on cDNA was performed using an MX3000P
machine (Stratagene) and the DyNAmo HS SYBR Green qPCR
kit (NEB). Amplification values for all primer sets were normalized
to actin (Invitrogen, Cat. No. 459250) was used to verify equal loading.

Protein samples were then obtained
from rabbit (Sigma, Cat. No. C3956) was used to detect Myc-
protein pairs, but it is unlikely that the rank order of divergences
among pairs would be significantly affected.

Evolutionary Analyses
Orthologous S. cerevisiae and S. bayanus genes were identified
on the basis of sequence similarity and syntenic context (D. Scannell
and M. B. Eisen, unpublished). Percent identities between 4,981
orthologous S. cerevisiae and S. bayanus proteins were then obtained
by running BLASTP [73] with default parameters, imposing an E-
value cutoff of 1 × 10⁻⁵, harvesting percent identities for each HSP
and calculating a length-weighted average. This will necessarily
lead to some underestimation of the true divergence between
protein pairs, but it is unlikely that the rank order of divergences
among pairs would be significantly affected.

For PAML and sliding window analyses, protein alignments
were produced using FSA with default parameters [74], and DNA
alignments were obtained by back translation with RevTrans [75].
All site and branch models were fit using codeml in the PAML
package [76]. To test for positive selection we compared model
M8 to M7 using a
2 test with two degrees-of-freedom. Posterior
parameters were estimated from the data.
We estimated the level of selective constraint operating on SIR4 on each branch of the Saccharomyces sensu stricto phylogeny by computing branch-specific ratios of non-synonymous to synonymous substitutions (dN/dS, or ω). Briefly, we performed protein-space alignments of orthologous SIR4 coding sequences with FSA [74] and then used codeml in the PAML package [76] to fit a “free-ratio” model (model = 1, NSSites = 0) to the alignment and obtain independent estimates of ω for each branch.

Supporting Information

Figure S1 Cross-species complementation analysis of sir2A and sir3A mutations in S. cerevisiae/S. bayanus interspecies hybrids. Top panel: Ten-fold serial dilutions of hybrid strains bearing a URA3 reporter gene at the S. cerevisiae HMR locus (Sc-HMR::URA3) were grown on medium counter-selective for URA3 expression (FOA), selective for URA3 expression (CSM/-Ura), or rich medium (YPD). Genotypes of both species’ sIR2 or sIR3 genes are indicated at left. Bottom panel: Hybrid strains bearing a URA3 reporter gene at the S. bayanus HMR locus (Sb-HMR::URA3), with genotypes of both species’ sIR2 or sIR3 genes indicated at left. Found at: doi:10.1371/journal.pbio.1000550.s001 (1.75 MB TIF)

Figure S2 SIR4 expression analysis in S. cerevisiae, S. bayanus, and S. cerevisiae/S. bayanus interspecies hybrids. (A) Sc-SIR4 and Sb-SIR4 RNA analysis by quantitative RT-PCR. Amplification values for SIR4 cDNA were normalized to those of actin (ACT1), as indicated in Methods. Left to right: Sc-SIR4 expression in S. cerevisiae haploid [JRY1012]; Sb-SIR4 expression in S. bayanus haploid [JRY8829]; expression of Sb-SIR4 replacement allele in S. bayanus haploid [JRY9049]; expression of Sb-SIR4 replacement allele in S. cerevisiae haploid [JRY9027]; expression of either the Sc-SIR4 or Sb-SIR4 allele in a hybrid diploid [JRY9054]. Note that because equivalent amounts of total cDNA were added to all qRT-PCR reactions, the apparent expression levels of Sc-SIR4 and Sb-SIR4 in this hybrid diploid were expected to be 50% of their levels in haploids. Error bars show standard deviations (n = 3). (B) Sc-Sir4 and Sb-Sir4 protein expression analysis by immunoblot. Left panel: A hybrid diploid with no Myc tag (lane 1), and Sc-Sir4-myc expression in S. cerevisiae diploids (lanes 2 and 3) and hybrid diploids (lanes 4 and 5). Right panel: Sb-Sir4-myc expression in S. bayanus diploids (lanes 6 and 7) and hybrid diploids (lanes 8 and 9). Phosphoglucokinase (Pgk1) expression is shown as a loading control. Found at: doi:10.1371/journal.pbio.1000550.s002 (0.85 MB TIF)

Figure S3 Genetic interaction analysis of ORC, Rap1, and Abf1 silencing functions at Sc-HMR in interspecies hybrids. Silencing of the Sc-HMR::URA3 reporter gene in S. cerevisiae/S. bayanus hybrids each lacking a single allele of the RAP1, ORC1, or ABF1 genes (dilutions, plating, and photography performed as in Figure 9B). Found at: doi:10.1371/journal.pbio.1000550.s003 (2.20 MB TIF)

Table S1 Complete genotypes of strains used in this study. Unless otherwise indicated, all strains originated from this study. For all genes in S. cerevisiae/S. bayanus hybrids, allele configurations are given as S. cerevisiae/S. bayanus. Found at: doi:10.1371/journal.pbio.1000550.s005 (0.12 MB DOC)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: OAZ JR. Performed the experiments: OAZ DS. Analyzed the data: OAZ DS LT. Contributed reagents/materials/analysis tools: OAZ LT. Wrote the paper: OAZ DS JR.

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