# Transcriptional silencing functions of the yeast protein Orc1/Sir3 subfunctionalized after gene duplication

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Edited by Jasper Rine, University of California, Berkeley, CA, and approved September 30, 2010 (received for review May 7, 2010)

**The origin recognition complex (ORC) defines origins of replication and also interacts with heterochromatin proteins in a variety of species, but how ORC functions in heterochromatin assembly remains unclear. The largest subunit of ORC, Orc1, is particularly interesting because it contains a nucleosome-binding BAH domain and because it gave rise to Sir3, a key silencing protein in *Saccharomyces cerevisiae*, through gene duplication. We examined whether Orc1 possessed a Sir3-like silencing function before duplication and found that Orc1 from the yeast *Kluyveromyces lactis*, which diverged from *S. cerevisiae* before the duplication, acts in conjunction with the deacetylase Sir2 and the histone-binding protein Sir4 to generate heterochromatin at telomeres and a mating-type locus. Moreover, the ability of KlOrc1 to spread across a silenced locus depends on its nucleosome-binding BAH domain and the deacetylase Sir2. Interestingly, KlOrc1 appears to act independently of the entire ORC, as other subunits of the complex, Orc4 and Orc5, are not strongly associated with silenced domains. These findings demonstrate that Orc1 functioned in silencing before duplication and suggest that Orc1 and Sir2, both of which are broadly conserved among eukaryotes, may have an ancient history of cooperating to generate chromatin structures, with Sir2 deacetylating histones and Orc1 binding to these deacetylated nucleosomes through its BAH domain.**

heterochromatin | replication | SIR

The origin recognition complex (ORC) not only defines origins of replication but also interacts with heterochromatin proteins in a broad range of species. For example, human and *Drosophila* ORC bind to HP1 (heterochromatin protein 1) (1–4) and human ORC associates with telomeric and pericentromeric heterochromatin (5–7). Orc1 and the deacetylase Sir2 also repress genes near telomeres in the evolutionarily distant organism *Plasmodium falciparum*, which causes malaria (8). However, it remains unclear how ORC influences the assembly of heterochromatin. The largest subunit of ORC, Orc1, is particularly interesting because it contains a nucleosome-binding BAH domain, which potentially enables it to associate with chromatin. In addition, Orc1 gave rise to Sir3, a key silencing protein in *Saccharomyces cerevisiae*, via gene duplication, suggesting that before this duplication Orc1 had properties that predisposed it to become a silencing protein.

Gene duplication is an important force in evolution, as it provides new genetic material free of selective constraint. Preservation of a duplicated gene pair can result in conservation of function, neofunctionalization, or subfunctionalization (9). Neofunctionalization occurs when one duplicate gene evolves a new function, while the other copy retains the original function. In such a case, the gene with the new function is predicted to display a more rapid change in sequence, i.e., "accelerated evolution." An alternative paradigm, subfunctionalization, predicts that if the ancestral gene had multiple, independent functions, those functions could be partitioned between the duplicates (10, 11). Although these models have been elaborated theoretically, there are relatively few duplicated gene pairs for which the path of divergence is known (12, 13). We previously demonstrated that the duplicated deacetylases Sir2 and Hst1 subfunctionalized (14, 15), and in this study, we propose that the *SIR3–ORC1* gene pair subfunctionalized and then specialized after duplication.

In *S. cerevisiae*, SIR-mediated silencing occurs at telomeres and the mating-type loci, *HMLα* and *HMRa* (16). Transcriptional silencing of the mating-type loci is required to maintain cell-type identity in haploid cells. In contrast, SIR-silenced chromatin at the telomeres is thought to serve a structural role (17). The Sir proteins are recruited to the mating-type loci through silencer sequences that bind ORC, Rap1, and Abf1, which in turn recruit the Sir proteins. At telomeres, Sir proteins are recruited via multiple molecules of Rap1, whose binding sites are embedded within the telomere repeats (18). Once recruited, the Sir proteins spread along the chromosome to form an extended silenced domain. Sir2 is a $NAD^+$-dependent deacetylase, and Sir3 and Sir4 bind preferentially to deacetylated histones H3 and H4 (19, 20). The deacetylation of nucleosomes by Sir2 is thought to create high-affinity binding sites for Sir3 and Sir4, which in turn recruit additional Sir2, enabling the propagation of Sir-silenced chromatin (21–23). A fourth Sir protein, Sir1, stabilizes the other Sir proteins at silencers by interacting with Orc1 but is not thought to spread (24–27).

