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Epistatic Interaction Between the K-Homology Domain Protein *HEK2* and *SIR1* at *HMR* and Telomeres in Yeast

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Abstract

In budding yeast, telomeres, the ribosomal DNA array, and *HM* loci are transcriptionally silenced by chromatin complexes containing Sir proteins. Hek2, a protein containing three evolutionary conserved RNA-binding K-homology domains, was identified as a suppressor of telomeric silencing [telomeric position effect (TPE)]. To explore the mechanisms of Hek2p action in gene silencing, we examined its relationship with Sir proteins. This search revealed an epistatic interaction between *HEK2* and *SIR1* at telomeres. Both single mutations, *sir1Δ* and *hek2Δ*, enhanced TPE, whereas the effect of double mutation, *sir1Δ hek2Δ*, did not exceed that of the single mutations. The results of chromatin immunoprecipitation analysis demonstrate that the TPE enhancement observed in these mutants is associated with increased binding of Sir2 protein to telomeres. At the *HMR* locus, *hek2Δ* rescues the silencing defect caused by *sir1Δ* mutation and reverses the loss of Sir2p and Sir3p. These data suggest that the epistatic interaction of *HEK2* and *SIR1* reflects competition between telomeres and *HMR* for Sir2/3 factors where *HEK2* acts to suppress silencing. Because chromatin immunoprecipitation analysis reveals the presence of Hek2p at a subtelomeric region and *HMR*, its silencing effects at these loci are likely direct. These observations suggest that *HEK2* regulates the composition of Sir complexes at *HMR* and telomeres.

Keywords

Hek2; Gene silencing; Sir proteins; Telomeres; *HMR*

Introduction

Chromatin was initially defined as two structures seen in eukaryotic nuclei under the microscope: transparent euchromatin and dense granular heterochromatin.¹ Expressed genes are localized within euchromatin, whereas silenced genes are restricted to heterochromatin. In budding yeast nuclei, there is no microscopically recognizable chromatin domain; however, chromatin complexes found at constitutively silenced loci are functionally similar to heterochromatin required for transcriptional silencing in both artificial² and natural³ contexts.

Many heterochromatin factors were initially characterized in yeast (reviewed in Ref. 4). Protein composition of heterochromatin is defined by the arrangement of silencer DNA elements and the availability of silencing factors. In yeast, several factors, such as Rap1, Abf1, and origin of replication complex (ORC), constitutively bind silencers at *HM* loci where they nucleate formation of silenced chromatin. In turn, these factors recruit Sir proteins that have a property of spreading along the arrays of nucleosomes with deacetylated histones.^{5,6} Different silenced

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loci have different protein compositions; for example, Sir2p is shared by ribosomal DNA, telomeres, and *HM* loci, Sir3p and Sir4p are found at telomeres and *HM* loci but not in ribosomal DNA, and Sir1p seems to act predominantly at *HM* loci.⁴

Histone modifications play a fundamental role in chromatin organization.^{6–8} In yeast, for example, Sir3p and Sir4p bind to hypoacetylated N-terminal tails of histones H3 and H4.^{6–8} Furthermore, the extent of spreading of silenced chromatin complexes containing Sir2–4 proteins is determined by an equilibrium between the activities of histone acetyltransferase Sas2p and deacetylase Sir2p.^{9,10} Because differently modified histones are recognized by different factors, histone modifications control chromatin composition and, therefore, function.^{11,12} These observations provide an important insight into the mechanisms of chromatin organization and dynamics.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a conserved family of proteins that bind to DNA and RNA and serve in many processes that compose gene expression.^{13–20} hnRNP K, a protein containing three K-homology domains, is recruited to the promoter and coding regions of several genes, modulates transcription of these genes,^{21,22} and binds transcripts, regulates their processing, and most likely follows them to the cytoplasm.²⁰ In addition to nucleic acids, K protein also interacts with a number of protein partners.²⁰ A general model predicts that K protein serves to organize protein complexes at nucleic acid sites in a process regulated by kinases and other enzymes.²⁰

Several lines of evidence suggested a role for K protein in chromatin function. For instance, the mammalian K protein interacts with the chromatin factor Polycomb group protein Eed¹⁶ and DNA methyltransferase Dnmt1.²³ In addition, K protein homologues in *Saccharomyces cerevisiae* *HEK2* and *PBP2* behave as suppressors of telomeric position effect (TPE); that is, *hek2Δ/pbp2Δ* mutations enhance silencing of the *URA3* marker gene at a subtelomeric but not an internal chromosomal location.¹⁷ The fact that Hek2p was detected in a complex with subtelomeric regions¹⁷ suggested direct involvement of this protein in telomeric chromatin rearrangements. Scp160, another K-homology domain protein in yeast, has recently been identified as an enhancer of silencing at telomeres and *HMR*, suggesting a role for this family of factors in gene silencing.²⁴ In this study, we show that *HEK2* regulates the composition of silenced chromatin at *HMR* and telomeres.

