The DNA replication protein Orc1 from the yeast *Torulaspora delbrueckii* is required for heterochromatin formation but not as a silencer-binding protein

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ABSTRACT

To understand the process by which new protein functions emerge, we examined how the yeast heterochromatin protein Sir3 arose through gene duplication from the conserved DNA replication protein Orc1. Orc1 is a subunit of the origin recognition complex (ORC), which marks origins of DNA replication. In *Saccharomyces cerevisiae*, Orc1 also promotes heterochromatin assembly by recruiting the structural proteins Sir1-4 to silencer DNA. In contrast, the paralog of Orc1, Sir3, is a nucleosome-binding protein that spreads across heterochromatic loci in conjunction with other Sir proteins. We previously found that a non-duplicated Orc1 from the yeast *Kluyveromyces lactis* behaved like ScSir3 but did not have a silencer-binding function like ScOrc1. Moreover, *K. lactis* lacks Sir1, the protein that interacts directly with ScOrc1. Here, we searched for the presumed intermediate state in which non-duplicated Orc1 possesses both the silencer-binding and spreading functions. In the non-duplicated species *Torulaspora delbrueckii,* which has an ortholog of Sir1 (TdKos3), we found that TdOrc1 spreads across heterochromatic loci independently of ORC, as ScSir3 and KlOrc1 do. This spreading is dependent on the nucleosome binding BAH domain of Orc1 and on Sir2 and Kos3. However, TdOrc1 does not have a silencer-binding function: *T. delbrueckii* silencers do not require ORC binding sites to function, and Orc1 and Kos3 do not appear to interact. Instead, Orc1 and Kos3 both spread across heterochromatic loci with other Sir proteins. Thus, Orc1 and Sir1/Kos3 originally had different roles in heterochromatin formation than they do now in *S. cerevisiae.*

INTRODUCTION

Gene duplication enables functionally distinct proteins to emerge without loss of ancestral functions. For multifunctional proteins, a common outcome after duplication is subfunctionalization, in which the ancestral functions are partitioned between the two duplicated copies (FORCE *et al.* 1999; CONANT AND WOLFE 2008; FROYD AND RUSCHE 2011). In some cases, subfunctionalization is followed by further change in one or both paralogs, which allows for optimization of activities that were constrained within the multifunctional ancestor (HITTINGER AND CARROLL 2007). Although various models have been proposed to account for the evolutionary trajectories after duplication, less attention has been given to the molecular events that give rise to multifunctional proteins in the first place. Here, we searched for an example of a multifunctional protein that is predicted to be an intermediate in the evolution of the heterochromatin protein Sir3 from the DNA replication protein Orc1.

In the budding yeast lineage, *ORC1* gave rise to *SIR3* after a whole genome duplication approximately 100 million years ago (WOLFE AND SHIELDS 1997; SHEN *et al.* 2018). Both Orc1 and Sir3 contribute to heterochromatin formation in the well-studied yeast *Saccharomyces cerevisiae*. This heterochromatin is composed of a complex of Sir proteins (Silent Information Regulator) that bind nucleosomes and spread across several kilobases of DNA, resulting in the repression of transcription. Sir heterochromatin forms at the silent mating-type loci, which encode extra copies of mating-type genes that must be repressed to maintain proper cell-type identity. Sir heterochromatin also forms near telomeres, where it is thought to stabilize the ends of chromosomes.

Orc1 is the largest subunit of the origin recognition complex (ORC), which marks origins of DNA replication (BELL AND STILLMAN 1992). In *S. cerevisiae*, ScOrc1 also helps establish heterochromatin at the silent mating-type loci. As part of ORC, Orc1 binds to DNA sequences called silencers and then recruits the Sir proteins. Specifically, Orc1 interacts with Sir1 (TRIOLO AND STERNGLANZ 1996; GARDNER *et al.* 1999), which in turn binds other Sir proteins. In contrast, the paralog of Orc1, Sir3, is a nucleosome-binding protein that spreads across the silent mating-type loci in conjunction with Sir2 and Sir4 (HOPPE *et al.* 2002; LUO *et al.* 2002; RUSCHE *et al.* 2002; ONISHI *et al.* 2007). Orc1 and Sir3 have similar domain structures,

with an N-terminal BAH (Bromo Adjacent Homology) domain and a C-terminal AAA+ domain, separated by a disordered linker (LI *et al.* 2018). The BAH domain of ScOrc1 specifically binds ScSir1 (HOU *et al.* 2005; HSU *et al.* 2005) and is therefore critical for Orc1's role as a silencer-binding protein. For ScSir3, the BAH domain binds nucleosomes and is critical for the Sir proteins to spread (BUCHBERGER *et al.* 2008; ARMACHE *et al.* 2011). The ScOrc1 BAH domain also binds nucleosomes (DE IOANNES *et al.* 2019), but this property is thought to be more important for origin binding than heterochromatin formation.

We previously showed that *ORC1* and *SIR3* subfunctionalized after duplication (HICKMAN AND RUSCHE 2010). We found that in a non-duplicated yeast species, *Kluyveromyces lactis*, KlOrc1 behaves like ScSir3, facilitating the spreading of Sir proteins through nucleosome binding. Thus, Orc1 already had Sir3-like behavior prior to duplication. Additionally, we found that after duplication the function of the Sir3 AAA+ base subdomain changed, presumably leading to optimization of its ability to promote heterochromatin assembly (HANNER AND RUSCHE 2017). However, one puzzle that remains is when Orc1 acquired a role as a silencer-binding protein. We found that KlOrc1 does not act as a silencer binding protein (HICKMAN AND RUSCHE 2010). Moreover, *K. lactis* lacks Sir1, which acts as a bridge between ScOrc1 and the Sir proteins and would presumably be required for Orc1 to act as a silencer-binding protein. Thus, the silencer-binding role of Orc1 likely arose after or in conjunction with the emergence of Sir1.

The first recognizable *SIR1* gene appeared more recently than the ancestors of the other *SIR* genes. The appearance of *SIR1* slightly pre-dates the whole-genome duplication, as homologs are found in the clade of non-duplicated yeasts that shares most sequence similarity to duplicated species (Figure 1A). Over evolutionary time, the *SIR1* gene has undergone expansions and contractions in copy number, likely facilitated by its subtelomeric location in many species (GALLAGHER *et al.* 2009). For example, among duplicated yeast species, *S. cerevisiae* has a single *SIR1* gene, *Saccharomyces bayanus* has four functional *SIR1* homologs (called *KOS* or Kin Of Sir1), and *Candida glabrata* has no *SIR1* homologs (GALLAGHER *et al.* 2009). In addition to variation in copy number, the *SIR1* gene has undergone an internal tandem duplication. The C-terminal portion of the protein consists of an ORC Interacting Region (OIR) that binds to Orc1 (TRIOLO AND STERNGLANZ 1996; GARDNER *et al.* 1999). The N-terminal portion has a similar

70 sequence, called the OIR', whose function remains unclear (CONNELLY *et al.* 2006; HOU *et al.* 2009). Only
71 a single OIR occurs in the presumed ancestral version of the protein, called Kos3. All non-duplicated
72 species that have a Sir1 ortholog have Kos3 but no other Sir1-like proteins (GALLAGHER *et al.* 2009;
73 ELLAHI AND RINE 2016).

74 In addition to having appeared more recently than other Sir proteins, Sir1 differs functionally from
75 the other Sir proteins, at least in *S. cerevisiae*. ScSir1 promotes the establishment of heterochromatin by
76 recruiting the other Sir proteins to the silencer. However, it does not spread across the silent mating-type
77 loci with the other Sir proteins and thus is not required to maintain silencing (RUSCHE *et al.* 2002; ELLAHI
78 AND RINE 2016). Consequently, *sir1Δ S. cerevisiae* cells can exist in two epigenetically stable states, in
79 which the mating-type loci are either silenced or expressed (PILLUS AND RINE 1989). Unlike ScSir1,
80 TdKos3 is not restricted to the silencers and is required for silencing (ELLAHI AND RINE 2016). Therefore,
81 despite being homologous proteins, ScSir1 and TdKos3 make different contributions to heterochromatin.
82 This different behavior could be related to the presence of a single Orc1 that potentially behaves both as a
83 silencer-binding protein and a spreading protein and therefore recruits Kos3 both to silencers and to sites
84 of spreading.

85 To search for an ancestral Orc1 that has both silencer-binding and spreading functions, we
86 investigated the properties of Orc1 in *Torulaspora delbrueckii*. This species belongs to the
87 *Zygosaccharomyces-Torulaspora* (ZT) clade of budding yeast (Figure 1A), which has the most sequence
88 similarity to duplicated species and encodes the Sir1 ortholog Kos3. Thus, *T. delbrueckii* could represent
89 an intermediate stage in the evolution of Sir silencing, in which Orc1 already had two distinct roles in
90 heterochromatin formation. However, we found that despite *T. delbrueckii* having a Sir1-like protein,
91 TdOrc1 does not act as a silencer binding protein. In contrast, it does serve as a nucleosome-binding protein
92 that spreads across the heterochromatic loci independently of the ORC complex. Given the absence of ORC
93 binding sites at silencers, we also investigated which proteins do bind these sequences. We found that, Rap1
94 and Abf1, which bind to *S. cerevisiae* silencers, are also present at *T. delbrueckii* silencers. In contrast,

95 proteins that bind to *K. lactis* silencers were not associated with *T. delbrueckii* silencers. Taken together,

96 our data indicate that a silencer binding function for Orc1 does not go hand-in-hand with a *SIR1* homolog.

97 **MATERIALS AND METHODS**

98 **Yeast strain construction**

99 Yeast strains used in this study were derived from JRY10156 (ELLAHI AND RINE 2016), a
100 *Torulaspora delbrueckii MATα ura3Δ0 trp3-1(G466A)* strain descended from NRRL Y-866. Yeast strains
101 are listed in the Reagent Table, plasmids used for strain construction are described in Table S1 and the
102 Reagent Table, and oligos used for strain construction are listed in Table S2. The details of strain
103 construction are provided in the supplemental materials.

104

105 **Yeast growth and transformation**

106 Yeast were grown at 30˚C in YPD (1% yeast extract, 2% peptone, 2% glucose) or YM (0.67%
107 yeast nitrogen base without amino acids, 2% glucose). *T. delbrueckii* cells were transformed by
108 electroporation (HICKMAN AND RUSCHE 2009), and *S. cerevisiae* cells were transformed using polyethylene
109 glycol and lithium acetate (SCHIESTL AND GIETZ 1989). Transformation details are provided in the
110 supplemental materials.

111

112 **Silencing assay using *URA3* reporter**

113 To assess transcriptional silencing, a previously described (ELLAHI AND RINE 2016) reporter
114 plasmid was used, in which the entire *HMR2* locus was cloned onto a plasmid with a *T. delbrueckii*
115 centromere, an *S. cerevisiae* origin, and a hygromycin resistance gene. To evaluate silencing, the **a**1 open
116 reading frame was replaced with *URA3*. We replaced the hygromycin resistance gene with a kanamycin
117 resistance gene using NEBuilder HiFi DNA assembly master mix to create pLR1377. This plasmid was
118 then modified (Table S3) by replacing the *HMR2* silencer with the corresponding region from *HMR1*
119 (pLR1384), deleting the ORC binding motif (pLR1382), deleting the ORC binding motif plus the Abf1
120 binding sites (pLR1399), or deleting the *S. cerevisiae* derived plasmid origin (pLR1400 and pLR1401).