Interestingly, both Sir2 and Sir3 have paralogs that arose in a whole-genome duplication ≈100 million years ago (28–30). The paralog of Sir2 is the deacetylase Hst1, which is part of the promoter-specific SUM1 repressor complex. The paralog of Sir3 is Orc1, the largest subunit of ORC. The sequences of Sir3 and Orc1 have diverged considerably, and these proteins cannot complement each other (31). However one domain, the nucleosome-binding BAH domain, is 50% identical and 65% similar between ScOrc1 and ScSir3 and has a highly conserved tertiary structure (31–33). Nonduplicated orthologs of Orc1 and Sir3 display more sequence similarity to ScOrc1 than to ScSir3, and this accelerated sequence divergence in Sir3 has led to the hypothesis that the silencing function of Sir3 arose through neofunctionalization (29). However, others have argued that Orc1 and Sir3 subfunctionalized (34).

Despite their common ancestry, Orc1 and Sir3 have distinct roles in the formation of silenced chromatin in *S. cerevisiae*. Orc1, as part of ORC, binds silencers (16), and the BAH domain of Orc1 interacts with Sir1 (26, 27) to stabilize other Sir proteins at silencers. This recruitment of Sir1 is thought to be the only function of ORC in silencing because ORC can be bypassed by

Author contributions: M.A.H. and L.N.R. designed research, performed research, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1006436107/-/DCSupplemental.

tethering Sir1 to the silencer (27, 35). Interestingly, the BAH domain of Orc1 is not essential for DNA replication in *S. cerevisiae* (31), although it is conserved in most eukaryotic orthologs of Orc1. In contrast to Orc1, Sir3 is critical for the spreading of the SIR complex (21, 23), presumably due to its ability to bind histones through the BAH domain and a second C-terminal histone binding domain (19, 20).

The evolution of *SIR3* from *ORC1* could indicate that before duplication, Orc1 functioned with Sir2 to generate heterochromatin and that this relationship is ancient, consistent with the association of both ORC and Sir2 with heterochromatin in many organisms. In this model, after the duplication, the replication and silencing functions of Orc1 were partitioned between *ORC1* and *SIR3*. The alternative hypothesis is that the ability to assemble and spread with Sir2 was acquired by Sir3 after duplication. To distinguish between these models, we examined the function of Orc1 in the yeast *Kluyveromyces lactis*, which diverged from *S. cerevisiae* before the duplication. *K. lactis* is the only nonduplicated budding yeast species in which silencing has been examined experimentally. *K. lactis* has orthologs of *SIR2* and *SIR4*, and deletion of either gene derepresses mating-type loci (15, 36–38). In addition, the SUM1 complex, which is not associated with Sir-silenced domains in *S. cerevisiae*, is essential for silencing both mating-type loci in *K. lactis* (15). Interestingly, the characterized silencers in *K. lactis* do not contain an ORC-binding sequence (39), and Sir1 is not identifiable in the *K. lactis* genome (40). Thus, KlOrc1 probably does not have the same function in silencing as ScOrc1.

We have examined the function of the single Orc1/Sir3 protein from *K. lactis* as a proxy for the ancestral Orc1 and found that KlOrc1 does indeed act in conjunction with the deacetylase Sir2 to generate silenced chromatin at telomeres and a mating-type locus.

## Results

**KlOrc1 Associated with the Silenced *HMLα* Locus.** To determine whether the nonduplicated KlOrc1 (*KLLA0B05016g*) functions strictly as a replication factor, as predicted by the neofunctionalization model, or also functions as a silencing factor capable of spreading, as predicted by the subfunctionalization model, we assessed the association of KlOrc1 with the silenced mating-type locus *HMLα* and the known replication origin *KlARS503* (41, 42) by chromatin immunoprecipitation. As anticipated, KlOrc1 associated with the origin, and its maximum enrichment coincided with the autonomously replicating sequence (Fig. 1*A*). Importantly, KlOrc1 also associated with *HMLα* and was distributed across the entire 6-kb locus in a pattern similar to that observed for other silencing factors, such as Sir2 (Fig. 1*B*). This result suggests either that KlOrc1 is a component of silenced chromatin at *HMLα* or that an unusually large replication origin occurs at *HMLα*. To distinguish between these possibilities, we assessed the association of two additional subunits of ORC, Orc4 (*KLLA0C16984g*) and Orc5 (*KLLA0C02607g*), with *HMLα*. If a replication origin occurs at *HMLα*, Orc4 and Orc5 should also associate with the locus. However, these subunits were only slightly enriched above background at *HMLα* (Fig. 1*B*), although they associated robustly with the origin *KlARS503* (Fig. 1*A*). Therefore, KlOrc1 appears to have a role at *HMLα* that is independent of the entire ORC.