Results

Epistatic interaction between *HEK2* and *SIR1* in TPE

HEK2 was described as a modifier of TPE.¹⁷ In TPE assay, *hek2Δ* mutation enhances silencing of subtelomeric genes. To explore the mechanisms of *HEK2* action, we set out to identify other factors that mediate its TPE effects. In these experiments, we used strains with the *URA3* marker gene inserted near the 5R telomere, as shown in Fig. 1a.²⁵ Strains were grown on agar plates supplemented with 1 g/l of 5-fluoroorotic acid (5FOA) to estimate the efficiency of *URA3* silencing. This reagent is toxic to cells that express orotidine 5'-monophosphate decarboxylase (*URA3*); therefore, the frequency of 5FOA-resistant (5FOA^R) colonies is proportional to the efficiency of *URA3* silencing.²⁶ Surprisingly, in the course of these experiments, we found that *sir1Δ* mutation enhances TPE (Fig. 1a). Results of reverse transcription (RT)-PCR analysis of *URA3* mRNA levels in these strains complement changes in TPE efficiency (Fig. 1b), suggesting transcriptional control. *SIR1* is a factor required for efficient silencing at *HML* and *HMR*, but little or no role for this protein in telomeric silencing has been reported.^{2,27–30} TPE enhancement observed in *sir1Δ* strains may reflect relocation of limiting chromatin factors to subtelomeric regions. To test this suggestion, we analyzed the density of Sir2 at *URA3* in the same strains by using chromatin immunoprecipitation (ChIP) assay.³¹

³² Results of ChIP analysis demonstrate that, in agreement with TPE data (Fig. 1), *hek2Δ* and *sir1Δ* mutations increase the density of Sir2p at the subtelomeric *URA3* (Fig. 2).

HEK2 regulates silencing at the *HMR* locus

The TPE enhancement of the double mutant *hek2Δ sir1Δ* does not exceed that of the single mutant *sir1Δ* (Figs. 1 and 2), indicating that *HEK2* and *SIR1* belong to the same gene-silencing pathway. Sir1p binds to ORC at *HM* loci, attracts Sir proteins, and stabilizes silenced chromatin at these loci.^{29,33} Deletion of the *SIR1* gene has been shown to partially derepress *HMR* and *HML*.^{33,34} Interaction between *HEK2* and *SIR1* in TPE raises a possibility that they also interact at *HM* loci. Therefore, we next examined interaction between *hek2Δ* and *sir1Δ* mutations in *HMR* silencing by using mating assay. In the mating assay, α -type strains that express $\alpha 1$ and $\alpha 2$ factors from the *MAT α* locus mate with **a**-type strains that express the **a1** factor from the *MAT α* locus. However, if *HMR α* is de-repressed in *MAT α* strains, then cells express both $\alpha 1$ and **a1** factors, imitating diploids, and therefore cannot mate. As expected, in the *HML α MAT α HMR α* strain, *sir1Δ* mutation caused a several-fold decrease in mating efficiency compared with the wild-type (WT) strain (Fig. 3a). *hek2Δ* mutation rescues the mating defect in *sir1Δ* mutants, indicating that *hek2Δ* mutation enhances *HMR* silencing in *sir1Δ* mutants. Results of RT-PCR analysis of *HMR $\alpha 1$* mRNA levels indicate that the effect of *hek2Δ* mutation on *HMR* silencing in *sir1Δ* mutants involves transcriptional control (Fig. 3b). Expression of the *STE4* gene, a target of the **a1**/ $\alpha 2$ repressor complex, was downregulated in the *sir1Δ HEK2* strain (Fig. 3b, compare lane 1 with lane 3) but recovered in the *sir1Δ hek2Δ* strain (Fig. 3b, compare lane 3 with lane 4). These results rule out a possibility that changes in *HMR $\alpha 1$* mRNA processing and/or translation play a role in the observed phenomena.

Our data show that the *HEK2* and *SIR1* genes have opposite roles in *HMR* silencing. To further characterize the effect of *HEK2* on *HMR*, we used strains where the *URA3* reporter gene was inserted at *HMR* to the BglII site at the 3' end of the *HMR $\alpha 1$* gene.³⁵ As shown in Fig. 4a, while *sir1Δ* mutation causes substantial loss of *URA3* silencing, there is partial recovery of *URA3* silencing in the double mutant *sir1Δ hek2Δ*. These data support the role of *HEK2* in silencing of the *HMR* locus. Surprisingly, in contrast to the results shown above (Fig. 3b), we did not detect any difference in *URA3* mRNA levels between the *sir1Δ HEK2* and *sir1Δ hek2Δ* strains (Fig. 4b), raising a possibility of a posttranscriptional effect of *hek2Δ* mutation on *URA3* expression. Alternatively, such a result could reflect the all-or-none nature of *URA3* transcription at *HMR* in these strains. In fact, most cells in the *sir1Δ HEK2* and *sir1Δ hek2Δ* strains are sensitive to the 5FOA (*URA3*-ON) (Fig. 4a); therefore, 5FOA^R (*URA3*-OFF) cells represent an insignificant fraction of RNA prepared from the entire population of cells. To define the mechanism, we compared *URA3* mRNA levels in 5FOA^R-enriched and unselected cell cultures, expecting that in case of posttranscriptional control, the levels of *URA3* mRNA in 5FOA^R and unselected cells would be the same. In contrast, lack of *URA3* mRNA expression in 5FOA^R cells would suggest transcriptional regulation. To enrich cells in the 5FOA^R state, we transferred colonies growing on 5FOA plates to yeast extract/peptone/dextrose agar plates, from where individual colonies were analyzed for 5FOA resistance and for the level of *URA3* mRNA. As shown in Fig. 4c, such a procedure significantly enriches *sir1Δ HEK2* and *sir1Δ hek2Δ* cells in the 5FOA^R state. Northern blot analysis (Fig. 4d) demonstrates that in contrast to unselected cells, 5FOA^R state-enriched *sir1Δ HEK2* and *sir1Δ hek2Δ* mutants express very low levels of *URA3* mRNA. Little or no change was detected in the level of *ura3*-52 transcript serving as a control for specificity (Fig. 4d). Thus, these results rule out posttranscriptional events and support the role of *HEK2* in transcriptional silencing of *HMR*. We wondered if *HEK2* modulates the composition of silenced chromatin at *HMR*. This possibility was tested next.