121 To assess silencing of the *URA3* reporter, yeast were transformed with the reporter plasmids
122 described above. Transformed cells were grown overnight at 30˚C in 3 mL YPD with 0.3 mg/mL geneticin.
123 Next day, 1 OD_{600} equivalent of cells was harvested and resuspended in 100 µL YM. Five 10X serial
124 dilutions were prepared, and 3 µL of each dilution was plated on CSM (YM supplemented with amino acids
125 and nutrients Sunrise Science 1001), CSM-Ura (YM supplemented with amino acids and nutrients but
126 lacking uracil; Sunrise Science 1004) and CSM+5FOA (YM supplemented with 0.1% 5-fluoroorotic acid,
127 0.005% uracil, 0.01% tryptophan, and pH adjusted to 3.35). All three media were supplemented with 0.3
128 mg/mL geneticin. Plates were imaged after 3 days of incubation at 30˚C.
129
130 **Chromatin immunoprecipitation**
131 Chromatin immunoprecipitation was performed as previously described (RUSCHE AND RINE 2001).
132 Cells were grown in YPD at 30˚C, harvested at an OD_{600} of ~1, and cross-linked for 1 hour in 1%
133 formaldehyde. For immunoprecipitation, 4 µl of anti Myc antibody (Millipore 06-549) or anti V5 antibody
134 (Millipore AB3792) were used. The immunoprecipitation was conducted with 10 µL of Protein A agarose
135 beads. For qPCR analysis, immunoprecipitation was conducted in the presence of 0.25 mg/mL BSA and
136 0.1 mg/mL salmon sperm DNA. For ChIP-Seq analysis immunoprecipitation was conducted without BSA
137 or salmon sperm DNA. Following immunoprecipitation, samples were reverse crosslinked overnight at
138 65˚C and digested with proteinase K at 45˚C.
139 For ChIP-Seq, library preparation (Takara Bio SMARTer ThruPlex DNA seq kit) and sample
140 barcoding was done at the Next-Generation Sequencing facility at University at Buffalo. The samples were
141 then sequenced on an Illumina 1.9 sequencer using 76 bp single-end sequencing.
142 For ChIP-qPCR, samples were quantified relative to a standard curve prepared from input DNA.
143 Then, the ratio of the experimental locus to the control locus (*ATS1*, TDEL0H03720) was calculated and
144 presented as relative enrichment. Oligonucleotides used for qPCR are listed in Reagent Table.
145
146

147 **Pipeline for processing ChIP-Seq data**

148 Raw single-end reads were aligned to the *T. delbrueckii* genome (CBS1146 (GCA_000243375.1))
149 (GORDON *et al.* 2011) using Bowtie2/2.3.4.1 (LANGMEAD *et al.* 2009). Samtools/1.9 was used to convert
150 .sam output files from Bowtie2/2.3.4.1 to .bam, -sorted.bam and index files (LI *et al.* 2009). To identify
151 statistically significant Orc1 and Kos3 genome wide association over background, **M**odel-based **A**nalysis
152 of **C**hIP-**S**eq, MACS2/2.2.7.1 was used to call peaks using one million randomly selected high quality reads
153 with genomic input as control for all samples with the parameters -B, --nomodel, --keep-dup 3, - -extsize
154 200 (ZHANG *et al.* 2008). For visualization bedGraph files generated from MACS2 were processed using
155 bedClip against chromosome sizes extracted from ASM24337v1 (GORDON *et al.* 2011) and converted to
156 .bigwig files using bedGraphToBigWig (KENT *et al.* 2010). ChIP-Seq bigwig files were visualized on IGV
157 (ROBINSON *et al.* 2011).

158

159 **Bioinformatic analysis of Kos3 sequences**

160 Sequences of Kos3 proteins were obtained from NCBI using tblastn to search whole-genome
161 shotgun sequences of Saccharomycetaceae species. For each genus, Kos3 sequences from representative
162 species were used as queries. OIR domains (light grey boxes) were identified by submitting each protein as
163 a query to NCBI blastp. The program returned the boundaries of the "Sir1 superfamily" domain that
164 corresponds to the OIR. Other conserved regions (colored boxes) were identified using the MEME suite
165 (BAILEY *et al.* 2009) to search for sequence motifs present zero or one time (ZOOPS model) in each member
166 of the protein set. Search parameters were to search for six motifs per sequence, with a minimum of six and
167 a maximum of 50 amino acids per motif. A zero order Markov model of the background was used.

168

169 **Identification of binding sites in silencers**

170 The recognition sequences for Rap1, Abf1, and ORC were predicted at each of the mating-type loci
171 (Table 1) and are indicated by colored boxes in schematics of chromosomal features. For ORC, genome-
172 wide binding sites and motifs were previously reported (MARIA *et al.* 2021). For Rap1, we identified motifs

173 associated with ChIP-Seq peaks (unpublished) and then located matches to these motifs within mating-type
174 loci. For Abf1, we identified matches to the known binding site for *S. cerevisiae* Abf1, 5'-
175 TnnCGTnnnnnnTGAT (BEINORAVICIUTE-KELLNER *et al.* 2005).
176
177 **Construction of species tree**
178 To construct the species tree, we selected 10 protein-coding genes (Table S4) found in single copy
179 across the nine species (AGUILETA *et al.* 2008). The protein sequences were obtained from the yeast gene
180 order browser (BYRNE AND WOLFE 2005) and the candida gene order browser (MAGUIRE *et al.* 2013) and
181 then concatenated, resulting in a combined alignment of 10092 positions. The concatenated sequences were
182 aligned in MAFFT v7.490 using E-INS-i with default settings (KATOH AND STANDLEY 2013). MAFFT
183 alignment was input into IQTree (TRIFINOPOULOS *et al.* 2016) to determine the best amino acid substitution
184 model: LG+F+I+G4 (HOANG *et al.* 2018). Finally, a maximum likelihood phylogenetic tree was
185 constructed using PhyML v3.1 using the LG+F+I+G4 model as implemented in Seaview v5.0.5 (GOUY *et*
186 *al.* 2010). Node support was assessed with approximate likelihood ratio tests (aLRT). Subtree pruning and
187 regrafting (SPR) was used as a search algorithm with five random starts.
188
189 **DATA AVAILABILITY**
190 Strains and plasmids are available upon request. The raw Illumina reads for Orc1 and Kos3 ChIP-
191 sequencing and related data that underly this article were uploaded to the NCBI GEO database (Accession
192 number: GSE182111). Supplemental files available at FigShare contain additional methods and a reagent
193 table.

194 **RESULTS**

195 **TdOrc1 associated with the silent mating-type loci as well as replication origins.** To investigate whether
196 Orc1 contributes to the formation of heterochromatin, we determined its distribution across the *T.*
197 *delbrueckii* genome using chromatin immunoprecipitation followed by high throughput sequencing (ChIP-
198 Seq). For this purpose, we generated a tagged strain with *ORC1*-Myc and *KOS3*-V5 integrated at their
199 genomic loci. Both proteins were detectable by immunoblotting (Figure 1B). Next, we immunoprecipitated
200 and sequenced chromatin fragments associated with Orc1 and Kos3. We also sequenced input chromatin
201 as a control for regions over-represented in the starting material. For comparison, we examined previously
202 published ChIP-Seq data for Sir2, Sir4, Orc2 and Orc4 (ELLAHI AND RINE 2016; MARIA *et al.* 2021). We
203 found that TdOrc1 was associated with all three silent mating-type loci: *HMR1* (chromosome VII), *HMR2*
204 (chromosome V), and *HML* (chromosome III). At these loci, Orc1 was broadly distributed, spanning ~1.5
205 kb and overlapping the predicted silencers (Figure 1D-F). Note that *T. delbrueckii* has two *HMR* loci that
206 are nearly identical across the **a**1 and **a**2 genes but different in the silencer regions. The pattern of
207 enrichment for Orc1 was similar to that of the Sir proteins (Sir2, Sir4, and Kos3) at all three loci. In contrast,
208 the Orc2 and Orc4 subunits were absent from *HMR1* and *HML* and only associated with *HMR2* at the
209 previously described ORC binding sequence, ARS-V75 (MARIA *et al.* 2021). For comparison, at a
210 representative ORC binding site not at a mating-type locus, TdARS-VII69 (MARIA *et al.* 2021), all three
211 ORC subunits were enriched but the Sir proteins were not (Figure 1C). These findings indicate that TdOrc1
212 has an ORC-independent role at the silent mating-type loci, where it colocalizes with other Sir proteins.

213

214 **TdSir2 and TdKos3 facilitated the recruitment and spreading of TdOrc1 at *HMR1* and *HMR2*.** In *S.*
215 *cerevisiae*, ScSir3 requires ScSir2 and ScSir4 to spread across the mating-type loci (HOPPE *et al.* 2002;
216 RUSCHE *et al.* 2002). To determine if the association of TdOrc1 with the silent mating-type loci also
217 depends on the Sir proteins, we first investigated whether Orc1 associated with *HMR* in the absence of Sir2
218 by conducting ChIP-Seq on Orc1-Myc in a *sir2Δ* strain. We found TdOrc1 association at *HMR1* was
219 substantially reduced in the absence of Sir2 (Figure 2A, right panel). In contrast, Orc1 association at the

220 nearby origin, ARS-VII22, was not affected (Figure 2A, left panel). Similarly, at *HMR2* Orc1 association
221 was reduced in the absence of Sir2, although it remained associated with the ORC binding site ARS-V75
222 (Figure 2C, track 3). Thus, Sir2 was required for recruitment of Orc1 to *HMR1* and spreading of Orc1
223 beyond the ORC binding site at *HMR2*.

224 Although in *S. cerevisiae* ScSir3 does not require ScSir1 for association with the mating-type loci
225 (HOPPE *et al.* 2002; RUSCHE *et al.* 2002), the situation may differ in *T. delbrueckii* because the Sir1 ortholog
226 TdKos3 is essential for transcriptional silencing at *HMR2* (ELLAHI AND RINE 2016). To test if association
227 of TdOrc1 depends on the presence of TdKos3 we conducted ChIP-Seq on Orc1-Myc in a *kos3Δ*
228 background. At *HMR1*, Orc1 association was substantially reduced in the absence of Kos3 (Figure 2B, right
229 panel) but unaltered at the neighboring origin (Figure 2B, left panel). Similarly, at *HMR2* Orc1 was reduced
230 in the absence of Kos3, although it remained associated with the ORC binding site (Figure 2C, track 4).
231 Therefore, unlike ScSir1, TdKos3 is important for the recruitment and spreading of Orc1 across *HMR1* and
232 *HMR2*.

233 Taken together, these results indicate that both Sir2 and Kos3 are required for TdOrc1 recruitment
234 to *HMR1* as well as its spreading across *HMR2*. Thus, the behavior of TdOrc1 is more like ScSir3, which
235 requires ScSir2 and ScSir4 for spreading, than like ScOrc1, which associates with DNA independently.