**BAH Domain of KlOrc1 Was Required for Silencing but Not Viability.** To determine whether KlOrc1 contributes to transcriptional silencing at *HMLα*, we sought a separation-of-function mutation that would disrupt the silencing but not the essential replication properties of KlOrc1. We focused on the nucleosome-binding BAH domain, which is essential for the silencing functions of both ScSir3 and ScOrc1 but is dispensable for DNA replication in *S. cerevisiae* (26, 31, 43). We truncated the genomic copy of *KlORC1*, removing the sequence encoding the first 217 amino

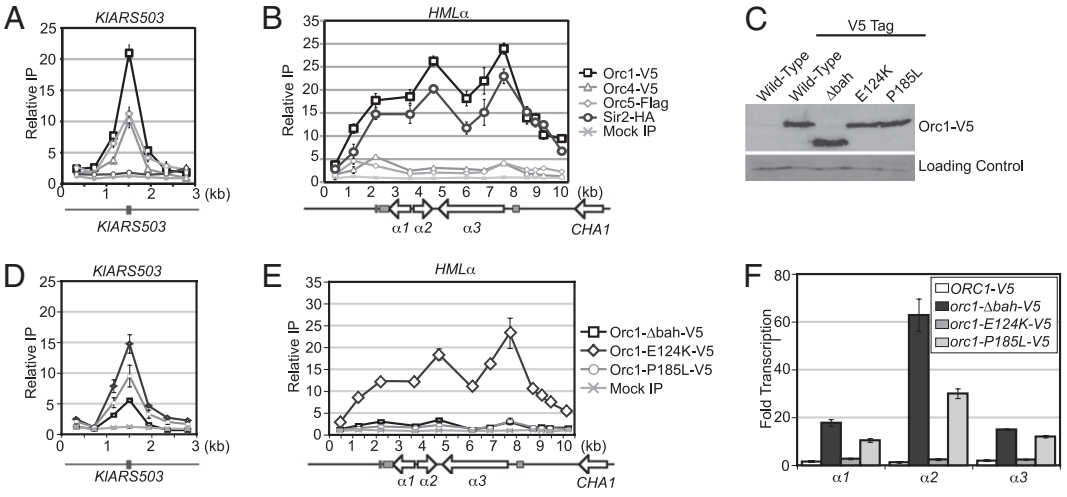


**Fig. 1.** KlOrc1 associates with replication origins and *HMLα*. (*A*) The associations of KlOrc1–V5 (LRY2561), KlOrc4–V5 (LRY2711), KlOrc5–Flag (LRY2235), and KlSir2–HA (LRY2566) with the replication origin *KlARS503* (previously known as *KARS12*) were assessed by chromatin IP followed by quantitative PCR. The gray box denotes the autonomously replicating sequence. The *y* axis represents the relative enrichment normalized to a control locus, *RRP7*, which is not detectably associated with KlOrc1, KlOrc4, KlOrc5, or KlSir2. Mock precipitations using the V5, HA, and Flag antibodies were conducted from a strain expressing untagged KlOrc1, KlOrc4, KlOrc5, and KlSir2 (CK213). (*B*) The associations of KlOrc1–V5, KlSir2–HA, and KlOrc5–Flag with *HMLα* were assessed using the same chromatin IP samples analyzed in *A*. The characterized silencer at *HMLα* is indicated by a dark gray box, and sequences conserved at all three mating-type loci are indicated in light gray. (*C*) KlOrc1–V5 (2561), KlOrc1–Δbah–V5 (LRY2562), KlOrc1–E124K–V5 (LRY2656), and KlOrc1–P185L–V5 (LRY2657) were detected by immunoblotting using an antibody against the V5 tag. A sample from a strain expressing untagged KlOrc1 (CK213) was also included. As a loading control, the same blot was probed with an antibody against H3-K4$^{me3}$. (*D*) The associations of KlOrc1–Δbah–V5 (LRY2562), KlOrc1–E124K–V5 (LRY2656), and KlOrc1–P185L–V5 (LRY2657) with *KlARS503* were analyzed as for *A*. (*E*) The associations of KlOrc1–Δbah–V5, KlOrc1–E124K–V5, and KlOrc1–P185L–V5 with *HMLα* were assessed using the same chromatin IP samples analyzed in *D*. (*F*) Quantitative RT-PCR analysis of *HMLα1*, *HMLα2*, and *HMLα3* mRNA derived from the same strains analyzed in *A*–*E*. The amount of cDNA was first normalized to the control locus *ACT1* and then compared with the untagged *ORC1* strain.

acids, which correspond to the BAH domain. KlOrc1–Δbah was expressed at a level comparable to wild-type KlOrc1 (Fig. 1*C*), and cells harboring *orc1–Δbah* as the only copy of *ORC1* were viable and displayed no growth defects or gross perturbations of the cell cycle (Figs. S1 and S2), indicating that the replication function of KlOrc1 was largely intact. Moreover, KlOrc1–Δbah still associated with the origins *KlARS503* (Fig. 1*D*) and *KlARS406* (Fig. S3), although its enrichment was reduced, consistent with observations in *S. cerevisiae* (44). In contrast, KlOrc1–Δbah no longer associated with *HMLα* (Fig. 1*E*).