The effect of *hek2Δ* mutation on composition of silenced chromatin at the *HMR* locus

Several chromatin factors, including Sir proteins, are essential for silencing at *HM* loci. To test if *HEK2* interferes with chromatin organization at *HMR*, we next examined the effect of *hek2* and *sir1* mutations on the density of Sir2p and Sir3p factors at this locus. It has been previously shown that partial loss of *HMR* silencing in *sir1Δ* mutants is associated with reduced density of other Sir proteins recruited to the locus.³³ Therefore, rescued silencing of *HMR* in the double mutant *sir1Δ hek2Δ* (Figs. 3 and 4) may reflect improved recruitment of Sir proteins to the locus. To test this suggestion, we analyzed the effect of *hek2Δ* mutation on Sir3p and Sir2p recruitment to *HMR* using ChIP assay. While *hek2Δ* mutation alone shows little or no effect on the density of these factors at the locus, the amount of Sir3p protein bound to *HMR* decreases in *sir1Δ HEK2* strains (Fig. 5a). Nearly WT levels of Sir3p were observed at *HMR* in *sir1Δ hek2Δ* strains, showing that *HEK2* is involved in the regulation of chromatin composition at this locus. No significant change in the total cellular level of Sir3p was found in these strains (Fig. 5b), arguing against the possibility that *hek2Δ* mutation regulates expression/stability of this factor. Parallel changes were observed in the level of Sir2 protein recruited to *HMR* in the same strains (Fig. 5a and b). The similar behaviors of two factors, Sir2 and Sir3, suggest that *hek2Δ* mutation enhances recruitment of these factors to *HMR* rather than altering the efficiency of their immunoprecipitation in ChIP assay.

These results support a model in which *HEK2* destabilizes Sir2/3-containing complexes at *HMR* in *sir1Δ* mutants. We next tested if Hek2 regulates recruitment of Sir1p to *HMR*. Sir1p, like other Sir proteins, was detected along the entire *HMR* locus²⁹; we also found it in the middle of *HMR* (*X/Ya* site) and at the *I* silencer (Fig. 6). As expected,²⁹ we detected no presence of Sir1p at the subtelomeric region (5R), a result that supports an indirect role for *SIR1* in TPE. In the *hek2Δ* mutants, no change could be found in the cellular levels of Sir1p and its density at *HMR*. These results imply that neither expression of Sir1p nor its recruitment to *HMR* is regulated by *HEK2*.

Hek2p is recruited to the *HMR* locus

To test if Hek2p is engaged at the *HMR* locus, we used ChIP assay in strains containing Flag-tagged *HEK2*. Results of ChIP analysis show that the Hek2p-Flag protein is enriched in the middle of the *HMR* locus (*X/Ya* site) but not detected at the *HMR-I* silencer (Fig. 7a). Enrichment of this factor at *HMR* is not as robust as that of Sir proteins (Figs. 5 and 6), suggesting that either Hek2p is inefficiently cross-linked to the locus due to lack of direct contact with DNA or its recruitment to *HMR* is transient. For example, it was estimated that the efficiency of cross-linking of nucleosome-remodeling complex RSC to DNA was ~10-fold lower than that of a DNA-binding protein; therefore, enrichment of this complex at target loci over other genomic sites was relatively small (1.5-fold).³⁶ Nonetheless, results of ChIP experiments suggest that at least some of the silencing effects of Hek2 at *HMR* are direct consequences of its binding to the locus. Differences in the binding profiles of Sir proteins (Figs. 5 and 6) and Hek2p (Fig. 7) along the *HMR* locus suggest that these proteins are components of different complexes.

Discussion

Through this study, we have shown that yeast hnRNP K-like protein Hek2p regulates silencing efficiency at telomeres and at the *HMR* locus. In these processes, *HEK2* appears to act in the same pathway as *SIR1*. Sir1p binds ORC at *HML* and *HMR*, recruits other Sir proteins, and stabilizes chromatin complexes at these loci.^{29,33} Because Sir1p is not known to bind telomeres (Fig. 6), its TPE suppressor activity (Fig. 1) is most likely an indirect result of competition between the telomeres and *HM* loci for limiting silencing factors. Regardless of the mechanism, a global consequence of *SIR1* action is redistribution of chromatin factors

between silenced loci (Figs. 2 and 5). The effect of *sir1Δ* mutation on TPE was examined before;³⁰ however, in contrast to the data presented here, little or no effect was observed. This discrepancy could be explained by the different reporter systems used. Strains reported here contained the *URA3* gene inserted near the truncated 5R chromosomal end, where all natural subtelomeric sequences were removed. Substantial distance between the *URA3* gene and telomere weakens silencing (Fig. 1) and makes these strains suitable for the detection of TPE enhancement.¹⁷ In contrast, in the previous study,³⁰ the effect of *sir1Δ* mutation on TPE was tested in strains containing efficiently silenced *URA3* gene, allowing detection of silencing loss rather than enhancement. It is also possible that TPEs of *sir1Δ* mutation depend on the composition of subtelomeric elements present at the chromosomal end, such as autonomous replication sequences consensus sequence, Abf1-binding sites, X element, etc.^{37,38}

As *hek2Δ* mutation shows epistatic interaction with *sir1Δ* mutation in regulating TPE and *HMR* silencing, *HEK2* may also be involved in partition of chromatin factors between the silenced loci. Indeed, the modes of action of these two factors appear closely interrelated. In *sir1Δ* cells, silencing factors are lost from *HMR* but somewhat enriched at telomeres (Figs. 2 and 5), an effect that is partially reversed in *sir1Δ hek2Δ* mutants where the density of Sir proteins is decreased at telomeres (Fig. 2) but increased at the *HMR* locus (Fig. 5). As a result, the efficiency of *HMR* silencing in the double mutant *sir1Δ hek2Δ* resembles that of the WT cells (Figs. 3 and 4a). We suggest that *HEK2* either destabilizes silencing complexes or stabilizes transcription at *HMR* and subtelomeric regions.