236

237 **TdSir2 and TdKos3 were required for transcriptional silencing at *HMR1*.** The requirement of Sir2 and
238 Kos3 for Orc1 association with *HMR1*, suggests that they are also required for transcriptional repression at
239 *HMR1*, as was shown previously for *HMR2* (ELLAHI AND RINE 2016). To test this expectation, we used an
240 established plasmid-based assay, in which the entire *HMR* locus was cloned onto a plasmid, and the **a**1
241 open reading frame was replaced with *URA3*. Expression of this *URA3* reporter was assessed by growth of
242 cells on medium lacking uracil, which is required for cells that do not express *URA3*, and by growth on
243 medium containing 5-FOA, which is toxic to cells expressing *URA3*. We modified the previous plasmid by
244 replacing the *HMR2* silencer with the corresponding region from *HMR1* (Figure 2E). It was not necessary
245 to swap the remainder of the *HMR* locus, because the sequence is nearly identical between *HMR1* and

246 *HMR2* (3939 identities over 3978 aligned nucleotides). Wild-type cells transformed with this plasmid
247 displayed minimal growth in the absence of uracil (Figure 2F, middle panel, row 2) and robust growth in
248 the presence of 5-FOA (Figure 2F, right panel, row 2), indicating silencing of *URA3* within the *HMR* locus.
249 In contrast, *kos3*Δ and *sir2*Δ cells bearing the plasmid displayed robust growth in the absence of uracil
250 (Figure 2F, middle panel, rows 3 and 4) and poor growth on 5-FOA (Figure 2F, right panel, rows 3 and 4),
251 indicating de-repression of the silent locus. A control empty vector with no *HMR2* or *URA3* did not enable
252 growth on plates lacking uracil (Figure 2F, middle panel, row 1) but permitted growth on plates with 5-
253 FOA (Figure 2F, right panel, row 1). Together, these data indicate that both Sir2 and Kos3 are required for
254 repressing *HMR1*.
255
256 **The nucleosome-binding BAH domain of TdOrc1 was required for its association and spreading at**
257 ***HMR1* and *HMR2*.** The BAH (bromo adjacent homology) domain of ScSir3 binds nucleosomes, and this
258 ability is critical for the propagation of Sir proteins across silent loci (ONISHI *et al.* 2007; ARMACHE *et al.*
259 2011). To investigate if the BAH domain of TdOrc1 also contributes to silencing, we constructed two
260 mutant alleles of Td*ORC1* designed to abrogate nucleosome binding. First, we deleted the entire bromo
261 adjacent homology (BAH) domain (A2-V213). Second, we replaced proline 179 with alanine (Figure 3A).
262 In ScSir3, a P179A substitution disrupts nucleosome binding and spreading of ScSir3 (BUCHBERGER *et al.*
263 2008). This is presumably because the main-chain carbonyl of P179 makes an electrostatic interaction with
264 histidine 18 of histone H4 and van der Waals interactions with other residues on the histone H4 tail
265 (ARMACHE *et al.* 2011). Both mutant Orc1 proteins were expressed at levels similar to the wild-type protein
266 (Figure 3B), and neither mutation affected cell viability or growth (data not shown). Consistent with the
267 behavior of ScSir3, the *orc1-bahΔ* (Figure 3C, right panel) and *P179A* (Figure 3D, right panel) alleles both
268 disrupted the association of TdOrc1 with *HMR1*, although both mutant proteins retained association with
269 the nearby ORC-binding site (Figure 3C-D, left panels).
270 The presence of Orc1 at *HMR1* suggests that it is required for transcriptional repression. To test
271 this idea, we used the plasmid-based expression assay with the *HMR1* silencer and *URA3* integrated in place

272 of ***a1***. Cells bearing this reporter plasmid and expressing the *orc1-bahΔ* or *orc1-P179A* alleles grew robustly
273 in medium lacking uracil (Figure 3E, middle panel, rows 3 and 4) but poorly on 5-FOA (Figure 3E, right
274 panel, rows 3 and 4). In contrast, in a wild-type background there was only weak growth in the absence of
275 uracil and robust growth in the presence of 5-FOA (Figure 3E, row 2). These results indicate that TdOrc1
276 is required for silencing *HMR1*.

277 Because the *orc1-bahΔ* and *orc1-P179A* alleles disrupt recruitment of Orc1 to *HMR1*, it was not
278 possible to assess whether these alleles also reduce spreading across the locus. However, at *HMR2* Orc1 is
279 recruited as part of ORC, and therefore we were able to assess spreading. In both *bah*Δ and *P179A* strains,
280 Orc1 associated with the ORC binding site within the *HMR2* silencer (Figure 3F). However, spreading
281 across the *HMR2* locus was substantially reduced for *orc1-bahΔ* (Figure 3F, track 3, light blue) and *orc1-*
282 *P179A* (Figure 3F, track 4, teal). These findings suggest that the nucleosome-binding BAH domain is
283 important for spreading of Orc1 across a silenced region, but that it is less important for ORC binding to
284 origins.

285 To determine if loss of nucleosome binding by TdOrc1 affects association of other Sir proteins with
286 the silent mating-type loci, we investigated the distribution of Kos3-V5 in the same *orc1-bah*Δ and *P179A*
287 cells. Indeed, these *orc1* alleles reduced Kos3 association at both *HMR1* (Figure 4A) and *HMR2* (Figure
288 4B).

289

290 **An ORC binding site was not required for Orc1 association with *HMR1*.** In *S. cerevisiae*, Orc1
291 contributes to silencing by recruiting Sir1, which in turn recruits other Sir proteins to the silent locus (FOX
292 *et al.* 1997; GARDNER *et al.* 1999). In this role, ScOrc1 acts as part of the ORC complex, binding to silencers
293 flanking the silent mating-type loci (FOSS *et al.* 1993). A similar function for TdOrc1 seems unlikely at
294 *HMR1* given the lack of Orc2 and Orc4 enrichment (Figure 1D). Nevertheless, we examined whether the
295 nearest ORC binding site (~14 kb away) recruits Sir proteins to *HMR1*. We deleted this ORC binding site,
296 ARS-VII22, in a strain lacking *HMR2* so that the ChIP signal would be derived solely from *HMR1.* To
297 confirm the disruption of ORC binding at this locus, we immunoprecipitated Orc1-Myc and analyzed the

298 co-precipitated DNA by qPCR. Deleting ARS-VII22 abolished Orc1 binding at this locus, as assessed using
299 primers adjacent to the deleted region (Figure 5B, left panel). However, deleting this ORC binding site did
300 not decrease binding at another control origin, ARS-V931 (Figure 5A). Importantly, Orc1 remained
301 associated with *HMR1* in *arsvii22Δ* cells (Figure 5B, right panel). Therefore, Orc1 association at *HMR1*
302 was not dependent on ORC binding at ARS-VII22. This finding, combined with the lack of ORC binding
303 sites within *HMR1* (Figure 1), indicates that TdOrc1 is not a silencer binding protein at *HMR1.*

304 **Rap1 binding sites were required for Orc1 association with *HMR1*.** For comparison with the ORC
305 binding site deletion, we deleted the binding sites for Rap1, another protein known to bind silencers in *S.*
306 *cerevisiae* (MORETTI *et al.* 1994). Rap1 binding sites are essential for establishing silencing at *HMR2* in *T.*
307 *delbrueckii* (ELLAHI AND RINE 2016) and three of four silencers in *S. cerevisiae* (SHORE AND NASMYTH
308 1987; BUCHMAN *et al.* 1988). We therefore deleted the two putative Rap1 binding sites at *HMR1* (Figure
309 5C, panel two, red boxes) in a strain lacking *HMR2.* We then immunoprecipitated Orc1-Myc and analyzed
310 the DNA by qPCR in both wild-type (Figure 5C, right panel, dark blue) and $rap1_{bs}\Delta$ (Figure 5C, right panel,
311 grey) backgrounds. Deleting Rap1 binding sites significantly reduced Orc1 association at *HMR1* compared
312 to wild-type cells, while Orc1 enrichment at the neighboring ORC binding site, ARS-VII22, was unaffected
313 (Figure 5C, left panel, grey and dark blue). These results indicate that the Rap1 binding sites, but not the
314 nearby ORC binding site, are required for Orc1 recruitment to *HMR1*.

315

316 **An ORC binding site at *HMR2* was dispensable for transcriptional silencing.** Unlike *HMR1*, there is
317 an ORC binding site in the silencer of *HMR2* (Figure 1E). Therefore, it was possible that TdOrc1 acts as a
318 silencer-binding protein at this locus. To investigate this possibility, we adapted the plasmid based *URA3*
319 reporter assay (ELLAHI AND RINE 2016). Starting with a plasmid containing the full-length *HMR2* locus,
320 we deleted the 17 bp conserved ORC binding motif (MARIA *et al.* 2021) in the silencer (Figure 6A). To
321 assess whether this deletion (*arsV75Δ*) reduced ORC binding, we conducted chromatin
322 immunoprecipitation on Orc2, which is part of ORC but not involved in silencing. We observed reduced

323 Orc2 association near the ORC binding site for the *arsV75Δ* plasmid compared to a plasmid with an intact
324 silencer (Figure 6B, right panel, red box). However, there was residual Orc2 association that we attributed
325 to the plasmid origin (*ScARS209*), ~3.4 kb away. Indeed, Orc2 was associated with *ScARS209* on plasmids
326 with and without the ORC binding site at the silencer (Figure 6B, left panel). As a second way to assess
327 whether the *arsV75Δ* deletion eliminated ORC binding, we also deleted the plasmid origin. We found that
328 when *T. delbrueckii* cells were transformed with a plasmid lacking both *ScARS209* and the ORC binding
329 motif in the silencer, only tiny yeast colonies were obtained, consistent with the plasmid not being
330 replicated. In contrast, when cells were transformed with a plasmid that had the silencer ORC binding motif
331 but lacked *ScARS209*, the normal sized colonies were observed (Figure 6C). Therefore, the silencer ORC
332 binding site can serve as an origin, and this capacity is lost in the *arsV75Δ* plasmid. Thus, the *arsV75Δ*
333 deletion successfully eliminated ORC binding.

334 Next, we assessed *URA3* silencing on the *arsV75Δ* plasmid. Cells containing this plasmid grew
335 similarly to those containing a plasmid with the wild-type silencer; they grew poorly on CSM-Ura (Figure
336 6C, middle panel, rows 2 and 3) and well on 5-FOA (Figure 6C, right panel, rows 2 and 3). This result
337 indicates that loss of the ORC binding site did not affect the silencing of *URA3* and therefore that an ORC
338 binding site at *HMR2* is not required to establish silencing. However, it remained possible that there is
339 redundancy in the silencer and that the ORC binding site is not needed when other silencer binding proteins
340 are present. In fact, for the *S. cerevisiae HMR-E* silencer, two of three binding sites must be deleted to
341 produce a loss of silencing (BRAND *et al.* 1987). We therefore created a larger deletion in which the ORC
342 binding site and both Abf1 binding sites were deleted. Cells containing this plasmid also grew poorly on
343 CSM-Ura (Figure 6C, middle panel, row 4) and well on 5-FOA (Figure 6C, right panel, row 4), indicating
344 that even loss of the ORC and Abf1 binding sites did not derepress *URA3*. A similar result was previously
345 observed (ELLAHI AND RINE 2016). Therefore, the ORC binding site in the *HMR2* silencer is not important
346 for silencing. In contrast, Orc1 itself is required to silence *HMR2*; in an *orc1-bah*Δ strain *URA3* is expressed

347 from the plasmid with the wild-type silencer (Figure 6C, row 5). Together, these results indicate that
348 TdOrc1 is important at *HMR2* but not as a silencer-binding protein.

349

350 **Some Kos3 proteins from *Torulaspora* species lack an ORC interacting region (OIR).** Two of our
351 findings suggest that for TdKos3, binding to Orc1 at the silencer is not its primary role in silencing. First,
352 TdKos3 has the same distribution at mating-type loci as TdSir2, TdSir4, and TdOrc1, rather than being
353 restricted to an ORC binding site (Figure 1D-F). Second, ORC does not act as a silencer-binding protein at
354 *HMR1* (Figure 5) or *HMR2* (Figure 6). To explore whether an interaction between TdKos3 and TdOrc1 is
355 likely, we compared the ORC interacting regions (OIR) of Sir1 and Kos3 proteins from species across the
356 *Saccharomycetaceae* family (Figure 7; see Figure 1A for species relationships). To identify the OIR, each
357 protein was submitted to NCBI blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which returned the
358 boundaries of a pre-defined domain that corresponds to the OIR (called "Sir1 superfamily," architecture ID
359 11189688). Interestingly, for several Kos3 proteins from the *Torulaspora* and *Zygotorulaspora* genera,
360 including *T. delbrueckii,* this "Sir1 superfamily" domain was not identified by the blastp analysis (Figure
361 7), suggesting the OIR is absent or highly diverged from the consensus. Nevertheless, other conserved
362 sequences that are shared among Kos3/Sir1 proteins could be identified in these proteins using the motif
363 finder MEME (BAILEY *et al.* 2009), demonstrating that these proteins are Kos3 homologs. We also found
364 that the Kos3 proteins lacking an OIR aligned poorly to other Kos3 proteins and had lower sequence
365 similarity. Therefore, unlike ScSir1, TdKos3 does not appear to have an OIR that would enable it to bind
366 to the BAH domain of TdOrc1.