To determine whether the loss of KlOrc1 at *HMLα* affects silencing, we measured the amount of the α*1*, α*2*, and α*3* transcripts in wild-type and *orc1–Δbah* strains by quantitative RT-PCR. In the absence of the BAH domain, all three genes were derepressed (Fig. 1*F*), demonstrating that KlOrc1 contributes to the transcriptional silencing of *HMLα*.

**BAH Domain of KlOrc1 Contributes to Silencing in a Sir3-Like Manner.** The BAH domains of ScSir3 and ScOrc1 contribute to silencing in different ways. The ScSir3 BAH domain enables the spreading of the Sir complex by binding nucleosomes (45, 46), whereas the ScOrc1 BAH domain recruits Sir1 to silencers. Given that there is no identifiable homolog of Sir1 in *K. lactis* (40), it is more likely that KlOrc1 acts at *HMLα* in a manner analogous to ScSir3. To explore this hypothesis, we generated two point mutations in *KlORC1*. The P185L mutation is analogous to a mutation in ScSir3 that disrupts nucleosome binding and the ability of Sir proteins to spread (46), and the E124K mutation occurs in the H domain, which interacts with Sir1 in *S. cerevisiae* (26) (Fig. S4). If KlOrc1 acts similarly to ScSir3, the P185L mutation should reduce its ability to associate with and maintain repression of *HMLα*, whereas if KlOrc1 acts similarly to ScOrc1 and interacts with a currently unknown Sir1-like protein, the E124K mutation might be disruptive. We found that the association of KlOrc1 with *HMLα* was greatly reduced by the P185L mutation but was only slightly affected by the E124K mutation (Fig. 1*E*). In addition, the P185L mutation derepressed *HMLα*, whereas the E124K mutation had only a slight effect (Fig. 1*F*). Both proteins were expressed at levels similar to wild-type Orc1 (Fig. 1*C*) and still associated with replication origins (Fig. 1*D* and Fig. S3), indicating that the observed phenotypes are not due to instability of the proteins. Therefore, KlOrc1 most likely contributes to silencing of *HMLα* by binding to nucleosomes in a Sir3-like manner.

**KlOrc1 BAH Domain Promoted the Distribution of Sir2 and Sir4 Across *HMLα*.** To investigate the mechanism by which KlOrc1 contributes to transcriptional silencing, we determined whether KlOrc1 was required for the association of other silencing factors. If KlOrc1 acts similarly to ScSir3, it would not be required for the initial recruitment of other silencing proteins to *HMLα*, but it would be required for them to spread. In contrast, if KlOrc1 acts like ScOrc1, it would be required for the recruitment of other proteins to silencers. In the absence of the BAH domain of KlOrc1, the association of Sir2 (*KLLA0F14663g*) was reduced across most of *HMLα*, although a peak of enrichment remained at the *α3* promoter (Fig. 2*A*). The association of Sir4 (*KLLA0F13420g*) was also reduced across the *HMLα* locus (Fig. 2*B*). In contrast, there was little change in the association of Sum1 (*KLLA0C14696g*) in the absence of the KlOrc1 BAH domain (Fig. 2*C*). Therefore, all three proteins associated with *HMLα*, at least to some extent, in the absence of the BAH domain, inconsistent with KlOrc1 acting as a primary recruiting factor. However, the BAH domain of KlOrc1 was important for Sir2 and Sir4 to spread across *HMLα*, consistent with KlOrc1 acting similarly to ScSir3.

**Sir2 and Sir4 Were Important for the Distribution of KlOrc1 Across HMLα.** In *S. cerevisiae*, ORC binds directly to silencers independently of the Sir proteins. In contrast, ScSir3 depends on silencer binding proteins and Sir4 for recruitment. To determine whether KlOrc1 requires known silencing proteins for recruitment to *HMLα*, KlOrc1 was immunoprecipitated from strains lacking Sir4, Sir2, or Sum1. In the absence of Sir2 or Sir4, individually or in combination, there was a severe reduction of KlOrc1 over most of the *HMLα* locus, except for the *α3* promoter (Fig. 3*A*). These results suggest that KlOrc1 is recruited independently of Sir2 and Sir4 to a putative silencer, but requires Sir2 and Sir4 to spread over the rest of the locus.