ChIP analysis reveals that Hek2p is bound to *HMR* (Fig. 7). Together with the previous finding that Hek2p binds to subtelomeric regions,¹⁷ this observation supports direct involvement of this factor in the silencing processes. The pattern of Hek2 binding to the *HMR* locus is unusual as it is present in the middle of *HMR* (*X/Ya*) but not at the flanking regions, silencers *I* (Fig. 7) and *E* (not shown). This observation identifies *HEK2* as a factor with potentially novel function in *HMR* silencing. The mechanism of Hek2p recruitment to *HMR* remains to be determined.

We have found that Hek2p regulates recruitment of Sir2/3 proteins to telomeres and *HMR*. Among several possible scenarios of the mechanism of *HEK2* action at telomeres/*HMR*, we favor a possibility that *HEK2* regulates activity/occupancy of histone-modifying enzymes at silenced loci. Sir2p, a NAD-dependent histone deacetylase that facilitates spreading of silenced domains, is one such putative direct target of Hek2p. Alternatively, recruitment of Sir2 could be regulated by Hek2p indirectly through other enzymes. For example, histone deacetylase complex Sin3–Rpd3 is another potential target for *HEK2* as loss of these genes causes silencing phenotypes similar to those of *hek2Δ* mutation. *rp3Δ/sin3Δ* mutations enhance TPE as well as suppress silencing defects at *HMR* in either *sir1Δ* or *hmrΔA* mutants^{39–41} and rescue telomeric silencing in *scp160Δ* mutants.²⁴ However, it seems unlikely that *HEK2* and the *RPD3* complex work in the same pathway because (i) in contrast to *rp3Δ* (also *sin3Δ* or *sds3Δ*) mutation, which suppresses loss of *HMR* silencing in *rif1Δ* mutants,³⁹ *hek2Δ* mutation shows no such suppressor activity (data not shown) and (ii) mutations in the components of the Rpd3 complex result in telomere shortening⁴² but *hek2Δ* mutation slightly increases telomere size.¹⁷ There may be other histone-modifying enzymes that interact with *HEK2* at silenced loci. In support of this notion, hnRNP K protein, the mammalian homologue of Hek2p, interacts with Eed, a chromatin factor that exists in a complex with histone methyltransferase Ezh2.^{16,43} Therefore, in the mammalian system, K protein may regulate chromatin organization by recruiting Eed/Ezh2 histone-modifying complex to the target loci.

In another scenario, *HEK2* may regulate long-range interactions between genomic loci (e.g., between *HMR/HML* and telomeres)⁴⁴ and restrict spreading of silenced state in trans between these loci. Involvement of the yeast Hek2p and the mammalian hnRNP K proteins in long-range interactions was suggested earlier.^{17,45} This type of interaction was also proposed as

an important function of the nuclear matrix, an unexplored RNA–protein network serving as a scaffold for the spatial organization of nuclear processes.⁴⁶ In this regard, hnRNP K has been identified as one of the major components of the nuclear matrix in mammalian cells.^{47,48} It would be revealing to find out if Hek2p has a similar affiliation in yeast.

The above studies provide evidence for the involvement of yeast hnRNP K-like protein Hek2p in the regulation of chromatin composition. Because Hek2 and hnRNP K proteins directly bind to specific RNA motifs and regulate mRNA translation and localization in cytoplasm,^{17,20,49} it is possible that the more general role of this family of factors is to coordinate nuclear and cytoplasmic stages of gene expression.

Materials and Methods

Yeast strains, plasmids, and methods

Media used for the growth of *S. cerevisiae* were as previously described;⁵⁰ cells were grown at 30 °C. Strains that were used in this study are listed in Table 1. Yeast transformation was performed by the lithium acetate procedure as described in Ref. 51. Resistance to 5FOA was determined as described in Ref. 52. 5FOA was obtained from Zymo Research (Orange, CA) and used at 0.1%. PCR-mediated gene disruption was performed as described in Ref. 50. The primers used in this study are as follows: CTAAATCGCATTTCTTTTCGTCCAC, HMR-E-S; TAACAAAAACCAGGAGTACCTGCGC, HMR-E-A; TGTCACCAACATTTTCGTATATGGCG, HMR-I-S; ACCACATTATCAATCCTTGCATCCAG, HMR-I-A; GGATGG A T C T A G G G T T T T A T G C C, H M L - E - S; TAGATTTGGCCCCCGAAATCG, HML-E-A; CAGCTGAGTAACTACTCTCATGG, HML-I-S; GCTGTTACGGAGATGCAAAGC, HML-I-A; TACCAACCCATCCGCCG, X/Ya-S; TCCGCCATACTACAAATATCATCC, X/Ya-A; ATGTCCACTGATTCTATTGT, RPL14B-S; GAAGGAGTTTGCCAGTAAC, RPL14B-A; GATAAATCATGTGCGAAAGCTAC, URA3-S; CCGTGTGCATTTCGTAATGTC, URA3-A2; CAGAACTCTGTTTAACATTC, a1-S; TCATA C G TT TAT TTAT G A AC, a 1 - A; G C TT TT G G GTTACCACATTC, 5RR-S; GACTGAACGCCTTTAATCTC, 5RR-A; GGATCTTCTACTACATCAGC, ACT1-S; AGTACATGAGACTTAGTAAC, ACT1-A3; GATGGACTCGATAACGTATTC, STE4-S; and GACGCAACGTTTTGCGCTAC, STE4-A.