367

368 **Silencers at the mating-type loci were associated with TdRap1 and TdAbf1 but not TdSum1, TdReb1,**
369 **or TdUme6.** Given that ORC does not serve as a silencer-binding protein in *T. delbrueckii,* we were
370 interested to identify other proteins that do. In *T. delbrueckii* only Rap1 bindings sites are documented to

371 be required for silencing ((ELLAHI AND RINE 2016) and Figure 5). Whereas, in *S. cerevisiae*, three silencer-
372 binding proteins are known: ORC, Rap1, and Abf1. The other species in which silencer-binding proteins
373 have been studied is *K. lactis,* which uses different DNA-binding proteins to initiate silencing, namely
374 Reb1, Ume6 and Sum1 (SJOSTRAND *et al.* 2002; HICKMAN AND RUSCHE 2009; BARSOUM *et al.* 2010). To
375 elucidate which proteins associate with *T. delbrueckii* silencers, we tagged the five DNA-binding proteins
376 that act at silencers in *S. cerevisiae* or *K. lactis* and then conducted chromatin IP to determine whether these
377 proteins are present at predicted silencers. Silencers coincide with the regions of highest Sir protein
378 association and are defined at *HML*, *HMR2* (ELLAHI AND RINE 2016) and *HMR1* (Figures 1 and 5).

379 TdRap1 and TdAbf1 were both enriched at *HMR1* (Figure 8B) and *HMR2* (Figure 8C), whereas only
380 TdRap1 was enriched at *HML* (Figure 8D). For the other DNA-binding proteins, we did not observe
381 significant enrichment at the silencers, although the immunoprecipitation was successful based on
382 enrichment at control loci: TdUme6 at *SPO11*, TdReb1 at *RDN25-1* and TdSum1 at *CDA2* (Figure 8E). To
383 better define the silencers, we identified putative binding sites Rap1 and Abf1. For TdRap1, we determined
384 a consensus binding motif using ChIP-Seq data (unpublished) and located matches to this motif that
385 correspond to peaks of Rap1 enrichment as assessed by ChIP-Seq (unpublished) and ChIP-qPCR (Figure
386 8). Moreover, deletion of these motifs at *HMR1* lead to loss of Orc1 association (Figure 5C). We also
387 identified putative binding sites for Abf1 as matches to the known motif for *S. cerevisiae* Abf1. At *HMR1*
388 and *HMR2* these putative binding sites correspond to the locations of TdAbf1 enrichment (Figures 8B and
389 C). Overall, these results indicate that TdRap1 and TdAbf1 are present at the silencers of *T. delbrueckii*
390 mating-type loci.

391 **DISCUSSION**

392 In this study, we examined the hypothesis that the appearance of a Sir1-like protein prior to the
393 whole genome duplication enabled Orc1 to become a silencer-binding protein. To do so, we characterized
394 the role of Orc1 in heterochromatin formation in the non-duplicated yeast species *T. delbrueckii,* which
395 belongs to the earliest clade of yeast to encode a Sir1-like protein, Kos3. However, we did not observe a
396 silencer binding function for TdOrc1. Nevertheless, we did find that TdOrc1 contributed to heterochromatin
397 formation through its nucleosome-binding BAH domain, much as ScSir3 and KlOrc1 do. In keeping with
398 TdOrc1 not serving as a silencer-binding protein, we extended previous observations (ELLAHI AND RINE
399 2016) that TdKos3 plays a different role in heterochromatin formation than ScSir1 does. Namely, TdKos3
400 and other Sir proteins depend on one another to spread across the silenced loci. Thus, the protein
401 composition of the heterochromatin in *T. delbrueckii* is distinct compared to other species that have been
402 examined (Figure 9).

403 Several lines of evidence support the conclusion that TdOrc1 does not behave as a silencer-binding
404 protein. First, there are no ORC binding sites at two of the mating-type loci (*HMR1* and *HML,* Figure 1D
405 and F), and the ORC binding site at *HMR2* was not required for silencing (Figure 6). In addition, TdOrc1
406 did not associate with the mating-type loci independently of other silencing proteins, but instead required
407 Sir2 and Kos3 (Figure 2). Moreover, recruitment of Orc1 to *HMR1* required the Rap1 binding sites in the
408 silencer but not the nearest ORC binding site (Figure 5). Thus, TdOrc1 does not behave as a silencer-
409 binding protein. We had speculated that the emergence of a Sir1-like protein would coincide with Orc1
410 acting as a silencer binding protein. However, for *T. delbrueckii*, having a Sir1-like protein is not sufficient
411 for Orc1 to serve as a silencer binding protein.

412 To better understand the lack of silencer binding function in TdOrc1, we compared the domain
413 structure of TdKos3 to other Kos3 orthologs and found that TdKos3 lacks an ORC interacting region (OIR)
414 (Figure 7). This domain was identified and characterized in *S. cerevisiae* Sir1 to interact with the Orc1 BAH
415 domain (GARDNER *et al.* 1999; HOU *et al.* 2005). Thus, without an OIR, TdKos3 is unlikely to bind to the

416 TdOrc1 BAH domain. Given the widespread distribution of the OIR among other Kos3 orthologs and Sir1-
417 like proteins (Figure 7 and (GALLAGHER *et al.* 2009)), we infer that the ancestral Kos3 had an OIR, which
418 was subsequently lost in the *Torulaspora* lineage. Thus, it remains possible that other non-duplicated Orc1
419 proteins are silencer-binding proteins.

420 Despite lacking an OIR, TdKos3 plays an important role in transcriptional silencing. Deletion of
421 *KOS3* derepresses transcription and reduces the association of Sir2, Sir4, and Orc1 with the mating type
422 loci ((ELLAHI AND RINE 2016) and Figure 2). Moreover, unlike ScSir1, TdKos3 is distributed across the
423 mating-type loci in the same pattern as Sir2, Sir4 (ELLAHI AND RINE 2016), and Orc1 (Figure 1), and it
424 requires the Orc1 BAH domain for association with these loci (Figure 4). Taken together these findings
425 suggest that Kos3 forms a complex with Sir2, Sir4, and Orc1, and that these proteins depend on one another
426 to assemble and spread across silenced loci (Figure 9). Thus, TdKos3 appears to be an integral part of the
427 silencing complex rather than a bridge between ORC and the other silencing proteins, as ScSir1 is. It
428 remains to be determined whether other Kos3 orthologs behave similarly to TdKos3, and if so whether
429 having an OIR that binds Orc1 facilitates assembly with Sir proteins, bridging to ORC at a silencer, or both.

430 It is notable that the set of heterochromatin proteins that spreads differs in *S. cerevisiae*, *T.*
431 *delbrueckii*, and *K. lactis*. In *S. cerevisiae* Sir2, Sir3, and Sir4 but not Sir1 spread away from silencers
432 (HOPPE *et al.* 2002; RUSCHE *et al.* 2002). In *K. lactis* Sum1 spreads with Sir2, Orc1, and Sir4 (HICKMAN
433 AND RUSCHE 2009; HICKMAN AND RUSCHE 2010). We show here that in *T. delbrueckii* Kos3 joins Sir2,
434 Orc1, and Sir4. Hence, we postulate that prior to the emergence of Sir3 through duplication of *ORC1*, the
435 Sir2/Orc1/Sir4 complex was insufficient on its own and required other proteins, such as Kos3 or Sum1, for
436 stability. For example, because ORC is required for DNA replication and viability, Orc1 may not have been
437 able to evolve optimal interactions with other Sir proteins while maintaining surfaces for assembly into the
438 ORC complex. However, after duplication, Sir3 acquired new properties in the AAA+ base subdomain
439 (HANNER AND RUSCHE 2017), which may have strengthened the Sir2/Sir3/Sir4 complex such that there
440 was no longer a need for a protein such as Kos3 or Sum1. Such a scenario could account for TdKos3 being
441 essential for silencing, whereas ScSir1 is not.

442 Our finding that TdOrc1 promotes the spreading of heterochromatin through its nucleosome-
443 binding BAH domain (Figure 3) reinforces the notion that this property arose prior to silencer binding. We
444 previously found that Orc1 from *K. lactis* also promotes spreading of heterochromatin but does not bind
445 silencers (HICKMAN AND RUSCHE 2010). It is therefore probable that the common ancestor of *T. delbrueckii*
446 and *K. lactis* also employed Orc1 in this way. Thus, considering this ancient role of the Orc1 BAH domain
447 in promoting the spreading of Sir proteins and that the BAH domain of Orc1 is conserved across eukaryotes,
448 the BAH domain might play a similar role in species beyond yeast. Moreover, ORC binding of HP1
449 (heterochromatin protein 1) in *Drosophila* and *Xenopus* (PAK *et al.* 1997; SHAREEF *et al.* 2001) may not be
450 analogous to the ORC-assisted Sir1 binding in *S. cerevisiae*.

451 Finally, we found that the silencer architecture of *T. delbrueckii* more resembles that of *S. cerevisiae*
452 than *K. lactis*. *S. cerevisiae* silencers have binding sites for Rap1, Abf1, and ORC, whereas *K. lactis*
453 silencers have binding sites for Reb1, Ume6, and potentially Sum1. All three presumed *T. delbrueckii*
454 silencers have Rap1 binding sites (Figure 8), and Rap1 is critical for silencer function at *HML* and *HMR2*
455 (ELLAHI AND RINE 2016) as well as *HMR1* (Figure 5). Abf1 binding sites are found at both *HMR* silencers
456 (Figure 8), and ORC binds to the *HMR2* silencer (Figure 1E). However, there is no evidence that either
457 Abf1 or ORC contributes to silencing in *T. delbrueckii*. Thus, their presence at silencers may be vestigial.
458 We did not detect the proteins associated with *K. lactis* silencers at *T. delbrueckii* silencers (Figure 8). The
459 shift in silencer architecture between *K. lactis* and *T. delbrueckii* could indicate that there is no adaptive
460 advantage to having a particular set of silencer binding proteins. On the other hand, the similarity between
461 *S. cerevisiae* and *T. delbrueckii* silencers suggests that there is an evolutionary advantage to maintaining a
462 Rap1-Abf1-ORC type silencer.

463 In summary, we found that the presence of a Sir1-like protein does not necessarily coincide with
464 Orc1 having a silencer-binding function. Thus, both Orc1 and Sir1/Kos3 originally had different roles in
465 heterochromatin formation than they do now in *S. cerevisiae,* illustrating the flexibility in how these
466 proteins achieve silencing.

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600

601 **TABLES**

602 **Table 1: Presumed binding sites at mating-type silencers**

Binding site	**Sequence**
HMR1-Abf1	actcgtaggtcatgat
HMR1-Rap1a	tggaggtactgatgc
HMR1-Rap1b	acatccacacacacc
HMR2-Abf1a	aaacgtgccaaatgat
HMR2-Abf1b	aaacgtctatagtgat
HMR2-ORC	ttttaatgtttggtaga
HMR2-Rap1	acatccatacacacc
HML-Rap1a	gtggtgtacggatgc
HML-Rap1b	gaggtgtacaggtct

603

604

605 **FIGURE LEGENDS**

606 **Figure 1: TdOrc1 associated with the mating-type loci and origins of replication. (A)** Evolutionary
607 relationship of yeast species discussed. WGD denotes whole genome duplication. *SIR1* indicates the
608 appearance of the *SIR1*/*KOS* gene family. The tree was built using 10 genes found in single copy in all
609 species (AGUILETA *et al.* 2008). The protein sequences were concatenated and aligned in MAFFT using
610 E-INS-I model, and a maximum likelihood tree was constructed with PhyML using the LG+F+I+G4
611 model. Node support was assessed with approximate likelihood ratio tests (aLRT). **(B)** Immunoblot of
612 TdOrc1-Myc and TdKos3-V5 (LRY3211). The parent strain (JRY10156) was included as a no tag
613 control. Endogenous 3-phosphoglycerate kinase (Pgk1) was detected as a loading control. **(C-F)** The
614 distribution of TdOrc1-Myc (LRY3211), TdKos3-V5 (LRY3211), TdSir2-V5 and TdSir4-V5 (ELLAHI
615 AND RINE 2016), and TdOrc2-V5 and TdOrc4-V5 (MARIA *et al.* 2021) at **(C)** a replication origin ARS-
616 VII69, **(D)** *HMR1*, **(E)** *HMR2***,** and **(F)** *HML* were determined using chromatin immunoprecipitation
617 followed by sequencing (ChIP-Seq). Each track represents the read depth of ChIP-Seq sequences from a
618 single sample. Replicate samples gave similar results. To assess potential variation in sequencing across
619 the genome, read depth for either input chromatin (Orc1, Kos3, Sir2, Sir4) or mock IP (Orc2, Orc4) is
620 overlaid in black. The genomic features are shown below the x-axis. Silencers are marked with solid
621 black lines. Predicted binding sites for potential silencer-binding proteins are represented as boxes for
622 Rap1 (orange), Abf1 (yellow) and ORC (grey).