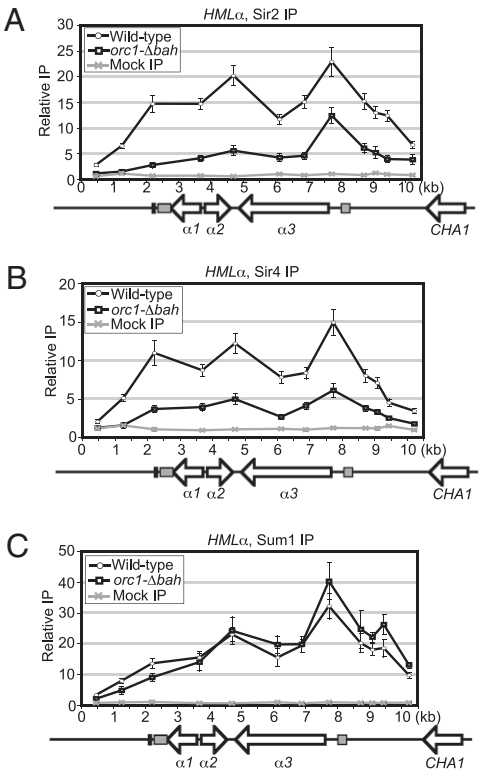


**Fig. 2.** KlSir2, KlSir4, and KSum1 associate with *HMLα* in an *orc1–Δbah* strain. (*A*) The association of KlSir2–HA with *HMLα* in *ORC1* (LRY2566) and *orc1–Δbah* (LRY2563) strains. (*B*) The association of KlSir4–Flag with *HMLα* in *ORC1* (LRY2566) and *orc1–Δbah* (LRY2563) strains. (*C*) The association of myc–KlSum1 with *HMLα* in *ORC1* (LRY2566) and *orc1–Δbah* (LRY2563) strains.

In contrast, the deletion of Sum1 did not result in a reduction of KlOrc1 with any region of *HMLα* (Fig. 3*B*). To determine whether Sir2/Sir4 and Sum1 act in parallel pathways to recruit KlOrc1, the association of KlOrc1 with *HMLα* was examined in *sum1Δ sir2Δ* and *sum1Δ sir4Δ* strains. In both strains, KlOrc1 was reduced over the entire locus (Fig. 3*B*). Therefore, KlOrc1 did not associate with *HMLα* independently of other silencing proteins, as *S. cerevisiae* ORC does, but instead required either the SUM1 complex or the Sir4/Sir2 complex for recruitment. Thus, KlOrc1 is recruited to *HMLα* in a manner resembling that of ScSir3.

**KlOrc1 Was Detected at Telomeres but Not the Silenced Mating-Type Locus *HMRa*.** To investigate whether KlOrc1 contributes to silencing at other genomic loci, we examined the association of KlOrc1 with *HMRa* and telomeres, loci associated with Sir proteins in *S. cerevisiae*. *KlHMRa* is silenced by Sir2 and Sum1 but not by Sir4 (15). Similarly, KlOrc1 was not associated with *KlHMRa* (Fig. 4*A*). Furthermore, there was no detectable change in expression of *HMRa1* or *a2* when the BAH domain was deleted (Fig. 4*B*), indicating that KlOrc1 does not contribute to the silencing of *HMRa*. Thus, in contrast to *S. cerevisiae*, the two silenced mating-type loci in *K. lactis* are repressed by different

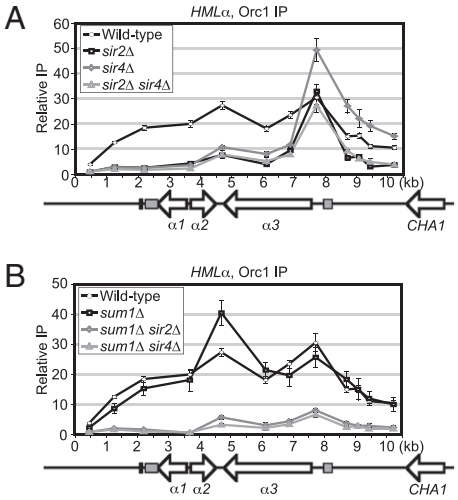


**Fig. 3.** KlSir2 and KlSir4, but not KlSum1, facilitate the spreading of KlOrc1 across *HMLα*. (*A*) The association of KlOrc1–V5 with *HMLα* in wild-type (LRY2561), *sir2*Δ (LRY2572), *sir4*Δ (LRY2573), and *sir2*Δ *sir4*Δ (LRY2577) strains. (*B*) The association of KlOrc1–V5 with *HMLα* in wild-type (LRY2561), *sum1*Δ (LRY2574), *sum1*Δ *sir2*Δ (LRY2578), and *sum1*Δ *sir4*Δ (LRY2576) strains. The wild-type data are the same as in Fig. 1*B*.