Efficiency of mating determination

Cells were mated as described in Ref. 28 with some modifications. Tester strains and experimental strains were grown overnight in yeast extract/peptone/dextrose. Fixed amounts of α or α tester cultures (10 μ l) were mixed with 10 μ l of 10-fold serial dilutions of experimental cultures, brought to 100 μ l with water, and 10- μ l aliquots were plated on synthetic dextrose minimal medium (SD) agar plates, where only diploid but neither one of haploid strains can grow. The same dilutions of experimental strains were plated on synthetic complete medium (SC) agar plates to estimate the number of viable cells present in the cultures. Efficiency of mating was calculated as the number of colonies growing on SD plates divided by the number of viable cells plated.

Western blot analysis

Western blot analysis of total cell extracts with anti-FLAG mouse monoclonal antibody (2.5 μ g/ml, M2, Sigma), anti-Sir3p (1:1000, a gift from J. Rine), and anti-Sir2p (1:1000, a gift from J. Rine) rabbit sera was performed as described elsewhere.³³ Secondary antibodies were conjugated with alkaline phosphatase, and membranes were developed with NBT/BCIP substrate.

RT-PCR and Northern blot analysis

RNA was purified from mid-log-phase yeast cultures (5 ml) as described in Ref. 52. RNA samples were treated with RNase-free DNase I (1 U/10 µg of RNA; Epicentre Technologies, Madison, WI) for 15 min at 37 °C and then deproteinized with phenol/chloroform mixture and precipitated with ethanol. One microgram of DNA-free RNA was reverse transcribed by Superscript II (200 U per reaction; Invitrogen, Carlsbad, CA) with random hexanucleotide primer mixture (1 µM) in a 10-µl final volume for 1 h at 42 °C. One-tenth of the reaction mixture was then amplified by PCR with relevant sets of primers (primer sequences are available upon request). PCR was performed in a 25-µl final volume with 0.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA) for 25–30 cycles. Five microliters of the reaction mixtures was analyzed by agarose gel electrophoresis, followed by staining with ethidium bromide (0.1 µg/ml).

ChIP and PCR analysis of precipitated DNA

To test if the tagging procedure interfered with the silencing activity of *HEK2*, we tested *SIR1* and *sir1Δ* strains with Flag-tagged *HEK2* (DY305 and DY306, *MATα*) for the levels of *HMRa1* and *STE4* expression by using RT-PCR (please see Fig. 2 for details). No change in the level of expression of these genes was found in tagged strains as compared with the corresponding untagged strains, showing that Flag tag has no effect on the activity of *HEK2* in *HMR* silencing. Formaldehyde cross-linking, whole-cell extract preparation, immunoprecipitation, DNA purification, and PCR analysis were performed as described in Ref. 32 with some modifications.^{31,53} Antibodies that were used in immunoprecipitations include anti-Flag (2 µg), anti-Sir2p (2 µl of whole serum), and anti-Sir3p (2 µl of whole serum). Sonication treatment resulted in average DNA fragment sizes of 0.5–1 kb. PCR (25–30 cycles) was carried out in a 25-µl volume with 1/50 of the immunoprecipitated material and 1/10,000 of the input material.¹⁷ Primer sequences are available upon request. PCR products were resolved in 1% agarose gel and visualized with 0.1 µg of ethidium bromide per milliliter. Densitometry was done using OptiQuant software (Packard).

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Abbreviations

TPE	telomeric position effect
ChIP	chromatin immunoprecipitation
RT	reverse transcription
ORC	origin of replication complex
hnRNP	heterogeneous nuclear ribonucleoprotein
5FOA	

5-fluoroorotic acid

5FOA^R

5FOA resistant

WT

wild type

References

1. van Holde, K. Chromatin. Springer-Verlag, Berlin; Germany: 1988.
2. Aparicio OM, Billington BL, Gottschling DE. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* 1991;66:1279–1287. [PubMed: 1913809]
3. Vega-Palas MA, Venditti S, Di Mauro E. Telomeric transcriptional silencing in a natural context. *Nat Genet* 1997;15:232–233. [PubMed: 9054930]
4. Rusche LN, Kirchmaier AL, Rine J. The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. *Annu Rev Biochem* 2003;72:481–516. [PubMed: 12676793]
5. Venditti S, Vega-Palas MA, Di Mauro E. Heterochromatin organization of a natural yeast telomere. Recruitment of Sir3p through interaction with histone H4 N terminus is required for the establishment of repressive structures. *J Biol Chem* 1999;274:1928–1933. [PubMed: 9890947]
6. Carmen AA, Milne L, Grunstein M. Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein *SIR3*. *J Biol Chem* 2002;277:4778–4781. [PubMed: 11714726]
7. Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, Kouzarides T. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 2001;410:120–124. [PubMed: 11242054]
8. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 2001;410:116–120. [PubMed: 11242053]
9. Kimura A, Umehara T, Horikoshi M. Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. *Nat Genet* 2002;32:370–377. [PubMed: 12410229]
10. Suka N, Luo K, Grunstein M. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine 16 and spreading of heterochromatin. *Nat Genet* 2002;32:378–383. [PubMed: 12379856]
11. Jenuwein T, Allis CD. Translating the histone code. *Science* 2001;293:1074–1080. [PubMed: 11498575]
12. Turner BM. Cellular memory and the histone code. *Cell* 2002;111:285–291. [PubMed: 12419240]
13. Cortes A, Huertas D, Fanti L, Pimpinelli S, Marsellach FX, Pina B, Azorin F. DDP1, a single-stranded nucleic acid-binding protein of *Drosophila*, associates with pericentric heterochromatin and is functionally homologous to the yeast Scp160p, which is involved in the control of cell ploidy. *EMBO J* 1999;18:3820–3833. [PubMed: 10393197]
14. Konkel LMC, Enomoto S, Chamberlain EM, McCune-Zierath P, Iyadurai SJP, Berman J. A class of single-stranded telomeric DNA-binding proteins required for Rap1p localization in yeast nuclei. *Proc Natl Acad Sci USA* 1995;92:5558–5562. [PubMed: 7777547]
15. LaBranche H, Dupuis S, Ben-David Y, Bani MR, Wellinger RJ, Chabot B. Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase [see comments]. *Nat Genet* 1998;19:199–202. [PubMed: 9620782]
16. Denisenko ON, Bomsztyk K. The product of the murine homolog of the *Drosophila extra sex comb* gene displays transcriptional repressor activity. *Mol Cell Biol* 1997;17:4707–4717. [PubMed: 9234727]
17. Denisenko O, Bomsztyk K. Yeast hnRNP K-like genes are involved in regulation of the telomeric position effect and telomere length. *Mol Cell Biol* 2002;22:286–297. [PubMed: 11739741]
18. Huertas D, Cortes A, Casanova J, Azorin F. *Drosophila* DDP1, a multi-KH-domain protein, contributes to centromeric silencing and chromosome segregation. *Curr Biol* 2004;14:1611–1620. [PubMed: 15380062]