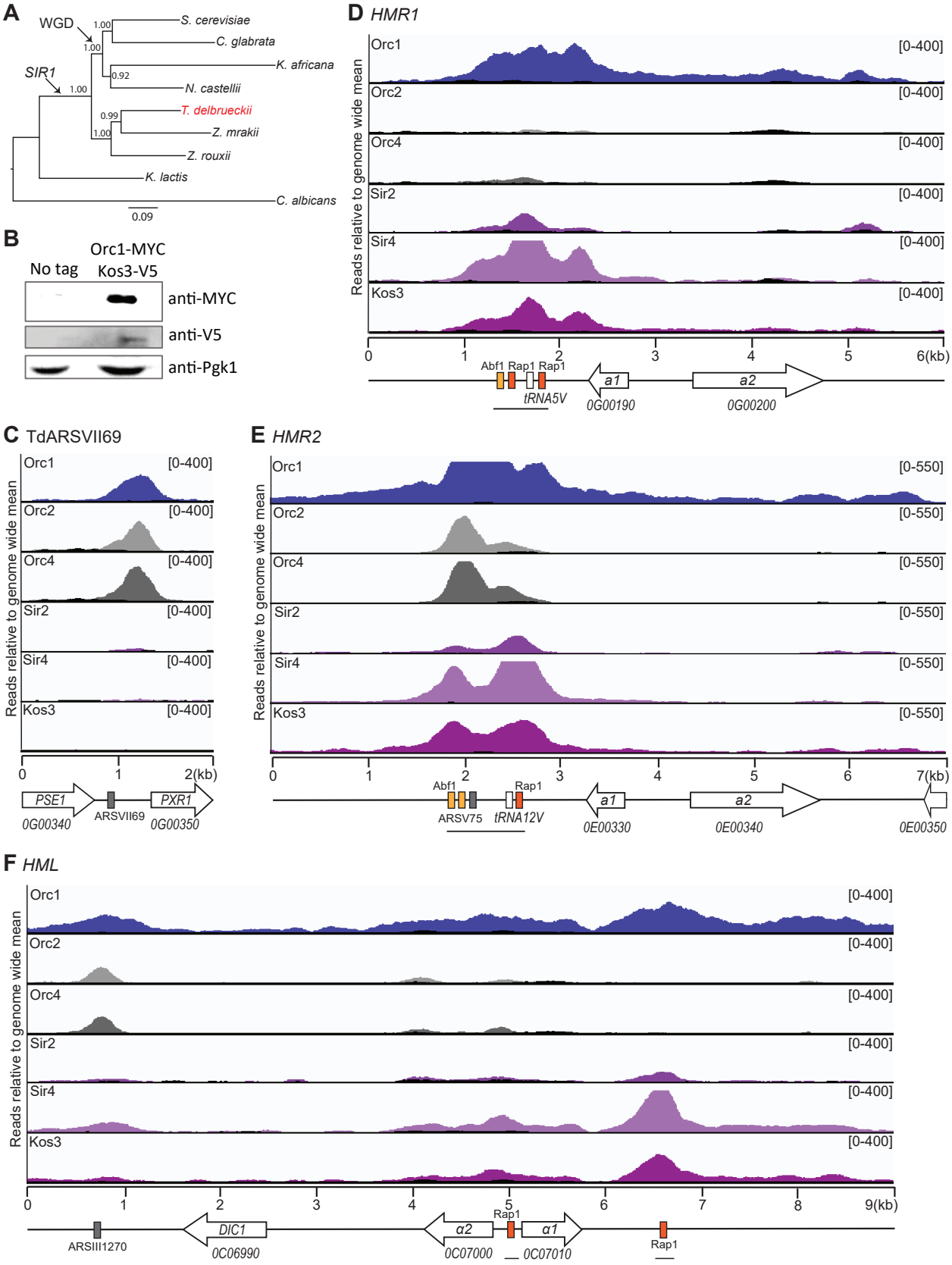
623

624 **Figure 2: TdSir2 and TdKos3 facilitated the recruitment and spreading of TdOrc1 at *HMR1* and**
625 ***HMR2.* (A, B)** Distribution of Orc1-Myc at *HMR1* (right) and the nearest ORC binding site (ARS-VII22;
626 left) in wild-type (LRY3204, blue), *sir2Δ* (LRY3206, red) and *kos3Δ* (LRY3205, green) strains. The ORC
627 binding site is ~14 kb from the Abf1 binding site at *HMR1*. Signal from input chromatin is shown in black.
628 **(C)** Association of Orc1-Myc at *HMR2* in *sir2Δ* (red) and *kos3Δ* (green) backgrounds and distribution of
629 Orc2-V5, and Orc4-V5 at *HMR2* (MARIA *et al.* 2021). **(D)** Immunoblot of Orc1-Myc in wild-type
630 (LRY3204), *kos3Δ* (LRY3205) and *sir2Δ* (LRY3206) backgrounds. The parent strain (JRY10156) is a no
631 tag control, and endogenous 3-phosphoglycerate kinase (Pgk1) is a loading control. **(E)** Diagram of plasmid
632 (pLR1384) used to evaluate transcriptional silencing. The plasmid consists of ~5 kb fragment of *HMR2*
633 with the **a**1 open reading frame replaced with Kl*URA3* and *HMR2* silencer replaced with *HMR1* silencer
634 (*HMR1s*). The plasmid bears a kanamycin resistance gene for selection. Diagram was created using
635 BioRender.com. **(F)** Expression of *URA3* integrated in place of ***a****1* and adjacent to *HMR1* silencer (*HMR1s*;
636 pLR1384) was measured in wild-type (JRY10156), *kos3Δ* (LRY3205) and *sir2Δ* (LRY3206) backgrounds
637 by assessing growth on media lacking uracil (CSM-Ura, growth requires *URA3* expression) or with 5-FOA
638 (CSM+5FOA, growth requires *URA3* silencing). Empty vector (pRS41K-TdCEN3) containing no *URA3*

639 was used as a control. CSM (left panel) indicates overall growth and equivalent dilutions. All media were
640 supplemented with geneticin to select for plasmids.
641
642 **Figure 3: The BAH domain facilitated the recruitment and spreading of TdOrc1 at *HMR1* and**
643 ***HMR2*. (A)** Alignment of *T. delbrueckii* Orc1, *S. cerevisiae* Orc1 and Sir3, and *K. lactis* Orc1 showing
644 conservation of the Proline (P) that was mutated (red). **(B)** Immunoblot of wild-type (LRY3211), *bahΔ*
645 (LRY3212), and *P179A* (LRY3215) Orc1-Myc. The anti-V5 band shows the simultaneous expression of
646 Kos3-V5. The parent strain (JRY10156) is a no tag control, and endogenous 3-phosphoglycerate kinase
647 (Pgk1) is a loading control. (**C-D**) Distribution of wild-type Orc1-Myc (LRY3211, blue), *orc1-bahΔ*-Myc
648 (**C**, LRY3212, light blue), and *orc1-P179A*-Myc (**D**, LRY3215, teal) at *HMR1* (right) and the nearest ORC
649 binding site to *HMR1*, ARS-VII22 (left). **(E)** Expression of *URA3* integrated in place of ***a1*** and adjacent to
650 *HMR1* silencer (*HMR1s*; pLR1384) was measured in wild-type *ORC1* (LRY3211), *orc1-bahΔ* (LRY3212),
651 and *orc1-P179A* (LRY3215) backgrounds by assessing growth on media lacking uracil (CSM-Ura, growth
652 requires *URA3* expression) or with 5-FOA (CSM+5FOA, growth requires *URA3* silencing). Empty vector
653 (pRS41K) containing no *URA3* was used as a control. CSM (panel one) was used to assess overall growth
654 and equivalent dilutions. All growth media were supplemented with geneticin to select for plasmids. **(F)**
655 Distribution at *HMR2* of wild-type Orc1-Myc (LRY3211, blue), *orc1-bahΔ*-Myc (LRY3212, light blue),
656 *orc1-P179A*-Myc (LRY3215, teal), Orc2-V5, and Orc4-V5 (MARIA *et al.* 2021).
657
658 **Figure 4: TdOrc1 BAH domain was important for TdKos3 association with *HMR1* and *HMR2*.**
659 Distribution of Kos3-V5 association in wild-type (LRY3211, purple), *orc1-bahΔ*-Myc (LRY3212, pink)
660 and *orc1P179A*-Myc (LRY3215, orange) strains at *HMR1* **(A)** and *HMR2* **(B)**. Distribution of Orc1-Myc
661 (blue), Orc2-V5 (light grey) and Orc4-V5 (dark grey) are shown for comparison (MARIA *et al.* 2021). Signal
662 from input chromatin is shown in black.
663
664 **Figure 5: Binding sites for Rap1, but not ORC, were required for Orc1 association with *HMR1*. (A)**
665 TdOrc1 enrichment at a control origin, ARS-V931, was determined by chromatin IP followed by
666 quantitative PCR (ChIP-qPCR) in strains with (dark blue, LRY3208 and 3209) and without (grey,
667 LRY3334 and 3335) the ORC binding site nearest to *HMR1*, ARS-VII22. **(B)** TdOrc1 enrichment at *HMR1*
668 (right) and the nearest ORC-binding site (ARS-VII22, left) in the presence (dark blue) and absence (grey)
669 of ARS-VII22. **(C)** TdOrc1 enrichment at ARS-VII22 (left) and *HMR1* (right) in strains with wild-type
670 *HMR1* (dark blue, LRY3208 and 3209) or lacking the Rap1 binding sites (*rap1bsΔ*, red, LRY3406 and
671 3407). The strains used for these experiments lacked *HMR2*. Mock immunoprecipitation using anti-Myc
672 antibody and the untagged parent strain (JRY10156) is shown in light blue. The y-axis represents the