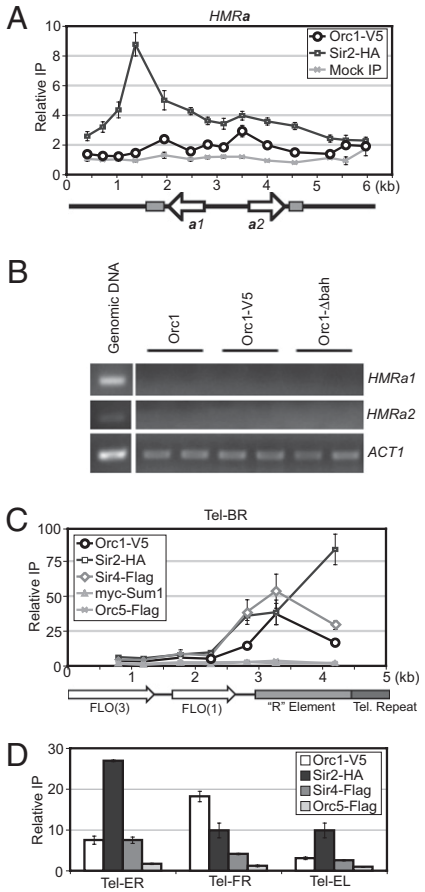


**Fig. 4.** KlOrc1 associates with telomeres, but not with *HMRa*. (*A*) The associations of KlOrc1–V5 (LRY2581) and KlSir2–HA (LRY2285) with ***HMRa***. A mock precipitation using the V5 antibody was conducted from a strain expressing untagged KlOrc1 (SAY538). Sequences found at *HMLα*, *MAT*, and *HMRa* are represented by light gray boxes. Data for KlSir2–HA were previously reported (15). (*B*) RT-PCR analysis of ***HMRa1***, ***HMRa2***, and *ACT1* in KlOrc1 (SAY538), KlOrc1–V5 (LRY2581) and KlOrc1–Δbah–V5 (LRY2709) strains. (*C*) The association of KlOrc1–V5 (LRY2561), KlOrc5–Flag (LRY2235), KlSir2–HA (LRY2239), KlSir4–Flag (LRY2239), and myc–KlSum1 (LRY2239) with Tel–BR. Telomeres in *K. lactis* contain a 1.5- to 2-kb element, termed the R element, immediately adjacent to the telomeric repeat (58). The R element, as well as the telomeric repeat sequence and the ORFs *FLO3* and *FLO1* are indicated. (*D*) Association of KlOrc1–V5, KlOrc5–Flag, KlSir2–HA, and KlSir4–Flag with Tel–ER, Tel–FR, and Tel–EL was assessed using the same chromatin IP samples as in *C*.

mechanisms, with the Sum1–Sir2 complex repressing both ***HMRa*** and *HMLα* and KlOrc1 and Sir4 acting only at *HMLα*.

We also examined the association of KlOrc1 with subtelomeric regions, focusing on the right arm of chromosome B, which has a unique sequence. We observed a gradient of Sir2 enrichment, with the greatest association nearest the telomere (Fig.4*C*), consistent with the pattern of Sir protein association at *S. cerevisiae* telomeres. KlOrc1 and Sir4 were also associated with this telomere, but peaked at an internal site. As was observed at *HMLα*, KlOrc1 was recruited to this telomere independently of the entire ORC, as Orc5 was not detected (Fig. 4*C*). Interestingly, Sum1 was also absent from this telomere (Fig. 4*C*). We also observed Sir2 adjacent to three other telomeres, and KlOrc1 and Sir4 associated with two of these telomeres (Fig. 4*D*). Thus, KlOrc1, Sir2, and Sir4 colocalize at subtelomeres.