19. Burd CG, Dreyfuss G. Conserved structures and diversity of functions of RNA-binding proteins. *Science* 1994;265:615–621. [PubMed: 8036511]
20. Bomsztyk K, Denisenko O, Ostrowski J. hnRNP K: one protein multiple processes. *BioEssays* 2004;26:629–638. [PubMed: 15170860]
21. Michelotti EF, Michelotti GA, Aronsohn AI, Levens D. Heterogeneous nuclear ribonucleoprotein K is a transcription factor. *Mol Cell Biol* 1996;16:2350–2360. [PubMed: 8628302]
22. Ostrowski J, Kawata Y, Schullery DS, Denisenko ON, Bomsztyk K. Transient recruitment of the hnRNP K protein to inducibly transcribed gene loci. *Nucleic Acids Res* 2003;31:3954–3962. [PubMed: 12853611]
23. Shnyreva M, Schullery DS, Suzuki H, Higaki Y, Bomsztyk K. Interaction of two multifunctional proteins. Heterogeneous nuclear ribonucleoprotein K and Y-box-binding protein. *J Biol Chem* 2000;275:15498–15503. [PubMed: 10809782]
24. Marsellach FX, Huertas D, Azorin F. The multi-KH domain protein of *Saccharomyces cerevisiae* Scp160p contributes to the regulation of telomeric silencing. *J Biol Chem* 2006;281:18227–18235. [PubMed: 16632467]
25. Renaud H, Aparicio OM, Zierath PD, Billington BL, Chhablani SK, Gottschling DE. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by *SIR3* dosage. *Genes Dev* 1993;7:1133–1145. [PubMed: 8319906]
26. Boeke JD, Trueheart J, Natsoulis G, Fink GR. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* 1987;154:164–175. [PubMed: 3323810]
27. Pillus L, Rine J. Epigenetic inheritance of transcriptional states in *S. cerevisiae*. *Cell* 1989;59:637–647. [PubMed: 2684414]
28. Rine J, Strathern JN, Hicks JB, Herskowitz I. A suppressor of mating-type locus mutations in *Saccharomyces cerevisiae*: evidence for and identification of cryptic mating-type loci. *Genetics* 1979;93:877–901. [PubMed: 397913]
29. Gardner KA, Fox CA. The Sir1 protein's association with a silenced chromosome domain. *Genes Dev* 2001;15:147–157. [PubMed: 11157772]
30. Pryde FE, Louis EJ. Limitations of silencing at native yeast telomeres. *EMBO J* 1999;18:2538–2550. [PubMed: 10228167]
31. Nelson JD, Denisenko O, Bomsztyk K. Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nature Protocols* 2006;1:179–185.
32. Kuo MH, Allis CD. *In vivo* cross-linking and immunoprecipitation for studying dynamic protein:DNA associations in a chromatin environment. *Methods* 1999;19:425–433. [PubMed: 10579938]
33. Rusche LN, Kirchmaier AL, Rine J. Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2002;13:2207–2222. [PubMed: 12134062]
34. Rine J, Herskowitz I. Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. *Genetics* 1987;116:9–22. [PubMed: 3297920]
35. Singer MS, Kahana A, Wolf AJ, Meisinger LL, Peterson SE, Goggin C, et al. Identification of high-copy disruptors of telomeric silencing in *Saccharomyces cerevisiae*. *Genetics* 1998;150:613–632. [PubMed: 9755194]
36. Ng HH, Robert F, Young RA, Struhl K. Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes Dev* 2002;16:806–819. [PubMed: 11937489]
37. Fourel G, Revardel E, Koering CE, Gilson E. Cohabitation of insulators and silencing elements in yeast subtelomeric regions. *EMBO J* 1999;18:2522–2537. [PubMed: 10228166]
38. Fourel G, Lebrun E, Gilson E. Protosilencers as building blocks for heterochromatin. *BioEssays* 2002;24:828–835. [PubMed: 12210519]
39. Vannier D, Balderes D, Shore D. Evidence that the transcriptional regulators *SIN3* and *RPD3*, and a novel gene (*SDS3*) with similar functions, are involved in transcriptional silencing in *S. cerevisiae*. *Genetics* 1996;144:1343–1353. [PubMed: 8978024]
40. Sun ZW, Hampsey M. A general requirement for the Sin3–Rpd3 histone deacetylase complex in regulating silencing in *Saccharomyces cerevisiae*. *Genetics* 1999;152:921–932. [PubMed: 10388812]