673 relative enrichment to the control locus *ATS1*. Solid black lines represent the position of the qPCR primers.
674 The bars represent the average of six independent IPs with error bars representing SEM. Asterisks show
675 statistically significant differences (**$p < 0.005$, *$p < 0.01$, ns = $p > 0.05$) determined using two-tailed
676 Student's t-test.
677
678 **Figure 6: An ORC binding site at *HMR2* was dispensable for transcriptional silencing. (A)** Diagram
679 of reporter plasmid to evaluate role of Orc1 binding at the *HMR2* silencer in gene expression. The plasmid
680 carries a ~5 kb fragment of *HMR2* with the **a**1 open reading frame replaced with Kl*URA3.* The plasmid
681 bears a Kanamycin resistance gene for selection in geneticin. Diagram was created using BioRender.com.
682 **(B)** ChIP-qPCR showing TdOrc2-V5 enrichment at the plasmid origin, *ScARS209* (left) or the *HMR2*-
683 associated ORC binding site, *TdARS-V75* (right), in the presence (dark blue, pLR1377) or absence (light
684 blue, pLR1382) of the ORC binding site. An Orc2-V5 strain (LRY3142) was transformed with both
685 plasmids. No antibody controls (grey) are overlayed on respective IP signals. The y-axis represents the
686 relative enrichment to the control locus *ATS1*. The bars represent the average of six independent IPs with
687 error bars representing SEM. Asterisks show statistically significant differences (**$p < 0.005$, *$p < 0.01$,
688 ns = $p > 0.05$) determined using two-tailed Student's t-test. Black lines in the schematic represent the
689 position of qPCR primers. **(C)** Plasmid stability assay to assess contribution of ORC binding sites. Wild-
690 type *T. delbrueckii* cells (JRY10156) were transformed with plasmids with both the plasmid and silencer
691 ORC binding sites (pLR1377), *arsV75Δ* (pLR1382), *ars209Δ* (pLR1401), or both *arsV75Δ* and *ars209Δ*
692 (pLR1400). Transformants were selected on geneticin and imaged after two days. **(D)** Expression of *URA3*
693 integrated in place of ***a1*** and adjacent to *HMR2* silencer in wild-type *ORC1* (JRY10156) or *orc1-bahΔ*
694 (LRY3212) strains. Yeast were transformed with plasmids containing no *URA3* (empty vector, pRS41K-
695 TdCEN3, row 1), *HMR2**a**1::URA3* with wild-type silencer (pLR1377, rows 2 and 5), no ORC binding site
696 in the silencer (*arsV75Δ*, pLR1382, rows 3 and 6), or no ORC or Abf1 binding sites (*arsΔ+abf1*$_{bs}$*Δ*,
697 pLR1399, row 4). Cells were grown on CSM (left) to assess growth and dilution, CSM-Ura (middle panel,
698 growth requires *URA3* expression), and CSM+5FOA (right panel, growth requires *URA3* silencing). All
699 plates were supplemented with geneticin to select for the plasmids.
700
701 **Figure 7: Some Kos3 orthologs lack an Orc Interacting Region (OIR).** Sequences of Kos3 orthologs
702 were obtained from NCBI, and analyzed for the presence of conserved domains. OIR domains (light grey
703 boxes) were identified by submitting each protein as a query to NCBI blastp. and the program returned the
704 boundaries of the defined "Sir1 superfamily" domain that corresponds to the OIR. Other conserved regions
705 (colored boxes) were identified using the MEME suite (BAILEY *et al.* 2009) to search for sequence motifs
706 present zero or one time in each member of the protein set. Lengths of the proteins are relative to their sizes.