## Discussion

This study demonstrates that KlOrc1, a subunit of the origin recognition complex, functions in the formation of silenced chromatin at *HMLα* and telomeres. Interestingly, the role of KlOrc1 in silencing is more like that of its duplicated homolog ScSir3 than like ScOrc1. In particular, KlOrc1 associated with silenced loci independently of the entire ORC and was distributed across the entire *HMLα* locus. Moreover, the BAH domain was required for the spreading of KlOrc1, KlSir2, and KlSir4 across the locus, and a single point mutation predicted to disrupt nucleosome binding perturbed transcriptional repression as well as the association of KlOrc1 with *HMLα*. In contrast, KlOrc1 did not appear to be a silencer binding protein, as it required other silencing proteins to associate with *HMLα*.

Data presented here and previously (15) suggest the existence of two distinct repressive modules acting at *HMLα*. One module, akin to the *S. cerevisiae* SIR complex, is composed of KlOrc1, Sir2, and Sir4 and associates with telomeres as well. These three proteins depend on one another to assemble on the telomere-proximal side of *HMLα*. The other module, akin to the *S. cerevisiae* SUM1 complex, is composed of Sum1, Sir2, and Rfm1 and also associates with *HMRa*. Interestingly, these two modules appear to assemble independently of one another at *HMLα*, as Sum1 still associated with *HMLα* in the *orc1–Δbah* strain, and Orc1 still associated in the *sum1Δ* strain (Figs. 2*C* and 3*B*).

The capacity of KlOrc1 to spread and promote the spreading of other silencing proteins implies that the common ancestor of KlOrc1 and ScSir3 also had this ability and that subfunctionalization of the replication and spreading functions occurred after duplication. There are different ways duplicated genes can subfunctionalize. In the duplication, degeneration, and complementation (DDC) model, duplicated genes each lose one of the original functions and together retain the entire set of ancestral functions (47, 48), whereas in the specialization model the divergence of functions among paralogs also involves the accumulation of advantageous mutations in at least one of the duplicated genes, enabling it to outperform the ancestral gene (10, 11, 49, 50). An earlier study investigating the nonduplicated Orc1 from *Saccharomyces kluyveri* concluded that Orc1 subfunctionalized through the DDC pathway, based on the ability of SkOrc1 to complement both *orc1* and *sir3* mutations in *S. cerevisiae* (34). However, we suggest that although the *SIR3–ORC1* gene pair did subfunctionalize, it is more likely a case of specialization. In particular, the

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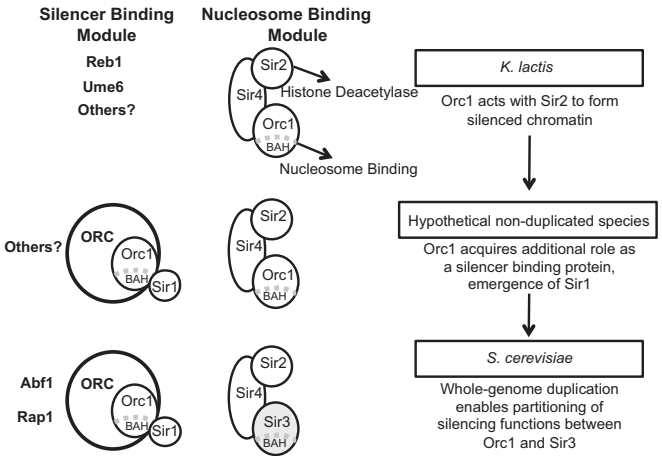


**Fig. 5.** Proposed sequence of events in the evolution of Orc1/Sir3. In the ancestral state (*Top*) Orc1 acted with Sir2 to generate extended silenced domains. The first transition (*Middle*) was the acquisition of a Sir1-like protein and the emergence of a role for Orc1 as a silencer-binding protein. Subsequently (*Bottom*), a whole-genome duplication enabled the partitioning of functions between Orc1 and Sir3.

accelerated sequence divergence of *SIR3* compared with *ORC1* implies that *SIR3* continued to evolve after duplication. A prediction of the specialization model is that the ancestral gene did not function as well as the duplicated genes. Although it is difficult to compare the silencing efficiencies of KlOrc1 and ScSir3, the requirement for Sum1 to silence the mating-type loci of *K. lactis* indicates that the KlOrc1–Sir4–Sir2 complex cannot maintain transcriptional repression on its own and therefore may be less efficient than the ScSIR complex.