41. Smith JS, Caputo E, Boeke JD. A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors. *Mol Cell Biol* 1999;19:3184–3197. [PubMed: 10082585]
42. Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, Coker C, et al. A genome-wide screen for *Saccharomyces cerevisiae* deletion mutants that affect telomere length. *Proc Natl Acad Sci USA* 2004;101:8658–8663. [PubMed: 15161972]
43. Denisenko O, Shnyreva M, Suzuki H, Bomsztyk K. Point mutations in the WD40 domain of Eed block its interaction with Ezh2. *Mol Cell Biol* 1998;18:5634–5642. [PubMed: 9742080]
44. Lebrun E, Fourel G, Defossez PA, Gilson E. A methyltransferase targeting assay reveals silencer–telomere interactions in budding yeast. *Mol Cell Biol* 2003;23:1498–1508. [PubMed: 12588971]
45. Tomonaga T, Michelotti GA, Libutti D, Uy A, Sauer B, Levens D. Unrestraining genetic processes with a protein–DNA hinge. *Mol Cell* 1998;1:759–764. [PubMed: 9660959]
46. He DC, Martin T, Penman S. Localization of heterogeneous nuclear ribonucleoprotein in the interphase nuclear matrix core filaments and on perichromosomal filaments at mitosis. *Proc Natl Acad Sci USA* 1991;88:7469–7473. [PubMed: 1881885]
47. Mattern KA, Humbel BM, Muijsers AO, de Jong L, van Driel R. hnRNP proteins and B23 are the major proteins of the internal nuclear matrix of HeLa S3 cells. *J Cell Biochem* 1996;62:275–289. [PubMed: 8844407]
48. Samuel SK, Spencer VA, Bajno L, Sun JM, Holth LT, Oesterreich S, Davie JR. In situ cross-linking by cisplatin of nuclear matrix-bound transcription factors to nuclear DNA of human breast cancer cells. *Cancer Res* 1998;58:3004–3008. [PubMed: 9679963]
49. Irie K, Tadauchi T, Takizawa PA, Vale RD, Matsumoto K, Herskowitz I. The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1 mRNA in yeast. *EMBO J* 2002;21:1158–1167. [PubMed: 11867544]
50. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 1998;14:115–132. [PubMed: 9483801]
51. Gietz RD, Woods RA. Transformation of yeast by LiAc/SS carrier DNA/PEG method. *Methods Enzymol* 2002;350:87–96. [PubMed: 12073338]
52. Adams, A.; Gottschling, DE.; Kaiser, CA.; Stearns, T., editors. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press; 1997.
53. Nelson JD, Denisenko O, Sova P, Bomsztyk K. Fast chromatin immunoprecipitation assay. *Nucleic Acids Res* 2006;34:e2. [PubMed: 16397291]

Fig. 1.

Interaction between *sir1Δ* and *hek2Δ* mutations in TPE and *HMR* silencing. (a) Silencing efficiency of the *URA3* gene inserted near the 5R telomere was estimated in the WT *HEK2 SIR1* (DY71), *hek2Δ* (DY73), *sir1Δ* (DY215), and *hek2Δ sir1Δ* (DY216) strains. Each data point represents the frequency of 5FOA^R cells that was estimated by growing 10-fold dilutions of overnight cultures on plates with synthetic complete medium (SC) agar supplemented with or without 5FOA (1 mg/ml). Data points represent ratios of the number of cells growing on a 5FOA plate to the number of cells growing on an SC plate. Results of seven experiments in duplicates are shown. Bars represent mean values. (b) RT-PCR analysis of *URA3* mRNA levels. RNA purified from the strains shown in (a) was analyzed by RT-PCR with primers to

URA3 or *ACT1* genes. PCR products were resolved by electrophoresis in agarose gel, stained with ethidium bromide, and analyzed by densitometry. Results of three experiments in duplicates are shown. Bars represent mean $\pm$ SD.

Fig. 2.

Sir2p recruitment to silenced loci. (a) ChIP analysis was performed with antibodies to Sir2p in the *sir2Δ* (Mock, UCC3248), WT (DY71), *hek2Δ* (DY73), *sir1Δ* (DY215), and *hek2Δ sir1Δ* (DY216) strains. Purified DNA was analyzed by PCR with primers to *URA3*. Ethidium bromide-stained gels with representative results of one of three independent experiments are shown. Input, total DNA used in precipitations was analyzed by PCR with primers to *URA3* that are shown as a black bar in the cartoon below. (b) Gel band intensities (a) were analyzed by densitometry and normalized to the input DNA and Mock IP (no tag). Graph shows mean $\pm$ SD ($n=3$ independent experiments). p values are <0.02 ; p value for the *hek2Δ-sir1Δ* pair was 0.07 (t test).

Fig. 3. *hek2Δ* mutation rescues loss of mating efficiency in *sir1Δ* strains. (a) Mating efficiency was measured in the WT (DY92α), *SIR1 hek2Δ* (DY294), *sir1Δ HEK2* (DY295), and *sir1Δ hek2Δ* (DY296) *HMLα MATα HMRα* strains. Tenfold dilutions of overnight cultures were mixed with fixed amounts of *MATa* tester strains and spotted on synthetic dextrose minimal medium (SD) agar. The same dilutions were spotted on SC agar to estimate amounts of viable cells ($n=4$). (b) RT-PCR analysis of *MATa1* expression. RNA purified from the strains shown in (a) was analyzed by RT-PCR with primers to *MATa1* or *ACT1* genes. PCR products were resolved by electrophoresis in agarose gel and stained with ethidium bromide. Representative results of one of three experiments are shown.