707 *S. cerevisiae* Sir1 is included for comparison. ScSir1 has a C-terminal OIR (light grey) and N-terminal OIR'
708 (dark grey).
709
710 **Figure 8: Rap1 and Abf1 associated with the silencers at the *T. delbrueckii* mating-type loci. (A)**
711 Immunoblot of V5 tagged Rap1 (JRY10207), Sum1 (LRY3172), Abf1 (LRY3336), Reb1 (LRY3338) and
712 Ume6 (LRY3340) proteins. The parent strains (JRY10156 and LRY3208) were included as no V5 tag
713 controls. Endogenous 3-phosphoglycerate kinase (Pgk1) was detected as a loading control. **(B-D)**
714 Association of the silencer binding candidates, Rap1 (orange, JRY10207) Abf1 (yellow, LRY3336 and
715 LRY3337), Sum1 (grey, LRY3172 and 3173), Reb1 (blue, LRY3338 and 3339), and Ume6 (green,
716 LRY3340 and 3341) at *HMR1* **(B)**, *HMR2* **(C)**, and *HML* **(D)** loci. Solid black lines represent the
717 positions of the qPCR primers. **(E)** Association of Sum1 (grey), Reb1 (blue) and Ume6 (green) with other
718 genomic loci to confirm successful immunoprecipitation. The y-axis represents ratio of IP over mock IP:
719 IP enrichments were first calculated relative to the control locus *ATS1* and then normalized to the mock IP
720 Mock IPs were anti-Myc and anti-V5 antibody pulldowns in untagged parent strains (JRY10156 and
721 LRY3208). The bars represent the average of six independent IPs with error bars representing SEM.
722 Asterisks show statistically significant differences (**$p < 0.005$, *$p < 0.01$, ns = $p > 0.05$) when IP sample
723 was compared to mock IP using two-tailed Student's t-test.
724
725 **Figure 9: Composition of SIR heterochromatin in three budding yeast.** Model for SIR
726 heterochromatin at *S. cerevisiae HMR* (top), *T. delbrueckii HMR2* (middle; model based on this study),
727 and *K. lactis HML* (bottom). *ORC1* duplication – duplication resulting in *ORC1* and *SIR3*. *SIR1* –
728 appearance of *SIR1* family. ORC – Origin Recognition Complex (violet), 1 – Sir1 (yellow), 2 – Sir2
729 (grey), 4 – Sir4 (grey), 3 – Sir3 (teal), R – Rap1 (brick red), A – Abf1 (green), Ac – acetyl group on
730 H4K16 (grey), O1 – Orc1 (blue), K – Kos3 (yellow), R_{eb1} – Reb1 (coral), U – Ume6 (dark violet), and S –
731 Sum1 (pink). Figure was created using BioRender.com.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1.00
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T. delbrueckii
Z. mrakii
Z. rouxii
K. lactis
C. albicans
0.09
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Reads relative to genome wide mean
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Orc2
Orc4
Sir2
Sir4
Kos3
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a1
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Sir2
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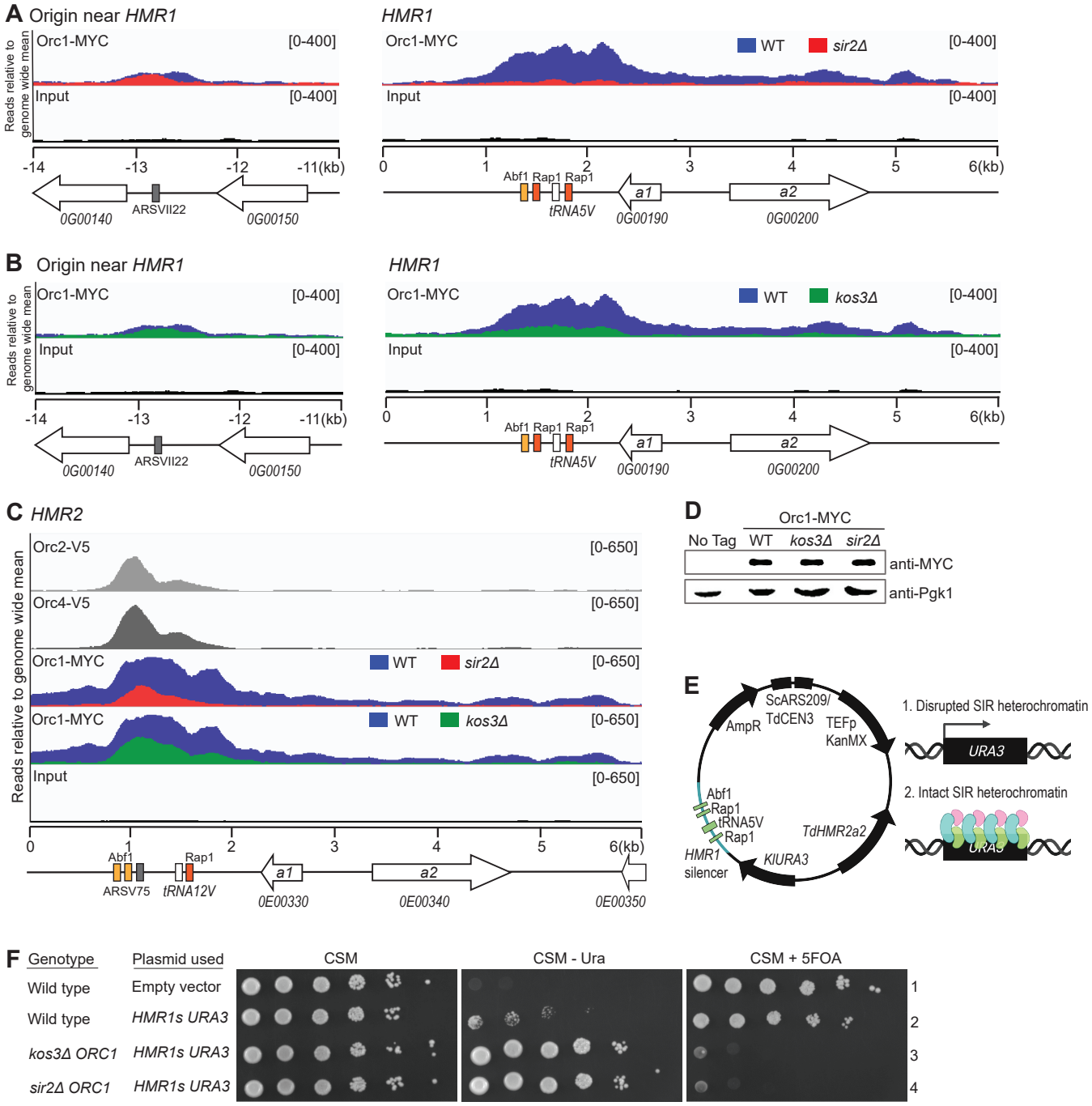
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a1
a2
0G00190
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HMR1
kos3Δ
C HMR2
Orc2-V5
Orc4-V5
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anti-Pgk1
E
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TdCEN3
AmpR
TEFp
KanMX
Abf1
Rap1
tRNA5V
Rap1
TdHMR2a2
HMR1
silencer
KlURA3
1. Disrupted SIR heterochromatin
URA3
2. Intact SIR heterochromatin
F
Genotype
Plasmid used
CSM
CSM - Ura
CSM + 5FOA
Wild type
Empty vector
Wild type
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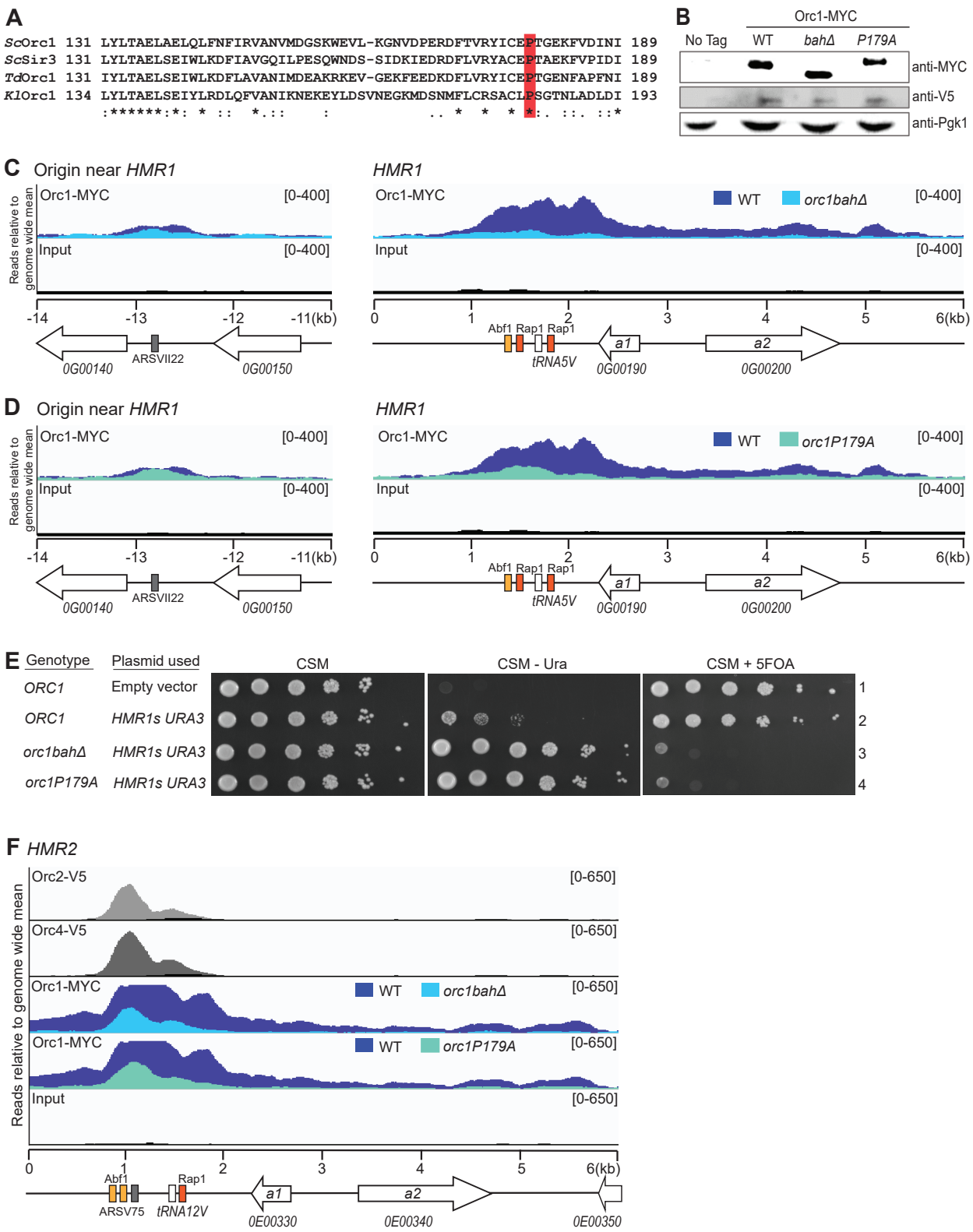
2
3
4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KlOrc1 134 LYLTAELSEIYLRDLQFVANIKNEKEYLDSVNEGKMDSNMFLCRSACLPSGTNLADLDI 193
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Orc1-MYC
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Orc1-MYC
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Genotype
Plasmid used
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CSM + 5FOA
ORC1
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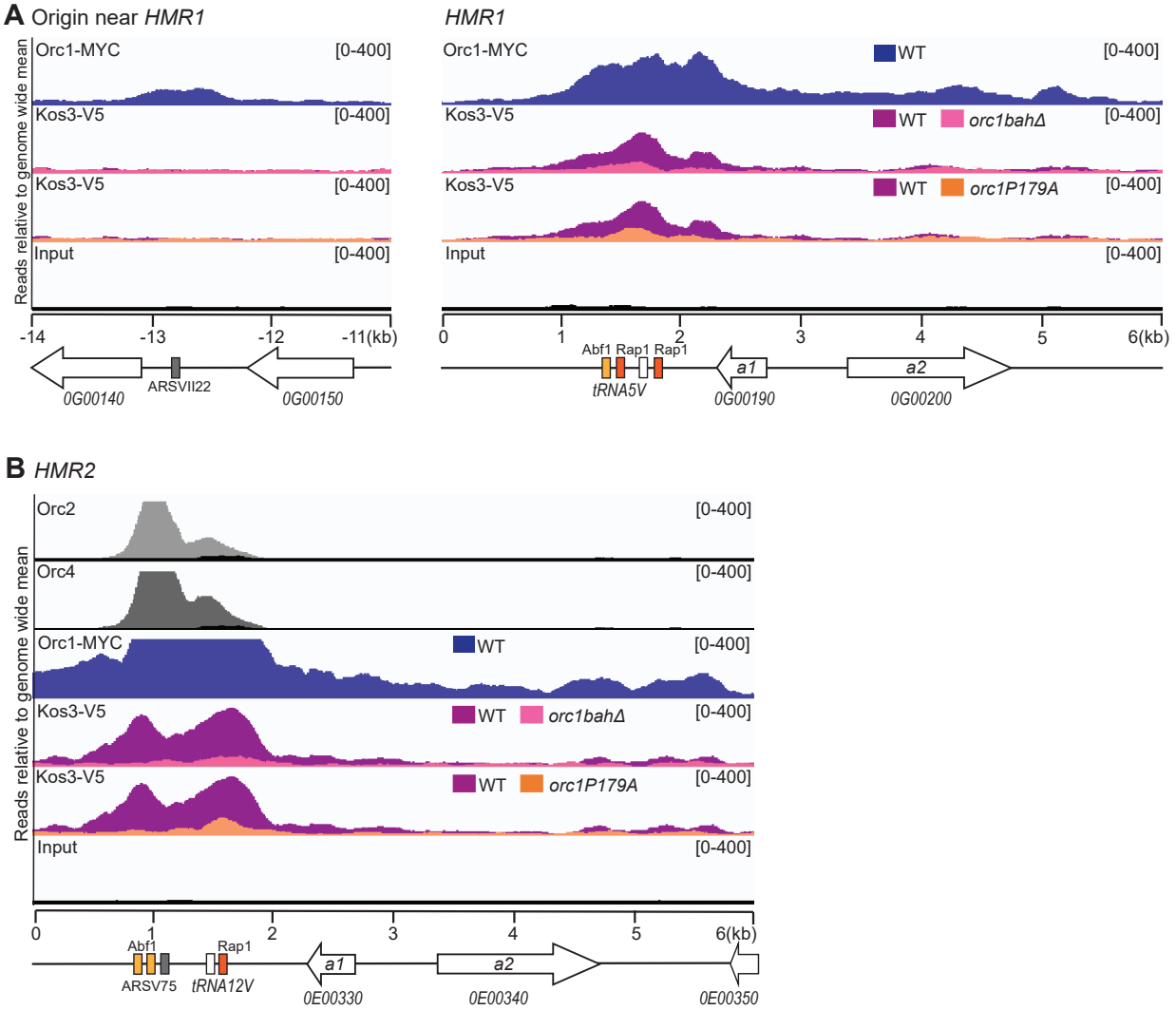
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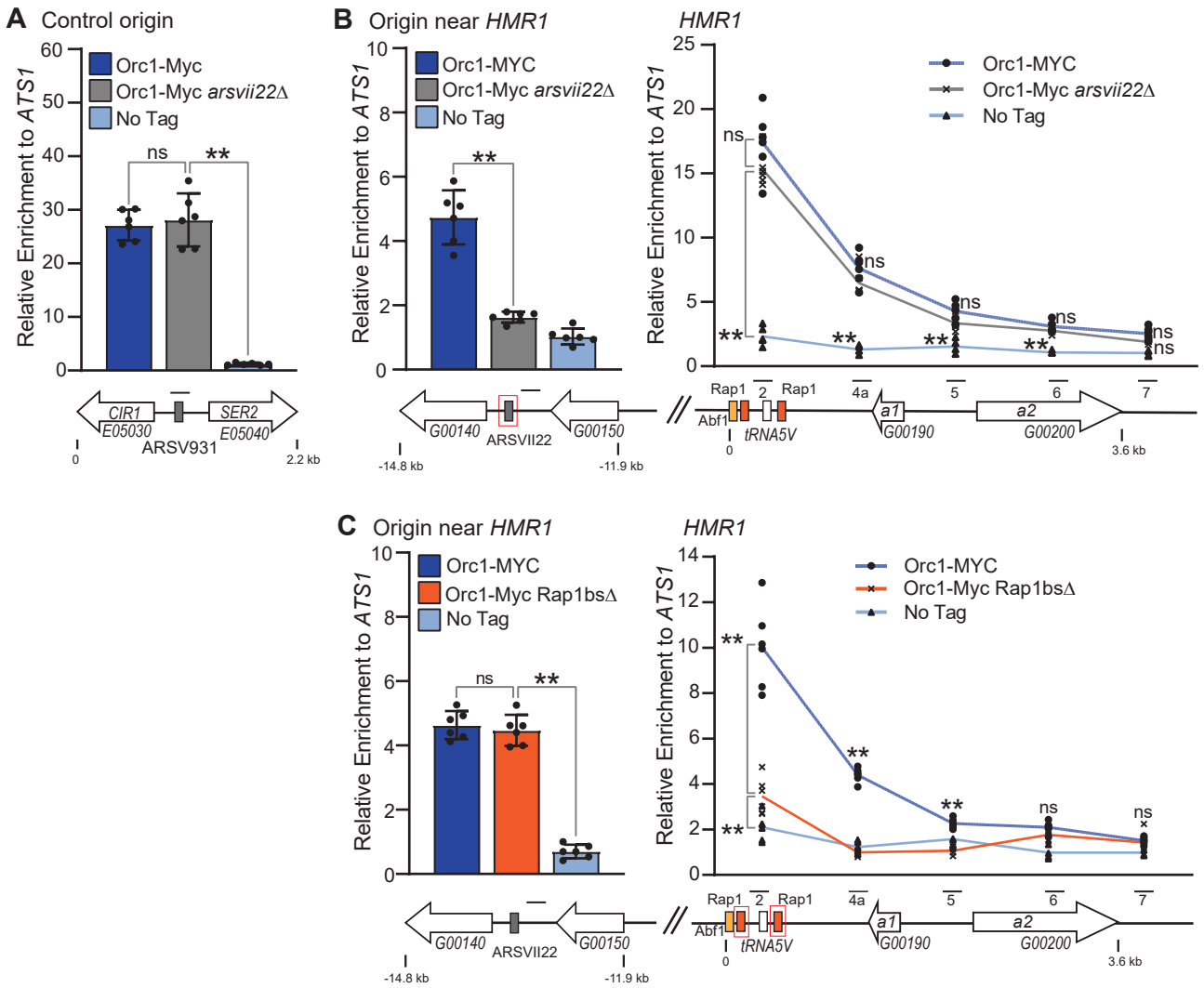
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Rap1
2
Rap1
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6
7
Abf1
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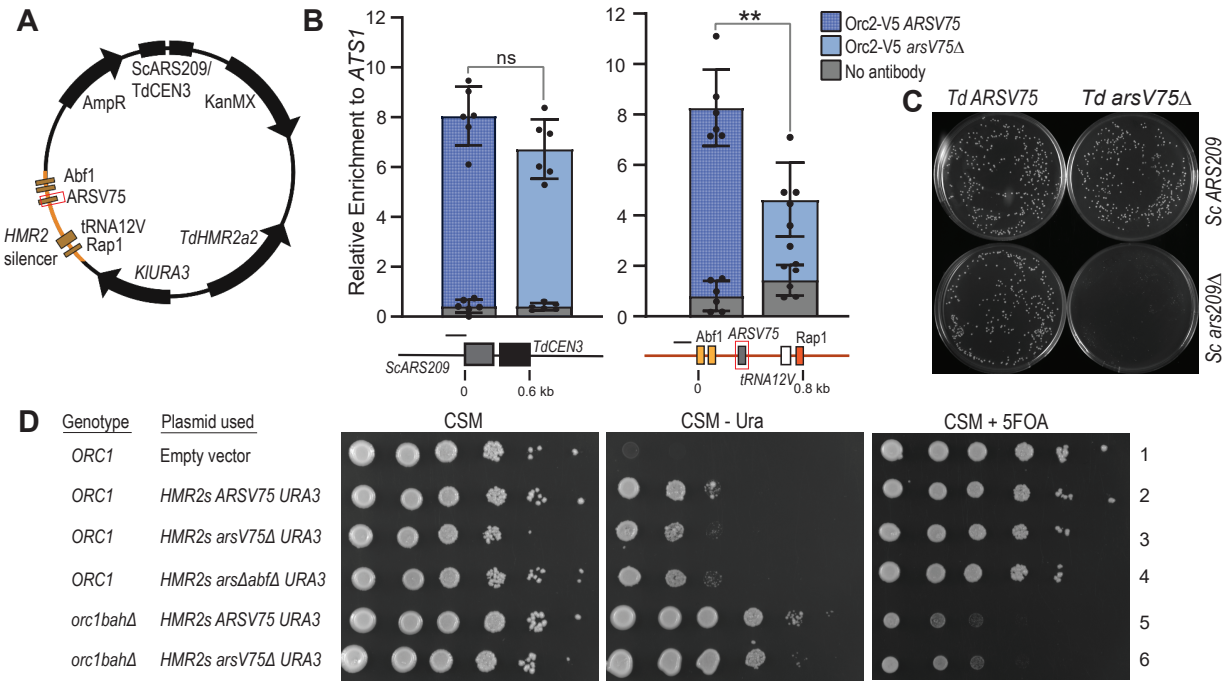