The observation that KlOrc1 does not appear to have a silencing function similar to that of ScOrc1, which interacts with Sir1 (25–27) is best explained by the absence of *SIR1* in *K. lactis* (40, 51). The only nonduplicated species in which an ortholog of *SIR1* has been detected is *Zygosaccharomyces rouxii* (51), which is thought to be closer to the duplication event than *K. lactis* is. It will be interesting to determine whether ZrOrc1 has both the capacity to bind nucleosomes like ScSir3 and the capacity to recruit Sir1 (called ZrKos3), as ScOrc1 does. If so, the *ORC1* gene that became duplicated most likely also had both properties. In species such as *K. lactis* that lack Sir1, other mechanisms must exist to recruit silencing proteins to the chromosome, and indeed two silencer-binding proteins distinct from those that act in *S. cerevisiae*, KlReb1 and KlUme6, have been described (39, 52). The recent discovery of RNAi in budding yeasts (53) also raises the possibility that small RNAs could play a role in the formation of heterochromatin, as occurs in *Schizosaccharomyces pombe*, although neither argonaute nor dicer-like proteins have been detected in *K. lactis*.

In summary, we propose that two important transitions occurred in the mechanism by which yeast Orc1/Sir3 functions in silencing (Fig. 5). In the ancestral state, as in *K. lactis*, Orc1 acted with Sir2 to generate extended silenced domains, with Sir2 deacetylating histones and Orc1 binding to these deacetylated nucleosomes through its BAH domain. As Sir2 and Orc1 are both associated with heterochromatin in a variety of eukaryotes, this type of cooperation may be quite ancient. The first transition was the acquisition of a Sir1-like protein, which could interact with the BAH domain of Orc1. Thus, Orc1 gained the ability to act as a silencer-binding protein. Subsequently, a whole-genome duplication enabled the partitioning of these two silencing functions between the *ORC1* and *SIR3* paralogs, as observed in *S. cerevisiae*. Once Sir3 was no longer constrained to act in DNA replication, it may have acquired additional beneficial properties. For example, the second histone-binding domain in the C terminus of Sir3 (20) or the modification of the ATPase domain to bind the Sir2 product O-acetyl-ADP ribose (54) could have arisen after duplication. It is also possible that even after subfunctionalization, Orc1 retained some ability to propagate along chromatin, as recently suggested (55).

## Materials and Methods

**Strains and Media.** Strains used in this study were derived from CK213 and SAY538 (Table S1) and were grown at 30° in YPD medium containing 1% yeast extract, 2% peptone, and 2% glucose.

**Gene Expression Analysis.** RNA was isolated from two independent logarithmically growing cultures of each strain using a hot phenol method (56), and cDNA was synthesized as previously described (14). The resulting cDNA was quantified by real-time PCR in the presence of SYBR Green on a Bio-Rad iCycler. A standard curve was generated with genomic DNA isolated from the wild-type strain (CK213). Oligonucleotide sequences are provided in Table S2. Transcript levels of queried genes were first normalized to the *KlACT1* mRNA for each genetic background. The fold induction was then calculated by normalizing to the wild-type strain. The SE measurement (SEM) was calculated from the differences in fold induction of two or more independent cultures from the mean.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitations were performed as previously described (15) using 10 optical density equivalents of cell lysate and 4 μL anti-HA tag antibody (Sigma, H6908), anti-myc tag antibody (Upstate Biotechnology, 06–549), anti-FLAG antibody (Sigma, F3165), or anti-V5 antibody (Millipore, AB3792). Dimethyl adipimidate was used as a second cross-linking agent (57). The amounts of immunoprecipitated DNA at experimental loci and a control locus, *KlRRP7*, were quantified by real-time PCR relative to a standard curve prepared from input DNA, and the relative enrichments of the experimental loci compared with the control locus were calculated. Oligonucleotide sequences are provided in Table S2. The data presented represent results from two or more independent cultures of each strain, and the SEM was calculated from differences in the relative enrichment from the mean.

**Immunoblotting.** Protein samples were prepared from 40 optical density equivalents of cells fixed in 10% trichloroacetic acid for 20 min. Cell pellets were washed with 1 M Tris pH 8.0 and lysed by vortexing 5 min in the presence of glass beads in 40 μL lysis buffer (10 mM hepes pH 7.9, 150 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, protease inhibitors). The volume of the lysate was increased to 160 μL, proteins were denatured by the addition of 3× sample buffer (30% glycerol, 15% β-mercaptoethanol, 6% SDS, 200 mM Tris pH 6.8, 0.08 mg/mL bromophenol blue) at 95° for 5 min, and the samples were clarified by centrifugation. Samples were fractionated on 7.5% polyacrylamide-SDS gels, transferred to nitrocellulose membranes (Amersham), and probed with rabbit α-V5 (Millipore, AB3792).

**ACKNOWLEDGMENTS.** We thank S. Haase for help with the FACS analysis and D. L. Bailey, H. Chang, and K. Cocce for technical assistance. This research was supported by a grant from the National Institutes of Health (GM073991) (to L.N.R.).

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