Fig. 4. *hek2Δ* mutation exerts all-or-none effect on the expression of *HMR*. (a) WT (DY110), *hek2Δ* (DY112), *sir1Δ* (DY235), and *sir1Δ hek2Δ* (DY236) strains with the *URA3* gene inserted to the 3' end of the *HMRa1* gene were used. Tenfold dilutions of overnight cultures were plated on SC agar with or without 5FOA. Representative results of one of six experiments are shown. (b) RT-PCR analysis of *URA3* gene expression. RNA purified from the strains shown in (a) was analyzed by RT-PCR with primers to *URA3* or *ACT1* genes. PCR products were resolved by electrophoresis in agarose gel and stained with ethidium bromide. Representative results of one of three experiments are shown. (c) Enrichment of cells in the 5FOA^R state. Colonies of *sir1Δ* and *sir1Δ hek2Δ* strains growing on 5FOA plate shown in (a) were transferred on yeast extract/peptone/dextrose agar plates from where individual colonies were analyzed for growth on 5FOA plates as described in (a). WT strain was not selected for the 5FOA^R state. Representative results of one of four experiments are shown. (d) Northern blot analysis of *URA3* gene expression in 5FOA^R cells. RNA purified from 5FOA^R cells or the same strains that were not selected for the 5FOA^R state (NS) were analyzed by Northern blot analysis with ³²P-labeled probe to the *URA3* gene. A fragment of the membrane with 25S and 18S RNAs stained with methylene blue is shown in the lower panel. Results of one of two experiments are shown.

Fig. 5.

The effect of *hek2Δ* mutation on Sir2p and Sir3p recruitment to *HMR*. (a) ChIP analysis was performed with antibodies to Sir3p (left panel) or Sir2p (right panel) in the WT (*Mock* and *WT*) (DY292), *hek2Δ* (DY294), *sir1Δ* (DY295), and *hek2Δ sir1Δ* (DY296) strains. *Mock*, no antibody was added. Ethidium bromide-stained gels with representative results of one of three independent experiments are shown. *Input*, total DNA used in precipitations was analyzed by PCR with primers to *HMR-I*. Positions of the *HML* and *HMR* primers are shown as black bars in the cartoons below. (b) Western blot analysis of Sir2 and Sir3 protein levels. Equal amounts of total protein extracted from exponentially growing cells were analyzed by Western blot analysis with antibodies to Sir2p or Sir3p. Strains are the same as those shown in (a), *sir2Δ* (UCC3248), and *sir3Δ* (DY309). *Load*, a fragment of Amido black-stained membrane is shown.

Fig. 6.

Sir1p recruitment to *HMR* locus. Upper panel, ChIP assay was performed with anti-Flag monoclonal antibodies (M2) in *SIR1 HEK2* (DY92α), *SIR1-Flag HEK2* (DY319), and *SIR1-Flag hek2Δ* (DY321) strains. PCR analysis was performed with the primers to *HMR* that are shown in Fig. 5a. Ethidium bromide-stained gels are shown. *Input*, twofold dilutions of input DNA were amplified by *HMR-I* primers. Representative results of one of three experiments are shown. Lower panel, equal amounts of whole-cell proteins extracted from strains shown in the upper panel were resolved by SDS-PAGE, transferred on polyvinylidene difluoride membrane, and stained with anti-Flag monoclonal antibodies (M2). Asterisk marks unspecific band recognized by M2 antibodies in yeast extracts.

Fig. 7.

Analysis of Hek2p recruitment to *HMR* locus. (a) ChIP assay was performed with anti-Flag monoclonal antibodies (M2) in *HEK2* (DY110) and *HEK2-Flag* (DY311) strains. PCR analysis was performed with the primers to *HMR* shown in the cartoon below gels. Ethidium bromide-stained gels are shown. *Input*, twofold dilutions of input DNA were amplified by *HMR-I* primers. Representative results of one of four experiments are shown. (b) Results of densitometric analysis are presented as ratios of *X/Ya* to *HMR-I* band intensities (*X/Ya*:*I*) and shown as mean $\pm$ SD [$n=4$, $p=0.03$ (t test)]. (c) Western blot analysis of Hek2-Flag protein levels. Equal amounts of whole-cell proteins extracted from strains shown in (a) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and stained with anti-Flag monoclonal antibodies (M2). Asterisk marks unspecific band recognized by M2 antibodies in yeast extracts.

Table 1

Strains used in this study

Name	Relevant genotype	Reference
DY71	UCC509, <i>MATa HMLa HMRa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1Δ-1 ura3-52 URA3-TEL5R</i>	25
DY73	DY71, <i>hek2::HIS3</i>	17
DY92	<i>MATa HMLa HMRa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1Δ-1 ura3-52</i>	25
DY110	UCC3511, <i>MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 hmr::URA3</i>	25
DY112	DY110, <i>hek2::HIS3</i>	This study
DY215	DY71, <i>sir1::kanMX6</i>	This study
DY216	DY73, <i>sir1::kanMX6</i>	This study
DY235	DY110, <i>sir1::kanMX6</i>	This study
DY236	DY235, <i>hek2::HIS3</i>	This study
DY92a	<i>MATa HMLa HMRa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1Δ-1 ura3-52</i>	This study
DY294	DY92a, <i>hek2::HIS3</i>	This study
DY295	DY92a, <i>sir1::HIS3</i>	This study
DY296	DY92a, <i>sir1::TRP1 hek2::HIS3</i>	This study
DY309	DY295, <i>sir3::kanMX6</i>	This study
DY311	DY110, <i>HEK2-3XFlag-kanMX6</i>	This study
DY312	DY311, <i>sir1::HIS3</i>	This study
DY319	DY92a, <i>SIR1-3XFlag-kanMX6</i>	This study
DY321	DY294, <i>SIR1-3XFlag-kanMX6</i>	This study
UCC3248	<i>MATa ura3-52 ade2-101 lys2-801 trp1Δ-1 leu2-Δ1 his3-Δ200 adh4::URA3-TEL-VIIL sir2::HIS3</i>	Gottschling Lab