D

Genotype	Plasmid used
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ORC1	*HMR2s arsV75Δ URA3*
ORC1	*HMR2s arsΔabfΔ URA3*
orc1bahΔ	*HMR2s ARSV75 URA3*
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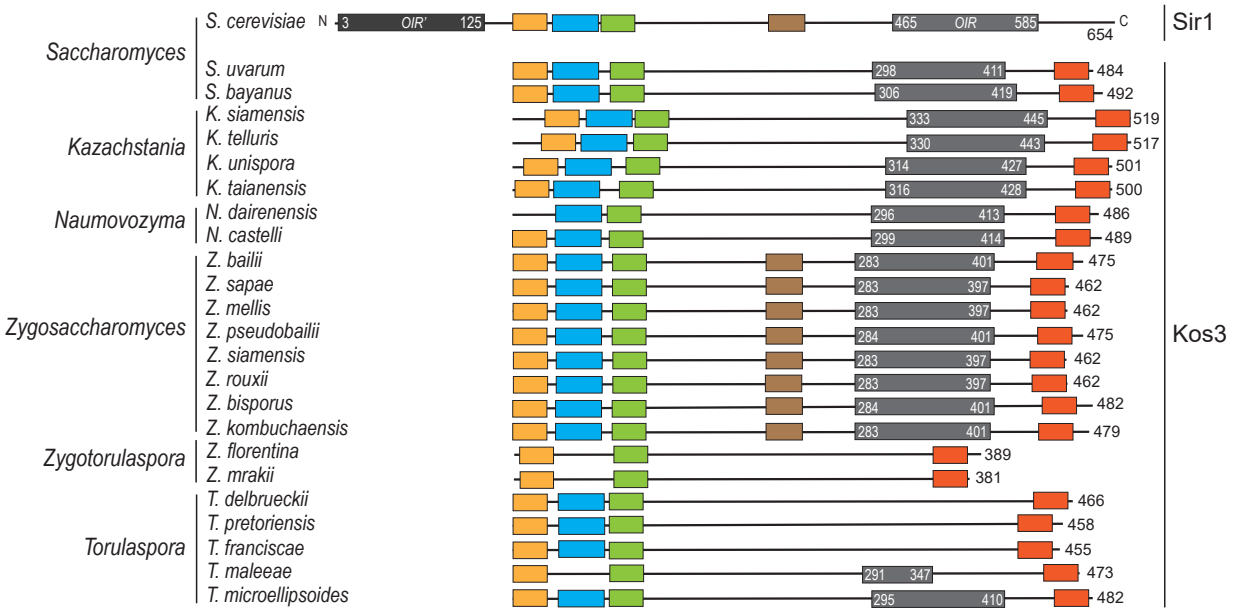
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3
OIR'
125
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OIR
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C
654
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K. unispora
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K. taianensis
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Z. bailii
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Z. mellis
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Kos3
Zygotorulaspora
Z. florentina
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Z. mrakii
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Torulaspora
T. delbrueckii
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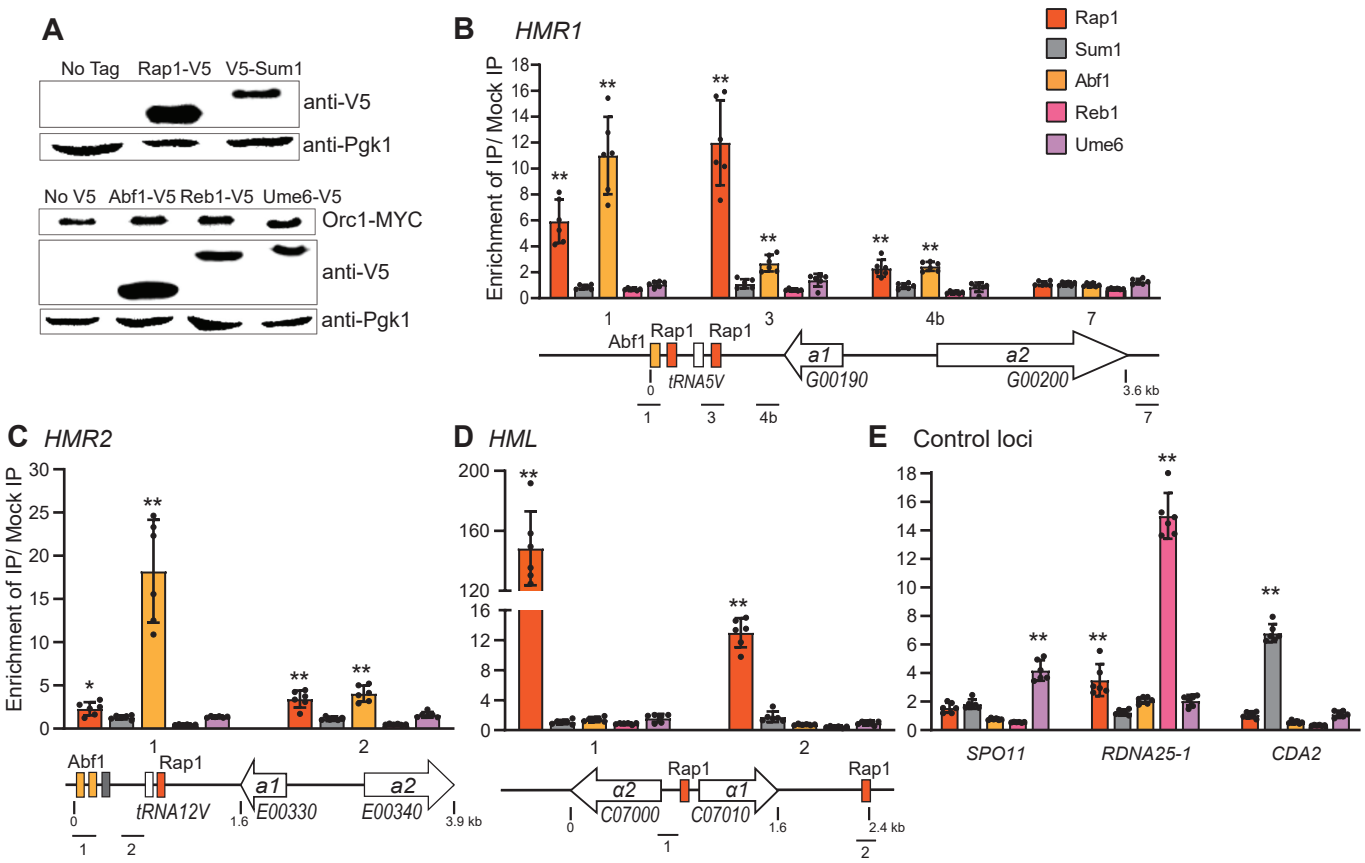
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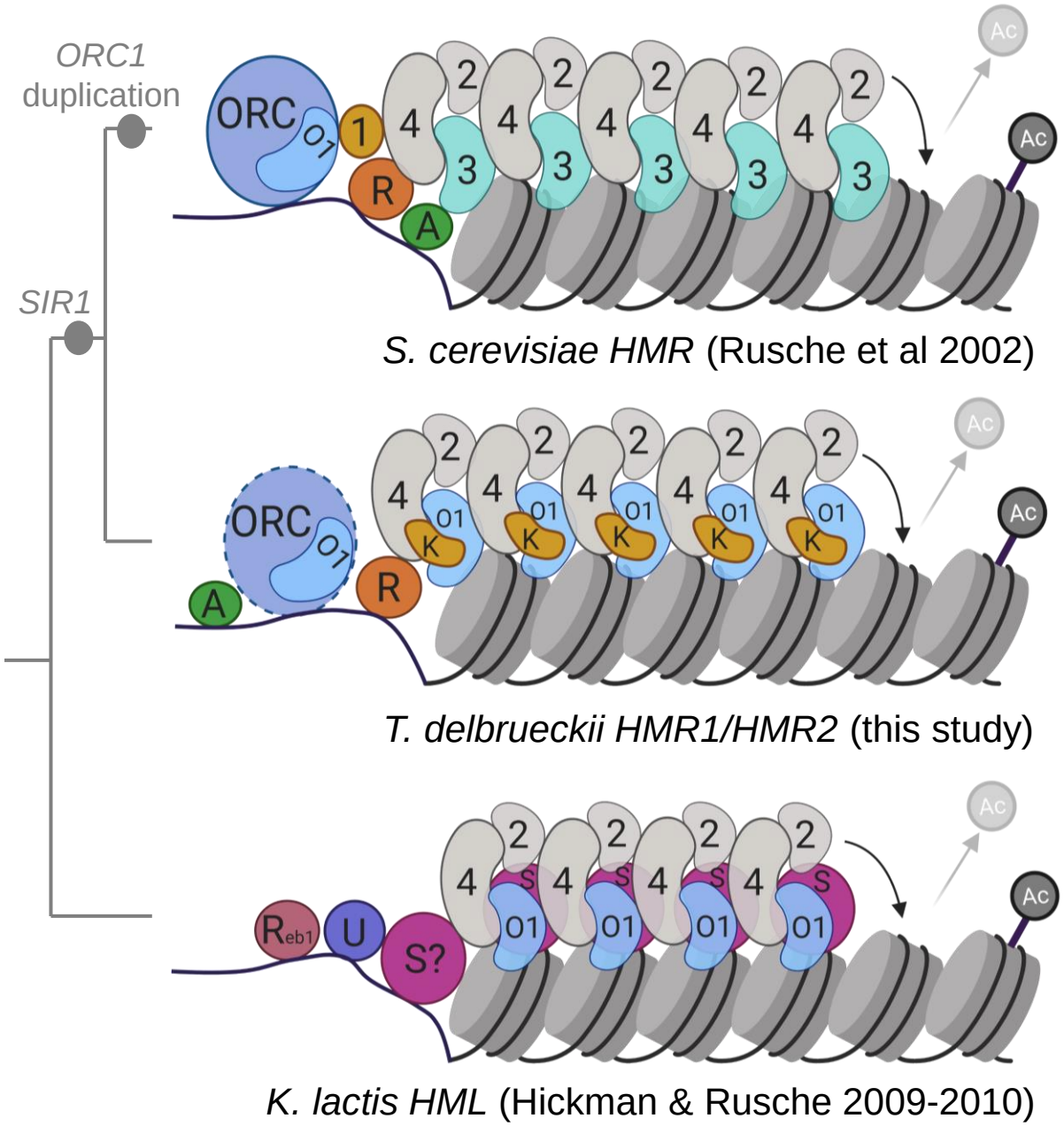
Orc1-MYC
anti-V5
anti-Pgk1
B
HMR1
Rap1
Sum1
Abf1
Reb1
Ume6
Enrichment of IP/ Mock IP
Abf1
Rap1
Rap1
tRNA5V
a1
G00190
a2
G00200
3.6 kb
C
HMR2
Enrichment of IP/ Mock IP
Abf1
Rap1
tRNA12V
a1
E00330
a2
E00340
3.9 kb
D
HML
α2
C07000
Rap1
α1
C07010
Rap1
2.4 kb
E
Control loci
SPO11
RDNA25-1
CDA2


ORC1
duplication
SIR1
ORC
O1
1
R
A
4
2
3
Ac
S. cerevisiae HMR (Rusche et al 2002)
K
T. delbrueckii HMR1/HMR2 (this study)
Reb1
U
S?
S
K. lactis HML (Hickman & Rusche 2009-2010)